PROGRESS IN ECOLOGICAL STOICHIOMETRY

EDITED BY: Dedmer B. Van de Waal, James J. Elser, Adam C. Martiny, Robert W. Sterner and James B. Cotner

PUBLISHED IN: Frontiers in Microbiology, Frontiers in Ecology and Evolution, Frontiers in Environmental Science, Frontiers in Marine Science, Frontiers in Earth Science and Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-621-5
DOI 10.3389/978-2-88945-621-5

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

PROGRESS IN ECOLOGICAL STOICHIOMETRY

Topic Editors:
Dedmer B. Van de Waal, Netherlands Institute of Ecology,Netherlands
James J. Elser, Arizona State University, University of Montana, United States
Adam C. Martiny, University of California, Irvine, United States
Robert W. Sterner, University of Minnesota Duluth, United States
James B. Cotner, University of Minnesota, United States

'A Countryside Landscape with Tree and Reflection in Water' by Binkski/Shutterstock.com

Ecological stoichiometry concerns the way that the elemental composition of organisms shapes their ecology. It deals with the balance or imbalance of elemental ratios and how that affects organism growth, nutrient cycling, and the interactions with the biotic and abiotic worlds. The elemental composition of organisms is a set of constraints through which all the Earth's biogeochemical cycles must pass. All organisms consume nutrients and acquire compounds from the environment proportional to their needs. Organismal elemental needs are determined in turn by the energy required to live and grow, the physical and chemical constraints of their environment, and their requirements for relatively large polymeric biomolecules such as RNA, DNA, lipids, and proteins, as well as for structural needs including stems, bones, shells, etc. These materials together constitute most of the biomass of living organisms. Although there may be little variability in elemental ratios of many of these biomolecules, changing the proportions of different biomolecules can have important effects on organismal elemental composition. Consequently, the variation in elemental composition both within and across organisms can be tremendous, which has important implications for Earth's biogeochemical cycles.

It has been over a decade since the publication of Sterner and Elser's book, Ecological Stoichiometry (2002). In the intervening years, hundreds of papers on stoichiometric topics ranging from evolution and regulation of nutrient content in organisms, to the role of stoichiometry in populations, communities, ecosystems and global biogeochemical dynamics have been published. Here, we present a collection of contributions from the broad scientific community to highlight recent insights in the field of Ecological Stoichiometry.

Citation: Van de Waal, D. B., Elser, J. J., Martiny, A. C., Sterner, R. W., Cotner, J. B., eds (2018). Progress in Ecological Stoichiometry. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88945-621-5

Table of Contents

HERBIVORES

PATHOGENS

CROSSING SCALES

BEYOND C:N:P

frontiers in Microbiology

EDITORIAL
published: 05 September 2018
doi: 10.3389/fmicb.2018.01957

Editorial: Progress in Ecological Stoichiometry

Dedmer B. Van de Waal[1], James J. Elser[2,3], Adam C. Martiny[4,5], Robert W. Sterner[6] and James B. Cotner[7]*

[1] Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, [2] School of Life Sciences, Arizona State University, Tempe, AZ, United States, [3] Flathead Lake Biological Station, University of Montana, Polson, MT, United States, [4] Department of Earth System Science, University of California, Irvine, Irvine, CA, United States, [5] Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA, United States, [6] Large Lakes Observatory, University of Minnesota Duluth, Duluth, MN, United States, [7] Department of Ecology, Evolution and Behavior, University of Minnesota, Saint Paul, MN, United States

Keywords: biological stoichiometry, elemental composition, ecological theory, C:N:P, ecological scaling

Editorial on the Research Topic

Progress in Ecological Stoichiometry

INTRODUCTION

Ecological Stoichiometry is the study of the balance of energy and multiple chemical elements in ecological interactions (Sterner and Elser, 2002). With a deep ancestry extending to J. Liebig and A. J. Lotka, during the twentieth century stoichiometric foundations were laid by A. C. Redfield, M. Droop, D. Tilman, W. Reiners, V. Smith, J. Urabe, and D. O. Hessen, among others. In their book, Sterner and Elser sought to assemble and articulate some of the core concepts of Ecological Stoichiometry. These include a formalized approach to variation in the strength of stoichiometric homeostasis among biota (weak in photoautotrophs, tight in consumers), extension of Urabe's Threshold Elemental Ratio (TER) approach, elucidation of the rules governing differential nutrient recycling by consumers, and organization of materials that lay the basis for the Growth Rate Hypothesis (GRH), linking the C:N:P stoichiometry of an organism to its growth rate due to the P-rich signature of the ribosomal growth machinery. Since the appearance of Sterner and Elser (2002), these core concepts of the stoichiometric framework have been extended widely. This is manifested in citation statistics. For example, as of 10 July 2018, Sterner and Elser (2002) has been cited a total of 3,728 times, including 566 citations since 2017 (Google Scholar). In Google Scholar, a search on *Ecological Stoichiometry* returns more than 40,000 records, with citations increasing at a ∼8% rate annually during recent years.

Indeed, much has happened in the field of Ecological Stoichiometry in the past 15 or so years and an update is needed. The collection of papers in this Frontiers Research Topic is a cross-section of the diversity of applications of stoichiometric theory that have emerged. From this compilation we see how stoichiometric perspectives have been further applied in some areas (microbial processes and consumer-resource interactions), and extended in several novel directions to other areas, such as studies of host-pathogen interactions, of the role of pollen in trophic webs, and of fungi. While most stoichiometric work has focused on the core macroelements (C, N, and P), increasing attention is now being paid to other essential elements that play a role in organismal biology and ecosystem functioning. This broader view of the Periodic Table is seen in several papers in this collection. Importantly, in nearly all papers we see signs of the integrative thinking that stoichiometric theory catalyzes: the fact that chemical elements both make up the fundamental molecules of life and constitute one of the core foci of ecosystem ecology. This integration

OPEN ACCESS

Edited by:
George S. Bullerjahn, Bowling Green State University, United States

Reviewed by:
Steven Wilhelm, University of Tennessee, Knoxville, United States

****Correspondence:***
Dedmer B. Van de Waal
d.vandewaal@nioo.knaw.nl

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *17 July 2018*
Accepted: *02 August 2018*
Published: *05 September 2018*

Citation:
Van de Waal DB, Elser JJ, Martiny AC, Sterner RW and Cotner JB (2018) Editorial: Progress in Ecological Stoichiometry. Front. Microbiol. 9:1957. doi: 10.3389/fmicb.2018.01957

provides a means to create a seamless fabric across almost all of biology. We encourage readers to find their own common threads that connect these papers to their own research. Since pretty much everything that is a thing is, at its core, elemental, novel applications of the stoichiometric framework await.

MICROBES

A diverse set of papers are included in this Research Topic on microbial stoichiometry, spanning terrestrial to aquatic systems and including the human microbiome. Manzoni used a theoretical approach to follow C and N dynamics during litter degradation. Carbon use efficiency is flexible and increased under N depletion while decreased when N was replete. These findings suggest that decomposer community traits depend on litter stoichiometry and shift from N to C-limited conditions during decomposition. Zhang and Elser used a meta-analysis to examine the stoichiometry of fungi, a little-studied but incredibly diverse group. An important observation was that C content varied widely while N:P ratios were similar to the Redfield ratio. However, latitude and temperature affected N:P ratios while temperature also affected C:P ratios. In a study looking at microbial diversity in Cuatro Cienegas (Mexico), Lee et al. found that adding nutrients to this ultra-oligotrophic system disrupted community structure and promoted the growth of rare phototrophic species, while enriching at different N:P ratios had an impact above and beyond fertilization *per se*. In a paper examining temperature and nutrient effects on heterotrophic bacteria stoichiometry, Phillips et al. found that nutrients had a strong effect on stoichiometry and, perhaps more importantly, that temperature effects on stoichiometry were greatest when nutrient levels were lowest. At the ecosystem scale, They et al. observed that microbial biomass and the stoichiometry of dissolved pools diverged from each other in lakes with the longest residence times, suggesting that microbial influence on ecosystem stoichiometry is maximized at long residence times. In another ecosystem, humans, Vecchio-Pagan et al. used protein sequences to infer differences in microbial stoichiometry in the human microbiome. They observed that the skin and nares had higher N and O content while the gut microbiome was high in S. In a similar vein, Dittberner et al. found that open ocean microbial communities optimized the protein N use as an adaptation to low nutrient availability. Lastly, Linzner et al. examined the evolution of stoichiometry in *Escherichia coli*. They found that adaptation to increased temperature influenced the cellular stoichiometry but was mediated by the specific evolutionary pathway.

Although this collection of studies of microbes is diverse in approaches and habitats, these studies emphasize the important roles of temperature and nutrients in influencing microbial stoichiometry at different scales. The use of "omics" information to infer stoichiometry of communities will be an important tool going forward.

PRIMARY PRODUCERS

The elemental composition of phytoplankton and other primary producers has been an area of intense research. Traditionally, the elemental stoichiometry of especially marine phytoplankton had been viewed as having limited variability (Redfield, 1958). Nonetheless, numerous physiological experiments have revealed strong stoichiometric responses to variations in light and nutrient availability (Geider and La Roche, 2002). However, several key questions are currently unanswered including the impact of producer diversity, the role of temperature as well as the interaction between temperature and nutrient limitation, and how changes in biochemistry is ultimately linked to overall cellular elemental composition (Moreno and Martiny, 2018). Several studies in this Research Topic aimed at addressing these three major unknowns.

Garcia et al. showed high variation in the elemental composition of marine eukaryotic phytoplankton, particularly within the group of Bacillariophyceae where variation was comparable to that among seven investigated classes. The authors tested different variables and showed that temperature accounted for a significant yet small portion of the variation. Yvon-Durocher et al. found that experimental as well as seasonal warming of freshwater ponds led to species sorting, nutrient drawdown, and increased C:P and N:P ratios of the community. Moreover, isolates of the green alga *Chlamydomonas* from the warm treatments exhibited higher C:P and N:P ratios, indicating that the direction and magnitude of stoichiometric shifts by local adaptation in response to warming were comparable to the overall shifts in seston stoichiometry. Velthuis et al. examined aquatic plants and showed, in support of theory, that nutrient addition consistently resulted in decreased carbon:nutrient ratios. However, elevated temperature did not change the elemental stoichiometry in a consistent manner. Villar-Argaiz et al. placed nutrient impacts within a broad context of multiple stressors including UV radiation, temperature, CO_2, and others. Their main conclusion was that cellular compositional responses to these various stressors was most often non-additive, stressing the importance of context in exploring how ecological systems change over time. Using an elegant microplate factorial design, Hessen et al. also found that nutrient limitation had a relatively stronger impact than temperature on the elemental stoichiometry. However, the experiment also demonstrated how the macromolecular composition changed in response to both environmental factors. Changes in biochemical composition were similarly examined in Grosse et al. Here, it was shown that the cellular concentrations of amino acids, fatty acids and carbohydrates were sensitive to light and types of nutrient limitation. Fernandes et al. show how to use phytoplankton to effectively remove N and P from wastewater and found that P was assimilated faster than N. Thus, the stoichiometric requirement and nutrient uptake mechanisms are important to consider when using phytoplankton for wastewater treatment. The above-described studies demonstrate how diversity, temperature, and the biochemical regulation of macromolecules are important for understanding the elemental stoichiometry of producers.

HERBIVORES

Studies on the plant-animal interface were central to the development of Ecological Stoichiometry and new insights continue to emerge. The plant-herbivore interaction is notable

for its varying degree of stoichiometric mismatch. Sometimes primary producers offer a nutritious, elementally balanced forage for the next trophic level, and sometimes they are far from doing so. Studies specifically looking at controls and dynamics of element content and potential mismatches include Moody et al., who examined body composition and growth rate of two neotropical stream grazers (a mayfly and a tadpole) with an eye toward looking for indications consistent with the Growth Rate Hypothesis. Focusing on the temperature differences among streams at different elevations, the authors observed that among the mayflies, highest body P as well as highest growth occurred in the warmest, lowland stream, but overall the study emphasized that the GRH likely does not apply for temperature-based differences in growth. Most considerations of stoichiometric dimensions of food quality take a temporal perspective appropriate to assessing generation-long effects of plant element content on animal growth. However, studies are beginning to consider how to incorporate changing food within an animal's lifetime. Wagner et al. shifted *Daphnia magna* grazers from high to low P-content food and vice versa, and then looked at how body composition (P, RNA, ATP), and organism mass changed over several days following the shift. *Daphnia* did not react instantly, but they responded measurably to changing food conditions after 12–36 h. Changes in body P were first observed, then changes in RNA followed by changes in mass. These results help us understand how organisms integrate over variable food conditions.

Work continues to address the fundamental question of how much the biochemical forms of elements affect ecological dynamics. At its purest level, Ecological Stoichiometry is concerned only with elements, not with the forms they are found in. We all recognize that not all carbon or phosphorus atoms are equal, that in biological material some molecules are reactive while others are recalcitrant, etc., but the question for stoichiometrists is how much this biochemical variation, layered on top of elemental variation, matters. Zhou et al. report the interesting findings that P recently (<90 min) incorporated by algae does not support growth as well as P that has been part of the algal cell for longer periods. Living beings are not bags of elements but we continue to ask how much we can understand with that simplified view. Two other contributions seek to understand how stoichiometric relationships revealed in one system carry over to other systems. Bracken reports on a geographic study with results that point to an importance of stoichiometric mismatch in rocky intertidal suspension feeders, and Sitters et al. point out that the vast literature on terrestrial herbivores has remarkably little to offer in terms of simultaneous analysis of N and P. There clearly is much yet to do to assemble relevant data sets to test stoichiometric theory in a wide diversity of habitats.

PATHOGENS

Pathogens such as viruses and fungal parasites require nutrients from their hosts, and alterations in host stoichiometry may thus affect disease dynamics. Lacroix et al. studied the impact of plant stoichiometry on the infection dynamics of two viruses, by exposing the plant to a range of N:P supply ratios. Although nutrient supply and plant stoichiometry did not alter the titer of one of the viruses, infection by the other virus reduced the total titer, indicating within host nutrient competition. Moreover, higher nutrient supply rates affected host traits and caused an increase in infection and coinfection rates. Also the infection dynamics of a cyanobacterial fungal parasite were shown to depend on N:P supply ratios, as shown by Frenken et al. Their findings demonstrate how parasite N:P ratios follow that of their host, and increased with N:P supply. Moreover, production of parasite zoospores were shown to increase with host N:P ratios. It thus seems that these fungal parasites have relatively high N, but low P requirements. Together, these studies demonstrate how shifts in primary producer stoichiometry may alter infection dynamics of their pathogens, with possible consequences for higher trophic levels.

CROSSING SCALES

Ecological Stoichiometry spans a range of organizational scales. Cherif et al. describe an operational framework where they identify the processes connecting stoichiometry across a wide range of biological levels in order to characterize the consequences of stoichiometric imbalances at one level for all other levels. The review specifically highlights advances, potential for further development, and integration of theories from the genome to the biosphere level, including processes from gene expression to atmospheric and oceanic circulation of elements, thereby connecting all biological levels. At the organism level, the balance of carbon (energy) and nutrients is affected by traits. Meunier et al. cluster these traits in four main groups including acquisition, body stoichiometry, storage, and excretion. Their review provides a general description on the stoichiometry of traits, both in autotrophs and heterotrophs. Moreover, they highlight the role of trade-offs in determining the dominance of distinct traits in response to shifts in resource supply loads and ratios. Traits define the interactions between one organism and another, and between organisms and their environment. In turn, the environment determines the ecological niche of organisms. González et al. provide a multidimensional elemental view on this ecological niche, thus focusing on elemental niches rather than trait based niches. They propose an approach visualizing the stoichiometry of individuals in multivariate space in order to quantify niche dimensions within and between organisms. Their analysis integrates stoichiometric niches occupied by terrestrial and freshwater food webs, trophic groups, individual species, and individuals with species. With complementarity tests, this method allows the assessment of unoccupied stoichiometric niche space.

Ecological Stoichiometry can also be applied for connecting ecology with biogeochemistry and ecosystem metabolism, which is highlighted by Welti et al. They describe how trophic interactions (and nutrient requirements) link biogeochemistry to food webs, how carbon: nutrient ratios (and nutrient limitation) link food webs to ecosystem metabolism, and how elemental

fluxes and transformation rates link ecosystem metabolism to biogeochemistry. Global fluxes of N and P have been perturbed over the last century, and Guignard et al. explore the impacts of these changes on the stoichiometry and genomic traits of organisms. Responses of organisms to changes in N and P loads and ratios may depend on their genome size, as larger genomes have higher N and P demands, yet genome size is an often overlooked trait. Importantly, the authors highlight that we are close to the planetary boundary of a safe operating space for P flowing into the ocean (Rockström et al., 2009), which calls for a more sustainable use of fertilizers. To this end, applying Ecological Stoichiometry to agricultural practices may enable the maintenance of agricultural productivity, while conserving biodiversity and thereby supporting the wide range of services that ecosystems provide across the world.

Organisms can transport elements between different environments. Luhring et al. developed a framework to describe the stoichiometry of elemental fluxes among ecosystems. Specifically, they describe how life histories of amphibians and salmon determine the relative fluxes of elements between freshwater-terrestrial and freshwater-marine ecosystems, respectively. Their work shows how fluxes may differ between elements and depend on life-history, leading to simultaneous imports and exports of different elements depending on ontogeny and the movement of organisms between systems. Cross-system transfer of elements not only occurs via animals, but also by plants. Indeed, Filipiak describes how temporal nutrient limitation by detritivores may be alleviated by pine pollen, in both terrestrial but also aquatic ecosystems. Compared to litter, pollen is relatively balanced in nitrogen and phosphorus and contains a range of additional valuable elements. Consequently, pollen rains may substantially add nutrients to ecosystems, particularly aquatic ones. Thus, pollen may play an important role in nutrient cycling both within and across ecosystems. The stoichiometry of decomposition may differ from that of primary production. Indeed, Evans-White and Halvorson describe how detritus based "brown" aquatic food webs differ from autotroph-based "green" food webs with respect to C quality and nutrient contents. In a meta-analysis, they show how N and P availabilities largely limit both detritivores and herbivores following general stoichiometric principles, but also show distinct differences in the mechanisms of limitation due to distinct consumer regulatory processes in both types of food webs. Together, these studies demonstrate how Ecological Stoichiometry can be used to connect a wide range of scales, from subcellular processes to ecosystem dynamics and services.

BEYOND C:N:P

Since the days of Redfield (1958), stoichiometric approaches have had a predominant focus on three elements: C, N, and P. This makes sense, as C is the architectural linchpin of biomass and a very useful proxy for tracking energy, while N and P are key constituents of vital biomolecules (protein and nucleic acids) and often limiting to biota of all kinds. However, more than 2–3 dozen elements are essential for living things and a number of these can also be limiting to different biota at various times and under various conditions. So, it would make sense to extend stoichiometric thinking to other essential elements in the Periodic Table. Such efforts are reflected here. Karl and Grabowski bring attention to a much-neglected element, hydrogen (H), perhaps the "plain brown wrapper" of the elements: ubiquitous in biota, an essential regulator of organic matter redox state, and, while frequently measured in CHN analyzers, generally ignored. Karl and Grabowski call for more accurate determinations of the H content of organic matter and argue that such data will yield better estimates of the energy state of organic matter and thus its impacts in ecosystems. Jeyasingh et al. move to broad swaths of the Periodic Table, arguing for an "ionomic" perspective, highlighting key tradeoffs that exist because of the coupling of elements in biological processes. Evidence is presented for characteristic and coupled shifts in sets of chemical elements. For example, Mg, Na, and K were seen to be associated with each other in experimental studies of numerous strains of freshwater bacterial heterotrophs grown under different conditions of P supply. Much work remains to illuminate these complex interactions and Jeyasingh et al. sketch out some promising paths forward.

Of course, elements are used to make molecules and the relative balance of major molecules (e.g., proteins, carbohydrates, lipids) and essential biochemical components (e.g., essential fatty acids) can also play an important role in determining the nutritional quality of food. Often similar analytical and conceptual frameworks can be used for analyzing such biochemical dimensions as are used in elemental stoichiometry studies. Anderson et al. take such an approach to detritus-based food webs in the ocean, analyzing "trophic upgrading" of C:PUFA (polyunsaturated fatty acids) ratios by bacteria and their protist consumers, pointing to a complex interplay between the abundance of food and its quality in supporting mesopelagic copepods.

OUTLOOK

The field of Ecological Stoichiometry has matured considerably in the 15 years or so since the publication of Sterner and Elser (2002), which is evident from the studies presented in this Research Topic. This collection of papers covers the application of Ecological Stoichiometry in a wide range of topics, from microbes, to primary producers, herbivores, pathogens and entire ecosystems, that are tied together by Ecological Stoichiometry alone, or coupled to existing ecological frameworks, including C, N, and P, as well as the remaining Periodic Table. Ecological Stoichiometry was even shown to have societal relevance by application in agriculture, wastewater treatment and assessing ecosystem services. Clearly, there is still a lot more work that needs to be done! The presented papers not only provide a tremendous infilling of facts and details, but also extend in new exciting areas.

Novel areas where we see that Ecological Stoichiometry can and most likely will contribute substantially in the future include, for instance, human health via food nutrition (Myers et al.,

2014), and the understanding of disease virulence or suppression in the human microbiome (Bäumler and Sperandio, 2016). In agriculture, better understanding of the coupling of nutrient cycles is and will be increasingly important for sustainable food production, with simultaneous benefits toward ecosystem services. Relatedly, it is worth mentioning that humans are conducting a huge stoichiometric experiment through our manipulation of the carbon cycle, with important implications to all living things (Loladze, 2002). Moreover, with an eye toward balancing production and recovery of elements in waste streams, we may more effectively close multiple elemental cycles. There are clearly many more areas where Ecological Stoichiometry will provide useful contributions, both in our basic understanding of ecosystems, as well as applications to societal issues. As the papers assembled in this collection demonstrate, Ecological Stoichiometry continues to provide a useful lens with which to study the world with nature's infinite complexity. Sterner and Elser's tome presented a huge scaffolding for understanding macromolecules, organisms, communities and ecosystems in light of Ecological Stoichiometry. The details of what lies within that framework are incredibly fascinating and will continue to be important, interesting and relevant for a long time.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Bäumler, A. J., and Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 535, 85–93. doi: 10.1038/nature18849

Geider, R. J., and La Roche, J. (2002). Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37, 1–17 doi: 10.1017/S0967026201003456

Loladze, I. (2002). Rising atmospheric CO_2 and human nutrition:toward globally imbalanced plant stoichiometry? *Trends Ecol. Evolu.* 17, 457–461 doi: 10.1016/S0169-5347(02)02587-9

Moreno, A. R., and Martiny, A. C. (2018). Ecological stoichiometry of Ocean Plankton. *Annu. Rev. Mar. Sci.* 10, 43–69 doi: 10.1146/annurev-marine-121916-063126

Myers, S. S., Zanobetti, A., Kloog, I., Huybers, P., Leakey, A. D. B., Usui, A. J., et al. (2014). Increasing CO_2 threatens human nutrition. *Nature* 510, 139–142. doi: 10.1038/nature13179

Redfield, A. C. (1958). The biological control of chemical factors in the environment. *Am. Sci.* 46, 205–221.

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F. S., III., Lambin, E., et al. (2009). Planetary boundaries:exploring the safe operating space for humanity. *Ecol. Soc.* 14:32. doi: 10.5751/ES-03180-140232

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 26 April 2017
doi: 10.3389/fmicb.2017.00661

Flexible Carbon-Use Efficiency across Litter Types and during Decomposition Partly Compensates Nutrient Imbalances—Results from Analytical Stoichiometric Models

Stefano Manzoni [1, 2]*

[1] Department of Physical Geography, Stockholm University, Stockholm, Sweden, [2] Bolin Centre for Climate Research, Stockholm University, Stockholm, Sweden

Mathematical models involving explicit representations of microbial processes have been developed to infer microbial community properties from laboratory and field measurements. While this approach has been used to estimate the kinetic constants related to microbial activity, it has not been fully exploited for inference of stoichiometric traits, such as carbon-use efficiency (CUE). Here, a hierarchy of analytically-solvable mass-balance models of litter carbon (C) and nitrogen (N) dynamics is developed, to infer decomposer CUE from measured C and N contents during litter decomposition. The models are solved in the phase space—expressing litter remaining N as a function of remaining C—rather than in time, thus focusing on the stoichiometric relations during decomposition rather than the kinetics of degradation. This approach leads to explicit formulas that depend on CUE and other microbial properties, which can then be treated as model parameters and retrieved via nonlinear regression. CUE is either assumed time-invariant or as a function of the fraction of remaining litter C as a substitute for time. In all models, CUE tends to increase with increasing litter N availability across a range of litter types. When temporal trends in CUE are considered, CUE increases during decomposition of N-poor litter cohorts, in which decomposers are initially N-limited, but decreases in N-rich litter possibly due to C-limitation. These patterns of flexible CUE that partly compensate stoichiometric imbalances are robust to moderate shifts in decomposer C:N ratio and hold across wide climatic gradients.

Keywords: carbon-use efficiency, C:N ratio, nitrogen mineralization, nitrogen immobilization, stoichiometric model

OPEN ACCESS

Edited by:
James Cotner,
University of Minnesota, USA

Reviewed by:
Zhanfei Liu,
University of Texas at Austin, USA
André Megali Amado,
Federal University of Rio Grande do Norte, Brazil

***Correspondence:**
Stefano Manzoni
stefano.manzoni@natgeo.su.se

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 December 2016*
Accepted: *31 March 2017*
Published: *26 April 2017*

Citation:
Manzoni S (2017) Flexible Carbon-Use Efficiency across Litter Types and during Decomposition Partly Compensates Nutrient Imbalances—Results from Analytical Stoichiometric Models. Front. Microbiol. 8:661. doi: 10.3389/fmicb.2017.00661

INTRODUCTION

Microbial decomposers play a key role in global carbon (C) and nutrient cycles by driving the degradation and mineralization of organic matter. C from decomposing compounds is partly used for growth of new cells and partly respired for energy production. The partitioning of C between these anabolic and catabolic processes has consequences on the rates of C accumulation in soils and sediments, because C allocated to microbial growth may remain in the system, whereas respired C is lost (Six et al., 2006; Sinsabaugh et al., 2013). The decomposer C-use efficiency (CUE), defined as the ratio of decomposer growth rate over the rate of organic matter uptake,

integrates these processes into a single parameter (Manzoni et al., 2012b; Geyer et al., 2016). High values of CUE characterize systems where C is effectively retained in new biomass, whereas low values are suggestive of a leaky system. Patterns in CUE thus become useful indicators of trends in potential C storage along environmental and stoichiometric gradients.

Empirically estimating CUE, however, is challenging because this parameter integrates processes occurring at different time scales and carried out by different fractions of the microbial community (Geyer et al., 2016). As a consequence, estimated CUE values are sensitive to the chosen experimental approach (e.g., length of the measurement procedure, addition of labile C), possibly masking its "true" variability caused by ecological and biogeochemical factors (e.g., abiotic factors such as temperature and moisture, community status and composition, nutrient availability). These empirical approaches are mainly based on the definition of CUE as ratio of biomass production over substrate consumption. Rates of microbial production can be measured either directly as change in microbial biomass or indirectly as difference between consumption and respiration. Similarly, consumption rates can be measured directly or indirectly as sum of production and respiration. In any case, the type and amount of C amendment, length of the experiment, and measurement uncertainties affect the estimated CUE values, potentially complicating the comparison and interpretation of results across experiments.

As an alternative and complement to these empirical approaches, CUE could be estimated by fitting analytical models where CUE appears as an explicit parameter to biogeochemical data (pools or fluxes). This approach is conceptually equivalent to inferring microbial traits with kinetic models (Panikov, 1995; Pansu et al., 2004; Manzoni et al., 2012a). Using this rationale, CUE has been estimated along gradients in temperature (Wetterstedt and Ågren, 2011), inorganic nutrient availability (Ågren et al., 2001), and litter elemental composition (Nicolardot et al., 2001; Manzoni et al., 2008a, 2010). Compared to the empirical approaches, these methods exploit coarser data that might be less prone to uncertainties, such as total soil or litter C and nutrient content. The most obvious disadvantage is that they all rely on the simplified mathematical representation of our conceptual understanding of microbial functioning. The main purpose of this contribution is to further develop these analytical methods to reduce their level of simplification and thus offer more realistic estimates of CUE and its trends during decomposition.

These biogeochemical models are based on mass balance equations (as described in Section Theory), which are solved analytically to predict C or nutrient changes through time during decomposition of a cohort of litter. To allow comparison across incubation environmental conditions, the analytical solutions can be expressed in terms of nutrient content as a function of C content instead of time (Ågren and Bosatta, 1987; Manzoni et al., 2008a). This representation removes environmental effects (e.g., temperature and moisture) and compartment size effects (decomposition is faster when there is more C available) that alter the kinetics of decomposition, and focuses on the stoichiometric relations among elements using C content as a proxy of time. Even after removing temporal effects, these models should still account for changes in decomposer community traits as decomposition progresses, but as of now, they all assume time-invariant traits. For example, CUE is expected to vary during decomposition because it is sensitive to changes in nutrient availability and C quality (Cotrufo et al., 2013; Frey et al., 2013; Sinsabaugh et al., 2013). Other traits such as microbial biomass elemental composition might also shift, partly tracking trends in substrate nutrient concentration, thereby reducing stoichiometric imbalances. The microbial P:C ratio is particularly flexible in aquatic bacterial communities grown along wide gradients of P availability (Cotner et al., 2010; Godwin and Cotner, 2015). Also the microbial N:C ratio can increase with widening organic matter N:C in both aquatic and terrestrial systems (Tezuka, 1990; Wagener and Schimel, 1998), but in general decomposer communities can be regarded as nearly homeostatic with respect to N (Cotner et al., 2010; Fanin et al., 2013; Xu et al., 2013).

In this contribution, trends in CUE and other decomposer traits are taken into account in a new set of analytical stoichiometric models. Different from previous approaches that assumed time-invariant CUE (Manzoni et al., 2010; Wetterstedt and Ågren, 2011; Ågren et al., 2013), the aim here is to quantify trends in CUE during decomposition of litter cohorts, and identify the stoichiometric and environmental drivers of such trends. Moreover, results assuming weakly homeostatic decomposer communities are compared to those assuming strict homeostasis, thus providing a complete picture of trait variability effects on decomposition patterns. The analytical models are employed to estimate decomposer traits via nonlinear fitting to litterbag C and N content data. Finally, results are compared to observations and previous CUE estimates using the simpler model with time-invariant traits, and litter quality and climatic conditions are considered as potential drivers of the observed trends.

METHODS

Estimating CUE from decomposition data requires a mathematical model that explicitly includes CUE as a fitting parameter. In Section Theory, a minimal process-model of C and N dynamics during decomposition is presented, and analytical equations suitable for CUE estimation are derived considering: (i) time-invariant decomposer traits (model I, Section Time-Invariant Decomposer Traits—Model I), (ii) flexible CUE and time-invariant decomposer elemental composition (model II, Section Variable Decomposer Traits—Models II and III), and (iii) flexible CUE and decomposer elemental composition (model III, Section Variable Decomposer Traits—Models II and III). The fitting procedure and the datasets used are described in Section Data Analysis and Model Parameterization. Symbols are defined in **Table 1**.

Theory

A cohort of organic matter constitutes the modeled system and is followed during decomposition between an initial state (indicated by subscripts "0") to a generic time t. The system is

TABLE 1 | Definition of symbols and units.

Symbol	Description	Units
a, b	Fitting parameters (**Table 3**)	Multiple
C, C_0	Litter C content, initial litter C content	gC
$(C:N)_0$	Initial litter C:N ratio	gC gN^{-1}
$(C:N)_B$	Decomposer biomass C:N ratio	gC gN^{-1}
D	Decomposition rate	gC y^{-1}
e	C-use efficiency (growth rate over uptake rate)	–
e_0	Initial C-use efficiency (at $x = 1$; Equation 9)	–
N, N_0	Litter N content, initial litter N content	gN
p, q	Auxiliary functions (Equation 5)	–
r_0	Initial litter N:C ratio ($r_0 = (C:N)_0^{-1}$)	gN gC^{-1}
r_B	Decomposer biomass N:C ratio ($r_B = (C:N)_B^{-1}$)	gN gC^{-1}
$r_{B,0}$	Initial decomposer N:C ratio (at $x = 1$; Equation 10)	gN gC^{-1}
t	Time	y
u	Dummy variable of integration (Equation 5)	–
x	Fraction of remaining C, $x = C/C_0$	–
y	Fraction of remaining N, $y = N/N_0$	–
α	Coefficient for preferential N uptake	–
β	Slope of $r_B(x)$ relation (Equation 10)	gN gC^{-1}
ε	Slope of $e(x)$ relation (Equation 9)	–
ν	Parameter group, $\nu = \alpha/(1 - e_0 + \varepsilon)$ (Equations 11, 12)	–
ξ	Parameter group, $\xi = \varepsilon/(1 - e_0 + \varepsilon)$ (Equations 11, 12)	–

assumed to be open to exchanges of mineralized products, but closed to new inputs of organic matter. As described in Section Data Analysis and Model Parameterization, the first phase of decomposition, dominated by leaching of soluble organic C and nutrients is removed from the data sets, so that leaching can be neglected in the model as well. Even though this mathematical approach can be applied with some modifications to any nutrient associated to organic matter, here the focus is on nitrogen. Mass balance equations can then be written to describe the temporal evolution of total organic carbon $[C(t)]$ and nitrogen $[N(t)]$ in the organic matter cohort, for a given set of initial conditions and decomposer traits (Manzoni et al., 2010),

$$\frac{dC(t)}{dt} = -D\left[1 - e\left(\frac{C}{C_0}\right)\right], \tag{1}$$

$$\frac{dN(t)}{dt} = -D\left[\alpha\frac{N}{C} - e\left(\frac{C}{C_0}\right) r_B\left(\frac{C}{C_0}\right)\right], \tag{2}$$

where D is the decomposition rate in C units, e is the decomposer community carbon-use efficiency, r_B is the N:C ratio of the decomposer biomass, and α is a coefficient taking into account the chemical heterogeneity of the substrate ($\alpha > 1$ indicates preferential assimilation of compounds richer in N than the bulk organic matter). The two parameters representing microbial traits (e and r_B) are written as generic functions of the degree of decomposition, expressed in terms of the fraction of remaining C instead of time (C/C_0, where subscript "0" indicates the initial mass of C). It is important to emphasize that as time progresses, C/C_0 decreases from 1 to 0; that is, time and C/C_0 change in opposite directions during decomposition. Linking traits to C/C_0 allows comparing litter decomposition across climatic gradients that affect the degradation rates, but not the underlying relations between microbial community and litter amounts and quality.

With the above assumptions, the only loss in the C balance (Equation 1) is due to decomposer respiration, modeled as the product of the decomposition rate and the fraction of assimilated C not used for growth (i.e., $1 - e$). The N balance (Equation 2) accounts for the net exchanges of N between decomposers and the inorganic N pool, given by the difference between N supply from organic matter (i.e., $D\alpha\frac{N}{C}$) and the decomposer stoichiometric demand (Der_B); the minus sign indicates that N in excess of demand is released (so that $\frac{dN(t)}{dt} < 0$), whereas in case of N shortage, inorganic N is immobilized ($\frac{dN(t)}{dt} > 0$). Similar stoichiometric arguments have already been presented in the context of decomposition in both terrestrial and aquatic systems (Bosatta and Berendse, 1984; Goldman et al., 1987). These equations have the same form as in previous contributions (Manzoni et al., 2010), except for the explicit dependence of decomposer traits (e and r_B) on remaining C (which introduces nonlinearities in the system of equations) and a slight notation simplification (N:C ratios are used instead of C:N ratios, allowing more compact equations).

It is important to emphasize that Equation (2) is exact only when r_B is time-invariant, but it is an accurate approximation of the exact mass balance as long as $\left|\frac{dr_B}{dt}C_B\right| \ll |r_B D|$. Here, a dependence of r_B on the decomposition state (and thus time) will also be considered. However, at the annual time scale at which the model is interpreted, these changes in r_B are small compared to the decomposition rate D, supporting the use of Equation (2) in the following derivations. Moreover, possible climatic effects on r_B are neglected, assuming that inter-annual variations in temperature and water availability have small effect on the mean annual r_B values.

Dividing Equation (2) by Equation (1), a single equation linking organic matter N to organic matter C (instead of t) is readily found,

$$\frac{dN}{dC} = \frac{\alpha\frac{N}{C} - \mathrm{e}\left(\frac{C}{C_0}\right) r_B\left(\frac{C}{C_0}\right)}{1 - e\left(\frac{C}{C_0}\right)}. \tag{3}$$

Defining for convenience the fraction of remaining carbon, $x = C/C_0$, the fraction of remaining nitrogen, $y = N/N_0$, and the initial litter N:C ratio, $r_0 = N_0/C_0$, Equation (3) becomes,

$$\frac{dy}{dx} = \frac{\alpha y r_0 - x e(x) r_B(x)}{x r_0 [1 - e(x)]}. \tag{4}$$

Equation (4) is a linear in y, non-autonomous ordinary differential equation that can be solved for the initial condition $y(x = 1) = 1$ using the integrating factor method (Boyce and DiPrima, 2009),

$$\begin{aligned} y(x) &= e^{-p(x)} \int e^{p(x)} q(u)\, du, \\ p(x) &= -\int \frac{\alpha}{x[1 - e(x)]} dx, \\ q(x) &= -\frac{e(x) r_B(x)}{r_0 [1 - e(x)]}, \end{aligned} \tag{5}$$

TABLE 2 | Relations between C-use efficiency and fraction of remaining C, $e(x)$, for different assumptions on microbial biomass N:C ratio, $r_B(x)$, and references to the corresponding N release curves, $y(x)$.

Model	$e(x)$	$r_B(x)$	$y(x)$	% with highest R^2	% with lowest AIC
I	e = constant	r_B = constant	Equation 6	0	56.1
II	$e_0 - \varepsilon(1-x)$	r_B = constant	Equation 12	70.7	24.4
III	$e_0 - \varepsilon(1-x)$	$r_{B,0} - \beta(1-x)$	Equation 11	29.3	19.5

The right columns indicate the percentages of datasets in which each model had the highest fraction of explained variance, R^2, or the lowest Akaike information criterion score (AIC).

where u is a dummy variable of integration. The analytical solution of Equation (5) requires specific assumptions on the form of $e(x)$ and $r_B(x)$. In the following, the simplest case of time-invariant decomposer traits is considered first, as it allows for an illustrative derivation (Section Time-Invariant Decomposer Traits—Model I). Other cases are discussed in Section Variable Decomposer Traits—Models II and III, and all models are summarized in **Table 2**. The effects of different choices of $e(x)$ on the temporal trajectories of litter C are illustrated in Figure S1.

Time-Invariant Decomposer Traits—Model I

In the simplest case, CUE and decomposer N:C ratio are assumed to be time-invariant (i.e., $e(x) = e$ and $r_B(x) = r_B$; referred to as model I), although both traits could vary across litter types. This assumption has been the basis of previous derivations of analytical nutrient release curves (Bosatta and Staaf, 1982; Bosatta and Ågren, 1985; Manzoni et al., 2008a, 2010; Ågren et al., 2013). With these assumptions, $p(x) = -\alpha \ln(x)/(1-e)$, and after accounting for the initial condition $y(x=1) = 1$, the fraction of remaining nitrogen is found as (model I),

$$y(x) = x\frac{e}{\alpha - 1 + e}\frac{r_B}{r_0} + \left(1 - \frac{e}{\alpha - 1 + e}\frac{r_B}{r_0}\right)x^{\frac{\alpha}{1-e}}, \qquad (6)$$

recovering the equation derived by Manzoni et al. (2010). When $\alpha = 1$ (bulk litter N:C is representative of the N:C of microbial substrates), the earlier results cited above are also recovered,

$$y(x) = x\frac{r_B}{r_0} + \left(1 - \frac{r_B}{r_0}\right)x^{\frac{1}{1-e}}. \qquad (7)$$

Because $0 < e < 1$, the exponent of the second term in Equations (6, 7) is larger than one, so that the whole second term vanishes faster than the first toward the end of the decomposition process (i.e., in the limit for $x \to 0$). As a result, the asymptotic litter N:C ratio is given by,

$$\frac{N}{C}\underset{x\to 0}{\to}\frac{e}{\alpha - 1 + e}r_B \approx r_B, \qquad (8)$$

where the last approximation holds as long as $\alpha \approx 1$. Equation (8) implies that litter N:C ratio converges toward the microbial biomass N:C ratio as microbial turnover products become an increasingly larger litter fraction. When N is preferentially used, however, $\alpha > 1$ and hence litter N:C remains lower than the microbial biomass N:C.

Variable Decomposer Traits—Models II and III

For generic $e(x)$ and $r_B(x)$ relations, the analytical integration of Equation (5) becomes unfeasible. However, solutions can be found for various functional relations between microbial traits and the fraction of remaining C. For simplicity, linear functions are assumed here,

$$e(x) = e_0 - \varepsilon(1-x), \qquad (9)$$

$$r_B(x) = r_{B,0} - \beta(1-x), \qquad (10)$$

where e_0 and $r_{B,0}$ are the initial CUE and microbial biomass N:C ratio (at $x = 1$), and ε and β are the slopes of the linear relations. The signs of the slopes are not imposed a priori, so ε and β could be either positive (e and r_B decrease during decomposition, as x decreases) or negative (e and r_B increase) depending on the data. The parameterization assuming $\beta = 0$ (i.e., time-invariant r_B but flexible e) is referred to as model II, while that accounting also for changes in r_B is referred to as model III (**Table 2**).

With these linear relations and using Equation (5), the fraction of remaining nitrogen is obtained as (model III),

$$y(x) = x^{\nu}\left(\frac{1-e_0}{1-e_0+\varepsilon(1-x)}\right)^{\nu}\left\{1 + \frac{\varepsilon^{\nu-2}}{2r_0(1-e_0)^{\nu}}\left[\left((1-e_0+\varepsilon)(\alpha-2)\beta + \left(2\left(r_{B,0}-\beta\right)+\alpha\beta\right)\varepsilon\right)\mathrm{B}_{\xi}(1-\nu,\nu)\right.\right.$$
$$-\left((1-e_0+\varepsilon)(\alpha-2)\beta + \left(2\left(r_{B,0}-\beta\right)+\alpha\beta x\right)\varepsilon\right)\mathrm{B}_{x\xi}(1-\nu,\nu)$$
$$-(1-e_0+\varepsilon)\left(((\alpha-2)\beta + \left(2\left(r_{B,0}-\beta\right)+\beta\right)\varepsilon\right)\mathrm{B}_{\xi}(1-\nu,1+\nu)$$
$$\left.\left.-\left((\alpha-2)\beta + \left(2\left(r_{B,0}-\beta\right)+\beta x\right)\varepsilon\right)\mathrm{B}_{x\xi}(1-\nu,1+\nu)\right)\right]\right\}, \qquad (11)$$

where $\nu = \frac{\alpha}{1-e_0+\varepsilon}$, $\xi = \frac{\varepsilon}{1-e_0+\varepsilon}$, and $\mathrm{B}_z(a,b)$ is the incomplete Beta function of the variable z, with parameters a and b (Weisstein, 2016).

When the microbial biomass N:C ratio is set to a time-invariant value (i.e., $\beta = 0$), Equation (11) simplifies to (model II),

$$y(x) = x^{\nu}\left(\frac{1-e_0}{1-e_0+\varepsilon(1-x)}\right)^{\nu}$$
$$\left\{1 + \frac{r_{B,0}}{r_0}\frac{\varepsilon^{\nu-1}}{(1-e_0)^{\nu}}\left[\mathrm{B}_{\xi}(1-\nu,\nu)\right.\right.$$
$$-\mathrm{B}_{x\xi}(1-\nu,\nu) - (1-e_0+\varepsilon)$$
$$\left.\left.\left(\mathrm{B}_{\xi}(1-\nu,1+\nu) - \mathrm{B}_{x\xi}(1-\nu,1+\nu)\right)\right]\right\}. \qquad (12)$$

Finally, when also the microbial CUE is time-invariant (i.e., $\varepsilon = 0$), Equation (6) is recovered. Equations (6, 7, 11, and 12) thus represent a hierarchy of models of increasing complexity. In the

following, only the latter three equations will be considered, as α is in general higher than one.

Data Analysis and Model Parameterization

Litterbag decomposition datasets reporting C and N mass decay over time were collected from the literature and online databases. For each litter type and field incubation site, data were further screened to select datasets representative of the whole decomposition process, and ensure a meaningful fitting of the theoretical $y(x)$ curves. To this aim, datasets in which the fractions of remaining C and N reached values lower than 0.2 and 0.4, respectively, were selected. Moreover, to avoid the risk of overfitting, only datasets with more than eight measurement points were retained. These criteria are more restrictive compared to those adopted in previous studies—a choice justified by the higher flexibility of the $y(x)$ curves derived here. This selection resulted in 41 datasets including litter from angiosperm grasses, angiosperm trees, and conifer trees, with initial C:N ratios ranging from 15 to 125; i.e., r_0 ranging from 0.008 to 0.067 (Table S1). Litter samples were incubated in field conditions in a range of ecosystems, with mean annual temperature (MAT) ranging from 6 to 28°C, and mean annual precipitation from 300 to 4,000 mm/year (top right panel of Figure S2).

Because the model presented in Section Theory focuses on biological processes and neglects leaching of organic C and N, the initial leaching phase was removed from each dataset (as in Manzoni et al., 2010). Measurement points during the initial leaching phase were identified as those with larger N losses than C losses and simply removed. The initial C and N amounts were updated accordingly. Based on this criterion, significant initial leaching was detected in three out of 41 datasets. It should be emphasized that the stoichiometric model presented here is meant to assess decomposer traits, not predict the rates of litter mass loss, for which a detailed accounting of leaching would be necessary. A single data point characterized by an unrealistically high N concentration (N:C $>$ 0.2) was also excluded, as deemed contaminated.

Litter C and N amounts were normalized by their initial condition, to obtain fractions of remaining C and N to be used for fitting the $y(x)$ curves. The same normalization was conducted for datasets in which the initial leaching phase had been removed, by recalculating the fractions of remaining C and N based on the litter C and N contents after the end of the leaching phase. The nonlinear least square fitting was conducted in Mathematica environment, using the function NonlinearModelFit (Wolfram Mathematica version 10.0.0.0). Only parameter e was obtained by fitting $y(x)$ curves for model I (Equation 6), whereas both e_0 and ε were obtained by fitting $y(x)$ curves for models II and III (Equations 11, 12). Values of e_0 and ε were further constrained so that $0 < e < 1$. Goodness of fit was evaluated by the coefficient of determination and by the finite sample-corrected Akaike information criterion (AIC) scores, which account for both the goodness of fit and the number of fitting parameters (Burnham and Anderson, 2002). The best fitting model has the highest coefficient of determination, while the model that best balances performance and simplicity has the lowest AIC score.

Three parameters were not obtained via nonlinear fitting: the coefficient representing preferential N uptake, α, and the parameters of the $r_B(x)$ relation, β and $r_{B,0}$. The former parameter was set to $\alpha = 1.25$, based on the observation that the long-term litter N:C ratio is smaller than the microbial biomass N:C, thus requiring $\alpha > 1$ [Section Time-Invariant Decomposer Traits—Model I; see details in Manzoni et al. (2010)]. The parameters of the $r_B(x)$ relation were estimated from measured microbial biomass N:C ratios in decomposing litter. In models I and II, $\beta = 0$ and r_B was assumed equal to the long-term average litter microbial N:C ratio of 0.1 [i.e., $(C:N)_B \cong 10$]. In model III, r_B was assumed to change linearly from the minimum value $r_{B,0} = 0.083$ at $x = 1$ (i.e., $(C:N)_B = 12$ at the beginning of decomposition) to the maximum value $r_B = 0.125$ at $x = 0$ (i.e., $(C:N)_B = 8$ at the end of decomposition). With these values at the beginning and at the end of the decomposition process, the slope is found as $\beta = -0.04$. This trend implies an increasing microbial biomass N:C as decomposition progresses and litter N:C ratio increases, as suggested by data (Wagener and Schimel, 1998; van Meeteren et al., 2008; Brandstäetter et al., 2013; Toberman et al., 2014). Moreover, with this parameterization of model III, the value of r_B at $x = 0.5$ is consistent with the long-term average $r_B = 0.1$ assumed for models I and II.

To assess if the obtained C-use efficiency values are reasonable (although without a rigorous validation), literature data were collected on fungal decomposer C-use efficiency estimates from field and laboratory studies. Four studies specifically investigating litter decomposition in either terrestrial or aquatic systems were found (Frankland et al., 1978; Kominkova et al., 2000; Boberg et al., 2014; Lashermes et al., 2016); the data retrieved from these studies are reported in Table S2.

RESULTS

The role of decomposer traits and initial litter N availability on the N release curves is illustrated first (Section Effects of Decomposer Traits on the N Release Curves). Second, C-use efficiency and related parameters are estimated for the selected litter decomposition datasets (Section C-Use Efficiency Estimates from N Release Data). Finally, patterns in the estimated traits as a function of stoichiometric and climatic factors are assessed and results from the different C-use efficiency models are compared (Sections Relation between C-Use Efficiency and Litter Stoichiometry and Relation between C-Use Efficiency and Climate). In most analyses, N:C ratios are reported, except when linking C-use efficiency estimates and initial litter stoichiometry, where C:N ratios are used to facilitate comparison with earlier works.

Effects of Decomposer Traits on the N Release Curves

The sensitivity of N release curves to various assumptions on C-use efficiency and microbial biomass C:N ratios is illustrated

in **Figure 1**, starting with the assumption that microbial traits are time-invariant in **Figures 1A,B**, and then allowing for C-use efficiency and microbial N:C flexibility in **Figures 1C,D**. In general, lower initial litter N:C ratios require larger N immobilization rates to sustain a homeostatic microbial biomass, resulting in a net increment of N amounts in the litter bags (fraction of remaining N, $y > 1$; **Figure 1A**). For a given initial litter N:C ratio, microbial stoichiometric traits also affect the shape of the N release curves (**Figures 1B–D**). Increasing C-use efficiency allows growing more biomass per unit C taken up, but for a given microbial biomass N:C ratio, this translates into higher N requirements and more intense N immobilization (solid vs. dashed curves in **Figure 1B**). A lower microbial biomass N:C ratio allows growth with lower N supply, because less N is required per unit of C taken up. Hence, N release curves resulting from decomposition by microbial communities with low biomass N:C ratios exhibit a shorter N immobilization phase compared to those resulting from communities with high N:C (gray vs. black curves in **Figure 1B**).

When the assumptions of time-invariant C-use efficiency and microbial N:C ratio are relaxed, the N release curves do not change their general qualitative behavior, but some quantitative changes emerge (**Figures 1C,D**). When C-use efficiency increases during decomposition ($\varepsilon < 0$), the N demand in the early decomposition phase is reduced due to relatively low CUE, compared to the case of time-invariant CUE (dotted vs. solid curve in **Figure 1D**). In contrast, when C-use efficiency decreases during decomposition ($\varepsilon > 0$), the pattern is reversed, and initial N demand is enhanced, resulting in high initial N immobilization (dashed vs. solid curve in **Figure 1D**). Trends in microbial biomass N:C ratios are compounded with trends in CUE, resulting in less intense N immobilization when microbial N:C is lower in the early decomposition phase (gray vs. black curves in **Figure 1D**).

C-Use Efficiency Estimates from N Release Data

As suggested by **Figure 1**, the N release curves based on variable CUE can exhibit a wider range of shapes compared to curves based on time-invariant CUE. This flexibility is required to capture subtle or hidden patterns in the data, as shown in **Figure 2**. When time-invariant traits are assumed, the best fit $y(x)$ curve, despite capturing the main trend, overestimates the fraction of remaining N in the early phase of decomposition, while underestimating it in the later phase (black dashed curve). When considering variable CUE (with time-invariant or variable microbial biomass N:C), the model fitting yields $\varepsilon < 0$ (**Figure 2B**), suggesting that CUE was lower in the early phase than predicted by model I. In turn, lower CUE results in relatively lower N requirements and thus flatter $y(x)$ (in **Figure 2A**, compare the solid black and dot-dashed gray curves based on

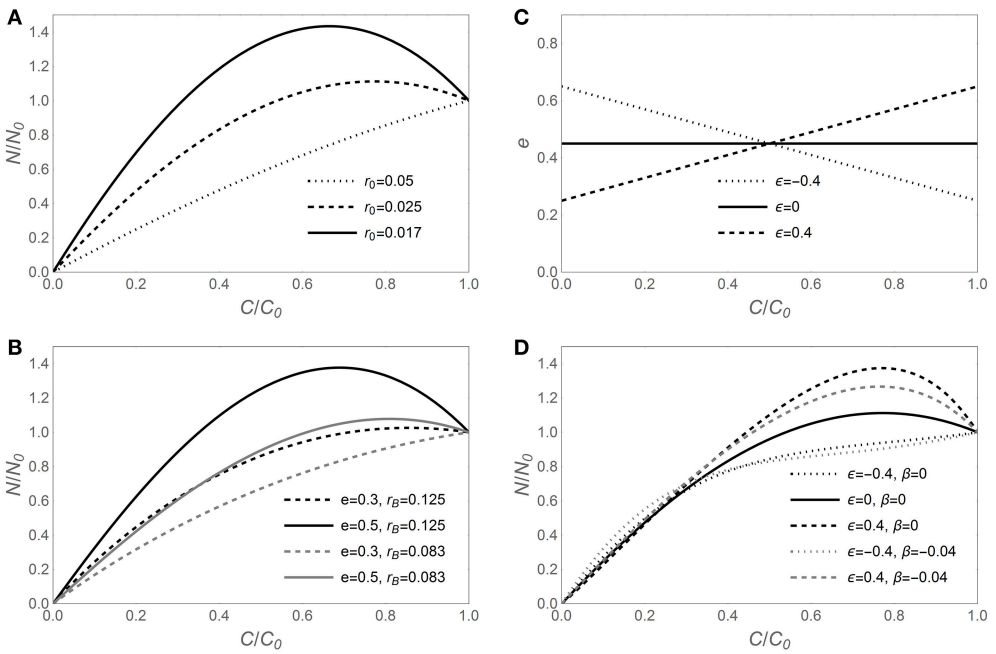

FIGURE 1 | Effect of contrasting C-use efficiency parameterizations on modeled nitrogen release from decomposing litter, expressed by the fraction of remaining nitrogen ($y = N/N_0$) as a function of the fraction of remaining carbon ($x = C/C_0$). Left panels show the predicted $y(x)$ curves for time-invariant decomposer traits (model I, Equation 6) when varying **(A)** litter N:C ratio (r_0), or **(B)** C-use efficiency (e) and decomposer biomass N:C ratio (r_B). **(C)** Assumed $e(c)$ relations (Equation 9) and **(D)** corresponding $y(x)$ curves when r_B is either time-invariant [$\beta = 0$ in Equation (10); $y(x)$ from Equation (12); model II] or decreasing during decomposition [$\beta = -0.04$ in Equation (12); $y(x)$ from Equation (11); model III]. The same dashing styles are applied in **(C,D)** for the same C-use efficiency model. Unless otherwise specified in the legends of each panel, $e = 0.45$, $r_B = 0.1$ [$(C:N)_B = 10$], and $r_0 = 0.025$ [$(C:N)_0 = 40$]; in all panels, $\alpha = 1.25$. Note that the temporal development in these graphs is from right ($C/C_0 = 1$) to left ($C/C_0 = 0$).

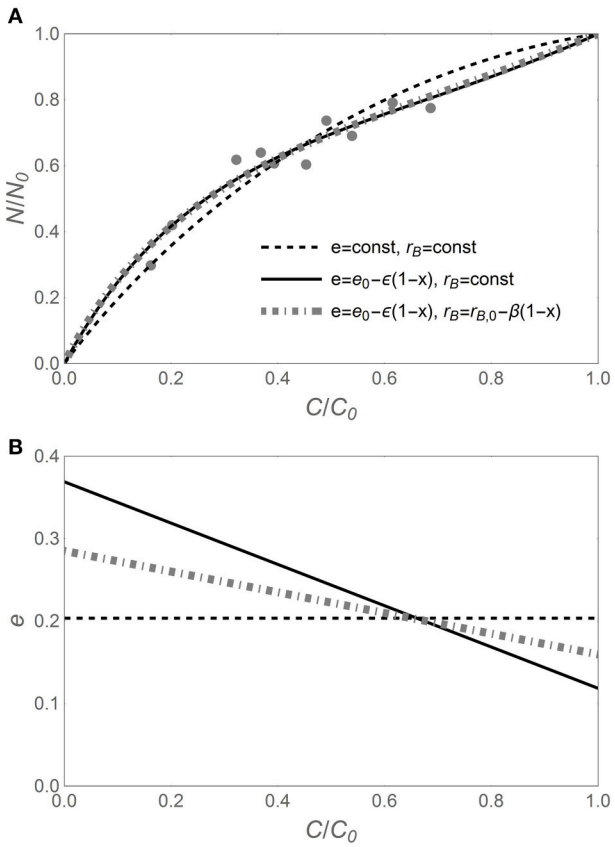

FIGURE 2 | (A) Example of nitrogen release trajectory during litter decomposition, expressed as fraction of remaining nitrogen ($y = N/N_0$) vs. fraction of remaining carbon ($x = C/C_0$). Light gray symbols represent observations (*Macaranga kingii* data from Hirobe et al., 2004), and curves refer to analytical $y(x)$ relations based on different assumptions on the decomposer traits (see legend). **(B)** Predicted relations between C-use efficiency (e) and x for the different $y(x)$ relations. C-use efficiency parameters are obtained via nonlinear least square fitting of the data in A [e in Equation (6), and e_0 and ε in Equations (11, 12)]; other parameters: $\alpha = 1.25$, $r_{B,0} = 0.083$ [$(C:N)_B = 12$], and $\beta = -0.04$. As in **Figure 1**, the temporal development is from right ($C/C_0 = 1$) to left ($C/C_0 = 0$).

models II and III to the dashed curve of model I). Comparing models II and III, it is evident that trends in microbial N:C ratio partly compensate for trends in CUE, resulting in less negative ε (**Figure 2B**). In other cases—primarily with N-rich litter—CUE is found to decrease during decomposition ($\varepsilon > 0$), also indicating that the slope parameter ε can be important. Thus, the $y(x)$ curves based on flexible traits can be more accurate, at the expense of an additional fitting parameter.

When fitting the three N release curves to all datasets, some general patterns begin to emerge (**Figure 3**; all regression results are reported in Table S1). First, all models perform well, with coefficients of determination higher than 0.98 for 75% or more of the datasets (left panels in **Figure 3**). As expected by the higher number of parameters, models II and III performed marginally better than model I (**Table 2**). Model II performs better than model III in 70% of the datasets, but differences between the N release curves obtained from the two models are small (as exemplified by **Figure 2A**). When evaluating model performance by means of AIC scores, model I emerges as the best model in 56% of the cases, followed by model II and III (**Table 2**). Second, the predicted slopes ε vary between -0.5 and 0.5 when using model II, and between -0.3 and 0.8 when using model III (consistent with the compensation effect of flexible microbial N:C), resulting in a range of $e(x)$ curves (**Figures 3D,F**). In general, the slopes ε tend to be negative in most litter types, except for N-rich litter (light-colored lines).

Relation between C-Use Efficiency and Litter Stoichiometry

Patterns in time-invariant e (model I) and $e(x)$ parameters (models II and III) as a function of litter stoichiometry are illustrated in **Figure 4**. To ease comparisons with previous results, litter C:N ratios are used to characterize litter stoichiometry, instead of N:C ratios. In model I, the estimated e strongly decreases with initial litter C:N, $(C:N)_0$ (**Figure 4A**), consistent with observations (open square symbols). When using models II and III, both the initial CUE (e_0; **Figures 4C,F**) and the slopes (ε; **Figures 4D,G**) decrease with increasing $(C:N)_0$ (regression parameters and statistics are reported in **Table 3**). The initial C-use efficiency for models II and III follows the same pattern as the time-invariant C-use efficiency estimated from model I, as also confirmed by high correlation coefficients between the values of e_0 and those of the time-invariant e (**Figure 5**; correlation coefficients = 0.84 and 0.90, respectively). The three observed initial C-use efficiency values (open square symbols in **Figures 4C,F**) follow the decreasing trend of the estimated e_0, but tend to be higher in N-poor litter.

The slopes of the $e(x)$ relations are predominantly positive in N-rich litter and negative in N-poor litter (**Figures 4D,G**), suggesting that in N-rich litter CUE decreases as decomposition progresses, whereas CUE increases in N-poor litter types. These increasing trends of ε with increasing initial litter N concentration are statistically significant for both models, as indicated by $a < 0$ in the $\varepsilon\left[(C:N)_0\right]$ regression models reported in **Table 3**. The C:N ratio at the transition between positive and negative slopes is given by parameter b in the $\varepsilon\left[(C:N)_0\right]$ relations. The C:N ratios at the transition are estimated as 42 and 66 for models II and III, respectively, and both values are significantly higher than zero, confirming that there is a statistically significant shift from increasing to decreasing CUE along a gradient of litter N availability.

Finally, **Figures 4B,E,H** show the trajectories of CUE and litter C:N as emerging from the modeled $y(x)$ curves. To draw these curves, C:N ratios are calculated as $x\ (C:N)_0/y(x)$ (these are the actual variable C:N ratios, not the constant initial value), and CUE trends as $e(x)$. Thus, each curve describes trajectories of CUE and C:N as the fraction of remaining C decreases from 1 to 0. While in **Figure 4B** CUE remains stable due to the underlying assumption of model I, in **Figures 4E,H** trajectories are more complex. The dark and orange curves referring to litter with initial C:N ratio above ~50, exhibit increasing CUE as litter C:N decreases (N:C increases) during decomposition. In contrast, light colored, "C-shaped" curves

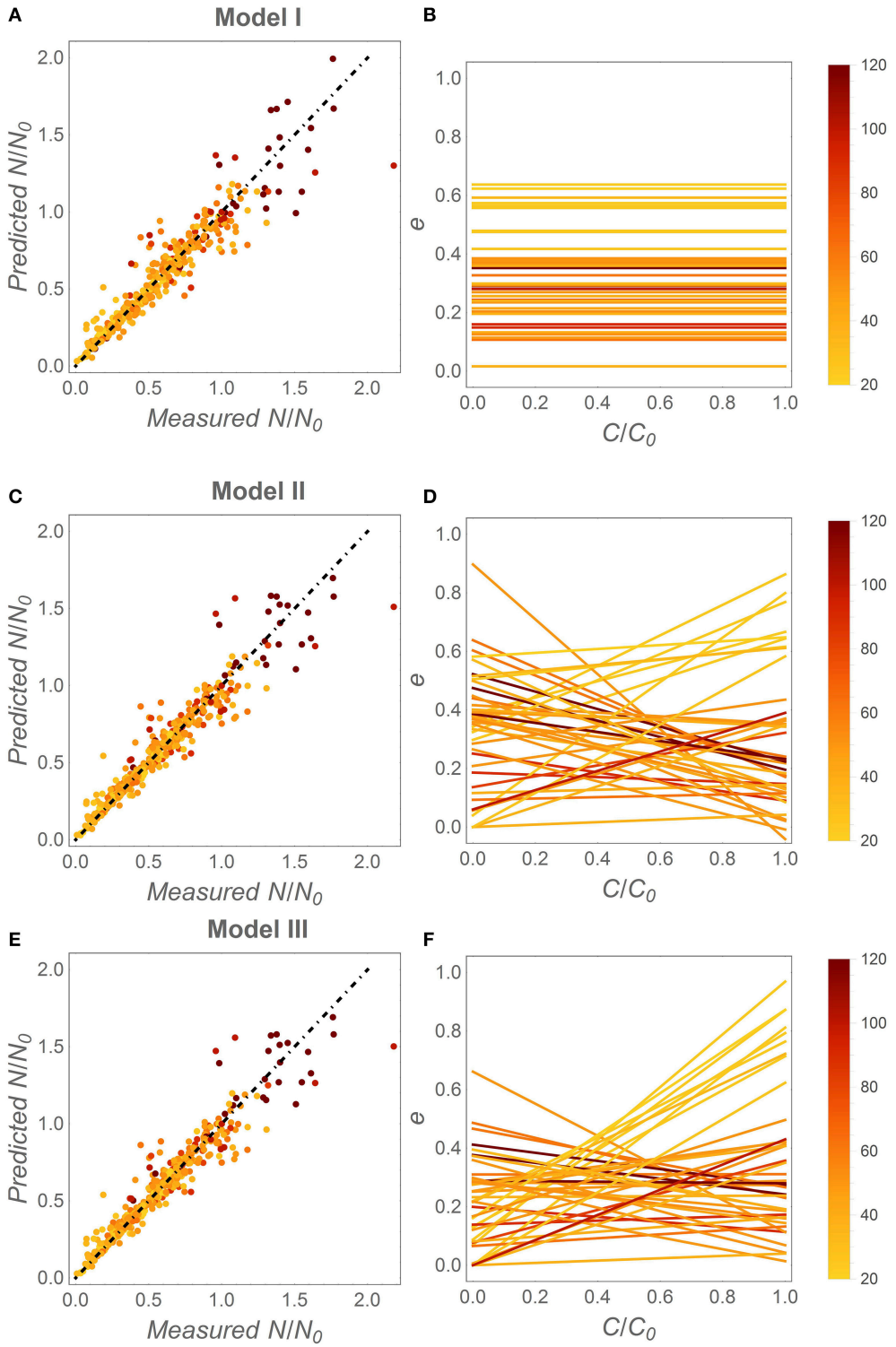

FIGURE 3 | Fitting results for the different C-use efficiency (*e*) parameterizations. Left panels: fitting performance by comparing measured and modeled fractions of remaining N (**Table 2**, Table S1). Right panels: inferred *e* as a function of the fraction of remaining C (x). **(A,B)** model I (time-invariant *e* and microbial N:C); **(C,D)** model II [variable *e* (x) and time-invariant microbial N:C]; **(E,F)** model III [variable *e* (x) and microbial N:C]. Symbols and lines are color coded as a function of the litter initial C:N ratio (color-bars); the dot-dashed lines have unitary slope. Fixed parameters: $\alpha = 1.25$, $r_{B,0} = 0.083$ [$(C:N)_B = 12$], and $\beta = -0.04$. As in **Figure 1**, the temporal development in the right panels is from right ($C/C_0 = 1$) to left ($C/C_0 = 0$).

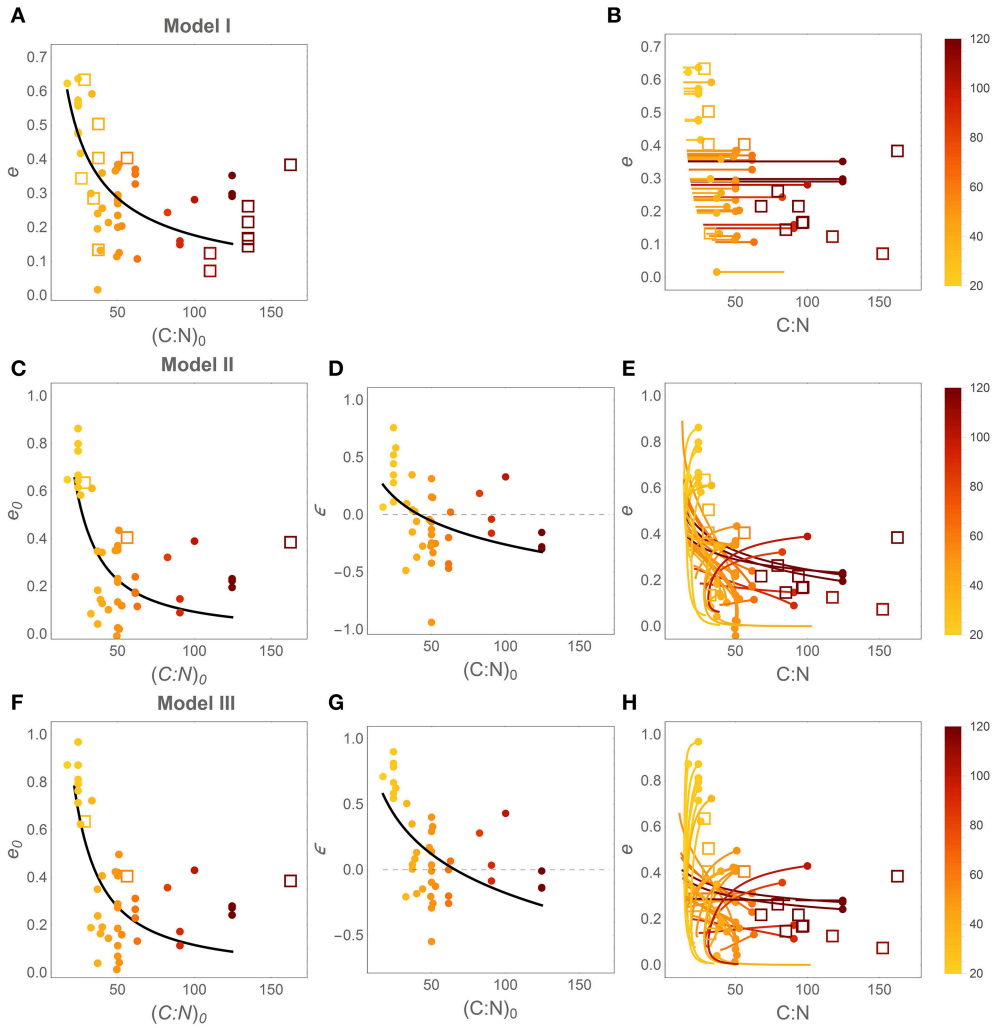

FIGURE 4 | Effect of litter C:N ratio on C-use efficiency (e). Left and central panels: relations between initial litter C:N ratio, $(C:N)_0 = 1/r_0$, and the parameters of the different CUE models. Right panels: modeled e as a function of litter C:N during decomposition (initial points are identified by filled circles). **(A,B)** model I (time-invariant e and microbial N:C); **(C–E)** model II [variable e (x) and time-invariant microbial N:C]; **(F–H)** model III [variable e (x) and microbial N:C]. Symbols and lines are color coded as a function of $(C:N)_0$ (color-bars). Closed symbols refer to estimates from model fitting; open symbols refer to observations (Table S2). Regression statistics for the curves in **(A,C,D,F,G)** are reported in **Table 3** (all slopes are significantly different from zero). Thin dashed lines in **(D)** and **(G)** indicate $\varepsilon = 0$.

corresponding to N-rich litter exhibit decreasing CUE as the C:N ratio initially decreases, but then mildly increases toward the final phase of decomposition (N:C first increases and then decreases). Observed C-use efficiency values broadly overlap with the estimated $e\,(C:N)$ trajectories (compare open symbols and solid curves in **Figures 4B,E,H**).

Relation between C-Use Efficiency and Climate

In contrast to the clear patterns of CUE and related parameters in relation to litter stoichiometry, mean annual temperature (MAT) and precipitation (MAP) do not appear to have an effect on CUE. The slopes of linear relations between e (model I) or e_0 and ε (models II and III), and MAT or MAP are not significantly different from zero, suggesting lack of significant climatic effects (Figure S3). Climatic effects are also weak or inconsistent when considering specific litter types incubated at different sites (results not shown).

TABLE 3 | Relations between C-use efficiency parameters and initial litter C:N ratio, $(C:N)_0$.

Model	Fitting function	*a*	*b*	R^2
I	$e = a\,(C:N)_0^b$	4.4 [0.22, 8.5]	−0.70 [−0.96, −0.43]	0.89
II	$\varepsilon = a\ln\left[\frac{(C:N)_0}{b}\right]$	−0.30 [−0.50, −0.094]	42 [28, 56]	0.19
	$e_0 = a\,(C:N)_0^b$	35 [−13, 83]	−1.3 [−1.7, −0.89]	0.81
III	$\varepsilon = a\ln\left[\frac{(C:N)_0}{b}\right]$	−0.43 [−0.62, −0.24]	66 [49, 83]	0.44
	$e_0 = a\,(C:N)_0^b$	37 [−6.9, 81]	−1.3 [−1.6, −0.90]	0.85

Fitting parameters are reported with 95% confidence intervals in square brackets, and fitting performance is quantified by the coefficient of determination, R^2.

DISCUSSION

A Stoichiometric Approach for Decomposer Trait Estimation

The analysis of microbial growth kinetics is based on the idea that microbial traits can be mapped into parameters of process models. These parameters can then be obtained by fitting the model results (either analytical equations or numerical solutions) to observations (Panikov, 1995). While mapping traits into model parameters can be relatively simple in idealized laboratory conditions with minimal microbial and substrate diversity, it can be challenging in complex soil systems, where both traits and model parameters must be interpreted as "macroscopic" properties that integrate the underlying heterogeneities (Manzoni et al., 2008b). Nevertheless, microbial community traits have also been obtained by fitting models to soil incubation studies (Nicolardot et al., 2001; Pansu et al., 2004) and litter decomposition datasets (Wetterstedt and Ågren, 2011; Manzoni et al., 2012a). Here the same conceptual approach is applied, but instead of fitting model results as a function of time, analytical relations between two elements are obtained (Manzoni et al., 2008a; Ågren et al., 2013). This method focuses on stoichiometry rather than kinetics and reduces the degrees of freedom of the problem, thereby allowing more robust trait estimation.

The microbial community C-use efficiency is particularly challenging to measure and interpret (Geyer et al., 2016), motivating the use of the proposed stoichiometric approach as an alternative tool for CUE estimation. CUE has been hypothesized to vary in response to nutrient availability (Sterner and Elser, 2002; Manzoni et al., 2008a), but also microbial elemental composition could change as cells try to compensate nutrient imbalances. Unfortunately, the roles played by decomposer elemental composition and CUE in the nutrient release curves employed here to estimate microbial traits are difficult to disentangle. In fact, either decreasing microbial biomass N:C or decreasing e reduces the nutrient demand per unit C consumed (as demonstrated analytically by Ågren et al., 2013, and as illustrated in **Figure 1**). In two studies where both parameters were fitted via nonlinear regression (Ågren et al., 2001; Nicolardot et al., 2001), contrasting results were found. In one case, microbial N:C increased significantly with amendment N:C ratio, while e tended to reach the upper bound set as a constraint for parameter optimization (Nicolardot et al., 2001). In the other case, microbial N:C remained stable, while e increased with increasing inorganic nutrient availability and litter N:C ratio (Ågren et al., 2001). These opposite results confirm the covariation of e and r_B and suggest strong sensitivity to the fitting procedure, unless some additional constraints are imposed. This issue has been dealt with in different ways: by imposing a time-invariant r_B and letting e vary (Manzoni et al., 2008a, 2010), or by estimating the product er_B (corresponding to the N:C ratio at incipient N immobilization) without distinguishing between the two parameters (Ågren et al., 2013). Because r_B values are relatively more constrained than CUE values, here a time-invariant r_B (models I and II) or a pre-defined relation between r_B and the fraction of remaining C (model III) is assumed. Comparing models II and III suggests little impact of r_B trends on CUE, as also discussed in Section Stoichiometric Drivers of Decomposer Trait Flexibility.

Two other confounding factors could complicate the interpretation of the results: leaching and variations in the coefficient α. The former could contribute to losses of organic C and N from the litter system independently of microbial traits, thereby affecting the estimated CUE. Because the proposed modeling framework focuses on microbial-driven decomposition and cannot capture decomposition trajectories when physical processes dominate, the initial leaching phase was removed when evident in the data. This procedure only affected three out of 41 datasets, suggesting that in the selected decomposition trajectories initial leaching was not as important as microbial processes. Besides the initial leaching, it is possible that residual leaching losses occurred in wet sites throughout the decomposition process. This residual leaching (referred to simply as "leaching" in the following) had been included in a previous work employing a comparable model (Manzoni et al., 2010), but was neglected here for simplicity. An increase in leaching rate would increase the estimated CUE, because losses of organic C via leaching occur "in parallel" with respiration. Thus, for a given (measured) C loss, assuming larger leaching implies lowering the contribution of respiration, which translates into higher CUE estimates. Therefore, if leaching was higher in the early decomposition phase, the initial CUE was overestimated. As a consequence, the actual CUE trends should be weaker when the slope $\varepsilon > 0$, but stronger when $\varepsilon < 0$, because initial CUE was overestimated. However, mean annual precipitation, which could be expected to be correlated to leaching rates, has no significant effect on any of the CUE parameters (Figure S3), suggesting that CUE estimates are not significantly biased by neglecting leaching.

Increasing decomposer preference for N (higher α), by improving organic N availability, reduces N immobilization (Figure S2). Hence, for a given (measured) N release curve, higher α results in higher estimated CUE. However, it is not clear how α could change during decomposition. Leachate elemental ratios, which could be hypothesized to be more representative of microbial substrate than the bulk litter ratios, are variable (Magill and Aber, 2000; Michalzik et al., 2001; Lashermes et al., 2016) and in some cases leachate N:C ratios are even lower than in the bulk litter (Fanin et al., 2013). Without more specific information on temporal trends in α, the constant value $\alpha = 1.25$ appears to be a reasonable choice. As an alternative, more complex models including a dissolved organic matter pool could be useful. However, increased model complexity would also introduce further uncertainties.

Despite difficulties in isolating the effects of CUE from those of other traits, the proposed method (i) provides a highly accurate description of N release trajectories (**Table 2**), and (ii) identifies trait patterns that are consistent with current conceptual understanding of decomposition (as discussed in Section Stoichiometric Drivers of Decomposer Trait Flexibility). Empirical quantification of microbial community CUE and elemental composition in long-term litter incubations could help

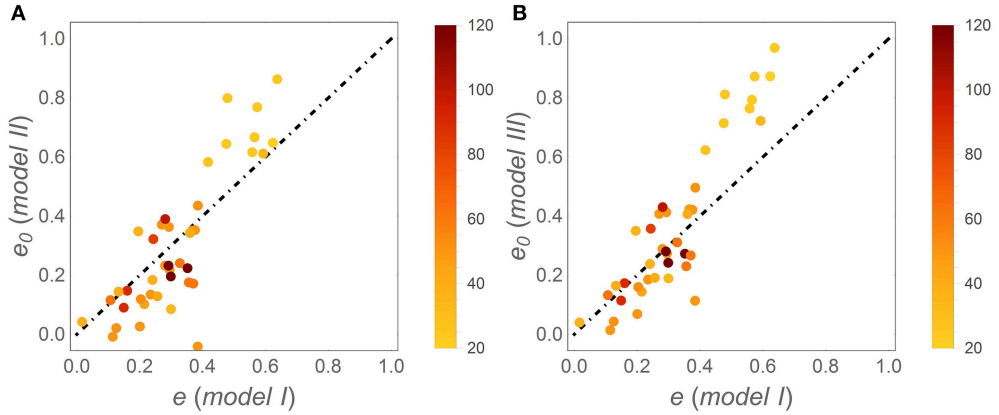

FIGURE 5 | Correlations between the initial C-use efficiency (e_0) estimated using (A) model II and **(B)** model III, and the time-invariant C-use efficiency estimated from model I (simply denoted as *e*); the dot-dashed lines have unitary slope. Symbols are color coded as a function of $(C:N)_0$ (color-bars).

validate this approach and disentangle the effects of possible confounding factors.

Stoichiometric Drivers of Decomposer Trait Flexibility

Microbial traits associated with metabolic processes often vary along gradients of nutrient availability in both microbial isolates and communities. This trait flexibility at the community level is required to reduce imbalances in nutrient supply (e.g., by tuning extracellular enzyme expression; Sinsabaugh et al., 2014), and to compensate stoichiometric imbalances by changing body composition (Tezuka, 1990; Godwin and Cotner, 2015), altering metabolism (Sterner and Elser, 2002; Manzoni et al., 2008a), or shifting community composition (Cotner et al., 2010; Godwin and Cotner, 2014). While trait variations have been mainly studied across nutrient availability treatments, temporal trends in traits along nutrient availability trajectories are less clear. In this context, litter decomposition represents a useful model system thanks to the wide range of nutrient conditions during degradation of a single cohort and the abundance of data tracking the temporal changes of litter C and nutrient pools.

Even though N:C ratios of individual microbial strains can vary significantly (Mouginot et al., 2014), decomposer communities appear homeostatic with respect to N across wide ranges of organic matter elemental composition (Fanin et al., 2013; Xu et al., 2013). However, in decomposing litter undergoing strong nutrient enrichment, some trends in microbial biomass N:C have been found. In some studies, microbial N:C increases with increasing litter N:C as decomposition progresses (Wagener and Schimel, 1998; van Meeteren et al., 2008; Brandstätter et al., 2013; Toberman et al., 2014), but in others no trends are apparent (Mooshammer et al., 2014a). Along the extreme stoichiometric gradient between a decaying log and the nearby soil (assuming that the latter is representative of the final phases of wood decomposition), only small differences in microbial biomass elemental composition were found, despite the four-fold increase in N:C ratio (Hart, 1999). Overall, this evidence suggests that N availability during decomposition of a litter cohort might not always be a good predictor of microbial biomass elemental composition. Here, r_B flexibility is accounted for in model III, but does not affect the main patterns predicted for CUE (**Figures 3**, **4**), suggesting that stoichiometric flexibility of litter microbial communities may not be sufficient to compensate strong nutrient imbalances.

The assumed increase from $r_B = 0.083$ to 0.125 (i.e., $(C:N)_B$ decreases from 12 to 8) is consistent with observed changes, even though lower N:C ratios have also been found. Had a steeper decrease in r_B with decreasing fraction of remaining C been considered, stronger differences between results from model II and III would have emerged. However, the lower N:C ratios observed in some litter microbial communities might not be representative of the actively growing (and relatively nutrient-rich) fraction of the microbial community that the stoichiometric model is meant to describe.

If the cellular composition is too stable to compensate stoichiometric imbalances, it can be surmised that metabolic processes might provide the required flexibility (Manzoni et al., 2008a; Mooshammer et al., 2014b). Observed C-use efficiency values support this view by strongly decreasing as the initial litter C:N ratio (**Figures 4A,C,F**) as well as the instantaneous litter C:N (**Figures 4B,E,H**) increase across litter types. Evidence from an experiment designed to test this hypothesis is supportive (Lashermes et al., 2016), although the measured CUE values are higher than expected from model results in N-poor litter. In this dataset, however, fungal biomass—and consequently CUE—might have been overestimated (Lashermes et al., 2016), thus explaining the mismatch. The expected decline in CUE at high substrate C:N ratio is not always observed in culture studies, suggesting that some organisms may exhibit sufficient flexibility in their cellular composition to compensate nutrient imbalances and that the effects of compound C- and nutrient-limitation are not easy to predict (Keiblinger et al., 2010).

The temporal trends in C-use efficiency are potentially more complex to interpret than the trends in decomposer biomass elemental composition. If CUE is reduced in response to nutrient limitation, it can be hypothesized that CUE increases during

decomposition, as the nutrient availability increases (in this framework, this trend would be characterized by $\varepsilon < 0$). However, such an effect would be apparent only in litter types with high initial C:N ratio. In contrast, as recalcitrant material and microbial by-products accumulate in the late decomposition phases (Berg and McClaugherty, 2003), acquiring C could require larger investments in extracellular enzymes (Ågren and Bosatta, 1987), and microbial populations would spend more time in relatively inactive states associated with higher respiration per unit C taken up and thus lower CUE. Experimental evidence indeed showed that decomposers degrading lignin exhibit lower CUE than communities feeding on higher quality substrates (Bahri et al., 2008). Moreover, increased N concentration in this late phase is often associated with reduced decomposition rates, due to inhibition of extracellular enzymes (Berg and McClaugherty, 2003; Hobbie et al., 2012). As a result, in such conditions CUE could decrease with progressing decomposition ($\varepsilon > 0$). This effect would be apparent only in N-rich litter where C would become limiting. A similar pattern of higher initial CUE in N-rich litter than in N-poor litter, followed by a reversed pattern in the late decay phase had also been hypothesized by Cotrufo et al. (2013). As shown in **Figure 4**, the patterns in ε estimated with models II and III are in line with these expectations, with positive ε values in N-rich litter types, transitioning to negative ε values at high initial litter C:N ratios.

The initial CUE values of models II and III follow the same decreasing pattern with increasing initial litter C:N as the time-invariant CUE of model I (**Figure 5**). It can thus be concluded that regardless of the specific time trajectory of CUE, the initial CUE compensates litter stoichiometric imbalances by reducing the decomposer growth rate and nutrient demand in N-poor litter. Moreover, the initial CUE values are representative of the first and most active phase of decomposition, so that the same pattern remains evident when considering the long-term average CUE, as in model I. Hence, this result lends some support to the use of time-invariant CUE to detect stoichiometric effects on CUE across broad litter quality gradients (Manzoni et al., 2008a), although temporal trends are key to understanding the interplay between N- and C-limitation during degradation of a single litter cohort.

Climatic Drivers of Decomposer Trait Flexibility

Mean annual temperature and precipitation explain a large fraction of the observed variability in litter decay constants (Aerts, 1997; Adair et al., 2008). For a given litter type, incubation under conditions ranging from cold or dry to warm and moist causes a more than 10-fold variation in the decay constants (Adair et al., 2008). Such a large climatic effect could be expected to also appear when investigating broad-scale trends in C-use efficiency and related parameters. However, climatic factors do not play any significant role (Figure S3), at least at the scale of this analysis, as previously noted by Manzoni et al. (2008a). Thus, two complementary effects of climate on decomposition are occurring. While warm and moist conditions are favorable for decomposers and thus promote rapid litter decomposition, the way C is partitioned between growth and respiration does not seem to be affected, suggesting a separation between the drivers of microbial metabolic rates (climate) and those affecting metabolic efficiency (litter stoichiometry). Hence, climate is expected to affect C sequestration by altering the balance of inputs to the soil and respiration rates, while litter elemental composition largely affects the patterns of nutrient release and the metabolic efficiency of the decomposers.

Had an increase in leaching rate with mean annual precipitation been accounted for, the CUE estimates would have been higher for the wetter sites, potentially introducing a positive correlation between CUE and precipitation. Dissolved organic C production from litterbags incubated in a lake amounted to about 20% of mass loss (Kominkova et al., 2000). Even assuming that this value is representative for leaching in the wettest terrestrial ecosystems (10% is a more reasonable figure, Michalzik et al., 2001), variability in our CUE estimates would still be driven by litter stoichiometry rather than precipitation.

Both short-term fluctuations in soil moisture (e.g., Tiemann and Billings, 2011) and incubation at different temperatures (e.g., Frey et al., 2013) have been shown to affect CUE of soil microbial communities in laboratory conditions. In particular, CUE tends to decline with increasing temperature, although its temperature sensitivity is still a matter of debate, and when discounting the effect of mortality, stable CUE values have been found (Hagerty et al., 2014). In contrast, positive temperature effects on CUE were found in a global-scale study, in which CUE was estimated using a model driven by the stoichiometric ratios of substrates and ecoenzymatic activities (Sinsabaugh et al., 2016). It is conceivable that when integrating responses to environmental fluctuations and microbial community dynamics at the annual time scale considered here, more stable CUE values than under laboratory conditions are achieved. Furthermore, physiological factors such as temperature acclimation, or shifts in microbial community composition, can contribute to reducing temperature sensitivity in the long-term (Frey et al., 2013; Allison, 2014; Sinsabaugh et al., 2016). These processes could explain why the CUE values of decomposer communities degrading the same litter type along a climatic gradient (datasets from the LIDET study) are weakly or inconsistently dependent on climate, but strongly dependent on litter quality.

On the Interpretation of Community-Scale C-Use Efficiency

The estimated CUE values are to be interpreted at the microbial community scale (sensu Geyer et al., 2016) and should be regarded as "effective," lumped parameters that capture the average behavior of a biologically and chemically heterogeneous system (Manzoni et al., 2008b). In fact, the proposed model integrates the contributions of all decomposer to the bulk community metabolism. However, the CUE estimates presented here are not confounded by microbial turnover, which is implicitly accounted for as a recycling flux into the litter pool. Community dynamics can generate patterns in CUE that differ from those of the individual constituents or a homogeneous

community (Kaiser et al., 2014). Results from an individual-based model show that CUE can remain high regardless of litter C:N ratio, because turnover of N-rich microbial products allows at least part of the community to feed on substrates with substantially higher N:C than the bulk litter (Kaiser et al., 2014). This effect could be captured by higher values of α in this framework, but such an adjustment would not be supported by independent information. While considering lumped processes aids in extracting information from relatively coarse data (such as C and N mass in litter samples), it also represents a limitation of this approach, as it precludes the possibility of further disentangling the mechanistic drivers of flexible community-level traits. It would be fruitful to combine litter decay data with detailed community composition and physiological measurements that can assist in interpreting patterns in the estimated community-level traits.

CONCLUSIONS

A stoichiometric model is presented as a tool for quantifying variations in decomposer traits. Specifically, changes in microbial community C-use efficiency across litter types and through time during decomposition of individual litter cohorts are estimated by fitting analytical N release curves to litter C and N mass data. This method offers insights on decomposer traits that would be difficult to measure, and allows generating specific hypotheses that could be targets of more detailed empirical studies. C-use efficiency is found to be flexible, showing a continuum of responses during decomposition. In general, C-use efficiency increases along a gradient of litter types with increasing N availability. Temporal patterns in a single litter cohort are more complex. N-poor litter types tend to exhibit increasing CUE possibly due to large stoichiometric imbalances and N-limitation in the early phase of decomposition, followed by improved N status and consequently more efficient biomass production. In contrast, N-rich litter types exhibit lowering CUE, possibly driven by C-limitation, increased chemical complexity and inhibiting effects of high N availability in the late phase of decomposition. Hence, these data-driven analysis suggests that trajectories of decomposer community traits depend in a strongly nonlinearly way on litter stoichiometry, as the decomposers transition from N- to C-limited conditions.

AUTHOR CONTRIBUTIONS

SM designed the study, developed the theory, conducted the analyses, and wrote the manuscript.

FUNDING

Funding was provided by the Bolin Centre for Climate Research (Research Area 4), through the project "Scaling carbon-use efficiency from the organism- to the global-scale," and by the Swedish Research Councils, Formas (2015-468), and VR (2016-04146). Significant funding for the LIDET data was provided by the National Science Foundation Long-Term Ecological Research program.

ACKNOWLEDGMENTS

I would like to thank Göran Ågren for providing in-depth feedback on the theory and comments on an early version of the manuscript, and two reviewers for their constructive critiques. The Bolin Centre for Climate Research workshop "Carbon-use efficiency across scales," held in 2016 at Stockholm University, was instrumental for developing ideas on the C-use efficiency of decomposer communities. The LIDET dataset was provided by the Forest Science Data Bank, a partnership between the Department of Forest Science, Oregon State University, and the U.S. Forest Service Pacific Northwest Research Station, Corvallis, Oregon.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00661/full#supplementary-material

REFERENCES

Adair, E. C., Parton, W. J., Del Grosso, S. J., Silver, W. L., Harmon, M. E., Hall, S. A., et al. (2008). Simple three-pool model accurately describes patterns of long-term litter decomposition in diverse climates. *Glob. Chang. Biol.* 14, 2636–2660. doi: 10.1111/j.1365-2486.2008.01674.x

Aerts, R. (1997). Climate, leaf litter chemistry and leaf litter decomposition in terrestrial ecosystems: a triangular relationship. *Oikos* 79, 439–449. doi: 10.2307/3546886

Ågren, G. I., and Bosatta, E. (1987). Theoretical-analysis of the long-term dynamics of carbon and nitrogen in soils. *Ecology* 68, 1181–1189. doi: 10.2307/1939202

Ågren, G. I., Bosatta, E., and Magill, A. H. (2001). Combining theory and experiment to understand effects of inorganic nitrogen on litter decomposition. *Oecologia* 128, 94–98. doi: 10.1007/s004420100764

Ågren, G. I., Hyvonen, R., Berglund, S. L., and Hobbie, S. E. (2013). Estimating the critical N:C from litter decomposition data and its relation to soil organic matter stoichiometry. *Soil Biol. Biochem.* 67, 312–318. doi: 10.1016/j.soilbio.2013.09.010

Allison, S. D. (2014). Modeling adaptation of carbon use efficiency in microbial communities. *Front. Microbiol.* 5:571. doi: 10.3389/fmicb.2014.00571

Bahri, H., Rasse, D. P., Rumpel, C., Dignac, M. F., Bardoux, G., and Mariotti, A. (2008). Lignin degradation during a laboratory incubation followed by (13)C isotope analysis. *Soil Biol. Biochem.* 40, 1916–1922. doi: 10.1016/j.soilbio.2008.04.002

Berg, B., and McClaugherty, C. A. (2003). *Plant Litter. Decomposition, Humus Formation, Carbon Sequestration.* Berlin: Springer.

Boberg, J. B., Finlay, R. D., Stenlid, J., Ekblad, A., and Lindahl, B. D. (2014). Nitrogen and carbon reallocation in fungal mycelia during decomposition of boreal forest litter. *PLoS ONE* 9:e92897. doi: 10.1371/journal.pone.0092897

Bosatta, E., and Ågren, G. I. (1985). Theoretical-analysis of decomposition of heterogeneous substrates. *Soil Biol. Biochem.* 17, 601–610. doi: 10.1016/0038-0717(85)90035-5

Bosatta, E., and Berendse, F. (1984). Energy or nutrient regulation of decomposition-implications for the mineralization immobilization response to perturbations. *Soil Biol. Biochem.* 16, 63–67. doi: 10.1016/0038-0717(84)90127-5

Bosatta, E., and Staaf, H. (1982). The control of nitrogen turn-over in forest litter. *Oikos* 39, 143–151. doi: 10.2307/3544478

Boyce, W. E., and DiPrima, R. C. (2009). *Elementary Differential Equations and Boundary Value Problems*. New York, NY: John Wiley & Sons, Inc.

Brandstäetter, C., Keiblinger, K., Wanek, W., and Zechmeister-Boltenstern, S. (2013). A closeup study of early beech litter decomposition: potential drivers and microbial interactions on a changing substrate. *Plant Soil* 371, 139–154. doi: 10.1007/s11104-013-1671-7

Burnham, K. P., and Anderson, D. R. (2002). *Model Selection and Multimodel Inference. A Practical Information-Theoretic Approach.* New York, NY: Springer.

Cotner, J. B., Hall, E. K., Scott, T., and Heldal, M. (2010). Freshwater bacteria are stoichiometrically flexible with a nutrient composition similar to seston. *Front. Aquat. Microbiol.* 1:132. doi: 10.3389/fmicb.2010.00132

Cotrufo, M. F., Wallenstein, M. D., Boot, C. M., Denef, K., and Paul, E. (2013). The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: do labile plant inputs form stable soil organic matter? *Glob. Chang. Biol.* 19, 988–995. doi: 10.1111/gcb.12113

Fanin, N., Fromin, N., Buatois, B., and Hättenschwiler, S. (2013). An experimental test of the hypothesis of non-homeostatic consumer stoichiometry in a plant litter–microbe system. *Ecol. Lett.* 16, 764–772. doi: 10.1111/ele.12108

Frankland, J. C., Lindley, D. K., and Swift, M. J. (1978). Comparison of two methods for estimation of mycelial biomass in leaf litter. *Soil Biol. Biochem.* 10, 323–333. doi: 10.1016/0038-0717(78)90030-5

Frey, S. D., Lee, J., Melillo, J. M., and Six, J. (2013). The temperature response of soil microbial efficiency and its feedback to climate. *Nat. Clim. Chang.* 3, 395–398. doi: 10.1038/nclimate1796

Geyer, K. M., Kyker-Snowman, E., Grandy, A. S., and Frey, S. D. (2016). Microbial carbon use efficiency: accounting for population, community, and ecosystem-scale controls over the fate of metabolized organic matter. *Biogeochemistry* 127, 173–188. doi: 10.1007/s10533-016-0191-y

Godwin, C. M., and Cotner, J. B. (2014). Carbon:phosphorus homeostasis of aquatic bacterial assemblages is mediated by shifts in assemblage composition. *Aquat. Microb. Ecol.* 73, 245–258. doi: 10.3354/ame01719

Godwin, C. M., and Cotner, J. B. (2015). Stoichiometric flexibility in diverse aquatic heterotrophic bacteria is coupled to differences in cellular phosphorus quotas. *Front. Microbiol.* 6:159. doi: 10.3389/fmicb.2015.00159

Goldman, J. C., Caron, D. A., and Dennett, M. R. (1987). Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C-N ratio. *Limnol. Oceanogr.* 32, 1239–1252. doi: 10.4319/lo.1987.32.6.1239

Hagerty, S. B., van Groenigen, K. J., Allison, S. D., Hungate, B. A., Schwartz, E., Koch, G. W., et al. (2014). Accelerated microbial turnover but constant growth efficiency with warming in soil. *Nat. Clim. Chang.* 4, 903–906. doi: 10.1038/nclimate2361

Hart, S. C. (1999). Nitrogen transformations in fallen tree boles and mineral soil of an old-growth forest. *Ecology* 80, 1385–1394. doi: 10.1890/0012-9658(1999)080[1385:NTIFTB]2.0.CO;2

Hirobe, M., Sabang, J., Bhatta, B. K., and Takeda, H. (2004). Leaf-litter decomposition of 15 tree species in a lowland tropical rain forest in Sarawak: dynamics of carbon, nutrients, and organic constituents. *J. For. Res.* 9, 347–354. doi: 10.1007/s10310-004-0088-9

Hobbie, S. E., Eddy, W. C., Buyarski, C. R., Adair, E. C., Ogdahl, M. L., and Weisenhorn, P. (2012). Response of decomposing litter and its microbial community to multiple forms of nitrogen enrichment. *Ecol. Monogr.* 82, 389–405. doi: 10.1890/11-1600.1

Kaiser, C., Franklin, O., Dieckmann, U., and Richter, A. (2014). Microbial community dynamics alleviate stoichiometric constraints during litter decay. *Ecol. Lett.* 17, 680–690. doi: 10.1111/ele.12269

Keiblinger, K. M., Hall, E. K., Wanek, W., Szukics, U., Hämmerle, I., Ellersdorfer, G., et al. (2010). The effect of resource quantity and resource stoichiometry on microbial carbon-use-efficiency. *FEMS Microbiol. Ecol.* 73, 430–440. doi: 10.1111/j.1574-6941.2010.00912.x

Kominkova, D., Kuehn, K. A., Busing, N., Steiner, D., and Gessner, M. O. (2000). Microbial biomass, growth, and respiration associated with submerged litter of Phragmites australis decomposing in a littoral reed stand of a large lake. *Aquat. Microb. Ecol.* 22, 271–282. doi: 10.3354/ame022271

Lashermes, G., Gainvors-Claisse, A., Recous, S., and Bertrand, I. (2016). Enzymatic strategies and carbon use efficiency of a litter-decomposing fungus grown on maize leaves, stems, and roots. *Front. Microbiol.* 7:1315. doi: 10.3389/fmicb.2016.01315

Magill, A. H., and Aber, J. D. (2000). Dissolved organic carbon and nitrogen relationships in forest litter as affected by nitrogen deposition. *Soil Biol. Biochem.* 32, 603–613. doi: 10.1016/S0038-0717(99)00187-X

Manzoni, S., Jackson, R. B., Trofymow, J. A., and Porporato, A. (2008a). The global stoichiometry of litter nitrogen mineralization. *Science* 321, 684–686. doi: 10.1126/science.1159792

Manzoni, S., Pineiro, G., Jackson, R. B., Jobbagy, E. G., Kim, J. H., and Porporato, A. (2012a). Analytical models of soil and litter decomposition: solutions for mass loss and time-dependent decay rates. *Soil Biol. Biochem.* 50, 66–76. doi: 10.1016/j.soilbio.2012.02.029

Manzoni, S., Porporato, A., and Schimel, J. P. (2008b). Soil heterogeneity in lumped mineralization-immobilization models. *Soil Biol. Biochem.* 40, 1137–1148. doi: 10.1016/j.soilbio.2007.12.006

Manzoni, S., Taylor, P. G., Richter, A., Porporato, A., and Ågren, G. I. (2012b). Environmental and stoichiometric controls on microbial carbon-use efficiency in soils. *New Phytol.* 196, 79–91. doi: 10.1111/j.1469-8137.2012.04225.x

Manzoni, S., Trofymow, J. A., Jackson, R. B., and Porporato, A. (2010). Stoichiometric controls dynamics on carbon, nitrogen, and phosphorus in decomposing litter. *Ecol. Monogr.* 80, 89–106. doi: 10.1890/09-0179.1

Michalzik, B., Kalbitz, K., Park, J. H., Solinger, S., and Matzner, E. (2001). Fluxes and concentrations of dissolved organic carbon and nitrogen – a synthesis for temperate forests. *Biogeochemistry* 52, 173–205. doi: 10.1023/A:1006441620810

Mooshammer, M., Wanek, W., Hämmerle, I., Fuchslueger, L., Hofhansl, F., Knoltsch, A., et al. (2014a). Adjustment of microbial nitrogen use efficiency to carbon:nitrogen imbalances regulates soil nitrogen cycling. *Nat. Commun.* 5, 3694–3694. doi: 10.1038/ncomms4694

Mooshammer, M., Wanek, W., Zechmeister-Boltenstern, S., and Richter, A. (2014b). Stoichiometric imbalances between terrestrial decomposer communities and their resources: mechanisms and implications of microbial adaptations to their resources. *Front. Microbiol.* 5:22. doi: 10.3389/fmicb.2014.00022

Mouginot, C., Kawamura, R., Matulich, K. L., Berlemont, R., Allison, S. D., Amend, A. S., et al. (2014). Elemental stoichiometry of Fungi and Bacteria strains from grassland leaf litter. *Soil Biol. Biochem.* 76, 278–285. doi: 10.1016/j.soilbio.2014.05.011

Nicolardot, B., Recous, S., and Mary, B. (2001). Simulation of C and N mineralisation during crop residue decomposition: a simple dynamic model based on the C: N ratio of the residues. *Plant Soil* 228, 83–103. doi: 10.1023/A:1004813801728

Panikov, N. S. (1995). *Microbial Growth Kinetics*. London: Chapman & Hall.

Pansu, M., Bottner, P., Sarmiento, L., and Metselaar, K. (2004). Comparison of five soil organic matter decomposition models using data from a ^{14}C and ^{15}N labeling field experiment. *Global Biogeochem. Cycles* 18:GB4022. doi: 10.1029/2004GB002230

Sinsabaugh, R. L., Belnap, J., Findlay, S. G., Shah, J. J. F., Hill, B. H., Kuehn, K. A., et al. (2014). Extracellular enzyme kinetics scale with resource availability. *Biogeochemistry* 121, 287–304. doi: 10.1007/s10533-014-0030-y

Sinsabaugh, R. L., Manzoni, S., Moorhead, D. L., and Richter, A. (2013). Carbon use efficiency of microbial communities: stoichiometry, methodology and modelling. *Ecol. Lett.* 16, 930–939. doi: 10.1111/ele.12113

Sinsabaugh, R. L., Turner, B. L., Talbot, J. M., Waring, B. G., Powers, J. S., Kuske, C. R., et al. (2016). Stoichiometry of microbial carbon use efficiency in soils. *Ecol. Monogr.* 86, 172–189. doi: 10.1890/15-2110.1

Six, J., Frey, S. D., Thiet, R. K., and Batten, K. M. (2006). Bacterial and fungal contributions to carbon sequestration in agroecosystems. *Soil Sci. Soc. Am. J.* 70, 555–569. doi: 10.2136/sssaj2004.0347

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry. The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ; Oxford: Princeton University Press.

Tezuka, Y. (1990). Bacterial regeneration of ammonium and phosphate as affected by the carbon - nitrogen - phosphorus ratio of organic substrates. *Microb. Ecol.* 19, 227–238. doi: 10.1007/BF02017167

Tiemann, L. K., and Billings, S. A. (2011). Changes in variability of soil moisture alter microbial community C and N resource use. *Soil Biol. Biochem.* 43, 1837–1847. doi: 10.1016/j.soilbio.2011.04.020

Toberman, H., Chen, C., Lewis, T., and Elser, J. J. (2014). High-frequency fire alters C: N: P stoichiometry in forest litter. *Glob. Chang. Biol.* 20, 2321–2331. doi: 10.1111/gcb.12432

van Meeteren, M. M., Tietema, A., van Loon, E. E., and Verstraten, J. M. (2008). Microbial dynamics and litter decomposition under a changed climate in a Dutch heathland. *Appl. Soil Ecol.* 38, 119–127. doi: 10.1016/j.apsoil.2007.09.006

Wagener, S. M., and Schimel, J. P. (1998). Stratification of soil ecological processes: a study of the birch forest floor in the Alaskan taiga. *Oikos* 81, 63–74. doi: 10.2307/3546468

Weisstein, E. W. (2016). *Incomplete Beta Function [Online]. MathWorld - A Wolfram Web Resource.* Available online at: http://mathworld.wolfram.com/IncompleteBetaFunction.html (Accessed August 1, 2016).

Wetterstedt, J. A. M., and Ågren, G. I. (2011). Quality or decomposer efficiency - which is most important in the temperature response of litter decomposition? A modelling study using the GLUE methodology. *Biogeosciences* 8, 477–487. doi: 10.5194/bg-8-477-2011

Xu, X., Thornton, P. E., and Post, W. M. (2013). A global analysis of soil microbial biomass carbon, nitrogen and phosphorus in terrestrial ecosystems. *Glob. Ecol. Biogeogr.* 22, 737–749. doi: 10.1111/geb.12029

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 July 2017
doi: 10.3389/fmicb.2017.01281

Carbon:Nitrogen:Phosphorus Stoichiometry in Fungi: A Meta-Analysis

Ji Zhang[1,2] *and James J. Elser*[2,3]*

[1] Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences, Kunming, China, [2] School of Life Sciences, Arizona State University, Tempe, AZ, United States, [3] Flathead Lake Biological Station, University of Montana, Polson, MT, United States

Surveys of carbon:nitrogen:phosphorus ratios are available now for major groups of biota and for various aquatic and terrestrial biomes. However, while fungi play an important role in nutrient cycling in ecosystems, relatively little is known about their C:N:P stoichiometry and how it varies across taxonomic groups, functional guilds, and environmental conditions. Here we present the first systematic compilation of C:N:P data for fungi including four phyla (Ascomycota, Basidiomycota, Glomeromycota, and Zygomycota). The C, N, and P contents (percent of dry mass) of fungal biomass varied from 38 to 57%, 0.23 to 15%, and 0.040 to 5.5%, respectively. Median C:N:P stoichiometry for fungi was 250:16:1 (molar), remarkably similar to the canonical Redfield values. However, we found extremely broad variation in fungal C:N:P ratios around the central tendencies in C:N:P ratios. Lower C:P and N:P ratios were found in Ascomycota fungi than in Basidiomycota fungi while significantly lower C:N ratios ($p < 0.05$) and higher N:P ratios ($p < 0.01$) were found in ectomycorrhizal fungi than in saprotrophs. Furthermore, several fungal stoichiometric ratios were strongly correlated with geographic and abiotic environmental factors, especially latitude, precipitation, and temperature. The results have implications for understanding the roles that fungi play in function in symbioses and in soil nutrient cycling. Further work is needed on the effects of actual *in situ* growth conditions of fungal growth on stoichiometry in the mycelium.

Keywords: elemental composition, fungus, guild, homeostasis, Redfield ratios, stoichiometry

OPEN ACCESS

Edited by:
Télesphore Sime-Ngando, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Michael Danger, Université de Lorraine, France
Marcela Claudia Pagano, Universidade Federal de Minas Gerais, Brazil

***Correspondence:**
James J. Elser
jim.elser@flbs.umt.edu

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *28 February 2017*
Accepted: *26 June 2017*
Published: *14 July 2017*

Citation:
Zhang J and Elser JJ (2017) Carbon:Nitrogen:Phosphorus Stoichiometry in Fungi: A Meta-Analysis. Front. Microbiol. 8:1281. doi: 10.3389/fmicb.2017.01281

INTRODUCTION

Ecological stoichiometry is the study of the balance of multiple chemical elements in ecological interactions and processes (Sterner and Elser, 2002). By using the perspective of ecological stoichiometry, we can better understand the coupling of energy and material flows at key interfaces in Earth's diverse habitats (Elser et al., 2000a). For example, the features of the stoichiometry of algae–zooplankton interactions have been shown to affect trophic transfer efficiencies and consumer-driven nutrient recycling at the ecosystem scale (Frost et al., 2002; Andersen et al., 2004). Moreover, the extension of ecological stoichiometry to biological phenomena, referred to as biological stoichiometry (Elser et al., 2000c), provides a mechanistic theory linking cellular and biochemical features of co-evolving biota with constraints imposed by, and impacts on, ecosystem energy and nutrient flows (Elser et al., 2003).

To date, studies involving ecological stoichiometry and biological stoichiometry have mostly been done in aquatic systems (McGroddy et al., 2004), but recent work has extended it to new research areas beyond the aquatic realm (Hessen et al., 2013) such as study of terrestrial vegetation (Kerkhoff et al., 2006), insects (Woods et al., 2004), and soils (Cleveland and Liptzin, 2007). As nutrient cycling in natural ecosystems is largely driven by microorganisms (Spohn, 2016), the carbon:nitrogen:phosphorus ratios of soil microbial biomass and ecoenzymatic activity ratios related to resource acquisition have begun to be surveyed (Cleveland and Liptzin, 2007; Sinsabaugh et al., 2009), with a primary emphasis on bacteria. Differences in fungal and bacterial physiology may have important influences on large scale C and N cycling (Waring et al., 2013). However, relatively little is known about fungal stoichiometry despite the important role they play in biogeochemical cycling in ecosystems (Gadd, 2004, 2007). It is still not clear that how these processes might be influenced by the C:N:P stoichiometric requirements of the fungi themselves. In one of the first studies of fungal C:N:P stoichiometry, Danger et al. (2016) assessed the ecological stoichiometry of aquatic fungi and several species of terrestrial fungi, finding that the variation of C:N:P ratios of fungal biomass exceeded variations found for bacteria. However, fungal biodiversity is considerably higher in terrestrial than in aquatic ecosystems (Bärlocher and Boddy, 2016) but our knowledge of their stoichiometry remain lacking.

The role of fungi in symbiosis, e.g., ectomycorrhizae, is of particular interest. Most plant species form symbioses with soil fungi, and up to 80% of plant N and P is provided by mycorrhizal fungi (Behie and Bidochka, 2014; van der Heijden et al., 2015). Mycorrhizal fungi can use organic nitrogen and phosphorus forms, which would otherwise remain largely unavailable to plant roots (Landeweert et al., 2001). Intriguingly, the reciprocity of benefits to host plant and fungus are highly dependent upon the C:P stoichiometry of the transaction (Schwartz and Hoeksema, 1998). Since mycorrhizal fungi explore the soil volume for nutrients as part of this symbiotic transaction, it is important to better understand the resource requirements of fungi symbiosis. In particular, what are N and P requirements of the fungal partner? Answering such simple questions is difficult because our understanding of fungal C, N, and P stoichiometry and how it varies across taxonomic groups, functional guilds, and environmental conditions is poorly developed.

To address these gaps in our knowledge, here we present the first systematic compilation of C:N:P data for fungi. Our questions are as follows. What range and average values of C:N:P ratios are exhibited by fungi? Does fungal stoichiometry differ among phyla or functional guilds? Do environmental factors affect fungal stoichiometry? Answering these questions will allow stoichiometric theory to be brought to bear on key ecological processes driven by fungi.

MATERIALS AND METHODS

We performed a systematic literature search in ISI Web of Science and Google Scholar using combinations of key words including stoichiometry, element, carbon, nitrogen, phosphorus, chemical composition, mineral, nutrient, fungi, mushroom, yeast, Saccharomyces, fruit body, sporocarp, mycorrhizal, mycelium, and hyphae. We also followed cited references in the identified literature to find additional relevant studies.

Contents (percent of dry mass) or ratios of C, N, and P were extracted from published studies, either from tables or from figures by WebPlotDigitizer[1]. Studies that directly reported either absolute protein and RNA (or rRNA) content or protein:RNA ratio were also selected. In this case, studies that measured macromolecular content only under severe limitation and far from optimal growth conditions were excluded (Loladze and Elser, 2011). The protein:RNA ratio was converted to N:P ratio (28 entries for 18 species) according to a model developed by Loladze and Elser (2011). For studies that did not report %C, we used the average of C content (%C) data from the literature (44.0) in order to calculate C:N or C:P ratios from N content and P content values. Records without detailed taxonomic information (e.g., unidentified fungi) were excluded. See Supplementary Materials for the full dataset and references (Appendix S1, S2).

Fungal nomenclature followed the Index Fungorum[2]. Information for 377 fungi species (101 genera from 82 families) was assembled across a broad range of diversity including four phyla (Ascomycota, Basidiomycota, Glomeromycota, and Zygomycota). We used Funguild, a new open annotation tool, to assign the fungal species to the functional guilds (Nguyen et al., 2016). Ectomycorrhizal fungi, pathotrophs (receiving nutrients by harming host cells), and saprotrophs were selected for comparison of C:N:P stoichiometry among functional guilds (Appendix S3).

To assess possible correlations of fungal C:N:P ratios with climatic conditions, mean annual temperature, and mean annual precipitation data from 1950 to 2000 for the sampling sites of Agaricomycetes species were obtained from the WorldClim database[3] at a spatial resolution of 30 arc-seconds (ca.1 km) using Diva-GIS 7.5 software[4] (Hijmans et al., 2005). Agaricomycetes were the taxonomic group for which we had the most data, providing sufficient basis for assessment of climatic and geographic factors. Most of the element data for the correlation analysis are from fruiting bodies due to the lack of data for mycelia.

Elemental concentration and stoichiometric ratio data (%C, %N, %P, C:N, C:P, and, N:P) were log10-transformed before analyses to improve the normality of residuals. Student's *t*-test was used to compare the concentration and ratios in fungus species for two phyla, Ascomycota and Basidiomycota, for which we had a large number of observations. ANOVA followed by Tukey's *post hoc* test were used to assess the statistical significance of differences in the variables among different functional guilds. Pearson's correlation analysis was performed to check for correlations between fungal C:N:P ratios and various geographic

[1]http://arohatgi.info/WebPlotDigitizer/

[2]http://www.indexfungorum.org

[3]http://worldclim.org/current

[4]http://www.diva-gis.org

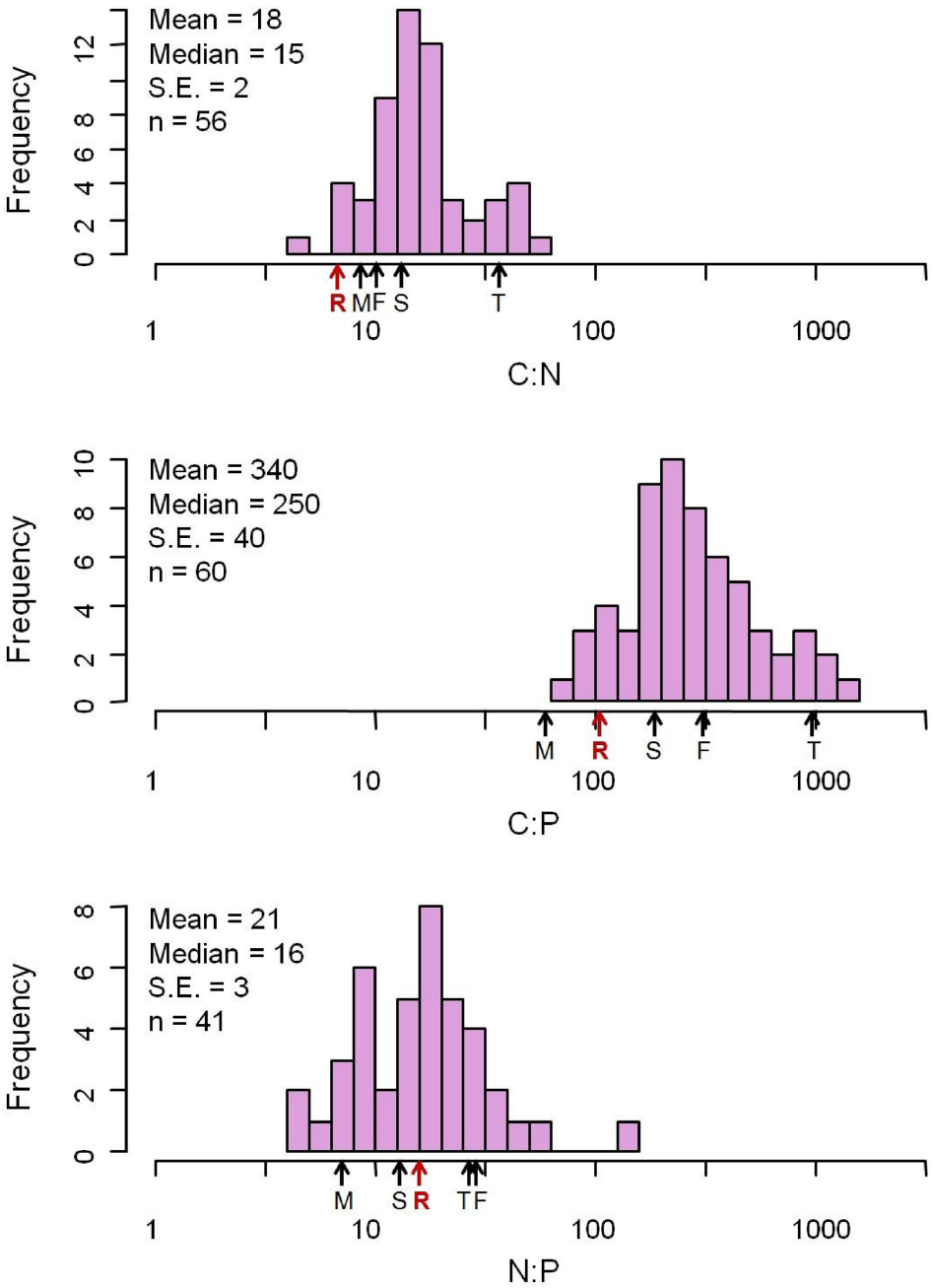

FIGURE 1 | Distribution of C:N:P molar ratios from 377 fungus species. The mean C:N:P ratios for major biota and ecosystems from previous studies are marked with arrows. F, freshwater autotrophs (Elser et al., 2000a); M, soil microbial biomass (Cleveland and Liptzin, 2007); R, Redfield (Redfield, 1958); T, terrestrial autotrophs (Elser et al., 2000a); S, soil (Cleveland and Liptzin, 2007).

and abiotic environmental factors. All analyses were performed using R 3.1.3 program (R Core Team, 2015).

RESULTS

The elemental concentrations of fungal biomass varied considerably: from 38 to 57% for %C, 0.23 to 15% for %N, and 0.040 to 5.5% for %P (Appendix S2). The median C:N:P ratio was 250:16:1 (molar, **Figure 1** and **Table 1**), a value with N:P ratio remarkably close to the canonical Redfield ratio of 16:1 (Redfield, 1958).

At the family level, significant differences ($p < 0.05$) between Ascomycota and Basidiomycota fungi in C content and C:P and N:P ratios were found (**Figure 2**). Among the three fungal guilds considered, significantly lower C:N ratio

TABLE 1 | Contents and ratios of C, N, and P in fungi at different taxonomic levels.

Taxonomic level		%C	%N	%P	C:N	C:P	N:P
Species	Mean	43.75	3.95	0.61	17.23	322.44	19.64
	Median	43.40	3.70	0.53	13.65	222.88	15.06
	Minimum	38.19	0.25	0.05	3.98	47.56	2.80
	Maximum	57.10	12.90	2.39	203.95	2085.63	141.71
	Number	128	238	232	252	246	105
	SD	2.32	1.74	0.39	16.75	303.97	18.48
Genus	Mean	44.08	3.96	0.59	18.71	327.13	19.74
	Median	43.88	3.73	0.52	14.13	242.41	15.83
	Minimum	38.80	0.52	0.06	4.50	76.70	2.80
	Maximum	57.10	11.40	1.76	126.19	1854.72	141.71
	Number	62	97	116	110	128	70
	SD	2.88	2.00	0.34	15.96	265.55	17.98
Family	Mean	44.04	3.66	0.57	18.47	344.68	21.21
	Median	44.09	3.57	0.52	15.16	249.74	16.05
	Minimum	39.73	0.97	0.10	4.55	76.70	4.39
	Maximum	50.80	8.18	1.55	52.92	1284.01	141.71
	Number	37	48	54	56	60	41
	SD	2.32	1.56	0.31	11.31	271.69	22.24

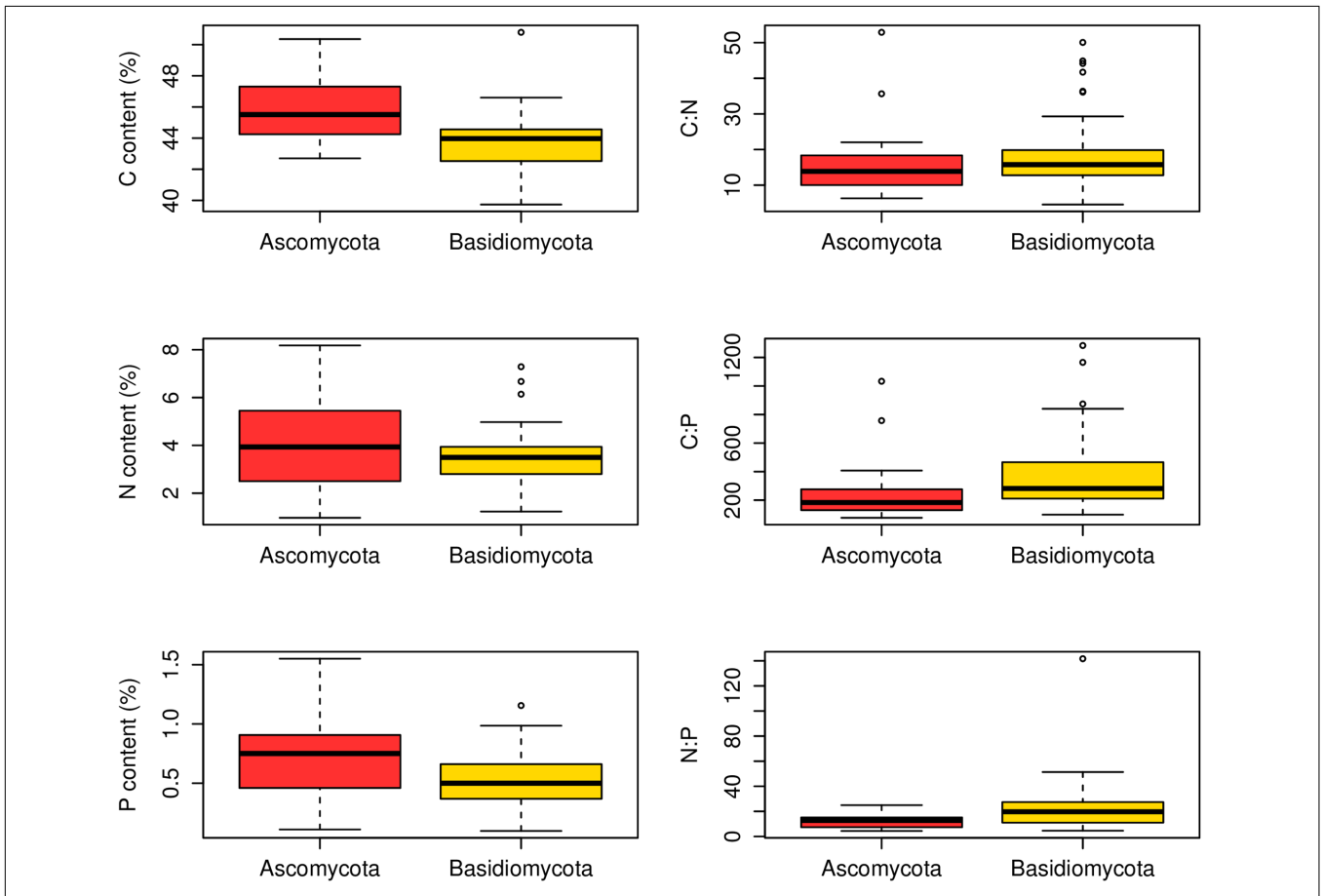

FIGURE 2 | Contents and ratios of fungi C, N, and P between different phyla. Significant differences ($p < 0.05$) between Ascomycota (23 families) and Basidiomycota (49 families) fungi were found in C content and C:P and N:P ratios.

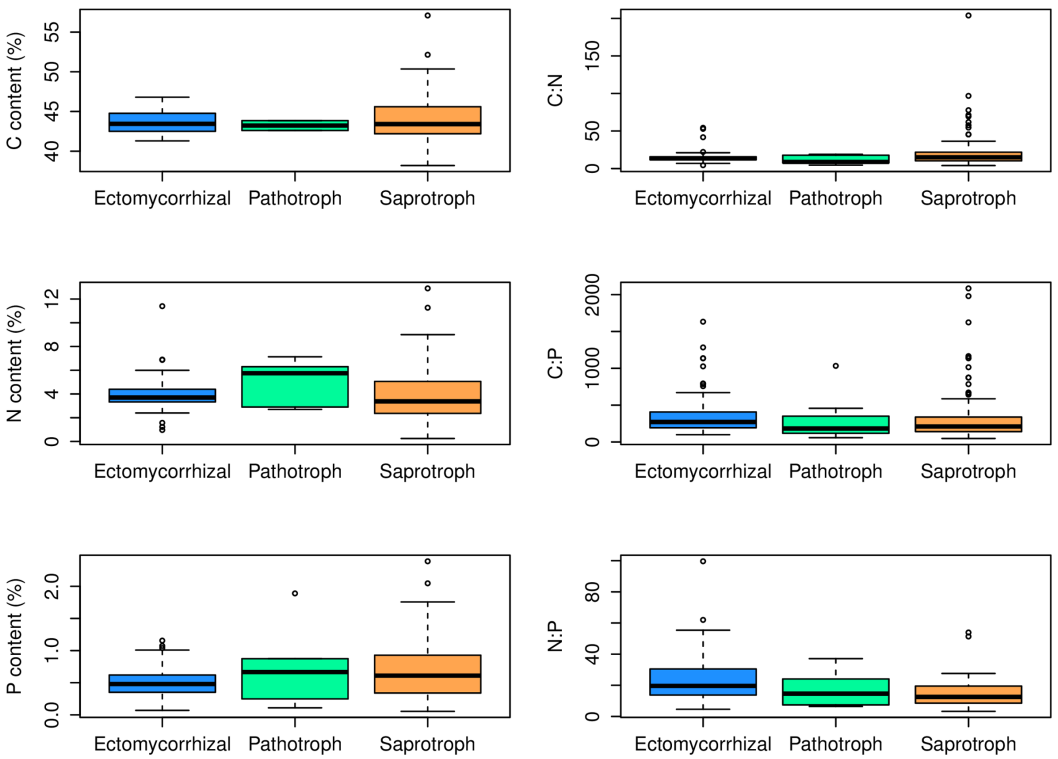

FIGURE 3 | Contents and ratios of fungi C, N, and P among different functional guilds. Significant lower C:N ratio ($p < 0.05$) and higher N:P ratio ($p < 0.01$) were found in the ectomycorrhizal fungi rather than in saprotrophs.

($p < 0.05$) and higher N:P ratio ($p < 0.01$) were observed in ectomycorrhizal species compared to saprotrophic species (**Figure 3** and **Table 2**).

N:P ratios of Agaricomycete fungi increased toward the equator, coincident with increases in average temperature and precipitation (**Table 3**). However, elemental contents and stoichiometric ratios in this group showed little direct correlation with elevation (**Table 3**).

DISCUSSION

Large-Scale Patterns of Fungal N:P

Our data reveal that elemental ratios of fungal biomass vary from 3.3 to 220 (C:N), 21 to 2800 (C:P), and 1.5 to 140 (N:P) (Appendix S2). The variation of these ratios is considerable and exceeds that reported for fungi, bacteria or whole microbial communities in previous studies (Cleveland and Liptzin, 2007; Mouginot et al., 2014; Danger et al., 2016), although the highest N:P ratio we report is less than the extremely high N:P ratio (400) of microbes from hot springs of Yellowstone National Park (Neveu et al., 2016). Overall, however, our data for fungi bear remarkable resemblance to stoichiometric properties of other taxa. Most notable is that the median N:P ratio of fungal biomass was 16:1, identical to the canonical Redfield ratio for marine phytoplankton (Redfield, 1958).

This remarkable outcome highlights the influence of a strong central tendency in N:P ratio due to the core biochemical investments associated with N-rich proteins and P-rich protein synthesis machinery (e.g., ribosomal RNA) that holds across all biota (Loladze and Elser, 2011). However, there was extremely broad variation in C:N:P ratios around this central value, variation that likely reflects both local biochemical variation due to physiological adjustment to environmental conditions (Klausmeier et al., 2004) but also broader phylogenetic influences of different fungal taxa exhibiting different life history strategies in particular environments.

The poleward decline of N:P in our data (**Table 3**) also bears a striking similarity to patterns seen for other biota and ecosystems. For example, leaf N:P ratios decline with latitude (McGroddy et al., 2004; Reich and Oleksyn, 2004; Kerkhoff et al., 2005) as do N:P ratios in multicellular and unicellular photoautotrophs in freshwater and marine ecosystems (Borer et al., 2013; Martiny et al., 2013). Elser et al. (2000b) reported a similar poleward increase in P demands in the crustacean *Daphnia*, invoking selection for rapid growth (and thus P-rich RNA) due to the short growing season as an explanation. Decreases in soil microbial N:P ratios on the Tibetan Plateau as a function of latitude were reported by (Chen et al., 2016) and were associated with changes in soil microbial community structures. However, Cleveland and Liptzin (2007) found that microbial N:P ratios in soil microbial biomass were constrained at the global scale and did not show a latitudinal pattern.

TABLE 2 | Contents and ratios of fungal C, N, and P among different functional guilds.

Functional guild		%C	%N	%P	C:N	C:P	N:P
Ectomycorrhizal fungi	Mean	43.67	3.89	0.58	14.44	348.30	24.61
	Median	43.45	3.70	0.48	13.65	270.13	19.75
	Minimum	41.30	0.97	0.07	4.50	10.63	4.58
	Maximum	46.80	11.40	4.60	54.33	1633.14	99.64
	Number	84	135	90	135	91	37
	SD	1.41	1.10	0.64	6.08	271.92	18.28
Pathotroph	Mean	42.60	4.41	6.90	13.04	317.77	11.92
	Median	42.60	4.33	0.87	13.09	195.46	7.38
	Minimum	42.60	2.70	0.11	7.13	58.37	6.39
	Maximum	42.60	6.30	43.85	19.01	1033.33	24.06
	Number	1	4	7	6	7	5
	SD		1.88	16.30	5.63	341.38	7.55
Saprotroph	Mean	43.86	5.66	1.28	21.19	312.04	55.69
	Median	43.30	3.29	0.65	14.16	192.27	13.12
	Minimum	38.19	0.25	0.055	0.30	1.17	3.24
	Maximum	57.10	47.40	42.30	203.95	2085.63	473.33
	Number	28	71	102	86	112	62
	SD	3.83	9.01	4.22	27.02	360.57	106.69

TABLE 3 | Pearson's correlation coefficients for the Agaricomycete fungi C, N, and P contents and ratios and four abiotic environmental variables.

Trait	Absolute latitude	Elevation	Mean annual precipitation	Mean annual temperature
C	−0.174**	−0.089	−0.125*	0.147**
N	−0.075	−0.025	−0.133*	0.144**
P	−0.062	−0.006*	−0.244**	−0.075
C:N	−0.099	−0.020	−0.244**	0.025
C:P	−0.071	−0.143*	−0.101	0.229**
N:P	−0.246**	−0.108	−0.191*	0.341**

*$p < 0.05$, **$p < 0.01$.

Foliar nutrient contents tend to increase with altitude (Körner, 1989). In contrast to studies of foliar N:P ratios in plants along mountain slopes (Morecroft and Woodward, 1996; van de Weg et al., 2009), we found no correlation of fungal N:P ratios with elevation. This may have been the result of a confounding effect driven by the negative relationship between absolute latitude and elevation of the sampling sites in our dataset (Appendix S2).

In general, foliar N:P ratios increase with temperature across large geographic gradients (Reich and Oleksyn, 2004). We found a similar trend in fungal N:P ratio (**Table 3**). However, while temperature explained a substantial proportion of the total variability in leaf P (Reich and Oleksyn, 2004), variation in fungal P showed no detectable association with temperature. This may be due, in part, to the smaller temperature range from 1.5°C to 26.6°C in our data set compared with the −12.8°C to 28.0°C range considered by Reich and Oleksyn (2004). As most of the element data for the correlation analysis in our study are from fruiting bodies, further work is needed on the effects of actual *in situ* growth conditions (temperature and humidity) of fungal growth on stoichiometry in the mycelium.

While fungal N:P ratios changed with environmental conditions, it is unclear if the patterns we observe reflect shifts in the species present (with each species stoichiometrically homeostatic around a particular value) or if the ratios might vary if a given species was grown across the full range of environmental (soil, temperature, N, P, etc) conditions. This issue requires further investigation. How resource stoichiometry affects fungi is of particular interest (Danger et al., 2016) but data on nutrient availability are found in only a few studies. For instance, in terrestrial fungi, phylogenetically related strains can have distinct stoichiometric ratios despite the same growth conditions (Mouginot et al., 2014). In aquatic fungi, Leach and Gulis (2010) reported that fungal biomass is homeostatic with respect to C:N and N:P but is weakly homeostatic with respect to C:P. However, Danger and Chauvet (2013) found highly plastic C:P and N:P ratios in aquatic hyphomycetes as a function of external nutrient supply.

Stoichiometry in Mycorrhizal Symbioses

While the central similarity of average fungal C:N:P stoichiometry to previous observations is notable, the differences of N:P among fungal phyla that we observed needs further investigation. At least some of this variation may be associated with different functional guilds within fungal groups (Talbot et al., 2015). For example, in this study, ectomycorrhizal fungi had lower C:N ratio and higher N:P ratio compared with

saprotrophs, supporting a role for trophic guild in influencing fungal C:N:P stoichiometry. The ectomycorrhizal fungal lifestyle has evolved multiple times from saprotrophic lineages of wood and litter decomposers through convergent evolution (van der Heijden et al., 2015). Contrasting effects of nitrogen availability on plant carbon supply to mycorrhizal fungi and saprotrophs may exist (Högberg et al., 2003). A considerable amount of research has been aimed at assessing the ability of ectomycorrhizal fungi to use organic nitrogen sources (Chalot and Brun, 1998). Ectomycorrhizal fungi benefit from organic matter decomposition primarily through increased nitrogen mobilization (Lindahl and Tunlid, 2015). Compared to saprotrophs, mycorrhizal fungi have a stable supply of C from their hosts, so they may exploit organic substrates selectively for N and other nutrients (Bödeker et al., 2016). A recent study has shown that ectomycorrhizal species have lower N content than saprotrophic fungal species (Trocha et al., 2016). However, we found that the N content of saprotrophic fungal species had a wider range than ectomycorrhizal species but the average N content did not differ significantly between the two guilds. We suggest that additional information about the C:N:P stoichiometry of functional groups of fungi may shed considerable light on the nature of various symbioses as well as on the functioning of various fungi as a reflection of their functional group.

Implications for Soil Nutrient Cycling

Soil microbial communities strongly affect element cycling at the ecosystem scale by adjusting the rates of various element acquisition processes (organic matter decomposition, N_2 fixation, and P solubilization) to acquire limiting resources or by adjusting partitioning and turnover times in the microbial biomass (Spohn, 2016). For instance, global nitrogen-release patterns can be explained by fundamental stoichiometric relationships of decomposer activity (Manzoni et al., 2008). Heterotrophic microbial communities, which drive much of the nutrient cycling in soils, have received increasing interest in recent years (Zechmeister-Boltenstern et al., 2015). Indeed, Cleveland and Liptzin (2007) suggested that an average soil microbial N:P ratio might be a more appropriate index of ecosystem nutrient limitation than plant N:P ratios. A recent study showed that the biogeochemical consequences of N deposition in temperate forests may be driven by the stoichiometry of the dominant trees and their associated microbes (Midgley and Phillips, 2016) while van Diepen et al. (2017) found that fungi exposed to chronic nitrogen enrichment are less able to decay leaf litter. All of these studies point to the importance of multi-resource interactions in nutrient cycling in soils. To advance our understanding of how fungi contribute to these processes, further studies are needed of the relationship between fungal and soil C:N:P ratios as a function of nutrient supply, temperature, and precipitation in order to establish the strength of C:N:P stoichiometric homeostasis in soil fungi. This information will help in many areas of terrestrial ecosystem ecology, including in improving our models of the impacts of fungi on N and P immobilization vs. mineralization.

Our analysis indicates extremely broad variation in fungal C:N:P ratios around a core central tendency in N:P ratio that is notably similar to observations for other biota. Our analyses suggest that variation in fungal C:N:P stoichiometry likely reflects both local biochemical variation due to physiological adjustment to environmental conditions but also broader phylogenetic influences of different fungal guilds in particular environments.

AUTHOR CONTRIBUTIONS

JZ and JE conceived and designed the study; JZ analyzed the data; JZ and JE wrote the paper.

FUNDING

This work was supported by the National Natural Science Foundation of China (31460538) and by the J. Bierman fund at the Flathead Lake Biological Station.

ACKNOWLEDGMENT

We thank Diana Six for her helpful comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01281/full#supplementary-material

REFERENCES

Andersen, T., Elser, J. J., and Hessen, D. O. (2004). Stoichiometry and population dynamics. *Ecol. Lett.* 7, 884–900. doi: 10.1111/j.1461-0248.2004.00646.x

Bärlocher, F., and Boddy, L. (2016). Aquatic fungal ecology – How does it differ from terrestrial? *Fungal Ecol.* 19, 5–13. doi: 10.1016/j.funeco.2015.09.001

Behie, S. W., and Bidochka, M. J. (2014). Nutrient transfer in plant–fungal symbioses. *Trends Plant Sci.* 19, 734–740. doi: 10.1016/j.tplants.2014.06.007

Bödeker, I. T. M., Lindahl, B. D., Olson, Å., and Clemmensen, K. E. (2016). Mycorrhizal and saprotrophic fungal guilds compete for the same organic substrates but affect decomposition differently. *Funct. Ecol.* 30, 1967–1978. doi: 10.1111/1365-2435.12677

Borer, E. T., Bracken, M. E. S., Seabloom, E. W., Smith, J. E., Cebrian, J., Cleland, E. E., et al. (2013). Global biogeography of autotroph chemistry: is insolation a driving force? *Oikos* 122, 1121–1130. doi: 10.1111/j.1600-0706.2013.00465.x

Chalot, M., and Brun, A. (1998). Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. *FEMS Microbiol. Rev.* 22, 21–44. doi: 10.1111/j.1574-6976.1998.tb00359.x

Chen, Y., Chen, L., Peng, Y., Ding, J., Li, F., Yang, G., et al. (2016). Linking microbial C:N:P stoichiometry to microbial community and abiotic factors along a 3500-km grassland transect on the Tibetan Plateau. *Glob. Ecol. Biogeogr.* 25, 1416–1427. doi: 10.1111/geb.12500

Cleveland, C. C., and Liptzin, D. (2007). C:N:P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? *Biogeochemistry* 85, 235–252.

Danger, M., and Chauvet, E. (2013). Elemental composition and degree of homeostasis of fungi: are aquatic hyphomycetes more like metazoans, bacteria or plants? *Fungal Ecol.* 6, 453–457. doi: 10.1016/j.funeco.2013.05.007

Danger, M., Gessner, M. O., and Bärlocher, F. (2016). Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. *Fungal Ecol.* 19, 100–111. doi: 10.1016/j.funeco.2015.09.004

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000a). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580. doi: 10.1038/35046058

Elser, J. J., O'Brien, W. J., Dobberfuhl, D. R., and Dowling, T. E. (2000b). The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* 13, 845–853. doi: 10.1046/j.1420-9101.2000.00215.x

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., et al. (2000c). Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* 3, 540–550. doi: 10.1111/j.1461-0248.2000.00185.x

Frost, P. C., Stelzer, R. S., Lamberti, G. A., and Elser, J. J. (2002). Ecological stoichiometry of trophic interactions in the benthos: understanding the role of C:N:P ratios in lentic and lotic habitats. *J. N. Am. Benthol. Soc.* 21, 515–528. doi: 10.2307/1468427

Gadd, G. M. (2004). Mycotransformation of organic and inorganic substrates. *Mycologist* 18, 60–70. doi: 10.1017/S0269915X04002022

Gadd, G. M. (2007). Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. *Mycol. Res.* 111, 3–49. doi: 10.1016/j.mycres.2006.12.001

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G., and Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *Int. J. Climatol.* 25, 1965–1978. doi: 10.1002/joc.1276

Högberg, M. N., Bååth, E., Nordgren, A., Arnebrant, K., and Högberg, P. (2003). Contrasting effects of nitrogen availability on plant carbon supply to mycorrhizal fungi and saprotrophs - a hypothesis based on field observations in boreal forest. *New Phytol.* 160, 225–238. doi: 10.1046/j.1469-8137.2003.00867.x

Kerkhoff, A. J., Enquist, B. J., Elser, J. J., and Fagan, W. F. (2005). Plant allometry, stoichiometry and the temperature-dependence of primary productivity. *Glob. Ecol. Biogeogr.* 14, 585–598. doi: 10.1111/j.1466-822X.2005.00187.x

Kerkhoff, A. J., Fagan, W. F., Elser, J. J., and Enquist, B. J. (2006). Phylogenetic and growth form variation in the scaling of nitrogen and phosphorus in the seed plants. *Am. Nat.* 168, E103–E122. doi: 10.1086/507879

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2004). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174. doi: 10.1038/nature02454

Körner, C. (1989). The nutritional status of plants from high altitudes. *Oecologia* 81, 379–391. doi: 10.1007/BF00377088

Landeweert, R., Hoffland, E., Finlay, R. D., Kuyper, T. W., and van Breemen, N. (2001). Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends Ecol. Evol.* 16, 248–254. doi: 10.1016/S0169-5347(01)02122-X

Leach, D., and Gulis, V. (2010). *Microbial Stoichiometry and Homeostasis of Nutrient Ratios in Fungi. Bridges 5*. Available at: http://citeseerx.ist.psu.edu/viewdoc/download?

Lindahl, B. D., and Tunlid, A. (2015). Ectomycorrhizal fungi - potential organic matter decomposers, yet not saprotrophs. *New Phytol.* 205, 1443–1447. doi: 10.1111/nph.13201

Loladze, I., and Elser, J. J. (2011). The origins of the Redfield nitrogen-to-phosphorus ratio are in a homoeostatic protein-to-rRNA ratio. *Ecol. Lett.* 14, 244–250. doi: 10.1111/j.1461-0248.2010.01577.x

Manzoni, S., Jackson, R. B., Trofymow, J. A., and Porporato, A. (2008). The global stoichiometry of litter nitrogen mineralization. *Science* 321, 684–686. doi: 10.1126/science.1159792

Martiny, A. C., Pham, C. T. A., Primeau, F. W., Vrugt, J. A., Moore, J. K., Levin, S. A., et al. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci.* 6, 279–283. doi: 10.1038/NGEO1757

McGroddy, M. E., Daufresne, T., and Hedin, L. O. (2004). Scaling of C:N:P stoichiometry in forests worldwide: implications of terrestrial Redfield-type ratios. *Ecology* 85, 2390–2401. doi: 10.1890/03-0351

Midgley, M. G., and Phillips, R. P. (2016). Resource stoichiometry and the biogeochemical consequences of nitrogen deposition in a mixed deciduous forest. *Ecology* 97, 3369–3378. doi: 10.1002/ecy.1595

Morecroft, M. D., and Woodward, F. I. (1996). Experiments on the causes of altitudinal differences in the leaf nutrient contents, size and δ13C of *Alchemilla alpina*. *New Phytol.* 134, 471–479. doi: 10.1111/j.1469-8137.1996.tb04364.x

Mouginot, C., Kawamura, R., Matulich, K. L., Berlemont, R., Allison, S. D., Amend, A. S., et al. (2014). Elemental stoichiometry of fungi and bacteria strains from grassland leaf litter. *Soil Biol. Biochem.* 76, 278–285. doi: 10.1016/j.soilbio.2014.05.011

Neveu, M., Poret-Peterson, A. T., Anbar, A. D., and Elser, J. J. (2016). Ordinary stoichiometry of extraordinary microorganisms. *Geobiology* 14, 33–53. doi: 10.1111/gbi.12153

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., et al. (2016). FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* 20, 241–248. doi: 10.1016/j.funeco.2015.06.006

R Core Team (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Redfield, A. C. (1958). The biological control of chemical factors in the environment. *Am. Sci.* 46, 205–221.

Reich, P. B., and Oleksyn, J. (2004). Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11001–11006. doi: 10.1073/pnas.0403588101

Schwartz, M. W., and Hoeksema, J. D. (1998). Specialization and resource trade: biological markets as a model of mutualisms. *Ecology* 79, 1029–1038.

Sinsabaugh, R. L., Hill, B. H., and Follstad Shah, J. J. (2009). Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. *Nature* 462, 795–798. doi: 10.1038/nature08632

Spohn, M. (2016). Element cycling as driven by stoichiometric homeostasis of soil microorganisms. *Basic Appl. Ecol.* 17, 471–478. doi: 10.1016/j.baae.2016.05.003

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Talbot, J. M., Martin, F., Kohler, A., Henrissat, B., and Peay, K. G. (2015). Functional guild classification predicts the enzymatic role of fungi in litter and soil biogeochemistry. *Soil Biol. Biochem.* 88, 441–456. doi: 10.1016/j.soilbio.2015.05.006

Trocha, L. K., Rudy, E., Chen, W., Dabert, M., and Eissenstat, D. M. (2016). Linking the respiration of fungal sporocarps with their nitrogen concentration: variation among species, tissues and guilds. *Funct. Ecol.* 30, 1756–1768. doi: 10.1111/1365-2435.12688

van de Weg, M. J., Meir, P., Grace, J., and Atkin, O. K. (2009). Altitudinal variation in leaf mass per unit area, leaf tissue density and foliar nitrogen and phosphorus content along an Amazon-Andes gradient in Peru. *Plant Ecol. Divers.* 2, 243–254. doi: 10.1080/17550870903518045

van der Heijden, M. G. A., Martin, F. M., Selosse, M., and Sanders, I. R. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytol.* 205, 1406–1423. doi: 10.1111/nph.13288

van Diepen, L. T. A., Frey, S. D., Landis, E. A., Morrison, E. W., and Pringle, A. (2017). Fungi exposed to chronic nitrogen enrichment are less able to decay leaf litter. *Ecology* 98, 5–11. doi: 10.1002/ecy.1635

Waring, B. G., Averill, C., and Hawkes, C. V. (2013). Differences in fungal and bacterial physiology alter soil carbon and nitrogen cycling: insights from meta-analysis and theoretical models. *Ecol. Lett.* 16, 887–894. doi: 10.1111/ele.12125

Woods, H. A., Fagan, W. F., Elser, J. J., and Harrison, J. F. (2004). Allometric and phylogenetic variation in insect phosphorus content. *Funct. Ecol.* 18, 103–109. doi: 10.1111/j.1365-2435.2004.00823.x

Zechmeister-Boltenstern, S., Keiblinger, K. M., Mooshammer, M., Peñuelas, J., Richter, A., Sardans, J., et al. (2015). The application of ecological stoichiometry to plant–microbial–soil organic matter transformations. *Ecol. Monogr.* 85, 133–155. doi: 10.1890/14-0777.1

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 30 May 2017
doi: 10.3389/fmicb.2017.00949

Nutrient Stoichiometry Shapes Microbial Community Structure in an Evaporitic Shallow Pond

*Zarraz M.-P. Lee[1], Amisha T. Poret-Peterson[2†], Janet L. Siefert[3], Drishti Kaul[4], Ahmed Moustafa[5], Andrew E. Allen[4,6], Chris L. Dupont[4], Luis E. Eguiarte[7], Valeria Souza[7] and James J. Elser[1,8]**

[1] School of Life Sciences, Arizona State University, Tempe, AZ, United States, [2] School of Earth and Space Exploration, Arizona State University, Tempe, AZ, United States, [3] Department of Statistics, Rice University, Houston, TX, United States, [4] J. Craig Venter Institute, La Jolla, CA, United States, [5] Department of Biology and Biotechnology Graduate Program, American University in Cairo, New Cairo, Egypt, [6] Integrative Oceanography Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA, United States, [7] Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Ciudad de México, Mexico, [8] Flathead Lake Biological Station, University of Montana, Polson, MT, United States

Nutrient availability and ratios can play an important role in shaping microbial communities of freshwater ecosystems. The Cuatro Ciénegas Basin (CCB) in Mexico is a desert oasis where, perhaps paradoxically, high microbial diversity coincides with extreme oligotrophy. To better understand the effects of nutrients on microbial communities in CCB, a mesocosm experiment was implemented in a stoichiometrically imbalanced pond, Lagunita, which has an average TN:TP ratio of 122 (atomic). The experiment had four treatments, each with five spatial replicates – unamended controls and three fertilization treatments with different nitrogen:phosphorus (N:P) regimes (P only, N:P = 16 and N:P = 75 by atoms). In the water column, quantitative PCR of the 16S rRNA gene indicated that P enrichment alone favored proliferation of bacterial taxa with high rRNA gene copy number, consistent with a previously hypothesized but untested connection between rRNA gene copy number and P requirement. Bacterial and microbial eukaryotic community structure was investigated by pyrosequencing of 16S and 18S rRNA genes from the planktonic and surficial sediment samples. Nutrient enrichment shifted the composition of the planktonic community in a treatment-specific manner and promoted the growth of previously rare bacterial taxa at the expense of the more abundant, potentially endemic, taxa. The eukaryotic community was highly enriched with phototrophic populations in the fertilized treatment. The sediment microbial community exhibited high beta diversity among replicates within treatments, which obscured any changes due to fertilization. Overall, these results showed that nutrient stoichiometry can be an important factor in shaping microbial community structure.

Keywords: stoichiometry, community structure, beta diversity, bacteria, algae, rRNA gene copy number, growth rate hypothesis

OPEN ACCESS

Edited by:
George S. Bullerjahn, Bowling Green State University, United States

Reviewed by:
Ryan J. Newton, University of Wisconsin–Milwaukee, United States
Benjamin R. K. Roller, ETH Zurich, Switzerland

****Correspondence:***
James J. Elser
j.elser@asu.edu

†Present address:
Amisha T. Poret-Peterson, Crops Pathology and Genetics Research Unit, University of California, Davis – United States Department of Agriculture–Agricultural Research Service, Davis, CA, United States

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *13 December 2016*
Accepted: *11 May 2017*
Published: *30 May 2017*

Citation:
Lee ZM-P, Poret-Peterson AT, Siefert JL, Kaul D, Moustafa A, Allen AE, Dupont CL, Eguiarte LE, Souza V and Elser JJ (2017) Nutrient Stoichiometry Shapes Microbial Community Structure in an Evaporitic Shallow Pond. Front. Microbiol. 8:949. doi: 10.3389/fmicb.2017.00949

INTRODUCTION

The absolute and relative supplies of nitrogen (N) and phosphorus (P) in the environment have a major influence on the diversity of species at macro- and microscopic scales (Elser et al., 2005b; Leflaive et al., 2008). Hence, the availabilities and ratios of key limiting nutrients, such as N and P, have been suggested to be fundamental in understanding microbial diversity (Torsvik et al., 2002; Newton et al., 2011; Groszkopf and Soyer, 2016). Indeed, studies involving natural gradients or *in situ* experimental manipulation across different time scales and environments show that nutrient availability affects biodiversity. When responses are observed, effects range from little impact to large alterations in community structure, reflected as changes in species richness often accompanied by shifts in dominance/evenness (Claire Horner-Devine et al., 2003; Hewson et al., 2003; Bowen et al., 2011; Van Horn et al., 2011; Logue et al., 2012; Soininen and Meier, 2014). Microbial community responses to nutrients are likely rooted in the metabolic diversity and ecological strategies of the responsive taxa (Carbonero et al., 2014); therefore, attention should also be given to the ability of individual taxonomic groups or specific taxa to access and use nutrient inputs (Haukka et al., 2006; Nelson and Carlson, 2011; Peura et al., 2012; Corman et al., 2016). However, our ability to predict how various microbial taxa respond to nutrient enrichment is still limited.

More recently, the theory of biological stoichiometry had been used to integrate evolutionary biology and ecosystem ecology in both macro- and microbiology (Elser, 2003; Hall et al., 2011). Biological stoichiometry provides a mechanistic theory that links cellular and biochemical features of biota with the environmental constraints imposed by the supplies of multiple limiting nutrients, especially N and P (Elser et al., 2006; Hillebrand et al., 2014). Thus, understanding variation in biomass carbon:nitrogen:phosphorus (C:N:P) stoichiometry provides an avenue to understand community responses to nutrient supply. In particular, the Growth Rate Hypothesis, GRH (Elser et al., 2000), postulates that an organism's C:N:P stoichiometric requirements are dependent on its growth rate because elevated growth rate depends on increased production and maintenance of P-rich ribosomes. Various field and laboratory studies have shown that growth, RNA content (percent of dry mass), and biomass P content are often tightly coupled within and across species, and especially under physiological P limitation (Elser et al., 2003; Makino and Cotner, 2004; Klausmeier et al., 2007; Chan et al., 2012; Hessen et al., 2013). Furthermore, it has been proposed that maximum growth rate, as a key life history parameter, and ribosome production capacity have a genomic basis in the multiplicity of ribosomal RNA operons (rRNA gene copy number) (Weider et al., 2005; Stoddard et al., 2014; Roller et al., 2016). This is consistent with studies that have shown that microbes with fewer copies of rRNA genes tend to be more competitive in oligotrophic or low nutrient conditions due to their high efficiency in resource use, whereas copiotrophs with higher rRNA gene copy numbers tend to respond more rapidly and are adapted to high nutrient supply and episodic availability (Klappenbach et al., 2000; Lauro et al., 2009; Nemergut et al., 2016; Roller et al., 2016). Thus, it is expected that P availability should have an especially significant effect on fast-growing taxa and/or those with elevated rRNA gene copy number and, consequently, overall community structure. However, the rRNA gene copy number hypothesis has not yet been experimentally tested under field conditions. Hence, assessing impacts of nutrient supply and stoichiometry in P-limited ecosystems may help in understanding the underlying mechanism of how nutrients shape microbial community structure.

Microbe-dominated, P-limited aquatic ecosystems suitable for such tests are found in the Cuatro Ciénegas basin (CCB), an oasis of oligotrophic springs in the Chihuahuan desert in the state of Coahuila in northwest Mexico. CCB contains diverse and often endemic microbes and macroorganisms (Souza et al., 2006, 2012) that persist in aquatic ecosystems with very low available P concentrations as well as strong stoichiometric imbalance with nitrogen (N:P ratios commonly exceed 100:1 by atoms). Hence, these ecosystems are strongly limited by P but with secondary impacts of N (Elser et al., 2005a; Lee et al., 2015), making them an excellent system to study the effects of nutrient ratios and to evaluate the GRH (Elser et al., 2000, 2005a,b). In this study, we use spatially replicated *in situ* mesocosms to investigate the effects of altered nutrient stoichiometry on bacterial and microbial eukaryotic communities in Lagunita, an evaporative pond in the CCB. We previously reported large effects of this fertilization on nutrient pools, biomass concentrations, and biomass C:N:P stoichiometry (Lee et al., 2015). Here, we describe the impact of nutrient stoichiometry on microbial community composition using high-throughput pyrosequencing of bacterial and eukaryotic small subunit (SSU) rRNA genes.

MATERIALS AND METHODS

Study Location and Experimental Design

The *in situ* nutrient enrichment experiment was conducted during summer 2011 in a small (∼12 m × 4 m on average) shallow (<0.33 m) evaporitic pond, Lagunita (26.84810° N, 102.14160° W), lateral to the main Churince flow system in CCB in the state of Coahuila, Mexico. The Churince system is located at the western region of CCB and is dominated by gypsum-rich sediments. Lagunita is characterized by low P concentrations (PO_4^{3-} as low as 0.1 μM) but relatively high concentrations of inorganic N and thus high N:P ratios (>200:1 by atoms) (Lee et al., 2015). Lagunita is also low in macrophyte abundance, reducing the potential confounding factor of plant–microbe interactions.

The mesocosm experiment was described in detail in Lee et al. (2015). Briefly, the mesocosms consisted of clear plastic cylinders (40-cm diameter) that were inserted into the pond sediments and extended above the water surface by 20 cm. Five replicated blocks of four treatments were established along an east–west transect of the pond. The treatments were unenriched (U), P-only (P), N and P at N:P = 16 (NP16), and N:P = 75 (NP75). P was applied as KH_2PO_4 while N was applied as NH_4NO_3. Nutrients were re-applied every 3–4 days to maintain a soluble reactive phosphorus (SRP) concentration of 1 μM (an

approximate 16-fold increase over initial SRP concentration of 0.06 ± 0.02 μM) and appropriate N:P ratio. This fertilization regime was maintained for 21 days. Pre-fertilization values of total P and total N in the water column were 1.79 ± 0.20 μM and 187 ± 8.58 μM, respectively. After 3 weeks of periodic fertilization, P addition increased total P in fertilized treatments by >3.5-fold, while total N in the NP16 and NP75 mesocosms increased by >40% and >3-fold, respectively (Lee et al., 2015).

Sample Collection and DNA Extraction

Water column and sediment samples were collected on day 21. Water column samples were collected by filtering 120 – 140 mL of water onto sterile GF/F filters (0.7-μm nominal pore size, Whatman, Piscataway, NJ, United States). GF/F filters were used in order to capture sufficient amount of biomass for DNA extraction. Sediment samples were collected by scooping the top ∼2 mm of the surface with a plastic spatula into cryovials. Both water column and sediment samples were flash-frozen in liquid N_2 and stored at −80°C until extraction.

DNA was extracted from water column samples using the MO BIO PowerWater DNA Isolation kit, with one modification (Mo Bio Laboratories, Carlsbad, CA, United States). The volume of PW1 solution was increased to 1.5 mL due to the high absorbency of the GF/F filters. The DNA extraction method for sediment samples was modified from Purdy (2005). Briefly, frozen sediment was thawed by centrifugation to remove pore water. The sediment was then transferred into a MO BIO Bead tube for mechanical lysis in a FastPrep® -24 (MP Biomedicals, Solon, OH, United States) in a solution of Tris-buffered phenol and acid washed polyvinylpolypyrrolidone (PVPP). The extracted DNA was purified by column filtration through Sephadex G-50 (Sigma, St. Louis, MO, United States) and Bio-Gel HTP hydroxyapatite (Bio-Rad, Hercules, CA, United States) followed by ethanol precipitation. DNA yield and quality were assessed by Picogreen assay (Life Technologies, Carlsbad, CA, United States) and PCR amplification as described below.

qPCR Analysis

A measure of 16S rRNA gene copy number of water column samples was determined using quantitative PCR (qPCR) with primers targeting the V6 region of the gene (967F and 1046R) (Huber et al., 2009). qPCR was performed on 10-fold dilutions of DNA extracts. A standard curve was constructed from plasmid containing a cloned V6 region of 16S rRNA gene from *Bacillus* sp. m3-13 and ranged from 9.5×10^2 to 9.5×10^7 copies μL^{-1} with efficiencies of 98% or higher. Triplicate 10-μL qPCR reactions were performed for each sample in a PikoReal 96 Real-Time PCR system (Thermo Scientific, Inc., Waltham, MA, United States). Each reaction contained 1 μl DNA template, 1X DyNAmo ColorFlash SYBR Green qPCR master mix and 500 nM of each primer. The cycling parameters were as follows: (1) initial denaturation at 95°C for 7 min, (2) 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s with fluorescence capture after extension, (3) final extension at 72°C for 30 s, and (4) melt curve analysis from 55 to 95°C. The qPCR results were used to calculate the abundance 16S rRNA gene copies as gene copies per ml water (copies mL^{-1}). Gene copy number was then divided by the number of cells per ml water (cells ml^{-1}) obtained from epifluorescence microscopy.

Differences in 16S rRNA gene copy number (normalized to bacterial cell counts) between the unenriched and fertilized treatments were assessed by analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* tests to compare individual groups. Only water samples were analyzed due to interferences with qPCR of sediment-derived DNA.

SSU rRNA Gene Sequence Processing and Analyses

The 16S rRNA gene V4-V5 region and 18S rRNA gene V4 region was PCR amplified from all DNA samples with barcoded primers. The 16S forward primer combined the 357F primer (CCTACGGGAGGCAGCAG) with the Titanium B adapter (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG). The 16S reverse primer combined the 926R primer (CCGTCAATTCMTTTRAGT) with the Titanium A adapter (CCATCTCATCCCTGCGTGTCTCCGACTCAG) with a 10 nucleotide barcode in between. The primer set 357F/926R targets >96% of bacteria; however, it does not capture 16S rRNA genes from Epsilonproteobacteria and Archaea when tested using SILVA TestPrime (Klindworth et al., 2013; see Supplementary Figure 3 in Dupont et al., 2014). The 18S primers were similarly designed but use the EukV4F (CCAGCASCYGCGGTAATTCC) and EukV4R (ACTTTCGTTCTTGATYRA) primers. The amplicons were checked by gel electrophoresis and submitted to JCVI for sequencing. Amplicons for each sample were quantified by qPCR and pooled prior to pyrosequencing using the 454 Titanium pipeline (454 Life Sciences, Branford, CT, United States). The sequences are available at NCBI under the accession number PRJNA311559.

Reads were de-multiplexed according to the barcodes and trimmed of barcodes and adapters. Taxonomic affiliation of the aligned reads was determined using the SILVA database by BLAST search to generate phylotypes, which is specific to the genus level (Quast et al., 2013). All sequences that passed the sequence processing screen, including those that could only be classified as Bacteria or Eukarya, were included in downstream analyses. To compare the difference in community structure, relative abundance of each phylotype was calculated as percent sequence abundance for each library. The 50 most abundant phylotypes among all the samples are presented as a heatmap using the ggplot2 package v 2.1.0 in R (Wickham, 2009).

To determine if fertilization had significant effects on planktonic and sediment community composition or structure, several statistical approaches were applied on normalized libraries using the cumulative sum scaling method (Paulson et al., 2013). Phylotype data were used for all statistical analyses to allow the same method of sequence analysis for both bacterial and eukaryotic sequence libraries. The more conservative phylotype-based approach was selected because the sequence similarity value for operational taxonomic unit (OTU) calling of 18S rRNA gene sequences into OTUs is

still debatable due to the heterogenous evolution rate for this gene among different eukaryotic taxa (Caron et al., 2009; Bik et al., 2012). Community alpha diversity metrics were calculated in mothur and analyzed via ANOVA with *post hoc* Tukey's test ($p \leq 0.05$). The Bray–Curtis calculated distance matrices for planktonic and sediment communities were analyzed via permutational MANOVA (perMANOVA) to test for significance in overall differences in community composition between treatments. Bray–Curtis distance matrices were also used for beta diversity analysis and statistical testing using the QIIME (v.1.9.1) make_distance_boxplot.py script (Caporaso et al., 2010). EdgeR was used to detect significant changes in abundances of phylotypes using the edgeR package v 3.12.1 in R (Robinson et al., 2010). Log_2 ratio of fold-changes (log_2FC) in abundance of each phylotype were calculated for each fertilized treatment relative to the unenriched treatment. The diversity metric and relative abundance calculations were done on a replicate-by-replicate basis but for the sake of clarity, the visualizations (e.g., **Figures 1, 3, 6, 8**) used replicates pooled by treatment.

Phylotypes are genus-specific taxonomic units identified based on a supervised classification method using reference sequences. However, each phylotype may contain sequences from microbes with differing ecology that can be further resolved based on sequence similarity. Phylotypes of interest were explored further by binning sequences within a phylotype into OTUs. OTU identification is an unsupervised clustering method in which sequences with ≥97% sequence similarity are clustered into the same OTU. Aligned sequences were clustered using the average neighbor method in mothur (Schloss et al., 2009). To construct the maximum-likelihood phylogenetic trees for OTUs from phylotypes of interest (**Figure 5**), reference sequences were identified using the SILVA SINA aligner (Pruesse et al., 2012) and constructed using MEGA 7 (Kumar et al., 2016).

RESULTS

Here, we summarize the biogeochemical responses to enrichment reported in Lee et al. (2015). After the 21 days of periodic nutrient enrichment, soluble reactive P and total dissolved P concentrations in the water column of fertilized treatments were similar to (P-only and NP16) or significantly lower than (NP75) those in the unenriched control. Seston carbon and chlorophyll *a* concentrations in the water column of NP16 and NP75 treatments increased significantly. In contrast, the P-only treatment experienced marginal changes in measures of biomass. Planktonic (e.g., sestonic) C:P and N:P ratios decreased drastically in all fertilized treatments but were lowest in the P-only treatment; no differences in seston C:N ratios were observed. In the sediment, all treatments had significantly increased total P content (percent of dry mass) but sediment total N content did not change. Microbial cells extracted from sediment had three-fold to four-fold lower C:P and N:P ratios in the NP16 and P-only treatments than in the unenriched control while cells from the NP75 treatment had C:P and N:P ratios similar to the control and cells from all treatments had similar C:N ratios (Lee et al., 2015).

Bacterial Responses

To assess changes in the relative abundances of bacteria with low versus high rRNA gene copy number as a function of nutrient enrichment in the water column (plankton or planktonic hereafter) communities, planktonic bacterial abundance was assessed by microscopy-based cell counts and qPCR of 16S rRNA gene. The unenriched treatment had both the lowest cell abundances and 16S rRNA gene copies (**Table 1**). With P enrichment, 16S rRNA gene copies mL^{-1} increased significantly and considerably more strongly than did cell counts (14-fold versus 2-fold); thus, 16S rRNA gene copies $cell^{-1}$ ($F_{3,18} = 18.3$, p-value < 0.0001) were significantly higher than the control. Both NP16 and NP75 treatments had higher cell abundances and 16S rRNA gene copies ml^{-1} but the 16S rRNA gene copies $cell^{-1}$ were not significantly different from the unenriched treatment.

Sequencing of 16S rRNA genes showed significant change in overall planktonic community structure in response to nutrient amendment, based on perMANOVA of the Bray–Curtis distance matrix ($F_{3,12} = 13.8$, p-value < 0.001). This change was also evident in the principal coordinates analysis (PCoA) visualization of the Bray–Curtis distance matrix beta diversity (**Figure 1**). Quantitatively, beta diversity was lower within treatments than between treatments. **Figure 2A** shows the distribution of beta diversity when comparing samples within treatment and between treatments. Each treatment exhibited reproducible changes in community composition observed as a tight range of beta diversity (**Figure 2A**). Distribution of beta diversity between treatments was more spread out and had higher values (**Figure 2A**).

In the water column, fertilization (all treatments) significantly decreased community richness ($F_{3,15} = 15.5$, p-value < 0.001) but had only minimal effects on evenness and Simpson diversity index (**Table 2**). While there was no significant change in

TABLE 1 | Water column 16S rRNA gene copies and cell counts normalized to the sample volume.

Treatment	Cell counts (10^6 cells mL^{-1} ± SD)	16S rRNA genes (10^6 copies mL^{-1} ± SD)	16S rRNA genes (copies $cell^{-1}$ ± SE)
U	0.74 ± 0.13^{a}	0.92 ± 0.35^{a}	1.29 ± 0.26^{a}
P	1.65 ± 0.78^{b}	13.35 ± 5.76^{b}	9.64 ± 2.67^{b}
NP16	5.50 ± 1.50^{c}	7.02 ± 2.70^{bc}	1.35 ± 0.26^{a}
NP75	7.02 ± 1.91^{c}	4.29 ± 3.21^{c}	0.63 ± 0.19^{a}

The values represent the average of five samples from each treatment. Different letters indicate a significant treatment effect based on ANOVA followed by Tukey's HSD post hoc test.

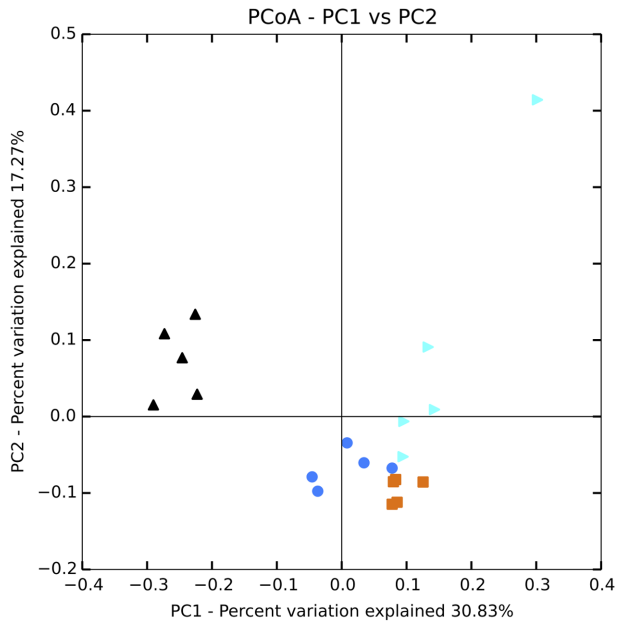

FIGURE 1 | Principal coordinates analysis (PCoA) plot of Bray–Curtis distances for bacterial communities in the water column.
Black = unenriched, orange = P-only, light blue = NP16, dark blue = NP75.

community evenness, the dominant phylotype shifted in all the fertilized treatments. In particular, some of the rare phylotypes (<1% relative abundance) detected in the unenriched treatment decreased to below detection limit in the fertilized treatment while several rare phylotypes became more abundant (**Figure 3**). Across all planktonic samples, the bacterial community was mainly dominated by Proteobacteria and Bacteroidetes, which together made up more than 80% of the population (**Figure 3**). The Proteobacteria consisted primarily of *Porphyrobacter*, a common freshwater bacterium and *Rubribacterium*, an aerobic anoxygenic phototroph. The Bacteroidetes consisted mostly of *Lewinella* and sequences that can only be mapped to the Bacteroidetes VC2.1 Bac22 (**Figure 3**).

EdgeR analysis identified two phylotypes that were significantly affected in the water column of all three fertilized treatments (**Figure 4**). Significance was defined as $\log_2$FC with a false discovery rate (FDR) or 5% or less. *Lewinella* (B0330) significantly decreased in all three fertilized treatments by more than four-fold while an uncultured member of the Rhodobacteraceae (B1001) increased in dominance in all three fertilized treatments (**Figure 4**). The enriched Rhodobacter had a relative abundance of 1% or less in the unenriched treatment but contributed over 20% in all the fertilized treatments (**Figure 3**). The OTUs within this phylotype are most phylogenetically similar to Rhodobacteraceae of marine origin (**Figure 5**).

Relative to controls, the P-only and NP16 treatments also displayed a significant decrease in Actinobacteria PeM15 sequences (B0088) while *Phenylobacterium* (B0802) sequences increased. The P-only treatment also experienced a significant enrichment of *Algoriphagus* (B0172) with a $\log_2$FC of 5.05. Both *Phenylobacterium* and *Algoriphagus* were present at less than 1% in the unenriched treatment. We also note that *Porphyrobacter* (B1131) and Bacteroidetes VC2.1 (B0127), two dominant phylotypes in the unamended treatment, were negatively affected by nutrients, especially in the P-only and NP75 treatments, respectively.

In the surficial sediment, no significant changes in the community structure were detected based on analysis of the Bray–Curtis distance between the treatments (perMANOVA p-value > 0.05). PCoA visualizations of beta diversity similarly failed to show reproducible community patterns (**Supplementary Figure S1**). Quantitatively, the replicates within each treatment in the sediment had similar beta diversity in comparison with samples from different treatments, even without nutrient amendment. Essentially, each spatial replicate was already quite different from each of the others. This observation is supported by comparison of the distribution of beta diversity between and within treatment for water (**Figure 2A**) and sediment (**Figure 2B**) samples. The ranges of distances for sediment samples between and within treatment are similar, while the water samples showed a larger range of beta diversity between treatments than within treatment. Alpha diversity measures of the sediment bacterial community also did not appear to be significantly affected by nutrient enrichment (**Table 2**). As expected, the sediment bacterial community was more diverse than the planktonic community, with over three times as many phylotypes (**Table 2**). Phototrophs such as Chloroflexi and Cyanobacteria were more dominant in the sediment with relative abundance of 4.5–6.3% and 4.2–5.8%, respectively (**Figure 3**).

Eukaryotic Responses

Planktonic eukaryotic community structure among replicates within a treatment was also highly variable but a significant change in community structure (perMANOVA: $F_{3,12} = 9.59$, p-value $= 0.001$) was still apparent (**Figure 6**). Nutrient enrichment tended to decrease phylotype richness, although this effect was not significant due to large variation within treatments (**Table 3**). The high variability between replicates was captured in the beta diversity analysis. Nevertheless, beta diversity of the planktonic communities between treatments was still higher than within treatment (**Figure 7A**).

Planktonic eukaryotic community was highly uneven with two phylotypes of green algae (Chlorophyceae) and a rotifer (Flosculariacea) making up over 80% of the 18S rRNA gene sequence library (**Figure 8**). Nonetheless, EdgeR analysis identified three phylotypes that were significantly responsive to nutrient addition (either P alone or N and P at both ratios) in the water column (**Figure 9**). Two green alga OTUs (E064, E090) significantly increased in dominance in the fertilized treatments while a rotifer (E260) was negatively affected by nutrient addition. Indeed, the two-fold increase in the green algae led to this group contributing more than 90% of the 18S sequence library in the fertilized treatments. It should also be noted that the protist Perkinsidae (E395) was present between 0.4 and 6.3% in the unenriched treatment but decreased to below detection in most of the mesocosms receiving both N and P (**Figures 8, 9**).

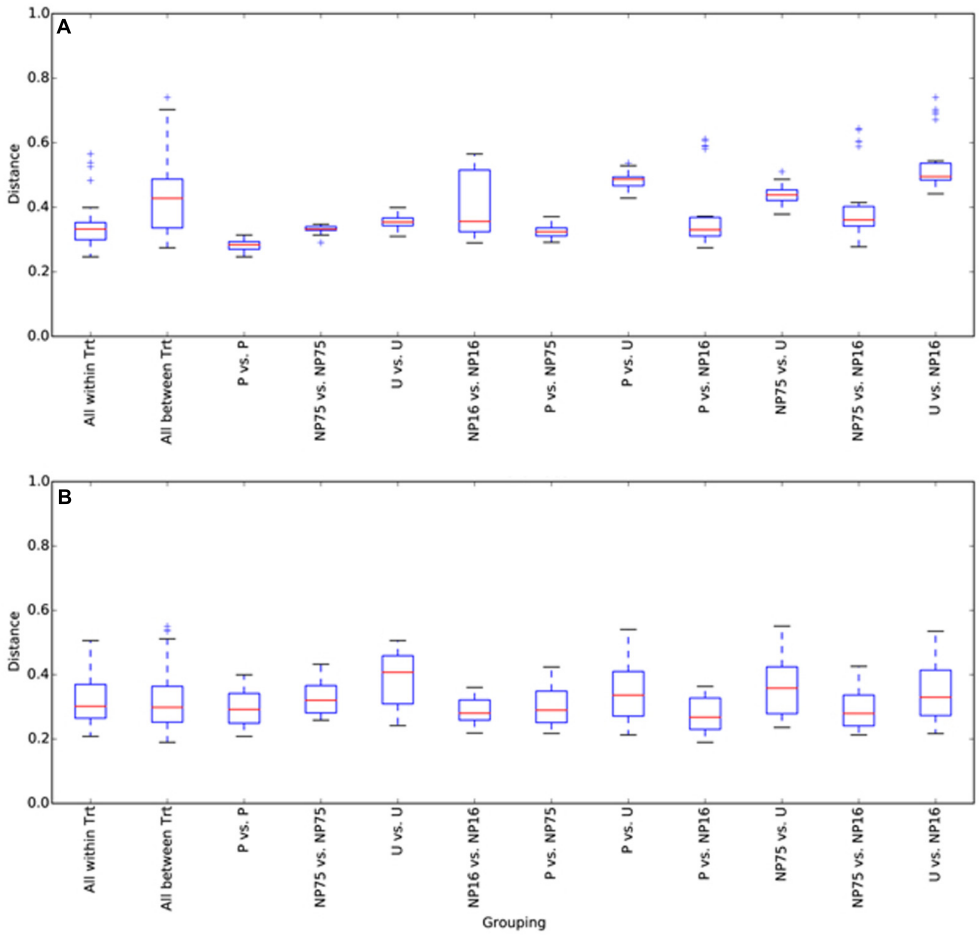

FIGURE 2 | Box and whisker plots for beta diversity distances of 16S rRNA gene libraries within and between treatments for (A) water and **(B)** sediment samples. The plot was constructed using the lower and upper quartile of the data ("whiskers" extending from either end of the box; one going from the first quartile to the smallest non-outlier and the other going from third quartile to the largest non-outlier), the inter-quartile range (width of the "box"; the bottom and the top being the lower and upper quartile, respectively), median (red line) and outliers (+ sign). Trt, treatment.

TABLE 2 | Phylotype-based alpha diversity indices for 16S rRNA gene libraries after normalization to 1038 and 575 sequences for sediment and water libraries, respectively.

Location	Treatment	Shared phylotypes[a]	Observed phylotypes	Chao richness estimator	Simpson evenness	Simpson diversity (1/D)
Water	U	21	66 ± 1.9^{a}	118 ± 2.4^{a}	0.14 ± 0.03	8.9 ± 1.9
	P	22	50 ± 2.5^{b}	90 ± 4.6^{b}	0.13 ± 0.01	6.3 ± 0.6
	NP16	18	50 ± 3.8^{b}	87 ± 9.8^{b}	0.13 ± 0.05	6.5 ± 2.7
	NP75	19	53 ± 4.2^{b}	99 ± 12^{b}	0.12 ± 0.02	6.4 ± 1.6
Sediment	U	55	199 ± 33	319 ± 47	0.20 ± 0.05	40.4 ± 14.3
	P	67	194 ± 22	317 ± 34	0.16 ± 0.06	31.3 ± 14.0
	NP16	69	211 ± 27	349 ± 45	0.21 ± 0.04	43.6 ± 9.2
	NP75	62	210 ± 38	347 ± 65	0.23 ± 0.02	47.8 ± 12.1

The values (except for shared phylotypes) represent the average of five samples from each treatment ± SEM. Different letters represent a significant treatment effect based on post hoc Tukey's test. [a]*Number of phylotypes shared between replicates.*

In the sediment, no distinct eukaryotic community structure was observed for different treatments, with all the samples exhibiting high beta diversity (**Figure 7B**) and similar measures of alpha diversity (**Table 3**). Hence, no clustering of samples from the same treatment is observed in the PCoA plot (**Supplementary Figure S2**). Sediment eukaryotic community consisted mostly of benthic diatoms, nematodes, and dinoflagellates (**Figure 8**). EdgeR analysis also did not identify any eukaryotic sediment

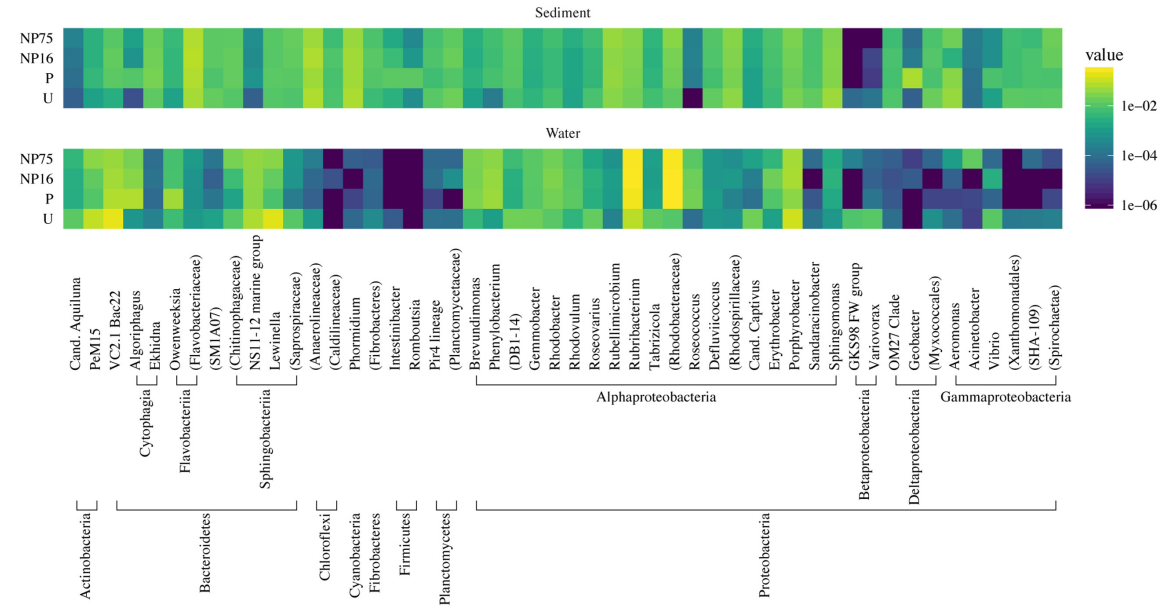

FIGURE 3 | Taxonomic heat map of the 50 most abundant phylotypes demonstrating the effect of nutrient application on the composition of bacterial communities in the water column and sediment of the mesocosms. The color scale represents relative abundance as the average percent read totals of five replicates within a treatment, shown in log scale.

phylotypes that were significantly influenced by nutrient enrichment.

DISCUSSION

The CCB hosts diverse water bodies with unique microbiota (Souza et al., 2006) and it has been hypothesized that low phosphorus availability itself maintains high diversity of microorganisms, some potentially endemic, in the basin (Souza et al., 2008). In this study, we evaluated the effects of nutrient stoichiometry on both the bacterial and eukaryotic community in P-deficient Lagunita Pond by conducting an *in situ* mesocosm experiment. Most freshwater studies investigating the effects of nutrients focus on nutrient availability *per se* but the ratio of available nutrients can also play an important role in shaping the microbial community (Elser et al., 2003; Hessen et al., 2013). Based on the GRH, we expected that phylotypes that are responsive to P enrichment would have high rRNA gene copy number that allows high ribosome production capacity. We also sought to determine if the N:P ratio of nutrient enrichment itself was important in shaping microbial responses and driving community structure.

Nutrient enrichment, either P alone or both N and P at two different ratios, altered community structure as shown in the beta diversity analysis but had minimal effect on alpha diversity of the microbial community. In particular, there was a significant change in the structure of Lagunita's planktonic microbial community, enriching for phylotypes that were rare in the unenriched treatment. P addition (without N) resulted in the highest number of significantly affected bacterial phylotypes followed by enrichment at 16:1 and 75:1. This indicates that the ratio of the added nutrient plays an important role in shaping the planktonic microbial community in this system. The extent of change in community structure is also congruent with previously observed changes in the N:P ratio of these treatments, where P-only mesocosms experienced the largest change in dissolved N:P ratio (Lee et al., 2015). We suspect that only the P-alone treatment sufficiently reduced the overall N:P ratio to levels where P was no longer limiting; enrichment at 16:1 and (especially) 75:1 ratios likely did not eliminate P-limitation given the extremely high ambient N:P ratios characteristic of Lagunita Pond.

Importantly, we found a significant nutrient enrichment effect on 16S rRNA gene copy number per cell (**Table 3**) in the P-only treatment. While the absolute magnitudes of the rRNA gene copy number per cell values we report likely have some associated error due to uncertainties in cell counts and PCR (e.g., primer coverage), these uncertainties likely held across all treatments and thus should not impact among-treatment comparisons. The closest available reference genomes for responsive phylotypes in the P-only treatment were analyzed for ribosome production capability, and each have rRNA gene copy number between 1 and 3 copies. *Algoriphagus* sp., which is highly enriched in the P-only treatment had the highest rRNA gene copy number of 3 (Stoddard et al., 2014). However, the majority of phylotypes in the samples are not very well characterized. Thus, it is likely that the reference genomes are simply not appropriate for inferring the rRNA copy number of related microbes in nature, especially in a poorly studied and unique environment such as Cuatro Cienegas. Overall, the data provide some of the first field evidence supporting the hypothesized connection

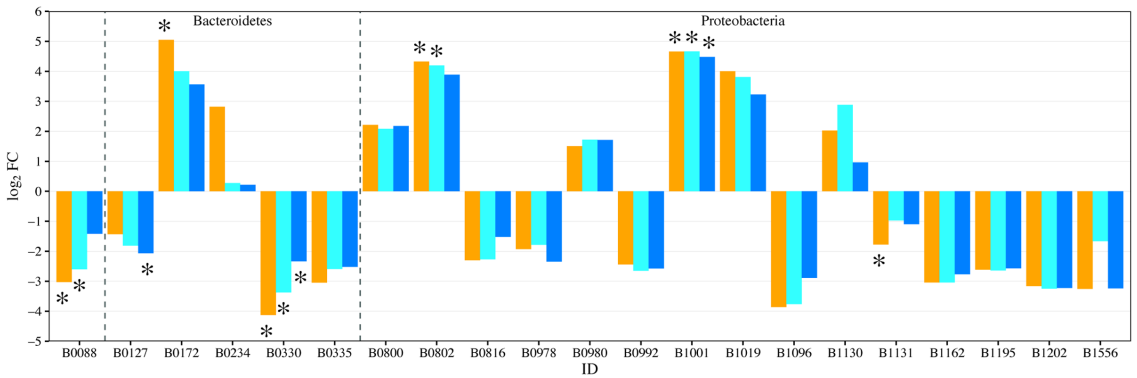

FIGURE 4 | Fold change (log_2FC) in bacterial phylotype abundance between U and each of the fertilized treatments (orange = P-only, light blue = NP16, dark blue = NP75). Only genera with fold change with *p*-value ≤ 0.05 are represented in the figure. *Represents change with false discovery rate (FDR) ≤ 0.05. B0088: Actinobacteria; Actinobacteria; PeM15
B0127: Bacteroidetes; VC2.1 Bac22
B0172: Bacteroidetes; Cytophagia; Algoriphagus
B0234: Bacteroidetes; Flavobacteriia; Owenweeksia
B0330: Bacteroidetes; Sphingobacteriia; Lewinella
B0335: Bacteroidetes; Sphingobacteriia; uncultured Saprospiraceae
B0800: Proteobacteria; Alphaproteobacteria; Brevundimonas
B0802: Proteobacteria; Alphaproteobacteria; Phenylobacterium
B0816: Proteobacteria; Alphaproteobacteria; DB1-14
B0978: Proteobacteria; Alphaproteobacteria; Roseovarius
B0980: Proteobacteria; Alphaproteobacteria; Rubribacterium
B0992: Proteobacteria; Alphaproteobacteria; Tabrizicola
B1001: Proteobacteria; Alphaproteobacteria; uncultured Rhodobacteraceae
B1019: Proteobacteria; Alphaproteobacteria; Roseococcus
B1096: Proteobacteria; Alphaproteobacteria; Candidatus Captivus
B1130: Proteobacteria; Alphaproteobacteria; Erythrobacter
B1131: Proteobacteria; Alphaproteobacteria; Porphyrobacter
B1162: Proteobacteria; Betaproteobacteria; GKS98 freshwater bacteria
B1195: Proteobacteria; Betaproteobacteria; Polaromonas
B1202: Proteobacteria; Betaproteobacteria; Variovorax
B1556: Proteobacteria; Gammaproteobacteria; Vibrio

between P supply and ribosomal RNA genome organization under the GRH and suggests that P enrichment at low N:P ratio favors bacteria with higher rRNA gene copy number. The result is congruent with previous work investigating ecological strategies of bacteria with low and high rRNA gene copy numbers (Klappenbach et al., 2000; Nemergut et al., 2016). It also warrants further investigation of the rRNA gene copy numbers of the other positively responding phylotypes that have yet to be classified. The lack of significant change in the rRNA gene copy number per cell in the NP16 and NP75 treatments compared to the unenriched treatment may indicate that growth of bacteria with high rRNA gene copy number remained limited by P as argued above. Moreover, NP75 treatment may even have increased P limitation in relation to N because both sediment and seston N:P ratios in the NP75 treatments were still at least twice the Redfield ratio (Lee et al., 2015).

Given that there were only two phylotypes that responded uniquely in the P-only treatment, change in community structure may not be a sufficient explanation for the increased rRNA gene copies/cell observed in the P-only treatment. An alternative explanation is that the high rRNA gene copy number members have a higher growth rate in the P-only treatment and thus contain more copies per cell due to ongoing DNA replication in each cell. The method used to measure rRNA gene content does not distinguish cells with higher rRNA gene copy number in the genome from cells with higher rRNA gene content due to multiple DNA replication forks. At a high growth rate, cells initiate DNA replication prior to cell division, creating multiple replication forks that consequently increase the effective copy number of genes located near the origin of replication (*oriC*) by up to eight-fold (Skarstad et al., 1986). Highly transcribed genes involved in transcription and translation, such as rRNA genes, are often found to be in close proximity to the *oriC*, especially in fast-growing bacteria (Couturier and Rocha, 2006; Vieira-Silva and Rocha, 2010). If the responsive phylotypes have two copies of rRNA operons near the origin of replication, the measured rRNA gene content/cell could increase by another two-fold. We cannot resolve this question with the data we currently have available.

Variation in organismal stoichiometry of microbial eukaryotes, especially phytoplankton, has also been attributed to the allocation of resources to growth machinery (i.e., ribosomes) vs. resource acquisition machinery (i.e., chloroplasts) (Arrigo, 2005; Klausmeier et al., 2007). The planktonic eukaryotic community response pattern was more representative of a

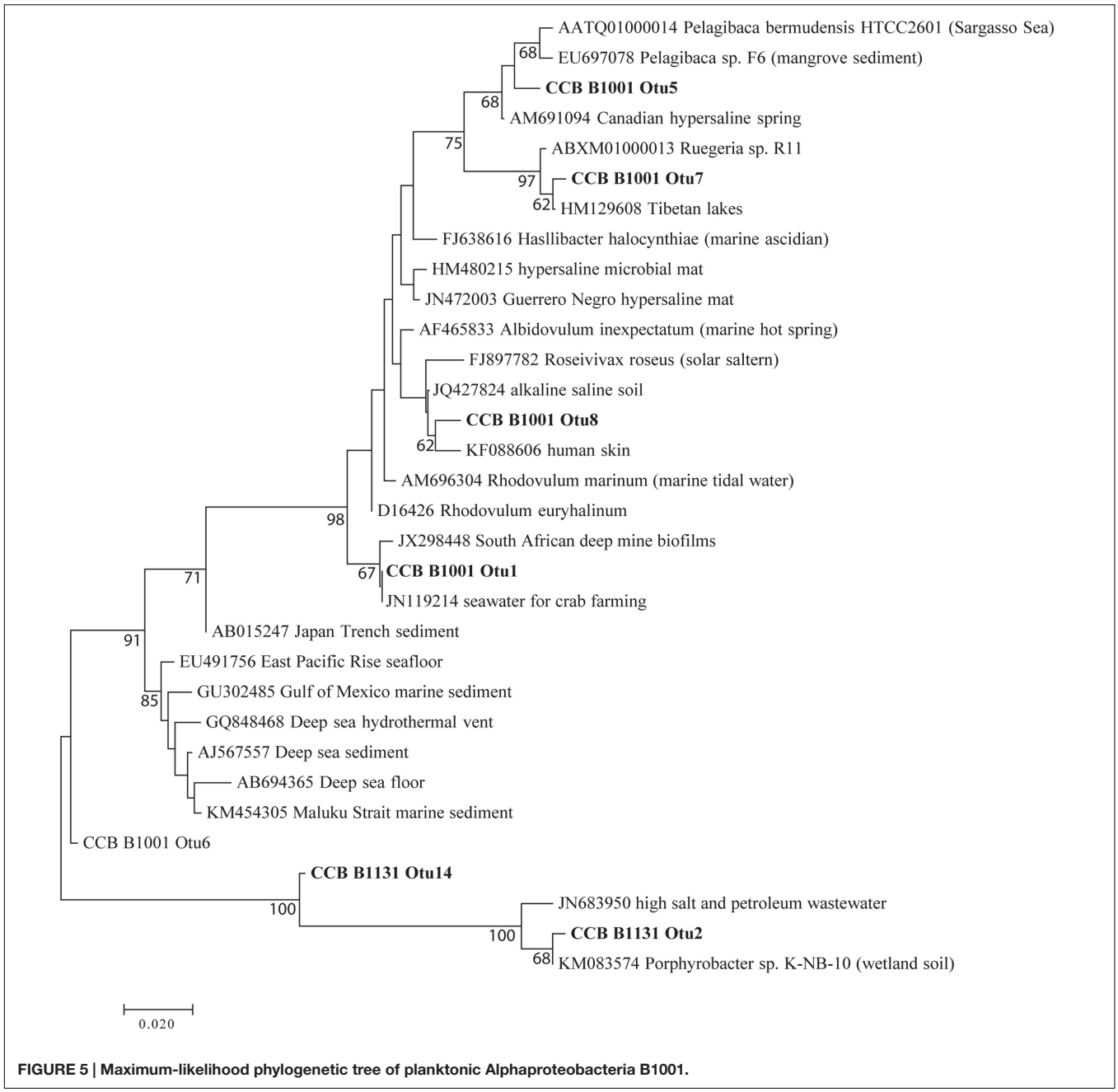

FIGURE 5 | Maximum-likelihood phylogenetic tree of planktonic Alphaproteobacteria B1001.

general nutrient limitation. The stimulation of green algae we observed is consistent with the observed increase in chl *a* concentration in the fertilized treatments (Lee et al., 2015). A previous study in the CCB region found that P enrichment enhanced the proliferation of diatoms rather than green algae (Elser et al., 2005a). Diatoms are also expected to be more successful in environments with low N:P regime (Arrigo, 2005). However, most of the diatoms in Lagunita are found in the sediment, where they might have had limited accessibility to the added phosphorus.

No statistically significant changes in the microbial community were observed in the sediment in response to nutrient enrichment. However, it cannot be concluded that sediment microbial communities are resistant to nutrient perturbation because highly variable community structure was observed for all the samples (**Figures 2, 7**). The high variation in the sediment community is not surprising due to high heterogeneity in the local habitat (Fenchel, 2002). The evaporitic nature of Lagunita further increases habitat heterogeneity, which can lead to high variation in community structure as the communities recover independently (Shade et al., 2008). The high diversity between the different samples within each treatment highlights the critical need for replication in microbial community analysis, especially for field samples.

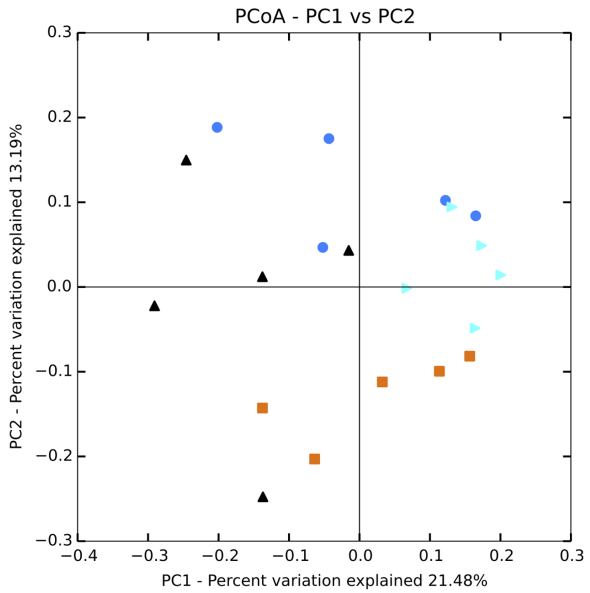

FIGURE 6 | Principal coordinates analysis plot of Bray–Curtis distances for eukaryote communities in the water column.
Black = unenriched, orange = P-only, light blue = NP16, dark blue = NP75.

Further investigation targeting the responsive phylotypes will be required to determine other mechanisms of response elicited by changes in nutrient ratio. While the qPCR results from the P-only treatment supports the GRH, it is unlikely to be the only biochemical/physiological response to alterations in nutrient supply ratios. In fact, the GRH was not supported in another study exploring the large range of rRNA gene copy number in *Bacillus* sp. isolated from CCB (Valdivia-Anistro et al., 2015). Consistent with their study, *Bacillus* sp., which was found to be less than 0.5% of the sequence libraries, did not exhibit any treatment effect (data not shown). Although *Bacillus* sp. is not a dominant phylotype in CCB, the use of cultivation-dependent methods in Valdivia-Anistro et al. (2015) suggests that species with high rRNA gene copy number can survive and remain active in such a P-limited environment. In fact, the discovery of cultivable *Bacillus* sp. in CCB at different times suggests that high rRNA gene copy number may be a more important trait for rapid response to changes in rapidly fluctuating physical factors, such as moisture. Furthermore, Ponce-Soto et al. (2015) found that antibiotic resistance decreased in the Lagunita bacterial community after nutrient enrichment. Decreases in antibiotic resistance suggest that antagonistic interactions between microbes decrease, which may create new niche space for the rare phylotypes. Antagonistic activity was found to be more common for particle-associated bacteria (Long and Azam, 2001). The enrichment with dissolved nutrient may have selected for free-living species that are more efficient at taking up the added nutrient and have less antagonistic activity. One particular phylotype that warrants further investigation is the appearance of uncultured Rhodobacteraceae in the fertilized treatment, while *Rubribacterium* was the sole dominant Rhodobacteraceae in the unenriched treatment.

It was previously discovered that a large number of bacterial phylotypes in CCB are closely related to bacteria of marine origin (Souza et al., 2006). We note that the planktonic bacterial community in Lagunita is highly enriched with aerobic anoxygenic phototrophic (AAP) bacteria. High AAP abundance is typical of oligotrophic temperate lakes, especially during the summer (Koblížek, 2015). The importance of light-sensing mechanisms in CCB was previously suspected when *Bacillus coahuilensis* was found to carry a constitutive bacteriorhodopsin gene (Alcaraz et al., 2011). Phototrophic metabolism provides a competitive advantage in the macrophyte-free Lagunita, where dissolved organic carbon sources are limited while the use of light as energy source allows AAP bacteria to have higher growth efficiency compared to heterotrophs (Koblížek, 2015). Unlike most freshwater ecosystems where the phylum Actinobacteria is often the most abundant (Newton et al., 2011; Ghai et al., 2012), Alphaproteobacteria and Bacteroidetes dominated the Lagunita water column. Bacteroidetes are known for their ability to degrade secondary metabolites from eukaryotes and hence have a dynamic relationship with eukaryotic primary producers (Eiler and Bertilsson, 2004; Schauer et al., 2005). Carbon cycling in Lagunita is likely driven by eukaryotic phototrophs but their relationship with the Bacteroidetes remains to be elucidated because nutrient addition had opposing effects on these two groups of microorganisms. Lagunita is also dominated by non-Flavobacteriales Bacteroidetes that are not commonly found in

TABLE 3 | Alpha diversity indices for 18S rRNA gene libraries.

Location	Treatment	Shared phylotypes[a]	Observed phylotypes	Chao richness estimator	Simpson evenness	Simpson diversity (1/D)
Water	U	16	60 ± 14	71 ± 15	0.05 ± 0.01	3.20 ± 0.88
	P	18	50 ± 12	64 ± 16	0.05 ± 0.01	2.57 ± 0.37
	NP16	16	36 ± 5.2	48 ± 4.8	0.08 ± 0.01	2.70 ± 0.28
	NP75	13	43 ± 14	59 ± 21	0.07 ± 0.04	2.56 ± 0.37
Sediment	U	12	89 ± 19	103 ± 20	0.10 ± 0.08	9.56 ± 8.71
	P	15	86 ± 24	105 ± 28	0.08 ± 0.05	6.07 ± 3.27
	NP16	30	83 ± 10	101 ± 11	0.06 ± 0.03	4.95 ± 1.74
	NP75	16	69 ± 16	82 ± 19	0.07 ± 0.03	4.28 ± 1.35

The libraries were normalized to 10,521 and 9,299 sequences for sediment and water libraries, respectively. The values represent the average of five samples from each treatment ± SEM. [a]*Number of phylotypes shared between replicates.*

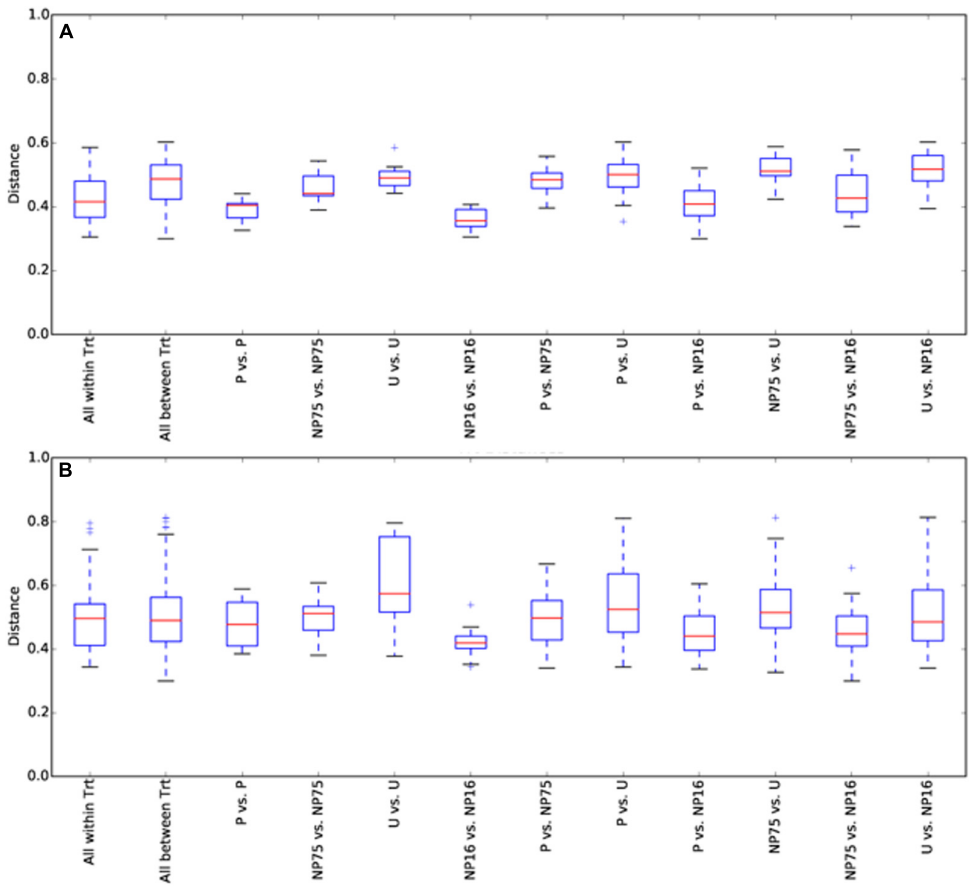

FIGURE 7 | Box and whisker plot for beta diversity distances of 18S rRNA gene libraries within and between treatments for (A) water and **(B)** sediment samples. The plot was constructed using the lower and upper quartile of the data ("whiskers" extending from either end of the box; one going from the first quartile to the smallest non-outlier and the other going from third quartile to the largest non-outlier), the inter-quartile range (width of the "box"; the bottom and the top being the lower and upper quartile, respectively), median (red line) and outliers (+ sign). Trt, treatment.

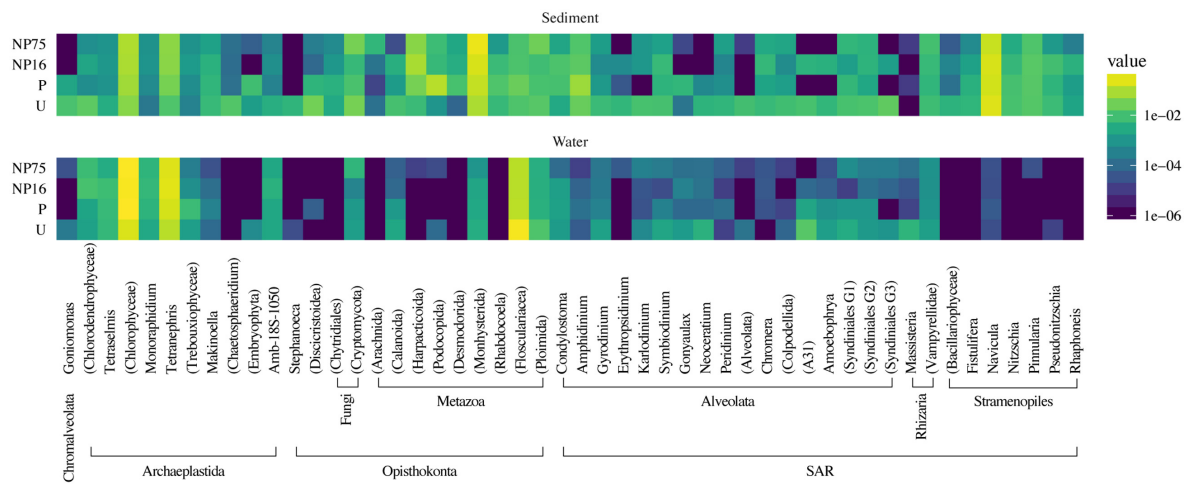

FIGURE 8 | Taxonomic heat map of the 50 most abundant phylotypes demonstrating the effect of nutrient application on the composition of eukaryotic communities in the water column and sediment of the mesocosms. The color scale represents relative abundance as the average percent read totals of five replicates within a treatment, shown in log scale.

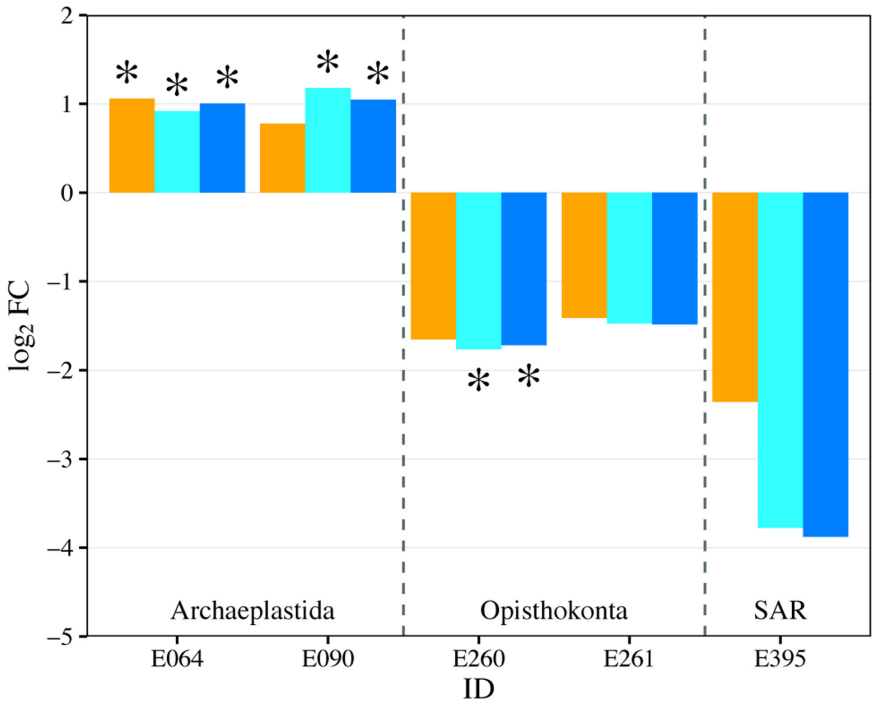

FIGURE 9 | Fold change (log_2FC) in eukaryotic genera abundance between the control treatment (U) and each of the fertilized treatments (orange = P-only, light blue = NP16, dark blue = NP75). Only genus with fold change that has p-value $\leq$ 0.05 is represented in the figure. *Represents change with FDR $\leq$ 0.05. E064: Archaeplastida; Chloroplastida; Tetranephris
E090: Archaeplastida; Chloroplastida; unclassified
E260: Opisthokonta; Metazoa; Floscullariacea
E261: Opisthokonta; Metazoa; Ploimida
E395: SAR; Alveolata; A31

freshwater ecosystems. For example, phylotype B0127 can only be mapped to Bacteroidetes VC2.1 Bac22, which consists of sequences obtained mostly from marine environments such as hydrothermal vents, marine snow, and alkaline lakes (Zhang et al., 2013). The physiology of this cluster remains to be investigated because there is still no genomic or cultured representative. In addition, the dominant Actinobacteria in Lagunita is a more closely related to the marine PeM15 than to the more common acl1 lineage (Hahn, 2009). The nutrient-responsive uncultured Rhodobacteraceae phylotype also consists of sequences that are closely related to sequences obtained from marine ecosystems. Further investigation targeting these unique phylotypes will be required to understand how some of these phylotypes persist under P-limitation and strong stoichiometric imbalance.

PERSPECTIVES AND CONCLUSION

Our study is among the first to use molecular surveys within a replicated field experiment to evaluate how the bacterial and microbial eukaryotic communities of a severely- P-limited ecosystem respond to perturbation of supplies and ratios of key nutrients. Our data highlight the importance of marine-affiliated taxa such as Bacteroidetes cluster VC2.1 Bac22 and uncultured Rhodobacteraceae in support of the surprising inference of Souza et al. (2006) that CCB's biological history has retained a signature of its ancient geologic marine history. High-throughput sequencing of both the bacterial and eukaryotic community further confirmed that CCB is rich in microbial biodiversity and especially in major phylotypes that are not well-described. At the community level, our results suggest that nutrient enrichment had relatively modest impacts on overall taxonomic diversity but led to significant shifts in community structure, particularly in the water column. This provides further evidence that nutrient ratios play an important role in shaping the structure of both bacterial and eukaryotic communities. Finally, we provide evidence that, contributing to these nutrient-driven community shifts, are changes in the abundances of taxa that differ in rRNA gene copy number, consistent with the GRH. Future studies using metagenomic approaches coupled to *in situ* nutrient enrichment may shed further light on the underlying mechanisms, and functional consequences, of nutrient enrichment in oligotrophic and stoichiometrically imbalanced ecosystems.

AUTHOR CONTRIBUTIONS

ZL was involved in conducting the sampling, analyzing samples, analyzing the data, and writing the manuscript. JS was involved

in designing the study conducting the sampling, analyzing the data, and writing the manuscript. AP-P was involved in analyzing samples, analyzing the data, and writing the manuscript. DK was involved in analyzing the data. AM was involved in analyzing the data. AA was involved in analyzing the data. CD was involved in designing the data analysis plan, analyzing the data, and writing the manuscript. LE was involved in designing the study, conducting the sampling, and writing the manuscript. VS was involved in designing the study, conducting the sampling, analyzing the data, and writing the manuscript. JE was involved in envisioning the original study, designing the experiment, conducting the sampling, advising on data analysis, and writing the manuscript.

FUNDING

This study was conducted with financial support from NSF (DEB-0950175) and NASA (NAI5-0018) grants awarded to JE, WWF-FCS to VS, and NSF (1536546) and NAI (NNH05ZDA001C, NNH12ZDA002C, NNA08CN87A, NNA13AA93A) to JS.

ACKNOWLEDGMENTS

We thank N. Macias and J. Ramos for their assistance in the field experiment and Jason Artigas for laboratory assistance. This study was made possible with the sampling permit from Vida Silvestre-SEMARNAT, granted to VS (09762). We also thank Matthew Church for his helpful comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00949/full#supplementary-material

FIGURE S1 | PCoA plot of Bray–Curtis distances for bacterial communities in the sediment. Black = unenriched, orange = P-only, light blue = NP16, dark blue = NP75.

FIGURE S2 | PCoA plot of Bray–Curtis distances for eukaryotic communities in the sediment. Black = unenriched, orange = P-only, light blue = NP16, dark blue = NP75.

REFERENCES

Alcaraz, L. D., López-Ramírez, V., Moreno-Letelier, A., Herrera-Estrella, L., Souza, V., and Olmedo-Alvarez, G. (2011). "Genomics of bacteria from an ancient marine origin: clues to survival in an oligotrophic environment," in *Earth and Environmental Sciences*, ed. I. A. Dar (Rijeka: INTECH Open Access Publisher).

Arrigo, K. R. (2005). Marine microorganisms and global nutrient cycles. *Nature* 437, 349–355. doi: 10.1038/nature04159

Bik, H. M., Porazinska, D. L., Creer, S., Caporaso, J. G., Knight, R., and Thomas, W. K. (2012). Sequencing our way towards understanding global eukaryotic biodiversity. *Trends Ecol. Evol.* 27, 233–243. doi: 10.1016/j.tree.2011.11.010

Bowen, J. L., Ward, B. B., Morrison, H. G., Hobbie, J. E., Valiela, I., Deegan, L. A., et al. (2011). Microbial community composition in sediments resists perturbation by nutrient enrichment. *ISME J.* 5, 1540–1548. doi: 10.1038/ismej.2011.22

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Carbonero, F., Oakley, B. B., and Purdy, K. J. (2014). Metabolic flexibility as a major predictor of spatial distribution in microbial communities. *PLoS ONE* 9:e85105. doi: 10.1371/journal.pone.0085105

Caron, D. A., Countway, P. D., Savai, P., Gast, R. J., Schnetzer, A., Moorthi, S. D., et al. (2009). Defining DNA-based operational taxonomic units for microbial-eukaryote ecology. *Appl. Environ. Microbiol.* 75, 5797–5808. doi: 10.1128/AEM.00298-09

Chan, L.-K., Newton, R. J., Sharma, S., Smith, C. B., Rayapati, P., Limardo, A. J., et al. (2012). Transcriptional changes underlying elemental stoichiometry shifts in a marine heterotrophic bacterium. *Front. Microbiol.* 3:159. doi: 10.3389/fmicb.2012.00159

Claire Horner-Devine, M., Leibold, M. A., Smith, V. H., and Bohannan, B. J. M. (2003). Bacterial diversity patterns along a gradient of primary productivity. *Ecol. Lett.* 6, 613–622. doi: 10.1046/j.1461-0248.2003.00472.x

Corman, J. R., Poret-Peterson, A. T., Uchitel, A., and Elser, J. J. (2016). Interaction between lithification and resource availability in the microbialites of Rio Mesquites, Cuatro Ciénegas, México. *Geobiology* 17, 176–189. doi: 10.1111/gbi.12168

Couturier, E., and Rocha, E. P. C. (2006). Replication-associated gene dosage effects shape the genomes of fast-growing bacteria but only for transcription and translation genes. *Mol. Microbiol.* 59, 1506–1518. doi: 10.1111/j.1365-2958.2006.05046.x

Dupont, C. L., Larsson, J., Yooseph, S., Ininbergs, K., Goll, J., Asplund-Samuelsson, J., et al. (2014). Functional tradeoffs underpin salinity-driven divergence in microbial community composition. *PLoS ONE* 9:e89549. doi: 10.1371/journal.pone.0089549

Eiler, A., and Bertilsson, S. (2004). Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environ. Microbiol.* 6, 1228–1243. doi: 10.1111/j.1462-2920.2004.00657.x

Elser, J. J. (2003). Biological stoichiometry: a theoretical framework connecting ecosystem ecology, evolution, and biochemistry for application in astrobiology. *Int. J. Astrobiol.* 2, 185–193. doi: 10.1017/S1473550403001563

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/J.1461-0248.2003.00518.X

Elser, J. J., Schampel, J. H., Garcia-Pichel, F., Wade, B. D., Souza, V., Eguiarte, L., et al. (2005a). Effects of phosphorus enrichment and grazing snails on modern stromatolitic microbial communities. *Freshw. Biol.* 50, 1808–1825. doi: 10.1111/J.1365-2427.2005.01451.X

Elser, J. J., Schampel, J. H., Kyle, M., Watts, J., Carson, E. W., Dowling, T. E., et al. (2005b). Response of grazing snails to phosphorus enrichment of modern stromatolitic microbial communities. *Freshw. Biol.* 50, 1826–1835. doi: 10.1111/j.1365-2427.2005.01453.x

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., et al. (2000). Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* 3, 540–550. doi: 10.1111/j.1461-0248.2000.00185.x

Elser, J. J., Watts, J., Schampel, J. H., and Farmer, J. (2006). Early Cambrian food webs on a trophic knife-edge? A hypothesis and preliminary data from a modern stromatolite-based ecosystem. *Ecol. Lett.* 9, 295–303. doi: 10.1111/j.1461-0248.2005.00873.x

Fenchel, T. (2002). Microbial behavior in a heterogeneous world. *Science* 296, 1068–1071. doi: 10.1126/science.1070118

Ghai, R., McMahon, K. D., and Rodriguez-Valera, F. (2012). Breaking a paradigm: cosmopolitan and abundant freshwater actinobacteria are low GC. *Environ. Microbiol. Rep.* 4, 29–35. doi: 10.1111/j.1758-2229.2011.00274.x

Groszkopf, T., and Soyer, O. S. (2016). Microbial diversity arising from thermodynamic constraints. *ISME J.* 10, 2725–2733. doi: 10.1038/ismej.2016.49

Hahn, M. W. (2009). Description of seven candidate species affiliated with the phylum Actinobacteria, representing planktonic freshwater bacteria. *Int. J. Syst. Evol. Microbiol.* 59, 112–117. doi: 10.1099/ijs.0.001743-0

Hall, E. K., Maixner, F., Franklin, O., Daims, H., Richter, A., and Battin, T. (2011). Linking microbial and ecosystem ecology using ecological stoichiometry: a synthesis of conceptual and empirical approaches. *Ecosystems* 14, 261–273. doi: 10.1007/s10021-010-9408-4

Haukka, K., Kolmonen, E., Hyder, R., Hietala, J., Vakkilainen, K., Kairesalo, T., et al. (2006). Effect of nutrient loading on bacterioplankton community composition in lake mesocosms. *Microb. Ecol.* 51, 137–146. doi: 10.1007/s00248-005-0049-7

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Hewson, I., Vargo, G. A., and Fuhrman, J. A. (2003). Bacterial diversity in shallow oligotrophic marine benthos and overlying waters: effects of virus infection, containment, and nutrient enrichment. *Microb. Ecol.* 46, 322–336. doi: 10.1007/s00248-002-1067-3

Hillebrand, H., Cowles, J. M., Lewandowska, A., Van de Waal, D. B., and Plum, C. (2014). Think ratio! A stoichiometric view on biodiversity–ecosystem functioning research. *Basic Appl. Ecol.* 15, 465–474. doi: 10.1016/j.baae.2014.06.003

Huber, J. A., Morrison, H. G., Huse, S. M., Neal, P. R., Sogin, M. L., and Mark Welch, D. B. (2009). Effect of PCR amplicon size on assessments of clone library microbial diversity and community structure. *Environ. Microbiol.* 11, 1292–1302. doi: 10.1111/j.1462-2920.2008.01857.x

Klappenbach, J. A., Dunbar, J. M., and Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of bacteria. *Appl. Environ. Microbiol.* 66, 1328–1333. doi: 10.1128/aem.66.4.1328-1333.2000

Klausmeier, C. A., Litchman, E., and Levin, S. A. (2007). A model of flexible uptake of two essential resources. *J. Theor. Biol.* 246, 278–289. doi: 10.1016/j.jtbi.2006.12.032

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Koblížek, M. (2015). Ecology of aerobic anoxygenic phototrophs in aquatic environments. *FEMS Microbiol. Rev.* 39, 854–870. doi: 10.1093/femsre/fuv032

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lauro, F. M., McDougald, D., Thomas, T., Williams, T. J., Egan, S., Rice, S., et al. (2009). The genomic basis of trophic strategy in marine bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15527–15533. doi: 10.1073/pnas.0903507106

Lee, Z. M., Steger, L., Corman, J. R., Neveu, M., Poret-Peterson, A. T., Souza, V., et al. (2015). Response of a stoichiometrically imbalanced ecosystem to manipulation of nutrient supplies and ratios. *PLoS ONE* 10:e0123949. doi: 10.1371/journal.pone.0123949

Leflaive, J., Danger, M., Lacroix, G., Lyautey, E., Oumarou, C., and Ten-Hage, L. (2008). Nutrient effects on the genetic and functional diversity of aquatic bacterial communities. *FEMS Microbiol. Ecol.* 66, 379–390. doi: 10.1111/j.1574-6941.2008.00593.x

Logue, J. B., Langenheder, S., Andersson, A. F., Bertilsson, S., Drakare, S., Lanzen, A., et al. (2012). Freshwater bacterioplankton richness in oligotrophic lakes depends on nutrient availability rather than on species-area relationships. *ISME J.* 6, 1127–1136. doi: 10.1038/ismej.2011.184

Long, R. A., and Azam, F. (2001). Antagonistic interactions among marine pelagic bacteria. *Appl. Environ. Microbiol.* 67, 4975–4983. doi: 10.1128/aem.67.11.4975-4983.2001

Makino, W., and Cotner, J. B. (2004). Elemental stoichiometry of a heterotrophic bacterial community in a freshwater lake: implications for growth-and resource-dependent variations. *Aquat. Microb. Ecol.* 34, 33–41. doi: 10.3354/ame034033

Nelson, C. E., and Carlson, C. A. (2011). Differential response of high-elevation planktonic bacterial community structure and metabolism to experimental nutrient enrichment. *PLoS ONE* 6:e18320. doi: 10.1371/journal.pone.0018320

Nemergut, D. R., Knelman, J. E., Ferrenberg, S., Bilinski, T., Melbourne, B., Jiang, L., et al. (2016). Decreases in average bacterial community rRNA operon copy number during succession. *ISME J.* 10, 1147–1156. doi: 10.1038/ismej.2015.191

Newton, R. J., Jones, S. E., Eiler, A., McMahon, K. D., and Bertilsson, S. (2011). A guide to the natural history of freshwater lake bacteria. *Microbiol. Mol. Biol. Rev.* 75, 14–49. doi: 10.1128/mmbr.00028-10

Paulson, J. N., Stine, O. C., Bravo, H. C., and Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nat Methods* 10, 1200–1202. doi: 10.1038/nmeth.2658

Peura, S., Eiler, A., Hiltunen, M., Nykänen, H., Tiirola, M., and Jones, R. I. (2012). Bacterial and phytoplankton responses to nutrient amendments in a boreal lake differ according to season and to taxonomic resolution. *PLoS ONE* 7:e38552. doi: 10.1371/journal.pone.0038552

Ponce-Soto, G. Y., Aguirre-von-Wobeser, E., Eguiarte, L. E., Elser, J. J., Lee, Z. M. P., and Souza, V. (2015). Enrichment experiment changes microbial interactions in an ultra-oligotrophic environment. *Front. Microbiol.* 6:246. doi: 10.3389/fmicb.2015.00246

Pruesse, E., Peplies, J., and Glöckner, F. O. (2012). SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28, 1823–1829. doi: 10.1093/bioinformatics/bts252

Purdy, K. J. (2005). Nucleic acid recovery from complex environmental samples. *Environ. Microbiol.* 397, 271–292. doi: 10.1016/s0076-6879(05)97016-x

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140. doi: 10.1093/bioinformatics/btp616

Roller, B. R. K., Stoddard, S. F., and Schmidt, T. M. (2016). Exploiting rRNA operon copy number to investigate bacterial reproductive strategies. *Nat. Microbiol.* 1:16160. doi: 10.1038/nmicrobiol.2016.160

Schauer, M., Kamenik, C., and Hahn, M. W. (2005). Ecological differentiation within a cosmopolitan group of planktonic freshwater bacteria (SOL Cluster, *Saprospiraceae, Bacteroidetes*). *Appl. Environ. Microbiol.* 71, 5900–5907. doi: 10.1128/aem.71.10.5900-5907.2005

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/aem.01541-09

Shade, A., Jones, S. E., and McMahon, K. D. (2008). The influence of habitat heterogeneity on freshwater bacterial community composition and dynamics. *Environ. Microbiol.* 10, 1057–1067. doi: 10.1111/j.1462-2920.2007.01527.x

Skarstad, K., Boye, E., and Steen, H. B. (1986). Timing of initiation of chromosome replication in individual *Escherichia coli* cells. *EMBO J.* 5, 1711–1717.

Soininen, J., and Meier, S. (2014). Phytoplankton richness is related to nutrient availability, not to pool size, in a subarctic rock pool system. *Hydrobiologia* 740, 137–145. doi: 10.1007/s10750-014-1949-7

Souza, V., Eguiarte, L. E., Siefert, J., and Elser, J. J. (2008). Microbial endemism: does phosphorus limitation enhance speciation? *Nat. Rev. Microbiol.* 6, 559–564. doi: 10.1038/nrmicro1917

Souza, V., Espinosa-Asuar, L., Escalante, A. E., Eguiarte, L. E., Farmer, J., Forney, L., et al. (2006). An endangered oasis of aquatic microbial biodiversity in the Chihuahuan desert. *Proc. Nat. Acad. Sci. U.S.A.* 103, 6565–6570. doi: 10.1073/pnas.0601434103

Souza, V., Siefert, J. L., Escalante, A. E., Elser, J. J., and Eguiarte, L. E. (2012). The Cuatro Ciénegas basin in Coahuila, Mexico: an astrobiological precambrian park. *Astrobiology* 12, 641–647. doi: 10.1089/ast.2011.0675

Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. K., and Schmidt, T. M. (2014). *rrnDB*: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res.* 43, D593–D598. doi: 10.1093/nar/gku1201

Torsvik, V., Øvreås, L., and Thingstad, T. F. (2002). Prokaryotic diversity–magnitude, dynamics, and controlling factors. *Science* 296, 1064–1066. doi: 10.1126/science.1071698

Valdivia-Anistro, J. A., Eguiarte-Fruns, L. E., Delgado-Sapien, G., Marquez-Zacarias, P., Gasca-Pineda, J., Learned, J., et al. (2015). Variability of

rRNA operon copy number and growth rate dynamics of *Bacillus* isolated from an extremely oligotrophic aquatic ecosystem. *Front. Microbiol.* 6:1486. doi: 10.3389/fmicb.2015.01486

Van Horn, D., Sinsabaugh, R., Takacs-Vesbach, C., Mitchell, K., and Dahm, C. (2011). Response of heterotrophic stream biofilm communities to a gradient of resources. *Aquat. Microb. Ecol.* 64, 149–161. doi: 10.3354/ame01515

Vieira-Silva, S., and Rocha, E. P. C. (2010). The systemic imprint of growth and its uses in ecological (meta)genomics. *PLoS Genet.* 6:e1000808. doi: 10.1371/journal.pgen.1000808

Weider, L. J., Elser, J. J., Crease, T. J., Mateos, M., Cotner, J. B., and Markow, T. A. (2005). The Functional significance of ribosomal (r)DNA variation: impacts on the evolutionary ecology of organisms. *Ann. Rev. Ecol. Syst.* 36, 219–242. doi: 10.2307/30033803

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag. doi: 10.1007/978-0-387-98141-3

Zhang, R., Wu, Q., Piceno, Y. M., Desantis, T. Z., Saunders, F. M., Andersen, G. L., et al. (2013). Diversity of bacterioplankton in contrasting Tibetan lakes revealed by high-density microarray and clone library analysis. *FEMS Microbiol. Ecol.* 86, 277–287. doi: 10.1111/1574-6941.12160

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 September 2017
doi: 10.3389/fmicb.2017.01692

The Effects of Nutrient Imbalances and Temperature on the Biomass Stoichiometry of Freshwater Bacteria

Katherine N. Phillips[1,2], *Casey M. Godwin*[1,3] *and James B. Cotner*[1]*

[1] *Department of Ecology, Evolution and Behavior, University of Minnesota, St. Paul, MN, United States,* [2] *Science Department, Saint Paul College, Saint Paul, MN, United States,* [3] *School of Natural Resources and Environment, University of Michigan, Ann Arbor, MI, United States*

Two contemporary effects of humans on aquatic ecosystems are increasing temperatures and increasing nutrient concentrations from fertilizers. The response of organisms to these perturbations has important implications for ecosystem processes. We examined the effects of phosphorus (P) supply and temperature on organismal carbon, nitrogen and phosphorus (C, N, and P) content, cell size and allocation into internal P pools in three strains of recently isolated bacteria (*Agrobacterium* sp., *Flavobacterium* sp., and *Arthrobacter* sp.). We manipulated resource C:P in chemostats and also manipulated temperatures from 10 to 30°C. Dilution rates were maintained for all the strains at ∼25% of their temperature-specific maximum growth rate to simulate low growth rates in natural systems. Under these conditions, there were large effects of resource stoichiometry and temperature on biomass stoichiometry, element quotas, and cell size. Each strain was smaller when C-limited and larger when P-limited. Temperature had weak effects on morphology, little effect on C quotas, no effect on N quotas and biomass C:N, but had strong effects on P quotas, biomass N:P and C:P, and RNA. RNA content per cell increased with increasing temperature at most C:P supply ratios, but was more strongly affected by resource stoichiometry than temperature. Because we used a uniform relative growth rate across temperatures, these findings mean that there are important nutrient and temperature affects on biomass composition and stoichiometry that are independent of growth rate. Changes in biomass stoichiometry with temperature were greatest at low P availability, suggesting tighter coupling between temperature and biomass stoichiometry in oligotrophic ecosystems than in eutrophic systems. Because the C:P stoichiometry of biomass affects how bacteria assimilate and remineralize C, increased P availability could disrupt a negative feedback between biomass stoichiometry and C availability.

Keywords: temperature, phosphorus, carbon, nitrogen, stoichiometry, nucleic acids, morphology

OPEN ACCESS

Edited by:
George S. Bullerjahn, Bowling Green State University, United States

Reviewed by:
Michael Paul Manzella, Colorado State University, United States
Robert (Mike) McKay, Bowling Green State University, United States

***Correspondence:**
James B. Cotner
cotne002@umn.edu

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *13 February 2017*
Accepted: *22 August 2017*
Published: *08 September 2017*

Citation:
Phillips KN, Godwin CM and Cotner JB (2017) The Effects of Nutrient Imbalances and Temperature on the Biomass Stoichiometry of Freshwater Bacteria. Front. Microbiol. 8:1692. doi: 10.3389/fmicb.2017.01692

INTRODUCTION

Freshwaters are increasingly recognized as important regulators of exported terrestrial organic carbon through effects on mineralization and burial (Tranvik et al., 2009). Aquatic heterotrophic bacteria play an important role in aquatic systems as the "gatekeepers" to aquatic food webs through effects on the mineralization of nutrients and organic carbon (Cotner and Wetzel, 1992; Cotner and Biddanda, 2002) with important feedbacks to ecosystem productivity and global change processes.

Therefore, it is important to understand how heterotrophic bacteria respond to two important and simultaneous anthropogenic drivers of freshwater ecosystem processes: global warming and eutrophication (Baron et al., 2002; Carpenter, 2005).

Temperature plays a critical role in microbial metabolism. Early work by Pomeroy and others indicated that temperature has important effects on interactions between marine heterotrophic bacteria and phytoplankton, with implications for export production and subsequent burial in the polar oceans (Pomeroy and Deibel, 1986). Increasing temperatures decrease the growth efficiency of heterotrophic bacteria in both marine and freshwater ecosystems, leading to higher nutrient regeneration efficiencies at high temperatures (Rivkin and Legendre, 2001; Biddanda and Cotner, 2002; Apple et al., 2006; Hall and Cotner, 2007). Another way that temperature affects nutrient regeneration and carbon cycling by bacteria is through effects on biomass stoichiometry (Cotner et al., 2006; Chrzanowski and Grover, 2008; Hall et al., 2009). Previous work with rapidly growing *Escherichia coli* in a nutrient replete chemostat culture at a constant dilution rate demonstrated that increasing temperature led to decreased P content, and increased carbon (C) to phosphorus (P) ratios, which we argued was partially due to increased translational efficiency of ribosomal RNA at higher temperatures (Dicks and Tempest, 1966; Cotner et al., 2006). This work was consistent with a large survey of ectotherms from microbes to higher plants and animals indicating that P content and RNA content decreased at higher temperatures (Woods et al., 2003).

However, the effects of temperature on bacterial metabolism and stoichiometry can be confusing due to interactive effects of temperature and resource availability and adaptation by communities to a given thermal regime (Hall et al., 2008; Martiny et al., 2016). A problem with interpreting temperature effects on microorganisms is that the maximum growth rate generally increases with temperature, which means that at a fixed dilution rate, increasing temperature would decrease the relative growth rate, i.e., the ratio between realized growth rate and the maximal growth rate. Therefore, experiments that use a fixed dilution rate to examine the effect of temperature run the risk of inadvertently manipulating relative growth rate, which has been shown to affect biomass stoichiometry, element quotas, and growth efficiency (Godwin et al., 2017). Furthermore, the extent to which the realized growth rate, but not the maximal growth rate, increases with temperature is very much dependent on resource availability. To address some of these concerns, we isolated strains of heterotrophic bacteria from lakes in northern Minnesota and examined their macromolecular and biomass elemental content at varying temperatures and nutrient (P) availability. Rather than maintaining a constant dilution rate in chemostats, we determined the maximum growth rate at each temperature and ran the chemostats at 25% of their temperature-specific maximum growth rate, maintaining a uniform relative growth rate by varying the dilution rate at all temperatures. We hypothesized that normalizing the relative growth rate across temperatures would minimize the effects of temperature on stoichiometry but would also allow us to understand how nutrients and carbon affect biomass composition independent of growth rate.

MATERIALS AND METHODS

Water was collected from three lakes in northern Minnesota (Itasca State Park, Hubbard and Clearwater counties) and bacterial cultures were established using the agar streak plate method onto complex culture media (Difco nutrient agar, cellulose + Difco nutrient agar, or LB agar medium). Individual colonies were picked from plates after visible growth was observed and this process was repeated several times with the goal of isolating individual bacterial strains. Each unique strain was transferred to plates containing defined nutrient rich basic minimal media (BMM; Tanner, 2007) with 23.88 mmol C L^{-1} as glucose and then grown in the same media as broth until late log phase. These cultures were stored at −70°C in 15% glycerol until use. The bacterial strains examined in this study were identified using partial 16S rDNA sequence analysis (Ghosh and LaPara, 2007) as: *Agrobacterium*, sp. (Phylum: Alpha-proteobacteria; Gram negative), *Flavobacterium*, sp. (Phylum: Bacteriodetes; Gram negative), and *Arthrobacter*, sp. (Phylum: Actinobacteria; Gram positive) and were isolated from Deming, Itasca and Elk lakes, respectively. We chose these strains because they represent a wide diversity from classes that are well-represented in freshwaters (Newton et al., 2011), and previous work with two of the strains indicated that they differed in how they regulate their biomass composition (Scott et al., 2012).

Experiments were conducted at 10, 15, 20, 25, and 30°C, which nearly encompasses the annual temperature range experienced in these lakes. Because the maximal growth rate (μ_{max}) is temperature dependent (Kuhn et al., 1980), we determined μ_{max} of each strain at each temperature by measuring the optical density at 600 nm during logarithmic growth in batch culture in a nutrient replete medium (in BMM with 2.4 mM phosphate and 19 mM ammonium). We cultured each strain in a complete factorial design of temperature and P availability. At each combination of temperature and P availability, each strain was grown in triplicate chemostat cultures at 25% μ_{max}. The phosphate concentration of the medium was manipulated to achieve molar resource C:P ratios (C:P_R) of 50, 250, and 1,000:1. The sterile 80 mL acid-soaked glass chemostats were mixed continuously with filtered air until the cell densities reached steady state and then measurements were made.

It is known that relative growth rate (proportional to a temperature-specific maximum growth rate) is a strong driver of elemental content and stoichiometry (Godwin et al., 2017). In order to isolate the unknown effect of temperature from the known effect of relative growth rate, we adjusted the dilution rate to a fixed proportion of the maximum growth rate observed at each temperature. If we had used a single dilution rate at all temperatures, the relative growth rate would be different for each temperature. As a result, we would not be able to separate the effect of temperature from relative growth rate.

Cell Abundance and Cell Morphology

A sample of cells harvested from each chemostat was preserved in formalin (final concentration of 3.7%) and stored at 4°C.

The fixed cells were diluted in 118 mM pyrophosphate to reduce clumping. Also, after a 30 min period of shaking at 200 rpm, the cells were sonicated on ice for 2 min in 30-s intervals, then stained with acridine orange, filtered onto 0.2 μm pore-size black polycarbonate filters and examined by epifluorescence microscopy (Hobbie et al., 1977). Cell abundance was estimated by counting a minimum of 10 fields. The bacteria were photographed with a SPOT digital camera (Diagnostics Instruments, Inc) for image analysis. Mean cell length and width were measured for a minimum of 75 randomly selected cells from each chemostat using Image Pro Plus software (Media Cybernetics, version 4.5.1). Cell volume and surface area of *Agrobacterium* sp. and *Flavobacterium* sp. were determined using equations assuming the cell shape was a cylinder capped by two hemispheres (Sun and Liu, 2003). Cell volume and cell surface area of *Arthrobacter* sp. was determined by using equations derived for a prolate spheroid (Sun and Liu, 2003).

Elemental Analysis

Elemental analyses for carbon and phosphorus were performed on biomass samples from the chemostats to assess the elemental composition of each strain. Bacteria were harvested and filtered ($<5 * 10^{-5}$ atm) onto pre-combusted Whatman GF/F filters for particulate carbon analysis and onto acid-rinsed GF/F filters for particulate phosphorus analysis. All samples were collected and analyzed in triplicate. The particulate organic carbon content was determined using a CHN analyzer (PerkinElmer, model 2400). The P content was determined spectrophotometrically following an acid-persulfate digestion, reacting with molybdenum blue and absorption measurements at 880 nm (APHA, 1992).

Nucleic Acids

Cells from each chemostat were collected on 0.2 μm white polycarbonate filters in triplicate. Bacterial nucleic acids were determined after extraction via sonication and staining with the fluorochrome RiboGreen (Molecular Probes), which reacts with both DNA and RNA (Gorokhova and Kyle, 2002). Bacterial samples on the filters, negative control samples (containing all reagents but no bacteria), and standards of RNA (*E. coli*, Invitrogen) and DNA (calf thymus, Sigma) were prepared for analysis as described in Makino et al. (2003). Two identical 96-well black microplates were prepared with one plate receiving an addition of RNase (Promega; conc in 1X * TE buffer) before the 30-min digestion period. After the digestion reaction period, 75 μL of RiboGreen was added to both plates followed by a second reaction period of 10 min. The fluorescence of the samples was measured on both plates (excitation 480 nm and emission at 520 nm) on a Micromax 384 plate reader (Fluormax, Horbia Jobin Yvon) using DataMax software. The fluorescence of the digested sample was used to determine DNA concentration and differences between the undigested and digested plates were used to determine RNA concentrations.

Statistical Analysis

The effect of varying C:P_R and temperature on the molar ratio of biomass C:P in the replicated chemostats was evaluated using analysis of variance (ANOVA) using the JMP® statistical software (version 11.2). ANOVAs were conducted on biomass parameters with temperature, resource C:P and strain as fixed effects. We did a three-way ANOVA initially and if there were statistically significant interactions, we conducted a two-way ANOVA for each strain (with temperature and P as the independent variables). If there was a significant interaction in the two-way ANOVA, then we did a one-way test for each variable at each level of the other variable. We used a *p*-value of less than or equal to 0.05 to determine statistical significance.

RESULTS

The maximum growth rate of the three strains varied greatly with temperature, with all three strains having a μ_{max} <0.08 hr^{-1} at 10°C and a μ_{max} greater or equal to 0.2 hr^{-1} at the highest temperature (**Figure 1**). Two of the strains (*Agrobacterium* sp. and *Arthrobacter* sp.) had very similar temperature-dependent growth curves with the highest maximum growth rate at the highest temperature used in this study (30°C). However, *Flavobacterium* reached its highest growth rate at 25°C and μ_{max} varied only from 0.07 to 0.25 hr^{-1} from 10 to 30°C.

Morphology and Biovolume

Three-way ANOVAs on cell volume and length-to-width ratios indicated that there were significant effects of both C:P_R ratio and different strains but not temperature (**Table 1**, **Figure 2**, Table S1) on morphology. There was a strain*temperature interaction with length to width ratios but not biovolume, and there was a strain*C:P_R interaction with both length to width ratios and biovolume. Two-way ANOVAs revealed significant effects of temperature only on the biovolume of *Arthrobacter* and the length to width ratio of both *Agrobacterium* and *Arthrobacter* (**Table 1**, Table S1). C:P_R ratio had significant effects on both morphometric variables in all three strains and there were no interactions between C:P_R ratio and temperature (**Table 1**, Table S1). As C:P_R increased, the biovolume and length to width ratios also increased. There was a significant effect of C:P_R on both biovolume and L:W ratios in the 3-way ($p < 0.0001$)

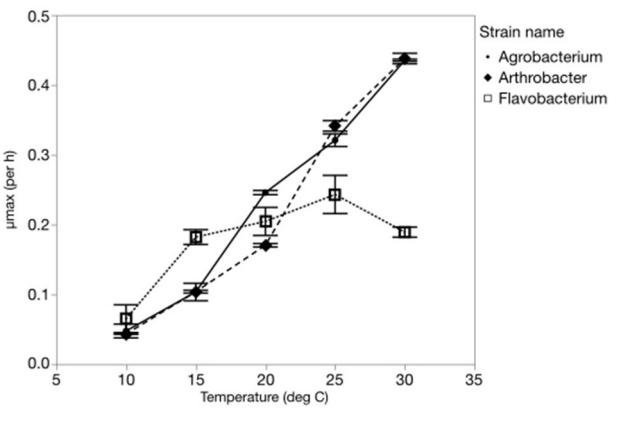

FIGURE 1 | The effects of temperature on the maximum growth rates (μ_{max}) of each strain from 10 to 30°C.

TABLE 1 | Three-way and two-way ANOVAs.

	Three-way ANOVA						*Agrobacterium*			*Arthrobacter*			*Flavobacterium*		
	S	**T**	**P**	**S*T**	**S*P**	**T*P**	**T**	**P**	**T*P**	**T**	**P**	**T*P**	**T**	**P**	**T*P**
C quota	***	ns	***	ns	**	***	ns	***	***	ns	***	ns	*	***	**
N quota	***	ns	***	ns	*	ns	ns	***	*	ns	***	ns	ns	***	*
P quota	ns	***	**	ns	ns	**	ns	**	ns	***	ns	ns	*	ns	*
Volume	***	ns	***	*	**	ns	ns	*	ns	*	***	ns	ns	***	ns
L:W	***	ns	***	*	**	ns	**	***	ns	***	**	ns	ns	***	ns
C:N	***	ns	***	ns	ns	ns	ns	***	ns	ns	ns	ns	ns	**	ns
N:P	***	***	***	ns	*	***	**	***	ns	ns	***	ns	*	***	*
C:P	**	***	***	ns	*	***	**	***	*	**	***	ns	**	***	*
RNA	***	*	***	ns	ns	ns	*	*	ns	ns	*	ns	ns	*	ns
DNA	***	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns
Total ns	1	6	1	8	4	6	6	0	7	6	3	10	6	2	5
Total sig	9	4	9	2	6	4	4	10	3	4	7	0	4	8	5

*Statistically insignificant results are denoted by "ns" and significant results ($p < 0.05$) are denoted with asterisks, with more asterisks representing lower p-values. Units for each parameter are the same as discussed in the paper. S, strain; T, temperature; P, C:P_R; S*T, strain by temperature interaction; S*P, strain by C:P_R interaction; T*P, temperature by C:P_R interaction. There were no three-way interactions (S*T*P). We summed the non-significant and significant observations at the bottom of the table.*

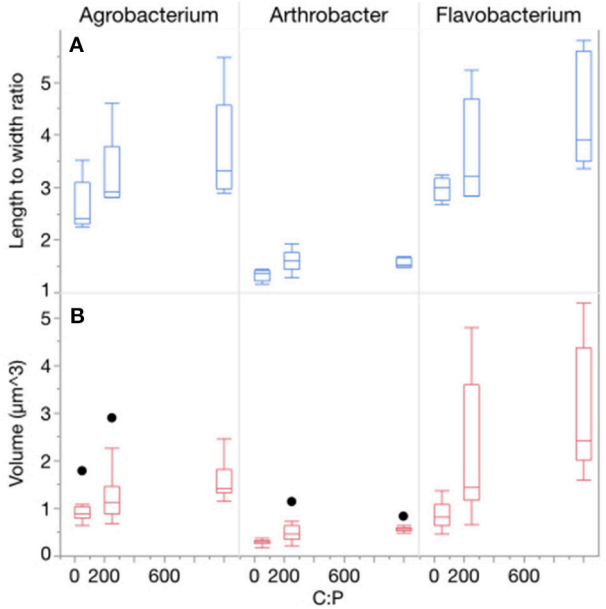

FIGURE 2 | (A) Changes in the morphology (length to width ratios) of each strain across C:P supply ratios. Bar depict the median with the 25th and 75th percentiles and error bars are the 95% confidence interval with outliers represented by points. **(B)** Changes in the biovolume (μm^3) of each strain across C:P supply ratios.

and two-way ANOVAs (all $p < 0.006$). There was a great deal of variability in both the volume and the L:W ratios among the different strains, however (**Figure 2**). There were large differences in the mean length: width ratios of the three strains with *Flavobacterium* sp. and *Agrobacterium* sp., having higher ratios (3.68 and 3.20, respectively) than *Arthrobacter* sp. (1.50). *Arthrobacter* also had the lowest volume (mean = 0.46 μm^3) followed by *Agrobacterium* (1.30 μm^3) and *Flavobacterium* (2.03 μm^3).

Internal Pools and Ratios

Temperature and P availability interactively affected biomass element content. Consistent with differences in the sizes of the three strains, the strains also differed in terms of C and N content per cell, but not P content per cell (**Table 1**, **Figure 3**, Table S2; C quota: $p < 0.0001$; N quota: $p = 0.0003$; P quota: $p = 0.068$). Temperature had no effect on C and N cell quotas but it did affect P quotas ($p < 0.0001$), with increasing quotas with temperature increases and at the lowest C:P_R (**Table 1**, **Figure 3**). C, N, and P cell quotas were strongly affected by C:P_R and there were significant interactions between temperature and C:P_R for C, N, and P quotas, especially C and N quotas for *Agrobacterium* and *Flavobacterium* (**Table 1**). C:P_R had effects on the C and N quotas of all three strains. P quotas were affected by different factors among the strains. C:P_R had a significant effect on P quotas for *Agrobacterium* ($p = 0.006$) while the other two strains were affected more by temperature ($p < 0.0001$ and $p = 0.0069$ in *Arthrobacter* and *Flavobacterium*, respectively). There was also a temperature*C:P_R interaction in *Flavobacterium*.

The changes in cell quotas were not consistent among C, N, and P, leading to substantial variation in the effects of temperature and P on biomass stoichiometry (**Table 1**, Table S3). The biomass C:P (C:P_B) ratios were high at a C:P_R of 1000:1, with all three strains having ratios near 500:1 at the lowest temperatures and decreasing with increased temperature (**Figure 4**). The median biomass C:P for the three strains was similar, varying from 144 in *Flavobacterium* to 181 in *Agrobacterium* and 202 *Arthrobacter*. Although, biomass C:P decreased with temperature at all levels of C:P_R, the response of biomass C:P to temperature was strongest at the highest C:P_R, indicating that biomass C:P_B ratios were the most sensitive to temperature changes when P was least available. A similar pattern

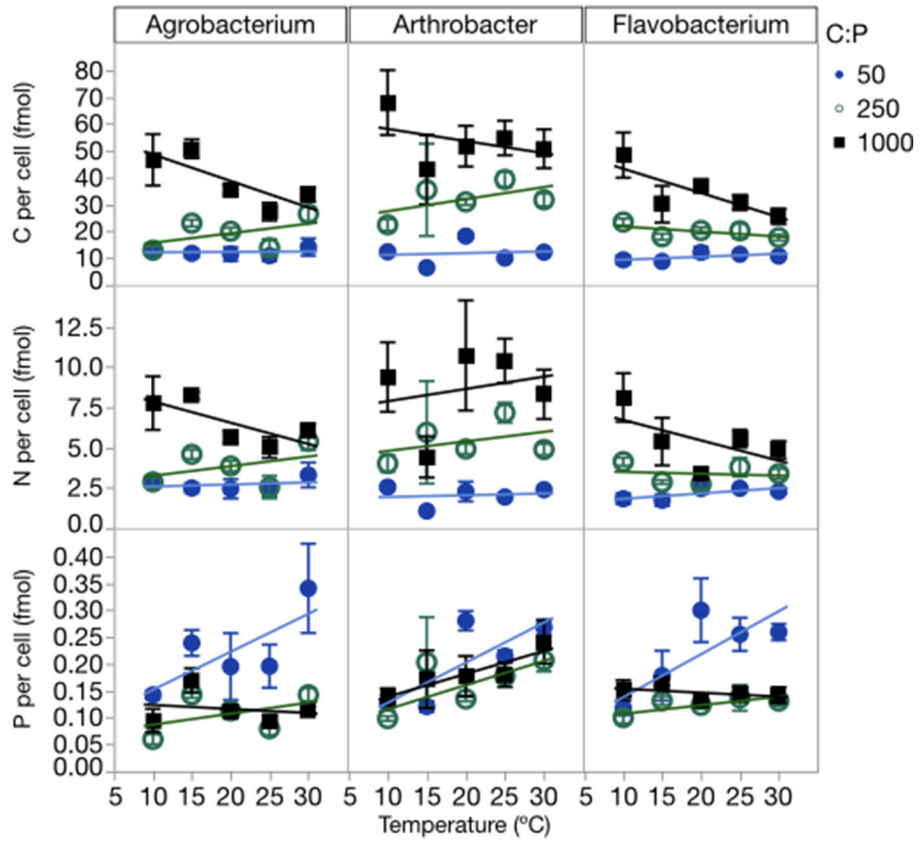

FIGURE 3 | Changes in cellular C, N, and P quotas of each strain across temperatures, with different symbols for each level of $C:P_R$. Error bars represent one standard error of the mean.

was observed for N:P but less so for C:N. The mean N:P biomass ratio for the three strains varied from 25 in *Flavobacterium* to 31 in *Arthrobacter* and 36 in *Agrobacterium*. The mean $C:N_B$ for the three strains varied much less among strains, with a mean of 5.9 in *Flavobacterium, to* 6.3 in *Arthrobacter* and 5.1 in *Agrobacterium*.

Consistent with the cell quota observations, temperature did not affect biomass C:N ratios, rather only biomass N:P and C:P ratios (**Figure 4**, **Table 1**). There were significant effects of temperature on both $C:P_B$ and $N:P_B$ in all three strains, with the exception of *Arthrobacter* biomass N:P. $C:P_R$ also had significant effects on all three biomass ratios in each of the strains, with the exception of the C:N of *Arthrobacter*.

RNA per cell varied by strain, temperature and $C:P_R$, while DNA per cell varied only among strains (**Figure 5**, **Table 1**, Table S4). There were no interactions for either of these factors. RNA per cell was highest when $C:P_R$ was highest (lowest P supply) in two of the strains (*Agrobacterium* and *Flavobacterium)*, perhaps due to increased cell size when P was least available. In addition, RNA per cell increased with increasing temperatures in *Agrobacterium* and *Arthrobacter*. *Agrobacterium*, *Arthrobacter,* and *Flavobacterium* had mean values of 4.8, 4.7, and 9.5 fg RNA $cell^{-1}$, respectively. Across all levels of temperature and $C:P_R$, *Agrobacterium*, *Arthrobacter,* and *Flavobacterium* had mean values of 9.9, 9.8, and 16.8 fg DNA $cell^{-1}$, respectively, giving RNA/DNA ratios of 0.48, 0.48, and 0.56, respectively.

The proportion of cellular P in nucleic acids (RNA and DNA) increased with increasing $C:P_R$ particularly at 1,000:1 and varied among strains (**Figure 6**). There was an effect of temperature on the proportion of total P in RNA ($p = 0.0139$) but no effect on total nucleic acid content per cell. The percentage of P in nucleic acids varied from a low of 10% (*Agrobacterium*, high P supply and 10°C) to 92% (*Flavobacterium*, low P supply, 25°C) with mean percentages of 32% for both *Agrobacterium* and *Arthrobacter* and 48% for *Flavobacterium*.

DISCUSSION

Here we show that temperature and resource stoichiometry both affect the biomass stoichiometry and physiology of aquatic heterotrophic bacteria and illustrate that these factors had a strong interactive effect that was independent of the relative growth rate. We observed important effects of C:P supply on all of these parameters, particularly cell size and shape, as well as internal pools of C, N, and RNA. One of the key observations was that the biomass C:P stoichiometry in these three strains was quite responsive to varying temperatures and nutrient supply rates, going from about 40:1 at high temperatures and high

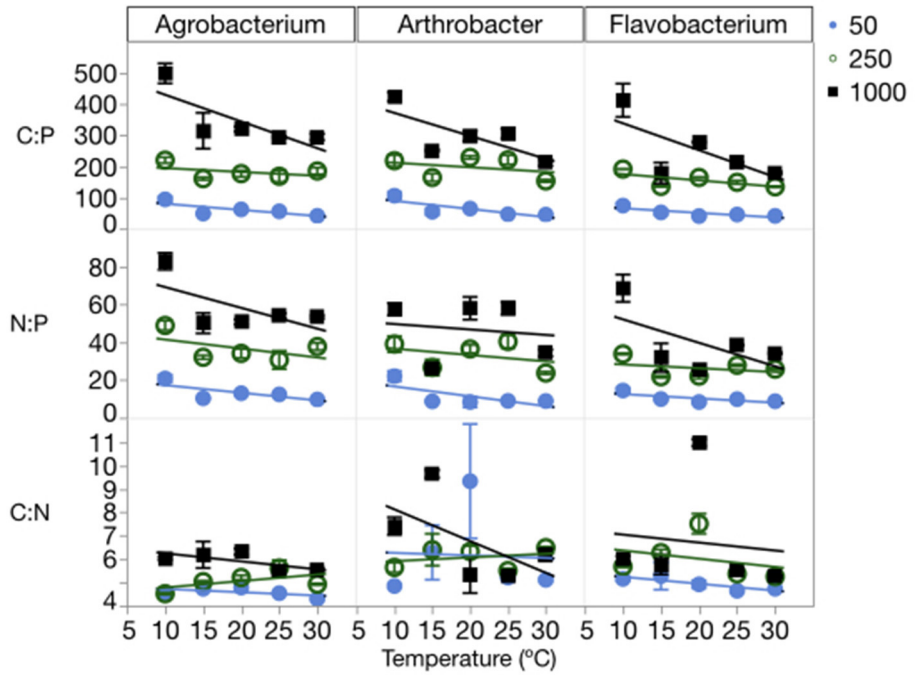

FIGURE 4 | Changes in the biomass stoichiometry (molar ratios of C:P, C:N, and N:P) of each strain across temperatures, with different symbols for each level of supply C:P. Error bars represent one standard error of the mean. The three-way ANOVA for C:N showed significant effects of strain and supply C:P only ($p < 0.001$). The ANOVA for C:P showed significant effects of strain, temperature, supply C:P, an interaction between strain and supply C:P, and an interaction between temperature and supply C:P ($p < 0.01$). The ANOVA for N:P showed significant effects of strain, temperature, supply C:P, and an interaction between strain and supply C:P ($p < 0.05$).

P availability to over 500:1 at cooler temperatures and low P, confirming recent observations that both bacterial strains and bacterial communities can be quite flexible in their C and P content (Hochstädter, 2000; Cotner et al., 2010; Scott et al., 2012; Godwin and Cotner, 2014, 2015).

Perhaps as important as the ability of these strains to vary internal P content was their capacity to vary C content, primarily mediated via changes in cell size (Garcia et al., 2016). Temperature has important influences on the stoichiometry of ectotherms as well as endotherms in freshwater, terrestrial and marine systems (Gillooly et al., 2002; Woods et al., 2003; Reich and Oleksyn, 2004; Borer et al., 2013). The general pattern of decreased cell size at higher temperature (Bergmann's Rule) likely mediates many of these interactions but our study seems to indicate that, at least in heterotrophic bacteria, decreased cell size at increased temperatures may be mediated in part by the effects of temperature on relative growth rates. Specifically, we only observed effects of temperature on the morphology of *Arthrobacter*.

Related to the morphology of these three strains, it is curious that we more frequently found temperature and C:P_R effects on *Agrobacterium* and *Flavobacterium* (both Gram negative) than on *Arthrobacter* which is Gram positive. It is speculative given the small number of strains examined here, but the thicker and more rigid structure of Gram positive bacterial cell walls may constrain their morphology (Cabeen and Jacobs-Wagner, 2005), thereby restricting their capacity to significantly change cell quotas and stoichiometry. To the best of our knowledge, these connections between cell wall structure and stoichiometry in bacteria have not been examined but could provide an alternative explanation for why some strains are more homeostatic than others (Godwin and Cotner, 2015).

This work provides important insights on the growth rate hypothesis (GRH), a tenet of ecological stoichiometry (Elser et al., 2000). By maintaining a uniform relative growth rate while changing the temperature, we were able to observe how temperature and nutrient limitation affect biomass composition independent of relative growth rate. Cell size and morphology were strongly affected by nutrient limitation, i.e., C:P_R ratios, with accompanying changes in cell quotas and stoichiometry in all three strains. Moving forward, it is important to keep in mind that limitation by different nutrients can impose changes in morphology, with effects on stoichiometry, that are independent of growth rate. In contrast, temperature had mixed effects on morphology (effects on cell volume and length to width ratios in *Arthrobacter,* effect on cell volume for *Agrobacterium*, but no effects on *Flavobacterium*), but had effects on P quotas in two of the three strains and effects on biomass N:P in two strains and C:P ratios in three strains. The fact that these effects were observed at uniform relative growth rate indicates that there can be important effects of nutrients and temperature that are unrelated to the GRH.

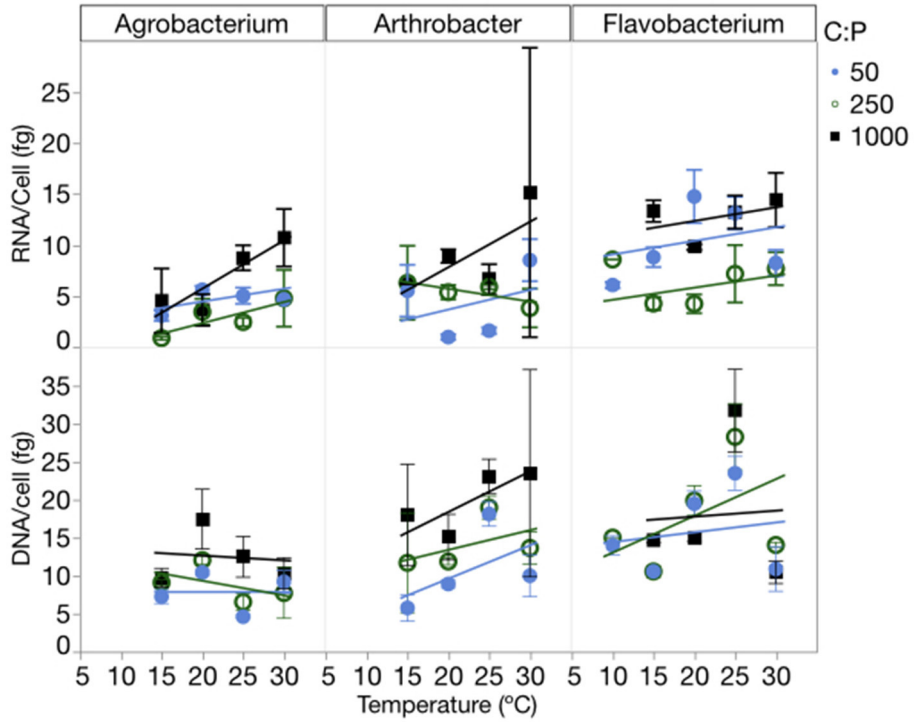

FIGURE 5 | Changes in the RNA and DNA content (fg cell^{-1}) of each strain across temperatures, with different symbols for each level of supply C:P. Error bars represent one standard error of the mean.

By minimizing the influence of relative growth rate in the present study, we observed what more closely represents the effects of temperature on internal pools of C, N, and P (**Table 1**, Table S2). Relative growth rate is an important concept particularly for ecological stoichiometry because it describes what portion of the organismal biomass should be focused on growth vs. other cellular activities (resource acquisition; structural materials, maintenance, etc.; Klausmeier et al., 2004). As the relative growth rate increases, the concentration of P-rich ribosomes should increase and therefore the C:P and N:P ratios should decrease (Elser et al., 2003). The GRH (Elser et al., 2000) predicts that RNA content is proportional to growth rate, so it was somewhat surprising that we observed increased RNA with increasing temperatures at uniform relative growth rates which may have been due to changes in cell size or differences in ribosome translation efficiency or degradation that vary with temperature (Dicks and Tempest, 1966) and/or nutrient limitation (Deutscher, 2003).

Temperature and Relative Growth Rate

As mentioned above, there were mixed effects of temperature on elemental quotas and stoichiometry. In previous studies, temperature has been shown to have important effects on cell size and stoichiometry in diverse organisms, from bacteria to fish and terrestrial, freshwater and marine systems (Daufresne et al., 2009; Forster et al., 2012; Borer et al., 2013; Morán et al., 2015). Specifically, past studies have shown that increasing temperature generally leads to *increased* C:P, N:P, and C:N ratios (Woods et al., 2003; Cotner et al., 2006; Martiny et al., 2013), but in the present study we found that increasing temperatures often led to *decreased* biomass C:P and N:P ratios ($p < 0.05$ for N:P in two strains and $p < 0.05$ for C:P in all strains). This contradiction is likely due to differences in growth and temperature manipulations. In a chemostat at a constant dilution rate, as temperature increases, the relative growth rate will decrease due to a uniform realized growth with increasing μ_{max}, in contrast to the uniform relative growth in the present experiments.

Of the previous studies that have examined the effects of temperature on biomass stoichiometry, Chrzanowski and Grover (2008) manipulated both the dilution rate and temperature in a chemostat study using *Pseudomonas*. They observed that growth rate had a larger effect on biomass stoichiometry than did temperature and this observation was one of the motivations for why we maintained uniform relative growth rates in the present study. However, unlike the present study, Chrzanowski and Grover (2008) observed that cell quotas for C, N, and P were highest at either low temperature and low dilution rate or high temperature and high dilution rate. At low dilution rates, C, N, and P content decreased with increasing temperatures, but in the present study there was no significant effect of temperature on C and N cell quotas, while P cell quotas increased with increasing temperatures, particularly when P availability was high. Also, in their study, at high dilution rates, C, N, and P quotas increased with temperature, similar to our observations with *E. coli* for C and P (Makino et al., 2003).

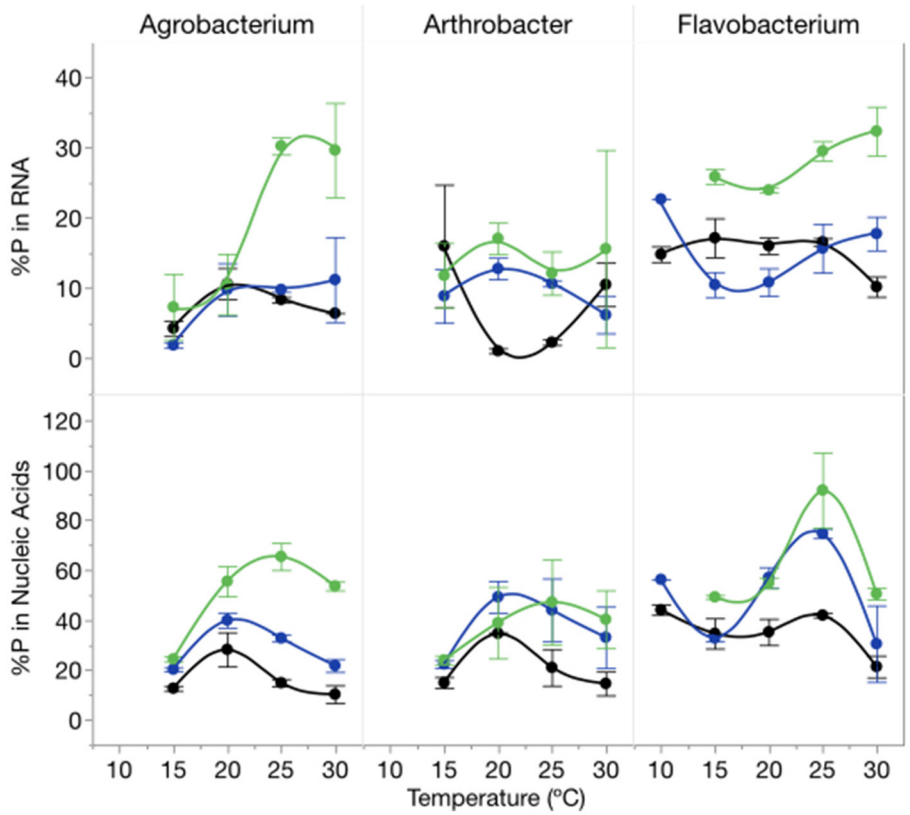

FIGURE 6 | Changes in %P in RNA (top panel) and %P in nucleic acids (bottom panel) for each strain across temperatures and C:P_R with different symbols for each level of supply C:P (black-50:1; blue: 250:1; green: 1000:1). Error bars represent one standard error of the mean.

The lack of a consistent effect of temperature on cell size (Table S1) and quotas (Table S2) was also surprising in light of past work showing an inverse relationship between temperature and cell size. Among species, there is a general decline in maximal growth rates and metabolic rates with increasing size (Sommer, 1989; Niklas and Enquist, 2001) but variation within a species is influenced by the relative growth rate, due to the fact that growth and structural components scale differently with size (Clark et al., 2013). As relative growth rates increase, single cell organisms tend to become larger and more dilute internally (Kempes et al., 2012), whereas increasing temperatures tend to make cells smaller (Woods et al., 2003; Morán et al., 2015). We have recently observed that there is a great deal of variability in how different bacterial strains regulate their C:P and N:P ratios (Godwin and Cotner, 2015) and some of the capacity to do this may be coupled with the capacity of cells to change their morphology.

An obvious question that needs to be addressed is whether, in natural systems the effects of temperature on organism stoichiometry are mediated largely through the effects of temperature on cell size and thus, internal pools as we observed here, or through effects on relative growth rate, as observed in our recent work (Godwin et al., 2017). Biomass stoichiometric ratios (C:P, N:P, or C:N) generally decrease with latitude, a pattern that has been documented in marine plankton (Martiny et al., 2013), multiple ecosystem types (Borer et al., 2013), plant leaf tissue (Reich and Oleksyn, 2004; Kerkhoff et al., 2005), marcrophyte biomass (Xia et al., 2014), and freshwater plankton (Dobberfuhl and Elser, 2000), but many variables other than temperature change along a latitudinal gradient (Martiny et al., 2016), complicating interpretation of these data.

C:P Supply

Although temperature had important effects on several internal pools and some stoichiometric ratios, C:P_R affected nearly all of these parameters. This result suggests that, across the range of temperatures and C:P_R, nutrient availability was the more important determinant of bacterial stoichiometry and physiology at uniform relative growth rates. While it is not surprising that C:P_R had a strong effect on the C:P and N:P ratios of the bacterial strains (and to a less extent C:N), the temperature*C:P_R interaction suggests that temperature modified the response to C:P_R (**Figure 3**). Specifically, biomass C:P was more responsive to differences in C:P_R at the lowest temperatures.

Some of the effect of supply stoichiometry on biomass stoichiometry and elemental quotas was attributable to changes in RNA content. There was an increase in RNA content at the highest C:P supply ratio (**Figure 5**) which is similar to other

studies looking at the GRH when P was not limiting (Elser et al., 2003). This suggests that there may be significant changes in RNA unrelated to the growth rate. One possibility is that changes in RNA were caused by changes in cell size. If the cellular ribosome concentration varied little with C:P supply ratios, but there were increases in cell size, it would have increased the RNA content per cell. Biovolumes increased with increasing C:P supply ratios from 56% for *Agrobacterium*, and 108% for *Arthrobacter*, to 255% for *Flavobacterium*.

Temperature had more significant effects on the P quotas of the three strains ($p < 0.05$ for *Arthrobacter* and *Flavobacterium* and $p = 0.08$ in *Agrobacterium*) than did C:P_R, while C:P_R had stronger effects on C and N cell quotas (**Figure 3**, **Table 1**). These differences were likely due in a large part to the strong effects that C:P_R had on morphology in all three strains (**Table 1**). The lack of a strong C:P_R effect on P quotas likely reflects the compensating effects of P availability on cell volume. Higher P availability leads to decreased cell volume but higher internal concentrations of some pools, while decreased P availability has the opposite effect, leading to a "buffered" P quota per cell. Similar observations have been made by others examining the effects of C and P limitation on cell morphology with P limitation leading to larger and more elongate cells (Løvdal et al., 2008; Godwin and Cotner, 2015). But Løvdal et al. also noted in their study of *Vibrio splendidus* that C, N, and P scaled differently, with larger cells having proportionately less P than C and N.

Implications for Climate Change and Eutrophication

Temperature is a fundamental environmental variable that affects the behavior of everything from molecules to ecosystems. The metabolic theory of ecology (Brown et al., 2004) demonstrated that metabolic rates vary in predictable ways based on organismal size (mass) and temperature. In a literature survey of organisms from bacteria and algae to plants and animals, Woods et al. (2003) observed that most organisms were more N-, P-, and RNA-rich at colder temperatures. More recently, it was shown that bacterial size (Morán et al., 2010) and plankton stoichiometry (Martiny et al., 2013) in the oceans may both be influenced by temperature. Although temperature has effects on both metabolism and biomass composition, the present study shows that there are important effects of temperature on stoichiometry independent of growth rates. This mechanism is not widely recognized in biogeochemical modeling, but has important implications for predicting how the pools and fluxes will respond to warming temperatures and increased availability of nutrients.

One thing that was clear in examining the effects of temperature on biomass stoichiometry was that there was much greater change in stoichiometry at different temperatures when P was most limiting. In particular, C:P and N:P slopes relative to temperature were much steeper at the lowest P availability. These results imply that temperature effects on nutrient cycling are likely to be greatest in the least productive (oligotrophic) ecosystems such as large lakes and the open ocean. Furthermore, increased eutrophication in freshwater and marine ecosystems is likely to dampen feedbacks between climate change and biogeochemical processing, due to this decreased sensitivity at high nutrient levels.

AUTHOR CONTRIBUTIONS

JC designed the research plan and wrote the manuscript; CG implemented portions of the research and wrote the manuscript and KP helped design the research plan, implement the research plan and helped writing the manuscript.

FUNDING

This work was funded by NSF IGERT grant DGE-0504195 and NSF-IOS award 1257571 to JC.

ACKNOWLEDGMENTS

Our work was partially supported by funding from the National Science Foundation (DEB: 0519041). KP and CG were partially supported by a National Science Foundation IGERT grant (DGE: 0504195) and KP received additional support from the University of Minnesota's Institute on the Environment (IREE Program).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01692/full#supplementary-material

REFERENCES

APHA (1992). *Standard Methods for the Examination of Water and Wastewater*. Washington, DC: American Public Health Association.

Apple, J. K., Del Giorgio, P. A., and Kemp, W. (2006). Temperature regulation of bacterial production, respiration, and growth efficiency in a temperate salt-marsh estuary. *Aqua. Microbial. Ecol.* 43, 243–254. doi: 10.3354/ame043243

Baron, J. S., Poff, N. L., Angermeier, P. L., Dahm, C. N., Gleick, P. H., Hairston N. G. Jr., et al. (2002). Meeting ecological and societal needs for freshwater. *Ecol. Appl.* 12, 1247–1260. doi: 10.1890/1051-0761(2002)012[1247:MEASNF]2.0.CO;2

Biddanda, B. A., and Cotner, J. B. (2002). Love handles in aquatic ecosystems: the role of dissolved organic carbon drawdown, resuspended sediments, and terrigenous inputs in the carbon balance of lake michigan. *Ecosystems* 5, 431–445. doi: 10.1007/s10021-002-0163-z

Borer, E. T., Bracken, M. E. S., Seabloom, E. W., Smith, J. E., Cebrian, J., Cleland, E. E., et al. (2013). Global biogeography of autotroph chemistry: is insolation a driving force? *Oikos* 122, 1121–1130. doi: 10.1111/j.1600-0706.2013.00465.x

Brown, J. H., Gilooly, J. F., Allen, A. P., Savage, V. M., and West, G. B. (2004). Toward a metabolic theory of ecology. *Ecology* 87, 1771–1779. doi: 10.1890/03-9000

Cabeen, M. T., and Jacobs-Wagner, C. (2005). Bacterial cell shape. *Nat. Rev. Microbiol.* 3, 601–610. doi: 10.1038/nrmicro1205

Carpenter, S. R. (2005). Eutrophication of aquatic ecosystems: bistability and soil phosphorus. *Proc. Natl. Acad. Sci. U.S.A.* 102, 10002–10005. doi: 10.1073/pnas.0503959102

Chrzanowski, T. H., and Grover, J. P. (2008). Element content of *Pseudomonas fluorescens* varies with growth rate and temperature: a replicated chemostat study addressing ecological stoichiometry. *Limnol. Oceanogr.* 53, 1242–1251. doi: 10.4319/lo.2008.53.4.1242

Clark, J. R., Lenton, T. M., Williams, H. T. P., and Daines, S. J. (2013). Environmental selection and resource allocation determine spatial patterns in picophytoplankton cell size. *Limnol. Oceanogr.* 58, 1008–1022. doi: 10.4319/lo.2013.58.3.1008

Cotner, J. B., and Biddanda, B. A. (2002). Small players, large role: microbial influence on biogeochemical processes in pelagic aquatic ecosystems. *Ecosystems* 5, 105–121. doi: 10.1007/s10021-001-0059-3

Cotner, J. B., Hall, E. K., Scott, T., and Heldal, M. (2010). Freshwater bacteria are stoichiometrically flexible with a nutrient composition similar to seston. *Front. Microbiol.* 1:0132. doi: 10.3389/fmicb.2010.00132

Cotner, J. B., Makino, W., and Biddanda, B. A. (2006). Temperature affects stoichiometry and biochemical composition of *Escherichia coli*. *Microb. Ecol.* 52, 26–33. doi: 10.1007/s00248-006-9040-1

Cotner, J. B., and Wetzel, R. G. (1992). Uptake of dissolved inorganic and organic phosphorus compounds by phytoplankton and bacterioplankton. *Limnol. Oceanogr.* 37, 232–243. doi: 10.4319/lo.1992.37.2.0232

Daufresne, M., Lengfellner, K., and Sommer, U. (2009). Global warming benefits the small in aquatic ecosystems. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12788–12793. doi: 10.1073/pnas.0902080106

Deutscher, M. P. (2003). Degradation of stable RNA in bacteria. *J. Biol. Chem.* 278, 45041–45044. doi: 10.1074/jbc.R300031200

Dicks, J. W., and Tempest, D. W. (1966). The influence of temperature and growth rate on the quantitative relationship between potassium, magnesium, phosphorus and ribonucleic acid of aerobacter aerogenes growing in a chemostat. *J. Gen. Microbiol.* 45, 547–557. doi: 10.1099/00221287-45-3-547

Dobberfuhl, D. R., and Elser, J. J. (2000). Elemental stoichiometry of lower food web components in arctic and temperate lakes. *J. Plankton Res.* 22, 1341–1354. doi: 10.1093/plankt/22.7.1341

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., et al. (2000). Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* 3, 540–550. doi: 10.1046/j.1461-0248.2000.00185.x

Forster, J., Hirst, A. G., and Atkinson, D. (2012). Warming-induced reductions in body size are greater in aquatic than terrestrial species. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19310–19314. doi: 10.1073/pnas.1210460109

Garcia, N. S., Bonachela, J. A., and Martiny, A. C. (2016). Interactions between growth-dependent changes in cell size, nutrient supply and cellular elemental stoichiometry of marine *Synechococcus*. *ISME J.* 10, 2715–2724. doi: 10.1038/ismej.2016.50

Ghosh, S., and LaPara, T. M. (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J.* 1, 191–203. doi: 10.1038/ismej.2007.31

Gillooly, J. F., Charnov, E. L., West, G. B., Savage, V. M., and Brown, J. H. (2002). Effects of size and temperature on developmental time. *Nature* 417, 70–73. doi: 10.1038/417070a

Godwin, C., and Cotner, J. (2014). Carbon:Phosphorus homeostasis of aquatic bacterial assemblages is mediated by shifts in assemblage composition. *Aquatic. Microbial. Ecol.* 73, 245–258. doi: 10.3354/ame01719

Godwin, C. M., and Cotner, J. B. (2015). Stoichiometric flexibility in diverse aquatic heterotrophic bacteria is coupled to differences in cellular phosphorus quotas. *Front. Microbiol.* 6:159. doi: 10.3389/fmicb.2015.00159

Godwin, C. M., Whitaker, E. A., and Cotner, J. B. (2017). Growth rate and resource imbalance interactively control biomass stoichiometry and elemental quotas of aquatic bacteria. *Ecology* 98, 820–829. doi: 10.1002/ecy.1705

Gorokhova, E., and Kyle, M. (2002). Analysis of nucleic acids in daphnia: development of methods and ontogenetic variations in RNA-DNA content. *J. Plankton Res.* 24, 511–522. doi: 10.1093/plankt/24.5.511

Hall, E. K., and Cotner, J. B. (2007). Interactive effect of temperature and resources on carbon cycling by freshwater bacterioplankton communities. *Aquatic. Microbial. Ecol.* 49, 35–45. doi: 10.3354/ame01124

Hall, E. K., Dzialowski, A. R., Stoxen, S. M., and Cotner, J. B. (2009). The effect of temperature on the coupling between phosphorus and growth in lacustrine bacterioplankton communities. *Limnol. Oceanogr.* 54, 880–889. doi: 10.4319/lo.2009.54.3.0880

Hall, E. K., Neuhauser, C., and Cotner, J. B. (2008). Toward a mechanistic understanding of how natural bacterial communities respond to changes in temperature in aquatic ecosystems. *ISME. J.* 2, 471–481. doi: 10.1038/ismej.2008.9

Hobbie, J. E., Daley, R. J., and Jasper, S. (1977). Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 33, 1225–1228.

Hochstädter, S. (2000). Seasonal changes of C: P ratios of seston, bacteria, phytoplankton and zooplankton in a deep, mesotrophic lake. *Freshw. Biol.* 44, 453–463. doi: 10.1046/j.1365-2427.2000.00597.x

Kempes, C. P., Dutkiewicz, S., and Follows, M. J. (2012). Growth, metabolic partitioning, and the size of microorganisms. *Proc. Natl. Acad. Sci. U.S.A.* 109, 495–500. doi: 10.1073/pnas.1115585109

Kerkhoff, A. J., Enquist, B. J., Elser, J. J., and Fagan, W. F. (2005). Plant allometry, stoichiometry and the temperature-dependence of primary productivity. *Global. Ecol. Biogeogr.* 14, 585–598. doi: 10.1111/j.1466-822X.2005.00187.x

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2004). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174. doi: 10.1038/nature02454

Kuhn, H. J., Cometta, S., and Fiechter, A. (1980). Effects of growth temperature on maximal specific growth rate, yield, maintenance, and death rate in glucose-limited continuous culture of the thermophilic bacillus caldotenax. *Appl. Microbiol. Biotechnol.* 10, 303–315. doi: 10.1007/BF00498727

Løvdal, T., Skjoldal, E. F., Heldal, M., Norland, S., and Thingstad, T. F. (2008). Changes in morphology and elemental composition of vibrio splendidus along a gradient from carbon-limited to phosphate-limited growth. *Microb. Ecol.* 55, 152–161. doi: 10.1007/s00248-007-9262-x

Makino, W., Cotner, J. B., Sterner, R. W., and Elser, J. J. (2003). Are bacteria more like plants or animals? Growth rate and resource dependence of bacterial C: N: P *stoichiometry*. Funct. Ecol. 17, 121–130. doi: 10.1046/j.1365-2435.2003.00712.x

Martiny, A. C., Ma, L., Mouginot, C., Chandler, J. W., and Zinser, E. R. (2016). Interactions between thermal acclimation, growth rate, and phylogeny influence *Prochlorococcus* elemental stoichiometry. *PLoS ONE* 11:e0168291. doi: 10.1371/journal.pone.0168291

Martiny, A. C., Pham, C. T. A., Primeau, F. W., Vrugt, J. A., Moore, J. K., Levin, S. A., et al. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci.* 6, 279–283. doi: 10.1038/ngeo1757

Morán, X. A., Alonso-Sáez, L., Nogueira, E., Ducklow, H. W., González, N., López-Urrutia, Á., et al. (2015). More, smaller bacteria in response to ocean's warming? *Proc. Biol. Sci.* 282:20150371. doi: 10.1098/rspb.2015.0371

Morán, X. A. G., Lopez-Urrutia, Á., Calvo-Diaz, A., and Li, W. K. W. (2010). Increasing importance of small phytoplankton in a warmer ocean. *Glob. Change Biol.* 16, 1137–1144. doi: 10.1111/j.1365-2486.2009.01960.x

Newton, R. J., Jones, S. E., Eiler, A., McMahon, K. D., and Bertilsson, S. (2011). A guide to the natural history of freshwater lake bacteria. *Microbiol. Mol. Biol. Rev.* 75, 14–49. doi: 10.1128/MMBR.00028-10

Niklas, K. J., and Enquist, B. J. (2001). Invariant scaling relationships for interspecific plant biomass production rates and body size. *Proc. Natl. Acad. Sci. U.S.A.* 98, 2922–2927. doi: 10.1073/pnas.041590298

Pomeroy, L. R., and Deibel, D. (1986). Temperature regulation of bacterial activity during the spring bloom in newfoundland coastal waters. *Science* 233, 359–361. doi: 10.1126/science.233.4761.359

Reich, P. B., and Oleksyn, J. (2004). Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11001–11006. doi: 10.1073/pnas.0403588101

Rivkin, R. B., and Legendre, L. (2001). Biogenic carbon cycling in the upper ocean: effects of microbial respiration. *Science* 291, 2398–2400. doi: 10.1126/science.291.5512.2398

Scott, J. T., Cotner, J. B., and Lapara, T. M. (2012). Variable stoichiometry and homeostatic regulation of bacterial biomass elemental composition. *Front. Microbiol.* 3:42. doi: 10.3389/fmicb.2012.00042

Sommer, U. (1989). Maximal growth rates of antarctic phytoplankton: only weak dependence on cell size. *Limnol. Oceanogr.* 34, 1109–1112. doi: 10.4319/lo.1989.34.6.1109

Sun, J., and Liu, D. (2003). Geometric models for calculating cell biovolume and surface area for phytoplankton. *J. Plankton Res.* 25, 1331–1346. doi: 10.1093/plankt/fbg096

Tanner, R. S. (2007). *Manual of Environmental Microbiology*. Washington, DC: ASM Press.

Tranvik, L. J., Downing, J. A., Cotner, J. B., Loiselle, S. A., Striegl, R. G., Ballatore, T. J., et al. (2009). Lakes and reservoirs as regulators of carbon cycling and climate. *Limnol. Oceanogr.* 54, 2298–2314. doi: 10.4319/lo.2009.54.6_part_2.2298

Woods, H. A., Makino, W., Cotner, J. B., Hobbie, S. E., Harrison, J. F., Acharya, K., et al. (2003). Temperature and the chemical composition of poikilothermic organisms. *Funct. Ecol.* 17, 237–245. doi: 10.1046/j.1365-2435.2003.00724.x

Xia, C., Yu, D., Wang, Z., and Xie, D. (2014). Stoichiometry patterns of leaf carbon, nitrogen and phosphorous in aquatic macrophytes in eastern china. *Ecol. Eng.* 70, 406–413. doi: 10.1016/j.ecoleng.2014.06.018

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer RM and handling Editor declared their shared affiliation.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 08 August 2017
doi: 10.3389/fmicb.2017.01505

Redfield Ratios in Inland Waters: Higher Biological Control of C:N:P Ratios in Tropical Semi-arid High Water Residence Time Lakes

Ng H. They[1], André M. Amado[1,2] and James B. Cotner[3]*

[1] Graduate Program in Ecology, Limnology Laboratory, Department of Oceanography and Limnology, Universidade Federal do Rio Grande do Norte, Natal, Brazil, [2] Department of Biology, Universidade Federal de Juiz de Fora, Juiz de Fora, Brazil, [3] Department of Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, MN, United States

The canonical Redfield C:N:P ratio for algal biomass is often not achieved in inland waters due to higher C and N content and more variability when compared to the oceans. This has been attributed to much lower residence times and higher contributions of the watershed to the total organic matter pool of continental ecosystems. In this study we examined the effect of water residence times in low latitude lakes (in a gradient from humid to a semi-arid region) on seston elemental ratios in different size fractions. We used lake water specific conductivity as a proxy for residence time in a region of Eastern Brazil where there is a strong precipitation gradient. The C:P ratios decreased in the seston and bacterial size-fractions and increased in the dissolved fraction with increasing water retention time, suggesting uptake of N and P from the dissolved pool. Bacterial abundance, production and respiration increased in response to increased residence time and intracellular nutrient availability in agreement with the growth rate hypothesis. Our results reinforce the role of microorganisms in shaping the chemical environment in aquatic systems particularly at long water residence times and highlights the importance of this factor in influencing ecological stoichiometry in all aquatic ecosystems.

Keywords: nutrients, metabolism, bacteria, ecological stoichiometry, semi-arid, tropical, lakes, water residence time

OPEN ACCESS

Edited by:
Télesphore Sime-Ngando, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Dag O. Hessen, University of Oslo, Norway
Michael Danger, Université de Lorraine, France

****Correspondence:***
André M. Amado
amado@ufrnet.br

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *28 February 2017*
Accepted: *27 July 2017*
Published: *08 August 2017*

Citation:
They NH, Amado AM and Cotner JB (2017) Redfield Ratios in Inland Waters: Higher Biological Control of C:N:P Ratios in Tropical Semi-arid High Water Residence Time Lakes. Front. Microbiol. 8:1505. doi: 10.3389/fmicb.2017.01505

INTRODUCTION

Life has had a strong impact in influencing the availability of elements on the Earth. About 2.3 billion years ago the atmosphere was drastically changed to an oxidative state with the rise of O_2 and fall of CO_2 concentrations that were preceded by the 'invention' of photosynthesis (Kasting and Siefert, 2002). As the redox state of the Earth changed, organisms had to adapt to the changing steady state. Similar processes occur in the oceans today, whereby micro-organisms alter their external chemical environment, which is reflective of their own elemental biomass composition, i.e., stoichiometry. The mean elemental composition of ocean plankton with respect to the macroelements carbon (C), nitrogen (N) and phosphorus (P) was first discussed by Redfield (1934, 1958) and the ratio of 106C: 16N: 1P is referred today as the 'Redfield ratio.'

In marine waters, it is now clear that there is variability around the Redfield ratio due to latitudinal changes and nutrient availability effects (Martiny et al., 2013), whereas in inland waters the C:N:P ratios are often higher in magnitude and with greater variability (Hecky et al., 1993; Hassett et al., 1997; Sterner et al., 2008). The seston C:N:P ratios are influenced by species composition (e.g., eukaryotes *vs.* prokaryotes, N-fixing cyanobacteria, diatoms, etc.), temperature, organic matter inputs (quality and quantity), light:nutrient ratio, total P, chlorophyll, POC (particulate organic carbon): chlorophyll ratio and water residence time (WRT) (Hecky et al., 1993; McGroody et al., 2004; Hessen, 2006; Geider and La Roche, 2002; Martiny et al., 2013). Particularly in inland waters, it is often assumed that the income of large amounts of organic matter from terrestrial environments with typically high C:N:P ratios, as well as a variable share of phytoplankton (autochthonous production) relative to total seston (Hessen et al., 2003), and a tendency of lower environmental stability in space and time could cause the great variability in the seston elemental ratios when compared to the oceans (but see Hessen et al., 2003 and Hessen, 2006).

The residence times of substances in the oceans are quite long. The water has a residence (WRT) time of >3,000 years on average, while NO_3^- and PO_4^{3-} have a much longer residence time ($\sim 10^4$ years) (Falkowski and Davis, 2004). In contrast, in inland waters the WRT can be as low as days to 10s of 1000s years (Bell et al., 2002), but are typically much shorter than in the oceans. The residence times of elements such as P are comparable to WRT, usually less than a year to a few years (Sonzogni et al., 1976). Nitrogen retention time also is highly dependent on WRT, but has a more complex cycle and its retention depends on the type and abundance of vegetation, nitrification and denitrifications rates (Saunders and Kalff, 2001). Even though it has been debated about the general applicability of the classical Redfield ratio in the oceans (Koeve and Kähler, 2010; Martiny et al., 2013) and organisms (Geider and La Roche, 2002), the central tenet of Redfield's idea remains valid today (Falkowski and Davis, 2004; Gruber and Deutsch, 2014).

Microbes including virus, archaea, autotrophic and heterotrophic bacteria and algae play a central role in shaping aquatic ecosystem nutrient ratios (e.g., C, N, and P) and bacteria are the major consumers of P and N-based compounds in both marine and inland waters (Kirchman, 1994; Cotner and Biddanda, 2002). Nutrient concentrations and availability in the water column in turn also affect bacteria through constraints on metabolism and biomass composition. Bacteria growing under P-rich conditions (expressing high growth rates) are expected to exhibit low biomass C:P. High growth rates are associated with major allocation of P for nucleic acids in ribosomes, which is known as the Growth Rate Hypothesis (GRH) (Elser et al., 1996). Conversely, when growing under P-limiting conditions bacteria may either present high C:P ratios in the biomass (Cotner et al., 2010; Godwin and Cotner, 2015) or exhibit high respiration rates (Cimbleris and Kalff, 1998), possibly as a mechanism to eliminate the excess carbon from organic matter and to concentrate the P to an adequate amount (Hessen and Anderson, 2008). However, bacteria can also take up inorganic nutrients directly from the water (Cotner and Wetzel, 1992; Kirchman, 1994), which could possibly explain a compensation for low quality (high C:nutrients) substrates.

Most studies on ecological stoichiometry have examined the whole seston rather than examining the different planktonic groups (e.g., phytoplankton and bacterioplankton size fractions) separately and usually ignore the dissolved organic matter fraction (but see Hessen et al., 2003). For instance, the dissolved organic matter fraction and bacteria fraction are not well studied despite accounting for the largest part of the organic matter in many aquatic ecosystems (Wetzel, 1984; Simon et al., 1992; Gasol et al., 1997). Moreover, differences are expected in terms of stoichiometric flexibility (ability to adjust the internal C:N:P compositions in relation to the sources) among the planktonic components. The zooplankton have been reported to be quite inflexible, (Andersen and Hessen, 1991; Persson et al., 2010; Godwin and Cotner, 2015) and the phytoplankton community is more variable and more flexible (Geider and La Roche, 2002), responding to a wide gradient of light:nutrients ratios (Sterner et al., 1997; Hessen et al., 2002). However, a recent study has shown that the bacterial community can be the most flexible group of heterotrophic organisms known so far (Godwin and Cotner, 2015), with a high potential for adjusting and affecting environmental nutrient availability. The dissolved organic nutrient pools can buffer fluctuations in planktonic nutrient requirements depending on the rate of supply from external environments and their nutrient ratios. Heterotrophic bacteria grow relatively fast and consume primarily dissolved pools of inorganic and organic matter and thus are an important factor determining the elemental stoichiometry of both the dissolved and particulate pools.

The great variability in residence time in inland water ecosystems relative to oceans may be a key feature explaining the departure of freshwaters from Redfield ratios, i.e., a higher decoupling between bacteria and their substrates in inland water ecosystems due to variable WRT. To examine this hypothesis, we tested the effect of increasing WRT on the stoichiometry of seston, bacterial and dissolved fractions, as well as on bacterial metabolism in low latitude coastal and semi-arid lakes encompassing a wide range of trophic states and WRT. The sampling of aquatic systems in Eastern Brazil coincided with a strong El Niño event that began in June 2014 and extended through 2016.[1] This phenomenon is associated with droughts in Northeastern Brazil, particularly in the semi-arid zone (Rodrigues et al., 2011). Semi-arid lakes in eastern Brazil may experience extended periods of drought and high rates of water evaporation. Since these semi-arid lakes receive only the contribution of intermittent rivers, the inflow rates are negligible (close to zero) during drought, particularly for severe episodes such as the El Niño period that occurred during this study. Hence, drought has then an indirect effect on increasing WRT. This is paralleled by increasing concentrations of nutrients and increased specific conductivity, which in turn may be used as a proxy for WRT (Curtis and Adams, 1995; Rennella and Quirós, 2006; Anderson and Stedmon, 2007).

[1] http://www.bom.gov.au/climate/enso/#tabs=SOI

MATERIALS AND METHODS

Study Area

We sampled 15 ecosystems that consisted of both natural lakes and artificial reservoirs located in northeastern Brazil in Rio Grande do Norte state that encompass a trophic gradient ranging from oligotrophy to hypereutrophy and a wide range of environmental conditions (**Table 1**). These ecosystems are found in a relatively narrow latitudinal (from 06°24′36.5″ S to 06°41′42.9″ S) and longitudinal (from 35°05′59.4″ W to 36°37′43.6″ W) range that, nonetheless encompass a strong climatic gradient from the humid coastal zone (oligo- to mesotrophic lakes) to the inner, semi-arid zone (mostly eutrophic lakes and with longer WRT).

The coastal region climate is classified as humid with average annual temperature above 26°C. The historical annual average precipitation is 1,230 mm with 90% of rainfall between January and August. The vegetation in this area is classified as Atlantic rain forest (see Cestaro and Soares, 2004), which contributes with significant organic matter to aquatic ecosystems in the rainy season (Kosten et al., 2010). The inner region climate is classified as tropical and semi-arid (BS'h' according to Kottek et al., 2006) with annual average temperature higher than 25°C. It is characterized by irregular rainfall, high evapotranspiration rates, and negative water balance during most of the year, particularly in the last 3 years. The mean annual precipitation is 733 mm in the previous 20 years and the aquatic ecosystems have long residence times (e.g., water residence time calculated in 2004 from 780 days in Boqueirão reservoir to 1460 days in Gargalheiras reservoir, both sampled in the current study; Chellappa et al., 2009). However, the annual precipitation was below the average (between 200 and 600 mm per year) and the aquatic ecosystems have experienced extreme droughts since 2012 (Costa et al., 2016). Because of that, these ecosystems have experienced extreme reductions in volume (Medeiros et al., 2015).

Sampling

Each lake was sampled once close to its central point between October and December in 2014. In each lake, 10 L of sub-surface water (20–30 cm) was collected in acid-rinsed (HCl 10%) polyethylene bottles. The water transparency was determined with a secchi disk and the temperature and conductivity were measured *in situ* using a multi-parameter probe (Horiba U-22). The depth of the sampling point was determined with a depth meter or manually with a weight tied to a rope. Two subsamples were taken in plastic bottles without headspace for the determination of alkalinity. All samples were processed within 24 h; when the time between sampling and handling exceeded 4 h, the samples were kept refrigerated.

Limnological Variables and Fractionation

In the laboratory, the pH (Hanna HI-221) and alkalinity by Gran titration (H_2SO_4 0.0125 M) were immediately measured. The water was fractionated by sequential filtration through 1.6 μm mean retention pore-size glass fiber filters (Whatman

TABLE 1 | Main limnological characteristics of the 15 low latitude lakes sampled along a trophic gradient.

Lake	Location	Sampling point depth (m)	Secchi (cm)	Temp. (°C)	pH	Alkalinity (μEq L^{-1})	Conductivity (mS cm^{-1})	Chla (μg L^{-1})	TP (μM)	DOC:TDP (mol: mol)
Bonfim	06°02′18.4″S/35°12′50.0″W	11.8	580	28.23	5.7	90.0	0.090	0.24	0.96	104
EAJ	05°53′23.3″S/35°21′32.6″W	6.8	308	28.85	6.1	441.6	0.095	0.24	0.68	1938.3
Extremoz	05°42′45.1″S/35°17′06.3″W	4.25	230	28.56	6.7	960.1	0.162	1.08	0.51	382.7
Arituba	06°04′42.3″S/35°06′17.9″W	2.4	240	28.66	5.7	5.1	0.066	1.87	0.60	311.1
Carcará	06°03′41.2″S/35°09′35.4″W	3.9	300	28.29	5.7	25.9	0.040	2.07	0.57	490.8
Jiqui	05°55′08.3″S/35°11′14.0″W	3.0	230	29.79	6.2	287.7	0.073	2.33	0.68	121.8
Lagoa Azul	05°42′48.2″S/35°15′56.6″W	2.55	69	28.03	6.6	717.7	0.174	3.25	1.21	389.1
Jambeiro	05°52′06.3″S/35°20′25.2″W	6.0	81	29.34	6.7	1473.0	0.200	2.03	0.85	811.7
Ilhota	05°59′26.7″S/35° 07′34.1″W	1.3	130	29.22	5.9	50.5	0.045	5.93	0.68	332.3
Cruzeta	06°24′36.5″S/35°47′44.4″W	3.1	56	26.86	8.1	3923.2	0.353	6.27	2.52	444.1
Caliman	06°04′00.1″S/35°05′59.4″W	7.3	73	29.18	6.5	840.0	0.129	6.27	1.01	801.1
Boqueirão	06°41′42.9″S/36°37′43.6″W	9.9	45	25.40	8.4	7576.5	2.364	8.05	1.04	6259.8
Gargalheiras	06°25′30.6″S/36°36′07.9″W	10.4	33	28.40	8.5	6795.3	1.400	13.42	4.49	1886.7
Passagem das Traíras	06°30′52.6″S/36°56′32.2″W	5.1	20	28.14	8.7	10967.4	2.496	24.40	2.77	2925.4
Dourado	06°14′47.2″S/36°30′33.8″W	2.0	14	24.99	7.8	3770.1	1.133	44.53	5.80	1175.3

Temp, temperature; Chla, chlorophyll a; TP, total phosphorus; DOC, dissolved organic carbon (TOC < 0.7 μm); TDP, total dissolved phosphorus (total phosphorus < 0.7 μm).

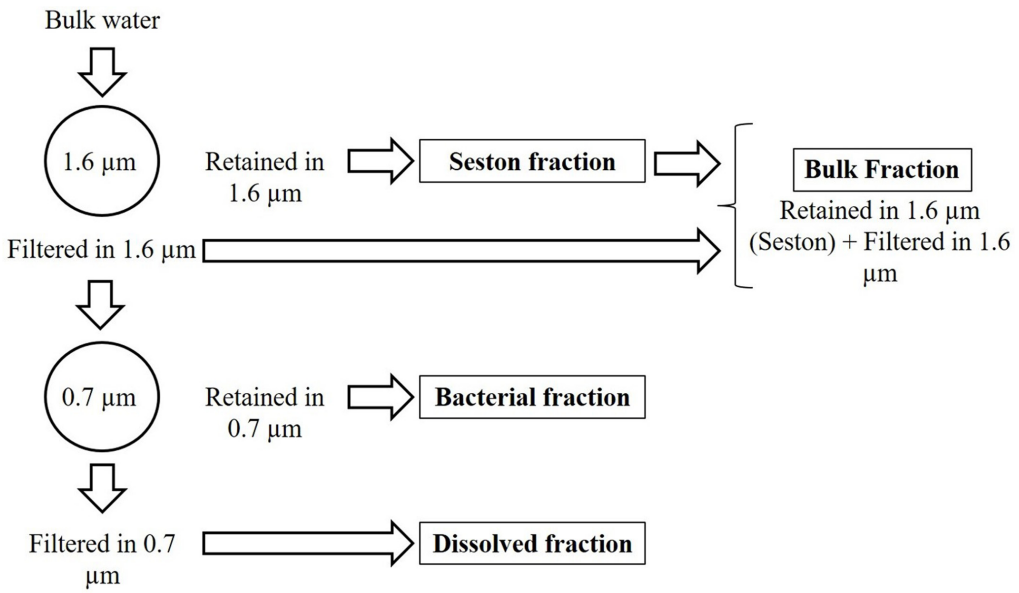

FIGURE 1 | Scheme of the size fractionation of the water.

GF/A) and 0.7 µm mean retention pore-size glass fiber filters (Whatman GF/F). This procedure was adopted to separate the seston (here assumed as > 1.6 µm), bacterial (here assumed as particles between 1.6 and 0.7 µm) and dissolved fractions (here assumed as < 0.7 µm). The bulk fraction was estimated as the sum of seston (>1.6 µm) and the <1.6 µm fraction (**Figure 1**). The bacterial fraction possibly includes archaeal cells, but since Archaea are in general a minor component (<6%) of pelagic prokaryote communities (Glöckner et al., 1999), we refer to this fraction as bacteria. The 1.6–0.7 µm fraction included on average 79% of the bacteria. Even though the 0.7 µm filter allowed some bacteria to pass, the glass fiber filter can be used for the analysis in the TOC equipment without interference of the filter components (such as C and N from 0.2 µm porosity membrane filters often made of C, N and P-containing polymers) and has been successfully employed in similar studies for the same purpose (Cotner et al., 2010). The bacterial density (BD) was determined in the bulk, bacterial and dissolved fractions (see below), and appropriate corrections were applied by estimating mean bacterial cell C, N, and P content in the bacterial fraction and summing up the nutrients amounts in the retained or filtered bacteria (Supplementary Table 1). It is noteworthy to mention that our understanding of the stoichiometry of bacteria is somewhat biased by the fact that we did not have appropriate tools for distinguishing the composition and abundance of free and attached bacteria, with the last being part of the seston. This should have a little impact on the results found, since typically attached bacteria represent a small proportion (<30%) of the total bacterial pool (Simon, 1987).

Chlorophyll *a* was measured after filtration of bulk water through a 1.2 µm mean retention pore size glass fiber filter (GF/C Whatman). The filters were kept at −80°C and the chlorophyll *a* was extracted with ethanol (90%) at −20°C in the dark (Nusch and Palme, 1975; APHA, 1999). The chlorophyll was measured without acidification at 665 nm after correction for turbidity (750 nm) and calculated according to Salonen and Sarvala (1995) and Schilling et al. (2006).

C, N, and P

In the four water fractions, we measured the total C, total N, and total P concentrations. The total and filtered (dissolved) fractions of the organic carbon (TOC and DOC) and nitrogen (TN and DN) were measured in a Total Organic Carbon Analyzer (TOC-V CPN Shimadzu). The different fractions of particulate organic carbon in the filters were measured using the solid sample module (SSM-5000 A) of the TOC analyzer. Particulate nitrogen and phosphorus on the filters and total phosphorus (TP) in the liquid samples were measured after potassium persulfate digestion (Carmouze, 1994). After digestion, the TN was measured in the TOC-V CPN (Shimadzu), whereas the TP was measured spectrophotometrically (SP2000UV) by the ascorbic acid method (Mackereth et al., 1978). All filters were combusted (550°C for 4 h) prior to filtration and after the filtration and before the analysis were oven dried (60°C for >24 h) and weighed.

All bulk seston, bacteria and dissolved C:N, C:P, and N:P ratios were calculated on a molar basis (mol: mol).

Bacterial Density and Metabolism

The BD was estimated in the bulk, <1.6 and <0.7 µm fractions via flow cytometry, which allowed the determination of the number of cells in each fraction and the number of cells retained on the filters. The samples were fixed with buffered formaldehyde (4% final concentration) and kept at −80°C until analysis in a BD FACSCalibur flow cytometer (within 5–8 months). The cells were stained with Syto 13 and the cytograms analyzed in the FlowJo X

10.0.7r2 software (Gasol and Del Giorgio, 2000; Sarmento et al., 2008).

Bacterial production (BP) was measured via the [^{3}H]-leucine incorporation and microcentrifugation method (Smith and Azam, 1992), incubating bulk water samples for 2.5 h. We assumed the molar percentage of leucine in the protein pool was equal to 0.073, the intracellular isotopic dilution was equal to 2 and a carbon:protein ratio of 0.86.

Bacterial respiration rates (BR) were measured in the $<$1.6 μm filtered water by following the oxygen consumption in 6 mL exetainer vials without headspace in the dark over variable periods of time (incubations were terminated when O_2 concentrations were $\leq$8% of initial O_2 concentrations; from 12 to 72 h of incubation). The oxygen concentrations were measured with a gold tip microprobe connected to a OXY-Meter (UNISENSE) and were converted to carbon using a respiratory quotient = 1.0 (Briand et al., 2004).

Specific bacterial production and respiration (BP and BR cell^{-1}) were calculated by dividing the respiration rates from each lake by the number of cells in the bulk and $<$1.6 μm fractions, respectively. The bacterial carbon demand was calculated as the sum of BP and BR (Alonso-Sáez et al., 2007).

Statistical Analysis

The $\log_{10}$ of the absolute concentrations of C, N, and P (μM) were linearly regressed against each other and compared to the expected Redfield ratios (106:16:1) by ANCOVA. Significant interactions indicated differences in slopes and significant intercepts indicated differences in the magnitude of the ratios.

The $\log_{10}$ of the C:N:P ratios of seston, bacteria and the dissolved fractions and of BP, BP cell^{-1}, BR, BR cell^{-1}, BGE (BP/[BP + BR]) and BCD (BP + BR) were linearly regressed against conductivity in order to assess the effect of residence time on the variation of these ratios, assuming conductivity as a proxy of water residence time (Rennella and Quirós, 2006; Anderson and Stedmon, 2007).

The $\log_{10}$ of the BD, BP, BR, BGE, and BCD were also linearly regressed against $\log_{10}$ of the bacterial C:N, C:P, and N:P ratios in order to determine whether the bacterial internal stoichiometry was coupled to bacterial metabolism. Conversely, the bacterial C:N, C:P, and N:P ratios were linearly regressed against potential substrates (seston and dissolved) C:N, C:P, and N:P ratios, respectively, in order to determine whether the substrate stoichiometry of these pools were correlated.

All statistics were carried out in R 3.2.2. (R Core Team, 2016) and all regressions assumptions were tested with the *gvlma* package (Pena and Slate, 2014).

RESULTS

The C:N:P ratios were variable for all fractions with means across all lakes being higher than expected by the classical Redfield ratio (mean seston C:N:P = 771:59:1; mean bacterial C:N:P = 320:34:1; mean dissolved C:N:P = 1225:173:1). Considering the extreme values, the lowest ratios were found in the bacterial fraction (90:19:1) and the highest in the dissolved fraction (1225:173:1; **Table 2**).

All bivariate regressions between the absolute concentrations of C, N, and P of all fractions were positive and significant (C to N; C to P; N to P), except for C × P and N × P of the dissolved fraction ($P > 0.05$) (**Table 3**). The comparison via ANCOVA of these relationships with those expected by the Redfield ratio revealed differences for all fractions with the exception of the dissolved C × P and N × P. All seston measured slopes and the bacterial C × P and N × P slopes were higher than Redfield ratios, whereas the bacterial C × N and the dissolved C × N slopes were lower than Redfield. The intercepts of these regressions were also significantly higher than zero, except for seston and bacterial C × N, the bacterial N × P and the dissolved C × P and N × P intercepts (**Table 3** and **Figure 2**).

The seston C:N and C:P ratios decreased significantly with water conductivity, and the bacterial C:P followed the same pattern, although with a marginally insignificant relationship ($P = 0.082$) (**Figures 3A–C**). The dissolved organic C:P and N:P, however, increased with increasing conductivity (**Figures 3D,E**). All other regressions of C:N:P of the fractions (Seston N:P, Bacterial C:N and N:P, and dissolved organic C:N) with conductivity were not significant (not shown).

BP, BP cell^{-1}, BD and BCD increased with specific conductivity (**Figures 4A,B,D,E**), while BR cell^{-1} displayed the opposite pattern, decreasing with conductivity (**Figures 4C**).

BD, BP and BCD were negatively correlated to bacterial C: P and N: P ratios (**Figure 5**). No significant relationships were found between BR or BR cell^{-1} and bacterial C:N:P ratios.

The C:N:P ratios in the dissolved organic pool and the seston pool had contrasting relationships when regressed against bacterial ratios. Bacterial C:P decreased with dissolved C:P (**Figure 6A**), whereas bacterial N:P increased with seston N:P (**Figure 6B**).

DISCUSSION

As expected for inland waters, the C:N:P ratios in the low-latitude lakes surveyed in the current study were mostly higher than the Redfield ratios. Nonetheless, seston ratios became more similar to Redfield ratios with increasing conductivity, with decreased C:N and C:P ratios. We argue that specific conductivity is a useful proxy for WRT in these systems and therefore, longer WRT may provide microbiota greater opportunity to extract nutrients from dissolved organic materials. Consistent with this argument, we observed positive correlations between C:P and N:P in the dissolved fraction (i.e., DOC:TDP and TDN:TDP) with increasing conductivity (**Figure 3**). We also observed faster specific growth rates in systems with higher conductivity (**Figure 4**), suggesting that part of the explanation for higher P content in systems with increased conductivity may have been the GRH (Sterner and Elser, 2002; Makino et al., 2003). Thus, we confirmed our hypothesis that the increasing WRT corresponded with decreased C:N and C:P in seston and bacterial biomass.

Our observations for freshwater seston stoichiometry in the tropics were similar to the mean seston estimates from a survey

TABLE 2 | Molar ratios of carbon (C), nitrogen (N) and phosphorus (P) for bacteria and the three substrates fractions (bulk, seston and dissolved) for the 15 lakes along a trophic gradient.

Lakes	Bacterial per cell (femtomol:femtomol)			Bulk (Seston + <1.6 μm) (μmol: μmol)			Seston (>1.6 μm) (μmol: μmol)			Dissolved (<0.7 μm) (μmol: μmol)		
	C:N	C:P	N:P	C:N	C:P	N:P	C:N	C:P	N:P	C:N	C:P	N:P
Bonfim	20.4	1675.3	82.1	11.5	156.3	13.6	8.1	431.7	53.1	14.6	104.0	7.1
EAJ	5.0	286.2	57.0	14.9	766.5	51.4	13.0	1502.7	115.2	17.1	1938.3	113.5
Extremoz	3.2	89.9	28.0	10.3	811.6	78.8	9.7	653.2	67.3	11.7	382.7	32.8
Arituba	12.3	289.1	23.5	9.6	430.0	44.8	16.2	915.3	56.6	7.3	311.1	42.7
Carcará	10.2	342.0	33.5	11.6	554.0	47.7	14.8	779.4	52.6	11.0	490.8	44.7
Jiqui	13.9	262.5	18.9	1.1	247.3	228.4	19.9	683.4	34.4	0.6	121.8	197.7
Lagoa Azul	8.1	395.6	48.9	1.5	902.8	584.4	13.5	2134.4	157.6	0.5	389.1	805.6
Jambeiro	2.8	108.7	39.1	13.1	1113.4	85.2	14.7	651.6	44.4	12.8	811.7	63.6
Ilhota	11.8	447.6	37.8	13.9	502.2	36.1	22.6	1350.8	59.9	10.2	332.3	32.7
Cruzeta	8.0	212.9	26.6	10.9	350.5	32.0	10.1	233.7	23.2	11.8	444.1	37.7
"Caliman"	7.4	148.7	20.1	15.2	563.6	37.1	19.2	681.8	35.6	12.9	801.1	61.9
Boqueirão	5.2	163.7	31.3	10.5	1632.5	156.1	7.8	614.7	79.2	11.1	6259.8	565.5
Gargalheiras	5.7	116.1	20.4	10.2	654.0	64.4	7.5	226.0	30.2	11.9	1886.7	158.7
Traíras	6.3	164.0	26.3	9.3	1057.2	114.2	8.0	456.2	57.0	10.0	2925.4	293.6
Dourado	9.3	220.1	23.6	12.0	565.1	47.1	11.3	244.2	21.5	8.6	1175.3	136.1
Mean	8.5	319.9	34.5	10.4	687.1	108.1	13.1	770.6	59.2	10.1	1224.9	172.9
Min.	2.8	89.9	18.9	1.1	156.3	13.6	7.5	226	21.5	0.5	104	7.1
Max.	20.4	1675.3	82.1	15.2	1632.5	584.4	22.6	2134.4	157.6	17.1	6259.8	805.6

TABLE 3 | Comparison of slopes and intercepts between measured and expected Redfield relationships of pairs of nutrients (carbon, C, nitrogen, N and phosphorus, P) for seston, bacterial and dissolved fractions for the 15 lakes along the trophic gradient.

	C × N		C × P		N × P	
	Measured	*P*-value	Measured	*P*-value	Measured	*P*-value
Seston						
slope	**9.22**	**<0.01**	**218.11**	**<0.01**	**22.80**	**<0.05**
intercept	37.19	>0.05	**136.24**	**<0.05**	**11.39**	**<0.05**
Bacteria						
slope	**5.74**	**<0.05**	**135.31**	**<0.05**	**23.39**	**<0.0001**
intercept	4.04	>0.05	**6.82**	**<0.05**	0.51	>0.05
Dissolved						
slope	**1.06**	**<0.001**	554.4	>0.05	68.96	>0.05
intercept	**611.99**	**<0.05**	308.2	>0.05	65.69	>0.05

The expected Redfield slopes are 106 for C × P, 6.625 for C × N and 16 for N × P whereas the expected Redfield intercepts are equal to 0. Significantly different from Redfield slopes and intercepts are highlighted in bold.

of 226 lakes from primarily temperate North America, and including some lakes from Europe, Africa, and Asia (Elser et al., 2000a). The mean seston C:N:P in this synthesis was 307:30:1, which is similar to the ratio that we observed in the bacterial size fraction (mean C:N:P 320:35:1) but lower than the mean values we observed in seston (C:N:P 607:108:1; **Table 2**). The highest seston C:N and C:P values were found at the lowest specific conductivities, perhaps indicating a strong terrigenous influence on the seston in the lower conductivity systems (from watershed input by rainfall).

Because our results were based on field data, numerous factors could have been affecting the stoichiometric behavior in these different lakes. Certainly, temperature is one factor that can affect seston and biomass stoichiometry (Cotner et al., 2006; Martiny et al., 2013; Phillips et al., under review) but we observed no significant differences in temperatures along the conductivity gradient (**Table 1**). However, there were differences in rainfall across the conductivity gradient that we observed, with greater rainfall occurring in the eastern, more ocean-influenced region and less in the semi-arid, inland region (as description in the study area section). Our observations were made when this region was being influenced by El Nino which was one of the factors that contributed to the semi-arid region lakes experiencing an unusual prolonged drought and drastically reducing their volumes (Medeiros et al., 2015; Costa et al., 2016; Mendonça et al., in press).

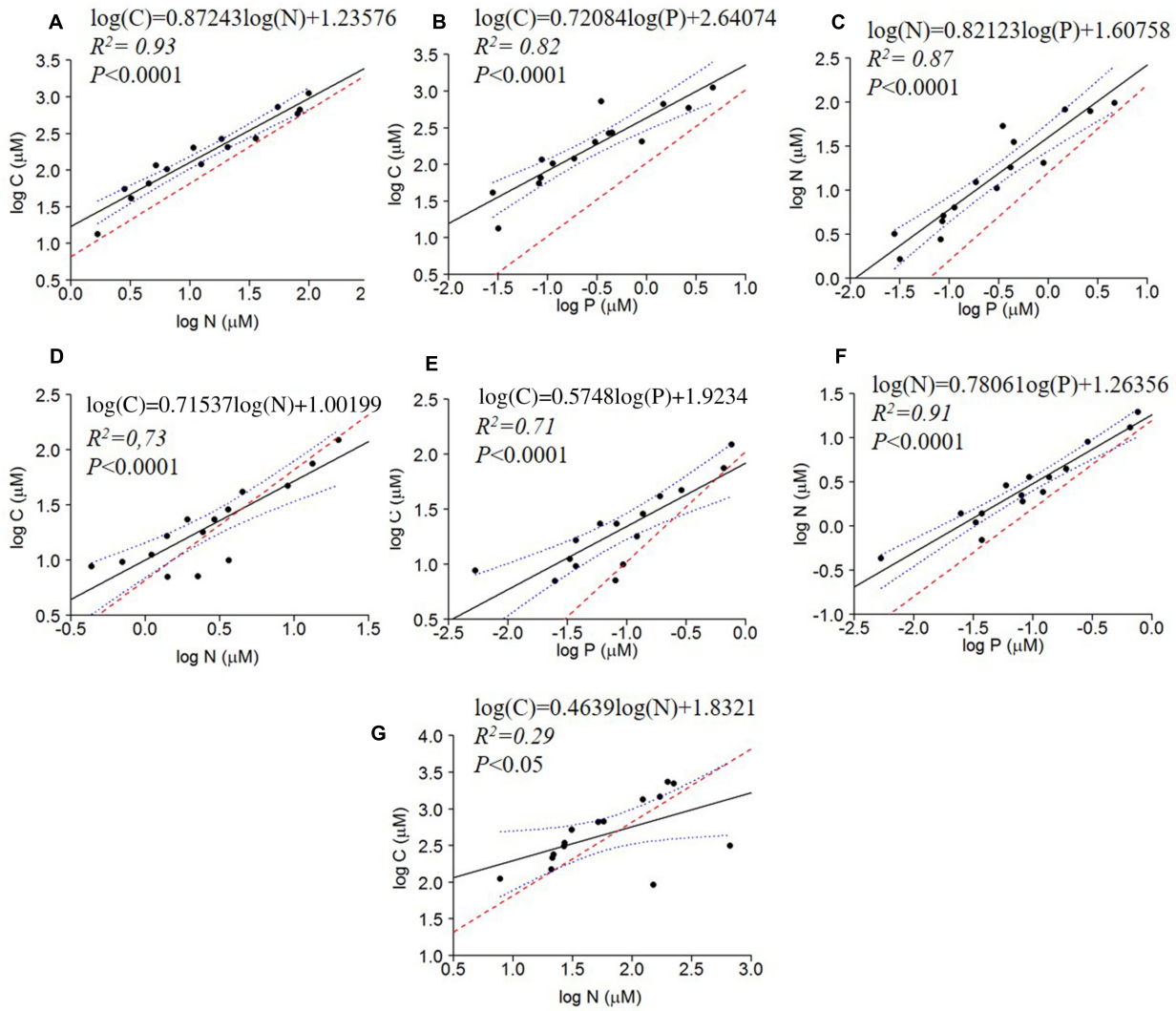

FIGURE 2 | Regressions of log of the absolute concentrations of C × N, C × P and N × P of seston **(A–C)**, bacterial **(D–F)** and dissolved fractions **(G)** compared to the Redfield ratio (dashed red line). The regression fit is indicated by black solid lines and 95% confidence intervals by blue dotted lines.

Therefore, it seems more likely that the patterns we observed between dissolved and particulate stoichiometry and specific conductivity may have been coupled to changes in residence times among the different systems. Few studies have examined relationships between water residence times and seston stoichiometry in freshwaters. One that did, however, in the Experimental Lakes of Canada (ELA), found that longer residence times correlated positively, not negatively as we found, with C:N:P (Hecky et al., 1993). Differences may have arisen from the fact that in Hecky et al.'s (1993) study a small number of lakes (<10) were included and only lakes with very short residence times (<3 months) were compared to longer residence time systems (>6 months). Also, the lakes were not influenced by a precipitation gradient and even the long WRT lakes were oligotrophic. On the other hand, the semi-arid area lakes in our study were all human-made reservoirs built in a way that the water outflow is only possible when large inputs of water enables dam overflow, and with WRT usually greater than 24 months.

The overall pattern is a bit more complicated in freshwaters due to differences in source material that are often coupled to differences in stoichiometry. Terrestrial stoichiometric signatures tend to be higher than microbial biomass signatures due to increased concentrations of structural carbohydrates and lignin (Elser et al., 2000a; Schlesinger and Bernhardt, 2013). In lakes with short residence times, the input of terrestrial material is high and tends to dominate the organic matter composition (Cole et al., 2002; Pace et al., 2004; Wilkinson et al., 2013). However, at longer residence times, there is less input of these components and increased influence from aquatic microbes and aquatic plants. Due to more rapid growth of aquatic plants and microbes in aquatic systems and lower amounts of structural material, their stoichiometry tends to be richer in N and P (lower

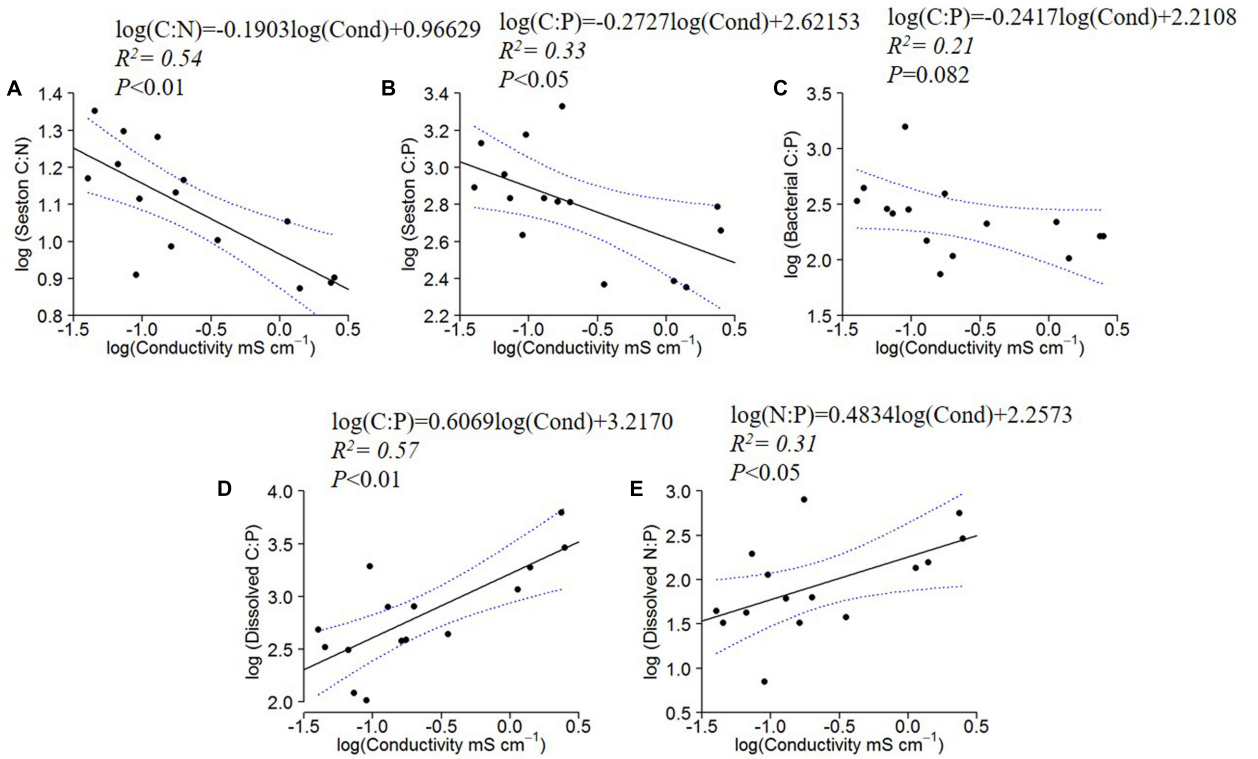

FIGURE 3 | Log_{10} linear regressions of seston C:N and C:P against specific conductivity **(A,B)**, bacterial C:P against specific conductivity **(C)** and dissolved C:P **(D)** and N:P ratios **(E)** against specific conductivity (residence time). The regression fit is indicated by black solid lines and 95% confidence intervals by blue dotted lines.

C:N and C:P) than terrestrial plants and they also degrade at faster rates (Enriquez et al., 1993), which means that long residence time systems should have nutrient signatures that reflect this. That seems to be the case of the lakes in the semiarid region.

Perhaps relatedly, Kellerman et al. (2014) recently demonstrated that the composition of DOM was strongly correlated with water residence times in boreal lakes. Short residence time systems had DOM composition that reflected the surrounding watershed but as residence times increased, the terrestrial signature was increasingly lost. In these boreal systems, organic matter in the longer residence time systems showed increased N content. The N-containing DOM compounds were either tightly recycled or resistant to decomposition processes (Kellerman et al., 2015). Our observation of increased TDN:TDP (i.e., N:P in the dissolved fraction) ratios with specific conductivity may reflect this N enrichment (**Figure 3E**), but it is also likely that there was tight recycling of both N and P due to the limiting nature of these elements in lakes as DOC:TDP (i.e., C:P in the dissolved fraction) also increased with specific conductivity (**Figure 3D**).

The decreasing seston C:P, C:N and bacterial C:P with increasing conductivity indicated P enrichment of the organisms with increasing WRT (**Figure 3**). This enrichment was concomitant with increasing dissolved C:P and N:P ratios, which suggests that the plankton may have been 'mining' P from this dissolved pool. Although this enrichment of the particulate pool via the dissolved pool was not sufficient to bring the seston ratios to values similar to Redfield ratios, it does illustrate the central mechanism envisioned by Redfield through which planktonic organisms can modify their environment given that they are provided with sufficient residence time to process that material (Redfield, 1934; 1958). Nutrients that are not incorporated into biomass are 'discarded' to the sediments or lost to the atmosphere in the oceans. Similar dynamics are likely occurring in all lakes, but the effects are more obvious at longer residence times.

The fact that bacterial N:P ratios were positively correlated with seston N:P ratios was consistent with other studies showing a tendency for bacterial biomass to reflect some of the same stoichiometric tendencies of the larger plankton pool. In a survey of lakes in the Midwest of the United States, Cotner et al. (2010) demonstrated that the bacterial pool had stoichiometry similar to the Redfield ratio with the total seston pool showing a similar pattern, but with slightly higher C:N, C:P, and N:P ratios. Higher N and P content in the microbial pool is often attributed to higher growth rates in these organisms (Bratbak and Dundas, 1984; Makino et al., 2003; Godwin et al., 2016), but somewhat surprisingly, the stoichiometry of the bacterial and seston pools do not often differ a great deal, perhaps due to the fact that they are drawing from the same dissolved and particulate nutrient pools and/or that large portions of the microbial community are dormant (Lennon and Jones, 2011).

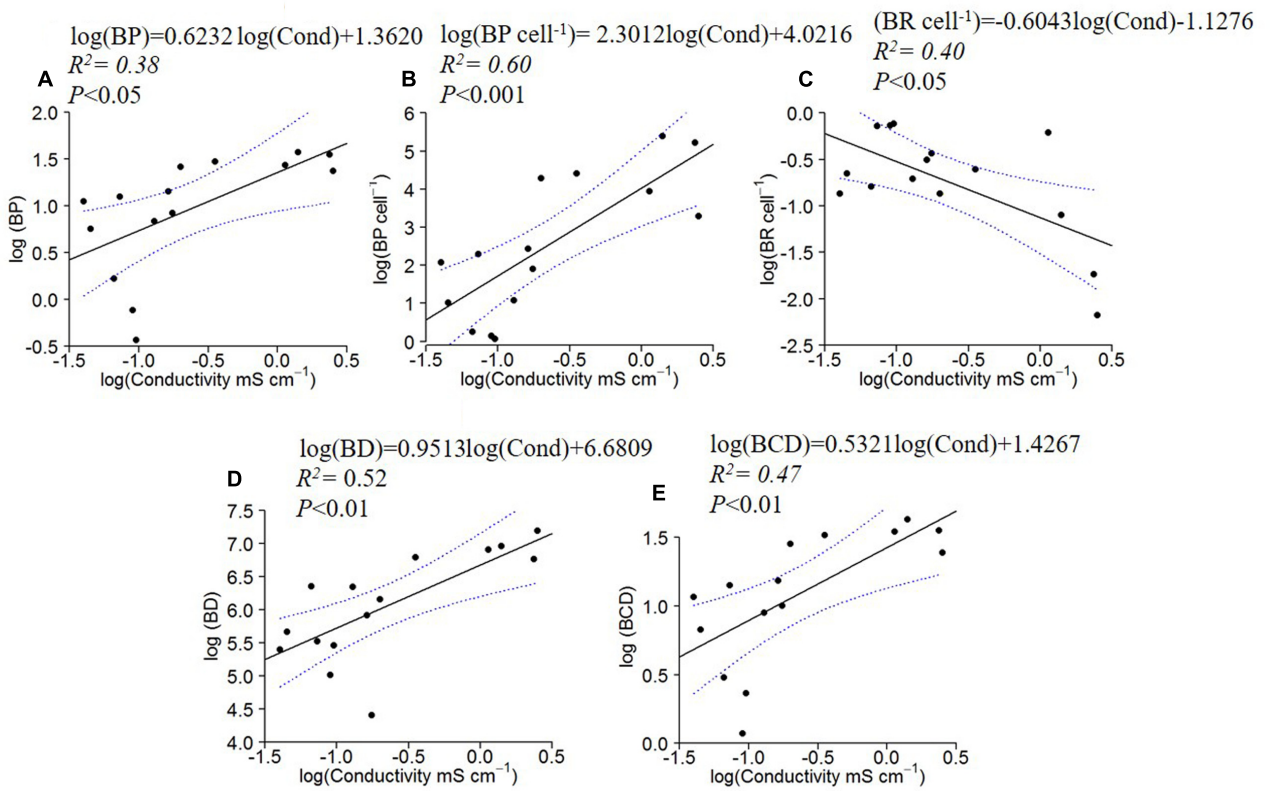

FIGURE 4 | Log_{10} linear regressions of bacterial production (BP; **A**), specific bacterial production **(B)** and specific bacterial respiration **(C)**, bacterial density (BD; **D**) and carbon demand (BCD; **E**) against specific conductivity. The regression fit is indicated by black solid lines and 95% confidence intervals by blue dotted lines.

Alternatively, the bacteria could be responding positively to the seston pool due to nutrients that are being provided directly from the phytoplankton. The seston in many of these lakes is dominated by cyanobacteria and zooplankton (rotifers and copepods, Eskinazi-Sant'Anna et al., 2007). Among the cyanobacteria, several N-fixing species like *Cylindrospermopsis raciborskii*, *Anabaena circinalis, Aphanizomenon gracile* (Fonseca et al., 2015; Medeiros et al., 2015), *Microcystis aeruginosa* and *Oscillatoria* sp. (Chellappa and Costa, 2003) have been reported to occur and dominate the phytoplankton community of these eutrophic lakes, i.e., the high WRT . The appearance/dominance of cyanobacteria in the phytoplankton community can have a strong impact in seston stoichiometry, with a tendency of lowering C:N ratios. Also, the release of dissolved organic N-based compounds could be an important source of both organic matter and N for bacteria in these lakes, especially semi-arid ones during El Niño events when there are very limited external organic matter inputs (Glibert and Bronk, 1994).

Another factor contributing to lower stoichiometric ratios in the longer residence time systems could be changes in the bacterial community composition. Although we did not explicitly address this issue, BD and bacterial production both increased along the conductivity gradient, perhaps reflecting an increased influence of relatively rapidly growing organisms (**Figure 4**). Nonetheless, there was no significant relationship between the seston-POC: bacterial-POC ratio and conductivity, suggesting that similar changes in the larger seston community may have also been occurring along the gradient. The GRH hypothesizes that rapidly growing organisms should increase their RNA and ribosome content (Elser et al., 2000b; Makino et al., 2003). Because RNA is P-rich relative to the mean composition of organisms, rapidly growing organisms should be more P-rich, with decreased C:P and N:P ratios. Recently, Godwin et al. (2017) demonstrated that the relative growth rate also plays an important role in this dynamic. Although it is difficult to assess the relative growth rate of organisms in natural environments, the increase in bacterial production and cell specific production that we observed (**Figure 4**) is consistent with the idea that growth rates and relative growth rates increased with increasing conductivity.

Relatedly, Goldman et al. (1979) argued that phytoplankton in the open ocean are growing at or near maximal rates because the C:P and C:N ratios are near Redfield ratios. They argued that the long residence time open ocean is much like a chemostat in that the biomass does not change much over time, yet there can be variation in nutrient supply that is drawn down by the biomass that is present with the main loss being grazing. Despite fundamental differences between open ocean and lakes, semi-arid lakes become more similar to open ocean during extended

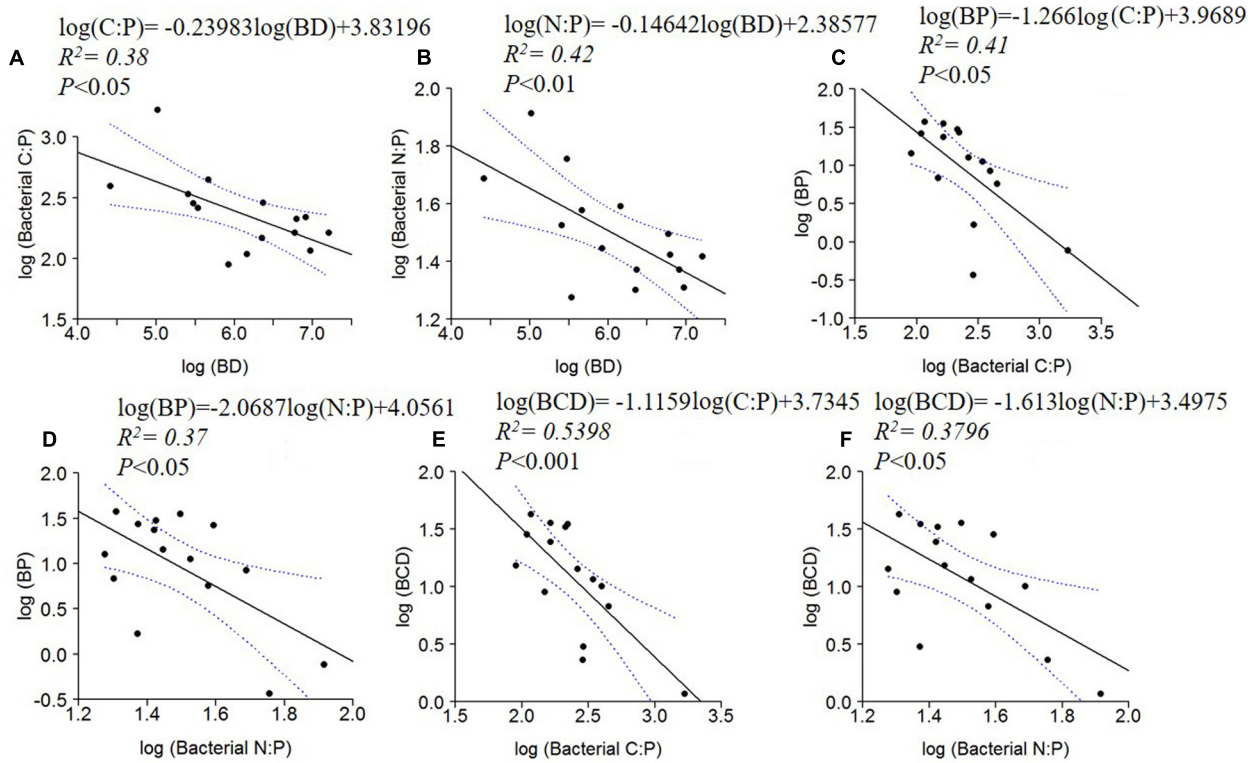

FIGURE 5 | Log_{10} linear regressions of ratios of bacterial C:P **(A)** and N:P **(B)** against bacterial density (BD), BP against bacterial C:P **(C)** and N:P **(D)** and BCD against bacterial C:P **(E)** and bacterial N:P **(F)**. The regression fit is indicated by black solid lines and 95% confidence intervals by blue dotted lines.

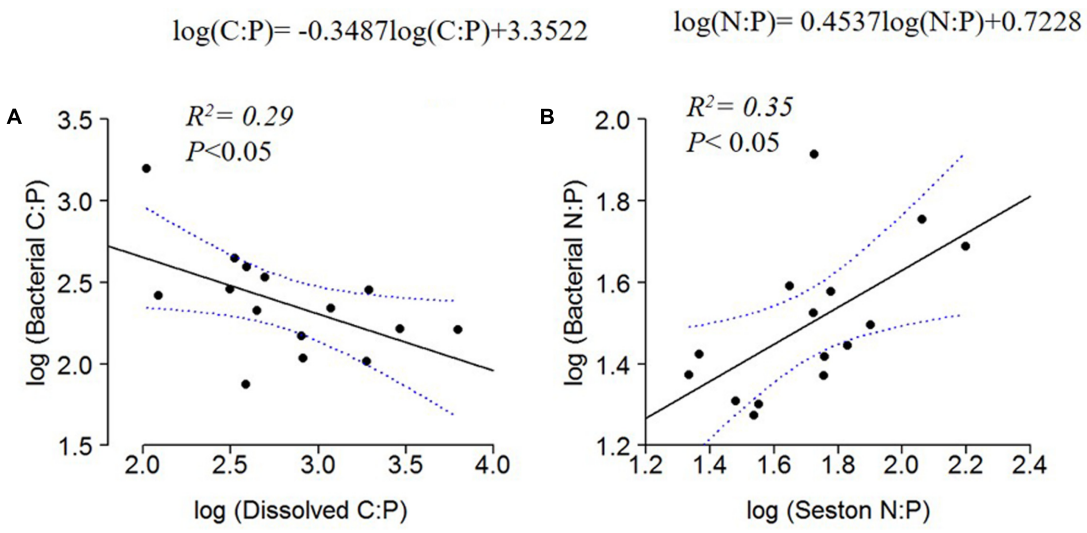

FIGURE 6 | Log_{10} linear regressions of bacterial C:P against dissolved C:P **(A)** and bacterial N:P against seston N:P ratio **(B)**. The regression fit is indicated by black solid lines and 95% confidence intervals by blue dotted lines.

drought periods when there is negligible input of allochthonous organic matter and the WRT increases. These lakes will likely have more variable biomass concentrations over time (still to be tested) due to greater variation in the supply rates due to episodic events such as changes in mixing depths, pulses due to storms, etc. Nevertheless, our results suggest that the mechanism envisioned by Redfield may be universal for all aquatic ecosystems under appropriate conditions.

Even though we observed a strong effect of WRT on bacterial and seston stoichiometry in these lakes, other factors not addressed here and that may co-vary with changes in environmental conditions may also be important. Grazing pressure on bacteria by protists is stronger on larger, fast dividing cells (Sherr et al., 1992), thus potentially favoring smaller, relatively more carbon rich cells (Norland, 1993). Also, weaker top–down effects of zooplankton have also been found to favor low-quality phytoplankton, with important potential repercussions for food webs (Hessen et al., 2005).

CONCLUSION

Our observations indicated WRT can be an important factor affecting the stoichiometry of plankton in freshwater ecosystems. As WRT increased, the biomass stoichiometry of seston and the bacterial size-fraction became more enriched with N and P. The dissolved organic nutrient pool stoichiometry demonstrated a negative correlation with residence time, suggesting that organic N and P pools may have been important sources of nutrients in long residence time systems. Water residence time is a parameter that has been little explored in freshwaters with respect to stoichiometry and needs to be considered in the future studies.

AUTHOR CONTRIBUTIONS

NT Performed field and lab work and writing. AA planned, performed field and lab work and contributed to the writing. JC planned, performed field and lab work and contributed to the writing.

FUNDING

This work was partially supported by a grant to JC from NSF (IOS No. 1257571) and by a grant to AA from CAPES (PVE – No. 88881.030384/2013-01).

ACKNOWLEDGMENTS

The authors thank B. Wanderley, V. Ferreira, C. Godwin and A. Little for field and laboratory support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01505/full#supplementary-material

REFERENCES

Alonso-Sáez, L., Gasol, J. M., Arístegui, J., Vilas, J. C., Vaqué, D., Duarte, C. M., et al. (2007). Large-scale variability in surface bacterial carbon demand and growth efficiency in the subtropical northeast Atlantic Ocean. *Limnol. Oceanogr.* 52, 553–546. doi: 10.4319/lo.2007.52.2.0533

Andersen, T., and Hessen, D. O. (1991). Carbon, nitrogen and phosphorus content of freshwater zooplankton. *Limnol. Oceanogr.* 36, 807–814. doi: 10.4319/lo.1991.36.4.0807

Anderson, N. J., and Stedmon, C. A. (2007). The effect of evapoconcentration on dissolved organic carbon concentration and quality in lakes of SW Greenland. *Freshw. Biol.* 52, 280–289. doi: 10.1111/j.1365-2427.2006.01688.x

APHA (1999). *Standard Methods for the Examination of Water and Wastewater*, 20th Edn. Washington, DC: American Public Health Association.

Bell, R. E., Studinger, M., Tikku, A. A., Clarke, G. K. C., Gutner, M. M., and Meertens, C. (2002). Origin and fate of Lake Vostok water frozen to the base of the East Antarctic ice sheet. *Lett. Nat.* 416, 307–310. doi: 10.1038/416307a

Bratbak, G., and Dundas, I. (1984). Bacterial dry matter content and biomass estimations. *Appl. Environ. Microbiol.* 48, 755–757.

Briand, E., Pringault, O., Jacquet, S., and Terréton, J. P. (2004). The use of oxygen microprobes to measure bacterial respiration for determining bacterioplankton growth efficiency. *Limnol. Oceanogr* 2, 406–416. doi: 10.4319/lom.2004.2.406

Carmouze, J. P. (1994). *O Metabolismo dos Ecossistemas Aquáticos - Fundamentos Teóricos, Métodos de Estudo e Análises Químicas*. São Paulo: FAPESP.

Cestaro, L. A., and Soares, J. J. (2004). Variações florística e estrutural e relações fitogeográficas de um fragmento de floresta decídua no Rio Grande do Norte, Brasil. *Acta Bot. Brasilica* 18, 203–218. doi: 10.1590/S0102-33062004000200001

Chellappa, N. T., Chellappa, T., Câmara, F. R. A., Rocha, O., and Chellappa, S. (2009). Impact of stress and disturbance factors on the phytoplankton communities in Northeastern Brazil reser- voir. *Limnologica* 39, 273–282. doi: 10.1016/j.limno.2009.06.006

Chellappa, N. T., and Costa, M. A. M. (2003). Dominant and co-existing species of Cyanobacteria from a eutrophicated reservoir of Rio Grande do Norte State, Brazil. *Acta Oecol.* 24, S3–S10. doi: 10.1016/S1146-609X(03)00005-5

Cimbleris, A. C. P., and Kalff, J. (1998). Planktonic bacterial respiration as a function of C:N:P ratios across temperate lakes. *Hydrobiologia* 384, 89–100. doi: 10.1023/A:1003496815969

Cole, J. J., Carpenter, S. R., Kitchell, J. F., and Pace, M. L. (2002). Pathways of organic carbon utilization in small lakes: results from a whole-lake C-13 addition and coupled model. *Limnol. Oceanogr.* 47, 1664–1675. doi: 10.4319/lo.2002.47.6.1664

Costa, M. R. A., Attayde, J. L., and Becker, V. (2016). Effects of water level reduction on the dynamics of phytoplankton functional groups in tropical semi-arid shallow lakes. *Hydrobiologia* 778, 75–89. doi: 10.1007/s10750-015-2593-6

Cotner, J. B., and Biddanda, B. A. (2002). Small players, large role: microbial influence on biogeochemical processes in pelagic aquatic ecosystems. *Ecosystems* 5, 105–121. doi: 10.1007/s10021-001-0059-3

Cotner, J. B., Hall, E. K., Scott, J. T., and Heldal, M. (2010). Freshwater bacteria are stoichiometrically flexible with a nutrient composition similar to seston. *Front. Microbiol.* 1:132. doi: 10.3389/fmicb.2010.00132

Cotner, J. B., Makino, W., and Biddanda, B. (2006). Temperature affects stoichiometry and biochemical composition of *Escherichia coli*. *Microb. Ecol.* 52, 26–33. doi: 10.1007/s00248-006-9040-1

Cotner, J. B., and Wetzel, R. G. (1992). Uptake of dissolved inorganic and organic phosphorus compounds by phytoplankton and bacterioplankton. *Limnol. Oceanogr.* 37, 232–243. doi: 10.4319/lo.1992.37.2.0232

Curtis, P. J., and Adams, H. E. (1995). Dissolved organic matter quantity and quality from freshwater and saltwater lakes in east-central Alberta. *Biogeochemistry* 30, 59–76. doi: 10.1007/BF02181040

Elser, J. J., Dobberfuhl, D. R., MacKay, N. A., and Schampel, J. H. (1996). Organism size, life history, and N:P stoichiometry. *Bioscience* 46, 674–684. doi: 10.2307/1312897

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000a). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580.

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., et al. (2000b). Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* 3, 540–550. doi: 10.1046/j.1461-0248.2000.00185.x

Enriquez, S., Duarte, C. M., and Sandjensen, K. (1993). Patterns in decomposition rates among photosynthetic organisms: the importance of detritus C:N:P content. *Oecologia* 94, 457–471. doi: 10.1007/BF00566960

Eskinazi-Sant'Anna, E. M., Menezes, R., Costa, I. S., Panosso, R. F., Araújo, M. F., and Attayde, J. L. (2007). Composição da comunidade zooplanctônica em reservatórios eutróficos do semi-árido do Rio Grande do Norte. *Oecol. Brasiliensis* 11, 410–421. doi: 10.4257/oeco.2007.1103.10

Falkowski, P. G., and Davis, C. S. (2004). Redfield ratios: the uniformity of elemental ratios in the oceans and life they contain underpins our understanding of marine biogeochemistry. *Nature* 431:131. doi: 10.1038/431131a

Fonseca, J. R., Vieira, P. C. S., Kujbida, P., and Costa, I. A. S. (2015). Cyanobacterial occurrence and detection of microcystins saxitoxins in reservoirs of the Brazilian semi-arid. *Acta Limnol. Brasiliensia* 27, 78–92. doi: 10.1590/S2179-975X2814

Gasol, J. M., and Del Giorgio, P. A. (2000). Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci. Mar.* 64, 197–224. doi: 10.3989/scimar.2000.64n2197

Gasol, J. M., del Giorgio, P. A., and Duarte, C. M. (1997). Biomass distribution in marine planktonic communities. *Limnol. Oceanogr.* 42, 1353–1363. doi: 10.4319/lo.1997.42.6.1353

Geider, R. J., and La Roche, J. (2002). Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37, 1–17. doi: 10.1017/S0967026201003456

Glibert, P. M., and Bronk, D. A. (1994). Release of dissolved organic nitrogen by marine diazotrophic cyanobacteria, *Trichodesmium* spp. *Environ. Microbiol.* 60, 3996–4000.

Glöckner, F. O., Fuchs, B. M., and Amann, R. (1999). Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl. Environ. Microbiol* 65, 3721–3726.

Godwin, C. M., and Cotner, J. B. (2015). Aquatic hetrotrophic bacteria have highly flexible phosphorus content and biomass stoichiometry. *ISME J.* 9, 2324–2327. doi: 10.1038/ismej.2015.34

Godwin, C. M., Whitaker, E. A., and Cotner, J. B. (2016). Growth rate and resource imbalance interactively control biomass stoichiometry and elemental quotas of aquatic bacteria. *Ecology* 98, 820–829. doi: 10.1002/ecy.1705

Godwin, C. M., Whitaker, E. A., and Cotner, J. B. (2017). Growth rate and resource imbalance interactively control biomass stoichiometry and elemental quotas of aquatic bacteria. *Ecology* 98, 820–829. doi: 10.1002/ecy.1705

Goldman, J. C., McCarthy, J. J., and Peavey, D. G. (1979). Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature* 279, 210–215. doi: 10.1038/279210a0

Gruber, N., and Deutsch, C. A. (2014). Redfield's evolving legacy. *Nat. Geosci.* 7, 853–855. doi: 10.1038/ngeo2308

Hassett, R. P., Cardinale, B., Stabler, L. B., and Elser, J. J. (1997). Ecological stoichiometry of N and P in pelagic ecosystems: comparison of lakes and oceans with emphasis on the zooplankton-phytoplankton interaction. *Limnol. Oceanogr.* 42, 648–662. doi: 10.4319/lo.1997.42.4.0648

Hecky, R. E., Campbell, P., and Hendzel, L. (1993). The stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. *Limnol. Oceanogr.* 38, 709–724. doi: 10.4319/lo.1993.38.4.0709

Hessen, D. O. (2006). Determinants of seston C: P-ratio in lakes. *Freshw. Biol.* 51, 1560–1569. doi: 10.1111/j.1365-2427.2006.01594.x

Hessen, D. O., Andersen, T., Brettum, P., and Faafeng, A. (2003). Phytoplankton contribution to sestonic mass and elemental ratios in lakes: implications for zooplankton nutrition. *Limnol. Oceanogr.* 48, 1289–1296. doi: 10.4319/lo.2003.48.3.1289

Hessen, D. O., and Anderson, T. R. (2008). Excess carbon in aquatic organisms and ecosystems: physiological, ecological, and evolutionary implications. *Limnol. Oceanogr.* 53, 1685–1696. doi: 10.4319/lo.2008.53.4.1685

Hessen, D. O., Donk, E. V., and Gulati, R. (2005). Seasonal seston stoichiometry: effects on zooplankton in cyanobacteria:dominated lakes. *J. Plankton Res.* 27, 449–460. doi: 10.1093/plankt/fbi018

Hessen, D. O., Færovig, P. J., and Andersen, T. (2002). Light, nutrients, and P:C ratios in algae: grazer performance related to food quality and quantity. *Ecology* 83, 1886–1898. doi: 10.1890/0012-9658(2002)083[1886:LNAPCR]2.0.CO;2

Kasting, J. F., and Siefert, J. L. (2002). Life and evolution of Earth's atmosphere. *Science* 296, 1066–1068. doi: 10.1126/science.1071184

Kellerman, A. M., Dittmar, T., Kothawala, D. N., and Tranvik, L. J. (2014). Chemodiversity of dissolved organic matter in lakes driven by climate and hydrology. *Nat. Commun.* 5:3804. doi: 10.1038/ncomms4804

Kellerman, A. M., Kothawala, D. N., Dittmar, T., and Tranvik, L. J. (2015). Persistence of dissolved organic matter in lakes related to its molecular characteristics. *Nat. Geosci.* 8, 454–457. doi: 10.1038/ngeo2440

Kirchman, D. L. (1994). The uptake of inorganic nutrients by heterotrophic bacteria. *Microb. Ecol.* 28, 255–271. doi: 10.1007/BF00166816

Koeve, W., and Kähler, P. (2010). Balancing ocean nitrogen. *Nat. Geosci.* 3, 383–384. doi: 10.1038/ngeo884

Kosten, S., Roland, F., Marques, D., Van Nes, E. H., Mazzeo, N., Sternberg, L. D. L., et al. (2010). Climate-dependent CO2 emissions from lakes. *Glob. Biogeochem. Cycles* 24:GB2007.

Kottek, M., Grieser, J., Beck, C., Rudolf, B., and Rubel, F. (2006). World map of the Koppen-Geiger climate classification updated. *Meteorol. Z.* 15, 259–263. doi: 10.1127/0941-2948/2006/0130

Lennon, J. T., and Jones, S. E. (2011). Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nat. Rev. Microbiol.* 9, 119–130. doi: 10.1038/nrmicro2504

Mackereth, F. J. H., Heron, J., and Talling, J. F. (1978). *Water Analysis: Some Revised Methods for Limnologists*. Ambleside: Freshwater Biological Association.

Makino, W., Cotner, J. B., Sterner, R. W., and Elser, J. J. (2003). Are bacteria more like plants or animals? Growth rate and resource dependence of bacterial C : N : P stoichiometry. *Funct. Ecol.* 17, 121–130. doi: 10.1046/j.1365-2435.2003.00712.x

Martiny, A. C., Pham, C. T. A., Primeau, F. W., Vrugt, J. A., Moore, J. K., Levin, S. A., et al. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci.* 6, 279–283. doi: 10.1038/ngeo1757

McGroody, M. E., Daufresne, T., and Hedin, L. O. (2004). Scaling of C:N:P stoichiometry in forests worldwide: implications of terrestrial redfield-type ratios. *Ecology* 85, 2390–2401. doi: 10.1890/03-0351

Medeiros, L. C., Mattos, A., Lürling, M., and Becker, V. (2015). Is the future blue-green or brown? The effects of extreme events on phytoplankton dynamics in semi-arid man-made lake. *Aquat. Ecol.* 49, 293–307. doi: 10.1007/s10452-015-9524-5

Mendonça, J. R., Amado, M. A., and Becker, V. (in press). Extreme droughts drive tropical semi-arid eutrophic reservoirs towards CO2 sub-saturation. *Acta Limnol. Brasil.*

Norland, S. (1993). "The relationship between biomass and volume of bacteria," in *Handbook of Methods in Aquatic Microbial Ecology*, eds P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (Boca Raton, FL: Lewis Publishers), 339–345.

Nusch, E. A., and Palme, G. (1975). Biologische methoden für die praxis der gewisseruntersuchung 1. Bestimmung des chlorophyll a und phaeopigmentgehaltes in oberflachenwasser. *GWF* 116, 562–565.

Pace, M. L., Cole, J. J., Carpenter, S. R., Kitchell, J. F., Hodgson, J. R., Van De Bogert, M. C., et al. (2004). Whole-lake carbon-13 additions reveal terrestrial support of aquatic food webs. *Nature* 427, 240–243. doi: 10.1038/nature02227

Pena, E. A., and Slate, E. H. (2014). *gvlma: Global Validation of Linear Models Assumptions*. Available at: http://CRAN.R-project.org/package=gvlma

Persson, J., Fink, P., Hood, J. M., Jonas, J., and Kato, S. (2010). To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos* 119, 741–751. doi: 10.1111/j.1600-0706.2009.18545.x

R Core Team (2016). *R: A Language and Environment for Statistical Computing Author R Foundation for Statistical Computing*. Available at: https://www.R-project.org

Redfield, A. C. (1934). "On the proportions of organic derivatives in the sea water and their relation to the composition of plankton," in *James Johnstone Memorial Volume*, ed. R. J. Daniel (Liverpool: University Press of Liverpool), 176–192.

Redfield, A. C. (1958). The biological control of chemical factors in the environment. *Am. Sci.* 46, 205–221.

Rennella, A. M., and Quirós, R. (2006). The effects of hydrology on plankton biomass in shallow lakes of the Pampa Plain. *Hydrobiologia* 556, 181–191. doi: 10.1007/s10750-005-0318-y

Rodrigues, R. R., Haarsma, R. J., Campos, E. J. D., and Ambrizzi, T. (2011). The impacts of inter-El Niño variability on the tropical atlantic and northeast Brazil climate. *J. Clim.* 24, 3402–3422. doi: 10.1175/2011JCLI3983.1

Salonen, K., and Sarvala, J. (1995). *Field Manual for the Determination of Chlorophyll a, and Primary Production in the Lake Tanganyika Research*. Rome: FAO, 14.

Sarmento, H., Unrein, F., Isumbisho, M., Stenuite, S., Gasol, J. M., and Descy, J.-P. (2008). Abundance and distribution of picoplankton in tropical, oligotrophic Lake Kivu, eastern Africa. *Freshw. Biol.* 53, 756–771. doi: 10.1111/j.1365-2427.2007.01939.x

Saunders, D. L., and Kalff, J. (2001). Nitrogen retention times in wetlands, lakes and rivers. *Hydrobiologia* 443, 205–212. doi: 10.1023/A:1017506914063

Schilling, P., Powilleit, M., and Uhlig, S. (2006). Chlorophyll a determination: results of an interlaboratory comparison. *Accred. Qual. Assur.* 11, 462–469. doi: 10.1007/s00769-006-0158-0

Schlesinger, W. H., and Bernhardt, E. S. (2013). *Biogeochemistry: An Analysis of Global Change*. Cambridge, MA: Academic Press. doi: 10.1093/obo/9780199830060-0111

Sherr, B. F., Sherr, E. B., and McDaniel, J. (1992). Effect of protistan grazing on the frequency of dividing cells in bacterioplankton assemblages. *Appl. Environ. Microbiol.* 58, 2381–2385.

Simon, M. (1987). Biomass and production of small and large free-living and attached bacteria in Lake constance. *Limnol. Oceanogr.* 32, 591–607. doi: 10.1007/BF02011709

Simon, M., Cho, B. C., and Azam, F. (1992). Significance of bacterial biomass in lakes and the ocean: comparison to phytoplankton biomass and biogeochemical implications. *Mar. Ecol. Progr. Ser.* 86, 103–110. doi: 10.3354/meps086103

Smith, D. C., and Azam, F. (1992). A simple, economical method for measuring bacterial protein synthesis rates in sea water using 3H-leucine. *Mar. Microbial. Food Webs* 6, 107–114.

Sonzogni, W. C., Uttormark, P. C., and Lee, G. F. (1976). A phosphorus residence time model: theory and application. *Water Res.* 10, 429–435. doi: 10.1016/0043-1354(76)90061-0

Sterner, R. W., Andersen, T., Elser, J. J., Hessen, D. O., Hood, J. M., McCauley, E., et al. (2008). Scale-dependent carbon:nitrogen:phosphorus seston stoichiometry in marine and freshwaters. *Limnol. Oceanogr.* 53, 1169–1180. doi: 10.4319/lo.2008.53.3.1169

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press, 438.

Sterner, R. W., Elser, J. J., Fee, E. J., Guildford, S. J., and Chrzanowski, T. H. (1997). The light: nutrient ratio in lakes: the balance of energy and materials affects ecosystem structure and process. *Am. Nat.* 150, 663–684. doi: 10.1086/286088

Wetzel, R. G. (1984). Detrital dissolved and particulate organic carbon functions in aquatic ecosystems. *Bull. Mar. Sci.* 35, 503–509.

Wilkinson, G. M., Pace, M. L., and Cole, J. J. (2013). Terrestrial dominance of organic matter in north temperate lakes. *Glob. Biogeochem. Cycles* 27, 43–51. doi: 10.1029/2012GB004453

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 July 2017
doi: 10.3389/fmicb.2017.01119

A Stoichioproteomic Analysis of Samples from the Human Microbiome Project

Briana Vecchio-Pagan[1], Sharon Bewick[2], Kumar Mainali[2], David K. Karig[1] and William F. Fagan[2]*

[1] Research and Exploratory Development Department, Johns Hopkins Applied Physics Laboratory, Laurel, MD, United States, [2] Department of Biology, University of Maryland, College Park, MD, United States

Ecological stoichiometry (ES) uses organism-specific elemental content to explain differences in species life histories, species interactions, community organization, environmental constraints and even ecosystem function. Although ES has been successfully applied to a range of different organisms, most emphasis on microbial ecological stoichiometry focuses on lake, ocean, and soil communities. With the recent advances in human microbiome research, however, large amounts of data are being generated that describe differences in community composition across body sites and individuals. We suggest that ES may provide a framework for beginning to understand the structure, organization, and function of human microbial communities, including why certain organisms exist at certain locations, and how they interact with both the other microbes in their environment and their human host. As a first step, we undertake a stoichioproteomic analysis of microbial communities from different body sites. Specifically, we compare and contrast the elemental composition of microbial protein samples using annotated sequencing data from 690 gut, vaginal, oral, nares, and skin samples currently available through the Human Microbiome Project. Our results suggest significant differences in both the median and variance of the carbon, oxygen, nitrogen, and sulfur contents of microbial protein samples from different locations. For example, whereas proteins from vaginal sites are high in carbon, proteins from skin and nasal sites are high in nitrogen and oxygen. Meanwhile, proteins from stool (the gut) are particularly high in sulfur content. We interpret these differences in terms of the local environments at different human body sites, including atmospheric exposure and food intake rates.

Keywords: human microbiome project (HMP), stoichioproteomics, gut, oral, skin, vaginal, nasal, C:N ratio

OPEN ACCESS

Edited by:
James Joseph Elser, University of Montana, United States

Reviewed by:
Jay T. Lennon, Indiana University Bloomington, United States
Peggy Baudouin-Cornu, Commissariat á l'Énergie Atomique et aux Énergies Alternatives (CEA), France
Claudia Acquisti, Universität Münster, Germany

****Correspondence:***
Sharon Bewick
sharon_bewick@hotmail.com

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *02 February 2017*
Accepted: *01 June 2017*
Published: *18 July 2017*

Citation:
Vecchio-Pagan B, Bewick S, Mainali K, Karig DK and Fagan WF (2017) A Stoichioproteomic Analysis of Samples from the Human Microbiome Project. Front. Microbiol. 8:1119. doi: 10.3389/fmicb.2017.01119

INTRODUCTION

The study of ecological stoichiometry (Sterner and Elser, 2002) derives from observed commonalities and differences in the elemental compositions of organisms (Elser et al., 1996), populations and communities (Moe et al., 2005). That is, whereas all life is primarily composed of carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), and sulfur (S), there is nonetheless large variation in the ratios of these elements across different species

(Andersen and Hessen, 1991; Hessen and Lyche, 1991; Elser et al., 1996) and systems (Cebrian and Kingsolver, 1999; Elser et al., 2000a). Such variation is important because it provides valuable biological understanding into both the nature of the organisms in a particular ecosystem (Elser et al., 2000a,c; Fagan et al., 2002; Denno and Fagan, 2003), and the environmental conditions that these organisms face (Cebrian and Kingsolver, 1999; Elser et al., 2000a). Of particular note, because all organisms require C, H, O, N, P, and S, biomass ratios of these elements can provide insight into which are limiting (i.e., prevent further growth) for specific types of taxa or for taxa from specific regions.

Much of the work in the field of ecological stoichiometry has focused on C:N:P ratios (Elser et al., 2000b,c, 2003). Species growth rate, for example, has been associated with an organism's absolute C:N ratio, while C:N ratio variances have been linked to both growth rate and trophic guild (Rhee, 1978; Urabe, 1993; DeMott et al., 1998; Elser et al., 2000a,c; Denno and Fagan, 2003; Lemoine et al., 2014). Broad trends in organismal elemental composition have also been documented as a function of ecosystem type. Marine autotrophs, for instance, are more nutrient rich (lower C:P and C:N ratios) than freshwater autotrophs, which are more nutrient rich than terrestrial autotrophs (Elser and Hassett, 1994; Elser et al., 2000a; Sterner and Elser, 2002), while marine autotrophs exhibit smaller ranges in elemental ratios as compared to autotrophs from terrestrial systems (Elser et al., 2000a, 2006).

As primary nutrient remineralizers (Barsdate et al., 1974; Goldman et al., 1987; Chrzanowski and Kyle, 1996), and some of the most N- and P-rich of all living things (Sterner and Elser, 2002), the ecological stoichiometry of bacteria is particularly interesting. Some of the first studies of bacterial stoichiometry focused on the elemental composition of the bacterial cell (Herbert, 1961, 1976; Herbert et al., 1971). More recently, however, the emphasis has shifted to environment driven differences. Tezuka (1990), for example, studied the relationship between C:N ratios in culture medium and in bacterial biomass for an assemblage of lake bacteria. His study, and others (Nakano, 1994; Chrzanowski and Kyle, 1996) showed that there is a positive correlation between nutrient supply rates and bacterial biomass in microbial communities, and that this relationship is largely driven by shifts in the relative ratios of different bacterial populations (Makino et al., 2003).

Although understudied relative to classic ecology systems (e.g., oceans, lakes, and soils), it is reasonable to hypothesize that the relative abundances of bacteria across different human microbiomes (Turnbaugh et al., 2007; Peterson et al., 2009; Proctor, 2011; Gevers et al., 2012; Human Microbiome Project Consortium, 2012a) might be similarly governed by nutrient supply rates. Naively, one might expect that all human microbiomes should be relatively nutrient rich. This is because the human tissues and byproducts that provide the substrates for these microbes should be nutrient rich by virtue of the human lifestyle and trophic guild (Fagan et al., 2002). However, while human microbiomes may be overall more nutrient rich than those from plants or lakes or other nutrient-poor environments, differing nutrient supply ratios across body sites could still lead to different nutrients being limiting in one region relative to another. Nutrient availability in the gut, for instance, is at least partially a function of food intake, whereas nutrient availability on the skin depends primarily on host tissue and host produced compounds (Belkaid and Segre, 2014). Indeed, some body sites are typically thought of as providing more or less nutrients, with the gut being traditionally considered a nutrient-rich site, and the skin being considered a nutrient-poor site (Belkaid and Segre, 2014; Belkaid and Tamoutounour, 2016). It is already well known that bacterial abundances vary strongly among body sites (Human Microbiome Project Consortium, 2012a). If this is at least partially a result of differing nutrient limitations, then the elemental compositions of microbial populations should vary from one body site to the next. As an example, body sites with large aerobic niches (e.g., skin and nares) might be expected to contain microbial biomass with a higher oxygen content (Acquisti et al., 2007). By contrast, the anaerobic vagina contains nitrogen-rich urea and protein secretions (Geshnizgani and Onderdonk, 1992), and thus might be expected to exhibit microbial biomass with high nitrogen ratios.

As a first step toward testing the hypothesis that variation in human microbiomes reflects underlying differences in nutrient availability, we consider the elemental compositions of microbial proteins from different body sites. That is, we take a stoichioproteomic approach (Baudouin-Cornu et al., 2001; Elser et al., 2006, 2011) wherein we analyze the C, N, O, and S content of microbial proteins among gut, oral, nasal, vaginal and skin microbiome samples. Previous work has shown that even single atom changes in the amino acid (AA) composition of microbial proteins can have large fitness consequences under nutrient-limiting conditions (Bragg and Wagner, 2009), suggesting that variation in microbial protein composition across body sites could reflect differing selective forces resulting from varying nutrient supplies. Further supporting this claim, organisms suffering chronic deficiency in certain nutrients (e.g., C, N or S) are known to preferentially substitute AAs that are low in the limiting nutrients. For example, S and C assimilatory pathways in both *Escherichia coli* and *Saccharomyces cerevisiae* show evidence for depletion of S and C, respectively (Baudouin-Cornu et al., 2001). Likewise, under conditions of sulfur limitation, certain cyanobacteria express sulfur-depleted versions of abundant proteins (Mazel and Marliére, 1989). Thus, our expectation is that if there are differences in C, N, S or O limitation across different human body sites, then we should see body site differences in the content of microbial proteins as well.

Unfortunately, whereas a lack of body site variation in protein elemental composition would provide strong evidence that nutrient limitation does not differ across body sites, the inverse is not necessarily true – i.e., differences in protein composition do not necessarily imply differences in nutrient supply. One reason for this is that protein elemental composition is known to depend on the GC content of the underlying coding sequences (CDSs). The GC content of CDSs, however, is under different selective forces (Rocha and Danchin, 2002; Musto et al., 2006) that are completely distinct from protein composition and, by extension, nutrient limitation. Thus, body site differences in protein composition could indicate differing selective forces on GC content, rather than protein composition *per se*. For

similar reasons, chemical and/or structural constraints on protein shape and function might also select for body site differences in elemental composition. Acidic sequences, for instance, are higher in oxygen content, while basic AAs are higher in nitrogen content. Requirements for these functional groups and others at certain body sites may thus bias element usage, independent of nutrient supply.

Interestingly, we do find significant body site differences in the C, N, S, and O content of microbial proteins. Supporting the hypothesis that this is a result of selection acting at the level of proteins, rather than GC content, we find that body site differences in protein composition remain significant, even after accounting for the dependencies of protein composition on GC content. Further supporting our hypothesis that body site differences are a result of nutrient limitation, we find that AAs with similar functional groups are often used differentially at different body sites based on specific AA element ratios. Although it is impossible to rule out other forces of selection that might indirectly select for differences in element composition across body sites (e.g., thermal stability requirements of proteins), our results provide strong preliminary evidence that nutrient limitation may vary across the human body and that this, in turn, may impact the associated structure of the human microbiome.

MATERIALS AND METHODS

Input Data

The Human Microbiome Project's (HMP) gene indices dataset was downloaded from their ftp server on 07/10/2016[1]. At the time of download, 690 samples representing 15 body sites were available. In this dataset, person identity is masked. However, based on sample counts, samples were collected from a minimum of 139 people. The HMP dataset description suggests that samples came from a much larger group (Human Microbiome Project Consortium, 2012a). That said, at least some of the samples in our dataset are likely from different body sites on the same individual. Although we cannot statistically account for this form of non-independence because of the masking of identities, we do not expect it to significantly impact our predictions, because taxonomic body site differences are known to be large relative to interpersonal variation at a single body site (Human Microbiome Project Consortium, 2012b).

Samples were sequenced at four different institutions, specifically Baylor College of Medicine, the Broad Institute, the J. Craig Venter Institute and the Washington University Genome Sequencing Center. Since HMP sample collection and processing followed a standard protocol at all institutions, we did not account for potential institution differences. Supporting this decision, analysis of a reduced sample set considering only those samples processed at Washington University Genome Sequencing Center gave similar results to our full analysis (see Appendix I, Figure A.1.1). More information on the methods used for sample collection, and specific details about sample characteristics are available from the HMP website[2].

TABLE 1 | Body sites used in study; two-letter codes for minor body sites are shown in brackets.

Major body site	Samples	Minor body site	Samples
Skin	26	Left retroauricular crease (Lr)	9
		Right retroauricular crease (Rr)	17
Stool/Gut	139	Stool (St)	139
Nasal	87	Anterior nares (Na)	87
Vagina	56	Vaginal Introitus (Vi)	3
		Mid-vaginal (Mv)	2
		Posterior fornix (Pf)	51
Oral	382	Saliva (Sa)	3
		Throat (Th)	7
		Buccal mucosa (Bm)	107
		Palatine tonsils (To)	6
		Tongue dorsum (Td)	128
		Subgingival plaque (Sb)	7
		Supragingival plaque (Sp)	118
		Attached keratinized gingiva (Ak)	6

The HMP gene indices dataset was previously processed using HMP's Metagenomics Prokaryotic Annotation Pipeline[3]. This yielded putative protein sequences for each of the 690 shotgun sequencing samples. These putative protein sequences were downloaded both in nucleotide and AA multi-FASTA formats.

Table 1 shows the breakdown in sample numbers by body sites.

Protein Elemental Content

Our baseline goal was to determine differences in protein elemental composition across body sites. To this end, we used the AA sequences available for all identified CDSs in the HMP gene indices dataset. AA sequences for the CDSs in any given sample were used to infer the average protein elemental composition (C, O, N, and S) of that sample based on the known elemental compositions of each AA. Specifically:

$$X = \frac{1}{N}\sum_{\mathrm{i}=1}^{\mathrm{N}} \frac{\sum_{\mathrm{j}=1}^{20} x_{\mathrm{j}} n_{i,j}}{\sum_{\mathrm{j}=1}^{20} T_{\mathrm{j}} n_{i,j}}$$

where X is the average fraction of the focal element in proteins from the sample, N is the total number of CDSs in the sample, $n_{\mathrm{i,j}}$ is the number of AAs of type j in CDS i, x_{j} is the number of atoms of the focal element in an AA of type j and T_{j} is the total number of atoms in an AA of type j. For the analysis presented in the main text, we use x_{j} and T_{j} reflective of full AAs, i.e., including both the side chain and the backbone (but see Appendix I, Figures A.1.2–A.1.6 for analysis with just the side chains).

Protein Amino Acid Content

To further explore underlying chemical constraints that might govern body site differences in protein composition, we examined

[1] ftp://public-ftp.ihmpdcc.org/HMGI/

[2] http://hmpdacc.org/HMGI/

[3] https://www.ncbi.nlm.nih.gov/pubmed/21304707

AA use, focusing on AAs with N, O, and S, because these elements are present in the side-chains of less than half of common AAs. From the AA sequences provided, we determined the average AA content of a sample as follows:

$$Z_{\mathrm{j}} = \frac{1}{N}\sum_{\mathrm{i}=1}^{\mathrm{N}} \frac{n_{i,j}}{A_i}$$

where Z_{j} is the fraction of AAs of type j, $n_{\mathrm{i,j}}$ is the number of AAs of type j in CDS i, A_{i} is the total number of AAs in CDS i, and N is the total number of CDSs in the sample.

Coding Sequence GC Content

Because protein elemental composition is known to vary with GC content of the corresponding CDSs, we also examined CDS GC content. Using nucleotide sequences provided in the HMP gene indices dataset, we inferred the average GC content of a sample as follows:

$$Y = \frac{100}{N}\sum\nolimits_{\mathrm{i}=1}^{\mathrm{N}} \frac{\sum_{\mathrm{j}=1}^{\mathrm{B_i}} \mathbf{1}_{\mathrm{GC}}(y_{i,j})}{B_i}$$

where Y is the average GC content of the CDSs in the sample, N is the total number of CDSs in the sample, $\mathbf{1}_{\mathrm{GC}}(y) = \begin{cases} 1 & y = G, C \\ 0 & y = A, T \end{cases}$ is an indicator function, $y_{\mathrm{i,j}}$ is the jth nucleotide in the ith CDS, and B_{i} is the length of the ith CDS (i.e., total number of nucleotides).

Body Site Comparison

To determine whether protein elemental fractions or AA composition differed from one body site to another, we compared elemental ratios across the 15 specific body sites in **Table 1**. We also grouped these body sites into five broader categories, also shown in **Table 1**. Differences in median elemental/AA fractions among body sites were determined based on Dunn's test, which is a pairwise multiple comparisons procedure based on rank sums. False Discovery Rate (FDR) was controlled using the Benjamini-Hochberg adjustment. Differences in elemental fraction variances among body sites were also determined. This was done using Levene's test (only for the five major body sites). Again, FDR was controlled using the Benjamini-Hochberg adjustment. In the main text, we use boxplots to display differences in protein elemental/AA composition among body sites. Following standard convention, the center line of the boxplot is the median elemental/AA fraction (or ratio), the box defines the lower and upper quartiles of observed elemental fractions, and the 'whiskers' define the minimum and maximum elemental fractions, excluding outliers. Outliers are taken as any points greater (less) than 1.5× the upper (lower) quartile.

Element versus GC Analysis

To determine whether body site differences in protein elemental composition were significant, even after accounting for body site variation in GC content, we used analysis of covariance (ANCOVA) models. Type I sums of squares was used to calculate an F-value and to determine the significance of GC content and major body site on each elemental fraction and the C:N ratio.

All code from our work is available at: http://www.clfs.umd.edu/biology/faganlab/ecological-stoichiometry.html.

RESULTS

Carbon Content

Figure 1 shows boxplots of the average carbon fraction (see "Materials and Methods") of microbial proteins across the 5 major (**Figure 1A**) and 15 minor (**Figure 1B**) body sites in our study. Interestingly, all major body sites differed in carbon content except for those from nasal and skin regions and those from vaginal sites and stool (see Appendix II, Table A.2.1). Carbon content was lowest at nasal and skin sites, higher at oral sites, and reached a maximum at vaginal sites and in stool ($C_{\mathrm{stool}} \sim C_{\mathrm{vaginal}} > C_{\mathrm{oral}} > C_{\mathrm{skin}} \sim C_{\mathrm{nasal}}$). Similar trends held for minor body sites (see Appendix II, Table A.2.2). For example, the nares (nasal) had lower carbon content than any minor body site except for the left and right retroauricular creases (skin). Nevertheless, analysis of minor body sites did uncover the existence of additional, fine-scale variation that was not obvious from the five major body sites. Most notably, in the oral microbiome, there were significant differences in the carbon content of proteins from the buccal mucosa as compared to

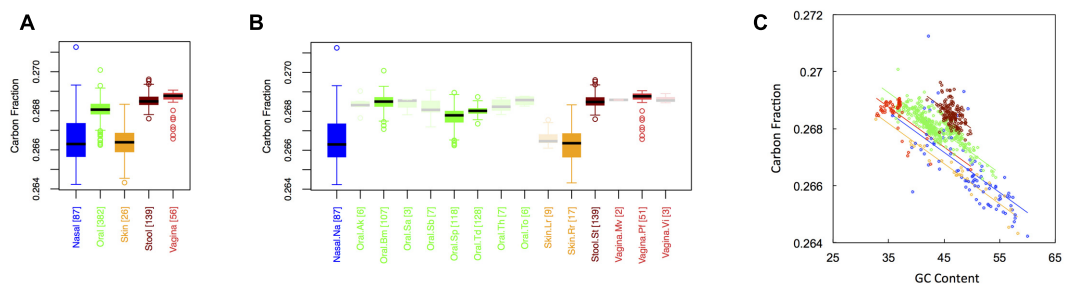

FIGURE 1 | Boxplots showing the average carbon content of microbial proteins as a function of human body site for **(A)** the 5 major sites and **(B)** the 15 minor sites in our study. The number of samples from each site is indicated in square brackets on axis labels. Faded boxes are used for sites with fewer than 10 samples. Differences in carbon content were tested with Dunn's test. Statistics are summarized in Appendix II, Tables A.2.1, A.2.2; **(C)** Scatterplot of carbon content versus GC content with associated regression lines from our ANCOVA analysis for major body sites: nasal (blue), oral (green), skin (yellow), stool (brown), and vaginal (red).

those from both supragingival plaque and the tongue. Likewise, proteins from supragingival plaque were significantly different from proteins on the tongue. It is worth noting that the buccal mucosa, supragingival plaque and the tongue are the three oral sites with the largest numbers of samples (see **Table 1** and **Figure 1B**), suggesting that more intense sampling of some of the other minor sites may uncover fine-scale differences in carbon content as well. For analysis of minor body sites in the remainder of the paper, we restrict our discussion to the three oral sites with sufficient sampling to obtain statistical significance, noting that further exploration of differences across other body sites would be an interesting extension of the current work.

As has been documented in other studies (Baudouin-Cornu et al., 2004; Bragg and Hyder, 2004), there was a negative relationship between the carbon content of protein sequences, and the GC content of the corresponding coding regions (**Figure 1C**). Nevertheless, the effect of major body site remained significant (ANCOVA; Type I ss; $F = 273.3$, $df = 4$, $p < 2 \times 10^{-16}$) when we included GC content as a covariate (ANCOVA; $F = 1719.4$, $df = 1$, $p < 2 \times 10^{-16}$). This suggests that selection forces driving variation in GC content across body sites cannot fully explain differences in protein carbon fractions. Similar to findings from large-scale ecological systems, we found that it was not only the median, but also the variance in C content that changed from one body site to another (see Appendix II, Table A.2.3, and Figure A.2.1). Skin and nasal sites, for example, had significantly larger variances in C content as compared to the other three body sites, with oral and vaginal sites having intermediate variances and stool having the smallest variance (though not significantly different from the variance of vaginal sites).

Oxygen Content

In **Figure 2**, we show boxplots of the average oxygen fraction of microbial proteins from major (**Figure 1A**) and minor (**Figure 1B**) body sites. As was the case with carbon, nasal and skin samples were not significantly different from one another. All other body site comparisons, however, indicated broad scale variation in oxygen use (see Appendix II, Table A.2.4). Nasal and skin microbiomes had the highest oxygen content, followed by oral, stool and, finally, vaginal microbiomes ($O_{nasal} \sim O_{skin} > O_{oral} > O_{stool} > O_{vaginal}$). Similar to carbon content, oxygen content at minor sites was largely predictable based on major site differences (see Appendix II, Table A.2.5). Once again, however, there were fine-scale trends apparent among oral sites. For example, the oxygen content the buccal mucosa differed from both supragingival plaque and the tongue, while the oxygen content of the tongue differed from supragingival plaque.

Overall, we found a positive relationship between the oxygen content of protein sequences, and the GC content of the corresponding coding regions (**Figure 2C**). As with carbon, however, the effect of major body site remained significant (ANCOVA; Type I ss; $F = 43.2$, $df = 4$, $p < 2 \times 10^{-16}$) when we included GC content as a covariate (ANCOVA; $F = 738.4$, $df = 1$, $p < 2 \times 10^{-16}$). Also similar to carbon, oxygen content showed differences in variance among body sites (see Appendix II, Table A.2.6, and Figure A.2.2), being largest at nasal sites and smallest in stool.

Nitrogen Content

Average nitrogen fraction of microbial proteins from major (**Figure 1A**) and minor (**Figure 1B**) body sites is shown in **Figure 3**. Comparing **Figures 2**, **3** indicates that, at least for major body sites, trends observed for nitrogen were quite similar to those observed for oxygen. That is, differences between skin and nasal sites were non-significant, with both the nares and skin showing elevated nitrogen as compared to the other three body locations. All other body site comparisons indicated significant differences (see Appendix II, Table A.2.7), with vaginal sites being particularly nitrogen poor ($N_{nasal} \sim N_{skin} > N_{stool} > N_{oral} > N_{vaginal}$). For minor body sites (see Appendix II, Table A.2.8), differences in the nitrogen content of proteins once again appeared between the buccal mucosa, supragingival plaque and the tongue. Unexpectedly, though, whereas major body sites that were relatively high in oxygen were also high in nitrogen, the same was not true for minor body sites. Within the oral microbiome, for example, supragingival plaques exhibited relatively low oxygen content. By contrast, this was one of the most nitrogen-rich sites in the mouth. At the same time, the buccal mucosa, which was relatively oxygen-rich, was the most nitrogen poor. Thus, whereas oxygen and nitrogen content

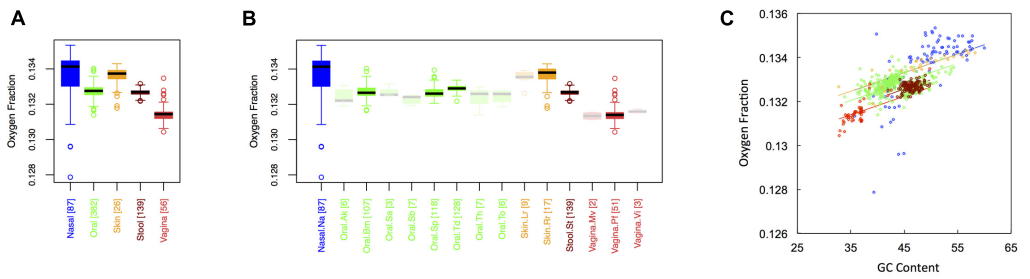

FIGURE 2 | Boxplots showing the average oxygen content of microbial proteins as a function of human body site for **(A)** the 5 major sites and **(B)** the 15 minor sites in our study. The number of samples from each site is indicated in square brackets on axis labels. Faded boxes are used for sites with fewer than 10 samples. Differences in oxygen content were tested with Dunn's test. Statistics are summarized in Appendix II, Tables A.2.4, A.2.5; **(C)** Scatterplot of oxygen content versus GC content with associated regression lines from our ANCOVA analysis for major body sites: nasal (blue), oral (green), skin (yellow), stool (brown), and vaginal (red).

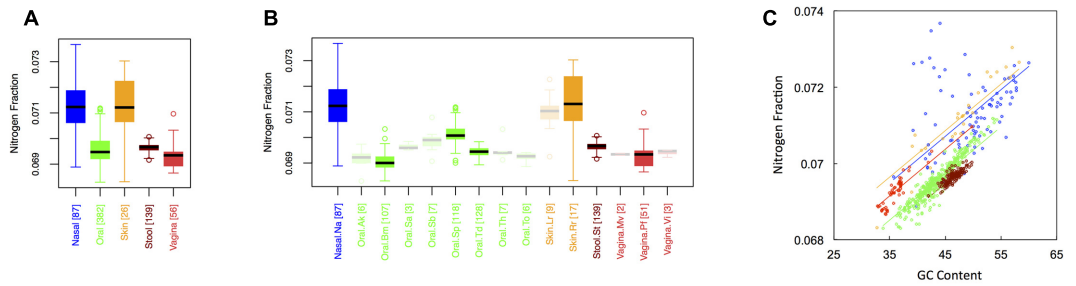

FIGURE 3 | Boxplots showing the average nitrogen content of microbial proteins as a function of human body site for **(A)** the 5 major sites and **(B)** the 15 minor sites in our study. The number of samples from each site is indicated in square brackets on axis labels. Faded boxes are used for sites with fewer than 10 samples. Differences in nitrogen content were tested with Dunn's test. Statistics are summarized in Appendix II, Tables A.2.7, A.2.8; **(C)** Scatterplot of nitrogen content versus GC content with associated regression lines from our ANCOVA analysis for major body sites: nasal (blue), oral (green), skin (yellow), stool (brown), and vaginal (red).

appear to exhibit broad scale similarities across major body sites, these similarities seem to break down at finer scales of resolution.

Like carbon, the nitrogen content of proteins is known to depend on the GC content of coding regions (Bragg and Hyder, 2004). Specifically, nitrogen content increases with increasing GC content – a trend that we also observed (**Figure 3C**). However, once again, we found that there was still an effect of major body site (ANCOVA; Type I ss; $F = 218.64$, $df = 4$, $p < 2 \times 10^{-16}$) when we included GC content as a covariate (ANCOVA, $F = 1901.1$, $df = 1$, $p < 2 \times 10^{-16}$). Differences in the variability of nitrogen content among body sites were also apparent (see Appendix II, Table A.2.9, and Figure A.2.3). As before, nasal and skin sites were not significantly different, and exhibited the largest variances of any of the five body sites. Likewise, the variance of the nitrogen content of stool was significantly smaller than any of the other body sites.

Sulfur Content

Figure 4 shows boxplots of the average sulfur fraction of microbial proteins from major (**Figure 1A**) and minor (**Figure 1B**) body sites. With respect to sulfur, stool had the highest fraction, followed by skin and nasal regions, and then by oral and vaginal sites ($S_{stool} > S_{skin} \sim S_{nasal} > S_{oral} \sim S_{vaginal}$). For minor sites, we again saw the buccal mucosa differing from supragingival plaque and the tongue, while supragingival plaque differed from the tongue. Interestingly, sulfur was lowest in the buccal mucosa, but was particularly high in the tongue. Again, ANCOVA indicated an effect of major body site (ANCOVA; Type I ss; $F = 511.7$, $df = 4$, $p < 2 \times 10^{-16}$), even after accounting for the influence of GC content (ANCOVA, $F = 221.6$, $df = 1$, $p < 2 \times 10^{-16}$). In terms of variance, nasal and skin sites exhibited the highest variation, while vaginal and stool sites exhibited the lowest (see Appendix II, Table A.2.12, and Figure A.2.4).

C:N Ratio

In keeping with the historical importance of the C:N ratio in ecological stoichiometry, we considered the C:N ratio of microbial proteins across the human body. This is shown in **Figure 5** for both major (**Figure 1A**) and minor (**Figure 1B**) body sites. Like the carbon fraction, C:N ratios were lowest at skin and nasal sites, which were not significantly different from one another. Interestingly, we found that stool and oral microbiomes exhibited intermediate C:N ratios (which were not significantly different), while the vaginal microbiome exhibited the highest ($CN_{vaginal} > CN_{oral} \sim CN_{stool} > CN_{nasal} \sim CN_{skin}$). This is in contrast to carbon content, where stool was highest. Notice, however, that the vagina was extremely nitrogen poor, explaining its inflated C:N ratio. In other words, the high C:N ratio in the vagina is driven by both high carbon content and low nitrogen content of vaginal proteins. Again, major body site remained an important determinant of sample C:N ratio (ANCOVA; Type I ss; $F = 405.82$, $df = 4$, $p < 2 \times 10^{-16}$), even after accounting for dependence on GC content (ANCOVA; $F = 3878.3$, $df = 1$, $p < 2 \times 10^{-16}$). Similar to both C content and N content, C:N ratios exhibited the greatest variance at skin and nasal sites, intermediate variance at oral and vaginal sites, and the smallest variance in stool (see Appendix II, Table A.2.15, and Figure A.2.5).

Amino Acid Compositions

Six AAs have nitrogen in their side chain. **Figure 6** shows boxplots for site-specific usage of these AAs. From **Figure 6**, it is clear that the overabundance of nitrogen in proteins from the skin and nares is primarily a result of higher usage of arginine and histidine at these locations. While both arginine and histidine are positively charged and basic, it does not appear that this feature is driving differences among body sites. Indeed, lysine, which is also positively charged and basic is actually utilized significantly less on skin and in the nares. Moreover, because the pKa of lysine falls between that of histidine and arginine, it suggests that acidity of the protein residues is also not the primary factor under selection. Notably, however, lysine provides the chemical properties of a positively charged, basic AA with only two nitrogen atoms, as opposed to the three found in histidine and the four found in arginine. Thus, organisms trying to conserve nitrogen may favor lysine in place of histidine or arginine, whereas organisms living in environments where nitrogen is not limiting are likely to use histidine and arginine freely. This supports our hypothesis that

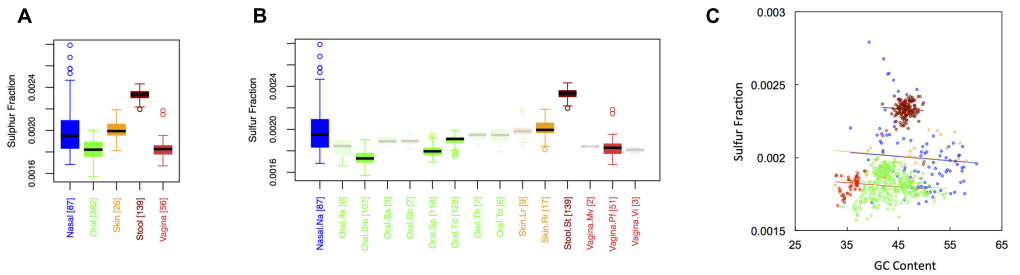

FIGURE 4 | Boxplots showing the average sulfur content of microbial proteins as a function of human body site for **(A)** the 5 major sites and **(B)** the 15 minor sites in our study. The number of samples from each site is indicated in square brackets on axis labels. Faded boxes are used for sites with fewer than 10 samples. Differences in sulfur content were tested with Dunn's test. Statistics are summarized in Appendix II, Tables A.2.10, A.2.11; **(C)** Scatterplot of sulfur content versus GC content with associated regression lines from our ANCOVA analysis for major body sites: nasal (blue), oral (green), skin (yellow), stool (brown), and vaginal (red).

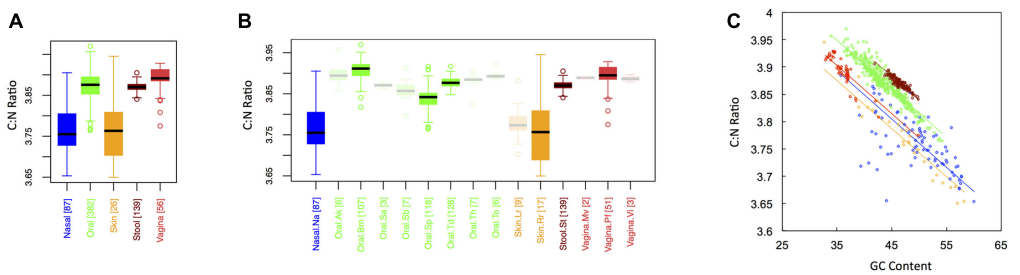

FIGURE 5 | Boxplots showing the average C:N ratio of microbial proteins as a function of human body site for **(A)** the 5 major sites and **(B)** the 15 minor sites in our study. The number of samples from each site is indicated in square brackets on axis labels. Faded boxes are used for sites with fewer than 10 samples. Differences in C:N ratio were tested with Dunn's test. Statistics are summarized in Appendix II, Tables A.2.13, A.2.14; **(C)** Scatterplot of C:N ratio versus GC content with associated regression lines from our ANCOVA analysis for major body sites: nasal (blue), oral (green), skin (yellow), stool (brown), and vaginal (red).

nutrient limitation, rather than specific structural or chemical requirements, may be driving body site differences in protein elemental composition.

By contrast to the variable usage of different nitrogen-rich AAs across body sites, both of the sulfur-rich AAs are incorporated in qualitatively similar patterns, except that methionine exhibits a relatively higher usage than cysteine in the vagina (**Figure 7**). Although both cysteine and methionine play roles in protein stability, cysteine is polar and forms disulfide bridges, whereas methionine is non-polar and generally non-reactive. Thus, if functionality alone were driving selective incorporation of sulfur-rich AAs in the gut, one might expect differences in the usages of cysteine and methionine in the gut relative to other body sites. Because we do not see this (except in vaginal samples), it suggests that elemental availability, rather than chemical function or specific structural requirements, may be responsible for body site differences. On the other hand, the differing usage patterns of cysteine relative to methionine in the vagina hint that function, rather than nutrient availability, may be driving usage patterns at this site.

In **Figure 8**, we show boxplots for site-specific usage of the seven oxygen-rich AAs. From these, it appears that the low oxygen content of vaginal proteins is largely a result of reduced use of glutamate, whereas the increased oxygen content of the skin and nares seems to stem from increased use of aspartate and serine, with threonine also being enriched in the nares. Notably, glutamate provides an acidic side-chain for a higher C:O ratio than aspartate, thus the patterns that we observe could be explained by glutamate and aspartate substituting for one another in oxygen-poor and oxygen-rich environments, respectively. By contrast, if functionality was driving selection, one would expect both aspartate and glutamate to be over-represented at the same sites. This argument, however, is weakened by the observed patterns in asparagine and glutamine. In particular, both asparagine and glutamine provide a carboxamide functionality, with glutamine exhibiting the higher C:O ratio. Thus, if oxygen availability was truly driving selective incorporation of specific oxygen-rich AAs, we would expect to see similar patterns in glutamate and glutamine and in aspartate and asparagine. In fact the opposite is true. Consequently, while functionality alone does not appear to govern body site differences in oxygen-rich AA incorporation, it is not clear that this is driven by oxygen availability either.

DISCUSSION

With the recent expansion of human microbiome research, scientists are rapidly acquiring more and more information about the identities of the species living on the human body. This information has important relevance for human health and disease (Cho and Blaser, 2012). However, the biological factors

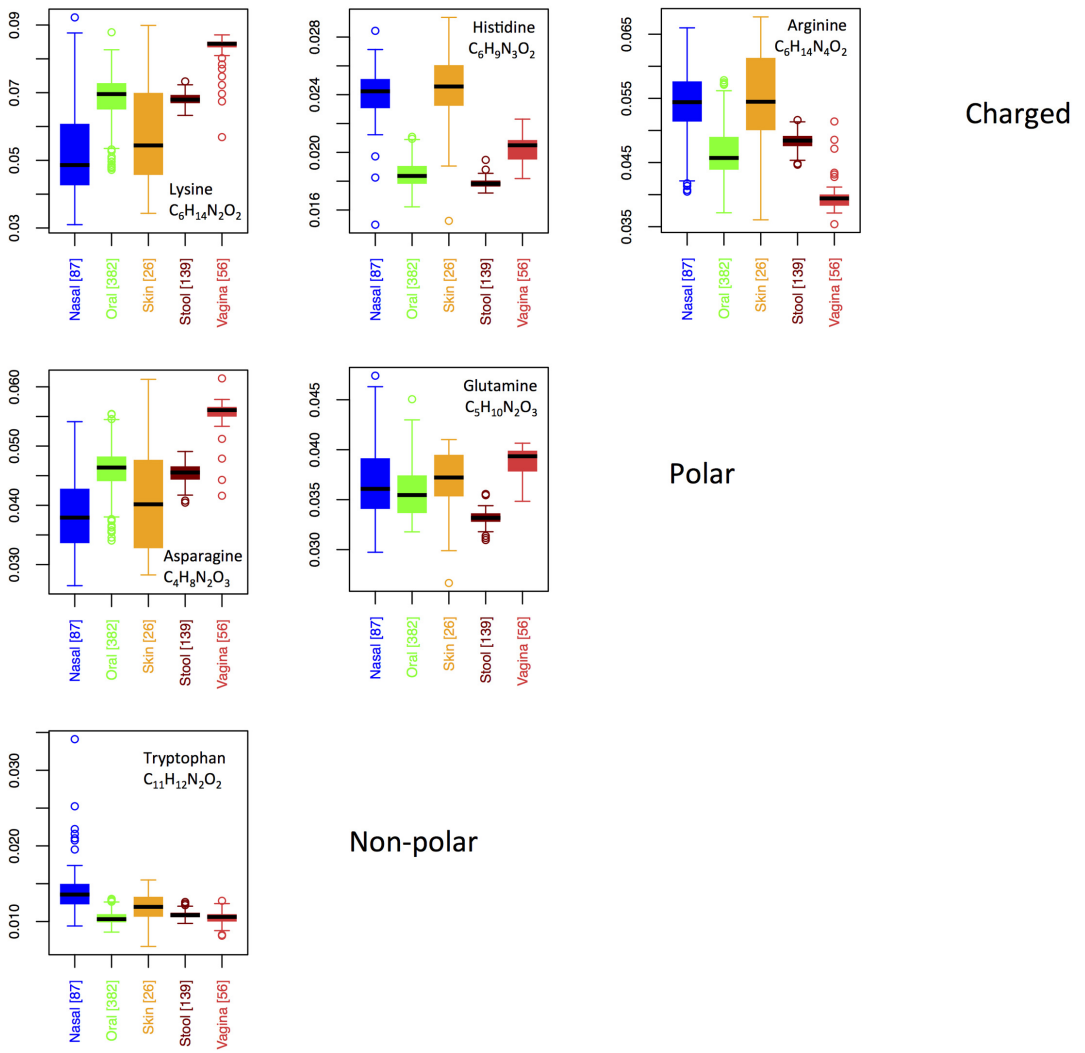

FIGURE 6 | Boxplots showing the fractions of specific N-rich amino acids as a function of body site. Amino acids are grouped according to their chemical properties.

and mechanisms governing microbial community composition, both at different human body sites and across different people, remain largely unknown. Lacking such insight, it is difficult to understand why specific organisms inhabit specific body niches, how this breaks down in the context of dysbiosis, and how dysbiosis might be anticipated, or even prevented, to support human health and well-being.

One framework for interpreting ecosystem function in macroscopic communities is ecological stoichiometry (Sterner and Elser, 2002). Reductionist in nature, ecological stoichiometry seeks to explain community structure, including species interactions, species life histories and even selection for one species over another, within the context of chemical requirements and constraints. By applying ecological stoichiometry to the human microbiome, the goal is to begin to address the chemical underpinnings of microbiome function. This is important, because it takes the focus off of taxonomic/biological mechanisms and moves it to chemical mechanisms. Whereas biological mechanisms are complex, and often compounded by sophisticated or incompletely characterized biological behaviors, chemical mechanisms are more straightforward, primarily limited by stoichiometric and energetic constraints. Further, whereas biology is frequently typified by redundancy, chemistry, at least at an elemental level, is not. Because elements cannot substitute for one another without directly altering mechanism/function, it is often easier to detect underlying patterns from chemical versus biological data. Finally, a chemical approach provides a more straightforward path to therapeutic strategies. In particular, determining how to remove certain problematic organisms, or cultivate beneficial ones requires an understanding of community assembly and the various species interactions involved in community organization. Indeed, simply 'adding' a beneficial organism is likely to fail, because that organism will usually be out-competed by the rest of the community. By contrast, determining how to alter the underlying chemical constraints of a system may be as simple

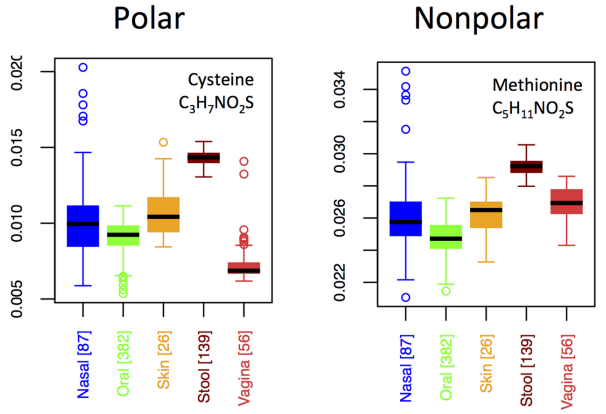

FIGURE 7 | Boxplots showing the fractions of the two S-rich amino acids as a function of body site.

as eating specific foods with the desired chemical ratios (gut microbiome) or applying certain nutrient-rich creams to the skin or vagina.

Interestingly, we find that different body sites harbor microbes with distinctly different protein compositions. Skin and nasal sites, for example, are characterized by proteins with high nitrogen and oxygen content. By contrast, carbon and sulfur contents are highest in proteins from stool. Surprisingly, vaginal sites are relatively nutrient-poor, having low nitrogen, oxygen, and sulfur content, and relatively high carbon content (though less than stool). Oral sites tend to be intermediate in carbon, nitrogen, oxygen, and sulfur. The differences that we observe in protein elemental content across body sites provide evidence that the microbiomes at these sites may be differentially limited by nutrient supply rates. However, additional factors beyond nutrient supply are known to impact protein composition. Most obvious is the relationship between protein elemental content and the GC content of CDSs. Because of this relationship, body site differences in protein composition may be generated indirectly as a result of differing selective forces acting on the nucleotide sequence, rather than the protein sequence. The selective forces acting on the nucleotide sequence might have little to do with nutrient supply rates. For example, high GC content appears to be favored in organisms exposed to UV radiation (Mcewan et al., 1998) – something that is likely at skin sites. Consequently, stoichioproteomic trends at skin sites may be driven by UV exposure, rather than nutrient supply rates. In particular, UV radiation may favor higher GC content on the skin, which then automatically translates into the lower carbon content, and higher oxygen and nitrogen contents that we observe at this site. Although this is a compelling mechanistic argument, our analysis shows that GC content alone is insufficient to explain protein elemental variation across body sites. Indeed, while all elements show the expected trends as a function of GC content of their CDSs, there is additional body site variation not explained by GC content. This suggests that selective forces acting on factors other than the nucleotide sequence are at least partially responsible for the protein elemental trends that we observe across different body sites. Further, while we cannot fully rule out structural or chemical requirements as the source of this selection, differing patterns of use of AAs with similar chemical properties across different body sites provides preliminary evidence against the chemical/structural argument and for the argument of differential nutrient supplies.

In addition to the absolute elemental content of proteins at different body sites, another interesting property is variation in protein composition. By and large, the most variation is observed at skin and nasal sites. The least variation is observed in stool. To some extent, this is not surprising, and likely derives in part from relative differences in CDS numbers across sites (see Appendix III, Figure A.3.1). In particular, stool has the largest number of CDSs per sample (median 183712), whereas the nares has the least (median 2188). As a consequence, estimates of sample elemental content at stool sites will generally include a larger number of data points as compared to nasal sites, tightening the precision of stool estimates relative to nasal estimates. Nevertheless, trends in variation are not fully explained by CDS numbers. Skin sites, for example, have approximately 10× as many CDSs as nasal sites, but exhibit similar levels of protein elemental variation. Meanwhile, vaginal sites have CDS counts similar to nasal sites (median 2907), but exhibit much lower variation in elemental content. Indeed, the elemental variation at vaginal sites is often on par with the variation at oral sites, which have much larger numbers of CDS per sample (median 129778). Larger variation in elemental content at a site might indicate larger habitat heterogeneity. For example, the larger variation in nasal versus vaginal sites might indicate a greater degree of variation in nutrient supply rates (or other selective factors) in the nose relative to the vagina. Because much of this variation comes from measures across individuals, this habitat heterogeneity is likely a result of greater inter-individual variation at these sites.

While many studies in ecological stoichiometry seek to define differences in the elemental compositions of organisms across habitats (Hecky et al., 1993; Elser and Hassett, 1994; Cardinale et al., 1997; Elser et al., 2000a; Sterner and Elser, 2002), a broader goal is to use this information to try to understand ecosystem function. With this in mind, we briefly discuss the various different habitats that we considered in our study, attempting to interpret our findings within the context of the specific environmental conditions present at these sites.

Skin and Nasal Sites

Skin and nasal sites are remarkably similar in all stoichiometric aspects studied here. Indeed, the only significant differences between these two sites were that skin tended to have proteins with a more tightly constrained oxygen content and a more tightly constrained sulfur content (possibly due to less interpersonal variation in oxygen and sulfur supply rates, see above). Perhaps it should not be surprising that the skin and nares are alike. After all, both locations are lined with a similar type of human cell – i.e., stratified squamous keratinizing epithelium – suggesting that the 'detritus' supplied to both locations may be similar, at least from the host. Nevertheless, nasal and skin sites (including the retroauricular crease) are known to differ in their

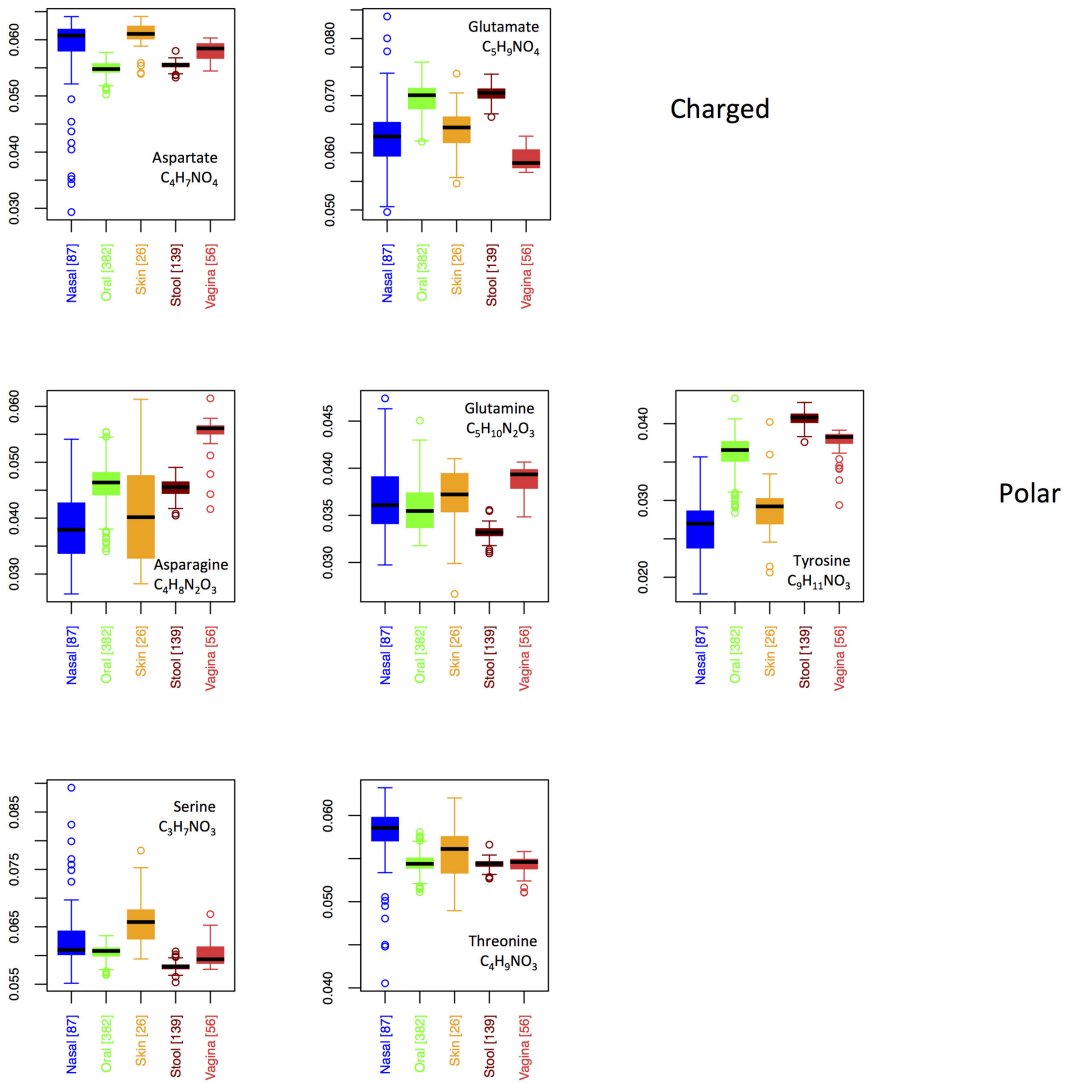

FIGURE 8 | Boxplots showing the fractions of the O-rich amino acids as a function of body site. Amino acids are grouped according to their chemical properties.

microbiome communities (Grice et al., 2009). Most notably, the nares is a common reservoir for *Staphylococcus aureus* which, though also present on exposed skin, is less abundant (Kluytmans et al., 1997). Thus, despite their similarities in host interface, skin and nasal sites still exhibit microbiome differences. For this reason, it is somewhat unexpected that their underlying elemental chemistries are so similar. While we cannot rule out the effects of chemical constraints other than those studied here (e.g., the presence of more complex chemicals like sebum), our preliminary findings suggest that factors other than nutrient availability may be dictating taxonomic differences in nasal versus skin colonization.

One observation that is hard to avoid is that both skin and nasal samples – the two air-exposed sites – are rich in nitrogen and oxygen. It is likely that oxygen limitation at these locations is minimized as a result of their interface with the atmosphere. Indeed, this is in keeping with our hypothesis that body sites associated with large aerobic niches should harbor microbial biomass that is richer in oxygen. While it is also conceivable that nitrogen limitation is minimized as a result of contact with nitrogen in the atmosphere, the high energetic costs of nitrogen fixation make this less probable. Thus, while species like *Klebsiella pneumoniae* – commonly found in the anterior nares (Wilson and Hamilos, 2014) – are known to have nitrogen fixing capabilities (Dixon et al., 1980), it is more likely that the high nitrogen content in skin microbiomes is supplied as nitrates, ammonia, and urea through human sweat (Gilchrist and Benjamin, 2011; Scharschmidt and Fischbach, 2013) or as other nitrogen-rich proteins common on epithelial surfaces.

Stool

The gut microbiome – rich in carbon and sulfur – should primarily receive nutrients through feeding behavior of the human host (Turnbaugh et al., 2009). However, food taken in

by the host is not universally transferred to gut bacteria. Instead, the host must absorb a good deal of the nutrients, setting up competition for potentially limiting elements (Bäckhed et al., 2005). With this in mind, it is perhaps not surprising that carbon and sulfur are in excess in stool. As omnivores, humans will generally exhibit stoichiometric mismatch, such that they consume food with a much higher C:N ratio than their own tissue (Denno and Fagan, 2003). Consequently, in the process of fulfilling human dietary requirements, carbon will be recycled to the environment. Within the human gut, this means that there will be an excess of carbon, whereas nitrogen may be limiting. Bacteria that can accommodate a high C:N ratio may be better able to live in symbiosis with their host, and thus may have been selected over years of human-microbe co-evolution. For similar reasons, it is likely that sulfur will also be in excess, and thus available to gut microbes. In particular, adult dietary protein requirements generally exceed adult sulfur requirements, which are almost universally met in Western diets (Shils and Shike, 2006; Masella and Mazza, 2009). Again, this means excess sulfur is left to be recycled back to the environment, including to the symbiotic microbiota living in the human stomach and intestines. Indeed, microbial sulfur metabolism appears to be common and of great importance within the human intestine, potentially regulating health and disease (Carbonero et al., 2012). That said, the high sulfur content of stool could also reflect the fact that anaerobic organisms – which are known to have higher protein sulfur contents than aerobic organism (Bragg et al., 2006) – form the majority of the gut microbiome. If this is true, however, it is not clear why the vagina, another site with a large fraction of anaerobes (Ravel et al., 2011), is one of the more sulfur-poor microbiomes (see **Figure 4A**).

While the high carbon and sulfur content of gut proteins may be easily rationalized, one finding that is less obvious is the observed tight regulation over ecological stoichiometry in the gut in general. This is somewhat surprising because the supply rate of nutrients to gut microbes should depend on human diet – something that is expected to be highly variable across individuals. An interesting and open question is why the protein elemental stoichiometry of the gut is so tightly regulated, while the elemental stoichiometry of other sites – most notably skin and the nares – is not. This may be because absolute bacterial biomass recovered from the skin and nares is low, as are CDS counts, making stochastic effects on protein elemental ratios more prominent. However, it may also be driven by as yet undetermined constraints on gut elemental protein content. Ultimately, finding an answer to this question may help to elucidate both the structuring principles guiding assembly of gut microbiomes, and the symbiotic host–microbe interactions that these microbiomes undertake.

Vaginal

The protein content of the vaginal microbiome is remarkably nutrient-poor. This is somewhat unexpected, given the presence of rich vaginal secretions containing proteins and immunoglobulins at concentrations of 15–26 μg/mL, as well as nitrogen-rich urea at concentrations of 49 mg/100 mL (Geshnizgani and Onderdonk, 1992). Whether the low nutrient content of vaginal proteins reflects additional constraints of the vaginal environment [e.g., its low pH (Ravel et al., 2011)], whether this is evidence of competing interests with host processes (e.g., the need for certain sulfur compounds during reproduction), or whether this is simply a result of the carbohydrate component of vaginal secretions being high relative to other components is unclear. Nevertheless, understanding nutrient constraints and exchange in this particular microbial niche could prove fruitful. Indeed, dysbiosis is relatively common in the vagina (Huth, 1989), and may be a result of nutrient imbalances that allow certain bacterial taxa to bloom.

Oral

Microbial proteins from the oral microbiome are intermediate in nutrient content between those of stool and those of the vagina. In particular, the oral microbiome has a similar nitrogen and oxygen content to stool, but a similar sulfur content to the vagina. Notably, the oral region is the only microbiome in the HMP where more than one sub-site was surveyed extensively (see **Table 1**). Interestingly, we do see fine-scale variation across different oral microbiomes for almost all elements considered. In particular, the buccal mucosa often differs from supragingival plaque which, itself, differs from the tongue. Most notably, supragingival plaque has a relatively higher nitrogen content. While salivary nitrate concentrations are quite high (Granli et al., 1989; Pannala et al., 2003), and could serve as nitrogen sources, it is unclear why nitrate should be particularly available in plaque. A more likely hypothesis is that the high nitrogen content in plaque comes from nitric oxide formed by gingival cells (Schreiber et al., 2010). Interestingly, denitrification in these locations has been documented (Schreiber et al., 2010).

CONCLUSION AND FUTURE DIRECTIONS

In this paper, we have taken a step toward untangling the complex ecological stoichiometry of the human microbiome. In particular, we have studied microbial stoichiometry as reflected in the protein composition of the various microbial proteins from different body sites. Although this is a promising start, showing significant variation between both major and minor body sites, a more complete picture will only emerge with further study. Indeed, just as microbial stoichiometry in oceans and soils has focused on whole cell analysis, extending the work presented in the current study to molecules beyond proteins could yield exciting results. At the very least, examining phosphorus content of the various human microbiomes could prove insightful.

Intermediate between the stoichioproteomic approach that we have taken here, and the type of whole-cell ecological stoichiometric analysis that has been applied to microbiomes from oceans, lakes, and rivers, an alternate option would be to consider transcriptomics. Though an RNA analysis would still be restricted to proteins, it would better reflect protein expression rates in specific environments. Indeed, just because an organism has DNA coding for a particular nutrient-rich protein does

not mean that protein is expressed under conditions where the nutrient is limiting (Gilbert and Fagan, 2011). By applying a stoichioproteomic analysis to transcriptomics datasets, it would be possible to determine more absolute metrics of nutrient use across the human microbiome. Although the types of transcriptomics datasets that this approach requires are currently limited, this is likely to change in the near future.

Another complicating factor that we have not considered in the current work is the possibility that the trends we observe are driven by phylogenetic conservation of traits. Indeed, elemental contents of proteins are known to be similar across related taxa, while different microbiomes are known to be preferentially populated with organisms from certain taxonomic groups. Although there are undoubtedly selective forces determining the phylogenetic relationships among and between microbial constituents from different microbiomes, these forces may act on factors independent of nutrient use and availability. In other words, nutrient composition may be indirectly selected for as a result of selection acting on some other phylogenetically conserved trait that is differentially important for survival at various body site locations. It would be interesting to try to determine the extent to which nutrient constraints are driving phylogenetic differences among body sites and vice versa.

A final avenue of research would be to better define stoichiometric mismatches between nutrient supply rates and bacterial biomass across the different body sites. Although we have suggested largely speculative explanations for observed differences in C, N, O, and S content of proteins, a true understanding of which elements are limiting at which locations, and how this depends on both host biology and the biology of the microbes in the community, is necessary for a mechanistic understanding of nutrient exchange and nutrient cycling in these systems. Although not an easy task, development of such studies could provide valuable information about host–microbe interactions and host influence on microbiome composition. This, in turn, could be useful for devising therapeutic strategies aimed at maintaining healthy microbiomes in order to preserve the beneficial services that they provide. It could also be used to develop understanding of microbiome dysbiosis, which is known to play a role in a number of diseases, including psoriasis and vaginal bacteriosis. Indeed, ecological stoichiometry may be a particularly advantageous framework for understanding the human microbiome because it brings sequencing studies together with a multiple currency approach to understanding ecosystem function. Ultimately, this allows for a rigorous chemical interpretation of microbial processes. In a time when microbiome data is exploding, but methods for making sense of the data lag behind, ecological stoichiometry may be just the tool that we need for taking the next step in human microbiome science.

AUTHOR CONTRIBUTIONS

SB, DK, and WF conceived of the idea; BV-P and SB performed all microbiome and bioinformatics analysis necessary to determine the elemental fractions of the proteins at different body sites; KM, BV-P, and SB performed statistical analysis on protein elemental fractions; BV-P, SB, KM, DK, and WF discussed and interpreted results; SB wrote the paper.

ACKNOWLEDGMENTS

This material is based upon work supported by, or in part by, the U.S. Army Research Laboratory and the U.S. Army Research Office under contract/grant number #W911NF-14-1-0490.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01119/full#supplementary-material

REFERENCES

Acquisti, C., Kleffe, J., and Collins, S. (2007). Oxygen content of transmembrane proteins over macroevolutionary time scales. *Nature* 445, 47–52. doi: 10.1038/nature05450

Andersen, T., and Hessen, D. O. (1991). Carbon, nitrogen, and phosphorus content of freshwater zooplankton. *Limnol. Oceanogr.* 36, 807–814. doi: 10.4319/lo.1991.36.4.0807

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science* 307, 1915–1920. doi: 10.1126/science.1104816

Barsdate, R., Prentki, R., and Fenchel, T. (1974). Phosphorus cycle of model ecosystems: significance for decomposer food chains and effect of bacterial grazers. *Oikos* 25, 239–251. doi: 10.2307/3543942

Baudouin-Cornu, P., Schuerer, K., Marlière, P., and Thomas, D. (2004). Intimate evolution of proteins. Proteome atomic content correlates with genome base composition. *J. Biol. Chem.* 279, 5421–5428. doi: 10.1074/jbc.M306415200

Baudouin-Cornu, P., Surdin-Kerjan, Y., Marliere, P., and Thomas, D. (2001). Molecular evolution of protein atomic composition. *Science* 293, 297–300. doi: 10.1126/science.1061052

Belkaid, Y., and Segre, J. A. (2014). Dialogue between skin microbiota and immunity. *Science* 346, 954–959. doi: 10.1126/science.1260144

Belkaid, Y., and Tamoutounour, S. (2016). The influence of skin microorganisms on cutaneous immunity. *Nat. Rev. Immunol.* 16, 353–366. doi: 10.1038/nri.2016.48

Bragg, J. G., and Hyder, C. L. (2004). Nitrogen versus carbon use in prokaryotic genomes and proteomes. *Proc. R. Soc. Lond. B Biol. Sci.* 271, S374–S377. doi: 10.1098/rsbl.2004.0193

Bragg, J. G., Thomas, D., and Baudouin-Cornu, P. (2006). Variation among species in proteomic sulphur content is related to environmental conditions. *Proc. R. Soc. Lond. B Biol. Sci.* 273, 1293–1300. doi: 10.1098/rspb.2005.3441

Bragg, J. G., and Wagner, A. (2009). Protein material costs: single atoms can make an evolutionary difference. *Trends Genet.* 25, 5–8. doi: 10.1016/j.tig.2008.10.007

Carbonero, F., Benefiel, A. C., Alizadeh-Ghamsari, A. H., and Gaskins, H. R. (2012). Microbial pathways in colonic sulfur metabolism and links with health and disease. *Front. Physiol.* 3:448. doi: 10.3389/fphys.2012.00448

Cardinale, B., Stabler, L., and Elser, J. (1997). Ecological stoichiometry of N and P in pelagic ecosystems: comparison of lakes and oceans with emphasis on

the zooplankton-phytoplankton interaction. *Limnol. Oceanogr.* 42, 648–662. doi: 10.4319/lo.1997.42.4.0648

Cebrian, J., and Kingsolver, J. G. (1999). Patterns in the fate of production in plant communities. *Am. Nat.* 154, 449–468. doi: 10.1086/303244

Cho, I., and Blaser, M. J. (2012). The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* 13, 260–270. doi: 10.1038/nrg3182

Chrzanowski, T. H., and Kyle, M. (1996). Ratios of carbon, nitrogen and phosphorus in *Pseudomonas fluorescens* as a model for bacterial element ratios and nutrient regeneration. *Aquat. Microb. Ecol.* 10, 115–122. doi: 10.3354/ame010115

DeMott, W. R., Gulati, R. D., and Siewertsen, K. (1998). Effects of phosphorus-deficient diets on the carbon and phosphorus balance of *Daphnia magna*. *Limnol. Oceanogr.* 43, 1147–1161. doi: 10.4319/lo.1998.43.6.1147

Denno, R. F., and Fagan, W. F. (2003). Might nitrogen limitation promote omnivory among carnivorous arthropods? *Ecology* 84, 2522–2531. doi: 10.1890/02-0370

Dixon, R., Eady, R. R., Espin, G., Hill, S., Iaccarino, M., Kahn, D., et al. (1980). Analysis of regulation of *Klebsiella pneumoniae* nitrogen fixation (nif) gene cluster with gene fusions. *Nature* 286, 128–132. doi: 10.1038/286128a0

Elser, J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate–stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Acquisti, C., and Kumar, S. (2011). Stoichiogenomics: the evolutionary ecology of macromolecular elemental composition. *Trends Ecol. Evol.* 26, 38–44. doi: 10.1016/j.tree.2010.10.006

Elser, J. J., Dobberfuhl, D. R., MacKay, N. A., and Schampel, J. H. (1996). Organism size, life history, and N: P stoichiometry toward a unified view of cellular and ecosystem processes. *BioScience* 46, 674–684. doi: 10.2307/1312897

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000a). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580.

Elser, J. J., Fagan, W. F., Subramanian, S., and Kumar, S. (2006). Signatures of ecological resource availability in the animal and plant proteomes. *Mol. Biol. Evol.* 23, 1946–1951. doi: 10.1093/molbev/msl068

Elser, J. J., and Hassett, R. P. (1994). A stoichiometric analysis of the zooplankton-phytoplankton interaction in marine and freshwater ecosystems. *Nature* 370, 211–213. doi: 10.1038/370211a0

Elser, J. J., O'brien, W., Dobberfuhl, D., and Dowling, T. (2000b). The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* 13, 845–853. doi: 10.1046/j.1420-9101.2000.00215.x

Elser, J. J., Sterner, R., Gorokhova, E., Fagan, W., Markow, T., Cotner, J., et al. (2000c). Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* 3, 540–550. doi: 10.1046/j.1461-0248.2000.00185.x

Fagan, W. F., Siemann, E., Mitter, C., Denno, R. F., Huberty, A. F., Woods, H. A., et al. (2002). Nitrogen in insects: implications for trophic complexity and species diversification. *Am. Nat.* 160, 784–802. doi: 10.1086/343879

Geshnizgani, A., and Onderdonk, A. B. (1992). Defined medium simulating genital tract secretions for growth of vaginal microflora. *J. Clin. Microbiol.* 30, 1323–1326.

Gevers, D., Knight, R., Petrosino, J. F., Huang, K., McGuire, A. L., Birren, B. W., et al. (2012). The Human Microbiome Project: a community resource for the healthy human microbiome. *PLoS Biol.* 10:e1001377. doi: 10.1371/journal.pbio.1001377

Gilbert, J. D., and Fagan, W. F. (2011). Contrasting mechanisms of proteomic nitrogen thrift in *Prochlorococcus*. *Mol. Ecol.* 20, 92–104. doi: 10.1111/j.1365-294X.2010.04914.x

Gilchrist, M., and Benjamin, N. (2011). *From Atmospheric Nitrogen to Bioactive Nitrogen Oxides, Nitrite and Nitrate in Human Health and Disease*. Berlin: Springer, 9–19. doi: 10.1007/978-1-60761-616-0_2

Goldman, J. C., Caron, D. A., and Dennett, M. R. (1987). Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C: N ratio. *Limnol. Oceanogr.* 32, 1239–1252. doi: 10.4319/lo.1987.32.6.1239

Granli, T., Dahl, R., Brodin, P., and Bøckman, O. (1989). Nitrate and nitrite concentrations in human saliva: variations with salivary flow-rate. *Food Chem. Toxicol.* 27, 675–680. doi: 10.1016/0278-6915(89)90122-1

Grice, E. A., Kong, H. H., Conlan, S., Deming, C. B., Davis, J., Young, A. C., et al. (2009). Topographical and temporal diversity of the human skin microbiome. *Science* 324, 1190–1192. doi: 10.1126/science.1171700

Hecky, R., Campbell, P., and Hendzel, L. (1993). The stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. *Limnol. Oceanogr.* 38, 709–724. doi: 10.4319/lo.1993.38.4.0709

Herbert, D. (1961). The chemical composition of micro-organisms as a function of their environment Symp. *Soc. Gen. Microbiol.* 11, 391.

Herbert, D. (1976). "Stoichiometric aspects of microbial growth," in *Continuous Culture 6: Applications and New Fields*, eds A. C. R. Dean, D. C. Ellwood, C. G. T. Evans, and J. Melling (Chichester: Ellis Harwood), 1–30.

Herbert, D., Phipps, P., and Strange, R. (1971). Chapter III chemical analysis of microbial cells. *Methods Microbiol.* 5, 209–344. doi: 10.1016/S0580-9517(08)70641-X

Hessen, D., and Lyche, A. (1991). Inter-and intraspecific variations in zooplankton element composition. *Arch. Hydrobiol.* 121, 343–353.

Human Microbiome Project Consortium (2012a). A framework for human microbiome research. *Nature* 486, 215–221.

Human Microbiome Project Consortium (2012b). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Huth, E. J. (1989). Style notes: bacterial vaginosis or vaginal bacteriosis? *Ann. Intern. Med.* 111, 553–554. doi: 10.7326/0003-4819-111-7-553

Kluytmans, J., Van Belkum, A., and Verbrugh, H. (1997). Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin. Microbiol. Rev.* 10, 505–520.

Lemoine, N. P., Giery, S. T., and Burkepile, D. E. (2014). Differing nutritional constraints of consumers across ecosystems. *Oecologia* 174, 1367–1376. doi: 10.1007/s00442-013-2860-z

Makino, W., Cotner, J., Sterner, R., and Elser, J. (2003). Are bacteria more like plants or animals? Growth rate and resource dependence of bacterial C: N: P stoichiometry. *Funct. Ecol.* 17, 121–130. doi: 10.1046/j.1365-2435.2003.00712.x

Masella, R., and Mazza, G. (2009). *Glutathione and Sulfur Amino Acids in Human Health and Disease*. Hoboken, NJ: John Wiley & Sons. doi: 10.1002/9780470475973

Mazel, D., and Marliére, P. (1989). Adaptive eradication of methionine and cysteine from cyanobacterial light-harvesting proteins. *Nature* 341, 245–248. doi: 10.1038/341245a0

Mcewan, C. E., Gatherer, D., and Mcewan, N. R. (1998). Nitrogen-fixing aerobic bacteria have higher genomic GC content than non-fixing species within the same genus. *Hereditas* 128, 173–178. doi: 10.1111/j.1601-5223.1998.00173.x

Moe, S. J., Stelzer, R. S., Forman, M. R., Harpole, W. S., Daufresne, T., and Yoshida, T. (2005). Recent advances in ecological stoichiometry: insights for population and community ecology. *Oikos* 109, 29–39. doi: 10.1111/j.0030-1299.2005.14056.x

Musto, H., Naya, H., Zavala, A., Romero, H., Alvarez-Valín, F., and Bernardi, G. (2006). Genomic GC level, optimal growth temperature, and genome size in prokaryotes. *Biochem. Biophys. Res. Commun.* 347, 1–3. doi: 10.1016/j.bbrc.2006.06.054

Nakano, S.-I. (1994). Carbon: nitrogen: phosphorus ratios and nutrient regeneration of a heterotrophic flagellate fed on bacteria with different elemental ratios. *Arch. Hydrobiol.* 129, 257–271.

Pannala, A. S., Mani, A. R., Spencer, J. P., Skinner, V., Bruckdorfer, K. R., Moore, K. P., et al. (2003). The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. *Free Radic. Biol. Med.* 34, 576–584. doi: 10.1016/S0891-5849(02)01353-9

Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J. A., et al. (2009). The NIH human microbiome project. *Genome Res.* 19, 2317–2323. doi: 10.1101/gr.096651.109

Proctor, L. M. (2011). The human microbiome project in 2011 and beyond. *Cell Host Microbe* 10, 287–291. doi: 10.1016/j.chom.2011.10.001

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., et al. (2011). Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4680–4687. doi: 10.1073/pnas.1002611107

Rhee, G.-Y. (1978). Effects of N: P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. *Limnol. Oceanogr.* 23, 10–25. doi: 10.4319/lo.1978.23.1.0010

Rocha, E. P., and Danchin, A. (2002). Base composition bias might result from competition for metabolic resources. *Trends Genet.* 18, 291–294. doi: 10.1016/S0168-9525(02)02690-2

Scharschmidt, T. C., and Fischbach, M. A. (2013). What lives on our skin: ecology, genomics and therapeutic opportunities of the skin microbiome. *Drug Discov. Today Dis. Mech.* 10, e83–e89. doi: 10.1016/j.ddmec.2012.12.003

Schreiber, F., Stief, P., Gieseke, A., Heisterkamp, I. M., Verstraete, W., de Beer, D., et al. (2010). Denitrification in human dental plaque. *BMC Biol.* 8:24. doi: 10.1186/1741-7007-8-24

Shils, M. E., and Shike, M. (2006). *Modern Nutrition in Health and Disease*. Philadelphia, PA: Lippincott Williams & Wilkins.

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Tezuka, Y. (1990). Bacterial regeneration of ammonium and phosphate as affected by the carbon: nitrogen: phosphorus ratio of organic substrates. *Microb. Ecol.* 19, 227–238. doi: 10.1007/BF02017167

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C., Knight, R., and Gordon, J. I. (2007). The human microbiome project: exploring the microbial part of ourselves in a changing world. *Nature* 449, 804–810. doi: 10.1038/nature06244

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., and Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* 1, ra14–ra16. doi: 10.1126/scitranslmed.3000322

Urabe, J. (1993). N and P cycling coupled by grazers' activities: food quality and nutrient release by zooplankton. *Ecology* 74, 2337–2350. doi: 10.2307/1939586

Wilson, M. T., and Hamilos, D. L. (2014). The nasal and sinus microbiome in health and disease. *Curr. Allergy Asthma Rep.* 14:485. doi: 10.1007/s11882-014-0485-x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 July 2018
doi: 10.3389/fmicb.2018.01590

Stoichio-Metagenomics of Ocean Waters: A Molecular Evolution Approach to Trace the Dynamics of Nitrogen Conservation in Natural Communities

Hannes Dittberner†, Niklas Ohlmann† and Claudia Acquisti†*

Institute for Evolution and Biodiversity, University of Münster, Münster, Germany

OPEN ACCESS

Edited by:
Adam Martiny, University of California, Irvine, United States

Reviewed by:
Catherine Ann Pfister, University of Chicago, United States
Jason Bragg, National Herbarium of New South Wales, Royal Botanic Garden and Domain Trust, Australia

***Correspondence:**
Claudia Acquisti
claudia.acquisti@uni-muenster.de

†Present address:
Hannes Dittberner, Institute for Botany, University of Cologne, Cologne, Germany
Niklas Ohlmann, Forest Research Institute Baden-Württemberg, Freiburg im Breisgau, Germany
Claudia Acquisti, Department of Biology, University of Iowa, Iowa City, IA, United States

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *28 February 2017*
Accepted: *26 June 2018*
Published: *18 July 2018*

Citation:
Dittberner H, Ohlmann N and Acquisti C (2018) Stoichio-Metagenomics of Ocean Waters: A Molecular Evolution Approach to Trace the Dynamics of Nitrogen Conservation in Natural Communities. Front. Microbiol. 9:1590. doi: 10.3389/fmicb.2018.01590

Nitrogen is crucially limiting in ocean surface waters, and its availability varies substantially with coastal regions typically richer in nutrients than open oceans. In a biological stoichiometry framework, a parsimonious strategy of nitrogen allocation predicts nitrogen content of proteins to be lower in communities adapted to open ocean than to coastal regions. To test this hypothesis we have directly interrogated marine microbial communities, using a series of metagenomics datasets with a broad geographical distribution from the Global Ocean Sampling Expedition. Analyzing over 20 million proteins, we document a ubiquitous signal of nitrogen conservation in open ocean communities, both in membrane and non-membrane proteins. Efficient nitrogen allocation is expected to specifically target proteins that are expressed at high rate in response to nitrogen starvation. Furthermore, in order to preserve protein functional efficiency, economic nitrogen allocation is predicted to target primarily the least functionally constrained regions of proteins. Contrasting the NtcA-induced pathway, typically up-regulated in response to nitrogen starvation, with the arginine anabolic pathway, which is instead up-regulated in response to nitrogen abundance, we show how both these predictions are fulfilled. Using evolutionary rates as an informative proxy of functional constraints, we show that variation in nitrogen allocation between open ocean and coastal communities is primarily localized in the least functionally constrained regions of the genes triggered by NtcA. As expected, such a pattern is not detectable in the genes involved in the arginine anabolic pathway. These results directly link environmental nitrogen availability to different adaptive strategies of genome evolution, and emphasize the relevance of the material costs of evolutionary change in natural ecosystems.

Keywords: stoichiogenomics, nitrogen limitation, marine microbial communities, molecular evolution, material costs

INTRODUCTION

Recent investigations in "stoichiogenomics," projecting biological stoichiometry into molecular evolution, have indicated that the atomic composition of proteins and genes has adaptive significance (reviewed in Elser et al., 2011). These studies have explored the material cost of evolutionary change, showing how environmental nutrient limitations have affected the

composition of the genetic material (Baudouin-Cornu et al., 2004; Bragg and Hyder, 2004; Bragg et al., 2006; Elser et al., 2006; Bragg and Wagner, 2007, 2009; Acquisti et al., 2009a,b; Gilbert and Fagan, 2011; Read et al., 2017). In particular, nitrogen limitation has been the focus of many of these analyses because nitrogen is an essential component of amino acids and nucleotides, and it is often limiting in natural environments. The impact of selection for nitrogen conservation in shaping evolutionary change remains, however, elusive. One of the problems is that previous analyses have primarily relied on model organisms, leaving the relevance of adaptation to nutrient availability in natural environments only partially addressed. The aim of this paper is to bridge this gap, and to directly quantify the role of selection for nitrogen conservation in a natural ecosystem, combining the power of metagenomics and biogeochemistry in an evolutionary framework.

Covering almost three quarters of the Earth's surface and more than half of the planetary total net primary productivity, oceans play a profound role in tuning the global dynamics of nutrient pools in the biosphere. In the complex regulation of the oceanic bio-geochemical cycles, the severe depletion of inorganic nitrogen in surface waters is one of the factors critically limiting growth and reproduction in seawaters (Capone and Hutchins, 2013). Nutrient regimes are however highly variable in oceans. For example, the severity of nitrogen limitation in surface waters follows a clear spatial pattern, as it decreases dramatically in coastal regions due to upwelling, and to terrestrial and riverine nutrient inputs (Capone and Hutchins, 2013). In the last decades this effect has been on the rise, as nutrient enrichment of coastal zones has substantially increased due to anthropogenic activity. Due to the intrinsic variation of nitrogen availability between coastal and open ocean surface waters, the different marine biota adapted to these two habitats provide an ideal set of related ecosystems to study the effect of environmental nitrogen limitation on the evolution of proteins.

The Global Ocean Sampling Expedition (Rusch et al., 2007; Yooseph et al., 2007), a comprehensive catalog of marine surface water metagenomics sampling across the globe, offers a useful dataset to address the role of nitrogen limitation in shaping evolutionary change in marine microbial communities. Under persistent conditions of severe nitrogen limitation, growth and reproduction of individuals with lower allocation of nitrogen in their proteins should be favored by natural selection. Thus, species adapted to oligotrophic open ocean waters are expected to exhibit a progressive evolutionary shift in the frequencies of amino acids toward an enrichment of amino acids with a lower relative concentration of nitrogen. Indeed a previous analysis (Grzymski and Dussaq, 2012) has shown that the average nitrogen content of proteins is significantly lower in open ocean than in coastal microbial communities.

Merging the perspective of molecular evolution and biological stoichiometry, we say that selection for the fittest is expected to shape nitrogen allocation while preserving protein structure and function. However, protein structural constraints alone strongly affect amino acid composition, and consequently protein nitrogen content. This is independent from adaptation to environmental nitrogen scarcity. As such, it is compelling to tell apart the contribution of these two forces in shaping protein nitrogen content. To shed light on these critical points, here we take an evolutionary approach to analyze the dynamics of nitrogen allocation in different functional and structural classes of proteins in natural communities.

Membrane proteins, in order to be inserted into the lipidic cellular membranes, are bound to be rich in hydrophobic amino acids. Due to the specific stoichiometry of hydrophobic amino acids (Berg et al., 2002), this structural constraint alone leads to a low nitrogen content. Given that membrane proteins typically cover 12% of marine metagenomes (Patel et al., 2010), their weight in shaping nitrogen allocation in natural communities is particularly relevant. In order to address this important aspect, we have identified (Käll et al., 2004) all membrane proteins in the dataset, and studied nitrogen allocation separately in membrane and non-membrane proteins. We have found that protein nitrogen content is lower in communities adapted to oligotrophic open oceans in both membrane and non-membrane proteins. Membrane proteins are further shaped by structural constraints that result in a sharp distribution of charged residues between the two sides of cellular membranes (von Heijne, 1992). This in turn defines striking differences in the nitrogen content of the intracellular, transmembrane, and periplasmic domains. We have followed nitrogen allocation along membrane protein topology and we show a widespread signature of nitrogen limitation across their three different structural domains.

A parsimonious strategy of nitrogen allocation driving genetic variation in natural communities is expected to specifically target proteins that are expressed at high rate in response to nitrogen starvation. The metagenomics approach used, while relying on a thorough and widespread geographical sampling and a large amount of data, lacks the power to directly measure relative expression levels. However, insights on the stoichiometry of the proteins specifically up-regulated during nitrogen deficiency, can be gained from the well established knowledge on the metabolic response triggered by nitrogen starvation in photosynthetic bacteria. The key player is the gene NtcA, also known as the global nitrogen regulator, a transcriptional activator that triggers a signaling cascade of genes subject to nitrogen control (García-Domínguez et al., 2000; Schwarz and Forchhammer, 2005). Glutamine synthase (glnA) is then up-regulated (Tanigawa et al., 2002) to control the intracellular nitrogen flow, along with an augmented expression of the transporters involved in nitrogen uptake from the environment, such as the urtABCDE operon (Beckers et al., 2004) and the amt gene family (Paz-Yepes et al., 2008). In the opposite scenario, when nitrogen is instead particularly abundant, a completely different metabolic network is activated to exploit the abundance of this important nutrient. Photosynthetic organisms store nitrogen as arginine (the most nitrogen-rich amino acid) by relieving the feedback mechanisms of arginine biosynthesis (Llácer et al., 2008), resulting in a downstream up-regulation of the Arg gene family involved in the different steps of arginine biosynthesis (Cunin et al., 1986). We contrasted therefore the NtcA-induced cascade to the arginine biosynthetic pathway. We have used a highly reliable functional gene prediction approach based on hidden Markov models (HMMs) (Eddy, 2011) to identify these proteins in the

dataset, and compared nitrogen allocation in these two metabolic networks along with the environmental nitrogen gradient from coastal regions to open oceans.

In the context of an adaptive process that preserves functional efficiency while parsimoniously allocating nitrogen, it is particularly relevant to address the role of functional and structural constraints in shaping nitrogen allocation in those two metabolic networks. To achieve this, we have used evolutionary rates as an informative proxy for functional constraints (Graur, 1985), following the rationale that sites highly constrained by structural and functional requirements will be under strong purifying selection, and tend to evolve slower than sites with a less stringent role in protein function (Kimura, 1983). After estimating the evolutionary rate of each residue in each protein using (Ashkenazy et al., 2010), we report here a clear signature of nitrogen limitation along the NtcA-induced cascade up-regulated in response to nitrogen starvation. Furthermore, we show that this signal is indeed localized in sites with lower functional constrains. As expected in the context of an adaptive and specific response to nitrogen limitation, such a pattern is not detectable in the arginine anabolic apparatus. The results presented indicate a fundamental role of selection for parsimonious nitrogen allocation in constraining protein composition in open ocean microbial communities, and strongly advocate for material costs as a key factor shaping natural selection in natural ecosystems.

MATERIALS AND METHODS

Global Ocean Sampling Metagenome

More than 40 million assembled peptides of the Global Ocean Sampling metagenome (Rusch et al., 2007; Yooseph et al., 2007) were downloaded from the CAMERA portal (Sun et al., 2011) (April 30, 2012). Along with providing latitude and longitude, the authors have classified the sampling location as open ocean, coastal, and estuary. We have relied on these classification and worked with sequences from surface water samples with locations "open ocean," "coastal," and "estuary" (**Supplementary Table S1**). Estuary regions are a specific type of coastal regions where a river or a stream meets the ocean. Therefore, due to the very low number of estuary samples, they were merged with coastal samples. Only proteins longer than 100 amino acids were analyzed.

Transmembrane Protein Topology Prediction

Membrane proteins were identified using Phobius 1.01 (Käll et al., 2004). This approach is highly sensitive, and it is based on a HMM that models the different sequence regions of a signal peptide and the different regions of a transmembrane protein in a series of interconnected states (Käll et al., 2004). Sequences with at least one transmembrane domain were classified as transmembrane proteins, and further dissected into "Cytoplasmic," "Transmembrane," and "Periplasmic/Extracellular" domains based on the topology prediction (signal peptides were not included in the analysis). Analyses of the three topological domains (intracellular, transmembrane, and extracellular) were performed on proteins longer than 100 amino acids and with at least 20 amino acids in each type of domain.

HMM-Based Functional Annotation

The HMMER3 (Eddy, 2011), a software suite for protein sequence similarity searches based on probabilistic methods, was used for the functional annotation of the genes involved in the NtcA-mediated response to nitrogen scarcity, and in arginine biosynthesis. The HMMER3 package hmmscan (Eddy, 2011) was used with TIGRFAM models[1] (Selengut et al., 2007), a collection of protein families featuring curated multiple sequence alignments, and HMMs designed to support the automated functional identification of proteins by sequence homology. For a list of genes, see **Supplementary Table S2**. Sequences were filtered based on the model-specific noise-cutoffs.

Estimation of Evolutionary Rates

Evolutionary rates were estimated for each amino acid residue using ConSurf1.0 (Ashkenazy et al., 2010), a method based on empirical Bayesian inference. This approach explicitly accounts for the stochastic process underlying sequence evolution and takes into account their phylogenetic relationships, enabling high sensitivity in discriminating sequence conservation due to short evolutionary time from conservation due to purifying selection (Celniker et al., 2013). For each protein a set of homologs was retrieved using Swiss-Prot and UniProtKB/TrEMBL as first and second pass BLAST database, respectively. A multiple sequence alignment was performed and a phylogenetic tree reconstructed with a neighbor joining approach. Referring to the software settings, position-specific evolutionary rates where calculated based on (i) Bayesian inference, (ii) JTT substitution model, (iii) Jukes–Cantor model for the tree search, and (iv) no branch length optimization. As in the default settings, all positions that in the multiple alignment had less than 10% ungapped amino acids were excluded from the analysis. Sites were classified based on the normalized scores (average score for all residues is 0, and the standard deviation is 1) with −0.75 as a cutoff for slow evolving sites and 0.75 for fast evolving sites.

Nitrogen Content Analyses

Nitrogen content Nc was calculated for each sequence as follows:

$$\mathrm{Nc} = (\Sigma n_i)/L$$

where n_i is the number of nitrogen atoms in the i^{th} residue side chain and L is the length of the sequence ($n = 1$ for asparagine, glutamine, lysine, and tryptophan; $n = 2$ for histidine; $n = 3$ for arginine; and $n = 0$ for the rest).

Plots and statistics were done with R (version 2.15), and nitrogen content calculation and sequence parsing with rpy (Python 2.7).

[1] ftp://ftp.jcvi.org/pub/data/TIGRFAMs/13.0_Release/

RESULTS

We have analyzed the nitrogen content of membrane and non-membrane proteins in natural communities adapted to open ocean or coastal regions. The comparison of over 20 million sequences shows a statistically significant (one-sided Wilcoxon rank sum test) difference accounting for a decrease in nitrogen content of 4–7% in open ocean communities (**Figure 1**). After predicting protein topology for each membrane protein using (Käll et al., 2004), we have studied nitrogen allocation separately in transmembrane, intracellular, and periplasmic domains. We document here a widespread and consistent signal in each of these three structurally different domains (**Figure 2**), further reinforcing the idea of a persistent and ubiquitous signature of selection for nitrogen conservation.

In order to have a relevant impact on the total nitrogen budget, an adaptive strategy of thrifty nitrogen allocation predicts that the largest difference between open ocean and coastal communities will be in genes up-regulated in response to nitrogen limitation. Furthermore, natural selection is expected to preserve functional efficiency while parsimoniously allocating nitrogen. Using evolutionary rates as a proxy for functional constraints, we expect faster evolving sites to be the primary targets of selection for nitrogen conservation, while slow evolving sites tend to be frozen by severe functional constraints (Tourasse and Li, 2000). To test these predictions, we have narrowed the focus on the pathways that enables cells to specifically respond to critical nitrogen scarcity. We have contrasted NtcA, the critical metabolic switch up-regulated in response to nitrogen starvation, to ArgF, a key enzyme involved in the biosynthesis of arginine and down-regulated in response to nitrogen scarcity in primary producers. Using a highly reliable functional prediction approach based on high quality sequence models (see section "Materials and Methods"), we have identified over 5000 homologs for nine proteins up-regulated via NtcA in response to nitrogen scarcity (NtcA, glnA, pII, urtB, urtC, urtD, urtE, recD, and amt1) and six proteins down-regulated in response to nitrogen starvation (argC, argJ, argH, argE, argG, and argF). With a phylogenetic reconstruction approach, we have then estimated evolutionary rates for each position in each sequence in the set (see section "Materials and Methods").

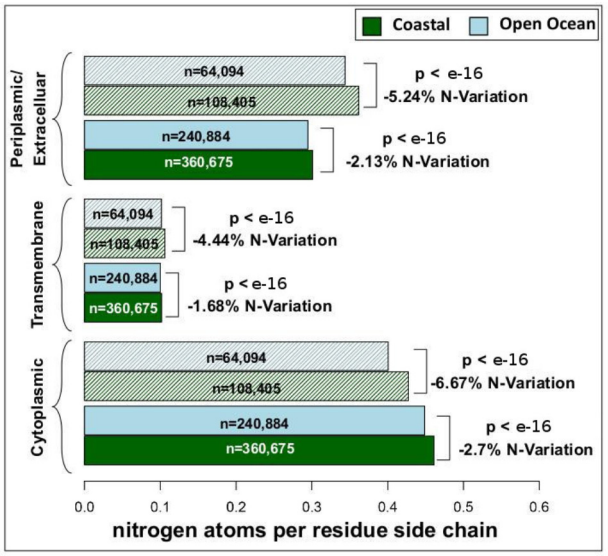

FIGURE 2 | Nitrogen content and transmembrane protein topology. Mean nitrogen content of cytoplasmic, transmembrane, and periplasmic/extracellular domains from coastal and open ocean samples. Solid bars represent transmembrane proteins with more than one transmembrane domain, while striped bars represent transmembrane proteins with only one transmembrane domain. The overall variation between open ocean and coastal samples is estimated as the relative difference in mean nitrogen content. Statistical significance is based on unpaired, one-sided approximative Wilcoxon–Mann–Whitney test. Standard errors of the mean are <0.001 for all samples (thus not visible in the present plot).

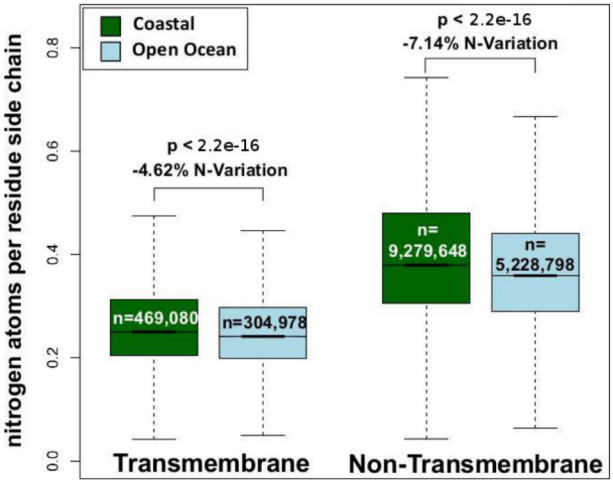

FIGURE 1 | Nitrogen content of transmembrane and non-transmembrane proteins from coastal and open ocean samples: a box plot visualization. The overall variation between open ocean and coastal samples is estimated as the relative difference in mean nitrogen content. Statistical significance is based on unpaired, one-sided approximative Wilcoxon–Mann–Whitney test.

The analysis of the 140 NtcA homologs distributed across open ocean and coastal regions indicates that the differences in nitrogen allocation are specifically localized in the fast evolving sites (**Figure 3A**), where they account for over 5% variation in nitrogen content between the two environments. Instead, the portions that are most severely constrained by functional requirements do not show any significant variation (**Figure 3A**). As expected, no significant nitrogen content variation is detectable between the natural communities adapted to coastal and open ocean regions in ArgF, neither in fast, nor in slow evolving sites (**Figure 3B**). In the present context, it is worth to note that, while different amino acids might tend to show different average evolutionary rates (Graur, 1985), the analysis performed here does not aim at comparing nitrogen composition between fast and slow evolving regions.

To test the relevance of the results obtained beyond NtcA and ArgF, we have expanded the same approach along the

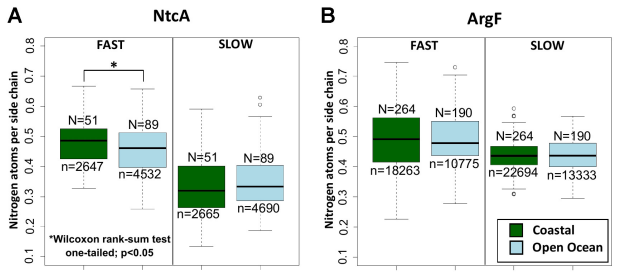

FIGURE 3 | Nitrogen content allocation in NtcA and ArgF. Nitrogen content in fast and slow evolving residues (for details, see section "Materials and Methods") in **(A)** NtcA and in **(B)** ArgF. *N* indicates the number of sequences, and *n* the number of sites in each dataset. Statistical significance is based on unpaired, one-sided Wilcoxon rank sum test.

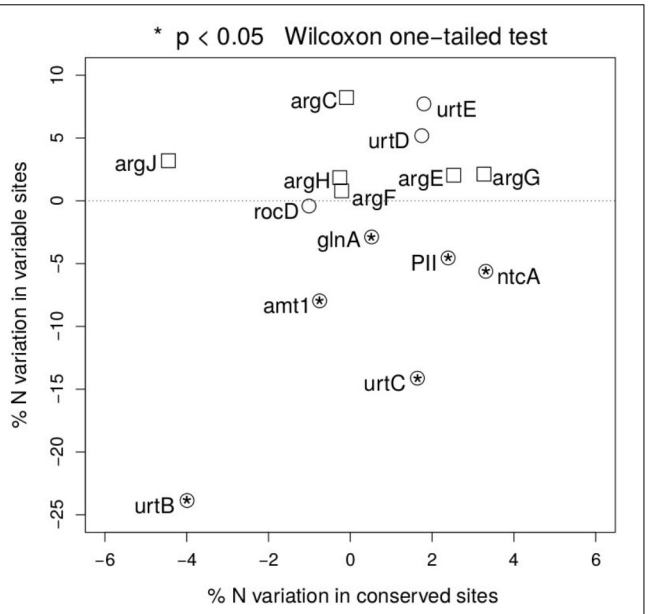

FIGURE 4 | Nitrogen content of proteins up- and down-regulated in response to nitrogen depletion. Scatter plot of the variation in mean nitrogen content in open ocean relative to coastal samples in fast and slow evolving residues for the different set of proteins (circles for the NtcA-induced cascade, and squares for the arginine biosynthetic pathway). Functional identification is based on TIGRFAM model (see section "Materials and Methods"). Statistical significance is reported for variable residues, and is based on unpaired, two-sided Wilcoxon rank sum test.

two pathways, and measured the percentage nitrogen content variation between open ocean and coastal regions in fast and slow evolving sites in each gene (**Figure 4**). Following the NtcA-induced signaling cascade fired by nitrogen deprivation, we have found that, with the exception of one of the domains of the urea transporter, overall stoichiometric variation is selectively localized in fast evolving regions (**Figure 4**), where it accounts for a 5–23% statistically significant (one-sided Wilcoxon rank sum test) decrease in nitrogen content in open ocean communities. In conserved regions nitrogen content variation ranges instead between 2 and 5% (**Figure 4**.) We show a signature of nitrogen conservation in variable regions of the ammonium transporter (amt1) and in two subunits (urtB and urtC) of the ABC transporter permease proteins associated with transport of urea (Valladares et al., 2002) (**Figure 4**), while the urtD and urtE subunits do not show the same signal. Furthermore, a similar signal is detectable in the glutamine synthetase (glnA) (**Figure 4**), a key player in the condensation of inorganic nitrogen into amino acids, directly up-regulated by NtcA (Schwarz and Forchhammer, 2005). As expected, such patterns of parsimonious nitrogen allocation are not detectable along the arginine biosynthetic pathway, typically down-regulated during nitrogen scarcity (**Figure 4**), and used here as a metabolic control to the NtcA-induced pathway.

DISCUSSION

In microorganisms, membrane proteins are key players in the interaction between cells and their environment. Consistent with this fact, it has been shown that across marine environments membrane protein content co-varies substantially with oceanographic variables including nutrient concentration and pollution levels (Patel et al., 2010). Furthermore, due to the strong structural constraints that allow their insertion in cellular membranes, membrane proteins harbor a high percentage of hydrophobic residues, resulting in an overall low nitrogen content. Therefore, the possibility exists that the variation in the relative amount of membrane proteins alone could be a factor driving differences in the nitrogen allocation in the proteomes of natural communities adapted to open ocean and coastal environments. In order to address this potentially confounding factor we have analyzed membrane and non-membrane proteins separately, and shown a decreased nitrogen allocation in open ocean communities in both functional groups (**Figure 1**). The differences between the two environments survive and increase when addressing the role of protein structural constraints in shaping the patterns observed. Intracellular, transmembrane, and extracellular membrane protein domains separately show a statistically significant (one-sided Wilcoxon rank sum test) difference of 1–7% in nitrogen content between the two environments (**Figure 2**). This signal is particularly relevant when considering that proteins are the most nitrogen-rich component of cells, and that they constitute about 40% of the cellular dry mass (Sterner and Elser, 2002).

If evolutionary pressures for strategic nitrogen investment were a biologically relevant force able to significantly affect the cellular nitrogen budget, it is expected that they preferentially impact the stoichiometry of highly expressed proteins, especially in response to nitrogen-specific nutritional stress. Previous work on few model organisms supports this prediction, indicating that the catabolic machinery shows substantially lower N content than the anabolic machinery (Acquisti et al., 2009b). However, an understanding of the specific target of selection for parsimonious resource allocation in the genetic material of natural communities is still missing. In a regime of efficient material costs allocation, natural selection for parsimonious

nitrogen usage is expected to act in synergy with other evolutionary forces, such as those that preserve the functional and structural integrity of proteins. In this case, the most effective signature of "nutrient-allocation driven" selection is expected to be located in the protein regions that are less functionally constrained.

In order to validate these predictions, we have employed a highly reliable functional prediction approach to identify proteins up- and down-regulated in response to severe nitrogen limitation in the metagenomics sequences analyzed. Furthermore, we have addressed the role of functional constraints as an alternative explanation for the pattern of nitrogen allocation observed. In the best tradition of molecular evolution (Kimura, 1983), we have used evolutionary rates as a proxy for the level of functional constraints in each protein residue. This approach has enabled us to study the variation in nitrogen allocation between natural communities adapted to open ocean and to coastal regions in more stringent evolutionary context. Finally, we have used a "metabolic control" to test the specificity of the patterns of nitrogen limitation along the NtcA-induced cascade. This was done by focusing on arginine biosynthesis, one of the key cellular routes activated by photosynthetic species to store nitrogen during nitrogen abundance. An adaptive response that shapes nitrogen material costs in proteins predicts that the NtcA-mediated pathway and the arginine anabolic pathway will show contrasting patterns of nitrogen allocation in open ocean communities. Indeed, we see that decreased nitrogen allocation is selectively localized in the functionally least constrained regions of the NtcA-induced metabolic apparatus up-regulated in response to nitrogen starvation, while it does not affect the Arg genes (**Figures 3**, **4**). While the environmental data analyzed are not well suited to assess the phylogeny of different metabolic networks, it is worth to note that NtcA is known to be ubiquitous in Cyanobacteria (Frias et al., 1993). Similarly, based on KEGG database[2], ArgE is present in the genome of marine model species in the genus *Synechococcus*, *Synechocystis*, and *Prochlorococcus*.

The work presented addresses the role of material costs of evolutionary change in shaping protein evolution in natural communities, taking into account the role of functional constraints. The results confirm previous observations (Grzymski and Dussaq, 2012) and indicate that the history of nitrogen availability has directly constrained the molecular architecture of the metabolic apparatus that enable cells to respond to this ecologically relevant environmental cue. Further analyses of environmental samples will be paramount in shedding further light on the mode and tempo of natural selection for parsimonious nutrient allocation in shaping the dynamics of genome evolution in natural communities.

[2]https://www.genome.jp/kegg/

AUTHOR CONTRIBUTIONS

CA designed the study, analyzed the data, and wrote the manuscript. HD performed the analyses and contributed to the writing of the manuscript. NO performed the analyses.

FUNDING

This work has been financially supported through a grant to CA by the Volkswagen Foundation (Grant number I/85373).

ACKNOWLEDGMENTS

We thank Jörn Scharsack, Tabea Höhmann, Florian Wünnemann, Andrew Moore, John Vollmers, Wolfgang Riss, Harald Strauss, and Thomas Wiehe for scientific discussions, and Robert Fürst for technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01590/full#supplementary-material

TABLE S1 | List of the sampling locations from the global ocean sampling metagenome.

TABLE S2 | Sequences analysed in **Figure 4**: TIGRfam gene Ids, and counts.

REFERENCES

Acquisti, C., Elser, J. J., and Kumar, S. (2009a). Ecological nitrogen limitation shapes the DNA composition of plant genomes. *Mol. Biol. Evol.* 26, 953–956. doi: 10.1093/molbev/msp038

Acquisti, C., Kumar, S., and Elser, J. J. (2009b). Signatures of nitrogen limitation in the elemental composition of the proteins involved in the metabolic apparatus. *Proc. Biol. Sci.* 27, 2605–2610. doi: 10.1098/rspb.2008.1960

Ashkenazy, H., Erez, E., Martz, E., Pupko, T., and Ben-Tal, N. (2010). ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res.* 38, 529–533. doi: 10.1093/nar/gkq399

Baudouin-Cornu, P., Schuerer, K., Marlière, P., and Thomas, D. (2004). Intimate evolution of proteins proteome atomic content correlates with genome base composition. *J. Biol. Chem.* 279, 5421–5428. doi: 10.1074/jbc.M306415200

Beckers, G., Bendt, A. K., Krämer, R., and Burkovski, A. (2004). Molecular identification of the urea uptake system and transcriptional analysis of urea transporter- and urease-encoding genes in *Corynebacterium glutamicum*. *J. Bacteriol.* 186, 7645–7652. doi: 10.1128/JB.186.22.7645-7652.2004

Berg, J. M., Tymoczko, J. L., and Stryer, L. (2002). *Biochemistry*. New York, NY: W.H. Freeman.

Bragg, J. G., and Hyder, C. L. (2004). Nitrogen versus carbon use in prokaryotic genomes and proteomes. *Proc. Biol. Sci.* 271, S374–S377. doi: 10.1098/rsbl.2004.0193

Bragg, J. G., Thomas, D., and Baudouin-Cornu, P. (2006). Variation among species in proteomic sulphur content is related to environmental conditions. *Proc. Biol. Sci.* 273, 1293–1300. doi: 10.1098/rspb.2005.3441

Bragg, J. G., and Wagner, A. (2007). Protein carbon content evolves in response to carbon availability and may influence the fate of duplicated genes. *Proc. Biol. Sci.* 274, 1063–1070. doi: 10.1098/rspb.2006.0290

Bragg, J. G., and Wagner, A. (2009). Protein material costs: single atoms can make an evolutionary difference. *Trends Genet.* 25, 5–8. doi: 10.1016/j.tig.2008.10.007

Capone, D. G., and Hutchins, D. A. (2013). Microbial biogeochemistry of coastal upwelling regimes in a changing ocean. *Nat. Geosci.* 6, 711–717. doi: 10.1038/ngeo1916

Celniker, G., Nimrod, G., Ashkenazy, H., Glaser, F., Martz, E., Mayrose, I., et al. (2013). ConSurf: using evolutionary data to raise testable hypotheses about protein function. *Isr. J. Chem.* 53, 199–206. doi: 10.1002/ijch.201200096

Cunin, R., Glansdorff, N., Pierard, A., and Stalon, V. (1986). Biosynthesis and metabolism of arginine in bacteria. *Microbiol. Rev.* 50, 314–352.

Eddy, S. R. (2011). Accelerated profile HMM searches. *PLoS Comput. Biol.* 7:e1002195. doi: 10.1371/journal.pcbi.1002195

Elser, J. J., Acquisti, C., and Kumar, S. (2011). Stoichiogenomics: the evolutionary ecology of macromolecular elemental composition. *Trends Ecol. Evol.* 26, 38–44. doi: 10.1016/j.tree.2010.10.006

Elser, J. J., Fagan, W. F., Subramanian, S., and Kumar, S. (2006). Signatures of ecological resource availability in the animal and plant proteomes. *Mol. Biol. Evol.* 23, 1946–1951. doi: 10.1093/molbev/msl068

Frias, J. E., Merida, A., Herrero, A., Martin-Nieto, J., and Flores, E. (1993). General distribution of the nitrogen control gene ntcA in cyanobacteria. *J. Bacteriol.* 175, 5710–5713. doi: 10.1128/jb.175.17.5710-5713.1993

García-Domínguez, M., Reyes, J. C., and Florencio, F. J. (2000). NtcA represses transcription of gifA and gifB, genes that encde inhibitors of glutamine synthetase type I from *Synechocystis* sp. PCC6803. *Mol. Microbiol.* 35, 1192–1201. doi: 10.1046/j.1365-2958.2000.01789.x

Gilbert, J. D., and Fagan, W. F. (2011). Contrasting mechanisms of proteomic nitrogen thrift in *Prochlorococcus*. *Mol. Ecol.* 20, 92–104. doi: 10.1111/j.1365-294X.2010.04914.x

Graur, D. (1985). Amino acid composition and the evolutionary rates of protein-coding genes. *J. Mol. Evol.* 22, 53–62. doi: 10.1007/BF02105805

Grzymski, J. J., and Dussaq, A. M. (2012). The significance of nitrogen cost minimization in proteomes of marine microorganisms. *ISME J.* 6, 71–80. doi: 10.1038/ismej.2011.72

von Heijne, G. (1992). Membrane protein structure prediction. *J. Mol. Biol.* 225, 487–494. doi: 10.1016/0022-2836(92)90934-C

Käll, L., Krogh, A., and Sonnhammer, E. L. L. (2004). A combined transmembrane topology and signal peptide prediction method. *J. Mol. Biol.* 338, 1027–1036. doi: 10.1016/j.jmb.2004.03.016

Kimura, M. (1983). The neutral theory of molecular evolution. Cambridge: Cambridge University Press. doi: 10.1017/CBO9780511623486

Llácer, L. J., Fita., I., and Rubio, V. (2008). Arginine and nitrogen storage. *Curr. Opin. Struct. Biol.* 8, 673–681. doi: 10.1016/j.sbi.2008.11.002

Patel, P. V., Gianoulis, T. A., Bjornson, R. D., Yip, K. Y., Engelman, D. M., Gerstein, M. B., et al. (2010). Analysis of membrane proteins in metagenomics: networks of correlated environmental features and protein families. *Genome Res.* 20, 960–971. doi: 10.1101/gr.102814.109

Paz-Yepes, J., Merino-Puerto, V., Herrero, A., and Flores, E. (2008). The amt gene cluster of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 190, 6534–6539. doi: 10.1128/JB.00613-08

Read, R. W., Berube, P. M., Biller, S. J., Neveux, I., Cubillos-Ruiz, A., Chisholm, S. W., et al. (2017). Nitrogen cost minimization is promoted by structural changes in the transcriptome of N-deprived *Prochlorococcus* cells. *ISME J.* 11, 2267–2278. doi: 10.1038/ismej.2017.88

Rusch, D. B., Halpern, A. L., and Sutton, G. et al. (2007). The sorcerer II global ocean sampling expedition: northwest Atlantic through eastern tropical Pacific. *PLoS Biol.* 5:e77. doi: 10.1371/journal.pbio.0050077

Schwarz, R., and Forchhammer, K. (2005). Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. *Microbiology* 151, 2503–2514. doi: 10.1099/mic.0.27883-0

Selengut, J. D., Haft, D. H., and Davidsen, T., et al. (2007). TIGRFAMs and genome properties: tools for the assignment of molecular function and biological process in prokaryotic genomes. *Nucleic Acids Res.* 35, D260–D264. doi: 10.1093/nar/gkl1043

Sterner, R., and Elser, J. J. (2002). *Ecological Stoichiometry*. Princeton, NJ: Princeton University Press.

Sun, S., Chen, J., and Li, W., et al. (2011). Community cyberinfrastructure for advanced microbial ecology research and analysis: the CAMERA resource. *Nucleic Acids Res.* 39, D546–D551. doi: 10.1093/nar/gkq1102

Tanigawa, R., Shirokane, M., Maeda Si, S., Omata, T., Tanaka, K., and Takahashi, H. (2002). Transcriptional activation of NtcA-dependent promoters of *Synechococcus* sp. PCC 7942 by 2-oxoglutarate in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 99, 4251–4255. doi: 10.1073/pnas.072587199

Tourasse, N. J., and Li, W. H. (2000). Selective constraints, amino acid composition, and the rate of protein evolution. *Mol. Biol. Evol.* 17, 656–664. doi: 10.1093/oxfordjournals.molbev.a026344

Valladares, A., Montesinos, M. L., Herrero, A., and Flores, E. (2002). An ABC-type, high-affinity urea permease identified in cyanobacteria. *Mol. Microbiol.* 43, 703–715. doi: 10.1046/j.1365-2958.2002.02778.x

Yooseph, S., Sutton, G., Rusch, DB., Halpern, AL., Williamson, SJ., Remington, K., et al. (2007). The sorcerer II global ocean sampling expedition: expanding the universe of protein families. *PLoS Biol.* 5:e16. doi: 10.1371/journal.pbio.0050016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 11 January 2018
doi: 10.3389/fevo.2017.00173

Evolutionary Pathway Determines the Stoichiometric Response of *Escherichia coli* Adapted to High Temperature

Krista A. Linzner[1], *Alyssa G. Kent*[1]* *and Adam C. Martiny*[1,2]

[1] Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA, United States, [2] Department of Earth System Science, University of California, Irvine, Irvine, CA, United States

Microorganisms exhibit shifts in elemental stoichiometry in response to short-term temperature increases due to varying growth rate, biochemical reactions, and protein degradation. Yet, it is unknown how an organism's elemental stoichiometry will respond to temperature change on evolutionary timescales. Here we ask how cellular elemental stoichiometry and physiology change in *Escherichia coli* that have adapted to high temperature over 2,000 generations compared to their low temperature adapted ancestor. Cell lines evolved to a temperature of 42.2°C via two, negatively epistatic adaptive pathways leading to mutations in either the RNA polymerase complex or the termination factor *rho*. Compared to the ancestor, high temperature adapted cell lines overall had 14% higher N:P ratios, but did not differ significantly in C:N or C:P. However, cell lines with mutations in the *rho* gene had 13% lower C:N and 34% higher N:P. Furthermore, the two adaptive strategies of the *rho* and RNA polymerase mutations varied significantly from one another, cell lines with the *rho* mutation had lower C:N, higher N:P and higher protein content compared to cell lines with the RNA polymerase mutation. Thus, specific adaptive pathways modulate the effect of temperature on the cellular elemental stoichiometry and may explain why the elemental composition of specific lineages is differentially affected by temperature changes.

Keywords: stoichiometry, experimental evolution, adaptation, temperature, *Escherichia coli*

OPEN ACCESS

Edited by:
Patrick S. Fitze, Museo Nacional de Ciencias Naturales (CSIC), Spain

Reviewed by:
Bopi Biddanda, Grand Valley State University, United States
Olivier A. Tenaillon, Institut National de la Santé et de la Recherche Médicale, France

***Correspondence:**
Alyssa G. Kent
agkent@uci.edu

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *01 March 2017*
Accepted: *22 December 2017*
Published: *11 January 2018*

Citation:
Linzner KA, Kent AG and Martiny AC (2018) Evolutionary Pathway Determines the Stoichiometric Response of Escherichia coli Adapted to High Temperature. Front. Ecol. Evol. 5:173. doi: 10.3389/fevo.2017.00173

INTRODUCTION

The possibility of stoichiometric changes, or changes in cellular elemental composition, has important implications for understanding how evolving organisms will respond to anthropogenic change (Arrigo, 2005; Dijkstra et al., 2012). Microorganisms, especially, present an interesting group capable of responding through natural selection to environmental change on ecological timescales due to their short generation times and generally large populations (Lenski et al., 1991). Prokaryotic life has adapted to a vast range of different temperatures over the course of evolutionary history (Price and Sowers, 2004). While various microorganisms are adapted to different ranges of temperatures, many biological and biochemical processes are directly impacted by temperature and are disrupted when temperatures change extensively (Burra et al., 2010). To deal with short-term changes, *Escherichia coli* is capable of quickly modifying gene expression with a heat shock response when thermally stressed (Arsene et al., 2000). Moreover, studies have shown that microorganisms

are capable of evolving to environmental factors such as temperature, pH, etc. in a relatively rapid period of time (Bennett et al., 1990; Hughes et al., 2007). The evolutionary microbial response to temperatures above their growth optimum includes shifts in gene expression, mutations in transcription, and translation-related genes, maintaining increased heat shock proteins in the cell, etc. (Mongold et al., 1996; Riehle et al., 2003; Rudolph et al., 2010). Adaptive changes in macromolecular content and overall resource allocation can drive shifts in cellular stoichiometry (Sterner and Elser, 2002). Thus, changes in temperature can affect the elemental composition of bacteria through mechanisms of adaptation.

A previous hypothesis for the impact of temperature on stoichiometry predicts a lower phosphorus cell quota and higher carbon-to-phosphorus and nitrogen-to-phosphorus ratios for growth at elevated temperature (Toseland et al., 2013). Ribosomes and protein biosynthesis in bacteria are more efficient at elevated temperature (Broeze et al., 1978; VanBogelen and Neidhardt, 1990). This improved efficiency can result in in a lower requirement for P-rich ribosomes to maintain a specific biosynthesis rate (Toseland et al., 2013; Yvon-Durocher et al., 2015). Assuming the same growth rate, this should lead to higher C:P and N:P in warming environments. This theory applies to both acclimation (i.e., cells growing across a temperature gradient) and adaptation (cells adapted to different temperature levels) (Moreno and Martiny, 2018). Accordingly, *E. coli* grown at higher temperatures show higher levels of cellular C and N and decreased P, yielding these predicted results (Cotner et al., 2006). However, there are a multitude of ways that microbes adapt to increased temperature (Mongold et al., 1996; Riehle et al., 2003; Rudolph et al., 2010). Several bacterial strains, including psychrophiles, show variation in biomass stoichiometry independent of phylogenetic relatedness, suggesting small genetic differences can yield variable stoichiometry (Zimmerman et al., 2014). Moreover, *Prochlorococcus* ecotypes with different temperature optima (Johnson et al., 2006) are variable in their elemental ratios at the strain level (Martiny et al., 2016). Thus, it is still unclear if there is a consistent relationship between adaptation to temperature and how microbes alter their allocation of chemical resources.

There are two broad conceptual models for how adaptation might affect cellular biochemistry and stoichiometry: adaptations could restore the organism to it normal pre-stressed physiological state or acclimation phenotypes could be reinforced by adaptations (Hug and Gaut, 2015). Physiologically in *E. coli*, protein degradation as well as peptide elongation rates increase at high temperature, resulting in a higher turnover of peptides (Farewell and Neidhardt, 1998). However, their genome contains a number of heat inducible genes, encoding for chaperone proteins and proteases, which prevent aggregation of proteins at high temperature and refold denatured proteins (Arsene et al., 2000). While the heat-shock response of *E. coli* to short-term changes in temperature has been well-studied, recent research has also emerged concerning the long-term evolutionary response of microbes to high temperature. *E. coli* grown at 42.2°C had significant fitness gains after only 200 generations, compared to its ancestor (Bennett et al., 1990). An expanded experiment analyzed *E. coli* after being exposed to 42.2°C over 2,000 generations and subsequently sequenced a clonal genome from 115 adapted cell lines. Here, a total of 1,331 mutations were present (Tenaillon et al., 2012) and two of the most frequent mutations were within the *rho* and RNA polymerase β subunit *(rpo*B*)* genes. Out of the 115 cell lines sequenced, 17 lines had mutations in *rho* codon 15 and 18 had mutations in *rpo*B codon 966. Every cell line in the study was found to have either a mutation in the *rho* gene or the *rpo*BC operon. Additionally, mutations in these genes were in negative epistasis with one another, meaning mutations occurring in both genes at the same time happened less often than by random chance (Tenaillon et al., 2012). Nonetheless, there were some lines that did have mutations in both genes, although they did not include either of the most prevalent *rho* and *rpo*B mutations. Phenotypic differences between cells with *rho* and *rpo*B mutations were identified using Biolog plates (Hug and Gaut, 2015). The strongest differences between *rho* and *rpo*B lines were among chemical sensitivity, but also included differential amino acid usage, suggesting there may be differences in their resource requirements (Hug and Gaut, 2015). The gene *rpo*B encodes the β subunit of RNA polymerase and may increase the capacity of cell lines to adapt (Barrick et al., 2010). Rho is a ubiquitous prokaryotic termination factor that ends transcription, but also functions in maintaining chromosomal integrity in some circumstances (Washburn and Gottesman, 2011; Grylak-Mielnicka et al., 2016). Mutations in both of these transcriptional regulators have reduced susceptibility to antibiotics in other bacterial genera (Taniguchi et al., 1996; Lee and Helmann, 2014). While their broad cellular function may be a part of the reason for their adaptive role in new environments, the elemental resource allocation outcomes of these adaptations to growth at high temperature are currently unknown.

We propose two competing hypotheses for how cellular macromolecule content and C:N:P may shift in cell lines with adaptations to high temperature (**Figure 1**). The first is a compensation mechanism, which suggests cells produce a large amount of macromolecules (protein and ribosomes) to offset the heat related degradation (Farewell and Neidhardt, 1998). If cells compensate for degradation through mutations allowing the cell to elevate macromolecular levels, this would predict lower carbon-to-nutrient ratios (C:N and C:P) and an elevated N:P ratio in adapted lines due to high amounts of N-rich protein (**Figure 1**). Here a fitness tradeoff occurs because the increase in growth rate through production of additional ribosomes or other proteins will incur a heavy energetic cost. Alternatively, adaptation of enzymes and ribosomes may increase their efficiency at higher temperature leading to a lower need for proteins and ribosomes in the adapted lines compared to the ancestor at 42.2°C. This would lead to increased C:N and C:P ratios in high temperature adapted cells (**Figure 1**). A fitness cost occurs because the increased efficiency that comes from tuning an enzyme to a specific environment however, may reduce an organism's phenotypic plasticity.

To identify how adaptation to elevated temperature affects the elemental stoichiometry of heterotrophic bacteria, we analyzed

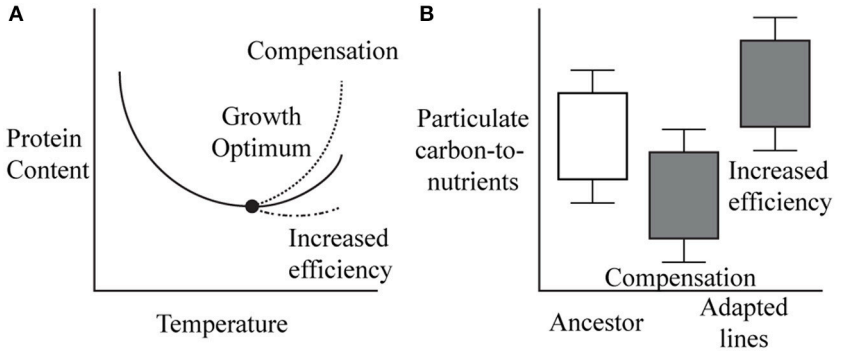

FIGURE 1 | (A) The compensation model predicts that cells will adapt to high temperature by producing a larger amount of protein and ribosome compared to the ancestor. **(B)** This would likely result in a depressed carbon to nutrients (nitrogen and phosphorus) ratio due to higher levels of nitrogen rich protein or phosphorus rich ribosomes. The increased efficiency model predicts macromolecule content to be lower in adapted cell lines than in the ancestor, due to adaptations that allow cells to better maintain proteins at a higher temperature or increase the efficiency of ribosomes. This would correspond to an increased carbon to nutrients (nitrogen and phosphorus) ratio.

E. coli lines experimentally adapted to 42.2°C (Tenaillon et al., 2012). We first ask if lines adapted to higher temperature have different C:N, C:P, and N:P ratios compared to their ancestral lineage. Second, we ask whether changes in the C:N:P ratio correspond to changes in total cellular protein content as changes in protein levels could alter cellular nitrogen. Third, we ask if different genetic pathways (*rpo*B vs. *rho*) for adaption to high temperature lead to differences in C:N:P ratio and protein content.

MATERIALS AND METHODS

Lines and Experimental Design

A lab adapted *E. coli* strain REL1206 and eight lines adapted from this common ancestor at 42.2°C were obtained from Dr. Brandon Gaut's Lab (Tenaillon et al., 2012) (Supplementary Tables 1, 2). Lines 5, 7, and 70 had mutations in both *rpo*B and *rho* genes, lines 75 and 135 had the prevalent mutation in *rpo*B and lines 20, 60, and 73 had the prevalent mutation in *rho* (Tenaillon et al., 2012). All adapted cell lines had additional background mutations, some of which were shared across lines, for example the cation/proton antiporter (ybaL) or a cardiolipin synthase (cls) were found in 5 of the 9 assayed lines (Supplementary Table 3). All cell lines, including the ancestor, were frozen from single colony cultures at −80°C storage then revived in lysogeny broth for 24 h at 37°C to acclimate cells to culture conditions. Next, they were transferred into a modified Davis Mingioli (MDM) media with a C:N:P ratio of 220:16:1 to yield a higher density of cells, for 24 h at 37°C. Finally, they were transferred into MDM media for 48 h at 42.2°C, the same temperature at which they were adapted for 2,000 generations, The length of time for growth after the second MDM transfer was increased to 48 instead of 24 h to reach higher density. It is possible that mutations may have arisen during the course of this experiment. Several biological replicates were used to reduce this noise. With separate populations new mutations will have a smaller effect on the overall signal.

Cellular Composition Analyses

After reaching sufficient population density of around 10^7 cells/mL, 5 mL of bacterial culture were filtered onto pre-combusted 25 mm glass fiber filters and rinsed with 5 mL of 0.17 M anhydrous Na_2SO_4 for particulate organic phosphorus (POP) and particulate organic carbon (POC) plus particulate organic nitrogen (PON) assays. Culture subsamples were preserved in glutaraldehyde and frozen for flow cytometry analysis at the time of filtration. Cells were stained with SYBR green and read at a wavelength of 488 nm with a BD Accuri C6 flow cytometer for cell counts.

Particulate organic phosphorus was determined in each sample using a modified ash-hydrolysis method (Lomas et al., 2010). Filters were placed in glass vials with 2 mL 0.017 M $MgSO_4$ and dried at 80–90°C overnight. Vials were transferred to a muffle furnace and combusted for 2 h at 500°C. After cooling, 5 mL of 0.2 M HCl were added and samples were heated at 80°C for 30 min. A mixed reagent of 2:5:1:2 ratio of 0.024 M $(NH_4)_6Mo_7O_{24}$, 5N H_2SO_4, 0.002 M $K_2Sb_2(C_4H_2O_6)_2{\cdot}3\ H_2O$, and 0.3 M $C_6H_8O_6$ was added to samples. After 30 min the samples were read in a spectrophotometer at a wavelength of 885 nm. A standard curve of KH_2PO_4 was used to calculate the concentration of phosphate in samples based on their absorbance.

Particulate organic carbon and particulate organic nitrogen assays were dried overnight and packed into tin capsules. Standards were created by packing pre-weighed quantities of atropine and peach leaf into capsules. POC and PON concentrations were determined using a FlashEA1112 nitrogen and carbon analyzer (Sharp, 1974).

Protein content was quantified using a Coomassie (Bradford) assay kit with bovine serum albumin standards (Bradford, 1976). One milliliter culture samples were aliquoted into microtubes with 0.2 mm glass beads and beat in a bead-beater for 5 min to disrupt cell membranes. These tubes were spun in a centrifuge and the supernatant was collected. Coomassie dye was added to bovine serum albumin standards and samples in a microplate. The microplate was read at 595 nm in a plate reader and

protein concentration was calculated based on standards and absorbance.

Statistical Analyses

Carbon to nitrogen, nitrogen to phosphate, and carbon to phosphate ratios were calculated from POC, PON, and POP results. Outliers were eliminated using Tukey's method and the average of two technical replicates for each sample was calculated. Data were square-root transformed before all statistical analyses. A Welch's *t*-test was performed to determine the differences in mean C:P, C:N, and N:P ratios and protein content between the ancestral line and all adapted lines. Levene's test was performed to determine homogeneity of variances. A single factor ANOVA test was used to analyze the differences in mean C:P, C:N, and N:P ratios and protein content between groups of cells sharing the same mutation and between cell lines. *Post-hoc* analysis

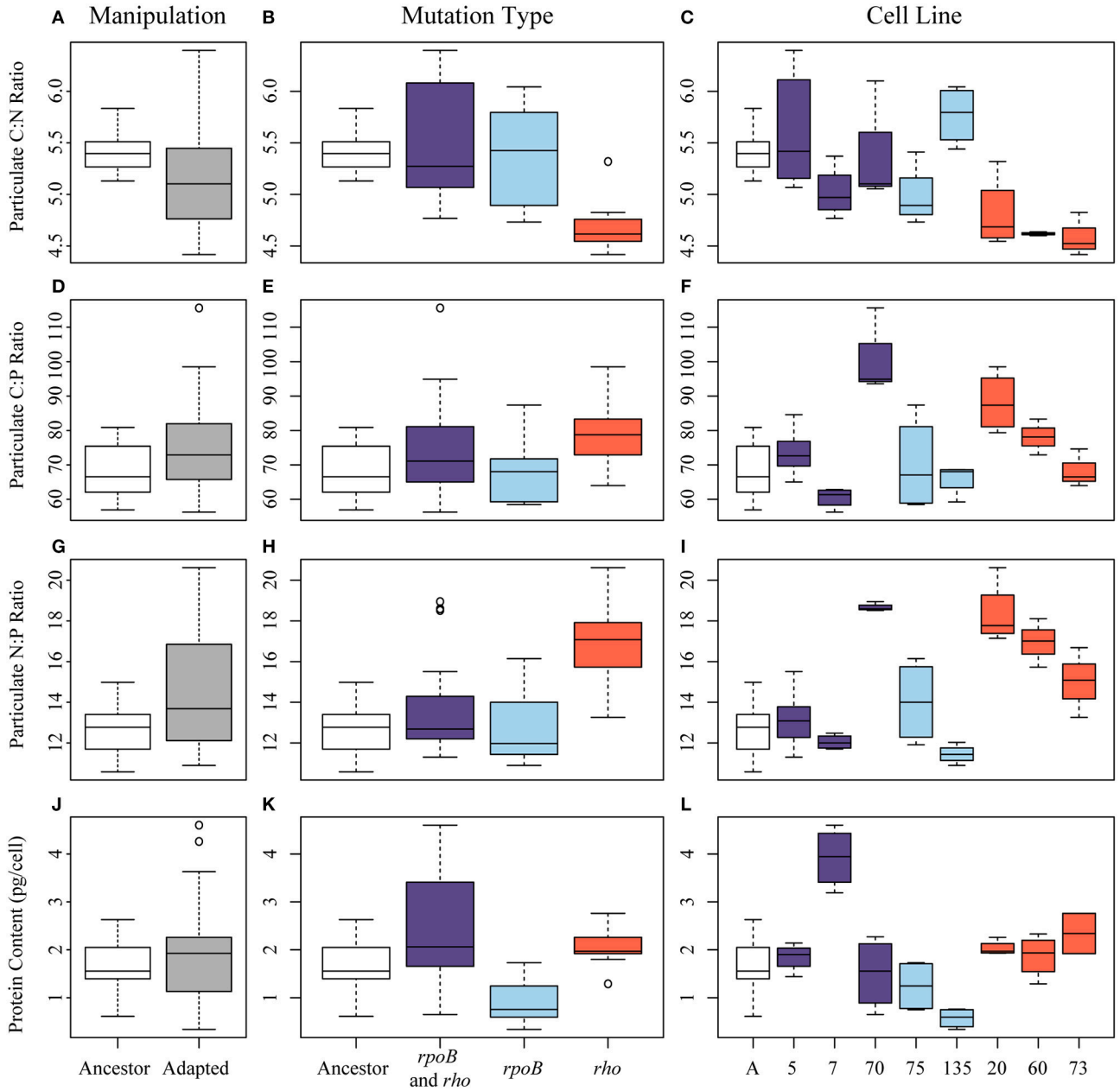

FIGURE 2 | Elemental ratios and protein content of high temperature adapted *E. coli*. Boxplots representing the median value and upper and lower quartiles for particulate C:N of **(A)** the ancestor (white) and all adapted cell lines (gray); **(B)** by the mutation type either the ancestor, having both *rho* and *rpoB*, only *rpoB*, or only *rho* mutations; **(C)** or by line and colored by mutation type (white-ancestor, purple-both *rpoB* and *rho* mutations, light blue-*rpoB* mutation, red-*rho* mutation. Similarly for particulate C:P ratios in **(D–F)**, particulate N:P ratios in **(G–I),** and protein content measured as picograms/cell in **(J–L)**.

was performed using a Tukey's HSD test to analyze the relative contributions of each pair of means to the overall variation. The Pearson product-moment correlation coefficient was calculated for the protein content vs. C:N, N:P, and C:P ratios of individual lines and to compare epimutations with the *rho* and *rpo*B mutations. All analyses were done in R (R Development Core Team, 2016).

RESULTS

To identify the elemental outcome of adaptation to high temperature, we compared the C:N:P of the ancestral and adapted *E. coli* lines. We found that adaptation to high temperature did not have a single consistent effect on C:N, C:P, and N:P ratios (**Figure 2**). Although the C:N ratio of the adapted lines was slightly lower than the ancestral line (5.21 vs. 5.41), it was not significantly different (Welch's *t*-test, **Table 1**). The average N:P ratio of the adapted lines was significantly higher than the ancestor (14.48 vs. 12.65, Welch's *t*-test, **Table 1**). C:N variance was significantly different between ancestor and adapted lines and N:P variance was marginally but not significantly different (Levene's test, **Table 1**). To determine whether or not the differences in stoichiometric ratios could be explained by differences in protein level, the average protein content per cell was compared. Protein content was slightly higher in adapted cell lines (1.89 compared to 1.62 pg/cell), although not significantly (Welch's *t*-test, **Table 1**). Protein content vs. C:N had the strongest relationship of the elemental ratios with a negative correlation ($r = -0.36$, **Table 2**), but was not significant.

The specific genetic pathways for adaptation to high temperature had a significant effect on the elemental stoichiometry of *E. coli* (ANOVA, **Table 3**). Cell lines with the *rpo*B mutation had C:N, N:P, and C:P ratios nearly identical to the ancestor (**Table 3**). In contrast, cell lines with the *rho* mutation had significantly decreased C:N (4.69 vs. 5.42, TukeyHSD, **Table 3**) and increased N:P (16.92 vs. 12.65, TukeyHSD, **Table 3**) compared to the ancestor. Cell lines with both the *rho* and *rpo*B mutations had average stoichiometric ratios with values in between the *rho* and *rpo*B lines, but did not differ significantly from the ancestor. Protein content varied significantly between the mutation groups (ANOVA, **Table 3**; **Figure 2**). Cell lines with the *rpo*B mutation had the lowest protein content (0.91 pg/cell), while cell lines with both mutations had the highest (2.42 pg/cell) (**Table 3**). The *rho* group had significantly higher average protein content compared to the *rpo*B group (2.03 vs. 0.91 pg/cell, TukeyHSD, **Table 3**) and *rho* protein content was also higher than the ancestor, but not significantly higher (**Table 3**).

There was substantial variation in stoichiometric ratios among cell lines with the same mutation type. While cell lines with the *rho* mutation were consistent in the direction in which they varied from the ancestor, cell lines with the *rpo*B mutation or both mutations were sometimes higher than the ancestor and sometimes lower (**Figures 2C,F,I,L**). Thus, when pooled by mutation, those cell lines had average ratios similar to the ancestor. All lines have several additional mutations, 9 of which were shared between at least two lines (Supplementary Table 3). Some mutations were significantly associated with *rho* or *rpo*B mutations, e.g., line 75 and 135 both have mutations in *yif*B, a gene encoding a magnesium chelatase family protein, while other epimutations were less specific (Supplementary Table 4). Thus, the specific underlying genetic makeup of adaptation to high temperature can influence the cellular elemental ratios.

TABLE 1 | Differences between ancestral vs. adapted lines for elemental ratios and protein content.

	C:N	C:P	N:P	Protein (pg/cell)
MEAN VALUE(STANDARD DEVIATION)				
Ancestor lines ($n = 7$)	5.42 (0.24)	68.48 (9.18)	12.65 (1.46)	1.62 (0.60)
Adapted lines ($n = 35$)	5.21 (0.56)	74.79 (13.13)	14.48 (2.73)	1.89 (1.04)
WELCH'S *T*-TEST				
P-value	0.11	0.17	0.029	0.47
T-value	1.66	−1.47	−2.43	0.73
Degrees of freedom	21.59	10.72	14.32	30.69
LEVENE TEST FOR HOMOGENEITY OF VARIANCES				
P-value	0.039	0.48	0.069	0.24
F-value	4.55	0.52	3.50	1.45
Degrees of freedom	1	1	1	1

Elemental ratios of C:N, C:P, and N:P and the cellular protein content measured in picograms of protein/cell in the ancestral lines and the adapted lines in aggregate. A Welch's t-test and Levene test for homogeneity of variances were performed on square-root transformed data.

TABLE 2 | Relationship between elemental ratios and protein content.

	C:N vs. Protein	C:P vs. Protein	N:P vs. Protein
Correlation coefficient r	−0.36	−0.08	0.08
P-value	0.34	0.84	0.84
T statistic	−1.025	−0.20	0.21
Degrees of freedom	7	7	7

Pearson correlation of elemental ratios C:N, C:P, and N:P with protein content (pg protein/cell).

DISCUSSION

Overall, there was strong variation in the complex phenotype of cellular stoichiometry between the high temperature adapted cell lines derived from a common ancestor. Thus, this study quantifies how the specific adaptive pathways underlying adaptation to high temperature are important for the stoichiometric outcome of the cell. Cells may adapt in many ways to increased temperature (Mongold et al., 1996; Riehle et al., 2003; Rudolph et al., 2010). Thus, adaptive mechanisms (i.e., compensation for degradation or increased macromolecular efficiency) may introduce unique changes in

TABLE 3 | Differences between mutational types for elemental ratios and protein content.

	C:N	C:P	N:P	Protein (pg/cell)
MEAN VALUE (STANDARD DEVIATION)				
Ancestor ($n = 7$)	5.42 (0.24)	68.48 (9.18)	12.65 (1.46)	($n = 12$) 1.62 (0.60)
Both *rho* and *rpoB* ($n = 17$)	5.44 (0.52)	75.41 (15.00)	13.87 (2.53)	($n = 12$) 2.42 (1.24)
rpoB ($n = 8$)	5.38 (0.50)	67.98 (9.76)	12.73 (1.94)	($n = 8$) 0.91 (0.52)
rho ($n = 10$)	4.69 (0.25)	79.20 (10.64)	16.92 (1.97)	($n = 10$) 2.03 (0.38)
ANOVA				
Degrees of freedom	3	3	3	3
Sum of squares	0.20	2.68	1.87	1.85
Mean squares	0.07	0.89	0.62	0.62
F-value	8.02	1.849	7.97	7.48
P-value	2.91e-4	0.16	3.04e-4	4.71e-4
***POST-HOC* TUKEY TEST: DIFFERENCE (*P*-VALUE)**				
Ancestor-Both	−0.03 (1.00)	−6.93 (0.61)	−1.22 (0.59)	−0.81 (0.13)
Ancestor-*rpoB*	0.04 (1.00)	−0.49 (1.00)	−0.09 (1.00)	0.71 (0.076)
Ancestor-*rho*	0.73 (4.8e-3)	−10.72 (0.28)	−4.27(1.4e-3)	−0.42 (0.51)
Both-*rpoB*	0.07 (0.98)	7.42 (0.50)	1.14 (0.59)	1.52 (3.6e-4)
Both-*rho*	0.76 (2.8e-4)	−3.79 (0.83)	−3.05 (5.4e-3)	0.39 (0.88)
rpoB-rho	0.69 (6.3e-3)	−11.22 (0.21)	−4.18 (1.0e-3)	−1.12 (4.1e-3)

Average elemental ratios of C:N, C:P, N:P in the ancestor, both rho and rpoB mutations, rpoB mutation, and rho mutation and protein content measured in picograms of protein/cell for the ancestor, both, rpoB, and rho. Mean value shown with standard deviation and number of replicates shown for elemental and protein analyses. Differences of the average cellular stoichiometric ratios and protein content among mutational groups: ancestor, both rho and rpoB mutations, rpoB mutation, and rho mutation. Results from initial ANOVA and a post-hoc TukeyHSD analysis of square-root transformed data were performed and adjusted p-values are in parentheses.

cellular stoichiometry. This work supports the idea that *rho* cell lines may compensate for the lower efficiency of proteins by accumulating a higher protein concentration than the ancestor and the *rpo*B type. There is a strong relationship between cellular nitrogen and protein content (Jones, 1941; Mariotti et al., 2008). Thus, a shift in protein concentration is likely the underlying cause for how the C:N and N:P ratio changes as a response to adaptation to high temperature in *rho* lines. The increase of N:P in *rho* lines relative to the ancestor suggests both mechanisms could be operating simultaneously. In contrast, lines with the *rpo*B mutation had significantly lower cellular amounts of protein compared to *rho* and slightly lower than the ancestor, although not significantly different. Considering how the stoichiometric ratios of these cell lines were nearly identical to the ancestor, this result does not follow either of the two initial hypotheses. Additionally, cell lines with both the *rho* and *rpo*B mutation did not vary much from the ancestor in stoichiometric ratios nor protein content. It is therefore difficult to align them with either model. Moreover, it suggests that stoichiometry need not change as a consequence of temperature adaptation.

In other efforts to characterize these pathways, the fitness tradeoffs of growing at different temperatures were assessed. A niche shift was observed associated with mutations in *rpo*B, while a niche expansion was observed in lines with a mutation in *rho* (Rodríguez-Verdugo et al., 2014). The pleiotropic effects of mutations in *rpo*B vs. *rho* may explain some of the results in this study. RNA polymerase affects every single gene during its function while *rho* only affects the transcription termination of a subset of genes (20–50%) (Zhu and Von Hippel, 1998; Peters et al., 2009). If a *rpo*B mutation influences multiple processes, the net effect on stoichiometry may result in the similarity we see between these lines and the ancestor. Single amino acid changes within *rpo*B have caused significant gene expression shifts. Specifically, changes in codon 572 of *rpo*B restored gene expression back toward the ancestral pre-stress conditions (Rodríguez-Verdugo et al., 2016). This supports a "compensation" response and may underlie the limited stoichiometric differences between the ancestor and the *rpo*B mutation lines.

There is a strong fitness advantage of the evolved lines compared to the ancestor when grown at 42.2°C. The ancestor also displays a strong difference in gene expression when grown at its optimal temperature of 37°C compared to 42.2°C (Rodríguez-Verdugo et al., 2016). Moreover, gene expression of engineered lines carrying either the rho or *rpo*B mutations in isolation also display differential gene expression from one another and from the ancestor (González-González et al., 2017) also some of the *rpo*B mutations appeared to be heading toward a pre-stressed ancestral state (Rodríguez-Verdugo et al., 2016). Yet the fact that different mutation types display differential CNP suggests that there is more happening than simply growth restoration. Some of the difference may have to do with co-occurring mutations, e.g., cardiolipin synthetase (cls) association with rho or rod-shape genes, which affect cell shape, and their association with *rpo*B (Tenaillon et al., 2012). Size of the cell may directly impact the cell stoichiometry, as seen in marine bacteria (Garcia et al., 2016). These affiliated mutations may be affecting

the composition of cell membranes, for example, resulting in differential C:N:P.

Temperature influences both microbial community composition and physiology (Bennett et al., 1990; Zinser et al., 2007; Bradford et al., 2008), but we currently have a limited understanding of the stoichiometric outcomes of adaptation vs. acclimation to this environmental factor (Yvon-Durocher et al., 2015; Martiny et al., 2016). The work presented here uses a laboratory strain but demonstrates that unique evolutionary "biochemical outcomes" of short-term adaptation to a specific temperature regime can have a significant impact on the elemental composition. Microbes do experience sustained environmental changes, for example migration from one environment to another or movement from the external environment to mammalian intestines. Different shifts in environment will likely differ in the resource and stoichiometric mechanisms that are favored. Studies with other organisms are needed to evaluate if our observations apply more broadly to other microorganisms. Due to the broad effect of temperature on most, if not all, cellular processes, perhaps this variation in outcomes should be expected. It may further partly explain the extensive variation in how temperature affects closely related bacterial lineages (Martiny et al., 2016). Thus, there may be some overall trends in how different temperature conditions influence stoichiometry (Yvon-Durocher et al., 2015), but our data suggest that specific microbial lineages adapted to a particular temperature condition will likely have varying elemental stoichiometry. As such, there is not a uniform link between adaptation to elevated temperature and stoichiometry. Instead, the specific genetic and biochemical modifications underlying adaptation to a temperature regime need to be considered.

AUTHOR CONTRIBUTIONS

AK and AM were involved in the conception and design of the project. KL conducted, analyzed, and interpreted the results. All authors helped write and revise the manuscript and provided important intellectual content.

FUNDING

KL was supported by the Undergraduate Research Opportunities Program at the University of California, Irvine. AK was supported by the National Science Foundation-Graduate Research Fellowship Program (DGE-1321846) and the National Institute of Biomedical Imaging and Bioengineering, National Research Service Award EB009418 from the University of California, Irvine, Center for Complex Biological Systems. AM was supported by the National Science Foundation (OCE-0928544 and OCE-1046297).

ACKNOWLEDGMENTS

We thank Rich Puxty for assisting with flow cytometry, Brandon Gaut for many helpful comments on the manuscript, and other members of the Martiny lab for their support and encouragement.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2017.00173/full#supplementary-material

REFERENCES

Arrigo, K. R. (2005). Marine microorganisms and global nutrient cycles. *Nature* 437, 343–348. doi: 10.1038/nature04159

Arsene, F., Tomoyasu, T., and Bukau, B. (2000). The heat shock response of *Escherichia coli*. *Int. J. Food Microbiol.* 55, 3–9. doi: 10.1016/S0168-1605(00)00206-3

Barrick, J. E., Kauth, M. R., Strelioff, C. C., and Lenski, R. E. (2010). *Escherichia coli* rpoB mutants have increased evolvability in proportion to their fitness defects. *Mol. Biol. Evol.* 27, 1338–1347. doi: 10.1093/molbev/msq024

Bennett, A. F., Dao, K. M., and Lenski, R. E. (1990). Rapid evolution in response to high-temperature selection. *Nature* 346, 79–81. doi: 10.1038/346079a0

Bradford, M. A., Davies, C. A., Frey, S. D., Maddox, T. R., Melillo, J. M., Mohan, J. E., et al. (2008). Thermal adaptation of soil microbial respiration to elevated temperature. *Ecol. Lett.* 11, 1316–1327. doi: 10.1111/j.1461-0248.2008.01251.x

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Broeze, R. J., Solomon, C. J., and Pope, D. H. (1978). Effects of low temperature on *in vivo* and *in vitro* protein synthesis in *Escherichia coli* and *Pseudomonas fluorescens*. *J. Bacteriol.* 134, 861–874.

Burra, P. V., Kalmar, L., and Tompa, P. (2010). Reduction in structural disorder and functional complexity in the thermal adaptation of prokaryotes. *PLoS ONE* 5:e12069. doi: 10.1371/journal.pone.0012069

Cotner, J. B., Makino, W., and Biddanda, B. A. (2006). Temperature affects stoichiometry and biochemical composition of *Escherichia coli*. *Microb. Ecol.* 52, 26–33. doi: 10.1007/s00248-006-9040-1

Dijkstra, F. A., Pendall, E., Morgan, J. A., Blumenthal, D. M., Carrillo, Y., Lecain, D. R., et al. (2012). Climate change alters stoichiometry of phosphorus and nitrogen in a semiarid grassland. *New Phytol.* 196, 807–815. doi: 10.1111/j.1469-8137.2012.04349.x

Farewell, A., and Neidhardt, F. C. (1998). Effect of temperature on *in vivo* protein synthetic capacity in *Escherichia coli*. *J. Bacteriol.* 180, 4704–4710.

Garcia, N. S., Bonachela, J. A., and Martiny, A. C. (2016). Interactions between growth-dependent changes in cell size, nutrient supply and cellular elemental stoichiometry of marine *Synechococcus*. *ISME J.* 10, 2715–2724. doi: 10.1038/ismej.2016.50

González-González, A., Hug, S. M., Rodríguez-Verdugo, A., Patel, J. S., and Gaut, B. S. (2017). Adaptive mutations in RNA polymerase and the transcriptional terminator Rho have similar effects on *Escherichia coli* gene expression. *Mol. Biol. Evol.* 34, 2839–2855. doi: 10.1093/molbev/msx216

Grylak-Mielnicka, A., Bidnenko, V., Bardowski, J., and Bidnenko, E. (2016). Transcription termination factor Rho: a hub linking diverse physiological processes in bacteria. *Microbiology* 162, 433–447. doi: 10.1099/mic.0.000244

Hug, S. M., and Gaut, B. S. (2015). The phenotypic signature of adaptation to thermal stress in *Escherichia coli*. *BMC Evol. Biol.* 15:177. doi: 10.1186/s12862-015-0457-3

Hughes, B. S., Cullum, A. J., and Bennett, A. F. (2007). Evolutionary adaptation to environmental pH in experimental lineages of *Escherichia coli*. *Evolution* 61, 1725–1734. doi: 10.1111/j.1558-5646.2007.00139.x

Johnson, Z. I., Zinser, E. R., Coe, A., McNulty, N. P., Woodward, E. M. S., and Chisholm, S. W. (2006). Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. *Science* 311, 1737–1740. doi: 10.1126/science.1118052

Jones, D. B. (1941). Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins. *Circ. US Dep. Agric.* 183, 1–22.

Lee, Y. H., and Helmann, D. (2014). Mutations in the primary sigma factor σ A and termination factor Rho that reduce susceptibility to cell wall. *Antibiotics* 196, 3700–3711. doi: 10.1128/JB.02022-14

Lenski, R. E., Rose, M. R., Simpson, S. C., and Tadler, S. C. (1991). Long-term experimental evolution in *Escherichia coli*. I. adaptation and divergence during 2,000 generations. *Am. Nat.* 138, 1315–1341. doi: 10.1086/285289

Lomas, M. W., Burke, A. L., Lomas, D. A., Bell, D. W., Shen, C., Dyhrman, S. T., et al. (2010). Sargasso 321Sea phosphorus biogeochemistry: an important role for dissolved organic phosphorus (DOP). *Biogeosciences* 7, 695–710. doi: 10.5194/bg-7-695-2010

Mariotti, F., Tomé, D., and Mirand, P. P. (2008). Converting nitrogen into protein–beyond 6.25 and Jones' factors. *Crit. Rev. Food Sci. Nutr.* 48, 177–184. doi: 10.1080/10408390701279749

Martiny, A. C., Ma, L., Mouginot, C., Chandler, J. W., and Zinser, E. R. (2016). Interactions between thermal acclimation, growth rate, and phylogeny influence Prochlorococcus elemental stoichiometry. *PLoS ONE* 11:e0168291. doi: 10.1371/journal.pone.0168291

Mongold, J. A., Bennett, A. F., and Lenski, R. E. (1996). Evolutionary adaptation to temperature. IV. Adaptation of *Escherichia coli* at a niche boundary. *Evolution* 50, 35–43.

Moreno, A. R., and Martiny, A. C. (2018). Ecological stoichiometry of ocean plankton. *Annu. Rev. Mar. Sci.* 10, 1–27. doi: 10.1146/annurev-marine-121916-063126

Peters, J. M., Mooney, R. A., Kuan, P. F., Rowland, J. L., Keles, S., and Landick, R. (2009). Rho directs widespread termination of intragenic and stable RNA transcription. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15406–15411. doi: 10.1073/pnas.0903846106

Price, P. B., and Sowers, T. (2004). Temperature dependence of metabolic rates for microbial growth, maintenance, and survival. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4631–4636. doi: 10.1073/pnas.0400522101

R Development Core Team (2016). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org/

Riehle, M. M., Bennett, A. F., Lenski, R. E., and Long, A. D. (2003). Evolutionary changes in heat-inducible gene expression in lines of *Escherichia coli* adapted to high temperature. *Physiol. Genomics* 14, 47–58. doi: 10.1152/physiolgenomics.00034.2002

Rodríguez-Verdugo, A., Carrillo-Cisneros, D., Gonzalez-Gonzalez, A., Gaut, B. S., and Bennett, A. F. (2014). Different tradeoffs result from alternate genetic adaptations to a common environment. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12121–12126. doi: 10.1073/pnas.1406886111

Rodríguez-Verdugo, A., Tenaillon, O., and Gaut, B. S. (2016). First-Step mutations during adaptation restore the expression of hundreds of genes. *Mol. Biol. Evol.* 33, 25–39. doi: 10.1093/molbev/msv228

Rudolph, B., Gebendorfer, K. M., Buchner, J., and Winter, J. (2010). Evolution of *Escherichia coli* for growth at high temperatures. *J. Biol. Chem.* 285, 19029–19034. doi: 10.1074/jbc.M110.103374

Sharp, J. H. (1974). Improved analysis for "particulate" organic carbon and nitrogen from seawater. *Limnol. Oceangr.* 19, 984–989.

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: the Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Taniguchi, H., Aramaki, H., Nikaido, Y., Mizuguchi, Y., Nakamura, M., Koga, T., et al. (1996). Rifampicin resistance and mutation of the rpoB gene in Mycobacterium tuberculosis. *FEMS Microbiol. Lett.* 144, 103–108. doi: 10.1111/j.1574-6968.1996.tb08515.x

Tenaillon, O., Rodríguez-Verdugo, A., Gaut, R. L., McDonald, P., Bennett, A. F., Long, A. D., et al. (2012). The molecular diversity of adaptive convergence. *Science* 335, 457–461. doi: 10.1126/science.1212986

Toseland, A., Daines, S. J., Clark, J. R., Kirkham, A., Strauss, J., Uhlig, C., et al. (2013). The impact of temperature on marine phytoplankton resource allocation and metabolism. *Nat. Clim. Chang.* 3, 979–984. doi: 10.1038/nclimate1989

VanBogelen, R., a, and Neidhardt, F. C. (1990). Ribosomes as sensors of heat and cold shock in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5589–5593. doi: 10.1073/pnas.87.15.5589

Washburn, R. S., and Gottesman, M. E. (2011). Transcription termination maintains chromosome integrity. *Proc. Natl. Acad. Sci. U.S.A.* 108, 792–797. doi: 10.1073/pnas.1009564108

Yvon-Durocher, G., Dossena, M., Trimmer, M., Woodward, G., and Allen, A. P. (2015). Temperature and the biogeography of algal stoichiometry. *Glob. Ecol. Biogeogr.* 24, 562–570. doi: 10.1111/geb.12280

Zhu, A. Q., and Von Hippel, P. H. (1998). Rho-dependent termination within the trp t' terminator. I. Effects of Rho loading and template sequence. *Biochemistry* 37, 11202–11214.

Zimmerman, A. E., Allison, S. D., and Martiny, A. C. (2014). Phylogenetic constraints on elemental stoichiometry and resource allocation in heterotrophic marine bacteria. *Environ. Microbiol.* 16, 1398–1410. doi: 10.1111/1462-2920.12329

Zinser, E. R., Johnson, Z. I., Coe, A., Karaca, E., Veneziano, D., and Chisholm, S. W. (2007). Influence of light and temperature on *Prochlorococcus* ecotype distributions in the Atlantic Ocean. *Limnol. Oceanogr.* 52, 2205–2220. doi: 10.4319/lo.2007.52.5.2205

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 27 March 2018
doi: 10.3389/fmicb.2018.00543

High Variability in Cellular Stoichiometry of Carbon, Nitrogen, and Phosphorus Within Classes of Marine Eukaryotic Phytoplankton Under Sufficient Nutrient Conditions

Nathan S. Garcia[1], *Julie Sexton*[2], *Tracey Riggins*[2], *Jeff Brown*[2], *Michael W. Lomas*[2] and *Adam C. Martiny*[1,3]*

[1] Department of Earth System Science, University of California, Irvine, Irvine, CA, United States, [2] Bigelow Laboratory for Ocean Sciences, National Center for Marine Algae and Microbiota, East Boothbay, ME, United States, [3] Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA, United States

OPEN ACCESS

Edited by:
Hongyue Dang,
Xiamen University, China

Reviewed by:
Edward Hall,
Colorado State University, United States
Connie Lovejoy,
Laval University, Canada
Robert Ptacnik,
WasserCluster Lunz, Austria
Gabriel Yvon-Durocher,
University of Exeter, United Kingdom

***Correspondence:**
Adam C. Martiny
amartiny@uci.edu

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: 10 November 2016
Accepted: 09 March 2018
Published: 27 March 2018

Citation:
Garcia NS, Sexton J, Riggins T, Brown J, Lomas MW and Martiny AC (2018) High Variability in Cellular Stoichiometry of Carbon, Nitrogen, and Phosphorus Within Classes of Marine Eukaryotic Phytoplankton Under Sufficient Nutrient Conditions. Front. Microbiol. 9:543. doi: 10.3389/fmicb.2018.00543

Current hypotheses suggest that cellular elemental stoichiometry of marine eukaryotic phytoplankton such as the ratios of cellular carbon:nitrogen:phosphorus (C:N:P) vary between phylogenetic groups. To investigate how phylogenetic structure, cell volume, growth rate, and temperature interact to affect the cellular elemental stoichiometry of marine eukaryotic phytoplankton, we examined the C:N:P composition in 30 isolates across 7 classes of marine phytoplankton that were grown with a sufficient supply of nutrients and nitrate as the nitrogen source. The isolates covered a wide range in cell volume (5 orders of magnitude), growth rate ($<$0.01–0.9 d^{-1}), and habitat temperature (2–24°C). Our analysis indicates that C:N:P is highly variable, with statistical model residuals accounting for over half of the total variance and no relationship between phylogeny and elemental stoichiometry. Furthermore, our data indicated that variability in C:P, N:P, and C:N within Bacillariophyceae (diatoms) was as high as that among all of the isolates that we examined. In addition, a linear statistical model identified a positive relationship between diatom cell volume and C:P and N:P. Among all of the isolates that we examined, the statistical model identified temperature as a significant factor, consistent with the temperature-dependent translation efficiency model, but temperature only explained 5% of the total statistical model variance. While some of our results support data from previous field studies, the high variability of elemental ratios within Bacillariophyceae contradicts previous work that suggests that this cosmopolitan group of microalgae has consistently low C:P and N:P ratios in comparison with other groups.

Keywords: eukaryote, protist, diatom, dinoflagellate, prymnesiophyte, temperature, cell size, growth

INTRODUCTION

The average ratio of elements in marine plankton has traditionally been thought to center on the Redfield ratio at 106 moles of carbon (C):16 moles of nitrogen (N):1 mole of phosphorus (P) (Redfield, 1958). However, the stoichiometry of elements within marine organic particles is variable between biogeographical provinces (Martiny et al., 2013; DeVries and Deutsch, 2014; Teng et al., 2014), and within phytoplankton isolates (Geider and La Roche, 2002), which suggests that the average oceanic C:N:P is plastic, and perhaps changes over time as a function of interacting physical and biological factors. Environmental factors like light, temperature, and nutrients influence phytoplankton physiology and cellular elemental content, potentially molding relationships between phytoplankton phylogeny and cellular elemental stoichiometry (Rhee, 1978; Laws and Bannister, 1980; Urabe et al., 2002; Finkel et al., 2006; Toseland et al., 2013; Garcia et al., 2016; Lopez et al., 2016). In order to understand how factors contribute to regional differences in elemental stoichiometry and field microbial populations, analyses need to separate physical and biological factors.

Systematic relationships between phylogeny and cellular elemental stoichiometry have been linked to the evolutionary and environmental history of major phytoplankton lineages (Ho et al., 2003; Quigg et al., 2003). For example, Quigg et al. (2003) suggests that algae with green plastids have higher C:P and N:P ratios than other groups with red plastids, which may be related to the evolution of ocean chemistry. Aside from this relationship, field studies have identified differences in C:P and N:P ratios between two lineages of cold-water phytoplankton with red plastids near Antarctica (Arrigo et al., 1999, 2002) that are as large as differences observed between laboratory cultures of phytoplankton with green and red plastids (Quigg et al., 2003); where *Phaeocystis* (Prymnesiophyceae) has high C:P and N:P ratios in comparison with diatoms (Bacillariophyceae). Although previous laboratory studies (Quigg et al., 2003) focus on high growth rate conditions to minimize potential effects of variable physiology on elemental stoichiometry, physiological variability may be very different between major phytoplankton groups in the natural environment. Linking physiological variability with variability in elemental stoichiometry between taxonomic groups in field studies may be key to identifying how taxonomic shifts in phytoplankton communities might influence biogeochemical cycles within large biogeographical provinces. Thus, determining the relationship between phylogenetic structure, environmental growth conditions and cellular elemental stoichiometry is key to understanding how phytoplankton interact with biogeochemical cycles through time (Deutsch and Weber, 2012).

To identify systematic relationships between environmental gradients and cellular elemental stoichiometry, analyses need to separate phylogenetically correlated traits from other effects (Finkel et al., 2005, 2006, 2007, 2010; Mouginot et al., 2015). For example, small phytoplankton belonging to marine Cyanobacteria may have high C:P and N:P ratios relative to eukaryotic lineages with larger cells (Bertilsson et al., 2003; Martiny et al., 2013). However, laboratory data indicate that eukaryotes can also have high C:P and N:P ratios (Goldman et al., 1979). To gain a more in-depth understanding of how phylogenetic structure is related to cellular elemental stoichiometry of marine eukaryotic phytoplankton, we analyzed the relationship between cellular C:N:P ratios and the 18S ribosomal RNA sequence of marine eukaryotic phytoplankton isolates. We asked the question: Does phylogeny structure relationships between cellular elemental stoichiometry and gradients like cell size, growth rate, and temperature? Our isolate selection includes wide ranges in phylogeny, cell volume, and temperature habitats from which phytoplankton cells were originally isolated. With respect to variability in cellular elemental stoichiometry, our data suggest that deep phylogenetic structure may not be as important as other factors that influence cellular elemental stoichiometry of marine eukaryotic phytoplankton, such as environmental controls on physiology and other biological factors.

METHODS

We measured the elemental composition of 30 isolates from the National Center for Marine Algae and Microbiota (NCMA) culture collection representing 7 classes within the kingdoms Chromista and Plantae. For taxonomic nomenclature and hierarchical organization, we utilized the World Register of Marine Species (www.marinespecies.org). Cultures within the class Bacillariophyceae were grown in L1 medium (mole N:mole P = 24.4) and others were grown in L1 medium without silicate. We grew isolate cultures at temperatures that were close to the ambient ocean temperature from which isolates were originally collected, yielding 4 groups based on temperature, ranging between 2 and 24°C. Not all cultures were axenic although we used stringent culturing methods to prevent contamination. Cultures were maintained at temperatures very close to the ambient temperatures from which they were collected. Light was supplied with daylight white fluorescent lamps between 50 and 80 μmol quanta m^{-2} s^{-1} on a 13 h light:11 h dark incubation cycle. We monitored cultures daily with *in vivo* fluorescence of Chl *a* (**Figure S1**) and growth rates were calculated over 2-day periods and plotted in **Figure S2**. Cultures were terminally harvested for the analysis of cellular elemental composition, cell size measurements, and 18S rRNA sequence analysis approximated 1–2 weeks after they were initiated. For the analysis of growth rate, we used fluorescence data from the last 2 days before cultures were terminally sampled. This time frame did not necessarily align with the maximum observed growth rate (**Figure S2**). All samples were collected 3–4 h after the beginning of the photoperiod. Triplicate samples for the analysis of carbon and nitrogen (20–25 ml) and phosphorus (20–25 ml) were collected onto precombusted GF/F filters (450°C, 4 h) under low pressure vacuum filtration. Samples for DNA extraction were collected either by filtering 10–20 ml onto a polycarbonate filter or by pelleting cells with a centrifuge. Particulate organic carbon and nitrogen were analyzed with an elemental analyzer (Flash EA 1112 NC soil analyzer, Thermo Scientific) with acetanilide as a standard. Particulate organic phosphorus was analyzed with

a spectrophotometer using methods described in Garcia et al. (2016).

For 17 isolates, we used 18S rRNA sequence data from the National Center for Biotechnology Information database and we sequenced this region from the remaining cultures. DNA was extracted using a DNA extraction kit (D6001; Zymo, Irvine, CA). Primers for PCR were prepared by Integrated DNA Technologies, Inc. (Coralville, IA) and selected based on eukaryotic 18S rRNA sequence data as provided by Wang et al. (2014), which amplified the region between the ∼300 and ∼1500th position on the 18S rRNA sequence. Primer sequences are: forward−5′CGGAGAGGGAGCMTGAG3′; reverse−5′GCATCACAGACCTGTTATTGCC3′, and had a melting point between 56.0–56.4°C. The sequences of the PCR products were determined with Sanger sequencing by Laragen, Inc. (Culver City, CA). The consensus sequence for the 30 isolates was determined with Geneious 9.0.4 (Biomatters, Inc., Newark, NJ) and aligned with the SINA aligner (Pruesse et al., 2012) provided by Silva (www.arb-silva.de). We built a phylogenetic tree of the 30 isolates using Phylip 3.695 (Felsenstein, 1989; 100 bootstrap, F84 distance model, and neighbor joining with *Schizosaccharomyces pombe* as an outgroup). The phylogenetic position of each lineage matched past phylogenetic analyses.

To broadly compare the phylogeny of our cultures with their cellular elemental ratios, we used the Mantel test (from the "vegan" package in "R"; Oksanen et al., 2015), to compare distance matrices of the 18S rRNA sequence computed as above and of elemental ratios compared as a Euclidean matrix. To further separate phylogenetic relationships between the 18S rRNA sequence and cellular elemental stoichiometry with other factors including growth rate, temperature, and cell size, we used the comparative analysis of phylogenetic relationships with a phylogenetic generalized linear model from the "caper" package in "R," with lambda set at maximum likelihood and kappa and delta fixed at 1.0 (Orme, 2013).

RESULTS

We used two statistical tools (i.e., phylogenetic least squares regression model and the Mantel test to compare distance matrices) to examine how phylogeny and physiology affect cellular elemental stoichiometry within broad and narrow ranges of phylogenetic groups of marine eukaryotic phytoplankton. First we examined general statistical characteristics such as means and ranges of mole ratios of C:N:P, cell volume and growth rate data across isolates (**Table 1**). We then quantified how cellular C:N:P within the isolates varied as a function of phylogenetic diversity with a matrix correlation (Mantel test) and as a function of the interaction between phylogeny and physiology with phylogenetically corrected linear statistical models. We designed our isolate selection so that diatoms covered a large fraction of the selection in order to determine how factors like cell size might control cellular elemental stoichiometry within this globally-abundant and biogeochemically-important phytoplankton lineage.

The mean growth rate relative to the mean maximum observed growth rate (μ:μ_{max}; from triplicate cultures) was highly variable between isolates at the time of sampling (**Table 1**). For example, this ratio was below 0.10 for 3 of the 30 isolates, suggesting that the physiology of some of the isolates was in poor condition. The mean particulate organic nitrogen and phosphorus concentrations in cultures were well below the concentrations of nitrate and phosphate in the L1 medium (882 μM nitrate and 36 μM phosphate, see **Table 1**), indicating that cultures were not limited by these nutrients. Mean C:P, N:P, and C:N of all isolates were 107.3 ± 31.9 (s.d.), 16.2 ± 5.0 (s.d.), and 6.7 ± 1.1 (s.d.), respectively, reflecting ratios proposed by Redfield (1958) (**Figure 1**). Among specific isolates, we observed the highest mean C:P and N:P within the classes Cryptophyceae [*Chroomonas mesostigmatica*, (CCMP1168); C:P = 182.4 ± 3.9 s.d., N:P = 26.8 ± 1.2 s.d.] and Bacillariophyceae [*Thalassiosira rotula* (CCMP1018); C:P = 181.8 ± 54.2 s.d., N:P = 29.6 ± 1.1 s.d.] and the lowest within the class Mamiellophyceae [*Micromonas pusilla* (CCMP1723); C:P = 56.9 ± 9.0 s.d., N:P = 8.9 ± 1.2 s.d.] (**Table 1**). Mean C:N among isolates was highest within the class Dinophyceae [*Prorocentrum mexicanum* (CCMP687) C:N = 9.4 ± 0.3 s.d.] and lowest within the class Bacillariophyceae [*Thalassiosira oceanica* (CCMP1005); C:N = 4.8 ± 0.2 s.d.]. We observed the highest variability in C:P, N:P, and C:N within Bacillariophyceae (**Figures 2**, **3**). We measured the largest cells within Dinophyceae [the isolate of *Karenia brevis* (CCMP687) had the largest mean cell volume, 13.1×10^3 μm^{-3}] and the mean cell volume within *Ostreococcus lucimarinus* (CCMP2972; Mamiellophyceae) was smallest (5.6×10^{-3} μm^{-3}). Despite the strong contrast in cell size, mean growth rates, under these conditions, were lowest and nearly identical in Dinophyceae (0.08 ± 0.07 d^{-1} s.d.) and Mamiellophyceae (0.08 ± 0.1 d^{-1} s.d.) and highest in Bacillariophyceae (0.38 ± 0.30 d^{-1} s.d.) and Prymnesiophyceae (0.38 ± 0.29 d^{-1} s.d.).

To determine how phylogenetic structure was related to cellular elemental stoichiometry of phytoplankton, we compared the phylogenetic relationship of the 18S rRNA to the ratios of elements within cells. To broadly examine this relationship, we compared matrices of the 18S rRNA sequences (dissimilarity distance matrix) and stoichiometric ratios (Euclidean distance matrix) using the Mantel test (Mantel, 1967). The Mantel correlation was low and not significant for C:P (0.11, $p = 0.10$), N:P (0.11, $p = 0.10$), and C:N = (0.11, p = 0.08), indicating no relationship between phylogeny of the 18S rRNA sequence and cellular C:N:P stoichiometry within our 30 isolates.

To determine how phylogenetic class, cell volume, growth rate, and temperature contribute to cellular CNP ratios, we fitted a general linear model (glm) to our data with the form $f(x) =$ (x) (class + cell volume + growth rate + temperature) + ε. In general, stoichiometric variability of C:P, N:P, and C:N within classes was high (**Figures 2**, **3**). Residuals form our statistical model were responsible for over half of the model variance for C:P (51.5%), N:P (57.3%), and C:N (70.4%) (ANOVA test on the glm). Phylogenetic class was identified as a significant contributor to the overall variance of C:P (43.3%, $p < 0.05$), but not on N:P (39.4, $p > 0.05$) or C:N (13.2%, $p > 0.05$). Tukey's analysis of means, however, did not identify significant differences in

TABLE 1 | Molar ratios of cellular elements within isolates of eukaryotic phytoplankton curated at the National Center for Marine Algae and Microbiota.

Class	Species	Isolate	Temp (°C)	μ (d^{-1})	s.d.	μ_{max} (d^{-1})	s.d.	μ: μ_{max}	Cell volume (μm^{-3})	s.d.	C:N	s.d.	N:P	s.d.	C:P	s.d.
Cryptophyceae	*Chroomonas mesostigmatica*	CCMP1168	20	0.31	0.01	0.58	0.04	0.54	150.7	65.3	6.81	0.22	26.81	1.20	182.4	3.9
Dinophyceae	*Heterocapsa neie*	CCMP448	20	0.11	0.02	0.41	0.02	**0.27**	3,513.3	1,366.8	7.31	0.10	9.99	0.24	72.9	0.9
	Amphidinium carterae	CCMP121	24	0.00	0.03	0.52	0.00	**0.00**	870.2	318.7	7.85	1.74	17.67	4.39	133.7	1.4
	Prorocentrum mexicanum	CCMP687	24	0.11	0.01	0.24	0.04	0.46	8,441.5	2,611.1	9.44	0.28	10.39	1.27	98.3	15.1
	Karenia brevis	**CCMP2281**	24	0.11	0.01	0.33	0.03	0.33	13,129.0	3,873.5	7.18	0.11	16.40	1.11	117.8	9.5
Prymnesiophyceae	*Phaeocystis antarctica*	CCMP1374	2	0.06	0.02	0.28	0.05	**0.22**	94.0	55.0	6.08	0.30	18.48	2.66	111.7	10.9
	Gephyrocapsa oceanica	**CCMP2051**	20	0.22	0.02	0.53	0.02	0.41	87.1	43.4	7.04	0.14	17.88	2.32	126.1	18.3
	Gephyrocapsa oceanica	CCMP2054	20	0.43	0.04	0.52	0.12	**0.83**	142.0	60.0	5.69	0.58	25.57	8.79	142.4	38.2
	Emiliania huxleyi	CCMP2090	20	0.36	0.01	0.71	0.03	0.51	390.9	213.8	7.39	0.10	18.17	0.43	134.3	2.8
	Phaeocystis globosa	CCMP1528	24	0.83	0.03	0.95	0.03	**0.87**	79.8	4.2	6.37	0.16	16.49	3.94	105.0	24.1
Bacillariophyceae	*Pseudo-nitzschia sp.*	CCMP1309	2	0.12	0.05	0.32	0.02	0.39	200.3	63.6	5.89	0.03	13.90	1.03	81.9	6.5
	Fragilariopsis cylindrus	CCMP1102	2	0.14	0.07	0.28	0.08	0.48	38.9	21.1	5.72	0.13	10.10	0.36	57.8	1.7
	Thalassiosira minima	CCMP991	2	0.11	0.05	0.40	0.10	**0.27**	790.4	243.9	5.76	0.21	11.75	1.71	67.7	10.0
	Thalassiosira nordenskioleldii	CCMP992	2	0.10	0.02	0.28	0.04	0.35	1,156.2	478.7	6.97	0.12	14.57	0.50	101.6	2.2
	Thalassiosira rotula	**CCMP1018**	14	0.12	0.05	0.54	0.01	**0.23**	2,190.4	1,206.3	6.11	1.59	29.58	1.13	181.8	54.2
	Thalasiosira guillardii	**CCMP988**	14	0.17	0.03	0.58	0.00	**0.29**	117.5	44.8	6.61	0.16	17.33	0.27	114.5	2.4
	Thalassiosira pseudonana	CCMP1335	20	0.76	0.01	1.04	0.02	**0.73**	42.4	28.6	8.89	0.08	13.61	0.76	121.0	6.0
	Thalassiosira weissflogii	CCMP1587	20	0.56	0.10	0.79	0.09	**0.71**	564.7	154.1	7.22	0.22	19.45	8.11	139.4	53.8
	Phaeodactylum tricornutum	**CCMP633**	20	0.45	0.04	0.64	0.04	0.70	154.5	84.2	5.64	0.31	12.83	2.60	71.8	10.6
	Phaeodactylum tricornutum	CCMP2561	20	0.69	0.01	0.77	0.01	**0.89**	229.1	366.4	5.23	0.06	15.61	1.21	81.6	5.3
	Thalassiosira oceanica	**CCMP1005**	24	0.92	0.04	1.04	0.03	**0.88**	91.5	3.2	4.85	0.18	12.20	3.04	75.9	30.9
	Thalassiosira weissflogii	CCMP1050	24	0.46	0.10	0.91	0.02	0.51	734.0	207.4	8.21	0.39	14.36	0.64	117.8	7.2
Dictyophyceae	*Aureococcus anophogefferens*	**CCMP1790**	20	0.30	0.02	0.60	0.05	0.49	14.3	10.2	7.36	0.26	13.33	1.32	98.0	7.3
	Pelagomonas calceolata	**CCMP1865**	20	0.20	0.02	0.55	0.03	0.36	13.2	7.3	6.06	0.16	18.06	1.86	109.4	9.8
	Pelagomonas calceolata	**CCMP1756**	24	0.34	0.03	0.88	0.03	0.39	10.5	6.3	5.60	0.26	20.35	3.35	113.5	14.4
Mamiellophyceae	*Micromonas pusilla*	**CCMP485**	14	0.22	0.01	0.59	0.06	0.38	5.3	3.0	7.37	0.34	12.38	2.77	90.7	16.9
	Micromonas pusilla	**CCMP1723**	20	0.01	0.02	0.54	0.00	**0.02**	5.9	4.2	6.39	0.19	8.89	1.22	56.9	9.0
	Ostreococcus lucimarinus	**CCMP2972**	20	0.07	0.02	0.55	0.02	**0.13**	0.1	0.0	5.25	0.17	22.81	2.13	119.5	7.3
	Micromonas pusilla	CCMP2709	24	0.00	0.04	0.47	0.10	**0.01**	4.6	3.7	5.63	0.53	12.20	4.56	67.1	18.3
Prasinophyceae	*Pycnococcus provasoli*	**CCMP3400**	20	0.21	0.05	0.51	0.03	0.42	5.9	3.8	7.87	0.30	15.85	1.94	125.1	19.3

*Isolates were grown and curated near field temperatures from which they were collected at 50–80 μmol quanta m^{-2} s^{-1}. Growth rate (μ) data were collected during the 2-day period just prior to sampling triplicate cultures for cellular elemental ratios. Observed maximum growth rate data (μmax, dependent on culture conditions) were calculated from the maximum change in relative fluorescence (**Figure S1**) over a 2-day period and are plotted in **Figure S2**. The standard deviation (s.d.) is reported. Phylogenetic nomenclature and heirarchical organization is based on information from the World Registry of Marine Species. Bold isolate identification numbers indicate isolates from which DNA was extracted, amplified with 18S rRNA primers and the PCR product sequenced. Other sequence data were collected from the National Center for Biotechnology Information. Mean μ relative to the observed mean μmax was not constant between isolates. Values of mean μ: mean μmax below 0.30 are bolded and highlighted in blue and values above 0.70 are bolded and highlighted in red.*

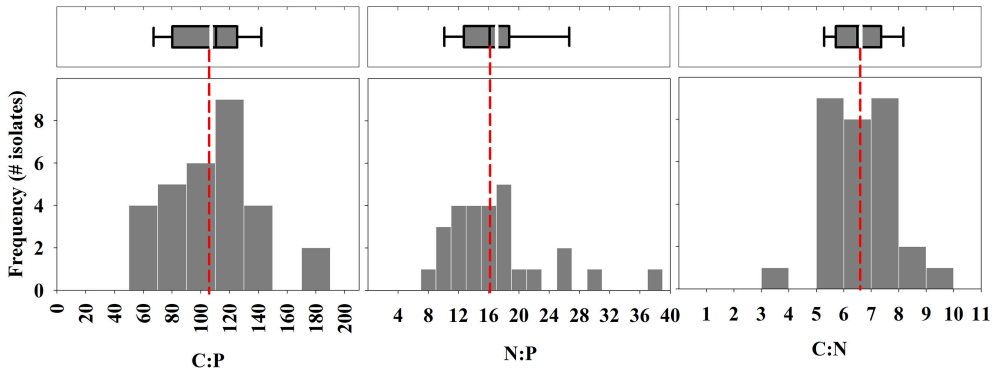

FIGURE 1 | Frequency of isolates within binned intervals of molar elemental ratios of carbon, nitrogen, and phosphorus. Bin intervals for C:P, N:P, and C:N are 20, 2, and 1, respectively. Top box-whisker plots include the mean (white line) and median (black line) of ratios for all 30 isolates. The box indicates quartiles and the dotted line provides a reference to the Redfield ratio of C:N:P (106:16:1).

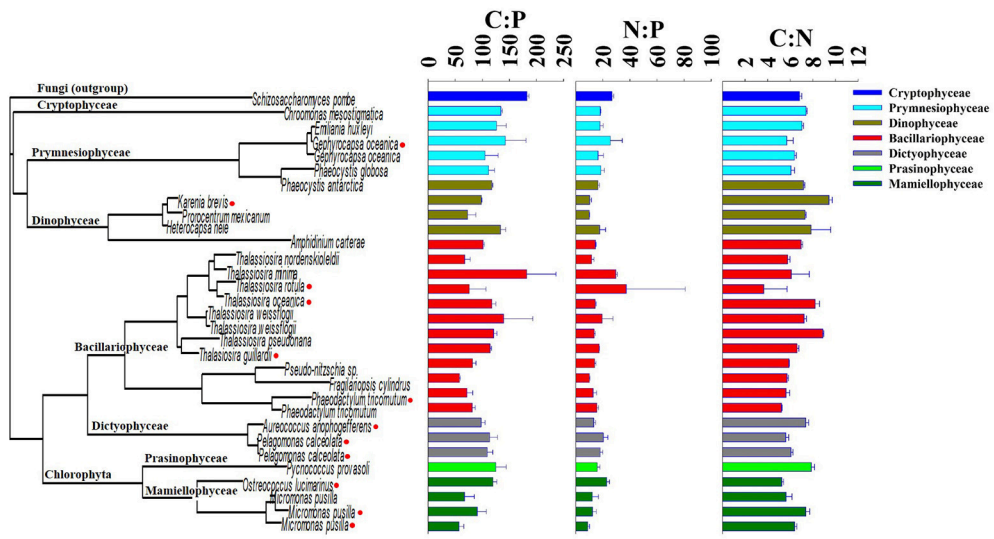

FIGURE 2 | Phylogenetic tree of the 18S rRNA sequence of 30 eukaryotic phytoplankton isolates in comparison with the molar ratio of cellular elements of carbon, nitrogen, and phosphorus. Red markers on the tree indicate isolates that were sequenced by Laragen, Inc., vs. others that were collected from the National Center for Biotechnology Information database.

cellular C:N:P ratios between classes except for a difference in C:P between Mamiellophyceae ($n = 4$ isolates) and Cryptophyceae ($n = 1$ isolate). Although the glm indicated that temperature had a significant effect on C:P ($p < 0.05$; **Figure 4**), temperature only explained 5.1% of the statistical model variance, suggesting that temperature had a minor effect. Growth rate and cell volume were not significant predictors of C:P, N:P, or C:N ($p > 0.05$).

We also used the phylogenetic least squares (pgls) statistical model to constrain phylogenetic structure of the isolates (using the 18S rRNA sequence) and determine how cell volume, growth rate and temperature might interact with the phylogenetic relationship of the 18S rRNA sequence to influence cellular CNP stoichiometry in the 30 isolates. The pgls model ($f(x) = x$(cell volume + growth rate + temperature)) however, did not identify significant trends between cellular elemental ratios and any of these factors ($p > 0.05$; **Figure 4**).

We selected phytoplankton isolates to include a wide range in cell volume within Bacillariophyceae. Within this class, we also selected isolates that were collected from environments that have a wide range in temperature. Thus, 12 of the 30 isolates that we examined were diatoms, representing 40% of our analysis. Within Bacillariophyceae, mean C:P, N:P and C:N were close to Redfield values (101.1 ± 35.8 s.d.; 15.4 ± 5.1 s.d.; 6.3 ± 1.4 s.d., respectively; **Figure 5**). The glm ($f(x) = (x)$ (cell volume + growth rate + temperature) + ε) indicated that cell volume had a significant positive effect on C:P and N:P, ($p < 0.05$; **Figure 5**; the effect on C:N was not significant $p > 0.05$) and accounted for a significant portion of the statistical model variance ($p < 0.05$; 46.6 and 59.4%, respectively). The pgls model also indicated that

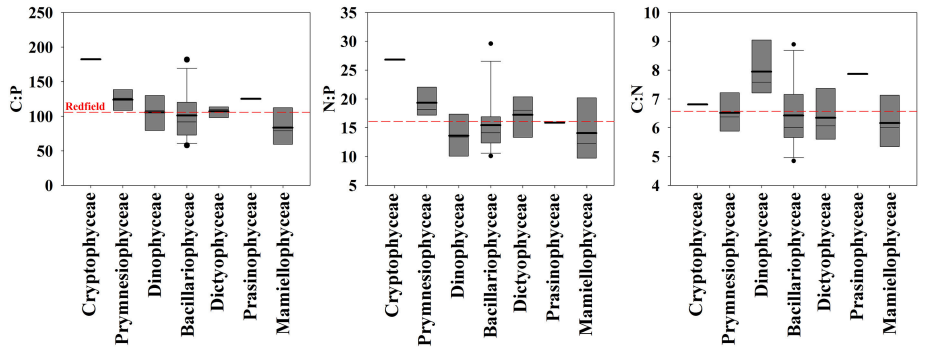

FIGURE 3 | Comparison of cellular molar elemental ratios of carbon, nitrogen, and phosphorus among classes of 30 isolates of eukaryotic phytoplankton with box plots. The mean (bold line) and median (thin line) are plotted within the box which indicates quartiles. Whiskers indicate 10 and 90% quantiles and dots are outliers.

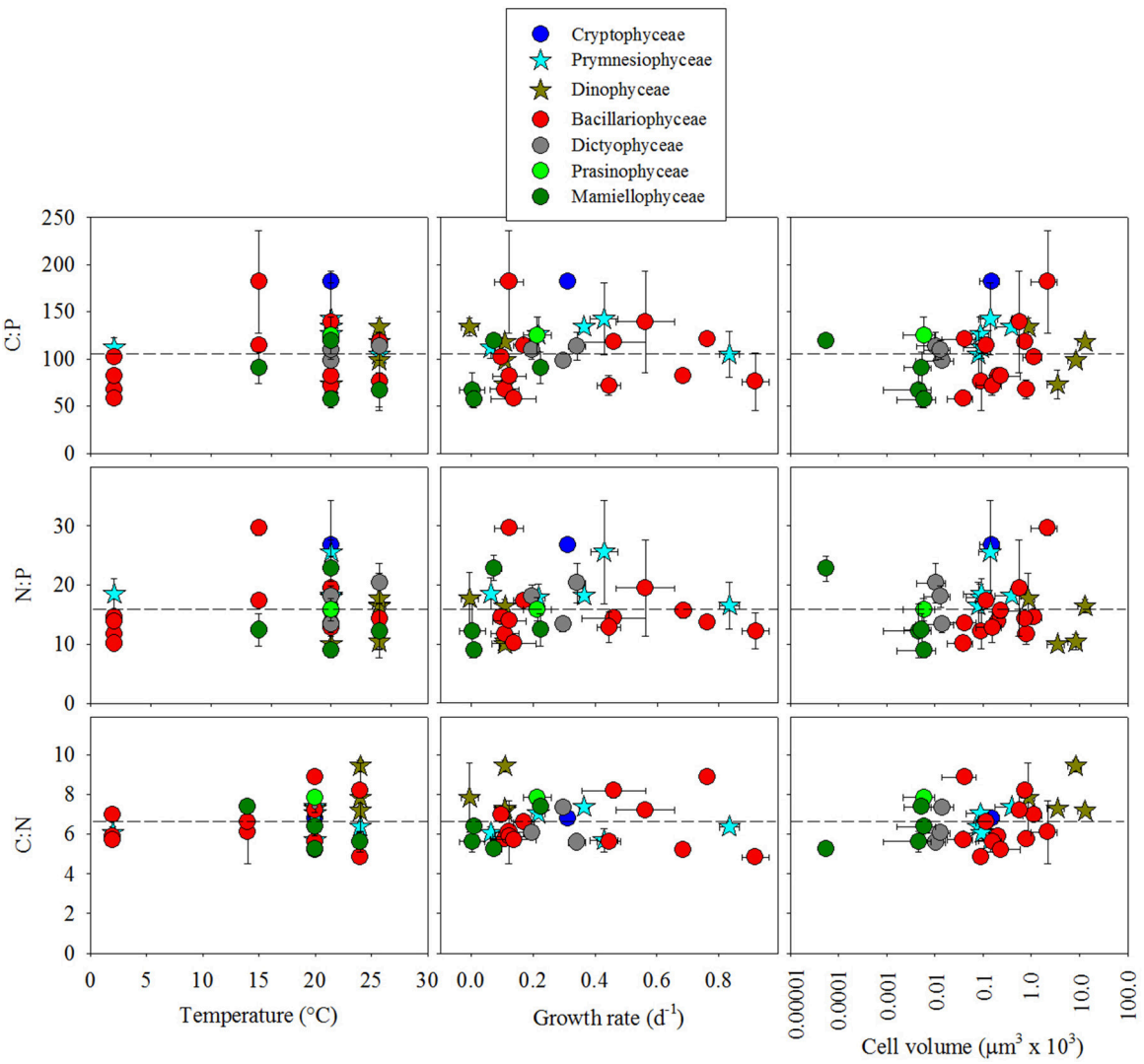

FIGURE 4 | Cellular molar elemental ratios of carbon, nitrogen, and phosphorus among classes of 30 isolates of eukaryotic phytoplankton as a function of temperature, growth rate, and cell volume. Different colors separate classes and dotted lines indicate reference to Redfield ratios.

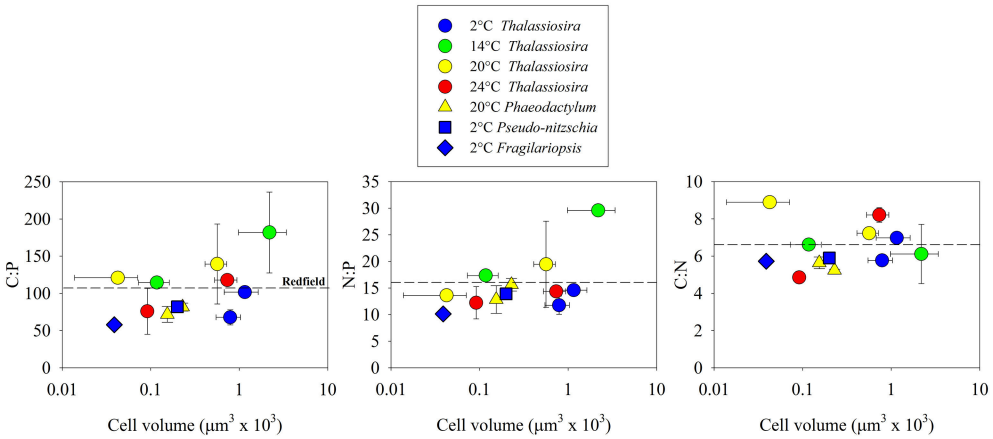

FIGURE 5 | Cellular molar elemental ratios of carbon, nitrogen, and phosphorus within diatoms (Bacillariophyceae) as a function of cell volume, temperature, and genus. Dotted lines indicate reference to Redfield ratios.

the robust effect of cell volume was the only significant factor affecting C:P and N:P ($p < 0.05$) within Bacillariophyceae.

DISCUSSION

We observe high variability in cellular elemental stoichiometry (C:N:P) within classes of phytoplankton (**Figure 3**). Furthermore, the phylogenetic relationship of the 18S rRNA sequence is not correlated with C:P, N:P, or C:N within our 30 isolates of eukaryotic phytoplankton, reflecting the absence of a relationship between phylogeny and elemental stoichiometry. Although we did not implement stringent physiological controls in our analysis, the effects of growth rate (i.e., μ/μ_{max}) on elemental stoichiometry may be important in identifying relationships between phylogeny and elemental stoichiometry as identified previously (Quigg et al., 2003). We note, however, that environmental populations grow at variable rates. Thus, broad physiological ranges may be more important in identifying class-specific variability in elemental stoichiometry in the natural environment.

In comparison with other studies, our results corroborate recent findings but conflict with some current hypotheses. In our study, C:P and N:P variation among isolates within Bacillariophyceae is as large as the total variation of elemental ratios among all of the isolates that we examined (**Figures 2**, **3**). This result is similar to findings from Finkel et al. (2016) where the species level was the largest source of variation in their hierarchical Bayesian analysis of macromolecular composition among lineages of phytoplankton. Our results also partially support previous studies that identify low C:P and N:P in cold-water diatoms (Arrigo et al., 1999, 2002). However, by including warm water diatom isolates, our data conflict with the hypothesis that all taxa within Bacillariophyceae have low C:P and N:P relative to other classes (Ho et al., 2003; Quigg et al., 2003). Thus, low cellular elemental ratios within diatoms may be common in cold-water isolates but elemental ratios were not consistently low in all of the diatom isolates that we examined and did not scale linearly with temperature (**Figure 4**). Our analysis, however, could be improved by including more isolates to account for larger variability within groups outside of Bacillariophyceae.

Our data also do not align with the general trend associated with the *growth rate hypothesis* (Sterner and Elser, 2002), whereby C:P and N:P ratios are expected to decline with increasing growth because of the high growth dependency on ribosomal P. Although several studies indicate that growth rate can have a strong relationship with C:P and N:P, there are many issues associated with this hypothesis in microalgae (Flynn et al., 2010) and may be restricted to phosphate-limited growth as identified previously (Goldman et al., 1979; Klausmeier et al., 2008; Garcia et al., 2016). This hypothetical change in C:P and N:P, for example, could be masked by cellular P storage in environments with a high P supply, as modeled by Klausmeier et al. (2004) but more generally, the growth-dependent change in the cellular ribosomal protein concentration seems to be a small fraction of a larger protein pool that changes as a function of microbial growth (Barenholz et al., 2016). The N and P input concentrations in our growth medium (L1) were considerably higher than the particulate concentrations of N and P in our cultures, indicating that growth rates were not limited by nutrients. Thus, the growth rate hypothesis may find more support in some natural environments where P is depleted relative to N.

Another related hypothesis suggests that temperature affects cellular elemental stoichiometry by modulating ribosome efficiency and hence the demand for ribosomal cellular P (Toseland et al., 2013). Their data suggest that cells compensate for low ribosomal efficiency at low temperatures by increasing the cellular concentration of ribosomes and hence cellular P (Toseland et al., 2013). Although our statistical model (glm) identified a significant effect of temperature on C:P and N:P, temperature only accounted for a small portion of the statistical model variance (5%). Thus, the effect of temperature on C:N:P variability within isolates might be more important than the effect on whole communities and may be more relevant for low P environments where P-storage does not interfere with

the underlying effect on P-rich ribosome concentrations. With regard to the temperature-dependent translation efficiency hypothesis, the specific question about how temperature affects cellular elemental stoichiometry might be more thoroughly investigated with a full-factorial experimental design focusing on temperature with account for broad physiological effects like P-limited growth. Such an experimental design might consider an observable maximum growth rate and account for covariable relationships between temperature and growth rate (Boyd et al., 2013) and cell size and growth rate (Marañón et al., 2013). In addition to these considerations, however, adaptive mechanisms (e.g., such as those identified by Toseland et al., 2013) may further complicate relationships between cellular elemental stoichiometry, temperature, cell size and growth rate.

Although silicate concentrations in general, can be high in glass culturing flasks (50–100 μM.—M. Lomas unpublished data) we did not control for possible effects of silicate addition on C:N:P ratios. Such additional environmental factors may contribute to the variation in observed C:N:P ratios in microalgae including diatoms. The supply of silicate to diatoms, for example, may influence the proportion of cell volume that is occupied by other elements (Raven and Waite, 2004).

Cell Volume and C:N:P Within Diatoms

A large portion (40%) of our analysis focused on Bacillariophyceae to identify how cell size, growth rate, and temperature might contribute to C:N:P variability as diatoms represent a large portion of marine net primary production (Nelson et al., 1995; Armbrust et al., 2002). Of these 3 factors, our analysis identified a significant positive relationship between cell volume and C:P and N:P (**Figure 5**). Because of the limited number of isolates that we analyzed in other classes of phytoplankton, we cannot directly compare this trend in Bacillariophyceae with other classes but one feature that is unique to Bacillariophyceae is the low carbon and nitrogen investments in the protective layer surrounding cells, as this group depends on silica for major support in addition to the silicolemma. This is in contrast to other phytoplankton lineages, like chlorophytes that have high concentrations of glycoproteins in the cell wall (Northcote and Goulding, 1958; Gerken et al., 2013). Another factor that might contribute to a large portion of C:N:P variability in some diatoms is β-chitin. Of the 12 isolates that we examined, 8 belong to the genus *Thalassiosira*, which is known to invest C and N at a ratio of 8:1 in β-chitin spines that extend through the silica shell to reduce sinking rates (McLachlan et al., 1965; McLachlan and Craigie, 1966; Round et al., 1990; Durkin et al., 2009). Thus, the positive relationship between cell volume and C:N:P within Bacillariophyceae could result from a combination of silica-based cell structure and C:N-enriched β-chitin, specifically within *Thalassiosira*. Although not unique to Bacillariophyceae, lipids are an important carbon storage mechanism (McGinnis et al., 1997), which could also contribute to the positive relationship between C:P and and cell volume. This positive relationship within Bacillariophyceae is in contrast to other data supporting the hypothesis that isolates with small cells have high C:P and N:P (Bertilsson et al., 2003; Martiny et al., 2013) and should be investigated further because of the projected surface-ocean warming and its associated effect of declining nutrient concentrations on phytoplankton cell size (Finkel et al., 2005, 2007, 2010). Further investigations might identify more complex effects of vacuoles on this relationship.

In summary, we identified a high degree of variability in C:P and N:P and C:N within the class Bacillariophyceae that is as large as the stoichiometric variability between the 7 classes that we examined. While some of our data support previous studies in this regard, this high variability is in contrast to several studies that identify only low ratios of C:P and N:P in diatoms in comparison with other lineages. Whereas, previous studies focus on a single factor relationship between phylogeny and cellular elemental stoichiometry, our analysis includes effects from multiple variables that are currently thought to affect cellular elemental stoichiometry. Overall, our study highlights the complexity of variability in cellular elemental ratios among marine phytoplankton. Generally, our results suggest that the link between changes in ocean phytoplankton community composition and C:N:P is complex and we cannot simply assume that the presence of diatoms leads to low ratios in ocean regions. Our study further identifies the need to control for physiological effects to test current hypotheses relating to potential trends in phytoplankton elemental stoichiometry. Future studies that use different sources of nutrients (e.g., ammonium vs. nitrate) might improve our understanding of variability in physiology and elemental stoichiometry between phytoplankton groups.

AUTHOR CONTRIBUTIONS

NG and AM contributed to the experimental design, statistical analyses, and writing. ML contributed to the experimental design, sample collection and writing. JS, JB, and TR contributed to the experimental design, culturing phytoplankton, and sample collection.

ACKNOWLEDGMENTS

We thank members at the Bigelow Laboratory for Ocean Sciences and the National Center for Marine Algae and Microbiota for providing the facilities, resources, and cultures for this work (DBI-1349350). We also thank the National Science Foundation (OCE 1046297 and OCE-1559002) and the UCI Chancellor's Postdoctoral Fellowship Program for providing funding to support this project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00543/full#supplementary-material

Figure S1 | Relative fluorescence units of *in vivo* Chl *a* in 30 isolates of marine eukaryotic phytoplankton. Symbols are color-coded by class: dark yellow, Dinophyceae; dark green, Mamiellophyceae; dark blue, Cryptophyceae; light blue, Prymnesiophyceae; gray, Dictyophyceae; light green, Prasinophyceae. Species names are color-coded by temperature. Growth rates were calculated between the 2-day period before terminal sampling of cultures.

Figure S2 | Observed growth rates in 30 isolates of marine eukaryotic phytoplankton. Symbols are color-coded by class: dark yellow, Dinophyceae; dark green, Mamiellophyceae; dark blue, Cryptophyceae; light blue, Prymnesiophyceae; gray, Dictyophyceae; light green, Prasinophyceae. Species names are color-coded by temperature. Growth rates were calculated between the 2-day period before terminal sampling of cultures. Black numbers in the lower left of the panel are cell volumes estimates in $\mu m^3 \times 10^3$ (estimates correspond with data in **Figures 4**, **5**).

REFERENCES

Armbrust, V. E., Berges, J. A., Bowler, C., Green, B. R., Martinez, D., Putman, N. H., et al. (2002). The genome of the diatom *Thalassiosira pseudonana*: ecology evolution, and metabolism. *Science* 306, 79–86. doi: 10.1126/science.1101156

Arrigo, K. R., Dunbar, R. B., Lizotte, M. P., and Robinson, D. H. (2002). Taxon-specific difference in C/P and N/P drawdown for phytoplankton in the Ross Sea, Antarctica. *Geophys. Res. Lett.* 29:1938. doi: 10.1029/2002GL015277

Arrigo, K. R., Robinson, D. H., Worthen, D. L., Dunbar, R. B., DiTullio, G. R., VanWoert, M., et al. (1999). Phytoplankton community structure and the drawdown of nutrients and CO_2 in the southern Ocean. *Science* 283, 365–367. doi: 10.1126/science.283.5400.365

Barenholz, U., Keren, L., Segal, E., and Milo, R. (2016). A minimalistic resource allocation model to explain ubiquitous increase in protein expression with growth rate. *PLoS ONE* 11:e0153344. doi: 10.1371/journal.pone.0153344

Bertilsson, S., Berglund, O., Karl, D. M., and Chisholm, S. W. (2003). Elemental composition of marine *Prochlorococcus* and *Synechococcus*: implications for the ecological stoichiometry of the sea. *Limnol. Oceanogr.* 48, 1721–1731. doi: 10.4319/lo.2003.48.5.1721

Boyd, P. W., Rynearson, T. A., Armstrong, E. A., Fu, F., Hayashi, K., Zhangxi, H., et al. (2013). Marine phytoplankton temperature vs. growth responses from polar to tropical waters outcome of a scientific community wide study. *PLoS ONE* 8:e63091. doi: 10.1371/journal.pone.0063091

Deutsch, C., and Weber, T. (2012). Nutrient ratios as a tracer and driver of ocean biogeochemistry. *Annu. Rev. Mar. Sci.* 4, 113–141. doi: 10.1146/annurev-marine-120709-142821

DeVries, T., and Deutsch, C. (2014). Large-scale variations in the stoichiometry of marine organic matter respiration. *Nat. Geosci.* 7, 890–894. doi: 10.1038/ngeo2300

Durkin, C. A., Mock, T., and Armbrust, E. V. (2009). Chitin in diatoms and its association with the cell wall. *Eukaryot. Cell* 8, 1038–1050. doi: 10.1128/EC.00079-09

Felsenstein, J. (1989). PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics* 5, 164–166.

Finkel, Z. V., Beardall, J., Flynn, K. J., Quigg, A., Rees, A. V., and Raven, J. A. (2010). Phytoplankton in a changing world: cell size and elemental stoichiometry. *J. Plankton Res.* 32, 119–137. doi: 10.1093/plankt/fbp098

Finkel, Z. V., Follows, M. J., Liefer, J. D., Brown, C. M., Benner, I., and Irwin, A. J. (2016). Phylogenetic diversity in the macromolecular composition of microalgae. *PLoS ONE* 11:e0155977. doi: 10.1371/journal.pone.0155977

Finkel, Z. V., Katz, M. E., Wright, J. D., Schofield, O. M. E., and Falkowski, P. G. (2005). Climatically driven macroevolutionary patterns in the size of marine diatoms over the Cenozoic. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8927–8932. doi: 10.1073/pnas.0409907102

Finkel, Z. V., Quigg, A., Raven, J. A., Reinfelder, J. R., Scholfiled, O. E., and Falkowski, P. G. (2006). Irradiance and the elemental stoichiometry of marine phytoplankton. *Limnol. Oceanogr.* 51, 2690–2701. doi: 10.4319/lo.2006.51.6.2690

Finkel, Z. V., Sebbo, J., Feist-Burkhardt, S., Irwin, A. J., Scholfield, O. M. E., Young, J. R., et al. (2007). A universal driver of macroevolutionary change in the size of marine phytoplankton over the Cenozoic. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20416–20420. doi: 10.1073/pnas.0709381104

Flynn, K. A., Raven, J. A., Rees, T. A. V., Finkel, Z., Quigg, A., and Beardall, J. (2010). Is the growth rate hypothesis applicable to microalgae? *J. Phycol.* 46, 1–12. doi: 10.1111/j.1529-8817.2009.00756.x

Garcia, N. S., Bonachela, J. A., and Martiny, A. C. (2016). Interactions between growth-dependent cell size, nutrient availability and cellular elemental stoichiometry of marine *Synechococcus*. *ISME J.* 10, 2715–2724. doi: 10.1038/ismej.2016.50

Geider, R. J., and La Roche, J. (2002). Redfield revisted: variability of C:N:P of marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37, 1–17. doi: 10.1017/S0967026201003456

Gerken, H. G., Donohoe, B., and Knoshaug, E. P. (2013). Enzymatic cell wall degradation for *Chlorella vulgaris* and other microalgae for biofuels production. *Planta* 237, 239–253. doi: 10.1007/s00425-012-1765-0

Goldman, J. C., McCarthy, J. J., and Peavey, D. G. (1979). Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature* 279, 210–215. doi: 10.1038/279210a0

Ho, T.-Y., Quigg, A., Finkel, Z. V., Milligan, A. J., Wyman, K., Falkowski, P. G., et al. (2003). The elemental composition of some marine phytoplankton. *J. Phycol.* 39, 1145–1159. doi: 10.1111/j.0022-3646.2003.03-090.x

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2008). Phytoplankton stoichiometry. *Ecol. Res.* 23, 479–485. doi: 10.1007/s11284-008-0470-8

Klausmeier, C. A., Litchman, E., and Levin, S. A. (2004). Phytoplankton growth and stoichiometry under multiple nutrient limitation. *Limnol. Oceanogr.* 49, 1463–1470. doi: 10.4319/lo.2004.49.4_part_2.1463

Laws, E. A., and Bannister, T. T. (1980). Nutrient- and light-limited growth of *Thalassiosira fluviatilis* in continuous culture, with implications for phytoplankton growth in the ocean. *Limnol. Oceanogr.* 25, 457–473. doi: 10.4319/lo.1980.25.3.0457

Lopez, J., Garcia, N. S., Talmy, D., and Martiny, A. C. (2016). Diel variability in the elemental composition of the marine cyanobacterium *Synechococcus*. *J. Plankton Res.* 38, 1052–1061. doi: 10.1093/plankt/fbv120

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Res.* 27, 209–220.

Marañón, E., Cermeño, P., López-Sandoval, D. C., Rodríguez-Ramos, T., Sobrino, C., Huete-Ortega, M., et al. (2013). Unimodal size scaling of phytoplankton growth and the size dependence of nutrient uptake and use. *Ecol. Lett.* 16, 371–379. doi: 10.1111/ele.12052

Martiny, A. C., Pham, C. T. A., Primeau, F. W., Vrugt, J. A., Moore, J. K., Levin, S. A., et al. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci.* 6, 279–283. doi: 10.1038/ngeo1757

McGinnis, K. M., Dempster, T. A., and Sommerfeld, M. R. (1997). Characterization of the growth and lipid content of the diatom *Chaetoceros muelleri*. *J. Appl. Phycol.* 9, 19–24. doi: 10.1023/A:1007972214462

McLachlan, J., and Craigie, J. S. (1966). "Chitan fibres in *Cyclotella cryptica* and growth of *C. cryptica* and *Thalassiosira fluviatilis*," in *Some Contemporary Studies in Marine Science*, ed H. Barnes (London: George Allen and Unwin Ltd), 511–517.

McLachlan, J., McInnes, A. G., and Falk, M. (1965). Studies on the chitan (chitin: poly-N-acytylglucosamine fibers of the diatom *Thalassiosira fluviatilis* Hustedt. *Can. J. Bot.* 43, 707–713. doi: 10.1139/b65-079

Mouginot, C., Zimmerman, A. E., Bonachela, J. A., Fredricks, H., Allison, S. D., Van Mooy, B. A. S., et al. (2015). Resource allocation by the marine cyanobacterium *Synechococcus* WH8102 in response to different nutrient supply ratios. *Limnol. Oceanogr.* 60, 1634–1641. doi: 10.100/lno10123

Nelson, D. M., Treguer, P., Brzezinski, M. A., Leynaert, A., and Queguiner, B. (1995). Production and dissolution of biogenic silica in the ocean: revised global estimates, comparison with regional data and relationship to biogenic sedimentation. *Global Biogechem. Cycles* 9, 359–372. doi: 10.1029/95GB01070

Northcote, D. H., and Goulding, K. J. (1958). The chemical composition and structure of the cell wall of *Chlorella pyrenoidosa*. *Biochem. J.* 70, 391–397. doi: 10.1042/bj0700391

Oksanen, J., Blanchet, F. G., Kindt, R.,Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2015). *vegan: Community Ecology Package*. R package version 2.2-1. Available online at: http://CRAN.R-project.org/package=vegan

Orme, D. (2013). *The Caper Package: Comparative Analysis of Phylogenetics And Evolution in R*.

Pruesse, E., Peplies, J., and Glöckner, F. O. (2012). SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28, 1823–1829. doi: 10.1093/bioinformatics/bts252

Quigg, A., Finkel, Z. V., Irwin, A. J., Rosenthal, Y., Ho, T.-Y., Reinfelder, J. R., et al. (2003). The evolutionary inheritance of elemental stoichiometry in marine phytoplankton. *Nature* 425, 291–294. doi: 10.1038/nature01953

Raven, J. A., and Waite, A. M. (2004). The evolution of silification in diatoms: inescapable sinking and sinking as escape? *New Phytol.* 162, 45–61. doi: 10.1111/j.1469-8137.2004.01022.x

Redfield, A. C. (1958). The biological control of chemical factors in the environment. *Am. Sci.* 46, 205–221.

Rhee, G. Y. (1978). Effects of N:P atomic ratios nitrate limitation on algal growth, cell composition, nitrate uptake. *Limnol. Oceanogr.* 23, 10–25. doi: 10.4319/lo.1978.23.1.0010

Round, F. E., Crawford, R. M., and Mann, D. G. (1990). *The Diatoms: Biology and Morphology of the Genera*. Cambridge: Cambridge University Press.

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Teng, Y.-C., Primeau, F. W., Moore, J. K., Lomas, M. W., and Martiny, A. C. (2014). Global-scale variations of the ratios of carbon to phosphorus in exported marine organic matter. *Nat. Geosci.* 7, 895–898. doi: 10.1038/ngeo2303

Toseland, A., Daines, S. J., Clark, J. R., Kirkham, A., Strauss, J., Uhulig, C., et al. (2013). The impact of temperature on marine phytoplankton resource allocation and metabolism. *Nature* 3, 979–984. doi: 10.1038/nclimate1989

Urabe, J., Kyle, M., Makino, W., Yoshida, T., Andersen, T., and Elser, J. J. (2002). Reduced light increases herbivore production due to stoichiometric effects of light/nutrient balance. *Ecology* 83, 619–627. doi: 10.1890/0012-9658(2002)083[0619:RLIHPD]2.0.CO;2

Wang, Y., Tian, M. R., Gao, Z. M., Bougouffa, S., and Qian, P.-Y. (2014). Optimal eukaryotic 18S and universal 16S/18S ribosomal RNA primers and their application in a study of symbiosis. *PLoS ONE* 9:e90053. doi: 10.1371/journal.pone.0090053

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 October 2017
doi: 10.3389/fmicb.2017.02003

The Temperature Dependence of Phytoplankton Stoichiometry: Investigating the Roles of Species Sorting and Local Adaptation

Gabriel Yvon-Durocher[1], Charlotte-Elisa Schaum[1] and Mark Trimmer[2]*

[1] Environment and Sustainability Institute, University of Exeter, Penryn, United Kingdom, [2] School of Biological and Chemical Sciences, Queen Mary University of London, London, United Kingdom

The elemental composition of phytoplankton (C:N:P stoichiometry) is a critical factor regulating nutrient cycling, primary production and energy transfer through planktonic food webs. Our understanding of the multiple direct and indirect mechanisms through which temperature controls phytoplankton stoichiometry is however incomplete, increasing uncertainty in the impacts of global warming on the biogeochemical functioning of aquatic ecosystems. Here, we use a decade-long warming experiment in outdoor freshwater ponds to investigate how temperature-driven turnover in species composition and shifts in stoichiometric traits within species through local thermal adaptation contribute to the effects of warming on seston stoichiometry. We found that experimental warming increased seston C:P and N:P ratios, while the C:N ratio was unaffected by warming. Temperature was also the dominant driver of seasonal variation in seston stoichiometry, correlating positively with both C:P and N:P ratios. The taxonomic composition of the phytoplankton community differed substantially between the warmed and ambient treatments indicating that warming resulted in differential sorting of species from the regional pool. Furthermore, taxonomic composition also changed markedly over the year within each of the warmed and ambient treatments, highlighting substantial temporal turnover in species. To investigate whether local adaptation also played an important role in shaping the effects of warming on seston stoichiometry, we isolated multiple strains of the cosmopolitan alga, *Chlamydomonas reinhardtii* from across the warmed and ambient mesocosms. We found that warmed isolates had higher C:P and N:P ratios, shifts that were comparable in direction and magnitude to the effects of warming on seston stoichiometry. Our results suggest that both species sorting and local adaptation are likely to play important roles in shaping the effects of warming on bulk phytoplankton stoichiometry and indicate that major shifts in aquatic biogeochemistry should be expected in a warmer world.

Keywords: global warming, phytoplankton, stoichiometry, rapid evolution, species sorting

OPEN ACCESS

Edited by:
Adam Martiny, University of California, Irvine, United States

Reviewed by:
David Talmy, Massachusetts Institute of Technology, United States
George Isaac Hagstrom, Princeton University, United States

****Correspondence:***
Gabriel Yvon-Durocher
g.yvon-durocher@exeter.ac.uk

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 March 2017*
Accepted: *29 September 2017*
Published: *23 October 2017*

Citation:
Yvon-Durocher G, Schaum C-E and Trimmer M (2017) The Temperature Dependence of Phytoplankton Stoichiometry: Investigating the Roles of Species Sorting and Local Adaptation. Front. Microbiol. 8:2003. doi: 10.3389/fmicb.2017.02003

INTRODUCTION

The stoichiometry of carbon (C), nitrogen (N), and phosphorous (P) in phytoplankton biomass set important constraints on the biogeochemistry of aquatic ecosystems, shaping patterns of nutrient limitation (Elser et al., 2009; Bonachela et al., 2013; Alexander et al., 2015), recycling (Sterner and Elser, 2002), material transfer, and C sequestration in planktonic ecosystems (Galbraith and Martiny, 2015). Until recently it was assumed that the ratios of these elements were maintained in relatively fixed proportions (i.e., the Redfield ratio, C:N:P = 106:16:1) and exhibit tight coupling between organic and inorganic pools (Geider and La Roche, 2002). It is now widely recognized that the C:N:P stoichiometry of phytoplankton is highly variable across multiple spatial, temporal and organizational scales (Geider and La Roche, 2002). Such variation has been linked to directional changes in abiotic factors (e.g., light, CO_2 and nutrients), with temperature often cited as a key determinant of phytoplankton stoichiometry (Woods et al., 2003; Hessen, 2005; Martiny et al., 2013; Toseland et al., 2013; Yvon-Durocher et al., 2015b). However, despite much recent progress, we still lack a detailed understanding of the multiple direct and indirect mechanisms through which temperature controls phytoplankton stoichiometry across scales of time, space and biological organization, limiting our ability to forecast impacts of global warming on macronutrient cycles.

The elemental stoichiometry of a phytoplankton cell is the result of resource allocation to different subcellular constituents that vary in their C, N, and P content and together determine the cell's macromolecular composition (Shuter, 1979; Daines et al., 2014). For example, polysaccharides, lipids and carbohydrates are major sinks for C allocation; proteins represent the major fraction of the cell's investment of N, while ribosomal RNA and phospholipids account for a large part of P allocation (Geider and La Roche, 2002). Temperature is a key driver phytoplankton metabolism (Raven and Geider, 1988; Thomas et al., 2012; Sal et al., 2015; Padfield et al., 2016) and recent work suggests that changes in temperature alter the cell's optimal allocation to C, N and P pools via phenotypic plasticity (i.e., acclimation; Toseland et al., 2013; Daines et al., 2014). The "*temperature-dependent physiology*" hypothesis predicts that organisms growing at higher temperatures should have higher N:P ratios because they require fewer P-rich ribosomes, relative to N-rich proteins, to sustain growth and maintenance (Woods et al., 2003; Toseland et al., 2013; Yvon-Durocher et al., 2015b). In phytoplankton, such a shift could occur if the rates of photosynthesis by N-rich photosynthetic proteins exhibit weaker temperature dependence than protein synthesis by P-rich ribosomes (Yvon-Durocher et al., 2015b). A recent meta-analysis of temperature manipulation experiments on 9 species of marine and freshwater phytoplankton demonstrated a positive association between temperature and C:P and N:P ratios, but not C:N, suggesting that, in support of the "*temperature-dependent physiology*" hypothesis, changes in cellular stoichiometry were attributable to declines in P content as cells acclimate to warmer temperatures (Yvon-Durocher et al., 2015b). Furthermore, direct measurements of cellular allocation to RNA in chlorophytes and diatoms have also revealed rapid declines as populations acclimate to warmer growth temperatures (Toseland et al., 2013; Hessen et al., 2017). Evidence in support of the "*temperature-dependent physiology*" hypothesis is not unequivocal however, with acclimation experiments on the marine cyanobacterium *Prochlorococcus* revealing that increases in C:P and N:P at higher temperatures were driven by elevated C and N contents, rather than declines in P, in warm acclimated cells (Martiny et al., 2016a). Nevertheless, the current weight of evidence indicates that physiological plasticity in response to rapid changes in temperature can cause substantial shifts in algal stoichiometry within species that are at least as large as those observed across species, irrespective of the underlying molecular and biochemical mechanisms.

Phytoplankton stoichiometry exhibits substantial variation among the major lineages, presumably reflecting their divergent evolutionary histories (Quigg et al., 2003; Litchman et al., 2007; Litchman and Klausmeier, 2008; Finkel et al., 2009). Red lineage algae, which include the diatoms and coccolithophores and dominate the eukaryotic contribution to contemporary global marine primary production, have relatively low N:P ratios (Quigg et al., 2003). In contrast, green lineage algae, which include the chlorophytes and prasinophytes that dominated ocean productivity in the Proterozoic and Paleozoic, often have relatively high N:P ratios (Quigg et al., 2003). Thus, owing to the substantial differences in stoichiometric traits that exist among phytoplankton taxa, environmental filtering of species along thermal gradients has the potential to drive variation in the bulk stoichiometry of seston when species-level selection is systematic with respect to temperature and elemental stoichiometry (Martiny et al., 2016b).

Rapid evolutionary responses to directional environmental change could also play an important role in shaping the effects of warming on phytoplankton stoichiometry and biogeochemical macronutrient cycling. Indeed, recent work experimentally evolving both marine and freshwater phytoplankton under future temperature and CO_2 scenarios have shown that adaptation can be rapid (<1 year or a few hundred phytoplankton generations) and often involves changes in stoichiometric traits (e.g., C:N; Schlueter et al., 2014; Schaum et al., 2016). However, experiments explicitly investigating rapid evolutionary shifts in phytoplankton C:N:P stoichiometry in response to warming are currently lacking. We therefore have limited understanding of the direction, magnitude and tempo over which stoichiometric traits might evolve as phytoplankton adapt to warming and consequently, the contribution of rapid evolution to changes in biogeochemical cycles.

Here, we use a decade-long warming experiment in outdoor freshwater ponds to investigate the interplay between species sorting and rapid evolution in shaping the effects of temperature on phytoplankton stoichiometry. We first present a detailed analysis of both seasonal changes in phytoplankton stoichiometry within ponds as well as the long-term differences between treatments attributable to experimental warming. We then assess the role of local adaptation by quantifying changes in C:N:P stoichiometry in strains of the cosmopolitan alga, *Chlamydomonas reinhardtii*, isolated from both our ambient and

warmed ponds. We have previously shown that *C. reinhardtii* strains isolated from the warmed and ambient treatments are locally adapted to the different thermal regimes imposed by experimental warming and exhibit fitness trade-offs when reciprocally transplanted in the warmed and ambient treatments (Schaum et al., 2017). Based on work in both marine and freshwater systems over latitudinal and temporal (seasonal) thermal gradients, we expect the C:P and N:P ratios of the seston in our experiment to increase with warming and exhibit positive seasonal temperature dependence (Hessen, 2005; Martiny et al., 2013; Yvon-Durocher et al., 2015b). Given the highly dynamic nature of phytoplankton communities, we hypothesize that seasonal variation and treatment effects on bulk stoichiometry will emerge from temperature driven turnover in the taxonomic composition of the algal assemblages. However, we also predict that if temperature driven adjustments in sub-cellular allocation to C, N and P pools that increase C:P and N:P in warmer environments (as expected under the "*temperature-dependent physiology*" hypothesis) also increase fitness, then they will be reinforced through evolutionary adaptation. Consequently, we hypothesize that isolates of the cosmopolitan alga, *C. reinhardtii*, that have adapted to the thermal regimes in the warmed mesocosms will have higher C:P and N:P ratios than their counterparts from the ambient treatments.

MATERIALS AND METHODS

Mesocosm Experimental Design

The mesocosm facility was established in 2005 and consists of 20 artificial ponds of $\sim$1 m^3 volume, 50 cm depth, sited in southern England (Freshwater Biological Association Rivers Laboratory, East Stoke, 2°10′W, 50°13′N), designed to be broadly representative of mid-latitude shallow lakes (Yvon-Durocher et al., 2010). Warming of 4–5°C above ambient began in half of the ponds in 2006 by maintaining a constant differential between thermocouples in a pair of warmed and ambient ponds. The ponds contain well-established benthic and pelagic communities including assemblages of macrophytes, phytoplankton, algal biofilms, and invertebrates; for a detailed description of the community composition see previous publications from this facility (Yvon-Durocher et al., 2010, 2015a; Dossena et al., 2012). Sediments are comprised of 8–10 cm of fine sands with a developed organic layer of 1–3 cm (**Table 2**).

Phytoplankton Sampling

The plankton community in each of the mesocosms was sampled every 2 months between July 2011 and May 2012 (6 sampling occasions in total). The phytoplankton composition data presented here are reanalyzed from those in Yvon-Durocher et al. (2015a). The entire water column from the sediment surface to the water surface was sampled using a 0.8 m-length tube sampler (Volume: 2 L), which was positioned at random in each mesocosm on each date. Each sample was passed through a 100 μm aperture sieve to remove the zooplankton. A 100 mL sub-sample of <100 μm fraction was preserved in 1% Lugol's iodine for microscope analysis of the phytoplankton community composition, while the remaining material was filtered through a pre-ashed, Whatman GF/F filter (0.7 μm nominal pore size) in duplicate and then immediately frozen at −20°C prior to analysis of particulate nutrients.

Seston Stoichiometry

Filters (GF/F) were dried for 48 h at 60°C. The dry weight of particulate matter on the filter was calculated and used to standardize by the sample mass in further analyses. One of the two filters was acidified (1 M HCl) to remove carbonates and used for the analysis of particulate organic carbon and nitrogen using a Sercon 20-22 IRMS. The other was used for determining particulate organic phosphorous on a segmented flow auto-analyser (Skalar, San++, Breda, Netherlands) following complete oxidation with potassium persulfate.

Taxonomic Characterization of the Phytoplankton Community

Phytoplankton <100 μm were counted using a LEICA DMIRB inverted microscope at 400x magnification, following the Utermöhl method. The microscope was connected to an interactive image analysis system (LEICA EC3 camera and LAS software) to allow for a higher magnification. For each sample, at least 400 individuals (single cell, colony or filament) were counted, measured and identified. Counts were converted to volumetric estimates of abundance (organisms mL^{-1}) based on the volume of sample analyzed, which varied between 1 and 25 mL depending on the density of organisms. In total, 171 taxa were identified, 85% of which were identified to species level; the remaining 15% were identified to genus or class, or were undetermined.

Measuring Gross Primary Production

Rates of gross primary production (GPP) were measured over a 24 h diel cycle for each replicate mesocosm on the sampling months described above using the free water dissolved oxygen (DO) change technique (Staehr et al., 2010). Measurements of DO and temperature were taken every 15 min for 24 h at mid-depth (0.25 m) in the water column of each pond with YSI 600XLM multi-parameter Sondes, equipped with 6562 rapid pulse^{TM} dissolved oxygen sensors. Light intensity was measured using a Licor spherical quantum sensor (LI-193, Licor USA) positioned at mid-depth (0.5 m) in the water column of a single ambient mesocosm in the center of the pond array. These measurements allow for quantification of seasonal variation in light intensity but do not allow us to quantify potential differences among treatments in light penetration. Light intensity was measured every minute and logged as 15-min averages using a Licor LI-1500 data logger. Prior to deployment, the multi-parameter Sondes were calibrated in water-saturated air with a correction for barometric pressure. Calibration accuracy was verified by monitoring the DO concentration of water-saturated air for 10 min and checking against 100% O_2 saturation for the measured temperature and pressure. Measurements of DO, wind speed at 1.7 m (Cole-Parmer, WS-821), and light intensity at mid-depth in the water column were used to calculate GPP and R_{eco} following the methods outlined in Staehr et al. (2010).

Isolation and Characterization of *Chlamydomonas reinhardtii*

We isolated *Chlamydomonas*, a known cosmopolitan and highly abundant genus in the heated and ambient mesocosms (Yvon-Durocher et al., 2015a), by first passing water through a 45 μm and then a 20 μm filter, followed by serial dilution and streaking the isolate on to agar-filled pipette tips turned toward a light source (enabling identification and isolation of motile autotrophs). Organisms putatively identified as *Chlamydomonas* were then grown on agar plates infused with Bold's Basal Medium (BBM). Colonies were then picked under a microscope, transferred back into liquid culture (autoclaved, filtered water taken from rainwater holding tanks at the mesocosm experiment supplemented with BBM at 1/3 the standard concentration) and kept at 18°C (the average daytime temperature across treatments at the time of sampling) for 2 weeks in semi-continuous batch-culture. This yielded concentrations of NO_3^- at 1,000 μmol L^{-1} and PO_4^{3+} at 330 μmol L^{-1}. The rationale here was to grow the isolates under nutrient replete conditions (concentrations of N and P that were much larger than observed in the mesocosms) so (i) rates of growth and biomass yields were sufficient to quantify physiological traits, and (ii) the observed cellular stoichiometry would not depend on the availability of nutrients in the medium. Taxonomy of *Chlamydomonas* was confirmed by microscopy and using PCR followed by Sanger sequencing within the 18S sequence using a set of primers with forward sequence GAAG TCGTAACAAGGTTTCC and reverse sequence TCCTGGTTA GTTTCTTTTCC. Positive controls were run using p23 primers amplifying in the RuBisCO region, with forward GGACAGAAA GACCCTATGAA and reverse TYAGCCTGTTATCCCTAGAG. This yielded 18 out of the 20 isolates with an at least 99% BLAST match for *C. reinhardtii,* 8 from heated and 10 from ambient mesocosms. These were used throughout the experiments and the other 2 samples were discarded.

To determine intracellular C, N, and P content, *Chlamydomonas* cultures were grown to exponential phase and the cells counted. A total of 200 mL were spun down at 2,500 r.p.m, the supernatant decanted and the samples frozen immediately in liquid nitrogen until further analysis. Samples for CN content were freeze-dried, weighed out into tin capsules, and analyzed for C and N content using a Sercon 20-22 IRMS. Cell P content was determined via a colorimetric reaction on a Seal Analytics AA3 flow analyser. Pellets were washed in 0.17 M Na_2SO_4, transferred to scintillation vials and re-suspended in 4 ml 0.017 M $MnSO_4$. The samples were transferred to an autoclave (1 h, 121°C), shaken vigorously and centrifuged at 2,500 r.p.m for 30 min, the pellet discarded, and the supernatant brought to 10 ml with MillliQ purified water. The samples were immediately analyzed on the AA3 using the colorimetric molybdate/antimony method after (Murphy and Riley, 1958).

Dissolved Inorganic Nutrients

Water samples for measuring dissolved inorganic nutrient concentrations were collected from mid-depth in each mesocosm at 9 a.m. on each sampling occasion. Samples were filtered (Whatmann GF/F) and stored frozen at −20°C for subsequent determination of NO_3^-, NO_2^-, NH_4^+, and soluble reactive phosphorous (SRP) using a segmented flow auto-analyser (Skalar, San++, Breda, Netherlands) and the methods of Kirkwood (1996).

Statistical Analyses

Seasonal changes in biotic and abiotic variables often exhibit highly non-linear patterns of change, particularly in temperate regions. We therefore used generalized additive mixed effects models (GAMMs) to characterize the seasonal trends and overall treatment effects on temperature, light intensity, GPP, dissolved inorganic nutrients, particulate organic nutrients, and seston stoichiometry. GAMMs do not prescribe any particular functional form for the trend; rather its shape is estimated from the data using penalized regression. GAMMs further account for hierarchical data structures (Zuur et al., 2009). For example, our experimental design yielded replicate seasonal responses for each variable in each treatment. This hierarchical structure meant that measurements were non-independent—e.g., measurements from the same pond will be autocorrelated. We account for this by treating replicate pond as a random effect on the intercept of the model, which models deviations among ponds from the fixed effects as normally distributed with a mean of zero. The full models were specified as follows

$$\begin{aligned} y_{pt} &= \beta + \alpha_p + \mathrm{Treat}_t + f_{\mathrm{Treat}}\left(\mathrm{DOY}_t\right) + \varepsilon_{pt} \\ \varepsilon_{pt} &= N\left(0, \sigma^2\right) \\ \alpha_p &= N(0, \sigma^2) \end{aligned} \tag{1}$$

where y_{pt} is the response variable in pond, p, and time, t, β is the intercept, which characterizes the median value of the response variable, "Treat" captures differences in the intercept between treatments (e.g., "warmed" or "ambient"), α_p is a random effect that characterizes deviations among replicate ponds from the intercept, which we assume are normally distributed with a mean of zero and a variance, σ^2. The seasonal smooth function, $f_{\mathrm{Treat}}(\mathrm{DOY}_t)$, uses a cubic regression spline to model the seasonal trend in the response variable y, which is allowed to vary between warmed and ambient treatments. The model residuals, ε_{pt}, are assumed to be drawn from a normal distribution with a mean of zero and a variance, σ^2. Model selection entailed fitting a range of models to the data, starting with the full model and then a series of reduced models with interaction terms (e.g., different seasonal smooth functions for each treatment) and main effects removed to test hypotheses about the potential differences in seasonal changes in the response variables among treatments. For multi-model selection we computed small sample-size corrected AIC scores (AICc) and then compared between models by calculating delta AICc values and AIC weights using the "MuMIn" package. When candidate models deviated from the most parsimonious model (that with the lowest AICc score) by less than two AICc units, parameters were averaged across those candidate models using the "model.avg" function in the "MuMIn" package. The relative importance of the fixed factors in the averaged model was determined using the sum of their relative weights. GAMMs were

fitted to the data using the "gamm4" package and were conducted in R (v.3.23).

To assess the relative importance of putative abiotic drivers in shaping seasonal variation in seston stoichiometry, we fitted each stoichiometric ratio to the seasonal changes in temperature, light intensity, dissolved inorganic nitrogen (DIN), and SRP in a multiple regression mixed effects model using the "lme" function in the "nlme" package for R.

$$\begin{aligned} \ln(R_{ip}) &= \beta_0 + \alpha_p + \beta_1 T_{ip} + \beta_2 \ln\left(I_{ip}\right) + \beta_3 \ln\left(DIN_{ip}\right) \\ &\quad + \beta_4 \ln\left(SRP_{ip}\right) + \varepsilon_{ip} \\ \varepsilon_{ip} &= N\left(0, \sigma^2\right) \\ \alpha_p &= N(0, \sigma^2) \end{aligned} \tag{2}$$

where $\ln(R_{ip})$ is the natural logarithm of the ith observation of the stoichiometric ratio in pond p, β_0 is the intercept and α_p is a random effect that characterizes deviations among replicate ponds from overall the intercept, which we assume are normally distributed with a mean of zero and a variance, σ^2. The slope coefficients, $\beta_{1...n}$ characterize the response of $\ln(R_{ip})$ to the to the various predictor variables. The model residuals, ε_{ip}, are assumed to be drawn from a normal distribution with a mean of zero and a variance, σ^2. We natural logarithm transformed the stoichiometric ratios, light intensity, DIN and SRP to linearize all relationships and ensure data were normally distributed prior to statistical analyses. We tested for multi-colinearity by calculating the variance inflation factors (VIF) for each predictor. In each case VIFs were <2.5 indicating that multi-colinearity was low. As for the GAMM analyses, model selection entailed fitting a range of models to the data, starting with the full model (Equation 2) and then a series of reduced models with predictors removed to test hypotheses about the dominant abiotic drivers of seasonal changes in the stoichiometric ratios. Model selection and model averaging was conducted in the same way as described above for the GAMM analyses.

Variation in the taxonomic composition of the phytoplankton communities between treatments and among sampling months was indexed as the "score" for each mesocosm along the first axis of a non-metric multidimensional scaling (NMDS) ordination. NMDS ordination was conducted on each sampling month using the "metaMDS" function in the "vegan" package in R based on a Bray–Curtis dissimilarity matrix derived from $\log_{10}$ (X+1)-transformed total abundances of the taxa in each mesocosm. NMDS projected this matrix into a new coordinate space with a

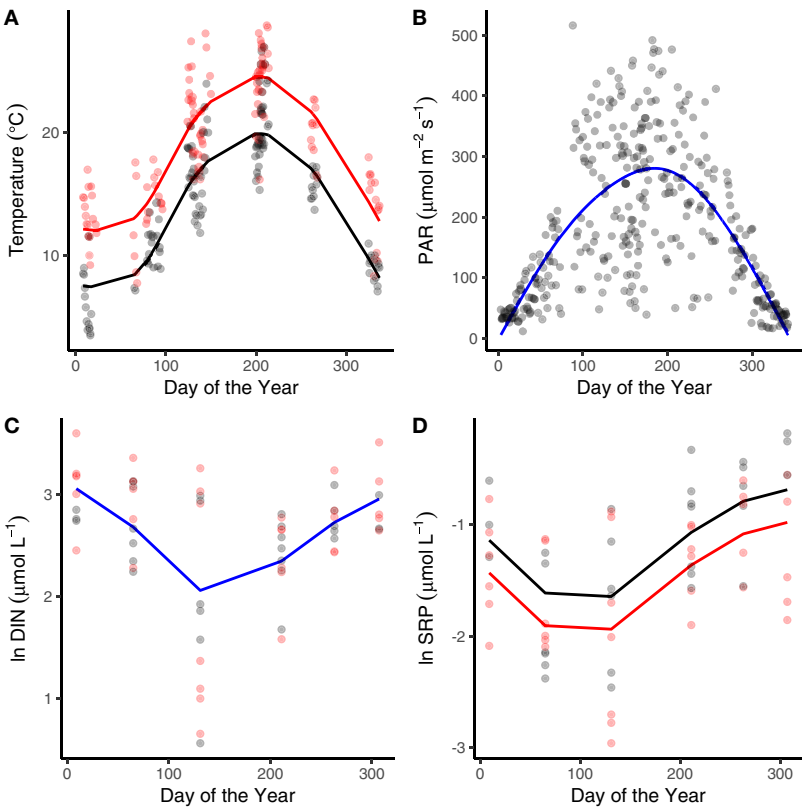

FIGURE 1 | Seasonal variation in abiotic variables. Seasonal changes and treatment effects on **(A)** average daily temperature, **(B)** average daily light intensity, **(C)** dissolved inorganic nitrogen, and **(D)** soluble reactive phosphorous (SRP). Black denotes ambient treatments, red indicates warmed treatments. Fitted lines are from the fixed effects of the best fitting mixed effects models. Where red and black fitted lines are present warmed and ambient treatments differed in either the median value and/or the seasonality of the response variable. Where the fitted line is blue a single function provided the best fit to the data from both treatments.

small number of dimensions (in this case, 10) while preserving the original Bray–Curtis dissimilarities among samples to the extent possible. Orthogonal rotation was applied to the axes in this new coordinate space so as to maximize the variance in "scores" among samples along the first NMDS axis. Thus, samples with more similar scores along the first NMDS axis are more similar to each other with respect to the dominant gradient in taxonomic composition. We used Permutational Multivariate Analysis of Variance (PERMANOVA) to test whether Bray–Curtis dissimilarities between treatments, months and their interaction were significant.

Because we were interested in assessing the relative importance of species sorting in shaping the seasonal variation and effects of experimental warming on seston stoichiometry, we used the methods described in Baselga and Orme (2012) to partition beta-diversity into its turnover and nested components. We quantify beta-diversity (e.g., spatial or temporal differences in taxonomic composition based on presence-absence data) using the Sorensen's Index (β_{sor}), which can be partitioned into components attributable to species turnover (e.g., spatial or temporal replacement of species, β_{turn}) and nestedness (e.g., where different sites or time points have species compositions that are nested subsets, β_{nes}).

$$\beta_{sor} = \beta_{turn} + \beta_{nes} \equiv \frac{b}{b+a} + \left(\frac{c-b}{2a+b+c}\right)\left(\frac{a}{b+a}\right) \quad (3)$$

where a is the number of species shared between two sites (or distinct time points), b is the number species unique to the poorest site and c is the number of species unique to the richest site. We then estimated the fraction of total beta-diversity attributable to turnover (e.g., spatial or temporal replacement of species) as $\beta_{frac} = \beta_{turn}/\beta_{sor}$. Using these metrics, we calculated two forms of beta-diversity. First, for each sampling month we estimated spatial beta-diversity as pond-to-pond differences in taxonomic composition, partitioning out variation among ambient, heated and ambient vs. heated ponds. Second, we estimated temporal beta-diversity for each replicate pond by assessing variation in taxonomic composition among sampling months. For both spatial and temporal beta-diversity we calculated $\beta_{frac} = \beta_{turn}/\beta_{sor}$ to assess the relative importance of taxonomic turnover between the warmed and ambient treatments as well as over seasonal variation within mesocosms.

RESULTS

Seasonal Variation and Treatment Effects on Abiotic Variables

The abiotic variables measured in the mesocosms showed characteristic seasonality, with temperature and surface water light intensity reaching maxima in July and minima in January (**Figures 1A,B**). The heated mesocosms were, on average, 4.1°C (±0.7°C) warmer than their ambient counterparts over the entire year (**Figure 1A**). The seasonality of DIN and SRP reflected drawdown in the spring and early summer, followed by regeneration through autumn and winter (**Figures 1C,D**).

TABLE 1 | Multi-model selection on generalized additive mixed effects models fitted to the seasonal data.

Model	*Df*	logLik	AICc	ΔAICc	Weight
TEMPERATURE					
T1 = treat + s(DOY)	**6.00**	**−156.54**	**326.54**	**0.00**	**1.00**
T0 = treat + s(DOY, by = treat)	8.00	−160.86	340.29	13.76	0.00
T3 = s(DOY)	5.00	−165.54	342.10	15.56	0.00
T2 = s(DOY, by = treat)	7.00	−169.84	355.65	29.11	0.00
DISSOLVED INORGANIC NITROGEN					
DIN3 = s(DOY)	**5.00**	**−54.06**	**119.13**	**0.00**	**0.74**
DIN1 = treat + s(DOY)	6.00	−53.95	121.34	2.21	0.25
DIN2 = s(DOY, by = treat)	7.00	−55.75	127.46	8.33	0.01
DIN0 = treat + s(DOY, by = treat)	8.00	−55.59	129.74	10.61	0.00
SRP					
SRP1 = treat + s(DOY)	**6.00**	**−51.69**	**116.82**	**0.00**	**0.73**
SRP3 = s(DOY)	5.00	−54.17	119.35	2.52	0.21
SRP0 = treat + s(DOY, by = treat)	8.00	−51.89	122.36	5.53	0.05
SRP2 = s(DOY, by = treat)	7.00	−54.47	124.90	8.08	0.01
GROSS PRIMARY PRODUCTION					
GPP1 = treat + s(DOY)	**6.00**	**−54.11**	**121.66**	**0.00**	**0.77**
GPP3 = s(DOY)	5.00	−56.64	124.31	2.64	0.21
GPP0 = treat + s(DOY, by = treat)	8.00	−55.29	129.14	7.48	0.02
GPP2 = s(DOY, by = treat)	7.00	−57.46	130.88	9.21	0.01
PARTICULATE CARBON					
PC3 = s(DOY)	**5.00**	**−56.31**	**123.63**	**0.00**	**0.89**
PC1 = treat + s(DOY)	6.00	−57.27	128.00	4.36	0.10
PC2 = s(DOY, by = treat)	7.00	−58.46	132.89	9.26	0.01
PC0 = treat + s(DOY, by = treat)	8.00	−59.36	137.28	13.65	0.00
PARTICULATE NITROGEN					
PN3 = s(DOY)	**5.00**	**−68.19**	**147.40**	**0.00**	**0.81**
PN1 = treat + s(DOY)	6.00	−68.90	151.24	3.84	0.12
PN2 = s(DOY, by = treat)	7.00	−68.28	152.53	5.13	0.06
PN0 = treat + s(DOY, by = treat)	8.00	−68.99	156.55	9.15	0.01
PARTICULATE PHOSPHOROUS					
PP3 = s(DOY)	**5.00**	**−51.52**	**114.06**	**0.00**	**0.54**
PP1 = treat + s(DOY)	**6.00**	**−50.81**	**115.06**	**1.01**	**0.33**
PP2 = s(DOY, by = treat)	7.00	−50.82	117.61	3.56	0.09
PP0 = treat + s(DOY, by = treat)	8.00	−50.32	119.20	5.15	0.04
C:N RATIO					
CN3 = s(DOY)	**5.00**	**−61.44**	**133.91**	**0.00**	**0.74**
CN1 = treat + s(DOY)	6.00	−61.32	136.09	2.18	0.25
CN2 = s(DOY, by = treat)	7.00	−63.25	142.46	8.55	0.01
CN0 = treat + s(DOY, by = treat)	8.00	−63.12	144.82	10.91	0.00
C:P RATIO					
CP1 = treat + s(DOY)	**6.00**	**−70.76**	**154.96**	**0.00**	**0.63**
CP3 = s(DOY)	**5.00**	**−72.51**	**156.03**	**1.08**	**0.37**
CP0 = treat + s(DOY, by = treat)	8.00	−73.53	165.63	10.68	0.00
CP2 = s(DOY, by = treat)	7.00	−75.33	166.63	11.67	0.00
N:P RATIO					
NP3 = s(DOY)	**5.00**	**−67.60**	**146.22**	**0.00**	**0.54**

(Continued)

TABLE 1 | Continued

Model	*Df*	logLik	AICc	ΔAICc	Weight
NP1 = treat + s(DOY)	**6.00**	**−66.58**	**146.61**	**0.39**	**0.44**
NP2 = s(DOY, by = treat)	7.00	−68.78	153.52	7.31	0.01
NP0 = treat + s(DOY, by = treat)	8.00	−67.78	154.14	7.92	0.01

A range of models testing hypotheses on the effects of the warming treatment ("treat") were fitted to the seasonal data; "treat" assess differences in median values warmed and ambient treatments, while comparisons between s(DOY) and s(DOY, by = treat) assess whether the shape of the seasonality differs among treatments. Models were compared via the small sample size corrected Akaike Information Criterion (AICc), delta AICc is the difference in AICc score relative to the model with the lowest value (most parsimonious model) and AICc Weight (Wt) is the relative support for the model. The best fitting models were selected as those returning the lowest AICc score and the highest AICc weight and are highlighted in bold. Where two models differed in <2 AICc units we averaged the coefficients between those models.

Median SRP levels over the year were lower in the warmed treatments (**Figure 1D**; **Table 1**).

Seasonal Variation in Primary Production, Particulate Nutrients and Seston Stoichiometry

Patterns in GPP and phytoplankton nutrient content reflected variation in the abiotic variables. GPP peaked in July in both the warmed and ambient treatments when temperatures and light levels were maximal (**Figure 2A**; **Table 1**). Rates of GPP were also elevated in the warmed treatments across all sampling occasions (**Figure 2A**; **Table 1**). Seston carbon content peaked in July, in alignment with the maximal rates of GPP, but exhibited no difference between the warmed and ambient treatments (**Figure 2B**; **Table 1**). The nitrogen and phosphorous content of

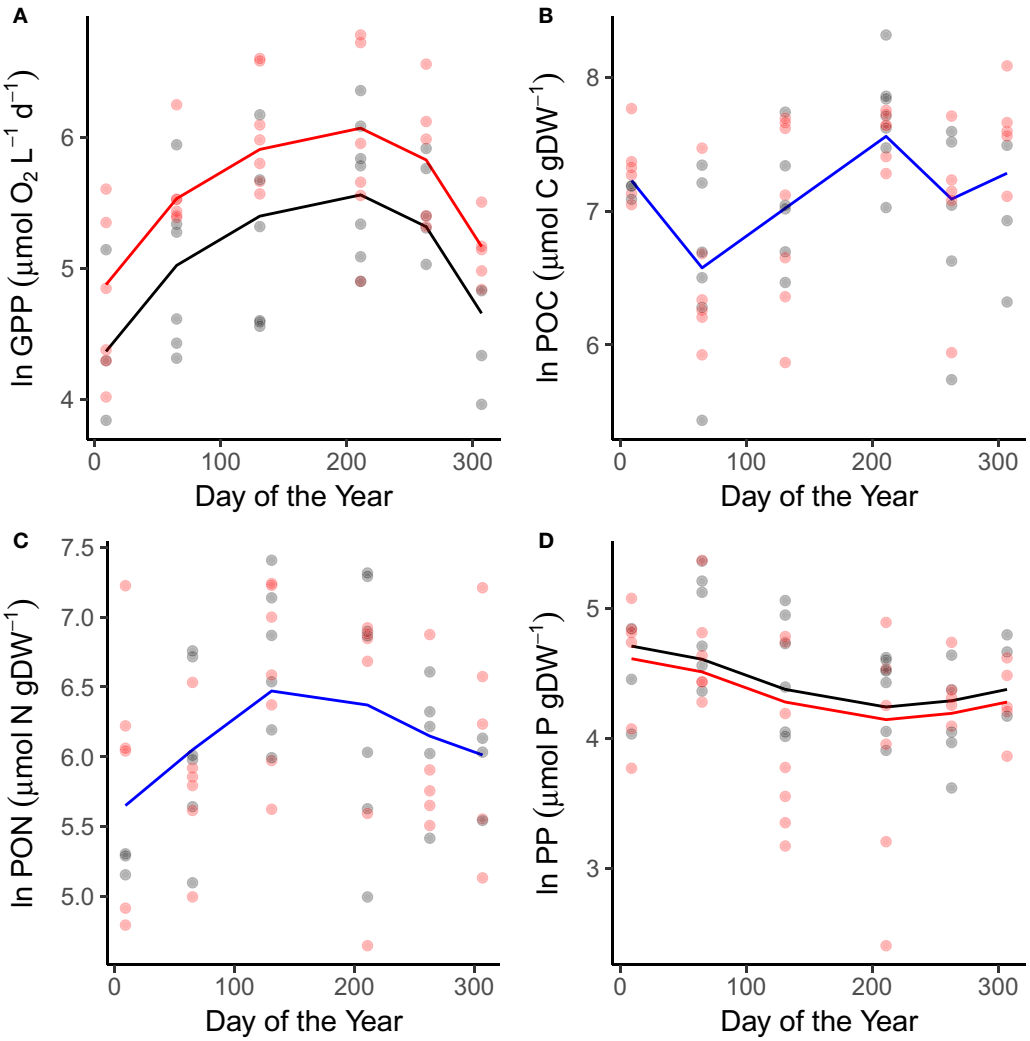

FIGURE 2 | Seasonal variation in primary production and particulate nutrients. Seasonal changes and treatment effects on **(A)** gross primary production, **(B)** particulate organic carbon, **(C)** particulate organic nitrogen, and **(D)** particulate organic phosphorous. Fitted lines are from the fixed effects of the best fitting mixed effects models. Where red and black fitted lines are present warmed and ambient treatments differed in either the median value and/or the seasonality of the response variable. Where the fitted line is blue a single function provided the best fit to the data from both treatments.

the phytoplankton peaked in May and March respectively, in line with the peaks in DIN and SRP drawdown (**Figures 2C,D**; **Table 1**). Seston phosphorous content was lower on average in the warmed treatments (**Figure 2D**; **Table 1**).

C:N, C:P and N:P ratios all exhibited seasonal variation (**Figure 3**; **Table 1**). C:N ratios were highest in winter and declined through spring and summer (**Figure 3A**; **Table 1**). C:P and N:P ratios exhibited the opposite seasonal trends, peaking in the spring and summer and declining through autumn and winter (**Figures 3B,C**; **Table 1**). In line with our predictions, both C:P and N:P ratios were elevated in the warmed treatments (**Figures 3B,C**; **Table 1**) while the C:N ratio was consistent between treatments (**Figure 3A**; **Table 1**).

Abiotic Drivers of Seston Stoichiometry

To investigate the factors shaping the seasonal variation in seston stoichiometry we fitted the data for each elemental ratio to the measured abiotic drivers (temperature, light, DIN, and SRP) using multiple regression in a mixed effects modeling framework. The best fitting model for the C:N ratio included temperature, light, and SRP as predictors (**Table 2**). C:N ratios were negatively related to light intensity and positively correlated with temperature and SRP (**Figure 4A**). Temperature, light, DIN, and SRP were all retained as predictors of the C:P ratio in the best fitting model (**Table 2**), though support for the inclusion of SRP and DIN were weak (i.e., they had low summed weights after model averaging, see **Table 2**). The C:P ratio was positively correlated with temperature, and negatively related to light (**Figure 4B**). For the N:P ratio, temperature, light and DIN were all retained in the most parsimonious model (**Table 2**). The N:P ratio was positively correlated with temperature and light, and negatively related to DIN (**Figure 4C**). Consistent with our hypotheses, temperature was an important predictor of all the stoichiometric ratios and was the most important predictor for the C:P and N:P ratio (i.e., it had the highest summed weight after model averaging; see **Table 2**).

Seasonal Variation and Treatment Effects on Phytoplankton Community Composition

The effects of seasonal variation in temperature and the other abiotic variables, as well as the effect of experimental warming on the bulk stoichiometry of the phytoplankton, will be mediated by a combination of factors, including (i) physiological acclimation of cellular stoichiometry within species; (ii) evolutionary change in response to the long-term warming treatment; (iii) environmentally driven species sorting; both in response to seasonal changes in temperature within ponds as well as the long-term temperature differential maintained between the warmed and ambient treatments. To assess the potential importance of species sorting and temperature-dependent variation in phytoplankton community composition on seston stoichiometry we quantified the taxonomic composition and relative abundance of the phytoplankton communities in the mesocosms on 6 sampling occasions over an annual cycle. We found significant variation in phytoplankton community composition both among treatments [**Figure 5A**; PERMANOVA, $F_{(1,\ 89)} = 9.2$; $P < 0.001$] as well as across sampling occasions [**Figure 5A**; PERMANOVA, $F_{(5,\ 89)} = 2.3$; $P < 0.001$],

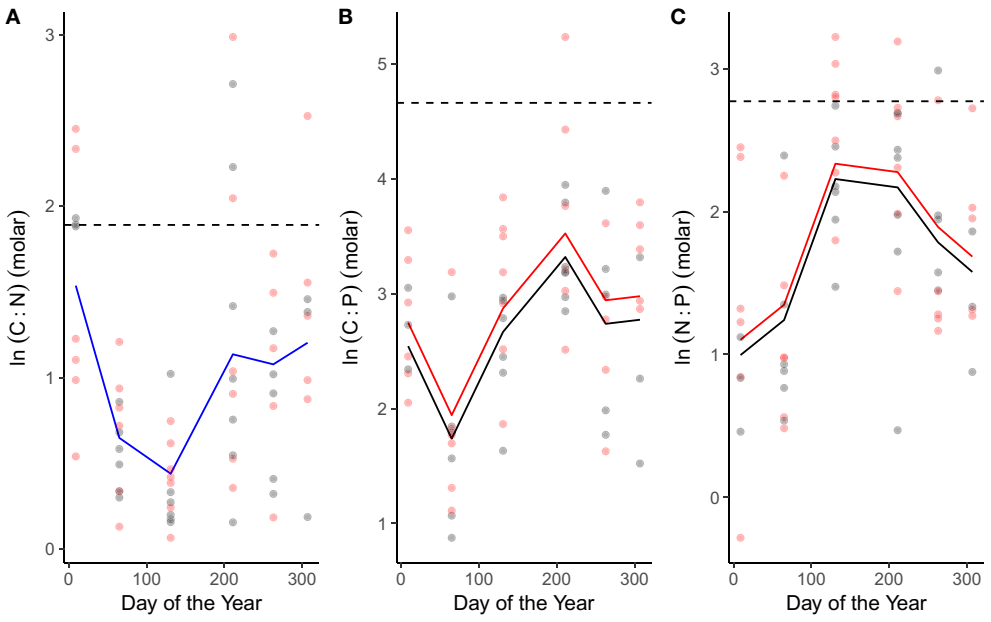

FIGURE 3 | Seasonal variation in phytoplankton stoichiometry. Seasonal changes and treatment effects on **(A)** the C:N ratio, **(B)** C:P ratio, and **(C)** N:P ratio. Fitted lines are from the fixed effects of the best fitting mixed effects models. Where red and black fitted lines are present warmed and ambient treatments differed in either the median value and/or the seasonality of the response variable. Where the fitted line is blue a single function provided the best fit to the data from both treatments. Dashed lines indicate Redfield ratios.

TABLE 2 | Model selection on multiple regression mixed effects models fitted to investigate abiotic drivers of seston stoichiometry.

Model	*Df*	logLik	AICc	ΔAICc	Weight
C:N RATIO					
PAR+SRP	5.00	−63.43	137.88	0.00	0.27
PAR+ SRP +Temp	6.00	−62.28	138.00	0.12	0.26
PAR+Temp	5.00	−64.01	139.03	1.15	0.15
PAR	4.00	−65.32	139.30	1.42	0.13
	PAR	**SRP**	**Temp**		
⟨β⟩	−0.39	0.24	0.03		
SW	1.00	0.65	0.50		
C:P RATIO					
PAR+Temp	5.00	−75.67	162.36	0.00	0.22
PAR+ SRP +Temp	6.00	−74.82	163.09	0.73	0.15
DIN+PAR+Temp	6.00	−74.83	163.11	0.75	0.15
DIN+PAR+ SRP +Temp	7.00	−73.58	163.13	0.77	0.15
SRP +Temp	5.00	−76.16	163.33	0.97	0.14
Temp	4.00	−77.39	163.45	1.09	0.13
	PAR	**Temp**	**SRP**	**DIN**	
⟨β⟩	−0.38	0.08	0.11	−0.08	
SW	0.72	1.00	0.47	0.32	
N:P RATIO					
Temp	4.00	−68.89	146.44	0.00	0.30
DIN+Temp	5.00	−67.82	146.66	0.22	0.27
PAR+Temp	5.00	−68.35	147.71	1.27	0.16
	Temp	**DIN**	**PAR**		
⟨β⟩	0.07	−0.07	0.04		
SW	1.00	0.37	0.22		

Models including all possible combinations of light (PAR) temperature (Temp), dissolved inorganic nitrogen (DIN), and soluble reactive phosphorous (SRP) as predictors of the stoichiometric rations were compared via the small sample size corrected Akaike Information Criterion (AICc), where delta AICc is the difference in AICc score relative to the model with the lowest value (most parsimonious model) and AICc Weight (Wt) is the relative support for the model. The best fitting models were selected as those returning the lowest AICc score and the highest AICc weight. Where models differed by <2 AICc units we averaged the coefficients between those models. All models with delta AICc < 2 are given in the table above with model averaged coefficients, ⟨β⟩ and the relative importance of each parameter, given by SW, which ranges from 0 (no models in the final set retain the parameter) to 1 (all models in the final set retain the parameter).

based on Bray–Curtis dissimilarities. To determine the extent to which pond-to-pond and month-to-month differences in species composition (e.g., spatial and temporal beta-diversity respectively) were driven by species replacements vs. species losses, we quantified the relative proportion of total beta-diversity attributable to taxonomic turnover and nestedness (Baselga and Orme, 2012). On all sampling months, spatial beta-diversity was driven primarily by turnover in species composition among ponds, which was consistent when comparing across treatments—e.g., on average, beta-diversity attributable to taxonomic turnover was responsible for 78% of total beta-diversity (**Figure 5B**). Similarly, temporal beta-diversity within ponds also predominantly reflected turnover in composition rather than nestedness (**Figure 5C**). These analyses demonstrate that ∼80% of the variation in taxonomic composition of the phytoplankton communities both in response to seasonal changes in temperature and the effects of experimental warming were driven by replacements of species.

Effects of Thermal Adaptation on Phytoplankton Stoichiometry

The green alga, *C. reinhardtii*, was one of the most abundant (top 10% of all species across the meta-community) and widely distributed taxa across the meta-community, with established populations in all warmed and ambient mesocosms. To investigate whether thermal adaptation resulted in changes in cellular stoichiometry that might contribute to the effects of warming on bulk seston stoichiometry we measured C:N:P ratios in strains of *C. reinhardtii* isolated from the warmed and ambient treatments. Differences in the stoichiometric ratios between the warmed and ambient isolates closely matched the treatment effects on bulk seston stoichiometry. The C:N ratios were not significantly different between warmed and ambient isolates [**Figure 6A**; ANOVA Type-III; $F_{(1, 16)} = 2.52$, $P = 0.13$]. The C:P ratio was significantly elevated in the warmed isolates [**Figure 6B**; ANOVA Type-III; $F_{(1, 16)} = 6.40$, $P = 0.02$], in line with the higher seston C:P ratios observed in the warmed treatments and the positive correlation between seasonal variation in temperature and bulk phytoplankton stoichiometry (**Figures 3**, **4**). The N:P ratio was marginally, but not significantly, elevated in the warmed isolates [**Figure 6C**; ANOVA Type-III; $F_{(1, 16)} = 2.49$, $P = 0.13$]. This weaker effect of warming on the N:P ratio was also consistent with the smaller effect size of warming on the seston N:P ratio (**Figure 3**) and the weaker seasonal temperature dependence of phytoplankton N:P (**Figure 4**), relative to the effects of warming and temperature on the C:P ratio.

DISCUSSION

Understanding how ecosystem-level properties, like the bulk stoichiometry of plankton, are shaped by selection on trait variation within and across species is key to improving predictions of global change on biogeochemical cycles (Hagstrom and Levin, 2017). Central to this issue is a grasp of the relative importance of rapid evolution (i.e., changes in the frequency of traits within species populations) and species sorting (i.e., changes in the frequency of traits attributable different species) in shaping how ecosystem-level properties respond to environmental change (Lomas et al., 2014). We tackled this issue by assessing the extent to which the effects of long-term experimental warming and seasonal changes in temperature on the C:N:P stoichiometry of phytoplankton in pond mesocosms reflected temperature-dependent changes in the composition of the communities vs. evolutionary shifts in stoichiometric traits within component species. We found that warming resulted in substantial shifts in phytoplankton community composition, consistent with temperature-driven species sorting. Furthermore, isolates of *C. reinhardtii* from warmed mesocosms had higher C:P and N:P ratios than their ambient counterparts, with shifts that were comparable in direction and magnitude to the effects of warming on seston stoichiometry. These analyses suggest that both species sorting and rapid local adaptation could have contributed to the effects of warming on bulk phytoplankton stoichiometry.

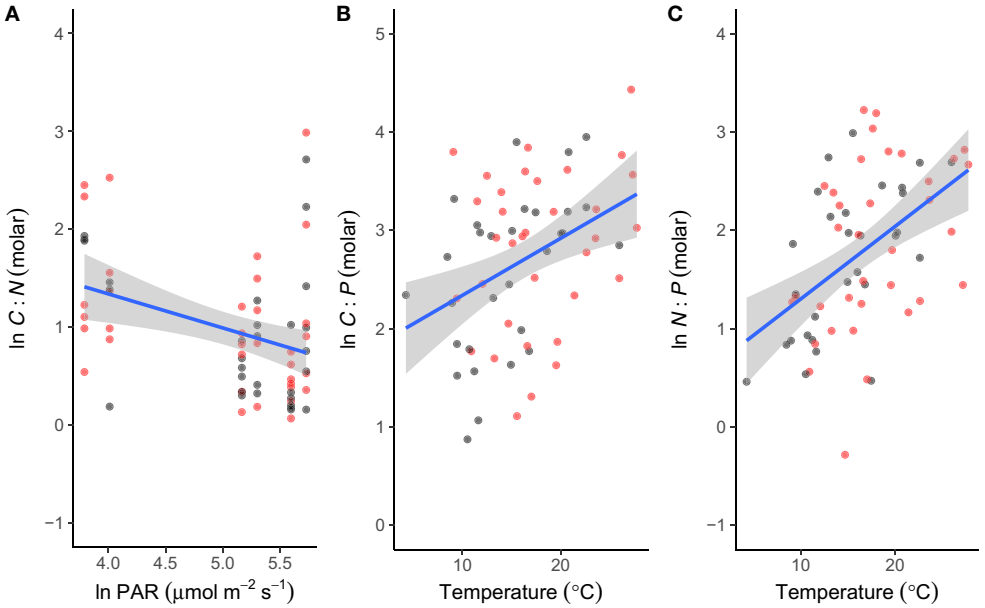

FIGURE 4 | Abiotic drivers of phytoplankton stoichiometry. Correlations between seasonal variation in **(A)** light intensity (PAR) and the C:N ratio, **(B)** temperature and the C:P ratio and **(C)** temperature and the N:P ratio. For each stoichiometric ratio the predictor with the highest summed weight is plotted (see **Table 2** for statistics). Fitted lines are from the fixed effects of the best fitting mixed effects models. Black denotes ambient treatments, red indicates warmed treatments.

We found higher average C:P and N:P ratios driven by lower particulate P content in the seston from the warmed mesocosms, as well as positive correlations between seasonal changes in temperature and C:P and N:P ratios in both the warmed and ambient treatments. These findings add to a growing body of evidence demonstrating positive covariance between temperature and C:P and N:P ratios in aquatic and terrestrial autotrophs (Reich and Oleksyn, 2004; Martiny et al., 2013; Toseland et al., 2013; Yvon-Durocher et al., 2015b). However, a critical question concerning the mechanisms underlying these patterns is the extent to which they are driven by evolutionary flexibility in stoichiometric traits within species vs. temperature driven turnover in taxonomic composition along thermal gradients.

To investigate the role of species sorting we quantified the extent to which differences in phytoplankton communities between the warmed and ambient treatments as well as across sampling months, reflected turnover in species composition (e.g., replacement of species across space and/or time). We found that ∼80% of the variation in phytoplankton composition among treatments and sampling months could be attributed to taxonomic replacements (e.g., turnover as opposed to nestedness). This result demonstrates that the composition of the phytoplankton communities were highly dynamic both in response to seasonal abiotic change and sustained increases in temperature between the treatments. Consequently, this result implies that species sorting and associated changes in community-wide traits could have played an important role in shaping the effects of temperature on bulk stoichiometry, consistent with recent work in marine ecosystems (Irwin et al., 2015; Edwards, 2016; Martiny et al., 2016b). Temperature-driven species sorting could affect community-level bulk stoichiometry in two ways. First, phytoplankton stoichiometry is known to exhibit substantial variation among the major taxonomic clades (Quigg et al., 2003; Finkel et al., 2016). For example, red lineage algae, which include the diatoms, have relatively low N:P ratios, while green lineage algae, which include the chlorophytes, tend have higher N:P ratios (Quigg et al., 2003). Thus, changes in phytoplankton community composition, where taxa with high average C:P and N:P ratios are favored in warmer environments and those with low values are abundant in cooler conditions could conceivably be an important factor shaping the effects of temperature on bulk phytoplankton stoichiometry. Second, it is also possible that C:N:P stoichiometry is phenotypically plastic with respect to temperature change in a consistent way across species (Yvon-Durocher et al., 2015b) and the observed turnover in community composition reflects selection for traits other than stoichiometry. In this case, the composition of the community changes with temperature and the stoichiometry of the constituent species also shift with temperature, not because different taxa have divergent stoichiometry, but because the reaction norm for temperature-driven stoichiometric plasticity is conserved across species. Unfortunately, our data do not allow us to differentiate between these two possibilities, as this would require detailed acclimation experiments to be conducted on a wide range of the species that comprise the phytoplankton communities in the experiment. It is important to note however, these two scenarios are not mutually exclusive and there is evidence for both conserved temperature driven stoichiometric plasticity across species (Yvon-Durocher et al., 2015b) as well as

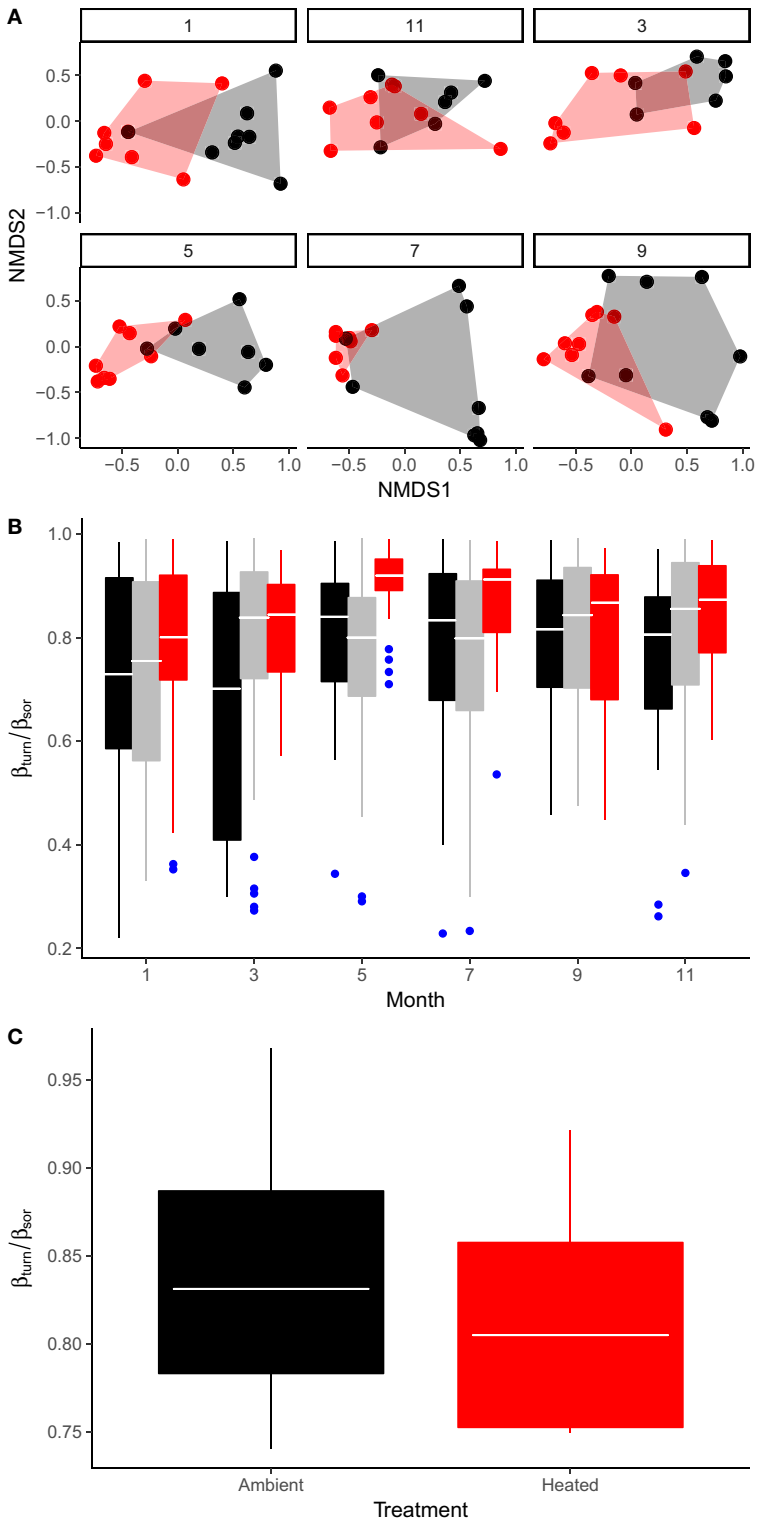

FIGURE 5 | Seasonal variation and treatment effects on phytoplankton community structure. **(A)** Non-metric multidimensional scaling (NMDS) of phytoplankton community composition comparing treatment effects across sampling months (1 = Jan, 3 = Mar, 5 = May, 7 = Jul, 9 = Sep, 11 = Nov). **(B)** Seasonal variation in the fraction of total beta-diversity among ponds that is attributable to taxonomic turnover (β_{turn}/β_{sor}), here black boxes encompass variation in β_{turn}/β_{sor} among ambient replicates, red show variation between warmed replicates and gray denotes variation in beta-diversity derived from comparisons among warmed vs. ambient replicates. **(C)** Treatment effects on β_{turn}/β_{sor} derived from temporal comparisons of community composition among sampling months within each mesocosm. Tops and bottoms of boxes in box-whisker plots correspond to the 25th and 75th percentiles, horizontal white lines correspond to medians, whisker extents correspond to 1.5 × the interquartile range.

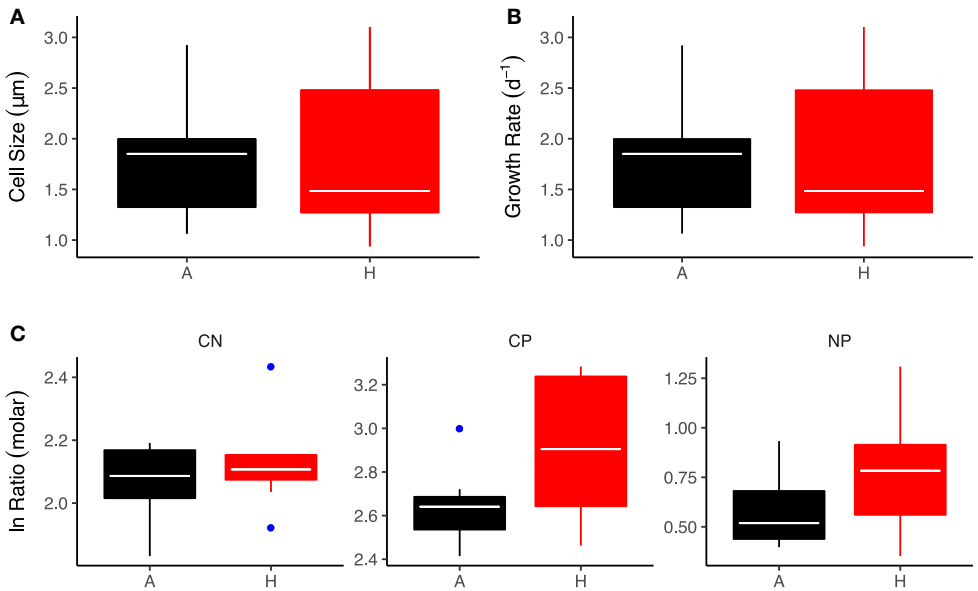

FIGURE 6 | Effects of experimental warming on physiological and stoichiometric traits of *C. reinhardtii* isolates. **(A)** Cell size at 18°C, **(B)** Growth rate at 18°C, **(C)** Stoichiometric ratios. Black indicates the ambient treatments and red the warmed. Tops and bottoms of boxes in box-whisker plots correspond to the 25th and 75th percentiles, horizontal white lines correspond to medians, whisker extents correspond to 1.5 × the interquartile range and blue points are outliers.

systematic taxonomic variation in average C:N:P ratios (Quigg et al., 2003; Finkel et al., 2016).

To investigate whether rapid evolutionary shifts in stoichiometric traits in response to warming played a role in the effects of warming on phytoplankton bulk stoichiometry we isolated the abundant and cosmopolitan alga *C. reinhardtii*, which was present across both warmed and ambient treatments. We have previously shown, through reciprocal transplants, that isolates of *C. reinhardtii* are locally adapted with respect to the warming treatment, with warmed isolates incuring a reduction in competitive fitness when transplanted to ambient temperatures and ambient isolates having reduced fitness when exposed to warming (Schaum et al., 2017). Our analyses here demonstrate that the warm-adapted isolates had C:P ratios that were 33% higher than their ambient counterparts. This within taxon response was remarkably close to the overall effect size on average bulk seston C:P with ratios that were 38% higher in the warmed treatments. The N:P ratio was marginally, but not significantly elevated in the warm-adapted isolates of *C. reinhardtii*. However, notwithstanding the absence of a significant treatment effect, the effect sizes between the isolates and the bulk community response to warming in the N:P ratio were also very similar, a 21 and 27% increase in response to warming in the isolates and the bulk seston respectively. These results demonstrate that selection on stoichiometric traits within and across species in response to warming are of a similar direction and magnitude, implying that that thermal adaptation also likely contributed the shifts in bulk seston stoichiometry in response to long-term experimental warming.

Our findings of elevated C:P and N:P ratios with increases in temperature, both at the species- and community-levels, are broadly consistent with the "*temperature-dependent physiology*" hypothesis, which predicts that fewer P-rich ribosomes are required at warmer temperatures owing to the increased efficiency of ribosomes at higher temperatures (Woods et al., 2003; Toseland et al., 2013; Yvon-Durocher et al., 2015b). Average C:P and N:P ratios both in the seston and in the isolates of *C. reinhardtii* were however very low (seston C:P = 16.6, seston N:P = 6.3; isolate C:P = 17.2, isolate N:P = 4.2), indicating that luxury uptake and storage of phosphorous may have contributed significantly to the cellular P content. Given the nanomolar concentrations of SRP in the mesocosms, luxury storage of P, which is known to be an adaptation to severe nutrient limitation, is a plausible explanation for the very low C:P and N:P ratios. Nevertheless, growth rates and cell sizes of the *C. reinhardtii* strains were comparable between the warmed and ambient isolates (see **Figure 6**) indicating that whilst luxury P storage might explain the overall low C:P and N:P ratios it is unlikely to be the underlying driver of the effects of warming.

The low C:P and N:P ratios in the seston appear at odds with the nanomolar concentrations of SRP in the mesocosms and raise the fundamental question of where the algae are sourcing the phosphorous required to sustain such high C:P and N:P ratios. It is notable, that many of the algae that are numerically dominant in both the warmed and the ambient mesocosms are capable of mixotrophic growth (e.g., *C. reinhardtii*, *Cryptomonas* spp., *Gymnodinium* spp.) and are known to supplement their nutritional requirements with resource uptake via osmotrophy or phagotrophy (Spero and Morée, 1981; Tranvik et al., 1989; Tittel et al., 2005). The fact that seston C:P and N:P ratios are only weakly correlated with seasonal changes in SRP suggests that uptake of P from organic sources (dissolved organic P or P

associated with bacterial prey) could be an important component of the phosphorous biogeochemistry of these oligotrophic systems.

Overall our experiments demonstrate a striking coherence between stoichiometric responses to temperature change between the long-term warmed and ambient mesocosms, across seasonal variations in temperature, and between strains of a cosmopolitan alga isolated from the long-term experiment. The consistency in the effects of temperature in driving increases in C:P and N:P stoichiometry both within species as an adaptive response to the long-term warming as well as at the community level via species sorting, implies that temperature driven adjustments in sub-cellular allocation to C, N and P pools that increase C:P and N:P in warmer environments increase fitness and can be reinforced through ecological and evolutionary processes. Our findings demonstrate highly conserved responses of elemental stoichiometry to temperature across multiple spatial, temporal and organizational scales and highlight that profound changes in aquatic biogeochemistry should be expected in a warmer world.

AUTHOR CONTRIBUTIONS

GYD and MT conceived the study. GYD and CS conducted the experiments. GYD analyzed the data. GYD wrote the manuscript and all authors contributed to revisions.

ACKNOWLEDGMENTS

This study was supported by a grant from the Natural Environment Research Council of the UK (NE/H022511/1) awarded to MT and GYD a Leverhulme Trust research grant (RPG-2013-335) awarded to GYD, and an ERC starting grant awarded to GYD (677278 TEMPDEP).

REFERENCES

Alexander, H., Jenkins, B. D., Rynearson, T. A., and Dyhrman, S. T. (2015). Metatranscriptome analyses indicate resource partitioning between diatoms in the field. *Proc. Natl. Acad. Sci. U.S.A.* 112, E2182–E2190. doi: 10.1073/pnas.1421993112

Baselga, A., and Orme, C. D. L. (2012). betapart: an R package for the study of beta diversity. *Methods Ecol. Evol.* 3, 808–812. doi: 10.1111/j.2041-210X.2012.00224.x

Bonachela, J. A., Allison, S. D., Martiny, A. C., and Levin, S. A. (2013). A model for variable phytoplankton stoichiometry based on cell protein regulation. *Biogeosciences* 10, 4341–4356. doi: 10.5194/bg-10-4341-2013

Daines, S. J., Clark, J. R., and Lenton, T. M. (2014). Multiple environmental controls on phytoplankton growth strategies determine adaptive responses of the N: P ratio. *Ecol. Lett.* 17, 414–425. doi: 10.1111/ele.12239

Dossena, M., Yvon-Durocher, G., Grey, J., Montoya, J. M., Perkins, D. M., Trimmer, M., et al. (2012). Warming alters community size structure and ecosystem functioning. *Proc. R. Soc. B Biol. Sci.* 279, 3011–3019. doi: 10.1098/rspb.2012.0394

Edwards, K. F. (2016). Community trait structure in phytoplankton: seasonal dynamics from a method for sparse trait data. *Ecology* 97, 3441–3451. doi: 10.1002/ecy.1581

Elser, J. J., Andersen, T., Baron, J. S., Bergstrom, A. K., Jansson, M., Kyle, M., et al. (2009). Shifts in Lake N:P stoichiometry and nutrient limitation driven by atmospheric nitrogen deposition. *Science* 326, 835–837. doi: 10.1126/science.1176199

Finkel, Z. V., Beardall, J., Flynn, K. J., Quigg, A., Rees, T. A. V., and Raven, J. A. (2009). Phytoplankton in a changing world: cell size and elemental stoichiometry. *J. Plankton Res.* 32, 119–137. doi: 10.1093/plankt/fbp098

Finkel, Z. V., Follows, M. J., Liefer, J. D., Brown, C. M., Benner, I., and Irwin, A. J. (2016). Phylogenetic diversity in the macromolecular composition of microalgae. *PLoS ONE* 11:e0155977. doi: 10.1371/journal.pone.0155977

Galbraith, E. D., and Martiny, A. C. (2015). A simple nutrient-dependence mechanism for predicting the stoichiometry of marine ecosystems. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8199–8204. doi: 10.1073/pnas.1423917112

Geider, R. J., and La Roche, J. (2002). Redfield revisited: variability of C: N: P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37, 1–17. doi: 10.1017/S0967026201003456

Hagstrom, G. I., and Levin, S. A. (2017). Marine ecosystems as complex adaptive systems: emergent patterns, critical transitions, and public goods. *Ecosystems* 20, 1–19. doi: 10.1007/s10021-017-0114-3

Hessen, D. O. (2005). Seasonal seston stoichiometry: effects on zooplankton in cyanobacteria-dominated lakes. *J. Plankton Res.* 27, 449–460. doi: 10.1093/plankt/fbi018

Hessen, D. O., Hafslund, O. T., Andersen, T., Broch, C., Shala, N. K., and Wojewodzic, M. W. (2017). Changes in stoichiometry cellular RNA and alkaline phosphatase activity of chlamydomonas in response to temperature and nutrients. *Front. Microbiol.* 8:18. doi: 10.3389/fmicb.2017.00018

Irwin, A. J., Finkel, Z. V., Mueller-Karger, F. E., and Ghinaglia, L. T. (2015). Phytoplankton adapt to changing ocean environments. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5762–5766. doi: 10.1073/pnas.1414752112

Kirkwood, D. (1996). *Nutrients: Practical Notes on Their Determination in Sea Water*. Copenhagen: International Council for the Exploration of the Sea.

Litchman, E., and Klausmeier, C. A. (2008). Trait-Based Community Ecology of Phytoplankton. *Annu. Rev. Ecol. Evol. Syst.* 39, 615–639. doi: 10.1146/annurev.ecolsys.39.110707.173549

Litchman, E., Klausmeier, C. A., Schofield, O. M., and Falkowski, P. G. (2007). The role of functional traits and trade-offs in structuring phytoplankton communities: scaling from cellular to ecosystem level. *Ecol. Lett.* 10, 1170–1181. doi: 10.1111/j.1461-0248.2007.01117.x

Lomas, M. W., Bonachela, J. A., Levin, S. A., and Martiny, A. C. (2014). Impact of ocean phytoplankton diversity on phosphate uptake. *Proc. Natl. Acad. Sci. U.S.A.* 111, 17540–17545. doi: 10.1073/pnas.1420760111

Martiny, A. C., Ma, L., Mouginot, C., Chandler, J. W., and Zinser, E. R. (2016a). Interactions between thermal acclimation, growth rate, and phylogeny influence prochlorococcus elemental stoichiometry. *PLoS ONE* 11:e0168291. doi: 10.1371/journal.pone.0168291

Martiny, A. C., Pham, C. T. A., Primeau, F. W., Vrugt, J. A., Moore, J. K., Levin, S. A., et al. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci.* 6, 279–283. doi: 10.1038/ngeo1757

Martiny, A. C., Talarmin, A., Mouginot, C., Lee, J. A., Huang, J. S., Gellene, A. G., et al. (2016b). Biogeochemical interactions control a temporal succession in the elemental composition of marine communities. *Limnol. Oceangr.* 61, 531–542. doi: 10.1002/lno.10233

Murphy, J., and Riley, J. P. (1958). A single solution method for the determination of soluble phosphate in sea water. *J. Mar. Biol. Assoc.* 37, 9–14. doi: 10.1017/S0025315400014776

Padfield, D., Yvon-Durocher, G., Buckling, A., Jennings, S., and Yvon-Durocher, G. (2016). Rapid evolution of metabolic traits explains thermal adaptation in phytoplankton. *Ecol. Lett.* 19, 133–142. doi: 10.1111/ele.12545

Quigg, A., Finkel, Z. V., Irwin, A. J., Rosenthal, Y., Ho, T. Y., Reinfelder, J. R., et al. (2003). The evolutionary inheritance of elemental stoichiometry in marine phytoplankton. *Nature* 425, 291–294. doi: 10.1038/nature01953

Raven, J. A., and Geider, R. J. (1988). Temperature and algal growth. *New Phytol.* 110, 441–461. doi: 10.1111/j.1469-8137.1988.tb00282.x

Reich, P. B., and Oleksyn, J. (2004). Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11001–11006. doi: 10.1073/pnas.0403588101

Sal, S., Alonso-Sáez, L., Bueno, J., Garcia, F. C., and López-Urrutia, Á. (2015). Thermal adaptation, phylogeny, and the unimodal size scaling of marine phytoplankton growth. *Limnol. Oceangr*. 60, 1212–1221. doi: 10.1002/lno.10094

Schaum, C. E., Barton, S., Bestion, E., Buckling, A., Garcia-Carreras, B., Lopez, P. (2017). Adaptation of phytoplankton to a decade of experimental warming linked to increased photosynthesis. *Nat. Ecol. Evol*. 1, 94. doi: 10.1038/s41559-017-0094

Schaum, C. E., Rost, B., and Collins, S. (2016). Environmental stability affects phenotypic evolution in a globally distributed marine picoplankton. *ISME J.* 10, 75–84. doi: 10.1038/ismej.2015.102

Schlueter, L., Lohbeck, K. T., Gutowska, M. A., Groeger, J. P., Riebesell, U., and Reusch, T. B. H. (2014). Adaptation of a globally important coccolithophore to ocean warming and acidification. *Nat. Clim. Chang.* 4, 1024–1030. doi: 10.1038/nclimate2379

Shuter, B. (1979). A model of physiological adaptation in unicellular algae. *J. Theor. Biol.* 78, 519–552. doi: 10.1016/0022-5193(79)90189-9

Spero, H. J., and Morée, M. D. (1981). Phagotrophic feeding and its importance to the life cycle of the holozoic dinoflagellate, *gymnodinium fungiforme*. *J. Phycol.* 17, 43–51. doi: 10.1111/j.1529-8817.1981.tb00817.x

Staehr, P. A., Bade, D., Van de Bogert, M. C., Koch, G. R., Williamson, C., Hanson, P., et al. (2010). Lake metabolism and the diel oxygen technique: state of the science. *Limnol. Oceanogr. Methods* 8, 628–644. doi: 10.4319/lom.2010.8.0628

Sterner, R., and Elser, J. J. (2002). *Ecological Stoichiometry the Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Thomas, M. K., Kremer, C. T., Klausmeier, C. A., and Litchman, E. (2012). A global pattern of thermal adaptation in marine phytoplankton. *Science* 338, 1085–1088. doi: 10.1126/science.1224836

Tittel, J., Bissinger, V., Gaedke, U., and Kamjunke, N. (2005). Inorganic carbon limitation and mixotrophic growth in *Chlamydomonas* from an acidic mining lake. *Protist* 156, 63–75. doi: 10.1016/j.protis.2004.09.001

Toseland, A., Daines, S. J., Clark, J. R., Kirkham, A., Strauss, J., Uhlig, C., et al. (2013). The impact of temperature on marine phytoplankton resource allocation and metabolism. *Nat. Clim. Chang.* 3, 979–984. doi: 10.1038/nclimate1989

Tranvik, L. J., Porter, K. G., and Sieburth, J. M. (1989). Occurrence of bacterivory in *Cryptomonas*, a common freshwater phytoplankter. *Oecologia* 78, 473–476. doi: 10.1007/BF00378736

Woods, H. A., Makino, W., Cotner, J. B., Hobbie, S. E., Harrison, J. F., Acharya, K., et al. (2003). Temperature and the chemical composition of poikilothermic organisms. *Funct. Ecol.* 17, 237–245. doi: 10.1046/j.1365-2435.2003.00724.x

Yvon-Durocher, G., Allen, A. P., Cellamare, M., Dossena, M., Gaston, K. J., Leitao, M., et al. (2015a). Five years of experimental warming increases the biodiversity and productivity of phytoplankton. *PLoS Biol.* 13:e1002324. doi: 10.1371/journal.pbio.1002324

Yvon-Durocher, G., Dossena, M., Trimmer, M., Woodward, G., and Allen, A. P. (2015b). Temperature and the biogeography of algal stoichiometry. *Global Ecol. Biogeogr.* 24, 562–570. doi: 10.1111/geb.12280

Yvon-Durocher, G., Jones, J. I., Trimmer, M., Woodward, G., and Montoya, J. M. (2010). Warming alters the metabolic balance of ecosystems. *Philos. Trans. R. Soc. B Biol. Sci.* 365, 2117–2126. doi: 10.1098/rstb.2010.0038

Zuur, A., Ieno, E., Walker, N., and Saveliev, A. (2009). *Mixed Effects Models and Extensions in Ecology with R*. New York, NY: Springer Verlag.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 May 2017
doi: 10.3389/fpls.2017.00655

Impact of Temperature and Nutrients on Carbon: Nutrient Tissue Stoichiometry of Submerged Aquatic Plants: An Experiment and Meta-Analysis

Mandy Velthuis[1], Emma van Deelen[1], Ellen van Donk[1,2], Peiyu Zhang[1] and Elisabeth S. Bakker[1]*

[1] *Department of Aquatic Ecology, Netherlands Institute of Ecology, Wageningen, Netherlands,* [2] *Department of Biology, Utrecht University, Utrecht, Netherlands*

Human activity is currently changing our environment rapidly, with predicted temperature increases of 1–5°C over the coming century and increased nitrogen and phosphorus inputs in aquatic ecosystems. In the shallow parts of these ecosystems, submerged aquatic plants enhance water clarity by resource competition with phytoplankton, provide habitat, and serve as a food source for other organisms. The carbon:nutrient stoichiometry of submerged aquatic plants can be affected by changes in both temperature and nutrient availability. We hypothesized that elevated temperature leads to higher carbon:nutrient ratios through enhanced nutrient-use efficiency, while nutrient addition leads to lower carbon:nutrient ratios by the luxurious uptake of nutrients. We addressed these hypotheses with an experimental and a meta-analytical approach. We performed a full-factorial microcosm experiment with the freshwater plant *Elodea nuttallii* grown at 10, 15, 20, and 25°C on sediment consisting of pond soil/sand mixtures with 100, 50, 25, and 12.5% pond soil. To address the effect of climatic warming and nutrient addition on the carbon:nutrient stoichiometry of submerged freshwater and marine plants we performed a meta-analysis on experimental studies that elevated temperature and/or added nutrients (nitrogen and phosphorus). In the microcosm experiment, C:N ratios of *Elodea nuttallii* decreased with increasing temperature, and this effect was most pronounced at intermediate nutrient availability. Furthermore, higher nutrient availability led to decreased aboveground C:P ratios. In the meta-analysis, nutrient addition led to a 25, 22, and 16% reduction in aboveground C:N and C:P ratios and belowground C:N ratios, accompanied with increased N content. No consistent effect of elevated temperature on plant stoichiometry could be observed, as very few studies were found on this topic and contrasting results were reported. We conclude that while nutrient addition consistently leads to decreased carbon:nutrient ratios, elevated temperature does not change submerged aquatic plant carbon:nutrient stoichiometry in a consistent manner. This effect is rather dependent on nutrient availability and may be species-specific. As

OPEN ACCESS

Edited by:
James Joseph Elser,
University of Montana, USA

Reviewed by:
Evelyn Elaine Gaiser,
Florida International University, USA
Paul Frost,
Trent University, Canada

****Correspondence:***
Mandy Velthuis
m.velthuis@nioo.knaw.nl

Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *27 January 2017*
Accepted: *10 April 2017*
Published: *04 May 2017*

Citation:
Velthuis M, van Deelen E, van Donk E, Zhang P and Bakker ES (2017) Impact of Temperature and Nutrients on Carbon: Nutrient Tissue Stoichiometry of Submerged Aquatic Plants: An Experiment and Meta-Analysis. Front. Plant Sci. 8:655. doi: 10.3389/fpls.2017.00655

changes in the carbon:nutrient stoichiometry of submerged aquatic plants can impact the transfer of energy to higher trophic levels, these results suggest that eutrophication may enhance plant consumption and decomposition, which could in turn have consequences for carbon sequestration.

Keywords: submerged freshwater and marine macrophytes, meta-analysis, microcosm experiment, *Elodea nuttallii*, eutrophication, global warming, carbon:nutrient stoichiometry, growth rate

INTRODUCTION

Human activity has led to rapid environmental changes on our planet (Vitousek et al., 1997; Steffen et al., 2015). Water temperatures in marine and freshwater systems have increased over the last decades and are expected to increase further over the course of the century (Mooij et al., 2008; Adrian et al., 2009; IPCC, 2014). Furthermore, agriculture and industrialization have a strong impact on nutrient cycles (Carpenter et al., 1998; Tilman et al., 2001) and are major sources of nitrogen and phosphorus input in freshwater and marine ecosystems. Changes in temperature and nutrient availability can have consequences for the abundance of submerged aquatic plants that occur in the shallow parts of aquatic ecosystems (Bornette and Puijalon, 2011).

Changes in plant abundances and growth rates can have effects on their nutrient demand and uptake and as such can influence their carbon:nutrient stoichiometry (Sterner and Elser, 2002). Alterations in internal stoichiometry in turn can have consequences for ecosystem functioning, as lower carbon:nutrient ratios can make aquatic plants more palatable to herbivores (Dorenbosch and Bakker, 2011), resulting in higher herbivory rates and stimulated top-down control (Olsen and Valiela, 2010; Bakker and Nolet, 2014) and leading to lowered carbon stocks in the form of plant biomass (Heithaus et al., 2014; van Altena et al., 2016).

However, contrasting hypotheses exist on how temperature and nutrient availability may affect carbon:nutrient ratios in aquatic plants. Elevated temperature can lead to an increase in plant biomass and a biomass dilution effect, where increased growth rates are accompanied by reduced tissue content (per unit of biomass) of a particular element (Taylor et al., 1991; Vermaat and Hootsmans, 1994). In terrestrial plant and phytoplankton research, this effect is referred to as enhanced nutrient-use efficiency (An et al., 2005; De Senerpont Domis et al., 2014). According to this hypothesis, elevated temperature would lead to reduced N and P content in aquatic plants and a subsequent increase in carbon:nutrient ratios. Alternatively, higher temperatures can increase the rate of cellular processes, but do not necessarily lead to an unbalanced nutrient uptake, provided that enough nutrients are available in the environment, and therefore would not result in changes in carbon:nutrient ratios.

Similarly, nutrient addition can positively affect the nutritional quality of aquatic plants (e.g., lower carbon:nutrient ratios; Burkholder et al., 2007; Bakker and Nolet, 2014) as they may take up relatively more nutrients compared to carbon. This fertilization effect is demonstrated for terrestrial plants in a recent meta-analysis (Sardans et al., 2012). Furthermore, the combined effect of elevated temperature and nutrient addition may be antagonistic under the hypotheses of enhanced nutrient-use efficiency and luxurious uptake, as the former would be expected to increase carbon:nutrient ratios, while the latter would decrease carbon:nutrient ratios.

Here, we aimed to quantify the effects of temperature and nutrient addition on the carbon:nutrient stoichiometry of submerged aquatic angiosperms. We hypothesized that (1) both elevated temperature and nutrient addition lead to enhanced growth rates of submerged angiosperms, (2) if the biomass-dilution effect applies, elevated temperature will lead to higher carbon:nutrient ratios, whereas (3) nutrient addition is expected to lead to decreased carbon:nutrient ratios. These hypothesized changes in carbon:nutrient ratios are expected to be driven by changes in nutrient contents as opposed to carbon (4). Furthermore, we hypothesized that elevated temperature and nutrient addition are antagonists in their combined effect on carbon:nutrient ratios (5).

We tested these hypotheses using two complementary approaches. First, we performed a full-factorial experiment on the effects of temperature and sediment nutrient content (and their interaction) on the growth and carbon:nutrient stoichiometry of the submerged freshwater angiosperm *Elodea nuttallii*. *E. nuttallii* is native to North America, but has become common throughout the northern hemisphere in the 1900s (Cook and Urmi-König, 1985). Subsequently, a meta-analytic approach was used to address the effect of elevated temperature and nutrient addition on submerged angiosperms in general. We performed a meta-analysis using experimental studies that simulated temperature rise and/or increased nutrient (nitrogen and phosphorus) input and documented the effects on plant growth and carbon:nutrient stoichiometry. In this analysis, we included both marine and freshwater plants. Whereas the responses of aquatic plants to environmental change in marine and freshwater systems are mostly discussed independently, we expected that responses in growth and carbon:nutrient stoichiometry similarly apply to both submerged marine and freshwater angiosperms alike.

MATERIALS AND METHODS

Elodea Laboratory Experiment

Experimental Set-Up

To test the effect of temperature and sediment nutrient content on *Elodea nuttallii*, a full-factorial microcosm experiment was set up. Shoots of *E. nuttallii* were collected from a small pond on the grounds of The Netherlands Institute of Ecology

(NIOO-KNAW), Wageningen, The Netherlands (51°59′15.0″N; 5°40′14.8″E) on 07-09-2015. After collection, the plants were rinsed and acclimatized at room temperature for 2 days prior to the start of the experiment.

The experiment was carried out in 4 L plastic microcosms (14 × 14 × 21 cm), which contained 1.1 L of sediment and 2.7 L of water. Nutrient treatments were achieved by mixing artificial pond sediment (20% organic matter, Velda, Enschede, The Netherlands) with sand and consisted of 12.5, 25, 50, and 100% (v/v) of pond sediment ($n = 5$), covered with a one centimeter layer of sand. The artificial pond sediment contained 31 ± 1.8, 0.80 ± 0.048, and 0.11 ± 0.0084% (mean ± SE) C, N and P respectively. One shoot fragment of *E. nuttallii* of ± 5.5 cm (C:N = 19 ± 1.4, C:P = 435 ± 72; mol:mol; $n = 5$) was placed in the middle of each microcosm and the microcosm was topped off with nutrient-poor tap water [3.5 ± 0.5 (mean ± SE) μM DIN and undetectable levels of DIP]. The microcosms were placed in four aquaria, which served as temperature-regulated water baths. The temperature treatments were 10, 15, 20, and 25°C, which were obtained by a computer-controlled (Specview 32/859, SpecView Ltd., Uckfield, UK) custom-made climate control system. These temperatures are within the range of natural temperatures *E. nuttallii* would encounter, as water temperatures in the Netherlands vary seasonally between 4 and 23°C (van Dam, 2009). Light (14:10 hours light:dark) was provided by two 28W TL5 HE lamps (Philips, Eindhoven, The Netherlands), hung above the aquaria with an average light intensity at the water surface of 30 μmol s^{-1} m^{-2}. To ensure equal light conditions between treatments, position of the microcosms in the water bath was randomized once a week and evaporation losses were compensated by additions of demi-water. To prevent excessive periphyton and phytoplankton growth during the experiment, one periphyton-grazing snail (*Planorbarius corneus*) and one filtering mussel (*Dreissena polymorpha*) were put in each microcosm. In pilot tests, *P. corneus* did not feed on *E. nuttallii* (Peiyu Zhang, personal observation) and no grazing on the plants was observed during the experiment. The snails were retrieved from the same pond as the plants, and the mussels were collected from the Nether Rhine, Wageningen, the Netherlands (51°57′12.9″N 5°39′48.2″E). In case either snail or mussel died, another one was added.

Harvest

After 58 days the experiment was terminated. From the middle of each microcosm, water samples were taken to determine dissolved nutrient concentrations, filtered over prewashed GF/F filters (Whatman, Maidstone, U.K.) and stored at −20°C until further analysis. Samples for pore water nutrients were taken in each microcosm through a 10 cm Rhizon SMS (Rhizosphere, Wageningen, the Netherlands) and stored at −20°C until further analysis. Plants were cut at the sediment level, and the above- and belowground biomass was harvested and rinsed with demi-water. All plant materials (above- and belowground) were dried at 60°C until constant dry mass and weighed. During the harvest, basic parameters were measured that describe the growing conditions (pH, alkalinity and seston chlorophyll-a). Methods and results of these measurements can be found in Supplementary Material S1.

Chemical Analysis

Plant material was grinded to a fine powder on a microfine grinder (MF 10 basic, IKA-werke, Staufen, Germany) or in test tube with a 1/8" ball bearing (Weldtite, Lincolnshire, UK) on a Tissuelyser II (QIAGEN, Germantown, USA). For nitrogen (N) and carbon (C) content, 0.2–2 mg dry mass was analyzed on a NC analyser (FLASH 2000 NC elemental analyser, Brechbueler Incorporated, Interscience B.V., Breda, The Netherlands). For phosphorus (P) content, 1–4 mg dry mass was combusted in a Pyrex glass tube at 550°C for 30 min. Subsequently, 5 mL of persulfate (2.5%) was added and samples were autoclaved for 30 min at 121°C. Digested P (as PO_4^{3-}) was measured on a QuAAtro39 Auto-Analyzer (SEAL Analytical Ltd., Southampton, U.K.). Concentrations of dissolved nutrients (PO_4^{3-}, NO_2^-, NO_3^- and NH_4^+) of thawed water-samples were determined on a QuAAtro39 Auto-Analyzer (SEAL Analytical Ltd., Southampton, U.K.). Results for the dissolved nutrients in the water column can be found in Figure S1.2.

Calculations and Statistics

Plant specific growth rate (SGR) was calculated with the following formula:

$$SGR = \frac{Ln(DW_t) - Ln(DW_0)}{t}$$

Where DW_t is the plant aboveground dry weight at the end of the experiment, DW_0 the dry weight at the beginning of the experiment (determined by multiplying the initial wet weight with the plants wet weight/dry weight ratio) and t the experimental duration (=58 days).

Data on above- and belowground parameters (specific growth rate, above- and belowground biomass, carbon:nutrient stoichiometry and elemental contents) and dissolved nutrient concentrations (DIN and DIP) in the water column and the pore water were tested for effects of temperature, nutrients and their interaction with generalized linear models (function *glm* from stats package). Visual examination of the data distribution (function *hist*) led to the use of a gamma distribution. *Post-hoc* tests within treatment levels were carried out using Tukey contrasts [function glht from multcomp package (Hothorn et al., 2008)], with *P*-values corrected for multiple comparison as described by Benjamini and Hochberg (1995).

Meta-Analysis

Systematic Literature Review and Data Collection

A systematic literature review was carried out in Web of Science based on the guidelines described by the Collaboration for Environmental Evidence (2013). The search term ("submerged macrophyte*" OR "aquatic plant" OR isoetid OR macrophyte* OR "aquatic weed" OR seagrass*) AND (stoichiometr* OR "*chemical composition" OR "nutritional quality" OR "nutrient composition" OR "elemental composition" OR "nutrient content" OR "nutrient ratio*" OR C:N OR C:P OR N:P OR "plant nutrient concentration*") AND (warming OR eutrophication OR temperature* or enrichment or fertilis* or "nutrient availability") on 01-11-2016 gave 414 hits. Further selection based on abstracts, graphs and tables led to 47 papers

that contained information on temperature and/or nutrient effects on elemental composition of submerged angiosperms. Data originating from light limited conditions (as indicated in the paper itself) were excluded from analysis, as well as studies without reported standard errors or deviations, and studies with limited ($n < 2$) or non-reported sample size. From the selected papers, data on C:N and C:P ratios were extracted with use of Plotdigitizer and Engauge and converted to molar ratios when necessary. In addition, C, N and P contents, growth rates (above- or belowground), habitat (marine or freshwater), which part of the plant was analyzed (above- or belowground) and sample size were extracted when reported. If the described methodology indicated possible additional results that were not reported, corresponding authors were contacted to retrieve those data. If experiments reported several measurements over time, only the final measurement was extracted. If papers contained multiple experiments, on the same or on different species, these were extracted as being separate studies.

Data Selection

Control and elevated treatments were defined for both temperature and nutrient addition for each experiment separately. The lowest water temperature reported was defined as the control temperature treatment and 3–6°C above that temperature [equivalent to RCP scenario 8.5 from IPCC (2014)] was defined as the elevated temperature treatment. For the nutrient addition studies, those studies that manipulated both nitrogen and phosphorus simultaneously were selected. The lowest nutrient condition reported was defined as the control treatment and the highest as the elevated treatment. The data was then split up into above- and belowground plant responses, as different parts of plants were expected to respond differently (Bloom et al., 1985). These selection criteria led to a total of 50 studies on nutrient addition spread over 26 papers (of which 50 and 11 on above- and belowground responses respectively) and 3 studies on temperature (only on aboveground responses) originating from 3 papers. Temperature studies were all conducted in mesocosms, whereas nutrient studies included *in situ* fertilization experiments (38), mesocosm experiments (9) and laboratory experiments (3). Of the nutrient addition studies, 9 studies tested a range of nutrient concentrations of which the highest and lowest were selected, while the majority (41) specifically looked at the addition of nitrogen and phosphorus to the system relative to a control level. An overview of the dataset selection can be found in Figure S2 and an overview of the selected papers in Supplementary Material S3.

Response Factors and Statistics

Delta response ratios and their variances were calculated for each separate study according to Lajeunesse (2015):

$$RR\Delta = Ln\left(\frac{Xtreatment}{Xcontrol}\right) + \frac{1}{2}\left[\frac{(SDtreatment)^2}{Ntreatment * Xtreatment^2} - \frac{\left(SDcontrol\right)^2}{Ncontrol * Xcontrol^2}\right]$$

$$var\,(RR\Delta) = \frac{(SDtreatment)^2}{Ntreatment * Xtreatment^2} + \frac{\left(SDcontrol\right)^2}{Ntreatment * Xcontrolt^2} + \frac{1}{2}\left[\frac{(SDtreatment)^4}{Ntreatment^2 * Xtreatment^4} + \frac{SDcontrol^4}{Ncontrol * Xcontrolt^4}\right]$$

Where X denotes mean of the fixed factor of interest [C:N and C:P ratio, growth rate (μ) and C, N and P contents], SD the standard deviation of that mean and N the sample size.

All statistics were carried out in R (R Core Team, 2015). To test whether response ratios deviated from zero, mixed effect models were fitted to the response ratios and their variances with the function *rma.mv* [package metafor; Viechtbauer (2010)], incorporating reference and species as random effects. To test whether freshwater and marine systems differed in response ratio, separate models were compared for significant differences between the two habitat types (by adding habitat as a moderator to the function *rma.mv*).

RESULTS

Elodea Experiment

Biomass Responses

Temperature affected the specific growth rate and above- and belowground biomass of *E. nuttallii* (**Table 1**). As indicated by the interaction term, temperature only affected specific growth rate at intermediate sediment nutrient content (e.g., 25%), with optimal growth at 15 and 20°C ($P < 0.05$, Tukey *post-hoc* comparison; Figure S4). Similarly, aboveground biomass was highest at these temperatures, irrespective of nutrient treatment ($P < 0.05$; **Figure 1A**). Belowground biomass of *E. nuttallii* was affected by temperature, and this effect interacted with nutrient treatment (**Figure 1B**, **Table 1**). The effects of temperature on belowground biomass seemed strongest in the lowest nutrient treatments (e.g., 12.5%), where biomass tended to increase 3-fold between 15 and 20°C but these effects were not significant in *post-hoc* tests ($P = 0.09$).

Sediment nutrient content affected the specific growth rate and the above- and belowground biomass of *Elodea nuttallii* (**Table 1**). However, no significant differences between nutrient treatments could be observed for specific growth rate (Figure S4), nor for aboveground biomass (**Figure 1A**) in the *post-hoc* comparisons. Belowground biomass decreased with increasing sediment nutrient content, and this effect interacted with temperature treatment (**Table 1**; **Figure 1B**). Belowground biomass tended to decrease 6-fold over the entire range of nutrient treatments at 25°C, but this effect was not significant ($P = 0.08$).

Carbon:Nutrient Stoichiometry

Temperature negatively affected aboveground C:N ratios (**Table 1**, **Figure 2A**), which was most visible at intermediate sediment nutrient content (25%). In this treatment, aboveground C:N ratios decreased moderately but significantly between 10 and 25°C ($P < 0.001$, Tukey *post-hoc* comparison). No effects of temperature on aboveground C:P ratios were observed, nor on belowground C:N and C:P ratios (**Table 1**, **Figures 2B–D**).

TABLE 1 | Summary of generalized linear model analysis of the *Elodea* experiment, describing the effect of temperature treatment, nutrient treatment and their interaction on the biomass, carbon:nutrient stoichiometry and elemental contents of *Elodea nuttallii* and nutrient concentrations.

	Unit	Chi-square values		
		Temperature	Nutrients	Temperature × Nutrients
BIOMASS VARIABLES				
Specific growth rate	day^{-1}	**16.7*** **	**8.8***	7.3
Aboveground biomass	mg DW	**62.4*** **	**17.7*** **	13.1
Belowground biomass	mg DW	**10.8***	**18.6*** **	**20.8***
CARBON:NUTRIENT STOICHIOMETRY				
Aboveground C:N	mol:mol	**43.6*** **	3.5	**22.5****
Aboveground C:P	mol:mol	7.7	**45.6*** **	8.2
Belowground C:N	mol:mol	4.7	**8.8***	**21.8****
Belowground C:P	mol:mol	6.0	**12.0****	7.5
ELEMENTAL CONTENTS				
Aboveground C	mol g DW^{-1}	5.1	0.7	1.3
Aboveground N	mol g DW^{-1}	**15.5****	1.4	3.1
Aboveground P	mol g DW^{-1}	7.6	**83.4*** **	**45.7*** **
Belowground C	mol g DW^{-1}	**9.4***	**35.2*** **	9.8
Belowground N	mol g DW^{-1}	7.4	0.9	14.3
Belowground P	mol g DW^{-1}	6.1	1.2	**17.3***
NUTRIENT CONCENTRATIONS				
Pore water DIN	μM	**42.1*** **	**163.9*** **	**19.8***
Pore water DIP	μM	**47.9*** **	**317.6*** **	**29.3*** **

*Significant results are indicated in bold, with ***P < 0.001, **P < 0.01 and *P < 0.05.*

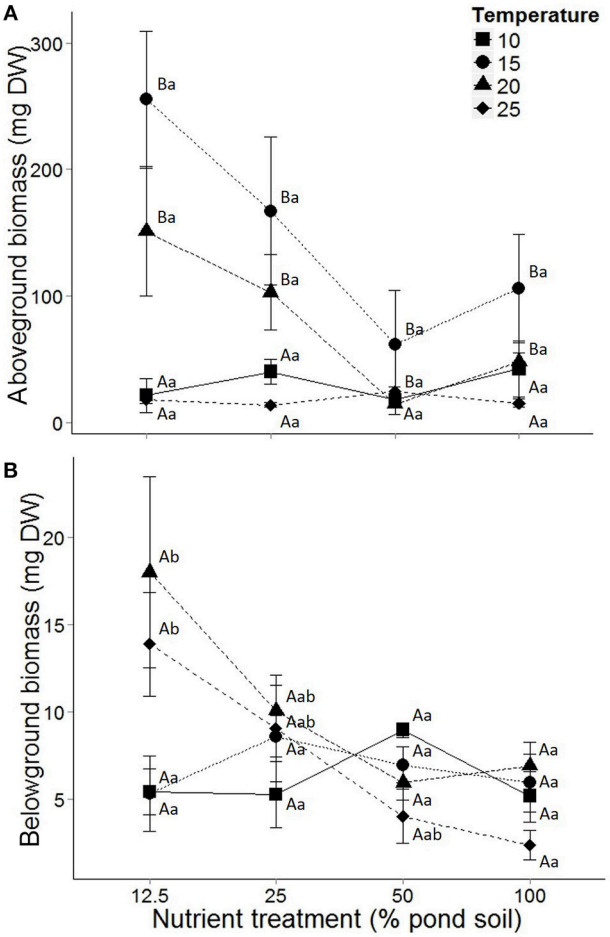

FIGURE 1 | Above—(A) and belowground (B) biomass of *Elodea nuttallii* grown at different temperatures and sediment nutrient content. Temperature treatments include 10 (■), 15 (●), 20 (▲) and 25 (♦)°C. Dots represent means and error bars standard error of the mean ($n = 5$). Capital and lower case letters indicate *post-hoc* differences between temperature and nutrient treatments, respectively.

Sediment nutrient content affected aboveground C:P ratio, while no effect on aboveground C:N ratios was observed (**Table 1**). Aboveground C:P ratios of *E. nuttallii* were negatively affected by increasing sediment nutrient content (**Figure 2B**, **Table 1**). This effect was most visible at 15°C, where the C:P ratio significantly decreased 4-fold the entire range of nutrient treatments ($P < 0.001$, Tukey *post-hoc* comparison). Belowground C:N and C:P ratios were affected by nutrient treatment, and the effect on C:N interacted with temperature (**Figure 2C**). Belowground C:N ratios significantly increased between 25 and 100% nutrient treatments at 20°C, while belowground C:P ratios increased 4-fold between those nutrient treatments at the same temperature ($P < 0.01$; **Figure 2D**).

Elemental Contents

Accompanied by the changes in aboveground C:N ratio, temperature seemed to affect aboveground N content (**Table 1**). However, no differences between any of the temperature treatments could be detected in *post-hoc* comparisons (Figure S5B). Belowground carbon content was affected by temperature, and halved between 10 and 25°C in the lowest sediment nutrient treatments (12.5 and 25%; $P < 0.05$). No effects of temperature on aboveground C and P content were observed, nor on belowground N and P contents (**Table 1**).

Sediment nutrient content affected aboveground P content, with a 3-fold increase over the entire range of nutrient treatments ($P < 0.05$; Figure S5C). No effect on aboveground C or N content was observed for nutrient content (Figures S5A,B). Belowground C content significantly increased 14% over the entire range of nutrient treatments at 25°C ($P < 0.01$; Figure S5D), while belowground N and P content were not affected by nutrient treatment (**Table 1**).

Abiotic Conditions

Temperature affected dissolved nutrient concentrations in the pore water, and this effect interacted with nutrient treatment (**Table 1**). Temperature effects on pore water DIN concentrations were strongest at intermediate sediment nutrient content (50%), where values significantly doubled from 10 to 15°C, and decreased at higher temperatures ($P < 0.01$, Tukey *post-hoc* comparison; **Figure 3A**). Pore

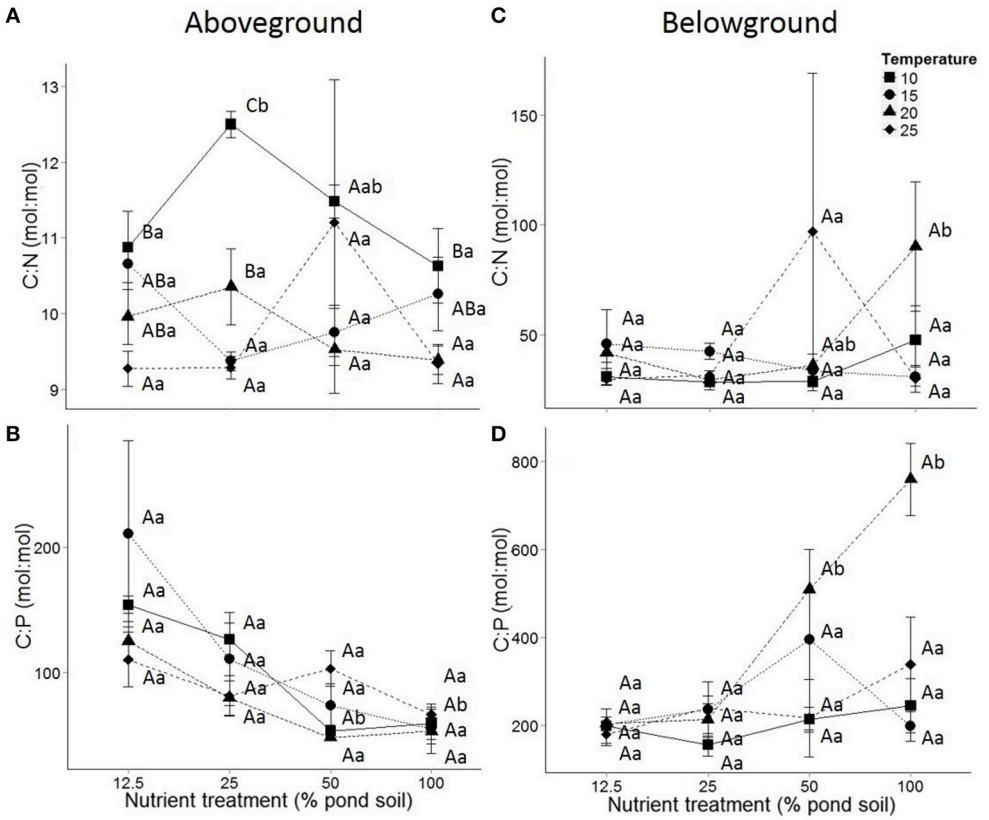

FIGURE 2 | Above—(A,B) and belowground (C,D) carbon:nutrient stoichiometry of *Elodea nuttallii* in response to sediment nutrient content, with **(A,C)** C:N and **(B,D)** C:P ratios. Temperature treatments include 10 (■), 15 (●), 20 (▲) and 25 (♦)°C. Dots represent means and error bars standard error of the mean. Capital and lower case letters indicate *post-hoc* differences between temperature and nutrient treatments, respectively.

water DIP concentrations were significantly higher at 15°C than other temperature treatments in the highest nutrient treatment ($P < 0.01$; **Figure 3B**). This response was less pronounced in other nutrient treatments. Similarly to temperature, sediment nutrient content affected DIN and DIP concentrations in the pore water. Pore water DIN and DIP increased 7- and 16-fold, respectively, from the 12.5 to 50% nutrient treatment irrespective of temperature ($P < 0.01$; **Figures 3A,B**).

Meta-Analysis

Effects of Elevated Temperature on Carbon:Nutrient Stoichiometry

No significant effects of elevated temperature were observed on aboveground C:N and C:P ratios (**Figure 4**), nor on aboveground C, N and P contents (Figure S6A) or belowground N and P contents (Figure S6B). Sample sizes were too low to analyze effects of elevated temperature on aboveground growth rates ($n = 0$), on belowground C:N and C:P ratios and C content ($n = 1$) or on potential differences between marine and freshwater ecosystems ($n = 1$ and 2 respectively).

Effects of Nutrient Addition on Carbon:Nutrient Stoichiometry

Nutrient addition significantly decreased aboveground carbon:nutrient ratios, with 24.7 and 21.9% for C:N and C:P ratios, respectively (**Figure 5A**). This decrease in aboveground carbon:nutrient ratios was accompanied by a 23.5% increase in aboveground N content and a tendency for increased P content (with 20.6%, $P = 0.06$), while C content remained unaffected (Figure S6C). Furthermore, aboveground growth rates tended to increase 83.1% with nutrient addition, but this effect was not significant ($P = 0.08$, **Figure 5A**). Similar to aboveground responses, belowground C:N ratio also declined 15.6% with nutrient addition, while no effect on belowground C:P ratios was observed (**Figure 5B**). This decline in C:N ratio was accompanied by an 18.2% increase in belowground N content (Figure S6D).

Aboveground carbon:nutrient stoichiometry of marine and freshwater plants responded qualitatively similar to nutrient addition, though the number of studies in the latter group was far lower (**Figure 6**). Quantitatively, responses in C:N and C:P were stronger for freshwater compared to marine plants ($P < 0.001$). Sample sizes were too low to analyze differences in aboveground

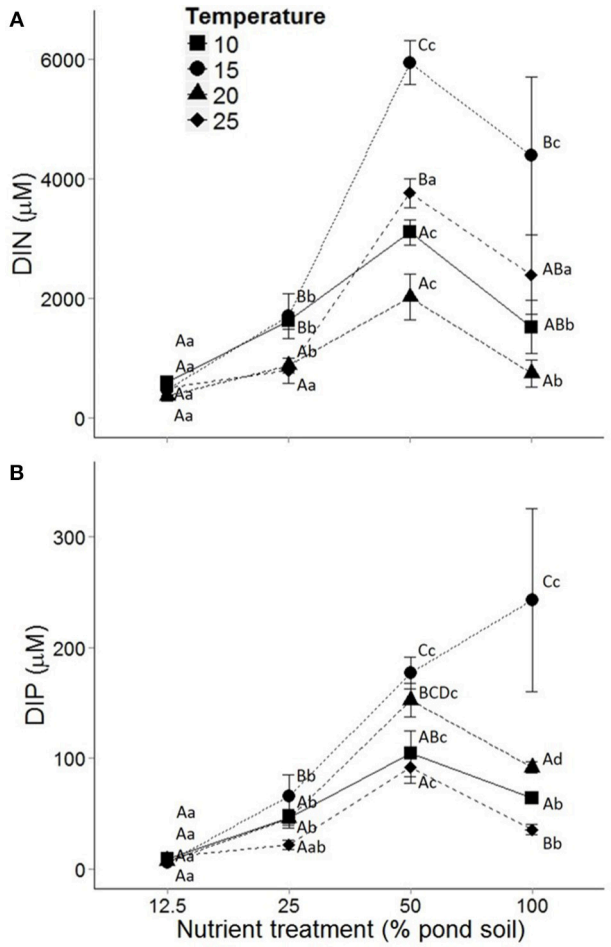

FIGURE 3 | Dissolved nutrient concentrations in the pore water in response to sediment nutrient content, with dissolved inorganic nitrogen (DIN) **(A)** and dissolved inorganic phosphorus (DIP) **(B)** at the end of the experiment. Temperature treatments include 10 (■), 15 (●), 20 (▲) and 25 (◆)°C. Dots represent means and error bars standard error of the mean. Capital and lower case letters indicate *post-hoc* differences between temperature and nutrient treatments, respectively.

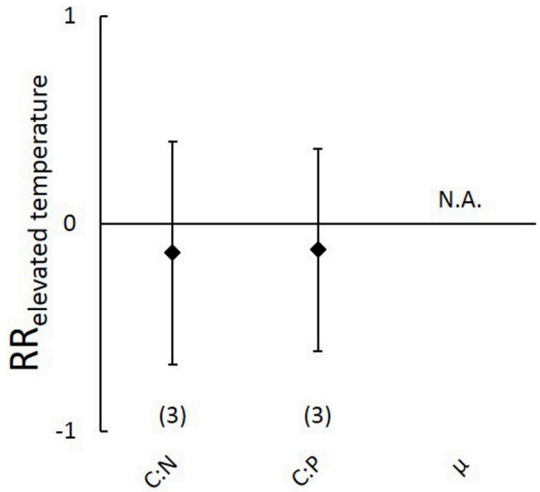

FIGURE 4 | Natural-log response ratios of aboveground carbon:nutrient stoichiometry and plant growth rates (μ) to 3-6 degrees elevated temperature from the meta-analysis on submerged aquatic plants. Values represent means, error bars 95% confidence intervals and sample size is indicated between brackets. No response ratios were significantly different from zero. N.A., indicates that data were not available.

growth rates between freshwater and marine plants ($n = 0$ for freshwater plants).

DISCUSSION

To address the impacts of temperature and nutrient availability on the growth and carbon:nutrient stoichiometry of aquatic plants, we performed a microcosm experiment and a meta-analysis. In line with our first hypothesis, elevated temperatures led to higher growth rates and standing stock biomass of the freshwater plant *Elodea nuttallii*, with an optimal growth at 15°C. In contrast to the biomass-dilution effect (second hypothesis), aboveground C:N ratios were negatively affected by temperature, and this effect interacted with nutrient treatment. Aboveground C:P ratios of E. *nuttallii* were lower with higher sediment nutrient content, in line with our third hypothesis. The observed decrease in aboveground C:P ratio coincided with an increase in P content, confirming our fourth hypothesis. However, in contrast to our third and fourth hypotheses, belowground C:N and C:P ratios as well as belowground C content increased with higher sediment nutrient content.

In the meta-analysis, elevated temperature did not lead to enhanced growth rates or increased carbon:nutrient ratios of submerged aquatic plants in general, in contrast to our first and second hypotheses. However, it should be noted that overall sample sizes were very low ($n = 3$), which may (partly) explain the lack of effect. In line with our first hypothesis, nutrient (e.g., nitrogen and phosphorus) addition tended to increase plant growth rates, though this effect was not significant. Nutrient addition led to decreased C:N and C:P ratios and increased N content, in agreement with the third and fourth hypotheses. The carbon:nutrient ratio declined in both marine and freshwater plants upon nutrient addition, although the absolute level of the response was stronger in freshwater systems.

Effects of Temperature on Plant Carbon:Nutrient Stoichiometry

Aboveground C:N ratio of *E. nuttallii* decreased moderately with increasing temperatures in our experiment. This is in direct contrast with the hypothesis of enhanced nutrient-use efficiency with elevated temperatures (2), which would lead to increased carbon:nutrient ratios [as is observed for other aquatic plants such as *Zostera marina* (Kaldy, 2014)]. The decrease in C:N ratios was most pronounced between 10 and 25°C, even though the aboveground biomass did not differ between those temperatures. Thus, temperature does not seem to indirectly affect C:N ratios through changes in biomass. Accompanied by

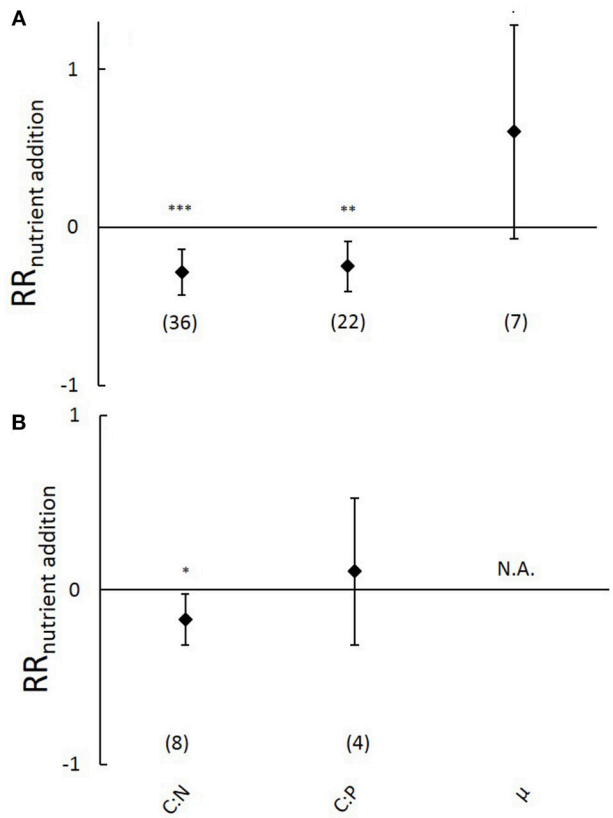

FIGURE 5 | Natural-log response ratios of carbon:nutrient stoichiometry and plant growth rates (μ) to nutrient (nitrogen and phosphorus) addition in (A) above- and **(B)** belowground biomass of submerged aquatic plants. Values represent means, error bars 95% confidence intervals and sample size is indicated between brackets. Response ratios significantly different from zero are indicated as follows: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{\cdot}P < 0.10$. N.A., indicates that data were not available.

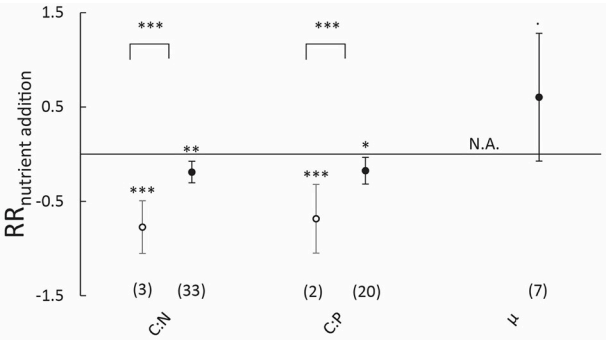

FIGURE 6 | Natural-log response ratios of aboveground carbon:nutrient stoichiometry and growth rates (μ) to nutrient (nitrogen and phosphorus) addition in freshwater (open circles) and marine (closed circles) submerged aquatic plants. Values represent means, error bars 95% confidence intervals and sample size is indicated between brackets. Significance levels are indicated as follows: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{\cdot}P < 0.10$. N.A., indicates that data were not available.

the decreased C:N ratios in our *Elodea* experiment with higher temperatures, N contents tended to be higher as well, but this effect was not significant. Increased N content over similar temperature ranges has been documented for *E. canadensis* (Ventura et al., 2008) and *Ruppia drepanensis* (Santamaria and Hootsmans, 1998) and could indicate resource allocation to nitrogen-rich compounds such as chlorophyll-a (Santamaria and Hootsmans, 1998). Furthermore, elevated temperature can increase nitrogen availability in the sediment pore water through enhanced nitrogen mobilization (Alsterberg et al., 2012), thereby indirectly leading to higher nitrogen availability for plant growth. In our experiment, the temperature treatments with highest aboveground biomass of *E. nuttallii* (e.g., 15 and 20°C) varied considerably in their pore water nitrogen availability, indicating that those are not directly related. Furthermore, as the temperature effect on C:N ratios was most pronounced at intermediate sediment nutrient content (as indicated by the temperature × nutrient interaction term), these results indicate that stoichiometric responses of plants to changes in temperature may be directly and indirectly altered by nutrient availability.

In our meta-analysis, we observed no overall effect of an 3–6°C elevated temperature on carbon:nutrient ratios of submerged aquatic plants, which contradicts findings in other groups of primary producers, such as phytoplankton (Toseland et al., 2013; De Senerpont Domis et al., 2014) and terrestrial plants (An et al., 2005). The number of studies in our analysis was rather low ($n = 3$) and included a positive (Zhang et al., 2016), negative (Ventura et al., 2008), and neutral (Touchette et al., 2003) response. The different directions of responses indicate that effects of temperature on the carbon:nutrient stoichiometry of aquatic plants are not necessarily linked to the temperature increments they are exposed to. Thus, it may indicate species-specific responses or possibly even a phylogenetic relationship considering the similar response of *E. nuttallii* in our experiment and *E. canadensis* (Ventura et al., 2008), which both have an optimal growth temperature of around 15°C (Olesen and Madsen, 2000). However, due to the limited sample size of each species ($n = 1$), we currently cannot distinguish between species-specific and study-specific responses (such as experimental set-up and environmental conditions) of carbon:nutrient stoichiometry in our analysis.

Effects of Nutrient Addition on Carbon:Nutrient Tissue Stoichiometry

Aboveground C:P ratios decreased about 4-fold with increasing sediment nutrient content in the *Elodea* experiment, confirming our third hypothesis. However, in contrast to this hypothesis, belowground C:P ratios of *E. nuttallii* and carbon content rather increased with sediment nutrient availability. Higher belowground carbon content can indicate thicker cell walls and thicker roots. Possibly, with sufficient nutrient availability, *Elodea* may shift from investment in root structures for nutrient uptake to thicker roots for anchorage in the sediment (Sand-Jensen and Madsen, 1991). In the meta-analysis, nutrient addition

led to a 25% and 22% decrease in aboveground C:N and C:P ratios of submerged aquatic plants, consistent with the results from the *Elodea* experiment. While some variability in response can be detected at the species level, responses are consistently either absent or negative (Figure S7). Similar to the aboveground responses, nutrient addition led to a 16% decrease in belowground C:N ratios. These decreases in above- and belowground carbon:nutrient ratios were accompanied by increased tissue N and P contents and demonstrate the flexibility in carbon:nutrient stoichiometry of aquatic plants under fluctuating nutrient availability (Sardans et al., 2012).

Increased plant nutrient content as observed in our meta-analysis and *Elodea* experiment may have resulted from excess or luxurious uptake of nutrients (Millard, 1988), as terrestrial plants can store excess P in cell vacuoles (Bieleski, 1973) and N in specialized storage organs (Aerts and Chapin, 2000). Similar to our results, meta-analytic studies on terrestrial plants observed elevated foliar N and P contents in response to nutrient addition (Yuan and Chen, 2015) and a decrease in C:N in photosynthetic tissues to N addition (Sardans et al., 2012). Combined with our results, this indicates that these effects are not ecosystem specific, but can be seen as a general qualitative response of primary producers to nutrient addition.

Our analysis indeed indicated qualitatively similar responses to nutrient addition in both marine and freshwater submerged plants, though the responses were stronger in the latter group. Sample sizes for freshwater plants were far lower than for marine plants, highlighting the potential for freshwater research to learn from physiological studies on marine plants. Mean C:N ratios of freshwater plants are lower than marine plants (Bakker et al., 2016) and could result from higher levels of fertilization as nutrient levels in freshwater are generally considered higher than in marine systems (Smith et al., 1999). However, as these ecosystems differ greatly in retention time, sediment characteristics and osmotic stress from salinity (Short et al., 2016), caution must be taken when interpreting these differences.

Possible Implications for Carbon Cycling and Food-Web Dynamics

Changes in plant carbon:nutrient stoichiometry in aquatic systems can have consequences for carbon cycling. In our meta-analysis, nutrient addition tended to increase plant growth rates, with positive (Murray et al., 1992; Udy et al., 1999; Peralta et al., 2003) and neutral (Erftemeijer et al., 1994; Holzer and McGlathery, 2016) responses reported. Thus, carbon sequestration in the form of plant standing stock biomass can be enhanced by nutrient addition (Armitage and Fourqurean, 2016). Furthermore, changes in carbon:nutrient stoichiometry can have consequences for the energy transfer to higher trophic levels as elevated nutrient content in aquatic plants can lead to increased herbivore grazing rates (Bakker and Nolet, 2014) and subsequent reduction in standing-stock biomass (van Altena et al., 2016). This may counteract positive effects of fertilization on plant growth rates and carbon sequestration. Furthermore, eutrophic conditions can enhance plant litter quality (Emsens et al., 2016) and plants with lower carbon:nutrient ratios decompose faster than those with higher ratios (Wang et al., 2017), indicating an accelerated release of sequestered carbon and nutrients. We therefore hypothesize that eutrophication can affect carbon stocks in submerged aquatic vegetation, through changes in their nutritional quality (e.g., reduced carbon:nutrient stoichiometry) and subsequent effects on grazing and decomposition. Given the current knowledge about the effects of temperature on carbon:nutrient stoichiometry of aquatic plants presented in this study, we cannot draw any general conclusions on the effect of global warming on aquatic carbon cycling. However, our results suggest species-specific responses, which indicates that given the community composition in an ecosystem, effects may be substantial. Our current analysis focuses on individual plant responses and their stoichiometric flexibility. On a community level, interspecific variability can drive changes in C:N:P stoichiometry (Frost and Hicks, 2012), with consequences for community composition under elevated nutrient availability and temperature. For instance, elevated temperature can shift aquatic plant community composition toward floating vegetation (Netten et al., 2010), while nutrient addition can lead to a decline in overall plant abundance at the expense of algae (Scheffer et al., 1993; Short and Neckles, 1999). Therefore, hypotheses on an ecosystem level should also take these changes into account.

CONCLUSION

We conclude that nutrient (e.g., nitrogen and phosphorus) addition decreases carbon:nutrient stoichiometry in submerged aquatic plants, while no consistent effects of elevated temperature on these ratios were observed. The latter could be an effect of low sample size or could indicate species-specific responses in carbon:nutrient stoichiometry to global warming, which is an interesting avenue for future research. Furthermore, our experiment shows that the impact of temperature on aquatic plant stoichiometry depends on the availability of nutrients for plant growth, which is seldom taken into account. The impact of temperature may thus be modified by nutrient availability. The observed decline in carbon:nutrient stoichiometry of aquatic plants in response to nutrient addition can stimulate the further energy transfer to herbivores and decomposers, leading to reduced carbon stocks. With ongoing global warming, the knowledge gap of temperature effects on carbon:nutrient stoichiometry of submerged aquatic plants is in urgent need for further investigation.

AUTHOR CONTRIBUTIONS

MV, EB, and EvDo conceived and designed the experiments. EvDe performed the experiments. MV, EvDe, and PZ analyzed the data. MV and EB wrote the manuscript; all other authors provided editorial contributions.

FUNDING

The work of MV is funded by the Gieskes-Strijbis Foundation and the work of PZ by the China Scholarship Council (CSC).

ACKNOWLEDGMENTS

The authors would like to thank Dennis de Raaij and Arjan Wiersma for their help with extracting papers and setting up the database and Nico Helmsing for the chemical analyses during the experiment. Furthermore, we would like to thank Joost Keuskamp for the development of statistical tools for the meta-analysis, Sven Teurlincx for his help with the harvest together with fruitful discussions and Inés Castejón, Kim Holzer and Marion Cambridge for contributing unpublished data to the meta-analysis database. This is NIOO publication number 6275.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00655/full#supplementary-material

REFERENCES

Adrian, R., O'Reilly, C. M., Zagarese, H., Baines, S. B., Hessen, D. O., Keller, W., et al. (2009). Lakes as sentinels of climate change. *Limnol. Oceanogr.* 54, 2283–2297. doi: 10.4319/lo.2009.54.6_part_2.2283

Aerts, R., Chapin, F. S. III. (2000). The mineral nutrition of wild plants revisited: a re-evaluation of processes and patterns. *Adv. Ecol. Res.* 30, 1–67. doi: 10.1016/S0065-2504(08)60016-1

Alsterberg, C., Sundbäck, K., and Hulth, S. (2012). Functioning of a shallow-water sediment system during experimental warming and nutrient enrichment. *PLoS ONE* 7:e51503. doi: 10.1371/journal.pone.0051503

An, Y., Wan, S., Zhou, X., Subedar, A. A., Wallace, L. L., and Luo, Y. (2005). Plant nitrogen concentration, use efficiency, and contents in a tallgrass prairie ecosystem under experimental warming. *Glob. Chang. Biol.* 11, 1733–1744. doi: 10.1111/j.1365-2486.2005.01030.x

Armitage, A. R., and Fourqurean, J. W. (2016). Carbon storage in seagrass soils: long-term nutrient history exceeds the effects of near-term nutrient enrichment. *Biogeosciences* 13, 313–321. doi: 10.5194/bg-13-313-2016

Bakker, E. S., and Nolet, B. A. (2014). Experimental evidence for enhanced top-down control of freshwater macrophytes with nutrient enrichment. *Oecologia* 176, 825–836. doi: 10.1007/s00442-014-3047-y

Bakker, E. S., Wood, K. A., Pagès, J. F., Veen, G. F., Christianen, M. J. A., Santamaría, L., et al. (2016). Herbivory on freshwater and marine macrophytes: a review and perspective. *Aquat. Bot.* 135, 18–36. doi: 10.1016/j.aquabot.2016.04.008

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300.

Bieleski, R. L. (1973). Phosphate pools, phosphate transport, and phosphate availability. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 24, 225–252. doi: 10.1146/annurev.pp.24.060173.001301

Bloom, A. J., Chapin, F. S., and Mooney, H. A. (1985). Resource limitation in plants - an economic analogy. *Annu. Rev. Ecol. Syst.* 16, 363–392. doi: 10.1146/annurev.es.16.110185.002051

Bornette, G., and Puijalon, S. (2011). Response of aquatic plants to abiotic factors: a review. *Aquat. Sci.* 73, 1–14. doi: 10.1007/s00027-010-0162-7

Burkholder, J. M., Tomasko, D. A., and Touchette, B. W. (2007). Seagrasses and eutrophication. *J. Exp. Mar. Biol. Ecol.* 350, 46–72. doi: 10.1016/j.jembe.2007.06.024

Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R. W., Sharpley, A. N., and Smith, V. H. (1998). Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecol. Appl.* 8, 559–568. doi: 10.1890/1051-0761(1998)008[0559:NPOSWW]2.0.CO;2

Collaboration for Environmental Evidence (2013). *Guidelines for Systematic Review and Evidence Synthesis in Environmental Management. Version 4.2. Environmental Evidence*: Available online at: www.environmentalevidence.org/Documents/Guidelines/Guidelines4.2.pdf

Cook, C. D. K., and Urmi-König, K. (1985). A revision of the genus Elodea (Hydrocharitaceae). *Aquat. Bot.* 21, 111–156. doi: 10.1016/0304-3770(85)90084-1

De Senerpont Domis, L. N., Van de Waal, D. B., Helmsing, N. R., Donk, E., and Mooij, W. M. (2014). Community stoichiometry in a changing world: combined effects of warming and eutrophication on phytoplankton dynamics. *Ecology* 95, 1485–1495. doi: 10.1890/13-1251.1

Dorenbosch, M., and Bakker, E. S. (2011). Herbivory in omnivorous fishes: effect of plant secondary metabolites and prey stoichiometry. *Freshw. Biol.* 56, 1783–1797. doi: 10.1111/j.1365-2427.2011.02618.x

Emsens, W. J., Aggenbach, C. J., Grootjans, A. P., Nfor, E. E., Schoelynck, J., Struyf, E., et al. (2016). Eutrophication triggers contrasting multilevel feedbacks on litter accumulation and decomposition in fens. *Ecology* 97, 2680–2690. doi: 10.1002/ecy.1482

Erftemeijer, P. L. A., Stapel, J., Smekens, M. J. E., and Drossaert, W. M. E. (1994). The limited effect of *in-situ* phosphorus and nitrogen additions to seagrass beds on carbonate and terrigenous sediments in South Sulawesi, Indonesia. *J. Exp. Mar. Biol. Ecol.* 182, 123–140. doi: 10.1016/0022-0981(94)90215-1

Frost, P. C., and Hicks, A. L. (2012). Human shoreline development and the nutrient stoichiometry of aquatic plant communities in Canadian Shield lakes. *Can. J. Fish. Aquat. Sci.* 69, 1642–1650. doi: 10.1139/f2012-080

Heithaus, M. R., Alcoverro, T., Arthur, R., Burkholder, D. A., Coates, K. A., Christianen, M. J. A., et al. (2014). Seagrasses in the age of sea turtle conservation and shark overfishing. *Front. Mar. Sci.* 1:28. doi: 10.3389/fmars.2014.00028

Holzer, K. K., and McGlathery, K. J. (2016). Cultivation grazing response in seagrass may depend on phosphorus availability. *Mar. Biol.* 163:88. doi: 10.1007/s00227-016-2855-5

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical J.* 50, 346–363. doi: 10.1002/bimj.200810425

IPCC (2014). *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Geneva: IPCC

Kaldy, J. E. (2014). Effect of temperature and nutrient manipulations on eelgrass *Zostera marina* L. from the Pacific Northwest, USA. *J. Exp. Mar. Biol. Ecol.* 453, 108–115. doi: 10.1016/j.jembe.2013.12.020

Lajeunesse, M. J. (2015). Bias and correction for the log response ratio in ecological meta-analysis. *Ecology* 96, 2056–2063. doi: 10.1890/14-2402.1

Millard, P. (1988). The accumulation and storage of nitrogen by herbaceous plants. *Plant Cell Environ.* 11, 1–8. doi: 10.1111/j.1365-3040.1988.tb01769.x

Mooij, W. M., De Senerpont Domis, L. N., and Hülsmann, S. (2008). The impact of climate warming on water temperature, timing of hatching and young-of-the-year growth of fish in shallow lakes in the Netherlands. *J. Sea Res.* 60, 32–43. doi: 10.1016/j.seares.2008.03.002

Murray, L., Dennison, W. C., and Kemp, W. M. (1992). Nitrogen versus phosphorus limitation for growth of an estuarine population of eelgrass (*Zostera marina* L.). *Aquat. Bot.* 44, 83–100. doi: 10.1016/0304-3770(92)90083-U

Netten, J. J. C., Arts, G. H. P., Gylstra, R., Nes, E. H. Scheffer, M., and Roijackers, R. M. M. (2010). Effect of temperature and nutrients on the competition between free-floating Salvinia natans and submerged *Elodea nuttallii* in mesocosms. *Fundam. Appl. Limnol.* 177, 125–132. doi: 10.1127/1863-9135/2010/0177-0125

Olesen, B., and Madsen, T. V. (2000). Growth and physiological acclimation to temperature and inorganic carbon availability by two submerged aquatic macrophyte species, *Callitriche cophocarpa* and *Elodea canadensis*. *Funct. Ecol.* 14, 252–260. doi: 10.1046/j.1365-2435.2000.00412.x

Olsen, Y. S., and Valiela, I. (2010). Effect of sediment nutrient enrichment and grazing on turtle grass *Thalassia testudinum* in Jobos Bay, Puerto Rico. *Estuaries Coasts* 33, 769–783. doi: 10.1007/s12237-009-9256-7

Peralta, G., Bouma, T. J., van Soelen, J., Perez-Llorens, J. L., and Hernandez, I. (2003). On the use of sediment fertilization for seagrass restoration:

a mesocosm study on *Zostera marina* L. *Aquat. Bot.* 75, 95–110. doi: 10.1016/S0304-3770(02)00168-7

R Core Team (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for statistical computing. Available online at: https://www.R-project.org/

Sand-Jensen, K., and Madsen, T. V. (1991). Minimum light requirements of submerged freshwater macrophytes in laboratory growth experiments. *J. Ecol.* 79, 749–764. doi: 10.2307/2260665

Santamaria, L., and Hootsmans, M. J. M. (1998). The effect of temperature on the photosynthesis, growth and reproduction of a Mediterranean submerged macrophyte, Ruppia drepanensis. *Aquat. Bot.* 60, 169–188. doi: 10.1016/S0304-3770(97)00050-8

Sardans, J., Rivas-Ubach, A., and Penuelas, J. (2012). The C:N:P stoichiometry of organisms and ecosystems in a changing world: a review and perspectives. *Perspect. Plant Ecol. Evol. Syst.* 14, 33–47. doi: 10.1016/j.ppees.2011.08.002

Scheffer, M., Hosper, S. H., Meijer, M. L., Moss, B., and Jeppesen, E. (1993). Alternative equilibria in shallow lakes. *Trends Ecol. Evol. (Amst).* 8, 275–279. doi: 10.1016/0169-5347(93)90254-M

Short, F. T., Kosten, S., Morgan, P. A., Malone, S., and Moore, G. E. (2016). Impacts of climate change on submerged and emergent wetland plants. *Aquat. Bot.* 135, 3–17. doi: 10.1016/j.aquabot.2016.06.006

Short, F. T., and Neckles, H. A. (1999). The effects of global climate change on seagrasses. *Aquat. Bot.* 63, 169–196. doi: 10.1016/S0304-3770(98)00117-X

Smith, V. H., Tilman, G. D., and Nekola, J. C. (1999). Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environ. Pollut.* 100, 179–196. doi: 10.1016/S0269-7491(99)00091-3

Steffen, W., Richardson, K., Rockström, J., Cornell, S. E., Fetzer, I., Bennett, E. M., et al. (2015). Planetary boundaries: guiding human development on a changing planet. *Science* 347:1259855. doi: 10.1126/science.1259855

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: the Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Taylor, W. D., Carey, J. H., Lean, D. R. S., and McQueen, D. J. (1991). Organochlorine concentrations in the plankton of lakes in southern Ontario and their relationship to plankton biomass. *Can. J. Fish. Aquat. Sci.* 48, 1960–1966. doi: 10.1139/f91-233

Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., et al. (2001). Forecasting agriculturally driven global environmental change. *Science* 292, 281–284. doi: 10.1126/science.1057544

Toseland, A., Daines, S. J., Clark, J. R., Kirkham, A., Strauss, J., Uhlig, C., et al. (2013). The impact of temperature on marine phytoplankton resource allocation and metabolism. *Nat. Clim. Chang.* 3, 979–984. doi: 10.1038/nclimate1989

Touchette, B. W., Burkholder, J. M., and Glasgow, H. B. (2003). Variations in eelgrass (*Zostera marina* L.) morphology and internal nutrient composition as influenced by increased temperature and water column nitrate. *Estuaries* 26, 142–155. doi: 10.1007/BF02691701

Udy, J. W., Dennison, W. C., Long, W. J. L., and McKenzie, L. J. (1999). Responses of seagrass to nutrients in the Great Barrier Reef, Australia. *Mar. Ecol. Prog. Ser.* 185, 257–271. doi: 10.3354/meps185257

van Altena, C., Bakker, E. S., Kuiper, J. J., and Mooij, W. M. (2016). The impact of bird herbivory on macrophytes and the resilience of the clear-water state in shallow lakes: a model study. *Hydrobiologia* 777, 197–207. doi: 10.1007/s10750-016-2779-6

van Dam, H. (2009). *Evaluatie Basismeetnet Waterkwaliteit Hollands Noorderkwartier: Trendanalyse Hydrobiology, Temperatuur en Waterchemie 1982–2007*. Edam: Hoogheemraadschap Hollands Noorderkwartier.

Ventura, M., Liboriussen, L., Lauridsen, T. M., SØndergaard, M., SØndergaard, and Jeppesen, E. (2008). Effects of increased temperature and nutrient enrichment on the stoichiometry of primary producers and consumers in temperate shallow lakes. *Freshw. Biol.* 53, 1434–1452. doi: 10.1111/j.1365-2427.2008.01975.x

Vermaat, J. E., and Hootsmans, M., J. M. (1994). "Growth of *Potamogeton pectinatus* L. in a temperature-light gradient," in *Lake Veluwe, a Macrophyte-Dominated System under Eutrophication Stress*, eds W. van Vierssen, M. Hootsmans, and J. Vermaat (Dordrecht: Springer Netherlands), 40–61.

Viechtbauer, W. (2010). Conducting meta-analyses in R with the metafor package. *J. Stat. Softw.* 36, 1–48. doi: 10.18637/jss.v036.i03

Vitousek, P. M., Mooney, H. A., Lubchenco, J., and Melillo, J. M. (1997). Human domination of earth's ecosystems. *Science* 277, 494–499. doi: 10.1126/science.277.5325.494

Wang, M., Hao, T., Deng, X. W., Wang, Z. X., Cai, Z. H., and Li, Z. Q. (2017). Effects of sediment-borne nutrient and litter quality on macrophyte decomposition and nutrient release. *Hydrobiologia* 787, 205–215. doi: 10.1007/s10750-016-2961-x

Yuan, Z. Y., and Chen, H. Y. H. (2015). Negative effects of fertilization on plant nutrient resorption. *Ecology* 96, 373–380. doi: 10.1890/14-0140.1

Zhang, P. Y., Bakker, E. S., Zhang, M., and Xu, J. (2016). Effects of warming on Potamogeton crispus growth and tissue stoichiometry in the growing season. *Aquat. Bot.* 128, 13–17. doi: 10.1016/j.aquabot.2015.08.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 30 January 2018
doi: 10.3389/fmicb.2018.00069

Predominant Non-additive Effects of Multiple Stressors on Autotroph C:N:P Ratios Propagate in Freshwater and Marine Food Webs

Manuel Villar-Argaiz[1]*, *Juan M. Medina-Sánchez*[1], *Bopaiah A. Biddanda*[2] and *Presentación Carrillo*[3]

[1] Departamento de Ecología, Facultad de Ciencias, Universidad de Granada, Granada, Spain, [2] Annis Water Resources Institute, Grand Valley State University, Muskegon, MI, United States, [3] Instituto del Agua, Universidad de Granada, Granada, Spain

A continuing challenge for scientists is to understand how multiple interactive stressor factors affect biological interactions, and subsequently, ecosystems–in ways not easily predicted by single factor studies. In this review, we have compiled and analyzed available research on how multiple stressor pairs composed of temperature (T), light (L), ultraviolet radiation (UVR), nutrients (Nut), carbon dioxide (CO_2), dissolved organic carbon (DOC), and salinity (S) impact the stoichiometry of autotrophs which in turn shapes the nature of their ecological interactions within lower trophic levels in streams, lakes and oceans. Our analysis from 66 studies with 320 observations of 11 stressor pairs, demonstrated that non-additive responses predominate across aquatic ecosystems and their net interactive effect depends on the stressor pair at play. Across systems, there was a prevalence of antagonism in freshwater (60–67% vs. 47% in marine systems) compared to marine systems where synergism was more common (49% vs. 33–40% in freshwaters). While the lack of data impeded comparisons among all of the paired stressors, we found pronounced system differences for the L × Nut interactions. For this interaction, our data for C:P and N:P is consistent with the initial hypothesis that the interaction was primarily synergistic in the oceans, but not for C:N. Our study found a wide range of variability in the net effects of the interactions in freshwater systems, with some observations supporting antagonism, and others synergism. Our results suggest that the nature of the stressor pairs interactions on C:N:P ratios regulates the "continuum" commensalistic-competitive-predatory relationship between algae and bacteria and the food chain efficiency at the algae-herbivore interface. Overall, the scarce number of studies with even more fewer replications in each study that are available for freshwater systems have prevented a more detailed, insightful analysis. Our findings highlighting the preponderance of antagonistic and synergistic effects of stressor interactions in aquatic ecosystems—effects that play key roles in the functioning of feedback loops in the biosphere—also stress the need for further studies evaluating the interactive effects of multiple stressors in a rapidly changing world facing a confluence of tipping points.

Keywords: interactive effects, C:N:P ratios, stoichiometry, autotroph, microbial loop, zooplankton

OPEN ACCESS

Edited by:
James Cotner,
University of Minnesota, United States

Reviewed by:
Valeria Souza,
Universidad Nacional Autónoma de México, Mexico
Nina Welti,
Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia

***Correspondence:**
Manuel Villar-Argaiz
mvillar@ugr.es

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *06 March 2017*
Accepted: *11 January 2018*
Published: *30 January 2018*

Citation:
Villar-Argaiz M, Medina-Sánchez JM, Biddanda BA and Carrillo P (2018) Predominant Non-additive Effects of Multiple Stressors on Autotroph C:N:P Ratios Propagate in Freshwater and Marine Food Webs. Front. Microbiol. 9:69. doi: 10.3389/fmicb.2018.00069

INTRODUCTION

"*An ecosystem is greater than the sum of its parts*."
–Eugene P. Odum (1964)

A paramount theme in the scientific and political arena is to better understand and predict the impact of human activities on the functioning of ecosystems, such as modification of biogeochemical cycles, climate change or species harvest and biodiversity loss (Carpenter et al., 2008; Cheung et al., 2009). We have greatly advanced our knowledge about the molecular and cellular basis of these impacts, but are still blind to the effects on the higher levels of integration of populations and ecosystems. Much of the higher complexity that arises as we scale up to higher trophic levels is a consequence of the interaction of species within their biotic and abiotic environment (Krebs, 2009; Boyd and Hutchins, 2012). While ecologists have traditionally focused on a particular level of integration (population, community, or ecosystem ecology), modern ecology is no longer viewed as isolated parts anymore, instead seeks to theoretically connect all levels of integration. One theoretical approach that has greatly advanced our knowledge on this systematic integration is Ecological Stoichiometry (ES). In short, ES is the scientific study of the balance of multiple chemical elements in ecological interactions (Sterner and Elser, 2002). Although, it can be lightly classified as a highly reductionistic approach, implications of stoichiometry span from atoms to all higher levels of integration: growth and reproduction rates, nutrient recycle, interspecific interactions or food quality and energy transfer in food webs are all subject to the limitations imposed by the availability and stoichiometry of elements. A core pillar of stoichiometry is that chemical composition is variable and reflects that of the available substrate in plants but is relatively tight in their consumer heterotrophs (Sterner and Elser, 2002). Herbivores with high somatic demands for nutrients face a world where plants are for the most part poor food quality resources (Hessen, 1992). As a result, nutrients and energy that flow through consumer-resource interactions obey to the fundamental constraints of a mass balance reaction and thermodynamics, with a myriad of consequences for organism's growth, population dynamics and ecological processes (Sterner and Elser, 2002).

Global change is happening now and has already affected numerous species and ecosystem processes (Pace et al., 2015). Relevant global factors include increased atmospheric CO_2 with consequences for global warming, alterations in biochemical cycles (e.g., N and P), local and regional eutrophication, habitat use and land use alterations or increased UV radiation, among others. A fundamental strength of ES in the study of global change is that the multiplicity of human activities and natural perturbations have an impact at the base of food webs because the elemental composition of algae and plants in general, reflect the resource availability in their environment, and can be traced as they indirectly influence secondary producers and predators at higher levels (Sterner and Elser, 2002). In fact, numerous observations suggest that the indirect-food chain mediated effects of a stressor can be far more significant than direct effects on organisms at any given trophic level (e.g., Durif et al., 2015). There is an increasing awareness among scientists that realistic scenarios of global changes need holistic approaches that include multiple interactive factors (Folt et al., 1999). For example, current climate change due to rising CO_2 concentrations are likely having an effect on photosynthetic rates in plants. At the same time, the associated rise in temperature can stimulate growth of many species, but enhance stratification of the water column, which can itself exacerbate nutrient exhaustion in surface waters leading to community changes and greater sensitivity to photosynthesis and UVR. Organisms are exposed to multiple stressors simultaneously, whose interactions can enhance or decrease the effect of a given stressor (Folt et al., 1999; Crain et al., 2008; Boyd et al., 2015). However, while numerous studies have documented how environmental conditions affect the elemental composition of primary producers, there is far less information on the combined effects of multiple global stressors, and even fewer studies have examined the role of stoichiometry on how multiple interactive effects impact ecological interactions. If examining the role of interactive effects on stoichiometry is not a trivial task, establishing the carry-on consequences of these effects on ecological interactions is even more difficult as it demands thorough work explicitly gauging cause-effect relationships. Studies on this incipient research area would be immensely valuable in our path to an improved predictive framework for interactive effects in nature.

The initial convention distinguished between interactions that increased stress or "synergistic" from those that decreased stress or "antagonistic" (Folt et al., 1999; Gunderson et al., 2016). While the study of interactions has been extremely active of late [see for example recent reviews by Jackson et al. (2015) and Piggott et al. (2015)], still a consensus is lacking in the literature regarding an operational definition of interactions. Our intention in this study is not to examine the appropriateness of a given classification method for the effects of multiple stressors. Instead, we adhered to a basic definition of synergism and antagonism to illustrate the nature of the net effect of multiple stressors in ecosystems with the purpose of improving our understanding of the mechanisms behind multiple stress effects and the habitat-specific prevalence of the various types of interactions in nature. On the basis of a literature survey, Crain et al. (2008) and Jackson et al. (2015) found that while the effect of paired stressors were consistently antagonistic or additive in freshwater systems, there was a greater prevalence of synergistic interactions in marine systems.

In this review, we hierarchically examine progress in these areas of ecological interactions and stoichiometry by answering several fundamentally related questions: What are the interactive effects of multiple stressors on the elemental composition of primary producers in aquatic ecosystems? Are there habitat differences in the prevalence of synergistic, antagonistic or additive responses? To address these questions, we will first compile field and laboratory investigations that examine the effect of paired stressors on the elemental stoichiometry of autotrophs in aquatic ecosystems and classify them as synergistic or antagonistic according to a modification of Allgeier et al.'s interaction effect index (IEI) (Allgeier et al., 2011), which compares the cumulative mean size effect of two paired stressors with the sum of their individual effects (see methods for further

explanation). With the resulting database, we will test whether patterns in the prevalence of a given interaction differ across systems and, more specifically, the general hypothesis that interactive effects across paired stressors are primarily synergistic in marine systems, but antagonistic or additive in freshwater ecosystems.

Finally, we discuss recent progress about the relevance of the effects of paired environmental drivers in fundamental food web relationships (microbial loop, algae-herbivore), identify gaps in the research of multiple stressors, and suggest new avenues for stressor interactions research.

DATA EXTRACTION AND STATISTICAL ANALYSES

Experimental literature on the impacts of multiple stressors on C:N:P ratios was searched using Web of Science (review to January 2017). The following criteria were applied to select articles:

1. Published studies on the research topic were identified using the following search keywords: "stoichiometry," "interactive effect*," "C:N*," and "C:P*." For each extracted study, we examined the cited references in search of additional suitable data, and used ResearchGate and Google Scholar as supplementary research tools in addition to Web of Science.
2. The dataset covered laboratory and *in situ* experiments in which the effects of paired stressors on the C:N:P composition of the autotrophs in aquatic food webs (plankton, epilithon or periphyton) were reported. Macrophytes were excluded from this analysis. As in Crain et al. (2008), a stressor was defined in a broad sense as any environmental global driver that can potentially exceed natural levels of variation.
3. The study belonged to any of habitat categories of streams, lakes (reservoirs or lakes) and marine systems, regardless of whether these were field or controlled laboratory studies using species from their natural habitats.

Sixty-six studies met the study selection criteria outlined above (full list in Table S1). Our database compiled a total of 320 observations, since most studies reported more than one observation. We investigated a total of 11 stressor pairs (**Figure 1**). Whenever possible, we obtained C:N:P values from the result section of the study. A PlotDigitizer software (Free Software Foundation, 2001) was used to acquire numbers from the figures when data were not readily available in the results. Care was taken to differentiate between observations testing the interactive effects of an increase or decrease in the main stressor. For example, studies covering L × Nut effects on C:N:P ratios were classified in two broad categories depending on whether experiments tested the increase (↑L × Nut) by supplementing light conditions or decrease (↓L × Nut) by shading or reducing light supplementation relative to that received by the organisms in their habitat (field research) or optimal level (laboratory assays). Also decreased or increased stressor scenarios could help differentiate between future and past scenarios in the various ratios, as well as future opposed predictions for a given stressor. For example, most studies agree upon lower-than-today CO_2 levels (∼150 ppm) for past glacial events and higher-than-today CO_2 levels for future CO_2 scenarios (500–1,000 ppm), although there is the potential for future scenarios of deprived CO_2 in local environments of markedly increased photosynthesis.

To illustrate the overall nature of the interactive effects of multiple stressor on C:N:P ratios, we modified the Interaction Effect Index (IEI) proposed by Allgeier et al. (2011) according to the following equation:

$$\text{IEI} = \ln\left[\text{Abs}(\text{effect AB}/(\text{effect A} + \text{effect B}))\right]$$

where A and B are the two stressors, "Abs" indicates absolute value, "effect AB" is the combined effect of AB calculated as (treatment_{A+B}−control), "effect A" is the main effect of A calculated as (treatment_{A}−control), and "effect B" is the main effect of B calculated as (treatment_{B}−control).

Taking the effect ratio as the natural logarithm of the absolute quotient between combined (effect AB) and additive effects (effect A + effect B), has important advantages compared to other forms of evaluating interactive effects: (i) it allows calculation of IEI for all integer numbers (negative, zero, and positive numbers), (ii) includes standardization of the responses, what reduces variability in the interactive effects and grants the use of parametric analysis for comparative purposes, and (iii) centers IEI values around zero. We used IEI in this study to identify the three broad categories of interactions types (as defined in Folt et al., 1999) on C:N:P ratios (see **Figure 2**). IEI values above one were defined as cases in which the interaction was synergistic, whereas IEI values below one indicated antagonistic interaction, whereas values non distinguishable from zero indicated the occurrence of an additive effect or the absence of a net interactive effect.

The frequency of interaction types was calculated for the various subsets corresponding with the C:N, C:P, and N:P ratios in the studied species. This graphic representation of these data in **Figure 3** contributed to identify differences in the prevailing interaction type among systems and C:N:P ratios. Finally, one-sample *t*-tests were used to evaluate whether mean C:N:P-IEI differed from zero for each stressor pair and system (**Figure 4**). If the null hypothesis is rejected, it demonstrates an interactive effect (antagonisms or synergisms). On the other hand, non-rejection of the null hypothesis was interpreted as the absence of significant interaction, which does not necessarily mean the presence of an additive effect because observations with opposing interactive effects could compensate each other such that their mean might not significantly differ from zero.

Significant differences in C:N:P-IEI between habitats and drivers were tested using one-way ANOVA, followed by *post-hoc* comparison of the means using Tukey's HSD (Sokal and Rohlf, 1995). Normality was test by Kolmogorov-Smirnov test and homocedasticity by Cochran's and Levene's tests. Although care was taken to differentiate data that originally tested interactions in our illustrations (see different bar colors in Figures S1–S6), we chose to include all observations in our intersystem comparison in order to increase the statistical confidence of our findings.

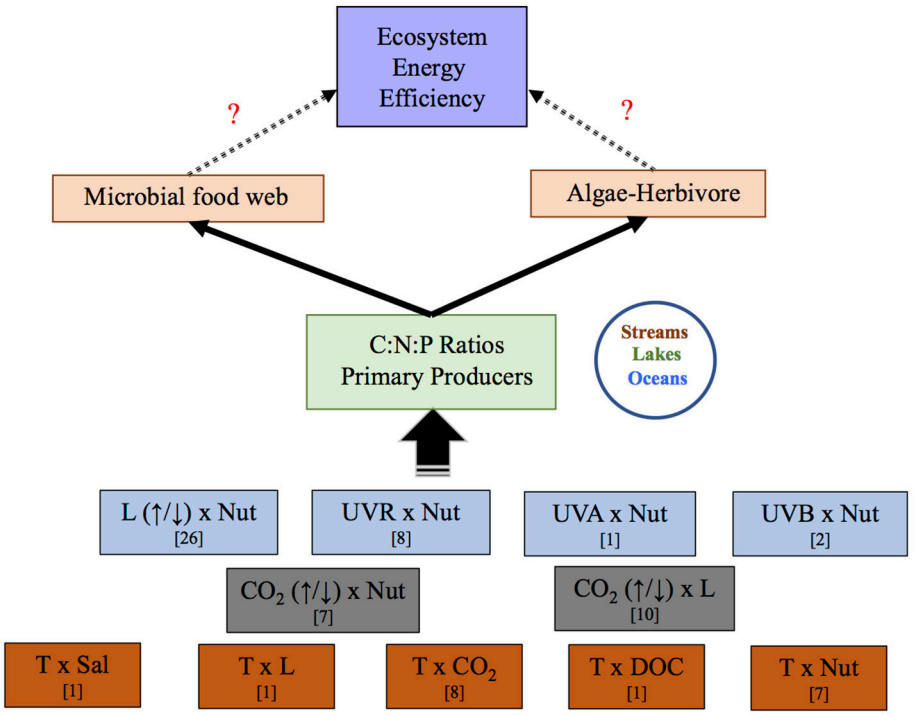

FIGURE 1 | Scheme illustrating the potential effects of stressor pairs in food web interactions via changes in C:N:P stochiometry. Eleven stressor-pairs were analyzed in this study. L, light; Nut, nutrients; UVR, ultraviolet radiation; T, temperature; Sal, salinity; CO_2, carbon dioxide; DOC, dissolved organic carbon. Question marks indicate pathways lacking information. In brackets is number of studies found for each stressor pair.

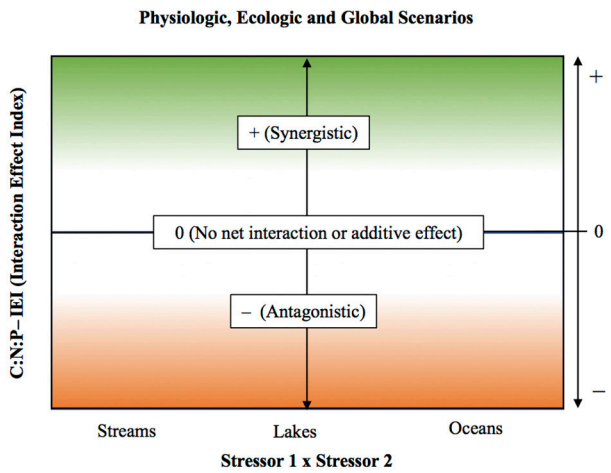

FIGURE 2 | Scheme illustrating the interpretation of the Interactive Effect Index (IEI) for each stressor pair. IEI was calculated as Ln [ABS (Combined/Additive effects)] (see statistical methods for details). The IEI in the y-axis reflects the nature of the interactive effect for C:N:P ratios, with values > 0 indicating a synergistic interaction, values < 0 indicating an antagonistic interaction, and values = 0 indicating the absence of a net interactive effect or the occurrence of an additive effect. Stressor pairs are shown in x-axis.

IBM SPSS Statistics (version 24) was used for all data analyses and a statistical significance level of $p < 0.05$ was applied to all tests.

INTERACTIVE EFFECTS OF GLOBAL CHANGE STRESSORS ON AUTOTROPH C:N:P RATIOS

Light × Nutrients

The key role of light and nutrients in the structure and functioning of ecosystems was first identified in a benchmark contribution by Lindeman (1942). Since then, numerous experimental and observational studies have shown that the production of algae and biomass of pelagic herbivores increases with nutrient enrichment (Murdoch et al., 1998). Aquatic autotrophs might also "compete" for the light availability. Therefore, the nutrient-phytoplankton coupled dynamics as well as changes in the mixing depth due to global warming are expected to have important consequences for the light availability in the upper layers of the water column (Carrillo et al., 2015). We now know that light and nutrient availability not only affect production of algae but also their C:nutrient ratio. This idea was elegantly formulated by Sterner et al. (1997) as the Light:nutrient hypothesis which predicts that low light conditions decrease not only production in primary producers but also their C:nutrient ratio, and potentially offsets the negative effects of decreased food availability for their pelagic consumers. Simultaneous algal controlled assays provided with experimental evidence for the chief role of these two factors on the elemental composition of phytoplankton (Urabe and Sterner, 1996). In addition, global change research has reported strong changes in the biogeochemical cycles of N, P, and C (Falkowski et al.,

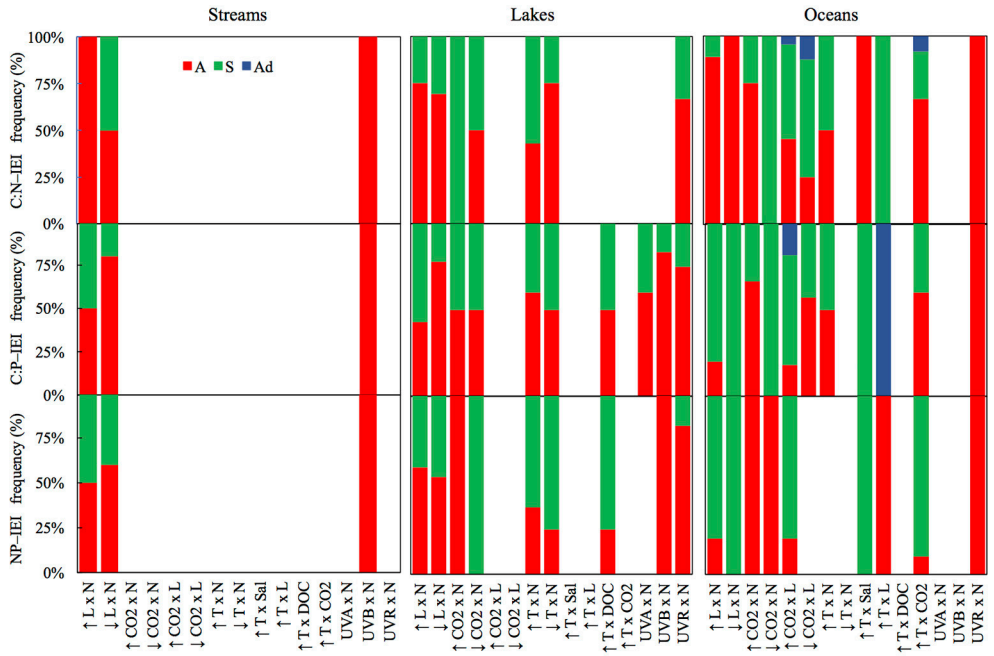

FIGURE 3 | Mean frequencies (%) of C:N:P-IEI interaction types for streams, lakes and oceans for the different stressor pairs tested in this study.

2000) and predict changes in the solar radiation underwater mainly due to increased temperature or land alterations and droughts that might regionally increase dust emissions to the atmosphere (IPCC, 2013). Therefore, anthropogenic impacts on ecosystems can drive changes in light and nutrient availability with consequences for the elemental composition of plants at the base of food webs.

Light and nutrients was the only stressor pair for which we found sufficient evidence to compare interactive effect among streams, lakes and oceans (Figure S1). While most observations consisted of antagonistic and synergistic effects, there were clear differences in IEI among habitats and C:N:P ratios (**Figure 3**). In an scenario of increased light and nutrients, IEI varied among habitats in C:N ratio, but not in C:P and N:P ratios (**Figure 3**, **Table 1**). More specifically, *Post-hoc* comparisons indicated that the effect on C:N-IEI was different between lakes and oceans, but neither of these habitats differed from streams (**Figure 4**). In agreement with our general hypothesis of synergism prevalence in oceanic systems, IEI was synergistic for C:P and N:P ratios in the oceans, but not for C:N. As for streams and lakes, IEI did not statistically differ from zero, which was a consequence of the low number of evidences ($n = 2$ in rivers) or a situation where stressor effects were opposing directions (i.e., synergism and antagonism).

As for the scenario of increased light, the effect of interactive effects of decreased light × nutrients on C:N deviated from our initial prediction and was also antagonistic in oceans. As for C:P and N:P ratios, we had no statistically discernible effects with a single study that yielded a positive synergism (**Figure 3**). As expected, interactive effects in both stream and lakes were either additive for C:N and N:P or antagonistic for C:P (**Figure 4**).

CO_2 × Nutrients

Climate change due to increased CO_2 is the most notable feature of global change. The extra CO_2 can directly fuel photosynthesis worldwide and indirectly impact the C:N:P stoichiometry of plankton by decreasing the nutrient content of autotrophs (increased C:P and C:N ratios) at the base of trophic webs (Riebesell et al., 2007; Verschoor et al., 2013). Elevated CO_2 concentrations only seem to change phytoplankton stoichiometry under specific conditions, for instance, at low nutrient availability (Gervais and Riebesell, 2001; Leonardos and Geider, 2005; Li et al., 2012) whereas rising CO_2 levels will increase phytoplankton biomass at high nutrient loads (Verspagen et al., 2014). The enrichment of aquatic systems with anthropogenic CO_2 is already having consequences on acidification and nutrient biogeochemistry (Gattuso et al., 2015). Since biogeochemical cycles are intrinsically linked, the change in carbon will have large consequences on the nitrogen cycle through microbial mediated processes such as increases in N fixation or denitrification and decreases in nitrification (Hutchins et al., 2009). At the same time, humans are altering planet's biogeochemical cycles at unprecedented rates (Falkowski et al., 2000). Consequently, the net impact of human on biochemical cycles may change ratios of nutrients at big scales that can unbalance the stoichiometry of ecological interactions and alter natural and managed ecosystems across the globe (Peñuelas et al., 2013). Anthropogenic alterations of N and P biochemical cycles are nearly 10 and 40-fold higher those of C cycle (Falkowski et al., 2000). Because the availability of N limits primary production in much of the ocean (Moore et al., 2013) and continental waters (Elser et al., 2007), human activities might be responsible for much of the nitrogen fertilization via N_2O emissions and

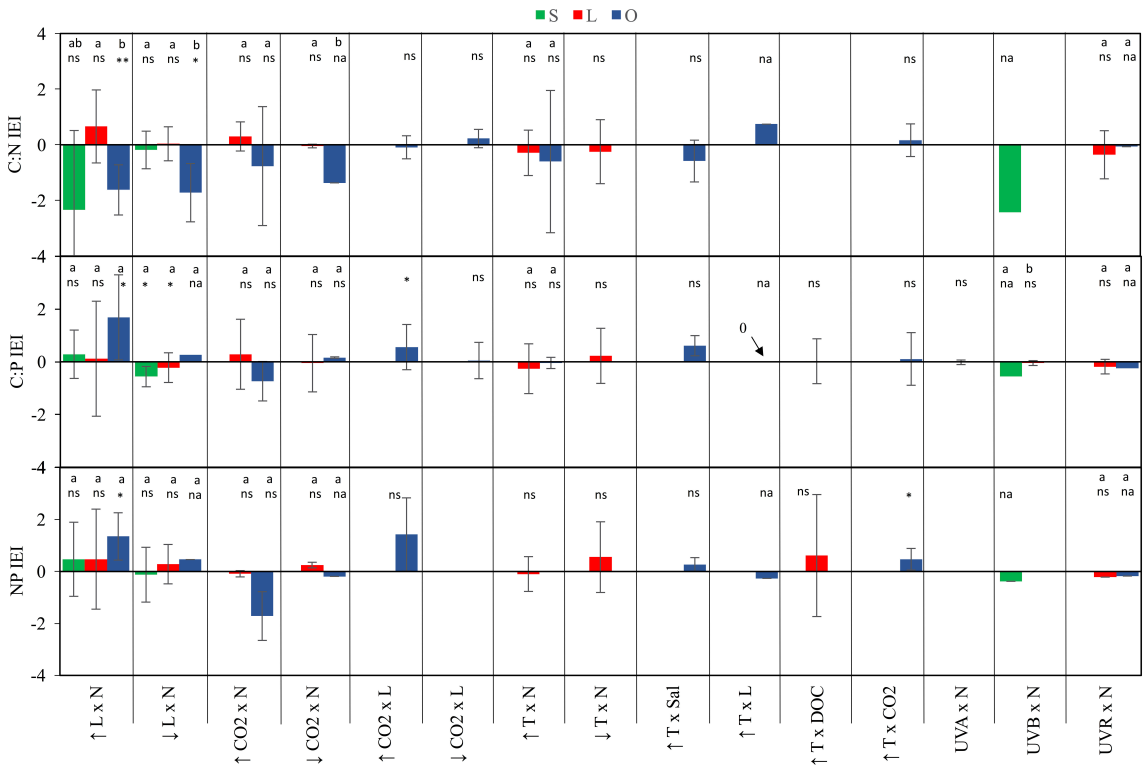

FIGURE 4 | IEI for C:N:P ratios in streams, lakes and oceans. Values represent IEI mean ± 95% confidence intervals (see statistical methods for further detail). Values > 0 indicate a synergistic interaction, values < 0 indicate an antagonistic interaction, and values = 0 indicate the absence of a net interactive effect or the occurrence of an additive effect. Significant differences among systems are denoted by different case letters according to Tuckey's *post-hoc* comparisons. Below letters and/or symbols are results for one-sample *t*-test testing IEI differences from the test value = 0 (ns, not significant; na, not applicable with less than two observations; $^{*}p < 0.05$; $^{**}p < 0.01$).

industrial, agriculture and wastewater discharges (Duce et al., 2008). Similarly, there has been an increase in P inputs in the biosphere due to mining of P compounds for fertilizer (Falkowski et al., 2000). While there is uncertainty on whether the higher mobilization of essential nutrient can boost up primary production and help mitigate CO_2 accumulation in Earth's atmosphere (Falkowski et al., 2000), there is still no conclusive evidence of whether the combined effects of increased CO_2 and nutrients would lead to increase or decrease in C:N:P ratios (Hutchins et al., 2009).

Only few studies in lakes and oceans covered the effects of this stressor pair. In the scenario of increased CO_2 there was a general prevalence of synergistic effects in lakes but antagonistic in oceans (**Figures 3**, **4**). The only two studies to statistically test the increased CO_2 × Nut effect were carried out in the ocean and were consistent with the general appreciation for the prevalence of antagonistic effects in the ocean, but only for the C:P and N:P ratio (Figure S2). Not surprisingly, IEI did not differ from zero in most comparisons due to the low number of observations.

While most studies to date examine the effects of future CO_2 levels, low CO_2 concentration represents a potential scenario for past glacial ages where this gas diminished on a global scale (Sigman and Boyle, 2000). Also decreased CO_2 availability is a likely future scenario in sites where nutrient eutrophication could boost autotroph growth depriving the concentration of this photosynthetic resource. Under the scenario of decreased CO_2, there was an antagonistic interaction on C:N (**Table 1**), but indistinguishable from zero in the rest of the cases, indicating that the effect of decreased CO_2 on C:nutrient ratio was not necessarily affected by nutrient availability (**Figures 3**, **4**).

CO_2 × Light

Phytoplankton have an essential role in sequestering CO_2 at global scales, and therefore play a key role in the partitioning of CO_2 between the atmosphere and the hydrosphere (Leonardos and Geider, 2005). However, the capacity of the ocean as a carbon sink is determined not only by CO_2 levels, but also by other environmental variables that affect photosynthesis CO_2 fixation such as nutrient availability or light. Light conditions can drastically vary in the future due to global changes in the upper mixed layer depth (Carrillo et al., 2015), and a crucial question is whether this might exacerbate or mitigate the effects of increasing CO_2 in the physiology of phytoplankton.

For this stressor pair, only ocean observations were available in the literature. The prevailing interactions for both the scenarios of increased and decreased CO_2 were synergistic, although some additive observations were observed (Figure S3, **Figure 3**). Overall, the synergistic interactions were statistically significantly

TABLE 1 | Results of ANOVA testing whether interactive effects for C:N:P ratios [ln (combined effect/additive effects)] differ across systems (streams, lakes and oceans).

Interactive effect	Factor 1	C: nutrient	d.f.	Sum of squares	*F*	*p*-value
L × Nut	↑ Light	C:N	**2, 13**	**20.77**	**4.80**	**0.028**
		C:P	2, 11	7.60	1.05	0.383
		N:P	2, 9	2.24	0.41	0.677
	↓ Light	C:N	**2, 15**	**11.26**	**5.31**	**0.012**
		C:P	2, 20	1.23	0.43	0.658
		N:P	2, 14	0.61	1.20	0.323
CO_2 × Nut	↑ CO_2	C:N	1, 2	0.06	0.016	0.912
		C:P	1, 2	2.12	2.35	0.265
		N:P	1, 2	2.69	10.69	0.082
	↓ CO_2	C:N	**1, 1**	**1.21**	**297.78**	**0.037**
		C:P	1, 2	0.04	0.071	0.815
		N:P	1, 1	0.13	27.00	0.121
T × Nut	↑ T	C:N	1, 7	0.17	0.11	0.748
		C:P	1, 10	0.07	0.08	0.778
		N:P	–	–	–	–
UVB × Nut	UVB	C:N	–	–	–	–
		C:P	**1, 4**	**0.17**	**16.30**	**0.016**
		N:P	–	–	–	–
UVR × Nut	UVR	C:N	1, 2	0.16	0.28	0.648
		C:P	1, 8	0.02	0.42	0.535
		N:P	1, 3	0.001	0.012	0.920

Only stressor pairs with observations for at least two systems (streams, lakes, oceans) were included in this analysis. In bold are significant differences.

different from zero only for C:P ratio in the scenario of increased CO_2, but indistinguishable from additive effect for the rest of comparisons (**Figure 4**).

Temperature × Nutrients

Recent unprecedented increase in atmospheric CO_2 is responsible for warming of lake and oceanic surface waters around the globe (Schlüter et al., 2014). Mean temperatures and heat waves that are expected to increase can have profound effects on autotrophs and heterotrophs, and thus ecosystem functioning (IPCC, 2013). Temperature is an all-embracing environmental factor that affects the growth, reproduction and survival of organisms, and the interactions among species (Kingsolver, 2009). Rising temperatures *per ser* can have an effect on organisms chemical composition (Woods et al., 2003), and favor the dense blooms of toxic cyanobacteria (Johnk et al., 2008). Additionally, higher temperatures can alter ambient conditions by reducing the duration of ice (IPCC, 2013), decreasing lake water levels (Hanrahan et al., 2010), changing patterns in phenology (Menzel et al., 2006) and reducing nutrient fluxes from the hypolimnion due to the greater stability of the stratification process (Huisman et al., 2006). The study of the joint effect of temperature and nutrients on autotrophs is of particular importance. First, these are two factors where pronounced global changes have already been detected (IPCC, 2013). Second, evidence exists that warming can reduce the effects of eutrophication on periphyton by altering C:N ratios (Shurin et al., 2012). However, while nutrient availability may decrease in C:N ratio, this effect could be counterbalanced by depletion of nutrients due to strengthening stratification as a result of global warming (van de Waal et al., 2010).

For this stressor pair, the small sample size only allowed for the comparison of differential responses on C:N and C:P between lakes and oceans in the scenario of increased temperature, in which no differences were detected (**Table 1**). In addition, IEI values for most ratios were equally distributed between antagonistic and synergistic cases (Figure S4, **Figure 3**), resulted in values of mean IEI that did not significantly differ from zero (**Figure 4**).

Temperature × Other Stressors

Future shifts in the chemical composition of algae are also anticipated given the continuous changes in environmental drivers that interact with temperature (Passow and Laws, 2015). For example, some studies have documented temperature pair interactions with salinity, light or CO_2 on the stoichiometry of marine phytoplankton yielding contrasting responses. Thus, for the temperature salinity stressor pair, the effects were antagonistic for C:N, but synergistic for C:P and N:P (Figure S5, **Figure 3**). As for the only study covering temperature and light effects, the interactions switched from synergistic for C:N to additive for C:P and antagonistic for N:P (Figure S5, **Figure 3**). We identified eight studies that focus on the temperature-CO_2 stressor pair in the ocean, which yielded a similar distribution of synergistic and antagonistic responses for C:N and C:P ratios, and a dominant, and significant synergistic response for N:P ratio (**Figures 3**, **4**, **Table 1**). Only one study in lakes covered the interactive effects of increased temperature and DOC availability yielding inconclusive findings for the nature of the interactions on the algal stoichiometry due to the low number of observations and their opposing interactive signs (**Figure 3**).

UVR × Nutrients

Compared to photosynthetically active radiation (PAR), exposure to UVR increases C:nutrient ratios in algae (Xenopoulos et al., 2002; Carrillo et al., 2008a; Korbee et al., 2012). As a result, the net outcome of the two opposing effects of UVR and PAR on algal composition depends on the optical properties of the water column (Hessen et al., 2008), as well as factors like nutrients that promote phytoplankton growth influencing the light climate in the water column.

Although empirical evidence for the nature of the combined effects of UVR radiation and nutrients on elemental ratio of autotrophs remains very limited, the compiled dataset allowed us to distinguish among the interactive effects of distinct spectral regions of UVR and nutrients. Thus, the combined effects of UVA and nutrients on algal C:P ratios in lakes, frequencies of interactions varied between synergistic and antagonistic (Figure S6, **Figure 3**). The only stream observation in the dataset for the effects of UVB and nutrients yielded antagonistic

effects on all C:N:P ratios (**Figure 3**), which differed from the additive effect found for C:P in lakes (**Table 1**). Finally, the type of interactions for UVR paired with nutrients did not significantly differ between lakes and oceans and were predominantly antagonistic (**Figures 3**, **4**, **Table 1**).

PROPAGATION OF INTERACTIVE EFFECTS ON FOOD WEBS

As we have seen, few studies have evaluated the impact of multiple stressors on the elemental composition of planktonic organisms (Table S1), but barely a handful of them examined the carry-on consequences of multiple interactive effects on the role of stoichiometry on ecological interactions. Unraveling the "ecological surprises" (*sensu* Jackson et al., 2015) that arise from the interactive impacts of multiple environmental stressors on C:N:P is a thrilling challenge that can help understand ecological responses to environmental change. In this section we will examine the few available studies to assess how multiple stressors impact ecological interactions within the microbial and grazing food chains via stoichiometry.

Microbial Food Web

The coevolution of algae and bacteria has shaped life on Earth in many aspects and their interaction has, not only defined the structure of their habitats (Ashen and Goff, 2000), but is responsible for the productivity of the biosphere (Ramanan et al., 2016). Numerous lines of evidence indicate that the long coevolution of algae and bacteria has given rise to diverse types of associations including mutualism, commensalism, competition or parasitism (Ramanan et al., 2016, and references therein). A significant step in the study of the relationship between algae and bacteria is the insight that their complex association points to a relation continuum, where the relationship between organisms transits from a commensal (one organism benefits with no detriment to the partner's fitness) to mutualistic (two organisms that benefit one another) to predatory/parasitic (one organisms benefits at the expense of the other) relationship. But what is the role of C:N:P ratios in shaping this continuum?

A first level of approach stems from one-factor experimental studies adding nutrients at definite N:P ratios in highly UVR-exposed systems, which changed algal and bacterial N:P ratios and modulated their interaction with consequences for the development of the microbial loop (Carrillo et al., 2008b). Variations in algal and bacterial N:P ratios revealed a shift from their commensalistic-mutualistic relationship to a competitive one for the available P when bacterial P-deficiency increased (N:P $>$ 20–24). Hence, the bacterial N:P ratio proved to be a key factor in understanding the algae—bacteria relationship. The development of ciliates occurred only when bacteria remained P-rich (N:P $<$ 20) and algae was close to Redfield proportions indicating that bacterial N:P ratio also served as an essential predictor for the ciliate and microbial loop development (Villar-Argaiz et al., 2002; Carrillo et al., 2008b).

A second level of approach stems from two-factor experimental studies manipulating resource N:P (by adding nutrients at definite N:P ratios) and another global-change stressor. Thus, Medina-Sánchez et al. (2006) set up three resource N:P treatments (N:P $>$ 180, N:P $=$ 16, and N:P $=$ 5) in presence vs. absence of UVR in two contrasting unbalanced scenarios in the algal and bacterial N:P ratio in a high mountain lake (i.e., middle of ice-free period: P-poor algae and P-sufficient bacteria; late ice-free period: P-rich algae and P-poor bacteria). Under such scenarios, these authors found antagonistic UVR $\times$ P effects (i.e., the effect of P enrichment attenuated the effect of UVR) that decreased autotroph N:P ratio, increased the excretion of organic carbon by algae, and in turn reinforced the commensalistic-predatory relationship between algae and bacteria (**Figure 5A**). This dual control exerted by mixotrophic algae implies a shift in the paradigm of the functioning of microbial food web in lakes (Medina-Sánchez et al., 2004; Cabrerizo et al., 2017), recently extended to marine ecosystems (Ptacnik et al., 2016). In a different experiment, Wohlers-Zöllner et al. (2011) set up two resource N:P treatments (N:P $=$ 9 as "N-deficient" vs. N:P $=$ 30 as "N-replete") and a temperature gradient simulating weak to strong warming predicted for winter in the Baltic Sea region at 2100. The pelagic algal-bacterial assemblage showed a synergistic interactive effect on autotroph N:P ratio which enhanced utilization of organic matter and bacterial growth and, ultimately, reinforced the development of the microbial food web (**Figure 5B**). These studies illustrate the key role of resource stoichiometry in determining how paired stressors modulate the interaction between algae and bacteria.

A third level of approach stems from experimental two-factor studies, manipulating UVR (presence vs. absence) and nutrient availability in mid to long term scales (days to months) and reporting pronounced changes in the autotrophs stoichiometry (N:P). For example, Delgado-Molina et al. (2009) showed that the antagonistic interaction between UVR and nutrients generated a gradual decrease in the algal N:P ratio that ultimately leads to unimodal responses in the heterotrophic microbial food web (bacteria, ciliates, and viruses), where the effect of UVR is maximum at intermediate P concentrations (Medina-Sánchez et al., 2013).

Grazing Food Web

The plant-herbivore interface is the level at which nutrient imbalances are among the highest in nature (Sterner and Elser, 2002), constraining the energy transfer and production of higher trophic levels in food webs (Power, 1992). Several potential factors affect the food quality for herbivores, and research has extensively addressed the carryover consequences of single stressors, primarily nutrient availability, on herbivore performance mediated through changes in plant chemical composition. To date, most research has covered freshwater species (DeMott et al., 1998; Villar-Argaiz and Sterner, 2002), but also included stream (Stelzer and Lamberti, 2002) and marine species (Schoo et al., 2010). We examine the few studies to systematically evaluate the interactive effect of paired stressors at the plant-herbivore interface.

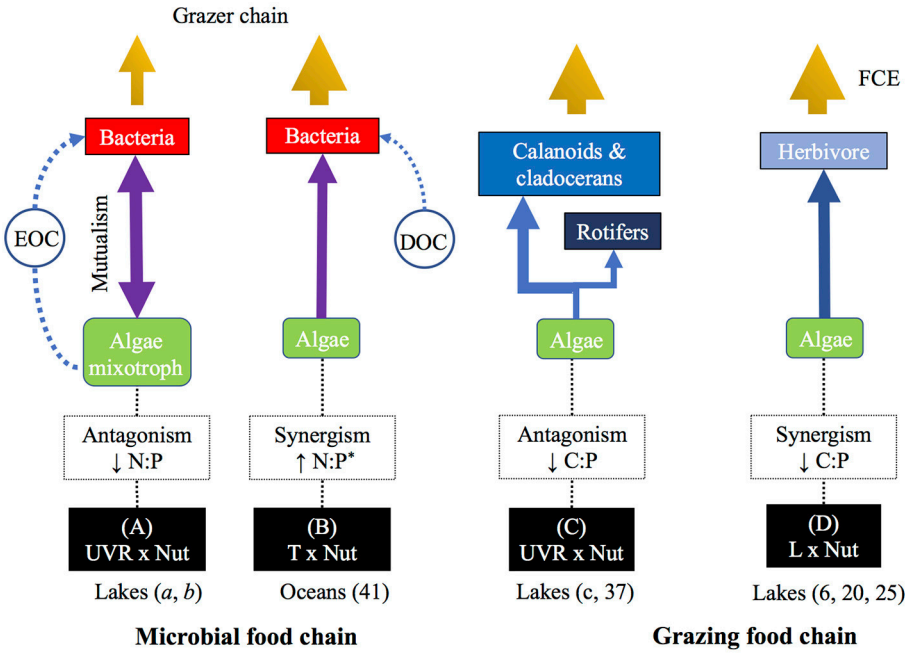

FIGURE 5 | Conceptual diagram illustrating the nature of the interactive effect between **(A)** UVR × Nut, **(B)** T × Nut, **(C)** UVR × Nut, and **(D)** L × Nut on autotroph C:N:P ratios and its propagation in microbial and grazing food chains. The figure summarizes the main food web effects analyzed in this study. In parenthesis are studies cited in the manuscript (*a*, Medina-Sánchez et al., 2004; *b*, Cabrerizo et al., 2017; *c*, Villar-Argaiz et al., 2012) or supplementary references (41, 6, 20, and 25). Arrow thinkness is proportional to magnitude of the effect. EOC, excretion of organic carbon, DOC, dissolved organic carbon, FCE, food chain efficiency. (*)Type of interactive effect calculated for N:P ratio from **Figure 2** in reference 41.

Food Quality × Temperature

Several studies have examined the joint role of food quality, as cultured algae to a nutrient replete or limited state, and temperature on zooplankton performance (McFeeters and Frost, 2011; Persson et al., 2011; Malzahn et al., 2016). These works generally agreed upon the conclusion that low food quality constraints on herbivore growth were strongest at low temperatures and decreased at high temperatures. In other words, the highest herbivore growth at low diet C:P ratios (high P availability) and elevated temperature demonstrated that P food quality and temperature synergistically increased herbivore performance. Studies like these, provide a very valuable perspective on how temperature can indirectly affect species worldwide by enhancing their likelihood to face P limitation.

Temperature × DOC

One test of the importance of multiple stressors on herbivore communities comes from the study by Weidman et al. (2014) in alpine lakes, where zooplankton were expected to respond strongly to increased water temperature. The effects of temperature × DOC on particulate C:P ratios were antagonistic on these lakes (see reference 46 in Figure S5). As for the carryover effects on zooplankton, while warming alone stimulated the growth of the cladoceran and suppressed that of the calanoid copepod, the combined effect of warming and DOC reversed these results. Therefore, the addition of DOC suppressed the detrimental effect of temperature on copepod and total zooplankton biomass. These findings imply that the antagonistic temperature × DOC effects resulted in food changes that contributed to overcome the negative effect of temperature on zooplankton in the alpine lakes.

UVR × Nutrients

In a field study using mesocosms, Carrillo et al. (2008a) demonstrated that the interactive UVR × Nut effects on seston C:P ratio were antagonistic, i.e., the addition of nutrients ameliorated the effect of UVR decreasing C:P and hence improving food quality for herbivores (see reference 37 in Figure S6). Villar-Argaiz et al. (2012) subsequently tested the effects of these two stressors on the growth of three species of zooplankton with contrasting life history traits in field-coupled bioassays. Their results, that allowed discrimination between food quality and food quantity, showed that interactive effects of UVR × Nut on algal C:P ratio were antagonistic, i.e., the addition of nutrients dampened the detrimental effects of UVR on the growth of herbivorous zooplankton (**Figure 5C**).

Light × Nutrients

Dickman et al. (2008) manipulated light and nutrients to test the general hypothesis that food chain efficiency (and hence herbivore production) was constrained by the nutritional quality of the food. The combined effect of light and nutrients yielded synergistic effects on seston C:N and C:P ratios in

treatments without fish (see reference 6 in Figure S1), which resulted in increased food quality that favored the production of zooplankton (**Figure 5D**). In a similar study, Plum et al. (2015) found that the nature of the interactive effects of light and nutrients on algal C:P and N:P ratios were mostly synergistic (four out of five algal bioassays; see reference 20 in Figure S1), and it was under high light and N reduced conditions when the highest copepod biomass was attained. Further, in a recent study, Rock et al. (2016) identified that carnivores can affect the mediated effects of light and nutrients on aquatic food chain efficiency. Interestingly, if we examine their carnivore-bluegill treatment (see reference 25 in Figure S1), the effects of light and nutrient on seston C:P were synergistic, and under these conditions herbivore efficiency was at its highest. Our analysis of the above studies suggest that synergistic effects of L × Nut resulted in enhanced food quality (decreased autotroph C:P) for zooplankton herbivores (**Figure 5D**).

From the heterogeneous paired-stressor studies analyzed above, the idea emerges that the identity of the stressor might mediate on the nature of the interaction with a second stressor, and in turn on herbivore performance. Thus, the harmful UVR antagonistic interaction with nutrients, and the synergistic interaction of light with nutrients on autotrophs, could both benefit zooplankton by decreasing C:P ratios in their food resources (**Figures 5C,D**). Altogether, these studies strongly highlight the importance of considering both the direction and magnitude of interactive impacts when evaluating ecological interactions if we are to advance in the theory of food chain in nature.

DISCUSSION AND CONCLUSION

Across all stressor pairs considered in the present study the following patterns emerged: (1) Antagonisms were the prevalent interaction in freshwaters, both streams and lakes, (2) both synergisms and antagonisms were co-dominant in marine systems with synergisms being slightly more common, and (3) overall non-additive effects were the dominant interactions in all aquatic ecosystems studied.

While the lack of data impeded comparisons among all of the paired stressors, we found pronounced system-specific differences. For example, with regards to the L × Nut interactions, our data for C:P and N:P is consistent with the initial hypothesis that the interaction was primarily synergistic in the oceans, but not in freshwater systems where interactions were more evenly distributed between synergism and antagonism. As for C:N, the present study showed no differences between stream and lakes, but a sharp contrast between freshwater systems and the ocean. We suggest that this could possibly be associated with differences in the nutrient limiting freshwater and marine systems (e.g., Moore et al., 2013). The inherent similarities that we find for freshwater systems are possibly due to the similar stoichiometric principles that apply in "green" autotroph-based food webs and the "brown" detritus-based systems that dominante in lakes and streams, respectively (Evans-White and Halvorson, 2017).

While discussing the interactive effects of changing precipitation, decreasing DOC ("sun screen"), increasing UV-penetration, and continuing acid-rain deposition/mobilization from sediments in the Canadian Shield Lakes, Gorham (1996) made the following observation: "Most of our diverse impacts on the environment are studied as separate problems, and only rarely do scientists examine appropriately their many and complex physical, chemical and biological interactions." As is now revealed by the analyses of interaction of stressors (Crain et al., 2008; Jackson et al., 2015, present study), non-additive responses predominate across aquatic ecosystems. Our study finds strong evidence that the nature of the interactive effect depends on the stressor pair at play, with a wide range of variability in the net effects of the interactions, with some observations supporting antagonism while others synergism.

Interesting questions arise from these observations regarding the basis for the commonalities as well as differences across ecosystems: Why are non-additive interactions prevalent across such a wide variety of aquatic ecosystems? Could the inherent heterogeneity of freshwater systems versus the relative uniformity of marine systems, and/or variability in the type and magnitude of stressor pairs, be responsible for the observed inter-ecosystem differences? How do we ascribe specific interaction effects of stressor pairs (e.g., light and nutrients on EOC-production in phytoplankton) with specific ecological phenomena (e.g., microbial growth and respiration)? Finally, how do we go about studying the interactive effects of all the stressors that are acting on an ecosystem at a given time?

Our findings highlight the importance of non-additive effects of interacting stressors on ecosystem processes and the need for further studies evaluating the interactive effects for developing a more rigorous comparative ecology. Serving as key feedback loops in ecosystems, the balance between these sensitive antagonistic and synergistic responses affects everything from small scale phenomena such as food chain efficiency to large scale phenomena such as ecosystem carbon and nutrient cycling (Schlesinger and Bernhardt, 2013). Of recent, there has been a resurgence of interest in role of multiple stressors on aquatic ecosystems (Allan et al., 2012). However, very few studies have explored the possibility of untangling the issue of interactions among these stressors (2 factor and more) on higher-order phenomena (Jackson et al., 2015; present study). The findings from our study suggesting how interactions of multiple stressors may result in subtle shifts in the net responses (synergistic or antagonistic) point to the importance of stressor interaction studies in the future. Today, ecologists are being challenged to predict dynamic tipping points of ecosystems shifting thresholds under a confluence of conditions where interactive effects play a key role (Scheffer et al., 2012; Duarte, 2014; Steffen et al., 2015; Costanza, 2017). As Odum (1964) pointed out decades ago, the challenging study of the emergent properties of ecosystems is the new ecology. If non-additive effects of interactions should be the norm in ecosystems, it now appears that an ecosystem is more than even the sum of its interactions. In a rapidly changing world, gaining a better understanding of how multiple stressors interact and the predictive modeling of their complex outcomes, should be an urgent interdisciplinary priority.

AUTHOR CONTRIBUTIONS

MV-A, JM-S, and PC conceived the original idea for this study, with inputs from BB. MV-A, JM-S, BB, and PC wrote, approved and equally contributed to the final version of the manuscript.

ACKNOWLEDGMENTS

We are thankful for support of this work by the University of Granada, Spain and Grand Valley State University, USA. This work was supported by the Ministry of Economy and Competitiveness and *Fondo Europeo de Desarrollo Regional* FEDER (CGL2015-67682-R to PC and JM-S), and Junta de Andalucía (P12-RNM327 to MV-A). BB participated in writing this review during sabbatical at *Departamento de Ecología* and *Instituto del Agua,* University of Granada, and was supported by grants from NASA-Michigan Space Grants Consortium, NSF-Geobiology Program, and NOAA-University of Michigan-Cooperative Institute for Great Lakes Research. Finally, we would like to dedicate this work to Andalucía Day (February 28, 2017) on which it was collaboratively finalized in Granada, Spain.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00069/full#supplementary-material

REFERENCES

Allan, J. D., McIntyre, P. B., Smith, S. D., Halpern, B. S., Boyer, G. L., Buchsbaum, A., et al. (2012). Joint analysis of stressors and ecosystem services to enhance restoration effectiveness. *Proc. Natl. Acad. Sci. U.S.A.* 110, 372–377. doi: 10.1073/pnas.1213841110

Allgeier, J. E., Rosemond, A. D., and Layman, C. A. (2011). The frequency and magnitude of non-additive responses to multiple nutrient enrichment. *J. Appl. Ecol.* 48, 96–101. doi: 10.1111/j.1365-2664.2010.01894.x

Ashen, J. B., and Goff, L. J. (2000). Molecular and ecological evidence for species specificity and coevolution in a group of marine algal–bacterial symbioses. *Appl. Environ. Microbiol.* 66, 3024–3030. doi: 10.1128/AEM.66.7.3024-3030.2000

Boyd, P. W., and Hutchins, D. A. (2012). Understanding the responses of ocean biota to a complex matrix of cumulative anthropogenic change. *Mar. Ecol. Prog. Ser.* 470, 125–135. doi: 10.3354/meps10121

Boyd, P. W., Lennartz, S. T., Glover, D. M., and Doney, S. C. (2015). Biological ramifications of climate-change mediated oceanic multi-stressors. *Nat. Clim. Change* 5, 71–79. doi: 10.1038/nclimate2441

Cabrerizo, M. J., Medina-Sánchez, J. M., Dorado-García, I., Villar-Argaiz, M., and Carrillo, P. (2017). Rising nutrient-pulse frequency and high UVR strengthen microbial interactions. *Sci. Rep.* 7:43615. doi: 10.1038/srep43615

Carpenter, K. E., Abrar, M., Aeby, G., Aronson, R. B., Banks, S., Bruckner, A., et al. (2008). One third of reef-building corals face elevated extinction risk from climate change and local impacts. *Science* 321, 560–563. doi: 10.1126/science.1159196

Carrillo, P., Delgado-Molina, J. A., Medina-Sánchez, J. M., Bullejos, F. J., and Villar-Argaiz, M. (2008a). Phosphorus inputs unmask negative effects of ultraviolet radiation on algae in a high mountain lake. *Glob. Change Biol.* 14, 423–439. doi: 10.1111/j.1365-2486.2007.01496.x

Carrillo, P., Medina-Sánchez, J. M., Herrera, G., Durán, C., Segovia, M., Cortés, D., et al. (2015). Interactive effect of UVR and phosphorus on the coastal phytoplankton community of the western Mediterranean Sea: unravelling eco-physiological mechanisms. *PLoS ONE* 10:e0142987. doi: 10.1371/journal.pone.0142987

Carrillo, P., Villar-Argaiz, M., and Medina-Sánchez,J. M., (2008b). Does microorganism stoichiometry predict microbial food web interactions after a phosphorus pulse? *Microb. Ecol.* 56, 350–363. doi: 10.1007/s00248-007-9353-8

Cheung, W. W., Lam, V. W., Sarmiento, J. L., Kearney, K., Watson, R., and Pauly, D. (2009). Projecting global marine biodiversity impacts under climate change scenarios. *Fish Fish.* 10, 235–251. doi: 10.1111/j.1467-2979.2008.00315.x

Costanza, R. (2017). Trump: a confluence of tipping points. *Nature* 542:295. doi: 10.1038/542295b

Crain, C. M., Kroeker, K., and Halpern, B. S. (2008). Interactive and cumulative effects of multiple human stressors in marine systems. *Ecol. Lett.* 11, 1304–1315. doi: 10.1111/j.1461-0248.2008.01253.x

Delgado-Molina, J. A., Carrillo, P., Medina-Sánchez, J. M., Villar-Argaiz, M., and Bullejos, F. J. (2009). Interactive effects of phosphorus loads and ambient ultraviolet radiation on the algal community in a high-mountain lake. *J. Plankton Res.* 31, 619–634. doi: 10.1093/plankt/fbp018

DeMott, W. R., Gulati, R. D., and Siewertsen, K. (1998). Effects of phosphorus-deficient diets on the carbon and phosphorus balance of *Daphnia magna*. *Limnol. Oceanogr.* 43, 1147–1161. doi: 10.4319/lo.1998.43.6.1147

Dickman, E. M., Newell, J. M., González, M. J., and Vanni, M. J. (2008). Light, nutrients, and food-chain length constrain planktonic energy transfer efficiency across multiple trophic levels. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18408–18412. doi: 10.1073/pnas.0805566105

Duarte, C. (2014). Global change and the future ocean: grand challenges for marine sciences. *Front. Marine Sci.* 1:63. doi: 10.3389/fmars.2014.00063

Duce, R. A., LaRoche, J., Altieri, K., Arrigo, K. R., Baker, A. R., Capone, D. G., et al. (2008). Impacts of atmospheric anthropogenic nitrogen on the open ocean. *Science* 5878, 893–897. doi: 10.1126/science.1150369

Durif, C. M. F., Fields, D. M., Browman, H. I., Shema, S. D., Enoae, J. R., Skiftesvik, A. B., et al. (2015). UV radiation changes algal stoichiometry but does not have cascading effects on a marine food chain. *J. Plankton Res.* 37, 1120–1136. doi: 10.1093/plankt/fbv082

Elser, J. J., Bracken, M. E., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Evans-White, M. A., and Halvorson, H. M. (2017). Comparing the ecological stoichiometry in green and brown food webs–a review and meta-analysis of freshwater food webs. *Front. Microbiol.* 8:1184. doi: 10.3389/fmicb.2017.01184

Falkowski, P., Scholes, J. R., Boyle, E., Canadell, J., Canfield, D., Elser, J., et al. (2000). The global carbon cycle: a test of our knowledge of earth as a system. *Science* 290, 291–296. doi: 10.1126/science.290.5490.291

Folt, C. L., Chen, C. Y., Moore, M. V., and Burnaford, J. (1999). Synergism and antagonism among multiple stressors. *Limnol. Oceanogr.* 44, 864–877. doi: 10.4319/lo.1999.44.3_part_2.0864

Gattuso, J.-P., Magnan, A., Billé, R., Cheung, W. W. L., Howes, E. L., Joos, F., et al. (2015). Contrasting contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. *Nature* 349:6243. doi: 10.1126/science.aac4722

Gervais, F., and Riebesell, U. (2001). Effect of phosphorus limitation on elemental composition and stable carbon isotope fractionation in a marine diatom growing under different CO_2 concentrations. *Limnol. Oceanogr.* 46, 497–504. doi: 10.4319/lo.2001.46.3.0497

Gorham, E. (1996). Lakes under a three-pronged attack. *Nature* 381, 109–110. doi: 10.1038/381109a0

Gunderson, A. R., Armstrong, E. J., and Stillman, J. H. (2016). Multiple stressors in a changing world: the need for an improved perspective on physiological responses to the dynamic marine environment. *Ann. Rev. Mar. Sci.* 8, 357–378. doi: 10.1146/annurev-marine-122414-033953

Hanrahan, J. L., Kravtsov, S. V., and Roebber, P. J. (2010). Connecting past and present climate variability to the water levels of Lakes Michigan and Huron. *Geophys. Res. Lett*. 37, L01701. doi: 10.1029/2009GL041707

Hessen, D. O. (1992). Nutrient element limitation of zooplankton production. *Am. Nat*. 140, 799–814. doi: 10.1086/285441

Hessen, D. O., Leu, E., Færøvig, P. J., and Petersen, S. F. (2008). Light and spectral properties as determinants of C:N:P-ratios in phytoplankton. *Deep Sea Res. II* 55, 2169–2175. doi: 10.1016/j.dsr2.2008.05.013

Huisman, J., Pham Thi, N. N., Karl, D. M., and Sommeijer, B. (2006). Reduced mixing generates oscillations and chaos in the oceanic deep chlorophyll maximum. *Nature* 439, 322–325. doi: 10.1038/nature04245

Hutchins, D. A., Mulholland, M. R., and Fu, F. (2009). Nutrient cycles and marine microbes in a CO_2-enriched ocean. *Oceanography* 22, 128–145. doi: 10.5670/oceanog.2009.103

IPCC. (2013). *Climate Change 2013. The Physical Science Basis*. New York, NY: Cambridge University Press.

Jackson, M. C., Loewen, C. J., Vinebrooke, R. D., and Chimimba, C. T. (2015). Net effects of multiple stressors in freshwater ecosystems: a meta-analysis. *Glob. Change Biol*. 22, 180–189. doi: 10.1111/gcb.13028

Johnk, K. D., Huisman, J., Sharples, J., Sommeijer, B., Visser, P. M., and Stroom, J. M. (2008). Summer heatwaves promote blooms of harmful cyanobacteria. *Glob. Change Biol*. 14, 495–512. doi: 10.1111/j.1365-2486.2007.01510.x

Kingsolver, J. G. (2009). The well-temperatured biologist. *Am. Nat*. 174, 755–768. doi: 10.1086/648310

Korbee, N., Carrillo, P., Mata, M. T., Rosillo, S., Medina-Sánchez, J. M., and Figueroa, F. L. (2012). Effects of ultraviolet radiation and nutrients on the structure–function of phytoplankton in a high mountain lake. *Photochem. Photobiol. Sci*. 11, 1087–1098. doi: 10.1039/c2pp05336e

Krebs, C. J. (2009). *Ecology*. San Francisco, CA: Pearson Benjamin Cummings.

Leonardos, N., and Geider, R. J. (2005). Elevated atmospheric carbon dioxide increases organic carbon fixation by *Emiliania huxleyi* (Haptophyta), under nutrient-limited high-light conditions. *J. Phycol*. 41, 1196–1203. doi: 10.1111/j.1529-8817.2005.00152.x

Li, W., Gao, K., and Beardall, J. (2012). Interactive effects of ocean acidification and nitrogen-limitation on the diatom *Phaeodactylum tricornutum*. *PLoS ONE* 7:e51590. doi: 10.1371/journal.pone.0051590

Lindeman, R. L. (1942). The trophic dynamic aspect of ecology. *Ecology* 23, 399–417. doi: 10.2307/1930126

Malzahn, A. M., Doerfler, D., and Boersma, M. (2016). Junk food gets healthier when it's warm. *Limnol. Oceanogr*. 61, 1677–1685. doi: 10.1002/lno.10330

McFeeters, B. J., and Frost, P. C. (2011). Temperature and the effects of elemental food quality on Daphnia. *Freshw. Biol*. 56, 1447–1455. doi: 10.1111/j.1365-2427.2011.02586.x

Medina-Sánchez, J. M., Delgado-Molina, J. A., Bratbak, G., Bullejos, F. J., Villar-Argaiz, M., and Carrillo, P. (2013). Maximum in the middle: nonlinear response of microbial plankton to ultraviolet radiation and phosphorus. *PLoS ONE* 8:e60223. doi: 10.1371/journal.pone.0060223

Medina-Sánchez, J. M., Villar-Argaiz, M., and Carrillo, P. (2004). Neither with nor without you: a complex algal control on bacterioplankton in a high-mountain lake. *Limnol. Oceanogr*. 49, 1722–1733. doi: 10.4319/lo.2004.49.5.1722

Medina-Sánchez, J. M., Villar-Argaiz, M., and Carrillo, P. (2006). Solar radiation-nutrient interaction enhances the resource and predation algal control on bacterioplankton: a short-term experimental study. *Limnol. Oceanogr*. 51, 913–924. doi: 10.4319/lo.2006.51.2.0913

Menzel, A., Sparks, T. H., Estrella, N., Koch, E., Aasa, A., Ahas, R., et al. (2006). European phenological response to climate change matches the warming pattern. *Glob. Change Biol*. 12, 1969–1976. doi: 10.1111/j.1365-2486.2006.01193.x

Moore, C. M., Mills, M. M., Arrigo, K. R., Berman-Frank, I., Bopp, L., Boyd, P. W., et al. (2013). Processes and patterns of oceanic nutrient limitation. *Nat. Geosci*. 6, 701–710. doi: 10.1038/ngeo1765

Murdoch, W. W., Nisbet, R. M., McCauley, E., deRoos, A. M., and Gurney, W. S. C. (1998). Plankton abundance dynamics across nutrient level: test of hypothesis. *Ecology* 79, 1339–1356. doi: 10.1890/0012-9658(1998)079[1339:PAADAN]2.0.CO;2

Odum, E. P. (1964). The new ecology. *Bioscience* 14, 14–16. doi: 10.2307/1293228

Pace, M. L., Carpenter, S. R., and Cole, J. J. (2015). With and without warning: managing ecosystems in a changing world. *Front. Ecol. Environ*. 13, 460–467. doi: 10.1890/150003

Passow, U., and Laws, E. A. (2015). Ocean acidification as one of multiple stressors: growth response of *Thalassiosira weissflogii* (diatom) under temperature and light stress. *Mar. Ecol. Progr. Ser*. 541, 75–90. doi: 10.3354/meps11541

Persson, J., Wojewodzic, M. W., Hessen, D. O., and Andersen, T. (2011). Increased risk of phosphorus limitation at higher temperatures for *Daphnia magna*. *Oecologia* 165, 123–129. doi: 10.1007/s00442-010-1756-4

Peñuelas, J., Poulter, B., Sardans, J., Ciais, P., van der Velde, M., Bopp, L., et al. (2013). Human-induced nitrogen–phosphorus imbalances alter natural and managed ecosystems across the globe. *Nat. Commun*. 4:2934. doi: 10.1038/ncomms3934

Piggott, J. J., Townsend, C. R., and Matthaei, C. D. (2015). Reconceptualizing synergism and antagonism among multiple stressors. *Ecol. Evol*. 5, 1538–1547. doi: 10.1002/ece3.1465

Plum, C., Hüsener, M., and Hillebrand, H. (2015). Multiple vs. single phytoplankton species alter stoichiometry of trophic interaction with zooplankton. *Ecology* 96, 3075–3089. doi: 10.1890/15-0393.1

Power, M. E. (1992). Top-down and bottom-up forces in food webs: do plants have primacy? *Ecology* 73, 733–746. doi: 10.2307/1940153

Ptacnik, R., Gomes, A., Royer, S.-J., Berger, S. A., Calbet, A., Nejstgaard, J. C., et al. (2016). A light-induce shortcut in the planktonic microbial loop. *Sci. Rep*. 6:29286. doi: 10.1038/srep29286

Ramanan, R., Kim, B.-H., Cho, D.-H., Oh, H.-M., and Kim, H.-S. (2016). Algae–bacteria interactions: evolution, ecology and emerging applications. *Biotech. Adv*. 34, 14–29. doi: 10.1016/j.biotechadv.2015.12.003

Riebesell, U., Schulz, K. G., Bellerby, R. G. J., Botros, M., Fritsche, P., Meyerhöfer, M., et al. (2007). Enhanced biological carbon consumption in a high CO_2 ocean. *Nature* 450, 545–549. doi: 10.1038/nature06267

Rock, A. M., Hall, M. R., Vanni, M. J., and Gonzalez, M. J. (2016). Carnivore identity mediates the effects of light and nutrients on aquatic food-chain efficiency. *Freshw. Biol*. 61, 1492–1508. doi: 10.1111/fwb.12790

Scheffer, M., Carpenter, S. R., Lenton, T. M., Bascompte, J., Brock, W., Dakos, V., et al. (2012). Anticipating critical transitions. *Science* 338, 344–348. doi: 10.1126/science.1225244

Schlesinger, W. H., and Bernhardt, E. S. (2013). *Biogeochemistry: An Analysis of Global Change*. Waltham, MA: Academic Press.

Schlüter, L., Lohbeck, K. T., Gutowska, M. A., Gröger, J. P., Riebesell, U., and Reusch, T. B. (2014). Adaptation of a globally important coccolithophore to ocean warming and acidification. *Nat. Climate Change* 4, 1024–1030. doi: 10.1038/nclimate2379

Schoo, K. L., Aberle, N., Malzahn, A. M., and Boersma, M. (2010). Does the nutrient stoichiometry of primary producers affect the secondary consumer *Pleurobrachia pileus*? *Aquat. Ecol*. 44, 233–242. doi: 10.1007/s10452-009-9265-4

Shurin, J. B., Clasen, J. L., Greig, H. S., Kratina, P., and Thompson, P. L. (2012). Warming shifts top-down and bottom-up control of pond food web structure and function. *Phil. Trans. R. Soc. B* 267, 30008–33017. doi: 10.1098/rstb.2012.0243

Sigman, D. M., and Boyle, E. A. (2000). Glacial/interglacial variations in atmospheric carbon dioxide. *Nature* 407, 859–869. doi: 10.1038/35038000

Sokal, R. R., and Rohlf, F. J. (1995). *Biometry, 3rd Edn*. New York, NY: Freeman.

Steffen, W., Richardson, K., Rockström, J., Cornell, S. E., Fetzer, I., Bennett, E. M., et al. (2015). Planetary boundaries: guiding human development on a changing planet. *Science* 347:1259855. doi: 10.1126/science.1259855

Stelzer, R. S., and Lamberti, G. A. (2002). Ecological stoichiometry in running waters: periphyton chemical composition and snail growth. *Ecology* 83, 1039–1051. doi: 10.1890/0012-9658(2002)083[1039:ESIRWP]2.0.CO;2

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry*. Princeton, NY: Princeton University Press.

Sterner, R. W., Elser, J. J., Fee, E. J., Guildford, S. J., and Chrzanowski, T. H. (1997). The light:nutrient ratio in lakes: the balance of energy and materials affects ecosystem structure and process. *Am. Nat*. 150, 663–684. doi: 10.1086/286088

Urabe, J., and Sterner, R. W. (1996). Regulation of herbivore growth by the balance of light and nutrients. *Proc. Natl. Acad. Sci. U.S.A*. 93, 8465–8469. doi: 10.1073/pnas.93.16.8465

van de Waal, D. B., Verschoor, A. M., Verspagen, J. M. H., van Donk, E., and Huisman, J. (2010). Climate-driven changes in the ecological stoichiometry of aquatic ecosystems. *Front. Ecol. Environ*. 8, 145–152. doi: 10.1890/080178

Verschoor, A. M., Van Dijk, M. A., Huisman, J., and Van Donk, E. (2013). Elevated CO_2 concentrations affect the elemental stoichiometry and species composition of an experimental phytoplankton community. *Freshw. Biol*. 58, 597–611. doi: 10.1111/j.1365-2427.2012.02833.x

Verspagen, J. M., van de Waal, D. B., Finke, J. F., Visser, P. M., and Huisman, J. (2014). Contrasting effects of rising CO_2 on primary production and ecological stoichiometry at different nutrient levels. *Ecol. Lett*. 17, 951–960. doi: 10.1111/ele.12298

Villar-Argaiz, M., Bullejos, F. J., Medina-Sánchez, J. M., Ramos-Rodríguez, E., Delgado-Molina, J. A., and Carrillo, P. (2012). Disentangling food quantity and quality effects in zooplankton response to P-enrichment and UV radiation. *Limnol. Oceanogr*. 57, 235–250. doi: 10.4319/lo.2012.57.1.0235

Villar-Argaiz, M., Medina-Sánchez, J. M., and Carrillo, P. (2002). Microbial plankton response to contrasting climatic conditions: insights from the community structure, productivity and fraction stoichiometry. *Aquat. Microb. Ecol*. 29, 253–266. doi: 10.3354/ame029253

Villar-Argaiz, M., and Sterner, R. W. (2002). Life history bottlenecks in *Diaptomus clavipes* induced by phosphorus-limited algae. *Limnol. Oceanogr*. 47, 1229–1233. doi: 10.4319/lo.2002.47.4.1229

Weidman, P. R., Schindler, D. W., Thompson, P. L., and Vinebrooke, R. D. (2014). Interactive effects of higher temperature and dissolved organic carbon on planktonic communities in fishless mountain lakes. *Freshw. Biol*. 59, 889–904. doi: 10.1111/fwb.12313

Wohlers-Zöllner, J., Breithaupt, P., Walther, K., Jürgens, K., and Riebesell, U. (2011). Temperature and nutrient stoichiometry interactively modulate organic matter cycling in a pelagic algal–bacterial community. *Limnol. Oceanogr*. 56, 599–610. doi: 10.4319/lo.2011.56.2.0599

Woods, H. A., Makino, W., Cotner, J. B., Hobbie, S. E., Harrison, J. F., Acharya, K., et al. (2003). Temperature and the chemical composition of poikilothermic organisms. *Func. Ecol*. 17, 237–245. doi: 10.1046/j.1365-2435.2003.00724.x

Xenopoulos, M. A., Frost, P. C., and Elser, J. J. (2002). Joint effects of UV radiation and phosphorus supply on algal growth rate and elemental composition. *Ecology* 83, 423–435. doi: 10.1890/0012-9658(2002)083[0423:JEOURA]2.0.CO;2

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 January 2017
doi: 10.3389/fmicb.2017.00018

Changes in Stoichiometry, Cellular RNA, and Alkaline Phosphatase Activity of *Chlamydomonas* in Response to Temperature and Nutrients

Dag O. Hessen, Ola T. Hafslund, Tom Andersen, Catharina Broch, Nita K. Shala and Marcin W. Wojewodzic*

Aquatic Ecology and Toxicology, Department of Biosciences, University of Oslo, Oslo, Norway

Phytoplankton may respond both to elevated temperatures and reduced nutrients by changing their cellular stoichiometry and cell sizes. Since increased temperatures often cause increased thermal stratification and reduced vertical flux of nutrients into the mixed zone, it is difficult to disentangle these drivers in nature. In this study, we used a factorial design with high and low levels of phosphorus (P) and high and low temperature to assess responses in cellular stoichiometry, levels of RNA, and alkaline phosphatase activity (APA) in the chlorophyte *Chlamydomonas reinhardtii.* Growth rate, C:P, C:N, N:P, RNA, and APA all responded primarily to P treatment, but except for N:P and APA, also temperature contributed significantly. For RNA, the contribution from temperature was particularly strong with higher cellular levels of RNA at low temperatures, suggesting a compensatory allocation to ribosomes to maintain protein synthesis and growth. These experiments suggest that although P-limitation is the major determinant of growth rate and cellular stoichiometry, there are pronounced effects of temperature also via interaction with P. At the ecosystem level, nutrients and temperature will thus interact, but temperatures would likely exert a stronger impact on these phytoplankton traits indirectly via its force on stratification regimes and vertical nutrient fluxes.

Keywords: alkaline phosphatase, cell size, growth, phosphorus, phytoplankton, RNA, stoichiometry, temperature

OPEN ACCESS

Edited by:
James Cotner,
University of Minnesota, USA

Reviewed by:
Michael R. Twiss,
Clarkson University, USA
Douglas Andrew Campbell,
Mount Allison University, Canada

****Correspondence:***
Dag O. Hessen
d.o.hessen@ibv.uio.no

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *03 November 2016*
Accepted: *04 January 2017*
Published: *23 January 2017*

Citation:
Hessen DO, Hafslund OT, Andersen T, Broch C, Shala NK and Wojewodzic MW (2017) Changes in Stoichiometry, Cellular RNA, and Alkaline Phosphatase Activity of Chlamydomonas in Response to Temperature and Nutrients. Front. Microbiol. 8:18. doi: 10.3389/fmicb.2017.00018

INTRODUCTION

Elemental composition and temperature are key factors that affect growth and stoichiometry in algae. The ambient concentrations and ratios of key elements, such as carbon (C), nitrogen (N), and phosphorus (P) will have major impacts of phytoplankton elemental ratios and thus growth (Sterner and Elser, 2002; Klausmeier et al., 2008). Nutrient uptake and demands in autotrophs do also depend on ambient temperatures. The direct responses of temperature related to growth rate and stoichiometry are primarily governed by kinetic responses, i.e., enzyme activity, cell division, and nutrient uptake that may occur at higher rates with elevated temperature. However, also macromolecular make-up, rate of protein synthesis, and storage of elements may respond to temperature, which in this way also indirectly affect growth and cellular stoichiometry (Woods et al., 2003; Klausmeier et al., 2004; Toseland et al., 2013).

In higher plants and multicellular algae, there has been observed a general decline in specific N and P contents when moving from cold, high latitudes, toward the warmer, equatorial regions (Reich and Oleksyn, 2004; Borer et al., 2013). The decrease in P content with elevated temperatures is higher than that of N, however, causing an increase in the overall N:P ratio with increased temperature (or decreased latitude). Several studies have revealed a similar positive correlation between the overall N:P ratio of marine phytoplankton and global temperature (Martiny et al., 2013; Toseland et al., 2013; Yvon-Durocher et al., 2015), but there are few comparative lake studies, despite the fact that a strong increase in lake temperatures has been recorded worldwide (O'Reilly et al., 2015). A higher N:P with elevated temperature is likely associated with the increased enzyme efficiency at higher temperatures that cause a lower cellular density of P-rich ribosomes because fewer ribosomes are then needed to maintain a certain level of protein synthesis (Toseland et al., 2013; Thrane et al., 2016, 2017). If true, levels of RNA would also be reduced with elevated temperature, but again this response could be confounded by ambient nutrient concentrations.

Decreased phytoplankton cell size is another proposed response to warming (Atkinson et al., 2003; Daufresne et al., 2009; Sheridan and Bickford, 2011; Forster et al., 2012). The causality for smaller cell size, notably at the intraspecific level, remains obscure however. Experimental studies on phytoplankton indicate contributions both from ambient nutrient levels and temperature *per se* to cell size (Peter and Sommer, 2013, 2015), but it is difficult to disentangle the drivers based on *in situ* studies because warming also will affect thermal stratification, mixing depth, and thus vertical nutrient fluxes in aquatic ecosystems (Galbraith and Martiny, 2015). Reduced concentrations of ambient nutrients in response of reduced mixing would promote smaller cells owing to their higher surface-to-volume ratios and thus higher nutrient affinities (Raven, 1998; Marañón et al., 2012; Marañón, 2015). Hence in a "global change" context, both temperature and nutrient fluxes will change, with expected effects on the stoichiometry, growth and size of phytoplankton, yet likely with several confounding interactions (Sommer et al., 2016).

With this study, we aim to disentangle the effects of temperature and nutrients on phytoplankton growth and stoichiometry under controlled experimental conditions. To assess the responses in stoichiometry and growth, and the related responses [RNA, alkaline phosphatase activity (APA), and cell size] we conducted a factorial experiment with the chlorophyte *Chlamydomonas reinhardtii,* under high and low temperatures and high and low concentrations of phosphorus.

MATERIALS AND METHODS

For the experiments, we used the unicellular chlorophyte *C. reinhardtii* (strain CC-1690 wild type mt+) obtained from the Chlamydomonas Resource Centre (University of Minnesota). The species, and notably this strain, is widely used for experimental studies. While this species clearly may not be representative for all phytoplankton responses, it is commonly found across a variety of freshwater habitats and widely used also in ecologically relevant experiments.

The experiment was designed as a cross factorial setup with two P treatments (5 μmol P L^{-1} or 25 μmol P L^{-1}), hereafter low P (LP) and high P (HP), and two temperature treatments (13 or 19°C), designated low temperature (LT) and high temperature (HT), respectively. While the concentrations of P only differ by a factor of 5, the use of chemostats and turbidostats produced P-limited and P-sufficient cultures by design (see details below), and hence the actual P-concentrations were not critical in this context. A wider temperature gradient would likely provide stronger temperature responses, but the applied temperature represent a "realistic" span in epilimnetic summer temperatures of temperate lakes. Each treatment had three replicates. The experiments were run as semicontinuous cultures in 40 ml tissue bottles (Nunclon Delta filtercap, Thermo Scientific). We used a modified version of Guillard and Lorenzen's (1972) WC medium with filtered water from a high-alkalinity lake as a base to minimize the risk of CO_2-deficiency. Excess N was ensured by keeping N:P well above Redfield ratio (Redfield, 1958). A concentration of 1000 μmol NO_3 was used in both the high and LP treatments yielding molar N:P-ratios of 40:1 and 200:1, respectively. The lake water was initially filtered on Whatman GF/F and then sterile filtered (0.2 μm pore width) prior to additions of macronutrients, trace elements, and vitamins according to the WC medium recipe.

The algae were cultivated in two climate-controlled rooms of LT and HT (13 and 19°C, respectively) with a 12:12 h light-dark cycle and a light insity of approximately 85 μE m^{-2} s^{-1} of PAR (both cool and warm white light). For the LP treatment, a semicontinuous culture with a fixed dilution of 50% 3 days per week was applied. In this chemostat-type of dilution the algae are kept in a stationary growth phase below the carrying capacity. For the HP treatment we used a turbidostat-type of dilution where the culture was diluted to a fixed cell number (50,000 cells ml^{-1}) 3 days per week. The turbidostat design is beneficial by a maintaining a fixed density of algae in a non-limited condition with regard to nutrients, light and CO_2, thus avoiding the pitfalls of high-nutrient chemostats.

For all dilutions, the cultures were transferred to new bottles to avoid or minimize "bottle effects" like wall growth. Analysis of cell number (for growth estimates) and cell size were done at each day of dilution (3 days a week), samples for elemental ratios (C:N:P) were taken 14 days after inoculation, while samples for RNA and APA were taken 21 days after inoculation. However, because the APA results for two of the replicates in the HT × LP treatment had to be discarded due to a mistake made in the experimental procedure, we included a second sampling and analysis both for APA and RNA.

The cell number and size was measured by an electronic cell counter (CASY TT, Schärfe, Germany). A regular 12:12 h light:dark cycle was applied to synchronize cell division, and samples for estimations of cell densities and sizes were taken at the same time point each harvesting day. The specific growth rate was determined as the ln of relative change in cell abundance between two points in time (see Supplement for formula), and averaged for the duration of the experiment. As measures of cell

size, we recorded both the mean and the peak (mode) of the size distribution of the algal samples.

For analysis of particulate C and N content, algae were collected on a GF/C filter (Whatman, Sigma-Aldrich), and analyzed using an element analyzer (Thermo Finnegan EA 1112 series flash, Thermo Fisher scientific). P-content was estimated by digesting the samples in a solution of potassium peroxydisulfate ($K_2S_2O_8$) before colorimetric analyses using an autoanalyzer (Bran Luebbe, Norderstedt Germany). In this case, the samples were soaked in 10 ml of a 1% solution of potassium peroxydisulfate for 30 min at 120°C.

Cellular contents of RNA were included in the study both as a proxy of growth rate and to judge the effect of P-limitation. In addition, the total RNA serves as an indicator of the amount of ribosomal RNA in the cell (Flynn et al., 2010). For the RNA analyses, we applied a modified version of the RiboGreen fluorescence protocol (Turner BioSystems) (cf. Gorokhova and Kyle, 2002). Depending on cell density, we sampled 1–4 ml from each culture. The sampled volume was filtered on a nitrocellulose membrane (0.65 μm DAWP, Millipore) and the filter stored in nuclease tubes before snap-frozen in liquid N. Prior to analysis, 120 μl of the extraction buffer was added (1% sarcosyl, Sigma) and, while still frozen, the samples were homogenized by ice-cold sonification (Branson Sonifier) for 2 min. The samples were again put on ice and diluted with TE buffer in a 1:5 ratio (10 mM Tris-HCL, 1 mM EDTA, pH 7.5). For each sample, we extracted 2 × 75 μl into two separate slots of a 96-well plate (655076 Greiner Bio-one, USA). The duplicates were inserted pairwise in the columns following the first, which was reserved for standard. In the first set of duplicate samples, we added a total of 20 μl RNase-free water (Gibco BRL1071), and in the other set of duplicates 20 μl of 0.1% RNase A (A7973, Promega). Immediately after the RNase mixture was added, the well plate was incubated at 37°C on a shaking table with the output 400RPM to ensure homogenous dispatch of the RNase and digestion of RNA. After the incubation, 75 μl of 100 × diluted RiboGreen dye (R-11490, Molecular Probes, USA) was added to each well by the use of an automatic eight-channel pipette. The RNA content was then analyzed by the use of a fluorescence microplate reader (Synergy MX, BioTek Instruments, USA) with an excitation wavelength of 480 nm and an emission wavelength of 525 nm.

Alkaline phosphatase activity was included in the study as an independent biomarker for P-limitation (Thingstad and Mantoura, 2005; Litchman and Nguyen, 2008; Wang and Liang, 2014). This enzyme is used to split the ester bound in phosphomonoesters, thus removing phosphate groups from macromolecules scaled with the degree of P-deficiency (Hoppe, 2003). APA was analyzed by the CDP-star chemo-luminescence method according to the protocol of Wojewodzic et al. (2011). Samples for APA were collected as in the RNA analysis and stored at −80°C prior to analysis. For analysis, 0.3 ml of Triton X-100 solution (T8787, Sigma) was added to each sample (while kept on ice) and then sonified corresponding to the RNA procedure described above. The standards for the calibration curve were then prepared by using a concentration gradient AP type VII-S from bovine intestinal mucosa (P5521, Sigma). After the preparation of the standards, 20 μl of both the standards and the samples was transferred to a pyrophosphate-free 96-well plate (Nunc, 236105) kept on ice. Next 20 μl of the 0.4 mM CDP-star solution was dispensed with and automatic eight-channel pipette to all the wells. The APA contents of the cells were then analyzed using a fluorescence microplate reader (Synergy Mx, BioTek Instruments, USA). All statistical analysis and plotting were performed in the R programming environment v3.2 (R Development Core Team, 2016). The full script with additional data is given in the Supplementary Material.

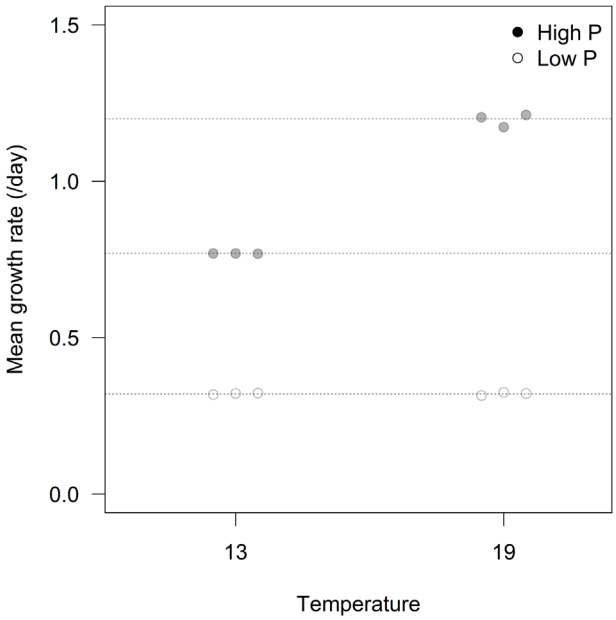

FIGURE 1 | Mean growth rates for the three replicates in each of the experimental treatments.

RESULTS

We found an overall strong effect of P-treatment on growth rates of algae, but with temperature and the interaction between P-treatment and temperature as important explanatory factors contributing to differences in growth (**Figure 1**; **Table 1**). At HP × HT, growth rates stabilized at ca. 1.2 d^{-1}, dropped to 0.77 d^{-1} at HP × LT, and further to 0.32 d^{-1} in the LP treatments. The growth rate responses remained stable during the 3 weeks course of the experiments (see Supplementary Figure S1).

The cell-specific content of C, N, and P all responded to P treatments, yet in different fashions (**Figure 2**; **Table 2**). The response in cell-specific C was modest, yet with somewhat lower C-content at LP × HT (**Figure 2A**). The same pattern was seen for cellular N, although in this case response was stronger, notably at HT, where cell-specific N was twice as high in the HP-treatment (**Figure 2B**), and for cellular P the differences were even stronger (**Figure 2C**). For N and P, there was also a positive interaction with temperature, where HP × HT yielded the highest cell-specific content of nutrients. This effect was most prominent for P. Since the cell sizes differed somewhat between

TABLE 1 | Fraction of total variance explained by temperature and P limitation treatments, and their interaction.

Variable	Growth rate (per day)	RNA (mg/ww)	Alkaline phosphatase activity (APA) (pU/ww)
P treatment	0.83	0.47	0.94
Temperature	0.08	0.21	–
P treatment × Temp.	0.08	–	–
R^2	0.99	0.68	0.94

w:w = weight fraction of wet weight (mg:mg), with wet weight estimated from total biovolume under the assumption of density = 1 g/cm^3. 1 pU = the amount of AP enzyme required to hydrolyse 1 pmol of 4-nitrophenyl phosphate per minute at pH 9.8 at 37°C. The analysis of RNA and APA includes the results from two sampling times, and the two response variables are in both cases corrected for cell size. See supplementary for the full ANOVA table.

treatments, and notably with reduced cell size at HT × HP (cf. Supplementary Figure S5), the estimates of cell specific elemental contents were corrected for cell size (using the mean of the cell size distribution). The correction was also applied for the other cell-specific responses (RNA and APA). The cell-specific results (not corrected for size) are given in the supplementary in the plot to the left in Supplementary Figures S6–S8 and S13 and S14. The size correction considerably changed the patterns for the C, N, and P contents, whereas RNA and APA basically displayed the same patterns as the non-corrected.

These responses to the T × P treatments yielded strong responses in elemental ratios, and unsurprisingly with the largest deviation in C:P, primarily in response to P-treatment, and for C:N and C:P also with modest contribution from temperature (**Figures 3A–C**; **Table 2**). The responses in N:P was consistent across temperature treatments with a fourfold decrease in N:P in the HP treatments. Also C:N responded strongly with lower ratios in the HP treatments, likely as a response to higher N-demands for protein synthesis with high access to P. In this case temperature also contributed, notably in the LP treatment with elevated C:N.

Cellular RNA responded strongly both to temperature and phosphorus with higher levels at HP and LT (**Figure 4A**; **Table 1**). While RNA corresponded well with cellular P (**Figure 2C**), it deviated from the growth rate responses (cf. **Figure 1**). Note that despite higher growth rates at HT (19°C), the RNA-concentration was higher at LT (13°C). APA ranged almost two orders of magnitude in response to P-treatment, with almost negligible contributions from temperature (**Figure 4B**; **Table 1**).

The combined response for all variables to temperature and phosphorus treatments can be summarized by a principal component analysis (**Figure 5**). The analysis clearly supports that P treatment was the experimental factor that explained most of the variation in RNA and nutrient contents (the separation of points along PCA axis 1, which captures 82% of the variance, primarily reflects P treatment). Growth rate and APA activity is included in the plot as passive variables (i.e., they did not influence the ordination) and clearly convey a strong positive relationship between P availability and growth rate, and a negative one between P availability and APA.

DISCUSSION

The overall conclusion from these experiments is that P-limitation is the major determinant of growth rate as well as stoichiometry and indicators thereof (RNA and APA), but that temperature also exerted a significant impact on most parameters. The cultures were maintained as chemostats (although semicontinuous) and turbidostats for the LP and HP treatments, respectively, to ensure chronically P-deficient and P-saturated cells. The APA analysis clearly showed that these premises were fulfilled with a striking deviation between HP and LP cultures under both temperatures. While APA only marginally responded to temperature, suggesting that this enzymatic response is not sensitive to a temperature span from 13 to 19°C, cellular RNA was much elevated at LT under both HP and LP, indicating a compensatory mechanism to maintain the rate of protein synthesis and thus growth at reduced temperature. Still growth rates were consistently lower at LT for the HP treatment (while not for LP).

The responses in RNA were also reflected in the cell quotas of P, and hence also the cellular stoichiometry. It is however

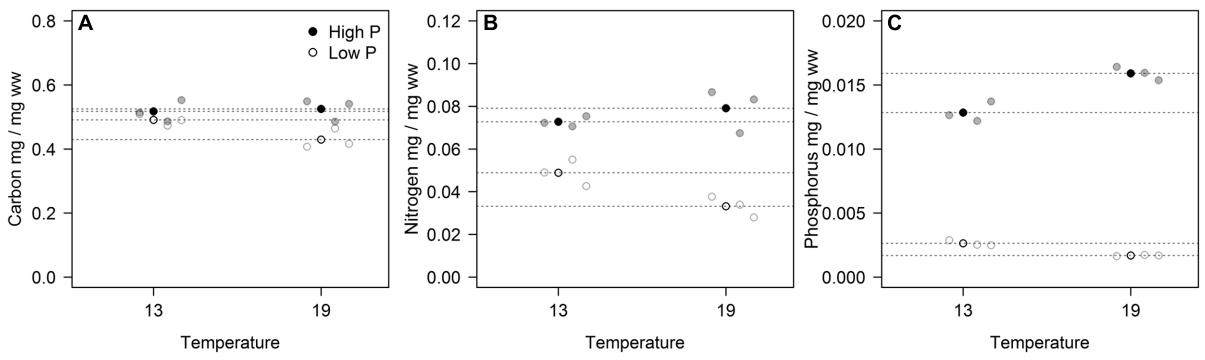

FIGURE 2 | Volume corrected contents of carbon (A), nitrogen **(B)**, and phosphorus **(C)** expressed as fraction of wet weight for each of the three replicates (gray circles) in the four experimental treatments. Points in black and dashed horizontal lines denote the mean in each group.

TABLE 2 | The proportion of variance explained (significantly) by the experimental variables.

Variable	C content (mg/ww)	N content (mg/ww)	P content (mg/ww)	C:N (molar)	C:P (molar)	N:P (molar)
P treatment	0.47	0.82	0.96	0.79	0.93	0.97
Temperature	–	0.01	0.01	0.03	0.03	–
P treatment × Temp.	–	0.08	0.03	0.08	0.04	–
R^2	0.47	0.91	0.99	0.90	0.99	0.97

See supplementary for the full ANOVA table. The nutrient contents are corrected for cell size (ww, wet weight), and the nutrients ratios are in molar units.

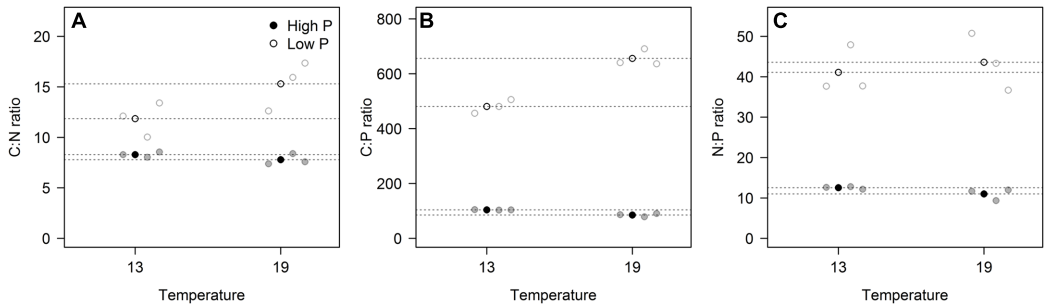

FIGURE 3 | The elemental ratios for carbon:nitrogen (A), carbon:phosphorus **(B)**, and nitrogen:phosphorus **(C)** for each of the three replicates (gray circles) in the four experimental treatments. Points in black and dashed horizontal lines denote the mean in each group. All ratios in molar units.

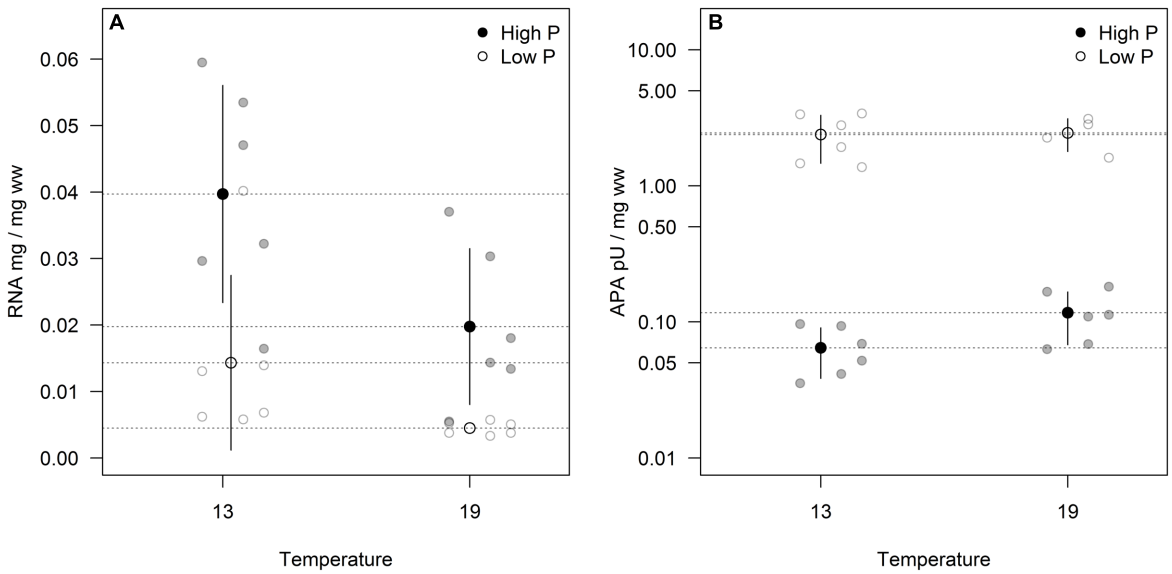

FIGURE 4 | Volume corrected RNA contents (A) and alkaline phosphatase activity (APA)/enzyme content **(B)** expressed as fraction of wet weight for two sampling times. Points in black and dashed horizontal lines denote the mean in each group (vertical lines = standard deviation).

noteworthy that the response in C:P was not only related to cellular quotas of P, but also of C (**Figure 2A**). The fact that also C:N were impacted by elevated P likely reflects its effect on growth and protein synthesis. Cellular processes, such as transcription and translation require a coupling of N and P, where P is needed for mRNA synthesis while N is required for protein synthesis.

The causal relationship between the close correlations typically found between growth rate, P-content and RNA in small heterotrophs, such as bacteria, crustacean zooplankton, and other invertebrates (Elser et al., 2000), is not straightforward, i.e., high growth rate could promote high RNA-content, but also be a consequence of other factors promoting elevated growth rates (in both cases this presupposes that sufficient P is available for making RNA). Also the availability of N would modify the relationship between RNA and growth rate, since the rate of protein synthesis may be constrained by the access to N (amino acids).

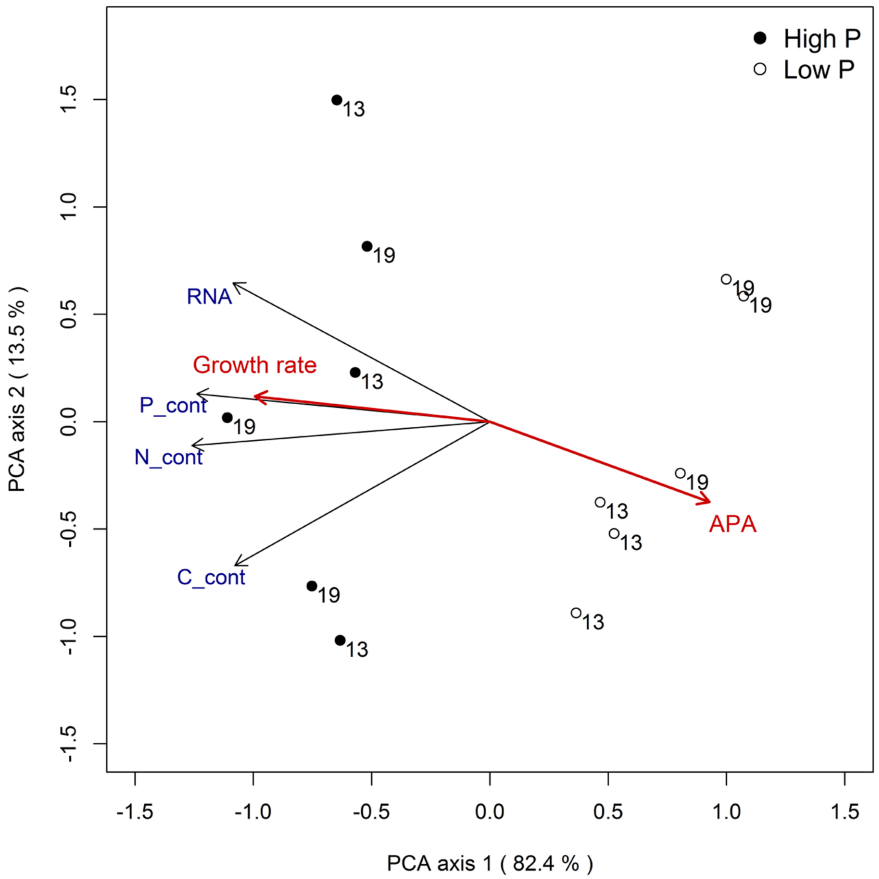

FIGURE 5 | Principal component analysis for responses related to RNA and elemental content (size corrected) with growth rate and APA as passive variables.

The contrasting temperature responses we found between cell-specific P-content relative to that of RNA is striking, however, and with the proviso that we here only tested one species, and that cryophilic species might respond differently, this support the "RNA-efficiency" hypothesis (cf. Woods et al., 2003; Cotner et al., 2006). This means higher demands of RNA at low temperature to maintain the levels of protein synthesis. This may further result in decreased N:P-ratios at lower temperatures, or thus *vice versa* as a response of warming (Martiny et al., 2013; Toseland et al., 2013; Yvon-Durocher et al., 2015), which is supported by the observed trend of decreased N:P in foliage or macroalgae when moving pole-wards (Reich and Oleksyn, 2004; Borer et al., 2013). For pelagic autotrophs, however, the stoichiometric responses may be confounded by the aforementioned change in mixing regimes along temperature gradients, and our study on *Chlamydomonas* suggest no impact at all of temperature on N:P. The chlorophyte *Chlamydomonas* may, however, not necessarily be representative of responses in other taxa. Different species and taxa of phytoplankton may have different strategies and responses with regard to N and P acquisition together with the N:P ratio of nutrient availability (Klausmeier et al., 2004; Thrane et al., 2016). In a more detailed assay with the same strain of *Chlamydomonas* grown in microwells along a wide gradient of temperatures and ambient N:P in the media, the optimum N:P ratio shifted from 27 to 37 (atomic ratio) over a temperature gradient from 11 to 18°C (Thrane et al., 2017). Yet, as pointed out in their paper, there are differences in measured optimal demand ratios for maximum growth and the ratio of total cellular pools of N:P in cells. Very strong deviations in N:P should basically not be expected, however, due to the mutual demands for N and P under transcription and translation as well as other cellular processes. I.e., also APA may ultimately be influenced by N-availability (Marklein and Houlton, 2012), and while photosynthetic capacity may be limited by the high N-demands of the photosynthetic machinery, so may P-limitation constrain the production of Rubisco (Reich et al., 2009). Corresponding temperature responses in stoichiometry and RNA has been observed in heterotrophic bacteria (Cotner et al., 2006).

Among the parameters tested in this study, RNA gave the strongest temperature response with elevated cellular concentrations at LT, but also growth rate and C:P showed a relatively strong response to temperature. Since growth rate was positively related to temperature, especially at HP (**Figure 1**), this does indeed suggest a stronger allocation of P to RNA under low temperature. The fact that it did not result in a corresponding decrease in N:P suggest

that N-uptake and protein synthesis kept pace with P-uptake and RNA synthesis. RNA did not correspond well with growth rate across temperatures, and to which extent the growth rate hypothesis (GRH) holds for autotrophs is still a matter of controversy (Ågren, 2004; Matzek and Vitousek, 2009; Flynn et al., 2010). It is, however, important to note that the GRH was explicitly formulated to hold within temperatures (Elser et al., 2000), hence within temperature (either 13 or 19°C) growth rate clearly match RNA content (and P).

Whether or not temperature *per se* affect cell size, or indirectly via lower ambient nutrient concentrations, is an issue of major concern in the context of global warming. In our experiments, smaller cells were found under HP × HT, but this response likely reflects the higher rate of cell division in this treatment. Still this implies that systems with higher temperatures could give smaller cells, but only when sufficient nutrients are available to promote strong growth.

Although temperature poses a direct impact on phytoplankton traits, notably on growth rate and cellular RNA, judged from these experiments, temperature would be expected to exert the strongest impact on phytoplankton indirectly via changes in stratification regimes and vertical nutrient fluxes.

AUTHOR CONTRIBUTIONS

DH had the idea of the experiment, and planned the design together with TA. OH was the main responsible for conducting the experiment with help from CB, NS, and MW, and was also instrumental in the analysis of the data together with input from the other authors. CB run the final scripts, stats, and figures in discussion with TA and DH. DH wrote up the manuscript with input from all co-authors.

FUNDING

This project received grants from the Norwegian Research Council "Genome" (project no. 196468) to DH.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00018/full#supplementary-material

REFERENCES

Ågren, G. I. (2004). The C:N:P stoichiometry of autotrophs – theory and observations. *Ecol. Lett.* 7, 185–191. doi: 10.1111/j.1461-0248.2004.00567.x

Atkinson, D., Ciotti, B. J., and Montagnes, D. J. S. (2003). Protists decrease in size linearly with temperature: ca. 2.5% ∘C-1. *Proc. Biol. Sci.* 270, 2605–2611. doi: 10.1098/rspb.2003.2538

Borer, E. T., Bracken, M. E. S., Seabloom, E. W., Smith, J. E., Cebrian, J., Cleland, E. E., et al. (2013). Global biogeography of autotroph chemistry: Is insolation a driving force? *Oikos* 122, 1121–1130. doi: 10.1111/j.1600-0706.2013.00465.x

Cotner, J. B., Makino, W., and Biddanda, B. A. (2006). Temperature affects stoichiometry and biochemical composition of *Escherichia coli*. *Microb. Ecol.* 52, 26–33. doi: 10.1007/s00248-006-9040-1

Daufresne, M., Lengfellner, K., and Sommer, U. (2009). Global warming benefits the small in aquatic ecosystems. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12788–12793. doi: 10.1073/pnas.0902080106

Elser, J. J., O'Brian, W. J., Dobberfuhl, D. R., and Dowling, T. E. (2000). The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* 13, 845–853. doi: 10.1046/j.1420-9101.2000.00215.x

Flynn, K. J., Raven, J. A., Rees, T. A. V., Finkel, Z., Quigg, A., and Beardall, J. (2010). Is the growth rate hypothesis applicable to microalgae? *J. Phycol.* 46, 1–12. doi: 10.1111/j.1529-8817.2009.00756.x

Forster, J., Hirst, A. G., and Esteban, G. F. (2012). Achieving temperature-size changes in a unicellular organism. *ISME J.* 7, 28–36. doi: 10.1038/ismej.2012.76

Galbraith, E. D., and Martiny, A. C. (2015). A simple nutrient-dependence mechanism for predicting the stoichiometry of marine ecosystems. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8199–8204. doi: 10.1073/pnas.1423917112

Gorokhova, E., and Kyle, M. (2002). Analysis of nucleic acids in Daphnia: development of methods and ontogenetic variations in RNA-DNA content. *J. Plankton Res.* 24, 511–522. doi: 10.1093/plankt/24.5.511

Guillard, R. R. L., and Lorenzen, C. J. (1972). Yellow-green algae with chlorophyllide C. *J. Phycol.* 8, 10–14. doi: 10.1111/j.1529-8817.1972.tb03995.x

Hoppe, H.-G. (2003). Phosphatase activity in the sea. *Hydrobiologia* 493, 187–200. doi: 10.1023/A:1025453918247

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. (2004). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174. doi: 10.1038/nature02454

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2008). Phytoplankton stoichiometry. *Ecol. Res.* 23, 479–485. doi: 10.1007/s11284-008-0470-8

Litchman, E., and Nguyen, B. L. V. (2008). Alkaline phosphatase activity as a function of internal phosphorus concentration in freshwater phytoplancton. *J. Phycol.* 44, 1379–1383. doi: 10.1111/j.1529-8817.2008.00598.x

Marañón, E. (2015). Cell size as a key determinant of phytoplankton metabolism and community structure. *Annu. Rev. Mar. Sci.* 7, 241–264. doi: 10.1146/annurev-marine-010814-015955

Marañón, E., Cermeño, P., Latasa, M., and Tadonléké, R. D. (2012). Temperature, resources, and phytoplankton size structure in the ocean. *Limnol. Oceanogr.* 57, 1266–1278. doi: 10.4319/lo.2012.57.5.1266

Marklein, A. R., and Houlton, B. Z. (2012). Nitrogen inputs accelerate phosphorus cycling rates across a wide variety of terrestrial ecosystems. *New Phytol.* 193, 696–704. doi: 10.1111/j.1469-8137.2011.03967.x

Martiny, A. C., Pham, C. T. A., Primeau, F. W., Vrugt, J. A., Moore, J. K., Levin, S. A., et al. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci.* 6, 279–283. doi: 10.1038/ngeo1757

Matzek, V., and Vitousek, P. M. (2009). N: P stoichiometry and protein : RNA ratios in vascular plants: an evaluation of the growth-rate hypothesis. *Ecol. Lett.* 12, 765–771. doi: 10.1111/j.1461-0248.2009.01310.x

O'Reilly, C. M., Sharms, S., Gray, D. K., Hampton, S. E., Read, J. S., Rowley, P., et al. (2015). Rapid and highly variable warming in lake surface waters around the globe. *Geophys. Res. Lett.* 42, 10773–10781. doi: 10.1002/2015GL066235

Peter, K. H., and Sommer, U. (2013). Phytoplankton cell size reduction in response to warming mediated by nutrient limitation. *PLoS ONE* 8:e71528. doi: 10.1371/journal.pone.0071528

Peter, K. H., and Sommer, U. (2015). Interactive effect of warming, nitrogen and phosphorus limitation on phytoplankton cell size. *Ecol. Evol.* 5, 1011–1024. doi: 10.1002/ece3.1241

R Development Core Team (2016). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Raven, J. A. (1998). The twelfth Tansley lecture. Small is beautiful: the picophytoplankton. *Funct. Ecol.* 12, 503–513. doi: 10.1046/j.1365-2435.1998.00233.x

Redfield, A. C. (1958). The biological control of chemical factors in the environment. *Am. Sci.* 46, 205–221.

Reich, P. B., and Oleksyn, J. (2004). Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11001–11006. doi: 10.1073/pnas.0403588101

Reich, P. B., Oleksyn, J., and Wright, I. J. (2009). Leaf phosphorus influences the photosynthesis–nitrogen relation: a cross-biome analysis of 314 species. *Oecologia* 160, 207–212. doi: 10.1007/s00442-009-1291-3

Sheridan, J. A., and Bickford, D. (2011). Shrinking body size as an ecological response to climate change. *Nat. Clim. Change* 1, 401–406. doi: 10.1038/nclimate1259

Sommer, U., Peter, K. H., Genitsaris, S., and Moustaka-Gouni, M. (2016). Do marine phytoplankton follow Bergmann's rule sensu lato? *Biol. Rev.* doi: 10.1111/brv.12266

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stochiometry*. Princeton, NJ: Princton University Press.

Thingstad, T. F., and Mantoura, R. F. C. (2005). Titrating excess nitrogen content of phosphorous-deficient eastern Mediterranean surface water using alkaline phosphatase activity as a bio-indicator. *Limnol. Oceanogr. Methods* 3, 94–100. doi: 10.4319/lom.2005.3.94

Thrane, J.-E., Hessen, D. O., and Andersen, T. (2016). The impact of irradiance on optimal and cellular nitrogen to phosphorus ratios in phytoplankton. *Ecol. Lett.* 19, 880–888. doi: 10.1111/ele.12623

Thrane, J.-E., Hessen, D. O., and Andersen, T. (2017). Temperature influences the nitrogen-to-phosphorus requirement ratio (the optimal N:P) of a unicellular alga. *Limnol. Oceanogr.* (in press).

Toseland, A., Daines, S. J., Clark, J. R., Kirkham, A., Strauss, J., Uhlig, C., et al. (2013). The impact of temperature on marine phytoplankton resource allocation and metabolism. *Nat. Clim. Change* 3, 979–984. doi: 10.1038/nclimate1989

Wang, Z.-H., and Liang, Y. (2014). Growth and alkaline phosphatase activity of *Chattonella marina* and *Heterosigma akashiwo* in response to phosphorus limitation. *J. Environ. Sci.* 27, 1–7. doi: 10.1016/j.jes.2014.04.015

Wojewodzic, M. W., Kyle, M., Elser, J. J., Hessen, D. O., and Andersen, T. (2011). Joint effect of phosphorus limitation and temperature on alkaline phosphatase activity and somatic growth in *Daphnia magna*. *Oecologia* 165, 837–846. doi: 10.1007/s00442-010-1863-2

Woods, H. A., Makino, W., Cotner, J. B., Hobbie, S. E., Harrison, J. F., Acharya, K., et al. (2003). Temperature and the chemical composition of poikilothermic organisms. *Funct. Ecol.* 17, 237–245. doi: 10.1046/j.1365-2435.2003.00724.x

Yvon-Durocher, G., Dossena, M., Trimmer, M., Woodward, G., and Allen, A. P. (2015). Temperature and the biogeography of algal stoichiometry. *Glob. Ecol. Biogeogr.* 24, 562–570. doi: 10.1111/geb.12280

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 July 2017
doi: 10.3389/fmicb.2017.01299

From Ecological Stoichiometry to Biochemical Composition: Variation in N and P Supply Alters Key Biosynthetic Rates in Marine Phytoplankton

Julia Grosse[1]†, Amanda Burson[2], Maayke Stomp[2], Jef Huisman[2] and Henricus T. S. Boschker[1†]*

[1] Department of Marine Microbiology and Biogeochemistry, Royal Netherlands Institute for Sea Research, and Utrecht University, Den Burg, Netherlands, [2] Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, Netherlands

OPEN ACCESS

Edited by:
Robert Warner Sterner,
University of Minnesota Duluth,
United States

Reviewed by:
Edward Hall,
Colorado State University,
United States
Irakli Loladze,
Bryan College of Health Sciences,
United States

***Correspondence:**
Julia Grosse
jgrosse@geomar.de

†Present address:
Julia Grosse,
Department of Biological Oceanography, GEOMAR Helmholtz Centre of Ocean Research Kiel, Kiel, Germany
Henricus T. S. Boschker,
Microbiology Section, Department of Bioscience, Aarhus University, Aarhus, Denmark

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: 26 January 2017
Accepted: 27 June 2017
Published: 12 July 2017

Citation:
Grosse J, Burson A, Stomp M, Huisman J and Boschker HTS (2017) From Ecological Stoichiometry to Biochemical Composition: Variation in N and P Supply Alters Key Biosynthetic Rates in Marine Phytoplankton. Front. Microbiol. 8:1299. doi: 10.3389/fmicb.2017.01299

One of the major challenges in ecological stoichiometry is to establish how environmental changes in resource availability may affect both the biochemical composition of organisms and the species composition of communities. This is a pressing issue in many coastal waters, where anthropogenic activities have caused large changes in riverine nutrient inputs. Here we investigate variation in the biochemical composition and synthesis of amino acids, fatty acids (FA), and carbohydrates in mixed phytoplankton communities sampled from the North Sea. The communities were cultured in chemostats supplied with different concentrations of dissolved inorganic nitrogen (DIN) and phosphorus (DIP) to establish four different types of resource limitations. Diatoms dominated under N-limited, N+P limited and P-limited conditions. Cyanobacteria became dominant in one of the N-limited chemostats and green algae dominated in the one P-limited chemostat and under light-limited conditions. Changes in nutrient availability directly affected amino acid content, which was lowest under N and N+P limitation, higher under P-limitation and highest when light was the limiting factor. Storage carbohydrate content showed the opposite trend and storage FA content seemed to be co-dependent on community composition. The synthesis of essential amino acids was affected under N and N+P limitation, as the transformation from non-essential to essential amino acids decreased at DIN:DIP $\leq$ 6. The simple community structure and clearly identifiable nutrient limitations confirm and clarify previous field findings in the North Sea. Our results show that different phytoplankton groups are capable of adapting their key biosynthetic rates and hence their biochemical composition to different degrees when experiencing shifts in nutrient availability. This will have implications for phytoplankton growth, community structure, and the nutritional quality of phytoplankton as food for higher trophic levels.

Keywords: marine phytoplankton, compound specific isotope analysis, nutrient competition, N:P ratios, ^{13}C-labeling, ecological stoichiometry

INTRODUCTION

Changes in nutrient availability affect the C:N:P ratio of primary producers, both through physiological acclimation and shifts in species composition. In turn, these shifts in the elemental composition of primary producers can have major implications for nutrient cycling and their quality as food for herbivores, which are key focal research areas of the rapidly expanding field of ecological stoichiometry (Sterner and Elser, 2002; Hessen et al., 2004; Vrede et al., 2004; Persson et al., 2010). However, although C:N:P ratios are easily measured, an often voiced criticism is that they do not provide detailed information on changes in the biochemical composition of primary producers in terms of, e.g., amino acids (AA), fatty acids (FA) and carbohydrates (CH), DNA and RNA (Anderson et al., 2004; Raubenheimer et al., 2009). The biochemical composition of primary producers is important for their own growth and survival, and plays a key role in many plant-herbivore interactions. For instance, most herbivores cannot synthesize all AA and FA themselves, but rely on the provision of essential AA and FA from the primary producers in their diet (Müller-Navarra, 1995; Fink et al., 2011). Therefore, a deeper understanding of how changes in environmental nutrient availability affect the biochemical composition of primary producers would be a major next step.

Many coastal waters have witnessed major changes in nutrient input during the past several decades. The North Sea provides a good example. Between the early 1960s and mid-1980s mean annual concentration of dissolved inorganic N tripled, while at the same time P concentrations doubled, resulting in coastal eutrophication (Hickel et al., 1993). Effects of eutrophication included an increase in phytoplankton biomass (Cadée and Hegeman, 2002), shifts in species composition (Philippart et al., 2000), the formation of toxic algal blooms (Riegman et al., 1992; Lancelot et al., 2007), changed trophic food web structures (Van Beusekom and Diel-Christiansen, 2009) and the development of hypoxia (Westernhagen and Dethlefsen, 1983). In response, members of the OSPAR Convention (Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic) agreed to lower riverine N and P inputs to the North Sea by at least 50% compared to the year 1985 (OSPAR, 1988). Nutrient reduction efforts resulted in an effective P removal from domestic and industrial wastewater. By 2002, many countries reached and even exceeded the goal for P, by decreasing P inputs by 50–70%. However, decreasing N inputs was less successful and N loads were only lowered by 20–30% (Lenhart et al., 2010; OSPAR, 2010; Passy et al., 2013). As a consequence, riverine N:P inputs to the coastal North Sea currently greatly exceed the Redfield ratio of 16:1 (Radach and Pätsch, 2007; Thieu et al., 2010; Grizzetti et al., 2012; Burson et al., 2016).

Similar patterns have been observed in other coastal waters. Effective P removal in combination with a global increase in the application of N fertilizers has increased the N:P ratios of many riverine nutrient inputs to coastal waters (Turner et al., 2003; Grizzetti et al., 2012; Glibert et al., 2014). Consequently, P limitation is currently becoming more prevalent in river-influenced coastal seas, not only in the North Sea (Burson et al., 2016) but also in, e.g., the Gulf of Mexico and the South China Sea (Sylvan et al., 2007; Xu et al., 2008), challenging the classical view that N is the main limiting nutrient in marine coastal systems (Hecky and Kilham, 1988; Howarth and Marino, 2006). In the North Sea, this pattern is further confirmed by high nearshore POC:POP ratios (400–700) during the phytoplankton spring bloom, indicative of severe P deficiency of coastal phytoplankton (Burson et al., 2016). Lab studies have shown that P-deficient phytoplankton may cause lower growth rates in marine zooplankton (Malzahn et al., 2007; Malzahn and Boersma, 2012; Schoo et al., 2013), and that the elevated C:P ratios of this zooplankton can, in turn, have detrimental effects on larval growth of economically valuable species such as herring (Malzahn et al., 2007) and European lobster (Schoo et al., 2014). So far, however, little is known about the implications of these changes in nutrient limitation for the biochemical composition of marine phytoplankton.

In recent years, advances in compound-specific isotope analysis by either gas chromatography (GC) or liquid chromatography (LC) in combination with isotopic ratio mass spectrometry (IRMS) have made it possible to obtain specific isotope information from a wide range of biomolecules in complex mixtures (e.g., McCullagh et al., 2006; Boschker et al., 2008; Veuger et al., 2012). Now, ^{13}C stable isotopes can be used in the same way as in primary production measurements but on a more detailed compound-specific level, by measuring the incorporation of photosynthetically fixed carbon into individual FA, AA, and CH (Grosse et al., 2015, 2017). This opens up opportunities to study the biochemical composition and nutritional quality of phytoplankton in much further detail.

In this study, we explore how changes in N and P loads may potentially affect the biochemical composition of coastal marine phytoplankton. As model system, we inoculated laboratory chemostats with mixed phytoplankton communities sampled from the North Sea. This experimental approach enabled a systematic investigation of the effects of different N:P supply ratios on resource limitation, biochemical composition and biosynthesis rates of the phytoplankton community using compound-specific isotope analysis.

MATERIALS AND METHODS

Collection of Inoculum

Samples for field inoculum were taken from eight stations along a 450 km long transect from the Dutch coast towards the center of the North Sea between 15 and 22 March 2013 onboard the Dutch research vessel RV Pelagia (Grosse et al., 2017). At each station a 20 L carboy was rinsed and filled with water collected at 7 m depth. Water was passed through a 200 μm mesh then bubbled for 30 min each with CO_2 and N_2 gas to eliminate grazers. The carboys were kept at 4°C until initiation of chemostat experiments at the University of Amsterdam. Equal portions of water from each station were combined, resulting in a single inoculum for the chemostat experiments containing a mix of phytoplankton from all eight stations along the entire 450 km transect (for additional details, see Burson et al. (2016).

TABLE 1 | Concentrations and ratios of nutrients in the different media and in chemostats when phytoplankton communities reached steady state conditions.

	MNHP	LNHP	MNMP	LNLP	HNHP	HNMP	HNLP
Nutrients							
$DIN:DIP_{Medium}$	1.28	0.512	16	16	16	200	500
DIN_{Medium} (μM)	160	64	160	64	2000	2000	2000
DIP_{Medium} (μM)	125	125	10	4	125	10	4
$DIN:DIP_{Chemostat}$	0.04	0.04	1	2	6	275	2380
$DIN_{Chemostat}$ (μM)	2	2	3	4	181	825	1190
$DIP_{Chemostat}$ (μM)	49	46	3	2	29	3	0.5
Community Composition							
Diatoms (%)	23	**81**	**84**	**86**	38	6	**86**
Green algae (%)	15	4	5	1	**57**	**87**	9
Cyanobacteria (%)	**62**	15	11	13	5	6	5
Light penetration I_{out} (μmol photons m^{-2} s^{-1})	17	24	19	26	0.4	9.5	23
Biochemical parameters							
POC (mM)	8.21	3.46	9.63	3.04	26.31	13.35	3.81
PON (mM)	0.334	0.180	0.365	0.127	3.152	1.144	0.485
POP (mM)	0.011	0.010	0.007	0.004	0.060	0.010	0.003
POC:PON	25	19	26	24	8	12	8
POC:POP	746	346	1376	760	439	1335	1270
PON:POP	30	18	52	32	53	114	162
C-fixation (nmol C μmol POC^{-1} d^{-1})	44	73	52	56	83	109	191
DIN requirement (nmol DIN μmol POC^{-1} d^{-1})	1.8	3.8	2.0	2.3	9.9	9.3	24.3
DIP requirement (nmol DIP μmol POC^{-1} d^{-1})	0.06	0.22	0.04	0.07	0.19	0.08	0.14

Chemostats are sorted left to right by increasing $DIN:DIP_{Chemostat}$ ratios. Contribution of the different phytoplankton groups to total biovolume is also shown, with the highest contributing group in bold. Light penetration indicates light availability and particulate organic carbon (POC), nitrogen (PON), and phosphorus (POP) and their ratios are shown. DIN and DIP requirements were calculated using C-fixation rates, POC:PON and POC:POP ratios.

Chemostat Set-Up

Within 2 days after the cruise ended, seven flat-walled chemostats (mixing depth: 5 cm) were set up according to Huisman et al. (2002), using full-spectra white fluorescent bulbs as light sources and magnetic stir bars to minimize accumulation of sticky and heavy species. The incident light intensity at the front surface of each chemostat was set at 40 μmol photons m^{-2} s^{-1} and the dilution rate at 0.2 d^{-1}. Irradiance passing through the chemostat vessel (I_{out}) was measured with a light meter (LI-250 LI-COR, NE, United States) at ten regularly spaced positions at the back surface of the chemostat. The seawater inoculum was added to fill half the chemostat's volume (0.5 L) and was topped off with one of seven artificial seawater media using peristaltic pumps. Media exhibited different combinations of dissolved inorganic nitrogen (DIN_{Medium}) and phosphate (DIP_{Medium}) at low (LN/LP), medium (MN/MP), or high (HN/HP) concentrations, hence also differed in their $DIN:DIP_{Medium}$ ratios (**Table 1** and **Figure 1**). Inorganic carbon was added in two ways; as sodium bicarbonate to media (0.5 mM final concentration) and as CO_2 in filtered air, which was bubbled through the chemostats. The chemostats were run as a competition experiment until phytoplankton communities established steady state conditions (91 days) and were then harvested for the carbon fixation experiment.

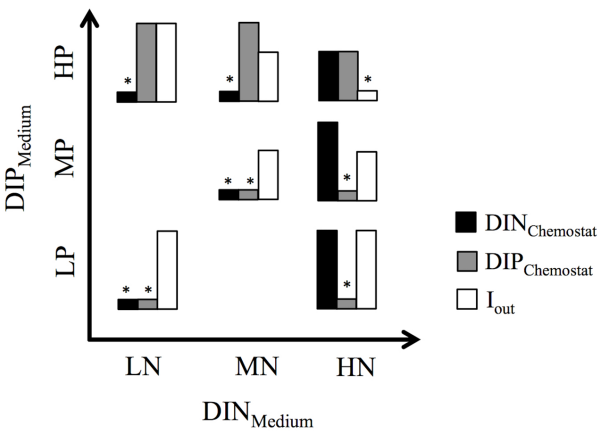

FIGURE 1 | Bar graphs show the relative distribution of nutrient concentrations ($DIN_{Chemostat}$, $DIP_{Chemostat}$) and light penetration (I_{out}) in the seven chemostat experiments. *above bars indicate the resulting resource limitation. Experimental set-up is based on three levels of dissolved inorganic nitrogen (DIN_{Medium}) and phosphorus (DIP_{Medium}) in the mineral medium supplied to the chemostats.

Carbon Fixation Experiment

Chemostats were harvested by transferring 1 L of culture into 1.2 L culture flasks. From there, initial and unlabeled subsamples were taken for dissolved inorganic carbon (DIC), nutrient concentrations, particulate organic C, N, and P (POC, PON, POP), and biomolecules (AA, FA, CH). Nutrient samples were filtered through a 0.2 μm Acrodisc filter and stored at 4°C until analysis. DIC samples were also filtered through a

0.2 μm Acrodisc filter, sealed bubble-free in a 10 mL crimp vial and stored at 4°C until analysis. Samples for POC/PON, POP and biomolecules were taken by filtering 30–100 mL per analysis (depending on biomass) over pre-combusted GF/F filters (Whatman, 4 h at 450°C). POC/PON and POP filters were stored at –20°C and biomolecule filters were stored at −80°C.

Carbon fixation experiments had to be carried out in batch cultures because ^{13}C-DIC labeling levels throughout the experiment had to be kept constant, which is difficult to achieve in air-flushed chemostats. Because the remaining culture in the flasks could not be air-bubbled for the same reason, we added additional unlabeled sodium bicarbonate to a final concentration of 2 mM in order to avoid DIC-limitation during the experiment. Thereafter, all culture flasks (volume between 450 and 650 mL) were enriched with ^{13}C-sodium bicarbonate (99% ^{13}C) to a final labeling concentration of ∼5% of total DIC concentration. Concentrations and absolute ^{13}C-DIC enrichment were measured as previously described (Grosse et al., 2015). Culture flasks were closed airtight and incubated for 24 h at a constant rotation (60 rpm), 20°C and 40 μmol photons m^{-2} s^{-1} light intensity, assuring conditions resembling those of the chemostats. After 24 h samples were taken stable isotope analysis of DIC, POC/PON, and biomolecules and stored as described above until analysis.

Laboratory Analysis and Biomolecule Extractions

Dissolved inorganic nitrogen (DIN = nitrate + nitrite) and DIP (phosphate) concentrations were analyzed using standard colorimetric methods (Grasshoff et al., 1983).

Detailed descriptions of POC/PON analysis, extraction protocols for biomolecules (CH, AA, and FA), EA-, LC-, and GC/C-IRMS systems as well as compound separation protocols and conditions have been published in detail elsewhere (Grosse et al., 2015 and references therein). In short, frozen POC/PON filters were lyophilized overnight, acidified, and subsequently packed into tin cups before the analysis of organic carbon and nitrogen content and δ^{13}C values by EA-IRMS. The POP content was quantified with inductive coupled plasma spectroscopy after digestion with 10 mL of 65% HNO_3 (ICPOES; Perkin Elmer Optima 3300 DV; Nieuwenhuize and Poley-Vos, 1989). CH samples were acid hydrolyzed and analyzed for concentrations and ^{13}C-labeling of individual CH by LC/IRMS using an Aminex HPX-87H column, which separates glucose from all other carbohydrates, while galactose, xylose, mannose, and fructose co-elute in a second peak. A third peak contains fucose, arabinose, and ribose. Glucose is also part of storage compounds and was therefore reported separately from all other CH, which are hereafter referred to as structural CH. AA samples were acid hydrolyzed and analyzed by LC/IRMS using a Primsep A column, which separates a total of 17 individual AA (McCullagh et al., 2006). Due to the analytical procedures glutamate and glutamine co-elute as do aspartate and asparagine, formed one peak each. Other detected AA included threonine, valine, methionine, isoleucine, leucine, lysine, histine, phenylalanine, argin, serine, glycine, alanine, proline, cystine, and tyrosine. Because of their very low concentrations, cysteine and methionine were excluded in the data analysis.

Fatty acid samples were extracted following the protocol of Bligh and Dyer (1959) and subsequently separated into storage lipids (triglycerides), glycolipids and phospholipids by silicate column chromatography. However, it has been shown that the phospholipid fraction also contains other non-P containing intact polar lipids (Heinzelmann et al., 2014). The glycolipid- and phospholipid fractions were therefore combined and are further referred to as structural, membrane-derived lipids. After derivatization to fatty acid methyl esters, they were analyzed and the ^{13}C measured by GC/C-IRMS using the column BPX-70.

Biosynthesis rates of each individual compound were calculated from ^{13}C incorporation rates according to Grosse et al. (2015), and were added up in order to obtain values for each biomolecule group (essential and non-essential AA, storage and structural FA, and storage (glucose) and structural CH) (see Supplementary Materials for details). Throughout this text, biomolecule concentrations and biosynthesis rates were reported relative to cumulative POC concentrations and C-fixation rates (AA + FA + CH = 100%), respectively. Unidentified biomolecules were included only in total POC concentrations and bulk C-fixation rates.

Statistical Analysis

To explore differences in amino acid composition between different nutrient limitations and communities, principle component analysis (PCA) was performed using AA data. Data for the relative contribution (%) of (*i*) individual AA concentrations to total AA concentrations (nmol C μmol POC^{-1}) and (*ii*) individual AA synthesis to total AA synthesis (nmol C μmol POC^{-1} d^{-1}) was used. The package CRAN:factoMineR in the open source software R was used for the PCA analysis using a correlation matrix.

RESULTS

Resource Limitation

Both the MNHP and LNHP chemostats received media with low DIN:DIP ratios of 1.28 and 0.5, respectively. Their communities decreased the DIN concentrations from 160 μM (MNHP) and 64 μM (LNHP) in the medium to 2 μM in the steady-state chemostat, while the DIP concentrations remained high at 49 and 46 μM, respectively. DIN:DIP ratios in both chemostats were decreased to 0.04, indicating that the communities were limited by N (**Table 1** and **Figure 1**).

The HNMP and HNLP chemostats received media with high DIN:DIP ratios of 200 and 500, respectively. Nutrient uptake by the phytoplankton increased DIN:DIP ratios in the chemostats further to 275 and 2380, respectively (**Table 1**). The DIP concentrations decreased from 10 μM (HNMP) and 4 μM (HNLP) in the medium to 3 and 0.5 μM, respectively, in the steady-state chemostats, while DIN concentrations remained

high at 825 and 1190 μM. The high DIN:DIP ratios, as well as the low DIP concentrations, point at P-limitation in these two chemostats (**Figure 1**).

Three chemostats received media with DIN:DIP ratios of 16 (LNLP, MNMP, HNHP) and nutrient uptake by the phytoplankton reduced the DIN:DIP ratios in the chemostats to 1, 2, and 6 for LNLP, MNMP, and HNHP, respectively. Although those ratios might be interpreted as N-limitation, both DIN and DIP concentrations were very low in the LNLP and MNMP chemostats (**Table 1**), and therefore suggested N+P co-limitation (**Figure 1**). In contrast, nutrients in the HNHP chemostat remained high with 181 μM DIN and 29 μM DIP (**Table 1**). At the same time, the high biomass (26.3 mM POC) decreased light levels, inducing light-limitation in this chemostat (**Figure 1**).

Phytoplankton biomass ranged from 3.0 to 26.3 mM POC and increased with increasing DIN and DIP concentrations in the mineral medium (**Figure 2A** and **Table 1**). POC:PON ratios in N-limited and N+P co-limited chemostats ranged between 19 and 26 and were lower in light- and P-limited chemostats (**Table 1**). Extremely high POC:POP ratios (>1000) were found in the P-limited chemostats. The MNMP chemostat also showed extremely high POC:POP ratios, consistent with the idea that this community was co-limited by N and P (**Table 1**). PON:POP ratios were >100 in P-limited chemostats and ranged between 18 and 53 in all others.

Based on these POC:PON and POC:POP ratios and total C-fixation rates, we calculated daily DIN and DIP requirements. The N-limited and N+P co-limited chemostats had lowest DIN requirements of only 1.8 nmol DIN μmol POC^{-1} d^{-1} for the MNHM chemostat and slightly higher values for the LNLP, LNHP and MNMP chemostats. The P-limited HNLP required 24.3 nmol DIN μmol POC^{-1} d^{-1}. The HNHP and HNMP chemostats required similar amounts of DIN with 9.9 and 9.3 nmol DIN μmol POC^{-1} d^{-1}.

Phytoplankton in the P-limited and N+P co-limited chemostats had DIP requirements ranging from 0.04 to 0.14 nmol DIP μmol POC^{-1} d^{-1}. The DIP-requirements in the other chemostats ranged from 0.06 to 0.22 nmol DIP μmol POC^{-1} d^{-1} (**Table 1**).

Community Composition

Differences in DIN and DIP concentrations in media, and resulting DIN:DIP ratios shaped the community structure in all seven chemostats. Based on microscopic observations and flow cytometry (Accuri C6 flow cytometer, BD Biosciences, San Jose, CA, United States) at least five species could be distinguished in the steady-state chemostats, representing three phytoplankton phyla. Green algae were represented by a *Chlorella* sp., while unicellular cyanobacteria (*Synechococcus* spp.) and diatoms (*Nitzschia agnita* and *N. pusilla)* were represented by at least two taxa each. *Chlorella* sp. and the two strains of *Synechococcus* spp. were distinguished by differences in their chlorophyll and phycocyanin fluorescence as well as their cell size, using flow cytometry. The two diatom species were identified microscopically.

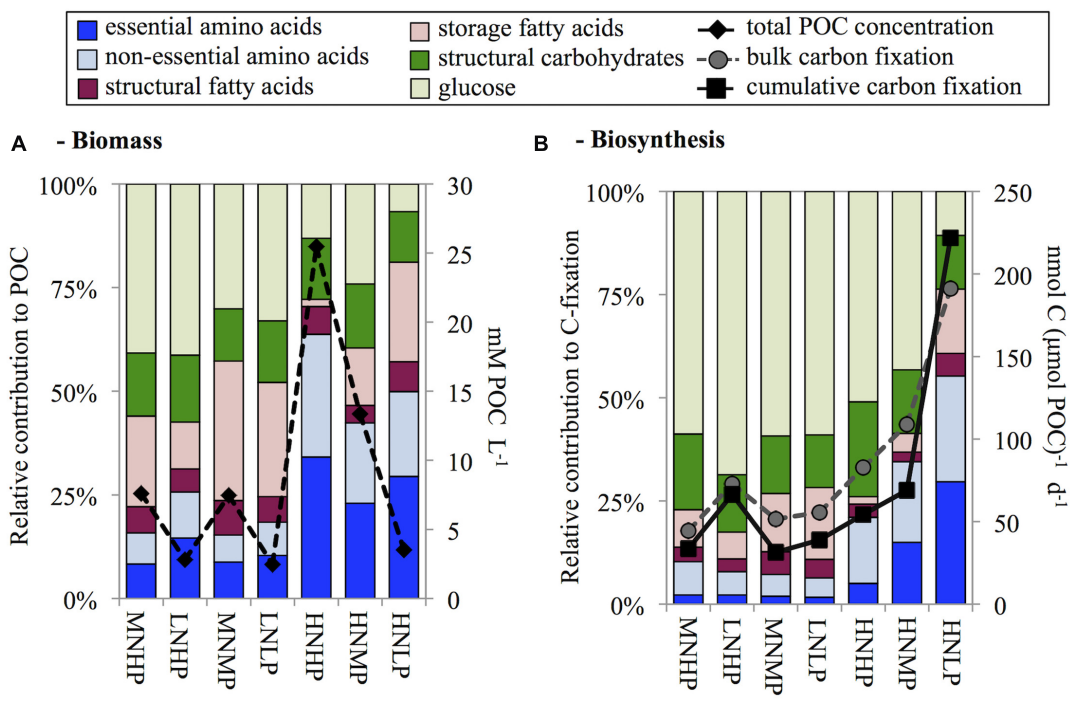

FIGURE 2 | Overview of biomolecule composition of biomass **(A)** and biosynthesis **(B)** sorted by DIN:DIP ratios in the chemostats before the ^{13}C addition experiment. Biomass concentrations are added to panel **A** as total particulate organic carbon (POC), and both bulk and cumulative carbon fixation are added to panel **B**.

Mixed communities developed in all chemostats. Diatoms dominated one of the N-limited chemostats (LNHP), both N+P co-limited chemostats (LNLP, MNMP), and one of the P-limited chemostats (HNLP), where *N. agnita* was more abundant under N-limited conditions and *N. pusilla* under P-limited conditions. Cyanobacteria dominated in the other N-limited chemostat with diatoms being second most abundant (MNHP). Green algae dominated in the other P-limited chemostat (HNMP), while the light-limited chemostat (HNHP) showed a more even co-dominance of green algae and diatoms (**Table 1**).

Biochemical Composition and Synthesis

AA contribution to biomass was highest in the light limited HNHP chemostat where it contributed 64% of POC concentration, intermediate in the P-limited HNMP and HNLP chemostats contributing 42% and 50% of POC concentration, respectively, and lowest in the remaining four N-(co-)limited chemostats (16–26% of POC concentration, **Figure 2A**). Glucose concentrations showed an opposite trend to that of total AA and contributed between 7 and 42% to POC concentration.

Storage FA contributions varied considerably (1.6–33% of POC concentration), being lowest in the HNHP chemostat and highest in the MNMP chemostat. However, no DIN:DIP ratio dependent increase or decrease was observed. Structural CH and structural FA showed little variation. Structural CH contributed 14 ± 2% to POC concentrations and structural FA contributed 6 ± 1% to POC concentration (averages ± standard deviation, $n = 7$).

With the dilution rate set to 0.2 d^{-1}, we would have expected to find biomass specific C-fixation rates to be ∼200 nmol C μmol POC^{-1} d^{-1}. However, only the HNLP chemostat showed expected value, while C-fixation rates of all other chemostats were considerably lower (**Figure 2B**). One likely explanation is the contribution of dead material to the biomass, leading to an underestimation of biomass specific C-fixation rates. The 24 h incubation of the C-fixation experiment induced further nutrient depletion, which may have also been a contributing factor to the decreased specific C-fixation rates. Alternatively, nutrient-stressed phytoplankton can exudate photosynthetically fixed carbon as DOC (Myklestad, 2000; Nagata, 2000), a pool that we have not quantified in this study.

The synthesis rates of all investigated biomolecules summed up to between 61% (MNMP) and 91% (LNHP) of bulk C-fixation (**Figure 2B**). This range was similar to field findings (Grosse et al., 2015) and suggested that 9–32% of bulk carbon fixation ends up in biomolecules that were not investigated in this study, such as nucleic acids (DNA and RNA) or pigments. In the HNLP chemostat, we found a value slightly above 100% (116%). This was also in accordance with field findings of P-limited, diatom-dominated stations in the North Sea (Grosse et al., 2015, 2017) and suggests that the de-novo synthesis of nucleic acids and pigments may have been low. Additionally, the diatoms also formed sticky aggregates and the subsequent splitting of the cultures into equal parts was difficult, which could have caused an experimental error.

With the exception of the HNLP chemostat, the majority of fixed C was still in the glucose fraction after 24 h (43–69% of C-fixation). AA synthesis was highest in the HNLP chemostat contributing 55% of C-fixation and decreased to values between 6 and 10% of C-fixation in chemostats with DIN:DIP ratios below ≤2. The HNHP chemostat also showed decreased AA synthesis, accounting for 21% of C-fixation (**Figure 2B**).

We investigated whether the contribution of biomolecules to biomass (% of POC concentration) was correlated with their contribution to biosynthesis (% of C-fixation). The contribution of total AA to biomass was not significantly correlated with the contribution of AA to biosynthesis ($R^2 = 0.46$, $n = 7$, n.s.; **Figure 3A**). The data suggest that the light-limited chemostat (HNHP) was an outlier, however. Possibly the N concentration was depleted during the 24 h incubation for the C-fixation measurements, thereby suppressing AA synthesis. Removal of this outlier resulted in a significant correlation between the AA contribution to biomass and to biosynthesis ($R^2 = 0.90$, $n = 6$, $p < 0.01$; **Figure 3A**). Structural FA showed a significant correlation between its contribution to biomass and its contribution to biosynthesis ($R^2 = 0.73$, $n = 7$, $p < 0.05$; **Figure 3B**). By contrast, structural CH did not show a significant correlation ($R^2 = 0.07$, $n = 7$, n.s.; **Figure 3C**). Instead, the contribution of structural CH to biomass did not show much variation (mean ± SD of 16 ± 4%), indicating that a fixed proportion of the phytoplankton biomass was invested in structural CH irrespective of nutrient availability. Storage CH (glucose) and storage FA both showed a significant correlation between their contribution to biomass and their contribution to biosynthesis (glucose: $R^2 = 0.71$, $n = 7$, $p < 0.05$, **Figure 3D**; storage FA: $R^2 = 0.79$, $n = 7$, $p < 0.01$; **Figure 3E**).

Individual Amino Acids

Principle component analysis of the relative contribution of individual AA to total AA concentration and C-fixation rates revealed differences between phytoplankton groups as well as nutrient limitations (**Figure 4**). PCA analysis of AA concentrations indicated that 62% of the variation was explained by the first two axes. The first axis separates the AA lysine, histine, proline, glutamate/glutamine, aspartate/asparagine, and alanine from all others and caused a separation of chemostats dominated by diatoms and cyanobacteria from chemostats dominated by green algae (HNHP, HNMP, **Figure 4A**), demonstrating a phytoplankton group specific separation of AA. The second axis showed that lysine, histine, and proline were associated with the HNHP chemostats, whereas glutamate/glutamine, aspartate/asparagine and alanine were associated with the HNMP chemostat, demonstrating a nutrient specific effect on AA distribution in chemostats with green algae dominance. A nutrient related separation was less distinct in diatom and cyanobacteria dominated chemostats, however, N-limited chemostats seemed to associate with serine and alanine, while the N+P-co-limited MNMP chemostat drifted towards Lys (**Figure 4A**).

A pronounced nutrient specific separation was visible in the AA biosynthesis data (**Figure 4B**). The first two axes in the PCA of AA biosynthesis explained 82% of the variation within the samples. A separation between nutrient limitations was visible, along the first axis. The P-limited chemostats

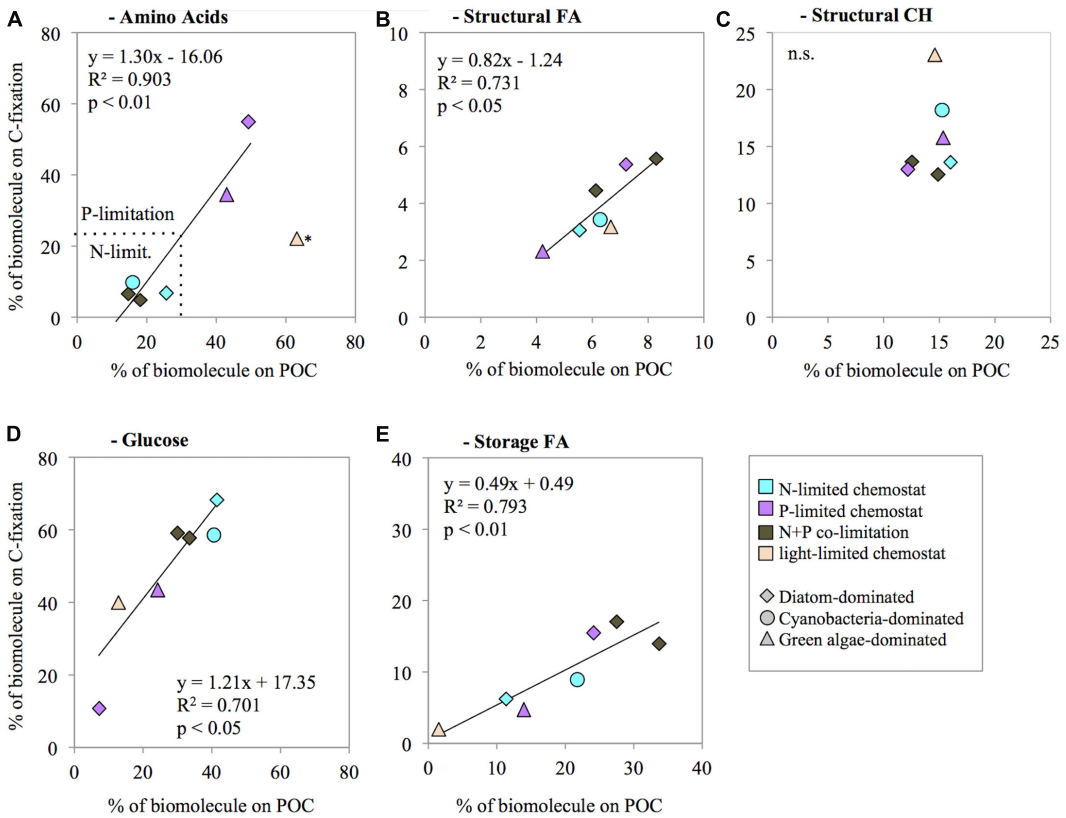

FIGURE 3 | Correlation between the contribution of biomolecules to biomass (% of POC concentration) and their contribution to biosynthesis (% of C-fixation), for amino acids **(A)**, structural fatty acids **(B)**, structural carbohydrates **(C)**, glucose **(D)**, and storage fatty acids **(E)**. The prevailing nutrient limitation is indicated by symbol color, the dominant phytoplankton group is indicated by symbol shape. *the light-limited chemostat was treated as an outlier in panel **A**.

(HNLP, HNMP) were associated with all essential AA and proline, while all other chemostats were associated with all non-essential AA (except proline). A separation along the second axis occurred as well; chemostats with DIN:DIP ratios of 0.04 and 2 (LNHP, MNHP, LNLP) associated with glutamate/glutamine and aspartate/asparagine and chemostats with DIN:DIP ratios of 1 and 6 (MNMP, HNHP) associated with alanine, serine, and glycine.

DISCUSSION

Effects on Phytoplankton Stoichiometry

Although chemostat experiments cannot reproduce the full complexity of marine ecosystems, they provide an excellent tool to study the response of marine phytoplankton to different N and P levels under highly controlled conditions. In our study, four different limitations were encountered among the chemostats (**Figure 1**). Chemostats receiving media with low DIN:DIP ratios became N-limited, whereas chemostats receiving media with high DIN:DIP ratios became P-limited. Chemostats that received DIN:DIP ratios at the optimal Redfield ratio (Redfield et al., 1963) developed a co-limitation by N+P at low and medium DIN and DIP concentrations. The relatively low DIN:DIP ratios in these two chemostats indicate that the N+P co-limitation might be tending somewhat more to N- than to P-limitation. The chemostat that received high DIN and DIP concentrations developed a high biomass that induced light-limitation through self-shading (Brauer et al., 2012).

Phytoplankton PON:POP ratios and POC:POP ratios in the HNMP and HNLP chemostats were very high, supporting the conclusion that these cultures were P-limited. Interestingly, though, storage CH (glucose) and storage FA contents of the phytoplankton were lower in these P-limited chemostats (**Figure 2A**) than in the N+P co-limited chemostat MNMP, which showed similar POC:POP ratios. This may indicate that the high POC:POP ratios under solely P-limited conditions were mainly due to low cellular P contents, while the high POC:POP ratio in the MNMP chemostat was additionally determined by a higher accumulation of C-rich storage compounds. Overall, P-deficient phytoplankton tend to have a low nutritional value for a variety of herbivorous zooplankton (Plath and Boersma, 2001), which can negatively affect growth rates of zooplankton and larvae of fish and shellfish (Malzahn and Boersma, 2012; Schoo et al., 2014) and consequently induces changes in the entire food web (Sterner et al., 1993).

Conversely, PON:POP ratios were lowest in the MNHP and LNHP chemostats but do not point at a severe N limitation, since

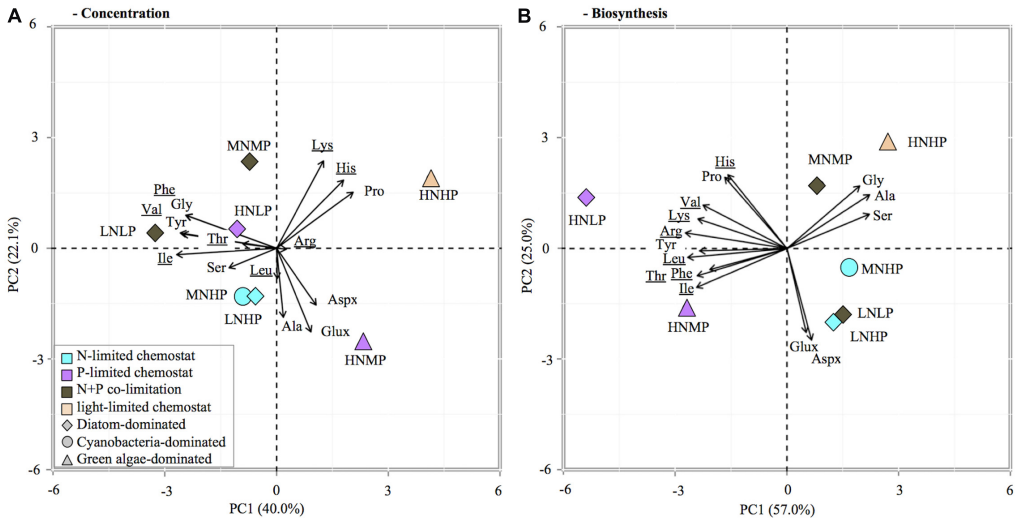

FIGURE 4 | Principle component analysis (PCA) biplot of relative contribution of individual AA to total AA concentration in POC **(A)** and total AA synthesis **(B)**. Symbol color indicates limitation, symbol shape refers to dominant phytoplankton group. The analysis included 15 amino acids: glutamate/glutamine (Glux), aspartate/asparagine (Aspx), threonine (Thr), valine (Val), isoleucine (Ile), leucine (Leu), lysine (Lys), histidine (His), phenylalanine (Phe), argin (Arg), serine (Ser), glycine (Gly), alanine (Ala), proline (Pro), and tyrosine (Tyr).

PON:POP ratios of 18 to 30 can also be found under nutrient replete conditions or in communities transitioning from N to P limitation (Geider and LaRoche, 2002). The depletion of DIN did, however, cause an accumulation of C-rich storage CH (glucose) and storage FA, and increased the POC:PON ratio to values typical for N-limited phytoplankton (Geider and LaRoche, 2002).

Biomolecule Dynamics under Different Resource Limitations

It should be noted that no nutrients were added during the ^{13}C-incubations, which will have resulted in a decrease of available nutrients (compared to chemostat conditions) and may have affected the outcome of these carbon fixation experiments to some extent. While the biomolecule concentration of cultures was determined by the conditions in the chemostats (long-term adaptation), the biomolecule synthesis rates will have been affected immediately by decreasing nutrient availabilities during the ^{13}C-incubations. N-, P-, and N+P co-limited cultures probably became exhausted of DIN and/or DIP. The light limited HNHP culture may have also exhausted the DIN concentration and probably became co-limited by N and light during the incubation experiments. This was most evident in AA biosynthesis rates, which were much lower than expected (see the outlier in **Figure 3A**). AA will be mainly used in protein synthesis, and nitrogen limitation causes changes in the levels of transcription and translation (Yang et al., 2011; Alipanah et al., 2015). Several studies have demonstrated that gene expression, especially of the photosystem and ribosomal genes, starts to change within a few hours after removal or addition of nitrogen (Morey et al., 2011; Krasikov et al., 2012), indicating that AA synthesis may indeed decline rapidly in response to a decrease in N availability.

AA contents and synthesis rates were closely linked to N availability. In particular, in N-limited and N+P co-limited phytoplankton AA contributions to POC and C-fixation were very low, whereas AA contents and synthesis rates were much higher under light-limited and P-limited conditions (**Figure 2**). The reduction of AA synthesis under N-limited conditions appears at odds with model predictions of Klausmeier et al. (2004), where *both* N-limited and P-limited phytoplankton invest in nutrient uptake proteins and hence have relatively high N:P ratios. Instead, our findings are more in agreement with the model of Loladze and Elser (2011), which predicts that N limitation slows down AA synthesis and thereby lowers organismal N:P ratios, whereas P limitation does not constrain AA synthesis and results in high cellular N:P ratios.

The correlation trendline between AA contribution to POC concentrations and AA contribution to C-fixation crosses the x-axis at a value of ∼12% (**Figure 3A**), which can be interpreted as the minimum AA concentration necessary in the POC under N-starvation. In other words, this is the minimum amount of AA needed to maintain cell functions under zero growth. We found slightly higher values in the North Sea, where required minimum concentrations of AA in POC were ∼17% (Grosse et al., 2017). The small difference between our laboratory results and these field observations may have been caused by the contribution of micro- and mesozooplankton and debris in the field, which can also be sources of AA.

Accumulation of storage CH (glucose) showed a pattern opposite to AA synthesis. CH contents were lowest under light-limited and P-limited conditions whereas high levels of storage CH accumulated in N-limited phytoplankton. A similar contrast between AA synthesis and CH accumulation was obtained in short-term experiments with natural phytoplankton during a series of research cruises on the North Sea, where N addition

increased AA synthesis of N-limited phytoplankton within 24 h while CH storage decreased concomitantly (Grosse et al., 2017).

A direct relationship between P availability and rRNA synthesis has been established previously (Hessen et al., 2004; Van Mooy and Devol, 2008) and with evidence of P-limitation becoming more prevalent in coastal seas (Sylvan et al., 2007; Xu et al., 2008; Burson et al., 2016) measurements of nucleic acids concentrations and biosynthesis should be included into future studies of biomolecule dynamics. A method to detect ^{13}C incorporation into DNA and RNA nucleotides recently became available (Moerdijk-Poortvliet et al., 2014). Biomass requirements for this method, however, are much higher than for the biomolecules investigated here and the culture volumes in our chemostats did not allow for the additional sampling of this parameter.

Effects on Amino Acid Composition

The different nutrient treatments in our experiments had considerable effects not only on the AA content, but also on the AA composition of marine phytoplankton. Similar results were found in a recent field study in the North Sea, where N-limited communities were associated especially with glutamate/glutamine and aspartate/asparagine, while P-limited communities were characterized by higher contributions of essential AA (Grosse, 2016). Together, these findings provide an interesting new perspective on individual AA dynamics in the water column. Previous studies on the geochemical composition of particulate organic matter assumed that the AA composition of marine phytoplankton is more or less constant (e.g., Dauwe et al., 1999). In contrast, our lab experiments and recent field study (Grosse, 2016) point at consistent variation in the AA composition of marine phytoplankton depending on the growth conditions.

Proline showed high contributions to the POC concentration in the light limited chemostat (HNHP, Supplementary Table S1). Under N-replete conditions, proline can be used as an osmoprotectant, which is replaced by compounds such as dimethylsulfoniopropionate under N-depleted conditions (Bromke et al., 2013), and only the HNHP chemostat had sufficient DIN available to suggest proline may have been important for osmoregulation. Glutamate/glutamine and aspartate/asparagine are directly synthesized from glycolysis and TCA intermediates, and thereby, constitute precursors for the synthesis of AA with longer synthesis pathways (especially essential AA). They were therefore first affected by changing N availabilities. The other non-essential AA (serine, alanine, and glycine) had higher contribution to C-fixation than they had in biomass (over-synthesis), indicating the transformation into AA "down the line" was not completed after 24 h, which was confirmed by all essential AA showing lower synthesis compared to their percentage contribution in biomass (under-synthesis). The results also showed that the degree of "over-" or "under-synthesis" was greater under N-limitation than under P-limitation, suggesting AA synthesis from non-essential to essential AA occurred slower under N-limitation. For example, the non-essential alanine contributed 12% to total AA synthesis in the two P-limited chemostats, whereas the synthesis contribution increased in N-limited phytoplankton to between 17 and 29% (Supplementary Table S1). Similarly, large differences were found in glutamate/glutamine, serine, glycine, and leucine, while differences in other AA were observed but at a much lower scale. N and P-limitation affected biosynthesis of total AA and in addition turnover times of precursor may be longer because the conversion of non-essential to essential AA relies on numerous additional enzymes, proteins themselves, and their production may be reduced under N-limitation as well.

The distribution of individual FA was also analyzed but we did not find any nutrient-dependent relationships. These were most likely concealed by pronounced differences in FA composition between different phytoplankton groups (Dijkman and Kromkamp, 2006).

CONCLUSION

The chemostat experiments showed that changes in N and P supply lead to substantial changes in the biochemical composition as well as species composition of phytoplankton communities. Although natural phytoplankton communities are clearly more complex than laboratory chemostats, the nutrient-dependent shifts in biomolecule composition and biosynthesis from these simplified chemostat experiments are generally in agreement with results from natural phytoplankton communities in the North Sea (Grosse, 2016; Grosse et al., 2017). In particular, our experimental results show that shifts from N limitation to P limitation, as observed in coastal waters like the North Sea, will not only increase the N:P and C:P stoichiometry of phytoplankton but will also increase their total amino acid content, alter their amino acid composition and reduce their cellular carbohydrate storage.

Future studies may build on this work, by expanding beyond the elemental stoichiometry of phytoplankton to further elucidate the range of adaptations in biochemical composition of different phytoplankton species, and their implications for, e.g., phytoplankton growth rates, DOM production, microbial loop activity, the production of secondary metabolites and the nutritional quality of phytoplankton as food for higher trophic levels.

AUTHOR CONTRIBUTIONS

JG and AB designed research; MS, JH, and HB supervised design and implementation, JG and AB performed research and data analysis; JG, AB, MS, JH, and HB interpreted the data and wrote the paper.

FUNDING

This research is part of the CHARLET project, funded by the Sea and Coastal Research (ZKO) program of the Netherlands Organization for Scientific Research (NWO) to HB (grant ZKO 839.10.511) and JH (grant ZKO 839.10.512).

ACKNOWLEDGMENTS

The authors thank Emma Greenwell and Jessica Koops for their skilled assistance with chemostat maintenance, and the reviewers for their helpful and constructive comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01299/full#supplementary-material

REFERENCES

Alipanah, L., Rohloff, J., Winge, P., Bones, A. M., and Brembu, T. (2015). Whole-cell response to nitrogen deprivation in the diatom *Phaeodactylum tricornutum*. *J. Exp. Bot.* 66, 6281–6296. doi: 10.1093/jxb/erv340

Anderson, T. R., Boersma, M., and Raubenheimer, D. (2004). Stoichiometry: linking elements to biochemicals. *Ecology* 85, 1193–1202. doi: 10.1890/02-0252

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917. doi: 10.1139/o59-099

Boschker, H. T. S., Moerdijk-Poortvliet, T. C. W., van Breugel, P., Houtekamer, M., and Middelburg, J. J. (2008). A versatile method for stable carbon isotope analysis of carbohydrates by high-performance liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* 22, 3902–3908. doi: 10.1002/rcm.3804

Brauer, V. S., Stomp, M., and Huisman, J. (2012). The nutrient-load hypothesis: patterns of resource limitation and community structure driven by competition for nutrients and light. *Am. Nat.* 179, 721–740. doi: 10.1086/665650

Bromke, M. A., Giavalisco, P., Willmitzer, L., and Hesse, H. (2013). Metabolic analysis of adaptation to short-term changes in culture conditions of the marine diatom *Thalassiosira pseudonana*. *PLoS ONE* 8:e67340. doi: 10.1371/journal.pone.0067340

Burson, A., Stomp, M., Akil, L., Brussaard, C. P. D., and Huisman, J. (2016). Unbalanced reduction of nutrient loads has created an offshore gradient from phosphorus to nitrogen limitation in the North Sea. *Limnol. Oceanogr.* 61, 869–888. doi: 10.1002/lno.10257

Cadée, G. C., and Hegeman, J. (2002). Phytoplankton in the Marsdiep at the end of the 20th century; 30 years monitoring biomass, primary production, and *Phaeocystis* blooms. *J. Sea Res.* 48, 97–110. doi: 10.1016/s1385-1101(02)00161-2

Dauwe, B., Middelburg, J. J., Herman, P. M. J., and Heip, C. H. R. (1999). Linking diagenetic alteration of amino acids and bulk organic matter reactivity. *Limnol. Oceanogr.* 44, 1809–1814. doi: 10.4319/lo.1999.44.7.1809

Dijkman, N. A., and Kromkamp, J. C. (2006). Phospholipid-derived fatty acids as chemotaxonomic markers for phytoplankton: application for inferring phytoplankton composition. *Mar. Ecol. Prog. Ser.* 324, 113–125. doi: 10.3354/meps324113

Fink, P., Pflitsch, C., and Marin, K. (2011). Dietary essential amino acids affect the reproduction of the keystone herbivore *Daphnia pulex*. *PLoS ONE* 6:e28498. doi: 10.1371/journal.pone.0028498

Geider, R. J., and LaRoche, J. (2002). Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37, 1–17. doi: 10.1017/S0967026201003456

Glibert, P. M., Maranger, R., Sobota, D. J., and Bouwman, L. (2014). The Haber Bosch–harmful algal bloom (HB–HAB) link. *Environ. Res. Lett.* 9:105001. doi: 10.1088/1748-9326/9/10/105001

Grasshoff, K., Ehrhardt, M., and Kremling, K. (1983). *Methods of Seawater Analysis*, 2nd Edn. Weinheim: Verlag Chemie.

Grizzetti, B., Bouraoui, F., and Aloe, A. (2012). Changes of nitrogen and phosphorus loads to European seas. *Glob. Change Biol.* 18, 769–782. doi: 10.1111/j.1365-2486.2011.02576.x

Grosse, J. (2016). *Resource Limitation and the Biochemical Composition of Marine Phytoplankton*. Ph.D. dissertation, University of Amsterdam, Amsterdam.

Grosse, J., van Breugel, P., and Boschker, H. T. S. (2015). Tracing carbon fixation in phytoplankton-compound specific and total 13C incorporation rates. *Limnol. Oceanogr. Methods* 13, 288–302. doi: 10.1002/lom3.10025

Grosse, J., van Breugel, P., Brussaard, C. P. D., and Boschker, H. T. S. (2017). A biosynthesis view on nutrient stress in coastal phytoplankton. *Limnol. Oceanogr.* 62, 490–506. doi: 10.1002/lno.10439

Hecky, R. E., and Kilham, P. (1988). Nutrient limitation of phytoplankton in freshwater and marine environments - A review of recent evidence on the effects of enrichment. *Limnol. Oceanogr.* 33, 796–822. doi: 10.4319/lo.1988.33.4part2.0796

Heinzelmann, S. M., Bale, N. J., Hopmans, E. C., Damste, J. S. S., Schouten, S., and van der Meer, M. T. J. (2014). Critical assessment of glyco- and phospholipid separation by using silica chromatography. *Appl. Environ. Microbiol.* 80, 360–365. doi: 10.1128/aem.02817-13

Hessen, D. O., Agren, G. I., Anderson, T. R., Elser, J. J., and De Ruiter, P. C. (2004). Carbon sequestration in ecosystems: the role of stoichiometry. *Ecology* 85, 1179–1192. doi: 10.1890/02-0251

Hickel, W., Mangelsdorf, P., and Berg, J. (1993). The human impact in the German Bight: eutrophication during three decades (1962–1991). *Helgol. Meeresuntersuchungen* 47, 243–263. doi: 10.1007/bf02367167

Howarth, R. W., and Marino, R. (2006). Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: evolving views over three decades. *Limnol. Oceanogr.* 51, 364–376. doi: 10.4319/lo.2006.51.1_part_2.0364

Huisman, J., Matthijs, H. C. P., Visser, P. M., Balke, H., Sigon, C. A. M., Passarge, J., et al. (2002). Principles of the light-limited chemostat: theory and ecological applications. *Antonie Van Leeuwenhoek* 81, 117–133. doi: 10.1023/a:1020537928216

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2004). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174. doi: 10.1038/nature02454

Krasikov, V., Aguirre von Wobeser, E., Dekker, H. L., Huisman, J., and Matthijs, H. C. (2012). Time-series resolution of gradual nitrogen starvation and its impact on photosynthesis in the cyanobacterium *Synechocystis* PCC 6803. *Physiol. Plant.* 145, 426–439. doi: 10.1111/j.1399-3054.2012.01585.x

Lancelot, C., Gypens, N., Billen, G., Garnier, J., and Roubeix, V. (2007). Testing an integrated river-ocean mathematical tool for linking marine eutrophication to land use: the *Phaeocystis*-dominated Belgian coastal zone (Southern North Sea) over the past 50 years. *J. Mar. Syst.* 64, 216–228. doi: 10.1016/j.jmarsys.2006.03.010

Lenhart, H.-J., Mills, D. K., Baretta-Bekker, H., Van Leeuwen, S. M., Van Der Molen, J., Baretta, J. W., et al. (2010). Predicting the consequences of nutrient reduction on the eutrophication status of the North Sea. *J. Mar. Syst.* 81, 148–170. doi: 10.1016/j.jmarsys.2009.12.014

Loladze, I., and Elser, J. J. (2011). The origins of the redfield nitrogen-to-phosphorus ratio are in a homoeostatic protein-to-rRNA ratio. *Ecol. Lett.* 14, 244–250. doi: 10.1111/j.1461-0248.2010.01577.x

Malzahn, A. M., Aberle, N., Clemmesen, C., and Boersma, M. (2007). Nutrient limitation of primary producers affects planktivorous fish condition. *Limnol. Oceanogr.* 52, 2062–2071. doi: 10.4319/lo.2007.52.5.2062

Malzahn, A. M., and Boersma, M. (2012). Effects of poor food quality on copepod growth are dose dependent and non-reversible. *Oikos* 121, 1408–1416. doi: 10.1111/j.1600-0706.2011.20186.x

McCullagh, J. S. O., Juchelka, D., and Hedges, R. E. M. (2006). Analysis of amino acid 13C abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* 20, 2761–2768. doi: 10.1002/rcm.2651

Moerdijk-Poortvliet, T. C. W., Brasser, J., de Ruiter, G., Houtekamer, M., Bolhuis, H., Stal, L. J., et al. (2014). A versatile method for simultaneous stable carbon isotope analysis of DNA and RNA nucleotides by liquid chromatography/isotope ratio mass spectrometry. *Rapid commun. Mass Spectrom.* 28, 1401–1411. doi: 10.1002/rcm.6919

Morey, J. S., Monroe, E. A., Kinney, A. L., Beal, M., Johnson, J. G., Hitchcock, G. L., et al. (2011). Transcriptomic response of the red tide dinoflagellate, *Karenia brevis*, to nitrogen and phosphorus depletion and addition. *BMC Genomics* 12:346. doi: 10.1186/1471-2164-12-346

Müller-Navarra, D. C. (1995). Evidence that a highly unsaturated fatty acid limits Daphnia growth in nature. *Arch. Hydrobiol.* 132, 297–307.

Myklestad, S. (2000). "Dissolved organic carbon from phytoplankton," in *Marine Chemistry*, ed. P. J. Wangersky (Berlin: Springer), 111–148.

Nagata, T. (2000). "Production mechanisms of dissolved organic matter," in *Microbial Ecology of the Oceans. Wiley Series in Ecological and Applied Microbiology*, ed. D. L. Kirchmann (New York, NY: Wiley & Sons), 121–152.

Nieuwenhuize, J., and Poley-Vos, C. (1989). A rapid microwave dissolution method for the determination of trace and minor elements in lyophilized plant material. *At. spectrosc.* 10, 148–153.

OSPAR (1988). *PARCOM recommendation 88/2: On the Reduction in Nutrients to the Paris Convention Area*. Publication number 88/2. London: OSPAR Commission.

OSPAR (2010). *Quality Status Report 2010*. London: OSPAR Commission, 176.

Passy, P., Gypens, N., Billen, G., Garnier, J., Thieu, V., Rousseau, V., et al. (2013). A model reconstruction of riverine nutrient fluxes and eutrophication in the Belgian Coastal Zone since 1984. *J. Mar. Syst.* 128, 106–122. doi: 10.1016/j.jmarsys.2013.05.005

Persson, J., Fink, P., Goto, A., Hood, J. M., Jonas, J., and Kato, S. (2010). To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos* 119, 741–751. doi: 10.1111/j.1600-0706.2009.18545.x

Philippart, C. J. M., Cadee, G. C., van Raaphorst, W., and Riegman, R. (2000). Long-term phytoplankton-nutrient interactions in a shallow coastal sea: algal community structure, nutrient budgets, and denitrification potential. *Limnol. Oceanogr.* 45, 131–144. doi: 10.4319/lo.2000.45.1.0131

Plath, K., and Boersma, M. (2001). Mineral limitation of zooplankton: stoichiometric constraints and optimal foraging. *Ecology* 82, 1260–1269. doi: 10.1890/0012-9658(2001)082[1260:MLOZSC]2.0.CO;2

Radach, G., and Pätsch, J. (2007). Variability of continental riverine freshwater and nutrient inputs into the North Sea for the years 1977-2000 and its consequences for the assessment of eutrophication. *Estuaries Coasts* 30, 66–81. doi: 10.1007/BF02782968

Raubenheimer, D., Simpson, S. J., and Mayntz, D. (2009). Nutrition, ecology and nutritional ecology: toward an integrated framework. *Funct. Ecol.* 23, 4–16. doi: 10.1111/j.1365-2435.2009.01522.x

Redfield, A. C., Ketchum, B. A., and Richards, F. A. (1963). "The influence of organisms on the composition of sea-water," in *The Sea*, ed. M. N. Hill (New York, NY: John Wiley), 26–77.

Riegman, R., Noordeloos, A. M., and Cadée, G. (1992). Phaeocystis blooms and eutrophication of the continental coastal zones of the North Sea. *Mar. Biol.* 112, 479–484. doi: 10.1007/BF00356293

Schoo, K. L., Aberle, N., Malzahn, A. M., Schmalenbach, I., and Boersma, M. (2014). The reaction of European lobster larvae (*Homarus gammarus*) to different quality food: effects of ontogenetic shifts and pre-feeding history. *Oecologia* 174, 581–594. doi: 10.1007/s00442-013-2786-5

Schoo, K. L., Malzahn, A. M., Krause, E., and Boersma, M. (2013). Increased carbon dioxide availability alters phytoplankton stoichiometry and affects carbon cycling and growth of a marine planktonic herbivore. *Mar. Biol.* 160, 2145–2155. doi: 10.1007/s00227-012-2121-4

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Sterner, R. W., Hagemeier, D. D., and Smith, W. L. (1993). Phytoplankton nutrient limitation and food quality for *Daphnia*. *Limnol. Oceanogr.* 38, 857–871. doi: 10.4319/lo.1993.38.4.0857

Sylvan, J. B., Quigg, A., Tozzi, S., and Ammerman, J. W. (2007). Eutrophication-induced phosphorus limitation in the Mississippi River plume: evidence from fast repetition rate fluorometry. *Limnol. Oceanogr.* 52, 2679–2685. doi: 10.4319/lo.2007.52.6.2679

Thieu, V., Garnier, J., and Billen, G. (2010). Assessing the effect of nutrient mitigation measures in the watersheds of the Southern Bight of the North Sea. *Sci. Total Environ.* 408, 1245–1255. doi: 10.1016/j.scitotenv.2009.12.031

Turner, R. E., Rabalais, N. N., Justic, D., and Dortch, Q. (2003). Global patterns of dissolved N, P and Si in large rivers. *Biogeochemistry* 64, 297–317. doi: 10.1023/a:1024960007569

Van Beusekom, J. E. E., and Diel-Christiansen, S. (2009). Global change and the biogeochemistry of the North Sea: the possible role of phytoplankton and phytoplankton grazing. *Int. J. Earth Sci.* 98, 269–280. doi: 10.1007/s00531-007-0233-8

Van Mooy, B. A. S., and Devol, A. H. (2008). Assessing nutrient limitation of *Prochlorococcus* in the North Pacific subtropical gyre by using an RNA capture method. *Limnol. Oceanogr.* 53, 78–88. doi: 10.4319/lo.2008.53.1.0078

Veuger, B., van Oevelen, D., and Middelburg, J. J. (2012). Fate of microbial nitrogen, carbon, hydrolysable amino acids, monosaccharides, and fatty acids in sediment. *Geochim. Cosmochim. Acta* 83, 217–233. doi: 10.1016/j.gca.2011.12.016

Vrede, T., Dobberfuhl, D. R., Kooijman, S., and Elser, J. J. (2004). Fundamental connections among organism C:N:P stoichiometry, macromolecular composition, and growth. *Ecology* 85, 1217–1229. doi: 10.1890/02-0249

Westernhagen, H. V., and Dethlefsen, V. (1983). North Sea oxygen deficiency 1982 and its effects on the bottom fauna. *Ambio* 12, 264–266.

Xu, J., Yin, K., He, L., Yuan, X., Ho, A. Y. T., and Harrison, P. J. (2008). Phosphorus limitation in the northern South China Sea during late summer: influence of the Pearl River. *Deep Sea Res. I* 55, 1330–1342. doi: 10.1016/j.dsr.2008.05.007

Yang, I., Beszteri, S., Tillmann, U., Cembella, A., and John, U. (2011). Growth- and nutrient-dependent gene expression in the toxigenic marine dinoflagellate *Alexandrium minutum*. *Harmful Algae* 12, 55–69. doi: 10.1016/j.hal.2011.08.012

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

PERSPECTIVE
published: 11 September 2017
doi: 10.3389/fmicb.2017.01742

Toward an Ecologically Optimized N:P Recovery from Wastewater by Microalgae

Tânia V. Fernandes, María Suárez-Muñoz, Lukas M. Trebuch, Paul J. Verbraak and Dedmer B. Van de Waal*

Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands

Global stores of important resources such as phosphorus (P) are being rapidly depleted, while the excessive use of nutrients has led to the enrichment of surface waters worldwide. Ideally, nutrients would be recovered from wastewater, which will not only prevent eutrophication but also provide access to alternative nutrient stores. Current state-of-the-art wastewater treatment technologies are effective in removing these nutrients from wastewater, yet they can only recover P and often in an insufficient way. Microalgae, however, can effectively assimilate P and nitrogen (N), as well as other macro- and micronutrients, allowing these nutrients to be recovered into valuable products that can be used to close nutrient cycles (e.g., fertilizer, bioplastics, color dyes, and bulk chemicals). Here, we show that the green alga *Chlorella sorokiniana* is able to remove all inorganic N and P present in concentrated toilet wastewater (i.e., black water) with N:P ratios ranging between 15 and 26. However, the N and P uptake by the algae is imbalanced relative to the wastewater N:P stoichiometry, resulting in a rapid removal of P but relatively slower removal of N. Here, we discuss how ecological principles such as ecological stoichiometry and resource-ratio theory may help optimize N:P removal and allow for more effective recovery of N and P from black water.

Keywords: decentralized black water treatment, algal photobioreactor, nutrient removal, *Chlorella*, nitrogen, phosphorus

OPEN ACCESS

Edited by:
Télesphore Sime-Ngando, Centre National de la Recherche Scientifique, France

Reviewed by:
Anja Engel, GEOMAR Helmholtz Centre for Ocean Research Kiel (HZ), Germany
William Sunda, University of North Carolina at Chapel Hill, United States

****Correspondence:***
Tânia V. Fernandes
t.fernandes@nioo.knaw.nl

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *28 February 2017*
Accepted: *28 August 2017*
Published: *11 September 2017*

Citation:
Fernandes TV, Suárez-Muñoz M, Trebuch LM, Verbraak PJ and Van de Waal DB (2017) Toward an Ecologically Optimized N:P Recovery from Wastewater by Microalgae. Front. Microbiol. 8:1742. doi: 10.3389/fmicb.2017.01742

INTRODUCTION

Welcome to the world of tomorrow, where waste no longer exists. In this world waste is converted into resources that can be reused. Today, essential resources are being depleted, literally flushed down our toilets. Among the main examples is phosphorus (P), which is a major element in life for it is involved in energy transfer (ATP), cellular structures (phospholipids), and storage and transfer of genetic information (DNA/RNA) (Sterner and Elser, 2002). Human waste contributes to 68% of the total P present in domestic wastewater (Kujawa-Roeleveld and Zeeman, 2006). If we were able to recover that P, human excreta could supply 22% of the global P demand (Mihelcic et al., 2011). This is particularly important because global reserves of P are becoming increasingly scarce, expensive, and unevenly distributed, which will have important societal consequences (Elser and Bennett, 2011; Cordell and White, 2014). Human waste contains between 1.8 and 10 g P kg^{-1} (Roy, 2017), but also many valuable macro- and micronutrients. Recovery of these elements alongside P may improve fertilizer quality (de Graaff et al., 2011).

Current wastewater treatment plants can effectively remove nitrogen (N) and P from wastewater, therefore preventing the enrichment of surface waters with nutrients. Despite this removal, actual recovery of increasingly important resources, such as P, is practically non-existent. In the few cases that P is recovered, as for example by struvite precipitation, this is often inefficient (Hao et al., 2013; Egle et al., 2016). Microalgae can effectively assimilate P, but also N, and other macro-/micronutrients that are present in the wastewater, allowing these nutrients to be utilized for the production of valuable products such as biofuels, bioplastics, dyes, and bulk chemicals (Wijffels and Barbosa, 2010; Wijffels et al., 2010; Zeller et al., 2013; Suganya et al., 2016). Microalgae can also be used as an enriched fertilizer, as they include a wide range of micronutrients (Mg, Fe, Co, etc.). These micronutrients are often missing in commonly used artificial fertilizers, leading to nutrient depletion of agricultural soils (Udo de Haes et al., 2012). Moreover, microalgae fertilizer can improve soil structure and water retention capacity (Metting, 1990; Maurya et al., 2015).

Wastewater N:P ratios depend on the origin of the wastewater. Municipal wastewater, which includes domestic wastewater with minor contributions of industrial wastewater (common in industrialized countries), have N:P ratios of approximately 10 (Henze and Comeau, 2008). Wastewater with animal manure and human excreta can reach N:P ratios up to 40 (Kumar et al., 2010; Tuantet et al., 2013). In concentrated toilet wastewater (i.e., black water), the N:P ratio usually varies from 20 to 30 (Vasconcelos Fernandes et al., 2015). Earlier work has demonstrated how the microalga *Chlorella sorokiniana* can fully remove N and P from black water with 76 mmol NH_4^+ L^{-1} and 3.4 mmol PO_4^{3-}, yielding an N:P ratio of 22. All P was removed from the black water in 4 days, but another 8 days were needed for all N to be removed (Vasconcelos Fernandes et al., 2015). This may be due to the high N:P ratio of the black water, as well as due to a low N relative to P removal and recovery by the microalgae. Here, we first tested how black water N:P stoichiometry can affect N:P removal and recovery ratios by the green alga *C. sorokiniana*. Additionally, we highlight how microalgae may optimize nutrient removal from wastewater by applying the ecological principles of ecological stoichiometry (Sterner and Elser, 2002) and resource-ratio theory (Tilman, 1982).

MATERIALS AND METHODS

Chlorella sorokiniana was cultivated in anaerobically treated black water (AnBW) with different initial N:P ratios (15, 17, 20, 23, and 26). The lower N:P ratios were achieved by addition of PO_4^{3-}. AnBW was collected from an upflow anaerobic sludge blanket (UASB) reactor fed with vacuum collected black water and operated at 35°C and a hydraulic retention time of 8 days. AnBW was thermally pre-treated at 55°C for 4 days to eliminate the interference of bacteria on C, N, and P uptake. The inoculum density of the experimental cultures was 0.5 g dry weight L^{-1}. The experiment was performed in 400 mL flat panel photobioreactors (PBRs) with a light path of 14 mm. The PBRs were illuminated with a maximum incident light intensity of 1,500 μmol photons m^{-2} s^{-1}, an average *in situ* light intensity of 945 μmol photons m^{-2} s^{-1}, and a light:dark cycle of 16 h:8 h. Temperature was set at 37°C and controlled by a water jacket placed between the light source and bioreactor. The pH was kept at 6.8 ± 0.1 by automated acid/base additions to prevent the conversion of ammonium ions (NH_4^+) to gaseous ammonia (NH_3), which is harmful to green microalgae (Abeliovich, 2005). The culture was mixed by aeration with compressed air (enriched with 10% CO_2) at a flow of 400 mL min^{-1}. Daily samples were analyzed for biomass and dissolved inorganic nutrients. Particulate C, N, and P were assessed at day 4 and at the end of each treatment (between days 9 and 18). Biomass was determined by dry weight. NH_4^+ and PO_4^{3-} were analyzed by a Seal QuAAtro Auto Analyzer (Seal Analytical Inc., Netherlands). Particulate N and C were measured on a FLASH 2000 NC Elemental Analyzer (Brechbuhler Incorporated, Interscience B.V., Netherlands). Particulate P was first combusted at 550°C for 30 min, then digested with persulfate (2.5%) at 121°C for 30 min, and subsequently analyzed on the Seal QuAAtro Auto Analyzer. The ratio at which N and P were removed from the black water was calculated from the linear relation between dissolved inorganic N and P concentrations over the first 4 days of the experiment.

RESULTS AND DISCUSSION

Algal biomass increased similarly in the different black water N:P ratio treatments, reaching stationary phase in about 10 days (**Figure 1A**). Almost all dissolved inorganic N and P were removed via algal assimilation, showing similar dynamics irrespective of initial wastewater N:P ratios, with full removal of P within 4 days and subsequent removal of remaining N in another 5–10 days (**Figure 1B** and Supplementary Figure 1). The N:P removal ratio, i.e., the ratio at which N and P were removed from the black water, was about 13 during the first 4 days of each treatment (i.e., the P replete phase), and lower than the initial black water N:P (**Figure 1C**). The N:P recovery, i.e., the cellular N:P of *Chlorella*, largely followed the N:P removal during the initial 4 days (see circles in **Figure 1D**). During the subsequent P deplete phase, algae continued to grow for another 3–6 days, which lead to increased N:P ratios at the end of each treatment compared to day 4 (compare triangles to circles in **Figure 1D**). An increase in cellular N:P as well as C:P ratios upon P limitation is commonly observed in phytoplankton (Klausmeier et al., 2004a; Hillebrand et al., 2013), and is caused by a continued uptake and assimilation of N and C after P is limited (Sterner and Elser, 2002; Supplementary Figure 2).

Our results show that both the initial N:P removal and N:P recovery ratio are independent of the initial black water N:P ratios, and demonstrate further cellular uptake and removal of N after P has been depleted from the medium. Overall, the observed biomass increase and nutrient removal from black water were very effective, with a final mean biomass yield of 12 g dry weight L^{-1} (**Figure 1A**), final mean biomass C:N:P ratio of 125:14:1, and mean biomass yield per light photon of 0.20 g dry weight $(mol\ photon)^{-1}$. The recovery efficiencies of

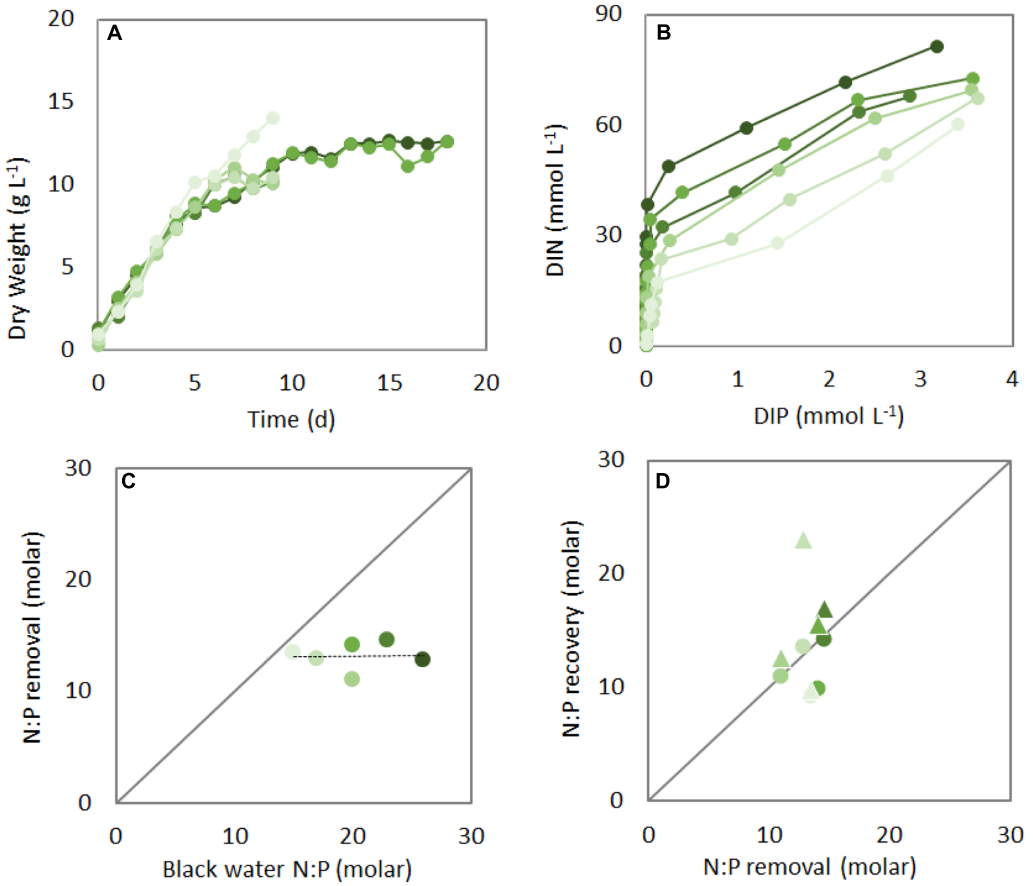

FIGURE 1 | Dynamics of biomass **(A)**, dissolved inorganic nitrogen (DIN) and phosphorus (DIP) **(B)**, N:P removal **(C)**, and N:P recovery **(D)** by *Chlorella sorokiniana* at different initial black water N:P ratios (green shades, darker colors represent higher N:P). Symbols in **(A)** and **(B)** indicate daily measurements. In **(B)** the highest N and P concentrations of the beginning of the experiment are at the top right corner. Symbols in **(C)** indicate the integrated N:P removal rates during the first 4 days of each experiment, and circles and triangles in **(D)** show the cellular N:P after the first 4 days and the end of the experiment, respectively.

N and P from the black water by algal assimilation were 75 and 100%, respectively. The lower recovery of N is presumably due to volatilization of N as NH_3 and N_2O (Fagerstone et al., 2011; Mezzari et al., 2013) and release of dissolved organic nitrogen compounds by the microalgae (Sipler and Bronk, 2015). Thus, all P and most of the N from the wastewater were converted into algal biomass.

The average nutrient removal rates over the period of P-sufficiency to P-depletion (i.e., day 0 to day 4) were 7.6 mmol N L^{-1} d^{-1} and 0.9 mmol P L^{-1} d^{-1}, respectively. Compared to an earlier study with continuous light, the observed yield of biomass on light, N removal rates, and P removal rates increased by about 25, 34, and 81%, respectively. This suggests that despite the high microalgal biomass and associated self-shading, the availability of light was not limiting and a shorter light:dark cycle even promoted a higher biomass yield on light. These findings support the application of microalgae for wastewater treatment using natural light with day:night cycling. The delayed removal of N relative to P, however, imposes a great challenge to the application of microalgae for treatment of wastewater with high N:P ratios. For instance, on a small-scale decentralized black water treatment system for ∼250 people where only 1 L of flush water is used, a 5-day delayed N removal is associated with a black water accumulation of 2–10 m^3 for each treatment cycle (depending on toilet usage – office or household). In practical terms, such a delay would lead to the need of a large AnBW storage capacity, which adds complexity to the treatment system. Thus, higher N:P removal and consequently N:P recovery ratios are essential for a more effective use of microalgae in wastewater treatment.

Previous studies have shown how ecological approaches (e.g., complementarity in resource use) may support industrial scale biomass, crop, and diesel production (Smith et al., 2010; Stockenreiter et al., 2012; Shurin et al., 2013). Comparable principles will apply to the removal and recovery of nutrients from wastewater. The rate at which nutrients can be removed and recovered from the wastewater is directly associated to key physiological traits of alga species, such as growth rate and nutrient demands. Higher growth rates are generally associated with higher nutrient uptake rates, and thereby result in faster removal of nutrients from wastewater. Yet, algal species with

high growth rates may also have higher P demands, and thereby lower N:P ratios (Geider and LaRoche, 2002; but see Flynn et al., 2010). Thus, although selection of single species with higher growth rates may enhance the overall removal rate of nutrients, it may not enhance the N:P removal ratio. On the other hand, selection of a single species with N:P uptake ratios resembling the N:P of the wastewater may accelerate the combined removal of N and P (**Figure 2A**). In our study, the cellular microalgal N:P ratio was 13 during the P replete phase of the experiment, which was lower compared to the black water. Phytoplankton species with higher optimal N:P ratios [i.e., N:P ratios under nutrient sufficient maximum growth rates (Klausmeier et al., 2004b)] should lead to higher N:P removal ratios and overall N removal rates. The black water N:P ratios are at the higher end of reported optimal phytoplankton N:P ratios (Geider and LaRoche, 2002), and are found in some species of cyanobacteria and green algae (Hillebrand et al., 2013). Thus, further screening of particularly these groups may provide the best chances at finding species with N:P ratios resembling that of black water and thus optimizing N:P removal and recovery from wastewater (**Figure 2A**).

Besides optimized N:P removal by selection of a single species, enhanced N:P removal and N:P recovery ratios could also be achieved using phytoplankton communities, where each species has a complementary N:P uptake ratio. In competition for two nutrients at steady state in a continuous culture system (e.g., a chemostat), trade-offs may lead to stable coexistence of two species, where both resources are depleted to the minimum requirements of either species (Tilman, 1982). This minimum required is indicated by R^*, and gives the amount of a resource where growth and losses are balanced (i.e., zero-net-growth-isoclines, **Figure 2B**). Thus, growth will be positive (biomass will increase) at concentrations above the R^* value, while growth will be negative (biomass will decrease) at concentrations below R^*. The R^* for a nutrient will generally be lower for species with a high affinity (i.e., a low half-saturation constant $K_{1/2}$) and high uptake rate for a particular nutrient (Litchman et al., 2007). For instance, if one species has a high affinity and uptake rate for N, it will likely have a low R^* for N as well, and will be a better competitor for N. A trade-off between competitive abilities for N and P between different species may facilitate coexistence at intermediate nutrient supply ratios, where neither species can competitively exclude the other (**Figure 2B**).

The chance for coexistence, and thereby optimized removal of both N and P, will increase with the number of species exhibiting trade-offs between the competitive abilities for N and P. Indeed, although mixtures of multiple algal species showed differential effects on algal production (Schmidtke et al., 2010; Shurin et al., 2013, 2014), enhanced diversity did show reduced residual concentrations of nutrients in various systems (Cardinale et al., 2006; Shurin et al., 2013) and may support higher nutrient use efficiencies (Ptacnik et al., 2008), and overall nutrient uptake rates (Cardinale, 2011). Thus, an enhanced functional trait diversity in algal mixtures, and thereby a higher complementarity in nutrient use, may favor the increased removal of nutrients compared to single algal cultures.

Mixtures of species with complementary light harvesting strategies may allow a more effective use of the light spectrum. For instance, green and red cyanobacterial species were shown to coexist in white-light (Stomp et al., 2004). Thus, co-culturing of distinct green algae and cyanobacteria together with red algal species (e.g., *Haematococcus*) would likely enhance the N:P removal and N:P recovery ratio, and further increase the biomass yield from wastewater. Moreover, a higher algal diversity may enhance the resilience of a system against variations in growth conditions, top-down control, and pathogen infections (Shurin et al., 2013).

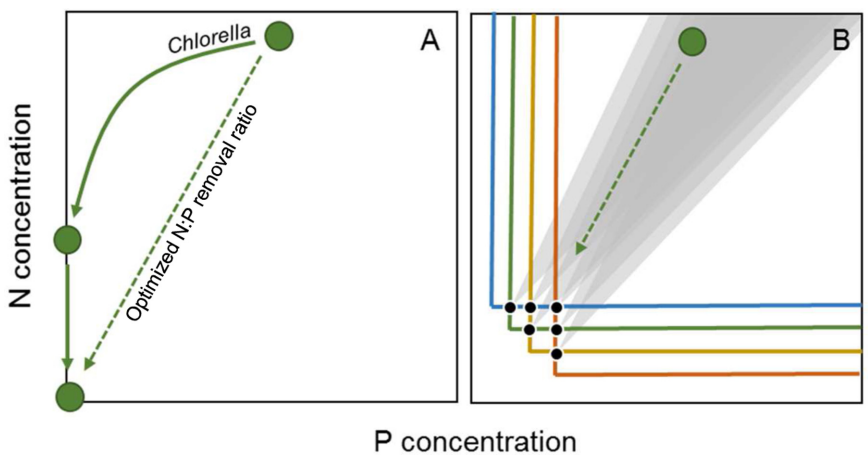

FIGURE 2 | Schematic representation of current N and P removal with *Chlorella* **(A)** and expected removal with multiple species in a continuous culture system **(B)**, where horizontal and vertical lines indicate zero net growth isoclines or R^* values, with colors denoting different species and the black circles indicating equilibrium points where two species can coexist. The gray areas in **(B)** indicate hypothesized ranges in consumption vectors, or N:P uptake ratios, of species coexisting at the associated equilibrium point. The diagonal dashed arrows indicate an optimized higher N:P removal ratio.

Application of ecological principles for technological microalgal application is still in its infancy, particularly for wastewater treatment. Here, we highlighted how our understanding of trade-offs and complementarity in resource acquisition and demands may support optimized N:P removal ratios and ultimately greater recovery of nutrients from wastewater. Connecting wastewater nutrient reuse to product cycles will support a more sustainable future, where a full understanding of phytoplankton eco-physiology provides an overarching guide to effective nutrient removal, recovery, and the production of valuable algal biomass-based products from wastewater.

AUTHOR CONTRIBUTIONS

TF, MS-M, and DW did the conception of the work; LT and PV performed the experimental work; TF supervised the experimental work; TF and DW wrote a first draft of the manuscript, which was revised by all co-authors.

FUNDING

This work was funded by NWO (Netherlands) and FAPESP (Brazil) Joint Research Projects Biobased Economy, project "Recovering nutrients and carbon from concentrated black water" (project number 729.004.004).

ACKNOWLEDGMENTS

We thank Nico Helmsing and Suzanne Wiezer for the technical support. We are grateful to Ted Harris for his comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01742/full#supplementary-material

REFERENCES

Abeliovich, A. (2005). *Handbook of Microalgal Culture, Biotechnology and Applied Phycology*. Oxford: Blackwell Publishing.

Cardinale, B. J. (2011). Biodiversity improves water quality through niche partitioning. *Nature* 472, 86–89. doi: 10.1038/nature09904

Cardinale, B. J., Srivastava, D. S., Emmett Duffy, J., Wright, J. P., Downing, A. L., Sankaran, M., et al. (2006). Effects of biodiversity on the functioning of trophic groups and ecosystems. *Nature* 443, 989–992. doi: 10.1038/nature05202

Cordell, D., and White, S. (2014). Life's bottleneck: sustaining the world's phosphorus for a food secure future. *Annu. Rev. Environ. Resour.* 39, 161–188. doi: 10.1146/annurev-environ-010213-113300

de Graaff, M. S., Temmink, H., Zeeman, G., and Buisman, C. J. N. (2011). Energy and phosphorus recovery from black water. *Water Sci. Technol.* 63, 2759–2765. doi: 10.2166/wst.2011.558

Egle, L., Rechberger, H., Krampe, J., and Zessner, M. (2016). Phosphorus recovery from municipal wastewater: an integrated comparative technological, environmental and economic assessment of P recovery technologies. *Sci. Total Environ.* 571, 522–542. doi: 10.1016/j.scitotenv.2016.07.019

Elser, J., and Bennett, E. (2011). Phosphorus cycle: a broken biogeochemical cycle. *Nature* 478, 29–31. doi: 10.1038/478029a

Fagerstone, K. D., Quinn, J. C., Bradley, T. H., De Long, S. K., and Marchese, A. J. (2011). Quantitative measurement of direct nitrous oxide emissions from microalgae cultivation. *Environ. Sci. Technol.* 45, 9449–9456. doi: 10.1021/es202573f

Flynn, K. J., Raven, J. A., Rees, T. A. V., Finkel, Z., Quigg, A., and Beardall, J. (2010). Is the growth rate hypothesis applicable to microalgae? *J. Phycol.* 46, 1–12. doi: 10.1111/j.1529-8817.2009.00756.x

Geider, R. J., and LaRoche, J. (2002). Redfield revisited: variability of C[ratio]N[ratio]P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* 37, 1–17. doi: 10.1017/S0967026201003456

Hao, X., Wang, C., van Loosdrecht, M. C. M., and Hu, Y. (2013). Looking beyond struvite for P-Recovery. *Environ. Sci. Technol.* 47, 4965–4966. doi: 10.1021/es401140s

Henze, M., and Comeau, Y. (2008). "Wastewater characterization," in *Biological Wastewater Treatment. Principles, Modeling and Design*, eds M. Henze, M. C. M. Van Loosdrecht, G. A. Ekama, and D. Brdjanovic (London: IWA publishing), 33.

Hillebrand, H., Steinert, G., Boersma, M., Malzahn, A., Meunier, C. L., Plum, C., et al. (2013). Goldman revisited: faster-growing phytoplankton has lower N : P and lower stoichiometric flexibility. *Limnol. Oceanogr.* 58, 2076–2088. doi: 10.4319/lo.2013.58.6.2076

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2004a). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174.

Klausmeier, C. A., Litchman, E., and Levin, S. A. (2004b). Phytoplankton growth and stoichiometry under multiple nutrient limitation. *Limnol. Oceanogr.* 49, 1463–1470. doi: 10.4319/lo.2004.49.4_part_2.1463

Kujawa-Roeleveld, K., and Zeeman, G. (2006). Anaerobic treatment in decentralised and source-separation-based sanitation concepts. *Rev. Environ. Sci. Biotechnol.* 5, 115–139. doi: 10.1007/s11157-005-5789-9

Kumar, M. S., Miao, Z. H., and Wyatt, S. K. (2010). Influence of nutrient loads, feeding frequency and inoculum source on growth of *Chlorella vulgaris* in digested piggery effluent culture medium. *Bioresour. Technol.* 101, 6012–6018. doi: 10.1016/j.biortech.2010.02.080

Litchman, E., Klausmeier, C. A., Schofield, O. M., and Falkowski, P. G. (2007). The role of functional traits and trade-offs in structuring phytoplankton communities: scaling from cellular to ecosystem level. *Ecol. Lett.* 10, 1170–1181. doi: 10.1111/j.1461-0248.2007.01117.x

Maurya, R., Chokshi, K., Ghosh, T., Trivedi, K., Pancha, I., Kubavat, D., et al. (2015). Lipid extracted microalgal biomass residue as a fertilizer substitute for *Zea mays* L. *Front. Plant Sci.* 6:1266. doi: 10.3389/fpls.2015.01266

Metting, B. (1990). Microalgae applications in agriculture. *Dev. Ind. Microbiol.* 31, 265–270.

Mezzari, M. P., da Silva, M. L. B., Nicoloso, R. S., Ibelli, A. M. G., Bortoli, M., Viancelli, A., et al. (2013). Assessment of N2O emission from a photobioreactor treating ammonia-rich swine wastewater digestate. *Bioresour. Technol.* 149, 327–332. doi: 10.1016/j.biortech.2013.09.065

Mihelcic, J. R., Fry, L. M., and Shaw, R. (2011). Global potential of phosphorus recovery from human urine and feces. *Chemosphere* 84, 832–839. doi: 10.1016/j.chemosphere.2011.02.046

Ptacnik, R., Solimini, A. G., Andersen, T., Tamminen, T., Brettum, P., Lepistö, L., et al. (2008). Diversity predicts stability and resource use efficiency in natural phytoplankton communities. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5134–5138. doi: 10.1073/pnas.0708328105

Roy, E. D. (2017). Phosphorus recovery and recycling with ecological engineering: a review. *Ecol. Eng.* 98, 213–227. doi: 10.1016/j.ecoleng.2016.10.076

Schmidtke, A., Gaedke, U., and Weithoff, G. (2010). A mechanistic basis for underyielding in phytoplankton communities. *Ecology* 91, 212–221. doi: 10.1890/08-2370.1

Shurin, J. B., Abbott, R. L., Deal, M. S., Kwan, G. T., Litchman, E., McBride, R. C., et al. (2013). Industrial-strength ecology: trade-offs and opportunities in algal biofuel production. *Ecol. Lett.* 16, 1393–1404. doi: 10.1111/ele.12176

Shurin, J. B., Mandal, S., and Abbott, R. L. (2014). Trait diversity enhances yield in algal biofuel assemblages. *J. Appl. Ecol.* 51, 603–611. doi: 10.1111/1365-2664.12242

Sipler, R. E., and Bronk, D. A. (2015). "Dynamics of dissolved organic nitrogen," in *Biochemistry of Marine Dissolved Oganic Matter*, eds D. A. Hansell and C. A. Carlson (Oxford: Elsevier).

Smith, V. H., Sturm, B. S. M., deNoyelles, F. J., and Billings, S. A. (2010). The ecology of algal biodiesel production. *Trends Ecol. Evol.* 25, 301–309. doi: 10.1016/j.tree.2009.11.007

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Stockenreiter, M., Graber, A.-K., Haupt, F., and Stibor, H. (2012). The effect of species diversity on lipid production by micro-algal communities. *J. Appl. Phycol.* 24, 45–54. doi: 10.1007/s10811-010-9644-1

Stomp, M., Huisman, J., de Jongh, F., Veraart, A. J., Gerla, D., Rijkeboer, M., et al. (2004). Adaptive divergence in pigment composition promotes phytoplankton biodiversity. *Nature* 432, 104–107. doi: 10.1038/nature03044

Suganya, T., Varman, M., Masjuki, H. H., and Renganathan, S. (2016). Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: a biorefinery approach. *Renew. Sustain. Energy Rev.* 55, 909–941. doi: 10.1016/j.rser.2015.11.026

Tilman, D. (1982). Resource competition and community structure. *Monogr. Popul. Biol.* 17, 1–296.

Tuantet, K., Janssen, M., Temmink, H., Zeeman, G., Wijffels, R., and Buisman, C. N. (2013). Microalgae growth on concentrated human urine. *J. Appl. Phycol.* 26, 287–297. doi: 10.1016/j.jenvman.2014.06.013

Udo de Haes, H. A., Voortman, R. L., Bastein, T., Bussink, W., Rougoor, C. W., and Weijden, W. J. V. D. (2012). *Scarcity of Micronutrients in Soil, Feed, Food, and Mineral Reserves – Urgency and Policy Options*. Culemborg: Platform Agriculture, Innovation & Society.

Vasconcelos Fernandes, T., Shrestha, R., Sui, Y., Papini, G., Zeeman, G., Vet, L. E. M., et al. (2015). Closing domestic nutrient cycles using microalgae. *Environ. Sci. Technol.* 49, 12450–12456. doi: 10.1021/acs.est.5b02858

Wijffels, R. H., and Barbosa, M. J. (2010). An outlook on microalgal biofuels. *Science* 329, 796–799. doi: 10.1126/science.1189003

Wijffels, R. H., Barbosa, M. J., and Eppink, M. H. M. (2010). Microalgae for the production of bulk chemicals and biofuels. *Biofuels Bioproducts Biorefining* 4, 287–295. doi: 10.1002/bbb.215

Zeller, M. A., Hunt, R., Jones, A., and Sharma, S. (2013). Bioplastics and their thermoplastic blends from Spirulina and Chlorella microalgae. *J. Appl. Polym. Sci.* 130, 3263–3275. doi: 10.1002/app.39559

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 18 April 2017
doi: 10.3389/fenvs.2017.00014

Does the Growth Rate Hypothesis Apply across Temperatures? Variation in the Growth Rate and Body Phosphorus of Neotropical Benthic Grazers

Eric K. Moody[1], Amanda T. Rugenski[1], John L. Sabo[1], Benjamin L. Turner[2] and James J. Elser[1,3]*

[1] School of Life Sciences, Arizona State University, Tempe, AZ, USA, [2] Smithsonian Tropical Research Institute, Ancón, Panama, [3] Flathead Lake Biological Station, University of Montana, Polson, MT, USA

The growth rate hypothesis predicts that organisms with higher maximum growth rates will also have higher body percent phosphorus (P) due to the increased demand for ribosomal RNA production needed to sustain rapid growth. However, this hypothesis was formulated for invertebrates growing at the same temperature. Within a biologically relevant temperature range, increased temperatures can lead to more rapid growth, suggesting that organisms in warmer environments might also contain more P per gram of dry mass. However, since higher growth rates at higher temperature can be supported by more rapid protein synthesis per ribosome rather than increased ribosome investment, increasing temperature might not lead to a positive relationship between growth and percent P. We tested the growth rate hypothesis by examining two genera of Neotropical stream grazers, the leptophlebiid mayfly *Thraulodes* and the bufonid toad tadpole *Rhinella*. We measured the body percent P of field-collected *Thraulodes* as well as the stoichiometry of periphyton resources in six Panamanian streams over an elevational gradient spanning approximately 1,100 m and 7°C in mean annual temperature. We also measured *Thraulodes* growth rates using *in situ* growth chambers in two of these streams. Finally, we conducted temperature manipulation experiments with both *Thraulodes* and *Rhinella* at the highest and lowest elevation sites and measured differences in percent P and growth rates. *Thraulodes* body percent P increased with temperature across the six streams, and average specific growth rate was higher in the warmer lowland stream. In the temperature manipulation experiments, both taxa exhibited higher growth rate and body percent P in the lowland experiments regardless of experimental temperature, but growth rate and body percent P of individuals were not correlated. Although we found that *Thraulodes* from warmer streams grew more rapidly and had higher body percent P, our experimental results suggest that the growth rate hypothesis does not apply across temperatures. Instead, our results indicate that factors other than temperature drive variation in organismal percent P among sites.

Keywords: ecological stoichiometry, elemental phenotype, thermal gradient, Panamá, neotropical stream, leptophlebiidae

OPEN ACCESS

Edited by:
Sergi Sabater, University of Girona, Spain

Reviewed by:
Daniel von Schiller, University of the Basque Country, Spain
Claudia Feijoó Feijoó, National University of Luján, Argentina

****Correspondence:***
Eric K. Moody
erickmoody@gmail.com

Specialty section:
This article was submitted to Freshwater Science, a section of the journal Frontiers in Environmental Science

Received: *28 January 2017*
Accepted: *29 March 2017*
Published: *18 April 2017*

Citation:
Moody EK, Rugenski AT, Sabo JL, Turner BL and Elser JJ (2017) Does the Growth Rate Hypothesis Apply across Temperatures? Variation in the Growth Rate and Body Phosphorus of Neotropical Benthic Grazers. Front. Environ. Sci. 5:14. doi: 10.3389/fenvs.2017.00014

INTRODUCTION

Somatic growth rate is a fundamental trait that integrates the effects of numerous environmental pressures (Arendt, 1997). Growth capacity is a key component of organismal fitness and as a result, is often strongly selected upon (Violle et al., 2007; Dmitriew, 2011). However, an organism's realized growth rate in nature rarely reaches its maximum capacity (Calow, 1982; Arendt, 1997). Realized growth rate varies with a number of environmental factors including predation, disturbance, food quality, and temperature (Reznick and Endler, 1982; Atkinson, 1994; Dmitriew, 2011). To achieve variation in realized growth rate over gradients in these variables, organisms must also vary in the biochemical processes that underlie growth. In most organisms except for retroviruses, new tissue is formed by the transcription of DNA into mRNA, which is then sent to ribosomes for protein synthesis. Nucleic acids are relatively phosphorus-rich molecules (8.7%; Sterner and Elser, 2002), and thus variation in their abundance in organismal cells could strongly affect phosphorus (P) demands. Although DNA concentration does not vary appreciably among individuals, RNA can vary considerably and thus represents a source of variation in whole organismal %P (Sterner and Elser, 2002). As a result, variation in growth capacity can lead to variation in organismal elemental demands.

The growth rate hypothesis (GRH) states that differences in maximum growth rate drive variation in organismal %P due to greater allocation to P-rich ribosomal RNA needed to support rapid growth (Elser et al., 1996, 2000a, 2003; Hessen et al., 2013). The GRH has been supported by empirical tests in a wide variety of organisms, particularly small invertebrates growing under the same conditions (Elser et al., 2003, 2006; Gillooly et al., 2005; see review in Hessen et al., 2013). However, the GRH was formulated specifically for differences in invertebrate consumer growth under P limitation and at constant temperature (Elser et al., 2000a; Lukas et al., 2011; Hessen et al., 2013) and has not been well-tested beyond these conditions. Ectotherms generally achieve higher realized growth rates at warmer temperatures (Atkinson, 1994); thus, their somatic %RNA and %P might be expected to increase as well (Cross et al., 2015). However, ectotherms also frequently exhibit countergradient variation in which maximum growth rate (i.e., growth capacity) is highest in organisms from cooler environments (Conover and Schultz, 1995; Arendt, 1997). Further, rapid growth at warmer temperatures is generally accomplished by more rapid protein synthesis per ribosome rather than increased ribosomal investment (Farewell and Neidhardt, 1998). As a result, %P should not increase with growth rate over a natural gradient in temperature. Supporting this hypothesis, RNA content of many organisms is actually lower when acclimated to warmer temperatures (Woods et al., 2003); thus, temperature-induced growth rate variation may not be useful in predicting consumer P demands under the growth rate hypothesis.

Changing thermal regimes could alter organismal %P through mechanisms other than the linkage between RNA and growth rate. Differences in temperature could alter P investment into other types of tissues, which may decouple growth rate and %P but could still lead to differences in somatic P storage (Sardans et al., 2012; Cross et al., 2015). This is particularly true in vertebrates, whose skeletal development involves a high P demand (Hendrixson et al., 2007). For this reason, the GRH was not expected to apply to vertebrates, a hypothesis supported by empirical tests with developing frogs (Sterner and Elser, 2002; Liess et al., 2013). However, tadpoles reared at warmer temperatures still had higher %P at metamorphosis, suggesting that developmental temperature could affect skeletal ossification (Liess et al., 2013). On the other hand, more rapidly growing fish exhibit reduced skeletal ossification (Arendt and Wilson, 2000), suggesting that warmer temperatures could reduce somatic %P in vertebrates. Elucidating these effects is important as alterations to the thermal regimes of aquatic ecosystems could lead to shifts in P cycling through the consumer-mediated storage and transport of this often limiting nutrient.

Animal consumers can play an important role in ecosystem P dynamics through the storage, transport, and recycling of P and other biologically important nutrients (Vanni et al., 2013). Much empirical work on this topic has been conducted in tropical streams, where grazers can substantially affect aquatic ecosystems through their feeding, recycling, and storage of nutrients (e.g., Taylor et al., 2006; McIntyre et al., 2008; Capps and Flecker, 2013). However, these consumer-mediated impacts are sensitive to changes in the biological community (Capps et al., 2015a). Throughout the neotropics, for example, the species richness and biomass of stream-dwelling amphibians have been drastically reduced by disease-driven amphibian declines, particularly in cooler highland streams (Lips, 1999). As a result, the dominant grazers in these affected streams are now aquatic insects, such as the immature stages of mayflies (Colón-Gaud et al., 2010; Rantala et al., 2015). This community shift has altered ecosystem function in affected streams (Connelly et al., 2008; Rugenski et al., 2012; Whiles et al., 2013), and can also affect riparian ecosystems through the movement of adults of both taxa to the terrestrial environment (Vanni et al., 2013). As a result, differential growth and P sequestration between aquatic insects and tadpoles in response to thermal variation could further alter the trajectory of stream and riparian ecosystems in the tropics affected by amphibian decline.

Here we investigate the relationships of consumer somatic %P with growth rate of grazing mayflies and development rate of grazing tadpoles across a thermal gradient in Panamá to test whether the growth rate hypothesis applies to temperature-induced growth rate differences. We conducted a survey of periphyton and consumer stoichiometry across a natural thermal gradient in combination with controlled temperature manipulation experiments. We predicted that organismal %P would not increase with growth rate over a thermal gradient based on the biochemical mechanisms by which more rapid growth is achieved at warmer temperatures. We especially did not expect this relationship in tadpoles, whose developing bones represent a substantial P reservoir. This work expands upon our understanding of the applicability of the growth rate hypothesis and sheds light on the ways in which temperature-induced growth rate differences may violate its assumptions.

METHODS

Study Sites

We conducted this study in six streams in Panamá ranging in elevation from 52 to 1,156 m and in mean temperature from approximately 18–25°C (**Figure 1**, **Table 1**, Table S1). The three lowland streams were located in Parque Nacional Soberanía near Gamboa (Mendoza, Macho, and Frijolito), while the three higher elevation streams were located in Parque Nacional Omar Torrijos (Guabal), Bosque Protector Palo Seco (Castillo), and Reserva Forestal Fortuna (Chorro). We measured temperature and light incidence hourly during the dry season of 2014 (February-May) at all sites using HOBO Pendant loggers (Onset Computer Corporation; Bourne, MA). Organisms from two streams, Río Frijolito and Quebrada Chorro, were studied more extensively through *in situ* growth measurements and temperature manipulation experiments.

Natural Thermal Gradient

At least six (range: 6–14) *Thraulodes* mayflies were collected from each stream by a combination of kick sampling with a D-frame net and hand searching on large rocks in riffles. The organisms were transported on ice and then frozen prior to determination of body %P. To examine whether variation in consumer %P could be driven by variation in food quality, we also measured carbon (C), nitrogen (N), and P concentrations of periphyton growing on rocks from all streams. Periphyton was collected from five replicate Loeb samples in both riffles and pools of each stream, and values were then averaged using methods described in Connelly et al. (2008) and Whiles et al. (2013).

We then measured *in situ* growth rates of *Thraulodes* using clear acrylic cylindrical chambers with fine mesh (500 μm) covering each end (described in Rugenski et al., 2012) in the two streams that provided the source populations for our experiments described below, Río Frijolito in Parque Nacional Soberanía and Quebrada Chorro in Reserva Forestal Fortuna. Mayflies were collected by hand by turning over rocks from stream riffles and then were placed into chambers containing four or five similar-sized individuals each with periphyton-covered rocks from the stream. We deployed five chambers per stream. Chambers were left in the stream for 4 days, after which mayflies were collected and re-measured. The total body length excluding cerci was measured to the nearest 0.1 mm from photographs taken immediately before and after deploying and retrieving the chambers using imageJ. Photographs were taken with a digital camera (Olympus TG-620) mounted above a petri dish on a flat surface. We then converted these values to dry mass using a length-mass regression for Leptophlebiidae (Benke et al., 1999). From these values we calculated specific growth rates (SGR) per day using the formula:

$$SGR = (\ln(Mass_{final}) - \ln(Mass_{initial}))/Time$$

Final sample size varied between sites based on number of mayflies recovered. We recovered approximately 60% of the mayflies initially placed into chambers at each site, but we excluded two chambers from the Frijolito site from further analysis because their corresponding labels were lost upon recovery.

Temperature Manipulation

We conducted temperature manipulation experiments at two sites with both *Thraulodes* and bufonid tadpoles in the genus *Rhinella* from the lowland Río Frijolito and the highland Quebrada Chorro. We collected organisms from each stream with hand dip nets and transported them in buckets of stream water via automobile to coolers filled with 45 L of aged tap water (Frijolito) or aged stream water (Chorro). Water was aged by filling coolers at least 24 h before adding organisms. For Frijolito experiments, coolers were housed in a climate-controlled room in Gamboa (ambient air temperatures were too high to maintain cooler temperatures in pilot trials) with a 12:12 light:dark cycle and on a covered outdoor patio at the Jorge L. Araúz research station for Chorro experiments (**Figure 2**). Both locations were located approximately 20 min driving from the source streams. Light recordings inside coolers were also somewhat higher than those recorded in-stream, where zero light was recorded for 13–14 h per day depending on weather.

We kept coolers either at ambient water temperature or heated them using 100 watt aquarium heaters (Eheim; Deizisau, Germany) to increase mean temperature by approximately 5°C for 3 days. To prevent acute thermal stress, temperature was slowly increased over the course of 12 h after adding organisms to heated treatments. We employed recirculating aquarium pumps (Technological Aquatic Associated Manufacturing; Taiwan) to homogenously heat the water and simulate stream flow in both ambient and heated coolers. Heaters were unplugged several hours after dark to mimic the slow overnight cooling of water experienced by the ambient treatments and the natural streams and then restarted in the morning after sunrise while pumps ran continuously throughout the experiments (**Figure 2**). The magnitude of the temperature manipulation was chosen based on the average sensitivity of stream temperature to changes in air temperature of 0.51/°C (Luce et al., 2014) and the range of predicted mean temperatures for Panamá under the A2 climate change scenarios (28–31°C, ensemble low and high, bottom and top 10% of all model predictions respectively, 2080–2099, World Bank Climate Change Knowledge Portal). Therefore, the experiment represents a short-term exposure to thermal regimes that these organisms may face for longer durations in the next century. We monitored water temperatures in the coolers using HOBO pendant loggers as above.

We placed five organisms in each cooler, conducting all treatments for a given species and site simultaneously. Organisms were fed periphyton *ad libitum* using either pre-colonized unglazed ceramic tiles from the stream of collection (Soberanía) or periphyton-covered rocks from the stream of collection (Fortuna). We replaced these with fresh tiles or rocks before starting with a new species and if periphyton appeared to have been substantially consumed during an experiment so that organisms could feed *ad libitum* and so the elemental composition of periphyton would not differ substantially from that in-stream. After 3 days, we measured change in body length as above, and for tadpoles, Gosner developmental stage (Gosner,

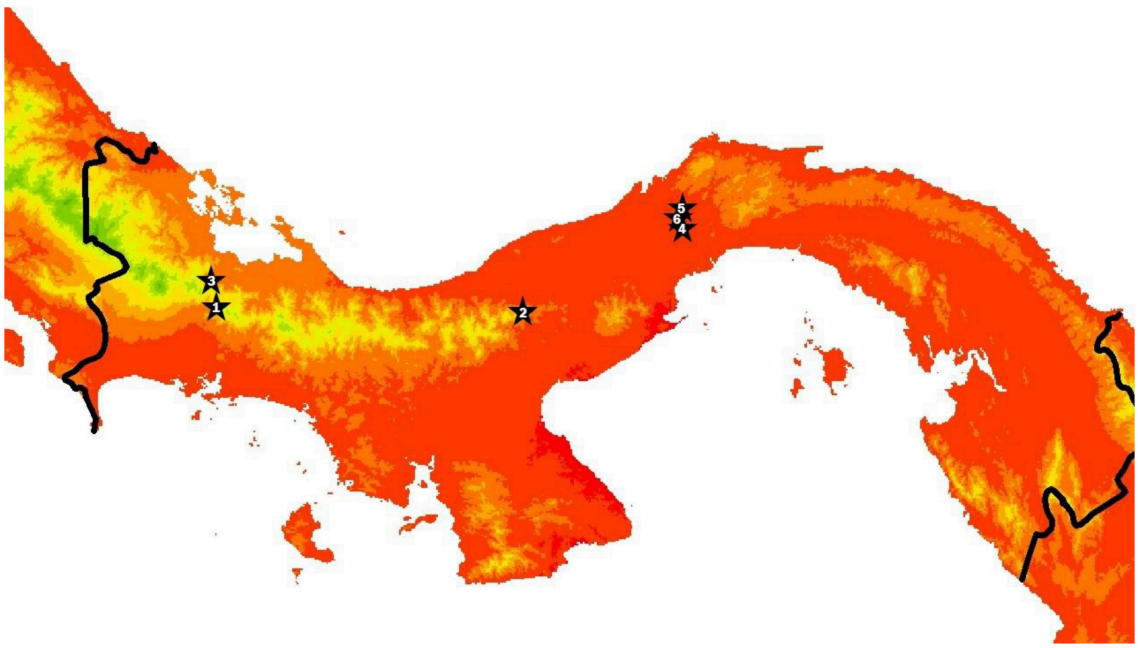

FIGURE 1 | Map of Panamá with stars indicating the locations of the six study streams. The color gradient shows mean annual air temperature from WORLDCLIM at 30 arcsecond resolution, with green to red indicating colder to warmer. Sites are ranked from coldest to warmest and correspond to site numbers in **Tables 1, 3**. Site 1 corresponds to where organisms were collected for Fortuna and Site 4 corresponds to where organisms were collected for Soberanía temperature manipulation experiments.

TABLE 1 | Temperature, light incidence, and elevation of the six study streams in Panamá.

Site	Mean temp (°C)	SD temp	Mean light (lx)	Elevation (m)
1. Quebrada Chorro	18.3	0.83	43.4	1,156
2. Río Guabal	21.2	0.54	242.6	679
3. Quebrada Castillo	21.3	0.87	156.0	533
4. Río Frijolito	24.7	0.93	156.5	52
5. Río Macho	25.1	0.60	373.8	134
6. Río Mendoza	25.3	0.86	370.0	80

1960). The Gosner developmental stage is based on the presence and developmental extent of limb buds, limbs, and toes, which we assessed visually under a dissecting scope after euthanizing tadpoles. We held all organisms without food for approximately 1 h to minimize the effect of food content on body %P to approximate the same methods used in field collections. We then euthanized organisms using an overdose of MS-222 and preserved them for determination of %P as described above. Half of the organisms were preserved for P analysis, while half were preserved for transcriptomic analysis (not included in this manuscript). Recovery rates were somewhat lower than field growth chambers. We recovered approximately 35% of mayflies and 60% of tadpoles at the Soberanía site and 50% of mayflies and 90% of tadpoles at the Fortuna site. Our observations indicated that both mortality and emergence of adults contributed to our failure to recover all organisms. Most observed mortalities occurred within 12 h of transport to coolers, thus we believe these were likely due to handling stress rather than unfavorable conditions within coolers.

Laboratory Analyses

To measure body %P, tissue was dried to a constant weight at 50°C. For mayflies and periphyton collected from streams, we digested homogenized tissues in 1N hydrochloric acid and then used inductively coupled plasma mass spectrometry to measure %P (Thermo iCAP 6300; ThermoFisher Scientific, Waltham, MA). Carbon and N concentrations of periphyton were measured using a CHN analyzer (Thermo Flash 2000 CHNS/O Elemental Analyzer; CE Elantech, New Brunswick, NJ). Body percent phosphorus of experimental organisms was measured using a similar protocol, but we instead used the colorimetric acid molybdate method following acid digestion (APHA, 2005).

Statistical Analysis

We used Spearman's rank correlation tests to test for differences in periphyton stoichiometry with temperature among streams. We then employed ANCOVA with body length as a covariate to test how mayfly body %P varied with stream temperature. As specific growth rate is age- and size-dependent, we also used ANCOVA with body length as a covariate to test whether it varied in mayflies grown in chambers between the high-elevation cool stream and the low-elevation warm stream. For the temperature manipulation experiment, we tested whether consumer %P varied among taxa, temperatures, sites, and the interaction of

the latter two using ANOVA. We used separate ANOVAs for tadpoles and mayflies to test whether growth or development rates varied with temperature, site, and/or their interaction as the growth metrics were not directly comparable between the two genera. Although the metric for tadpoles is a measure more of development rate than growth rate, we could not reliably measure specific growth rate in developing tadpoles as they lost size and mass at later Gosner stages. As faster rates of development also necessitate faster rates of protein synthesis, we believe this metric should produce results consistent with growth rate for the responses of interest. We also tested for a correlation between final Gosner stage and tadpole %P to test whether ontogenetic variation could explain variation in %P. Finally, we tested for significance of correlations between consumer %P and specific growth rate (in mayflies) or development rate (in tadpoles) by testing whether Pearson's correlation coefficients differed from zero using *t*-tests. For all analyses, we visually assessed normality and heterogeneity of variance of model residuals using normal quantile and residuals vs. fitted value plots, respectively, and log-transformed data to meet assumptions as needed. All statistical analyses were performed using R version 3.2.2 (R Core Team, 2015).

RESULTS

Natural Thermal Gradient

Mean water temperature during the dry season generally increased with decreasing elevation among the study sites, although the lowest site (Río Frijolito) was slightly cooler than the other two lowland sites (**Table 1**). The standard deviation of water temperature varied somewhat among streams but not systematically with elevation or the mean temperature (**Table 1**). Mean incident light also varied among streams but again there was no evident pattern related to temperature or elevation (**Table 1**).

Among the six study streams, *Thraulodes* body %P did not vary with body size [$F_{(2, 42)} = 0.98$, $p = 0.382$] but increased with mean water temperature [$F_{(1, 42)} = 6.81$, $p = 0.013$] (**Figure 3**). Periphyton %C, %N, and %P were all negatively correlated with water temperature ($p < 0.05$; **Figure 4**). Periphyton C:P was positively correlated with water temperature ($r = 0.39$, $S = 17{,}951$, $p = 0.003$), but periphyton C:N and N:P were not significantly correlated with water temperature ($p > 0.05$). In the *in situ* growth chambers, mean specific growth rate of mayflies decreased with initial body size [Size, $F_{(1, 11)} = 17.08$, $p = 0.002$], but for a given size did not differ among sites [Site, $F_{(1, 11)} = 4.09$, $p = 0.068$] (**Figure 5**). However, since mayflies were smaller on average in Río Frijolito, their average specific growth rate (0.059 mg^*d^{-1}) was higher than those in Quebrada Chorro (0.035 mg^*d^{-1}). We found no interactive effect of body size and stream site on specific growth rate [$F_{(1, 11)} = 0.03$, $p = 0.872$].

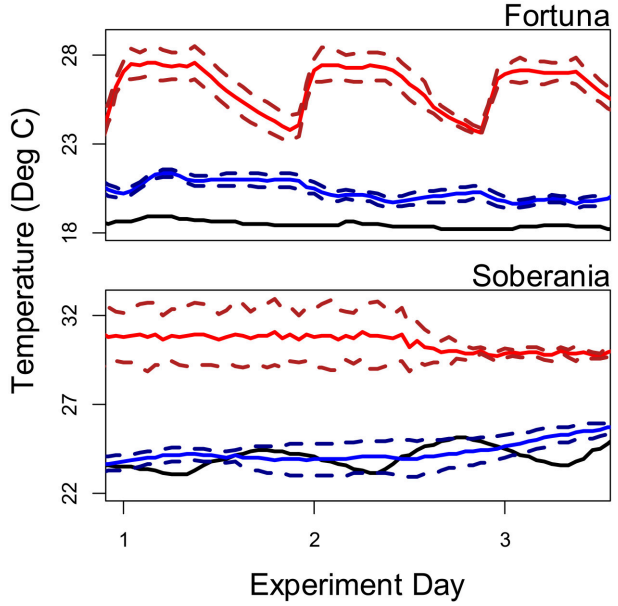

FIGURE 2 | Temperature regimes in source streams (black lines), ambient treatments (blue lines), and heated treatments (red lines) during the course of temperature manipulation experiments. For experimental treatments, solid lines represent average temperatures and dashed lines represent ± one standard deviation.

Temperature Manipulation

In mayflies, specific growth rate was higher at the warmer lowland site Frijolito [Site, $F_{(1, 20)} = 8.93$, $p = 0.007$) and in the warmer temperature treatment [Treatment, $F_{(1, 20)} = 8.56$, $p = 0.008$]. Tadpoles used in the experiments were initially at later Gosner developmental stages at the lowland Frijolito site, and thus ultimately reached later Gosner stages by the end of the experiment (**Table 2**). In tadpoles, development rate was also higher at the lowland Frijolito site [Site, $F_{(1, 35)} = 63.21$, $p < 0.001$] as well as in the warmed treatments [Treatment, $F_{(1, 35)} = 35.84$, $p < 0.001$],

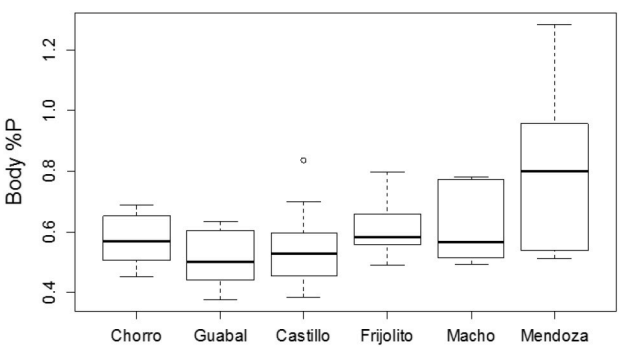

FIGURE 3 | Body %P by dry mass of six populations of *Thraulodes* mayflies in Panamá. Streams are ordered from coolest to warmest. Ln-transformed body %P did not vary with mayfly size [$F_{(2, 42)} = 0.98$, $p = 0.382$] but did increase with increasing average water temperature [$F_{(1, 42)} = 6.81$, $p = 0.013$]. This plot shows untransformed data for easier biological interpretation. In the boxplot, the thick line represents the median, the edges of the boxes represent the upper and lower quartiles, and the whiskers represent 1.5 times the interquartile range above and below the upper and lower quartiles.

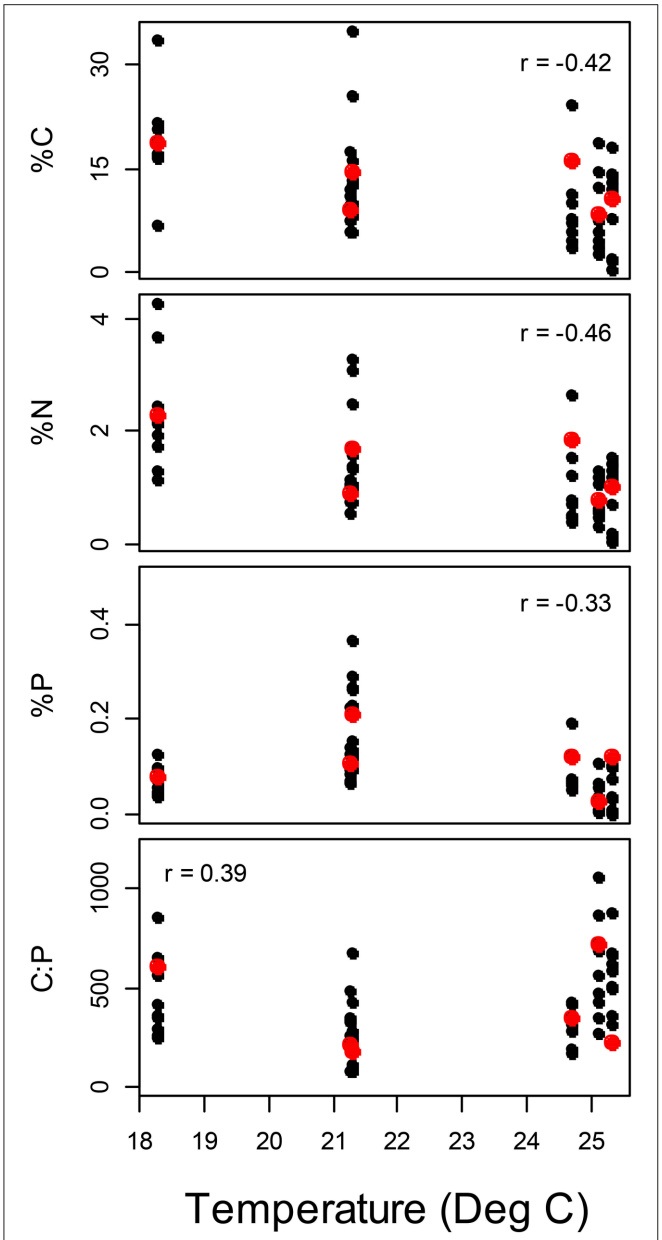

FIGURE 4 | Variation in periphyton elemental concentrations with temperature among streams. Spearman rank correlation coefficients significantly different from zero are presented. Red points represent the average value for each stream.

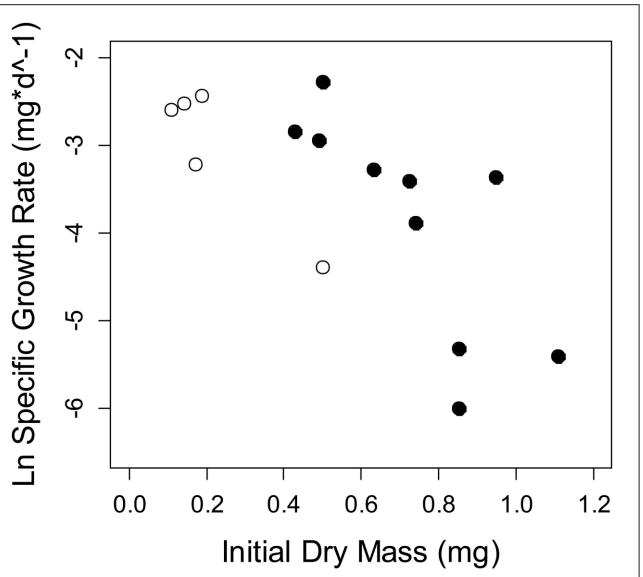

FIGURE 5 | Specific growth rate of *Thraulodes* measured from *in situ* chambers at two sites. Points in white are the lowland stream, Frijolito, and points in black are the highland stream, Chorro. Each point is an individual mayfly. There was a significant effect of the covariate initial dry mass [$F_{(1, 11)} = 17.08$, $p = 0.002$], but no site effect on specific growth rate after controlling for body size [$F_{(1, 11)} = 4.09$, $p = 0.068$].

TABLE 2 | Gosner developmental stages of *Rhinella* tadpoles used in the temperature manipulation experiments.

Site	Temperature	Initial avg.	Initial range	Final avg.	Final range
Chorro	Ambient	24.75	24–25	26.00	25–27
Chorro	Warm	25.00	25–25	25.67	25–27
Frijolito	Ambient	29.60	29–31	31.20	30–34
Frijolito	Warm	30.75	29–32	33.67	31–36

TABLE 3 | Average elemental concentrations and ratios of periphyton from the six sampled streams.

Site	%C	%N	%P	C:N	C:P	N:P
1. Quebrada Chorro	18.83	2.30	0.08	9.54	606.96	63.60
2. Río Guabal	9.04	0.90	0.11	11.71	211.92	18.10
3. Quebrada Castillo	14.45	1.69	0.21	9.97	177.44	17.80
4. Río Frijolito	16.07	1.86	0.12	10.07	345.33	34.29
5. Río Macho	8.33	0.79	0.03	12.29	716.01	58.25
6. Río Mendoza	10.65	1.03	0.12	12.05	228.86	18.99

Stoichiometric ratios are presented in molar form.

and there was also a significant interaction effect of site and temperature [Interaction, $F_{(1, 35)} = 39.73$, $p < 0.001$] such that tadpoles developed more rapidly in warmer water at Frijolito, but developmental rate did not vary with temperature at Chorro.

Body %P was not significantly correlated with final Gosner stage in tadpoles among sites and treatments ($r = 0.40$, $t_{13} = 1.59$, $p = 0.137$). Similar to specific growth rate, body %P was higher at the lowland Frijolito site in both taxa [Site, $F_{(1, 25)} = 20.83$, $p < 0.001$]. In contrast, body %P did not vary with temperature manipulation [Treatment, $F_{(1, 25)} = 2.72$, $p = 0.112$] nor did it vary between *Thraulodes* and *Rhinella* [Species, $F_{(1, 25)} = 0.01$, $p = 0.904$; **Figure 6**]. Body %P was not significantly correlated with ln-transformed development rate in tadpoles ($r = 0.32$, $t_{13} = 1.40$, $p = 0.238$) or ln-transformed specific growth rate in mayflies ($r = 0.42$, $t_{10} = 1.47$, $p = 0.173$; **Figure 7**).

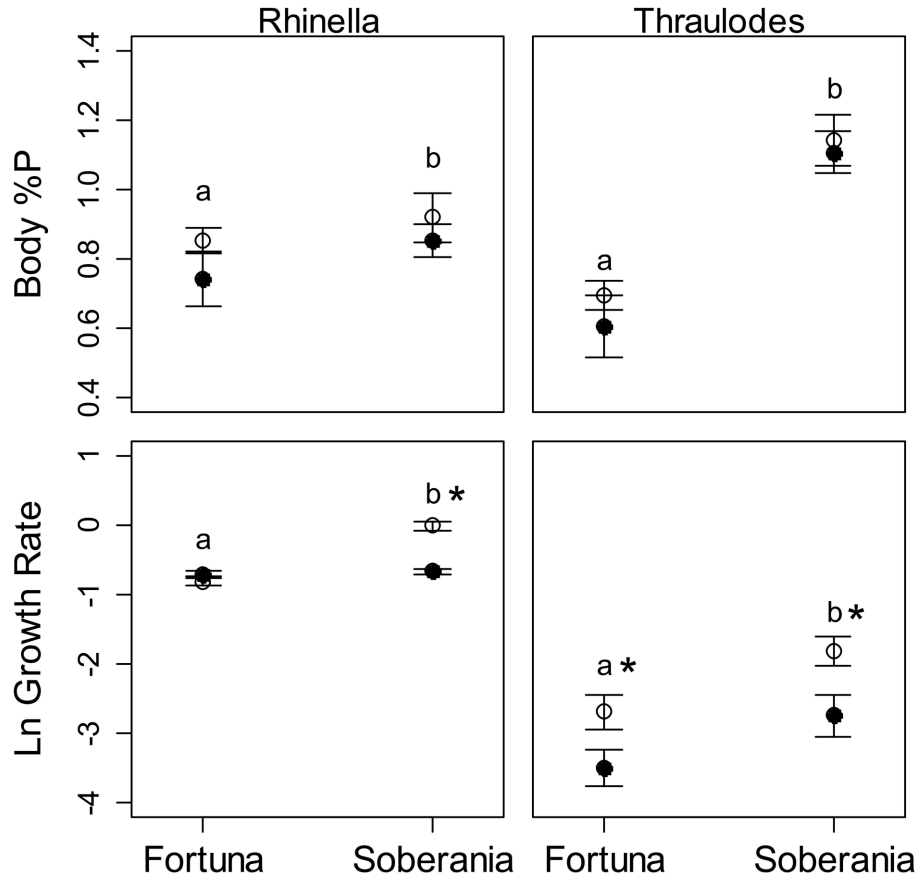

FIGURE 6 | Average body %P by dry mass and growth rate of *Rhinella* (left panels) and *Thraulodes* **(right panels)**. Growth of *Rhinella* is represented as Δ Gosner stage * d^{-1}, and for *Thraulodes* as specific growth rate based on body mass (mg * d^{-1}). White points are heated temperature treatments and black points are ambient temperature treatments. Error bars are ± one standard error. Letters above points represent significant differences in the response variable among sites and asterisks next to letters represent significant effects of temperature treatment at that site.

DISCUSSION

Variation in the somatic %P of invertebrates is well-explained by variation in rRNA production needed for protein synthesis to achieve somatic growth under the growth rate hypothesis (GRH), but the limits to its applicability, particularly in regards to developing vertebrates, have not been fully tested. In this study we sought to test the GRH in vertebrate and invertebrate benthic grazers over a thermal gradient in Panamá. Although our results meet predictions of the growth rate hypothesis, they do not support its application in explaining somatic %P variation with temperature. Across the six streams, average %P of *Thraulodes* increased with average water temperature. Since average SGR of *Thraulodes* were also higher in the warmer lowland Río Frijolito than the cooler highland Quebrada Chorro, these data conform to the predicted relationship between growth rate and somatic %P under the GRH. However, body %P did not increase with temperature in the manipulative experiment even though growth and developmental rates did in most cases. In fact, both body %P and growth/developmental rates were higher in the lowland Frijolito experiments regardless of temperature treatment (**Figure 6**). Our results suggest, at least in *Thraulodes*, body %P increases with growth rate among sites but thermal effects on growth rate did not drive this effect. This result supports the assumption of the GRH that it does not apply to growth rate variation driven by varying temperatures. Instead, we suggest that other factors drive variation in growth rate and body %P among sites.

The GRH was formulated with an assumption that organisms were growing at the same temperature (Elser et al., 1996, 2000a). We know from previously published work examining temperature-induced growth variation that it might not lead to variation in somatic %P if individual ribosomes synthesize proteins more rapidly, requiring a lower amount of ribosomal RNA to support rapid protein synthesis at higher temperatures (Farewell and Neidhardt, 1998; Gillooly et al., 2005). In fact, eukaryotic RNA content tends to decrease in warm-acclimated organisms (Woods et al., 2003). Therefore, in taxa for which RNA is the primary pool of somatic P, including the mayflies and early-stage tadpoles we studied, it is unlikely that increasing growth rates with warming temperatures will lead to higher somatic %P. If grazer P requirements do not change with

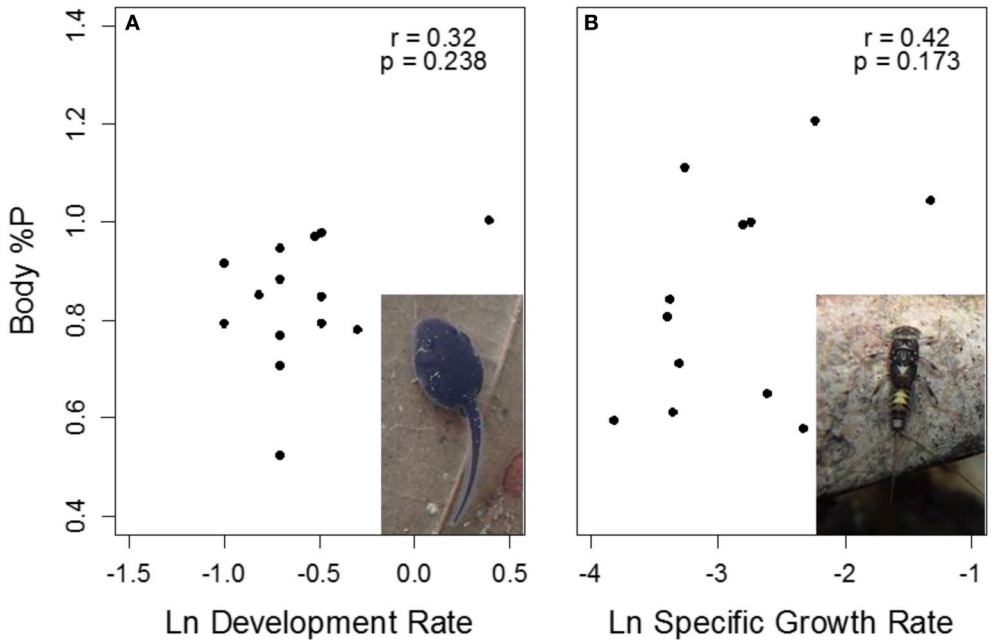

FIGURE 7 | Correlations of body %P with growth rate in *Rhinella* (A) and *Thraulodes* **(B)** from temperature manipulation experiments. For *Rhinella*, growth is represented as Δ Gosner stage * day^{-1}, while in *Thraulodes* it is represented as specific growth rate based on body mass. Neither correlation coefficients were significantly different from zero ($p > 0.05$).

warming temperatures, this will buffer them from potential P-limitation due to increased algal C:P with warming (Yvon-Durocher et al., 2010), a phenomenon we also observed with increasing temperature among streams in our study (**Figure 4**). Further, the storage of P by consumers can represent a substantial P sink in aquatic ecosystems, particularly when those consumers have biphasic life cycles and migrate to terrestrial ecosystems as adults (Vanni et al., 2013; Tiegs et al., 2016); thus, this finding has implications for ecosystem-scale fluxes of P in these streams. However, growth rate and %P of these grazers did vary among streams both in field collections and in our experiment; thus, it is important to discuss what may have driven this variation.

While it may be clear that temperature-driven effects did not affect the somatic %P of the grazers we studied, it is less clear why body %P and growth rate of both taxa varied among sites. As site-specific differences in %P were consistent in the temperature manipulation experiment in both *Thraulodes* and *Rhinella*, they may reflect some other important difference between lowland (<200 m) and highland (>500 m) streams (**Figure 3**). For example, the amphibian pathogen *Batrachochytridium dendrobatidis* is present across this entire elevational gradient, but has only led to catastrophic amphibian declines in highland streams (La Marca et al., 2005; Kilburn et al., 2010). If insect grazer densities increase in highland streams following amphibian declines, more intense intraspecific competition could lead to reduced individual growth rates. However, after reductions in tadpole densities following declines in one of our study streams, Río Guabal, densities of *Thraulodes* and other grazing invertebrate taxa did not significantly change (Colón-Gaud et al., 2010). Since *Thraulodes* should have less competition for algal resources in post-decline streams, we believe this would lead to increased growth rates if it affected them at all. Differences in the severity of amphibian decline do not effectively explain *Thraulodes* growth differences among sites, but other differences, such as disturbance frequency, resource quality, and predation pressure among streams may provide more insights.

The presence or absence of fishes was a defining characteristic between the upland and lowland streams in this study. The lowland streams in our study host diverse insectivorous fish communities including the aquatic insect-feeding genera *Astyanax*, *Brycon*, *Andinoacara*, *Geophagus*, *Pimelodella*, *Trichomycterus*, and *Gobiomorus* (Kramer and Bryant, 1995), while the upland streams either lack fish (Chorro and Castillo) or host only the primarily terrestrial insectivore *Brachyrhaphis* (Guabal) (Colón-Gaud et al., 2010). While all streams host predatory invertebrates such as pseudothelphusid crabs, dragonflies, and dobsonflies (Colón-Gaud et al., 2010; Múrria et al., 2015), fish and invertebrate predators can often invoke contrasting responses in prey due to differences in predator feeding strategies (Peckarsky and McIntosh, 1998; Touchon and Warkentin, 2008). Many fishes are visual predators and feed more actively during daylight hours while many of the invertebrate predators such as crabs and odonates are nocturnally active and rely more heavily on olfactory and tactile cues (Flecker, 1992; Peckarsky and McIntosh, 1998; Maitland, 2003). Unfortunately, which predators have greater effects on prey fitness is highly prey species-specific (e.g., Touchon and Vonesh, 2016), and we have insufficient data to infer whether fish predators have stronger fitness costs than invertebrate predators in *Thraulodes* and *Rhinella*.

Elevated predation pressure selects for more rapid growth and development to maturity in many organisms including mayflies and amphibians (Reznick and Endler, 1982; Peckarsky et al., 2001; Vonesh and Warkentin, 2006), so variation in predation pressure could explain higher growth rate and %P of lowland taxa if fish predators induce stronger selection for rapid growth. Selection for rapid growth and development to maturity in Arctic *Daphnia* populations developing under short growing seasons explained higher %P in those populations relative to temperate populations when reared at the same temperature (Elser et al., 2000b), and a similar scenario induced by predation risk may have occurred in our study. Although predation pressure is not a strong predictor of variation in body stoichiometry in Trinidadian guppies (El-Sabaawi et al., 2012), its effects on somatic %P via growth rate may be stronger in invertebrates and tadpoles. The latter develops P-rich bones only at later Gosner stages. As the streams we studied host genetically isolated populations of *Thraulodes* (Múrria et al., 2015), these effects could represent a potential evolutionary tradeoff in response to exposure to fish predators and merit further study.

Another possibility for the elevated %P of consumers at the lowland Frijolito site is that it is an artifact of our experimental design. Unfortunately, some methodological differences did occur among sites due to the remote location of the highland streams. In particular, our experiments at the highland Chorro site were kept on an open patio as opposed to in a climate-controlled room, which allowed temperatures to vary more than at the lowland Frijolito site (**Figure 2**). Thermal fluctuations often lead to more rapid development in ectotherms (e.g., Czarnoleski et al., 2013), yet we observed lower growth and developmental rates in organisms from Chorro. As a result, we do not believe this strongly affected our results. A further difference is that tadpoles at the lowland Frijolito site developed more rapidly and reached later stages by the end of our study (**Table 2**; **Figure 6**); thus, ontogenetic variation could explain variation in %P (Main et al., 1997; Pilati and Vanni, 2007). This is particularly true of developing Anurans, which exhibit extreme ontogenetic changes in %P as bone ossification begins at later Gosner stages (Tiegs et al., 2016). In the related European toad (*Bufo bufo*), tadpole bones begin ossification at Gosner stage 38 (Dunlap and Sanchiz, 1996). Some tadpoles at the lowland Frijolito site reached Gosner stage 38 by the end of the experiment, but we only included %P values for tadpoles at stages <38 in our analysis. The stage at which *Rhinella alata* or *R. marina* begin ossification is not known, but the fact that %P was not significantly correlated with final Gosner stage indicates that minimal ossification may occur before stage 38 in these species. Even if ontogenetic variation did not strongly influence our results, however, these changes are likely to affect the %P of developing tadpoles in natural ecosystems.

While our results provide insight into stoichiometric theory, they also have implications for Neotropical stream ecosystems facing disease-driven amphibian declines and future climatic change. The effects of the loss of amphibian communities on aquatic ecosystem functions have been well-studied (Connelly et al., 2008; Rugenski et al., 2012; Whiles et al., 2013; Rantala et al., 2015), but both amphibians and many aquatic insects have terrestrial adult stages. The movement of organisms from aquatic ecosystems to the terrestrial environment can represent a significant nutrient sink to aquatic ecosystems and an important subsidy to their surrounding terrestrial counterparts (Sabo and Power, 2002; Vanni et al., 2013; Capps et al., 2015b). Variation in %P among populations of *Rhinella* and *Thraulodes* can lead to variation in P export to the surrounding terrestrial ecosystem. However, the biphasic life cycles of these organisms could lead to dramatic changes in the %P of adults (Tiegs et al., 2016), which we did not consider in this study. *Rhinella* metamorphs in particular may exhibit strong differences in %P from pre-ossification tadpoles if bone density is unrelated to tadpole growth rates. Further, since the total consumer biomass remains significantly lower in post-decline streams (Whiles et al., 2013), this variation is unlikely to offset the P fluxes lost to amphibian extirpations.

One final caveat is that dietary stoichiometry of the study organisms likely varied among sites following variation in periphyton elemental concentrations (**Table 3**, **Figure 4**). The GRH is only successful at predicting somatic %P variation under P-limited consumer growth, because consumers can store P in tissues aside from ribosomal RNA when excess P is available (Hessen et al., 2013). This is particularly true of some vertebrates, in which bone tissues may serve as a flexible pool of body P (Benstead et al., 2014). Although periphyton C:P generally increased with water temperature (**Figure 4**), the highland Chorro site also had a relatively high average periphyton C:P. As a result, the particularly high C:P ratio of periphyton in the highland Chorro experiments relative to that in the lowland Frijolito experiments could explain why somatic %P of both taxa was not significantly correlated with growth rate (**Figure 7**) if consumer growth were limited by P in Chorro experiments and C in Frijolito experiments. A more detailed investigation of how threshold elemental ratios (TERs; Sterner and Hessen, 1994) vary with temperature is required to examine this mechanism. Variation in ontogeny and resource stoichiometry contributed to variation in consumer %P among sites in this study; thus, a carefully controlled study examining the effects of reduced competition for resources and predation pressure on the %P of these grazers is needed to understand the mechanisms behind the patterns we observed.

Although global climate change is expected to have a number of effects on stream ecosystems (Cross et al., 2015), our results do not provide any evidence that warming temperatures will alter nutrient storage and export by individual consumers. Instead, a mechanistic understanding of the drivers of variation in these stoichiometric traits is needed to predict how they will respond over environmental gradients. We suggest that more work investigating the drivers of growth rate variation and the relationship between juvenile growth and adult elemental composition in these taxa is needed to elucidate how consumers with biphasic life cycles such as these will alter nutrient dynamics in changing ecosystems.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Smithsonian Tropical Research Institute Institutional Animal Care and Use Committee. The

protocol was approved by the Smithsonian Tropical Research Institute Institutional Animal Care and Use Committee (Protocol 2013-1201-2016).

AUTHOR CONTRIBUTIONS

Conceived and designed the study: EM, AR, JS, BT, JE. Performed the study: EM, AR, BT. Analyzed the data: EM, AR. Wrote the paper: EM, AR, JS, BT, JE.

ACKNOWLEDGMENTS

We thank the Smithsonian Tropical Research Institute and Arizona State University for funding this research and providing logistical support through a collaborative grant to AR, JE, JS, and BT and a fellowship to EM. We specifically thank Dayana Agudo, Carlos Espinosa, Luis Fernando de León, and Diana Sharpe for their assistance with logistics in Panamá. Michael Angilletta, Krista Capps, James Collins, and Ren Ze provided feedback on earlier versions of this manuscript. Scott Brovarney and Ashley Sanders helped with laboratory work. This work was conducted under ANAM scientific collection permit SC/A-2-14. Work with *Rhinella* was approved by the STRI animal care use committee (STRI IACUC protocol 2013-1201-2016).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fenvs.2017.00014/full#supplementary-material

REFERENCES

APHA (2005). *Standard Methods for the Examination of Water and Wastewater*. Washington, DC: APHA-AWWA-WEF.

Arendt, J. D. (1997). Adaptive intrinsic growth rates: an integration across taxa. *Q. Rev. Biol.* 72, 149–177. doi: 10.1086/419764

Arendt, J., and Wilson, D. S. (2000). Population differences in the onset of cranial ossification in pumpkinseed (*Lepomis gibbosus*), a potential cost of rapid growth. *Can. J. Fish. Aquat. Sci.* 57, 351–356. doi: 10.1139/f99-250

Atkinson, D. (1994). Temperature and organism size – a biological law for ectotherms? *Adv. Ecol. Res.* 25, 1–58.

Benke, A. C., Huryn, A. D., Smock, L. A., and Wallace, J. B. (1999). Length-mass relationships for freshwater macroinvertebrates in North America with particular reference to the Southeastern United States. *J. North Am. Benthol. Soc.* 18, 308–343. doi: 10.2307/1468447

Benstead, J. P., Hood, J. M., Whelan, N. V., Kendrick, M. R., Nelson, D., Hanninen, A. F., et al. (2014). Coupling of dietary phosphorus and growth across diverse fish taxa: a meta-analysis of experimental aquaculture studies. *Ecology* 95, 2768–2777. doi: 10.1890/13-1859.1

Calow, P. (1982). Homeostasis and fitness. *Am. Nat.* 120, 416–419. doi: 10.1086/284001

Capps, K. A., Atkinson, C. L., and Rugenski, A. T. (2015a). Implications of species addition and decline for nutrient dynamics in fresh waters. *Freshw. Sci.* 34, 485–496. doi: 10.1086/681095

Capps, K. A., Berven, K. A., and Tiegs, S. D. (2015b). Modelling nutrient transport and transformation by pool-breeding amphibians in forested landscapes using a 21-year dataset. *Freshw. Biol.* 60, 500–511. doi: 10.1111/fwb.12470

Capps, K. A., and Flecker, A. S. (2013). Invasive aquarium fish transform ecosystem nutrient dynamics. *Proc. R. Soc. Lond. B* 280:20131520. doi: 10.1098/rspb.2013.1520

Colón-Gaud, C., Whiles, M. R., Brenes, R., Kilham, S. S., Lips, K. R., Pringle, C. M., et al. (2010). Potential functional redundancy and resource facilitation between tadpoles and insect grazers in tropical headwater streams. *Freshw. Biol.* 55, 2077–2088. doi: 10.1111/j.1365-2427.2010.02464.x

Connelly, S., Pringle, C. M., Bixby, R. J., Brenes, R., Whiles, M. R., Lips, K. R., et al. (2008). Changes in stream primary producer communities resulting from large-scale catastrophic amphibian declines: can small-scale experiments predict effects of tadpole loss? *Ecosystems* 11, 1262–1276. doi: 10.1007/s10021-008-9191-7

Conover, D. O., and Schultz, E. T. (1995). Phenotypic similarity and the evolutionary significance of countergradient variation. *Trends Ecol. Evol.* 10, 248–252. doi: 10.1016/S0169-5347(00)89081-3

Cross, W. F., Hood, J. M., Benstead, J. P., Huryn, A. D., and Nelson, D. (2015). Interactions between temperature and nutrients across levels of ecological organization. *Glob. Chang. Biol.* 21, 1025–1040. doi: 10.1111/gcb.12809

Czarnoleski, M., Cooper, B. S., Kierat, J., and Angilletta, M. J. (2013). Flies developed small bodies and small cells in warm and thermally fluctuating environments. *J. Exp. Biol.* 216, 2896–2901. doi: 10.1242/jeb.083535

Dmitriew, C. M. (2011). The evolution of growth trajectories: what limits growth rate? *Biol. Rev.* 86, 97–116. doi: 10.1111/j.1469-185X.2010.00136.x

Dunlap, K. D., and Sanchiz, B. (1996). Temporal dissociation between the development of the cranial and appendicular skeletons in Bufo bufo (Amphibia: Bufonidae). *J. Herpetol.* 30, 506–513. doi: 10.2307/1565693

El-Sabaawi, R. W., Zandonà, E., Kohler, T. J., Marshall, M. C., Moslemi, J. M., Travis, J., et al. (2012). Widespread intraspecific organismal stoichiometry among populations of the Trinidadian guppy. *Funct. Ecol.* 26, 666–676. doi: 10.1111/j.1365-2435.2012.01974.x

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Dobberfuhl, D. R., MacKay, N. A., and Schampel, J. H. (1996). Organism size, life history, and N:P stoichiometry. *Bioscience* 46, 674–684. doi: 10.2307/1312897

Elser, J. J., O'Brien, W. J., Dobberfuhl, D. R., and Dowling, T. E. (2000b). The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* 13, 845–853. doi: 10.1046/j.1420-9101.2000.00215.x

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., et al. (2000a). Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* 3, 540–550. doi: 10.1046/j.1461-0248.2000.00185.x

Elser, J. J., Watts, T., Bitler, B., and Markow, T. A. (2006). Ontogenetic coupling of growth rate with RNA and P contents in five species of Drosophila. *Funct. Ecol.* 20, 846–856. doi: 10.1111/j.1365-2435.2006.01165.x

Farewell, A., and Neidhardt, F. C. (1998). Effect of temperature on *in vivo* protein synthetic capacity in *Escherichia coli*. *J. Bacteriol.* 180, 4704–4710.

Flecker, A. S. (1992). Fish predation and the evolution of invertebrate drift periodicity: evidence from Neotropical streams. *Ecology* 73, 438–448. doi: 10.2307/1940751

Gillooly, J. F., Allen, A. P., Brown, J. H., Elser, J. J., Martínez del Río, C., Savage, V. M., et al. (2005). The metabolic basis of whole-organism RNA and phosphorus content. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11923–11927. doi: 10.1073/pnas.0504756102

Gosner, K. L. (1960). A simplified key for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16, 183–190.

Hendrixson, H. A., Sterner, R. W., and Kay, A. D. (2007). Elemental stoichiometry of freshwater fishes in relation to phylogeny, allometry, and ecology. *J. Fish Biol.* 70, 121–140. doi: 10.1111/j.1095-8649.2006.01280.x

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elemental approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Kilburn, V. L., Ibáñez, R., Sanjur, O., Bermingham, E., Suraci, J. P., and Green, D. M. (2010). Ubiquity of the pathogenic chytrid fungus, *Batrachochytridium* dendrobatidis, in Anuran communities in Panamá. *Ecohealth* 7, 537–548. doi: 10.1007/s10393-010-0634-1

Kramer, D. L., and Bryant, M. J. (1995). Intestine length in the fishes of a tropical stream: 2. Relationships to diet – the long and short of a convoluted issue. *Environ. Biol. Fish.* 42, 129–141. doi: 10.1007/BF00001991

La Marca, E., Lips, K. R., Lötters, S., Puschendorf, R., Ibáñez , R., Rueda-Almonacid, J. V., et al. (2005). Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica* 37, 190–201. doi: 10.1111/j.1744-7429.2005.00026.x

Liess, A., Rowe, O., Guo, J. W., Thomsson, G., and Lind, M. I. (2013). Hot tadpoles from cold environments need more nutrients – life history and stoichiometry reflects latitudinal adaptation. *J. Anim. Ecol.* 82, 1316–1325. doi: 10.1111/1365-2656.12107

Lips, K. R. (1999). Mass mortality and population declines of Anurans at an upland site in Western Panama. *Conserv. Biol.* 13, 117–125. doi: 10.1046/j.1523-1739.1999.97185.x

Luce, C., Staab, B., Kramer, M., Wenger, S., Isaak, D., and McConnell, C. (2014). Sensitivity of summer stream temperatures to climate variability in the Pacific Northwest. *Water Resour. Res.* 50, 3428–3443. doi: 10.1002/2013WR014329

Lukas, M., Frost, P. C., and Wacker, A. (2011). The neonate nutrition hypothesis: early feeding affects the body stoichiometry of *Daphnia* offspring. *Freshw. Biol.* 58, 2333–2344. doi: 10.1111/fwb.12213

Main, T. M., Dobberfuhl, D. R., and Elser, J. J. (1997). N: P stoichiometry and ontogeny of crustacean zooplankton: a test of the growth rate hypothesis. *Limnol. Oceanogr.* 42, 1474–1478. doi: 10.4319/lo.1997.42.6.1474

Maitland, D. P. (2003). Predation on snakes by the freshwater land crab Eudaniela garmani. *J. Crustacean Biol.* 23, 241–246. doi: 10.1163/20021975-99990331

McIntyre, P. B., Flecker, A. S., Vanni, M. J., Hood, J. M., Taylor, B. W., and Thomas, S. A. (2008). Fish distributions and nutrient cycling in streams: can fish create biogeochemical hotspots? *Ecology* 89, 2335–2346. doi: 10.1890/07-1552.1

Múrria, C., Rugenski, A. T., Whiles, M. R., and Vogler, A. P. (2015). Long-term isolation and endemicity of Neotropical aquatic insects limit the community responses to recent amphibian decline. *Divers. Distrib.* 21, 938–949. doi: 10.1111/ddi.12343

Peckarsky, B. L., and McIntosh, A. R. (1998). Fitness and community consequences of avoiding multiple predators. *Oecologia* 113, 565–576. doi: 10.1007/s004420050410

Peckarsky, B. L., Taylor, B. W., McIntosh, A. R., McPeek, M. A., and Lytle, D. A. (2001). Variation in mayfly size at metamorphosis as a development response to risk of predation. *Ecology* 82, 740–757. doi: 10.1890/0012-9658(2001)082[0740:VIMSAM]2.0.CO;2

Pilati, A., and Vanni, M. J. (2007). Ontogeny, diet shifts, and nutrient stoichiometry in fish. *Oikos* 116, 1663–1674. doi: 10.1111/j.0030-1299.2007.15970.x

Rantala, H. M., Nelson, A. M., Fulgoni, J. N., Whiles, M. R., Hall, R. O., Dodds, W. K., et al. (2015). Long-term changes in structure and function of a tropical headwater stream following a disease-driven amphibian decline. *Freshw. Biol.* 60, 575–589. doi: 10.1111/fwb.12505

R Core Team (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org/

Reznick, D. N., and Endler, J. A. (1982). The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). *Evolution* 36, 160–177. doi: 10.2307/2407978

Rugenski, A. T., Múrria, C., and Whiles, M. R. (2012). Tadpoles enhance microbial activity and leaf decomposition in a neotropical headwater stream. *Freshw. Biol.* 57, 1904–1913. doi: 10.1111/j.1365-2427.2012.02853.x

Sabo, J. L., and Power, M. E. (2002). River-watershed exchange: effects of riverine subsidies on riparian lizards and their terrestrial prey. *Ecology* 83, 1860–1869. doi: 10.2307/3071770

Sardans, J., Rivas-Ubach, A., and Peñuelas, J. (2012). The elemental stoichiometry of aquatic and terrestrial ecosystems and its relationships with organismic lifestyle and ecosystem structure and function: a review and perspectives. *Biogeochemistry* 111, 1–39. doi: 10.1007/s10533-011-9640-9

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry*. Princeton, NJ: Princeton University Press.

Sterner, R. W., and Hessen, D. O. (1994). Algal nutrient limitation and the nutrition of aquatic herbivores. *Annu. Rev. Ecol. Syst.* 25, 1–29. doi: 10.1146/annurev.es.25.110194.000245

Taylor, B. W., Flecker, A. S., and Hall, R. O. (2006). Loss of a harvested fish species disrupts carbon flow in a diverse tropical river. *Science* 313, 833–836. doi: 10.1126/science.1128223

Tiegs, S. D., Berven, K. A., Carmack, D. J., and Capps, K. A. (2016). Stoichiometric implications of a biphasic life cycle. *Oecologia* 180, 853–863. doi: 10.1007/s00442-015-3504-2

Touchon, J. C., and Vonesh, J. R. (2016). Variation in abundance and efficacy of tadpole predators in a Neotropical pond community. *J. Herpetol.* 50, 113–119. doi: 10.1670/14-111

Touchon, J. C., and Warkentin, K. M. (2008). Fish and dragonfly nymph predators induce opposite shifts in color and morphology of tadpoles. *Oikos* 117, 634–640. doi: 10.1111/j.0030-1299.2008.16354.x

Vanni, M. J., Boros, G., and McIntyre, P. B. (2013). When are fish sources vs. sinks of nutrients in lake ecosystems? *Ecology* 94, 2195–2206. doi: 10.1890/12-1559.1

Violle, C., Navas, M.-L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., et al. (2007). Let the concept of trait be functional! *Oikos* 116, 882–892. doi: 10.1111/j.0030-1299.2007.15559.x

Vonesh, J. R., and Warkentin, K. M. (2006). Opposite shifts in size at metamorphosis in response to larval and metamorph predators. *Ecology* 87, 556–562. doi: 10.1890/05-0930

Whiles, M. R., Hall, R. O., Dodds, W. K., Verburg, P., Huryn, A. D., Pringle, C. M., et al. (2013). Disease-driven amphibian declines alter ecosystem processes in a tropical stream. *Ecosystems* 16, 146–157. doi: 10.1007/s10021-012-9602-7

Woods, H. A., Makino, W., Cotner, J. B., Hobbie, S. E., Harrison, J. F., Acharya, K., et al. (2003). Temperature and the chemical composition of poikilothermic organisms. *Funct. Ecol.* 17, 237–245. doi: 10.1046/j.1365-2435.2003.00724.x

Yvon-Durocher, G., Jones, J. I., Trimmer, M., Woodward, G., and Montoya, J. M. (2010). Warming alters the metabolic balance of ecosystems. *Philos. Trans. R. Soc. Lond. B* 365, 2117–2126. doi: 10.1098/rstb.2010.0038

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 29 June 2017
doi: 10.3389/fenvs.2017.00036

Dynamic Responses of Phosphorus Metabolism to Acute and Chronic Dietary Phosphorus-Limitation in *Daphnia*

Nicole D. Wagner[1*†], *Clay Prater*[1] *and Paul C. Frost*[2]

[1] Environmental and Life Science Graduate Program, Trent University, Peterborough, ON, Canada, [2] Department of Biology, Trent University, Peterborough, ON, Canada

OPEN ACCESS

Edited by:
Dedmer B. Van de Waal, Netherlands Institute of Ecology (NIOO-KNAW), Netherlands

Reviewed by:
Cedric Leo Meunier, Alfred Wegener Institute, Germany
Steven Declerck, Netherlands Institute of Ecology (NIOO-KNAW), Netherlands

***Correspondence:**
Nicole D. Wagner
nicolegouldingwagner@gmail.com

†Present Address:
Nicole D. Wagner, Department of Environmental and Physical Science, University of Toronto, Toronto, ON, Canada

Specialty section:
This article was submitted to Freshwater Science, a section of the journal Frontiers in Environmental Science

Received: 01 February 2017
Accepted: 12 June 2017
Published: 29 June 2017

Citation:
Wagner ND, Prater C and Frost PC (2017) Dynamic Responses of Phosphorus Metabolism to Acute and Chronic Dietary Phosphorus-Limitation in Daphnia. Front. Environ. Sci. 5:36. doi: 10.3389/fenvs.2017.00036

Food quality is highly dynamic within lake ecosystems and varies spatially and temporally over the growing season. Consumers may need to continuously adjust their metabolism in response to this variation in dietary nutrient content. However, the rates of metabolic responses to changes in food nutrient content has received little direct study. Here, we examine responses in two metabolic phosphorus (P) pools, ribonucleic acids (RNA) and adenosine triphosphate (ATP), along with body mass and body P content in *Daphnia magna* exposed to chronic and acute dietary P-limitation. First, we examined food quality effects on animals consuming different food carbon (C):P quality over a 14 day period. Then, we raised daphnids on one food quality for 4 days, switched them to contrasting dietary treatments, and measured changes in their metabolic responses at shorter time-scales (over 48 h). Animal P, RNA, and ATP content all changed through ontogeny with adults containing relatively less of these pools with increasing body mass. Irrespective of age, *Daphnia* consuming high C:P diets had lower body %P, %RNA, %ATP, and mass compared to animals eating low C:P diets. Diet switching experiments revealed diet dependent changes in body %P, %RNA, %ATP, and animal mass within 48 h. We found that *Daphnia* switched from low to high C:P diets had some metabolic buffering capacity with decreases in body %P occurring after 24 h but mass remaining similar to initial diet conditions for 36 h after the diet switch. Switching *Daphnia* from low to high C:P diets caused a decrease in the RNA:P ratio after 48 h. *Daphnia* switched from high to low C:P diets increased their body P, RNA, and ATP content within 8–24 h. This switch from high to low C:P diets also led to increased RNA:P ratios in animal bodies. Overall, our study revealed that consumer P metabolism reflects both current and past diet due to more dynamic and rapid changes in P biochemistry than total body mass. This metabolic flexibility is likely linked to resource integration in *D. magna*, which reduces the negative effects of short-term or variable exposure to nutrient-deficient foods.

Keywords: *Daphnia* physiology, ecological stoichiometry, diet heterogeneity, P metabolite pools

INTRODUCTION

Consumers frequently face nutritional limitation in aquatic environments due to imbalances between limited supplies of elemental resources and the metabolic demands of consumers (Frost et al., 2005). Within lakes, the nutritional quality of seston (i.e., suspended particulate organic matter) varies across both short (e.g., weekly and possibly daily) and long (e.g., seasonally) timescales (Kreeger et al., 1997; Hessen et al., 2005). This variation in food quality should lead to fluctuating imbalances and elemental constraints on organismal performance and life-histories particularly for high-phosphorus (P) taxa such as *Daphnia*. Despite this, it remains unclear whether or how animals adjust metabolically to short- vs. long-term nutrient stress or how these adjustments would relate to consumer growth and body stoichiometry.

One well established effect of sustained periods of elemental limitation on consumers is slower growth rates as rates of P acquisition fail to match metabolic requirements to build new body tissues (Frost et al., 2005). However, when faced with heterogeneous dietary P supply, *Daphnia* appear to employ one of two growth mechanisms: growth or resource integration (Hood and Sterner, 2010). Growth integration is thought to occur in more homeostatic *Daphnia* species, where their growth rate is a function of the average C:P ratio of their fluctuating diets. This indicates when *Daphnia* encounter increasing or decreasing dietary nutrients their growth rates will match the supply of dietary P quickly (Hood and Sterner, 2010). Resource integration, on the other hand, may be used by less homeostatic *Daphnia* species, which possibly retain P when feeding on low C:P ratio diets for use when dietary C:P ratios subsequently increase (Hood and Sterner, 2010). Under this mechanism, growth will remain stable for a period of time after the nutrient quality of food is changed. For example, when switching from a low C:P to a high C:P diet, resource integrators should maintain growth for a period of time using stored nutrient pools to fuel growth. Once these pools are exhausted, growth integrators should decrease their growth to the new nutrient conditions. However, it is unknown how *Daphnia* carry over P from favorable environments as they are apparently unable to store significant quantities of P in vacuoles as documented for plants, algae, and bacteria (Frost et al., 2005).

One possible location of surplus P retention is within daphnid metabolic P pools, which may be adjustable over both long (days) and short (minutes to hours) time scales. These temporary pools could decouple animal nutrient content from growth requirements and alleviate growth constraints imposed by P deficient diets. Longer-term P retention might be achieved by altering tissue content of larger P pools such as ribosomes. As body ribonucleic acid (RNA) content is highly correlated with ribosome production and growth rate in zooplankton (Elser et al., 2003), decreasing dietary P content could lead to ribosome catabolism and the reallocation of released P toward the transcription of enzyme mRNA (e.g., alkaline phosphatase) needed to increase P-acquisition or for the maintenance of intracellular metabolic pools (Acquisti et al., 2009; Weiße et al., 2015). However, catabolizing ribosomes would not be an efficient response to short-term changes to dietary food quality, due to the high metabolic costs of ribosome synthesis and catabolism (Houseley and Tollervey, 2009). Alternatively, smaller P pools including energy metabolites (e.g., adenosine triphosphate; ATP) are known to rapidly change the energy status of the cell (Hardie, 2003), and changes in this pool may alleviate short-term responses to P-limitation (Wagner et al., 2015) that could be utilized for fine-scale variation (hours) in dietary P. The importance of these metabolite sources as P sources has not been generally considered from a stoichiometric perspective because ATP represents a very small amount of overall body P-content (Elser et al., 1996). However, as ATP has a role in P delivery in cells as the conversion of ATP to ADP yields an available phosphate molecule (Hardie, 2003), ATP thus may provide a mechanism, albeit a short-term one, for consumers to rapidly respond to changes in dietary C:P ratios. Alternatively, as ATP and other energy molecules are tightly regulated within the cells, they may be insensitive to dietary P and fluctuate little with changes in dietary P supply.

Here we examined responses of body P, RNA, and ATP content over short- (hours to days) and long-term (weeks) exposure to dietary P-limitation in the freshwater zooplankter, *Daphnia*. We hypothesized that body %P, %RNA, and %ATP are affected by both the duration and intensity of P-limitation due to imbalances between the dietary supply of and metabolic demand for P created by current and past diets. Under chronic limitation, we predicted that body mass, P, RNA, and ATP content would decrease throughout ontogeny in animals consuming high C:P diets. However, under acute dietary changes, we expected resource integration to be the predominant mechanism in *D. magna* as described by Hood and Sterner (2010). We predicted that animals switched from low to high C:P would continue to maintain their mass for a period of time and utilize their body P stores to fuel biomass production. After these body P pools decrease, daphnid biomass production (weight gain) should also decrease especially if compared to animals maintained in low C:P diets. If this true, we would expected to see body %P and ATP decrease before eventual changes to body RNA content. Whereas, when daphnids are switched from high to low C:P diets, we predicted their mass increase will also have a time lag. This lag between when animals encounter low C:P diets and mass increases is necessary to increase P pools to initiate ribosome production. Specifically, we predicted that body P content would change first, followed by ATP and RNA content, as P uptake is required before the production of these biomolecules can occur. Additionally, we predicted that more extreme dietary P switches would result in faster P pool changes. Alternatively, if growth integration is the main mechanism used by *D. magna* in heterogeneous dietary conditions we would expect the daphnids mass increase or decrease to the changing dietary P conditions at the same rate as the change in P-pools.

METHODS AND ANALYSIS

Algae and Daphnia Culturing

We varied *Scenedesmus obliquus* C:P content (Canadian Phycological Culture Centre strain 10, purchased as *S. acutus*)

by diluting semi-continuous cultures daily with differentially supplied P (Sterner et al., 1993; Wagner et al., 2015), to produce a range of C:P ratios (by mol). Low, intermediate and high C:P cultures contained 70, 30, and 7 μM of P, and was diluted by 55, 20, and 10% day^{-1}, respectively. Harvested algae were centrifuged at 3000 *g* and resuspended in P-free COMBO media (Kilham et al., 1998). The P-content of concentrated algal suspensions was determined on dry subsamples using the molybdate-blue colorimetric method after persulfate oxidation (APHA, 1992). Subsamples of concentrated algal cultures were also filtered onto ashed GF/C glass fiber filters to determine algal C and nitrogen (N) content using a CN Analyzer (Vario EL III, Elementar Incorporated, Mt Laurel NJ, USA). These data were used to mix nominal concentrations of the prescribed diets, and post-mixing verification was done to calculate the actual C:P ratios of each diet (Wagner and Frost, 2012; Wagner et al., 2015; Prater et al., 2016).

These diets were used to study the chronic and acute responses of animal mass and body %P, %RNA, and %ATP in *Daphnia magna*. Animals used in these experiments were clonal sisters who were held in groups of 10 animals in 400 ml of P free COMBO media (Kilham et al., 1998) and fed high nutrient quality (∼ C:P 80) *S. obliquus ad libitum*. On the morning of each experiment, we removed neonates (<24 h old) from brood jars ensuring only to use animals born in the second to fourth brood.

Responses to Chronic P-Limitation

Neonates were triple rinsed with P-free COMBO to remove residual nutrients and algal particles, and all animals were allocated to experimental treatments within an hour. Animals were grown in separate vials containing 20 mL of P-free COMBO from days 0–5 and in 40 mL of P-free COMBO from day 6–14. Daphnids were fed 4 mg CL^{-1} (day 0–5) to 8 mg CL^{-1} (day 6–14) of their prescribed diet (C:P 100 ± 8, 300 ± 24, 500 ± 45, and 700 ± 12) every other day. After 3, 6, 10, and 14 days of growth, animals were saved for body %P, %RNA, %ATP, and mass analyses to examine changes in the metabolic P-pools with chronic exposure to dietary P-limitation (Table S1).

Acute Changes in P Supply

To determine how short-term changes in dietary P supply affects animal mass, body %P, %RNA, and %ATP we grew *Daphnia* in separate 20 mL vials of P-free COMBO and fed them 4 mg C L^{-1} of a low C:P diet (C:P 100 ± 10) on days 0 and 2. On day 4, animals were rinsed, placed in 20 mL of P-free COMBO, and fed the same low C:P diets (C:P 92; nominal C:P 100) or were switched to intermediate or high C:P diets (C:P 289; nominal C:P 300 or C:P 679; nominal C:P 700, respectively). We also performed high to low C:P dietary switches by growing animals at intermediate (C:P 275 ± 20; nominal C:P 300) or high (C:P 710 ± 2; nominal C:P 700) food qualities for 4 days then switching half to low C:P diet (C:P 103 ± 9; nominal C:P 100) with the other half being maintained in the initial C:P 300/C:P 700 diets. Animal mass, body P, RNA, and ATP content were assessed for all contrasts in P-supply after 1, 2, 4, 8, 12, 24, 36, and 48 h (Table S1).

Animal Processing

After *D. magna* were individually grown, we saved animals for mass ($n = 10$), body %P, %RNA, and %ATP estimates ($n = 5$; Table S1). As dry weights could not be obtained for body %ATP and %RNA, we used a dissecting microscope to measure from the top of their eye spot to their tail spine the nearest μm using a digital camera and image software (iSolutions). The corresponding dry mass was calculated using a length-mass regression ($R^2 = 0.92$). Immediately after length measurements were taken, animals were transferred individually to 0.6 mL microfuge tubes and used to estimate body ATP or RNA content. *Daphnia* for body %RNA measurements were preserved with the addition of 100 μL RNAlater and stored at −80°C until analyzed, while animals used to quantify body %ATP were extracted right away to prevent ATP degradation.

Body P Content

Groups of 3–5 individual *Daphnia* from each sampling were placed in an aluminum cup and dried for 12 h at 60°C before being weighed on a microbalance to the nearest μg ($n = 5$). Samples were subsequently oxidized using persulfate and analyzed using the molydbate-blue colorimetric method (APHA, 1992). Animal P content was determined through visible spectrometry at 885 nm by comparing absorbance of samples to P standards.

Body RNA Content

Body %RNA content was determined following Wagner et al. (2015). Individual daphnids ($n = 5$) were removed from −80°C freezer and rinsed using TE buffer to remove RNAlater®. *Daphnia* was then transferred to a new 1.5 mL centrifuge tube containing 500 μL of TE buffer and 0.1 mm glass beads filled to the 0.1 mL mark on the centrifuge tube. Centrifuge tubes were placed in mechanical agitator (Bullet Blender 24, Next Advance) set on speed 8 for 3 min to fully homogenize *Daphnia*. Two subsamples of 50 μL were removed from each sample to determine nucleic acid fractions. To the DNA fraction, 50 μL of RNase (0.1 μg/mL) was added while 50 μL of TE buffer were added to the total nucleic acid fraction. Samples were incubated at 37°C for 15 min, and 75 μL of each sample was added to black 96 well microplate. To each well, 75 μL of RiboGreen was added and incubated for 5 min in the dark. RNA/DNA content was determined by fluorescence through the use of a microplate reader (BioTek SynergyHT, Gen5 software) with the excitation 485/20 nm and emission 528/20 nm. Body RNA content was determined by subtracting the DNA fluorescence fraction from the total nucleic acid fluorescence.

Body ATP Content

After length was determined for ATP animals, individual animals were transferred to a 0.6 mL centrifuge tube and the COMBO was removed with a pipette. We added 100 μL of −20°C methanol to each centrifuge tube to stop all enzymatic activity and preserve ATP. Daphnids were homogenized with a motorized pestle for 1 min to ensure cell lysis. An additional 400 μL of −20°C methanol was added to make the final volume of 500 μL. Body %ATP was analyzed by a stable luminescence assay CellTiter-Glo®

(Promega) following manufacturer's protocols. Briefly, samples were vortexed for 30 s, and 50 μL was pipetted in an opaque 96 well microplate with the addition of 50 μL of CellTiter-Glo®. ATP standards (Adenosine 5′-triphosphate disodium salt hydrate <99% purity) were prepared with methanol and were treated the same as samples to determine the ATP concentrations. After the addition of CellTiter-Glo®, the microplate was incubated in the dark for 5 min, placed into a microplate reader (BioTek SynergyHT, Gen5 software), and luminescence was measured with a sensitivity of 53.

Statistics

We analyzed the chronic and acute responses to food quality using 2-way ANOVAs with interaction examining the effects of age/time and diet for all response variables. *Post-hoc* differences between diets for each day/time were determined after Bonferroni corrections for multiple comparisons with a cumulative $\alpha < 0.05$ ($P < 0.0125$ chronic experiments, $P < 0.0062$ for acute experiments).

RESULTS

Chronic P-Limitation

We found significant interactive effects between age and dietary C:P ratio for animal mass, body %P, %RNA, and %ATP (**Table 1**). While weight increased throughout the chronic experiment for animals consuming all diets, significant dietary effects emerged after 6 days but only in animals consuming food with C:P ratios of 700 (**Figure 1A**, **Table 1**). By day 14, there were significant differences in mass among all dietary food C:P ratios (**Table 1**). After only 3 days of growth, lower body P content was observed for all P-limited diets with the lowest values found in C:P 500 and C:P 700 animals (**Figure 1B**). After 6–10 days, we found body P content was significantly different among all diet C:P ratios (**Table 1**). After 14 days, we found the same pattern in body P content as what emerged on day 3 with no differences between the P content of animals consuming food C:P ratios of 500 and 700. Lower dietary food P content resulted in decreased body RNA content after 3 days of growth in C:P 300 treatments and after 6 days at C:P 500 and 700 (**Figure 1C**). However, after 10 days body %RNA was similar among all food C:P ratios (**Table 1**). Relatively small changes were found in body %ATP content after 3 days of growth, but after 6–10 days, we found ATP content in *Daphnia* was affected by dietary P content (**Figure 1D**, **Table 1**). Body %ATP was reduced in animals consuming food C:P ratios 500 and 700 after 6 days and C:P ratios of 300 after 10 days (**Table 1**). These differences disappeared after 14 days of growth when no differences in ATP were observed among any of the dietary conditions.

Acute Diet Switches from Low to High C:P Diets

Daphnia switched from C:P 100 to high food C:P ratios exhibited similar changes in body mass as animals maintained in C:P 100 diets for the first 36 h (**Figure 2A**). However, after 48 h animals maintained in the C:P 100 diets weighed significantly more than animals switched to high food C:P ratios; thus the biomass production (net weight gain) was less in animals switched to high C:P diets. A significant time × diet interaction was also observed for body %P with animals rapidly decreasing their P content after 12 and 24 h when switched to C:P 300 and C:P 700 diets, respectively (**Figure 2B**). RNA content displayed a significant time × diet interaction, with the mean daphnid RNA content decreased steadily over time when animals were switched from C:P 100 to high C:P diets (**Figure 2C**). *Daphnia* switched from C:P 100 to higher C:P diets (C:P 300 and C:P 700) displayed a significant time x diet interaction with the RNA:P ratio decreasing after 48 h compared to animals maintained in C:P 100 diets (**Figure 2D**). Body %ATP displayed significant main effects for time and diet for animals switched from C:P 100 to C:P 300 and 700 diets (**Figure 2E**). When animals were switched to C:P 700 diets, their ATP content decreased significantly between 8 and 12 h, whereas animals switched to C:P 300 diets only showed slight decreases in body %ATP through time (**Figure 2E**).

TABLE 1 | Daphnid mass and P, RNA, and ATP content for animals grown on different C:P diets for 3, 6, 10, and 14 days.

	Animal mass (mg)		% P		%RNA		%ATP	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Day	286.4	<0.001	37.35	<0.001	35.3	<0.001	32.89	<0.001
Diet	142.91	<0.001	238.68	<0.001	3.04	0.036	10.18	<0.001
Day*Diet	39.68	**<0.001**	3.87	**0.001**	3.22	**0.031**	3.3	**0.002**

***Post-hoc* comparisons**

Diet	Animal mass (mg)	% P	%RNA	%ATP
DAY 3				
C:P 100	A	A	A	A
C:P 300	A	B	B	A
C:P 500	A	C	B	B
C:P 700	A	C	A	A
DAY 6				
C:P 100	A	A	A	A
C:P 300	A	B	A	AB
C:P 500	A	C	A	C
C:P 700	B	D	B	BC
DAY 10				
C:P 100	A	A	A	A
C:P 300	B	B	A	B
C:P 500	C	C	A	B
C:P 700	C	D	A	B
DAY 14				
C:P 100	A	A	A	A
C:P 300	B	B	A	A
C:P 500	C	C	A	A
C:P 700	D	C	A	A

*2-way ANOVA showing the main effects of Day and Diet with their interaction Day*Diet with the significant interaction terms in bold. Different letters indicate significant difference between diets using a post-hoc Bonferroni with a cumulative error of P < 0.05.*

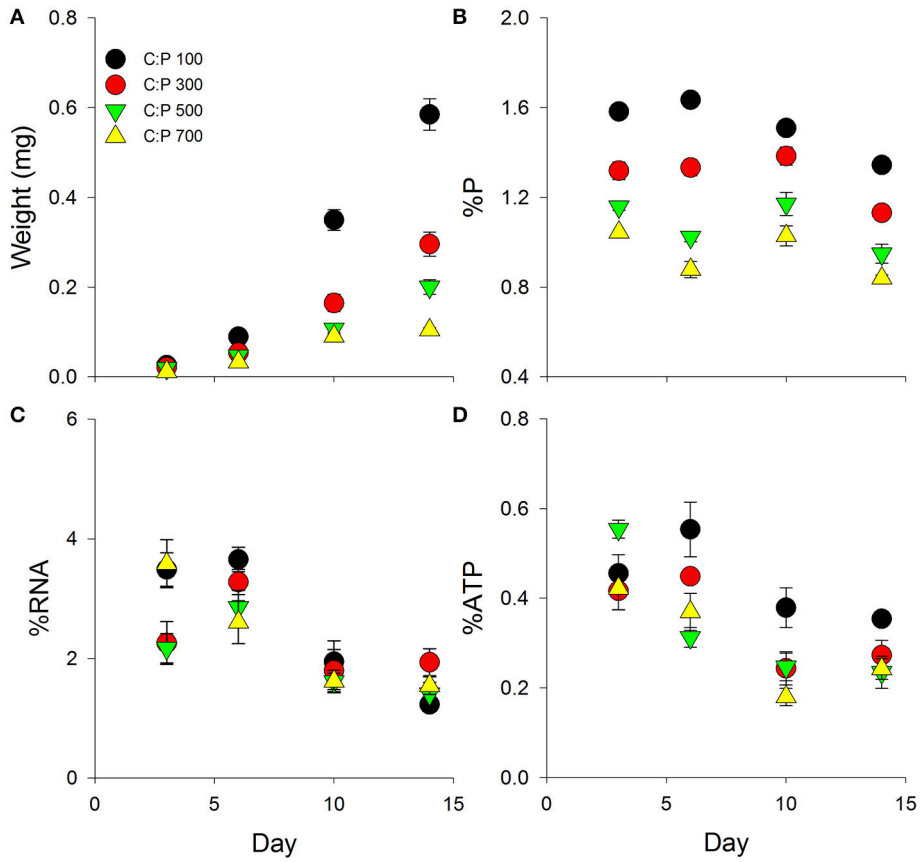

FIGURE 1 | Chronic averages ± standard error in *Daphnia* mass **(A)** and metabolic P-pools (**B**; body %P, **C**; body %RNA, **D**; body %ATP) for animals grown on the same C:P diet for 3, 6, 10, and 14 days. See **Table 1** for ANOVA results and *post-hoc* comparisons.

Acute Diet Switch from High to Low C:P Diets

Daphnia mass displayed a significant time × diet interaction with increases in biomass production in animals switched to C:P 100 diets (**Figures 3A**, **4A**). *Daphnia* body mass increased more rapidly (24 h) when switched from C:P 700 than from C:P 300 (48 h) to C:P 100 diets. Changes in body P content also differed depending on the magnitude of the dietary imbalance with animals switched from C:P 700 diets increasing after 12 h compared to animals switched from C:P 300 diets, which did not change until after 24 h (**Figures 3B**, **4B**). *Daphnia* %RNA, RNA:P ratios, and %ATP all displayed diet dependent changes. Body RNA content in animals switched from C:P 700 to C:P 100 diets increased significantly after 36 h (**Figure 4C**) but did not differ in animals switched from C:P 300 diets (**Figure 3C**). *Daphnia* switched from C:P 300 to C:P 100 diets showed no significant diet effects on their RNA:P ratios (**Figure 3D**). In contrast, the RNA:P ratio in animals switched from C:P 700 to C:P 100 diets displayed a significant time x diet interaction with ratios increasing after 48 h on low C:P food (**Figure 4D**). Animal body %ATP had significant diet and time effects with animals switched from C:P 300 diets displayed slightly higher average ATP content over time (**Figure 3E**). However, animals switched from C:P 700 diets showed much stronger changes as they differed significantly after 12 h and %ATP steadily increased over 48 h when we found the highest levels observed across all experiments (**Figure 4E**).

DISCUSSION

We found evidence to support our hypothesis that body %P, %RNA, and %ATP are affected by the duration and intensity of dietary P-limitation. As predicted, chronically P-limited animals were smaller and had lower body %RNA, %P, and %ATP. These metabolic P pools also decreased in aging animals with lower content found in larger animal bodies. Our results from short-term diet changes support findings of Hood and Sterner (2010) that resource integration mechanisms occur in *D. magna* in a heterogeneous dietary P-supply environment. For example, daphnids were able to maintain their biomass production (net weight gain) for 36 h when switched to higher C:P diets compared to animals maintained in C:P 100 diets. Additionally, when daphnids were switched to C:P 100 they incorporated P into their tissues before body %RNA and mass increases were observed. Overall, our results indicate the P pools are extremely sensitive and flexible under variable dietary P supply. These plastic metabolic responses are likely to be

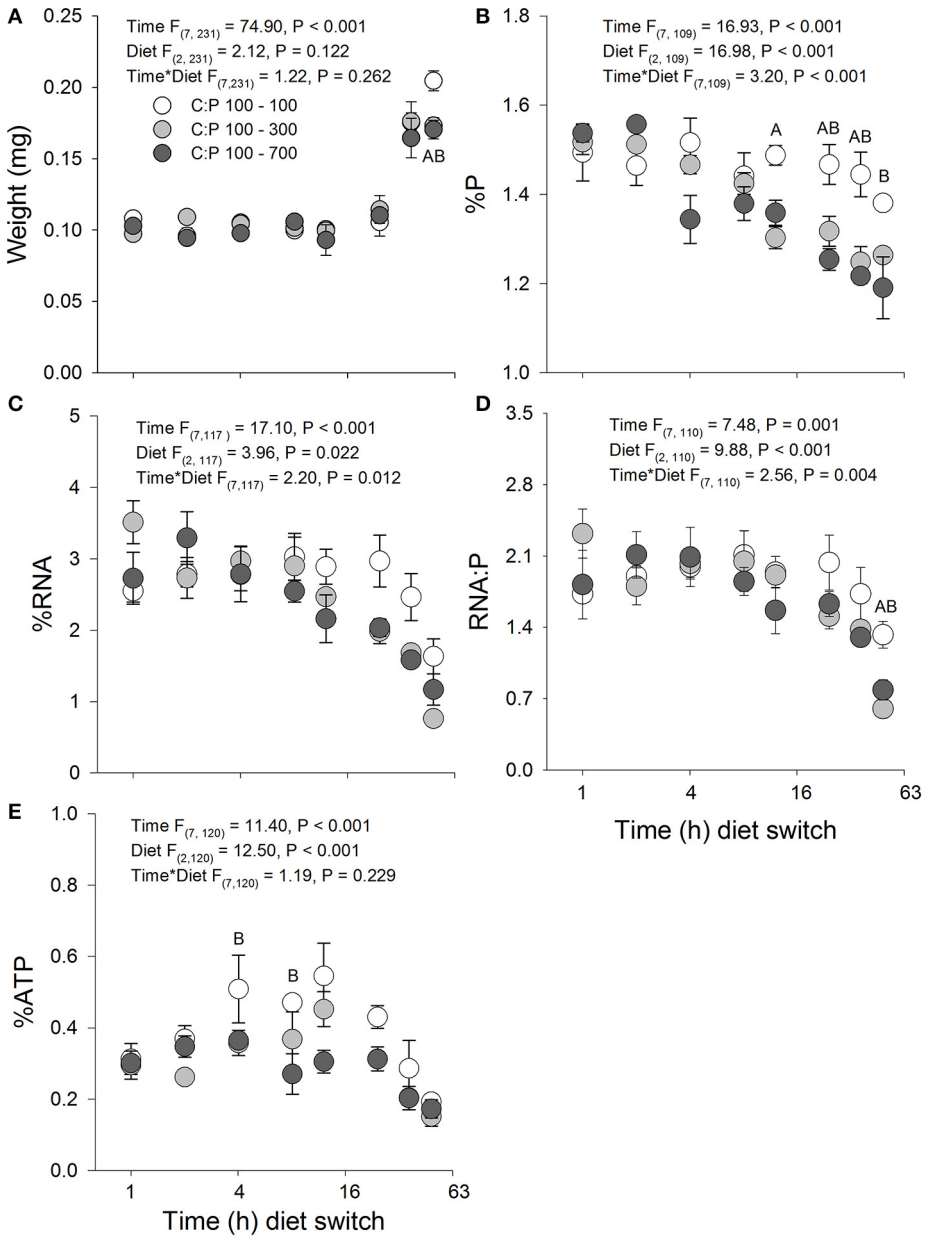

FIGURE 2 | Acute averages ± standard error in *Daphnia* mass **(A)** and metabolic P-pools (**B**; body %P, **C**; body %RNA, **D**; RNA:P, **E**; body %ATP). White circles are animals maintained at C:P 100 while light gray, and dark gray circles represent animals that were switched to C:P 300, and C:P 700, respectively. *Post-hoc* significant differences between C:P 100–300 represented with **(A)** and CP:100–700 represented with a **(B)** ($P < 0.006$).

highly beneficial in animals living in natural environments where they can frequently encounter large differences in food quality.

Consistent with previous results, *Daphnia* decreased their body %P and %RNA with high C:P diets during juvenile growth (Elser et al., 2003; Acharya et al., 2004; Wagner et al., 2015). We also found that ATP content decreased in animals provided high food C:P ratios during chronic P-limitation (Wagner et al., 2015). As predicted by the growth rate hypothesis, we saw that mass production decreased between neonate, juvenile, and adult stages concomitant with decreases in RNA and P content throughout ontogeny (Vrede et al., 1999; Elser et al., 2003). Body ATP content also decreased with age, but these changes were not linked to large declines in P content as ATP represented only 0.2–0.7% of daphnid dry mass. These dynamics are likely related to changes in life-history investment, as reduction in growth decreases the need for P-rich biomolecules (Vrede et al., 1999) and adult reproduction requires higher amounts of C-rich lipids (Tessier et al., 1983). Lipid accumulation causes the dilution of other elements and biomolecules leading to a decrease in overall P and

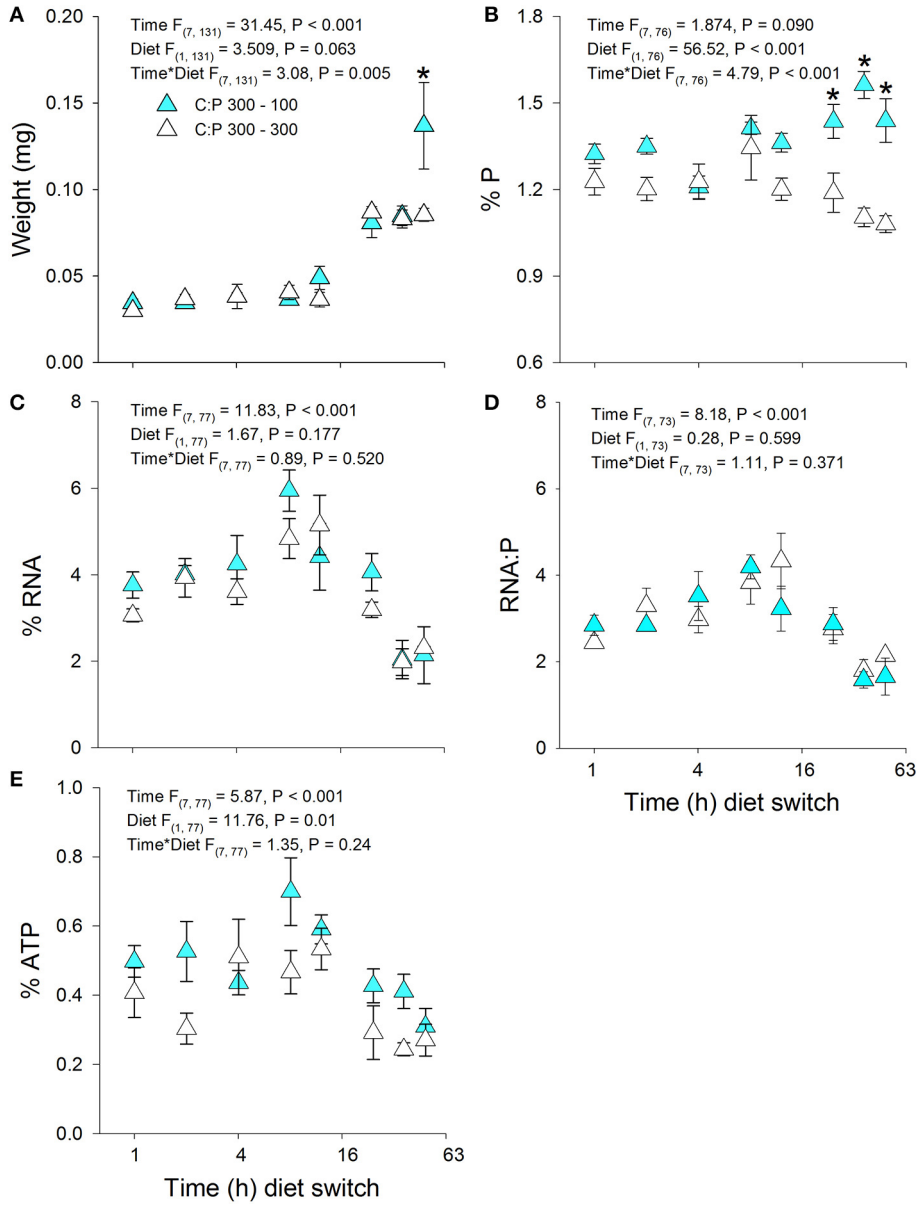

FIGURE 3 | Acute averages ± standard error in *Daphnia* mass **(A)** and metabolic P-pools (**B**; body %P, **C**; body %RNA, **D**; RNA:P, **E**; body %ATP). White triangles are animals maintained at C:P 300 while cyan triangles representing animals that were switched to C:P 100 * indicates significant changes between switched diets and non-switched diets ($P < 0.006$).

RNA content, thus altering daphnid stoichiometry under chronic P-limitation.

When *Daphnia* encounter high food C:P ratios in their diets, we found that their body P content declined within 12 h while their RNA content only started to decrease between 12 and 24 h. Gut contents can be largely excluded as a cause of body %P declines, because gut residence times are generally under 1 h (Wojewodzic et al., 2011). Some of this decrease in body %P can be attributed to the slight declines in body %RNA, as the RNA:P ratio remained constant for the first 36 h. However, animals switched to high C:P diets were able to maintain biomass production (net weigh gain) equivalent to low C:P diet animals for 36 h. Thus growth can be maintained even though there are slight decreases in RNA over the first 36 h. This result is further evidence for resource integration (Hood and Sterner, 2010). After 36 h, RNA:P ratios declined indicating there is more P per RNA and suggests that RNA catabolism is occurring. The P released from the RNA catabolism could be stored in organic P metabolites (e.g., α-glycerophosphate), which have been documented to be a significant P pools found in the hemolymph of an insect (Woods et al., 2002). If cladocerans can store organic P within their hemolymph, it could provide these

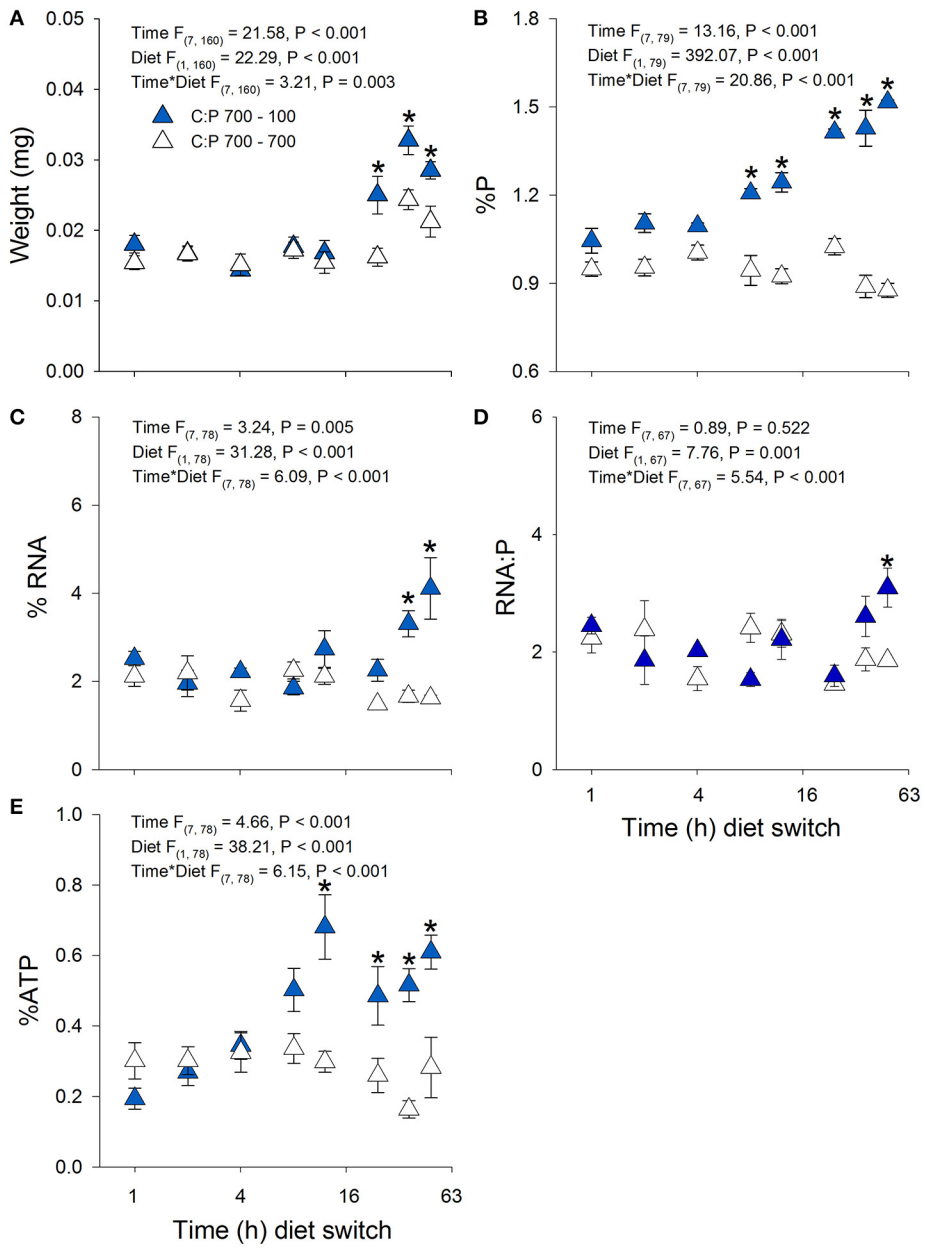

FIGURE 4 | Acute averages ± standard error in *Daphnia* mass **(A)** and metabolic P-pools (**B**; body %P, **C**; body %RNA, **D**; RNA:P, **E**; body %ATP). White triangles are animals maintained at C:P 700 while dark blue triangles representing animals that were switched to C:P 100 * indicates significant changes between switched diets and non-switched diets ($P < 0.006$).

animals with greater abilities to increase RNA synthesis if dietary C:P ratios increase again. However, it is unknown if these animals can initiate growth faster than animals switched from a high to low C:P diets.

Switching *Daphnia* from high to low C:P diets caused body %P to increase 24–40 h before RNA content increased, with faster changes in body P content seen in more severely P-limited animals. This result is especially surprising as ribosomal RNA accounts for up to 80% of the total body P content (Vrede et al., 1999; Sterner and Elser, 2002) and we would expect to see changes body %P coincide with changes in RNA content. However, it is possible physiological mechanisms to deal with low P and excess C in high C:P diets remain active for hours after animals are transferred to low C:P diets. When P-limited, *Daphnia* increase their P-acquisition enzymes (e.g., alkaline phosphatase; McCarthy et al., 2010; Elser et al., 2011; Wagner and Frost, 2012) and display increased feeding rates (Darchambeau et al., 2003). If these mechanisms remain active

for hours after animals are transferred to low C:P diets, they may contribute to the accumulation of P without direct changes in growth or RNA content. Dietary P enters through the gut lining as phosphate where it can be converted into phosphosugars (e.g., α-glycerophosphate) or metabolites such as ATP. We found evidence that P is incorporated into energy molecules as both P and ATP, increased after 8 and 12 h, respectively. These rapid changes in ATP could be priming molecular pathways with energy and P to restore P pool sizes needed to support the high material and energetic demands of ribosome and protein synthesis (Houseley and Tollervey, 2009). Altogether, these potential molecular mechanisms could allow for growth to initiate and animals to become significantly larger within 24 h when they are switched from C:P 700 to C:P 100 diets.

We found that animal body RNA:P ratios were fairly stable for the first 36 h after switching to contrasting diets. However, after 48 h the animals switched to high from low C:P diets decreased their RNA:P, while animals switched to low from high C:P diets had increased RNA:P ratios in their bodies. We suspect these changes in RNA:P ratios are caused by RNA being catabolized and contributing P into other body P pools when *Daphnia* are switched to high C:P diets. Whereas, increased RNA:P ratios when *Daphnia* are switched to low C:P diets may be due to the rapid synthesis of ribosomes that outstrips overall P accumulation by animals. Furthermore, these drastic changes in body %RNA could be caused by a change in the metabolic phenotype switching between fast and slow growth (Weiße et al., 2015). It may be important for these two distinct phenotypes to be resilient and avoid changing at the first signs of dietary heterogeneity. Delaying phenotype switches for short-time periods (i.e., up to 48 h) may prevent unnecessary energetic and resource costs of RNA anabolism and catabolism. Synthesizing large amounts of RNA when animals are switched from high to low C:P diets may have high costs associated if this process is initiated prematurely. These costs would include the resource and energetic demands of RNA synthesis and, if low C:P diets only remain for a few hours, the resource and energetic costs of breaking down RNA (Houseley and Tollervey, 2009). Premature RNA catabolism during short periods of high C:P diets could lead to slower growth and development and delayed maturity, which would have strong fitness consequences.

Overall, *Daphnia* responses to food quality differed with exposure duration and with diet. This variation in consumer metabolic responses has important implications for understanding consumer responses to nutrient limitation in nature. Consumers may frequently encounter spatio-temporal variation dietary food quality in nature (Kreeger et al., 1997; Interlandi et al., 1999), which would produce variable metabolic responses that are a function of the degree and direction of dietary switches. Despite this, most theory behind consumer-resource interactions has been built from studying consumer responses to chronic dietary P-limitation (but see Hood and Sterner, 2010). Thus, we lack a basic understanding of why differences in growth strategies (growth vs. resource integration) evolve and how these differences at the organismal level would alter higher order ecological processes at population and ecosystem-levels. Greater effort toward incorporating natural variation in food quality into laboratory experiments (Hood and Sterner, 2010, 2014) and in linking measurements of fine-scale resource variation and consumer metabolic and life-history responses (DeMott et al., 2004) would appear to be fertile areas of future work. In addition, our results highlight the fact that relationships between consumer growth, biochemistry, and elemental composition depend on both current and past dietary exposures. This reinforces the idea that consumer elemental composition should not be used as a proxy for demand (Frost and Elser, 2002; Hood and Sterner, 2014; Prater et al., 2016) and that calculations of consumer resource imbalances may not be sufficient to predict consumer responses to elemental limitation. Progress in understanding the ecological effects of consumer nutrient limitation therefore still hinges on the development of tools for directly assessing consumer nutrient status in natural ecosystems (Wagner et al., 2013; Frost et al., 2014).

We found the metabolic P pools and P content were plastic due to both chronic and acute exposure to in P-poor food. During chronic exposure to one dietary condition, animals in high C:P diets had lower body %P and RNA. While, over short-term exposure to contrasting C:P diets causes rapid changes in body %P, %RNA, and %ATP. Animal P content generally responded more rapidly compared to RNA and ATP content, with changes in biomass not occurring until 24–48 h after dietary switches which supports resource integration growth mechanisms. We offer a potential bottom-up molecular explanation for resource integration, where elements (i.e., P) must be present to make the biomolecules before altering growth rate. Additionally, delaying switching between slow and fast growing phenotypes is an efficient strategy for dealing with dietary elemental variation. In conclusion, we found *D. magna* has highly plastic metabolic P pools, which would provide growth advantages in heterogeneous food environments

ETHICS STATEMENT

This study is exempt from the animal care protocols as only invertebrates were used.

AUTHOR CONTRIBUTIONS

NW and PF designed the project, NW and CP performed the experiments and analyzed the data, all authors contributed to writing the manuscript.

ACKNOWLEDGMENTS

We would like to thank Andrea Conine, Hamaza Khattak, and Charlotte Narr for their assistance maintaining algae and *Daphnia* cultures. Both NW and CP would like to thank Ontario Graduate Scholarship for funding assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fenvs.2017.00036/full#supplementary-material

REFERENCES

Acharya, K., Kyle, M., and Elser, J. J. (2004). Effects of stoichiometric dietary mixing on *Daphnia* growth and reproduction. *Oecologia* 138, 333–340. doi: 10.1007/s00442-003-1444-8

Acquisti, C., Kumar, S., and Elser, J. J. (2009). Signatures of nitrogen limitation in the elemental composition of the proteins involved in the metabolic apparatus. *Proc. R. Soc. B. Biol. Sci.* 276, 2605–2610. doi: 10.1098/rspb.2008.1960

APHA (1992). *Standard Methods for the Examination of Water and Wastewater*. Washington, DC: APHA.

Darchambeau, F., Faerøvig, P. J., and Hessen, D. O. (2003). How *Daphnia* copes with excess carbon in its food. *Oecologia* 136, 336–346. doi: 10.1007/s00442-003-1283-7

DeMott, W. R., Pape, B. J., and Tessier, A. J. (2004). Patterns and sources of variation in *Daphnia* phosphorus content in nature. *Aquat. Ecol.* 38, 433–440. doi: 10.1023/B:AECO.0000035183.53389.66

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Dobberfuhl, D. R., Mackay, N., and Schampel, J. H. (1996). Organism size life history and N:P stoichiometry. *Bioscience* 46, 674–684. doi: 10.2307/1312897

Elser, J. J., Hayakawa, K., and Urabe, J. (2011). Nutrient limitation reduces food quality for zooplankton: *Daphnia* response to seston phosphorus enrichment. *Ecology* 82, 898–903. doi: 10.1890/0012-9658(2001)082[0898:NLRFQF]2.0.CO;2

Frost, P. C., and Elser, J. J. (2002). Growth responses of littoral mayflies to the phosphorus content of their food. *Ecol. Lett.* 5, 232–240. doi: 10.1046/j.1461-0248.2002.00307.x

Frost, P. C., Evans-white, M. A., Finkel, Z. V., Jensen, T. C., and Matzek, V. (2005). Are you what you eat? Physiological constraints on organismal stoichiometry in an elementally imbalanced world. *Okios* 109, 18–28. doi: 10.1111/j.0030-1299.2005.14049.x

Frost, P. C., Song, K., and Wagner, N. D. (2014). A beginner's guide to nutritional profiling in physiology and ecology. *Integr. Comp. Biol.* 54, 873–879. doi: 10.1093/icb/icu054

Hardie, D. G. (2003). Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology* 144, 5179–5183. doi: 10.1210/en.2003-0982

Hessen, D. O., Van Donk, E., and Gulati, R. (2005). Seasonal seston stoichiometry: effects on zooplankton in cyanobacteria-dominated lakes. *J. Plankton Res.* 27, 449–460. doi: 10.1093/plankt/fbi018

Hood, J. M., and Sterner, R. W. (2010). Diet mixing: do animals integrate growth or resources across temporal heterogeneity? *Am. Nat.* 176, 651–663. doi: 10.1086/656489

Hood, J. M., and Sterner, R. W. (2014). Carbon and phosphorus linkages in *Daphnia* growth are determined by growth rate, not species or diet. *Funct. Ecol.* 28, 1156–1165. doi: 10.1111/1365-2435.12243

Houseley, J., and Tollervey, D. (2009). The many pathways of RNA degradation. *Cell* 136, 763–776. doi: 10.1016/j.cell.2009.01.019

Interlandi, S. J., Kilham, S. S., and Theriot, E. C. (1999). Responses of phytoplankton to varied resource availability in large lakes of the Greater Yellowstone Ecosystem. *Limnol. Oceanogr.* 44, 668–682. doi: 10.4319/lo.1999.44.3.0668

Kilham, S. S., Kreeger, D., Lynn, S. G., Goulden, C. E., and Herrera, L. (1998). COMBO: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* 377, 147–159. doi: 10.1023/A:1003231628456

Kreeger, D., Goulden, C., Kilham, S. S., Lynn, S. G., Datta, S., Sebastian, J., et al. (1997). Seasonal changes in the biochemistry of lake seston. *Freshw. Biol.* 38, 539–554. doi: 10.1046/j.1365-2427.1997.00233.x

McCarthy, S. D., Rafferty, S. P., and Frost, P. C. (2010). Responses of alkaline phosphatase activity to phosphorus stress in *Daphnia magna*. *J. Exp. Biol.* 213, 256–261. doi: 10.1242/jeb.037788

Prater, C., Wagner, N. D., and Frost, P. C. (2016). Effects of calcium and phosphorus limitation on the nutritional ecophysiology of *Daphnia*. *Limnol. Oceanogr.* 61, 268–278. doi: 10.1002/lno.10208

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Sterner, R. W., Hagemeier, D. D., Smith, W. L., and Smith, R. F. (1993). Phytoplankton nutrient limitation and food quality for *Daphnia*. *Limnol. Oceanogr.* 38, 857–871. doi: 10.4319/lo.1993.38.4.0857

Tessier, A. J., Henry, L. L., Goulden, C. E., and Durand, M. W. (1983). Starvation in *Daphnia*: energy reserves and reproductive allocation. *Limnol. Oceanogr.* 28, 667–676. doi: 10.4319/lo.1983.28.4.0667

Vrede, T., Andersen, T., and Hessen, D. O. (1999). Phosphorus distribution in three crustacean zooplankton species. *Limnol. Oceanogr.* 44, 225–229. doi: 10.4319/lo.1999.44.1.0225

Wagner, N. D., and Frost, P. C. (2012). Responses of alkaline phosphatase activity in *Daphnia* to poor nutrition. *Oecologia* 170, 1–10. doi: 10.1007/s00442-012-2277-0

Wagner, N. D., Hillebrand, H., Wacker, A., and Frost, P. C. (2013). Nutritional indicators and their uses in ecology. *Ecol. Lett.* 16, 535–544. doi: 10.1111/ele.12067

Wagner, N. D., Lankadurai, B. P., Simpson, M. J., Simpson, A. J., and Frost, P. C. (2015). Metabolomic differentiation of nutritional stress in an aquatic invertebrate. *Physiol. Biochem. Zool.* 88, 43–52. doi: 10.1086/679637

Weiße, A. Y., Oyarzún, D. A., Danos, V., Swain, P. S. (2015). Mechanistic links between cellular trade-offs, gene expression, and growth. *Proc. Natl. Acad. Sci. U.S.A.* 112, E1038–E1047. doi: 10.1073/pnas.1416533112

Wojewodzic, M. W., Kyle, M., Elser, J. J., Hessen, D. O., and Andersen, T. (2011). Joint effect of phosphorus limitation and temperature on alkaline phosphatase activity and somatic growth in *Daphnia magna*. *Oecologia* 165, 837–846. doi: 10.1007/s00442-010-1863-2

Woods, H. A., Perkins, M., Elser, J., and Harrison, J. F. (2002). Absorption and storage of phosphorous by larval *Manduca sexta*. *J. Insect Physiol.* 48, 555–564. doi: 10.1016/S0022-1910(02)00060-4

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SD and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 09 February 2018
doi: 10.3389/fmicb.2018.00172

Direct and Indirect Effects of Resource P-Limitation Differentially Impact Population Growth, Life History and Body Elemental Composition of a Zooplankton Consumer

Libin Zhou[1], Kimberley D. Lemmen[1], Wei Zhang[1,2] and Steven A. J. Declerck[1]**

[1] Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands,
[2] Jiangxi Provincial Key Laboratory of Water Resources and Environment of Poyang Lake, Jiangxi Institute of Water Sciences, Nanchang, China

OPEN ACCESS

Edited by:
Robert Warner Sterner, University of Minnesota Duluth, United States

Reviewed by:
Shin-ichi Nakano, Center for Ecological Research, Kyoto University, Japan
Nicole Denise Wagner, University of Toronto Scarborough, Canada

***Correspondence:**
Libin Zhou
l.zhou@nioo.knaw.nl
Steven A. J. Declerck
s.declerck@nioo.knaw.nl

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *28 February 2017*
Accepted: *25 January 2018*
Published: *09 February 2018*

Citation:
Zhou L, Lemmen KD, Zhang W and Declerck SAJ (2018) Direct and Indirect Effects of Resource P-Limitation Differentially Impact Population Growth, Life History and Body Elemental Composition of a Zooplankton Consumer. Front. Microbiol. 9:172. doi: 10.3389/fmicb.2018.00172

One of the central tenets of ecological stoichiometry is that consumer growth rate is strongly determined by food phosphorus (P) content. In planktonic organisms population growth rates of zooplankton have repeatedly been shown to be reduced when fed with P-limited algal food sources. However, P-limitation may also affect other quality-related aspects of algae, such as biochemical composition or palatability. We studied the population growth, detailed life history and body elemental composition of the herbivorous rotifer, *Brachionus calyciflorus*, in response to three different food quality treatments: algae cultured in high phosphorus conditions (average algal molar C:P ≈ 112, 'HP'), algae cultured in low P conditions (molar C:P ≈ 631, 'LP') and low-P cultured algae spiked with P just before feeding (molar C:P ≈ 113, 'LP+P'). LP+P algae thus combined high P content with a history of growth under P-limited conditions. Total P content and the C:P ratio of rotifers in the LP+P treatment equaled those of rotifers in the HP treatment. Rotifer population growth rates were higher in HP than in LP and intermediate in the LP+P treatment. Similarly, many life history traits observed for animals in the LP+P treatment, such as somatic growth rate, age at maturity, and egg production rate were also intermediate to those observed in the LP and HP treatments. However, there were important deviations from this pattern: size at first reproduction and egg mortality in the LP+P treatment equaled the HP treatment, whereas size and development time of the first eggs equaled those of the LP treatment. Our results indicate that elemental limitation cannot fully explain reduced performance of consumers fed with P-limited algae and strongly suggest that indirect, non-stoichiometric effects of P-limitation, e.g., via changes in biochemical composition or morphology of the algae also play a major role. Furthermore, our study highlights that such indirect effects have a differential impact on major fitness components and may as such also determine the population dynamics and demographic structure of consumer populations.

Keywords: phosphorus limitation, *B. calyciflorus*, population growth, life history, organismal stoichiometry

INTRODUCTION

As a major component of the macromolecules DNA, RNA, and ATP, phosphorus (P) is an essential element for the growth and reproduction of organisms. Due to this dependence, the availability of P may strongly limit the productivity of primary producers and higher trophic levels (Hessen, 1992; DeMott and Gulati, 1999; McCarthy et al., 2006). Human activities increasingly alter the amounts and ratios of biogenic elements (e.g., carbon, nitrogen, and phosphorus) in natural systems and cause many freshwater systems to become P-limited (Stockner et al., 2000; Elser et al., 2009). A better mechanistic understanding of how P-limitation impacts the organisms in these ecosystems is therefore urgently needed.

Laboratory studies have shown strong reductions in the growth and reproduction of primary consumers when fed even high amounts of P-limited food (Sterner and Hessen, 1994; Sterner and Schulz, 1998). Such reduced performance has stimulated considerable debate about the underlying mechanisms. One potentially important cause of reduced consumer performance is pure mineral limitation: when the food resource has a very low P-content, the supply to a consumer may be too low even when food intake of the latter is at its maximum (Sterner and Hessen, 1994; DeMott, 1998). Furthermore, stoichiometric mismatches between the nutrient content of producers and consumers may also incur costs for the consumer, such as those associated with the disposal of excess C and other elements (Darchambeau et al., 2003). However, in addition to such direct effects, P-limitation may also affect the quality of producers indirectly. P-limitation in algae, for example, has been shown to decrease the amount of highly unsaturated fatty acids (Müller-Navarra, 1995; Weers and Gulati, 1997b; Spijkerman and Wacker, 2011; Challagulla et al., 2015) which are important components for consumer growth and reproduction (Weers and Gulati, 1997a; Ravet and Brett, 2006). P-limitation has also been shown to result in changes of algal cell size and cell wall morphology (van Donk and Hessen, 1995; van Donk et al., 1997). Lürling and van Donk (1997) and van Donk et al. (1997) explained reduced performance of *Daphnia* grown on P-limited algae by the lower digestibility of their thickened cell walls. DeMott (1998) demonstrated that the performance of *Daphnia* may be limited by energy even when fed high C:P algal food because of the low digestibility of P-deficient algae. These studies thus all indicate that food P-limitation may negatively affect consumers in direct as well as indirect, non-stoichiometric ways.

Ecological stoichiometry (Sterner and Elser, 2002; Hessen et al., 2013) has so far been the predominant framework contributing to a better understanding of the impact of nutrient limitation and stoichiometric mismatch on primary and secondary productivity (Malzahn et al., 2010), grazer top down control and nutrient cycling (Sistla and Schimel, 2012), the strength of trophic cascades (Hall, 2009) and trophic transfer efficiency (Rowland et al., 2015). Potentially, stoichiometric models still underestimate the full impact of nutrient limitation because indirect effects are typically not taken into account. The general lack of consideration of such indirect effects probably results from our poor understanding of the causal mechanisms underlying such effects, from the scarcity of information on their relative importance and from the difficulties inherent to incorporating these effects in mathematical models.

P-supplementation tests may provide us with a powerful experimental tool to address the relative importance of indirect, non-stoichiometric effects, even when knowledge about the causes is lacking. The approach makes use of the fact that P-limited algae are able to quickly absorb inorganic P from their environment (Lehman and Sandgren, 1982) and hinges on the assumption that the process of P-uptake is much faster than responses in other traits, such as abundance, biochemical composition or morphological features (Boersma, 2000; Elser et al., 2001). The relative importance of direct stoichiometric and indirect non-stoichiometric effects can be estimated through a comparison of the performance of consumers fed equal biomasses of P-replete (HP), P-limited (LP), and P-supplemented LP algae (LP+P). Equal performance of consumers in the LP+P as in the HP treatment indicates that direct P-limitation is the only cause of reduced performance in the LP treatment (**Figure 1**, Scenario I). Conversely, low consumer performance in the LP treatment can completely be attributed to indirect effects of P-limitation if P-supplementation results in no improved consumer performance compared to the LP treatment (**Figure 1**, Scenario III). If performance of consumers in the LP+P treatment is intermediate to the LP and HP treatments, then the relative importance of direct and indirect mechanisms can be inferred from the position of the LP+P treatment compared to LP and HP (**Figure 1**, Scenario II). A key requirement is that algae in the LP+P treatment acquire a C:P ratio equal to the HP algae.

Only few studies have used such experimental approach to evaluate the relative importance of direct and indirect effects of P-limitation on consumers. Rothhaupt (1995) found that although supplementation of P-limited algae enhanced the exponential population growth rate of the rotifer *Brachionus rubens*, it still remained considerably below that in P-rich algae and he suggested biochemical limitation as the mechanism underlying the observed indirect effect. DeMott (1998) found strong improvements of somatic growth to P-supplementation of P-limited algae in multiple *Daphnia* species; although growth of most species almost approximated the levels observed with P-rich algae, they still remained somewhat lower in most cases. Boersma (2000) and Becker and Boersma (2003) cross-combined P-treatments (LP, HP, and LP+P) with fatty acid supplementation treatments and concluded that biochemical limitation by fatty acids only becomes important when phosphorus is present in ample supply, and suggested that other factors were still at work since the joint effects of P and highly unsaturated fatty acids could not fully explain the higher growth rate observed in HP algae. Ravet and Brett (2006) demonstrated a stronger negative impact of indirect than direct P-limitation effects on *Daphnia* somatic growth and reproduction.

Nutritional requirements of a consumer organism differ between its life stages. This has been shown for stoichiometric (Urabe and Sterner, 2001; Villar-Argaiz et al., 2002; Færøvig and Hessen, 2003) as well as for biochemical requirements (Martin-Creuzburg and Von Elert, 2004; Boëchat and Adrian, 2006;

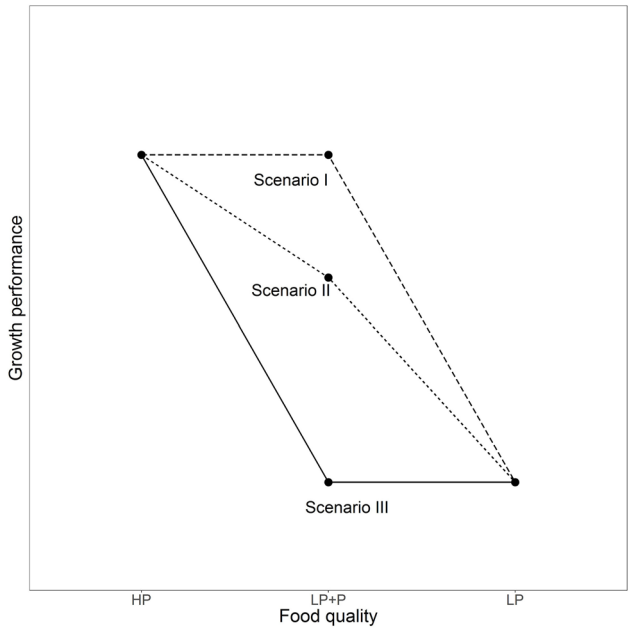

FIGURE 1 | Three potential scenarios of how the performance of consumers may respond to the food quality treatments in a P-supplementation experiment. Scenario I depicts a case where the growth reduction of consumers fed P-depleted food is uniquely caused by direct, stoichiometric effects of P-limitation. Conversely, in Scenario III, this reduction in growth performance is entirely due to non-stoichiometric indirect effects of P-limitation. In scenario II both direct and indirect effects are of large importance. HP: P-saturated food; LP: P-deficient food; LP+P: P-deficient food enriched with a P-supplement.

Wacker and Martin-Creuzburg, 2007). So far, P supplementation studies have mainly assessed the response of consumers to food quality treatments by considering general performance criteria, such as somatic growth (Boersma, 2000; Elser et al., 2001) or population growth (Rothhaupt, 1995). As a result, it remains unclear how the relative impacts of direct and indirect food quality effects vary among life history traits or major fitness components. Such information is, nevertheless, key to a better understanding of the consequences of nutrient limitation on the dynamics and demographic structure of consumer populations.

An implicit assumption of the P-supplementation method is that the accessibility of P to consumers should be equal in both LP+P and HP treatments. This may not necessarily be so. For example, a reduced digestibility of algae associated with P-limitation (van Donk et al., 1997) may result in a reduced availability of P to the consumers. Furthermore, when supplied to P-starved algal cells, anorganic phosphates may initially be stored under the form of polyphosphates in attendance of further metabolization (Eixler et al., 2006). If consumers are less able to take up and assimilate P from polyphosphates than from other P-containing biomolecules (e.g., DNA, RNA, ATP, phospholipids) then polyphosphate storage in LP+P algae could result in a reduced growth of consumers compared to those fed with HP food. To our knowledge, none of the P-supplementation studies so far have considered the possibility that a reduced accessibility of P in LP+P algae to consumers may unduly emphasize the importance of indirect effects.

With this study, using a P-supplementation approach we aimed at studying the relative importance of direct and indirect effects of P-limitation on population growth performance and a variety of life history traits, using the rotifer *B. calyciflorus* as consumer model. In an effort to evaluate whether differences exist in accessibility of P to consumers between LP+P and HP algae, we simultaneously studied the effect of food quality treatments on consumer elemental content and composition. Our results show that, whereas P-supplementation of P-limited algae enhanced P-content of algae as well as of rotifers to levels equal to those of P-replete conditions, population growth, somatic growth as well as individual fitness remained lower, indicating an important impact of non-stoichiometric, indirect effects. These effects seemed to have a differential impact on fitness components as life history traits responded in various ways to the supplementation treatment.

MATERIALS AND METHODS

Rotifer and Algae Cultures

Three clones of the rotifer *B. calyciflorus* were obtained from the resting egg banks of two Dutch lakes (D12 and D61 52°01′31.2″N, 4°11′16.8″E; E1 52°38′41.9″N, 4°43′81.7″E). *B. calyciflorus* consists of a species complex containing at least four putative species (Papakostas et al., 2016). Based on ITS1-sequences clones D12 and D61 belong to the evolutionary unit 'C' and E1 to 'D' as denoted by Papakostas et al. (2016). Stock cultures were maintained at room temperature under continuous light conditions and fed daily with the nutrient replete green alga *Chlamydomonas reinhardtii* (1000 μmol C L^{-1}). Every 3 days the rotifers were transferred to new containers with fresh medium.

All experiments were based on a comparison between three different food quality treatments: (1) algae cultured in high phosphorus conditions (molar C:P $=$ 112 $\pm$ 2.6 SE, further referred to as 'HP'), (2) algae cultured in low P conditions (molar C:P $=$ 631 $\pm$ 14.9 SE, 'LP') and (3) algae cultured in low-P media which was then spiked with inorganic phosphate prior to feeding to the rotifers (molar C:P = 113 $\pm$ 2.7 SE, LP+P). LP+P algae thus combined high P content with a history of growth under P-limited conditions.

Chlamydomonas reinhardtii was cultured in 10 continuous 2L-chemostats at 23 $\pm$ 1°C using modified WC (Woods Hole Chu-10) medium (Guillard and Lorenzen, 1972) at a dilution rate of 0.33/day (Appendix S1, Supplementary Figure S1). Five replicate chemostats with HP algae were cultured in media with 65 μmol L^{-1} P under $\approx$40 μmol quanta m^{-2} s^{-1} of continuous light. Five replicate chemostats with LP algae were cultured in media with 15 μmol L^{-1} P under $\approx$120 μmol quanta m^{-2} s^{-1} of continuous light. All chemostats were at steady state for at least 1 month prior to the experiments.

The algae for the HP and LP treatments were harvested daily from the chemostats, centrifuged (2500 rpm for 10 min) and resuspended in nutrient free WC medium. To create the LP+P treatment, inorganic phosphate (K_2HPO_4, 0.05 mol L^{-1}) was

added to centrifuged and resuspended LP algae 90 min before being fed to experimental rotifer cultures. The amount of added P was based on the algal C content estimated from cell counts (Multisizer 3^{TM} Coulter Counter, Beckman Coulter). For all three treatments, algae were kept in the dark for 90 min between their harvest and the feeding of the rotifers.

Population Level Growth Rate Experiment

Population growth rate in each food quality treatment (HP, LP, and LP+P) was estimated for all clones at *ad libitum* food concentrations. Each clone by food treatment had five resource replicates (45 experimental units, i.e., 3 clones × 3 food quality treatments × 5 chemostat replicates). Experimental units were initiated by randomly selecting 10 juvenile rotifers from a stock culture and transferring them into a 16 mL well filled with 8 mL of WC medium containing 1000 μmol C L^{-1} algae. Over the course of 22 days, wells were checked every 24 h and the number of females counted. After counting, each unit was reinitiated by transferring ten haphazardly selected individuals to a new plate with fresh medium. Only juveniles, females without eggs or females with parthenogenetic eggs were transferred, males or females with sexual eggs were not transferred. Plates were incubated at 23°C under continuous darkness.

Life Table Experiments

Using a life table experiment, we studied the effect of the three food quality treatments on rotifer life history. The design of the life table experiment consisted of a total of 225 experimental units, i.e., 3 resource qualities × 5 food chemostat replicates × 15 individuals. For reasons of feasibility and because all clones showed similar response patterns to the food quality treatments in the growth rate experiment, we only used one clone, D12.

To initiate the experiment, we used cultures as described for the growth rate experiment as a starting point. For each experimental unit in the life table design we isolated at least 10 females with parthenogenetic eggs from these cultures and transferred them to a new well with the corresponding food treatment. These wells were checked hourly for newly hatched neonates over the course of 8 h. Once observed, a neonate was individually transferred into a 3 mL well with 1 ml of algal suspension (1000 μmol C L^{-1}) of the same food quality and incubated at 23 ± 1°C in the dark at random locations in an incubator.

After the initial 8 h of their incubation, animals in all experimental units were checked every 2 h until the conclusion of the experiment. At each time point we recorded the number of eggs, the number of neonates produced during the latest interval (which were then removed), and survival. If an individual produced male eggs they were no longer monitored. In the HP and LP+P treatments individuals were monitored until the production of a fourth neonate. As development was much slower in the LP treatment these individuals were instead monitored for the first 62 h.

To obtain estimates on adult body and egg size at first reproduction, we conducted an additional but shortened version of a life table experiment. This experiment had the same design as the full life history experiment, except that only five individuals were used per resource replicate (75 experimental units, i.e., 3 resource qualities × 5 food chemostat replicates × 5 individuals). Neonates were collected in the same manner as in the life history experiment and checked hourly after 8 h. Gravid individuals were preserved in 4% formalin 2 h after the production of their first egg. Body and egg volume were measured manually under a microscope.

Algae and Rotifer Stoichiometry

Molar C:P ratios of phytoplankton in the food quality treatments were measured at day 1, 6, 11, 16, and 21 of the growth rate experiment. For the life table experiment, the algal C:P ratios were measured just before and after the experiment. Rotifer density was too low in the growth rate experiment to collect enough animals for elemental analysis. For this reason, we scaled up culture conditions of the growth rate experiment to 200 mL batch cultures. The design of this experiment consisted of 30 units, i.e., 2 clones (D12 and D61) × 3 food quality treatments × 5 food replicates. Flasks with 1000 μmol C L^{-1} of algae were initially seeded with rotifers at a density of 15 individuals mL^{-1}. Every other day rotifer density was estimated and a volume representing 3000 rotifers was transferred to a new flask, this volume was then reduced to 20 and 180 mL of fresh media was then added to the vessel. This method allowed rotifers to be cultured in a state of constant exponential growth with *ad libitum* food, similar to the cultures in the growth rate experiment. Prior to elemental analysis rotifer individuals with one egg were isolated in nutrient free WC medium for 1 h to allow emptying of the guts. C and N contents were determined using a FLASH 2000 organic element analyzer (Interscience B.V., Breda, Netherlands), while P content was determined by a QuAAtro segmented flow autoanalyzer (Beun de Ronde, Abcoude, Netherlands). Each of these analyses was based on a sample of 150 individuals. During this experiment we also measured molar C:P ratios of phytoplankton in the food quality treatments at two occasions.

Data Analysis

Exponential population growth rate was repeatedly calculated for each unit of the population level experiment as $R = \frac{\mathrm{InN_t - InN_0}}{t}$, where N_0 and N_t represent the population size at the start and end of each 24-h period. Growth rate for each unit was calculated as the mean growth rate for the last 16 days of the experiment (i.e., the period during which growth rates had stabilized).

Life table data was used to calculate mortality rate of focal individuals and of eggs, age at first egg production, egg development time, and egg production rate. Egg production rate was calculated as the total number of eggs produced per hour during a time interval encompassing at least two egg production events per individual. Finally, for each replicate we calculated the instantaneous population growth rate r using the Euler–Lotka equation $1 = \sum l_x * \mathrm{m}_x * e^{(-r*x)}$ (Stearns, 1992), where l_x represents the fraction of individuals surviving from birth to age class x, and m_x is the fraction of offspring in age class x.

Body volume at first reproduction was calculated as $V_b = \pi * L_b * (W_b/2)^2$, where L_b and W_b are body length and width at first reproduction, respectively. The volume of parthenogenetic eggs was calculated with the geometric formula for an ellipsoid: $V_e = \left(\frac{4}{3}\right) * \pi * (L_e/2) * (W_e/2)^2$, where L_e and W_e represent egg length and egg width (Appendix S2, Supplementary Figure S2). Somatic growth was estimated as the difference between the body volume of an individual at first reproduction and egg volume of the first egg for the same individual divided by the amount of time to mature from a juvenile to first egg production.

In all experiments, phytoplankton chemostats represented the true level of replication. For population growth rate, intrinsic rate of population increase *r*, phytoplankton and rotifer C:P we obtained one value for each independent replicate. Therefore, we analyzed the effect of food quality on *r* and phytoplankton C:P with one-way ANOVA whereas we evaluated the effect of food quality and its interaction with 'clone' on population growth rate and rotifer C:P with a two-way ANOVA. Whereas clone should in fact represent a random factor we still specified it as a fixed factor because it only comprises three levels. In contrast, for all other life history variables we collected data from multiple individuals per chemostat replicate. We accounted for the intrinsic dependency of these data using general linear mixed models. In these models, food chemostat replicates were specified as random factor and food quality as fixed factor. For all life history variables the significance of food quality was evaluated with a likelihood ratio test comparing the full model with the corresponding intercept model. All ANOVA and linear mixed models were studied in more detail with Tukey *post hoc* comparisons to assess the significance of differences among factor levels. All statistical analyses were performed in R software environment 3.3.1 (R Core Team, 2016). Mixed effects analyses were performed with the lme4-package (Bates et al., 2014) in R (R Core Team, 2016).

RESULTS

Growth Rate Experiment

Food quality had a strong effect on rotifer population growth rates (**Figure 2A**). A two-way ANOVA detected a significant interaction between food quality and clone identity for mean population growth rate (**Table 1**): growth rate differences among clones were clearly expressed in the HP and LP+P treatments, however, such differences proved relatively small in the LP treatment (**Figure 2B**). Yet, all clones showed a very similar response pattern to the food quality treatments: the HP treatment had the highest mean population growth rate, while the LP+P treatment was intermediate to the HP and LP treatments.

Life Table Experiments

The intrinsic rate of population increase *r* was significantly different between all treatment combinations (**Figure 3A** and **Table 1**). *r* was highest in the HP, lowest in the LP and intermediate in the LP+P treatment (*post hoc* test: HP-LP, $p < 0.001$, HP-LP+P, $p = 0.021$, LP+P-LP, $p = 0.012$). *r*-values were positive in the HP and LP+P treatments but negative in the LP treatment.

The mortality rate of experimental individuals was 8.0% in the LP, 1.4% in the LP+P and 0% in the HP treatment. Larger differences were observed in egg mortality where 23.1% of rotifer

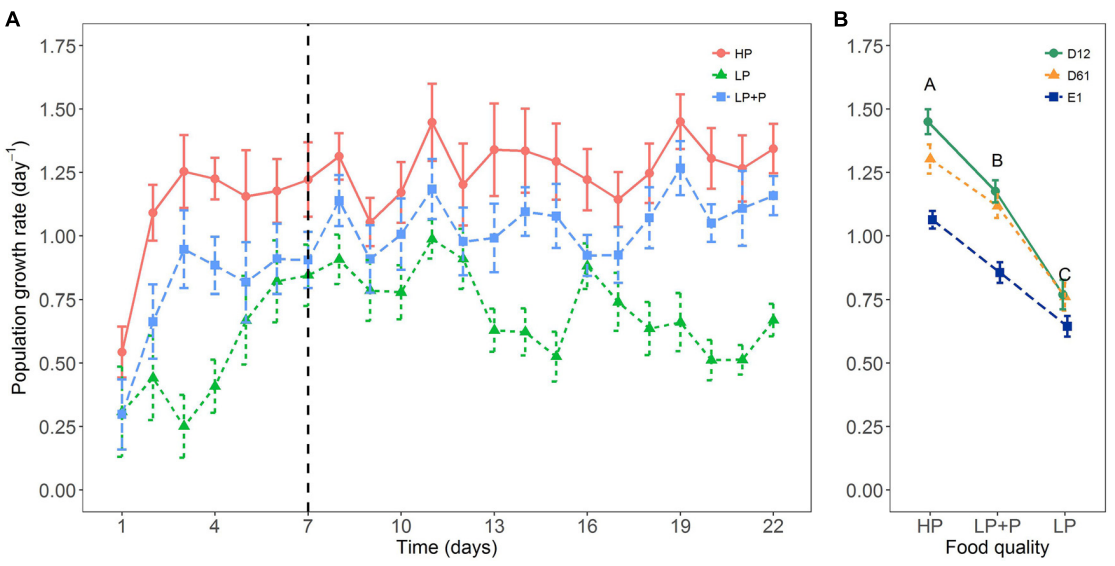

FIGURE 2 | Response of rotifer population growth rates to the three food quality treatments in the growth rate experiment. **(A)** Mean growth rate of the different food treatments for each day over the course of the experiment and **(B)** mean population growth rate (Days 7–22) of the three clone lines. Circles represent clone D12, triangles clone D61 and squares clone E1. HP: algal food cultured in P-replete conditions; LP: algal food cultured in P-depleted conditions; LP+P: LP algae spiked with inorganic phosphate just before feeding. Different letters indicate significant differences among food treatment levels as tested with a Tukey *post hoc* comparison across clones. Symbols and error bars represent the mean ± 2 standard error, respectively.

TABLE 1 | Summary of ANOVA results for population growth rate and algal and rotifer C:P ratios.

	SS	MS	df	*F*-value	*p*
Population-level growth rate experiment					
Population growth rate					
Food	2.27	1.14	2	478.4	<0.001
Clone	0.62	0.31	2	144.1	<0.001
Food * Clone	0.14	0.04	4	11.9	<0.001
Life table experiments					
Intrinsic growth rate *r*					
Food	0.04	0.02	2	21.9	<0.001
Algae and rotifer stoichiometry					
Algal C:P ratio					
Food	7.23×10^5	3.61×10^5	2	119.8	<0.001
Rotifer C:P ratio					
Food	3.37×10^4	1.69×10^4	2	179.6	<0.001
Clone	80.0	80.0	1	0.9	0.365
Food * Clone	1609	804	2	8.6	0.002

Note that an additional factor 'clone' was incorporated in the analyses of population growth rate and rotifer C:P. SS, sum of squares; MS, mean square; df, degrees of freedom.

TABLE 2 | Summary of mixed model analyses for life table results.

Fixed Effect	SS	MS	df	*F*-value
Life table experiment				
Age at first egg production				
Food quality	2372	1186	2	166.7
Development time of first egg				
Food quality	90.2	45.1	2	14.2
Egg production rate				
Food quality	1.68	0.84	2	604.5
Body size at first egg production				
Food quality	3.40	1.70	2	7.8
Size of first egg				
Food quality	0.18	0.09	2	7.8
Somatic growth rate				
Food quality	0.07	0.03	2	59.6

Food quality was specified as fixed effect in the models. SS, sum of squares; MS, mean square; df, degrees of freedom. P-values were obtained through application of the ratio likelihood test and are reported in the text of the section "Results."

eggs died before hatching in the LP treatment, in contrast to the HP and LP+P treatments where no eggs died.

The age at first egg production was lowest in the HP and highest in the LP treatment [**Figure 3B** and **Table 2**, $\chi^2(2) = 148.07$, $p < 0.001$]. Although values for this variable were higher in the LP+P treatment than in the HP treatment, they approached more those of the HP than of the LP treatment (**Figure 3B** and **Table 3**). A similar pattern was found for the ages at which subsequent eggs were produced. The development time of first egg was similar in the LP and LP+P treatments and longer than in the HP treatment [**Figure 3C**; $\chi^2(2) = 24.384$, $p < 0.001$; **Tables 2**, **3**]. The development time of subsequent eggs differed significantly among all treatments (**Figure 3C**). Egg production rate was highest in the HP and lowest in the LP [$\chi^2(2) = 338.67$, $p < 0.001$; **Table 2**]. Egg production rate in the LP+P treatment was intermediate but approached more that of the HP treatment (**Figure 3D** and **Table 3**).

Body size at first egg production in the HP did not differ significantly from the LP+P treatment (**Figure 4A**). However, in both treatments body size was significantly larger than in the LP treatment [$\chi^2(2) = 12.983$, $p < 0.002$; **Tables 2**, **3**]. In contrast, the size of first egg was not significantly different between the LP+P and LP treatments (**Figure 4B** and **Table 3**), but in both treatments it was significantly larger than in the HP treatment [$\chi^2(2) = 12.931$, $p < 0.002$; **Table 2**]. Somatic growth rate differed among all three treatments [**Figure 4C**; $\chi^2(2) = 51.508$, $p < 0.001$]. Somatic growth rate was highest in the HP treatment and intermediate in the LP+P treatment (**Tables 2**, **3**).

Algal and Rotifer Stoichiometry

Throughout the experiment the C:P ratio of the LP algae was much higher than in the other two treatments (**Figure 5A** and **Table 1**). No significant difference in the C:P ratio was observed between the HP and LP+P treatment.

A significant interaction between food quality treatment and clone was observed for rotifer body C:P ratio as well as body P and C content (**Figures 5B–D** and **Table 1**). However, both clones showed a very similar response to food quality and the majority of the variation was explained by the food quality treatment (**Table 1**). The body C:P of rotifers from the LP treatment was significantly higher than of rotifers from the HP and LP+P treatments. No significant difference in the C:P ratio was observed between the HP and LP+P treatment. These patterns were driven by variation in total body P (**Table 3**). Animals in the LP treatment contained less C than animals in the HP and LP+P treatments (**Table 3**). Nevertheless, their C:P values were higher due to a proportionally very low P content (**Figures 5C,D** and **Table 3**).

DISCUSSION

In line with previous work (Rothhaupt, 1995; DeMott, 1998; Boersma, 2000; Becker and Boersma, 2003), our P-supplementation study shows that P-limitation of primary producers negatively affects zooplankton consumers not only directly through a reduced availability of P, but also indirectly via non-stoichiometric, qualitative effects. Indeed, general performance measures of rotifers, such as somatic and population growth rates proved to be affected almost as strongly by indirect as by direct effects (**Table 3**). Novel to our study is that we were able to evaluate the relative importance of these direct and indirect effects on multiple life history traits simultaneously. Intriguingly, the response of these traits proved to differ very strongly. Some traits such as size and age at first reproduction and egg mortality were largely affected by the direct effects of P-shortage, whereas other traits (e.g., egg size and first egg development time) seemed only affected by indirect effects of P-limitation. The P content and C:P ratio of rotifers fed P-supplemented LP algae (LP+P) was equally high as in rotifers fed HP algae. This indicates that the observed reduction of rotifer performance in the LP+P compared to the HP treatment cannot be explained by a lower accessibility of P in LP+P food.

TABLE 3 | Overview table with estimates of the relative impact of direct and indirect effects of P limitation on the investigated traits of *B. calyciflorus*.

Traits	Effect source	Relative differences	*p*
Population growth rate			
(LP+P)-HP	Indirect	−17.5%	<0.001
LP-(LP+P)	Direct	−25.0%	<0.001
Somatic growth rate			
(LP+P)-HP	Indirect	−18.6%	0.001
LP-(LP+P)	Direct	−27.4%	<0.001
Age at first egg production			
(LP+P)-HP	Indirect	19.1%	<0.001
LP-(LP+P)	Direct	45.0%	<0.001
Development time of first egg			
(LP+P)-HP	Indirect	20.7%	<0.001
LP-(LP+P)	Direct	1.2%	0.940
Egg production rate			
(LP+P)-HP	Indirect	−33.8%	<0.001
LP-(LP+P)	Direct	−46.1%	<0.001
Egg mortality			
(LP+P)-HP	Indirect	0.0%	1
LP-(LP+P)	Direct	−23.1%	0.007
Body size at first egg production			
(LP+P)-HP	Indirect	−1.9%	0.889
LP-(LP+P)	Direct	−12.3%	0.007
Size of first egg			
(LP+P)-HP	Indirect	36.2%	0.005
LP-(LP+P)	Direct	2.9%	0.956
Rotifer C:P ratio			
(LP+P)-HP	Indirect	5.3%	0.67
LP-(LP+P)	Direct	90.6%	<0.001
Rotifer C content			
(LP+P)-HP	Indirect	14.6%	0.01
LP-(LP+P)	Direct	−39.8%	<0.001
Rotifer P content			
(LP+P)-HP	Indirect	8.3%	0.14
LP-(LP+P)	Direct	−70.3%	<0.001

The relative impact of direct effects was calculated as $(\mu_{LP}-\mu_{LP+P})/\mu_{HP}.100$, *whereas the relative impact of indirect effects was calculated as* $(\mu_{LP+P}-\mu_{HP})/\mu_{HP}.100$, *where* μ *refers to the mean value across replicates of a food quality treatment. Negative signs indicate reductions in trait values. P-values were obtained through Tukey post hoc comparisons.*

Animals provided with P-limited algae had lower somatic growth rate, older age of maturity, lower egg production rate, longer egg development time and higher egg mortality compared to animals grown with P-rich algae. These responses are largely in line with other studies reporting the effects of P-limitation on zooplankton life history, although most of such work has been done on *Daphnia* (Urabe and Sterner, 2001; Færøvig and Hessen, 2003; Lukas et al., 2013). To our knowledge, there are only two studies reporting on the impact of P-limitation on rotifer life history. When feeding *B. calyciflorus* P-limited algae, Jensen and Verschoor (2004) observed a lower somatic growth rate, an older age at first egg production and a shorter reproductive period compared to animals fed P-replete algae although egg mortality and total life span remained unaffected. Conversely, in a study of the rotifer *Keratella cochlearis*, Ramos-Rodríguez and Conde-Porcuna (2003) observed a lower offspring production, a higher age at maturity, and a lower life span in animals fed with P-replete compared to P-limited *Cryptomonas* algae. However, in this experiment the C:P of the nutrient sufficient *Cryptomonas* was higher than that of the P-limited *Cryptomonas*.

In our study, the enhancement of growth performance following supplementation of P-limited algae with inorganic P supports the idea that consumer productivity is strongly impacted by the quantitative lack of P and the associated stoichiometric imbalance. However, our results also indicate that such direct effects of P-limitation cannot fully explain the decreased performance of rotifers under P-limited food conditions. The C:P ratio of algae in the LP+P treatment was equal to that of the HP algae. Similarly, the body P content and the C:P ratio of adult rotifers fed LP+P food was similar to that of animals fed with HP food, and both were substantially different from rotifers in the LP treatment. We therefore conclude that it is unlikely that morphological changes induced by a history of P-limitation or that the form of P-storage in LP+P algae has reduced accessibility of P to the consumers. Nevertheless, population growth rate remained considerably lower than in rotifers fed HP algae. This result suggests that P-limitation induced non-stoichiometric qualitative changes in phytoplankton which negatively affected its suitability as food for zooplankton.

Our results are in line with a number of other P-supplementation studies (Rothhaupt, 1995; DeMott, 1998; Boersma, 2000; Becker and Boersma, 2003) which suggested important indirect effects of food P-limitation on zooplankton consumer performance. Furthermore, through our life table data, we are able to assess the relative importance of direct stoichiometric and indirect non-stoichiometric effects of algal

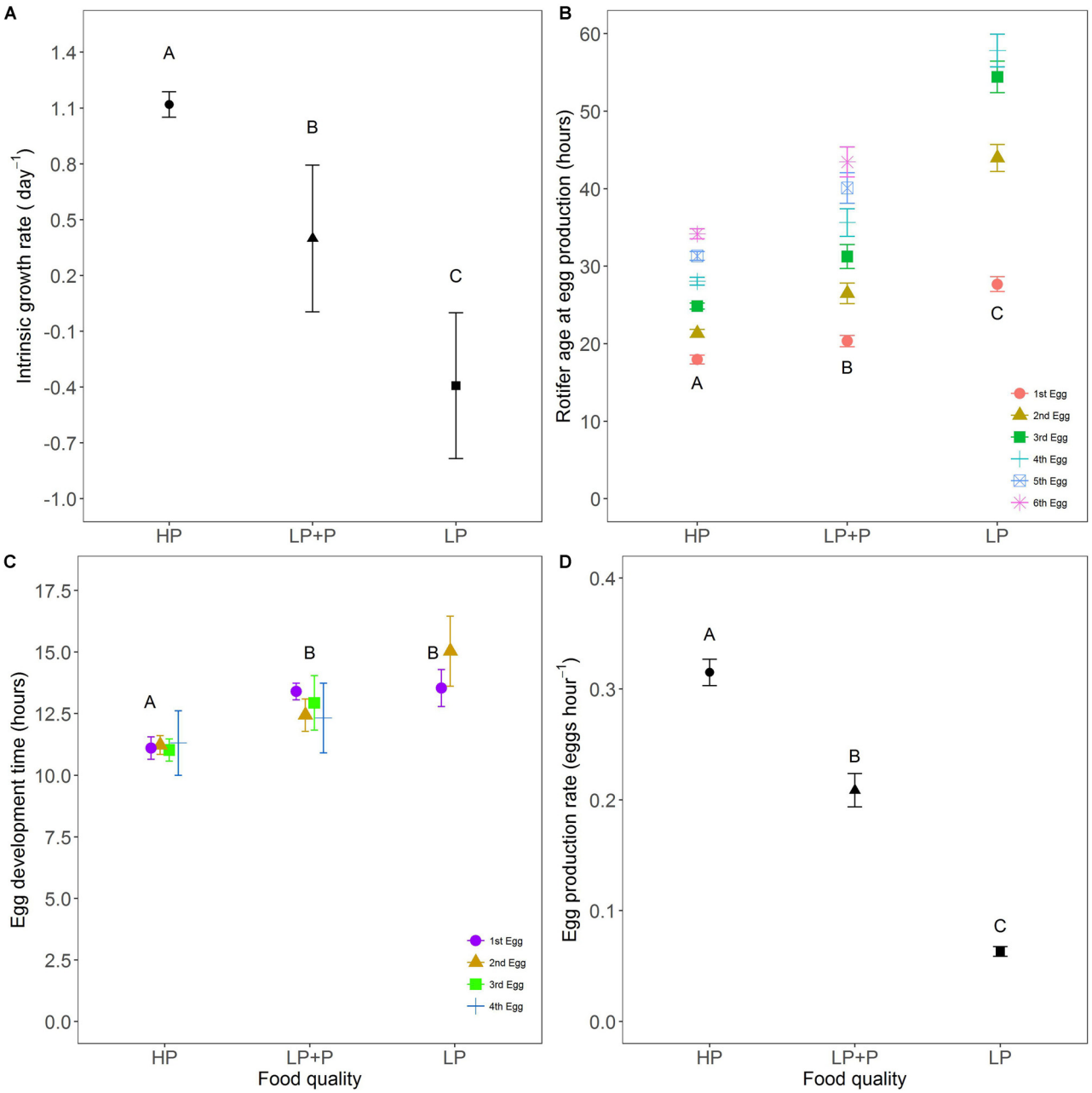

FIGURE 3 | Life history traits in response to food quality treatments. **(A)** Intrinsic population growth rate **(B)** age at egg production, **(C)** egg development time, and **(D)** egg production rate. HP: algal food cultured in P-replete conditions; LP: algal food cultured in P-depleted conditions; LP+P: LP algae spiked with inorganic phosphate just before feeding. Different letters indicate significant differences among food treatment levels as tested with a Tukey *post hoc* comparison. Letters in **(B,C)** only represent analysis results for the first eggs produced. Symbols and error bars represent the mean ± 2 standard error, respectively.

P-limitation on multiple fitness components, simultaneously. Most life history traits seemed to respond to P-addition, but still bore a clear signature of indirect effects of P-limitation. Similar to the population growth rates measured in the population-level culture experiment, somatic growth rate and intrinsic rate of population increase reached values in the LP+P treatment that were intermediate to that in the LP and HP treatments. Similarly, egg production rate and age at first egg production in the LP+P treatment were also intermediate to LP and HP although they appeared to be more strongly influenced by P addition because their values approached more those of the HP than the LP treatment.

However, other traits deviated strongly from such pattern. Both size at first reproduction and egg mortality in the LP+P treatment equaled that of the HP treatment, suggesting these traits are exclusively impacted by the direct effects of

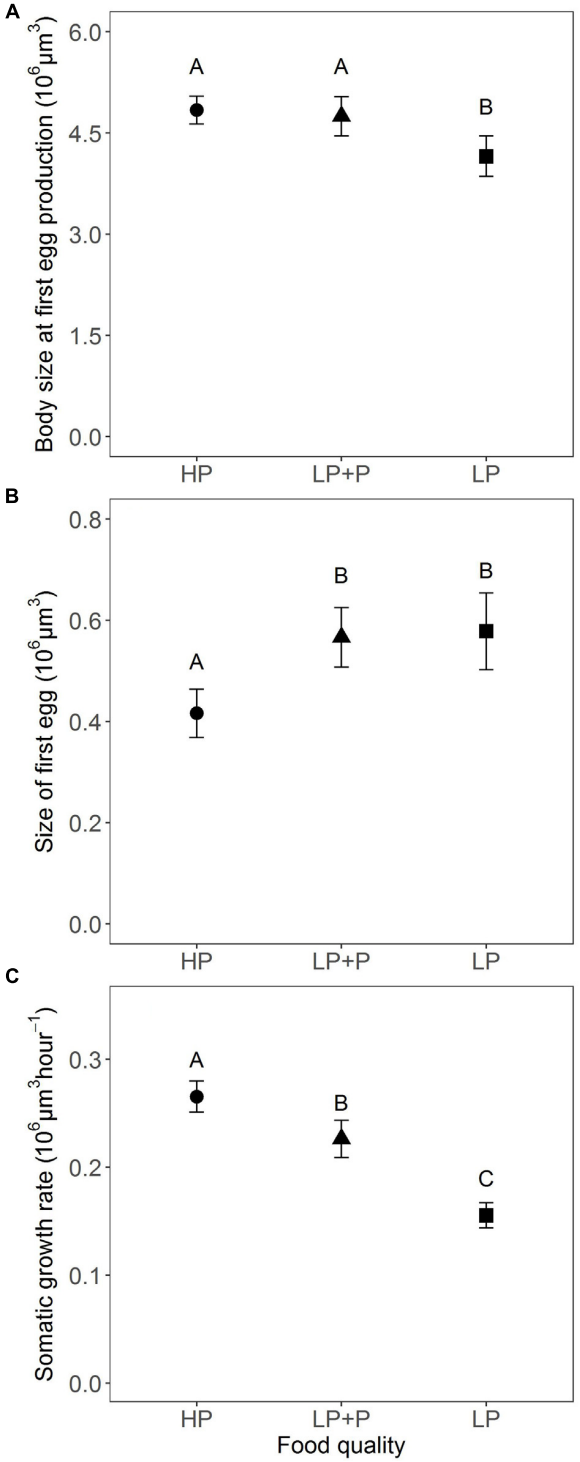

FIGURE 4 | Size-related traits in response to food quality treatments. **(A)** Estimated body size at first egg production, **(B)** estimated size of first egg, and **(C)** estimated somatic growth rate. HP: algal food cultured in P-replete conditions; LP: algal food cultured in P-depleted conditions; LP+P: LP algae spiked with inorganic phosphate just before feeding. Different letters indicate significant differences among food treatment levels as tested with a Tukey *post hoc* comparison. Symbols and error bars represent the mean ± 2 standard error, respectively.

P-limitation. Conversely, size and development time of the first egg showed no response to P-addition and appeared to be entirely controlled by indirect effects of P-limitation. Our results therefore clearly demonstrate a differential sensitivity of different fitness components to indirect and direct effects of P-limitation in the food resource. Likely this is reflective of the fact that both stoichiometric (Urabe and Sterner, 2001; Villar-Argaiz et al., 2002; Becker and Boersma, 2003; Færøvig and Hessen, 2003) and biochemical requirements (Martin-Creuzburg and Von Elert, 2004; Wacker and Martin-Creuzburg, 2007) vary among the different predominant physiological processes that characterize ontogenetic stages of the consumers. For example, fast somatic growth of juvenile stages is known to be highly dependent on the availability of P (cf. 'growth rate hypothesis,' Elser et al., 2003). In contrast, egg development may be more dependent on the availability of specific biochemical substances. For example, *Daphnia* eggs have been shown to contain disproportional amounts of fatty acids compared to somatic tissue (Wacker and Martin-Creuzburg, 2007), especially polyunsaturated fatty acids (PUFA's) such as eicosapentaenoic acid (EPA). Wacker and Martin-Creuzburg (2007) demonstrated that poor biochemical quality of food reduced the amount of these essential fatty acids in *Daphnia* eggs, and suggested an important role of biochemical compounds for egg development. Possibly, the slower development rate of eggs in the LP+P and LP treatments may have been the result of lower biochemical quality. We can only speculate about the mechanisms that may underlie our observation of larger eggs in the LP and LP+P treatments compared to the HP treatment. Larger eggs often reflect increased allocation of carbon resources of the mother to its progeny (Gliwicz and Guisande, 1992; Kirk, 1997). It is possible that mother animals in the LP treatment discarded excess C into their eggs (Urabe and Sterner, 2001). Rotifers of clone D12 contained more C in the LP+P treatment than in the HP treatment, despite equal C-availability and C:P ratio of these food treatments. Possibly, the larger egg size observed in the LP+P treatment also reflected a C allocation strategy of adults toward their eggs similar as in the LP treatment.

Morphological changes in phytoplankton have also been suggested to be the cause of reduced consumer performance under conditions of P-limitation. Algae have been reported to respond to nutrient limitation with an increase in cell size (van Donk and Hessen, 1995) and increased thickness of their cell wall (van Donk and Hessen, 1993; van Donk et al., 1997). In filter feeders like *Daphnia*, these morphological changes improve viable gut passage and explain reduced clearance and population growth rates of these grazers when fed P-limited algae (Lürling and van Donk, 1997; van Donk et al., 1997). However, although cell size increased in response to P-limitation in our experiment, they remained well within the limits of the food particle size range ingestible for *B. calyciflorus* (Rothhaupt, 1990). Additionally, in contrast to *Daphnia*, rotifers crush ingested food with a specialized stomach (mastax; Gilbert and Starkweather, 1977), hence, it is doubtful that cell wall thickening would allow gut passage of intact cells. Rothhaupt (1995) observed no reduction in grazing rates of *B. rubens* on P-limited algae, whereas P-limitation

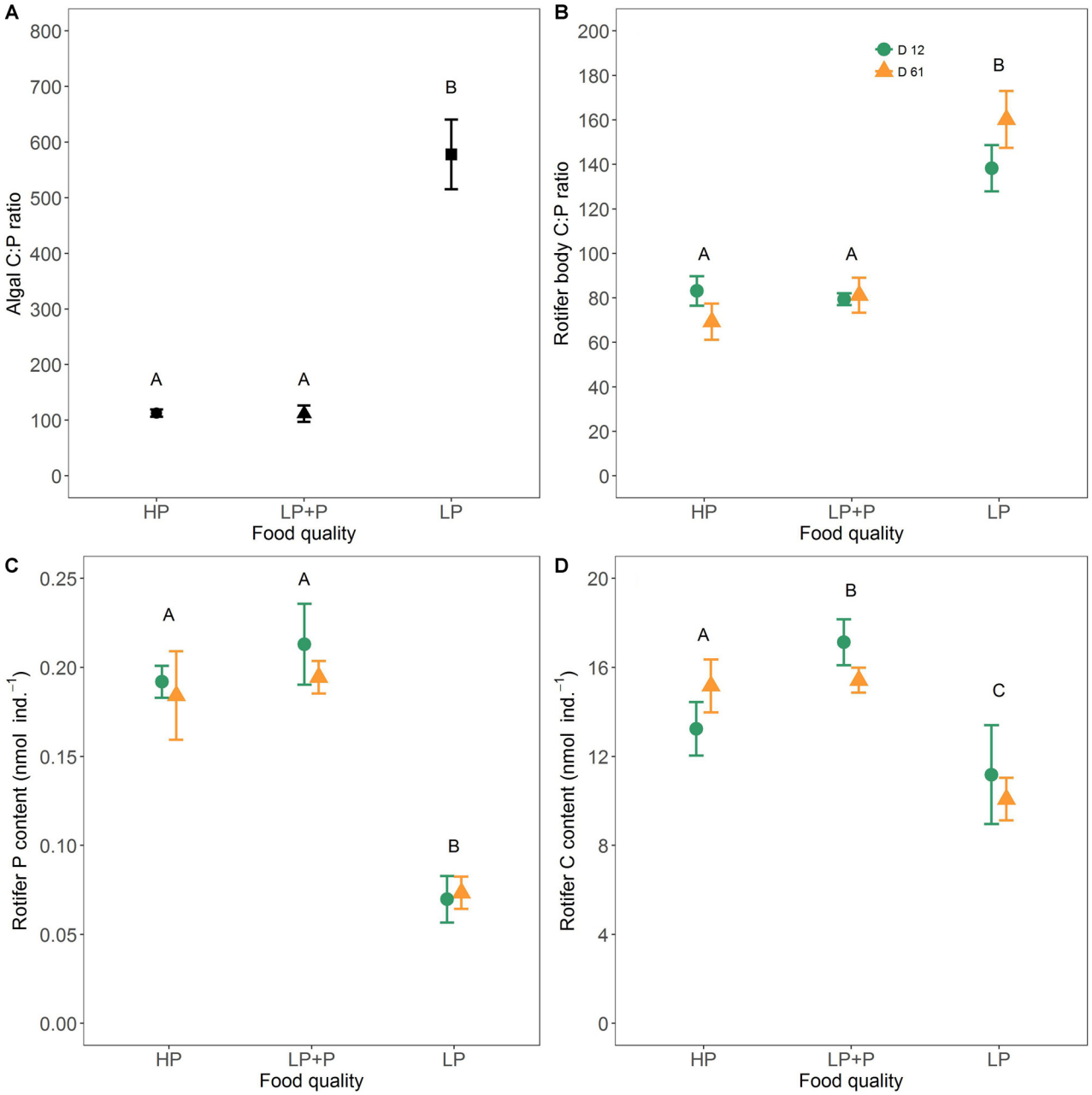

FIGURE 5 | Stoichiometric ratios of algal food and rotifers. **(A)** Averages over time of the molar C:P ratios of the three food quality treatments, **(B)** body C:P ratios of rotifers raised on the three food quality treatments, **(C)** body P content of rotifers raised on the three food treatments, and **(D)** body C content of rotifers raised on the three food treatments. Green circles represent clone D12, and yellow triangles clone D61. Different letters indicate significant differences among food treatment levels as tested with a Tukey *post hoc* comparison across clones. Symbols and error bars represent the mean ± 2 standard error, respectively.

has also been found to result in increased clearance rates (Suzuki-Ohno et al., 2012). Finally, in our experiment, rotifer body C and P content did not decrease in the LP+P compared to the HP treatment, suggesting no reduction in C and P ingestion and assimilation efficiencies.

Our study highlights that the performance of consumers provided with a phosphorus limited resource is not exclusively affected by the quantitative reduction of available P and the corresponding stoichiometric mismatch with their elemental requirements. Consumer performance was also impacted by the qualitative deterioration of the food as a result of the resource growth environment that acted independently of elemental content or stoichiometric ratios of the final food resource. In our study, such indirect qualitative effects proved to contribute strongly to the observed reductions in consumer population growth under P-limited conditions. Importantly, the

magnitude of the impact of these indirect effects seemed to differ between different key fitness components of consumers. Given the strong link between life history and population demography, this suggests that such effects may also have an important impact on the structure and dynamics of consumer populations. Furthermore, the relatively large impact of the indirect effects of P-limitation in our results highlight their potential importance in determining the strength of producer-consumer bottom-up control and the efficiency of energy transfer between trophic levels. A better knowledge of the consequences of non-stoichiometric food quality effects of P-limitation on consumer populations may therefore be crucial for a better understanding of the true nature of P-limitation effects in natural communities.

AUTHOR CONTRIBUTIONS

LZ and SD developed the idea and designed the experiments. LZ carried out the growth rate and batch culture experiments. LZ, KL, WZ, and SD conducted the life table experiment. Data analysis was mainly performed by LZ and KL. LZ, KL, and SD wrote the manuscript.

FUNDING

This work is funded by the Ph.D. grant of the China Scholarship Council (CSC) to LZ and by the Division for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO) through project 823.01.011.

ACKNOWLEDGMENTS

We wish to thank Dennis Waasdorp, Els Faassen, and Dedmer van de Waal for their advice regarding the set-up of the phytoplankton chemostats. We also thank Nico Helmsing and Erik Reichman for carrying out nutrient analyses as well as Caelen Miller and Laurens Verhage for their help in the lab.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00172/full#supplementary-material

REFERENCES

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2014). *lme4: Linear Mixed-Effects Models Using Eigen and S4*. Available at: http://CRAN.Rproject.org/package=lme4 [accessed September 18, 2014].

Becker, C., and Boersma, M. (2003). Resource quality effects on life histories of *Daphnia*. *Limnol. Oceanogr.* 48, 700–706. doi: 10.1016/j.aquatox.2009.06.011

Boëchat, I. G., and Adrian, R. (2006). Evidence for biochemical limitation of population growth and reproduction of the rotifer *Keratella quadrata* fed with freshwater protists. *J. Plankton Res.* 28, 1027–1038. doi: 10.1093/plankt/fbl036

Boersma, M. (2000). The nutritional quality of P-limited algae for *Daphnia*. *Limnol. Oceanogr.* 45, 1157–1161. doi: 10.4319/lo.2000.45.5.1157

Challagulla, V., Fabbro, L., and Nayar, S. (2015). Biomass, lipid productivity and fatty acid composition of fresh water microalga *Rhopalosolen saccatus* cultivated under phosphorous limited conditions. *Algal Res.* 8, 69–75. doi: 10.1016/j.algal.2015.01.010

Darchambeau, F., Faerøvig, P. J., and Hessen, D. O. (2003). How *Daphnia* copes with excess carbon in its food. *Oecologia* 136, 336–346. doi: 10.1007/s00442-003-1283-7

DeMott, W. R. (1998). Utilization of a cyanobacteria and a phosphorus-deficient green alga as complementary resources by *Daphnias*. *Ecology* 79, 2463–2481. doi: 10.1890/0012-9658(1998)079[2463:UOACAA]2.0.CO;2

DeMott, W. R., and Gulati, R. D. (1999). Phosphorus limitation in *Daphnia*, evidence from a long term study of three hypereutrophic Dutch lakes. *Limnol. Oceanogr.* 44, 1157–1164. doi: 10.4319/lo.1999.44.6.1557

Eixler, S., Karsten, U., and Selig, U. (2006). Phosphorus storage in *Chlorella vulgaris* (Trebouxiophyceae, Chlorophyta) cells and its dependence on phosphate supply. *Phycologia* 45, 53–60. doi: 10.2216/04-79.1

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Anderson, T., Baron, J. S., Bergström, A., Jansson, M., Kyle, M., et al. (2009). Shifts in lake N:P stoichiometry and nutrient limitation driven by atmospheric nitrogen deposition. *Science* 326, 835–837. doi: 10.1126/science.1176199

Elser, J. J., Hayakawa, K., and Urabe, J. (2001). Nutrient limitation reduces food quality for zooplankton: *Daphnia* response to seston phosphorus enrichment. *Ecology* 82, 898–903. doi: 10.2307/2680208

Færøvig, P. J., and Hessen, D. O. (2003). Allocation strategies in crustacean stoichiometry: the potential role of phosphorus in the limitation of reproduction. *Freshw. Biol.* 48, 1782–1792. doi: 10.1046/j.1365-2427.2003.01128.x

Gilbert, J. J., and Starkweather, P. L. (1977). Feeding in the rotifer *Brachionus calyciflorus*. I. Regulatory mechanisms. *Oecologia* 28, 125–131. doi: 10.1007/BF00345247

Gliwicz, Z. M., and Guisande, C. (1992). Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food webs. *Oecologia* 91, 463–467. doi: 10.1007/BF00650317

Guillard, R. R. L., and Lorenzen, C. J. (1972). Yellow-green algae with chlorophyllide C. *J. Phycol.* 8, 10–14.

Hall, S. R. (2009). Stoichiometrically explicit food webs: feedbacks between resource supply, elemental constraints, and species diversity. *Annu. Rev. Ecol. Syst.* 40, 503–528. doi: 10.1146/annurev.ecolsys.39.110707.173518

Hessen, D. O. (1992). Nutrient element limitation of zooplankton production. *Am. Nat.* 140, 799–814. doi: 10.1086/285441

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Jensen, T. C., and Verschoor, A. M. (2004). Effects of food quality on life history of the rotifer *Brachionus calyciflorus* Pallas. *Freshw. Biol.* 49, 1138–1151. doi: 10.1111/j.1365-2427.2004.01255.x

Kirk, L. K. (1997). Egg size, offspring quality and food level in planktonic rotifers. *Freshw. Biol.* 37, 515–521. doi: 10.1046/j.1365-2427.1997.00177.x

Lehman, J. T., and Sandgren, C. D. (1982). Phosphorus dynamics of the procaryotic nannoplankton in a Michigan lake. *Limnol. Oceanogr.* 27, 828–838. doi: 10.4319/lo.1982.27.5.0828

Lukas, M., Frost, P. C., and Wacker, A. (2013). The neonate nutrition hypothesis: early feeding affects the body stoichiometry of *Daphnia* offspring. *Freshw. Biol.* 58, 2333–2344. doi: 10.1111/fwb.12213

Lürling, M., and van Donk, E. (1997). Life history consequences for *Daphnia* pulex feeding on nutrient-limited phytoplankton. *Freshw. Biol.* 38, 693–709. doi: 10.1046/j.1365-2427.1997.00242.x

Malzahn, A. M., Hantzsche, F., Schoo, K. L., Boersma, M., and Aberle, N. (2010). Differential effects of nutrient-limited primary production on primary, secondary or tertiary consumers. *Oecologia* 162, 35–48. doi: 10.1007/s00442-009-1458-y

Martin-Creuzburg, D., and Von Elert, E. (2004). Impact of 10 dietary sterols on growth and reproduction of *Daphnia galeata*. *J. Chem. Ecol.* 30, 483–500. doi: 10.1023/B:JOEC.0000018624.94689.95

McCarthy, V., Donohue, I. A. N., and Irvine, K. (2006). Field evidence for stoichiometric relationships between zooplankton and N and P availability in a shallow calcareous lake. *Freshw. Biol.* 51, 1589–1604. doi: 10.1111/j.1365-2427.2006.01599.x

Müller-Navarra, D. C. (1995). Biochemical versus mineral limitation in *Daphnia*. *Limnol. Oceanogr.* 40, 1209–1214. doi: 10.1007/s004420100728

Papakostas, S., Michaloudi, E., Proios, K., Brehm, M., Verhage, L., Rota, J., et al. (2016). Integrative taxonomy recognizes evolutionary units despite widespread mitonuclear discordance: evidence from a rotifer cryptic species complex. *Syst. Biol.* 65, 508–524. doi: 10.1093/sysbio/syw016

R Core Team (2016). *R: A Language and Environment for Statistical Computing*. Available at: http://www.R-project.org [accessed February 27, 2017].

Ramos-Rodríguez, E., and Conde-Porcuna, J. M. (2003). Nutrient limitation of a planktonic rotifer: life history consequences and starvation resistance. *Limnol. Oceanogr.* 48, 933–938. doi: 10.4319/lo.2003.48.2.0933

Ravet, J. L., and Brett, M. T. (2006). Phytoplankton essential fatty acid and phosphorus content constraints on *Daphnia* somatic growth and reproduction. *Limnol. Oceanogr.* 51, 2438–2452. doi: 10.4319/lo.2006.51.5.2438

Rothhaupt, K. O. (1990). Differences in particle size-dependent feeding efficiencies of closely related rotifer species. *Limnol. Oceanogr.* 35, 16–23. doi: 10.4319/lo.1990.35.1.0016

Rothhaupt, K. O. (1995). Algal nutrient limitation affects rotifer growth rate but not ingestion rate. *Limnol. Oceanogr.* 40, 1201–1208.

Rowland, F. E., Bricker, K. J., Vanni, M. J., and González, M. J. (2015). Light and nutrients regulate energy transfer through benthic and pelagic food chains. *OIKOS* 124, 1648–1663. doi: 10.1111/oik.02106

Sistla, S. A., and Schimel, J. P. (2012). Stoichiometric flexibility as a regulator of carbon and nutrient cycling in terrestrial ecosystems under change. *New Phytol.* 196, 68–78. doi: 10.1111/j.1469-8137.2012.04234.x

Spijkerman, E., and Wacker, A. (2011). Interactions between P-limitation and different C conditions on the fatty acid composition of an extremophile microalga. *Extremophiles* 15, 597–609. doi: 10.1007/s00792-011-0390-3

Stearns, S. C. (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Sterner, R. W., and Hessen, D. O. (1994). Algal nutrient limitation and the nutrition of aquatic herbivores. *Annu. Rev. Ecol. Syst.* 25, 1–29. doi: 10.1146/annurev.es.25.110194.000245

Sterner, R. W., and Schulz, K. L. (1998). Zooplankton nutrition: recent progress and a reality check. *Aquat. Ecol.* 32, 261–279. doi: 10.1146/annurev.es.25.110194.000245

Stockner, J. G., Rydin, E., and Hyenstrand, P. (2000). Cultural oligotrophication: causes and consequences for fisheries resources. *Fisheries* 25, 7–14. doi: 10.1577/1548-84462000025<0007:CO<2.0.CO:2

Suzuki-Ohno, Y., Kawata, M., and Urabe, J. (2012). Optimal feeding under stoichiometric constraints: a model of compensatory feeding with functional response. *OIKOS* 121, 569–578. doi: 10.1111/j.1600-0706.2011.19320.x

Urabe, J., and Sterner, R. W. (2001). Contrasting effects of different types of resourse depletion on life history traits in *Daphnia*. *Funct. Ecol.* 15, 165–174. doi: 10.1046/j.1365-2435.2001.00511.x

van Donk, E., and Hessen, D. O. (1993). Grazing resistance in nutrient-stressed phytoplankton. *Oecologia* 93, 508–511. doi: 10.1007/BF00328958

van Donk, E., and Hessen, D. O. (1995). Reduced digestibility of UV-B stressed and nutrient-limited algae by *Daphnia* magna. *Hydrobiologia* 307, 147–151. doi: 10.1007/BF00032006

van Donk, E., Lürling, M., Hessen, D. O., and Lokhorst, G. M. (1997). Changed cell wall morphology in nutrient deficient phytoplankton and its impact on grazers. *Limnol. Oceanogr.* 42, 357–364. doi: 10.4319/lo.1997.42.2.0357

Villar-Argaiz, M., Medina-Sanchez, J. M., and Carrillo, P. (2002). Linking life history strategies and ontogeny in crustacean zooplankton: Implications for homeostasis. *Ecology* 83, 1899–1914. doi: 10.1890/0012-96582002083[1899:LLHSAO]2.0.CO;2

Wacker, A., and Martin-Creuzburg, D. (2007). Allocation of essential lipids in *Daphnia* magna during exposure to poor food quality. *Funct. Ecol.* 21, 738–747. doi: 10.1111/j.1365-2435.2007.01274.x

Weers, P. M. M., and Gulati, R. D. (1997a). Effect of the addition of polyunsaturated fatty acids to the diet on the growth and fecundity of *Daphnia galeata*. *Freshw. Biol.* 38, 721–729. doi: 10.1046/j.1365-2427.1997.00237.x

Weers, P. M. M., and Gulati, R. D. (1997b). Growth and reproduction of *Daphnia galeata* in response to changes in fatty acids, phosphorus and nitrogen in *Chlamydomonas reinhardtii*. *Limnol. Oceanogr.* 42, 1584–1589. doi: 10.4319/lo.1997.42.7.1584

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 12 July 2017
doi: 10.3389/fmicb.2017.01297

Stoichiometric Mismatch between Consumers and Resources Mediates the Growth of Rocky Intertidal Suspension Feeders

Matthew E. S. Bracken *

Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA, United States

The concept of ecological stoichiometry—the balancing of elemental ratios in ecological interactions—has transformed our thinking about processes in natural systems. Here, this perspective is applied to rocky shore ecosystems to explore the consequences of variation in internal nutrient ratios across two trophic levels. Specifically, I measured the internal concentrations of carbon (C) and nitrogen (N) in mussels (*Mytilus* spp.) and particulate organic matter (POM) to evaluate the effects of stoichiometric mismatch—the difference in the carbon-to-nitrogen ratio (C:N) between a consumer and its resources—on mussel growth at sites on the coasts of Oregon, USA, and the South Island of New Zealand. As POM quality (i.e., Chl *a*, a proxy for phytoplankton availability in the POM) increased, C:N of the POM declined, but C:N of mussels increased. This resulted in a greater mismatch in C:N between mussels and their food source at low Chl *a*. Mussel growth across sites was positively associated with Chl *a*, particulate organic carbon (POC), and particulate organic nitrogen (PON) but negatively associated with stoichiometric mismatch. Overall, as the elemental ratios of consumers became more different from those of their resources, growth declined, likely due to the energetic cost associated with processing lower quality food. Furthermore, the effect of food quantity on growth depended on stoichiometric mismatch. In New Zealand, where mismatch was high—i.e., consumer C:N differed substantially from resource C:N—consumer growth was strongly affected by resource quantity (Chl *a* or POC). However, in Oregon, where mismatch was low, the relationship between resource quantity and growth was considerably weaker. This interaction between resource quantity and mismatch was not apparent for PON, which is consistent with variation in PON underlying variation in POM C:N and highlights the role of N in limiting growth. Previous research has neglected the importance of ecological stoichiometry as a mediator of consumer-resource interactions in rocky intertidal communities. I show that resource quality and quantity interact to determine consumer growth, highlighting the utility of ecological stoichiometry in understanding spatial subsidies in benthic marine systems.

Keywords: Chl *a*, C:N, ecological stoichiometry, intertidal, marine, mussel, phytoplankton, stoichiometric mismatch

OPEN ACCESS

Edited by:
Robert Warner Sterner, University of Minnesota Duluth, United States

Reviewed by:
Zhanfei Liu, University of Texas at Austin, United States
Halvor Matthew Halvorson, University of Southern Mississippi, United States

***Correspondence:**
Matthew E. S. Bracken
m.bracken@uci.edu

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *31 January 2017*
Accepted: *27 June 2017*
Published: *12 July 2017*

Citation:
Bracken MES (2017) Stoichiometric Mismatch between Consumers and Resources Mediates the Growth of Rocky Intertidal Suspension Feeders. Front. Microbiol. 8:1297. doi: 10.3389/fmicb.2017.01297

INTRODUCTION

Understanding consumer-resource interactions is a fundamental goal of community ecology. This is particularly true for interactions between autotrophs and herbivores, which can serve as a bottleneck limiting the movement of carbon to higher trophic levels (Cebrian, 1999). The emergence of the theory of ecological stoichiometry over the past 15–20 years has provided a new and insightful lens through which to view consumer-resource interactions (Elser et al., 2000; Sterner and Elser, 2002). Ecological stoichiometry highlights the importance of balancing the supply and ratios of a variety of substances (e.g., energy, nutrients) in ecological interactions. Thus, for example, it is not just carbon (C) as an energy currency that mediates the interaction between herbivores and autotrophs. Nutrient [e.g., nitrogen (N) or phosphorus (P)] limitation affects both consumer growth and the efficiency of foraging (Denno and Fagan, 2003; Hillebrand et al., 2009).

The C:N and C:P ratios of consumers are often different from those of the resources they consume. This is particularly true for herbivores and autotrophs; C:N and C:P ratios of herbivores are typically much lower than the ratios of the autotrophs on which they feed (Elser et al., 2000). The magnitude of this stoichiometric mismatch between herbivores and autotrophs can mediate herbivore growth and consumption rates (Urabe and Sterner, 1996; Hillebrand et al., 2009). Ecological stoichiometry therefore highlights an important aspect of consumer-resource interactions: both the quantity and the quality of available resources matter to consumers (Frost and Elser, 2002).

Ecological stoichiometry takes an ecosystem-level approach to understanding community-level processes, allowing investigators to infer mechanism based on the balancing of elemental supply and ratios. For example, this perspective has been invoked to characterize the consumption of diverse microbial resources [e.g., particulate organic matter (POM) in aquatic environments] by larger organisms in the absence of detailed taxonomic information on POM composition (Elser et al., 2000). Determining C:N, C:P, and N:P ratios of the available POM—which consists of a mixture of microorganisms and detritus—can provide insights into the potential for those resources to meet the nutritional needs of consumers (Sterner and Elser, 2002). Here, this perspective is used to clarify how variation in the quantity and quality of available food mediates the growth of suspension feeders on rocky shorelines.

I specifically studied how stoichiometric mismatch—differences in the C:N of consumers and resources—affected the growth rates of mussels (*Mytilus californianus* and *M. galloprovincialis*) on rocky shorelines. Mussels are important resources at the bases of intertidal food webs (Paine, 1974; Navarrete and Menge, 1996; Menge et al., 2003), thereby mediating subsidies from nearshore pelagic to intertidal benthic ecosystems. Mytilid mussels, which are common inhabitants of temperate intertidal habitats worldwide (Seed, 1969; Koehn, 1991), capture POM from the nearshore ocean (Ward et al., 1998), and the quantity and quality of the resources available to these consumers are mediated by coastal oceanographic processes operating at meso-scales (e.g., sites separated by up to 100 km within a region) and macro-scales (e.g., upwelling vs. downwelling regimes spanning 100 s of km; Menge et al., 2003, 2004).

The coastlines of Oregon, USA, and the South Island of New Zealand, where I conducted my measurements, are characterized by gradients in oceanographic processes that underlie variation in POM quality and quantity. The Oregon coast, part of the California Current System, experiences strong, but intermittent, upwelling of cold, nutrient-rich water during the summer months (Huyer, 1983); the strength of upwelling increases from north to south (Menge et al., 2004; Broitman et al., 2008). Variation in the quality and quantity of POM around the South Island of New Zealand is primarily associated with two contrasting oceanographic regimes: a weak intermittent upwelling region on the west coast and a persistent downwelling region on the east coast (Stanton, 1976; Menge et al., 2003). At more local scales, availability of POM and phytoplankton is influenced by the interactions between upwelling and meso-scale attributes such as terrestrial and riverine inputs (Hill and Wheeler, 2002; McLeod and Wing, 2009; Bracken et al., 2012), headlands (Jenks et al., 1982), and the width of the continental shelf (Menge et al., 1997).

Previous work in Oregon and New Zealand identified phytoplankton availability as an important determinant of mussel growth, explaining ≥49% of the variance in mussels' growth in C (Bracken et al., 2012). Phytoplankton are a high-quality food source, and mussels sort the bulk POM, which includes a substantial amount of low-quality, terrestrially-derived detritus (Bracken et al., 2012), preferentially retaining phytoplankton, and rejecting detritus (Ward et al., 1998). This selectivity is both imperfect and energetically costly, suggesting that both POM quality (i.e., stoichiometric mismatch) and quantity [i.e., particulate organic carbon (POC), particulate organic nitrogen (PON), or phytoplankton availability (Chl *a*)] could affect mussel growth. Note that these different "quantitative" aspects of the POM also represent differences in quality, with POC describing the availability of low-quality resources and PON and Chl *a* describing the availability of high-quality resources.

I measured mussel growth rates at sites along the coastlines of Oregon and New Zealand and evaluated growth as a function of resource availability and stoichiometric mismatch in C:N. In line with previous work evaluating effects of resource quality (e.g., C:P) and quantity (e.g., Chl *a*) on consumer biomass (Qin et al., 2007), I hypothesized that consumer growth would increase with increasing food quantity but decline with decreasing food quality. Thus, I predicted that mussel growth would be higher at sites characterized by higher Chl *a*, POC, and PON availability but lower at sites characterized by greater stoichiometric mismatch between mussels and POM.

MATERIALS AND METHODS

Study Sites

Water-column attributes were quantified at nine sites along the coast of Oregon, USA, and eleven sites along the west and east coasts of the South Island of New Zealand (**Table 1**,

Figure 1). Sites were all characterized by rocky reefs on exposed open coastlines that supported substantial populations of congeneric intertidal mussels (*M. californianus* in Oregon and *M. galloprovincialis* in New Zealand). Sites spanned 475 km of the Oregon coastline from Cape Meares (CM) in the north to Cape Blanco (CB) in the south (**Figure 1A**). The five sites on the west coast of the South Island spanned 325 km from the mouth of the Nile River (NR) in the north to Jackson Bay (JB) in the south, and the six sites on the east coast spanned 475 km from Blue Duck Creek (BD) in the north to Sandfly Bay (SB) on the Otago Peninsula in the south (**Figure 1B**).

TABLE 1 | Characteristics of particulate organic matter (POM) and mussels at sites in Oregon, USA, and the South Island of New Zealand.

Site	Chl *a* (μg L^{-1})	POC (μg L^{-1})	PON (μg L^{-1})	C:Chl *a* (μg μg^{-1})	POM C:N	Mussel C:N	Growth (mg g^{-1} yr^{-1})
(A) OREGON							
Cape Meares (CM)	8.0	951.9	243.5	119.0	4.6	5.6	762.0
Fogarty Creek (FC)	6.2	860.5	181.2	139.3	5.5	5.3	960.3
Boiler Bay (BB)	5.4	757.6	179.0	140.3	4.9	5.3	757.0
Seal Rock (SR)	27.1	2150.9	422.0	79.3	5.9	5.7	1741.5
Yachats Beach (YB)	127.0	4565.3	1038.5	35.9	5.1	6.2	4082.6
Strawberry Hill (SH)	36.1	2569.0	598.2	71.1	5.0	5.7	1209.2
Tokatee Klootchman (TK)	43.8	2556.0	516.3	58.3	5.8	5.9	1927.6
Cape Arago (CA)	3.3	491.0	137.8	151.1	4.2	4.8	655.9
Cape Blanco (CB)	7.2	855.3	280.3	119.6	3.6	4.9	375.3
(B) NEW ZEALAND							
West Coast							
Nile River (NR)	2.1	1229.5	127.0	585.5	11.3	4.7	378.1
Woodpecker Bay (WB)	2.6	1072.2	118.5	412.4	10.6	4.6	706.3
Twelve Mile (TM)	1.9	1146.0	123.4	603.2	10.8	4.6	516.3
Nine Mile (NM)	3.4	1385.4	128.2	407.5	12.6	4.8	791.0
Jackson Bay (JB)	1.7	1370.0	136.4	805.9	11.7	4.7	301.7
East Coast							
Blue Duck (BD)	1.4	793.5	94.1	566.8	9.8	4.5	115.0
Raramai (RR)	0.7	512.6	56.4	732.3	10.6	4.5	88.9
Kie Kie (KK)	0.8	557.0	123.7	696.3	14.2	4.5	26.2
Box Thumb (BT)	1.8	787.9	93.5	437.7	9.8	5.1	389.4
Boulder Bay (BR)	1.9	757.4	92.0	398.6	9.6	5.2	210.2
Sandfly Bay (SB)	1.1	660.2	70.7	600.2	10.9	4.7	125.0

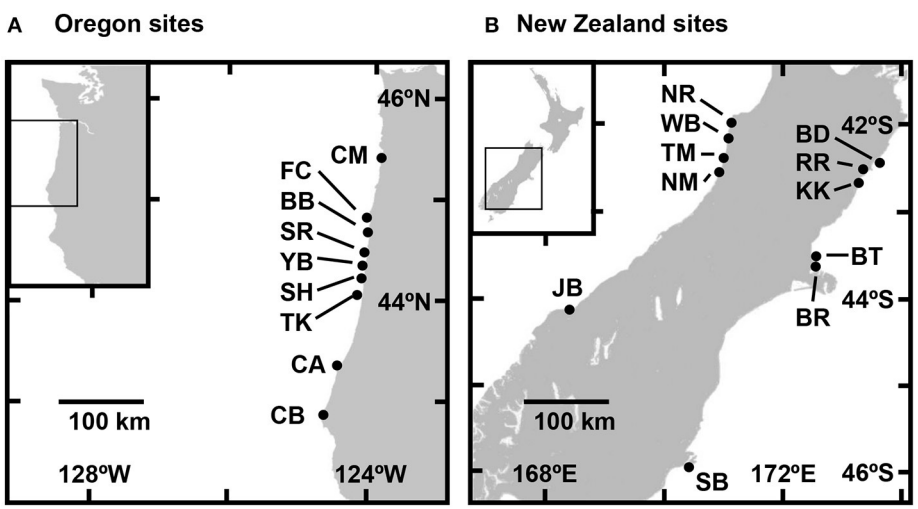

FIGURE 1 | Sampling locations on the coasts of **(A)** Oregon, USA, and **(B)** South Island, New Zealand. See **Table 1** for site abbreviations and characteristics.

Particulate Organic Matter

At each site, water samples were collected for measurement of POC ($\mu g\ L^{-1}$), PON ($\mu g\ L^{-1}$), and Chl *a* ($\mu g\ L^{-1}$). Oregon sites were visited monthly during the boreal summer (May through September) of 2003, and New Zealand sites were visited twice per month during the austral spring and summer (October through January) of 2003 and 2004. Samples were collected by filling 3 acid-washed opaque high-density polyethylene brown plastic bottles at ∼0.5 m depth in well-mixed water in the surf zone at each site at low tide. A 50 mL sample from each sample bottle was filtered through a 25 mm Whatman GF/F glass-fiber filter. These filters were transported to the laboratory on ice and analyzed for Chl *a* using a Turner Designs benchtop fluorometer after extraction in 90% HPLC acetone for 24 h at −20°C (Welschmeyer, 1994). Chl *a* was a reasonable proxy for phytoplankton concentrations based on relationships between Chl *a* and counts of phytoplankton and detritus in Lugol's-preserved water samples from a subset of sites. Analyses showed that Chl *a* concentrations were positively associated with phytoplankton cell counts [$R^2 = 0.68$; $F_{(1, 14)} = 10.9$, $p = 0.005$] and were unrelated to the amount of detritus in water samples [$R^2 = 0.01$; $F_{(1, 14)} = 0.2$, $p = 0.675$].

A 100 mL sample from each bottle was filtered through a pre-combusted 25 mm Whatman GF/F filter for analyses of POC and PON. These filters were transported to the laboratory on ice, dried (50°C for 72 h), acid fumed to remove carbonate, and analyzed for C and N using a PDZ Europa ANCA-GSL elemental analyzer at the UC Davis Stable Isotope Facility.

Mussel Growth and Tissue Analyses

Mussel growth rates were measured by marking five patches (20 × 20 cm) in the middle of the *Mytilus* zone at each site. The posterior edge of every mussel in every patch was carefully notched using a small triangular file. Mussels were collected ∼1 year later, and both the initial length from the umbo to the notch and the final length from the umbo to the posterior edge were measured (Menge, 2000; Menge et al., 2008; Bracken et al., 2012). An average of 19 ± 2 (mean ± SE) mussels were recovered per patch. To determine the relationship between shell length and mass, additional un-notched mussels were collected from each site, spanning a wide range of shell lengths. The shell length (umbo to posterior edge) of each mussel was measured, and the tissue from each mussel was dried to constant mass at 50°C. These measurements were used to determine the relationship between dry tissue mass and shell length at each site, then that relationship was used to estimate the initial and final dry tissue mass of each marked mussel at each site. These conversions were necessary because length-mass relationships differed at the different sites. The average *in situ* biomass-specific growth rate of mussels at each site (mg dry tissue $g^{-1}\ d^{-1}$) was calculated, then all values were normalized to an annual growth rate for each site (mg dry tissue $g^{-1}\ yr^{-1}$) based on the exact number of days between notching and collection.

Whole mussel tissue (3 randomly-selected individuals from each site) was carefully dissected from the shells, dried to constant mass at 50°C, and ground to a fine powder. Samples were analyzed for C and N (%) using a PDZ Europa ANCA-GSL elemental analyzer at the UC Davis Stable Isotope Facility.

Statistical Analyses

Particulate and mussel C:N mass ratios were multiplied by 1.167 to calculate molar ratios. All values (water-column: Chl *a*, POC, PON, C:Chl *a*, POM C:N; mussels: C:N, annual growth rate) for each site were averaged, and each site was treated as a single data point for analyses, resulting in measurements from $N = 20$ sites (9 sites in Oregon and 11 sites in New Zealand; **Table 1**) spanning a range of oceanographic and hydrodynamic conditions (Bracken et al., 2012). This averaging was necessary because mussel growth could only be calculated as an annual average for each site.

Stoichiometric mismatch was calculated for each site as the proportional difference between the carbon-to-nitrogen ratios (C:N) of the mussels ($C:N_{Consumer}$) and the POM ($C:N_{Resource}$; Hillebrand et al., 2009):

$$\text{Mismatch} = \ln\left(\frac{C:N_{Resource}}{C:N_{Consumer}}\right)$$

A positive mismatch value indicated that the resources at a site (i.e., POM) were characterized by higher C:N than the consumers (i.e., mussels), and a negative mismatch value indicated that the resources were characterized by lower C:N than the consumers. Log ratios are more effective at comparing C:N of resources and consumers than simple ratios because the natural log linearizes the ratio so that deviations in the numerator are equivalent to deviations in the denominator (Hedges et al., 1999).

General linear models (PROC GLM) and *t*-tests in SAS v. 9.4 (SAS Institute, Inc., Cary, North Carolina, USA) were used to evaluate relationships between site-level attributes after verifying assumptions of normality and homogeneity of variances. In particular, annual mussel growth rates were described as a function of resource quality (i.e., stoichiometric mismatch), resource quantity (i.e., Chl *a*, POC, or PON) a "quality × quantity" interaction (i.e., "Mismatch × Chl *a*," "Mismatch × POC," or "Mismatch × PON"), and region (i.e., Oregon vs. New Zealand; **Tables 2A**, **3A**, **4A**). Region was included as a factor in the models to account for geographic and taxonomic differences between Oregon and New Zealand. Additional analyses were conducted to evaluate relationships within each region separately, and these analyses evaluated growth as a function of stoichiometric mismatch and resource quantity (Chl *a*, POC, or PON; **Tables 2B**, **2C**, **3B**, **3C**, **4B**, **4C**). We only included main effects in these models, as no "Mismatch × Quantity" interactions were statistically significant ($p > 0.056$ in all cases). Models used Type III sums of squares, so the effect of every factor was evaluated after accounting for all other factors in the model. In most cases where variances were not homogeneous, data were natural-log transformed ($\ln[x]$ or $\ln[x+1]$). In some cases (e.g., comparisons between Oregon and New Zealand), heterogeneity of variances could not be corrected by transformation. These data were analyzed using generalized linear models (PROC GENMOD) in SAS v. 9.4, with log links and gamma distributions. All generalized linear models converged.

TABLE 2 | Effects of phytoplankton availability and stoichiometric mismatch on annual growth rates of mussels.

Source of variation	df	MS	Parameter estimates	*F*	*p*
(A) OVERALL					
Stoichiometric mismatch	1	2.79	−2.70	15.34	0.001
Chl *a*	1	7.88	+0.84	43.3	<0.001
Mismatch × Chl *a*	1	5.29	+1.74	29.0	<0.001
Region (OR vs. NZ)	1	0.29	+0.73	1.6	0.230
Error	15	0.18			
(B) OREGON					
Stoichiometric mismatch	1	0.38	+1.78	5.0	0.066
Chl *a*	1	3.01	+0.54	39.7	<0.001
Error	8	0.08			
(C) NEW ZEALAND					
Stoichiometric mismatch	1	0.37	−1.39	1.8	0.213
Chl *a*	1	7.30	+0.98	35.9	<0.001
Error	10	0.20			

Analyses are based on Type III Sums of Squares, so effects of each factor are determined after taking all other factors into account.

TABLE 3 | Effects of POC availability and stoichiometric mismatch on annual growth rates of mussels.

Source of variation	df	MS	Parameter estimates	*F*	*p*
(A) OVERALL					
Stoichiometric mismatch	1	2.80	−9.97	12.6	0.003
POC	1	5.00	+0.98	23.9	<0.001
Mismatch × POC	1	1.90	+1.15	10.7	0.005
Region (OR vs. NZ)	1	0.22	+1.14	<0.1	0.936
Error	15	0.24			
(B) OREGON					
Stoichiometric mismatch	1	0.28	+1.52	2.5	0.168
POC	1	2.80	+0.80	24.9	0.003
Error	8	0.11			
(C) NEW ZEALAND					
Stoichiometric mismatch	1	1.57	−2.78	6.8	0.030
POC	1	7.12	+2.38	31.7	<0.001
Error	10	0.22			

Analyses are based on Type III Sums of Squares, so effects of each factor are determined after taking all other factors into account.

TABLE 4 | Effects of PON availability and stoichiometric mismatch on annual growth rates of mussels.

Source of variation	df	MS	Parameter estimates	*F*	*p*
(A) OVERALL					
Stoichiometric mismatch	1	1.75	−9.52	3.1	0.101
PON	1	3.89	+0.95	6.8	0.020
Mismatch × PON	1	1.40	+1.51	2.5	0.138
Region (OR vs. NZ)	1	0.72	+1.73	1.3	0.280
Error	15	0.24			
(B) OREGON					
Stoichiometric mismatch	1	0.74	+2.48	6.0	0.050
PON	1	2.73	+0.88	22.1	0.003
Error	15	0.25			
(C) NEW ZEALAND					
Stoichiometric mismatch	1	3.50	−4.45	5.3	0.050
PON	1	3.66	+2.31	5.6	0.046
Error	15	0.24			

Analyses are based on Type III Sums of Squares, so effects of each factor are determined after taking all other factors into account.

RESULTS

Particulate Organic Matter and Mussel Characteristics

Despite spanning similar latitudes (**Figure 1**), water-column and mussel characteristics of sites on the coast of Oregon, USA, and the South Island of New Zealand differed substantially, creating a gradient in resource quantity and quality (Chl *a*, POC, PON, C:Chl *a*, POM C:N) and consumer condition (mussel C:N, mussel growth; **Table 1**). Overall, Chl *a* (GENMOD: Wald $\chi^2 = 52.1$, $p < 0.001$), POC (GENMOD: Wald $\chi^2 = 7.2$, $p = 0.007$), PON (GENMOD: Wald $\chi^2 = 41.1$, $p < 0.001$), mussel C:N (Wald $\chi^2 = 27.7$, $p < 0.001$), and mussel growth (Wald $\chi^2 = 17.8$, $p < 0.001$) were higher on the Oregon coast, whereas C:Chl *a* (Wald $\chi^2 = 129.8$, $p < 0.001$) and POM C:N (Wald $\chi^2 = 381.5$, $p < 0.001$) were higher on the coasts of the South Island (**Table 1**). PON availability was highly correlated with POC ($R^2 = 0.70$) and Chl *a* ($R^2 = 0.94$), and POC availability was highly correlated with Chl *a* ($R^2 = 0.73$).

Stoichiometric mismatch between mussels and POM was more pronounced in New Zealand ($t = 5.8$, df = 10, $p < 0.001$; **Figure 2A**) than in Oregon, where there was little to no difference in the C:N of mussels and POM ($t = 1.1$, df = 8, $p = 0.327$; **Figure 2B**). Overall, as phytoplankton availability (Chl *a*) in the nearshore ocean increased, the carbon-to-nitrogen ratio (C:N) of the POM declined [GLM: $R^2 = 0.47$; $F_{(1, 18)} = 15.8$, $p < 0.001$; **Figure 2C**]. Variation in the POM C:N was largely associated with variation in PON [GLM: $R^2 = 0.44$; $F_{(1, 18)} = 14.2$, $p = 0.001$]; there was no relationship between POM C:N and POC availability [GLM: $R^2 = 0.08$; $F_{(1, 18)} = 1.6$, $p = 0.229$]. Note, also, that the decline in the POM C:N with increases in Chl *a* represented a difference between New Zealand and Oregon. There was no relationship between Chl *a* and POM C:N in either New Zealand alone [$F_{(1, 9)} = 0.1$, $p = 0.761$; **Figure 2A**] or Oregon alone [$F_{(1, 7)} = 2.4$, $p = 0.163$; **Figure 2B**]. Multiple regression suggested that changes in POM C:N were associated with simultaneous changes in PON and POC in Oregon [PON: $F_{(1, 5)} = 440.2$, $p < 0.001$; POC: $F_{(1, 5)} = 251.0$, $p < 0.001$; Chl *a*: $F_{(1, 5)} = 0.3$, $p = 0.636$] and PON in New Zealand [PON: $F_{(1, 7)} = 5.3$, $p = 0.055$; POC: $F_{(1, 7)} = 0.3$, $p = 0.591$; Chl *a*: $F_{(1, 7)} = 0.6$, $p = 0.465$].

As Chl *a* increased, the C:N of the mussels increased [GLM: $R^2 = 0.85$; $F_{(1, 18)} = 103.1$, $p < 0.001$; **Figure 2**] Within regions,

this pattern held in Oregon [$F_{(1, 7)} = 31.9$, $p < 0.001$], but not in New Zealand [$F_{(1, 9)} = 2.3$, $p = 0.164$]. The difference in the relationships between Chl *a* and the C:N of the POM and mussels resulted in a divergence in the C:N of consumers and resources as Chl *a* increased, highlighting a decline in stoichiometric mismatch as the availability of high-quality food increased. Overall, C:N of the POM [coefficient of variation (c.v.) = 0.44] was much more variable than C:N of the mussels (c.v. = 0.11).

Overall Effects of Resource Quality and Quantity on Mussel Growth

Overall, stoichiometric mismatch negatively affected mussel growth rates (**Figure 3A**), but the strength of this relationship depended on the identity of the resource: Chl *a*, POC, or PON. After accounting for region (Oregon vs. New Zealand), Chl *a*, and the interaction between mismatch and Chl *a*, annual growth declined as mismatch increased [GLM: $F_{(1, 15)} = 15.3$, $p = 0.001$; **Table 2A**]. Similarly, after accounting for region, POC, and the interaction between mismatch and POC, growth declined as mismatch increased [GLM: $F_{(1, 15)} = 12.6$, $p = 0.003$; **Table 3A**]. However, after accounting for region, PON, and the interaction between mismatch and PON, there was little to no effect of mismatch on growth [GLM: $F_{(1, 15)} = 3.1$, $p = 0.101$; **Table 4A**].

Resource quantity enhanced growth, regardless of resource identity; mussel growth increased as Chl *a* [GLM: $F_{(1, 15)} = 43.3$, $p < 0.001$; **Table 2A**, **Figure 3B**], POC [GLM: $F_{(1, 15)} = 23.9$, $p < 0.001$; **Table 3A**, **Figure 3C**], and PON [GLM: $F_{(1, 15)} = 6.8$, $p = 0.020$; **Table 4A**, **Figure 3D**] increased. Furthermore, the effect of Chl *a* [GLM: "Mismatch × Chl *a*" interaction, $F_{(1, 15)} = 29.0$, $p < 0.001$; **Table 2A**, **Figure 3B**] and POC [GLM: "Mismatch × POC" interaction, $F_{(1, 15)} = 10.7$, $p = 0.005$; **Table 3A**, **Figure 3C**] on mussel growth depended on stoichiometric mismatch. The effects of both Chl *a* and POC availability on mussel growth were stronger (i.e., the slope of the relationship was steeper) in New Zealand, where mismatch was high, than in Oregon, where mismatch was low. In contrast, the effect of PON on growth was not affected by stoichiometric mismatch [GLM: "Mismatch × PON" interaction, $F_{(1, 15)} = 2.5$, $p = 0.138$; **Table 4A**, **Figure 3D**].

After accounting for the effects of stoichiometric mismatch, resource quantity, and the interaction between them, growth did not differ between Oregon and New Zealand. This lack of a difference between regions held regardless of resource identity: Chl *a* [$F_{(1, 15)} = 1.6$, $p = 0.230$; **Table 2A**], POC [$F_{(1, 15)} < 0.1$, $p = 0.936$; **Table 3A**], or PON [$F_{(1, 15)} = 1.3$, $p = 0.280$; **Table 4A**].

Comparisons within Oregon and New Zealand

Within each region, effects of resource quantity on mussel growth were universally positive. On the Oregon coast, growth increased with increases in Chl *a* [$F_{(1, 8)} = 39.7$, $p < 0.001$; **Table 2B**, **Figure 3B**], POC [$F_{(1, 8)} = 24.9$, $p = 0.003$; **Table 3B**, **Figure 3C**], and PON [$F_{(1, 8)} = 22.1$, $p = 0.003$; **Table 4B**, **Figure 3D**]. Similarly, in New Zealand, mussel growth was positively related to Chl *a* [$F_{(1, 10)} = 35.9$, $p < 0.001$; **Table 2C**, **Figure 3B**], POC [$F_{(1, 10)} = 31.7$, $p < 0.001$; **Table 3C**, **Figure 3C**], and PON [$F_{(1, 10)} = 5.6$, $p = 0.046$; **Table 2C**, **Figure 3D**].

Effects of stoichiometric mismatch on growth were neither as strong nor as consistent as effects of resource quantity, and they differed between regions. In Oregon, effects of mismatch on growth tended to be positive (**Tables 2B**, **3B**, **4B**, **Figure 3A**) and were generally weak [Chl *a*: $F_{(1, 8)} = 5.0$, $p = 0.066$; POC: $F_{(1, 8)} = 2.5$, $p =$ =; PON: $F_{(1, 8)} = 6.0$, $p = 0.050$]. In contrast, effects of mismatch on growth of New Zealand mussels tended to be negative (**Tables 2C**, **3C**, **4C**, **Figure 3A**) and were more consistent than those in Oregon [Chl *a*: $F_{(1, 10)} = 1.8$, $p = 0.213$; POC: $F_{(1, 10)} = 6.8$, $p = 0.030$; PON: $F_{(1, 10)} = 5.3$, $p = 0.050$].

DISCUSSION

Mussels are common, often dominant, suspension feeders on temperate rocky shorelines (Seed, 1969; Paine, 1974; Koehn, 1991; Menge et al., 2003). They therefore play an essential role as mediators of subsidies from nearshore pelagic into intertidal benthic ecosystems (Bracken et al., 2012). Understanding the factors underlying these subsidies is therefore crucial to understanding energy and nutrient flows into and within rocky shore systems. Here, I show that it is not just the quantity (i.e., Chl *a*, POC, or PON; **Figures 3B–D**), but also the quality (i.e., stoichiometric mismatch; **Figure 3A**) of the available particulate organic matter that mediates mussel growth in open-coast intertidal systems. This is in line with my initial predictions and previous research (e.g., Qin et al., 2007; Rowland et al., 2015); I predicted that growth would be higher where Chl *a*, POC, and PON availability was greater and lower where stoichiometric mismatch was greater.

Note that mussel growth is also related to water temperature, declining when and where temperatures are cooler (Menge et al., 2008). I did not measure temperatures during this study, but New Zealand coastal waters are warmer than those on the Oregon coast, and there is substantial within-region temperature variability due to site differences in nearshore oceanographic conditions (Menge et al., 2003, 2004). However, the factors included in my statistical models explained most of the variance in mussel growth (R^2 generally > 0.80), so temperature was not a strong mediator of growth relative to POM quality and quantity. Note also that many of the relationships between growth, mismatch, and POM quality and quantity were associated with differences between New Zealand sites (characterized by high mismatch) and Oregon sites (characterized by low mismatch). "Region" was included in statistical models to account for these differences, but it is important to note that differences between *M. californianus* and *M. galloprovincialis* could underlie some of the observed relationships. For example, *M. galloprovincialis* has been introduced to the coast of California, USA, where it now co-occurs with the native *M. californianus*. The introduced *M. galloprovincialis* grows more rapidly than its native congener under silty conditions, suggesting that it may be better adapted to poor-quality resources (Harger, 1970).

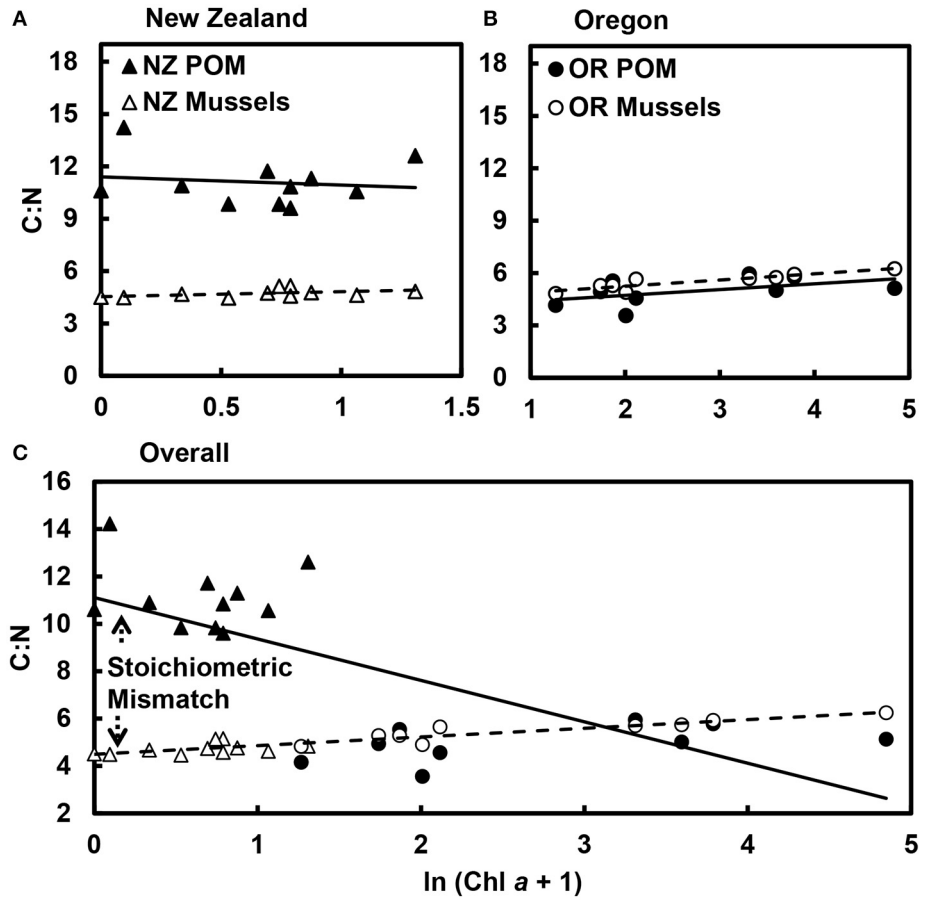

FIGURE 2 | Stoichiometric mismatch in C:N between mussels and particulate organic matter (POM) declined as phytoplankton abundance increased. Mismatch was much more pronounced in **(A)** New Zealand ($p < 0.001$) than in **(B)** Oregon ($p = 0.327$). **(C)** Overall, as phytoplankton abundance (ln Chl *a*) increased, the C:N of the POM declined ($R^2 = 0.47$, $p < 0.001$), whereas the C:N of the mussels increased ($R^2 = 0.85$, $p < 0.001$), resulting in mismatch between consumers and their resources. Data points are mean values for each site.

However, increases in stoichiometric mismatch were still associated with lower growth in New Zealand, particularly after accounting for the effects of POC and PON availability on growth (**Tables 3C**, **4C**, **Figure 3A**). In contrast, relationships between mismatch and growth actually tended to be positive in Oregon. Thus, whereas effects of mismatch on growth were relatively weak within each region, parameter estimates were universally positive in Oregon but negative in New Zealand. There was a weak, positive effect of mismatch on mussel growth in Oregon, particularly after accounting for effects of PON availability. Stoichiometric mismatch at most sites on the Oregon coast was actually negative (**Figures 2**, **3A**), meaning that mussels had higher C:N, on average, than the POM. An increase in mismatch therefore represented a shift toward balanced C:N of consumers and resources, which could also explain why mussel C:N increased with increasing Chl *a*. A similar result was found for mismatch in C:P of zebra mussels and POM in Swedish lakes; mussel tissue condition increased with increasing mismatch (Naddafi et al., 2009). Zebra mussels tended to have higher C:P than the POM, and increases in mismatch resulted in more balanced C:P of mussels and POM. The intriguing possibility that increases in mismatch could enhance growth if mismatch is negative would benefit from additional study, particularly in controlled mesocosm settings.

I did not, *a priori*, expect that the effect of resource quantity on consumer growth would depend on the stoichiometric mismatch between consumers and resources. If anything, I predicted that the effect of Chl *a* or POC on mussel growth would be greater where mismatch was lower. Previous work has shown that increases in food quantity have stronger effects on consumer growth where resource quality (e.g., C:P of algae) is high (Frost and Elser, 2002; Fink and Von Elert, 2006). In contrast, I found that increases in food quantity (Chl *a* or POC, but not PON) had stronger effects on mussel growth where resource quality was lower.

The interactions between resource quantity and quality described by Frost and Elser (2002) involved mayfly larvae feeding on benthic algae. In contrast, mussels are suspension feeders, feeding on POM in the water column, where assimilation efficiencies decline at high phytoplankton concentrations

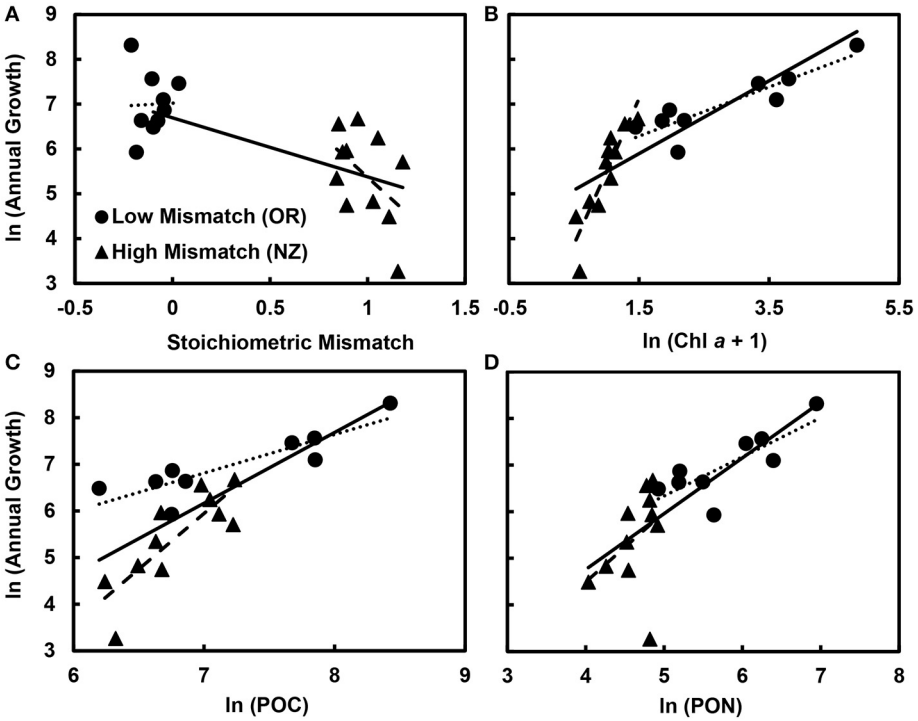

FIGURE 3 | Effects of resource availability and stoichiometric mismatch on mussel growth. Across both regions, growth (originally measured in mg g^{-1} yr^{-1}) declined with **(A)** stoichiometric mismatch ($p = 0.001$) and increased with **(B)** phytoplankton availability (Chl *a*, originally measured in $\mu g\ L^{-1}$; $p < 0.001$), **(C)** particulate organic carbon (POC, originally measured in $\mu g\ L^{-1}$; $p < 0.001$), and **(D)** particulate organic nitrogen (PON, originally measured in $\mu g\ L^{-1}$; $p < 0.001$). Within regions, for **(B)** Chl *a* ("Mismatch × Chl *a*" interaction, $p < 0.001$) and **(C)** POC ("Mismatch × POC" interaction, $p = 0.005$), the effect of resource quantity on growth was stronger in New Zealand, where stoichiometric mismatch was high, than in Oregon, where mismatch was low, so the slopes differed. However, for **(D)** PON ("Mismatch × PON" interaction, $p = 0.138$), the effect of resource quality on growth did not differ between regions. Data points are mean values for each site. Solid trendlines indicate relationships across both regions, dotted trendlines indicate relationships across Oregon sites, and dashed trendlines indicate relationships in New Zealand.

(Navarro and Winter, 1982). Scope for growth—the energy available for growth beyond that required for maintenance—in *Mytilus* is unimodally related to phytoplankton availability, declining at high concentrations due to optimization of assimilation efficiency at intermediate phytoplankton concentrations (Thompson and Bayne, 1974). The slope of the relationship between food availability and growth therefore tends to decline as Chl *a* concentrations increase (Bayne et al., 1989; **Figure 3B**). Because stoichiometric mismatch is closely related to Chl *a*—it declines with increasing phytoplankton availability (**Table 1**, **Figure 2**)—this translates to an interaction between mismatch and food availability (**Figures 3B,C**). Resource quantity at sites on the Oregon coast was exceptionally high, with average Chl *a* concentrations as high as 127 $\mu g\ L^{-1}$ (**Table 1**), which likely overwhelmed the ability of mussels to feed effectively. The fact that the effect of PON on growth did not change with mismatch highlights two important aspects of these interactions: (1) growth is likely limited by N and (2) stoichiometric mismatch is associated with variation in PON, not POC.

These relationships between food quality, food quantity, and growth are common attributes of planktonic consumer-resource interactions in both marine and freshwater systems (Mitra and Flynn, 2007), so the patterns I describe here are not just limited to benthic suspension feeders. In general, the patterns documented here suggest that stoichiometric mismatch can affect the growth of intertidal suspension feeders, but they are based on observations, not experiments. A controlled mesocosm study, where POM C:N could be held constant and POM quantity varied, would clarify the roles of POM quality and quantity in mediating mussel growth.

It is clear from my work and that of others that ecological stoichiometry can provide important insights into the mechanisms underlying consumer-resource interactions (Frost and Elser, 2002; Hessen et al., 2002; Sterner and Elser, 2002; Fink and Von Elert, 2006; Mitra and Flynn, 2007). However, this perspective is seldom invoked to explain consumption of POM by benthic suspension feeders (but see Carmichael et al., 2004, 2012). Mytilid mussels, particularly edible species in the genus *Mytilus*, have long served as a model system in marine physiology, and a large body of work has explored how the quality and quantity of suspended particulate material affects the growth of mussels and other benthic suspension feeders (e.g., Bayne et al., 1989, 1993; Arifin and Bendell-Young, 1997; Carmichael et al., 2004, 2012; Bracken et al., 2012). However, most of that research has considered "quality" in terms of the

amount of suspended organic vs. inorganic material. This is an important consideration, especially in coastal marine systems where suspension feeders must separate organic phytoplankton and detritus from inorganic silt and sand, but it has precluded a strictly stoichiometric perspective that examines seston quality in terms of elemental ratios in the organic fraction of the suspended particulate material.

I argue that our understanding of consumer-resource interactions in marine systems is incomplete without incorporating ecological stoichiometry. There is ample support for this perspective in phytoplankton-based open-water marine systems, where "stoichiometric modulation of predation" is an important component of models that explain the interactions between zooplankton and phytoplankton (Mitra et al., 2007). Models of climate-change impacts on pelagic marine systems explicitly invoke ecological stoichiometry to explain the effects of elevated CO_2 and reduced nutrient availability on trophic transfer of energy (van de Waal et al., 2010). However, ecological stoichiometry is seldom used to explain the magnitudes of spatial subsidies into benthic marine systems, despite the roles that these systems have played as experimental models of consumer-resource interactions (e.g., Paine, 1992; Navarrete and Menge, 1996). Here, I show that stoichiometric mismatch mediates an important subsidy into intertidal ecosystems and determines the growth of a foundational basal species in those systems. The interaction between nearshore phytoplankton and onshore suspension feeders is not amenable to the manipulative field experiments that are a hallmark of rocky intertidal research. However, understanding this spatial subsidy is key to our understanding of the dynamics of coastal marine systems (Menge et al., 2003).

More generally, it is not just the quantity, but also the quality, of resources that is important in determining trophic transfer of energy and the growth of consumers. My research, and that of others, demonstrates that resource quantity and quality interact to influence consumption and assimilation across terrestrial, freshwater, and marine systems and highlights the utility of ecological stoichiometry in understanding these interactions (Frost and Elser, 2002; Denno and Fagan, 2003; Fink and Von Elert, 2006; Mitra et al., 2007; Hillebrand et al., 2009).

AUTHOR CONTRIBUTIONS

MB conceived and designed the work; acquired, analyzed, and interpreted data; and drafted the manuscript. MB therefore agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This research was supported by the National Science Foundation (grants OCE-0351778 to J. Stachowicz and OCE-0549944 to S. Williams and MB), the Andrew W. Mellon Foundation (to J. Lubchenco, B. Menge, and D. Schiel), and the Partnership for Interdisciplinary Studies of Coastal Oceans (PISCO, to J. Lubchenco and B. Menge), a long-term, large-scale ecological consortium funded in part by the David and Lucile Packard and the Gordon and Betty Moore Foundations.

ACKNOWLEDGMENTS

I am grateful to J. van Berkel (Edward Percival Field Station, Kaikoura, New Zealand) and S. Williams (Bodega Marine Laboratory, Bodega Bay, California, USA) for laboratory space. F. Chan provided initial inspiration for this research. C. Cardoni, A. Carranza, F. Chan, A. Chaudoin, R. Dunmore, M. Dutton, M. Faubel, M. Foley, S. Lilley, J. Lubchenco, B. Menge, R. Milston-Clements, J. Pamplin, L. Petes, P. Reynolds, J. Ridley, M. Robart, R. Russell, J. Sapp, D. Schiel, C. Sorte, D. Taylor, and S. Wood cheerfully assisted with fieldwork, lab work, and/or logistical aspects of the research. This is publication number 476 from PISCO.

REFERENCES

Arifin, Z., and Bendell-Young, L. I. (1997). Feeding response and carbon assimilation by the blue mussel Mytilus trossulus exposed to environmentally relevant seston matrices. *Mar. Ecol. Prog. Ser.* 160, 241–253. doi: 10.3354/meps160241

Bayne, B. L., Hawkins, A. J. S., Navarro, E., and Iglesias, I. P. (1989). Effects of seston concentration on feeding, digestion, and growth in the mussel *Mytilus edulis*. *Mar. Ecol. Prog. Ser.* 55, 47–54. doi: 10.3354/meps055047

Bayne, B., Iglesias, J., Hawkins, A., Navarro, E., Heral, M., and Deslous-Paoli, J. M. (1993). Feeding behaviour of the mussel, *Mytilus edulis*: responses to variations in quantity and organic content of the seston. *J. Mar. Biol. Assoc. U. K.* 73, 813–829. doi: 10.1017/S0025315400034743

Bracken, M. E. S., Menge, B. A., Foley, M. M., Sorte, C. J. B., Lubchenco, J., and Schiel, D. R. (2012). Mussel selectivity for high-quality food drives carbon inputs into open-coast intertidal ecosystems. *Mar. Ecol. Progr. Ser.* 459, 53–62. doi: 10.3354/meps09764

Broitman, B. R., Blanchette, C. A., Menge, B. A., Lubchenco, J., Krenz, C., Foley, M., et al. (2008). Spatial and temporal patterns of invertebrate recruitment along the west coast of the United States. *Ecol. Monogr.* 78, 403–421. doi: 10.1890/06-1805.1

Carmichael, R. H., Shriver, A. C., and Valiela, I. (2004). Changes in shell and soft tissue growth, tissue composition, and survival of quahogs, Mercenaria mercenaria, and softshell clams, Mya arenaria, in response to eutrophic-driven changes in food supply and habitat. *J. Exp. Mar. Biol. Ecol.* 313, 75–104. doi: 10.1016/j.jembe.2004.08.006

Carmichael, R. H., Shriver, A. C., and Valiela, I. (2012). Bivalve response to estuarine eutrophication: the balance between enhanced food supply and habitat alterations. *J. Shellfish Res.* 31, 1–11. doi: 10.2983/035.031.0101

Cebrian, J. (1999). Patterns in the fate of production in plant communities. *Am. Nat.* 154, 449–468. doi: 10.1086/303244

Denno, R. F., and Fagan, W. F. (2003). Might nitrogen limitation promote omnivory among carnivorous arthropods? *Ecology* 84, 2522–2531. doi: 10.1890/02-0370

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580. doi: 10.1038/35046058

Fink, P., and Von Elert, E. (2006). Physiological responses to stoichiometric constraints: nutrient limitation and compensatory feeding in a freshwater snail. *Oikos* 115, 484–494. doi: 10.1111/j.2006.0030-1299.14951.x

Frost, P. C., and Elser, J. J. (2002). Growth responses of littoral mayflies to the phosphorus content of their food. *Ecol. Lett.* 5, 232–240. doi: 10.1046/j.1461-0248.2002.00307.x

Harger, J. R. E. (1970). Comparisons among growth characteristics of two species of sea mussel, *Mytilus edulis* and *Mytilus californianus*. *Veliger* 13, 44–56.

Hedges, L. V., Gurevitch, J., and Curtis, P. S. (1999). The meta-analysis of response ratios in experimental ecology. *Ecology* 80, 1150–1156. doi: 10.1890/0012-9658(1999)080[1150:TMAORR]2.0.CO;2

Hessen, D. O., Færøvig, P. J., and Andersen, T. (2002). Light, nutrients, and P:C ratios in algae: grazer performance related to food quality and quantity. *Ecology* 83, 1886–1898. doi: 10.1890/0012-9658(2002)083[1886:LNAPCR]2.0.CO;2

Hill, J. K., and Wheeler, P. A. (2002). Organic carbon and nitrogen in the northern California current system: comparison of offshore, river plume, and coastally upwelled waters. *Progr. Oceanogr.* 53, 369–387. doi: 10.1016/S0079-6611(02)00037-X

Hillebrand, H., Borer, E. T., Bracken, M. E. S., Cardinale, B. J., Cebrian, J., Cleland, E. E., et al. (2009). Herbivore metabolism and stoichiometry each constrain herbivory at different organizational scales across ecosystems. *Ecol. Lett.* 12, 516–527. doi: 10.1111/j.1461-0248.2009.01304.x

Huyer, A. (1983). Coastal upwelling in the California Current system. *Prog. Oceanog.* 12, 259–284. doi: 10.1016/0079-6611(83)90010-1

Jenks, W., Grindrod, J., and Peterson, J. (1982). Drift currents in the southern New Zealand region as derived from Lagrangian measurements and the remote sensing of sea-surface temperature distributions. *N. Z. J. Mar. Freshw. Res.* 16, 359–371. doi: 10.1080/00288330.1982.9515979

Koehn, R. K. (1991). The genetics and taxonomy of species in the genus Mytilus. *Aquaculture* 94, 125–145. doi: 10.1016/0044-8486(91)90114-M

McLeod, R. J., and Wing, S. R. (2009). Strong pathways for incorporation of terrestrially derived organic matter into benthic communities. *Estuar. Coast. Shelf Sci.* 82, 645–653. doi: 10.1016/j.ecss.2009.02.025

Menge, B. A. (2000). Top-down and bottom-up community regulation in marine rocky intertidal habitats. *J. Exp. Mar. Biol. Ecol.* 250, 257–289. doi: 10.1016/S0022-0981(00)00200-8

Menge, B. A., Blanchette, C., Raimondi, P., Freidenburg, T., Gaines, S., Lubchenco, J., et al. (2004). Species interaction strength: testing model predictions along an upwelling gradient. *Ecol. Monogr.* 74, 663–684. doi: 10.1890/03-4060

Menge, B. A., Chan, F., and Lubchenco, J. (2008). Response of a rocky intertidal ecosystem engineer and community dominant to climate change. *Ecol. Lett.* 11, 151–162. doi: 10.1111/j.1461-0248.2007.01135.x

Menge, B. A., Daley, B. A., Wheeler, P. A., Dahlhoff, E., Sanford, E., and Strub, P. T. (1997). Benthic-pelagic links and rocky intertidal communities: bottom-up effects on top-down control? *Proc. Natl. Acad. Sci. U.S.A.* 94, 14530–14535. doi: 10.1073/pnas.94.26.14530

Menge, B. A., Lubchenco, J., Bracken, M. E. S., Chan, F., Foley, M. M., Freidenburg, T. L., et al. (2003). Coastal oceanography sets the pace of rocky intertidal community dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12229–12234. doi: 10.1073/pnas.1534875100

Mitra, A., and Flynn, K. J. (2007). Importance of interactions between food quality, quantity, and gut transit time on consumer feeding, growth, and trophic dynamics. *Am. Nat.* 169, 632–646. doi: 10.1086/513187

Mitra, A., Flynn, K. J., and Fasham, M. J. (2007). Accounting for grazing dynamics in nitrogen-phytoplankton-zooplankton models. *Limnol. Oceanogr.* 52, 649–661. doi: 10.4319/lo.2007.52.2.0649

Naddafi, R., Eklöv, P., and Pettersson, K. (2009). Stoichiometric constraints do not limit successful invaders: zebra mussels in Swedish lakes. *PLoS ONE* 4:e5345. doi: 10.1371/journal.pone.0005345

Navarrete, S. A., and Menge, B. A. (1996). Keystone predation and interaction strength: interactive effects of predators on their main prey. *Ecol. Monogr.* 66, 409–429. doi: 10.2307/2963488

Navarro, J. M., and Winter, J. E. (1982). Ingestion rate, assimilation efficiency and energy balance in Mytilus chilensis in relation to body size and different algal concentrations. *Mar. Biol.* 67, 255–266. doi: 10.1007/BF00397666

Paine, R. T. (1974). Intertidal community structure: experimental studies on the relationship between a dominant competitor and its principal predator. *Oecologia* 15, 93–120. doi: 10.1007/BF00345739

Paine, R. T. (1992). Food-web analysis through field measurement of per capita interaction strength. *Nature* 355, 73–75. doi: 10.1038/355073a0

Qin, P., Mayer, C. M., Schulz, K. L., Ji, X., and Ritchie, M. E. (2007). Ecological stoichiometry in benthic food webs: effect of light and nutrients on periphyton food quantity and quality in lakes. *Limnol. Oceanogr.* 52, 1728–1734. doi: 10.4319/lo.2007.52.4.1728

Rowland, F. E., Bricker, K. J., Vanni, M. J., and González, M. J. (2015). Light and nutrients regulate energy transfer through benthic and pelagic food chains. *Oikos* 124, 1648–1663. doi: 10.1111/oik.02106

Seed, R. (1969). The ecology of *Mytilus edulis* L. (Lamellibranchiata) on exposed rocky shores. I. Breeding and settlement. *Oecologia* 3, 277–316. doi: 10.1007/BF00390380

Stanton, B. R. (1976). Circulation and hydrology off the west coast of the South Island, New Zealand. *N. Z. J. Mar. Freshw. Res.* 10, 445–467. doi: 10.1080/00288330.1976.9515629

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Thompson, R. J., and Bayne, B. L. (1974). Some relationships between growth, metabolism and food in the mussel *Mytilus edulis*. *Mar. Biol.* 27, 317–326. doi: 10.1007/BF00394367

Urabe, J., and Sterner, R. W. (1996). Regulation of herbivore growth by the balance of light and nutrients. *Proc. Natl. Acad. Sci. U.S.A.* 93, 8465–8469. doi: 10.1073/pnas.93.16.8465

van de Waal, D. B., Verschoor, A. M., Verspagen, J. M. H., van Donk, E., and Huisman, J. (2010). Climate-driven changes in the ecological stoichiometry of aquatic ecosystems. *Front. Ecol. Environ.* 8, 145–152. doi: 10.1890/080178

Ward, J. E., Levinton, J. S., Shumway, S. E., and Cucci, T. (1998). Particle sorting in bivalves: *in vivo* determination of the pallial organs of selection. *Mar. Biol.* 131, 283–292. doi: 10.1007/s002270050321

Welschmeyer, N. A. (1994). Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. *Limnol. Oceanogr.* 39, 1985–1992. doi: 10.4319/lo.1994.39.8.1985

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Earth Science

MINI REVIEW
published: 25 April 2017
doi: 10.3389/feart.2017.00032

The Stoichiometry of Nutrient Release by Terrestrial Herbivores and Its Ecosystem Consequences

Judith Sitters[1, 2, 3], Elisabeth S. Bakker[2], Michiel P. Veldhuis[4], G. F. Veen[3], Harry Olde Venterink[1] and Michael J. Vanni[5]*

[1] Ecology and Biodiversity, Department Biology, Vrije Universiteit Brussel, Brussels, Belgium, [2] Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, [3] Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, [4] Faculty of Science and Engineering, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, Netherlands, [5] Department of Biology, Miami University, Oxford, OH, USA

It is widely recognized that the release of nutrients by herbivores via their waste products strongly impacts nutrient availability for autotrophs. The ratios of nitrogen (N) and phosphorus (P) recycled through herbivore release (i.e., waste N:P) are mainly determined by the stoichiometric composition of the herbivore's food (food N:P) and its body nutrient content (body N:P). Waste N:P can in turn impact autotroph nutrient limitation and productivity. Herbivore-driven nutrient recycling based on stoichiometric principles is dominated by theoretical and experimental research in freshwater systems, in particular interactions between algae and invertebrate herbivores. In terrestrial ecosystems, the impact of herbivores on nutrient cycling and availability is often limited to studying carbon (C):N and C:P ratios, while the role of terrestrial herbivores in mediating N:P ratios is also likely to influence herbivore-driven nutrient recycling. In this review, we use rules and predictions on the stoichiometry of nutrient release originating from algal-based aquatic systems to identify the factors that determine the stoichiometry of nutrient release by herbivores. We then explore how these rules can be used to understand the stoichiometry of nutrient release by terrestrial herbivores, ranging from invertebrates to mammals, and its impact on plant nutrient limitation and productivity. Future studies should focus on measuring both N and P when investigating herbivore-driven nutrient recycling in terrestrial ecosystems, while also taking the form of waste product (urine or feces) and other pathways by which herbivores change nutrients into account, to be able to quantify the impact of waste stoichiometry on plant communities.

Keywords: autotroph productivity, aquatic ecosystems, C:N:P ratios, excretion, feces, herbivore-driven nutrient recycling, nitrogen, phosphorus

OPEN ACCESS

Edited by:
James Joseph Elser, University of Montana, USA

Reviewed by:
John Pastor, University of Minnesota, USA
Jacob Edward Allgeier, University of California, Santa Barbara, USA
Angelica L. Gonzalez, Rutgers University, USA

****Correspondence:***
Judith Sitters
judith.sitters@vub.be

Specialty section:
This article was submitted to Biogeoscience, a section of the journal Frontiers in Earth Science

Received: *30 January 2017*
Accepted: *07 April 2017*
Published: *25 April 2017*

Citation:
Sitters J, Bakker ES, Veldhuis MP, Veen GF, Olde Venterink H and Vanni MJ (2017) The Stoichiometry of Nutrient Release by Terrestrial Herbivores and Its Ecosystem Consequences. Front. Earth Sci. 5:32. doi: 10.3389/feart.2017.00032

INTRODUCTION

Herbivores are a major component of most ecosystems, ranging in size from zooplankton to elephants. All herbivores consume and digest autotroph biomass, and release nutrients, e.g., nitrogen (N) and phosphorus (P), in wastes through excretion (urine) or egestion (feces). Nutrient release by herbivores can strongly impact nutrient availability for autotrophs in terrestrial, marine,

and freshwater ecosystems (Pastor et al., 1993; McNaughton et al., 1997; Covich et al., 1999; Sirotnak and Huntly, 2000; Hunter, 2001; Vanni, 2002; Bardgett and Wardle, 2003; McIntyre et al., 2007; Cech et al., 2008; Roman and McCarthy, 2010; Metcalfe et al., 2014; Turner, 2015; Doughty et al., 2016). The ratio of N to P released (i.e., waste N:P) may be crucial for mediating ecosystem impacts of herbivore-driven nutrient recycling (Sterner, 1990; Urabe et al., 1995; Elser and Urabe, 1999). Two basic "stoichiometric rules" have been formulated, one based on how food and consumer body N:P determine waste N:P (rule 1), and the other on how waste N:P affects autotroph nutrient limitation and productivity (rule 2). Both rules allow for explicit predictions about the N:P stoichiometry of nutrient release and its ecosystem consequence (**Table 1**).

Thus far, evidence for these rules is mainly restricted to interactions between freshwater (pelagic) algae and invertebrate herbivores (Elser et al., 1988; Sterner, 1990; Sterner et al., 1992; Elser and Urabe, 1999; Sterner and Elser, 2002; Vanni, 2002), and to a lesser extent herbivorous fish (Schindler and Eby, 1997; Hood et al., 2005). However, herbivore-driven nutrient recycling also likely plays a major role in terrestrial ecosystems (Pastor et al., 1993; McNaughton et al., 1997; Hunter, 2001; Wardle et al., 2004; Metcalfe et al., 2014; Doughty et al., 2016). Indeed, the ratio of carbon (C) to nutrient (N and/or P) in plant tissues has long been recognized as an important determinant of herbivore feeding selectivity and subsequent nutrient cycling and availability in terrestrial ecosystems (Ritchie et al., 1998; Pastor et al., 2006; Bakker et al., 2009b). However, compared to aquatic systems, the role of terrestrial herbivores in mediating N:P ratios has received little attention so far. Because the ratio of N:P availability influences the type of growth limitation and the functional composition of terrestrial plant communities (Elser et al., 2007; Fujita et al., 2014), we hypothesize that the impact of terrestrial herbivores on this ratio has potentially strong ecosystem consequences.

In this review, we explore how we can apply these stoichiometric rules to terrestrial ecosystems, focusing on N:P ratios. We first explain the two rules derived from algae-invertebrate interactions in more detail. We then synthesize studies that applied these rules to terrestrial herbivores and ecosystems, identify research gaps, and suggest perspectives for future research.

RULE 1 (INDIVIDUAL CONSUMER LEVEL)—RELATIONSHIPS BETWEEN FOOD, BODY AND WASTE N:P

Rule 1 is based on mass balance and the assumption that consumers maintain elemental homeostasis in their tissues by differential release of N and P. Stoichiometry theory predicts a positive relationship between food N:P and waste N:P, assuming constant consumer body N:P (Sterner and Elser, 2002). Second, waste N:P is predicted to be negatively related to body N:P, if food N:P is constant (**Figure 1**). These predictions have been supported in lab studies using aquatic invertebrate herbivores (*Daphnia*) feeding on phytoplankton (Elser and Urabe, 1999;

TABLE 1 | Overview of the findings of the two stoichiometric rules, derived from interactions between algae and invertebrate herbivores in freshwater ecosystems, applied to terrestrial invertebrate (IV) and vertebrate (V) herbivores and ecosystems, including future research perspectives on each rule/prediction.

Stoichiometric rules	Terrestrial findings	Future research perspectives
Rule 1: To maintain homeostasis, a consumer will retain the element that most limits its growth, while releasing relatively large amounts of the element in excess relative to its needs.	See findings under prediction 1 and 2.	In general for rule 1 we are in need of studies that: • Measure both N and P in food, body and waste. • Quantify the relative degree of homeostasis. • Quantify total nutrient release, not only excretion or egestion. • Investigate the role of body size in determining body N:P both within and between invertebrate and vertebrate herbivore species.
Prediction 1: There is a positive relationship between food N:P and waste N:P (assuming body N:P is constant).	IV: Mixed findings for only N and only P (positive or unrelated), no correlation for N:P. V: True for only N and only P, no studies on N:P.	Specifically for prediction 1 of rule 1 we are in need of: • Experimental and field studies that measure both N and P in food and waste, preferably with single herbivore species (with constant body N:P).
Prediction 2: There is a negative relationship between herbivore body N:P and waste N:P (assuming food N:P is constant).	IV: No correlation for N:P. V: No studies on N:P.	Specifically for prediction 2 of rule 1 we are in need of: • Studies that measure both N and P in herbivore body and waste, while keeping food N:P equal.
Rule 2: The stoichiometry of nutrient release by herbivores can strongly affect autotroph nutrient limitation and primary production in ecosystems.	IV: True, but only on N. V: True, but often due to overall net effect of herbivores.	• Studies that focus on both N and P, and not only N. • Studies that quantify the different pathways by which herbivores impact the stoichiometry of plants. • Studies that compare the consequences of nutrient return through excretion (urine) or egestion (feces) for the stoichiometry of nutrient availability to plants. • Studies that incorporate the spatial return of N and P.

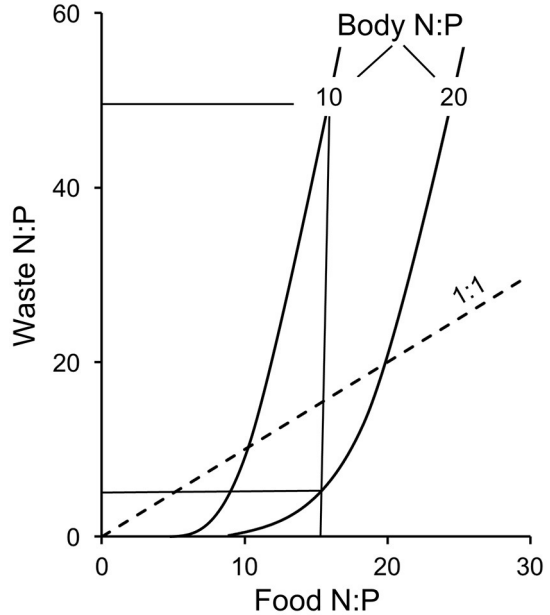

FIGURE 1 | Predicted relationships for the N:P of the consumer's waste products as a function of its food N:P and body N:P. Two curves for herbivores with body N:P values of 10 and 20 are shown. First, the predicted relationship between waste N:P and food N:P is positive; linear when food N:P > body N:P and curvilinear when food N:P < body N:P. This means that if the herbivore with a body N:P of 20 consumes food with an N:P of 15, it will release wastes with an N:P < 15. In contrast, if the same herbivore ingests food with an N:P of 25, it will release wastes that have an N:P > 25. Secondly, for any given food different consumers will recycle nutrients at different ratios depending on their body N:P ratio. If both herbivores feed on plants with an N:P ratio of 15, the herbivore with the low N:P (10) needs to sequester relatively more P, and will thus release wastes at a much higher N:P, than the herbivore with high body N:P. Redrawn from Sterner and Elser (2002) with permission of authors.

Sterner and Elser, 2002). However, support for these predictions from field data is mixed. For example, a strong negative correlation between consumer body N:P and waste N:P was found in systems with large variation among animal species in body N:P (e.g., Vanni et al., 2002; McManamay et al., 2011), while in other systems food N:P was more important in predicting waste N:P (e.g., Urabe, 1993; Torres and Vanni, 2007). Recent syntheses suggest that body size and temperature have much more influence than body nutrients on excretion rates and ratios (Allgeier et al., 2015; Vanni and McIntyre, 2016).

RULE 2 (ECOSYSTEM LEVEL)—IMPACT OF N:P RELEASE BY HERBIVORES ON PLANTS

Rule 2 states that the stoichiometry of nutrient release by herbivores strongly affects autotroph nutrient limitation and primary production (Elser and Urabe, 1999). For example, if a consumer feeding on N-limited plants (low plant N:P) releases waste products with an even lower N:P than that in plant tissue (following rule 1), the herbivore could render the plant community even more N-limited, which can then impact competitive interactions between plants and plant community composition (Sterner, 1990; Fujita et al., 2014). However, the impact on plant communities will depend on the proportion of nutrient demand met by consumer-driven recycling. In freshwater systems, for instance, it sustains anywhere from <5 to >80% of algal nutrient uptake (Taylor et al., 2015). So far, field evidence that nutrient recycling by herbivores can shift autotroph assemblages between N- and P-limitation is scant (e.g., Sterner et al., 1992; Knoll et al., 2009), and hence a general underpinning of rule 2 is still lacking.

Even though tests of these rules are still scarce, especially under field conditions, according to mass-balance principles the relative ratios of nutrient release by herbivores should be influenced by stoichiometric balance. In the following sections, we take on the challenge of applying this stoichiometric view to herbivore-driven nutrient recycling in terrestrial ecosystems.

APPLYING RULE 1 TO TERRESTRIAL HERBIVORES

Although rule 1 predicts a positive relationship between food N:P and waste N:P (**Figure 1**, **Table 1**), most studies with terrestrial herbivores focused on single nutrients. Mixed results are found for invertebrate herbivores; food N and waste N can be positively related (lepidopterans; Kagata and Ohgushi, 2012), or unrelated (grasshopper; Zhang et al., 2014). Similarly, food P and waste P were unrelated for caterpillars (Meehan and Lindroth, 2009), but positively related for a grasshopper species (Zhang et al., 2014). The latter study also investigated the N:P ratio of food and waste simultaneously (the only terrestrial study we know of), and the ratios were not correlated. The authors suggest that the lack of relationship between food and waste nutrients is likely because mechanisms other than excretion maintain N:P homeostasis, such as pre-ingestive regulation of the nutrient balance through food selection (Zhang et al., 2014) or compensatory feeding (Meehan and Lindroth, 2009). Work on large vertebrate herbivores is much more extensive—but again, measurements were often done on either N or P and not both—and generally finds positive correlations between food and waste nutrient contents. For example, diet N and fecal N were positively correlated for rabbits (Gil-Jimenez et al., 2015), roe deer (Verheyden et al., 2011), white-tailed deer (Osborn and Ginnett, 2001), blackbuck antelope (Jhala, 1997), and several African herbivores (Wrench et al., 1997). This positive relationship was also found for P for cattle (Zhang et al., 2016) and several African herbivores (Wrench et al., 1997). Furthermore, urinary N (excretion) of large ungulates increases with plant N concentration (Hobbs, 1996). These relationships seem so reliable that fecal N and P contents are used to predict food N and P contents (Wrench et al., 1997; Verheyden et al., 2011; Gil-Jimenez et al., 2015), although corrections for the presence of indigestible forms of N, e.g., tannins, are needed (Verheyden et al., 2011; Steuer et al., 2014).

The second prediction derived from rule 1 is a negative relationship between consumer body N:P and waste N:P

(**Table 1**). The only published study of terrestrial herbivores found no clear relationship between body and waste N:P for an invertebrate herbivore (grasshopper; Zhang et al., 2014); we found no studies on vertebrate herbivores.

This synthesis reveals the lack of studies examining relationships between food, body, and waste N:P (and not just N or P) in terrestrial herbivores, making it impossible to draw any general conclusions. Future work should include simultaneous measurements of N and P in food, bodies, and waste (**Table 1**). Controlled experiments where single herbivore species (constant body N:P) feed on food sources varying in N:P will provide a good test of the first prediction. Additionally, field studies on single species experiencing seasonal changes in food quality (food N:P) will be important, and again waste products and food sources of these herbivores need to be analyzed for both N and P. To test the second prediction, studies are needed where herbivores with a range of body N:P are fed a constant food N:P, and waste N:P is measured. Importantly, the predicted stoichiometric relationships between food, body and waste N:P might be impacted by several mitigating factors. These include the degree to which animals maintain homeostasis, which is variable and perhaps related to growth rate (Hood and Sterner, 2010; Downs et al., 2016), and which mechanisms they use to regulate this homeostasis (i.e., pre- or post-ingestive). Furthermore, the type of waste product (excretion or egestion; Halvorson et al., 2015) and the relationship between body size and body N:P are important mitigating factors, which should be taken into account and discussed below.

Animals that produce two types of wastes (feces and urine) can regulate their body composition pre-assimilation (by preferentially assimilating elements in short supply and releasing excess nutrients in feces) or post-assimilation (by excreting excess metabolic nutrients in urine before they reach toxic levels in the blood). Interestingly, the N:P stoichiometry of these two forms of nutrient release differ for terrestrial vertebrate herbivores; i.e., urine contains hardly any P but a high concentration of N in soluble form, while feces contain most of the P and some N (Morse et al., 1992; Hobbs, 1996). Relative concentrations of N in urine and feces depend on forage N, whereby herbivores that consume plants of high N (e.g., ungulate grazers consuming green grasses) return N to the soil mainly in the form of urine, while herbivores consuming plants of low N (e.g., ungulate browsers consuming tree twigs) need to extract as much N as possible and mainly produce feces of very low N (Pastor et al., 2006). However, very few studies quantify total nutrient release, instead of only excretion (often the case for aquatic animals) or egestion (often the case for terrestrial vertebrates) to test predictions of stoichiometry theory. This needs to be addressed in future studies, as the theory is based on mass-balance, and tests must therefore include all fluxes mediated by animal physiology (**Table 1**).

Generally, differences in body N:P are driven by patterns of investment in P-rich materials such as RNA and bone (Gillooly et al., 2005). Investments in RNA decrease significantly with body size (small organisms generally have higher growth rates), suggesting an increase in body N:P with increasing body size for invertebrates (Sterner and Elser, 2002; Back and King, 2013). However, more P is sequestered into supportive tissue like bones, suggesting that for vertebrates body N:P decreases with body size (Sterner and Elser, 2002) since skeleton mass scales allometrically (more than proportionally) with body mass (Anderson et al., 1979; Prange et al., 1979). This uncovers an important difference between aquatic and terrestrial systems, where terrestrial herbivores, especially larger individuals, need to invest more in P-rich structural tissue to counterbalance gravity. Hence, more studies investigating the role of body size in determining body N:P both within and between invertebrate and vertebrate herbivore species are needed (**Table 1**).

APPLYING RULE 2 TO TERRESTRIAL ECOSYSTEMS

Rule 2 states that the stoichiometry of nutrient release by herbivores can strongly affect autotroph nutrient limitation and primary production (Elser and Urabe, 1999). However, in terrestrial ecosystems it is hard to isolate the effect of herbivore-driven nutrient recycling, as the "net effect" of herbivores on nutrient cycling depends not only on direct effects of nutrient release through waste products, but also on indirect effects through modification of plant litter quantity and quality, and in the case of vertebrates, by alteration of soil physical properties through trampling (Ritchie et al., 1998; Belovsky and Slade, 2000; Hunter, 2001; Bardgett and Wardle, 2003; Schrama et al., 2013). Therefore, most empirical studies addressing how terrestrial herbivores shift plant assemblages between N- or P-limitation examined the "net effect" of herbivores (e.g., Carline et al., 2005; Frank, 2008; Zhang et al., 2011; Bai et al., 2012; Nitschke et al., 2015; Sitters et al., 2017) and not on the effects of nutrient release *per se*.

Many studies on herbivore-driven nutrient recycling in terrestrial ecosystems have focused on N, both for invertebrates (e.g., Seastedt and Crossley, 1984; Lovett and Ruesink, 1995; Belovsky and Slade, 2000; Reynolds and Hunter, 2001; Hunter et al., 2003; Metcalfe et al., 2014) and vertebrates (e.g., Pastor et al., 1988, 1993, 2006; McNaughton et al., 1988; Hobbs et al., 1991; Frank and McNaughton, 1993; Frank and Evans, 1997; McNaughton et al., 1997; Ritchie et al., 1998; Sirotnak and Huntly, 2000; Olofsson et al., 2001; Stark et al., 2003; Fornara and Du Toit, 2008). For invertebrate herbivores, the general view is that they speed up nutrient cycling in terrestrial systems by changing litter quantity and quality, modifying the nutrient content of throughfall, and releasing easily-available nutrients in frass and cadavers (Hunter, 2001). The direction of the impact of vertebrate herbivores on N cycling has traditionally been considered to depend on system fertility and corresponding plant N content; vertebrates have a positive effect on N availability and primary production in systems of high fertility, and a negative effect in low fertility systems (Hobbs, 1996; Bardgett and Wardle, 2003; Pastor et al., 2006). This is partly based on the proportion of N released, which is higher and mainly through urine when the nutrient content of plants is higher (Hobbs, 1996), but also on changes in plant litter quality, as herbivores feeding in systems with low plant N facilitate a shift toward litter of low N by

selectively consuming high-N plant tissue (Bardgett and Wardle, 2003).

Stoichiometry theory has challenged the traditional views for impacts of herbivores on nutrient cycling. Modeling results demonstrate that if herbivores promote microbial C-limitation through vegetation consumption and respiration, they will have a positive effect on N availability in sites with low plant N by decreasing microbial immobilization rates, and a positive effect in sites with high plant N by decreasing mineralization rates (Cherif and Loreau, 2013). For vertebrate herbivores these results are party supported by field data (Bakker et al., 2009a; Sitters et al., 2017). Also, for invertebrates, labile C in excreta can result in N immobilization and lower availability (Lovett and Ruesink, 1995). These studies again show the need for a more integrative framework to understand and quantify the different pathways by which vertebrate (Sitters and Olde Venterink, 2015) and invertebrate herbivores (Hunter, 2001) impact nutrient cycling and availability to plants.

To predict how the stoichiometry of nutrient release by herbivores affects autotroph nutrient limitation and primary production in terrestrial systems, we must expand studies on the role of P, because N- and P-limitation are both prevalent in terrestrial ecosystems (Elser et al., 2007). The effect of herbivores on N- and P-limitation depends first on the form in which N and P are returned to the soil (urine or feces). N in urine is soluble and directly available to plants, while feces contain a substantial amount of organic matter, which needs to be decomposed and mineralized to render the N and P available to plants (Hobbs, 1996). Furthermore, N in feces and urine is subject to a significant loss from the system via ammonia volatilization and leaching (Ruess and McNaughton, 1988; Frank and Zhang, 1997; Augustine, 2003), suggesting that terrestrial herbivores (that produce both types of waste products) may drive ecosystems to N-limitation through nutrient release (e.g., Cech et al., 2008). Very little data however exist, comparing the consequences of nutrient return through urine or feces for the stoichiometry of nutrient availability to plants.

Additionally, nutrient release by herbivores strongly increases the spatial heterogeneity of N:P availability across the landscape. Terrestrial herbivores typically do not graze and excrete randomly, but are attracted to landscape features, such as nutrient-rich areas with high food quality, resulting in a net import of nutrients and creating nutrient hotspots in the landscape. At the same time, large parts of the landscape with poorer quality vegetation experience a net removal of nutrients (McNaughton et al., 1997; Augustine et al., 2003; van der Waal et al., 2011). Water bodies may also induce spatial patterns; semi-aquatic herbivores such as hippopotamus, beaver or water birds can transport nutrients across ecosystem boundaries and thus strongly impact nutrient redistribution (Sitters et al., 2015; Subalusky et al., 2015; Bakker et al., 2016). Furthermore, social behavior may affect nutrient release. When herbivores defecate in common latrines, they concentrate nutrients in the landscape (e.g., rhinos, rabbits, horses), which function as hotspots of nutrients, possibly with a low N:P ratio, though data are scarce and not fully consistent regarding the effect on soil P (see Edwards and Hollis, 1982; Willott et al., 2000; Jewell et al., 2007). In this respect, there are similarities and differences between terrestrial and aquatic habitats. In both, animals can mediate a net translocation of nutrients across habitats and ecosystems (Vanni et al., 2001; Flecker et al., 2010; Ebel et al., 2015; Sitters et al., 2015). However, in aquatic habitats excreted nutrients may easily mix in the water, whereas in terrestrial habitats patches of released nutrients are much more spatially disconnected; this suggests that terrestrial animals may induce spatial variation in nutrient supply and stoichiometry more so than aquatic animals.

CONCLUDING REMARKS

The stoichiometric view of herbivore-driven nutrient recycling in terrestrial ecosystems has not yet received the attention it deserves. We were unable to find firm evidence for rule 1, as most studies investigating relationships between food, herbivore bodies, and wastes focused on single nutrients. At the same time, many studies consider the "net" effect of herbivores on nutrient cycling, making it impossible to determine the impact of waste stoichiometry on plant communities *per se* (rule 2). We therefore suggest several perspectives on future research, to increase our understanding of the stoichiometry of nutrient release by terrestrial herbivores, ranging from invertebrates to mammals, and its impact on ecosystem stoichiometry, plant nutrient limitation, and productivity (**Table 1**).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

JS was financially supported by a grant of the Research Foundation Flanders (FWO), grant 12N2615N. MPV has been financially supported by the AfricanBioServices project which received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 641918. MJV was supported by US National Science Foundation grants 0918993 and 1255159. Additional funding was provided by Strategic Resources of the Netherlands Institute of Ecology (NIOO-KNAW); this is publication number 6272.

REFERENCES

Allgeier, J. E., Wenger, S. J., Rosemond, A. D., Schindler, D. E., and Layman, C. A. (2015). Metabolic theory and taxonomic identity predict nutrient recycling in a diverse food web. *Proc. Natl. Acad. Sci. U.S.A.* 112, 2640–2647. doi: 10.1073/pnas.1420819112

Anderson, J. F., Rahn, H., and Prange, H. D. (1979). Scaling of supportive tissue mass. *Q. Rev. Biol.* 54, 139–148. doi: 10.1086/411153

Augustine, D. J. (2003). Long-term, livestock-mediated redistribution of nitrogen and phosphorus in an East African savanna. *J. Appl. Ecol.* 40, 137–149. doi: 10.1046/j.1365-2664.2003.00778.x

Augustine, D. J., McNaughton, S. J., and Frank, D. A. (2003). Feedbacks between soil nutrients and large herbivores in a managed savanna ecosystem. *Ecol. Appl.* 13, 1325–1337. doi: 10.1890/02-5283

Back, J. A., and King, R. S. (2013). Sex and size matter: ontogenetic patterns of nutrient content of aquatic insects. *Freshwater Science* 32, 837–848. doi: 10.1899/12-181.1

Bai, Y. F., Wu, J. G., Clark, C. M., Pan, Q. M., Zhang, L. X., Chen, S. P., et al. (2012). Grazing alters ecosystem functioning and C:N:P stoichiometry of grasslands along a regional precipitation gradient. *J. Appl. Ecol.* 49, 1204–1215. doi: 10.1111/j.1365-2664.2012.02205.x

Bakker, E. S., Knops, J. M. H., Milchunas, D. G., Ritchie, M. E., and Olff, H. (2009a). Cross-site comparison of herbivore impact on nitrogen availability in grasslands: the role of plant nitrogen concentration. *Oikos* 118, 1613–1622. doi: 10.1111/j.1600-0706.2009.17199.x

Bakker, E. S., Olff, H., and Gleichman, J. M. (2009b). Contrasting effects of large herbivore grazing on smaller herbivores. *Basic Appl. Ecol.* 10, 141–150. doi: 10.1016/j.baae.2007.10.009

Bakker, E. S., Pages, J. F., Arthur, R., and Alcoverro, T. (2016). Assessing the role of large herbivores in the structuring and functioning of freshwater and marine angiosperm ecosystems. *Ecography* 39, 162–179. doi: 10.1111/ecog.01651

Bardgett, R. D., and Wardle, D. A. (2003). Herbivore-mediated linkages between aboveground and belowground communities. *Ecology* 84, 2258–2268. doi: 10.1890/02-0274

Belovsky, G. E., and Slade, J. B. (2000). Insect herbivory accelerates nutrient cycling and increases plant production. *Proc. Natl. Acad. Sci. U.S.A.* 97, 14412–14417. doi: 10.1073/pnas.250483797

Carline, K. A., Jones, H. E., and Bardgett, R. D. (2005). Large herbivores affect the stoichiometry of nutrients in a regenerating woodland ecosystem. *Oikos* 110, 453–460. doi: 10.1111/j.0030-1299.2005.13550.x

Cech, P. G., Kuster, T., Edwards, P. J., and Olde Venterink, H. (2008). Effects of herbivory, fire and N2-fixation on nutrient limitation in a humid African savanna. *Ecosystems* 11, 991–1004. doi: 10.1007/s10021-008-9175-7

Cherif, M., and Loreau, M. (2013). Plant–herbivore–decomposer stoichiometric mismatches and nutrient cycling in ecosystems. *Proc. R. Soc. B. Biol. Sci.* 280:20122453. doi: 10.1098/rspb.2012.2453

Covich, A. P., Palmer, M. A., and Crowl, T. A. (1999). The role of benthic invertebrate species in freshwater ecosystems - Zoobenthic species influence energy flows and nutrient cycling. *Bioscience* 49, 119–127. doi: 10.2307/1313537

Doughty, C. E., Roman, J., Faurby, S., Wolf, A., Haque, A., Bakker, E. S., et al. (2016). Global nutrient transport in a world of giants. *Proc. Natl. Acad. Sci. U.S.A.* 113, 868–873. doi: 10.1073/pnas.1502549112

Downs, K. N., Hayes, N. M., Rock, A. M., Vanni, M. J., and Gonzalez, M. J. (2016). Light and nutrient supply mediate intraspecific variation in the nutrient stoichiometry of juvenile fish. *Ecosphere* 7:e01452. doi: 10.1002/ecs2.1452

Ebel, J. D., Leroux, S. J., Robertson, M. J., and Dempson, J. B. (2015). Ontogenetic differences in Atlantic salmon phosphorus concentration and its implications for cross ecosystem fluxes. *Ecosphere* 6, 1–18. doi: 10.1890/es14-00516.1

Edwards, P. J., and Hollis, S. (1982). The distribution of excreta on new forest grassland used by cattle, ponies and deer. *J. Appl. Ecol.* 19, 953–964.

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Elser, J. J., Elser, M. M., Mackay, N. A., and Carpenter, S. R. (1988). Zooplankton-mediated transitions between N-limited and P-limited algal growth. *Limnol. Oceanogr.* 33, 1–14.

Elser, J. J., and Urabe, J. (1999). The stoichiometry of consumer-driven nutrient recycling: theory, observations, and consequences. *Ecology* 80, 735–751. doi: 10.1890/0012-9658(1999)080[0735:tsocdn]2.0.co;2

Flecker, A. S., McIntyre, P. B., Moore, J. W., Anderson, J. T., Taylor, B. W., and Hall, R. O. (2010). Migratory fishes as material and process subsidies in riverine ecosystems. *Am. Fish. Soc. Symp.* 73, 559–592.

Fornara, D. A., and Du Toit, J. T. (2008). Browsing-induced effects on leaf litter quality and decomposition in a southern african savanna. *Ecosystems* 11, 238–249. doi: 10.1007/s10021-007-9119-7

Frank, D. A. (2008). Ungulate and topographic control of nitrogen: phosphorus stoichiometry in a temperate grassland; soils, plants and mineralization rates. *Oikos* 117, 591–601. doi: 10.1111/j.2007.0030-1299.16220.x

Frank, D. A., and Evans, R. D. (1997). Effects of native grazers on grassland N cycling in Yellowstone National Park. *Ecology* 78, 2238–2248. doi: 10.1890/0012-9658(1997)078[2238:eongog]2.0.co;2

Frank, D. A., and McNaughton, S. J. (1993). Evidence for the promotion of aboveground grassland production by native large herbivores in Yellowstone National Park. *Oecologia* 96, 157–161.

Frank, D. A., and Zhang, Y. M. (1997). Ammonia volatilization from a seasonally and spatially variable grazed grassland: Yellowstone National Park. *Biogeochemistry* 36, 189–203. doi: 10.1023/a:1005705121160

Fujita, Y., Olde Venterink, H., van Bodegom, P. M., Douma, J. C., Heil, G. W., Holzel, N., et al. (2014). Low investment in sexual reproduction threatens plants adapted to phosphorus limitation. *Nature* 505, 82–86. doi: 10.1038/nature12733

Gil-Jimenez, E., Villamuelas, M., Serrano, E., Delibes, M., and Fernandez, N. (2015). Fecal nitrogen concentration as a nutritional quality indicator for european rabbit ecological studies. *PLoS ONE* 10:e0125190. doi: 10.1371/journal.pone.0125190

Gillooly, J. F., Allen, A. P., Brown, J. H., Elser, J. J., del Rio, C. M., Savage, V. M., et al. (2005). The metabolic basis of whole-organism RNA and phosphorus content. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11923–11927. doi: 10.1073/pnas.0504756102

Halvorson, H. M., Fuller, C., Entrekin, S. A., and Evans-White, M. A. (2015). Dietary influences on production, stoichiometry and decomposition of particulate wastes from shredders. *Freshw. Biol.* 60, 466–478. doi: 10.1111/fwb.12462

Hobbs, N. T. (1996). Modification of ecosystems by ungulates. *J. Wildl. Manag.* 60, 695–713. doi: 10.2307/3802368

Hobbs, N. T., Schimel, D. S., Owensby, C. E., and Ojima, D. S. (1991). Fire and grazing in the tallgrass prairie - contingent effects on nitrogen budgets. *Ecology* 72, 1374–1382. doi: 10.2307/1941109

Hood, J. M., and Sterner, R. W. (2010). Diet mixing: do animals integrate growth or resources across temporal heterogeneity? *Am. Nat.* 176, 651–663. doi: 10.1086/656489

Hood, J. M., Vanni, M. J., and Flecker, A. S. (2005). Nutrient recycling by two phosphorus-rich grazing catfish: the potential for phosphorus-limitation of fish growth. *Oecologia* 146, 247–257. doi: 10.1007/s00442-005-0202-5

Hunter, M. D. (2001). Insect population dynamics meets ecosystem ecology: effects of herbivory on soil nutrient dynamics. *Agric. For. Entomol.* 3, 77–84. doi: 10.1046/j.1461-9563.2001.00100.x

Hunter, M. D., Linnen, C. R., and Reynolds, B. C. (2003). Effects of endemic densities of canopy herbivores on nutrient dynamics along a gradient in elevation in the southern Appalachians. *Pedobiologia* 47, 231–244. doi: 10.1078/0031-4056-00187

Jewell, P. L., Kauferle, D., Guesewell, S., Berry, N. R., Kreuzer, M., and Edwards, P. J. (2007). Redistribution of phosphorus by mountain pasture in cattle on a traditional the Alps. *Agric. Ecosyst. Environ.* 122, 377–386. doi: 10.1016/j.agee.2007.02.012

Jhala, Y. V. (1997). Seasonal effects on the nutritional ecology of blackbuck *Antelope cervicapra*. *J. Appl. Ecol.* 34, 1348–1358. doi: 10.2307/2405252

Kagata, H., and Ohgushi, T. (2012). Carbon to nitrogen excretion ratio in lepidopteran larvae: relative importance of ecological stoichiometry and metabolic scaling. *Oikos* 121, 1869–1877. doi: 10.1111/j.1600-0706.2012.20274.x

Knoll, L. B., McIntyre, P. B., Vanni, M. J., and Flecker, A. S. (2009). Feedbacks of consumer nutrient recycling on producer biomass and stoichiometry: separating direct and indirect effects. *Oikos* 118, 1732–1742. doi: 10.1111/j.1600-0706.2009.17367.x

Lovett, G. M., and Ruesink, A. E. (1995). Carbon and nitrogen mineralization from decomposing gypsy-moth frass. *Oecologia* 104, 133–138. doi: 10.1007/bf00328577

McIntyre, P. B., Jones, L. E., Flecker, A. S., and Vanni, M. J. (2007). Fish extinctions alter nutrient recycling in tropical freshwaters. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4461–4466. doi: 10.1073/pnas.0608148104

McManamay, R. A., Webster, J. R., Valett, H. M., and Dolloff, C. A. (2011). Does diet influence consumer nutrient cycling? Macroinvertebrate and fish

excretion in streams. *J. North Am. Benthol. Soc.* 30, 84–102. doi: 10.1899/09-152.1

McNaughton, S. J., Banyikwa, F. F., and McNaughton, M. M. (1997). Promotion of the cycling of diet-enhancing nutrients by African grazers. *Science* 278, 1798–1800. doi: 10.1126/science.278.5344.1798

McNaughton, S. J., Ruess, R. W., and Seagle, S. W. (1988). Large mammals and process dynamics in African ecosystems. *Bioscience* 38, 794–800. doi: 10.2307/1310789

Meehan, T. D., and Lindroth, R. L. (2009). Scaling of individual phosphorus flux by caterpillars of the whitemarked tussock moth, Orygia leucostigma. *J. Insect Sci.* 9:42. doi: 10.1673/031.009.4201

Metcalfe, D. B., Asner, G. P., Martin, R. E., Espejo, J. E. S., Huasco, W. H., Amezquita, F. F. F., et al. (2014). Herbivory makes major contributions to ecosystem carbon and nutrient cycling in tropical forests. *Ecol. Lett.* 17, 324–332. doi: 10.1111/ele.12233

Morse, D., Head, H. H., Wilcox, C. J., Vanhorn, H. H., Hissem, C. D., and Harris, B. (1992). Effects of concentration of dietary phosphorus on amount and route of excretion. *J. Dairy Sci.* 75, 3039–3049.

Nitschke, N., Wiesner, K., Hilke, I., Eisenhauer, N., Oelmann, Y., and Weisser, W. W. (2015). Increase of fast nutrient cycling in grassland microcosms through insect herbivory depends on plant functional composition and species diversity. *Oikos* 124, 161–173. doi: 10.1111/oik.01476

Olofsson, J., Kitti, H., Rautiainen, P., Stark, S., and Oksanen, L. (2001). Effects of summer grazing by reindeer on composition of vegetation, productivity and nitrogen cycling. *Ecography* 24, 13–24. doi: 10.1034/j.1600-0587.2001.240103.x

Osborn, R. G., and Ginnett, T. F. (2001). Fecal nitrogen and 2,6-diaminopimelic acid as indices to dietary nitrogen in white-tailed deer. *Wildl. Soc. Bull.* 29, 1131–1139. doi: 10.2307/3784136

Pastor, J., Cohen, Y., and Hobbs, N. T. (2006). "The role of large herbivores in ecosystem nutrient cycles," in *Large Herbivore Ecology, Ecosystem Dynamics and Conservation,* eds R. Dannell, R. Bergstrom, P. Duncan, and J. Pastor (Cambridge: Cambridge University Press), 289–319.

Pastor, J., Dewey, B., Naiman, R. J., McInnes, P. F., and Cohen, Y. (1993). Moose browsing and soil fertility in the boreal forests of Isle Royale National Park. *Ecology* 74, 467–480. doi: 10.2307/1939308

Pastor, J., Naiman, R. J., Dewey, B., and McInnes, P. (1988). Moose, microbes, and the boreal forest. *Bioscience* 38, 770–777. doi: 10.2307/1310786

Prange, H. D., Anderson, J. F., and Rahn, H. (1979). Scaling of skeletal mass to body-mass in birds and mammals. *Am. Nat.* 113, 103–122. doi: 10.1086/283367

Reynolds, B. C., and Hunter, M. D. (2001). Responses of soil respiration, soil nutrients, and litter decomposition to inputs from canopy herbivores. *Soil Biol. Biochem.* 33, 1641–1652. doi: 10.1016/s0038-0717(01)00085-2

Ritchie, M. E., Tilman, D., and Knops, J. M. H. (1998). Herbivore effects on plant and nitrogen dynamics in oak savanna. *Ecology* 79, 165–177. doi: 10.2307/176872

Roman, J., and McCarthy, J. J. (2010). The whale pump: marine mammals enhance primary productivity in a Coastal Basin. *PLoS ONE* 5:e13255. doi: 10.1371/journal.pone.0013255

Ruess, R. W., and McNaughton, S. J. (1988). Ammonia volatilization and the effects of large grazing mammals on nutrient loss from East African grasslands. *Oecologia* 77, 382–386. doi: 10.1007/bf00378047

Schindler, D. E., and Eby, L. A. (1997). Stoichiometry of fishes and their prey: implications for nutrient recycling. *Ecology* 78, 1816–1831.

Schrama, M., Veen, G. F., Bakker, E. S., Ruifrok, J. L., Bakker, J. P., and Olff, H. (2013). An integrated perspective to explain nitrogen mineralization in grazed ecosystems. *Perspect. Plant Ecol. Evolut. Syst.* 15, 32–44. doi: 10.1016/j.ppees.2012.12.001

Seastedt, T. R., and Crossley, D. A. (1984). The influence of arthropods on ecosystems. *Bioscience* 34, 157–161. doi: 10.2307/1309750

Sirotnak, J. M., and Huntly, N. J. (2000). Direct and indirect effects of herbivores on nitrogen dynamics: voles in riparian areas. *Ecology* 81, 78–87. doi: 10.1890/0012-9658(2000)081[0078:DAIEOH]2.0.CO;2

Sitters, J., Atkinson, C. L., Guelzow, N., Kelly, P., and Sullivan, L. L. (2015). Spatial stoichiometry: cross-ecosystem material flows and their impact on recipient ecosystems and organisms. *Oikos* 124, 920–930. doi: 10.1111/oik.02392

Sitters, J., te Beest, M., Cherif, M., Giesler, R., and Olofsson, J. (2017). Interactive effects between reindeer and habitat fertility drive soil nutrient availabilities in arctic tundra. *Ecosystems* 1–12. doi: 10.1007/s10021-017-0108-1

Sitters, J., and Olde Venterink, H. (2015). The need for a novel integrative theory on feedbacks between herbivores, plants and soil nutrient cycling. *Plant Soil* 396, 421–426. doi: 10.1007/s11104-015-2679-y

Stark, S., Tuomi, J., Strommer, R., and Helle, T. (2003). Non-parallel changes in soil microbial carbon and nitrogen dynamics due to reindeer grazing in northern boreal forests. *Ecography* 26, 51–59. doi: 10.1034/j.1600-0587.2003.03336.x

Sterner, R. W. (1990). The ratio of nitrogen to phosphorus resupplied by herbivores–zooplankton and the algal competitive arena. *Am. Nat.* 136, 209–229. doi: 10.1086/285092

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry*. Princeton, NJ: Princeton University Press.

Sterner, R. W., Elser, J. J., and Hessen, D. O. (1992). Stoichiometric relationships among producers, consumers and nutrient cycling in pelagic ecosystems. *Biogeochemistry* 17, 49–67.

Steuer, P., Sudekum, K. H., Tutken, T., Muller, D. W. H., Kaandorp, J., Bucher, M., et al. (2014). Does body mass convey a digestive advantage for large herbivores? *Funct. Ecol.* 28, 1127–1134. doi: 10.1111/1365-2435.12275

Subalusky, A. L., Dutton, C. L., Rosi-Marshall, E. J., and Post, D. M. (2015). The hippopotamus conveyor belt: vectors of carbon and nutrients from terrestrial grasslands to aquatic systems in sub-Saharan Africa. *Freshw. Biol.* 60, 512–525. doi: 10.1111/fwb.12474

Taylor, J. M., Vanni, M. J., and Flecker, A. S. (2015). "Top-down and bottom-up interactions in freshwater ecosystems: emerging complexities," in *Trophic Ecology: Bottom-Up and Top-Down Interactions Across Aquatic and Terrestrial Systems,* eds T. C. Hanley and K. J. La Pierre. (Cambridge: Cambridge University Press), 55–85.

Torres, L. E., and Vanni, M. J. (2007). Stoichiometry of nutrient excretion by fish: interspecific variation in a hypereutrophic lake. *Oikos* 116, 259–270. doi: 10.1111/j.2006.0030-1299.15268.x

Turner, J. T. (2015). Zooplankton fecal pellets, marine snow, phytodetritus and the ocean's biological pump. *Prog. Oceanogr.* 130, 205–248. doi: 10.1016/j.pocean.2014.08.005

Urabe, J. (1993). N-cycling and P-cycling coupled by grazer activities–food quality and nutrient release by zooplankton. *Ecology* 74, 2337–2350. doi: 10.2307/1939586

Urabe, J., Nakanishi, M., and Kawabata, K. (1995). Contribution of metazoan plankton to the cycling of nitrogen and phosphorus in Lake Biwa. *Limnol. Oceanogr.* 40, 232–241.

van der Waal, C., Kool, A., Meijer, S. S., Kohi, E., Heitkönig, I. M. A., de Boer, W. F., et al. (2011). Large herbivores may alter vegetation structure of semi-arid savannas through soil nutrient mediation. *Oecologia* 165, 1095–1107. doi: 10.1007/s00442-010-1899-3

Vanni, M. J. (2002). Nutrient cycling by animals in freshwater ecosystems. *Annu. Rev. Ecol. Syst.* 33, 341–370. doi: 10.1146/annurev.ecolysis.33.010802.150519

Vanni, M. J., Flecker, A. S., Hood, J. M., and Headworth, J. L. (2002). Stoichiometry of nutrient recycling by vertebrates in a tropical stream: linking species identity and ecosystem processes. *Ecol. Lett.* 5, 285–293. doi: 10.1046/j.1461-0248.2002.00314.x

Vanni, M. J., and McIntyre, P. B. (2016). Predicting nutrient excretion of aquatic animals with metabolic ecology and ecological stoichiometry: a global synthesis. *Ecology* 97, 3460–3471. doi: 10.1002/ecy.1582

Vanni, M. J., Renwick, W. H., Headworth, J. L., Auch, J. D., and Schaus, M. H. (2001). Dissolved and particulate nutrient flux from three adjacent agricultural watersheds: a five-year study. *Biogeochemistry* 54, 85–114. doi: 10.1023/a:1010681229460

Verheyden, H., Aubry, L., Merlet, J., Petibon, P., Chauveau-Duriot, B., Guillon, N., et al. (2011). Faecal nitrogen, an index of diet quality in roe deer *Capreolus capreolus*? *Wildlife Biol.* 17, 166–175. doi: 10.2981/10-111

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setala, H., van der Putten, W. H., and Wall, D. H. (2004). Ecological linkages between aboveground and belowground biota. *Science* 304, 1629–1633. doi: 10.1126/Science.1094875

Willott, S. J., Miller, A. J., Incoll, L. D., and Compton, S. G. (2000). The contribution of rabbits (*Oryctolagus cuniculus* L.) to soil fertility in semi-arid Spain. *Biol. Fertil. Soils* 31, 379–384. doi: 10.1007/s003749900183

Wrench, J. M., Meissner, H. H., and Grant, C. C. (1997). Assessing diet quality of African ungulates from faecal analyses: the effect of forage quality, intake and herbivore species. *Koedoe* 40, 125–136. doi: 10.4102/koedoe.v40i1.268

Zhang, B., Wang, C., Wei, Z. H., Sun, H. Z., Xu, G. Z., Liu, J. X., et al. (2016). The effects of dietary phosphorus on the growth performance and phosphorus excretion of dairy heifers. Asian-*Australas. J. Anim. Sci.* 29, 960–964. doi: 10.5713/ajas.15.0548

Zhang, G. M., Han, X. G., and Elser, J. J. (2011). Rapid top-down regulation of plant C:N:P stoichiometry by grasshoppers in an Inner Mongolia grassland ecosystem. *Oecologia* 166, 253–264. doi: 10.1007/s00442-011-1904-5

Zhang, Z. J., Elser, J. J., Cease, A. J., Zhang, X. M., Yu, Q., Han, X. G., et al. (2014). Grasshoppers regulate N:P stoichiometric homeostasis by changing phosphorus contents in their frass. *PLoS ONE* 9:e103697. doi: 10.1371/journal.pone.0103697

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 November 2017
doi: 10.3389/fmicb.2017.02116

Environmental Nutrient Supply Directly Alters Plant Traits but Indirectly Determines Virus Growth Rate

Christelle Lacroix†, Eric W. Seabloom and Elizabeth T. Borer*

Department of Ecology, Evolution, and Behavior, University of Minnesota, Saint Paul, MN, United States

OPEN ACCESS

Edited by:
Jonathan P. Zehr, University of California, Santa Cruz, United States

Reviewed by:
Gerald Moser, Justus Liebig Universität Gießen, Germany
Kerry Elizabeth Mauck, ETH Zurich, Switzerland

***Correspondence:**
Christelle Lacroix
christelle.lacroix@inra.fr

†Present Address:
Christelle Lacroix, UR0407 Plant Pathology, INRA, Montfavet, France

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 March 2017*
Accepted: *17 October 2017*
Published: *06 November 2017*

Citation:
Lacroix C, Seabloom EW and Borer ET (2017) Environmental Nutrient Supply Directly Alters Plant Traits but Indirectly Determines Virus Growth Rate. Front. Microbiol. 8:2116. doi: 10.3389/fmicb.2017.02116

Ecological stoichiometry and resource competition theory both predict that nutrient rates and ratios can alter infectious disease dynamics. Pathogens such as viruses hijack nutrient rich host metabolites to complete multiple steps of their epidemiological cycle. As the synthesis of these molecules requires nitrogen (N) and phosphorus (P), environmental supply rates, and ratios of N and P to hosts can directly limit disease dynamics. Environmental nutrient supplies also may alter virus epidemiology indirectly by changing host phenotype or the dynamics of coinfecting pathogens. We tested whether host nutrient supplies and coinfection control pathogen growth within hosts and transmission to new hosts, either directly or through modifications of plant tissue chemistry (i.e., content and stoichiometric ratios of nutrients), host phenotypic traits, or among-pathogen interactions. We examined two widespread plant viruses (BYDV-PAV and CYDV-RPV) in cultivated oats (*Avena sativa)* grown along a range of N and of P supply rates. N and P supply rates altered plant tissue chemistry and phenotypic traits; however, environmental nutrient supplies and plant tissue content and ratios of nutrients did not directly alter virus titer. Infection with CYDV-RPV altered plant traits and resulted in thicker plant leaves (i.e., higher leaf mass per area) and there was a positive correlation between CYDV-RPV titer and leaf mass per area. CYDV-RPV titer was reduced by the presence of a competitor, BYDV-PAV, and higher CYDV-RPV titer led to more severe chlorotic symptoms. In our experimental conditions, virus transmission was unaffected by nutrient supply rates, co-infection, plant stoichiometry, or plant traits, although nutrient supply rates have been shown to increase infection and coinfection rates. This work provides a robust test of the role of plant nutrient content and ratios in the dynamics of globally important pathogens and reveals a more complex relationship between within-host virus growth and alterations of plant traits. A deeper understanding of the differential effects of environmental nutrient supplies on virus epidemiology and ecology is particularly relevant given the rapid increase of nutrients flowing into Earth's ecosystems as a result of human activities.

Keywords: nutrient supply, stoichiometry, coinfection, plant traits, virus accumulation, transmission

INTRODUCTION

Ecological stoichiometry (Sterner and Elser, 2002; Hessen et al., 2013) and resource competition (MacArthur, 1972; Tilman, 1982; Miller et al., 2005) are two powerful theoretical frameworks for understanding the effects of altered nutrient supplies on the physiology and ecology of organisms. Both frameworks rest on the observation that species differ in their requirements for the supply rates and ratios of different elemental nutrients. Ecological stoichiometry primarily is founded on the assumption that the stoichiometric balance, or ratio, of multiple chemical elements (e.g., carbon [C], nitrogen [N], and phosphorus [P]) available to organisms is a driver of ecological processes (Sterner and Elser, 2002; Hessen et al., 2013; Hillebrand et al., 2014). In particular, this framework has been used to study the effects of C:N:P ratios in a resource (e.g., prey) on consumers' growth (Sterner and Elser, 2002; Hessen et al., 2013; Hillebrand et al., 2014). In contrast, resource competition theory predicts population persistence, population growth, and species coexistence based on both rates and ratios of nutrient resources and is based on the assumption of competitive interactions between species (e.g., consumers and prey) for shared resources (MacArthur, 1972; Miller et al., 2005). While originally grounded in aquatic and marine ecosystems (Redfield, 1958; Corner et al., 1976; Tilman, 1976, 1977), both ecological stoichiometry and resource competition have been used to assess the effects of nutrient addition on the abundance, diversity, and functional traits of coexisting species of free-living organisms in terrestrial ecosystems (Haddad et al., 2000; Cardinale et al., 2009; Zehnder and Hunter, 2009; Elser et al., 2010; Borer et al., 2014a,b; Seabloom et al., 2015a).

In addition to free-living organisms, ecological stoichiometry (Sterner and Elser, 2002) and resource competition (Miller et al., 2005) also can be used to interpret the effect of nutrient supply rates and ratios on microbe dynamics (Smith, 1993, 2007; Smith and Holt, 1996; Aalto et al., 2015). Both theoretical frameworks predict that changes in host nutrient supplies can alter microorganismal reproduction (i.e., titer or population size), because host nutrient content can limit the production of nutrient-demanding microbial cells and particles (Smith, 2007). Consistent with these predictions, the growth rate of human and animal microorganisms, algae viruses, and bacteria in crustaceans has been shown to be linked to nutrient stoichiometry (Elser et al., 2003; Karpinets et al., 2006; Clasen and Elser, 2007; Frost et al., 2008; Lange et al., 2014; Maat and Brussaard, 2016). Nutrient supply rates and ratios also can control the dynamics of various plant and insect infectious diseases (Mitchell et al., 2003; Bedhomme et al., 2004; Borer et al., 2010, 2014b; Seabloom et al., 2013, 2015b; Lacroix et al., 2014). However, whether environmental resource supplies influence the ecology of plant viruses at different stages of the infection cycle and through direct effects on virus growth or indirect effects on partners (i.e., host plant and competitors) of the interactive network leading to epidemics remains unclear.

Applying stoichiometric and resource competition theory to host-microbe interactions may not be straightforward, because environmental nutrient supplies and ratios available to hosts may alter disease dynamics at different stages of the epidemiological cycle (i.e., infection success after inoculation, within host-multiplication, and between-host transmission; Aalto et al., 2015; Seabloom et al., 2015b; Borer et al., 2016). In addition, each of these stages can be influenced through a variety of pathways (Borer et al., 2016) including: (i) direct effects of nutrient addition to hosts on the rates and ratios of limiting nutrients available for within-host pathogen replication (Smith et al., 2005), (ii) indirect effects of nutrient availability to the focal pathogen mediated by interactions with other competing pathogens (Smith and Holt, 1996; Smith, 2007; Lacroix et al., 2014; Lange et al., 2014), and (iii) indirect effects of nutrient supply mediated by changes in host growth rates, size, and other functional traits (Whitaker et al., 2015).

Ecological stoichiometry (Sterner and Elser, 2002) and resource competition (Miller et al., 2005) theory predict that the content and ratios of nutrients available in hosts could directly limit the production of molecules such as nucleic acids and proteins that are necessary for the infection cycle of micro-organisms. Obligate parasites such as plant viruses rely entirely on their host to complete multiple steps of their epidemiological cycle, including host entry and within-host accumulation; and within hosts, viruses hijack host nitrogen- and phosphorus- rich molecules and metabolic pathways (Maule et al., 2002; Sterner and Elser, 2002; Ahlquist et al., 2003; Elser et al., 2010). In controlled conditions, environmental supply rates, but not ratios, of N and P to plant hosts increase the probability of successful infection establishment (Bawden and Kassanis, 1950a; Lacroix et al., 2014; Smith, 2014). Within-host accumulation rate also can be controlled by host resources and host nutrient stoichiometry (Spencer, 1941; Bawden and Kassanis, 1950b; Adam et al., 1987; Eraslan et al., 2007; Dordas, 2009; Alexander, 2010; Rua et al., 2013). Further, as within-host pathogen population growth often has been correlated with transmission rate to new hosts (Froissart et al., 2010), changes in within-host growth driven by host nutrient supplies could alter secondary transmission events and disease dynamics.

Host nutrient supply rates and ratios also could act indirectly on pathogen within-host growth and between-host transmission by influencing coexistence between coinfecting pathogens because of inter-specific variation in stoichiometric C:N:P requirements (Jover et al., 2014; Smith, 2014; Aalto et al., 2015) and also could alter interactions among the community of pathogens within a host. Plants may host many micro-organisms (Seabloom et al., 2009; Roossinck, 2012), and inter-specific microbial interactions within a host can range from antagonistic to neutral to facilitative with various potential consequences for disease dynamics (Turner, 2005; Rigaud et al., 2010; Elena et al., 2014; Seabloom et al., 2015b). For example, increased supplies of nitrogen can reduce among-virus competition and increase infection success and coinfection rates (Lacroix et al., 2014). Nutrient competition among microbes sharing a host also can change disease dynamics by altering within host accumulation, virulence (i.e., detrimental effects of infection on host fitness), transmission rates and disease emergence (Smith and Holt, 1996; Al-Naimi et al., 2005; Pedersen and Fenton, 2007; Alizon et al., 2013; Hall and Little,

2013; Salvaudon et al., 2013; Lange et al., 2014; Borer et al., 2016).

Environmental nutrient supplies also may alter pathogen dynamics indirectly at various stages of the infection cycle by changing host functional traits. For example, N has been shown to increase the concentration of a plant virus through its impacts on host biomass, rather than via direct effects on the virus (Whitaker et al., 2015). Functional traits corresponding to host morphological, physiological, and phenological properties can ultimately impact organisms' fitness in varying environmental conditions via effects of growth, reproduction, and survival (Westoby, 1998; Westoby and Wright, 2006; Violle et al., 2007, 2014). Many of these traits reflect the influence of evolutionary history, environmental conditions, and trade-offs in the allocation of limited resources to each component of organismal fitness. In particular, differences in plant species strategy of acquisition, use and allocation of nutrient resources have been characterized based on measures of a suite of correlated functional traits (Craine et al., 2002; Wright et al., 2004; Reich, 2014). Along a "fast-slow" plant economics spectrum, fast-growing plants are generally associated with relatively low tissue C:P and N:P ratio (i.e., higher P) and increased allocation to P rich ribosomal RNA (Elser et al., 2010). Leaves of fast growing plants also tend to be short lived and structurally flimsy, with thin lamina, low leaf mass per area (LMA), and high photosynthetic capacity and dark respiration rates (Wright et al., 2004; Elser et al., 2010; Reich, 2014). Inter-specific differences in the average plant phenotype along the "fast-slow" economic spectrum have been shown to influence the ability of different plant species to act as efficient reservoirs of plant viruses (Cronin et al., 2010), and intra-specific variation in plant functional traits in response to environmental nutrient supply could also alter epidemiological parameters (Whitaker et al., 2015).

Microbial infection also can alter host phenotype, raising the possibility for feedbacks between nutrient supply and pathogen infection on plant traits. Obligate parasites such as viruses can be considered as consumers (Aalto et al., 2015) that compete with their host for nutrient resources, which can lead to increased virulence when host resources are depleted (Smith and Holt, 1996; Smith, 2007). In this case, pathogen virulence may evolve through a trade-off, if the benefits of increased within-host replication and correlated increases in between-host transmission come at the cost of increased detrimental effects on host fitness through exploitation of host resources (Alizon et al., 2009, 2013; Froissart et al., 2010; Doumayrou et al., 2013). Ultimately, the epidemiology of horizontally transmitted pathogens could be altered by these virulence effects on host growth and lifespan, which can be approximated by several traits associated with the plant trait economics spectrum (e.g., LMA, growth rate, leaf lifespan), and this feedback may alter the interaction between plant nutrient supply rates and the growth and spread of plant pathogens.

Overall, ecological stoichiometry (Sterner and Elser, 2002) and resource competition (Miller et al., 2005) theory predict that host nutrient supplies may drive the ecology of pathogens such as plant viruses at various stages of their cycle through direct effects of the rates or ratios of available nutrients in hosts. Each stage of the epidemiological cycle also could be influenced by changes in the dynamics of coinfecting pathogens and in host functional traits mediated by host nutrient supplies. Here, we experimentally tested the effect of N and P supply rate, plant nutrient content and functional traits, and the presence of a co-infecting microbe on within-host accumulation, virulence, and between-host transmission of two plant virus species, barley yellow dwarf virus-PAV (BYDV-PAV) and cereal yellow dwarf virus-RPV (CYDV-RPV). We used a factorial combination of two nutrient supply rates of N and P that created four nutrient treatments with stoichiometric ratios replicated at low and high nutrient supply rates. By measuring plant carbon and nutrient content as well as C:N:P stoichiometry, we were able to test whether processes were primarily dependent on plant tissue content or ratios of nutrients. We tested the role of a within-host competitor on infection dynamics by including singly- and co-infected hosts. Our focal host species was *Avena sativa* (*Poaceae*), a widely-cultivated host of this virus group. We used this design to answer the following questions:

- Do plant nutrient supplies and infection alter plant stoichiometry and traits?
- Can host nutrient supplies and host tissue stoichiometry predict within-host virus titer?
- What is the relative importance of plant nutrient supplies, plant stoichiometry and traits, and coinfection on within-host virus accumulation and between-host transmission?

MATERIALS AND METHODS

Study System

Barley and cereal yellow dwarf viruses (B/CYDVs, Luteoviridae) are host generalists and are known to infect at least 150 grass species in the Poaceae family (Irwin and Thresh, 1990; D'arcy and Burnett, 1995). Infection is systemic in plants but restricted to host phloem cells. Plant infection with B/CYDVs can be associated with the expression of various symptoms, including dwarfing, yellowing and reddening, and with severe crop yield losses (Irwin and Thresh, 1990; Perry et al., 2000). B/CYDVs can also alter various plant traits such as host fecundity and longevity and have been recognized as the precursors of a dramatic shift in plant species composition in natural California grasslands (Malmstrom et al., 2005; Borer et al., 2007).

The B/CYDV group is globally distributed and includes members of the genera *Luteovirus* (e.g., BYDV-PAV) and *Polerovirus* (e.g., CYDV-RPV), two of the common B/CYDVs virus species found in both crop and wild plants (Leclercq-Le Quillec et al., 2000; Robertson and French, 2007; Seabloom et al., 2010). These viruses are obligately transmitted from plant to plant via aphid vectors (Aphididae) in a persistent, circulative, and non-propagative manner (Miller and Rasochova, 1997; Gray and Gildow, 2003). At least 25 aphid species are known as vectors of B/CYDVs, and the transmission efficiency of each virus species differs strongly among vectors (Halbert and Voegtlin, 1995; Power and Gray, 1995; Miller and Rasochova, 1997). The aphid species *Rhopalosiphum padi* is an efficient vector for both BYDV-PAV and CYDV-RPV, the focal viruses of this study.

B/CYDVs Isolates and Aphid Vectors

We used one isolate of each of two virus species, BYDV-PAV and CYDV-RPV that were originally collected from cereal crops in New York State and maintained in Dr. Stewart Gray's lab (Cornell University, USA). In our laboratory, we maintained these isolates by inoculating new cultures of healthy 10 day old *A. sativa* cv. Coast Black oat (Poaceae; National plant germplasm system, USDA; USA; hereafter *A. sativa*) hosts planted in 15 × 15 cm pots containing Sunshine MVP potting soil (Sun Gro Horticulture, Massachusetts, USA) every 3 weeks following the inoculation procedure described below.

Non viruliferous *R. padi* aphids were raised in 15 × 15 cm pots, each planted with 15 healthy *A. sativa* in Sunshine MVP potting soil (Sun Gro Horticulture, Massachusetts, USA). Colonies were maintained in a separate growth chamber (27°C, 16 h day, 8 h night, 32 W fluorescent bulbs) and were watered twice a week with 300 ml tap water. Approximately 100 aphids were transferred every 2 weeks to healthy 10 days old *A. sativa* plants.

Host Plant Growth

Seeds of *A. sativa* were sown into 3.8 cm diameter by 21 cm depth, 164 ml pots containing a water saturated mixture of 70/30% (V/V) Sunshine, premium grade, medium vermiculite (Sun Gro Horticulture, Massachusetts, USA) and Turface MVP potting material (Turface Athletics, Illinois, USA). The pots were then placed under controlled conditions in a virus- and aphid-free growth chamber (23°C, 15 h day, 9 h night, 400 W high pressure sodium bulbs). The seeds were allowed a 10-day germination period during which seedlings were thinned to one plant per pot.

Experimental Design

Plants were randomly assigned to four groups that were mock-, singly-, or co- inoculated with BYDV-PAV or CYDV-RPV. In each group, we inoculated seven plants per fertilization treatment differing in N and P supply rate (Ctrl [7.5 μM, 1 μM]; N [375 μM, 1 μM]; P [7.5 μM, 50 μM] and NP [375 μM, 50 μM]; respectively; Table S1). The whole procedure was repeated three times. Thus, in each of our 16 experimental conditions, (4 inoculations types [Mock, BYDV-PAV, CYDV- RPV, BYDV-PAV + CYDV-RPV]) * (4 nutrient treatments [Ctrl, N, P, NP]), and due to loss of a few plants, we had between 19 and 21 plants.

Fertilization treatments represented thus a full factorial combination of two levels of N and P addition at concentrations equivalent to 0.2 and 10% of a half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1938; Downs and Hellmers, 1975), a range of nutrient supply rates known to alter virus infection success in this system (Lacroix et al., 2014), while concentrations of other macro- and micro- nutrients remained constant (Table S1). Each plant was fertilized twice each week with 30 ml nutrient solution.

Mock and Virus Inoculations

We performed inoculations of plants when they had two leaves following a previously published protocol (Lacroix et al., 2014) as modified from (Gray, 2008). Briefly, after a 2 h starvation period, non-viruliferous aphids were allowed a 48 h virus acquisition access period on leaves that were detached either from non-infected plants or hosts singly infected with BYDV-PAV or CYDV-RPV and that were placed in vertical 12 × 1.5 cm glass vials within a growth chamber (23°C, 16 h day, 8 h night, 40 W fluorescent bulbs). After this acquisition period, aphids that fed on detached leaves of the same infection type (non-, BYDV-PAV, and CYDV-RPV infected) were pooled together. After another 2 h starvation period, we transferred five aphids on each mock- and singly- inoculated plant and ten aphids on each co-inoculated plant (five individuals from each of the batch of aphids that fed on BYDV-PAV and CYDV-RPV infected tissue material). Aphids were enclosed into an 8 × 2.5 cm 118 μm polyester mesh cage (Sefar America Incorporated, Kansas City, Missouri, USA) affixed to the youngest leaf possible of each experimental plant (10 days old). Plants were then placed in a growth chamber (23°C, 16 h day, 8 h night, 40 W fluorescent bulbs) and aphids were killed using an insecticidal soap (Ortho) after a 72 h virus inoculation access period.

Virus Detection by RT-PCR

At 19 days post inoculation (dpi), i.e., a time point when virus infection has become systemic and virus titer is above detection threshold (Chain et al., 2005), a 20 cm piece of the first leaf of each plant was harvested and stored at −20°C for further virus detection. The infection status of each test plant was verified as described by Lacroix et al. (2014). Briefly, total RNA extraction was performed using Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. RNA extracts were then stored at −20°C until use. To assess each plant infection status, we used a multiplexed RT-PCR assay that yields different fragments size for BYDV-PAV (i.e., 298 bp) and CYDV-RPV (i.e., 447 bp) using specific primers for BYDV-PAV (PAVR1, ATTGTGAAGGAATTAATGTA; PAVL1, AGAGGAGGGGCAAATCCTGT) and CYDV-RPV (RPVR2 CTGCGTTCTGACAGCAGG, RPV L ATGTTGTACCGCTTGATCCAC). These primers were adapted from a previously published protocol (Deb and Anderson, 2008). The PCR products were visualized on SybrSafe (Invitrogen) stained 2% (W/V) agarose-1000 (Invitrogen) gel using a UV-light EZ doc system (Bio-Rad) and fragment size was checked comparatively to a 100 bp DNA ladder (Apex Bioresearch Products).

Virus Titer

Within-host accumulation of each virus species was determined based on the same RNA extracts obtained as described just above and using a real-time quantification PCR protocol. Absolute quantification of virus titer was performed based on a standard curve constructed for each virus species from 10-fold serial dilutions of RNA transcripts of known concentration. These transcripts were produced using the MEGAscript® T7 Kit (Life Technologies) and amplicons obtained from regular RT-PCR with specific primers (as described above). The transcripts were then purified using a phenol/chloroform and isopropanol precipitation protocol as specified by the manufacturer's instructions. The size of the obtained transcripts was checked on a regular 2% agarose gel relatively to a 100 bp DNA ladder (Apex

Bioresearch Products). RNA transcripts concentration (ng/µl) was determined using a nanodrop spectrophotometer (Thermo scientific). The obtained value was converted to mol/µl using the molecular weight of a ribonucleotide (340 g/mol) and the number of bases of each type of transcript (Nb). The following mathematical formula was applied: RNA concentration in mol/µl = RNA concentration in ng/µl * (10^{-9} ng/1 g) * (1 mol/340 g) * (1/Nb). The Avogadro's constant (6.023×10^{23} molecules/mol) was used to estimate the number of RNA transcript copies per µl.

TaqMan® RT-PCR reactions were performed in a final volume of 25 µl using one step RT-PCR Master Mix reagent kits (RNA-to-Ct 1-Step Kit, Applied Biosystems) and an Applied Biosystems One Plus Real-time PCR System. Three replicates of each serial dilution of RNA transcripts, of non-template control (i.e., sterile water instead of RNA), of non-amplification control (i.e., no enzymes in the reaction), and of RNA extract of each test sample were included in each run. The reactions were performed with 2.5 µl of sample (i.e., RNA extracts or sterile water) following the manufacturer's instructions. The reverse-transcription was performed during a 30 min 48°C cycle. After a 15 min 95°C activation period of the Taq polymerase, cDNA fragments were amplified following 45 cycles of denaturation (25 s 95°C), annealing and extension (1 min 60°C). cDNA amplification was performed using specific primers and probes attached to a minor groove binding (MGB) quencher and to a different reporter fluorescent dye for BYDV-PAV (forward primer, TGGTCGCCCAAAAAT CTAAAAC; reverse primer GGAGTAAGGCTCGCAGTAAAT TGCCGCATAAACAC; and probe, AGCAGCCTTCGTTTA TCCAGTGCCAGA, FAM) and CYDV-RPV (forward primer, GAGGTTAGCGAGGAGTTAGAATTC; reverse primer, AAC TACCTCAGAGTTGCCACATTC; and probe, ACATCTTCA AGACTCCTAACCTCGCCAT, VIC).

Standard curves were constructed using the known concentration of RNA transcripts ($\log_{10}$ of genomic copies in 2.5 µl) for each serial dilution and the corresponding cycle threshold (i.e., Ct, the number of amplification cycles required for a significant increase in the reporter's fluorescence). Quantification of RNA ($\log_{10}$ of genomic copies in 2.5 µl) for test samples was calculated based on the Ct threshold obtained for each sample and the standard curves. Virus titer was then expressed as the $\log_{10}$ of genomic copies per mg of fresh tissue.

Plant Traits

Plants were assessed for several functional traits that are commonly measured to describe plant species resource acquisition and use strategy along the plant economics spectrum (Craine et al., 2002; Wright et al., 2004; Reich, 2014). Measured plant traits include the number of days from mock- or virus-inoculation to the emergence of the third leaf (cf. NbDaysEmerg), the chlorophyll content of the second leaf averaged across three values per leaf measured 15 dpi with a SPAD-502 (Konica Minolta) instrument, the percent leaf area that was senescent (i.e., dry) averaged across the first and second leaf (i.e., Senes) 18 dpi, above (AG) and below-ground (BG) fresh biomass (g) and the ratio between these two values (ABG) 41 dpi, leaf dry mass per area (LMA, mg cm^{-2}) and water content measured as the difference between fresh and dry leaf mass per area (mg cm^{-2}) 41 dpi. We also recorded the average percent leaf area across two leaves that was covered by chlorotic symptoms characteristic of B/CYDVs infection (i.e., yellowing and reddening) 19 dpi.

Tissue chemistry data, i.e., phosphorus (P), nitrogen (N), and carbon (C) content, were obtained based on above-ground leaf tissue collected 41 dpi. For each sample, leaf tissue was oven dried at 65°C for 48 h and then ground using a Beadbeater (Biospec). For N and C content, 3 mg of dry and ground tissue from each sample was weighed in a tin capsule. N and C absolute quantification was performed using an elemental (C:H:N) analyzer. For P tissue content analysis, two replicates of 1–1.5 mg each were prepared in tin capsules. P quantification was performed using a sulfuric acid digestion protocol. A standard curve was constructed from data obtained from samples of apple NIST standard of various weights (from 0 to 6.9 mg) and known phosphorus content (i.e., 0.159% of dry tissue weight). All samples in tin capsules were introduced in previously acid washed and weighed glass vials, and were then placed in a muffle furnace for 30 min at 300°C, and then for 2 h at 550°C. Afterwards, 0.4 ml of sulfuric acid (10 N H_2SO_4, 10.8 M) and 5 ml of nanopure water was added to each vial. The capped vials were then autoclaved for 30 min at 121°C. Then, 1 ml of room temperature molybdate reagent (i.e., from a solution made with 0.208 g Antimony Potassium—Tartrate mixed with 9.6 g Ammonium Heptamolybdate 4-hydrate in 1 L nanopure water) was added to room temperature glass vials. Then, 0.4 ml ascorbic acid (2 g/ml) and 3.2 ml of Nanopure water was added to each vial to bring the final volume to 10 ml. Each tube was weighed and each sample was then analyzed at 880 nm using 1 cm cuvettes and a Varian spectrophotometer. N, P, and C tissue content was expressed in moles, from which tissue nutrient ratios (N:P, C:N, C:P) were calculated.

Transmission Rate

Fresh leaf tissue collected from experimental plants (i.e., source plants) 19 dpi was used to assess transmission rate. Following the inoculation protocol described above, non-viruliferous aphids were allowed a 48 h acquisition access period on leaf tissue of each source plant separately. After a 2 h starvation period, 5 aphids were transferred in a mesh cage affixed to the youngest possible leaf of each of seven 10 days old *A. sativa* recipient plants per source plant. Plants were placed in a growth chamber (23°C, 16 h day, 8 h night, 40 W fluorescent bulbs) and aphids were killed using an insecticidal soap (Ortho) after a 72 h virus inoculation access period. Recipient plants were fertilized twice a week with 30 ml of a Ctrl (7.5 µM N, 1 µM P) nutrient solution. Leaf tissue was collected 19 dpi to assess each recipient plant infection status using a RT-PCR protocol as described above. For each virus species, transmission rate was expressed as the proportion of infected recipient plants per source plant.

Statistical Analyses

All statistical analyses were performed using R version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

Trait data (i.e., raw values of all traits except for virus induced symptoms) of all experimental plants were analyzed using a principal component analysis (PCA) from the FactoMineR package. All plant trait variables were scaled to unit variance prior to analysis. Correlations between plant trait variables and PCA dimensions were determined based on the contribution of each variable to each dimension and based on the results (i.e., correlation coefficient and significance test) given by the dimdesc function. The principal components (i.e., orthogonal dimensions, $N = 5$) that altogether explained 80% of the variance included in the initial data set were retained for further analysis.

To assess the effect of fertilization and infection on plant traits, we analyzed the effects of host plant N and P supply rate and single- vs. co-infection on coordinates of individual plants on each of the five retained PCA dimension using model averaging (Grueber et al., 2011). This approach allowed us to take into account that explanatory variables could be covarying and that there could more than one pertinent model. All the variables were standardized prior to analysis using the standardize function in the arm R package. We used the dredge function in the MuMIn R package to fit all possible models. We estimated parameter values, errors, and AIC-weighted importance using the model.avg function in the MuMIn R package. The "Relative Importance" of each explanatory variable was estimated based on the relativized sum of the Akaike weights summed across all of the models in which the parameter appears that are within four AIC_C (i.e., AIC corrected for small sample size) units of the model with the lowest AIC. Importance ranges from 0 (parameter not given explanatory weight) to 1 (parameter in all top models).

For each virus species, differences in within-host accumulation in infected plants was assessed as a function of host plant N and P supply rate, the within-host presence vs. absence of a second virus species (i.e., coinfection), and plant traits as represented by coordinates of individual plants on each of the five retained PCA dimensions. We repeated this analysis of within-host titer as a function of host plant nutrient supply rates, coinfection and raw values of each measured plant trait instead of PCA coordinates. The variable corresponding to water content was removed from this latter analysis because of inherent correlation with LMA. We used a model averaging approach as described above for these two types of analyzes. We also fitted a linear model to assess the amount of virus induced symptoms, which was $\log_{10}$ transformed to normalize its distribution, as a function of within-host virus accumulation.

Finally, we assessed differences in transmission rate of each virus species separately as a function of host plant nutrient supply rate, coinfection and plant traits (i.e., represented either by coordinates of individual plants on each of the five retained PCA dimension, or raw measures of each plant trait except water) using model averaging.

RESULTS

Plant Traits

The first five principal components, taken together, explained 80.5% of phenotypic variation included in the initial data set (**Table 1**). Together, the first two principal components explained 53.7% of this phenotypic variation (**Table 1** and **Figure 1A**). Principal component 1 was associated with foliar P and above-ground growth traits. Samples with higher values on principal component 1 (i.e., Dim.1) corresponded to plants with lower P content, higher foliar C:P and N:P ratio, faster leaf emergence, higher chlorophyll content, less senescent tissue, higher above ground biomass, and above/below ground biomass ratio and higher water content (**Table 1** and **Figure 1A**).

TABLE 1 | Results of principal component analysis performed with values of traits measured on non-infected plants and plants singly- and co- infected with BYDV-PAV and/or CYDV-RPV.

	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
Eigenvalue	4.635	2.887	1.494	1.185	1.066
% variance	33.105	20.618	10.671	8.461	7.616
Cummulative % Variance	33.105	53.723	64.394	72.855	80.472
N	0.384	**−0.831**	0.150	0.217	0.060
P	**−0.735**	−0.039	0.480	−0.049	−0.338
C	0.229	0.456	−0.465	−0.314	−0.050
C:N	−0.382	**0.785**	−0.085	−0.136	0.016
C:P	**0.738**	0.145	**−0.525**	−0.053	0.306
N:P	**0.606**	**−0.695**	−0.070	0.183	0.216
NbDaysEmerg	**−0.534**	0.122	−0.387	0.395	0.281
Chlorophyll	**0.721**	0.143	0.162	−0.065	−0.071
Senes	**−0.554**	−0.194	−0.034	0.091	0.132
AG	**0.839**	0.139	0.306	−0.250	−0.049
BG	0.413	0.362	**0.581**	−0.206	**0.515**
ABG	**0.613**	−0.197	−0.293	−0.095	**−0.632**
LMA	0.303	**0.539**	0.105	**0.709**	−0.152
Water	**0.619**	**0.561**	0.176	0.444	−0.120

Shown are the eigenvalue of each principal component (Dim.), the corresponding individual and cumulative percentage of variance explained, as well as the coordinates of each variable (i.e., plant trait) on each principal component. Measured plant traits correspond to average tissue content in nitrogen (N), carbon (C), and phosphorus (P) expressed in moles, tissue nutrient ratios (C:N, C:P, and N:P per mole), number of days from the mock or virus inoculation to the emergence of the third leaf (NbDaysEmerg), chlorophyll content, average percent of dry leaf area (Senes), above- (AG) and below-ground (BG) biomass (g fresh tissue) and ratio (ABG), leaf dry mass per area (LMA, mg cm^{-2}) and water content (mg cm^{-2}). The coordinates of the variables that contributed the most to each principal component (according to contributions of variables to each dimension and correlation tests) are highlighted in bold.

Leaf traits, including tissue chemistry and biomass production differed along PCA dimensions 2 and 3. Plant individuals with higher coordinates on PCA dimension 2 had lower tissue N content and N:P ratio, but higher tissue C:N ratio, LMA and water content (**Table 1** and **Figure 1A**). PCA dimension 3 explained 10.7% of phenotypic variance and contrasted high leaf tissue C:P content with high below-ground biomass (**Table 1**). PCA dimension 4 and 5 explained together 16.1% of phenotypic variation (**Table 1** and **Figure 1B**). Higher values on these dimensions corresponded to individual plants with higher LMA values (cf. PCA dimension 4), and higher below-ground biomass and lower above/below ground biomass ratio (cf. PCA dimension 5; **Table 1** and **Figure 1B**). Nutrient ratios were significantly associated with the first three PC axes, but the plant trait most strongly correlated with each PCA dimension was above-ground biomass (AG), leaf N content (N), below-ground biomass (BG),

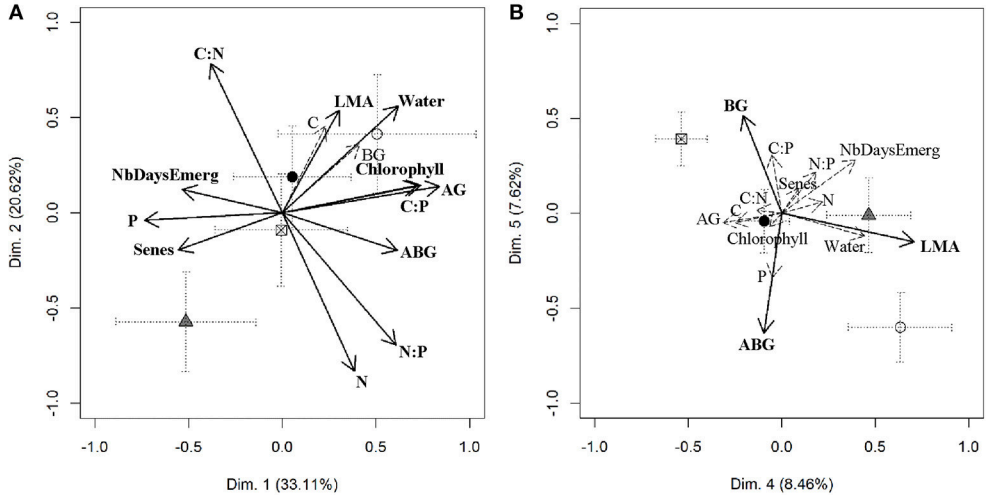

FIGURE 1 | Coordinates of initial variables (i.e., plant traits illustrated with arrows) and average coordinate of non- (crossed squares), CYDV-RPV singly- (white circles), BYDV-PAV singly- (black circles), and co- (gray triangles) infected plants along principal components (Dim.) 1 and 2 **(A)** and 4 and 5 **(B)**. Measured plant traits are abbreviated as in **Table 1**. The vector of variables that contributed the most to Dim. 1, 2, 4, and 5 are highlighted in bold. Error bars represent ± 1 SEM.

LMA, and above/below ground biomass ratio (ABG), respectively (**Table 1**).

Environmental Nitrogen Addition and Infection Altered Plant Traits

Elevated nitrogen supply rate to host plants increased ($p < 0.001$, relative importance = 1) individual plant values on PCA dimension 1 (e.g., higher AG) but decreased ($p = 0.006$, relative importance = 1) coordinates on dimension 4 (i.e., lower LMA; **Tables 2**, **3**). Increases in P supply rate did not significantly alter values of individual plants on any PCA dimension (**Tables 2**, **3**).

Single infection, either with BYDV-PAV or CYDV-RPV, did not significantly alter individual plant values on PCA dimension 1, 2, or 3 relative to mock-inoculated plants (**Tables 2**, **3**, and **Figure 1A**). However, coinfection reduced plant growth and altered plant chemistry, i.e., coinfection decreased coordinates on PCA dimension 1 ($p = 0.01$, relative importance = 1, e.g., lower AG and C:P) and 2 ($p = 0.042$, relative importance = 0.23, e.g., higher N) relative to mock-inoculated plants (**Table 2** and **Figure 1A**).

Infection by CYDV-RPV resulted in thicker (i.e., higher LMA) plant leaves, i.e., increased plant values on PCA dimension 4 ($p = 0.004$, relative importance = 0.1), and coinfection did not alter this relationship (**Table 3** and **Figure 1B**). In addition, CYDV-RPV infected plants were characterized by a higher above- to below- ground biomass ratio, i.e., associated with significantly lower coordinates on PCA dimension 5 ($p = 0.011$, relative importance = 1). However, the presence of BYDV-PAV increased coordinates of CYDV-RPV infected hosts ($p = 0.011$, relative importance = 1; **Table 3** and **Figure 1B**) on PCA dimension 5. Traits of BYDV-PAV infected plants did not differ from mock-inoculated plants on PCA dimensions 4 and 5 (**Table 3** and **Figure 1B**).

CYDV-RPV, But Not BYDV-PAV, Within-Host Accumulation Was Correlated with Changes in Plant Traits

Elevated N and P supply rates did not directly alter the within-host accumulation of CYDV-RPV (**Tables 4**, **5**). However, CYDV-RPV titer was increased within plants with higher coordinates PCA dimension 2 (e.g., higher plant C:N and LMA, $p = 0.039$, relative importance = 0.81, **Table 4** and **Figure 2A**). A detailed analysis of CYDV-RPV within-host accumulation as a function of nutrient supply rate, coinfection, and values of each plant trait revealed a positive correlation with LMA ($p = 0.001$, relative importance = 1; **Table 5** and **Figure 2B**), and a negative effect of coinfection ($p = 0.025$, relative importance = 0.91; **Table 5** and **Figure 3**). BYDV-PAV titer was not affected by environmental supply rates, co-infection, or plant traits (**Tables 4**, **5** and **Figure 3**).

CYDV-RPV Within-Host Accumulation Was Correlated with Chlorotic Symptoms

The percent area of infected leaf that was discolored (i.e., yellowing and reddening) was positively correlated with within-host titer for CYDV-RPV ($p = 0.004$, **Figure 4A**) but not for BYDV-PAV ($p = 0.95$, **Figure 4C**). Finally, within-host titer did not directly affect CYDV-RPV and BYDV-PAV virus transmission rate (Tables S2, S3 and **Figures 4B,D**). In addition, virus transmission rate was not significantly affected by nutrient supply rates, tissue chemistry, virus coinfection, or plant traits (Tables S2, S3).

DISCUSSION

The ecology of plant viruses may be influenced at various stages of their epidemiological cycle by multiple abiotic and

TABLE 2 | Summary of effects of N and P supply rate, single infection with BYDV-PAV or CYDV-RPV, and co-infection on plant traits (i.e., coordinates of individual plants on principal components [Dim.] 1 and 2) after model averaging.

Response	Variables[a]	Estimate[b]	Std.Error	Adjusted SE	z value	Pr(>|z|)[c]	Relative Importance	N containing models
Dim.1	(Intercept)	−2.326e+00	2.947e-01	2.974e-01	7.823	<2e-16	–	–
	N_{supply}	**1.020e-02**	**8.668e-04**	**8.742e-04**	**11.665**	**<2e16*** **	**1.00**	**11**
	P_{supply}	−1.845e-03	6.505e-03	6.559e-03	0.281	0.779	0.36	6
	N_{supply} :P_{supply}	−2.309e-05	2.784e-05	2.811e-05	0.821	0.411	0.05	1
	PAV	**1.314e-01**	**3.546e-01**	**3.577e-01**	**0.367**	**0.713**	**1.00**	**11**
	RPV	**2.046e-01**	**4.375e-01**	**4.412e-01**	**0.464**	**0.643**	**1.00**	**11**
	PAV:RPV	**−1.621e+00**	**6.233e-01**	**6.275e-01**	**2.584**	**0.010****	**1.00**	**11**
	PAV:N_{supply}	−8.329e-04	1.490e-03	1.502e-03	0.555	0.579	0.31	4
	RPV:N_{supply}	1.056e-03	1.867e-03	1.881e-03	0.562	0.574	0.25	4
	PAV:P_{supply}	−6.894e-03	9.496e-03	9.589e-03	0.719	0.472	0.05	1
	RPV:P_{supply}	2.265e-03	1.009e-02	1.019e-02	0.222	0.824	0.04	1
	PAV:RPV:N_{supply}	−4.969e-03	2.938e-03	2.967e-03	1.675	0.094	0.07	1
	PAV:RPV:P_{supply}	NA	NA	NA	NA	NA	NA	NA
Dim.2	(Intercept)	1.600e-01	3.630e-01	3.653e-01	0.438	0.662	–	–
	N_{supply}	**−1.838e-03**	**1.219e-03**	**1.227e-03**	**1.498**	**0.134**	**0.62**	**15**
	P_{supply}	**−5.459e-04**	**1.223e-02**	**1.229e-02**	**0.044**	**0.965**	**0.64**	**16**
	N_{supply}:P_{supply}	6.531e-05	3.614e-05	3.649e-05	1.790	0.074	0.31	6
	PAV	1.068e-01	4.038e-01	4.065e-01	0.263	0.793	0.39	13
	RPV	2.033e-01	5.325e-01	5.357e-01	0.379	0.704	0.41	14
	PAV:RPV	**−1.274e+00**	**6.217e-01**	**6.277e-01**	**2.029**	**0.042***	**0.23**	**8**
	PAV:N_{supply}	NA	NA	NA	NA	NA	NA	NA
	RPV:N_{supply}	NA	NA	NA	NA	NA	NA	NA
	PAV:P_{supply}	NA	NA	NA	NA	NA	NA	NA
	RPV:P_{supply}	1.308e-02	1.367e-02	1.380e-02	0.948	0.343	0.08	4
	PAV:RPV:N_{supply}	NA	NA	NA	NA	NA	NA	NA
	PAV:RPV:P_{supply}	NA	NA	NA	NA	NA	NA	NA

[a] *All variables were standardized prior to analysis. Variables of highest relative importance are highlighted in bold.*
[b] *NA is indicated for variables that were not included in any of the models selected within four AICc units of the model with the lowest AIC.*
[c] *Significance of effects is indicated according to a 0.05 (*), 0.01 (**), and 0.001 (***) threshold.*

biotic factors. The pathways by which nutrients influence virus population growth and transmission can include both direct effects of elevated host nutrient supplies and content on virus infection success and growth and indirect effects of nutrient addition through alterations of coinfecting pathogen dynamics and host phenotype. In contrast to predictions of ecological stoichiometry (Sterner and Elser, 2002) and resource competition (Miller et al., 2005) theories, we did not find any direct effects of rates and ratios of nutrients in environmental supplies or plant tissue on virus dynamics in our system. Increased nitrogen supply shifted plant phenotype toward higher plant growth rate and thinner leaves (i.e., reduced LMA), consistently with expected effects of N fertilization (Elser et al., 2007; Dordas, 2009; Reich, 2014), while infection with CYDV-RPV resulted in thicker plant leaves (i.e., higher LMA). CYDV-RPV titer was higher in plants with higher LMA. CYDV-RPV titer also was reduced by the presence of BYDV-PAV, further indicating within-host competition between these closely-related viruses (Lacroix et al., 2014). Chlorotic symptoms increased with CYDV-RPV titer, but transmission rate was independent of nutrient supply, tissue stoichiometry, structural plant traits, and virus titer. Our results reveal a more complex relationship between environmental nutrient supply, virus dynamics, alterations of plant phenotype, and within-host competition among pathogens.

Environmental Nutrient Supplies and Plant Tissue Chemistry Did Not Directly Alter Virus Titer

Ecological stoichiometry theory predicts that ratios in environmental nutrient supply or host nutrient content will limit infection success and the production of N and P demanding virus particles (Sterner and Elser, 2002), whereas resource competition theory predicts population growth, persistence and species coexistence based on both nutrient rates and ratios. Nutrient supply rates and ratios, in particular in carbon, nitrogen, and phosphorus, can limit virus multiplication in algae and phytoplankton hosts (Elser et al., 2003; Clasen and Elser, 2007; Frost et al., 2008; Maat and Brussaard, 2016). In contrast to these predictions and the tests in aquatic

TABLE 3 | Summary of effects of N and P supply rate, single infection with BYDV-PAV or CYDV-RPV, and co-infection on plant traits (i.e., coordinates of individual plants on principal component [Dim.] 3, 4, and 5) after model averaging.

Response	Variables[a]	Estimate[b]	Std.Error	Adjusted SE	z value	Pr(>|z|)[c]	Relative importance	N containing models
Dim.3	(Intercept)	6.273e-02	2.256e-01	2.272e-01	0.276	0.782	–	–
	N_{supply}	−3.109e-04	8.128e-04	8.186e-04	0.380	0.704	0.37	14
	P_{supply}	6.514e-03	4.852e-03	4.896e-03	1.330	0.183	0.58	16
	N_{supply}:P_{supply}	−5.979e-06	2.603e-05	2.628e-05	0.228	0.820	0.02	1
	PAV	3.084e-02	3.279e-01	3.300e-01	0.093	0.926	0.38	13
	RPV	**−3.680e-01**	**2.916e-01**	**2.938e-01**	**1.252**	**0.210**	**0.76**	**19**
	PAV:RPV	−6.375e-01	4.443e-01	4.485e-01	1.421	0.155	0.11	3
	PAV:N_{supply}	−1.925e-03	1.224e-03	1.236e-03	1.557	0.119	0.07	3
	RPV:N_{supply}	3.062e-04	1.376e-03	1.389e-03	0.220	0.826	0.04	2
	PAV:P_{supply}	5.609e-03	8.771e-03	8.856e-03	0.633	0.527	0.02	1
	RPV:P_{supply}	2.351e-03	9.387e-03	9.477e-03	0.248	0.804	0.06	2
	PAV:RPV:N_{supply}	NA	NA	NA	NA	NA	NA	NA
	PAV:RPV:P_{supply}	NA	NA	NA	NA	NA	NA	NA
Dim.4	(Intercept)	−1.276e-01	2.526e-01	2.542e-01	0.502	0.616	–	–
	N_{supply}	**−1.754e-03**	**6.324e-04**	**6.378e-04**	**2.750**	**0.006****	**1**	**29**
	P_{supply}	6.604e-03	5.943e-03	5.985e-03	1.103	0.270	0.52	19
	N_{supply}:P_{supply}	−3.363e-06	2.118e-05	2.138e-05	0.157	0.875	0.08	5
	PAV	**4.664e-01**	**2.914e-01**	**2.933e-01**	**1.590**	**0.112**	**0.80**	**24**
	RPV	**9.861e-01**	**3.403e-01**	**3.424e-01**	**2.880**	**0.004****	**1**	**29**
	PAV:RPV	−5.500e-01	3.658e-01	3.693e-01	1.489	0.136	0.41	12
	PAV:N_{supply}	−6.858e-04	1.014e-03	1.024e-03	0.670	0.503	0.19	8
	RPV:N_{supply}	1.125e-03	1.114e-03	1.124e-03	1.001	0.317	0.3	10
	PAV:P_{supply}	−1.106e-02	7.203e-03	7.273e-03	1.521	0.128	0.23	8
	RPV:P_{supply}	NA	NA	NA	NA	NA	NA	NA
	PAV:RPV:N_{supply}	NA	NA	NA	NA	NA	NA	NA
	PAV:RPV:P_{supply}	NA	NA	NA	NA	NA	NA	NA
Dim.5	(Intercept)	6.939e-01	2.443e-01	2.462e-01	2.818	0.005	–	–
	N_{supply}	**−9.388e-04**	**6.883e-04**	**6.940e-04**	**1.353**	**0.176**	**0.85**	**21**
	P_{supply}	**−6.699e-03**	**5.332e-03**	**5.375e-03**	**1.246**	**0.213**	**0.71**	**19**
	N_{supply}:P_{supply}	−7.176e-06	2.168e-05	2.189e-05	0.328	0.743	0.10	4
	PAV	**−4.949e-01**	**2.731e-01**	**2.754e-01**	**1.797**	**0.072**	**1.00**	**25**
	RPV	**−9.257e-01**	**3.597e-01**	**3.624e-01**	**2.555**	**0.011***	**1.00**	**25**
	PAV:RPV	**1.072e+00**	**4.171e-01**	**4.206e-01**	**2.550**	**0.011***	**1.00**	**25**
	PAV:N_{supply}	6.168e-04	1.091e-03	1.100e-03	0.561	0.575	0.25	9
	RPV:N_{supply}	−1.304e-03	1.274e-03	1.285e-03	1.015	0.310	0.29	9
	PAV:P_{supply}	7.094e-03	7.257e-03	7.328e-03	0.968	0.333	0.20	6
	RPV:P_{supply}	1.473e-03	7.857e-03	7.932e-03	0.186	0.853	0.12	5
	PAV:RPV:N_{supply}	2.935e-03	2.249e-03	2.271e-03	1.293	0.196	0.04	2
	PAV:RPV:P_{supply}	NA	NA	NA	NA	NA	NA	NA

[a] *All variables were standardized prior to analysis. Variables of highest relative importance are highlighted in bold.*

[b] *NA is indicated for variables that were not included in any of the models selected within four AIC c units of the model with the lowest AIC.*

[c] *Significance of effects is indicated according to a 0.05 (*), 0.01 (**), and 0.001 (***) threshold.*

ecosystems, we did not find any direct effects of nutrient supplies or plant tissue stoichiometry on the density (i.e., titer) of the two virus species examined in our study. Our study shows that alterations of plant phenotypic traits may be a key connection between nutrient rates and ratios and epidemiological rates.

Infection and Environmental Nutrient Supplies Altered Plant Phenotype

In our study, BYDV-PAV and CYDV-RPV did not reduce aboveground biomass, which contrasts with other studies of B/CYDV infection (Catherall, 1966; Malmstrom et al., 2005; Mordecai et al., 2015). However, we did find that leaf mass per area

TABLE 4 | Summary of effects of N and P supply rate, co-infection and plant traits (i.e., coordinates of individual plants on each principal component [Dim.]) on CYDV-RPV and BYDV-PAV titer after model averaging.

Response	Variables[a]	Estimate	Std. Error	AdjustedSE	Z value	Pr(>|z|)[b]	Relative importance	N containing models
RPV Titer	(Intercept)	4.54e+00	6.43e-02	6.63e-02	68.484	<2e-16	–	–
	N_{supply}	**−1.55e-03**	**7.86e-04**	**8.01e-04**	**1.93**	**0.054**	**0.67**	**23**
	P_{supply}	−1.29e-03	3.32e-03	3.42e-03	0.377	0.706	0.10	5
	Coinfection	−3.16e-01	1.70e-01	1.75e-01	1.808	0.071	0.58	20
	Dim.1	5.06e-01	2.61e-01	2.66e-01	1.903	0.057	0.59	20
	Dim.2	**3.24e-01**	**1.53e-01**	**1.58e-01**	**2.054**	**0.039***	**0.81**	**25**
	Dim.3	3.24e-02	1.80e-01	1.84e-01	0.176	0.861	0.14	7
	Dim.4	**2.87e-01**	**1.53e-01**	**1.57e-01**	**1.823**	**0.068**	**0.65**	**23**
	Dim.5	−2.85e-01	1.82e-01	1.87e-01	1.521	0.128	0.52	20
PAV Titer	(Intercept)	3.32e+00	5.40e-02	5.53e-02	59.903	<2e-16	–	–
	N_{supply}	2.35e-06	4.18e-04	4.26e-04	0.006	0.996	0.12	10
	P_{supply}	−2.92e-03	2.24e-03	2.29e-03	1.274	0.203	0.39	26
	Coinfection	−1.77e-01	1.37e-01	1.40e-01	1.263	0.207	0.40	27
	Dim.1	1.11e-01	1.22e-01	1.25e-01	0.893	0.372	0.12	15
	Dim.2	8.68e-02	1.11e-01	1.14e-01	0.763	0.445	0.17	13
	Dim.3	−1.20e-01	1.36e-01	1.39e-01	0.866	0.386	0.19	13
	Dim.4	1.49e-01	1.20e-01	1.22e-01	1.215	0.224	0.36	24
	Dim.5	−1.10e-01	1.16e-01	1.19e-01	0.93	0.353	0.22	15

[a] *All variables were standardized prior to analysis. Variables of highest relative importance are highlighted in bold.*
[b] *Significance of effects is indicated according to a 0.05 (*), 0.01 (**), and 0.001 (***) threshold.*

(i.e., LMA) was higher in CYDV-RPV infected plants, that CYDV-RPV titer was higher in plants with high LMA leaves, and that chlorotic symptoms induced on leaves increased with CYDV-RPV titer. Previous work on this virus group found reductions in fresh biomass and in yield of grass hosts infected with B/CYDV, and infected hosts also were characterized by higher leaf dry weight, which is similar to our findings for CYDV-RPV infected plants (Jensen, 1968, 1969, 1972). B/CYDV effects on plant phenotype arise from reduced translocation of photosynthates from leaves to the roots and apical meristem, resulting in the accumulation of soluble carbohydrates, starch and non-soluble proteins in diseased leaves, increased dry weight and dark respiration rates, decreased photosynthesis, and induction of chlorotic symptoms (Jensen, 1968, 1969, 1972; Malmstrom and Field, 1997). The increased non-soluble protein concentration in infected leaves may be due to the accumulation of non-soluble proteins, free amino acids, nucleic acids, amines, amides, and inorganic nitrogen (Jensen, 1969). These results in combination with our study suggest that the higher LMA associated with CYDV-RPV infection observed here could result from reduced nutrient translocation and subsequent accumulation of C-rich (e.g., carbohydrates and starch) and N-rich (e.g., non-soluble protein fraction) compounds in infected leaves. The accumulation of these resources in leaves of infected plants could induce stronger changes in phenotypes than nutrient supplies and could facilitate the CYDV-RPV multiplication cycle, increase virus titer, and lead to reduced photosynthate production and translocation to other plant parts causing chlorotic symptoms.

Possible Role of Carbon and Nitrogen Rich Compounds in Plant Tissues on Virus Titer

Our work further suggests that CYDV-RPV replication may not be directly limited by total tissue N, P, and C content and ratios, but by the concentration and ratios of specific C- and/ or N-rich molecules. Although, B/CYDV infection disrupts nutrient translocation and increases the content of C- and N- rich compounds in infected leaves, a greater amount of the carbohydrates accumulated during the day in leaves of infected vs. uninfected plants grown under elevated CO_2 conditions have been shown to be converted, exported and/or respired during the night due to decreases in sucrose, glucose and fructose concentration (Malmstrom and Field, 1997). This suggests that sugars could have been reallocated to fuel virus multiplication. Within-host BYDV-PAV accumulation was further found to increase in host plants exposed to elevated CO_2 levels, although this increase was unrelated to either increased plant growth or to the absolute dry weight (g) or mean leaf area (cm^{-2}) (Trebicki et al., 2015). However, specific leaf area ($cm^2\ g^{-1}$) was lower (i.e., higher LMA) in infected compared to uninfected plants (Trebicki et al., 2015), suggesting a potential positive correlation between BYDV-PAV titer and LMA. In contrast, increased host growth under conditions of elevated N supply led to increased BYDV-PAV titer in a different study (Whitaker et al., 2015), although this only occurred in small plants and this effect was reversed in larger plants (Whitaker et al., 2015), perhaps due to reduced allocation of nutrients by plants to cellular machinery with increasing plant size (Elser et al., 2010). Finally, carbohydrates

TABLE 5 | Summary of effects of N and P supply rate, co-infection and individual plant traits on CYDV-RPV and BYDV-PAV titer after model averaging.

Response	Variables[a,b]	Estimate	Std. Error	AdjustedSE	Z value	Pr(>|z|)[c]	Relative importance	N Containing models
RPV Titer	(Intercept)	4.542	0.063	0.065	70.426	–2e–16***	–	–
	Nsupply	–0.001	0.001	0.001	1.291	0.197	0.27	45
	Psupply	–0.002	0.003	0.003	0.727	0.467	0.08	13
	Coinfection	**–0.339**	**0.147**	**0.151**	**2.238**	**0.025***	**0.91**	**129**
	C	–0.188	0.166	0.171	1.103	0.270	0.17	28
	N	–0.637	0.555	0.561	1.136	0.256	0.52	74
	P	–0.063	0.444	0.450	0.140	0.889	0.08	15
	CN	0.202	0.197	0.202	1.003	0.316	0.22	35
	CP	–0.429	0.292	0.298	1.441	0.150	0.26	37
	NP	0.756	0.586	0.596	1.269	0.204	0.29	42
	ABG	0.196	0.223	0.228	0.858	0.391	0.09	15
	AG	0.157	0.277	0.281	0.561	0.575	0.10	19
	BG	–0.152	0.164	0.169	0.902	0.367	0.09	15
	Chlorophyll	–0.026	0.151	0.155	0.166	0.868	0.05	10
	LMA	**0.520**	**0.151**	**0.155**	**3.351**	**0.001*****	**1.00**	**146**
	NbDaysEmerg	0.006	0.146	0.150	0.040	0.968	0.04	7
	Senes	–0.205	0.146	0.151	1.360	0.174	0.32	49
PAV Titer	(Intercept)	3.315	0.053	0.054	60.949	<2e-16***	–	–
	Nsupply	–0.001	0.001	0.001	1.459	0.145	0.33	111
	Psupply	–0.003	0.002	0.002	1.281	0.200	0.30	109
	Coinfection	–0.167	0.146	0.149	1.116	0.265	0.18	67
	C	0.121	0.112	0.115	1.051	0.293	0.15	53
	N	–0.092	0.162	0.165	0.559	0.576	0.06	26
	P	0.001	0.161	0.164	0.005	0.996	0.05	22
	CN	–0.122	0.150	0.153	0.798	0.425	0.10	42
	CP	0.109	0.143	0.146	0.750	0.453	0.08	33
	NP	0.006	0.119	0.122	0.051	0.959	0.04	16
	ABG	0.065	0.222	0.225	0.290	0.772	0.17	65
	AG	0.397	0.283	0.286	1.388	0.165	0.62	215
	BG	–0.262	0.204	0.208	1.258	0.209	0.30	105
	Chlorophyll	–0.167	0.148	0.151	1.103	0.270	0.18	68
	LMA	0.159	0.113	0.115	1.378	0.168	0.38	138
	NbDaysEmerg	0.213	0.141	0.144	1.483	0.138	0.37	124
	Senes	0.003	0.135	0.18	0.021	0.983	0.04	19

[a] *All variables were standardized prior to analysis. Variables of highest relative importance are highlighted in bold.*

[b] *Because leaf water content was calculated based on fresh mass per area and LMA, the variable water content was removed from this analysis to avoid inherent correlations in explanatory variables.*

[c] *Significance of effects is indicated according to a 0.05 (*), 0.01 (**), and 0.001 (***) threshold.*

(e.g., fructose, mannitol, and trehalose) were higher in both healthy and B/CYDV infected plants under elevated CO_2 levels, and amino acid concentrations were higher in infected plants irrespective of atmospheric CO_2 levels (Vassiliadis et al., 2016). Taken together, these studies suggest that the titer of B/CYDVs may rely more on the content and ratios of particular C-rich (e.g., sugars like sucrose and fructose) and N-rich (e.g., amino acids) molecules than elemental nutrient concentration *per se*. The joint analysis of metabolic profiles in infected and healthy plants, of within-host virus accumulation and between-host transmission, and of the molecular mechanisms underlying these processes thus likely constitute a fruitful avenue for research.

Virus and Plant Species Differences in Functional Traits Might Drive Infection and Plant Responses to Environmental Nutrient Supplies

We did not find any relationship between BYDV-PAV titer or transmission rate and environmental host nutrient supplies, plant traits, or stoichiometry. In our experimental conditions, environmental and host tissue nutrient rates and ratios may not have been limiting for the BYDV-PAV multiplication cycle. As suggested previously, the question of potential differences in nutrient requirements for both CYDV-RPV and BYDV-PAV virus species remains open, and limitations in C-rich and N-rich

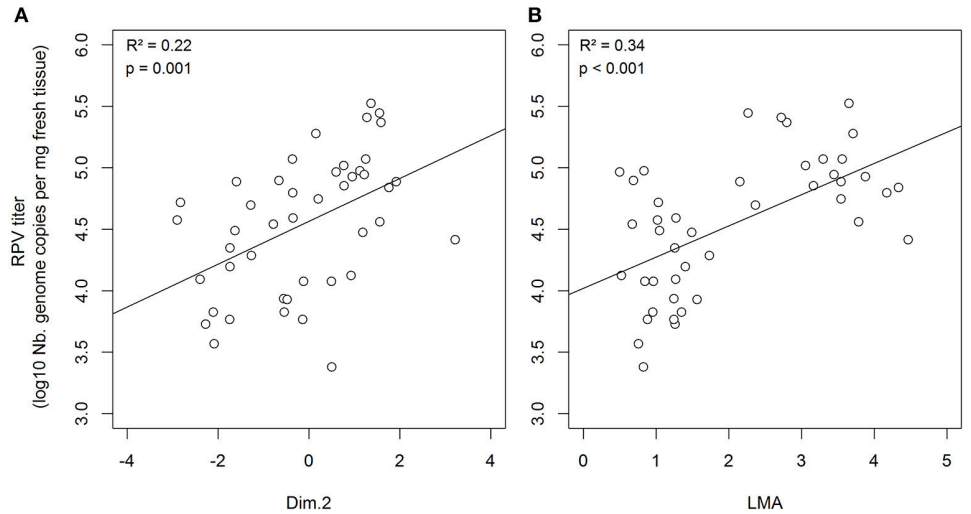

FIGURE 2 | Within-host CYDV-RPV accumulation measured based on leaf tissue 19 dpi as a function of individual plants coordinates on the second principal component **(A)** and of individual plant LMA values **(B)**. The R-square, *p*-value and slope is shown for significant linear bivariate relationships.

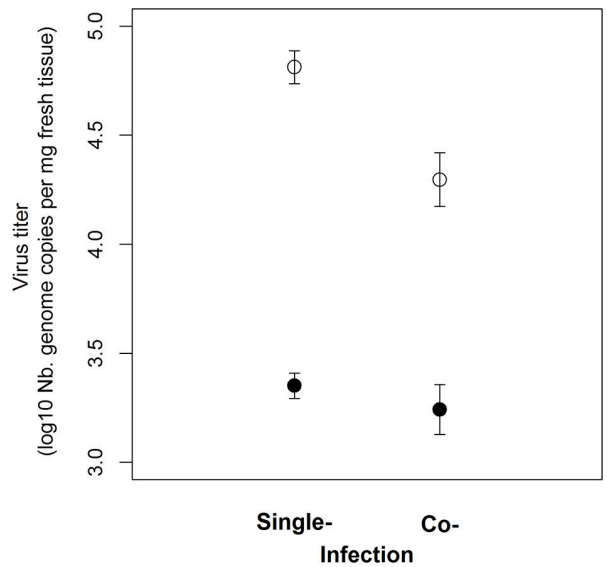

FIGURE 3 | Average virus accumulation in leaf tissue 19 dpi for CYDV-RPV (white circles) and BYDV-PAV (black circles) in single- and co-infection. Error bars represent ± 1 SEM.

metabolites might be stronger for CYDV-RPV than for BYDV-PAV (Lacroix et al., 2014; Smith, 2014). Moreover, Cronin et al. (2010) found inter-specific differences in plant species ability to act as reservoirs of BYDV-PAV, such that host susceptibility to the inoculation, within-host accumulation, and transmission rate of BYDV-PAV was higher on average for plant species characterized by a "faster" plant phenotype (Cronin et al., 2010). In addition, while previous field experiments have demonstrated a positive effect of P supply and N:P ratio on B/CYDV prevalence (Borer et al., 2010, 2014b), elevated P supply to grass hosts in controlled conditions decreased BYDV-PAV within-host accumulation in *Avena fatua* but not in *Bromus hordeaceus* grass hosts (Poaceae) (Rua et al., 2013). In our study, we found differences in the effects of CYDV-RPV- single, BYDV-PAV- single, and coinfection on plant phenotype, consistent with the emerging perspective of the diversity of possible host-virus interactions (Marquez et al., 2007; Roossinck et al., 2010; Roossinck, 2015). Although, BYDV-PAV alone did not significantly alter plant phenotype, coinfected plants were characterized by higher LMA, reduced above- to below- ground biomass ratio and plant growth, higher N and P content; higher N:P ratio; and more senescent tissue than uninfected or CYDV-RPV singly-infected hosts, suggesting that coinfection reduced plants' ability to allocate resources to plant growth. Overall, these results suggest that plant responses to infection, as well as virus epidemiological parameters at various stages of infection might be influenced by multiple factors including inter- and intra-specific virus and plant differences in functional traits, plant nutrient and metabolite stoichiometry, and environmental abiotic conditions.

Environmental Nutrient Supplies May Differentially Alter Various Steps of the Epidemiological Cycle

In terrestrial systems, elevated environmental nutrient supplies to grass species in natural ecosystems have been shown to increase the prevalence and co-infection rates of a group of generalist and aphid-vectored plant viruses (i.e., barley and cereal yellow dwarf viruses; Seabloom et al., 2009, 2013; Borer et al., 2010, 2014a,b). These responses observed in natural conditions may be the result of multiple interacting processes such as changes in the host plant community (Borer et al., 2009, 2010), altered vector behavior or performance (Borer et al., 2009; Seabloom et al., 2013), direct influences of plant nutrient supplies and plant stoichiometry on host susceptibility, virus inter-specific interactions, virus multiplication, and transmission (Smith, 1993, 2007; Smith and Holt, 1996), or through indirect influences on

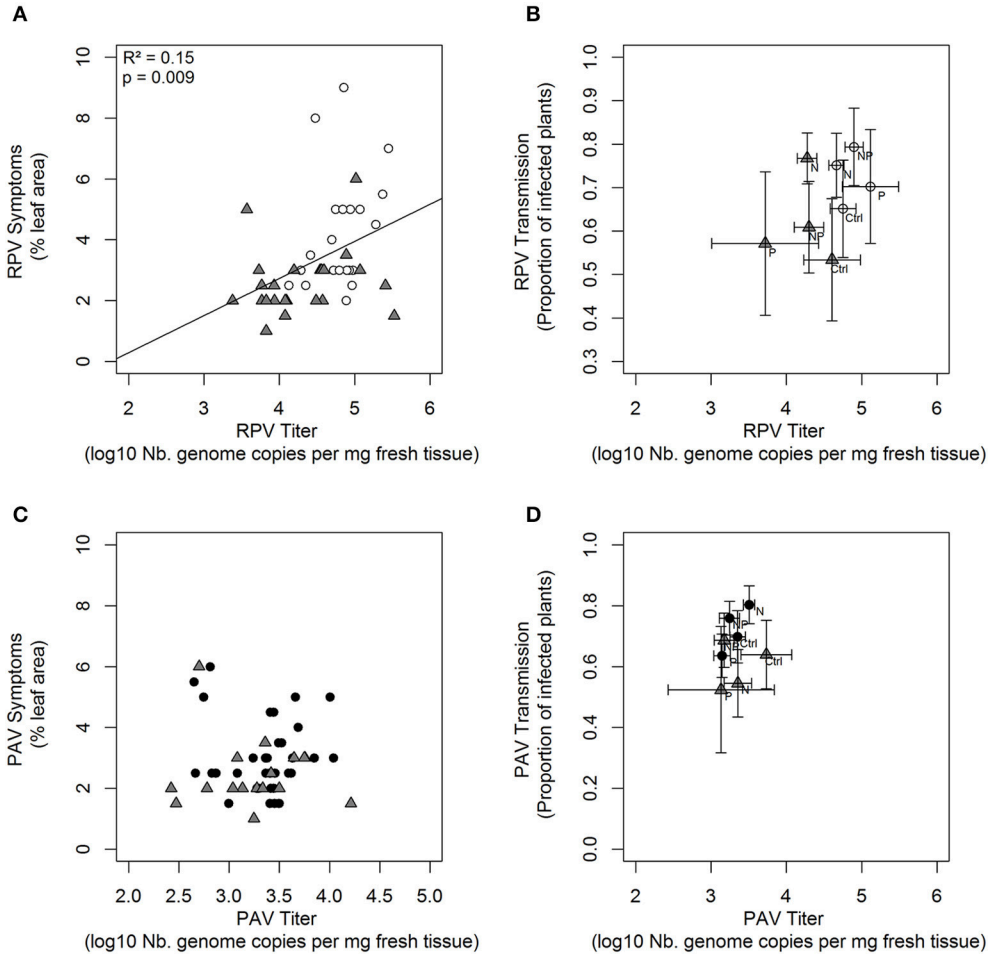

FIGURE 4 | Percent leaf area displaying symptoms associated with RPV **(A)** and PAV infection **(C)** and average proportion of plants infected after secondary transmission from differentially fertilized test plants as a function of RPV **(B)** and PAV **(D)** within-host accumulation measured based on leaf tissue 19 dpi. Virus associated titer, symptoms and transmission rate is shown for "source" RPV singly- (white circles), PAV singly- (black circles), and co- (gray triangles) infected plants. The R-square, *p*-value and line is shown for significant linear bivariate relationships. Nitrogen and phosphorus content of fertilization solutions Ctrl (Control), N (Nitrogen), P (Phosphorus), and NP (Nitrogen and Phosphorus) are as follows: 7.5/1, 375/1, 7.5/50, 375/50 μM, respectively. Error bars represent ± 1 SEM.

plant species diversity, composition, and functional traits (Liu et al., 2017).

Our work and that presented by Lacroix et al. (2014) now provide deeper insight into the host-level mechanisms that underlie nutrient effects on pathogen spread observed in natural ecosystems. Importantly, we have shown that infection success was differentially altered by nutrient supply rates and virus competition (Lacroix et al., 2014). CYDV-RPV infection rates were reduced by both P supply rates and competition with BYDV-PAV, but only at low N supply rates. After the establishment of infection, this study shows CYDV-RPV titer was affected through changes in plant phenotype, possibly through changes in rates and ratios of C-rich and N-rich metabolites, and titer was reduced by the presence of a competitor (coinfection). Higher virus titer led to increased expression of chlorotic symptoms, at least for CYDV-RPV. In our conditions, transmission, the final step in the epidemiological cycle, was independent of nutrient supplies, host phenotype, virus coinfection, and virus titer. The transmission experiment in our study was designed to assess effects of environmental nutrient supplies on host-to-host transmission through alterations of virus titer. Virus titer in all hosts and/or the acquisition access period allowed for the aphids to acquire virus particles from host tissue may have been high enough to maximize between-host transmission rate in our experimental conditions, which could explain the absence of correlation between titer and transmission often observed in plant-virus systems (Froissart et al., 2010). In addition to virus titer, host-to-host virus transmission can be strongly influenced by aphid vector behavior, especially for persistently transmitted viruses that require long acquisition time by aphids (Froissart et al., 2010; Ingwell et al., 2012; Smith, 2014; Blanc and Michalakis, 2016). Further studies investigating whether environmental nutrient supplies could influence among host virus transmission through alterations of host attractiveness

to insect vectors and aphid feeding behavior could deepen our knowledge on the multiple pathways through which host nutrient resources could influence plant virus dynamics.

GENERAL CONCLUSIONS

Our work highlights the challenges of understanding the implications of elevated nutrient deposition and altered global biogeochemical cycles (Tilman et al., 2001; Rockström et al., 2009) on disease ecology and epidemiology. While ecological stoichiometry and resource competition theory can provide a starting point to understand the effects of altered nutrient supply rates and ratios on disease and can effectively predict some of the links in the transmission chain, our work highlights the importance of disentangling the role of specific C-rich and N-rich metabolites on plant virus titer, rather than focusing solely on total elemental N, P, and C supply, content, or ratio. In addition, this work demonstrates that it is critical to understand the molecular mechanisms that lead to the host phenotypic changes that underlie host-virus interactions. With this stronger understanding of the direct and indirect pathways by which nutrient supply rates and ratios influence pathogen dynamics, we continue to build a predictive understanding of the effects of changing environmental conditions on virus multiplication and transmission.

AUTHOR CONTRIBUTIONS

CL, ES, and EB conceived and designed this research project. CL performed the experiments with the help of other lab members. CL analyzed data and drafted the paper, and ES and EB provided substantial contributions for statistical analysis and manuscript revisions. All authors approved this version of the manuscript and agreed to be accountable for all aspects of this work.

FUNDING

We received support from the NSF program in Ecology and Evolution of Infectious Disease (grant DEB-1015805 and EF-12-41895) to EB and ES.

ACKNOWLEDGMENTS

We thank Missy Rudeen, Kurra Renner, Abdulrahman Gamam, Amy Kendig, Aaron David, and Eric Lind as well as undergraduate and graduate students for help in the lab. We also thank Marty Dekkers (University of North Carolina) and Benham E. L. Lockhart (University of Minnesota) for sharing expertise and advice on methods for virus detection and transmission. We are grateful to Stewart Gray and Dawn M Smith (Cornell University), and to Dimitri Mollov (Plant Disease Clinic, University of Minnesota) for sharing and facilitating the reception of BYDV-PAV and CYDV-RPV virus isolates. We are also grateful to Georgiana May, Robert W. Sterner, and James B. Cotner for sharing the access to the qPCR, CHN analyser and Varian spectrophotometer piece of equipment. We thank Amy Kendig, Benoit Moury and reviewers for reading and providing useful comments on this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02116/full#supplementary-material

REFERENCES

Aalto, S. L., Decaestecker, E., and Pulkkinen, K. (2015). A three-way perspective of stoichiometric changes on host-parasite interactions. *Trends Parasitol.* 31, 333–340. doi: 10.1016/j.pt.2015.04.005

Adam, A., Gaborjanyi, R., Tobias, I., and Kiraly, Z. (1987). Effect of nitrogen nutrition on the concentration of viruses, phospholipids and galactolipids of barley leaves infected with barley stripe mosaic-virus (bsmv). *Ann. Appl. Biol.* 110, 313–319. doi: 10.1111/j.1744-7348.1987.tb03261.x

Ahlquist, P., Noueiry, A. O., Lee, W. M., Kushner, D. B., and Dye, B. T. (2003). Host factors in positive-strand rna virus genome replication. *J. Virol.* 77, 8181–8186. doi: 10.1128/JVI.77.15.8181-8186.2003

Alexander, H. M. (2010). Disease in natural plant populations, communities, and ecosystems: insights into ecological and evolutionary processes. *Plant Dis.* 94, 492–503. doi: 10.1094/PDIS-94-5-0492

Alizon, S., de Roode, J. C., and Michalakis, Y. (2013). Multiple infections and the evolution of virulence. *Ecol. Lett.* 16, 556–567. doi: 10.1111/ele.12076

Alizon, S., Hurford, A., Mideo, N., and Van Baalen, M. (2009). Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* 22, 245–259. doi: 10.1111/j.1420-9101.2008.01658.x

Al-Naimi, F. A., Garrett, K. A., and Bockus, W. W. (2005). Competition, facilitation, and niche differentiation in two foliar pathogens. *Oecologia* 143, 449–457. doi: 10.1007/s00442-004-1814-x

Bawden, F. C., and Kassanis, B. (1950a). Some effects of host nutrition on the susceptibility of plants to infection by certain viruses. *Ann. Appl. Biol.* 37, 46–57. doi: 10.1111/j.1744-7348.1950.tb00949.x

Bawden, F. C., and Kassanis, B. (1950b). Some effects of host-plant nutrition on the multiplication of viruses. *Ann. Appl. Biol.* 37, 215–228. doi: 10.1111/j.1744-7348.1950.tb01040.x

Bedhomme, S., Agnew, P., Sidobre, C., and Michalakis, Y. (2004). Virulence reaction norms across a food gradient. *Proc. R. Soc. B-Biol. Sci.* 271, 739–744. doi: 10.1098/rspb.2003.2657

Blanc, S., and Michalakis, Y. (2016). Manipulation of hosts and vectors by plant viruses and impact of the environment. *Curr. Opin. Insect Sci.* 16, 36–43. doi: 10.1016/j.cois.2016.05.007

Borer, E. T., Adams, V. T., Engler, G. A., Adams, A. L., Schumann, C. B., and Seabloom, E. W. (2009). Aphid fecundity and grassland invasion: invader life history is the key. *Ecol. Appl.* 19, 1187–1196. doi: 10.1890/08-1205.1

Borer, E. T., Hosseini, P. R., Seabloom, E. W., and Dobson, A. P. (2007). Pathogen-induced reversal of native dominance in a grassland community. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5473–5478. doi: 10.1073/pnas.0608573104

Borer, E. T., Laine, A. L., and Seabloom, E. W. (2016). A multiscale approach to plant disease using the metacommunity concept. *Annu. Rev. Phytopathol.* 54, 397–418. doi: 10.1146/annurev-phyto-080615-095959

Borer, E. T., Seabloom, E. W., Gruner, D. S., Harpole, W. S., Hillebrand, H., Lind, E. M., et al. (2014a). Herbivores and nutrients control grassland plant diversity via light limitation. *Nature* 508, 517–520. doi: 10.1038/nature13144

Borer, E. T., Seabloom, E. W., Mitchell, C. E., and Cronin, J. P. (2014b). Multiple nutrients and herbivores interact to govern diversity, productivity, composition, and infection in a successional grassland. *Oikos* 123, 214–224. doi: 10.1111/j.1600-0706.2013.00680.x

Borer, E. T., Seabloom, E. W., Mitchell, C. E., and Power, A. G. (2010). Local context drives infection of grasses by vector-borne generalist viruses. *Ecol. Lett.* 13, 810–818. doi: 10.1111/j.1461-0248.2010.01475.x

Cardinale, B. J., Hillebrand, H., Harpole, W. S., Gross, K., and Ptacnik, R. (2009). Separating the influence of resource 'availability' from resource 'imbalance' on productivity-diversity relationships. *Ecol. Lett.* 12, 475–487. doi: 10.1111/j.1461-0248.2009.01317.x

Catherall, P. L. (1966). Effects of barley yellow dwarf virus of the growth and yield of single plants and simulated swards of perennial rye-grass. *Annu. Appl. Biol.* 57, 155–162. doi: 10.1111/j.1744-7348.1966.tb06876.x

Chain, F., Riault, G., Trottet, M., and Jacquot, E. (2005). Analysis of accumulation patterns of barley yellow dwarf virus-pav (bydv-pav) in two resistant wheat lines. *Eur. J. Plant Pathol.* 113, 343–355. doi: 10.1007/s10658-005-7966-7

Clasen, J. L., and Elser, J. J. (2007). The effect of host chlorella nc64a carbon: phosphorus ratio on the production of paramecium bursaria chlorella virus-1. *Freshw. Biol.* 52, 112–122. doi: 10.1111/j.1365-2427.2006.01677.x

Corner, E. D. S., Head, R. N., Kilvingston, C. C., and Pennycuick, L. (1976). On the nutrition and metabolism of zooplankton. *J. Mar. Biol. Assoc.* 56, 345–358. doi: 10.1017/S0025315400018968

Craine, J. M., Tilman, D., Wedin, D., Reich, P., Tjoelker, M., and Knops, J. (2002). Functional traits, productivity and effects on nitrogen cycling of 33 grassland species. *Funct. Ecol.* 16, 563–574. doi: 10.1046/j.1365-2435.2002.00660.x

Cronin, J. P., Welsh, M. E., Dekkers, M. G., Abercrombie, S. T., and Mitchell, C. E. (2010). Host physiological phenotype explains pathogen reservoir potential. *Ecol. Lett.* 13, 1221–1232. doi: 10.1111/j.1461-0248.2010.01513.x

D'arcy, C. J., and Burnett, P. A. (1995). *Barley Yellow Dwarf, 40 Years of Progress*. St. Paul, MN: The American Phytopathological Society.

Deb, M., and Anderson, J. M. (2008). Development of a multiplexed pcr detection method for barley and cereal yellow dwarf viruses, wheat spindle streak virus, wheat streak mosaic virus and soil-borne wheat mosaic virus. *J. Virol. Methods* 148, 17–24. doi: 10.1016/j.jviromet.2007.10.015

Dordas, C. (2009). "Role of nutrients in controlling plant diseases in sustainable agriculture: a review," in *Sustainable Agriculture,* eds E. Lichtfouse, M. Navarrete, P. Debaeke, S. Véronique, and C. Alberola (Dordrecht: Springer), 443–460.

Doumayrou, J., Leblaye, S., Froissart, R., and Michalakis, Y. (2013). Reduction of leaf area and symptom severity as proxies of disease-induced plant mortality: the example of the cauliflower mosaic virus infecting two brassicaceae hosts. *Virus Res.* 176, 91–100. doi: 10.1016/j.virusres.2013.05.008

Downs, R. J., and Hellmers, H. (1975). *Environment and the Experimental Control of Plant Growth*. New York, NY: Academic Press.

Elena, S. F., Bernet, G. P., and Carrasco, J. L. (2014). The games plant viruses play. *Curr. Opin. Virol.* 8, 62–67. doi: 10.1016/j.coviro.2014.07.003

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Elser, J. J., Fagan, W. F., Kerkhoff, A. J., Swenson, N. G., and Enquist, B. J. (2010). Biological stoichiometry of plant production: metabolism, scaling and ecological response to global change. *New Phytol.* 186, 593–608. doi: 10.1111/j.1469-8137.2010.03214.x

Eraslan, F., Akbas, B., Inal, A., and Tarakcioglu, C. (2007). Effects of foliar sprayed calcium sources ontomato mosaic virus (tomv) infection in tomato plants grown in greenhouses. *Phytoparasitica* 35, 150–158. doi: 10.1007/BF02981110

Froissart, R., Doumayrou, J., Vuillaume, F., Alizon, S., and Michalakis, Y. (2010). The virulence-transmission trade-off in vector-borne plant viruses: a review of (non-)existing studies. *Philos. Trans. R. Soc. B Biol. Sci.* 365, 1907–1918. doi: 10.1098/rstb.2010.0068

Frost, P. C., Ebert, D., and Smith, V. H. (2008). Responses of a bacterial pathogen to phosphorus limitation of its aquatic invertebrate host. *Ecology* 89, 313–318. doi: 10.1890/07-0389.1

Gray, S. M. (2008). Aphid transmission of plant viruses. *Curr. Protoc. Microbiol.* Chapter 16:Unit 16B.1.1–16B.1.10. doi: 10.1002/9780471729259.mc16b01s10

Gray, S., and Gildow, F. E. (2003). Luteovirus-aphid interactions. *Annu. Rev. Phytopathol.* 41, 539–566. doi: 10.1146/annurev.phyto.41.012203.105815

Grueber, C. E., Nakagawa, S., Laws, R. J., and Jamieson, I. G. (2011). Multimodel inference in ecology and evolution: challenges and solutions. *J. Evol. Biol.* 24, 699–711. doi: 10.1111/j.1420-9101.2010.02210.x

Haddad, N. M., Haarstad, J., and Tilman, D. (2000). The effects of long-term nitrogen loading on grassland insect communities. *Oecologia* 124, 73–84. doi: 10.1007/s004420050026

Halbert, S., and Voegtlin, D. (1995). "Biology and taxonomy of vectors of barley yellow dwarf viruses," in *Barley Yellow Dwarf, 40 Years of Progress*, eds C. J. D'arcy and P. A. Burnett (St. Paul, MN: The American Phytopathological Society), 217–258.

Hall, G. S., and Little, D. P. (2013). Within-host competition between barley yellow dwarf-pav and –pas. *Virus Res.* 174, 148–151. doi: 10.1016/j.virusres.2013.03.013

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Hillebrand, H., Cowles, J. M., Lewandowska, A., Van de Waal, D. B., and Plum, C. (2014). Think ratio! A stoichiometric view on biodiversity-ecosystem functioning research. *Basic Appl. Ecol.* 15, 465–474. doi: 10.1016/j.baae.2014.06.003

Hoagland, D. R., and Arnon,. D. I. (1938). *The Water Culture Method for Growing Plants Without Soil.* California Agricultural Experiment Station, Circular 347, 1:32.

Ingwell, L. L., Eigenbrode, S. D., and Bosque-Perez, N. A. (2012). Plant viruses alter insect behavior to enhance their spread. *Sci. Rep.* 2:578. doi: 10.1038/srep00578

Irwin, M. E., and Thresh, J. M. (1990). Epidemiology of barley yellow dwarf - a study in ecological complexity. *Annu. Rev. Phytopathol.* 28, 393–424. doi: 10.1146/annurev.py.28.090190.002141

Jensen, S. G. (1968). Photosynthesis, respiration, and other physiological relationships in barley yeallow dwarf virus. *Phytopathology* 58, 204–208.

Jensen, S. G. (1969). Composition and metabolism of barley leaves infected with barley yellow dwarf virus. *Phytopathology* 59, 1694–1698.

Jensen, S. G. (1972). Metabolism and carbohydrate composition in barley yellow dwarf virus infected wheat. *Phytopathology* 62, 587–592. doi: 10.1094/Phyto-62-587

Jover, L. F., Effler, T. C., Buchan, A., Wilhelm, S. W., and Weitz, J. S. (2014). The elemental composition of virus particles: implications for marine biogeochemical cycles. *Nat. Rev. Microbiol.* 12, 519–528. doi: 10.1038/nrmicro3289

Karpinets, T. V., Greenwood, D. J., Sams, C. E., and Ammons, J. T. (2006). Rna: protein ratio of the unicellular organism as a characteristic of phosphorous and nitrogen stoichiometry and of the cellular requirement of ribosomes for protein synthesis. *BMC Biol.* 4:30. doi: 10.1186/1741-7007-4-30

Lacroix, C., Seabloom, E. W., and Borer, E. T. (2014). Environmental nutrient supply alters prevalence and weakens competitive interactions among coinfecting viruses. *New Phytol.* 204, 424–433. doi: 10.1111/nph.12909

Lange, B., Reuter, M., Ebert, D., Muylaert, K., and Decaestecker, E. (2014). Diet quality determines interspecific parasite interactions in host populations. *Ecol. Evol.* 4, 3093–3102. doi: 10.1002/ece3.1167

Leclercq-Le Quillec, F., Plantegenest, M., Riault, G., and Dedryver, C. A. (2000). Analyzing and modeling temporal disease progress of barley yellow dwarf virus serotypes in barley fields. *Phytopathology* 90, 860–866. doi: 10.1094/PHYTO.2000.90.8.860

Liu, X., Lyu, S., Sun, D., Bradshaw, C. J. A., and Zhou, S. (2017). Species decline under nitrogen fertilization increases community-level competence of fungal diseases. *Proc. R. Soc. B Biol. Sci.* 284:20162621. doi: 10.1098/rspb.2016.2621

Maat, D. S., and Brussaard, C. P. D. (2016). Both phosphorus- and nitrogen limitation constrain viral proliferation in marine phytoplankton. *Aquat. Microb. Ecol.* 77, 87–97. doi: 10.3354/ame01791

MacArthur, R. H. (1972). *Geographical Ecology: Patterns in the Distribution of Species*. Princeton, NJ: Princeton University Press.

Malmstrom, C. M., and Field, C. B. (1997). Virus-induced differences in the response of oat plants to elevated carbon dioxide. *Plant Cell Environ.* 20, 178–188. doi: 10.1046/j.1365-3040.1997.d01-63.x

Malmstrom, C. M., Hughes, C. C., Newton, L. A., and Stoner, C. J. (2005). Virus infection in remnant native bunchgrasses from invaded california grasslands. *New Phytol.* 168, 217–230. doi: 10.1111/j.1469-8137.2005.01479.x

Marquez, L. M., Redman, R. S., Rodriguez, R. J., and Roossinck, M. J. (2007). A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Science* 315, 513–515. doi: 10.1126/science.1136237

Maule, A., Leh, V., and Lederer, C. (2002). The dialogue between viruses and hosts in compatible interactions. *Curr. Opin. Plant Biol.* 5, 279–284. doi: 10.1016/S1369-5266(02)00272-8

Miller, T. E., Burns Jean, H., Munguia, P., Walters Eric, L., Kneitel Jamie, M., Richards Paul, M., et al. (2005). A critical review of twenty years' use of the resource-ratio theory. *Am. Nat.* 165, 439–448. doi: 10.1086/428681

Miller, W. A., and Rasochova, L. (1997). Barley yellow dwarf viruses. *Annu. Rev. Phytopathol.* 35, 167–190. doi: 10.1146/annurev.phyto.35.1.167

Mitchell, C. E., Reich, P. B., Tilman, D., and Groth, J. V. (2003). Effects of elevated co2, nitrogen deposition, and decreased species diversity on foliar fungal plant disease. *Glob. Change Biol.* 9, 438–451. doi: 10.1046/j.1365-2486.2003.00602.x

Mordecai, E. A., Hindenlang, M., and Mitchell, C. E. (2015). Differential impacts of virus diversity on biomass production of a native and an exotic grass host. *PLoS ONE* 10:e0134355. doi: 10.1371/journal.pone.0134355

Pedersen, A. B., and Fenton, A. (2007). Emphasizing the ecology in parasite community ecology. *Trends Ecol. Evol.* 22, 133–139. doi: 10.1016/j.tree.2006.11.005

Perry, K. L., Kolb, F. L., Sammons, B., Lawson, C., Cisar, G., and Ohm, H. (2000). Yield effects of barley yellow dwarf virus in soft red winter wheat. *Phytopathology* 90, 1043–1048. doi: 10.1094/PHYTO.2000.90.9.1043

Power, A. G., and Gray, S. M. (1995). "Aphid transmission of barley yellow dwarf viruses: interactions between viruses, vectors and host plants," in *Barley Yellow Dwarf: 40 Years of Progress*, eds C. J. D'arcy and P. A. Burnett (St. Paul, MN: The American Phytopathological Society), 259–289.

Redfield, A. C. (1958). The biological control of chemical factors in the environment. *Am. Sci.* 46, 205–221.

Reich, P. B. (2014). The world-wide 'fast-slow' plant economics spectrum: a traits manifesto. *J. Ecol.* 102, 275–301. doi: 10.1111/1365-2745.12211

Rigaud, T., Perrot-Minnot, M. J., and Brown, M. J. F. (2010). Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. *Proc. R. Soc. B Biol. Sci.* 277, 3693–3702. doi: 10.1098/rspb.2010.1163

Robertson, N. L., and French, R. (2007). Genetic structure in natural populations of barley/cereal yellow dwarf virus isolates from alaska. *Arch. Virol.* 152, 891–902. doi: 10.1007/s00705-006-0913-x

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F. S., Lambin, E. F., et al. (2009). A safe operating space for humanity. *Nature* 461, 472–475. doi: 10.1038/461472a

Roossinck, M. J. (2015). Plants, viruses and the environment: ecology and mutualism. *Virology* 479, 271–277. doi: 10.1016/j.virol.2015.03.041

Roossinck, M. J. (2012). Plant virus metagenomics: biodiversity and ecology. *Annu. Rev. Genet.* 46, 359–369. doi: 10.1146/annurev-genet-110711-155600

Roossinck, M. J., Saha, P., Wiley, G. B., Quan, J., White, J. D., Lai, H., et al. (2010). Ecogenomics: using massively parallel pyrosequencing to understand virus ecology. *Mol. Ecol.* 19, 81–88. doi: 10.1111/j.1365-294X.2009.04470.x

Rua, M. A., Umbanhowar, J., Hu, S., Burkey, K. O., and Mitchell, C. E. (2013). Elevated co2 spurs reciprocal positive effects between a plant virus and an arbuscular mycorrhizal fungus. *New Phytol.* 199, 541–549. doi: 10.1111/nph.12273

Salvaudon, L., De Moraes, C. M., and Mescher, M. C. (2013). Outcomes of co-infection by two potyviruses: implications for the evolution of manipulative strategies. *Proc. R. Soc. B Biol. Sci.* 280:20122959. doi: 10.1098/rspb.2012.2959

Seabloom, E. W., Borer, E. T., Buckley, Y. M., Cleland, E. E., Davies, K. F., Firn, J., et al. (2015a). Plant species' origin predicts dominance and response to nutrient enrichment and herbivores in global grasslands. *Nat. Commun.* 6, 7710. doi: 10.1038/ncomms8710

Seabloom, E. W., Borer, E. T., Gross, K., Kendig, A. E., Lacroix, C., Mitchell, C. E., et al. (2015b). The community ecology of pathogens: Coinfection, coexistence and community composition. *Ecol. Lett.* 18, 401–415. doi: 10.1111/ele.12418

Seabloom, E. W., Borer, E. T., Lacroix, C., Mitchell, C. E., and Power, A. G. (2013). Richness and composition of niche-assembled viral pathogen communities. *PLoS ONE* 8:e55675. doi: 10.1371/journal.pone.0055675

Seabloom, E. W., Borer, E. T., Mitchell, C. E., and Power, A. G. (2010). Viral diversity and prevalence gradients in north american pacific coast grasslands. *Ecology* 91, 721–732. doi: 10.1890/08-2170.1

Seabloom, E. W., Hosseini, P. R., Power, A. G., and Borer, E. T. (2009). Diversity and composition of viral communities: coinfection of barley and cereal yellow dwarf viruses in california grasslands. *Am. Nat.* 173, E79–E98. doi: 10.1086/596529

Smith, V. (2007). Host resource supplies influence the dynamics and outcome of infectious disease. *Integr. Comp. Biol.* 47, 310–316. doi: 10.1093/icb/icm006

Smith, V. H. (1993). Resource competition between host and pathogen. *Bioscience* 43, 21–30. doi: 10.2307/1312103

Smith, V. H. (2014). Nutrient supply differentially alters the dynamics of co-infecting phytoviruses. *New Phytol.* 204, 265–267. doi: 10.1111/nph.13019

Smith, V. H., and Holt, R. D. (1996). Resource competition and within-host disease dynamics. *Trends Ecol. Evol.* 11, 386–389. doi: 10.1016/0169-5347(96)20067-9

Smith, V. H., Jones, T. P., and Smith, M. S. (2005). Host nutrition and infectious disease: an ecological view. *Front. Ecol. Environ.* 3, 268–274. doi: 10.1890/1540-9295(2005)003[0268:HNAIDA]2.0.CO;2

Spencer, E. L. (1941). Influence of nitrogen supply on the rate of multiplication of tobacco mosaic virus. *Plant Physiol.* 16, 663–675. doi: 10.1104/pp.16.4.663

Sterner, R. W., and Elser, J. J. (eds.). (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Tilman, D. (1976). Ecological competition between algae - experimental confirmation of resource-based competition theory. *Science* 192, 463–465. doi: 10.1126/science.192.4238.463

Tilman, D. (1977). Resource competition between plankton algae: an experimental and theoretical approach. *Ecology* 58, 338–348. doi: 10.2307/1935608

Tilman, D. (1982). *Resource Competition and Community Structure*. Princeton, NJ: Princeton University Press.

Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., et al. (2001). Forecasting agriculturally driven global environmental change. *Science* 292, 281–284. doi: 10.1126/science.1057544

Trebicki, P., Nancarrow, N., Cole, E., Bosque-Perez, N. A., Constable, F. E., Freeman, A. J., et al. (2015). Virus disease in wheat predicted to increase with a changing climate. *Glob. Change Biol.* 21, 3511–3519. doi: 10.1111/gcb.12941

Turner, P. E. (2005). Parasitism between co-infecting bacteriophages. *Adv. Ecol. Res.* 37, 309–332. doi: 10.1016/S0065-2504(04)37010-8

Vassiliadis, S., Plummer, K. M., Powell, K. S., Trebicki, P., Luck, J. E., and Rochfort, S. J. (2016). The effect of elevated CO_2 and virus infection on the primary metabolism of wheat. *Funct. Plant Biol.* 43, 892–902. doi: 10.1071/FP15242

Violle, C., Navas, M. L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., et al. (2007). Let the concept of trait be functional!. *Oikos* 116, 882–892. doi: 10.1111/j.0030-1299.2007.15559.x

Violle, C., Reich, P. B., Pacala, S. W., Enquist, B. J., and Kattge, J. (2014). The emergence and promise of functional biogeography. *Proc. Natl. Acad. Sci. U.S.A.* 111, 13690–13696. doi: 10.1073/pnas.1415442111

Westoby, M. (1998). A leaf-height-seed (lhs) plant ecology strategy scheme. *Plant Soil* 199, 213–227. doi: 10.1023/A:1004327224729

Westoby, M., and Wright, I. J. (2006). Land-plant ecology on the basis of functional traits. *Trends Ecol. Evol.* 21, 261–268. doi: 10.1016/j.tree.2006.02.004

Whitaker, B. K., Rua, M. A., and Mitchell, C. E. (2015). Viral pathogen production in a wild grass host driven by host growth and soil nitrogen. *New Phytol.* 207, 760–768. doi: 10.1111/nph.13369

Wright, I. J., Reich, P. B., Westoby, M., Ackerly, D. D., Baruch, Z., Bongers, F., et al. (2004). The worldwide leaf economics spectrum. *Nature* 428, 821–827. doi: 10.1038/nature02403

Zehnder, C. B., and Hunter, M. D. (2009). More is not necessarily better: the impact of limiting and excessive nutrients on herbivore population growth rates. *Ecol. Entomol.* 34, 535–543. doi: 10.1111/j.1365-2311.2009.01101.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 June 2017
doi: 10.3389/fmicb.2017.01015

Changes in N:P Supply Ratios Affect the Ecological Stoichiometry of a Toxic Cyanobacterium and Its Fungal Parasite

Thijs Frenken[1], Joren Wierenga[1], Alena S. Gsell[1], Ellen van Donk[1,2], Thomas Rohrlack[3] and Dedmer B. Van de Waal[1]*

[1] Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, [2] Department of Biology, University of Utrecht, Utrecht, Netherlands, [3] Department of Plant and Environmental Sciences, Norwegian University of Life Sciences, Ås, Norway

Human activities have dramatically altered nutrient fluxes from the landscape into receiving waters. As a result, not only the concentration of nutrients in surface waters has increased, but also their elemental ratios have changed. Such shifts in resource supply ratios will alter autotroph stoichiometry, which may in turn have consequences for higher trophic levels, including parasites. Here, we hypothesize that parasite elemental composition will follow changes in the stoichiometry of its host, and that its reproductive success will decrease with host nutrient limitation. We tested this hypothesis by following the response of a host–parasite system to changes in nitrogen (N) and phosphorus (P) supply in a controlled laboratory experiment. To this end, we exposed a fungal parasite (the chytrid *Rhizophydium megarrhizum*) to its host (the freshwater cyanobacterium *Planktothrix rubescens*) under control, low N:P and high N:P conditions. Host N:P followed treatment conditions, with a decreased N:P ratio under low N:P supply, and an increased N:P ratio under high N:P supply, as compared to the control. Shifts in host N:P stoichiometry were reflected in the parasite stoichiometry. Furthermore, at low N:P supply, host intracellular microcystin concentration was lowered as compared to high N:P supply. In contrast to our hypothesis, zoospore production decreased at low N:P and increased at high N:P ratio as compared to the control. These findings suggest that fungal parasites have a relatively high N, but low P requirement. Furthermore, zoospore elemental content, and thereby presumably their size, decreased at high N:P ratios. From these results we hypothesize that fungal parasites may exhibit a trade-off between zoospore size and production. Since zooplankton can graze on chytrid zoospores, changes in parasite production, stoichiometry and cell size may have implications for aquatic food web dynamics.

Keywords: nutrients, harmful algal blooms, plankton, Chytridiomycota, disease, pathogen, microcystin

OPEN ACCESS

Edited by:
Alison Buchan, University of Tennessee, Knoxville, United States

Reviewed by:
Ludwig Jardillier, Université Paris-Sud, France
Emilie Lefevre, Duke University, United States

****Correspondence:***
Thijs Frenken
thijs.frenken@gmail.com

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *31 January 2017*
Accepted: *22 May 2017*
Published: *06 June 2017*

Citation:
Frenken T, Wierenga J, Gsell AS, van Donk E, Rohrlack T and Van de Waal DB (2017) Changes in N:P Supply Ratios Affect the Ecological Stoichiometry of a Toxic Cyanobacterium and Its Fungal Parasite. Front. Microbiol. 8:1015. doi: 10.3389/fmicb.2017.01015

INTRODUCTION

Human activities have substantially increased the flux of nutrients from land into receiving waters (Smith, 2003). This nutrient enrichment enhances aquatic primary production, and may lead to dramatic changes in the composition and structure of aquatic food webs (Schindler and Fee, 1974; Smith et al., 2006). Specifically, an increased nutrient supply might promote development

of harmful cyanobacterial blooms (Paerl et al., 2001, 2011; Smith and Schindler, 2009). Although nutrient loading has increased, primary production in aquatic ecosystems is often still limited by nitrogen (N) and/or phosphorus (P) (Elser et al., 2007; Bracken et al., 2014). This may be a result of an imbalanced nutrient supply (Carpenter et al., 1996; Sterner et al., 2007), as well as an increased nutrient demand associated to high phytoplankton densities (Carpenter et al., 1996). Nutrient limitation will alter the elemental composition of phytoplankton, and may specifically increase carbon:nutrient ratios (Sterner and Elser, 2002). As a consequence, nutritional quality of the phytoplankton decreases, thereby possibly constraining higher trophic levels (Sterner and Elser, 2002; Hessen et al., 2013). This may particularly apply to parasites that solely depend on their host as a food source (Smith, 2007).

Fungal parasites are very common pathogens infecting phytoplankton (Gerphagnon et al., 2015), which represent an important but yet overlooked ecological driving force in aquatic food web dynamics (Sime-Ngando, 2012). These parasites, belonging to the phylum Chytridiomycota and often referred to as chytrids, are host specific zoosporic fungi that can parasitize on phytoplankton and completely rely on their host to obtain energy and nutrients leading to death of the host (Sparrow, 1960, 1968; Barr, 2001). Thereby, they play an important role in natural aquatic ecosystems, in which chytrids can significantly change phytoplankton abundance and seasonal succession (Reynolds, 1973; Van Donk, 1984, 1989). Additionally, the free swimming stage of the chytrids (i.e., zoospores) may provide higher trophic levels with an alternative food source during blooms of large inedible diatoms (Kagami et al., 2007; Frenken et al., 2016) or cyanobacteria (Agha et al., 2016). Earlier work indicates that zoospores might find their host by chemotaxis (Muehlstein et al., 1988), and penetrate host cells using a rhizoidal system through which nourishment is conveyed to the zoospore (Van Donk and Ringelberg, 1983). After infection, the spore forms a sessile stage (i.e., sporangium) in which new zoospores (up to 60) are produced (Canter and Lund, 1951; Sparrow, 1960).

Chytrid zoospores generally contain a relatively high amount of nucleic acids that are particularly rich in P, but also contain substantial amounts of lipids, including fatty acids and sterols, which are rich in carbon (Barr and Hadland-Hartmann, 1978; Beakes et al., 1988, 1993; Elser et al., 1996; Kagami et al., 2007). Chytrids thus seem to have high P demands, as has been indicated by their low C:P as compared to their host (Kagami et al., 2007). As a consequence, limitation by P may affect a chytrid more than its host. Chytrid infections were indeed shown to be affected by host P limitation. More specifically, chytrid growth rate and the number of zoospores per sporangium decreased, and, as a function of lower host growth rate, zoospore loss as well as searching time increased, as compared to non-limited conditions (Bruning, 1991). If, however, P limitation impedes algal growth to a greater extent than that of the chytrid, epidemics may still occur (Bruning and Ringelberg, 1987; Bruning, 1991).

Nutrient limitation not only alters growth and reproduction of the parasite, it may also affect host defense. Freshwater cyanobacteria produce a wide range of oligopeptides including toxic microcystins (MC) (Welker and Von Döhren, 2006), which have been associated to chytrid defense (Rohrlack et al., 2013). These oligopeptides are N rich compounds, and their synthesis is typically constrained under N limitation (Van de Waal et al., 2010, 2014). Thus, during low N:P conditions, host defense may be reduced and thereby facilitate chytrid infections. In contrast, cellular N may accumulate under high N:P conditions and thereby enhance host defense. Limitation by N and P may thus have contrasting effects on chytrid infections of cyanobacteria. We hypothesized that parasite elemental composition will follow changes in the stoichiometry of its host, and that its reproductive success will decrease with host nutrient limitation. To test this hypothesis, we exposed the cyanobacterium *Planktothrix rubescens* to its chytrid *Rhizophydium megarrhizum* under control, low N and low P conditions, leading to a range of host N:P ratios. We predict that infections will decrease with increasing host N:P, as the availability of P for chytrid nutrition will decrease and the host defense by oligopeptides will increase.

MATERIALS AND METHODS

Description of Test Organisms

In this study the filamentous cyanobacterial host *P. rubescens* NIVA-CYA97/1 was used in combination with one of its parasites, the chytrid Chy-Lys2009 (photo provided in the Supplementary Material). This chytrid possesses identical morphological characteristics and infection patterns in agreement with *R. megarrhizum* described earlier by Canter and Lund (1951). More information on host specificity and virulence of the chytrid can be found in Sønstebø and Rohrlack (2011) and Rohrlack et al. (2013). All cultures used in this study were monoclonal and non-axenic.

Culture Maintenance

The *Planktothrix* and the chytrid Chy-Lys2009 cultures were grown in a temperature and light controlled incubator (Snijders Labs, Tilburg, The Netherlands) at 5 μmol photons m^{-2} s^{-1} in a 14:10 light:dark cycle, at 24 and 16°C, respectively. The applied low light levels resemble the conditions where the tested *Planktothrix* species was isolated, i.e., in the vicinity of the thermocline. All cultures were maintained in exponential growth in batch using 100 mL Erlenmeyer flasks with 50 mL suspension. Every other week, *Planktothrix* cultures were diluted using WC-medium (Guillard and Lorenzen, 1972) and chytrid cultures were diluted using host culture and WC-medium to 1/10 (v/v). Additionally, Erlenmeyer flasks were shaken every other day to prevent aggregation. The chytrid cultures were visually inspected for infection patterns and contaminations at least once a week.

Description of the Experiment

Culture Acclimatization and Inoculation

Prior to the experiment, *Planktothrix* was grown at 16°C on WC-medium at three distinct N:P supply ratios by modifying standard NO_3^- and PO_4^{3-} concentrations of 1000 and 50 μmol L^{-1} (N:P = 20) as control, to 200 and 50 μmol L^{-1} (N:P = 4) as the low N:P treatment, and 1000 and 10 μmol L^{-1} (N:P = 100) as the high N:P treatment. Cultures were acclimatized for about

18 generations to the distinct nutrient conditions by three consecutive transfers at late exponential phase. During each transfer, i.e., after each 7 days period, cultures were diluted back to half of maximum biovolume reached in order to maintain nutrient limited conditions. After acclimatization, *Planktothrix* was first grown without chytrids (unexposed treatment, 4 replicates per nutrient treatment, 12 experimental units) to late stationary phase to obtain uninfected host growth rates, stoichiometry and toxin composition. Subsequently, the host cultures were then pooled by nutrient treatment and used to inoculate the chytrid exposed treatments (4 replicates per nutrient treatment, 12 experimental units). At the start of the chytrid exposed treatment, *Planktothrix* cultures were inoculated together with a zoospore suspension that was obtained from a highly infected *Planktothrix* culture (with 58% Chy-Lys2009 infected filaments) by sieving gently over a 30 μm and a subsequent 5 μm nylon mesh to remove host cells, while collecting zoospores that have a typical size range of 2.5–3.5 μm (Sparrow, 1960). This zoospore suspension was washed with N and P free WC-medium and concentrated on a 1.2 μm cellulose acetate membrane filter (Whatman, Maidstone, United Kingdom), and used to inoculate to a final density of 18 zoospores mL^{-1}. The chytrid exposed cultures were grown for 7 days to obtain host and parasite growth rates, host and parasite stoichiometry, parasite zoospore production and toxin composition of parasite exposed host. Each treatment was performed in 500 mL Erlenmeyer flasks with 300 mL of culture.

Host and Parasite Quantification

During the experiment, cultures were sampled daily to determine biovolume using a CASY Cell Counter (Schärfe System GmbH, Reutlingen, Germany). Next, at least 5 mL of culture suspension was fixed with alkaline Lugol's iodine solution to a final concentration of 1.2% (v/v) and stored in the dark at room temperature. Prevalence of infected filaments was counted in duplicate (technical replicate) for each biological replicate within 2 weeks after the experiment by inspecting at least 50 filaments. Additionally, during the infection treatment, the number of free swimming zoospores was counted daily in duplicate, also for each biological replicate, in at least 15 fields of view (FOV) in fresh cultures. All microscopic counting was performed using a magnification of 200× on an inverted microscope (DMI 4000B, Leica Microsystems CMS GmbH, Mannheim, Germany). Cultures were harvested at the early stationary phase for the analyses of dissolved inorganic nutrients, elemental composition of the host and parasite, and the MC contents and composition of the host.

Elemental Analyses

Particulate organic carbon (C), N and P were determined in duplicate by collecting 5–15 mL of seston on a prewashed GF/F filter (Whatman, Maidstone, United Kingdom) applying gentle filtration (<1–2 psi). Filters were dried overnight at 60°C, and stored in a desiccator in the dark. For C and N analyses, a subsample (22%) of every filter was taken by a hole puncher, folded into a tin cup and analyzed on a FLASH 2000 organic elemental analyzer (Brechbueler Incorporated, Interscience B.V., Breda, The Netherlands). Particulate organic P was analyzed (Eaton, 2005) by first combusting the remainder of the filter (78%) for 30 min at 550°C in Pyrex glass tubes, followed by a digestion step with 2.5 mL persulfate (2.5%) for 30 min at 120°C. This digested solution was measured for PO_4^{3-} on the QuAAtro39 AutoAnalyzer (SEAL Analytical Ltd, Southampton, United Kingdom) following Armstrong et al. (1967). During the infection treatment, particulate organic C, N and P were determined for the seston fraction as well as for the zoospores. For this purpose, 90–160 mL infected culture suspension was gently filtered twice over a 30 μm nylon mesh filter to remove the larger cyanobacterial filaments. Subsequently, the smaller filaments were removed by an additional filtration over a 5 μm nylon mesh, and zoospores in the filtrate were collected on a prewashed GF/F filter (Whatman GF/F, Maidstone, United Kingdom). Organic C, N and P on the filters were analyzed as described above.

Microcystin Analyses

Extractions

Samples for MC analyses were collected in duplicate by filtering 5–15 mL of culture over a GF/C filter (Whatman, Maidstone, United Kingdom) applying low pressure after which the filters were stored at −20°C. Filters were lyophilized overnight before performing three rounds of extractions at 60°C using 2.5 mL 75% methanol-25% Millipore water (v/v) in 8 mL Pyrex glass tubes. After drying the samples with N_2, extracts were reconstituted in 900 μl methanol, filtered and centrifuged (Corning© Costar© Spin-X© polypropylene centrifuge tube filters with a 0.22 μm cellulose-acetate filter (Corning Inc., Corning, NY, United States) for 5 min at 16,000 × *g* (Sigma 1-15P, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). Filtrates were transferred to amber glass vials and analyzed by LC-MS/MS.

Analyses

Samples were analyzed for eight MC variants (dm-7-MC-RR, MC-RR, MC-YR, dm-7-MC-LR, MC-LR, MC-LY, MC-LW, and MC-LF). Calibration standards were obtained from the National Research Council (Ottawa, Canada) for dm-7-MC-LR, and from Enzo Life Sciences Inc. (Farmingdale, NY, United States) for the other variants. Measurements were performed on an Agilent 1260 LC and an Agilent 6460A QQQ (Agilent Technologies, Santa Clara, CA, United States). The compounds were separated on an Agilent Zorbax Eclipse XDB-C18 4.6 mm × 150 mm, 5 μm column using Millipore water with 0.1% formic acid (*v*/*v*, eluent A) and acetonitrile with 0.1% formic acid (*v*/*v*, eluent B). The elution program was set at 0–2 min 30% B, 6–12 min 90% B, with a linear increase of B between 2 and 6 min and a 5 min post run at 30% B. Sample injection volume was set at 10 μL, with a flow of 0.5 mL min^{-1} at a column temperature of 40°C. The LC-MS/MS was operated in positive mode with an ESI source, nitrogen was used as a drying, sheath and collision gas. For each compound, two transitions were monitored in MRM mode: *m/z* 491.3 to *m/z* 135.1 and *m/z* 981.5 to *m/z* 135.2 (dm-7-MC-LR, ratio between product ions 17%), *m/z* 498.3 to *m/z* 135.1 and *m/z* 995.6 to *m/z* 135.1 (MC-LR, ratio between product ions

16%). This protocol is based on the protocol earlier described by Faassen and Lürling (2013).

Data Analyses

Host population growth and zoospore production rates were calculated according to $\mu = \ln(B_{n+t}/B_n)/t$. In which μ is the maximum specific growth rate, B_n is the initial population density of non-infected or infected host (biovolume), or zoospores (counts), B_{n+t} is the final population density of these variables over time t in the exponential growth phase. Infected biomass was calculated by multiplying the proportion of infected filaments with biovolume. Zoospore production efficiency was calculated as the number of zoospores produced per infected host biovolume.

Host maximum specific growth rate, zoospore production rate, seston stoichiometry of the host and parasite and MC content of the host were tested for normality and equal variance using the Shapiro–Wilk and Brown-Forsythe tests, respectively. Data were transformed, log or reciprocal, if this improved normality. Host growth rates, zoospore production rate, seston and zoospore stoichiometry and host MC content were analyzed to test for effects of nutrient supply by performing a one-way ANOVA. Pairwise comparisons were conducted using the Holm-Sidak test (Sidak, 1967). The strength and direction of associations between variables were assessed by Pearson product-moment correlations. All analyses were performed using SigmaPlot version 13 (Systat Software Inc., London, United Kingdom). Detailed output of the different statistical tests can be found in the Supplementary Material.

RESULTS

Host Growth and Biovolume Build-Up

In the absence of the parasite, *Planktothrix* population growth rates were comparable in all treatments (**Table 1**), with replicates ranging between 0.30 and 0.59 d^{-1}. In the presence of the parasite, nutrient supply also had no clear effect on net population growth rate of the total biovolume (**Table 1**). After 4 days of infection, infected host biomass increased at the expense of susceptible host biomass (**Figure 1**). The total *Planktothrix* biomass build-up after 4–7 days was lower in the chytrid exposed cultures than in the unexposed cultures. The rate at which the infected biomass increased was highest under a high N:P supply, while it did not differ between the control and low N:P treatment (**Table 1**).

Elemental Composition

Host N:P ratios followed N:P supply ($r = 0.99$, $P < 0.001$; **Table 2**), and were lowest with 7.8 ± 0.1 (mean ± SE) under low N:P conditions, intermediate with 11.4 ± 0.4 in the control, and highest with 46.2 ± 2.7 under high N:P conditions (**Figure 2A**). This was also largely resembled in the overall N:P ratios of the cultures when the parasite was present (i.e., infected host + chytrids). The N:P ratio in the high N:P treatments was also highest with 44.2 ± 1.7, while the low N:P treatment and the control were not statistically different with an N:P ratio of 8.6 ± 0.22 and 10.3 ± 0.14, respectively. Similarly, N:P ratios of the chytrid zoospores increased with host N:P ratios ($r = 0.96$, $P < 0.001$). Specifically, chytrid N:P ratios increased from 12.6 ± 0.4 in the control to 25.9 ± 1.0 under high N:P, while remained largely unaltered in the low N:P treatment as compared to the control (**Figure 2B** and **Table 3**).

The observed shifts in host N:P ratios were mainly caused by changes in P contents ($r = -0.97$, $P < 0.001$), which decreased from 107.8 ± 2.5 pmol mm^{-3} at low N:P conditions down to 82.7 ± 4.3 pmol mm^{-3} in the control and 17.2 ± 1.0 pmol mm^{-3} under high N:P conditions, while C and N contents remained largely unaltered across all treatments (**Figure 2C** and **Table 2**). Zoospore C, N as well as P contents decreased with increasing host N:P ($r = -0.77$, $P = 0.003$, $r = -0.77$, $P = 0.003$ and $r = -0.84$, $P < 0.001$, respectively), with highest values under low N:P conditions, intermediate values in the control, and lowest values under high N:P conditions (**Figure 2D** and **Table 3**). The observed difference in N:P stoichiometry between high N:P conditions and the other nutrient supply treatments resulted from a stronger decline in P contents relative to N.

Parasite Prevalence and Production

Prevalence of infection on the last day of the experiment ranged between 30 ± 1.4% (mean ± SE) in the control up to 48 ± 2.1% and 56 ± 3.6% in high N:P and low N:P treatments, respectively (**Figure 3A**). Growth rates of the infection were highest in the high N:P cultures and lowest, although not significantly, in the low N:P cultures (**Table 1**). Comparably, zoospore concentrations, zoospore production rate and the amount of zoospores produced per unit of infected host biomass, i.e., the zoospore production efficiency, were all highest in the high N:P cultures and lowest in low N:P cultures (**Figures 3B**, **4A,B** and **Table 4**). Zoospore production rate and efficiency increased with host N:P ratio ($r = 0.61$, $P = 0.035$, and $r = 0.85$, $P < 0.001$, respectively), while production efficiency furthermore decreased with zoospore C contents ($r = -0.87$, $P < 0.001$; **Figure 4C**).

Microcystin

Four MC variants were detected, including dm-7-MC-RR, MC-YR, dm-7-MC-LR, and MC-LR. On average, dm-7-MC-RR was the dominant MC variant present, representing 56.6 ± 0.5% (mean ± SE) of the total amount of MC. The total cellular MC contents ranged between 60 and 250 μg mm^{-3}. MC concentrations were highest in the high N:P, lowest in the low N:P, and intermediate in the control treatment (**Figure 5**). Furthermore, in the chytrid exposed cultures, the total amount of intracellular MC seemed to be lower.

DISCUSSION

Although the different nutrient supply ratios had only minor effects on *Planktothrix* growth rates after acclimatization (**Table 1**), there were clear changes in the elemental composition of the cyanobacteria (**Figure 2A**). This indicates that nutrient depletion did affect host physiology, but not growth, at the time of sampling. Apparently, *Planktothrix* is able to maintain similar maximum growth rates as compared to the control at

TABLE 1 | *Planktothrix* maximum net population growth rates (d^{-1}) of the different biomass fractions in the unexposed and chytrid exposed cultures.

	Unexposed	Exposed		
Treatment	**Total**	**Total**	**Susceptible**	**Infected**
Low N:P	0.42 ± 0.05^{a}	0.06 ± 0.05^{a}	-0.05 ± 0.02^{a}	0.94 ± 0.09^{a}
Control	0.40 ± 0.04^{a}	0.11 ± 0.04^{a}	0.10 ± 0.03^{b}	0.92 ± 0.09^{a}
High N:P	0.40 ± 0.06^{a}	0.11 ± 0.03^{a}	0.10 ± 0.03^{b}	1.34 ± 0.09^{b}

Superscript letters denote significant differences between nutrient treatments based on One-way ANOVA and post hoc comparison of the means ($\alpha < 0.05$).

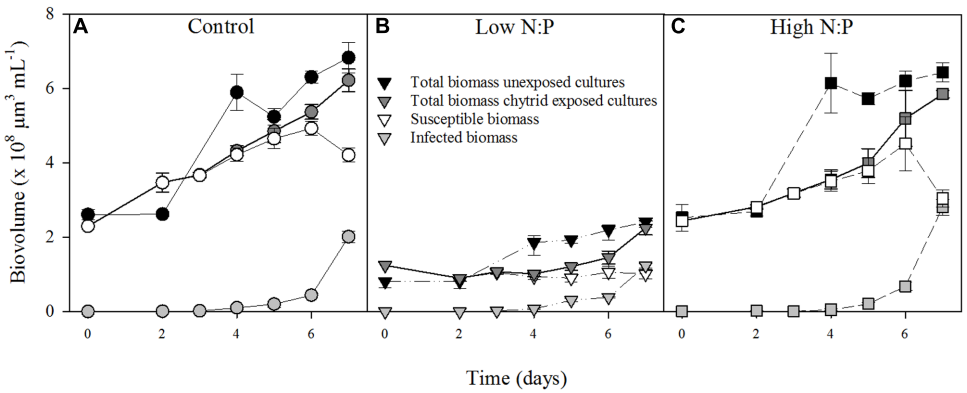

FIGURE 1 | Biovolumes of *Planktothrix* in the in the cultures with and without parasite exposure in the control **(A)**, low N:P **(B)** and high N:P **(C)** treatments. Symbols represent mean ± standard error ($n = 4$).

TABLE 2 | Host nutrient content and stoichiometry (mean ± SE) in unexposed cultures.

	Nutrient content (10^{-6} µmol mm^{-3})			Stoichiometry (molar)		
Treatment	**C**	**N**	**P**	**C:P**	**C:N**	**N:P**
Low N:P	5148 ± 268^{a}	837 ± 22^{ab}	108 ± 3^{a}	47.7 ± 1.9^{a}	6.1 ± 0.2^{a}	7.8 ± 0.1^{a}
Control	4098 ± 120^{b}	935 ± 26^{a}	83 ± 4^{b}	49.9 ± 1.9^{a}	4.4 ± 0.0^{b}	11.4 ± 0.4^{b}
High N:P	3705 ± 242^{b}	791 ± 48^{b}	17 ± 1^{c}	216.4 ± 11.7^{b}	4.7 ± 0.1^{b}	46.2 ± 2.7^{c}

Superscript letters denote significant differences between nutrient treatments based on One-way ANOVA and post hoc comparison of the means ($\alpha < 0.05$).

both a high and low N:P supply ratio (**Table 1**). The reduced host N:P under low N:P supply and increased host N:P under high N:P supply (**Figure 2A**) indicates nutrient limitation at the end of the exponential phase and/or early stationary phase. Moreover, population densities in the low N:P treatment at the end of the experiment were lower as compared to the control and high N:P treatment (**Figure 1B**). These lower population densities are mainly caused by a low N availability in the low N:P treatment, but may also result from the lower *Planktothrix* population densities at the start of the experiment. Differences in host population density may affect light availability in the cultures. A lowered population density, as observed in the low N:P treatment, may have resulted in an increased light availability due to reduced self-shading. Earlier studies have indicated that zoospores may find their host using chemical cues that are related to photosynthetic activity, since zoospores are generally attracted to carbohydrates, polysaccharides, proteins and amino acids (Muehlstein et al., 1988; Donaldson and Deacon, 1993; Moss et al., 2008). Some studies, however, also reported attachment of zoospores to new hosts during dark conditions (Barr and Hickman, 1967). Indirectly, the relative higher light availability in the low N:P treatment may thus have favored parasite attraction. Moreover, with a comparable amount of zoospores added at the start of the experiment, the zoospore-to-host ratio was also higher in the N limited treatment, which may favor infection rates. Yet, both the zoospore production rate and production efficiency were lower in the low N:P treatment, and did not lead to a different infection rate as compared to the control (**Table 1**). This suggests that higher relative light availabilities as well as higher initial zoospore-to-host ratios did not stimulate, and possibly even impeded the infection dynamics in our low N:P treatment.

In response to an increased N:P supply in the medium, host N:P increased as well. This resulted in a consecutive increase in the N:P of the zoospores (**Figure 2B**). Our results thus show that stoichiometry of a host can cascade to their parasites. In the host, changes in stoichiometry seem to be driven mainly by a change in P content, as host C and N remain constant with changing N:P supply (**Figure 2C**). In the parasite, changes

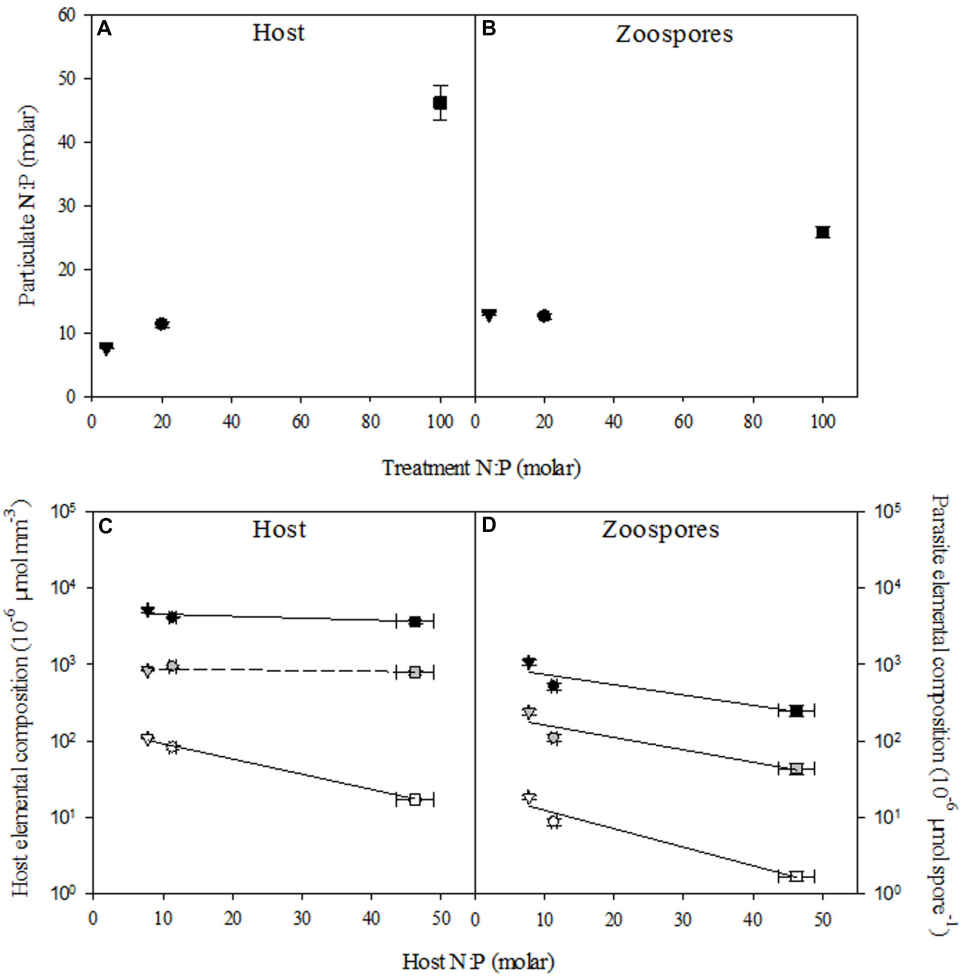

FIGURE 2 | N:P ratios of uninfected host **(A)** and zoospores **(B)**, and elemental content of the host **(C)** and zoospores **(D)** in the control (circle), low N:P (triangle) and high N:P (square) treatments. Symbols represent mean ± standard error (n = 4). In **(C,D)** black, gray, and white symbols indicate carbon, nitrogen and phosphorus content, respectively. Solid lines indicate significant correlations ($P < 0.05$).

TABLE 3 | Zoospore nutrient content and stoichiometry (mean ± SE).

	Nutrient content (×10^{-4} µmol per spore)			Stoichiometry (molar)		
Treatment	**C**	**N**	**P**	**C:P**	**C:N**	**N:P**
Low N:P	10.71 ± 0.77^{a}	2.41 ± 0.18^{a}	0.19 ± 0.01^{a}	57.8 ± 0.7^{a}	4.4 ± 0.0^{a}	13.0 ± 0.2^{a}
Control	5.27 ± 0.55^{b}	1.11 ± 0.11^{b}	0.09 ± 0.01^{b}	59.9 ± 1.7^{a}	4.7 ± 0.0^{b}	12.6 ± 0.4^{a}
High N:P	2.63 ± 0.49^{c}	0.45 ± 0.08^{c}	0.02 ± 0.00^{c}	149.9 ± 6.5^{b}	5.8 ± 0.1^{c}	25.9 ± 1.0^{b}

Superscript letters denote significant differences between nutrient treatments based on One-way ANOVA and post hoc comparison of the means ($\alpha < 0.05$).

in zoospore stoichiometry are also mainly driven by P content, but C and N contents decrease as well. Yet, P content decreases faster, suggesting a higher flexibility of the chytrid with respect to P (**Figure 2D**). These findings indicate that chytrid parasites can be stoichiometrically flexible, while maintaining their ability to infect along an N:P supply gradient. N:P and C:P ratios of the chytrid used in this experiment are relatively high as compared to two other studies using different chytrid species (Kagami et al., 2007, 2017), but fall well within the range of aquatic fungi reported before (Danger and Chauvet, 2013; Danger et al., 2015). These data furthermore suggest that fungal elemental homeostasis is indeed limited (Persson et al., 2010; Danger et al., 2015). Zoospore N:P ratios largely resembled that of the host under control conditions, but were different from the host in the low and high N:P treatment. We could not separate zoospores from the heterotrophic bacteria, and our results may therefore have been confounded by shifts in bacterial numbers. To prevent high bacterial numbers fueled by the lyses of

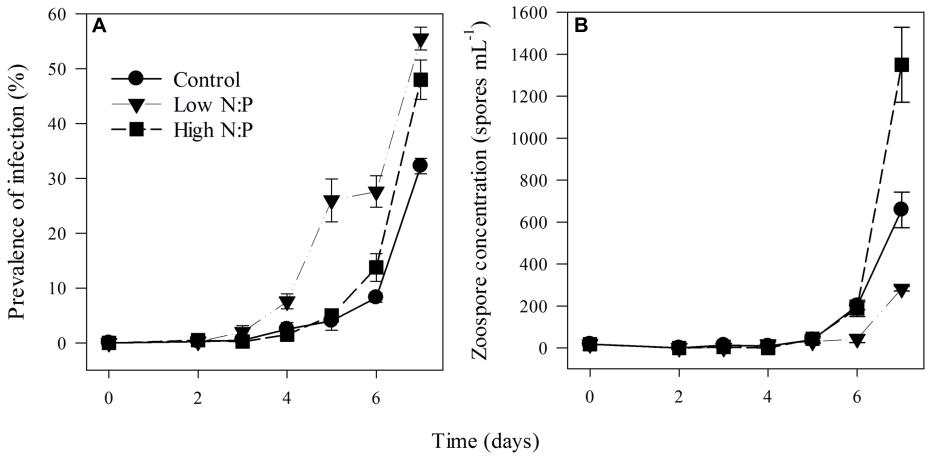

FIGURE 3 | Prevalence of infection **(A)** and zoospore concentration **(B)** in the control, low N:P and high N:P treatments. Symbols represent mean ± standard error (n = 4).

Planktothrix, we ran the experiments over a relatively short time period. Consequently, the overall biomass of bacteria and thereby their contribution to the elemental composition at the time of sampling is likely to be small.

Increasing N:P supply ratios resulted in an increased zoospore production rate and production efficiency (**Figures 4A,B**). These results are in contrast to earlier findings of an experiment that showed that under P-limitation (and presumably high N:P) zoospore production decreased (Bruning, 1991). It might be possible that the chytrid species (*R. planktonicum* Canter emend.) used in the experiment by Bruning (1991) has higher P-requirements, and therefore suffered more from P-limitation. Or, because the chytrid in our experiment can potentially infect and exploit multiple adjacent cells within one cyanobacterial filament (Canter and Lund, 1951), it might be less vulnerable to nutrient limitation. In other words, the chytrid might continue infecting adjacent cells until it has consumed sufficient nutrients to complete an infection cycle. However, this is only profitable if the energetic costs of growing rhizoids and producing degrading enzymes to invade host cells balance the gains with respect to resource acquisition.

Under low N:P conditions, the parasite zoospores were fewer but contained more C, as well as N and P as compared to the control (**Figure 2D**). Although we did not assess zoospore size in this experiment directly, increases in elemental contents do suggest that the chytrid produced larger zoospores. Zoospore size of the used chytrid was shown to vary from 2.84 to 5.36 μm under control growth conditions (Supplementary Material), and variation in spore size was also shown in other studies describing shifts in spore size with climatic conditions (Kauserud et al., 2008, 2011). Presumably, larger zoospores facilitate zoospore survival time, since they can contain more lipids and fatty acids that might represent an energy source to fuel zoospore metabolism (Steinhoff et al., 2011). A longer spore survival time may be particularly favorable at lower host densities, and may explain the unaltered chytrid infections in the low N:P treatment. Conversely, in the high N:P conditions, more zoospores were produced per host biomass but contained less C, N and P per zoospore, suggesting that they were smaller. These findings are supported by earlier observations indicating that the efficiency of spore production by a parasitic dinoflagellate is increased under high N:P conditions, which might result in a higher transmission to new hosts under high host density conditions (Yih and Coats, 2000).

At low N:P supply the chytrid seems to produce a low amount of large zoospores, while at high N:P supply it produced a higher amount of small zoospores. The chytrid thus possibly produced smaller spores with a higher production efficiency (**Figure 4C**), suggesting a trade-off between size and production rate as well as success of infection. In other words, larger spores may survive longer providing the chytrid more time to find a suitable host under low host density conditions, while smaller ones survive less long but due to their high numbers achieve a higher infection transmission in high host density conditions. A trade-off between organism size and growth rate has also been reported for various other organisms, including phytoplankton (Nielsen, 2006) and zooplankton (Stemberger and Gilbert, 1985). Moreover, a trade-off between zoospore survival time and production rate was observed in another chytrid, the amphibian killing fungus *Batrachochytrium dendrobatidis* (Woodhams et al., 2008). Thus, changes in host N:P stoichiometry may affect the growth strategy of the parasite, following a more general trade-off between cell size and production rates (**Figure 4C**). Such changes can have consequences not only for the infection dynamics, but also for higher trophic levels that are provided with either many smaller zoospores, or fewer larger ones.

As expected, intracellular MC content closely followed the relative availability of N, and thus increased with cellular N:P ratios (**Figure 5**). These results are in line with earlier work, showing a strong dependency of MC contents on N availability (Van de Waal et al., 2009, 2010). In the treatment with high N:P supply and high MC production, however, zoospore production (**Figure 4A**) and infection rate (**Table 1**) were highest. Additionally, in the low N:P treatment, MC contents was lowest

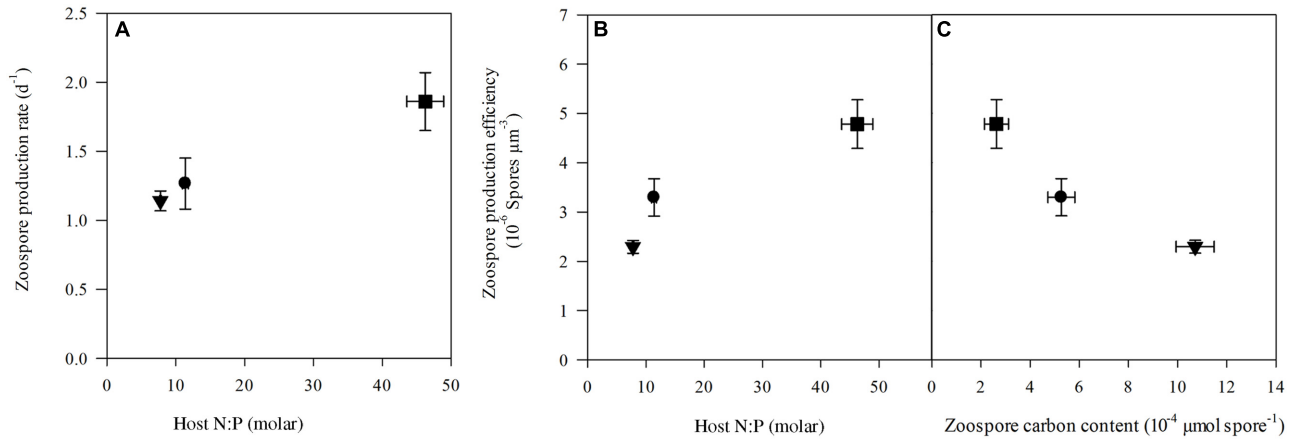

FIGURE 4 | Zoospore production rate **(A)** and production efficiency **(B)** plotted against host N:P and zoospore carbon content **(C)** in the control (circle), low N:P (triangle) and high N:P (square) treatments. Symbols represent mean ± standard error (n = 4).

TABLE 4 | Zoospore production rates and production efficiencies (mean ± SE).

Treatment	Rate (d^{-1})	Efficiency (10^{-6} spores μm^{-3})
Low N:P	1.14 ± 0.07^{a}	2.17 ± 0.04^{a}
Control	1.27 ± 0.18^{ab}	3.14 ± 0.42^{b}
High N:P	1.86 ± 0.21^{b}	5.02 ± 0.53^{c}

Superscript letters denote significant differences between nutrient treatments based on One-way ANOVA and post hoc comparison of the means ($\alpha < 0.05$).

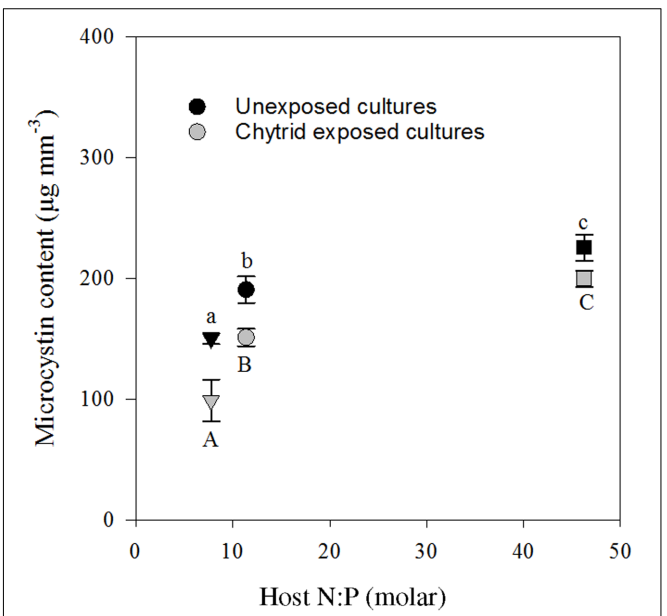

FIGURE 5 | Seston microcystin content in the cultures with and without parasite exposure, in the control (circle), low N:P (triangle) and high N:P (square) treatments. Symbols represent mean ± standard error (n = 4). Letters denote significant differences between treatments of unexposed (lower case) and chytrid exposed (upper case) cultures based on One-way ANOVA and *post hoc* comparison of the means ($\alpha < 0.05$).

while zoospore production and infection rate were not different from the control. So, there was no clear relation between intracellular MC content and chytrid proliferation. Possibly, a considerable fraction of the total MC might be bound to proteins of cyanobacterial cells (Zilliges et al., 2011), which were not included in our extraction processes. Furthermore, *Planktothrix* may produce other oligopeptides that play a role in parasite defense systems (Sønstebø and Rohrlack, 2011; Rohrlack et al., 2013), which were not analyzed here.

The intracellular MC seemed to be lower in the parasite exposed treatments as compared to the unexposed treatments. This may possibly result from leakage of MC from the cells into the liquid phase (Jones and Orr, 1994). Moreover, chytrid rhizoids that invade the host cells might use enzymes that are able to digest MC. Indeed, fungi were shown to be capable of degrading MC (Jia et al., 2012). In our experiment, however, extracellular MC concentrations nor chytrid MC contents were analyzed. If MC is released into the water column from cyanobacterial cells, it can have consequences for other organisms present (Carmichael, 1992; Zurawell et al., 2005). For instance, high MC concentrations in the water can accumulate in *Daphnia* (Chen et al., 2005) and have adverse effects on growth and development of fish (Jacquet et al., 2004). Yet, actual exposure of other organisms to MC in the water may be limited, as MCs can be rapidly biodegraded and detoxified by bacteria and adsorb to plants and sediments (Harada and Tsuji, 1998; Pflugmacher et al., 2001; Kato et al., 2007). Whether MCs can bind to- or be transported into zoospores is unknown. But, if this would occur, zooplankton might be exposed to MCs via this indirect route, since zoospores can serve as a food source for copepods, cladocerans and possibly rotifers (Kagami et al., 2004, 2007, 2011; Buck et al., 2011; Agha et al., 2016; Frenken et al., 2016).

Our results demonstrate an increase in infection rate with host N:P stoichiometry, thereby showing the opposite to what we hypothesized. Because chytrids seemed relatively more P rich as compared to their host, we initially predicted that host P content

would constrain chytrid growth more than it would constrain the host (Bruning and Ringelberg, 1987; Bruning, 1991). Our results suggest, however, that chytrid proliferation is much more sensitive to the relative availability of N. Specifically, if this increases (i.e., higher host N:P), infection rates increase, while if this decreases (i.e., lower host N:P), infection rates decrease. This is also shown by the lower flexibility of the parasite N content as compared to P, suggesting that spores are more likely to be constrained under low N conditions. It remains unclear why infection rates increase under P limitation and relative high N contents. Particularly as under these high N:P conditions, MC contents were highest as well. We initially expected that under such conditions, chytrid infections can be inhibited by MCs in its host. The increase in MC content with high N:P conditions, however, was relatively small and may therefore not have been sufficient to inhibit the chytrid infection. Possibly, regulation of other oligopeptides in response to N:P supply could have explained the observed responses, and should thus be included in future analyzes. Moreover, other metabolites synthesized by cyanobacteria under high N:P supply may have facilitated chytrid growth and reproduction. Further detailed biochemical analyses of chytrids and their distinct developmental stages would be required to fully understand the stoichiometric interactions with their hosts, and particularly the putative important role of N in controlling infections.

Our analysis revealed some still poorly understood effects of nutrient availability on the interaction of a host–parasite system. Shifts in nutrient supply ratios not only lead to a shift in host stoichiometry, but also to comparable changes in the parasite. Thereby, we show that elemental stoichiometry of a host can cascade to their parasites. We hypothesize that, in response to changes in nutrient supply, the parasite may exhibit a trade-off between size and zoospore production rate to optimize reproductive success. Therefore, nutrient limitation may indirectly affect parasite abundance and stoichiometry. Since chytrids can facilitate growth of zooplankton (Kagami et al., 2007; Agha et al., 2016), changes in parasite production, stoichiometry and cell size may have implications for aquatic food web dynamics.

AUTHOR CONTRIBUTIONS

TF, JW, and DVdW designed the study. JW and TF performed the experiment. TF, JW, AG, and DVdW analyzed and interpreted the data. TF and DVdW wrote a first draft of the manuscript which was corrected, revised and approved by all authors.

FUNDING

AG is supported by a NWO-veni grant (016.Veni.171.063).

ACKNOWLEDGMENTS

We are grateful to Nico Helmsing and Erik Reichman for assistance during the experiment and for analyses of MC and seston elemental composition. We also thank Suzanne Naus-Wiezer for help and advice with microscopic work and Els Faassen for advice on MC analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01015/full#supplementary-material

REFERENCES

Agha, R., Saebelfeld, M., Manthey, C., Rohrlack, T., and Wolinska, J. (2016). Chytrid parasitism facilitates trophic transfer between bloom-forming cyanobacteria and zooplankton (*Daphnia*). *Sci. Rep.* 6:35039. doi: 10.1038/srep35039

Armstrong, F. A. J., Stearns, C. R., and Strickland, J. D. H. (1967). The measurement of upwelling and subsequent biological process by means of the Technicon Autoanalyzer® and associated equipment. *Deep Sea Res. Oceanogr. Abstr.* 14, 381–389. doi: 10.1016/0011-7471(67)90082-4

Barr, D., and Hickman, C. (1967). Chytrids and algae: II. Factors influencing parasitism of *Rhizophydium sphaerocarpum* on Spirogyra. *Can. J. Bot.* 45, 431–440. doi: 10.1139/b67-043

Barr, D. J., and Hadland-Hartmann, V. (1978). Zoospore ultrastructure in the genus *Rhizophydium* (Chytridiales). *Can. J. Bot.* 56, 2380–2404. doi: 10.1139/b78-290

Barr, D. J. S. (2001). "Chytridiomycota," in *Systematics and Evolution: Part A*, eds D. J. Mclaughlin, E. G. Mclaughlin, and P. A. Lemke (Berlin: Springer), 93–112. doi: 10.1007/978-3-662-10376-0_5

Beakes, G. W., Canter, H. M., and Jaworski, G. H. (1988). Zoospore ultrastructure of *Zygorhizidium affluens* and *Z. planktonicum*, two chytrids parasitizing the diatom *Asterionella formosa*. *Can. J. Bot.* 66, 1054–1067. doi: 10.1139/b88-151

Beakes, G. W., Canter, H. M., and Jaworski, G. H. M. (1993). Sporangium differentiation and zoospore fine-structure of the chytrid *Rhizophydium planktonicum*, a fungal parasite of *Asterionella formosa*. *Mycol. Res.* 97, 1059–1074. doi: 10.1016/S0953-7562(09)80507-8

Bracken, M. E. S., Hillebrand, H., Borer, E. T., Seabloom, E. W., Cebrian, J., Cleland, E. E., et al. (2014). Signatures of nutrient limitation and co-limitation: responses of autotroph internal nutrient concentrations to nitrogen and phosphorus additions. *Oikos* 124, 113–121. doi: 10.1111/oik.01215

Bruning, K. (1991). Effects of phosphorus limitation on the epidemiology of a chytrid phytoplankton parasite. *Freshw. Biol.* 25, 409–417. doi: 10.1111/j.1365-2427.1991.tb01385.x

Bruning, K., and Ringelberg, J. (1987). The influence of phosphorus limitation of the diatom *Asterionella formosa* on the zoospore production of its fungal parasite *Rhizophydium Planktonicum*. *Hydrobiol. Bull.* 21, 49–54. doi: 10.1007/BF02255454

Buck, J. C., Truong, L., and Blaustein, A. R. (2011). Predation by zooplankton on *Batrachochytrium dendrobatidis*: biological control of the deadly amphibian chytrid fungus? *Biodivers. Conserv.* 20, 3549–3553. doi: 10.1007/s10531-011-0147-4

Canter, H. M., and Lund, J. (1951). Studies on plankton parasites III. Examples of the interaction between parasitism and other factors determining the growth of diatoms. *Ann. Bot.* 15, 359–371. doi: 10.1093/oxfordjournals.aob.a083287

Carmichael, W. (1992). Cyanobacteria secondary metabolites—the cyanotoxins. *J. Appl. Bacteriol.* 72, 445–459. doi: 10.1111/j.1365-2672.1992.tb01858.x

Carpenter, S. R., Kitchell, J. F., Cottingham, K. L., Schindler, D. E., Christense, D. L., Post, D. M., et al. (1996). Chlorophyll variability, nutrient input,

and grazing: evidence from whole- lake experiments. *Ecology* 77, 725–735. doi: 10.2307/2265497

Chen, W., Song, L., Ou, D., and Gan, N. (2005). Chronic toxicity and responses of several important enzymes in *Daphnia magna* on exposure to sublethal microcystin-LR. *Environ. Toxicol.* 20, 323–330. doi: 10.1002/tox.20108

Danger, M., and Chauvet, E. (2013). Elemental composition and degree of homeostasis of fungi: Are aquatic hyphomycetes more like metazoans, bacteria or plants? *Fungal Ecol.* 6, 453–457. doi: 10.1016/j.funeco.2013.05.007

Danger, M., Gessner, M. O., and Bärlocher, F. (2015). Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. *Fungal Ecol.* 19, 100–111. doi: 10.1016/j.funeco.2015.09.004

Donaldson, S. P., and Deacon, J. W. (1993). Effects of amino acids and sugars on zoospore taxis, encystment and cyst germination in *Pythium aphanidermatum* (Edson) Fitzp., *P. catenulatum* Matthews and *P. dissotocum* Drechs. *New Phytol.* 123, 289–295. doi: 10.1111/j.1469-8137.1993.tb03738.x

Eaton, A. D. (2005). *Standard Methods for the Examination of Water and Wastewater*. Washington, DC: APHA-AWWA-WEF.

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Elser, J. J., Dobberfuhl, D. R., Mackay, N. A., and Schampel, J. H. (1996). Organism size, life history, and N: P stoichiometry toward a unified view of cellular and ecosystem processes. *Bioscience* 46, 674–684. doi: 10.2307/1312897

Faassen, E., and Lürling, M. (2013). Occurrence of the microcystins MC-LW and MC-LF in Dutch surface waters and their contribution to total microcystin toxicity. *Mar. Drugs* 11, 2643–2654. doi: 10.3390/md11072643

Frenken, T., Velthuis, M., De Senerpont Domis, L. N., Stephan, S., Aben, R., Kosten, S., et al. (2016). Warming accelerates termination of a phytoplankton spring bloom by fungal parasites. *Glob. Change Biol.* 22, 299–309. doi: 10.1111/gcb.13095

Gerphagnon, M., Macarthur, D. J., Latour, D., Gachon, C. M. M., Van Ogtrop, F., Gleason, F. H., et al. (2015). Microbial players involved in the decline of filamentous and colonial cyanobacterial blooms with a focus on fungal parasitism. *Environ. Microbiol.* 17, 2573–2587. doi: 10.1111/1462-2920.12860

Guillard, R. R. L., and Lorenzen, C. J. (1972). Yellow-green algae with chlorophyllide C. *J. Phycol.* 8, 10–14.

Harada, K.-I., and Tsuji, K. (1998). Persistence and decomposition of hepatotoxic microcystins produced by cyanobacteria in natural environment. *J. Toxicol. Toxin Rev.* 17, 385–403. doi: 10.3109/15569549809040400

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Jacquet, C., Thermes, V., Luze, A. D., Puiseux-Dao, S., Bernard, C., Joly, J.-S., et al. (2004). Effects of microcystin-LR on development of medaka fish embryos (*Oryzias latipes*). *Toxicon* 43, 141–147. doi: 10.1016/j.toxicon.2003.11.010

Jia, Y., Du, J., Song, F., Zhao, G., and Tian, X. (2012). A fungus capable of degrading microcystin-LR in the algal culture of *Microcystis aeruginosa* PCC7806. *Appl. Biochem. Biotechnol.* 166, 987–996. doi: 10.1007/s12010-011-9486-6

Jones, G. J., and Orr, P. T. (1994). Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Res.* 28, 871–876. doi: 10.1016/0043-1354(94)90093-0

Kagami, M., Helmsing, N., and Van Donk, E. (2011). Parasitic chytrids could promote copepod survival by mediating material transfer from inedible diatoms. *Hydrobiologia* 659, 49–54. doi: 10.1007/s10750-010-0274-z

Kagami, M., Motoki, Y., Masclaux, H., and Bec, A. (2017). Carbon and nutrients of indigestible pollen are transferred to zooplankton by chytrid fungi. *Freshw. Biol.* 62, 954–964. doi: 10.1111/fwb.12916

Kagami, M., Van Donk, E., De Bruin, A., Rijkeboer, M., and Ibelings, B. W. (2004). Daphnia can protect diatoms from fungal parasitism. *Limnol. Oceanogr.* 49, 680–685. doi: 10.4319/lo.2004.49.3.0680

Kagami, M., Von Elert, E., Ibelings, B. W., De Bruin, A., and Van Donk, E. (2007). The parasitic chytrid, *Zygorhizidium*, facilitates the growth of the cladoceran zooplankter, *Daphnia*, in cultures of the inedible alga, *Asterionella*. *Proc. R. Soc. B Biol. Sci.* 274, 1561–1566. doi: 10.1098/rspb.2007.0425

Kato, H., Imanishi, S. Y., Tsuji, K., and Harada, K.-I. (2007). Microbial degradation of cyanobacterial cyclic peptides. *Water Res.* 41, 1754–1762. doi: 10.1016/j.watres.2007.01.003

Kauserud, H., Colman, J. E., and Ryvarden, L. (2008). Relationship between basidiospore size, shape and life history characteristics: a comparison of polypores. *Fungal Ecol.* 1, 19–23. doi: 10.1016/j.funeco.2007.12.001

Kauserud, H., Heegaard, E., Halvorsen, R., Boddy, L., Høiland, K., and Stenseth, N. C. (2011). Mushroom's spore size and time of fruiting are strongly related: Is moisture important? *Biol. Lett.* 7, 273–276. doi: 10.1098/rsbl.2010.0820

Moss, A. S., Reddy, N. S., Dortaj, I. M., and San Francisco, M. J. (2008). Chemotaxis of the amphibian pathogen *Batrachochytrium dendrobatidis* and its response to a variety of attractants. *Mycologia* 100, 1–5. doi: 10.1080/15572536.2008.11832493

Muehlstein, L. K., Amon, J. P., and Leffler, D. L. (1988). Chemotaxis in the marine fungus *Rhizophydium littoreum*. *Appl. Environ. Microbiol.* 54, 1668–1672.

Nielsen, S. L. (2006). Size-dependent growth rates in eukaryotic and prokaryotic algae exemplified by green algae and cyanobacteria: comparisons between unicells and colonial growth forms. *J. Plankton Res.* 28, 489–498. doi: 10.1093/plankt/fbi134

Paerl, H. W., Fulton, R. S., Moisander, P. H., and Dyble, J. (2001). Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *ScientificWorldJournal* 1, 76–113. doi: 10.1100/tsw.2001.16

Paerl, H. W., Hall, N. S., and Calandrino, E. S. (2011). Controlling harmful cyanobacterial blooms in a world experiencing anthropogenic and climatic-induced change. *Sci. Total Environ.* 409, 1739–1745. doi: 10.1016/j.scitotenv.2011.02.001

Persson, J., Fink, P., Goto, A., Hood, J. M., Jonas, J., and Kato, S. (2010). To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos* 119, 741–751. doi: 10.1111/j.1600-0706.2009.18545.x

Pflugmacher, S., Wiegand, C., Beattie, K. A., Krause, E., Steinberg, C. E. W., and Codd, G. A. (2001). Uptake, effects, and metabolism of cyanobacterial toxins in the emergent reed plant *Phragmites australis* (Cav.) Trin. ex steud. *Environ. Toxicol. Chem.* 20, 846–852. doi: 10.1002/etc.5620200421

Reynolds, C. (1973). The seasonal periodicity of planktonic diatoms in a shallow eutrophic lake. *Freshw. Biol.* 3, 89–110. doi: 10.1111/j.1365-2427.1973.tb00065.x

Rohrlack, T., Christiansen, G., and Kurmayer, R. (2013). Putative antiparasite defensive system involving ribosomal and nonribosomal oligopeptides in cyanobacteria of the genus *Planktothrix*. *Appl. Environ. Microbiol.* 79, 2642–2647. doi: 10.1128/AEM.03499-12

Schindler, D. W., and Fee, E. J. (1974). Experimental lakes area: whole-lake experiments in eutrophication. *J. Fish. Res. Board Can.* 31, 937–953. doi: 10.1139/f74-110

Sidak, Z. (1967). Rectangular confidence regions for the means of multivariate normal distributions. *J. Am. Stat. Assoc.* 62, 626–633. doi: 10.2307/2283989

Sime-Ngando, T. (2012). Phytoplankton chytridiomycosis: fungal parasites of phytoplankton and their imprints on the food web dynamics. *Front. Microbiol.* 3:361. doi: 10.3389/fmicb.2012.00361

Smith, V. (2007). Host resource supplies influence the dynamics and outcome of infectious disease. *Integr. Comp. Biol.* 47, 310–316. doi: 10.1093/icb/icm006

Smith, V. H. (2003). Eutrophication of freshwater and coastal marine ecosystems a global problem. *Environ. Sci. Pollut. Res.* 10, 126–139. doi: 10.1065/espr2002.12.142

Smith, V. H., Joye, S. B., and Howarth, R. W. (2006). Eutrophication of freshwater and marine ecosystems. *Limnol. Oceanogr.* 51, 351–355. doi: 10.4319/lo.2006.51.1_part_2.0351

Smith, V. H., and Schindler, D. W. (2009). Eutrophication science: Where do we go from here? *Trends Ecol. Evol.* 24, 201–207. doi: 10.1016/j.tree.2008.11.009

Sønstebø, J. H., and Rohrlack, T. (2011). Possible implications of chytrid parasitism for population subdivision in freshwater cyanobacteria of the genus *Planktothrix*. *Appl. Environ. Microbiol.* 77, 1344–1351. doi: 10.1128/AEM.02153-10

Sparrow, F. K. (1960). *Aquatic Phycomycetes*. Ann Arbor, MI: University of Michigan Press. doi: 10.5962/bhl.title.5685

Sparrow, F. K. (1968). "Ecology of freshwater fungi," in *The Fungi. An Advanced Treatise: The Fungal Population*, eds G. Ainsworth and A. Sussman (New York, NY: Academia Press), 41–93.

Steinhoff, F. S., Graeve, M., Wiencke, C., Wulff, A., and Bischof, K. (2011). Lipid content and fatty acid consumption in zoospores/developing gametophytes of *Saccharina latissima* (Laminariales, Phaeophyceae) as potential precursors for secondary metabolites as phlorotannins. *Polar Biol.* 34, 1011–1018. doi: 10.1007/s00300-011-0960-y

Stemberger, R. S., and Gilbert, J. J. (1985). Body size, food concentration, and population growth in planktonic rotifers. *Ecology* 66, 1151–1159. doi: 10.2307/1939167

Sterner, R. W., Anagnostou, E., Brovold, S., Bullerjahn, G. S., Finlay, J. C., Kumar, S., et al. (2007). Increasing stoichiometric imbalance in North America's largest lake: nitrification in lake superior. *Geophys. Res. Lett.* 34:L10406. doi: 10.1029/2006GL028861

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Van de Waal, D. B., Ferreruela, G., Tonk, L., Van Donk, E., Huisman, J., Visser, P. M., et al. (2010). Pulsed nitrogen supply induces dynamic changes in the amino acid composition and microcystin production of the harmful cyanobacterium *Planktothrix agardhii*. *FEMS Microbiol. Ecol.* 74, 430–438. doi: 10.1111/j.1574-6941.2010.00958.x

Van de Waal, D. B., Smith, V. H., Declerck, S. A. J., Stam, E. C. M., and Elser, J. J. (2014). Stoichiometric regulation of phytoplankton toxins. *Ecol. Lett.* 17, 736–742. doi: 10.1111/ele.12280

Van de Waal, D. B., Verspagen, J. M., Lürling, M., Van Donk, E., Visser, P. M., and Huisman, J. (2009). The ecological stoichiometry of toxins produced by harmful cyanobacteria: an experimental test of the carbon-nutrient balance hypothesis. *Ecol. Lett.* 12, 1326–1335. doi: 10.1111/j.1461-0248.2009.01383.x

Van Donk, E. (1984). Factors influencing phytoplankton growth and succession in Lake Maarsseveen I. *Hydrobiol. Bull.* 18, 69–71. doi: 10.1007/BF02256678

Van Donk, E. (1989). "The role of fungal parasites in phytoplankton succession," in *Plankton Ecology*, ed. U. Sommer (Berlin: Springer), 171–194. doi: 10.1007/978-3-642-74890-5_5

Van Donk, E., and Ringelberg, J. (1983). The effect of fungal parasitism on the succession of diatoms in Lake Maarsseveen I (The Netherlands). *Freshw. Biol.* 13, 241–251. doi: 10.1111/j.1365-2427.1983.tb00674.x

Welker, M., and Von Döhren, H. (2006). Cyanobacterial peptides — nature's own combinatorial biosynthesis. *FEMS Microbiol. Rev.* 30, 530–563. doi: 10.1111/j.1574-6976.2006.00022.x

Woodhams, D. C., Alford, R. A., Briggs, C. J., Johnson, M., and Rollins-Smith, L. A. (2008). Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. *Ecology* 89, 1627–1639. doi: 10.1890/06-1842.1

Yih, W., and Coats, D. W. (2000). Infection of *Gymnodinium sanguineum* by the dinoflagellate *Amoebophrya* sp.: effect of nutrient environment on parasite generation time, reproduction, and infectivity. *J. Eukaryot. Microbiol.* 47, 504–510. doi: 10.1111/j.1550-7408.2000.tb00082.x

Zilliges, Y., Kehr, J.-C., Meissner, S., Ishida, K., Mikkat, S., Hagemann, M., et al. (2011). The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of *Microcystis* under oxidative stress conditions. *PLoS ONE* 6:e17615. doi: 10.1371/journal.pone.0017615

Zurawell, R. W., Chen, H., Burke, J. M., and Prepas, E. E. (2005). Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. *J. Toxicol. Environ. Health B Crit. Rev.* 8, 1–37. doi: 10.1080/10937400590889412

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Marine Science

HYPOTHESIS AND THEORY
published: 08 September 2017
doi: 10.3389/fmars.2017.00286

An Operational Framework for the Advancement of a Molecule-to-Biosphere Stoichiometry Theory

Mehdi Cherif[1], Carolyn Faithfull[1], Junwen Guo[1], Cédric L. Meunier[2], Judith Sitters[3,4], Wojciech Uszko[1] and Francisco Rivera Vasconcelos[1]*

[1] Department of Ecology and Environmental Science, Umeå University, Umeå, Sweden, [2] Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, Biologische Anstalt Helgoland, Helgoland, Germany, [3] Ecology and Biodiversity, Department Biology, Vrije Universiteit Brussel, Brussels, Belgium, [4] Departments of Aquatic and Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands

Biological stoichiometry is an approach that focuses on the balance of elements in biological interactions. It is a theory that has the potential to causally link material processes at all biological levels—from molecules to the biosphere. But the lack of a coherent operational framework has so far restricted progress in this direction. Here, we provide a framework to help infer how a stoichiometric imbalance observed at one level impacts all other biological levels. Our framework enables us to highlight the areas of the theory in need of completion, development and integration at all biological levels. Our hope is that this framework will contribute to the building of a more predictive theory of elemental transfers within the biosphere, and thus, to a better understanding of human-induced perturbations to the global biogeochemical cycles.

Keywords: biological organization, biological stoichiometry, consumer-driven nutrient recycling, ecological theory, theory integration, growth-rate hypothesis, light:nutrient hypothesis

OPEN ACCESS

Edited by:
James Cotner,
University of Minnesota, United States

Reviewed by:
Ursula Felicitas Marianne Witte,
University of Aberdeen,
United Kingdom
Anna Villnäs,
University of Helsinki, Finland
Priscilla Licandro,
Plymouth Marine Laboratory,
United Kingdom

****Correspondence:***
Mehdi Cherif
mehdi.cherif@umu.se

Specialty section:
This article was submitted to Marine Ecosystem Ecology, a section of the journal Frontiers in Marine Science

Received: *30 November 2016*
Accepted: *22 August 2017*
Published: *08 September 2017*

Citation:
Cherif M, Faithfull C, Guo J, Meunier CL, Sitters J, Uszko W and Rivera Vasconcelos F (2017) An Operational Framework for the Advancement of a Molecule-to-Biosphere Stoichiometry Theory. Front. Mar. Sci. 4:286. doi: 10.3389/fmars.2017.00286

INTRODUCTION

Ecological stoichiometry studies the balance of chemical elements in ecological interactions (Sterner and Elser, 2002). Most hypotheses in ecology are born from the clever integration of previously unrelated assumptions and observations (Pickett et al., 2007d). Ecological stoichiometry is a great illustration of this principle since it came as a eureka combination of two observations: the demonstration that zooplankton can increase the growth rate of phytoplankton through the recycling of limiting nutrients (Sterner, 1986) and the shift in phytoplankton limitation between phosphorus (P) and nitrogen (N) that sometimes results from manipulations of the zooplankton community species composition (Elser et al., 1988). The result was a model of how zooplankton N and P composition could drive phytoplankton limitation through differential recycling of N and P (Sterner, 1990). The hypothesis was then called the Consumer-Driven Nutrient Recycling Hypothesis (Sterner et al., 1992). Hypotheses grow into fully-fledged theories through further integration of new observations, concepts, hypotheses and models, thus increasing their contribution to the understanding of the phenomena they are meant to explain (Pickett et al., 2007c; Marquet et al., 2014). Accordingly, the Consumer-Driven Nutrient Recycling hypothesis grew up into the field of ecological stoichiometry by incorporating more hypotheses, such as the growth rate and light:nutrient hypotheses (Sterner et al., 1997; Elser et al., 2000c). The depth

and breadth of ecological stoichiometry then expanded through the integration of empirical observations and experimental results from increasingly diverse habitats, ecosystems and trophic interactions (Sardans et al., 2012). One cannot help but be struck by the vitality of the theory, expressed in a large number of mechanistic hypotheses and testable predictions, as well as in the wide scope of biological phenomena it now encompasses: from freshwater plankton interactions to, for example, the evolution of terrestrial plant genomes (Acquisti et al., 2009), the growth of cancerous tumors (Elser et al., 2003) or the macroevolution of life during the early Cambrian period (Elser et al., 2006). Thus, the theory shows the greatest potential in the midst of many contemporary ecological theories to unify ecology across all biological levels, from molecules to the biosphere (Sterner and Elser, 2002; Elser, 2006; Hessen et al., 2013). Some even argue, with good reason, that the theory should now be called "Biological Stoichiometry," since it no longer restricts itself to the study of ecological patterns (Elser et al., 2000c).

But completeness—breadth of scope and diversity of components—is not the only axis along which a theory can grow (Sterner and Schulz, 1998). Two other axes are the development of each component toward more realism and applicability; and integration, the connection among components toward better articulation (Pickett et al., 2007c). The ultimate objective of a theory being to generate understanding, it must also be judged by the advancement it provides to our comprehension of natural patterns (Pickett et al., 2007a; Marquet et al., 2014). Only when organized into a logical framework that assembles its components into an explicit structure, can a theory correctly predict or explain occurring natural patterns (Pickett et al., 2007b). Natural patterns are generally the result of complex multi-scale hierarchical processes (O'Neill, 1986), and so, most of the fundamental and urgent questions in ecology nowadays are multi-scale and integrative (Irschick et al., 2013; Sutherland et al., 2013). The logical framework of an ecological theory should thus typically be nested and hierarchical as well as integrated, both in terms of its internal components and with other theories.

In the case of biological stoichiometry, foundations for such a logical framework have already been laid, notably in the canon book by Sterner and Elser (2002) and more recently in review articles (Hall, 2009; Sardans et al., 2012; Hessen et al., 2013). Despite offering a panorama of the insights gained from applying the stoichiometric approach to processes spanning from the physiological to the ecosystem level, those reviews do not provide the kind of framework that is needed to help the theory progress along the three axes of completeness, development and integration. Not enough efforts have been made to integrate and organize the various stoichiometric breakthroughs into one coherent framework. Moreover, links between mechanisms at the lower and higher ends of the biological organization levels are often inferred but seldom fully explicated (Schade et al., 2005).

Here, we offer a methodological framework that describes the processes that need to be investigated at each biological level in order to characterize the repercussions of a stoichiometric imbalance at one level over all the other levels. This framework is operational rather than descriptive. It explains "how to" derive consequences of stoichiometric imbalances rather than "what" will be those consequences. As such, it is adaptable to all biological systems and open to further development. Armed with this framework, we surveyed the literature, in order to assess the current understanding of stoichiometric patterns and processes at each biological level (see our framework on **Figure 1**). We focus particularly on the degree of completeness of current understanding (i.e., whether are there any important considerations missing or not), the degree of development (how reliable are the proposed concepts and hypotheses?) and the degree of integration (how are other theories, approaches and knowledge considered and included?). We selected readings from the stoichiometry literature, from seminal publications by Liebig and Playfair (1840) and Redfield (1934) to the most recent literature (>900 articles read). Articles were first selected using generic search engines (Web of Science, Scopus) and generic keywords (biological OR ecological AND stoichiometr*). Articles were then more selectively pursued according to their relevance to the component of the framework evaluated and the elements that we assessed as in need of completion, development or integration. Our evaluation of each framework component is discussed below in separate sections. We have also summarized the elements that we deemed in need of enhancement according to the three axes of completeness, development and integration in **Tables 1, 2**.

FRAMEWORK DEFINITION

Our framework is organized along a rendition of the classical biological levels, defined by their stoichiometry, the distribution of elements within them, and connected by the processes that mediate the repercussions of changes in the stoichiometry of one level upon another (**Figure 1**). We are aware that there is no universally agreed-upon description of hierarchical levels in biology, arguably because such a description depends to a large extent on the specificities of the system studied (Allen and Hoekstra, 1993). So, we open the possibility for adapting the choice and definition of levels to the specificities of the systems to which the framework is applied. Also, we acknowledge that the highest conventional biological levels can be defined at any spatial and temporal scale (Allen and Hoekstra, 1990). Ecosystems for example can be as small as a drop of water or as incommensurable as an ocean (O'Neill, 1986). So the landscape and biosphere levels in our framework should not be understood according to the common-sense definition of the terms. We define a landscape as a set of connected ecosystems, themselves operational units defined according to the aims of the investigators, be it a moss patch, a water pool or a continental forest. The biosphere is the surrounding medium that circulates unbounded between the ecosystem units. Populations are the sum of individuals from the same species (or any other relevant taxonomic unit) contained in one ecosystem unit that can reproduce or exchange genetic information at the timescale considered; community is the ensemble of populations present in a unit ecosystem. Levels from

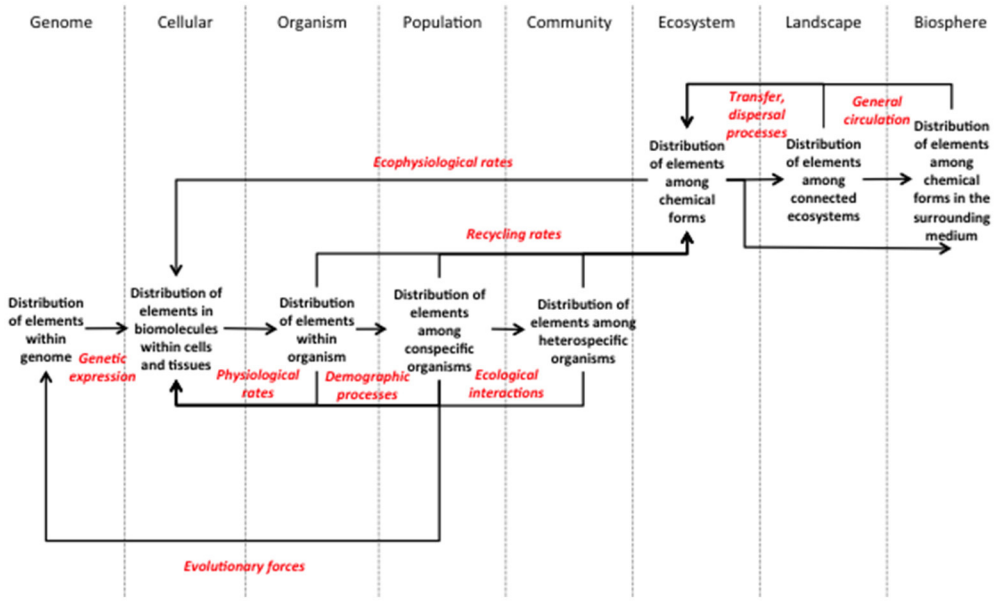

FIGURE 1 | Framework for a molecule-to-biosphere stoichiometry theory. This framework presents explicitly the biological levels and processes that need to be investigated in order to characterize the repercussions of a stoichiometric imbalance at one level over all the other levels. Each biological level should be described by the patterns of elemental distribution within it. As the framework shows, the processes investigated should go beyond the fluxes and rates of elements between components that are classically considered in stoichiometric studies. Any process affecting the structure of a biological level should be considered if the changes provoked relate to changes in the distribution of elements. Numerous examples are discussed within the text.

the organism and below are scale independent. We also include tissues as part of the cellular level to the extent that the cells that compose them answer uniformly to the factors affecting their stoichiometry.

The other elements of the framework are the processes that relate changes in the elemental distribution of a given level to the stoichiometric properties of the other levels (**Figure 1**). They are the medium by which imbalances in the stoichiometry of one level reverberate throughout all biological levels. In practical terms, they are the processes whose rates have to be measured by investigators interested in the multi-scale effects of stoichiometry. The framework reveals that the cellular, organism and ecosystem levels are essential nodes that cannot be bypassed when connecting high and low levels (**Figure 1**). Obviously, regional processes may affect individual organisms only if they influence the local ecosystem that embeds them. Within the local ecosystem, changes in the distribution of elements among chemical forms may impact organisms only if they affect at least one ecophysiological rate, altering the distribution of elements within cells. This highlights the central role of metabolism and ecosystem processes in connecting the biotic and abiotic cycles of elements. Ecophysiological rates and evolutionary forces are the only processes that enable the highest levels to reach the lowest levels (genomic and cellular). They are also the processes that allow organisms to adapt to changes in stoichiometry at larger scales. Biotic adaptation can then lead to further larger-scale changes through recycling processes, thus closing the feedback loop of stoichiometry from molecules to the biosphere.

BIOLOGICAL LEVELS

Stoichiometric studies can be found for all levels, some of them having acquired a foundational character in ecology, often solidifying into paradigms (e.g., Liebig and Playfair, 1840; Redfield, 1958; Tilman, 1982). The emergence of the biological stoichiometry approach made use of these paradigms, sometimes unaltered, sometimes developing them further, and sometimes challenging them (e.g., for the Redfield ratio paradigm, Cleveland and Liptzin, 2007; Sterner et al., 2008; Flynn, 2010; Loladze and Elser, 2011; Sardans et al., 2012). New paradigms have also been developed for various biological levels, and integrations with other ecological theories attempted. In this section, we briefly discuss the contribution of stoichiometric approaches to our understanding of the elemental distribution within each biological level, as well as the areas still in need of improvement, starting from genomes, the lowest level, to the biosphere.

Genome

Nucleic acids are known to be N-rich and, singularly, P-rich molecules (Sterner and Elser, 2002). But only recently has the new field of stoichiogenomics started working on a comparison between the elemental compositions of the genomes of various organisms (Elser et al., 2011). Intriguing differences in N content between wild plants and domesticated crops were revealed (Acquisti et al., 2009). As one Guanine-Cytosine (GC) pair contains 8 N atoms, one more than an Adenine-Thymine (AT) pair, correlations between the GC and N contents of genomes has been postulated (McEwan et al., 1998) but hotly

TABLE 1 | Brief summary of the major advances at each biological level of our framework for a molecule-to biosphere stoichiometry theory and of the elements of understanding identified as in need of improvement.

	Major advances	In need of development	Needed for completeness	Theories for integration
BIOLOGICAL LEVELS				
Genome	Genome elemental content reflects selective pressures	Stoichiometric differences within and between genomes	Elements more loosely associated to genomes such as transition metals	
Cellular	Association between given pools of elements, biomolecules and organelles (e.g., N and chloroplasts)	Contribution of storage organelles and molecules	Cellular components besides chloroplasts and ribosomes (mitochondria, intracellular membranes...)	Ionomics
Organisms	C:N:P composition of major taxa such as unicellular algae, vascular plants and crustacean zooplankton	Neglected taxonomic groups	Other elements besides C, N, and P	
Populations	Intraspecific variability mainly determined by environmental conditions, growth and body size in autotrophs	Mechanistic model for degree of homeostasis in heterotrophs	Major determinants of intraspecific variability in heterotrophs	Physiological homeostasis Developmental biology
Communities	Species in local competitive communities should converge toward similar stoichiometries Functional traits can explain some variations within communities Species diversity and body size are factors affecting community stoichiometries	Scale resolution from biomes to local communities More data on stoichiometric imbalances in realized interactions and across trophic levels	Patterns in stoichiometry-related traits (threshold elemental ratios, growth efficiencies...)	Community ecology theories (coexistence theory, neutral theory, etc.)
Ecosystems	Ecosystems should be close to co-limitation by multiple elements	Extensive monitoring of local elemental limitations for classically studied elements C, N, P and other elements such as K, Mo and Fe	Role of catchment properties and of the regional context	
Landscapes	Increasing role of watershed as water bodies decrease in size Important role for depth along the water column and for the biota along soil profile	Interaction between mixing and stoichiometry along the water column	Stoichiometry patterns along connected terrestrial landscapes	Hydrodynamics
Biosphere	Local heterogeneity in in elemental distribution on emerged lands and to a lesser extent in oceans	Global P distribution in the biosphere	Global distribution of other elements such as K, Fe and Mo	

The elements singled out here have been divided into those in need of development (if they have been already tackled in the literature, but insufficiently) and those needed for completeness (if they have not been tackled in the literature, or only rudimentarily). The integration column lists the relevant theories and fields whose integration would greatly promote understanding of stoichiometry at the corresponding level.

debated (Bragg and Hyder, 2004). Correlation between genome size and P content is also controversial (Hessen et al., 2010b; Vieira-Silva et al., 2010). There is another more subtle way for stoichiometric constraints to imprint on the genome of organisms. The N contents of codons and the amino acids they encode are positively correlated, resulting in a strong relationship between the nitrogen:carbon (N:C) ratio of genomes and proteomes across prokaryotic species (Bragg and Hyder, 2004). Hence, organisms under selection for lower N in their proteins should also see their genome N content decrease, although many other genomic processes may obscure the picture (Gunther et al., 2013). Regarding P, it is the causal association between rDNA structure, rRNA expression, cellular P content and growth rate that is likely to affect the structure of genomes (Weider et al., 2005). Despite these notable breakthroughs, the field of stoichiogenomics is at its early stages. Still lacking is information on potential differences in the stoichiometry of various types of sequences (eu- and heterochomatin, introns and exons, transposons, etc.), of distinct physical structures of genomes (chromosomes, telomeres, centromeres, etc.), as well as differences between the genomes of different organelles (e.g., chloroplasts and mitochondria), cell types (e.g., immune cells, or syncitia) and species (**Table 1**). Other elements beside C, N, and P are worth assessing too. For example, a rather old study by Kearns and Sigee (1979) suggests that some transition metals in cells, like nickel and copper, are mainly associated with chromosomes in dinoflagellates. Thus, differences in genome size among dinoflagellates may result in differences in transition metal contents.

Cellular

Genomes represent only a small fraction of a cell's biomass. Major pools of elements in cells are proteins and nucleotides for N, RNA and phospholipids for P, and carbohydrates and lipids for

TABLE 2 | Brief summary of the major advances at each biological level of our framework for a molecule-to biosphere stoichiometry theory and of the elements of understanding identified as in need of improvement for each process of our framework for a molecule-to biosphere stoichiometry theory.

	Major advances	In need of development	Needed for completeness	Theories for integration
PROCESSES				
Genetic expression	Fundamental links between rRNA production, rDNA gene structure, total P, and growth rate Relation between elemental content and level of expression of genes activated by a given elemental limitation	Organisms beyond classical models such as *E. coli*, *A. thaliana* and *Daphnia*	Interactive effects of multiple elements	Systems biology; Nutrigenomics
Ecophysiological rates	Feedback through increased investment in consumption of scarce resources, leading to co-limitation	Explore alternative resource acquisition strategies	Chemical side effects of elemental resources (e.g., O and anoxia; cations and salinity). Effects of the chemical environment.	Nutritional biology; Environmental chemistry
Physiological rates	Growth penalties for strictly homeostatic organisms under both limitation and excess of given elements The Growth rate Hypothesis and light:nutrient Hypothesis	Molecular and cellular mechanisms at the basis of empirical growth-stoichiometry patterns	A theory of homeostasis. Diseases linked to stoichiometric imbalances	Systems biology; Nutrigenomics
Demographic processes	Somatic growth and reproduction are affected by elemental limitation	Effects of stoichiometry on mortality and dispersal	Density-dependence and demographic stochasticity relationships with stoichiometry. Study of taxa beyond crustaceans	Population biology
Evolutionary forces	N limitation shape N content and gene expression of plant genomes	Measurement of natural selection mechanisms on stoichiometric properties	Other types of evolution (sexual selection, genetic drift, gene flow...)	Evolutionary biology
Ecological interactions	Limitation of herbivores and detritivores by mineral content of their resources Host-pathogen interactions are affected by the stoichiometry of the host	Growth limitation in trophic levels above primary consumers Other dimensions of food quality besides stoichiometry	Inclusion of non-trophic interactions	Multi-level framework
Recycling rates	Consumer-Driven Nutrient Recycling Hypothesis	Chemical and spatio-temporal variability in recycling. Effects of species diversity. Feedback loops between the chemical environment and recycling.	Effects of organisms on the stoichiometry of outputs and inputs in ecosystems	Biodiversity-Ecosystem Functioning theory
Transfer rates	Importance of dispersing organisms and of boundaries between ecosystems	Data reporting transfer rates of multiple elements simultaneously	Conceptual and predictive frameworks for coupled elemental transfer rates	Meta-ecosystems Nutrient spiraling Lagrangian models
General circulation	Dominant effects of plants and humans on global circulation of elements	Study of abiotic and biotic processes together	Study of multiple elements simultaneously	General circulation models

The elements singled out here have been divided into those in need of development (if they have been already tackled in the literature, but insufficiently) and those needed for completeness (if they have not been tackled in the literature, or only rudimentarily). The integration column lists the relevant theories and disciplines whose integration would greatly promote understanding of the stoichiometric role of the corresponding processes.

C (Sterner and Elser, 2002). Hence, the stoichiometry of cells is to all intents and purposes the result of the relative investment of the cell in those few dominant macromolecules (Vrede et al., 2004). This fundamental understanding allows us to predict the stoichiometry of cells in many cases. For example, actively photosynthesizing leaves should show high N:C ratios, because of their heavy investment in RubisCo and other photosynthesis proteins (Field and Mooney, 1986; Evans, 1989). The Growth Rate Hypothesis (Elser et al., 1996) states that fast growing cells should have high contents of ribosomal RNA, and thus also of P, such as in rapidly dividing cancerous cells (Elser et al., 2007b). There can also be a special association between a macromolecule type and a specific cellular structure, e.g., between chlorophyll and chloroplasts, lipids and membranes, or rRNA and ribosomes, resulting in an association between specific metabolic processes and specific elements (Sterner and Elser, 2002; Allen and Gillooly, 2009). Relatively neglected in stoichiometry are storage organelles and associated storage molecules (**Table 1**). In many organisms—like in most autotrophs and prokaryotes (Lee, 1996; Raven, 1997), storage is a major process that may disrupt the

functional link described above between cell stoichiometry and other organelles (Lukas et al., 2011), as well as play many other unforeseen roles (e.g., the many roles of polyphosphates, in Bjorkman, 2014). The role of other cellular components, such as mitochondria and intracellular membranes has been given very little consideration too. Elements beside C, N and P have seldom been analyzed at the cellular level in mainstream biological stoichiometry, outside the realm of unicellulars (Ho et al., 2003). They are however the major concern of the whole field of ionomics and both approaches certainly benefit from each other (Salt et al., 2008), revealing intriguing and potentially meaningful correlations among various elements (Loladze, 2014; Jeyasingh et al., 2017).

Organism

Knowledge of the C:N:P composition of species has greatly expanded since the first systematic measurements done on a couple of zooplankton taxa (Andersen and Hessen, 1991). The current datasets allow generalities to be drawn, as well as comparisons among various taxa, life forms, trophic levels and habitats to be performed (Sardans et al., 2012). However, besides the C, N, and P triptych, knowledge of other elements is still limited even though it is potentially of great ecological significance. For example, the oxygen content (O) of organisms may be a signature of their physiological conditions and of the type of molecules they store (Fagerbakke et al., 1996; **Table 1**; see also Han et al., 2011). Biases in the groups of organisms represented in datasets also exist, with unicellular algae, terrestrial vascular plants, crustacean zooplankton—mostly herbivorous—and insects overrepresented (but see Amatangelo and Vitousek, 2008; Martinson et al., 2008; Xia et al., 2014; Danger et al., 2016 for examples of recent attempts at evening the balance).

Population

Intra-specific variability in elemental stoichiometry is less well documented than inter-specific variability. There are different ways to measure variability within a population: according to ontogenetic stages (Main et al., 1997; Meunier et al., 2016), driven by a master trait such as body size or reproductive status (Mendez and Karlsson, 2005), controlled by genotypes, environmentally driven (DeMott et al., 2004), or as stochastic inter-individual variability. Teasing apart all these sources of variation is anything but straightforward and only a few attempts have been made so far, revealing a surprisingly large effect of abiotic conditions on supposedly homeostatic organisms (e.g., El-Sabaawi et al., 2012). Intraspecific variability in autotrophs is notoriously large and determined to a great extent by environmental conditions, with little difference between genotypes (Agren, 2008; Agren and Weih, 2012). Interestingly, ontogenetic stage and body size also strongly affect autotroph stoichiometry, reflecting the fundamental links between growth rate, biomolecules and elements as described in the Growth rate hypothesis (Agren, 2008; Elser et al., 2010). Heterotrophs are classically viewed as maintaining a strict stoichiometric homeostasis, but the paradigm is slowly shifting toward the view that the degree of homeostasis differs from strict to loose in heterotrophic species (Persson et al., 2010; Meunier et al., 2014). It is still unclear what determines the degree of homeostasis (**Table 1**). Potential candidates are ecological factors such as mortality rates (Wang et al., 2012) and the degree of osmotrophy (bacteria, fungi, and flagellates are often highly non-homeostatic, see Godwin and Cotner, 2015; Golz et al., 2015; Danger et al., 2016). Under natural conditions, non-homeostatic species, or conformers—to borrow the concept from physiological studies of homeostasis, are likely to show more intraspecific variability than homeostatic species, or regulators (Meunier et al., 2014). But the latter may still see substantial stoichiometric variability between different life stages or genotypes. It will be important to study the extent of these two sources of intraspecific variability and their effects on populations and nutrient fluxes in ecosystems, likely by integrating elements from the field of developmental biology.

Community

There are numerous studies that examine how elemental composition varies within local communities (Sterner and George, 2000; He et al., 2006; Hattenschwiler et al., 2008). Technical developments in electron microscopy (Gundersen et al., 2002) and microspectroscopy (Hall et al., 2011) allows for such studies in unicellular organisms too. Despite increasing data availability, comparatively few generalities on stoichiometric variability have been drawn (but see Sardans et al., 2012). One reason might be that such studies have not separated scales clearly, mixing data taken from one locality with other data from other localities (**Table 1**). For example, McGroddy et al. (2004) show that variability in N:P ratios is lower within than between biomes. Phylogeny may contribute to this pattern as was found in plants (Broadley et al., 2004) and insects (Woods et al., 2004). The resource-ratio theory may provide some theoretical underpinning as to why local communities feeding on shared resources might converge toward the same stoichiometry, the one ensuring co-limitation by the multiple shared resources (Cherif and Loreau, 2007; Danger et al., 2008). Functional traits may also explain some of the local variation in ratios. For example, N:P ratios are higher in graminoids and stress-tolerant plants than in forbs and ruderals (Gusewell, 2004). Body size is a recurring trait affecting stoichiometry distribution within local communities (Vanni et al., 2002). Other potential factors affecting inter-specific differences in stoichiometry within and between communities are reviewed in Carnicer et al. (2015). Alternatively, neutral processes might control stoichiometric patterns in communities as they may do for abundance distributions (Chave, 2004). Combination of multiple community ecology theories would certainly improve our understanding of community stoichiometry, since competition theory alone does not explain the high biodiversity found in, for example, phytoplankton communities (Passarge et al., 2006).

Species diversity is a factor that has received special interest recently, both as a factor affecting (Striebel et al., 2009; Abbas et al., 2013) and being affected by stoichiometry (Evans-White et al., 2009; Lewandowska et al., 2016). The study by Guiz et al. (2016) is unique in its attempts at causally explaining both the mean and variation in stoichiometry within local communities and suggests an intriguing convergence in the mean and variance

of C:N ratios between plant communities established on different soil fertilities.

Vertical diversity, the number of trophic levels, is another dimension of biodiversity that has garnered some attention from stoichiometry. Causal interactions between food chain length and variation in stoichiometry across trophic levels have been touched upon lately, but without any general conclusions drawn yet (Doi, 2012; Peace, 2015). Few generalities have been found regarding systematic stoichiometric differences across trophic levels, beyond the known fact that herbivores are generally poorer in C than their food, but show similar N:P ratios among habitats (Elser et al., 2000a; **Table 1**). For secondary consumers and higher trophic levels, studies have found increases, decreases or no change in N and P when climbing the food chain (Lemoine et al., 2014). Missing are more studies measuring the stoichiometric imbalances between predators and their prey in realized interactions (Malzahn et al., 2010; Lemoine et al., 2014), as well as measuring efficiencies and other important metabolic parameters if one wants to infer consequences on nutrient cycling (Doi et al., 2010).

Ecosystem

Generic knowledge of the balance of N and P in major ecosystems has been available for a long time. In terrestrial systems, chronosequence analyses suggest that terrestrial vegetation shifts from N limitation to P limitation as soils age, N_2 fixation increases N stocks, and erosion depletes soil P (Vitousek and Farrington, 1997). Hence, it is assumed that P is limiting in the tropics and on old bedrocks, while N is limiting in soils recently sedimented or newly exposed by deglaciation. In oceans, the classical work by Redfield has ingrained the view that total C:N:P ratio in pelagic waters is constant around a mean value of 105:15:1 (Redfield, 1958), with competition between N-fixer and non-fixer phytoplankton as the main regulating process (Lenton and Klausmeier, 2007). In freshwater systems, lakes are assumed to be P limited in temperate areas, because C and N can be incorporated into lakes from their vast atmospheric reservoirs (Schindler, 1977), while N limitation was noted for some large lakes in the tropics (Hecky and Kilham, 1988) and some humic lakes in Scandinavia (Jansson et al., 2001). But with biological stoichiometry, there was a renewed interest in measuring the balance of elements in ecosystems, and the classical paradigms depicted above has seen many challenges (Sterner, 2008). The current image emerging is that of ecosystems that are co-limited, or close to co-limitation, unless high imbalances or perturbations in the relative inputs of elements in the ecosystem occur (Elser et al., 2007a). Recent advances even advocate for light limitation in boreal lakes which are rich in colored dissolved organic matter (Karlsson et al., 2009). Anthropogenic alterations of the biogeochemical cycles may also push ecosystems toward limitation by new elements such as K or Mo (van Groenigen et al., 2006). Hence, the general rules about the balance of elements in ecosystems require constant revision as new studies at the local scale emerge (**Table 1**). Important lessons from the analysis of an extensive regional lake database (Hessen et al., 2009) are the significance of catchment properties (type and density of terrestrial vegetation), and of the regional context (N or S deposition intensity) in determining lake stoichiometry. The importance of the landscape context should prompt further distantiation from the "lake as a microcosm" paradigm (Jenkins, 2014).

Landscape

The stoichiometric focus in lake studies greatly highlighted the role of the watershed in determining the nutrient status of freshwater bodies (Frost et al., 2009; Hessen et al., 2009). But it also describes a decreasing influence of landscape as the sizes of water bodies increase, with an increasing convergence toward Redfield ratios from lakes to coastal areas to oceanic water bodies (Sterner et al., 2008). Depth is an equally important spatial dimension in aquatic ecosystems. Vertical profiles of nutrients along the water column, and the role of salinity, temperature and turbulence in their generation are classical material of hydrodynamics and aquatic ecology textbooks (Barnes et al.,, 1991). Additions taken from stoichiometry to these classical approaches look very promising already, indicating important interactions between water mixing and stoichiometric properties of organisms (Diehl et al., 2005; Frassl et al., 2014; **Table 1**).

Terrestrial ecology lags behind in terms of characterizing stoichiometry at the landscape level. Studies searching for differences in elemental availabilities between forests and grasslands for example yielded unresolved contrasting results (Cleveland and Liptzin, 2007; Bond, 2010). There are obvious factors that may affect soil nutrient availabilities between adjacent areas, such as age, vegetation type, or local climate. But there is a lack of knowledge about stoichiometric variation along connected terrestrial ecosystems, for example along a shared hill slope (Porder et al., 2005; **Table 1**). Stoichiometric approaches proved more beneficial when it comes to vertical profiles in soils, demonstrating a great effect of the biota on the relative distribution of elements along soil depth (Jobbagy and Jackson, 2001). Despite recent efforts, understanding landscape variation in ecosystem stoichiometry remains a challenge (Chadwick and Asner, 2016) and considering supplies from the biosphere is often necessary to balance large-scale elemental budgets (Chadwick et al., 1999).

Biosphere

Recent intensive sampling campaigns, global survey tools and data sharing allow for a better mapping of stoichiometry over continents and oceans (Cleveland and Liptzin, 2007; Elser et al., 2010; Weber and Deutsch, 2010), although P may require more data (Wang et al., 2010). Even less information is available on other elements (**Table 1**). The emerging picture so far is that local heterogeneity marks elemental distributions on emerged lands, and to a lesser extent in oceans (Key et al., 2004). Atmospheric circulation mixes gases very efficiently in the atmosphere despite persistent spatial and temporal variations (Wunch et al., 2011). But for other elements, like P and sulfur, homogenization is not fast enough compared to their turnover in the atmosphere, and so, deposition is localized around their sources of supply (Garland, 1978; Mahowald et al., 2008). Their radius of impact can still be surprisingly large as, for example, P from Saharan dust is known to fertilize American ecosystems (Okin et al., 2004).

PROCESSES

Processes are generally harder to measure than standing stocks of elements. They often require specific methods and equipment, as well as manipulative experiments, which may be different among elements that must be measured simultaneously in stoichiometric studies. Despite these complications, there are a large number of studies reporting on the various processes affecting the stoichiometry of all biological levels. The picture emerging from these studies, its beacons and shadows, are briefly discussed in this section.

Genetic Expression

Various genetic, biochemical and physiological methods have already been used in order to characterize genetic responses to stoichiometric imbalances (reviewed in Wagner et al., 2013). Studies of the genetic regulation of nutrient limitation has a strong tradition outside of the biological stoichiometry umbrella, mostly from the study of classical model organisms such as the bacterium *Escherichia coli*, the plant *Arabidopsis thaliana* and the yeast *Saccharomyces cerevisiae* (see, e.g., KEGG PATHWAY Database[1]; Broadley and White, 2009; Broach, 2012). The field seems now ready to move to the next step, from a reductionist to systemic approach, based on approaches such as systems biology and nutrigenomics (Muller and Kersten, 2003; Ruffel et al., 2010; **Table 2**). In parallel, the stoichiometric approach has provided its own novel perspective, by demonstrating, e.g., a fundamental causal link between the number of rDNA copies in a genome, the length and composition of the associated regulatory IGS regions, the cell ribosomal content, its total P content and somatic growth (Elser et al., 2000c; Weider et al., 2005). Stoichiometry sheds a unique light on the link between the economy of elements in organisms and genome structure by showing that the bulk elemental composition of the genetic material itself matters. Genes answering to a given elemental limitation tend to contain less of the limiting element and to code for proteins that do similarly (Gilbert et al., 2013). The two approaches of nutritional genetics and stoichiometry increasingly exchange methodologies (Wagner et al., 2013), but results and conclusions rarely flow in between. For example, a highly cited, comprehensive investigation of the interactive effects of C and N availability on gene expression regulation (Gutierrez et al., 2007) has gone un-cited in the stoichiometry literature so far.

Ecophysiological Rates

Rates of resource acquisition by organisms as modulated by elemental availability in their environment is an old topic in biology at least since the 1940s (Monod, 1947). The general rule is that of a negative feedback resulting from the availability of a given resource: the lower the availability, the higher the investment of the consumer to acquire it. Stoichiometry has made a substantial contribution to the subject by adding concerns about the potential role of other essential elements. On optimisation grounds, it is expected that organisms should use the excess availability of non-limiting elements to increase their acquisition of the limiting elements until co-limitation by all essential elements is reached (Darchambeau et al., 2003; Klausmeier et al., 2004a; Cherif and Loreau, 2007). However, a number of mechanisms may prevent co-limitation establishing in practice, like limits to physiological adaptation (Klausmeier et al., 2004b). Organism needs are complex, and resource acquisition strategies may have ultimate goals beyond just insuring immediate maximum growth for an organism (Flynn, 2009; **Table 2**). Besides, variations in the availabilities of various resources tend to be coupled under natural circumstances (Lemoine et al., 2014), sometimes in conjunction with under-appreciated limiting factors, such as water availability (Sardans et al., 2008). Hence multifactorial analyses beyond the usual N and P (sometimes also C) limitation experiments should be extended to include simultaneous variations in combined resources. Besides, the molecules that contain the essential resources may have peripheral chemical properties that otherwise affect growth, e.g., they may be toxic or change the pH of cells (McGrath and Quinn, 2000). They may also play other roles in the metabolism, for example as ions maintaining constant osmolality (e.g., Na, K, and Ca) or as electron donors/acceptors (e.g., O, N, and S). Such side effects have been considered in classical nutritional biology, but mostly ignored in stoichiometry. It is only recently that consideration of factors such as salinity and anoxia in stoichiometry emerged (Marino et al., 2006; Helton et al., 2015; Tadonleke et al., 2016). Even fewer studies have attempted to integrate the role of elements as biomass components with their other chemical functions (but see Payn et al., 2014). Environmental chemistry should thus be better integrated into the stoichiometric analyses of ecophysiological rates (**Table 2**).

Physiological Rates

Organisms have to match elemental demands set by genetic expression with the ecophysiological rates of resource uptake. They do this through various physiological processes that redistribute elements within the organism or between the organism and its environment, as well as through feedbacks that regulate the genetic expression. Elemental metabolism in primary producers and in some model microorganisms like *E. coli* and *S. cerevisiae* is an established part of research. So-called genome-scale metabolic models now offer the possibility to quantitatively predict the effects of changes in resource availabilities or of mutations in genes (e.g., Liu et al., 2010). On the other hand, stoichiometric studies generally concentrate on designing simple models describing the link between elements and growth in autotrophs and osmotrophs (Cherif, 2016). These models are mostly empirically-based and to a great extent divorced from the potential molecular and cellular underpinning mechanisms, which is both a strength and weakness for such models (Flynn, 2008). When it comes to consumers, classical stoichiometric theories assume strict homeostasis of elemental composition, resulting in strong constraints on the physiological fluxes of elements, particularly excretion (Sterner, 1990). This vision of homeostasis in heterotrophs is increasingly challenged (Persson et al., 2010). The quest is now for a mechanistic understanding

[1]KEGG PATHWAY Database. Available online at: http://www.genome.jp/kegg/pathway.html (Online).

of the determinants of the degree of homeostasis in organisms (Meunier et al., 2014; **Table 2**). The main physiological consequence of strict homeostasis is a decrease in growth rate because one element becomes limiting (Sterner and Hessen, 1994). Another consequence is an increase in the costs related to the regulation of the elements in excess, which may also lead to a decrease in growth (Boersma and Elser, 2006). Tolerating changes in stoichiometry by lowering the content of the limiting element, or storing the elements in excess, are strategies that dampen the sensitivity of a species to elemental deficiencies (Mulder and Bowden, 2007; Seidendorf et al., 2010) and may even prove competitively advantageous under both constant and variable environments (Grover, 1991; Grover and Chrzanowski, 2006). The stoichiometry theory has specifically emphasized two ecologically relevant stoichiometric rearrangements: the decrease in P content under P limitation, through a decrease in ribosome numbers (the "Growth rate hypothesis," see Elser et al., 1996) and the storage of excess C by plants when light and CO_2 are non-limiting (the "Light:nutrient hypothesis," see Sterner et al., 1997). Maintaining health and immunity in the face of a varying environment is a fundamental homeostatic feature, a fact acknowledged by nutrigenomics (Afman and Muller, 2006) but overlooked by stoichiometry (**Table 2**). If stoichiometric imbalances trigger diseases, then consequences for the organisms may go beyond a decrease in growth rate or reproductive output. More generally, stoichiometric adjustments that affect the life history traits of organisms, should display a variety of demographic and ecological consequences.

Demographic Processes

Classical population biology teaches us that life-history traits determine the demographic processes that set the structure and dynamics of populations (Tuljapurkar and Caswell, 1997). However, classical stoichiometric theories mainly considered the effects of elemental limitation on somatic growth, and more rarely its effects on life history traits (reviewed in Moe et al., 2005). Fecundity was generally found to be affected by poor diet quality (Sterner, 1998; Urabe and Sterner, 2001; Zandona et al., 2011). Few studies focussed on stoichiometry-related mortality (Sterner et al., 1993; Nelson et al., 2001; Declerck et al., 2015; **Table 2**). Only a couple of studies look into links between stoichiometry and dispersal or migration (Sterner and Schwalbach, 2001; Huberty and Denno, 2006). Empirically, much of this work concentrated on *Daphnia* and copepods at the expense of other taxa (Sterner and Schulz, 1998; Urabe and Sterner, 2001; Jeyasingh and Weider, 2005; but see Bertram et al., 2006). Hence, the effects of nutrient limitation, more specifically P limitation, on life-history traits in those organisms are reasonably well understood. But we know only of one study that put this information to use in a P-limited population model by Nakazawa (2011). One intriguing result from this study, but in line with most recent population ecology theory (Roos and Persson, 2013), is that, assuming that P limitation affects mainly somatic growth, more P-limited populations should be dominated by P-rich juveniles, and hence be more P-rich (Nakazawa, 2011). More models releasing simplifying assumptions are needed, as well as experimental data outside of the crustacean realm. Other fundamental concepts in population biology, such as demographic stochasticity and density dependence (feedbacks from the population to the organism and cellular levels), are virtually untouched by the stoichiometry theory so far. In short, a stoichiometric theory of demography is sorely lacking (**Table 2**).

Evolutionary Forces

If sustained over a sufficiently long time, changes in a population structure that are underlain by differences among genomes should result in evolution. Many studies have postulated evolution through adaptation to stoichiometric constraints. Evidence for N limitation shaping the DNA composition of plant genomes, and for P limitation decreasing genome size, P content (Hessen et al., 2010a) and P recycling efficiency (Elser et al., 2000b) has been presented. Evolutionary adaptation requires variability in traits, heritability and correlation to fitness. For a given trait, careful experiments and models examining the mechanisms of natural selection are required. Very few stoichiometric studies committed to examine these mechanisms, either through models (Branco et al., 2010; Yamamichi et al., 2015) or experiments (Declerck et al., 2015; Lind and Jeyasingh, 2015). The absence of mechanistic underpinnings to most evolutionary stoichiometric hypotheses exposes them to criticism (Worman and Kimbrell, 2008; Vieira-Silva et al., 2010; Gunther et al., 2013; **Table 2**). Moreover, other mechanisms for evolution besides natural selection, such as genetic drift and gene flow, are not yet addressed by any stoichiometric hypotheses (but see Morehouse et al., 2010 for sexual selection).

Ecological Interactions

Ecological interactions are the cornerstones of ecological stoichiometry. The theory established the idea that herbivores are most often limited in their growth by the mineral content of their resources, because most autotrophs are richer in C than their consumers (Elser et al., 2001). Later, mineral limitation was also demonstrated in detritivores (Frost et al., 2002). Subsequently, both deficits and excesses of mineral elements were found to adversely affect the growth rate of consumers (Boersma and Elser, 2006). Secondary consumers were first deemed to be limited by energy, since their prey have similar elemental contents, but given that excess elements can impact growth negatively and that consumer stoichiometry is more variable than previously thought, the jury is still out on whether limitation by resources other than energy is possible (Boersma et al., 2008; Lemoine et al., 2014; **Table 2**). Besides, imbalance in the stoichiometry of a prey may be associated with other changes in prey quality that may impact predators, an acknowledged but often neglected complication (Mitra and Flynn, 2005). The stoichiometric approach has extended its predictions beyond the immediate effect of stoichiometric imbalances on the growth of the consumer. For example, consumer-driven nutrient recycling predicts that primary consumers should affect the availability of elements at the ecosystem level (Elser and Urabe, 1999) and therefore influence coexistence criteria of primary producers (Grover, 2002); the light:nutrient hypothesis predicts that increased photosynthetic C fixation with

increasing light availability should result in widespread mineral limitation throughout the food web (Sterner et al., 1997); a stoichiometric approach to host-pathogen interactions predict that strength of infections should depend on the stoichiometry of the host resources (Smith et al., 2005; Aalto et al., 2015); and stoichiometrically-explicit food webs models suggest food quality-driven changes in trophic cascade strength (Hall et al., 2007). However, those derived predictions often proved difficult to demonstrate unequivocally in theory or experiments (e.g., Daufresne and Loreau, 2001; Sommer et al., 2004; Faithfull et al., 2011; Pulkkinen et al., 2014) probably because the conjectured mechanisms take place at various biological levels and thus need the type of multi-level framework we endeavor to sketch here. Another explanation is that neglecting non-trophic interactions (Kefi et al., 2012) and feedback loops between ecological interactions and physiological rates may have potent stoichiometric effects on higher-level processes (Leroux and Schmitz, 2015).

Recycling Rates

A clear conceptual advance gained from the stoichiometry theory is the connection between stoichiometric consumer-to-resource imbalances and the ratios of elements recycled by consumers (Sterner, 1990). This approach, however, has abstracted release rates as spatially, temporally and chemically uniform. But recycling by consumers is known to be spatially localized (Augustine, 2003), temporally fluctuating (Blackburn et al., 1998) and chemically variable (Anderson et al., 2005). This variability bears important consequences for the overall effects of recycling (Kato et al., 2007; Ramin et al., 2012; **Table 2**). Recycling rates are predominantly metabolic rates (i.e., excretion and egestion rates). As such, body size and temperature are important rate controllers in concert with stoichiometry (Cross et al., 2015). Recent integration of both stoichiometry and metabolic ecology approaches generally improved rate predictions (Vanni and McIntyre, 2016). The overall chemical conditions, such as anoxia levels, also interact with stoichiometry to determine recycling (Steenbergh et al., 2013). Oxygen minima zones are thus likely to affect and be affected by the recycling rates of essential elements (Stramma et al., 2010; Penn et al., 2016). This example points to the importance of considering the potential feedbacks between the chemical environment and recycling (**Table 2**). Research in biodiversity-ecosystem functioning relationships has otherwise shown that species diversity affects elemental rates within ecosystems (Hooper et al., 2005), as well as biomass production and stoichiometry of primary producers (Striebel et al., 2009). However, interactive effects of stoichiometry and biodiversity on recycling rates are still poorly understood and in need of further development (Hillebrand et al., 2014). In the long term, it has been shown that it is recycling of the forms that are not available to other organisms that determine the impact of a consumer on nutrient availability in its ecosystem (Paterson et al., 2002; Vanni et al., 2013). This impact depends on the quantity and quality of matter that it subtracts from its environment through, e.g., sedimented feces, recalcitrant carcasses or emigration. Potential control of the consumer on the inputs of elements in the ecosystem needs to be considered similarly (e.g., through N fixation, immigration). Hence, the effects of recycling are intimately related to transfer rates among ecosystems, because elements that are removed from an ecosystem are obligatorily added to another ecosystem thus affecting both ecosystems.

Transfer Rates

Translocation of elements among ecosystems has been an early scientific concern, defining the field of biogeochemistry (Gorham, 1991). However, the role of living organisms in the coupling of the fluxes of multiple elements has only been conceptualized and generalized to all ecosystems with the birth of the stoichiometric approach (Sterner and Elser, 2002). On the theoretical level, Schade et al. (2005) attempted to model the interaction of ecosystems through transfers of elements among them with an extension of the classical resource-ratio theory (Tilman, 1982), insisting on the importance of dispersing organisms and of boundaries in altering the stoichiometry of the transferred material and of the recipient ecosystem. Usage of this framework remains limited however, probably because of a relative paucity in data reporting the transfer rates of multiple elements simultaneously (reviewed in Sitters et al., 2015; **Table 2**). Other unifying frameworks, such as meta-ecosystems, are worth exploring as well (Loreau et al., 2003; Manzoni and Porporato, 2011; Marleau et al., 2015).

General Circulation

Shortcuts to localized transfers between adjacent ecosystems take place when elements are redistributed on larger scales by the atmospheric and oceanic circulations. Multiple processes put elements in general circulation: erosion, evapotranspiration, respiration, and most important, human-mediated processes such as fossil fuel burning. Other processes ensure that those elements fall back on local ecosystems. Some are physical (atmospheric deposition, precipitation, upwelling), others are mediated by living organisms (photosynthesis, N-fixation, fertilizer production by humans). Those processes are rarely studied together, or for multiple elements simultaneously (**Table 2**). Because of their sheer biomass and level of activity, plants (Wassen et al., 2013) and humans (Penuelas et al., 2013) are the biotic components that most affect the general circulation of elements. Explicit stoichiometric considerations increased the predictability of general circulation models that include the potential effects of these biotic drivers on the dynamics of the biosphere (Thornton et al., 2009; Penuelas et al., 2013).

CONCLUSION: A ROADMAP FOR A MULTILEVEL, COMPREHENSIVE THEORY OF STOICHIOMETRY

Our framework provides a roadmap of the way a given stoichiometric imbalance at a given biological level, or alteration in a given stoichiometric process can cascade to other biological levels by affecting higher- or lower-level processes. The survey

of the literature structured by our framework, although non-exhaustive, shows that the theory has proved astonishingly vigorous despite its relatively young age, as judged by its degree of completeness and development. But we also identified, for each level and each process of the framework, areas in need of completion, development, or articulation with other relevant non-stoichiometric theories (see **Tables 1, 2** for a summary). Stoichiometric theories for population dynamics and community composition appear to us as most urgently needed. Populations and communities obey constraints that are beyond physiology. These two biological levels may thus result in departures from the physiological rules that are used by stoichiometry theories to predict the flows of multiple elements. Various disciplines have already developed reliable theories, concepts and methodologies related to these areas in need of development, which are highly relevant to the stoichiometric approach. But lack of familiarity with other fields from the stoichiometry side, and lack of a comprehensive view on the stoichiometric approach on the other side, is likely to hinder integration, unless the links between fields are explicitly laid out, like in our framework.

By highlighting many areas in need of improvement, our framework indicates that the stoichiometry approach has not yet delivered a predictive theory of elemental transfers within the biosphere. But, we have every hope that our framework will help the theory reach a level of maturity such that it will be able to consistently relate local, microscopic processes to global, macroscopic processes. Current general circulation models often flagrantly ignore lower-scale biological processes for lack of a proper methodology, potentially restraining our capacity to accurately predict future global change. Our framework is one step in this direction.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study and to the selection, analysis and interpretation of the relevant literature. MC coordinated the writing of the manuscript. CF, CM, JS, WU, and FR revised the manuscript critically. All authors approve the manuscript in its current form and agree to be accountable for all aspects of the work.

ACKNOWLEDGMENTS

MC was supported by the strong research environment "Ecosystem Change" of Umeå University. JS was financially supported by a grant from the Research Foundation Flanders (FWO). CF was financially supported by a postdoctoral grant from Vetenskapsrådet (637-2013-7449). We thank Michel Loreau, Claire de Mazancourt, and Göran Englund for valuable comments on early versions of the manuscript.

REFERENCES

Aalto, S. L., Decaestecker, E., and Pulkkinen, K. (2015). A three-way perspective of stoichiometric changes on host-parasite interactions. *Trends Parasitol.* 31, 333–340. doi: 10.1016/j.pt.2015.04.005

Abbas, M., Ebeling, A., Oelmann, Y., Ptacnik, R., Roscher, C., Weigelt, A., et al. (2013). Biodiversity effects on plant stoichiometry. *PLoS ONE* 8:e58179. doi: 10.1371/journal.pone.0058179

Acquisti, C., Elser, J. J., and Kumar, S. (2009). Ecological nitrogen limitation shapes the DNA composition of plant genomes. *Mol. Biol. Evol.* 26, 953–956. doi: 10.1093/molbev/msp038

Afman, L., and Muller, M. (2006). Nutrigenomics: from molecular nutrition to prevention of disease. *J. Am. Diet. Assoc.* 106, 569–576. doi: 10.1016/j.jada.2006.01.001

Agren, G. I. (2008). Stoichiometry and nutrition of plant growth in natural communities. *Annu. Rev. Ecol. Evol. S.* 39, 153–170. doi: 10.1146/annurev.ecolsys.39.110707.173515

Agren, G. I., and Weih, M. (2012). Plant stoichiometry at different scales: element concentration patterns reflect environment more than genotype. *New Phytol.* 194, 944–952. doi: 10.1111/j.1469-8137.2012.04114.x

Allen, A. P., and Gillooly, J. F. (2009). Towards an integration of ecological stoichiometry and the metabolic theory of ecology to better understand nutrient cycling. *Ecol. Lett.* 12, 369–384. doi: 10.1111/j.1461-0248.2009.01302.x

Allen, T. F. H., and Hoekstra, T. W. (1990). The Confusion between scale-defined levels and conventional levels of organization in ecology. *J. Vegetation Sci.* 1, 5–12. doi: 10.2307/3236048

Allen, T. F. H., and Hoekstra, T. W. (1993). *Toward a Unified Ecology*. New York, NY: Columbia University Press.

Amatangelo, K. L., and Vitousek, P. M. (2008). Stoichiometry of ferns in Hawaii: implications for nutrient cycling. *Oecologia* 157, 619–627. doi: 10.1007/s00442-008-1108-9

Andersen, T., and Hessen, D. O. (1991). Carbon, nitrogen, and phosphorus-content of fresh-water zooplankton. *Limnol. Oceanogr.* 36, 807–814. doi: 10.4319/lo.1991.36.4.0807

Anderson, T. R., Hessen, D. O., Elser, J. J., and Urabe, J. (2005). Metabolic stoichiometry and the fate of excess carbon and nutrients in consumers. *Am. Nat.* 165, 1–15. doi: 10.1086/426598

Augustine, D. J. (2003). Long-term, livestock-mediated redistribution of nitrogen and phosphorus in an East African savanna. *J. Appl. Ecol.* 40, 137–149. doi: 10.1046/j.1365-2664.2003.00778.x

Barnes, R. S. K., Mann, K. H., and Wiley InterScience (Online service) (1991). *Fundamentals of Aquatic Ecology*. Oxford; Boston, MA: Blackwell.

Bertram, S. M., Schade, J. D., and Elser, J. J. (2006). Signalling and phosphorus: correlations between mate signalling effort and body elemental composition in crickets. *Anim. Behav.* 72, 899–907. doi: 10.1016/j.anbehav.2006.02.012

Bjorkman, K. M. (2014). Polyphosphate goes from pedestrian to prominent in the marine P-cycle. *Proc. Natl. Acad. Sci. U.S.A.* 111, 7890–7891. doi: 10.1073/pnas.1407195111

Blackburn, N., Fenchel, T., and Mitchell, J. (1998). Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. *Science* 282, 2254–2256. doi: 10.1126/science.282.5397.2254

Boersma, M., Aberle, N., Hantzsche, F. M., Schoo, K. L., Wiltshire, K. H., and Malzahn, A. M. (2008). Nutritional limitation travels up the food chain. *Int. Rev. Hydrobiol.* 93, 479–488. doi: 10.1002/iroh.200811066

Boersma, M., and Elser, J. J. (2006). Too much of a good thing: on stoichiometrically balanced diets and maximal growth. *Ecology* 87, 1325–1330. doi: 10.1890/0012-9658(2006)87[1325:TMOAGT]2.0.CO;2

Bond, W. J. (2010). Do nutrient-poor soils inhibit development of forests? A nutrient stock analysis. *Plant Soil* 334, 47–60. doi: 10.1007/s11104-010-0440-0

Bragg, J. G., and Hyder, C. L. (2004). Nitrogen versus carbon use in prokaryotic genomes and proteomes. *Proc. R. Soc. B Biol. Sci.* 271, S374–S377. doi: 10.1098/rsbl.2004.0193

Branco, P., Stomp, M., Egas, M., and Huisman, J. (2010). Evolution of nutrient uptake reveals a trade-off in the ecological stoichiometry of plant-herbivore interactions. *Am. Nat.* 176, E162–E176. doi: 10.1086/657036

Broach, J. R. (2012). Nutritional control of growth and development in yeast. *Genetics* 192:73. doi: 10.1534/genetics.111.135731

Broadley, M. R., Bowen, H. C., Cotterill, H. L., Hammond, J. P., Meacham, M. C., Mead, A., et al. (2004). Phylogenetic variation in the shoot mineral concentration of angiosperms. *J. Exp. Bot.* 55, 321–336. doi: 10.1093/jxb/erh002

Broadley, M. R., and White, P. J. (2009). *Plant Nutritional Genomics*. Oxford: Blackwell.

Carnicer, J., Sardans, J., Stefanescu, C., Ubach, A., Bartrons, M., Asensio, D., et al. (2015). Global biodiversity, stoichiometry and ecosystem function responses to human-induced C-N-P imbalances. *J. Plant Physiol.* 172, 82–91. doi: 10.1016/j.jplph.2014.07.022

Chadwick, K. D., and Asner, G. P. (2016). Tropical soil nutrient distributions determined by biotic and hillslope processes. *Biogeochemistry* 127, 273–289. doi: 10.1007/s10533-015-0179-z

Chadwick, O. A., Derry, L. A., Vitousek, P. M., Huebert, B. J., and Hedin, L. O. (1999). Changing sources of nutrients during four million years of ecosystem development. *Nature* 397, 491–497. doi: 10.1038/17276

Chave, J. (2004). Neutral theory and community ecology. *Ecol. Lett.* 7, 241–253. doi: 10.1111/j.1461-0248.2003.00566.x

Cherif, M. (2016). Stoichiometry and population growth in osmotrophs and non-osmotrophs. *eLS* 1–6. doi: 10.1002/9780470015902.a0026353

Cherif, M., and Loreau, M. (2007). Stoichiometric constraints on resource use, competitive interactions, and elemental cycling in microbial decomposers. *Am. Nat.* 169, 709–724. doi: 10.1086/516844

Cleveland, C. C., and Liptzin, D. (2007). C: N: P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? *Biogeochemistry* 85, 235–252. doi: 10.1007/s10533-007-9132-0

Cross, W. F., Hood, J. M., Benstead, J. P., Huryn, A. D., and Nelson, D. (2015). Interactions between temperature and nutrients across levels of ecological organization. *Glob. Chang. Biol.* 21, 1025–1040. doi: 10.1111/gcb.12809

Danger, M., Daufresne, T., Lucas, F., Pissard, S., and Lacroix, G. (2008). Does Liebig's law of the minimum scale up from species to communities? *Oikos* 117, 1741–1751. doi: 10.1111/j.1600-0706.2008.16793.x

Danger, M., Gessner, M. O., and Barlocher, F. (2016). Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. *Fungal Ecol.* 19, 100–111. doi: 10.1016/j.funeco.2015.09.004

Darchambeau, F., Faerovig, P. J., and Hessen, D. O. (2003). How Daphnia copes with excess carbon in its food. *Oecologia* 136, 336–346. doi: 10.1007/s00442-003-1283-7

Daufresne, T., and Loreau, M. (2001). Plant-herbivore interactions and ecological stoichiometry: when do herbivores determine plant nutrient limitation? *Ecol. Lett.* 4, 196–206. doi: 10.1046/j.1461-0248.2001.00210.x

Declerck, S. A. J., Malo, A. R., Diehl, S., Waasdorp, D., Lemmen, K. D., Proios, K., et al. (2015). Rapid adaptation of herbivore consumers to nutrient limitation: eco-evolutionary feedbacks to population demography and resource control. *Ecol. Lett.* 18, 553–562. doi: 10.1111/ele.12436

DeMott, W. R., Pape, B. J., and Tessier, A. J. (2004). Patterns and sources of variation in Daphnia phosphorus content in nature. *Aquatic Ecol.* 38, 433–440. doi: 10.1023/B:AECO.0000035183.53389.66

Diehl, S., Berger, S., and Wohrl, R. (2005). Flexible nutrient stoichiometry mediates environmental influences, on phytoplankton and its resources. *Ecology* 86, 2931–2945. doi: 10.1890/04-1512

Doi, H. (2012). Resource productivity and availability impacts for food-chain length. *Ecol. Res.* 27, 521–527. doi: 10.1007/s11284-012-0941-9

Doi, H., Cherif, M., Iwabuchi, T., Katano, I., Stegen, J. C., and Striebel, M. (2010). Integrating elements and energy through the metabolic dependencies of gross growth efficiency and the threshold elemental ratio. *Oikos* 119, 752–765. doi: 10.1111/j.1600-0706.2009.18540.x

El-Sabaawi, R. W., Kohler, T. J., Zandona, E., Travis, J., Marshall, M. C., Thomas, S. A., et al. (2012). Environmental and organismal predictors of intraspecific variation in the stoichiometry of a neotropical freshwater fish. *PLoS ONE* 7:e32713. doi: 10.1371/journal.pone.0032713

Elser, J. (2006). Biological stoichiometry: a chemical bridge between ecosystem ecology and evolutionary biology. *Am. Nat.* 168, S25–S35. doi: 10.1086/509048

Elser, J. J., Acquisti, C., and Kumar, S. (2011). Stoichiogenomics: the evolutionary ecology of macromolecular elemental composition. *Trends Ecol. Evol.* 26, 38–44. doi: 10.1016/j.tree.2010.10.006

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007a). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Elser, J. J., Dobberfuhl, D. R., MacKay, N. A., and Schampel, J. H. (1996). Organism size, life history, and N:P stoichiometry. *Bioscience* 46, 674–684. doi: 10.2307/1312897

Elser, J. J., Elser, M. M., Mackay, N. A., and Carpenter, S. R. (1988). Zooplankton-mediated transitions between N-limited and P-limited algal growth. *Limnol. Oceanogr.* 33, 1–14. doi: 10.4319/lo.1988.33.1.0001

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000a). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580. doi: 10.1038/35046058

Elser, J. J., Fagan, W. F., Kerkhoff, A. J., Swenson, N. G., and Enquist, B. J. (2010). Biological stoichiometry of plant production: metabolism, scaling and ecological response to global change. *New Phytol.* 186, 593–608. doi: 10.1111/j.1469-8137.2010.03214.x

Elser, J. J., Hayakawa, K., and Urabe, J. (2001). Nutrient limitation reduces food quality for zooplankton: Daphnia response to seston phosphorus enrichment. *Ecology* 82, 898–903. doi: 10.1890/0012-9658(2001)082[0898:NLRFQF]2.0.CO;2

Elser, J. J., Kyle, M. M., Smith, M. S., and Nagy, J. D. (2007b). Biological stoichiometry in human cancer. *PLoS ONE* 2:e1028. doi: 10.1371/journal.pone.0001028

Elser, J. J., Nagy, J. D., and Kuang, Y. (2003). Biological stoichiometry: an ecological perspective on tumor dynamics. *Bioscience* 53, 1112–1120. doi: 10.1641/0006-3568(2003)053[1112:BSAEPO]2.0.CO;2

Elser, J. J., O'Brien, W. J., Dobberfuhl, D. R., and Dowling, T. E. (2000b). The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* 13, 845–853. doi: 10.1046/j.1420-9101.2000.00215.x

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., et al. (2000c). Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* 3, 540–550. doi: 10.1046/j.1461-0248.2000.00185.x

Elser, J. J., and Urabe, J. (1999). The stoichiometry of consumer-driven nutrient recycling: theory, observations, and consequences. *Ecology* 80, 735–751. doi: 10.1890/0012-9658(1999)080[0735:TSOCDN]2.0.CO;2

Elser, J. J., Watts, J., Schampel, J. H., and Farmer, J. (2006). Early Cambrian food webs on a trophic knife-edge? A hypothesis and preliminary data from a modern stromatolite-based ecosystem. *Ecol. Lett.* 9, 292–300. doi: 10.1111/j.1461-0248.2005.00873.x

Evans, J. R. (1989). Photosynthesis and nitrogen relationships in leaves of C-3 plants. *Oecologia* 78, 9–19. doi: 10.1007/BF00377192

Evans-White, M. A., Dodds, W. K., Huggins, D. G., and Baker, D. S. (2009). Thresholds in macroinvertebrate biodiversity and stoichiometry across water-quality gradients in Central Plains (USA) streams. *J. North Am. Benthol. Soc.* 28, 855–868. doi: 10.1899/08-113.1

Fagerbakke, K. M., Heldal, M., and Norland, S. (1996). Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria. *Aquat. Microb. Ecol.* 10, 15–27. doi: 10.3354/ame010015

Faithfull, C. L., Wenzel, A., Vrede, T., and Bergstrom, A. K. (2011). Testing the light: nutrient hypothesis in an oligotrophic boreal lake. *Ecosphere* 2, 1–15. doi: 10.1890/ES11-00223.1

Field, C. B., and Mooney, H. A. (1986). "The photosynthesis-nitrogen relationship in wild plants," in *On the Economy of Plant Form and Function,* ed T. J. Givnish (Cambridge: Cambridge University Press), 25–55.

Flynn, K. J. (2008). The importance of the form of the quota curve and control of non-limiting nutrient transport in phytoplankton models. *J. Plankton Res.* 30, 423–438. doi: 10.1093/plankt/fbn007

Flynn, K. J. (2009). Going for the slow burn: why should possession of a low maximum growth rate be advantageous for microalgae? *Plant Ecol. Divers.* 2, 179–189. doi: 10.1080/17550870903207268

Flynn, K. J. (2010). Ecological modelling in a sea of variable stoichiometry: dysfunctionality and the legacy of Redfield and Monod. *Prog. Oceanogr.* 84, 52–65. doi: 10.1016/j.pocean.2009.09.006

Frassl, M. A., Rothhaupt, K. O., and Rinke, K. (2014). Algal internal nutrient stores feedback on vertical phosphorus distribution in large lakes. *J. Great Lakes Res.* 40, 162–172. doi: 10.1016/j.jglr.2013.11.001

Frost, P. C., Kinsman, L. E., Johnston, C. A., and Larson, J. H. (2009). Watershed discharge modulates relationships between landscape components and nutrient ratios in stream seston. *Ecology* 90, 1631–1640. doi: 10.1890/08-1534.1

Frost, P. C., Stelzer, R. S., Lamberti, G. A., and Elser, J. J. (2002). Ecological stoichiometry of trophic interactions in the benthos: understanding the role of C: N: P ratios in lentic and lotic habitats. *J. North Am. Benthol. Soc.* 21, 515–528. doi: 10.2307/1468427

Garland, J. A. (1978). "Dry and wet removal of sulphur from the atmosphere," in *Sulfur in the Atmosphere* (Pergamon), 349–362.

Gilbert, J. D. J., Acquisti, C., Martinson, H. M., Elser, J. J., Kumar, S., and Fagan, W. F. (2013). GRASP [Genomic Resource Access for Stoichioproteomics]: comparative explorations of the atomic content of 12 Drosophila proteomes. *BMC Genomics* 14:599. doi: 10.1186/1471-2164-14-599

Godwin, C. M., and Cotner, J. B. (2015). Aquatic heterotrophic bacteria have highly flexible phosphorus content and biomass stoichiometry. *Isme J.* 9, 2324–2327. doi: 10.1038/ismej.2015.34

Golz, A. L., Burian, A., and Winder, M. (2015). Stoichiometric regulation in micro- and mesozooplankton. *J. Plankton Res.* 37, 293–305. doi: 10.1093/plankt/fbu109

Gorham, E. (1991). Biogeochemistry—its origins and development. *Biogeochemistry* 13, 199–239. doi: 10.1007/BF00002942

Grover, J. P. (1991). Resource competition in a variable environment—phytoplankton growing according to the variable-internal-stores model. *Am. Nat.* 138, 811–835. doi: 10.1086/285254

Grover, J. P. (2002). Stoichiometry, herbivory and competition for nutrients: simple models based on planktonic ecosystems. *J. Theor. Biol.* 214, 599–618. doi: 10.1006/jtbi.2001.2488

Grover, J. P., and Chrzanowski, T. H. (2006). Stoichiometry and growth kinetics in the "smallest zooplankton"—phagotrophic flagellates. *Arch. Hydrobiol.* 167, 467–487. doi: 10.1127/0003-9136/2006/0167-0467

Guiz, J., Hillebrand, H., Borer, E. T., Abbas, M., Ebeling, A., Weigelt, A., et al. (2016). Long-term effects of plant diversity and composition on plant stoichiometry. *Oikos* 125, 613–621. doi: 10.1111/oik.02504

Gundersen, K., Heldal, M., Norland, S., Purdie, D. A., and Knap, A. H. (2002). Elemental C, N, and P cell content of individual bacteria collected at the Bermuda Atlantic Time-Series Study (BATS) site. *Limnol. Oceanogr.* 47, 1525–1530. doi: 10.4319/lo.2002.47.5.1525

Gunther, T., Lampei, C., and Schmid, K. J. (2013). Mutational bias and gene conversion affect the intraspecific nitrogen stoichiometry of the *Arabidopsis thaliana* transcriptome. *Mol. Biol. Evol.* 30, 561–568. doi: 10.1093/molbev/mss249

Gusewell, S. (2004). N: P ratios in terrestrial plants: variation and functional significance. *New Phytol.* 164, 243–266. doi: 10.1111/j.1469-8137.2004.01192.x

Gutierrez, R. A., Lejay, L. V., Dean, A., Chiaromonte, F., Shasha, D. E., and Coruzzi, G. M. (2007). Qualitative network models and genome-wide expression data define carbon/nitrogen-responsive molecular machines in Arabidopsis. *Genome Biol.* 8:R7. doi: 10.1186/gb-2007-8-1-r7

Hall, E. K., Singer, G. A., Polzl, M., Hammerle, I., Schwarz, C., Daims, H., et al. (2011). Looking inside the box: using Raman microspectroscopy to deconstruct microbial biomass stoichiometry one cell at a time. *Isme J.* 5, 196–208. doi: 10.1038/ismej.2010.115

Hall, S. R. (2009). Stoichiometrically Explicit Food Webs: feedbacks between resource supply, elemental constraints, and species diversity. *Ann. Rev. Ecol. Evol. Syst.* 40, 503–528. doi: 10.1146/annurev.ecolsys.39.110707.173518

Hall, S. R., Shurin, J. B., Diehl, S., and Nisbet, R. M. (2007). Food quality, nutrient limitation of secondary production, and the strength of trophic cascades. *Oikos* 116, 1128–1143. doi: 10.1111/j.0030-1299.2007.15875.x

Han, W. X., Fang, J. Y., Reich, P. B., Woodward, F. I., and Wang, Z. H. (2011). Biogeography and variability of eleven mineral elements in plant leaves across gradients of climate, soil and plant functional type in China. *Ecol. Lett.* 14, 788–796. doi: 10.1111/j.1461-0248.2011.01641.x

Hattenschwiler, S., Aeschlimann, B., Couteaux, M. M., Roy, J., and Bonal, D. (2008). High variation in foliage and leaf litter chemistry among 45 tree species of a neotropical rainforest community. *New Phytol.* 179, 165–175. doi: 10.1111/j.1469-8137.2008.02438.x

He, J. S., Fang, J. Y., Wang, Z. H., Guo, D. L., Flynn, D. F. B., and Geng, Z. (2006). Stoichiometry and large-scale patterns of leaf carbon and nitrogen in the grassland biomes of China. *Oecologia* 149, 115–122. doi: 10.1007/s00442-006-0425-0

Hecky, R. E., and Kilham, P. (1988). Nutrient Limitation of phytoplankton in freshwater and marine environments - a review of recent-evidence on the effects of enrichment. *Limnol. Oceanogr.* 33, 796–822.

Helton, A. M., Ardon, M., and Bernhardt, E. S. (2015). Thermodynamic constraints on the utility of ecological stoichiometry for explaining global biogeochemical patterns. *Ecol. Lett.* 18, 1049–1056. doi: 10.1111/ele.12487

Hessen, D. O., Andersen, T., Larsen, S., Skjelkvale, B. L., and de Wit, H. A. (2009). Nitrogen deposition, catchment productivity, and climate as determinants of lake stoichiometry. *Limnol. Oceanogr.* 54, 2520–2528. doi: 10.4319/lo.2009.54.6_part_2.2520

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Hessen, D. O., Jeyasingh, P. D., Neiman, M., and Weider, L. J. (2010a). Genome streamlining and the elemental costs of growth. *Trends Ecol. Evol.* 25, 75–80. doi: 10.1016/j.tree.2009.08.004

Hessen, D. O., Jeyasingh, P. D., Neiman, M., and Weider, L. J. (2010b). Genome streamlining in prokaryotes versus eukaryotes response. *Trends Ecol. Evol.* 25, 320–321. doi: 10.1016/j.tree.2010.03.003

Hillebrand, H., Cowles, J. M., Lewandowska, A., Van de Waal, D. B., and Plum, C. (2014). Think ratio! A stoichiometric view on biodiversity-ecosystem functioning research. *Basic Appl. Ecol.* 15, 465–474. doi: 10.1016/j.baae.2014.06.003

Ho, T. Y., Quigg, A., Finkel, Z. V., Milligan, A. J., Wyman, K., Falkowski, P. G., et al. (2003). The elemental composition of some marine phytoplankton. *J. Phycol.* 39, 1145–1159. doi: 10.1111/j.0022-3646.2003.03-090.x

Hooper, D. U., Chapin, F. S., Ewel, J. J., Hector, A., Inchausti, P., Lavorel, S., et al. (2005). Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecol. Monogr.* 75, 3–35. doi: 10.1890/04-0922

Huberty, A. F., and Denno, R. F. (2006). Consequences of nitrogen and phosphorus limitation for the performance of two planthoppers with divergent life-history strategies. *Oecologia* 149, 444–455. doi: 10.1007/s00442-006-0462-8

Irschick, D. J., Fox, C., Thompson, K., Knapp, A., Baker, L., and Meyer, J. (2013). Functional ecology: integrative research in the modern age of ecology. *Funct. Ecol.* 27, 1–4. doi: 10.1111/1365-2435.12037

Jansson, M., Bergstrom, A. K., Drakare, S., and Blomqvist, P. (2001). Nutrient limitation of bacterioplankton and phytoplankton in humic lakes in northern Sweden. *Freshw. Biol.* 46, 653–666. doi: 10.1046/j.1365-2427.2001.00703.x

Jenkins, D. G. (2014). Lakes and rivers as microcosms, version 2.0. *J. Limnol.* 73, 20–32. doi: 10.4081/jlimnol.2014.797

Jeyasingh, P. D., Goos, J. M., Thompson, S. K., Godwin, C. M., and Cotner, J. B. (2017). Ecological stoichiometry beyond redfield: an ionomic perspective on elemental homeostasis. *Front. Microbiol.* 8:722. doi: 10.3389/fmicb.2017.00722

Jeyasingh, P. D., and Weider, L. J. (2005). Phosphorus availability mediates plasticity in life-history traits and predator-prey interactions in Daphnia. *Ecol. Lett.* 8, 1021–1028. doi: 10.1111/j.1461-0248.2005.00803.x

Jobbagy, E. G., and Jackson, R. B. (2001). The distribution of soil nutrients with depth: global patterns and the imprint of plants. *Biogeochemistry* 53, 51–77. doi: 10.1023/A:1010760720215

Karlsson, J., Bystrom, P., Ask, J., Ask, P., Persson, L., and Jansson, M. (2009). Light limitation of nutrient-poor lake ecosystems. *Nature* 460, 506–580. doi: 10.1038/nature08179

Kato, S., Urabe, J., and Kawata, M. (2007). Effects of temporal and spatial heterogeneities created by consumer-driven nutrient recycling on algal diversity. *J. Theor. Biol.* 245, 364–377. doi: 10.1016/j.jtbi.2006.10.012

Kearns, L. P., and Sigee, D. C. (1979). High-levels of transition-metals in dinoflagellate chromosomes. *Experientia* 35, 1332–1334. doi: 10.1007/BF01963991

Kefi, S., Berlow, E. L., Wieters, E. A., Navarrete, S. A., Petchey, O. L., Wood, S. A., et al. (2012). More than a meal... integrating non-feeding interactions into food webs. *Ecol. Lett.* 15, 291–300. doi: 10.1111/j.1461-0248.2011.01732.x

Key, R. M., Kozyr, A., Sabine, C. L., Lee, K., Wanninkhof, R., Bullister, J. L., et al. (2004). A global ocean carbon climatology: results from Global

Data Analysis Project (GLODAP). *Global Biogeochem. Cycles* 18, 1–23. doi: 10.1029/2004GB002247

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2004a). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174. doi: 10.1038/nature02454

Klausmeier, C. A., Litchman, E., and Levin, S. A. (2004b). Phytoplankton growth and stoichiometry under multiple nutrient limitation. *Limnol. Oceanogr.* 49, 1463–1470. doi: 10.4319/lo.2004.49.4_part_2.1463

Lee, S. Y. (1996). Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.* 49, 1–14. doi: 10.1002/(SICI)1097-0290(19960105)49:1<1::AID-BIT1>3.0.CO;2-P

Lemoine, N. P., Giery, S. T., and Burkepile, D. E. (2014). Differing nutritional constraints of consumers across ecosystems. *Oecologia* 174, 1367–1376. doi: 10.1007/s00442-013-2860-z

Lenton, T. M., and Klausmeier, C. A. (2007). Biotic stoichiometric controls on the deep ocean N: P ratio. *Biogeosciences* 4, 353–367. doi: 10.5194/bg-4-353-2007

Leroux, S. J., and Schmitz, O. J. (2015). Predator-driven elemental cycling: the impact of predation and risk effects on ecosystem stoichiometry. *Ecol. Evol.* 5, 4976–4988. doi: 10.1002/ece3.1760

Lewandowska, A. M., Biermann, A., Borer, E. T., Cebrian-Piqueras, M. A., Declerck, S. A., De Meester, L., et al. (2016). The influence of balanced and imbalanced resource supply on biodiversity-functioning relationship across ecosystems. *Philos. Trans. R. Soc. Lond,. B Biol. Sci.* 371:20150283. doi: 10.1098/rstb.2015.0283

Liebig, J., and Playfair, L. P. (1840). *Organic Chemistry in its Applications to Agriculture and Physiology*. eds J. Liebig (London: Printed for Taylor and Walton).

Lind, P. R., and Jeyasingh, P. D. (2015). Genotypic differences in phosphorus use physiology in producers (*Chlamydomonas reinhardtii*) and consumers (*Daphnia pulex*) interact to alter primary and secondary production. *Evol. Ecol.* 29, 551–563. doi: 10.1007/s10682-015-9760-0

Liu, L. M., Agren, R., Bordel, S., and Nielsen, J. (2010). Use of genome-scale metabolic models for understanding microbial physiology. *FEBS Lett.* 584, 2556–2564. doi: 10.1016/j.febslet.2010.04.052

Loladze, I. (2014). Hidden shift of the ionome of plants exposed to elevated CO2 depletes minerals at the base of human nutrition. *Elife* 3:e02245. doi: 10.7554/eLife.02245

Loladze, I., and Elser, J. J. (2011). The origins of the redfield nitrogen-to-phosphorus ratio are in a homoeostatic protein-to-rRNA ratio. *Ecol. Lett.* 14, 244–250. doi: 10.1111/j.1461-0248.2010.01577.x

Loreau, M., Mouquet, N., and Holt, R. D. (2003). Meta-ecosystems: a theoretical framework for a spatial ecosystem ecology. *Ecol. Lett.* 6, 673–679. doi: 10.1046/j.1461-0248.2003.00483.x

Lukas, M., Sperfeld, E., and Wacker, A. (2011). Growth rate hypothesis does not apply across colimiting conditions: cholesterol limitation affects phosphorus homoeostasis of an aquatic herbivore. *Funct. Ecol.* 25, 1206–1214. doi: 10.1111/j.1365-2435.2011.01876.x

Mahowald, N., Jickells, T. D., Baker, A. R., Artaxo, P., Benitez-Nelson, C. R., Bergametti, G., et al. (2008). Global distribution of atmospheric phosphorus sources, concentrations and deposition rates, and anthropogenic impacts. *Global Biogeochem. Cycles* 22:GB4026. doi: 10.1029/2008GB003240

Main, T. M., Dobberfuhl, D. R., and Elser, J. J. (1997). N: P stoichiometry and ontogeny of crustacean zooplankton: a test of the growth rate hypothesis. *Limnol. Oceanogr.* 42, 1474–1478. doi: 10.4319/lo.1997.42.6.1474

Malzahn, A. M., Hantzsche, F., Schoo, K. L., Boersma, M., and Aberle, N. (2010). Differential effects of nutrient-limited primary production on primary, secondary or tertiary consumers. *Oecologia* 162, 35–48. doi: 10.1007/s00442-009-1458-y

Manzoni, S., and Porporato, A. (2011). Common hydrologic and biogeochemical controls along the soil-stream continuum. *Hydrol. Process.* 25, 1355–1360. doi: 10.1002/hyp.7938

Marino, R., Chan, F., Howarth, R. W., Pace, M. L., and Likens, G. E. (2006). Ecological constraints on planktonic nitrogen fixation in saline estuaries. I. nutrient and trophic controls. *Mar. Ecol. Prog. Ser.* 309, 25–39. doi: 10.3354/meps309025

Marleau, J. N., Guichard, F., and Loreau, M. (2015). Emergence of nutrient co-limitation through movement in stoichiometric meta-ecosystems. *Ecol. Lett.* 18, 1163–1173. doi: 10.1111/ele.12495

Marquet, P. A., Allen, A. P., Brown, J. H., Dunne, J. A., Enquist, B. J., Gillooly, J. F., et al. (2014). On theory in ecology. *Bioscience* 64, 701–710. doi: 10.1093/biosci/biu098

Martinson, H. M., Schneider, K., Gilbert, J., Hines, J. E., Hamback, P. A., and Fagan, W. F. (2008). Detritivory: stoichiometry of a neglected trophic level. *Ecol. Res.* 23, 487–491. doi: 10.1007/s11284-008-0471-7

McEwan, C. E. A., Gatherer, D., and McEwan, N. R. (1998). Nitrogen-fixing aerobic bacteria have higher genomic GC content than non-fixing species within the same genus. *Hereditas* 128, 173–178. doi: 10.1111/j.1601-5223.1998.00173.x

McGrath, J. W., and Quinn, J. P. (2000). Intracellular accumulation of polyphosphate by the yeast Candida humicola G-1 in response to acid pH. *Appl. Environ. Microbiol.* 66, 4068–4073. doi: 10.1128/AEM.66.9.4068-4073.2000

McGroddy, M. E., Daufresne, T., and Hedin, L. O. (2004). Scaling of C: N: P stoichiometry in forests worldwide: implications of terrestrial redfield-type ratios. *Ecology* 85, 2390–2401. doi: 10.1890/03-0351

Mendez, M., and Karlsson, P. S. (2005). Nutrient stoichiometry in Pinguicula vulgaris: nutrient availability, plant size, and reproductive status. *Ecology* 86, 982–991. doi: 10.1890/04-0354

Meunier, C. L., Boersma, M., Wiltshire, K. H., and Malzahn, A. M. (2016). Zooplankton eat what they need: copepod selective feeding and potential consequences for marine systems. *Oikos* 125, 50–58. doi: 10.1111/oik.02072

Meunier, C. L., Malzahn, A. M., and Boersma, M. (2014). A new approach to homeostatic regulation: towards a unified view of physiological and ecological concepts. *PLoS ONE* 9:e107737. doi: 10.1371/journal.pone.0107737

Mitra, A., and Flynn, K. J. (2005). Predator-prey interactions: is 'ecological stoichiometry' sufficient when good food goes bad? *J. Plankton Res.* 27, 393–399. doi: 10.1093/plankt/fbi022

Moe, S. J., Stelzer, R. S., Forman, M. R., Harpole, W. S., Daufresne, T., and Yoshida, T. (2005). Recent advances in ecological stoichiometry: insights for population and community ecology. *Oikos* 109, 29–39. doi: 10.1111/j.0030-1299.2005.14056.x

Monod, J. (1947). The phenomenon of enzymatic adaptation - and its bearings on problems of genetics and cellular differentiation. *Growth* 11, 223–289.

Morehouse, N. I., Nakazawa, T., Booher, C. M., Jeyasingh, P. D., and Hall, M. D. (2010). Sex in a material world: why the study of sexual reproduction and sex-specific traits should become more nutritionally-explicit. *Oikos* 119, 766–778. doi: 10.1111/j.1600-0706.2009.18569.x

Mulder, K., and Bowden, W. B. (2007). Organismal stoichiometry and the adaptive advantage of variable nutrient use and production efficiency in Daphnia. *Ecol. Modell.* 202, 427–440. doi: 10.1016/j.ecolmodel.2006.11.007

Muller, M., and Kersten, S. (2003). Nutrigenomics: goals and strategies. *Nature Rev. Genet.* 4, 315–322. doi: 10.1038/nrg1047

Nakazawa, T. (2011). The ontogenetic stoichiometric bottleneck stabilizes herbivore-autotroph dynamics. *Ecol. Res.* 26, 209–216. doi: 10.1007/s11284-010-0752-9

Nelson, W. A., McCauley, E., and Wrona, F. J. (2001). Multiple dynamics in a single predator-prey system: experimental effects of food quality. *Proc. R. Soc. B Biol. Sci.* 268, 1223–1230. doi: 10.1098/rspb.2001.1652

Okin, G. S., Mahowald, N., Chadwick, O. A., and Artaxo, P. (2004). Impact of desert dust on the biogeochemistry of phosphorus in terrestrial ecosystems. *Global Biogeochem. Cycles* 18:GB2005. doi: 10.1029/2003GB002145

O'Neill, R. V. (1986). *A Hierarchical Concept of Ecosystems*. Princeton, NJ: Princeton University Press.

Passarge, J., Hol, S., Escher, M., and Huisman, J. (2006). Competition for nutrients and light: stable coexistence, alternative stable states, or competitive exclusion? *Ecol. Monogr.* 76, 57–72. doi: 10.1890/04-1824

Paterson, M. J., Findlay, D. L., Salki, A. G., Hendzel, L. L., and Hesslein, R. H. (2002). The effects of Daphnia on nutrient stoichiometry and filamentous cyanobacteria: a mesocosm experiment in a eutrophic lake. *Freshw. Biol.* 47, 1217–1233. doi: 10.1046/j.1365-2427.2002.00842.x

Payn, R. A., Helton, A. M., Poole, G. C., Izurieta, C., Burgin, A. J., and Bernhardt, E. S. (2014). A generalized optimization model of microbially driven aquatic biogeochemistry based on thermodynamic, kinetic, and stoichiometric ecological theory. *Ecol. Modell.* 294, 1–18. doi: 10.1016/j.ecolmodel.2014.09.003

Peace, A. (2015). Effects of light, nutrients, and food chain length on trophic efficiencies in simple stoichiometric aquatic food chain models. *Ecol. Modell.* 312, 125–135. doi: 10.1016/j.ecolmodel.2015.05.019

Penn, J., Weber, T., and Deutsch, C. (2016). Microbial functional diversity alters the structure and sensitivity of oxygen deficient zones. *Geophys. Res. Lett.* 43, 9773–9780. doi: 10.1002/2016GL070438

Penuelas, J., Poulter, B., Sardans, J., Ciais, P., van der Velde, M., Bopp, L., et al. (2013). Human-induced nitrogen-phosphorus imbalances alter natural and managed ecosystems across the globe. *Nat. Commun.* 4:10. doi: 10.1038/ncomms3934

Persson, J., Fink, P., Goto, A., Hood, J. M., Jonas, J., and Kato, S. (2010). To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos* 119, 741–751. doi: 10.1111/j.1600-0706.2009.18545.x

Pickett, S. T. A., Kolasa, J., and Jones, C. G. (2007a). "2 - Understanding in Ecology," in *Ecological Understanding, 2nd Edn.,* eds S. T. A. Pickett, J. Kolasa and C. G. Jones (San Diego, CA: Academic Press), 33–58.

Pickett, S. T. A., Kolasa, J., and Jones, C. G. (2007b). "3 - The Anatomy of Theory," in *Ecological Understanding, 2nd Edn.,* eds S. T. A. Pickett, J. Kolasa and C. G. Jones (San Diego, CA: Academic Press), 61–96.

Pickett, S. T. A., Kolasa, J., and Jones, C. G. (2007c). "4 - The Ontogeny of Theory," in *Ecological Understanding 2nd Edn,* eds S. T. A. Pickett, J. Kolasa and C. G. Jones (San Diego, CA: Academic Press), 97–115.

Pickett, S. T. A., Kolasa, J., and Jones, C. G. (2007d). "7 - Integration and Synthesis," in *Ecological Understanding 2nd Edn,* eds S. T. A. Pickett, J. Kolasa and C. G. Jones (San Diego, CA: Academic Press), 146–167.

Porder, S., Paytan, A., and Vitousek, P. M. (2005). Erosion and landscape development affect plant nutrient status in the Hawaiian Islands. *Oecologia* 142, 440–449. doi: 10.1007/s00442-004-1743-8

Pulkkinen, K., Wojewodzic, M. W., and Hessen, D. O. (2014). Phosphorus limitation enhances parasite impact: feedback effects at the population level. *BMC Ecol.* 14:29. doi: 10.1186/s12898-014-0029-1

Ramin, M., Perhar, G., Shimoda, Y., and Arhonditsis, G. B. (2012). Examination of the effects of nutrient regeneration mechanisms on plankton dynamics using aquatic biogeochemical modeling. *Ecol. Modell.* 240, 139–155. doi: 10.1016/j.ecolmodel.2012.04.018

Raven, J. A. (1997). "The vacuole: a cost-benefit analysis," in *Advances in Botanical Research Incorporating Advances in Plant Pathology*, *Vol. 25*, *The Plant Vacuole*, eds R. A. Leigh and D. Sanders (San Diego, CA: Elsevier Academic Press Inc), 59–86.

Redfield, A. C. (1934). "On the proportions of organic derivatives in sea water and their relation to the composition of plankton," in *James Johnstone Memorial Volume,* ed R. J. Daniel (Liverpool: University Press of Liverpool).

Redfield, A. C. (1958). The biological control of chemical factors in the environment. *Am. Sci.* 46, 205–211.

Roos, A. M. D. E., and Persson, L. (2013). *Population and Community Ecology of Ontogenetic Development*. Princeton, NJ: Princeton University Press.

Ruffel, S., Krouk, G., and Coruzzi, G. M. (2010). A systems view of responses to nutritional cues in arabidopsis: toward a paradigm shift for predictive network modeling. *Plant Physiol.* 152, 445–452. doi: 10.1104/pp.109.148502

Salt, D. E., Baxter, I., and Lahner, B. (2008). Ionomics and the study of the plant ionome. *Annu. Rev. Plant Biol.* 59, 709–733. doi: 10.1146/annurev.arplant.59.032607.092942

Sardans, J., Penuelas, J., Estiarte, M., and Prieto, P. (2008). Warming and drought alter C and N concentration, allocation and accumulation in a Mediterranean shrubland. *Glob. Chang. Biol.* 14, 2304–2316. doi: 10.1111/j.1365-2486.2008.01656.x

Sardans, J., Rivas-Ubach, A., and Penuelas, J. (2012). The elemental stoichiometry of aquatic and terrestrial ecosystems and its relationships with organismic lifestyle and ecosystem structure and function: a review and perspectives. *Biogeochemistry* 111, 1–39. doi: 10.1007/s10533-011-9640-9

Schade, J. D., Espeleta, J. F., Klausmeier, C. A., McGroddy, M. E., Thomas, S. A., and Zhang, L. X. (2005). A conceptual framework for ecosystem stoichiometry: balancing resource supply and demand. *Oikos* 109, 40–51. doi: 10.1111/j.0030-1299.2005.14050.x

Schindler, D. W. (1977). Evolution of phosphorus limitation in lakes. *Science* 195, 260–262. doi: 10.1126/science.195.4275.260

Seidendorf, B., Meier, N., Petrusek, A., Boersma, M., Streit, B., and Schwenk, K. (2010). Sensitivity of Daphnia species to phosphorus-deficient diets. *Oecologia* 162, 349–357. doi: 10.1007/s00442-009-1452-4

Sitters, J., Atkinson, C. L., Guelzow, N., Kelly, P., and Sullivan, L. L. (2015). Spatial stoichiometry: cross-ecosystem material flows and their impact on recipient ecosystems and organisms. *Oikos* 124, 920–930. doi: 10.1111/oik.02392

Smith, V. H., Jones, T. P., and Smith, M. S. (2005). Host nutrition and infectious disease: an ecological view. *Front. Ecol. Environ.* 3, 268–274. doi: 10.1890/1540-9295(2005)003[0268:HNAIDA]2.0.CO;2

Sommer, U., Sommer, F., Feuchtmayr, H., and Hansen, T. (2004). The influence of mesozooplankton on phytoplankton nutrient limitation: a mesocosm study with northeast Atlantic plankton. *Protist* 155, 295–304. doi: 10.1078/1434461041844268

Steenbergh, A. K., Bodelier, P. L. E., Heldal, M., Slomp, C. P., and Laanbroek, H. J. (2013). Does microbial stoichiometry modulate eutrophication of aquatic ecosystems? *Environ. Microbiol.* 15, 1572–1579. doi: 10.1111/1462-2920.12042

Sterner, R., and Schulz, K. (1998). Zooplankton nutrition: recent progress and a reality check. *Aquat. Ecol.* 32, 261–279. doi: 10.1023/A:1009949400573

Sterner, R. W. (1986). Herbivores direct and indirect effects on algal populations. *Science* 231, 605–607. doi: 10.1126/science.231.4738.605

Sterner, R. W. (1990). The ratio of nitrogen to phosphorus resupplied by herbivores—zooplankton and the algal competitive arena. *Am. Nat.* 136, 209–229. doi: 10.1086/285092

Sterner, R. W. (1998). Demography of a natural population of Daphnia retrocurva in a lake with low food quality. *J. Plankton Res.* 20, 471–489. doi: 10.1093/plankt/20.3.471

Sterner, R. W. (2008). On the phosphorus limitation paradigm for lakes. *Int. Rev. Hydrobiol.* 93, 433–445. doi: 10.1002/iroh.200811068

Sterner, R. W., Andersen, T., Elser, J. J., Hessen, D. O., Hood, J. M., McCauley, E., et al. (2008). Scale-dependent carbon: nitrogen: phosphorus seston stoichiometry in marine and freshwaters. *Limnol. Oceanogr.* 53, 1169–1180. doi: 10.4319/lo.2008.53.3.1169

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Sterner, R. W., Elser, J. J., Fee, E. J., Guildford, S. J., and Chrzanowski, T. H. (1997). The light:nutrient ratio in lakes: the balance of energy and materials affects ecosystem structure and process. *Am. Nat.* 150, 663–684. doi: 10.1086/286088

Sterner, R. W., Elser, J. J., and Hessen, D. O. (1992). Stoichiometric relationships among producers, consumers and nutrient cycling in pelagic ecosystems. *Biogeochemistry* 17, 49–67. doi: 10.1007/BF00002759

Sterner, R. W., and George, N. B. (2000). Carbon, nitrogen, and phosphorus stoichiometry of cyprinid fishes. *Ecology* 81, 127–140. doi: 10.1890/0012-9658(2000)081[0127:CNAPSO]2.0.CO;2

Sterner, R. W., Hagemeier, D. D., and Smith, W. L. (1993). Phytoplankton nutrient limitation and food quality for Daphnia. *Limnol. Oceanogr.* 38, 857–871. doi: 10.4319/lo.1993.38.4.0857

Sterner, R. W., and Hessen, D. O. (1994). Algal nutrient limitation and the nutrition of aquatic herbivores. *Annu. Rev. Ecol. Syst.* 25, 1–29. doi: 10.1146/annurev.es.25.110194.000245

Sterner, R. W., and Schwalbach, M. S. (2001). Diel integration of food quality by Daphnia: luxury consumption by a freshwater planktonic herbivore. *Limnol. Oceanogr.* 46, 410–416. doi: 10.4319/lo.2001.46.2.0410

Stramma, L., Schmidtko, S., Levin, L. A., and Johnson, G. C. (2010). Ocean oxygen minima expansions and their biological impacts. *Deep Sea Res. Oceanog. Res. Pap.* 57, 587–595. doi: 10.1016/j.dsr.2010.01.005

Striebel, M., Behl, S., and Stibor, H. (2009). The coupling of biodiversity and productivity in phytoplankton communities: consequences for biomass stoichiometry. *Ecology* 90, 2025–2031. doi: 10.1890/08-1409.1

Sutherland, W. J., Freckleton, R. P., Godfray, H. C. J., Beissinger, S. R., Benton, T., Cameron, D. D., et al. (2013). Identification of 100 fundamental ecological questions. *J. Ecol.* 101, 58–67. doi: 10.1111/1365-2745.12025

Tadonleke, R. D., Pollet, T., Van Rijswijk, P., Leberre, B., and Middelburg, J. J. (2016). Effects of oxygen loss on carbon processing and heterotrophic prokaryotes from an estuarine ecosystem: results from stable isotope probing and cytometry analyses. *Estuar. Coasts* 39, 992–1005. doi: 10.1007/s12237-015-0053-1

Thornton, P. E., Doney, S. C., Lindsay, K., Moore, J. K., Mahowald, N., Randerson, J. T., et al. (2009). Carbon-nitrogen interactions regulate climate-carbon cycle feedbacks: results from an atmosphere-ocean general circulation model. *Biogeosciences* 6, 2099–2120. doi: 10.5194/bg-6-2099-2009

Tilman, D. (1982). *Resource competition and community structure*. Princeton, NJ: Princeton University Press.

Tuljapurkar, S., and Caswell, H. (1997). *Structured-Population Models in Marine, Terrestrial, and Freshwater Systems*. New York, NY: Springer.

Urabe, J., and Sterner, R. W. (2001). Contrasting effects of different types of resource depletion on life-history traits in Daphnia. *Funct. Ecol.* 15, 165–174. doi: 10.1046/j.1365-2435.2001.00511.x

van Groenigen, K. J., Six, J., Hungate, B. A., de Graaff, M. A., van Breemen, N., and van Kessel, C. (2006). Element interactions limit soil carbon storage. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6571–6574. doi: 10.1073/pnas.0509038103

Vanni, M. J., Boros, G., and McIntyre, P. B. (2013). When are fish sources vs. sinks of nutrients in lake ecosystems? *Ecology* 94, 2195–2206. doi: 10.1890/12-1559.1

Vanni, M. J., Flecker, A. S., Hood, J. M., and Headworth, J. L. (2002). Stoichiometry of nutrient recycling by vertebrates in a tropical stream: linking species identity and ecosystem processes. *Ecol. Lett.* 5, 285–293. doi: 10.1046/j.1461-0248.2002.00314.x

Vanni, M. J., and McIntyre, P. B. (2016). Predicting nutrient excretion of aquatic animals with metabolic ecology and ecological stoichiometry: a global synthesis. *Ecology* 97, 3460–3471. doi: 10.1002/ecy.1582

Vieira-Silva, S., Touchon, M., and Rocha, E. P. C. (2010). No evidence for elemental-based streamlining of prokaryotic genomes. *Trends Ecol. Evol.* 25, 319–320. doi: 10.1016/j.tree.2010.03.001

Vitousek, P. M., and Farrington, H. (1997). Nutrient limitation and soil development: experimental test of a biogeochemical theory. *Biogeochemistry* 37, 63–75. doi: 10.1023/A:1005757218475

Vrede, T., Dobberfuhl, D. R., Kooijman, S., and Elser, J. J. (2004). Fundamental connections among organism C: N: P stoichiometry, macromolecular composition, and growth. *Ecology* 85, 1217–1229. doi: 10.1890/02-0249

Wagner, N. D., Hillebrand, H., Wacker, A., and Frost, P. C. (2013). Nutritional indicators and their uses in ecology. *Ecol. Lett.* 16, 535–544. doi: 10.1111/ele.12067

Wang, H., Sterner, R. W., and Elser, J. J. (2012). On the "strict homeostasis" assumption in ecological stoichiometry. *Ecol. Modell.* 243, 81–88. doi: 10.1016/j.ecolmodel.2012.06.003

Wang, Y. P., Law, R. M., and Pak, B. (2010). A global model of carbon, nitrogen and phosphorus cycles for the terrestrial biosphere. *Biogeosciences* 7, 2261–2282. doi: 10.5194/bg-7-2261-2010

Wassen, M. J., de Boer, H. J., Fleischer, K., Rebel, K. T., and Dekker, S. C. (2013). Vegetation-mediated feedback in water, carbon, nitrogen and phosphorus cycles. *Landsc. Ecol.* 28, 599–614. doi: 10.1007/s10980-012-9843-z

Weber, T. S., and Deutsch, C. (2010). Ocean nutrient ratios governed by plankton biogeography. *Nature* 467, 550–554. doi: 10.1038/nature09403

Weider, L. J., Elser, J. J., Crease, T. J., Mateos, M., Cotner, J. B., and Markow, T. A. (2005). The functional significance of ribosomal (r)DNA variation: impacts on the evolutionary ecology of organisms. *Ann. Rev. Ecol. Evol. Syst.* 36, 219–242. doi: 10.1146/annurev.ecolsys.36.102003.152620

Woods, H. A., Fagan, W. F., Elser, J. J., and Harrison, J. F. (2004). Allometric and phylogenetic variation in insect phosphorus content. *Funct. Ecol.* 18, 103–109. doi: 10.1111/j.1365-2435.2004.00823.x

Worman, C. O., and Kimbrell, T. (2008). Getting to the hart of the matter: did antlers truly cause the extinction of the Irish elk? *Oikos* 117, 1397–1405. doi: 10.1111/j.0030-1299.2008.16608.x

Wunch, D., Toon, G. C., Blavier, J. F. L., Washenfelder, R. A., Notholt, J., Connor, B. J., et al. (2011). The total carbon column observing network. *Philos. Trans. R. Soc. Math. Phys. Eng. Sci.* 369, 2087–2112. doi: 10.1098/rsta.2010.0240

Xia, C. X., Yu, D., Wang, Z., and Xie, D. (2014). Stoichiometry patterns of leaf carbon, nitrogen and phosphorous in aquatic macrophytes in eastern China. *Ecol. Eng.* 70, 406–413. doi: 10.1016/j.ecoleng.2014.06.018

Yamamichi, M., Meunier, C. L., Peace, A., Prater, C., and Rua, M. A. (2015). Rapid evolution of a consumer stoichiometric trait destabilizes consumer-producer dynamics. *Oikos* 124, 960–969. doi: 10.1111/oik.02388

Zandona, E., Auer, S. K., Kilham, S. S., Howard, J. L., Lopez-Sepulcre, A., O'Connor, M. P., et al. (2011). Diet quality and prey selectivity correlate with life histories and predation regime in *Trinidadian guppies*. *Funct. Ecol.* 25, 964–973. doi: 10.1111/j.1365-2435.2011.01865.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Environmental Science

HYPOTHESIS AND THEORY
published: 08 May 2017
doi: 10.3389/fenvs.2017.00018

From Elements to Function: Toward Unifying Ecological Stoichiometry and Trait-Based Ecology

Cédric L. Meunier[1], Maarten Boersma[1,2], Rana El-Sabaawi[3], Halvor M. Halvorson[4], Emily M. Herstoff[5], Dedmer B. Van de Waal[6], Richard J. Vogt[7] and Elena Litchman[8]*

[1] Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung (AWI), Biologische Anstalt Helgoland, Helgoland, Germany, [2] Faculty Biology/Chemistry (FB2), University of Bremen, Bremen, Germany, [3] Department of Biology, University of Victoria, Victoria, BC, Canada, [4] Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS, USA, [5] Department of Ecology and Evolution, Stony Brook University, Stony Brook, NY, USA, [6] Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands, [7] Department of Biological Sciences, Trent University, Peterborough, ON, Canada, [8] W.K. Kellogg Biological Station, Michigan State University, Hickory Corners, MI, USA

The theories developed in ecological stoichiometry (ES) are fundamentally based on traits. Traits directly linked to cell/body stoichiometry, such as nutrient uptake and storage, as well as the associated trade-offs, have the potential to shape ecological interactions such as competition and predation within ecosystems. Further, traits that indirectly influence and are influenced by nutritional requirements, such as cell/body size and growth rate, are tightly linked to organismal stoichiometry. Despite their physiological and ecological relevance, traits are rarely explicitly integrated in the framework of ES and, currently, the major challenge is to more closely inter-connect ES with trait-based ecology (TBE). Here, we highlight four interconnected nutrient trait groups, i.e., acquisition, body stoichiometry, storage, and excretion, which alter interspecific competition in autotrophs and heterotrophs. We also identify key differences between producer-consumer interactions in aquatic and terrestrial ecosystems. For instance, our synthesis shows that, in contrast to aquatic ecosystems, traits directly influencing herbivore stoichiometry in forested ecosystems should play only a minor role in the cycling of nutrients. We furthermore describe how linking ES and TBE can help predict the ecosystem consequences of global change. The concepts we highlight here allow us to predict that increasing N:P ratios in ecosystems should shift trait dominances in communities toward species with higher optimal N:P ratios and higher P uptake affinity, while decreasing N retention and increasing P storage.

Keywords: food web, biological stoichiometry, functional trait, fitness, trade-off, resource

OPEN ACCESS

Edited by:
Michael M. Douglas, University of Western Australia, Australia

Reviewed by:
Wyatt F. Cross, Montana State University, USA
Joshua Hamilton, University of Wisconsin-Madison, USA

****Correspondence:***
Cédric L. Meunier
cedric.meunier@awi.de

Specialty section:
This article was submitted to Freshwater Science, a section of the journal Frontiers in Environmental Science

Received: *26 January 2017*
Accepted: *19 April 2017*
Published: *08 May 2017*

Citation:
Meunier CL, Boersma M, El-Sabaawi R, Halvorson HM, Herstoff EM, Van de Waal DB, Vogt RJ and Litchman E (2017) From Elements to Function: Toward Unifying Ecological Stoichiometry and Trait-Based Ecology. Front. Environ. Sci. 5:18. doi: 10.3389/fenvs.2017.00018

INTRODUCTION

Ecological stoichiometry (ES) is a framework that links an organism's metabolic demands with the relative supply of elements in the environment (Sterner and Elser, 2002; Hessen et al., 2013). It postulates a crucial relationship between the balance of elements, typically but not limited to carbon (C), nitrogen (N), and phosphorus (P), and their role in determining growth and reproduction of organisms as well as in ecological interactions. The recognition of the importance of stoichiometric constraints between consumer needs and prey nutrient content has substantially increased our

understanding of trophic interactions. For example, high C:P food is often of low-quality for a variety of organisms including molluscs (Stelzer and Lamberti, 2002; Fink and Elert, 2006), crustaceans (Boersma and Kreutzer, 2002; Meunier et al., 2012, 2016a), insects (Perkins et al., 2004), fish (Borlongan and Satoh, 2001; Vrede et al., 2011), and birds (Grone et al., 1995). ES has therefore proven to be a highly suitable framework in community ecology, explaining consumer responses to prey food quality (food intake, growth, as well as competition between consumer species, and consumer effects on prey nutrient composition (Sterner, 1990; Sterner et al., 1992; Sterner and Hessen, 1994).

Trait-based ecology (TBE) focuses on functional traits expressed by an organism allowing its growth and survival under distinct environmental conditions (McGill et al., 2006). Functional traits are the morphological, physiological, phenological, and behavioral characteristics of an organism that influence its performance or fitness. As such, TBE couples biological function to the success of species in a food-web (Litchman and Klausmeier, 2008; Litchman et al., 2013; Kremer et al., 2016). More specifically, TBE focuses on traits rather than taxa, providing a functional approach to understanding ecological interactions. For example, it has been shown that trait diversity can provide a better predictor of primary production as compared to taxonomic diversity (Vogt et al., 2010). Numerous studies indicate that abiotic parameters, such as temperature, precipitation, and nutrient concentrations, can directly influence spatial and temporal trait patterns both at the population and at the community levels (Brun et al., 2016). TBE therefore constitutes a powerful tool linking species functional characteristics to their distributions along environmental gradients, as well as to community interactions and ecosystem function.

The framework of ES is essentially, although not explicitly, based on traits such as homeostasis, growth rate, and nutrient uptake. For instance, the relationship between organism size and stoichiometry has been well studied (see below for more detail) but connecting these ES traits with more traditional TBE traits still remains a major challenge. Combining the framework of ES with TBE furthers the coupling of elements to functional traits from subcellular processes to species interactions and ultimately ecosystem dynamics. For example, linking ES with TBE could help studying how variation in traits related to elemental body composition influences organismal fitness (Leal et al., 2016). The focus of this paper is to explore and synthesize existing insights, and to develop novel connections between ES and TBE. We first review key traits and their elemental requirements, highlighting differences between trophic levels as well as ecosystems. Next, we describe trade-offs between traits that directly or indirectly affect the elemental composition or requirements of organisms. We also develop hypotheses on how different traits could be linked to life history trade-offs, and we outline community and ecosystem consequences of variation in ES traits and identify limitations and opportunities for future research further connecting TBE and ES. This framework will foster collaboration between scientists of different disciplines (freshwater, marine, and terrestrial ecologists) and enhance our understanding of fundamental ecological issues.

TRAITS AND ELEMENTAL BALANCES

Four interconnected major trait groups affect the balance of energy and elements in organisms: acquisition, cell/body stoichiometry, storage, and excretion. These traits define how organisms interact with their environment as well as with one another, and are, therefore, among the key determinants of ecological niches. This elemental trait framework has already been successfully used to identify how resource imbalances affect basic physiological processes (Frost et al., 2005). In the following sections, we describe different strategies utilized by autotrophs and heterotrophs to acquire and use C and nutrients, and we explain how these traits directly influence organismal stoichiometry. While traditional stoichiometric approaches are implicitly trait-based, we here aim to fully place life history trade-offs in a stoichiometric context. This conceptual framework should enhance our ability to predict how communities will respond to changes in nutrient conditions in the environment.

Autotrophs

Autotrophs obtain energy (mostly sunlight) and material from different, uncoupled sources within their environment. Therefore, plants needed to develop strategies to store these resources, as the availability of one resource does not guarantee another one. Nutrient uptake strategies are often linked to storage capacity and therefore to plasticity in cellular stoichiometry. When C or nutrients are taken up and fixed by autotrophs, they may be used immediately for growth or accumulate as storage pools for later use (Chapin, 1980; Reynolds, 2006). Due to their need and hence capacity to store nutrients, autotroph C:N:P ratios greatly vary with available nutrient ratios, indicating a high flexibility in chemical composition and a lack of homeostasis (Sterner and Elser, 2002; Meunier et al., 2014). This can result in different competitive strategies between various autotroph species. For instance, for phytoplankton, three major nutrient acquisition strategies have been proposed (Sommer, 1984): (1) velocity-adapted species, or r-strategists, with high maximum nutrient uptake rates and high maximum growth rates that are able to directly utilize nutrient pulses for growth, (2) storage-adapted species, with high rates of nutrient uptake but lower maximum growth rates that have the ability to store excess nutrients, and (3) affinity-adapted species, or K-strategists, with the ability to effectively take up and assimilate growth-limiting nutrients even at low concentrations, a strategy that is advantageous in oligotrophic environments. Comparable nutrient-acquisition strategies exist in terrestrial systems, and nutrient availability strongly influences interspecific plant interactions and community composition (Zemunik et al., 2015). For instance, tall-growing species with high growth rates are generally favored by high nutrient inputs at the expense of species with conservative growth strategies (Diekmann and Falkengren-Grerup, 2002). Physiological traits such as nutrient requirements also alter interspecific competition, giving a competitive advantage to species with high-nutrient affinity (Price and Morgan, 2007).

Such different strategies may affect the stoichiometry of autotrophs. For instance, high growth rates may lead

to higher demands for P (see below), storage will increase cellular quota of distinct elements, while high affinities will reduce the minimum nutrient requirements (**Figure 1**). Stoichiometric plasticity will furthermore be determined by physiological limits, as was shown for phytoplankton N:P ratios that increased non-linearly with increasing N:P supply ratios (Rhee, 1978; Persson et al., 2010). In other words, the upper limit of nutrient quota is determined by storage capabilities, while the lower limits are determined by minimal structural and functional requirements, as well as the affinity for a nutrient. These boundaries within which the organism's stoichiometry can fluctuate represent the "homeostatic capacity" parameter defined by Meunier et al. (2014). This parameter corresponds to the boundaries within which the organism's body stoichiometry can fluctuate and therefore characterizes storage capacity. Consequently, storage- and affinity-adapted species will be generally characterized by variable stoichiometric composition while velocity-adapted species with high P demands will have low cellular N:P ratios (Hillebrand et al., 2013).

Variation in N:P ratios between organisms often reflect functional differences, such as the ones that have been described for optimal N:P ratios (Güsewell, 2004; Hillebrand et al., 2013), i.e., the N:P ratio at which growth is maximized. In aquatic environment for example, a recent meta-analysis identified a scaling relationship between maximum growth rate and phytoplankton nutrient demand (Hillebrand et al., 2013). Phytoplankton N:P ratios decrease with increasing growth rates and simultaneously display decreasing variance, particularly driven by P limitation, which follows predictions of the Growth Rate Hypothesis (Sterner, 1995a; Elser et al., 1996; Sterner and Elser, 2002). This suggests that fast growing phytoplankton species, or within a species under less severe limiting conditions, phytoplankton are relatively more P-rich. Using a trait-based eco-evolutionary model, Klausmeier et al. (2004) showed that different environmental conditions select for species with different N:P ratios: P-rich conditions select for fast growers with low N:P, while P-limited conditions select for better P competitors with higher N:P ratios (due to their investment in resource acquisition proteins, **Figure 1**). Not only the N:P ratio itself but the form of N and P available to phytoplankton matters. While we may expect higher biomass of small phytoplankton at lower N:P supply due to their generally fast growth (and thus high P requirements), higher picophytoplankton densities are only observed under high N:P conditions (**Figure 1**; Glibert, 2016) which is likely caused by changes in N redox and relative proportions of reduced relative to oxidized N (Glibert et al., 2016). A review of interspecific differences in N:P ratios of terrestrial plants concluded that N:P ratios correlate negatively with maximum relative growth rate in herbaceous and woody plants (Güsewell, 2004). Species with inherently low N:P ratios are predicted to dominate N-limited communities and should be favored during P fertilization (**Figure 2**; Tilman, 1997). Therefore, nutrient supply and composition often shape terrestrial and aquatic autotroph community composition by species sorting as well as by dynamic shifts in species' C:N:P stoichiometry (Sterner and Elser, 2002; Persson et al., 2010; Meunier et al., 2014).

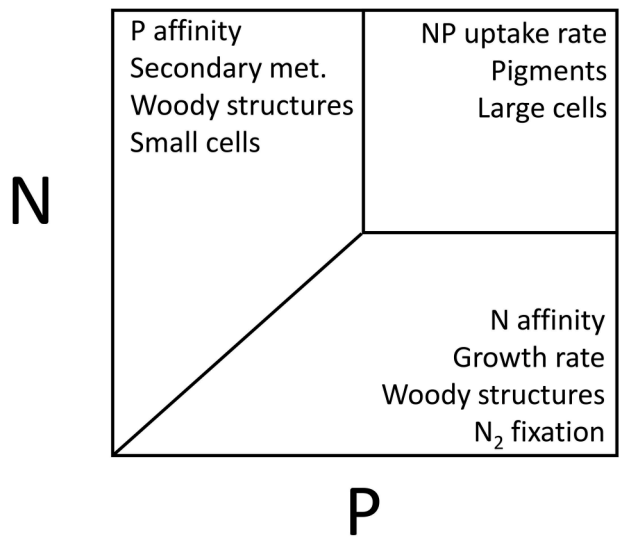

FIGURE 1 | Influence of N and Pavailabilities on trait dominance in autotrophs. Low N and P supplies will favor species with high affinities and may also facilitate synthesis of C-rich woody structures. Furthermore, species with C-rich woody structures may be facilitated by low N and P supplies. Relatively high N supplies may selectively favor synthesis of N-rich secondary metabolites, while relatively high P supplies may selectively favor faster growth, and N_2 fixation (in cyanobacteria). Both high N and P supplies may facilitate large species not limited by nutrients, and favor high maximum uptake rates. Furthermore, high N and P supplies will promote autotroph biomass and consequently self-shading, and thus may favor species with high pigment contents and/or species with accessory pigments.

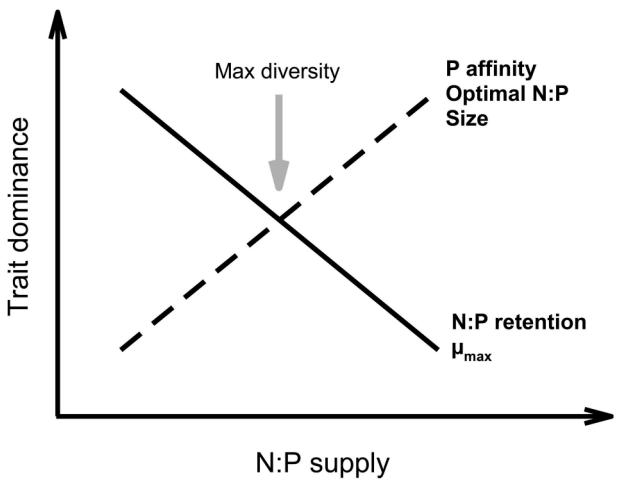

FIGURE 2 | Influence of N:P supply on trait dominance. With increasing N:P supply, an organism's optimal N:P ratio, P uptake affinity, and size are expected to increase, and organisms that selectively retain P over N should have higher maximal growth rates than those which do not, as predicted by the Growth Rate Hypothesis (Sterner, 1995b; Elser et al., 2000c). Further, Tilman's resource-ratio based theory of competition predicts that biodiversity should be maximal (gray arrow) at balanced supply ratios (Tilman, 1982).

Heterotrophs

Autotrophs' stoichiometry also reflects their quality as food for herbivores. Since autotrophs are usually more C-rich than animals, herbivores typically ingest a diet rich in C but deficient in nutrients (Sterner and Elser, 2002), which has implications for herbivore and detritivore performance and subsequent trophic dynamics (Elser et al., 2000b; Hessen et al., 2013). Grazers however possess a large range of adaptations that allow them to minimize the consequences of these nutrient imbalances. Besides selective feeding (e.g., Kagata and Ohgushi, 2011; Meunier et al., 2012, 2016a) and habitat choice (Winder et al., 2004; Reichwaldt, 2008), consumers may handle poor food quality by selectively retaining and excreting nutrients (Elser and Urabe, 1999; Knoll et al., 2009). These physiological adjustments are tightly linked with the stoichiometry of traits such as elemental ratios of recycled materials and body stoichiometric homeostasis.

Nutrient storage capacity is a much more confined trait in heterotrophs than in autotrophs resulting in more constrained body composition (Persson et al., 2010; Meunier et al., 2014). While the lack of homeostasis allows organisms to store nutrients (see above), the advantages of stoichiometric homeostasis remain unclear. It can be argued that the ability to store nutrients, which results in flexible body stoichiometry, is the more advantageous strategy, especially where food supply or food quality fluctuates (Meunier et al., 2014). It has been hypothesized that homeostasis is the optimal response strategy to fluctuations in food quality, as it should minimize the cost of adjusting to new conditions (e.g., selective nutrient retention and excretion) while maximizing growth rate (Giordano, 2013). Indeed, when feeding conditions are relatively stable within an organism's life span, homeostasis minimizes noise in physiological channels and establishes a stable environment for cellular processes (Woods and Wilson, 2013).

Stoichiometric homeostasis also results from adjustments in the elemental ratios of recycled material. The stoichiometry of excreted material is influenced by both the consumer's stoichiometry, e.g., low N:P consumers will have high N:P excretion, as well as by the resource stoichiometry, e.g., the excreted N:P ratio will increase with increasing resource N:P ratio (Vanni, 2002). However, several studies question the link between body stoichiometry and elemental ratios of excreted material (Allgeier et al., 2015; Vanni and McIntyre, 2016). The lack of evidence for the relationship between resource stoichiometry and consumer nutrient excretion is likely due to the fact that, in these studies, bulk stoichiometry was measured instead of the stoichiometry of assimilated materials (e.g., Dodds et al., 2014).

Interspecific variations in recycling traits will also have consequences at the ecosystem scale. For instance, experiments in lakes provided evidence that the replacement of the high body N:P copepods with low N:P *Daphnia* caused a transition from N to P limitation for primary producers (Sterner, 1990; Elser et al., 1996), likely driven by the higher N:P ratio of recycled material (Elser et al., 1988). In addition, intraspecific variation in nutrient recycling traits during copepod ontogeny has been suggested to influence biogeochemical cycling within marine systems (Meunier et al., 2016a). Like zooplankton, aquatic vertebrates such as fish and amphibians can also have important stoichiometric impacts on nutrient cycling and trophic dynamics (Vanni et al., 2002). Similarly, in terrestrial systems, consumers' body stoichiometry and nutrient recycling traits are expected to shape limitation patterns (Cherif and Loreau, 2009, 2013). However, it is important to consider the intrinsic differences between producer-consumer interactions in aquatic and terrestrial ecosystems. While planktonic herbivores may eat a very large percentage (70–80%) of the daily primary production (Calbet, 2008; Löder et al., 2011), in terrestrial ecosystems, plants are less nutritional and lose lower percentages of production to herbivores, and a higher level of C is channeled as detritus (Cebrian, 1999). Traits directly influencing herbivore stoichiometry should therefore play only a small role in the cycling of nutrients in forested ecosystems, with decomposers and detritivores playing a more important role (Cherif and Loreau, 2009, 2013). Given the importance of homeostasis for biogeochemical cycling in food webs, quantifying the contribution of this trait to ecosystem services related to biogeochemical cycles (i.e., C sequestration, water quality) represents a promising avenue for future research. The consequences of differences in homeostasis between autotrophic and heterotrophic microbes for nutrient cycling have been explored in a number of studies (e.g., Cross et al., 2005; Cotner et al., 2010). Further, exploring the importance of the stoichiometry of traits as both drivers of, and responses to, ecosystem changes will result in datasets that will advance our understanding of how biological communities will perform under different environmental conditions, including the changing climate. This approach could in turn be used to explore traits that both directly and indirectly affect a given ecosystem function or service.

TRAIT CONNECTIONS

Correlative Relationships

Several studies have linked elemental stoichiometry to distinct species traits in order to explain ecosystem structure (for an overview see **Table 1**). As we previously mentioned, the tight relationship between the body N:P ratio and organismal growth rate is formulated in the Growth Rate Hypothesis (**Table 1**), a central hypothesis in ES. It postulates that species with high growth rates have more ribosomal RNA content for rapid protein synthesis than slow-growing species (Sterner and Elser, 2002). Because ribosomal RNA is rich in P, faster growing species should have a higher P content and lower body N:P ratios (Sterner, 1995a; Elser et al., 1996; Sterner and Elser, 2002). Although the Growth Rate Hypothesis is supported for a large range of organisms, several studies have questioned its generality (Sardans et al., 2012, and reference therein). For instance, mass-balance modeling demonstrated that maintenance costs for high biomass P content can drive the relationship between P content and growth rate (Shimizu and Urabe, 2008). This has been confirmed in experiments showing that consumers release P at a substantial rate, even when fed high C:P food (Demott et al., 1998; Shimizu and Urabe, 2008). Despite these limitations associated with P losses, e.g., through molting (Shimizu and Urabe, 2008), a large body of literature points toward a negative relationship between

TABLE 1 | Relationships between elemental stoichiometry characteristics and physiological traits among autotrophs and heterotrophs, with abbreviated description of the linkage and associated citations.

ES characteristics	Physiological trait	Description	Citations
AUTOTROPHS			
Organismal C:P and N:P contents	Growth rate	Autotrophs of lower C:P and N:P contents exhibit greater maximum growth rates	Güsewell, 2004; Hillebrand et al., 2013
	Structure (wood investment)	Investment in woody structures reduces plant tissue N and P contents	Han et al., 2005
Homeostasis	Temporal stability	Homeostatic plant species show reduced temporal variation in grassland communities	Yu et al., 2015
	Maximum growth rate	Homeostatic phytoplankton exhibit faster growth rates	Hillebrand et al., 2013
Nutrient uptake	Cell size	Phytoplankton nutrient uptake affinities decline with increased cell size	Irwin et al., 2006; Edwards et al., 2012
	Growth rate	Higher phytoplankton maximum growth rates with greater nutrient uptake affinity	Edwards et al., 2012
	Resistance to grazing	Phytoplankton resistant to grazing may be poor competitors for limiting nutrients	Litchman and Klausmeier, 2008
	Symbioses (mycorrhizae)	Mycorrhizal symbionts increase host plant nutrient acquisition	Brundrett, 2009
HETEROTROPHS			
Organismal C:P and N:P contents	Growth rate	Heterotrophs of lower C:P and N:P exhibit greater maximum growth rates	Elser et al., 2003; Hood and Sterner, 2014
	Defense (bone investment)	Investment in bone decreases vertebrate body C:P contents	El-Sabaawi et al., 2016
	Ontogeny	Vertebrate C:P and N:P decline during development, whereas invertebrate C:P and N:P increase during development	Pilati and Vanni, 2007; González et al., 2011; Back and King, 2013; Tiegs et al., 2016
	Sex	Divergent P contents between males and females	Back and King, 2013
Homeostasis	Maximum growth rate	Species of flexible homeostasis tend to have high maximum growth rates	Hood and Sterner, 2014
	Sex	Divergent flexibility in body P contents between males and females	Goos et al., 2014
	Generalism vs. specialism?	Homeostatic consumers may exhibit greater nutritional specialism	Sperfeld et al., 2017
Nutrient assimilation	Gut residence time or gut length	Increased gut length confers increased nutrient assimilation efficiencies	Liess et al., 2015
Consumer-resource elemental imbalance	Trophic level	Higher trophic levels exhibit reduced consumer-resource elemental imbalances	Lemoine et al., 2014
	Omnivory	Selective feeding may reduce imbalances between consumers and resources	Snyder et al., 2015; Meunier et al., 2016a
Nutrient recycling	Growth rate	Faster-growing animals recycle less P	Elser et al., 2000a
	Body size	Higher mass-specific nutrient recycling by organisms of smaller body size	Allgeier et al., 2015; Vanni and McIntyre, 2016
	Phylogeny	Taxonomic identity affects size scaling and rates of N and P excretion	Allgeier et al., 2015

growth rate and body C:P or N:P (e.g., Carrillo et al., 2001; Meunier et al., 2016a).

Interestingly, organisms' size and growth rate are usually negatively correlated (**Table 1**), which implies that, based on the growth rate hypothesis, smaller heterotrophic organisms might generally have lower N:P ratios (**Figure 2**). These explicit connections between body size, ontogeny, and N:P stoichiometry have been well documented (Elser et al., 1996; Gillooly et al., 2005; Méndez and Karlsson, 2005; Meunier et al., 2016a). Further, larger cells have higher biomass-specific storage capacity due to smaller surface/volume ratios and higher minimum cellular metabolic requirement that selectively allows them to provide them with a competitive advantage over smaller cells under higher resource concentrations (**Figure 3**; Irwin et al., 2006).

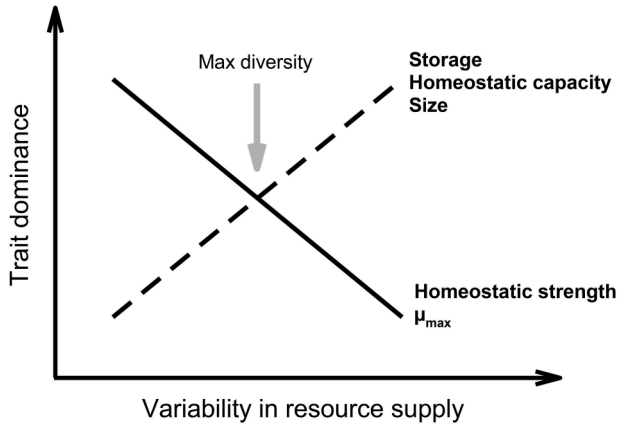

FIGURE 3 | Influence of variability in resource supply on traits. Increasing variability is expected to influence the trade-off between fast growth and investment in storage. Higher variability should therefore yield communities with larger organisms with greater storage and homeostatic capacities, but slower maximal growth rates. Moreover, the intermediate disturbance hypothesis suggests that local species diversity should be maximized when ecological disturbance is neither too rare nor too frequent (Wilkinson, 1999). Biodiversity should therefore be maximal at intermediate resource supply variability (gray arrow).

Experiments in lakes showed that the interaction between top-down and bottom-up controls can lead to the replacement of small zooplankton with high N:P by larger species with low N:P (Sterner et al., 1992; Hall et al., 2004). This community shift also causes a transition from N to P limitation for primary producers, likely caused by the higher N:P ratio of recycled material (Elser et al., 1988). However, the relationship between size and stoichiometry is expected to be opposite in vertebrates (i.e., decreasing N:P ratios with increasing body size) due to relatively higher P needs for bones (Elser et al., 1996; González et al., 2011). The interplay between traits and elemental requirements hence influences the success of an organism in response to different environmental conditions (**Table 1**), which, in turn, will shape community structure.

Changes in environmental conditions can also lead to evolutionary changes in traits. The mutual interactions between the evolved functional traits and environment characteristics therefore have gained increasing interest over the past two decades. In particular, understanding how genome and proteome adaptations are shaped by selection on growth-related traits and the parameters determining the extent of stoichiometrically relevant variation in genomes across taxa are increasingly studied (Kay et al., 2005). For example, genes that respond to changes in P availability have received much attention, and variation in the environmental supply of P has been associated with the expression of highly conserved genes (Jeyasingh and Weider, 2007; Frisch et al., 2014). Rotifer populations with a P limitation selection history yielded higher biomass and reduced food to lower levels when fed with P limited food (Declerck et al., 2015). Although this adaptation of populations did not involve changes in elemental composition, it did alter important population traits, including birth and death rates, population structure and grazing pressure. Both TBE and ES are at the intersection of evolution and ecology, and their integration will thus greatly help understanding eco-evolutionary dynamics (Jeyasingh et al., 2014; Declerck et al., 2015).

Trade-Offs

Natural selection balances traits associated with the three main missions of any organism, i.e., to eat, survive, and reproduce in order to maximize fitness. However, it is generally not possible to maximize all traits simultaneously, particularly as resources are often limiting. Trade-offs are therefore inevitable and different organisms specialize in various aspects of their life history. Such trade-offs are typically enabled by trait specialization and plasticity. For example, consumers with lower P requirements and higher body N:P ratios should be abundant in low P environments despite having reduced maximum growth rates (Sterner, 1995b; Elser et al., 2000c). The quantification of trade-offs associated with key traits will therefore yield a set of comparisons allowing the prediction of physiological, morphological, and behavioral responses of communities to environmental changes (Litchman and Klausmeier, 2008; Litchman et al., 2013). Particularly regarding stoichiometry, Montechiaro and Giordano (2010) discussed the conditions influencing the trade-off between stoichiometric homeostasis and maximal growth rate. Generally, when enduring periods of nutrient limitation, an organism should adopt a strategy that lessens the cost of adjusting to the new conditions (degree of homeostasis) and maximizes growth rate. This trade-off has been observed in experiments where phytoplankton were grown at different growth rates and subjected to a stress (Fanesi et al., 2014). The authors observed that, when the duration of the perturbation was long enough, cells with lower growth rate were more elementally homeostatic after a change in nutrient supply, which indicates that the trade-off between acclimation and homeostasis depends on the duration of the perturbation relative to cellular division rate (Fanesi et al., 2014). Based on these results, we hypothesize that homeostatic strength should decrease with increasing variability in resource supply (**Figure 3**). Similar patterns are expected in heterotrophs (Meunier et al., 2014) but, to our knowledge, no experiment has yet been conducted to test this prediction.

Similarly, low nutrient availability in the environment forces plants to adopt different strategies. For example, they can invest in storage traits and preferentially accumulate N rather than P (Chapin et al., 1990; Yu et al., 2015). Experimental work in freshwater (e.g., Sommer, 1984; Li and Stevens, 2012), marine (e.g., Smayda, 1997), and terrestrial systems (for review see Chapin et al., 1990) has shown that investing in storage traits provides a competitive advantage under highly variable and unpredictable nutrient availabilities (**Figure 3**). For example, organisms with high N affinity and storage capacity relative to other nutrients should have a competitive advantage under low N supply, as is often observed in N-limited terrestrial systems (Meunier et al., 2016b). These results indicate that the trade-offs between size, rapid growth, and storage capacity are strongly regulated by nutrient availability as well as the frequency and duration of nutrient pulses

relative to organismal growth rates (**Figure 3**). Thus, if nutrient pulses are regular, small velocity-adapted species should prosper due to their high maximum nutrient uptake rates and high maximum growth rates (**Figure 3**; Sommer, 1984). Along similar lines of reasoning, the intermediate disturbance hypothesis suggests that local species diversity should be maximized when ecological disturbance is neither too rare nor too frequent (Wilkinson, 1999), although the theoretical underpinnings of this hypothesis have been increasingly questioned (Fox, 2013). This implies that biodiversity is maximized when the resource supply is intermediately variable (**Figure 3**). Indeed, N pulses of intermediate frequencies may lead to coexistence of different strategies, i.e., velocity and storage specialists (Grover, 1991; Litchman et al., 2009).

In their review of phytoplankton traits and trade-offs, Litchman and Klausmeier (2008) suggest that the trade-off between nutrient competitive abilities and grazer resistance is key among phytoplankton. One strategy to obtain resistance to grazing is through a large cell size, which is associated with lower growth rate and reduced nutrient uptake abilities because of the decreased surface area to volume ratios (Reynolds, 1988) but increases the cell's storage capacity (Litchman et al., 2009; Wirtz, 2013). However, small size also offers several advantages to phytoplankton, including a more efficient acquisition of limiting nutrients (Sherwood et al., 1975; Ploug et al., 1999) and higher maximum growth rates (Banse, 1976), although it can increase susceptibility to grazing (Thingstad et al., 2005). This trade-off is one of the drivers for species succession during phytoplankton blooms as well as annual changes in community composition (Reynolds, 1984; Sommer et al., 1986). Organismal replacement along nutrient gradients can also be driven by this trade-off (Leibold, 1996), which ultimately has consequences for biodiversity within communities (Kneitel and Chase, 2004). The trade-off between cell size and competitive ability for limiting nutrients is therefore a key determinant of species dominance in phytoplankton communities. In addition to pairwise trade-offs, traits can be linked via multidimensional trade-offs. For example, Edwards et al. (2011) identified a three-way trade-off between N vs. P competitive abilities and cell size as a proxy for grazer resistance.

IMPLICATIONS

Understanding the link between nutrient stoichiometry and organismal traits can help predict how human-induced changes in biogeochemical cycles will alter the interaction between producers' stoichiometry and consumer elemental requirements. Human activities have altered the C, N, and P biogeochemical cycles on a global scale (Peñuelas et al., 2012). These changes are apparent from increasing atmospheric CO_2 concentrations and the large amounts of nutrients applied on land, subsequently also enriching lakes, rivers, and marine coastal waters. More specifically, the use of agricultural fertilizers has tremendously disturbed global biogeochemical cycles and increased the amount of N and P available to plants and animals (Bobbink et al., 2010). Not only do the C, N, and P supplies increase, also their relative abundances are changing both in aquatic and terrestrial environments (Grizzetti et al., 2012; Peñuelas et al., 2012; Sardans et al., 2012). Such shifts in the relative abundances of elements will alter autotroph stoichiometry and thereby their quality as food for herbivores (Van De Waal et al., 2010). For example, the N:P ratio available to plants has risen over the past decades in both terrestrial and aquatic systems (Sardans et al., 2012). The concepts we highlight here allow us to predict that such changes in nutrient availability should lead to communities dominated by species with higher optimal N:P ratios and higher P uptake affinity, while decreasing N retention and increasing P storage (**Figure 2**). The Growth Rate Hypothesis allows us to predict slower maximal growth rates at higher N:P supply and decreasing organismal size with increasing N:P supply, because body size is generally negatively correlated with growth rate (**Figure 2**). Linking TBE with ES therefore enables us to make predictions regarding the impact of human activities on both community structure and functioning. For example, it has been observed that communities dominated by species with higher optimal N:P ratios will substantially influence biogeochemical cycles by preferentially recycling N over P (Vanni et al., 2002; Knoll et al., 2009; El-Sabaawi et al., 2016).

CONCLUSION

Coupling functional traits to the stoichiometry of organisms allows a more general understanding of ecological interactions. Specifically, optimal body N:P ratios, nutrient uptake and storage traits, as well as their associated trade-offs, have the potential to drive species competition and thereby influence food web interactions and ecosystem dynamics. Quantifying the contribution of these traits to ecosystem services (i.e., C sequestration, water quality) represents a promising avenue for research into changes in biogeochemical cycling associated with global environmental change. At the same time, traits indirectly coupled to elemental demands, such as cell/body size and growth rate have a strong influence on, and are affected by, organismal stoichiometry. Therefore, combining observations and ideas from ES and TBE offers a unified framework that enables answering a wide array of complex ecological questions, for instance how biological communities will perform under changing environmental conditions. Linking and applying multiple ecological frameworks allows crosstalk between the various scientific disciplines, fostering the exchange of comparable efforts in understanding the complexity of ecosystem structure and functioning.

AUTHOR CONTRIBUTIONS

The manuscript was written by CM. **Table 1** was created by HH. **Figure 1** was created by DW. **Figures 2**, **3** were created by CM. All co-authors helped to evaluate and edit the manuscript.

ACKNOWLEDGMENTS

This article summarizes the work done in the "Workshop on Biological Stoichiometry and Trait-Based Ecology" that took

place during the Conference On Biological Stoichiometry (2015, Trent, Canada). This conference was supported by the David Schindler Professorship of Aquatic Sciences at Trent University and by Trent University. The workshop was supported by the Canadian Institute of Ecology and Evolution. We thank all workshop participants for their input into the discussions: Claudia Acquisti, Thomas Anderson, Jeff Back, Stephen Baines, Esteban Balseiro, Gergely Boros, Krista Capps, Sarah Collins, Richard Connon, James Cotner, Michael Danger, Sanatan Das Gupta, Steven Declerck, Lenore Doumas, Dan Durston, Jonathan Ebel, James Elser, Michelle Evans-White, Carolyn Faithfull, Isabel Fernandes, Laura Fidalgo, Michał Filipiak, Stephanie Fong, Paul Frost, Andrea Gall, Pat Glibert, Casey Godwin, Angelica L. Gonzalez, Jared Goos, Jin-Sheng He, Puni Jeyasingh, Susan Kilham, Ryan King, Jude Kong, James Larson, Cecilia Laspoumaderes, Kimberley Lemmen, Shawn Leroux, Patrick Lind, Paloma Lopes, Keeley MacNeill, Adam Martiny, Peter B. McIntyre, Katie Miller, Eric Moody, David Ott, Rachel Paseka, Angela Peace, Amber Rock, Anna Rożen, Amanda Rugenski, Andrew Sanders, Jennifer Schmitt, Kimberly Schulz, Ryan Sherman, Robert Sterner, Maren Striebel, Caroline Turner, Jotaro Urabe, Michael Vanni, Stoycho Velkovsky, Mandy Velthuis, Nicole Wagner, Wei Wang, Tanner Williamson, and Donald Yee.

REFERENCES

Allgeier, J. E., Wenger, S. J., Rosemond, A. D., Schindler, D. E., and Layman, C. A. (2015). Metabolic theory and taxonomic identity predict nutrient recycling in a diverse food web. *Proc. Natl. Acad. Sci. U.S.A.* 112, E2640–E2647. doi: 10.1073/pnas.1420819112

Back, J. A., and King, R. S. (2013). Sex and size matter: ontogenetic patterns of nutrient content of aquatic insects. *Freshw. Sci.* 32, 837–848. doi: 10.1899/12-181.1

Banse, K. (1976). Rates of growth, respiration and photosynthesis of unicellular algae as related to cell size—a review. *J. Phycol.* 12, 135–140.

Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., et al. (2010). Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. *Ecol. Appl.* 20, 30–59. doi: 10.1890/08-1140.1

Boersma, M., and Kreutzer, C. (2002). Life at the edge: is food quality really of minor importance at low quantities? *Ecology* 83, 2552–2561. doi: 10.1890/0012-9658(2002)083[2552:LATEIF]2.0.CO;2

Borlongan, I. G., and Satoh, S. (2001). Dietary phosphorus requirement of juvenile milkfish, Chanos chanos (Forsskal). *Aquac. Res.* 32, 26–32. doi: 10.1046/j.1355-557x.2001.00003.x

Brun, P., Payne, M. R., and Kiørboe, T. (2016). Trait biogeography of marine copepods–an analysis across scales. *Ecol. Lett.* 19, 1403–1413. doi: 10.1111/ele.12688

Brundrett, M. (2009). Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant Soil* 320, 37–77. doi: 10.1007/s11104-008-9877-9

Calbet, A. (2008). The trophic roles of microzooplankton in marine systems. *ICES J. Mar. Sci.* 65, 325–331. doi: 10.1093/icesjms/fsn013

Carrillo, P., Villar-Argaiz, M., and Medina-Sánchez, J. M. (2001). Relationship between N:P ratio and growth rate during the life cycle of calanoid copepods: an *in situ* measurement. *J. Plankton Res.* 23, 537–547. doi: 10.1093/plankt/23.5.537

Cebrian, J. (1999). Patterns in the fate of production in plant communities. *Am. Nat.* 154, 449–468. doi: 10.1086/303244

Chapin, F. S. (1980). The mineral nutrition of wild plants. *Ann. Rev. Ecol. Syst.* 11, 233–260. doi: 10.1146/annurev.es.11.110180.001313

Chapin, I. E., Schulze, A., and Mooney, H. A. (1990). The ecology and economics of storage in plants. *Annu. Rev. Ecol. Syst.* 21, 423–447. doi: 10.1146/annurev.es.21.110190.002231

Cherif, M., and Loreau, M. (2009). When microbes and consumers determine the limiting nutrient of autotrophs: a theoretical analysis. *Proc. R. Soc. Lond. B Biol. Sci.* 276, 487–497. doi: 10.1098/rspb.2008.0560

Cherif, M., and Loreau, M. (2013). Plant–herbivore–decomposer stoichiometric mismatches and nutrient cycling in ecosystems. *Proc. R. Soc. B Biol. Sci.* 280:20122453. doi: 10.1098/rspb.2012.2453

Cotner, J. B., Hall, E. K., Scott, T., and Heldal, M. (2010). Freshwater bacteria are stoichiometrically flexible with a nutrient composition similar to seston. *Front. Microbiol.* 1:132. doi: 10.3389/fmicb.2010.00132

Cross, W. F., Benstead, J. P., Frost, P. C., and Thomas, S. A. (2005). Ecological stoichiometry in freshwater benthic systems: recent progress and perspectives. *Freshw. Biol.* 50, 1895–1912. doi: 10.1111/j.1365-2427.2005.01458.x

Declerck, S. A., Malo, A. R., Diehl, S., Waasdorp, D., Lemmen, K. D., Proios, K., et al. (2015). Rapid adaptation of herbivore consumers to nutrient limitation: eco-evolutionary feedbacks to population demography and resource control. *Ecol. Lett.* 18, 553–562. doi: 10.1111/ele.12436

Demott, W. R., Gulati, R. D., and Siewertsen, K. (1998). Effects of phosphorus-deficient diets on the carbon and phosphorus balance of *Daphnia magna*. *Limnol. Oceanogr.* 43, 1147–1161. doi: 10.4319/lo.1998.43.6.1147

Diekmann, M., and Falkengren-Grerup, U. (2002). Prediction of species response to atmospheric nitrogen deposition by means of ecological measures and life history traits. *J. Ecol.* 90, 108–120. doi: 10.1046/j.0022-0477.2001.00639.x

Dodds, W. K., Collins, S., Hamilton, S., Tank, J., Johnson, S., Webster, J., et al. (2014). You are not always what we think you eat: selective assimilation across multiple whole-stream isotopic tracer studies. *Ecology* 95, 2757–2767. doi: 10.1890/13-2276.1

Edwards, K. F., Klausmeier, C. A., and Litchman, E. (2011). Evidence for a three-way tradeoff between nitrogen and phosphorus competitive abilities and cell size in phytoplankton. *Ecology* 92, 2085–2095. doi: 10.1890/11-0395.1

Edwards, K. F., Thomas, M. K., Klausmeier, C. A., and Litchman, E. (2012). Allometric scaling and taxonomic variation in nutrient utilization traits and maximum growth rate of phytoplankton. *Limnol. Oceanogr.* 57, 554–566. doi: 10.4319/lo.2012.57.2.0554

El-Sabaawi, R. W., Warbanski, M. L., Rudman, S. M., Hovel, R., and Matthews, B. (2016). Investment in boney defensive traits alters organismal stoichiometry and excretion in fish. *Oecologia* 181, 1209–1220. doi: 10.1007/s00442-016-3599-0

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate–stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Dobberfuhl, D. R., Mackay, N. A., and Schampel, J. H. (1996). Organism size, life history, and N:P stoichiometry. *Bioscience* 46, 674–684. doi: 10.2307/1312897

Elser, J. J., Elser, M. M., Mackay, N. A., and Carpenter, S. R. (1988). Zooplankton-mediated transitions between N- and P-limited algal growth. *Limnol. Oceanogr.* 33, 1–14. doi: 10.4319/lo.1988.33.1.0001

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000b). Nutritional constraints in terrestrial and freshwater foodwebs. *Nature* 408, 578–580. doi: 10.1038/35046058

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., et al. (2000c). Biological Stoichiometry from genes to ecosystem. *Ecol. Lett.* 3, 540–550. doi: 10.1046/j.1461-0248.2000.00185.x

Elser, J. J., and Urabe, J. (1999). The Stoichiometry of consumer-driven nutrient recycling: theory, observations, and consequences. *Ecology* 80, 735–751. doi: 10.1890/0012-9658(1999)080[0735:TSOCDN]2.0.CO;2

Elser, J. J., O'brien, W., Dobberfuhl, D., and Dowling, T. (2000a). The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* 13, 845–853. doi: 10.1046/j.1420-9101.2000.00215.x

Fanesi, A., Raven, J. A., and Giordano, M. (2014). Growth rate affects the responses of the green alga Tetraselmis suecica to external perturbations. *Plant Cell Environ.* 37, 512–519. doi: 10.1111/pce.12176

Fink, P., and Elert, E. V. (2006). Physiological responses to stoichiometric constraints: nutrient limitation and compensatory feeding in a freshwater snail. *Oikos* 115, 484–494. doi: 10.1111/j.2006.0030-1299.14951.x

Fox, J. W. (2013). The intermediate disturbance hypothesis should be abandoned. *Trends Ecol. Evol.* 28, 86–92. doi: 10.1016/j.tree.2012.08.014

Frisch, D., Morton, P. K., Chowdhury, P. R., Culver, B. W., Colbourne, J. K., Weider, L. J., et al. (2014). A millennial-scale chronicle of evolutionary responses to cultural eutrophication in Daphnia. *Ecol. Lett.* 17, 360–368. doi: 10.1111/ele.12237

Frost, P. C., Evans-White, M. A., Finkel, Z. V., Jensen, T. C., and Matzek, V. (2005). Are you what you eat? Physiological constraints on organismal stoichiometry in an elementally imbalanced world. *Oikos* 109, 18–28. doi: 10.1111/j.0030-1299.2005.14049.x

Gillooly, J. F., Allen, A. P., Brown, J. H., Elser, J. J., Del Rio, C. M., Savage, V. M., et al. (2005). The metabolic basis of whole-organism RNA and phosphorus content. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11923–11927. doi: 10.1073/pnas.0504756102

Giordano, M. (2013). Homeostasis: an underestimated focal point of ecology and evolution. *Plant Sci.* 211, 92–101. doi: 10.1016/j.plantsci.2013.07.008

Glibert, P. M. (2016). Margalef revisited: a new phytoplankton mandala incorporating twelve dimensions, including nutritional physiology. *Harmful Algae* 55, 25–30. doi: 10.1016/j.hal.2016.01.008

Glibert, P. M., Wilkerson, F. P., Dugdale, R. C., Raven, J. A., Dupont, C. L., Leavitt, P. R., et al. (2016). Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnol. Oceanogr.* 61, 165–197. doi: 10.1002/lno.10203

González, A. L., Fariña, J. M., Kay, A. D., Pinto, R., and Marquet, P. A. (2011). Exploring patterns and mechanisms of interspecific and intraspecific variation in body elemental composition of desert consumers. *Oikos* 120, 1247–1255. doi: 10.1111/j.1600-0706.2010.19151.x

Goos, J. M., French, B. J., Relyea, R. A., Cothran, R. D., and Jeyasingh, P. D. (2014). Sex-specific plasticity in body phosphorus content of Hyalella amphipods. *Hydrobiologia* 722, 93–102. doi: 10.1007/s10750-013-1682-7

Grizzetti, B., Bouraoui, F., and Aloe, A. (2012). Changes of nitrogen and phosphorus loads to European seas. *Glob. Chang. Biol.* 18, 769–782. doi: 10.1111/j.1365-2486.2011.02576.x

Grone, A., Swayne, D. E., and Nagode, L. A. (1995). Hypophosphatemic rickets in rheas (*Rhea americana*). *Vet. Pathol.* 32, 324–327. doi: 10.1177/030098589503200318

Grover, J. P. (1991). Resource competition in a variable environment: phytoplankton growing according to the variable-internal-stores model. *Am. Nat.* 138, 811–835. doi: 10.1086/285254

Güsewell, S. (2004). N: P ratios in terrestrial plants: variation and functional significance. *New Phytol.* 164, 243–266. doi: 10.1111/j.1469-8137.2004.01192.x

Hall, S. R., Leibold, M. A., Lytle, D. A., and Smith, V. H. (2004). Stoichiometry and planktonic grazer composition over gradients of light, nutrients, and predation risk. *Ecology* 85, 2291–2301. doi: 10.1890/03-0471

Han, W., Fang, J., Guo, D., and Zhang, Y. (2005). Leaf nitrogen and phosphorus stoichiometry across 753 terrestrial plant species in China. *New Phytol.* 168, 377–385. doi: 10.1111/j.1469-8137.2005.01530.x

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Hillebrand, H., Steinert, G., Boersma, M., Malzahn, A. M., Meunier, C. L., Plum, C., et al. (2013). Goldman revisited: faster growing phytoplankton has lower N:P and lower stoichiometric flexibility. *Limnol. Oceanogr.* 58, 2076–2088. doi: 10.4319/lo.2013.58.6.2076

Hood, J. M., and Sterner, R. W. (2014). Carbon and phosphorus linkages in Daphnia growth are determined by growth rate, not species or diet. *Funct. Ecol.* 28, 1156–1165. doi: 10.1111/1365-2435.12243

Irwin, A. J., Finkel, Z. V., Schofield, O. M., and Falkowski, P. G. (2006). Scaling-up from nutrient physiology to the size-structure of phytoplankton communities. *J. Plankton Res.* 28, 459–471. doi: 10.1093/plankt/fbi148

Jeyasingh, P. D., Cothran, R. D., and Tobler, M. (2014). Testing the ecological consequences of evolutionary change using elements. *Ecol. Evol.* 4, 528–538. doi: 10.1002/ece3.950

Jeyasingh, P. D., and Weider, L. J. (2007). Fundamental links between genes and elements: evolutionary implications of ecological stoichiometry. *Mol. Ecol.* 16, 4649–4661. doi: 10.1111/j.1365-294X.2007.03558.x

Kagata, H., and Ohgushi, T. (2011). Ecosystem consequences of selective feeding of an insect herbivore: palatability–decomposability relationship revisited. *Ecol. Entomol.* 36, 768–775. doi: 10.1111/j.1365-2311.2011.01327.x

Kay, A. D., Ashton, I. W., Gorokhova, E., Kerkhoff, A. J., Liess, A., and Litchman, E. (2005). Toward a stoichiometric framework for evolutionary biology. *Oikos* 109, 6–17. doi: 10.1111/j.0030-1299.2005.14048.x

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2004). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174. doi: 10.1038/nature02454

Kneitel, J. M., and Chase, J. M. (2004). Trade-offs in community ecology: linking spatial scales and species coexistence. *Ecol. Lett.* 7, 69–80. doi: 10.1046/j.1461-0248.2003.00551.x

Knoll, L. B., McIntyre, P. B., Vanni, M. J., and Flecker, A. S. (2009). Feedbacks of consumer nutrient recycling on producer biomass and stoichiometry: separating direct and indirect effects. *Oikos* 118, 1732–1742. doi: 10.1111/j.1600-0706.2009.17367.x

Kremer, C. T., Williams, A. K., Finiguerra, M., Fong, A. A., Kellerman, A., Paver, S. F., et al. (2016). Realizing the potential of trait-based aquatic ecology: new tools and collaborative approaches. *Limnol. Oceanogr.* 62, 253–271. doi: 10.1002/lno.10392

Leal, M. C., Seehausen, O., and Matthews, B. (2016). The ecology and evolution of stoichiometric phenotypes. *Trends Ecol. Evol.* 32, 108–117. doi: 10.1016/j.tree.2016.11.006

Leibold, M. A. (1996). A graphical model of keystone predators in food webs: trophic regulation of abundance, incidence, and diversity patterns in communities. *Am. Natural.* 147, 784–812. doi: 10.1086/285879

Lemoine, N. P., Giery, S. T., and Burkepile, D. E. (2014). Differing nutritional constraints of consumers across ecosystems. *Oecologia* 174, 1367–1376. doi: 10.1007/s00442-013-2860-z

Li, W., and Stevens, M. H. H. (2012). Fluctuating resource availability increases invasibility in microbial microcosms. *Oikos* 121, 435–441. doi: 10.1111/j.1600-0706.2011.19762.x

Liess, A., Guo, J., Lind, M. I., and Rowe, O. (2015). Cool tadpoles from Arctic environments waste fewer nutrients–high gross growth efficiencies lead to low consumer-mediated nutrient recycling in the North. *J. Anim. Ecol.* 84, 1744–1756. doi: 10.1111/1365-2656.12426

Litchman, E., and Klausmeier, C. A. (2008). Trait-based community ecology of phytoplankton. *Annu. Rev. Ecol. Evol. Syst.* 39, 615–639. doi: 10.1146/annurev.ecolsys.39.110707.173549

Litchman, E., Klausmeier, C. A., and Yoshiyama, K. (2009). Contrasting size evolution in marine and freshwater diatoms. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2665–2670. doi: 10.1073/pnas.0810891106

Litchman, E., Ohman, M. D., and Kiørboe, T. (2013). Trait-based approaches to zooplankton communities. *J. Plankton Res.* 35, 473–484. doi: 10.1093/plankt/fbt019

Löder, M. G. J., Meunier, C., Wiltshire, K. H., Boersma, M., and Aberle, N. (2011). The role of ciliates, heterotrophic dinoflagellates and copepods in structuring spring plankton communities at Helgoland Roads, North Sea. *Mar. Biol.* 158, 1551–1580. doi: 10.1007/s00227-011-1670-2

McGill, B. J., Enquist, B. J., Weiher, E., and Westoby, M. (2006). Rebuilding community ecology from functional traits. *Trends Ecol. Evol.* 21, 178–185. doi: 10.1016/j.tree.2006.02.002

Méndez, M., and Karlsson, P. S. (2005). Nutrient stoichiometry in *Pinguicula vulgaris* nutrient availability, plant size, and reproductive status. *Ecology* 86, 982–991. doi: 10.1890/04-0354

Meunier, C. L., Boersma, M., Wiltshire, K. H., and Malzahn, A. M. (2016a). Zooplankton eat what they need: copepod selective feeding and potential consequences for marine systems *Oikos* 125, 50–58. doi: 10.1111/oik.02072

Meunier, C. L., Gundale, M. J., Sánchez, I. S., and Liess, A. (2016b). Impact of nitrogen deposition on forest and lake food webs in nitrogen limited environments. *Glob. Chang. Biol.* 22, 164–179. doi: 10.1111/gcb.12967

Meunier, C. L., Hantzsche, F. M., Cunha-Dupont, A. Ö., Haafke, J., Oppermann, B., Malzahn, A. M., et al. (2012). Intraspecific selectivity, compensatory feeding, and flexible homeostasis in the phagotrophic flagellate *Oxyrrhis marina*: three ways to handle food quality fluctuations. *Hydrobiologia* 680, 53–62. doi: 10.1007/s10750-011-0900-4

Meunier, C. L., Malzahn, A. M., and Boersma, M. (2014). A new approach to homeostatic regulation: towards a unified view of physiological and ecological concepts. *PLoS ONE* 9:e107737. doi: 10.1371/journal.pone.0107737

Montechiaro, F., and Giordano, M. (2010). Compositional homeostasis of the dinoflagellate *Protoceratium reticulatum* grown at three different pCO_2. *J. Plant Physiol.* 167, 110–113. doi: 10.1016/j.jplph.2009.07.013

Peñuelas, J., Sardans, J., Rivas-Ubach, A., and Janssens, I. A. (2012). The human-induced imbalance between C, N and P in Earth's life system. *Glob. Chang. Biol.* 18, 3–6. doi: 10.1111/j.1365-2486.2011.02568.x

Perkins, M. C., Woods, H. A., Harrison, J. F., and Elser, J. J. (2004). Dietary phosphorus affects the growth of larval *Manduca sexta*. *Arch. Insect Biochem. Physiol. Behav.* 55, 153–168. doi: 10.1002/arch.10133

Persson, J., Fink, P., Goto, A., Hood, J. M., Jonas, J., and Kato, S. (2010). To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos* 119, 741–751. doi: 10.1111/j.1600-0706.2009.18545.x

Pilati, A., and Vanni, M. J. (2007). Ontogeny, diet shifts, and nutrient stoichiometry in fish. *Oikos* 116, 1663–1674. doi: 10.1111/j.0030-1299.2007.15970.x

Ploug, H., Stolte, W., Epping, E. H., and Jørgensen, B. B. (1999). Diffusive boundary layers, photosynthesis, and respiration of the colony-forming plankton alga. Phaeocystis sp. *Limnol. Oceanogr.* 44, 1949–1958. doi: 10.4319/lo.1999.44.8.1949

Price, J. N., and Morgan, J. W. (2007). Vegetation dynamics following resource manipulations in herb-rich woodland. *Plant Ecol.* 188, 29–37. doi: 10.1007/s11258-006-9145-0

Reichwaldt, E. S. (2008). Food quality influences habitat selection in Daphnia. *Freshw. Biol.* 53, 872–883. doi: 10.1111/j.1365-2427.2007.01945.x

Reynolds, C. (1988). *Functional Morphology and the Adaptive Strategies of Freshwater Phytoplankton. Growth and Reproductive Strategies of Freshwater Phytoplankton*. Cambridge: Cambridge University Press.

Reynolds, C. S. (1984). *The Ecology of Freshwater Phytoplankton*. Cambridge: Cambridge University Press

Reynolds, C. S. (2006). *The Ecology of Phytoplankton.* Cambridge; New York, NY: Cambridge University Press.

Rhee, G.-Y. (1978). Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. *Limnol. Oceanogr.* 23, 10–25. doi: 10.4319/lo.1978.23.1.0010

Sardans, J., Rivas-Ubach, A., and Peñuelas, J. (2012). The elemental stoichiometry of aquatic and terrestrial ecosystems and its relationships with organismic lifestyle and ecosystem structure and function: a review and perspectives. *Biogeochemistry* 111, 1–39. doi: 10.1007/s10533-011-9640-9

Sherwood, T. K., Pigford, R. L., and Wilke, C. R. (1975). *Mass Transfer*. McGraw-Hill.

Shimizu, Y., and Urabe, J. (2008). Regulation of phosphorus stoichiometry and growth rate of consumers: theoretical and experimental analyses with *Daphnia*. *Oecologia* 155, 21–31. doi: 10.1007/s00442-007-0896-7

Smayda, T. J. (1997). Harmful algal blooms: their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnol. Oceanogr.* 42, 1137–1153. doi: 10.4319/lo.1997.42.5_part_2.1137

Snyder, M. N., Small, G. E., and Pringle, C. M. (2015). Diet-switching by omnivorous freshwater shrimp diminishes differences in nutrient recycling rates and body stoichiometry across a food quality gradient. *Freshw. Biol.* 60, 526–536. doi: 10.1111/fwb.12481

Sommer, U. (1984). The paradox of the plankton: fluctuations of phosphorus availability maintain diversity of phytoplankton in flow-through cultures. *Limnol. Oceanogr.* 29, 633–636. doi: 10.4319/lo.1984.29.3.0633

Sommer, U., Gliwicz, Z. M., Lampert, W., and Duncan, A. (1986). The PEG-model of seasonal succession of planktonic events in fresh waters. *Arch. Hydrobiol.* 106, 433–471.

Sperfeld, E., Wagner, N. D., Halvorson, H. M., Malishev, M., and Raubenheimer, D. (2017). Bridging Ecological Stoichiometry and Nutritional Geometry with homeostasis concepts and integrative models of organism nutrition. *Funct. Ecol.* 31, 286–296. doi: 10.1111/1365-2435.12707

Stelzer, R. S., and Lamberti, G. A. (2002). Ecological stoichiometry in running waters: periphyton chemical composition and snail growth. *Ecology* 83, 1039–1051. doi: 10.1890/0012-9658(2002)083[1039:ESIRWP]2.0.CO;2

Sterner, R. W. (1990). The ratio of nitrogen to phosphorus resupplied by herbivores: zooplankton and the algal competitive arena. *Am. Nat.* 136, 209–229. doi: 10.1086/285092

Sterner, R. W. (1995a). "Elemental stoichiometry of species in ecosystems," in *Linking Species and Ecosystems*, eds C. G. Jones and J. H. Lawton (New York, NY: Chapman and Hall), 240–252.

Sterner, R. W. (1995b). *Linking Species and Ecosystems*. New York, NY: Chapman & Hall.

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton; Oxford: Princeton University Press.

Sterner, R. W., Elser, J. J., and Hessen, D. O. (1992). Stoichiometric relationships among producers, consumers and nutrient cycling in pelagic ecosystems. *Biogeochemistry* 17, 49–67. doi: 10.1007/BF00002759

Sterner, R. W., and Hessen, D. O. (1994). Algal nutrient limitation and the nutrition of aquatic herbivores. *Annu. Rev. Ecol. Syst.* 25, 1–29. doi: 10.1146/annurev.es.25.110194.000245

Thingstad, T. F., Øvreås, L., Egge, J. K., Løvdal, T., and Heldal, M. (2005). Use of non-limiting substrates to increase size; a generic strategy to simultaneously optimize uptake and minimize predation in pelagic osmotrophs? *Ecol. Lett.* 8, 675–682. doi: 10.1111/j.1461-0248.2005.00768.x

Tiegs, S. D., Berven, K. A., Carmack, D. J., and Capps, K. A. (2016). Stoichiometric implications of a biphasic life cycle. *Oecologia* 180, 853–863. doi: 10.1007/s00442-015-3504-2

Tilman, D. (1982). *Resource Competition and Community Structure*. Princeton, NJ: Princeton University Press.

Tilman, D. (1997). *8: Mechanisms of Plant Competition*. Oxford: Blackwell Science.

Van De Waal, D. B., Verschoor, A. M., Verspagen, J. M., Van Donk, E., and Huisman, J. (2010). Climate-driven changes in the ecological stoichiometry of aquatic ecosystems. *Front. Ecol. Environ.* 8, 145–152. doi: 10.1890/080178

Vanni, M. J. (2002). Nutrient cycling by animals in freshwater ecosystems. *Annu. Rev. Ecol. Syst.* 33, 341–370. doi: 10.1146/annurev.ecolsys.33.010802.150519

Vanni, M. J., Flecker, A. S., Hood, J. M., and Headworth, J. L. (2002). Stoichiometry of nutrient recycling by vertebrates in a tropical stream: linking species identity and ecosystem processes. *Ecol. Lett.* 5, 285–293. doi: 10.1046/j.1461-0248.2002.00314.x

Vanni, M. J., and McIntyre, P. B. (2016). Predicting nutrient excretion of aquatic animals with metabolic ecology and ecological stoichiometry: a global synthesis. *Ecology* 97, 3460–3471. doi: 10.1002/ecy.1582

Vogt, R. J., Beisner, B. E., and Prairie, Y. T. (2010). Functional diversity is positively associated with biomass for lake diatoms. *Freshw. Biol.* 55, 1636–1646. doi: 10.1111/j.1365-2427.2010.02397.x

Vrede, T., Drakare, S., Eklöv, P., Hein, A., Liess, A., Olsson, J., et al. (2011). Ecological stoichiometry of Eurasian perch – intraspecific variation due to size, habitat and diet. *Oikos* 120, 886–896. doi: 10.1111/j.1600-0706.2010.18939.x

Wilkinson, D. M. (1999). The disturbing history of intermediate disturbance. *Oikos* 84, 145–147. doi: 10.2307/3546874

Winder, M., Spaak, P., and Mooij, W. M. (2004). Trade-off in Daphnia habitat selection. *Ecology* 85, 2027–2036. doi: 10.1890/03-3108

Wirtz, K. (2013). Mechanistic origins of variability in phytoplankton dynamics: Part I: niche formation revealed by a size-based model. *Mar. Biol.* 160, 2319–2335. doi: 10.1007/s00227-012-2163-7

Woods, H. A., and Wilson, J. K. (2013). An information hypothesis for the evolution of homeostasis. *Trends Ecol. Evol.* 28, 283–289. doi: 10.1016/j.tree.2012.10.021

Yu, Q., Wilcox, K., Pierre, K. L., Knapp, A. K., Han, X., and Smith, M. D. (2015). Stoichiometric homeostasis predicts plant species dominance, temporal stability, and responses to global change. *Ecology* 96, 2328–2335. doi: 10.1890/14-1897.1

Zemunik, G., Turner, B. L., Lambers, H., and Laliberté, E. (2015). Diversity of plant nutrient-acquisition strategies increases during long-term ecosystem development. *Nature Plants* 1:15050. doi: 10.1038/nplants.2015.50

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

METHODS
published: 20 September 2017
doi: 10.3389/fevo.2017.00110

The Multidimensional Stoichiometric Niche

Angélica L. González[1]*, *Olivier Dézerald*[1], *Pablo A. Marquet*[2,3,4], *Gustavo Q. Romero*[5] and *Diane S. Srivastava*[6]

[1] Biology Department, Center for Computational and Integrative Biology, Rutgers University, Camden, NJ, United States, [2] Departamento de Ecología, Pontificia Universidad Católica de Chile, Santiago, Chile, [3] Institute of Ecology and Biodiversity, Santiago, Chile, [4] The Santa Fe Institute, Santa Fe, NM, United States, [5] Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, Brazil, [6] Department of Zoology, Biodiversity Research Centre, University of British Columbia, Vancouver, BC, Canada

The niche concept is essential to understanding how biotic and abiotic factors regulate the abundance and distribution of living entities, and how these organisms utilize, affect and compete for resources in the environment. However, it has been challenging to determine the number and types of important niche dimensions. By contrast, there is strong mechanistic theory and empirical evidence showing that the elemental composition of living organisms shapes ecological systems, from organismal physiology to food web structure. We propose an approach based on a multidimensional elemental view of the ecological niche. Visualizing the stoichiometric composition of individuals in multivariate space permits quantification of niche dimensions within and across species. This approach expands on previous elemental characterizations of plant niches, and adapts metrics of niche volume, overlap and nestedness previously used to quantify isotopic niches. We demonstrate the applicability of the multidimensional stoichiometric niche using data on carbon, nitrogen, and phosphorus of terrestrial and freshwater communities composed by multiple trophic groups. First, we calculated the stoichiometric niche volumes occupied by terrestrial and freshwater food webs, by trophic groups, by individual species, and by individuals within species, which together give a measure of the extent of stoichiometric diversity within and across levels of organization. Then we evaluated complementarity between these stoichiometric niches, through metrics of overlap and nestedness. Our case study showed that vertebrates, invertebrates, and primary producers do not overlap in their stoichiometric niches, and that large areas of stoichiometric space are unoccupied by organisms. Within invertebrates, niche differences emerged between freshwater and terrestrial food webs, and between herbivores and non-herbivores (detritivores and predators). These niche differences were accompanied by changes in the covariance structure of the three elements, suggesting fundamental shifts in organismal physiology and/or structure. We also demonstrate the sensitivity of results to sample size, and suggest that representative sampling is better than rarefaction in characterizing the stoichiometric niche occupied by food webs. Overall, our approach demonstrates that stoichiometric traits provide a common currency to estimate the dimensionality of stoichiometric niches, and help reduce and rationalize the number of axis required to characterize communities.

Keywords: chemical elements, ecological stoichiometry, food webs, multidimensional space, niche overlap, niche nestedness, traits

OPEN ACCESS

Edited by:
Robert Warner Sterner, University of Minnesota Duluth, United States

Reviewed by:
Shawn James Leroux, Memorial University of Newfoundland, Canada
Jared M. Goos, University of Texas at Arlington, United States

***Correspondence:**
Angélica L. González
angelica.gonzalez@rutgers.edu

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *01 March 2017*
Accepted: *01 September 2017*
Published: *20 September 2017*

Citation:
González AL, Dézerald O, Marquet PA, Romero GQ and Srivastava DS (2017) The Multidimensional Stoichiometric Niche. Front. Ecol. Evol. 5:110. doi: 10.3389/fevo.2017.00110

INTRODUCTION

The niche concept is fundamental to ecology. The idea of the ecological niche has been central to most theory explaining how biotic and abiotic factors affect the abundance and distribution of species, and how species utilize, modify and compete for resources in the environment. However, the ecological niche has been defined multiple times through the history of ecology (Leibold, 1995; Chase and Leibold, 2003). Grinnell (1917) defined a species' niche in terms of the match between the species' traits and its habitat, whereas Elton (1927) considered the niche to be a qualitative description of the resource requirements for species persistence, as well as the impacts of species on their environment. By contrast, the Hutchinsonian niche (Hutchinson, 1957) represents a more quantitative description of all biotic resources and abiotic conditions (i.e., multiple n-dimensions) that affect the fitness of a species. More recently, trait-based approaches have merged the Hutchinson n-dimensional niche perspective with the Eltonian niche to quantify a species' niche based on multiple functional traits. The main premise of trait-based ecology is that the niche can be characterized by the phenotypic space occupied by a group of organisms. Quantification of this functional trait space has provided insights into understanding the ecological and evolutionary processes that structure functional diversity, and the relationship between functional diversity and ecosystem processes (Carmona et al., 2016).

An alternative to trait based or Hutchinsonian niches are niches based on the chemical composition of organisms. As energy and material acquisition, storage and exchange are essential to life, and because the chemical composition of an organism has been shown to shape its ecology, it has been argued that biochemistry represents a key aspect of a species' niche (Sterner and Elser, 2002; Elser et al., 2003; Newsome et al., 2007; Carnicer et al., 2015). The chemical composition of an organism (isotopic, elemental, and molecular) is determined by the resources it consumed and the environmental conditions it experienced, as constrained by its nutritional and metabolic requirements. Importantly, these nutritional and metabolic requirements can often be expressed in only a few well-established dimensions, potentially resolving some of the ambiguity in selecting traits. In particular, isotopic ecology (Layman et al., 2007; Newsome et al., 2007; Jackson et al., 2011; Cucherousset and Villéger, 2015; Swanson et al., 2015), the geometric framework of nutrition (Simpson and Raubenheimer, 1993; Raubenheimer and Simpson, 2004; Raubenheimer et al., 2009, 2015; Kearney et al., 2010; Raubenheimer, 2011), and ecological stoichiometry (Elser et al., 2000; Peñuelas et al., 2008, 2010) have all represented the ecological niche using the chemistry of living organisms.

A number of metrics originally developed by functional ecologists to quantify trait functional diversity have been repurposed and applied in stable isotope ecology research (Rigolet et al., 2015; Comte et al., 2016). Like traditional traits, isotopic traits can be fit with a convex hull, the size of which has been extensively used as a proxy of the size of the trophic niche occupied by a group of individuals (Layman et al., 2007, 2012; Cucherousset and Villéger, 2015). Similarly, the geometric framework of nutrition (NGF) has been used to build multidimensional models of animal macronutrient budgets (i.e., multidimensional nutritional niche) in which information on food macronutrient contents (i.e., proteins, carbohydrates, lipids), animal macronutrient requirements, and animal nutritional processes such as macronutrient intake, growth and macronutrient use, are integrated, modeled and visualized as three macronutrient axes (Raubenheimer et al., 2015; Machovsky-Capuska et al., 2016. Although this approach has been extremely useful to improve our understanding of the foraging behavior, post-ingestion allocation of macronutrients, and the dietary niche breath of a large diversity of animals, it is a data hungry approach and some of the required nutritional descriptors are restricted to specific taxonomic groups—such as animals but not plants. Further, it does not allow for a formal empirical or theoretical application of the laws of conservation of matter to link the chemical niche of individual organisms to ecosystem-level processes (Sperfeld et al., 2016, 2017). This imposes some limitations on the use of NGF to integrate and describe entire ecological communities, and to identify links between chemical phenotypes and ecosystem processes (Sperfeld et al., 2016). Here we propose a new way to chemically describe the niche—the multidimensional stoichiometric niche, based on ecological stoichiometry theory—which may be even more useful.

THE MULTIDIMENSIONAL STOICHIOMETRIC NICHE

All living organisms share biochemistry based on carbon (C), nitrogen (N), phosphorus (P), and other chemical elements (Elser et al., 2000; Sterner and Elser, 2002). However, organisms differ widely in the proportion of these elements in their biomass, both across taxonomic groups and trophic groups (Elser et al., 2000; Sterner and Elser, 2002). Ecological stoichiometry (henceforth ES: Sterner and Elser, 2002) studies the balance of energy and multiple chemical elements in ecological interactions, and assumes that the elemental composition of living organisms reflects their demands for chemical elements and the degree of elemental homeostasis. The demands of chemical elements largely depend on the individual investment in structural resources such as C-rich cellulose and lignin by plants, C-and N-rich chitin and muscle by arthropods, and P-rich bones by vertebrates, but are also influenced by physiological processes such as growth and reproduction (Sterner and Elser, 2002; Elser et al., 2003; Leal et al., 2017). Similarly, the different degrees of elemental homeostasis among living organisms (e.g., plants and animals) can determine important variation in organismal stoichiometry (Sterner and Elser, 2002; Persson et al., 2010). Therefore, stoichiometric traits are defined here as the composition, assimilation, allocation, or excretion of elements at the individual level, quantities that are usually assessed by

the elemental content or elemental ratios of living organisms (Leal et al., 2017). Overall, stoichiometric trait variation, just like variation in any other trait, can be scaled-up from individuals to entire communities (Violle et al., 2007).

As with other quantitative traits, elemental composition and variation can be represented in a multivariate space (Sterner and Elser, 2002). Thus, each axis can represent the quantity (e.g., proportion) of a chemical element in the body of an organism, which allows a hypervolumetric visualization and analysis of the niche and trait distribution at multiple scales (groups of individuals, entire communities or regional pools of species; see Villéger et al., 2008; Cucherousset and Villéger, 2015; Carmona et al., 2016). This elemental view of a living organism has been called the elemental phenotype, which could include the ∼25 elements composing the biomass of living organisms (Jeyasingh et al., 2014; Leal et al., 2017). Each of these chemical elements play key roles in the chemistry of life ranging from biomolecules and organelle structure to its life history (Sterner and Elser, 2002), and consequently the elemental phenotype is thought to be shaped by the variation in classic traits (e.g., growth rates, body size) related to the structural and functional demands of individuals (Sterner and Elser, 2002; Hessen et al., 2013; Jeyasingh et al., 2014). Although the elemental phenotype approach goes beyond the mere quantification of the elemental composition of an organism, it relates to the niche by considering an integrated and multidimensional view of the elemental make-up of life. This multiscale approach—from individuals within a species to entire communities—allows ecologists to determine how much different levels of biological organization contribute to overall trophic and resource diversity.

The ES focus on chemical elements has significant advantages over the nutritional niche framework for analyses of ecological niches. The ES perspective provides a common currency that facilitates comparisons across diverse taxonomic groups, ecological levels of organization (individuals, populations, communities or regional pools of species), and habitats (e.g., terrestrial, freshwater), and links the elemental phenotype to community and ecosystem-level processes. The elemental phenotype view is not new; in fact plant ecologists previously described it as the "biogeochemical niche" (Peñuelas et al., 2008, 2010). The biogeochemical niche represents a species position in multivariate space as defined by elemental content (sensu Peñuelas et al., 2008). Much of the progress done by plant ecologists on this topic has been made possible because elemental traits in plants show strong responses to environmental gradients, with consequences for plant fitness. As with isotopic niches, biogeochemical niches have been quantified for individual species, based on the mean value of plant elemental traits (Peñuelas et al., 2008, 2010; Violle and Jiang, 2009), and scaled-up to describe entire plant communities (Kerkhoff and Enquist, 2006). Although the elemental composition of living organisms (plants, animals and microorganisms) is known to shape their ecology and influence the structure and functioning of ecological systems (Hall, 2009; Hawlena et al., 2012), similar analyses of the elemental niche of entire communities, including their size and overlap, remain largely unexplored. Therefore, further progress can be made by the development of an approach that allows the description and comparison of the biogeochemical niche across all taxa (e.g., bacteria, fungi, plants, and animals) and habitats (e.g., aquatic and terrestrial), as well as across observational gradients or experimental manipulation conditions.

Borrowing some ideas from the "biogeochemical niche" as defined by Peñuelas et al. (2008) and Sardans and Peñuelas (2013), and adapting metrics developed for the analysis of stable isotope data and functional trait diversity (Layman et al., 2007; Cucherousset and Villéger, 2015), we develop here the multidimensional stoichiometric niche approach for ecological stoichiometric research. Our approach expands from Peñuelas et al. (2008) who described and analyzed the "biogeochemical niche" for plants entirely based on the position of species in multivariate space using principal component analysis (PCA). We define the stoichiometric niche as the region of multivariate niche space occupied by a group of individuals where the axes represent their elemental content, but the stoichiometric niche could also be extended to incorporate nutrient recycling rates (**Table 1**). Incorporating element fluxes and transformation rates as additional or alternative axes describing the multidimensional stoichiometric niche, can provide a more complete picture of the relative importance of species redundancy in elemental cycling at the ecosystem level (**Table 1**).

We propose that the multidimensional stoichiometric niche offers a powerful and unified framework to perform analyses of elemental composition data across taxa and habitats in ecological stoichiometry. Importantly, this concept allows researchers to examine how different levels of biological organization affect the stoichiometric trait space occupied by entire food webs, how organisms differ in their stoichiometric niches based on ecological role (functional group, trophic level), and how entire food webs are affected by habitat and exposure to stressors (see section Discussion and Perspectives, **Table 1**). Here we convert some metrics developed earlier for stable isotope and functional trait data (mainly based on traditional traits such as growth, survival, photosynthetic rate; Violle et al., 2007) into a set of stoichiometric metrics based on the elemental content of individuals or groups of individuals within communities, which together describe the multidimensional stoichiometric niche. These metrics are as follows:

(i) Stoichiometric niche volume: Also known as convex hull volume, this metric measures the amount of elemental space filled by a group of organisms. This space represents the stoichiometric diversity of a group of organisms within a population, functional group or whole community. For example, a small stoichiometric niche volume would indicate that individuals of a particular group (e.g., species, trophic group) exhibit low intra-group variation regarding their elemental traits.

(ii) Stoichiometric niche overlap and nestedness: Both of these indices allow comparison of the position and size of the stoichiometric niches between groups of organisms. Basically, they provide information about how similar (i.e., redundant) organisms or groups of organisms are in terms of their stoichiometry. While stoichiometric niche

TABLE 1 | Examples of questions that could be addressed using the multidimensional stoichiometric niche approach, including the biological, spatial and temporal scales involved, and the associated tests to be conducted.

Biological scales	Spatial scales	Temporal scales	Tests in stoichiometric space
Intraspecific	Local	<life cycle	Compare rarefied and absolute species-specific niche sizes
Q1. *How much does intraspecific elemental variation contribute to the realized niche of a species?*			
Intraspecific	Local	>life cycle	Evaluate niche size, shape and position of the population before and after the stressor removal
Q2. *To what extent is the stoichiometric niche of a population impacted by a given stressor (e.g., drought, nutrient fertilization, species invasion)?*			
Intraspecific	Global	<life cycle	Determine changes in consumer-resource niche overlap across a broad spatial domain
Q3. *To what degree is the spatial distribution of a consumer's stoichiometric niche determined by that of its resource?*			
Intraspecific	Global	<life cycle	Assess the ratio of population-level niche size to metapopulation-level niche size
Q4. *How much does the stoichiometric niche of a population contribute to that of a metapopulation?*			
Interspecific	Local	<life cycle	Quantify the amount of overlap between the niches of consumers and resources
Q5. *How stoichiometrically balanced is a resource-consumer interaction?*			
Interspecific	Local	>life cycle	Evaluate niche size, shape and position of the food web before and after the stressor removal
Q6. *To what extent the stoichiometric niche of a food web is impacted by a given stressor (e.g., drought, nutrient fertilization, species invasion)?*			
Interspecific	Global	<life cycle	Compare niche sizes, shapes, and relative position across habitat types
Q7. *How do food webs differ among habitat types (e.g., aquatic vs. terrestrial, natural vs. agroecosystems)?*			
Interspecific	Global	<life cycle	Compare niche overlap among species, functional groups or trophic groups. Overlap indicates stoichiometric redundancy
Q8. *How does species, functional or trophic diversity within food webs affect the ability of food webs to exploit stoichiometric space?*			
Interspecific	Global	>life cycle	Compare niche overlap at varying taxonomic resolutions; compare niche size and shape between groups at each resolution
Q9. *How does phylogenetic relatedness constrain the stoichiometric niche of species (e.g., among genera, families, orders)?* How do phylogenetic differences between species result in niche differentiation (e.g., Chordata vs. Arthropoda)?			

To simplify, each scale has two modalities: biological = intraspecific (within and among individuals within a species including populations) vs. interspecific (among species including communities, metacommunities, and ecosystems); spatial = local (< plot scale) vs. global (including landscape, regional and global scales); temporal = < life cycle vs. > life cycle (relative to the lifespan of focal individuals at the intraspecific level or to the species with the longest lifespan in a given community at the interspecific level). Questions are arranged primarily according to increasing biological scales, and secondarily, according to increasing spatial scales.

overlap can reveal the degree to which certain elemental composition may be displayed by only few individuals or group of individuals in a community, stoichiometric nestedness allows differentiating between two potential stoichiometric niche overlap patterns. These two patterns are: (1) the overlap that occurs when groups or organisms share a similar portion of the stoichiometric niche volume; and (2) the overlap that occurs when one group of organisms occupies a subset of the stoichiometric volume occupied by the other group (see Carmona et al., 2016). For instance, a low overlap and/or nestedness between two groups of organisms indicate that they differ in their elemental traits (i.e., high elemental complementarity).

(iii) Stoichiometric niche shape: This refers to the contribution of variation in each of C, N and P to the stoichiometric niche of a group of organisms, and can be assessed by ordination techniques like PCA. When there is strong covariance between two elements, and the collinearity of these elements is important in defining niche shape (e.g., both elements load together on one PCA axis), the dimensionality of the niche may be reduced from three to two. For example, ES theory suggests that organisms have constrains in their elemental ratios, which would induce such collinearity. Thus, stoichiometric niche shape may be underlain by fundamental biological constraints. This metric reflects the "biogeochemical niche"approach developed by Peñuelas et al. (2008).

To illustrate the applicability of the multidimensional stoichiometric niche approach, we used data on C, N, and P content of several terrestrial and freshwater communities

composed by multiple trophic groups (from primary producers and detritivores to carnivores) and species. We use these data to answer Questions 1, 7, 8, and 9 in **Table 1**. First, we calculated the niche volumes in stoichiometric trait space occupied by the aquatic and terrestrial food webs, trophic groups, and individual morphospecies (hereafter "species"). Then we evaluated several aspects of the stoichiometric niche space (i.e., niche size, niche overlap and nestedness), which provide information of the stoichiometric complementarity within and among these groups. Finally, we identify key limitations of this approach, and discuss perspectives that may facilitate the development of quantitative intra and interspecific comparisons, and food-web approaches using stoichiometric data.

MULTIDIMENSIONAL STOICHIOMETRIC NICHE IN PRACTICE: DEMONSTRATION ANALYSIS USING TERRESTRIAL AND AQUATIC FOOD WEBS

As an example of the multidimensional stoichiometric niche, we analyzed entire terrestrial and aquatic food webs. The whole data set includes 73 species (35 families and 18 orders) belonging to multiple trophic positions along three axes: C, N, and P content. The terrestrial and aquatic food webs include, respectively, 33 and 40 species, 20 and15 families, and 13 and 7 orders.

Terrestrial invertebrates and vertebrates associated with *Tillandsia* bromeliads were collected from four sites in the coastal zone of the Atacama Desert (Chile; 20°13′S, 70°8′W) in 2007 and 2009 (González et al., 2011a). Low mean annual rainfall characterizes this area, as the only source of moisture is associated with fog events (Cereceda et al., 2008). The increased air moisture and nutrient deposition in the fog zone permits the development of isolated vegetation "islands." These "islands" are dominated by several terrestrial bromeliad species of the genus *Tillandsia*, which depend exclusively on fog inputs as their primary water source (Pinto et al., 2006), and sustain a diverse community of terrestrial invertebrates (e.g., Coleoptera, Araneae, Solifugae) and vertebrates (e.g., Squamata). At each of the four sites, we collected arthropods and lizards, which compose the terrestrial food webs associated with *Tillandsia* (see González et al., 2011a for sampling details).

Aquatic invertebrates were sampled from tank bromeliads from one site in each of two countries: Estacion Biologica Pitilla (Costa Rica; 10°59′N, 85°26′W), and Cardoso Island (Brazil; 25°03′S, 47°53′W) in 2012 and 2013. Costa Rica is dominated by primary and secondary tropical rain forests with sparse open clearings whereas Cardoso Island corresponds to a "resting" ecoregion (i.e., coastal vegetation located on nutrient-poor sand deposits; Magnago et al., 2012). Tank bromeliads are flowering plants that accumulate rainwater in their leaf rosettes creating an aquatic habitat—from a few milliliters up to several liters of water per plant—for a diverse community of macroinvertebrates (e.g., Diptera, Coleoptera, Haplotaxida, Ostracoda; Dézerald et al., 2013). Macroinvertebrates were sampled from tank bromeliads in each study site either by dissection of the plant or with a large-mouthed pipette.

Sample Preparation and Chemical Analyses

The guts of the aquatic and terrestrial invertebrates were not removed prior to chemical analyses due to their small size; therefore we kept them alive overnight to allow gut clearance (Evans-White et al., 2005). In contrast, we removed the digestive tract of the lizards prior to analyses. Before chemical analyses, the individuals were counted and identified to the species or morphospecies level (hereafter, "species"). We also classified all organisms by trophic groups (e.g., invertebrate detritivores, vertebrate predators) using information from the literature and field observations (Merritt et al., 2008; Dézerald et al., 2013). Most aquatic organisms were collected as larvae as this is the most common stage of invertebrates living inside tank bromeliads (except for holobiotic organisms: Ostracoda, Arhynchodbellida, and Haplotaxida) whereas terrestrial larvae/juveniles and adults were included in the analyses.

We determined dry mass of individuals using an electronic balance ($\pm 0.1\,\mu g$). We measured the phosphorus content on whole invertebrates (aquatic and terrestrial), and whole lizards, using the persulfate digestion and ascorbic acid methods (APA, 1992). Prior to digestion, we gently crushed samples with a Teflon-coated rod. Tissue C and N contents were measured with an elemental analyzer that involves complete combustion of samples (Model Carlo Erba NC2500). For smaller macroinvertebrates (<0.5 mg dry mass), we performed CN analyses on whole individuals. For larger individuals (invertebrates and lizards), we analyzed CN levels in homogenized subsamples from dried individuals that were first crushed with a mortar and pestle. CN analyses were conducted at the Stable Isotope Laboratory at Cornell University, Ithaca, NY. We determined the percent recovery in P assays in aquatic and terrestrial organisms by comparison to apple leaf and bovine muscle standards, respectively, from the National Institute of Standards and Technology, US (NIST-1515/8414). We performed all P analyses using a flow solution auto-analyzer. We use the term "C (or N or P) content" to describe C (or N or P) content as a percent of dry body mass. The C, N, and P contents of primary producers were estimated following the same protocols as described above for invertebrates and lizards.

Elemental-Body Size Scaling Relationships

Because all three elements (C, N, and/or P) could not be consistently obtained for small-sized individuals and/or species, we interpolated missing values using our own body size scaling relationships. About 31% of *C*-values (513/1,673), 29% of *N*-values (483/1,673), and 35% of *P*-values (589/1,673) were estimated, whereas the rest of the C, N, P values (∼75%) were directly measured. To estimate these elemental values, we performed ordinary least square regressions on each element (i.e., C, N, or P) and the log-transformed body size (mg dry mass) of individuals from any given species (Legendre, 2014; Finkel et al., 2016). Only significant relationships were used to interpolate missing values (Supplementary Tables S1–S3). When scaling relationships were not significant and/or the species had

less than five individuals, we used significant coefficient estimates from higher taxonomic levels (e.g., genus, family).

Individual-Based Analysis of Stoichiometric Niches

To evaluate the stoichiometric niche of species, trophic groups, and food-web types (aquatic vs. terrestrial), we calculated the respective convex hull volumes or stoichiometric niche volumes using individual-based coordinates in the multidimensional stoichiometric trait space. Specifically, we used a three-dimensional space constituted by three traits or axes, namely, C, N, and P contents. The relative size of niche volumes in functional space has broad ecological implications (Cornwell et al., 2006; Villéger et al., 2011). For instance, small niche volumes indicate that individuals are constrained to a limited range of the functional space and only represent a subset of all traits in the community or food-web type. In the context of stoichiometric niches, this would mean that those individuals exhibit low variation regarding their elemental content. In a three-dimensional stoichiometric space, as in our study, the convex hull volume of a stoichiometric niche may be represented as a polygon where individuals with extreme coordinates define its edges, vertices, faces and overall shape. However it is difficult to visually detect differences in polygon volumes when they also differ in shape (e.g., elongated *vs.* rectangular polygons). Instead, we chose to depict the convex hull volumes as spheres with volumes equal to those of the polygons, which are centered on the average coordinates of all individuals within a given group (e.g., trophic group, food web type).

We evaluated the redundancy of stoichiometric niches, between species, trophic groups, and food-web types, by measuring the percent overlap among niches (Villéger et al., 2011; Brandl and Bellwood, 2014). Specifically, niche overlap between two groups was calculated as the ratio of the niche volume in common to the combined unique niche volume (i.e., sum of the two niche volumes minus the volume of the intersection). Thus, individuals will have complementary niches (i.e., low redundancy), if they display a low percent overlap. A low percent overlap can, however, result from two large niche volumes overlapping marginally (pattern 1) or from a small volume being nested within a larger niche volume (pattern 2). Although in both cases the niche overlap may be low, the ecological implications of these two patterns are not. In pattern 1, most individuals of the two groups display dissimilar trait values, and only few individuals of each group share their traits values, suggesting that the groups do not need to compete for the same resources. In contrast, in pattern 2, most individuals of the group with the smaller niche size (group A) fill only a subset of the volume occupied by the group with the larger niche size (group B). This implies that individuals of group A display only a fraction of the trait values exhibited by the individuals of group B, suggesting that A displays less intraspecific trait variance. To discriminate between patterns 1 and 2, the nestedness of two niche volumes can be calculated as the ratio between the overlapped niche volume and the minimal niche volume occupied by a group (Villéger et al., 2013). The nestedness component varies between 0 and 1, where 0 means that niche volumes do not overlap, and tends to 1 when a given niche volume is nested and occupies a small portion of a larger niche volume displayed by a second group.

Finally, the last step in describing stoichiometric niche volumes is to assess which traits (C, N, P axes of the multidimensional stoichiometric space) or combination of traits drive variation in the overall shape of those niche volumes. We thus performed Principal Component Analyses (PCA) on the individuals of each species, functional feeding groups, and food webs. PCA allows multivariate data compression into its main orthogonal features by displaying the data into a lower dimensional space (Janžekovi and Novak, 2012). In other words, PCA axes are defined by the stoichiometric traits that best explain the niche shape in the multidimensional space. We thus expected spherical niche shapes to display an equal contribution of each axis to the overall variance in stoichiometric composition of a group. In contrast, other niche shapes could be driven by one or a combination of stoichiometric traits, which suggests that these organisms may have an increased allocation of a particular element or a combination of elements to structural or physiological processes (trait trade offs).

Intraspecific Analyses of Stoichiometric Niches

To further exemplify our approach, we repeated all analyses described above at the intraspecific level. To this end, we selected the most abundant morphospecies in our dataset (Arachnida, Solifugae, Ammotrechidae sp.; $N = 141$ individuals). This species is a terrestrial predator of ground-dwelling arthropods in arid environments, and so its elemental composition may reflect that of the food web below it. This species is also widespread, having been captured in all four sampled sites in the coastal Atacama Desert (northern Chile; see González et al., 2011a,b for site details). Ammotrechidae sp. thus presents ideal characteristics to evaluate variations in stoichiometric niches at the intraspecific level: high abundance and four geographically distinct populations. First, we determined whether the stoichiometric niche size of Ammotrechidae sp. significantly differs from random. Random niche volumes were generated by subsampling the elemental content (C, N, and P) of 141 individuals from the total pool of invertebrates, and by calculating the resulting niche volumes using 999 randomized permutations. Therefore, null models reflected the natural abundance of Ammotrechidae sp. (see next section). The observed niche volume was then compared to the cumulative distribution of the 999 random niche volumes and considered not significantly different from random if it fell between the 2.5 and 97.5th percentiles of that distribution. Second, we calculated all stoichiometric niche metrics (i.e., size, nestedness, and shape) for each of the four populations of Ammotrechidae sp., as previously described, in order to examine intraspecific variation in stoichiometric niches.

Effect of Sample Sizes on Niche Volumes

The total niche volume of a given species or functional group or food web is influenced by the number of individuals with the

most extreme positions in the multidimensional stoichiometric space. Theoretically, an increase in the number of randomly sampled individuals is associated with a higher probability of finding individuals with extreme positions, and thus of estimating the "true" population-level niche volumes of that species (Brandl and Bellwood, 2014). This may occur until a threshold number of individuals or is reached, after which randomly sampling individuals does not further influence the niche volume. Whether this threshold is common or unique to species remains unresolved, and is central to debates on the importance of intraspecific trait variation (Q1 in **Table 1**). Yet, the importance of sample size must be addressed as species in nature differ according to their relative abundances. A common method to account for different sample sizes among species is to sample a given subset of individuals (e.g., 30 individuals) and to calculate the resulting niche volume using 999 randomized permutations within each species (Brandl and Bellwood, 2014). This rarefaction method may, however, underestimate the hypothetically "true" niche volume (e.g., all individuals within a population) of more abundant species compared to species with fewer individuals. To tackle this issue, we first evaluated, with linear regression, whether the number of individuals in each species was positively correlated to the log-transformed niche volumes of species. It is important to note that we sampled species proportionally to their natural abundances, so the niche volumes uncorrected for sample size are our best estimate of population-level niche volume. Second, we subsampled an increasing number of individuals at regular intervals (e.g., every 20, 40, and 100 individuals from each species, trophic group, and food webs, respectively), and calculated the resulting niche volumes using 999 randomized permutations. We repeated this procedure until nearly all individuals from a species (or trophic group or food web) were sampled. We then quantified the range of variation in randomized niche volumes using 95% confidence interval (i.e., within ∼ 2 Standard Deviations from the mean of randomized permutations), and compared this range with the population-level niche volume based on all sampled individuals. Statistical analyses were performed using the packages *vegan*, *geometry*, *car*, *coin* and the function CHVintersect (Villéger et al., 2013) in the R-software V3.2.1 (R Development Core Team, 2015). Data from our case of study and all code (statistics and graphics) used to quantify the multidimensional stoichiometric niche is provided in the Supplementary Information to allow reproduction of our approach.

RESULTS OF A CASE STUDY: MULTIDIMENSIONAL STOICHIOMETRIC NICHE IN PRACTICE

Stoichiometric Niches of Primary Producers, Macroinvertebrates, and Vertebrates

We first examined phylogenetic effects on the stoichiometric niche (Q9 in **Table 1**). Primary producers, macroinvertebrates, and vertebrates occupied <1.0, 22.5, and 3.3% of the total niche volume, respectively, with ∼74.0% of the total elemental space being unoccupied by any group. The total stoichiometric niche volume was here calculated as the total convex hull volume encompassing all individuals regardless of taxonomic identity, trophic group and food web type (aquatic vs. terrestrial). This large amount of unoccupied space was due to the individuals being highly aggregated within each trophic group with no stoichiometric overlap among primary producers, macroinvertebrates, and vertebrates (**Figure 1**). Although we approximated niche volumes as spheres in **Figure 1**, there are a number of points that fall outside these spheres, especially for primary producers, so we used separate PCA analyses of each group to explore which elements determined the actual shape of niches. PCA analyses indicate that the stoichiometric niche volume of primary producers was driven by C and N content, which varied in opposite directions along Axis 1 (47.7% of explained variance), and by P content, which correlated with Axis 2 (**Figure 2**; Axis 2 = 30.9% of explained variance). For invertebrates, the niche volume was mainly driven by variation in N and P content (both negatively correlated to Axis 1), and by C content along the Axis 2 (Axis 1 and 2 = 44.9% and 31.8% of explained variance; **Figure 2**). In contrast, for vertebrates, both C and N content were negatively correlated to Axis 1 (61.8% of explained variance), while P content was positively correlated to Axis 2 (28.2% of explained variance; **Figure 2**).

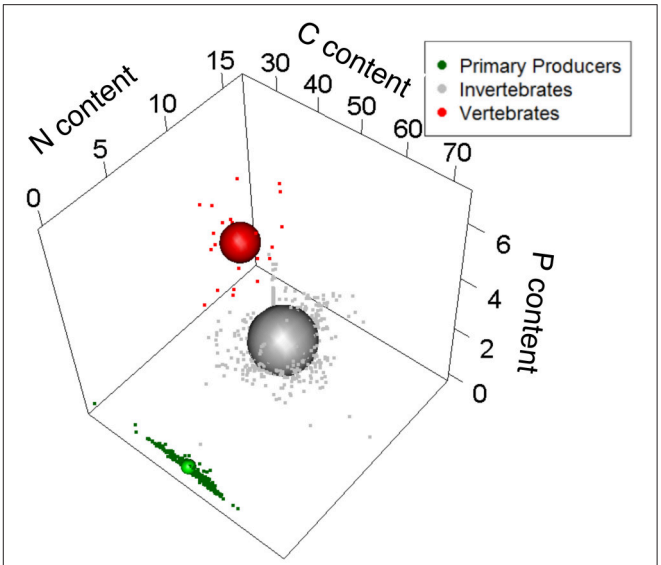

FIGURE 1 | Relative size of stoichiometric niche volumes of primary producers, invertebrates, and vertebrates (combining multiple terrestrial and aquatic food webs from bromeliads). Sphere sizes depicts the volumes of the stoichiometric niches, and are centered around the average C, N, and P contents of all individuals from those variables. Dots are individual coordinates and show the variability within a given stoichiometric niche. Axes represent each element as a percentage of dry body mass.

Stoichiometric Niches of Terrestrial vs. Aquatic Food Webs

To compare niche volumes among terrestrial and aquatic food webs (Q7 in **Table 1**), subsequent analyses were focused on

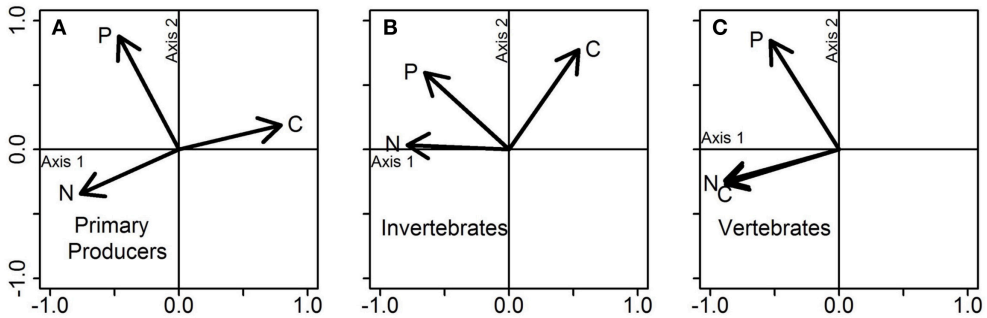

FIGURE 2 | PCA on the stoichiometric traits of **(A)** primary producers, **(B)** invertebrates, and **(C)** vertebrates. Each plot represents a PCA conducted separately for each group of organisms, using the data displayed jointly in **Figure 1**. Vectors show the influence of the three stoichiometric traits (C, N, and P contents) on each PCA axis.

invertebrate individuals. In this study, terrestrial organisms spend their entire life cycle on land. Aquatic organisms are represented by species that either spend their entire life cycle within the water or have complex life cycles (i.e., involving an aquatic larval stage and a terrestrial adult). For the later, we only have stoichiometric data for the aquatic stages. Hereafter, the total stoichiometric niche space refers to the total niche space occupied by all invertebrates regardless of taxonomic identity, trophic group and food-web type (aquatic or terrestrial). We found that terrestrial and aquatic invertebrate food webs shared *ca.* 38.0% of the volume occupied by their respective niches, where terrestrial individuals occupied a slightly larger volume (57.1%) of the total stoichiometric niche space than aquatic ones (50.0%; **Figure 3**). The low nestedness value (= 0.038, with 1 representing full nestedness and 0 no nestedness) between terrestrial and aquatic invertebrate food webs further confirms their relatively high complementarity in stoichiometric traits. In addition, the niche volume of terrestrial invertebrates was driven by covariation in N and P contents (both negatively correlated to Axis 1; 49.1% and Axis 2; 32.0% of total variance explained), while C and N content explained most of the variation in the stoichiometric composition of aquatic invertebrates (positively and negatively correlated to Axis 1; 53.2% and Axis 2; 32.7% of total explained variance; **Figure 4**).

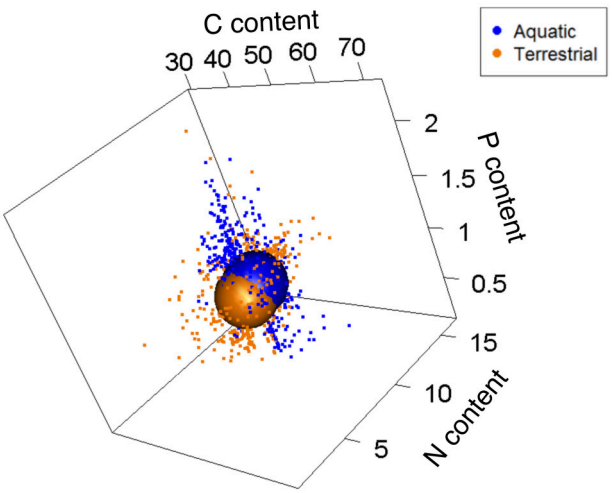

FIGURE 3 | Relative size of stoichiometric niche volumes of terrestrial and aquatic food webs (only invertebrates included). Sphere sizes depicts the volumes of the stoichiometric niches, and are centered around the average C, N, and P contents of all individuals from those variables. Dots are individual coordinates and show the variability within a given stoichiometric niche. Axes represent each element as a percentage of dry body mass.

Stoichiometric Niches of Invertebrate Trophic Groups

Our third example question concerns the contribution of trophic diversity to food web stoichiometric niches (Q8 in **Table 1**). Averaging over food-web type, the stoichiometric niche of herbivores (30.7% of total stoichiometric niche space) was smaller than the niche of detritivores (47.0%), while invertebrate predators displayed the highest niche volume (54.1%) (**Figure 5**). The highest percent overlap in stoichiometric niches was found between detritivores and predators (61.5%), while detritivores and herbivores shared 32.0% and herbivores and predators shared 29.1% of their niche space. Nestedness was low between the three trophic groups, with nestedness values between 0.07 and 0.16. Herbivores differed from detritivores and predators in their niche shape. The niche shape of herbivores was defined by correlations of C and N content with Axis 1 (51.5% of explained variance), while P content was positively correlated to the Axis 2 (28.7% of explained variance) (**Figure 6**). By contrast, the niche shape of detritivores and predators were driven primarily by the positive and negative correlations of C and N content, respectively, with Axis 1 (49.8 and 51.6% of explained variance for detritivores and predators respectively), and secondarily by P content (Axis 2; 33.3% and 26.0% of explained variance for detritivores and predators respectively).

Similar patterns were seen when we examined trophic groups within each food-web type (**Figure 7**). Overall, each trophic group in the various food-web types shared *ca.* one third of its stoichiometric niche with any other trophic group (mean pairwise percent overlap $\pm$ *SE* = 35.0 $\pm$ 2.6) and displayed relatively low nestedness values (mean pairwise nestedness values

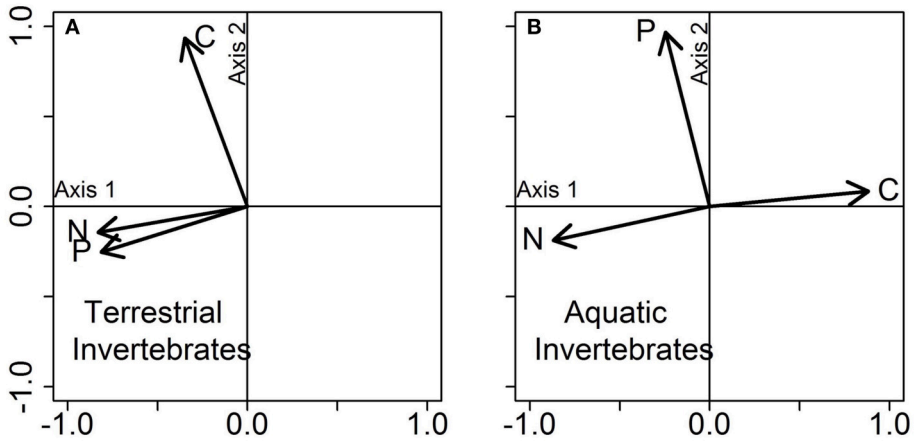

FIGURE 4 | PCA on the stoichiometric traits of **(A)** terrestrial and **(B)** aquatic food webs (only invertebrates included). Each plot represents a PCA conducted separately for each group of organisms, using the data displayed jointly in **Figure 3**. Vectors show the influence of the three stoichiometric traits (C, N, and P contents) on each PCA axis.

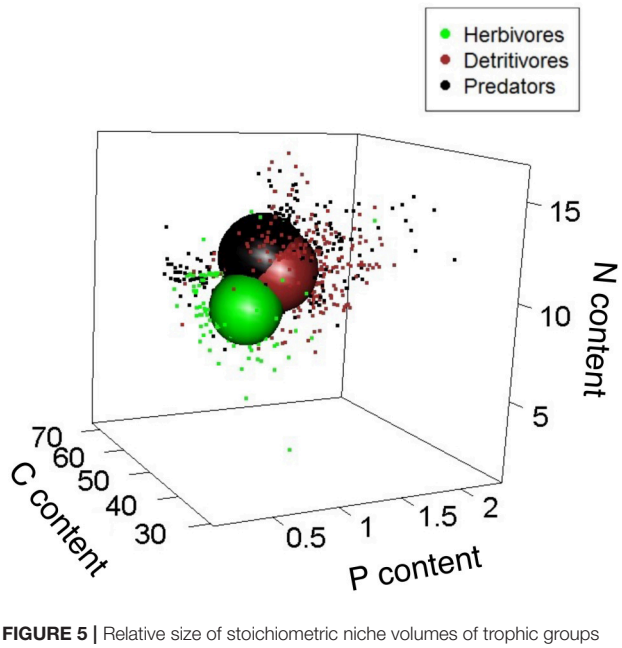

FIGURE 5 | Relative size of stoichiometric niche volumes of trophic groups (only invertebrates included). Sphere sizes depicts the volumes of the various grouping variables, and are centered around the average C, N, and P contents of all individuals from those variables. Dots are individual coordinates and show the variability within a given grouping variable. Axes represent each element as a percentage of dry body mass.

$\pm$ *SE* $= 0.08 \pm 0.02$). Within the terrestrial food web, herbivores (30.7%) and detritivores (18.3%) displayed the largest and smallest niche, respectively. Terrestrial detritivores and predators had the most niche overlap (51.1%) and the highest value of nestedness (0.28), indicating that most individuals of the former group fill only a subset of the space occupied by the latter (**Figure 7**). Within the aquatic food web, predators had larger niches (28.6%) than detritivores (26.1%); predators and detritivores shared 28.1% of their niche and more were weakly nested (nestedness value = 0.02) compared to their terrestrial counterparts.

Our analyses further indicate that the trophic groups could be divided into three groups according to the stoichiometric traits that best explained their differences in niche volumes. The first group is constituted by aquatic detritivores and aquatic predators, whose niche shapes were primarily driven by covarying C and N content, which both correlated negatively with Axis 1 (58.2% and 62.4.1 of the variance for detritivores and predators respectively), and secondarily by P content (32.7 and 24.4% of the variance, positively correlated to Axis 2; **Figures 8A,B**). The terrestrial detritivores and terrestrial predators composed the second group (**Figures 8C,D**). The niche shapes of both functional feeding groups were driven primarily by the positive correlations of N and P content with Axis 1 (43.1 and 40.1% of explained variance respectively for detritivores and predators), and secondarily by C content (Axis 2; 32.1 and 31.9% of explained variance respectively). Note that the niches of the first two groups (aquatic detritivores and predators, and terrestrial detritivores and predators) and the niches of the aquatic and terrestrial food webs are driven by similar stoichiometric traits. The third group was represented by terrestrial herbivores (**Figure 6A**). The niche shape of this group was defined by correlations of C and N content with Axis 1 (51.5% of explained variance), while P content was positively correlated to the Axis 2 (28.7% of explained variance).

Stoichiometric Niche Volumes of Individual Species

We next query the contribution of species diversity to the exploitation of stoichiometric space by food webs (Q8 in **Table 1**). Analyses at the species level were conducted on 37 invertebrate species (N = 22 and 15 terrestrial and aquatic species, respectively) for which we have > 4 individuals (>

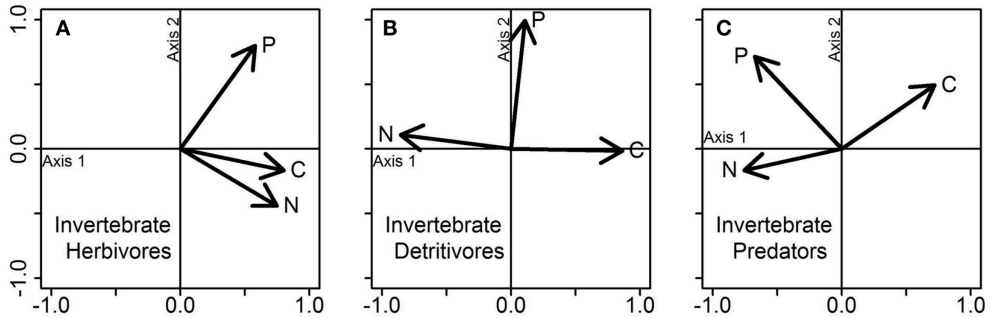

FIGURE 6 | PCA on the stoichiometric traits of **(A)** invertebrate herbivores, **(B)** invertebrate detritivores, and **(C)** invertebrate predators. Each plot represents a PCA conducted separately for each group of organisms, using the data displayed jointly in **Figure 5**. Vectors show the influence of the three stoichiometric traits (C, N, and P contents) on each PCA axis.

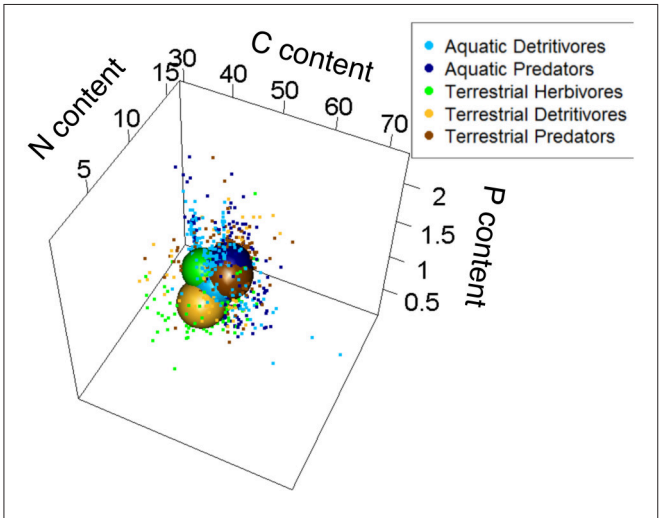

FIGURE 7 | Relative size of stoichiometric niche volumes of aquatic and terrestrial trophic groups (only invertebrates included). Sphere sizes depicts the volumes of the various grouping variables, and are centered around the average C, N, and P contents of all individuals from those variables. Dots are individual coordinates and show the variability within a given grouping variable. Axes represent each element as a percentage of dry body mass.

number of axes in the stoichiometric trait space). Of this 37 species dataset, any two species shared 5.0 ± 0.3% (mean pairwise percent overlap ± SE) of the total volume of their respective stoichiometric niches. The overlap was slightly but significantly higher within terrestrial (6.2 ± 0.7%) than within aquatic (5.7 ± 0.6%) species (permutation-based Wilcoxon and Mann-Whitney test: $Z = 2.86$, $P = 0.004$). Individual species niche volumes varied between <1% and 11.4% of the total stoichiometric niche space occupied by all invertebrates (2.4 ± 0.4% on average), and were not significantly different between terrestrial and aquatic food webs (permutation-based Wilcoxon and Mann-Whitney test: $Z = -0.35$; $P = 0.74$). These relatively small niche volumes are in accordance with a low mean nestedness between any two species (0.11 ± 0.009), suggesting high stoichiometric complementarity among species. Our PCA analyses did not show any consistent patterns in the main drivers of species-level stoichiometric niches.

Intraspecific Analyses of Stoichiometric Niches

Together, all individuals of Ammotrechidae sp. occupied 11.4% of the total stoichiometric niche space. This observed volume was significantly lower than niche volumes generated by chance as it fell outside the range between the 2.5 and 97.5th percentiles of the cumulative distribution of 999 random niche volumes (20.3 and 48.4% for the two percentiles, respectively). Each of the four populations of Ammotrechidae sp. occupied 1.4–3.6% of the total stoichiometric niche space (for populations in Guanacos and Huantajaya, respectively; 2.6 ± 0.47%, mean ± SE; **Figure 9**). These geographically distinct populations of Ammotrechidae sp. shared only ca. 26% of the volume occupied by their respective niches. Nestedness values among populations ranged from 0.04 to 0.26, indicating a wide range of complementarity of these populations in stoichiometric traits (0.12 ± 0.04, mean ± SE).

Finally, the niche volumes of all four populations were driven by different combinations of stoichiometric traits. For instance, N content was always correlated to the PCA Axis 1 of all populations, and explained most of the variations in stoichiometric composition of Huantajaya, Isla, and Pajonal populations (Axis 1: 55.4, 38.9, and 41.8% of total explained variance, for the three sites respectively), whereas the Guanacos population was driven by C content (Axis 1: 49.8% of total explained variance). In addition, P content was correlated with PCA Axis 2 in all but the Isla population, and, therefore, explained a large part of the total variance in those sites (Axis 2: 40.5, 33.0, and 32.5% in Guanacos, Huantajaya, and Pajonal populations, respectively). In contrast, C content of the Isla population was correlated to the PCA Axis 2, and this element explained 35.8% of the total variance (**Figure 10**).

Effect of Sampling Size on Niche Volumes

The population-level niche volume of any species is the result of both intraspecific niche differences as well as the number of individuals, and our last question concerns the relative importance of these two factors (Q1 in **Table 1**). The 35 species

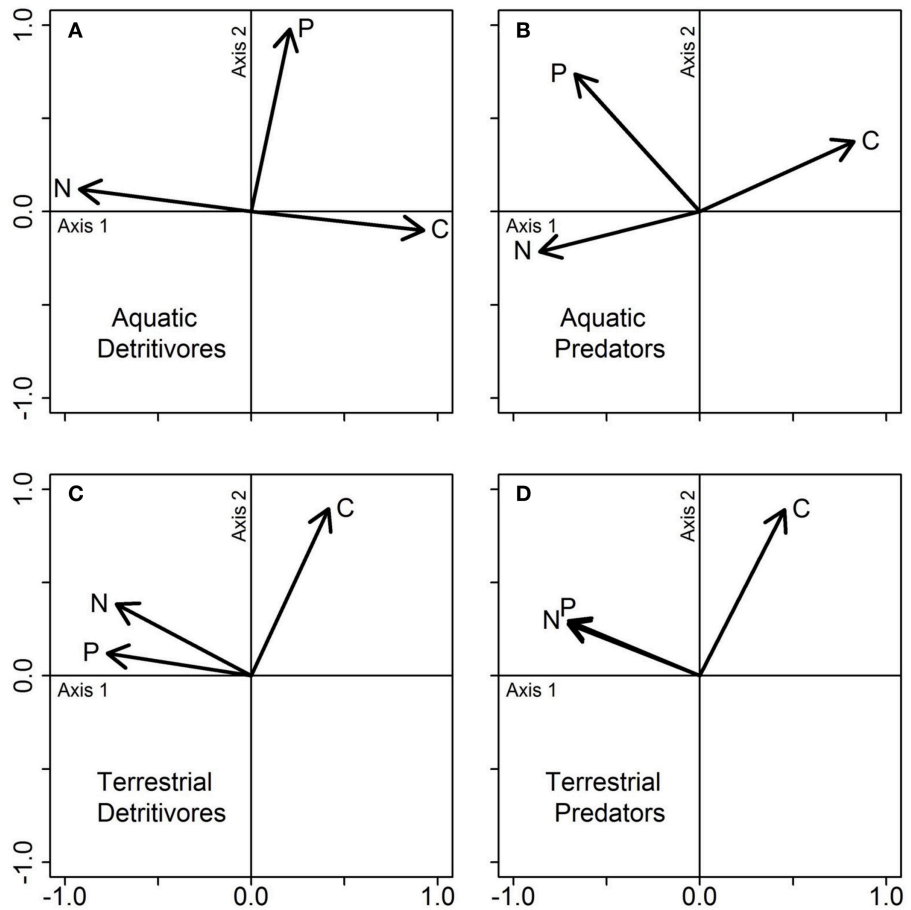

FIGURE 8 | PCA on the stoichiometric traits of aquatic **(A,B)** and terrestrial **(C,D)** detritivores, and predators. Note that terrestrial herbivores are displayed in **Figure 6**. Each plot represents a PCA conducted separately for each group of organisms, using the data displayed jointly in **Figure 7**. Vectors show the influence of the three stoichiometric traits (C, N, and P contents) on each PCA axis.

in our analyses were represented by a variable number of individuals (4–141 individuals/species). Our analyses showed that the number of individuals had a significant and positive influence on the log-transformed species niche volumes ($F = 34.23$; $df = 38$; $P < 0.0001$). This relationship is strongly influenced by species with very few (<19, $N_{sp} = 15$) or many (>120, $N_{sp} = 1$) individuals, as removing such species rendered the relationship insignificant ($N_{sp} = 24$; $F = 3.9$; $df = 22$; $P = 0.06$) despite a six-fold difference in the number of individuals. Our randomized permutation procedure further demonstrates that considering the number of individuals is crucial when evaluating the relative contribution of a set of species (or functional feeding group or habitat) to the total stoichiometric niche volumes. This can be seen as a reorganization of the rank order of groups in terms of niche volume as the number of sampled individuals increases toward the full sample (**Figure 11**). For instance, if we compare the species *Mecistogaster modesta* and *Lepisma* sp. by subsampling 20 individuals from each, we find that the average niche contribution of the former was larger than that of the latter (i.e., no overlap at 95% CI; **Figure 11A**). However, if we base the comparison on all samples (*Lepisma* sp. is more abundant than *M. modesta*, so there are more samples of the former), this difference is erased. In contrast, *Lepisma* sp. contributed more to the total stoichiometric niche space than Salticidae sp. despite having fewer individuals ($N = 91$ and 111, respectively; **Figure 11B**). Finally, the thresholds (i.e., minimum numbers of individuals beyond which the range of variation in randomized niche volumes always included the total volume) were different for every species (**Figure 11A**). Similar effects of sample size were not only found at the species but also at the level of functional feeding groups (**Figure 11B**) and food web types (**Figure 11C**) thus highlighting the importance of basing samples sizes on the natural variation in species abundances.

DISCUSSION AND PERSPECTIVES

The elemental composition of living organisms is of fundamental importance for population dynamics, consumer-resource interactions, food web structure, and nutrient cycling (i.e., effect traits) (Sterner and Elser, 2002; Hall, 2009; Yamamichi et al., 2015), and can mediate eco-evolutionary responses to environmental change (i.e., response traits) (Jeyasingh et al.,

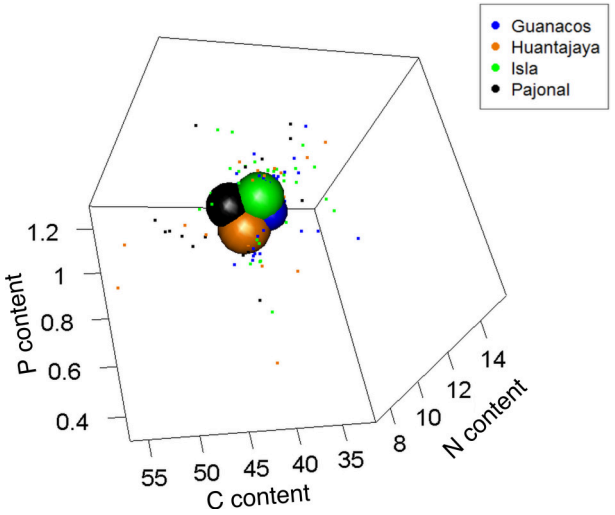

FIGURE 9 | Relative size of stoichiometric niche volumes of four geographically distinct populations of a predatory species (Arachnida, Solifugae, Ammotrechidae sp.). Sphere sizes depicts the volumes of the four populations, and are centered around the average C, N, and P contents of all individuals from those populations. Dots are individual coordinates and show the variability within a given population. Populations are labeled according to the sites where they were collected (Guanacos, Huantajaya, Isla, and Pajonal). Axes represent each element as a percentage of dry body mass.

2014; Leal et al., 2017). Stoichiometric traits, have been measured for a large number of very different species: primary producers (Reich and Oleksyn, 2004; Peñuelas et al., 2008, 2010; Reich, 2014), phytoplankton (Klausmeier et al., 2004; Litchman and Klausmeier, 2008; Quigg et al., 2011), microorganisms (Mouginot et al., 2014; Godwin and Cotner, 2015), invertebrates (Fagan et al., 2002; Woods et al., 2004; Hambäck et al., 2009; González et al., 2011a; Wiesenborn, 2011, 2013; Lemoine et al., 2014), and vertebrates (Torres and Vanni, 2007; González et al., 2011a), allowing across-species comparisons. For plants, studies on functional traits have revealed the existence of adaptive trait continua, which describes the phenotypic space of trait variation produced by evolutionary processes (Donovan et al., 2011; Carnicer et al., 2015; Diaz et al., 2016). The observed inter-specific variation in stoichiometric traits (i.e., C, N, and P contents) along these trait continua has shown strong trait co-variation between N and P (Carnicer et al., 2015). However, lacking in this framework are studies of whole communities focused on the relationships between major axes of variation in stoichiometric traits.

The multidimensional stoichiometric niche approach proposed here extends Hutchinson's (1957) concept of niches as n-dimensional hypervolumes in order to describe and compare the stoichiometry of groups of organisms at different scales using data from observational or experimental approaches. Overall, stoichiometric traits provide a common currency to estimate the dimensionality of trophic niche space, and help reduce the number of axis required to characterize community structures. All organisms interacting in an ecological community are composed by the same chemical elements, and these common currencies enable us to estimate trait similarities or differences across taxonomic groups. Several lines of evidence support the idea that differences in the elemental composition of living organisms reflect the evolution of internal and external structures (e.g., muscular, skeletal), as well as differences in organism life histories (e.g., Reiners, 1986; Sterner and Elser, 2002), and these stoichiometric differences exert strong influences on individual fitness, ecological interactions and ecosystem functioning (Hall, 2009; Leal et al., 2017). For example, the fitness of consumers can be directly related to the mismatch between their body's own elemental composition and that of their resources (Sterner and Elser, 2002). This mismatch can be elegantly encapsulated as the distance and consequent low overlap between the stoichiometric niches of consumers and resources (Q5 in **Table 1**). The multidimensional stoichiometric niche also provides a means of defining the elemental composition of an entire food web in more than one dimension, thereby capturing both the independent and interactive effects of nutrients on living organisms. This allows us to compare food webs in different habitats, locations, or points in time, in terms of their stoichiometric niches, specifically niche position, size and overlap (Q3, 4, 7, in **Table 1**). For example, Cross et al. (2007) reported the effects of experimental nutrient enrichment on biomass production and pathways of C, N, and P in a detritus-based stream food web. The multidimensional stoichiometric niche approach developed here could be used to re-analyze these and other data from experimental manipulations and evaluate the extent at which a particular or multiple stressors impact the stoichiometric niche (i.e., niche size, shape and position) of the food web in treated and reference systems. Importantly, this approach provides a novel way to quantitatively assess changes an ecosystem has experienced as a function of global change drivers, and to link such changes to shifts in the biogeochemical processes carried out by organisms (Q2 and Q6 in **Table 1**).

The idea of a multivariate description of the relative elemental content of organisms has been previously suggested and applied in plant ecology (Peñuelas et al., 2008, 2010), providing insights into plant trait differentiation and climate change responses. Here, we expand on those original ideas to describe the stoichiometric niche of entire communities from contrasting ecosystem types (i.e., aquatic vs. terrestrial) and trophic groups. Further, our approach includes novel ways to estimate the stoichiometric niche by borrowing and adapting metrics developed by isotopic ecologists to measure trophic niche (Layman et al., 2007; Jackson et al., 2011; Cucherousset and Villéger, 2015). These metrics have enabled us to compare the size, shape and position of the stoichiometric niche at different ecological levels, from a species niche to communities. We propose that this direct quantification of a multidimensional niche across ecological scales is possible from a set of elemental measurements of the individuals composing ecological communities, regardless of their taxonomic identity. Further, as stoichiometric differences within and between species are widely recognized, this approach enables us to quantify the stoichiometric niche of a single species up to that of entire communities.

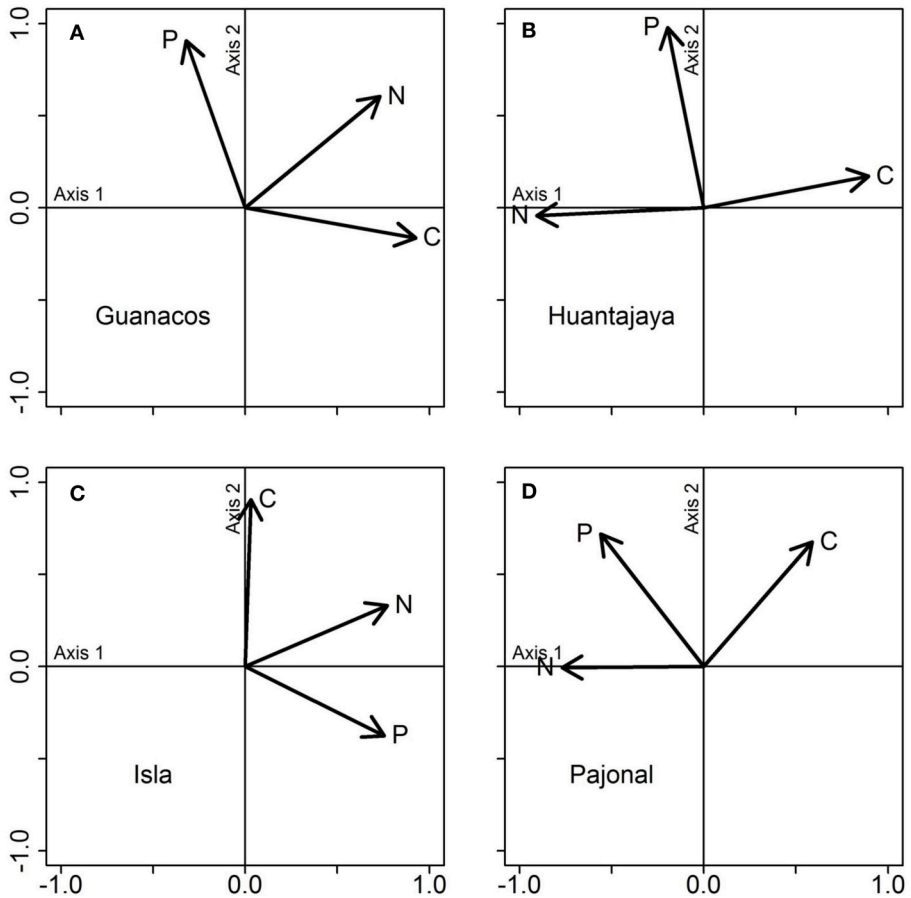

FIGURE 10 | PCA on the stoichiometric traits of four geographically distinct populations of a predatory species (Arachnida, Solifugae, Ammotrechidae sp.). Each plot represents a PCA conducted separately for each population, using the data displayed jointly in this figure. As in this figure, populations are labeled according to the sites where they were collected: Guanacos **(A)**, Huantajaya **(B)**, Isla **(C)**, and Pajonal **(D)**. Vectors show the influence of the three stoichiometric traits (C, N, and P contents) on each PCA axis.

Although the primary purpose of the case study was to demonstrate the use of the multidimensional stoichiometric niche approach, it also provides some insights into the concept of stoichiometric niche as a tool to describe and compare the stoichiometry of organisms at different scales. The questions we have addressed with this approach include those at the scales of individual morphospecies (intraspecific variation), trophic groups and food-web types, as well as questions that cross scales. Our analyses showed that species-level stoichiometric niches are relatively small and do not overlap much with each other. This suggests that species within terrestrial and aquatic systems exhibit large complementary in their stoichiometric niches. In addition, our analyses at the intraspecific level revealed similar patterns of small niche sizes, low overlap, and different niche shapes among geographically distinct populations of a predatory species (Ammotrechidae sp.). The observed variability in the elemental composition of those populations could be related to the fog nutrient supply that broadly varies across the coastal desert sites, thus affecting primary producers in those areas (González et al., 2011b), and potentially influencing the elemental composition of consumer populations such as Ammotrechidae sp. Finally, the niche size of Ammotrechidae sp. was significantly smaller than expected by chance, suggesting that strong deterministic factors may constrain the elemental composition of those organisms (including biological, environmental, and phylogenetic factors; Elser et al., 2000).

Our analyses also revealed that, although the terrestrial and aquatic food webs differ in both habitat and geographic location, the two food webs shared more than one third of their total niche volume. Thus, as with other quantitative traits, there are both gaps and crowded regions in the elemental space. The gaps suggest that there are fundamental constraints to stoichiometric evolution (Kay et al., 2005; Jeyasingh, 2007); macroscopic life (or at least that subset we sampled) may have only evolved a restricted set of morphological, physiological and cellular strategies for exploiting elemental resources. By contrast, the packed regions of phenotypic space suggest that certain trait combinations may have evolved either repeatedly or deep within phylogenetic trees (Pigliucci, 2007; Donovan et al., 2011). Interestingly, our findings are in agreement with recent worldwide studies on vascular plants, in which the trait hypervolume occupied by these plants is highly constrained

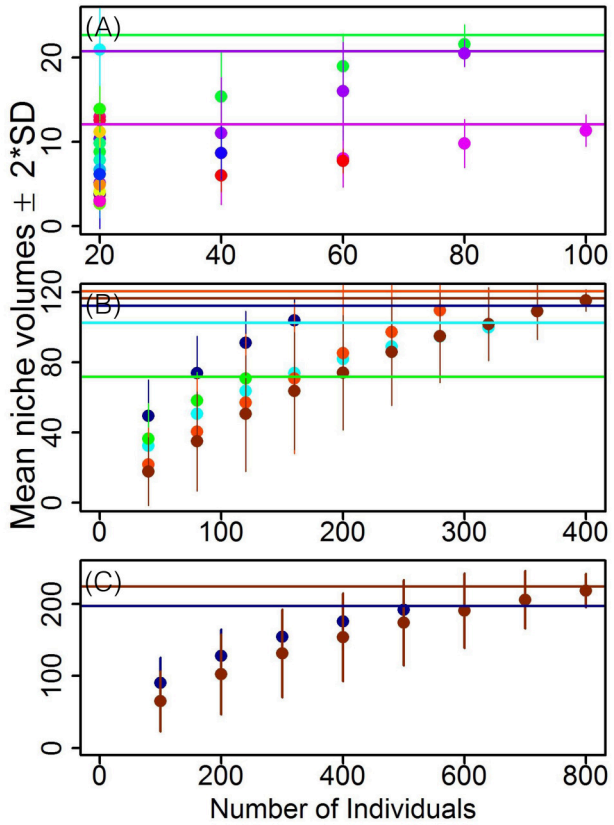

FIGURE 11 | Mean niche volumes (±2*SD ~ 95% CI) after 999 randomized permutations on increasing number of individuals of: **(A)** species; **(B)** trophic groups: aquatic detritivores (light blue) and predators (dark blue), terrestrial herbivores (green), detritivores, (red) and predators (dark brown); and **(C)** aquatic (dark blue) and terrestrial (dark brown) habitats. Horizontal lines indicate the total niche volume of a given grouping variable (i.e., species, trophic groups, habitats). Note that for the sake of visual clarity in **(A)** total niche volumes were shown only for three species that have >80 individuals (i.e., *Lepisma* sp.—green, Salticidae sp.—light purple, and *Pysogaster* sp.—dark purple). In addition, *Mecystogaster modesta* is in light blue (topleft in **A**; cf main text).

within a relatively small plane, representing a small set of successful trait combinations (Diaz et al., 2016). We also found that the size of the terrestrial and aquatic niche volumes are driven by contrasting stoichiometric traits, this is, by covariance in N and P for terrestrial food webs and opposing effects of C and N for aquatic food webs. Although previous analyses have shown differences in elemental compositions between aquatic and terrestrial primary producers (Elser et al., 2000; Cebrian and Lartigue, 2004), our results are remarkable in that aquatic invertebrate food webs in bromeliads are largely fueled by allochthonous inputs of terrestrial leaf litter (Farjalla et al., 2016). Therefore, despite both depending on terrestrial plants, aquatic and terrestrial invertebrates in bromeliads still show large differences in their stoichiometric niches. However, we caution that the terrestrial and aquatic food webs we examined also differ in terms of biome (desert vs. forest), ontogenetic stage (mostly larvae for aquatic and mostly adults for terrestrial) and geographic location (Pacific vs. Atlantic side of the Americas); we hope that the method we demonstrate here will inspire more detailed studies that compare terrestrial and aquatic food webs in the same location. Our analyses also showed that terrestrial herbivores represent a unique group in terms of their stoichiometric niche, distinct from animals that consume detritus or other animals. The differences in stoichiometric niche between herbivores and detritivores are curious, given that both groups consume vascular plants, but may reflect the importance of detrital-based bacteria and fungi in the diet of detritivores (terrestrial and aquatic), plus opportunistic consumption of dead or live animals by detritivores. Potential animal matter consumption by detritivores may explain the large stoichiometric overlap between them and predators.

Our findings also demonstrate that subsampling abundant species may substantially underestimate the actual (population-level) stoichiometric niche occupied by such species in food webs. We thus strongly recommend integrating the natural interspecific variability in abundances rather than trying to standardize the number of individuals per species, as the latter could lead to erroneous estimates of population-level niches and thus misleading estimates of species contributions to the niche of the entire community. This suggestion is contingent on researchers sampling species proportionally to their natural abundance. However, once species differences in stoichiometric niches are established, the role of intraspecific variation in determining niche differences could then be assessed by rarefying samples.

In our case study, we focused on the three elements (C, N, P) that have consistently been shown to constrain species interactions and limit production in ecological communities (Sterner and Elser, 2002). However, if other elements (e.g., Ca, K, S, Fe, Mg) or other physiological (e.g., growth and reproductive rates) and behavioral (e.g., microhabitat preferences) traits are found to further define particular ecological communities, then other dimensions would need to be considered. Hypervolumes that appear to overlap in low dimensions (e.g., C, N, P) may not overlap if more dimensions are included, and conversely, with the addition of extra redundant dimensions, estimates of overlap may be falsely inflated. We could thus expand our current method and include more elements by calculating our metrics in the PCA space. With this approach we can reduce any set of *n* elements to only three principal components, which usually explain most of the variance in the data (i.e., the first three PCA axes) and would allow the calculation of our metrics. The resulting metrics could, then, be compared across studies and ecosystem types if the same set of elements is used to construct the reduced PCA space. Therefore, our focus on C, N and P dimensions provides a useful starting point for broad comparisons.

We think these insights show only a fraction of the potential of possible applications of the stoichiometric niche in ecological stoichiometric research, and that the multidimensional stoichiometric niche can be a general approach for assessing and comparing stoichiometric variation within and among species, populations and communities (**Table 1**). There are numerous potential applications of stoichiometric data to understand

trophic structures especially when used in combination with other trophic-related functional traits, such as body size or with community-level properties such as species numerical abundances. This may offer a powerful and unified framework to assess niche partitioning and food web structures across contrasting ecosystem types and broad biogeographic scales. Such approaches could also be applied to explore individual specialization (Bolnick et al., 2002; Araujo et al., 2011), and to assess how populations and different genotypes vary in their fitness within a multidimensional stoichiometric space (Leal et al., 2017).

Recent research in ecological stoichiometry has highlighted the large intraspecific variation in elemental composition of plants and animals (e.g., González et al., 2011a; El-Sabaawi et al., 2012; Borer et al., 2013; Ebel et al., 2015). Within-species variation in stoichiometric traits can be driven by differences in morphological, physiological, behavioral, and life history traits, as well as predation pressure and spatial-temporal environmental heterogeneity in the quality of resources (Jeyasingh et al., 2014; Leal et al., 2017). The magnitude and origin of stoichiometric trait variation (i.e., phenotypic plasticity or genetic) within populations can be used to link the fitness of any population to changes in resource quality (Leal et al., 2017). For example, if directional selection is acting on stoichiometric traits to reduce elemental mismatches between a population and its resources, we should expect a high overlap between the stoichiometric niches of a consumer and its resources. In contrast, if fluctuating selection is acting upon stoichiometric traits along spatial or temporal variation in resource quality, then the stoichiometric niches of a population should reflect this variation by displaying a larger niche volume. Following Leal et al. (2017), we envision the assessment of how different genotypes vary in their fitness within a multidimensional space, in which stoichiometric trait axes determine the functional space.

Another exciting area of research in niche ecology is the analysis and mapping of stoichiometric niche-environment (i.e., elemental availability of resources) relationships, which can help understand and predict how well the stoichiometric niches of individuals are predicted by environmental gradients.

The integration of stoichiometric models and outcomes from the multidimensional stoichiometric niche could expand and strengthen predictions derived from traditional approaches linking stoichiometry traits to fitness consequences to trophic interactions. Further, as nutrient dynamics are tightly linked to the stoichiometry of living organisms, analyses of niche overlap in the multidimensional space can provide insights into species functional redundancy or complementarity in ecosystem function (e.g., nutrient recycling rates). The stoichiometric niche is therefore a concept with the potential to unify physiological, ecological and ecosystem approaches to understanding the biogeochemical role of life.

ETHICS STATEMENT

Procedures involving animals were approved by the ethics committee of the Pontificia Universidad Católica de Chile and by the Chilean Agriculture and Livestock Service (SAG).

AUTHOR CONTRIBUTIONS

AG developed the concept, designed of the manuscript, and wrote the manuscript. OD contributed on the development of the ideas presented here, analyzed the data, and helped writing the manuscript. PM, GR, and DS contributed to the writing of the manuscript. All authors made substantial contributions to the development of the ideas presented here and commented critically on drafts of the manuscript. This is a contribution of the Bromeliad Working Group.

ACKNOWLEDGMENTS

PM acknowledges support from Grants ICM-MINECON, P05-002 IEB and CONICYT PFB-0023 (Chile). DS acknowledges support from an NSERC E.W.R. Steacie Memorial Fellowship, the field assistance of S. Amundrud, T. Amundrud, P. Corvalan, and L. Zornig, and the logistical support of the Área de Conservación Guanacaste. OD received a postdoctoral fellowship provided by the Biology Department at Rutgers University-Camden. The authors thank the editors for inviting this contribution and the reviewers for helpful comments that improved the manuscript. The authors thank members of the Bromeliad Working Group who aided in sample collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00110/full#supplementary-material

REFERENCES

APA (1992). *Standard Methods for the Examination of Water and Wasterwater, 18th Edn*. Washington, DC: APHA Press.

Araujo, M. S., Bolnick, D. I., and Layman, C. A. (2011). The ecological causes of individual specialization. *Ecol. Lett.* 14, 948–958. doi: 10.1111/j.1461-0248.2011.01662.x

Bolnick, D. L., Yang, L. H., Fordyce, J. A., Davis, J. M., and Svanbäck, R. (2002). Measuring individual-level resource specialization. *Ecology* 83, 2936–2941. doi: 10.1890/0012-9658(2002)083[2936:MILRS]2.0.CO;2

Borer, E. T., Bracken, M. E. S., Seabloom, E. W., Smith, J. E., Cebrian, J., Cleland, E. E., et al. (2013). Global biogeography of autotroph chemistry: is insolation a driving force? *OIKOS* 122, 1121–1130. doi: 10.1111/j.1600-0706.2013.00465.x

Brandl, S. J., and Bellwood, D. R. (2014). Individual-based analyses reveal limited functional overlap in a coral reef fish community. *J. Anim. Ecol.* 83, 661–670. doi: 10.1111/1365-2656.12171

Carmona, C. P., de Bello, F., Wason, N. W. H., and Leps, J. (2016). Traits without borders: integrating functional diversity across scales. *Trends Ecol. Evol.* 31, 382–394. doi: 10.1016/j.tree.2016.02.003

Carnicer, J., Sardans, J., Stephanescu, J. S., Ubach, A., Bartrons, M., Asensio, D., et al. (2015). Global biodiversity, stoichiometry and ecosystem function

responses to human-induced C–N–P imbalances. *J. Plant Physiol.* 172, 82–91. doi: 10.1016/j.jplph.2014.07.022

Cebrian, J., and Lartigue, J. (2004). Patterns of herbivory and decomposition in aquatic and terrestrial ecosystems. *Ecol. Monogr.* 74, 237–259. doi: 10.1890/03-4019

Cereceda, P., Larrain, H., Osses, P., Farias, M., and Egaña, I. (2008). The spatial and temporal variability of fog and its relation to fog oases in the Atacama Desert, Chile. *Atmosph. Res.* 87, 312–323. doi: 10.1016/j.atmosres.2007.11.012

Chase, J., and Leibold, M. (2003). *Ecological Niches: Linking Classical and Contemporary Approaches*. Chicago, IL: University of Chicago Press. doi: 10.7208/chicago/9780226101811.001.0001

Comte, L., Cucherousset, J., Boulêtreau, S., and Olden, J. D. (2016). Resource partitioning and functional diversity of worldwide freshwater fish communities. *Ecosphere* 7:e01356. doi: 10.1002/ecs2.1356

Cornwell, W. K., Schwilk, D. W., and Ackerly, D. D. (2006). A trait-based test for habitat filtering: convex hull volume. *Ecology* 87, 1465–1471. doi: 10.1890/0012-9658(2006)87[1465:ATTFHF]2.0.CO;2

Cross, W. F., Wallace, J. B., and Rosemond, A. D. (2007). Nutrient enrichment reduces constraints on material flows in a detritus-based food web. *Ecology* 88, 2563–2575. doi: 10.1890/06-1348.1

Cucherousset, J., and Villéger, S. (2015). Quantifying the multiple facets of trophic diversity: new integra- tive metrics for stable isotope ecology. *Biol. Indicat.* 56, 152–160. doi: 10.1016/j.ecolind.2015.03.032

Dézerald, O., Leroy, C., Corbara, B., Carrias, J. F., Pélozuelo, L., Dejean, A., et al. (2013). Food-web structure in relation to environmental gradients and predator-prey ratios in tank-bromeliad ecosystems. *PLoS ONE* 8:e71735. doi: 10.1371/journal.pone.0071735

Diaz, S., Kattge, J., Johannes, H., Cornelissen, C., Wright, I. J., Lavorel, S., et al. (2016). The global spectrum of plant form and function. *Nature* 529, 167–171. doi: 10.1038/nature16489

Donovan, L. A., Maherali, H., Caruso, C. M., Huber, H., and de Kroon, H. (2011). The evolution of the worldwide leaf economics spectrum. *Trends Ecol. Evol.* 26, 88–95. doi: 10.1016/j.tree.2010.11.011

Ebel, J. D., Leroux, S. J., Robertson, M. J., and Dempson, J. B. (2015). Ontogenetic differences in Atlantic salmon phosphorus concentration and its implications for cross ecosystem fluxes. *Ecosphere* 6, 1–18. doi: 10.1890/ES14-00516.1

El-Sabaawi, R. W., Zandonà, E., Kohler, T. J., Marshall, M. C., Moslemi, J. M., Travis, J., et al. (2012). Widespread intraspecific organismal stoichiometry among populations of the Trinidadian guppy. *Funct. Ecol.* 26, 666–676. doi: 10.1111/j.1365-2435.2012.01974.x

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth-rate stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580. doi: 10.1038/35046058

Elton, C. S. (1927). *Animal Ecology*. London: Sidgwick and Jackson.

Evans-White, M. A., Stelzer, R. S., and Lamberti, G. A. (2005). Taxonomic and regional patterns in benthic macroinvertebrate elemental composition in streams. *Freshwater Biol.* 50, 1786–1799. doi: 10.1111/j.1365-2427.2005.01455.x

Fagan, W. F., Siemann, E., Mitter, C., Denno, R. F., Huberty, A. F., Woods, A. H., et al. (2002). Nitrogen in insects: implications for trophic complexity and species diversification. *Am. Nat.* 160, 784–802. doi: 10.1086/343879

Farjalla, V. F., González, A. L., Céréghino, R., Dézerald, O., Marino, N. A. C., Piccoli, G. C. O., et al. (2016). Terrestrial support of aquatic food webs depends on light inputs: a geographically-replicated test using tank bromeliads. *Ecology* 97, 2147–2156. doi: 10.1002/ecy.1432

Finkel, Z. V., Follows, M. J., and Irwin, A. J. (2016). Size-scaling of macromolecules and chemical energy content in the eukaryotic microalgae. *J. Plankton Res.* 38, 1151–1162. doi: 10.1093/plankt/fbw057

Godwin, C. M., and Cotner, J. B. (2015). Aquatic heterotrophic bacteria have highly flexible phosphorus content and biomass stoichiometry. *ISME J.* 9, 2324–2327. doi: 10.1038/ismej.2015.34

González, A. L., Fariña, J. M., Kay, A. D., Pinto, R., and Marquet, P. A. (2011a). Exploring patterns and mechanisms of interspecific and intraspecific variation in body elemental composition of desert consumers. *Oikos* 120, 1247–1255. doi: 10.1111/j.1600-0706.2010.19151.x

González, A. L., Fariña, J. M., Pinto, R., Pérez, C., Weathers, K. C., Armesto, J. J., et al. (2011b). Bromeliad growth and stoichiometry: responses to atmospheric nutrient supply in fog-dependent ecosystems of the hyper-arid Atacama Desert, Chile. *Oecologia* 167, 835–845. doi: 10.1007/s00442-011-2032-y

Grinnell, J. (1917). The niche relationships of the California thrasher. *Auk* 34, 427–433. doi: 10.2307/4072271

Hall, S. R. (2009). Stoichiometrically explicit food webs: feedbacks between resource supply, elemental constraints and species diversity. *Annu. Rev. Ecol. Evol. Syst.* 40, 503–528. doi: 10.1146/annurev.ecolsys.39.110707.173518

Hambäck, P. A., Gilbert, J., Schneider, K., Martinson, H. M., Kolb, G., and Fagan, W. F. (2009). Effects of body size, trophic mode and larval habitat on Diptera stoichiometry: a regional comparison. *Oikos* 118, 615–623. doi: 10.1111/j.1600-0706.2008.17177.x

Hawlena, D., Strickland, M. S., Bradford, M. A., and Schmitz, O. J. (2012). Fear of predation slows plant-litter decomposition. *Science* 336, 1434–1438. doi: 10.1126/science.1220097

Hessen, D., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Hutchinson, G. E. (1957). Concluding remarks. *Cold Spring Harbor Symp. Quant. Biol.* 22, 415–427. doi: 10.1101/SQB.1957.022.01.039

Jackson, A. L., Inger, R., Parnell, A. C., and Bearhop, S. (2011). Comparing isotopic niche widths among and within communities: SIBER—Stable Isotope Bayesian Ellipses in R. *J. Anim. Ecol.* 80, 595–602. doi: 10.1111/j.1365-2656.2011.01806.x

Janžekovi, F., and Novak, T. (2012). "PCA – a powerful method for analyze ecological niches," in *Principal Component Analysis - Multidisciplinary Applications*, eds P. Sanguansat, et al. (InTech), 212. Available online at: http://www.intechopen.com/books/principal-component-analysis-multidisciplinaryapplications/

Jeyasingh, P. D. (2007). Plasticity in metabolic allometry: the role of dietary stoichiometry. *Ecol. Lett.* 10, 282–289. doi: 10.1111/j.1461-0248.2007.01023.x

Jeyasingh, P. D., Cothran, R. D., and Tobler, M. (2014). Testing the ecological consequences of evolutionary change using elements. *Ecol. Evol.* 4, 528–538. doi: 10.1002/ece3.950

Kay, A. D., Ashton, I. W., Gorokhova, E., Kerkhoff, A. J., Liess, A., and Litchman, E. (2005). Toward a stoichiometric framework for evolutionary biology. *Oikos* 109, 6–17. doi: 10.1111/j.0030-1299.2005.14048.x

Kearney, M., Simpson, S. J., Raubenheimer, D., and Helmuth, B. (2010). Modelling the ecological niche from functional traits. *Phil. Trans. R. Soc. B* 365, 3469–3483. doi: 10.1098/rstb.2010.0034

Kerkhoff, A. J., and Enquist, B. J. (2006). Ecosystem allometry: the scaling of nutrient stocks and primary productivity across plant communities. *Ecol. Lett.* 9, 419–427. doi: 10.1111/j.1461-0248.2006.00888.x

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. (2004). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174, doi: 10.1038/nature02454

Layman, C. A., Araujo, M. S., Boucek, R., Hammerschlag-Peyer, C. M., Harrison, E., Jud, Z. R., et al. (2012). Applying stable isotopes to examine food-web structure: an overview of analytical tools. *Biol. Rev.* 87, 545–562. doi: 10.1111/j.1469-185X.2011.00208.x

Layman, C. A., Arrington, D. A., Montaña, C. G., and Post, D. M. (2007). Can stable isotope ratios provide for community-wide measures of trophic structure? *Ecology* 88, 42–48. doi: 10.1890/0012-9658(2007)88[42:CSIRPF]2.0.CO;2

Leal, M. C., Seehausen, O., and Matthews, B. (2017). The ecology and evolution of stoichiometric phenotypes. *Trends Ecol. Evol.* 32, 108–117. doi: 10.1016/j.tree.2016.11.006

Legendre, P. (2014). *lmodel2: Model II Regression*. R package version 1.7–2. Available online at: http://CRAN.R-project.org/package=lmodel2

Leibold, M. A. (1995). The niche concept revisited: mechanistic models and community context. *Ecology* 76, 1371–1382. doi: 10.2307/1938141

Lemoine, N. P., Giery, S. T., and Burkepile, D. E. (2014). Differing nutritional constraints of consumers across ecosystems. *Oecologia* 174, 1367–1376. doi: 10.1007/s00442-013-2860-z

Litchman, E., and Klausmeier, C. A. (2008). Trait-based community ecology of phytoplankton. *Annu. Rev. Ecol. Evol. Syst.* 39, 615–639. doi: 10.1146/annurev.ecolsys.39.110707.173549

Machovsky-Capuska, G., Senior, A., Simpson, S., and Raubenheimer, D. (2016). The multidimensional nutritional niche. *Trends Ecol. Evol.* 31, 355–365. doi: 10.1016/j.tree.2016.02.009

Magnago, L. F. S., Martins, S. V., Schaefer, C. E. G. R., and Neri, A. V. (2012). Restinga forests of the Brazilian coast: richness and abundance of tree species on different soils. *Anais da Academia Brasileira de Ciências* 84, 807–822. doi: 10.1590/S0001-37652012000300023

Merritt, R. W., Cummins, K. W., and Berg, M. B. (2008). *An Introduction to the Aquatic Insects of North America, 4th Edn*. Kendall Hunt Pub. Co.

Mouginot, C., Kawamura, R., Matulich, K. L., Berlemont, R., Allison, S. D., Amend, A. S., et al. (2014). Elemental stoichiometry of Fungi and Bacteria strains from grassland leaf litter. *Soil Biol. Biochem.* 76, 278–285. doi: 10.1016/j.soilbio.2014.05.011

Newsome, S. D., Martinez del Rio, C., Bearhop, S., and Phillips, D. L. (2007). A niche for isotopic ecology. *Front. Ecol. Environ.* 5, 429–436. doi: 10.1890/060150.1

Peñuelas, J., Sardans, J., Llusià, J., Owen, S. M., Carnicer, J., Giambelluca, T. W., et al. (2010). Faster returns on 'leaf economics' and different biogeochemical niche in invasive compared with native plant species. *Global Change Biol.* 16, 2171–2185. doi: 10.1111/j.1365-2486.2009.02054.x

Peñuelas, J., Sardans, J., Ogaya, R., and Estiarte, M. (2008). Nutrient stoichiometric relations and biogeochemical niche in coexisting plant species: effect of simulated climate change. *Pol. J. Ecol.* 56, 613–622.

Persson, J., Fink, P., Goto, A., Hood, J. M., Jonas, J., and Kato, S. (2010). To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos* 119, 741–751. doi: 10.1111/j.1600-0706.2009.18545.x

Pigliucci, M. (2007). Finding the way in phenotypic space: the origin and maintenance of constraints on organismal form. *Ann. Bot.* 100, 433–438. doi: 10.1093/aob/mcm069

Pinto, R., Barría, I., and Marquet, P. A. (2006). Geographical distribution of *Tillandsia* lomas in the Atacama Desert, Northern Chile. *J. Arid. Environ.* 65, 543–552. doi: 10.1016/j.jaridenv.2005.08.015

Quigg, A., Irwin, A. J., and Finkel, Z. V. (2011). Evolutionary inheritance of elemental stoichiometry in phytoplankton. *Proc. Biol. Sci.* 278, 526–534. doi: 10.1098/rspb.2010.1356

R Development Core Team (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Raubenheimer, D. (2011). Toward a quantitative nutritional ecology: the right-angled mixture triangle. *Ecol. Monogr.* 81, 407–427. doi: 10.1890/10-1707.1

Raubenheimer, D., Machovsky-Capuska, G., Chapman, C., and Rothman, J. (2015). Geometry of nutrition in field studies: an illustration using wild primates. *Oecologia* 177, 223–234. doi: 10.1007/s00442-014-3142-0

Raubenheimer, D., and Simpson, S. J. (2004). Organismal stoichiometry: quantifying non-independence among food components. *Ecology* 85, 1203–1216. doi: 10.1890/02-0248

Raubenheimer, D., Simpson, S. J., and Mayntz, D. (2009). Nutrition, ecology and nutritional ecology: toward an integrated framework. *Funct. Ecol.* 23, 4–16. doi: 10.1111/j.1365-2435.2009.01522.x

Reich, P. B. (2014). The world-wide'fast-slow' plant economics spectrum: a traits manifesto. *J. Ecol.* 102, 275–301. doi: 10.1111/1365-2745.12211

Reich, P. B., and Oleksyn, J. (2004). Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11001–11006. doi: 10.1073/pnas.0403588101

Reiners, W. A. (1986). Complementary models for ecosystems. *Am. Nat.* 127, 59–73. doi: 10.1086/284467

Rigolet, C., Thiébaut, E., Brind Amour, A., and Dubois, S. F. (2015). Investigating isotopic functional indices to reveal changes in the structure and functioning of benthic communities. *Func. Ecol.* 29, 1350–1360. doi: 10.1111/1365-2435.12444

Sardans, J., and Peñuelas, J. (2013). Tree growth changes with climate and forest type are associated with relative allocation of nutrients, especially phosphorus, to leaves and wood. *Glob. Ecol. Biogeogr.* 22, 494–507. doi: 10.1111/geb.12015

Simpson, S. J., and Raubenheimer, D. (1993). A multi-level analysis of feeding behaviour: the geometry of nutritional decisions. *Philos. Trans. R. Soc. Lond. B* 342, 381–402. doi: 10.1098/rstb.1993.0166

Sperfeld, E., Halvorson, H. M., Malishev, M., Clissold, F. J., and Wagner, N. (2016). Woodstoich III: integrating tools of nutritional geometry and ecological stoichiometry to advance nutrient budgeting and the prediction of consumer-driven nutrient recycling. *Oikos* 125, 1539–1553. doi: 10.1111/oik.03529

Sperfeld, E., Wagner, N. D., Halvorson, H. M., Malishev, M., and Raubenheimer, D. (2017). Bridging Ecological Stoichiometry and Nutritional Geometry with homeostasis concepts and integrative models of organism nutrition. *Funct. Ecol.* 31, 286–296. doi: 10.1111/1365-2435.12707

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Swanson, H. K., Lysy, M., Power, M., Stasko, A. D., Johnson, J. D., and Reist, J. D. (2015). A new probabilistic method for quantifying n-dimensional ecological niches and niche overlap. *Ecology* 96, 318–324. doi: 10.1890/14-0235.1

Torres, L. E., and Vanni, M. J. (2007). Stoichiometry of nutrient excretion by fish: interspecific variation in a hypereutrophic lake. *Oikos* 116, 259–270. doi: 10.1111/j.0030-1299.2007.15268.x

Villéger, S., Grenouillet, G., and Brosse, S. (2013). Decomposing functional β-diversity reveals that low functional β-diversity is driven by low functional turnover in European fish assemblages. *Glob. Ecol. Biogeogr.* 22, 671–681. doi: 10.1111/geb.12021

Villéger, S., Mason, N. W., and Mouillot, D. (2008). New multidimensional functional diversity indices for a multifaceted framework in functional ecology. *Ecology* 89, 2290–2301. doi: 10.1890/07-1206.1

Villéger, S., Novack-Gottshall, P. M., and Mouillot, D. (2011). The multidimensionality of the niche reveals functional diversity changes in benthic marine biotas across geological time. *Ecol. Lett.* 14, 561–568, doi: 10.1111/j.1461-0248.2011.01618.x

Violle, C., and Jiang, L. (2009). Towards a trait-based quantification of species niche. *J. Plant Ecol.* 2, 87–93. doi: 10.1093/jpe/rtp007

Violle, C., Navas, M. L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., et al. (2007). Let the concept of trait be functional. *Oikos* 116, 882–892. doi: 10.1111/j.0030-1299.2007.15559.x

Wiesenborn, W. D. (2011). Nitrogen content in riparian arthropods is most dependent on allometry and order. *Fla. Entomol.* 94, 71–80. doi: 10.1653/024.094.0110

Wiesenborn, W. D. (2013). Phosphorus contents in desert riparian spiders and insects vary among taxa and between flights capabilities. *Fla. Entomol.* 96, 424–432. doi: 10.1653/024.096.0206

Woods, H. A., Fagan, W. F., Elser, J. J., and Harrison, J. F. (2004). Allometric and phylogenetic variation in insect phosphorus content. *Funct. Ecol.* 18, 103–109. doi: 10.1111/j.1365-2435.2004.00823.x

Yamamichi, M., Meunier, C. L., Peace, A., Prater, A., and Rúan, M. (2015). Rapid evolution of a consumer stoichiometric trait destabilizes consumer–producer dynamics. *Oikos* 124, 960–969. doi: 10.1111/oik.02388

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 12 July 2017
doi: 10.3389/fmicb.2017.01298

Bridging Food Webs, Ecosystem Metabolism, and Biogeochemistry Using Ecological Stoichiometry Theory

Nina Welti[1,2†], Maren Striebel[3†], Amber J. Ulseth[4], Wyatt F. Cross[5], Stephen DeVilbiss[6], Patricia M. Glibert[7], Laodong Guo[6], Andrew G. Hirst[8,9], Jim Hood[10], John S. Kominoski[11], Keeley L. MacNeill[12], Andrew S. Mehring[13], Jill R. Welter[14] and Helmut Hillebrand[3,15]*

[1] Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland, [2] Agriculture and Food, Commonwealth Scientific and Industrial Research Organisation, Adelaide, SA, Australia, [3] Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, Oldenburg, Germany, [4] Stream Biofilm and Ecosystem Research, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, [5] Department of Ecology, Montana State University, Bozeman, MT, United States, [6] School of Freshwater Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI, United States, [7] University of Maryland Center for Environmental Science, Cambridge, MD, United States, [8] The Hirst Lab, Organismal Biology, School of Biological and Chemical Sciences, Queen Mary University of London, London, United Kingdom, [9] Centre for Ocean Life, National Institute for Aquatic Resources, Technical University of Denmark, Copenhagen, Denmark, [10] Department of Evolution, Ecology, and Organismal Biology, Aquatic Ecology Laboratory, The Ohio State University, Columbus, OH, United States, [11] The Kominoski Lab, Department of Biological Sciences, Florida International University, Miami, FL, United States, [12] Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, United States, [13] Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA, United States, [14] Department of Biology, St. Catherine University, Minneapolis, MN, United States, [15] Helmholtz-Institute for Functional Marine Biodiversity, Oldenburg, Germany

OPEN ACCESS

Edited by:
Robert Warner Sterner, University of Minnesota Duluth, United States

Reviewed by:
André Megali Amado, Federal University of Rio Grande do Norte, Brazil
Ian Salter, Alfred-Wegener-Institut für Polar- und Meeresforschung, Germany

***Correspondence:**
Maren Striebel
maren.striebel@uni-oldenburg.de

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *29 November 2016*
Accepted: *27 June 2017*
Published: *12 July 2017*

Citation:
Welti N, Striebel M, Ulseth AJ, Cross WF, DeVilbiss S, Glibert PM, Guo L, Hirst AG, Hood J, Kominoski JS, MacNeill KL, Mehring AS, Welter JR and Hillebrand H (2017) Bridging Food Webs, Ecosystem Metabolism, and Biogeochemistry Using Ecological Stoichiometry Theory. Front. Microbiol. 8:1298. doi: 10.3389/fmicb.2017.01298

Although aquatic ecologists and biogeochemists are well aware of the crucial importance of ecosystem functions, i.e., how biota drive biogeochemical processes and vice-versa, linking these fields in conceptual models is still uncommon. Attempts to explain the variability in elemental cycling consequently miss an important biological component and thereby impede a comprehensive understanding of the underlying processes governing energy and matter flow and transformation. The fate of multiple chemical elements in ecosystems is strongly linked by biotic demand and uptake; thus, considering elemental stoichiometry is important for both biogeochemical and ecological research. Nonetheless, assessments of ecological stoichiometry (ES) often focus on the elemental content of biota rather than taking a more holistic view by examining both elemental pools and fluxes (e.g., organismal stoichiometry *and* ecosystem process rates). ES theory holds the promise to be a unifying concept to link across hierarchical scales of patterns and processes in ecology, but this has not been fully achieved. Therefore, we propose connecting the expertise of aquatic ecologists and biogeochemists with ES theory as a common currency to connect food webs, ecosystem metabolism, and biogeochemistry, as they are inherently concatenated by the transfer of carbon, nitrogen, and phosphorous through biotic and abiotic nutrient transformation and fluxes. Several new studies exist that demonstrate the connections between food web ecology, biogeochemistry, and ecosystem metabolism. In addition to a general introduction into the topic, this paper presents examples of how these fields can be combined with a focus on ES. In this review, a series of concepts have

guided the discussion: (1) changing biogeochemistry affects trophic interactions and ecosystem processes by altering the elemental ratios of key species and assemblages; (2) changing trophic dynamics influences the transformation and fluxes of matter across environmental boundaries; (3) changing ecosystem metabolism will alter the chemical diversity of the non-living environment. Finally, we propose that using ES to link nutrient cycling, trophic dynamics, and ecosystem metabolism would allow for a more holistic understanding of ecosystem functions in a changing environment.

Keywords: nutrient dynamics, trophic interactions, energy transfer, ecosystem function, carbon quality, element cycling, ecological stoichiometry

INTRODUCTION

Aquatic ecologists and biogeochemists are well aware of the importance of biologically mediated ecosystem functions in driving biogeochemical cycling and its feedback (**Figure 1**). The magnitude of ecosystem fluxes and stoichiometric constraints on biogeochemical processes are determined by turnover of elements, including the most commonly studied, carbon (C), nitrogen (N), phosphorus (P). These basal resources can be governed by ecosystem metabolism, where the balance of gross primary production (GPP) and ecosystem respiration (ER) dictate net ecosystem production (NEP). In freshwater aquatic ecosystems, when GPP exceeds ER (NEP $>$ 0) the ecosystem is autotrophic and when ER $>$ GPP (NEP $<$ 0), it is heterotrophic indicating a reliance on imported C inputs, often of terrestrial origin, for respiration (Lovett et al., 2006). In other words, the biological processes of production, respiration, and excretion can drive biogeochemical cycles, therefore making it critical to understand how the elements (e.g., C, N, and P) required for these processes are coupled.

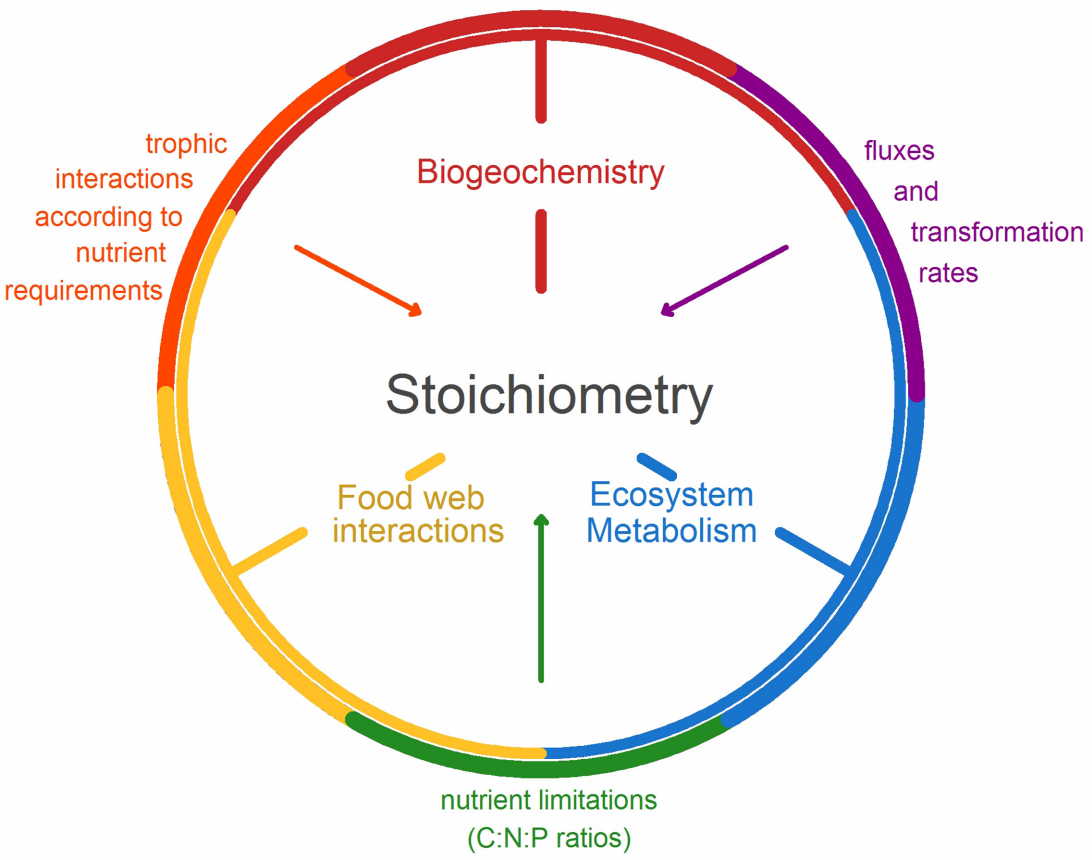

FIGURE 1 | Conceptual framework demonstrating the connection between biogeochemistry, food web interactions, ecosystem metabolism, and stoichiometry. Biogeochemistry and food webs are linked through trophic interactions according to nutrient requirements between trophic levels, food webs, and ecosystem metabolism according to the nutrient limitations (C:P or C:N ratios), and ecosystem metabolism and biogeochemistry through fluxes and transformation rates.

Chemical diversity in aquatic ecosystems is enormously high (Santos et al., 2008; Cai and Guo, 2009; Singer et al., 2012) and is a result of the high variety of metabolic pathways and abiotic reactions in the water column and sediment. Biological diversity can affect biogeochemical diversity, e.g., phytoplankton composition shapes the structure and functioning of the microbial loop by controlling dissolved organic matter (DOM) composition (Grossart et al., 2007; Murray et al., 2007; Passow et al., 2007; Pete et al., 2010), and thus the respective transformations and fluxes. As groups of organisms differ in terms of their elemental composition and turnover ratios, changes in the diversity of organisms are likely to affect the stoichiometry and patterns of different biogeochemical transformations and thus the flux of major elements. Scott et al. (2012) demonstrated that bacterial stoichiometry can provide a biogeochemical "set point" around which environmental variation is regulated from bottom-up controls. Furthermore, heterotrophic bacteria assemblages can have flexible and dynamic stoichiometric requirements, allowing for tight coupling and negative feedback between the bacterial requirements and the resource stoichiometry (Godwin and Cotner, 2015). Capps and Flecker (2013) showed that the growth of an introduced population of P-rich armored catfish significantly changed stream nutrient dynamics by altering nutrient storage and remineralization rates. This shows that changes in species composition can alter N and P cycling and C sequestration, producing large-scale effects on element fluxes and biogeochemical cycles.

Autotrophs and heterotrophs drive C and nutrient cycling in aquatic ecosystems. Therefore, the balance of GPP and ER controls the source and quality of C, thereby creating the basis for food webs (Marcarelli et al., 2011). Autochthonous material is usually higher in C quality than allochthonous material (Findlay et al., 1986) although terrestrial allochthonous material can have higher C:N and N:P ratios (Lennon and Pfaff, 2005). In terms of ecosystem metabolism, when NEP $>$ 0 (i.e., autotrophic), the bulk C source is likely of autochthonous origin, and hence of high quality. When an ecosystem is heterotrophic (i.e., NEP $<$ 0), allochthonous material subsidizes ER, indicating the potential for a lower quality C source (Findlay et al., 1986; Zhou et al., 2016). Most aquatic ecosystems are heterotrophic throughout the year (Vannote et al., 1980; Battin et al., 2008; Hoellein et al., 2013), resulting in high-flux, low-quality subsidies driving freshwater ecosystem dynamics (Marcarelli et al., 2011). However, the production of autochthonous material, including any window of autotrophy, is a key flux. The autochthonous fluxes are often low in quantity, but of high-quality, which support food webs and affect ecosystem processes (Marcarelli et al., 2011). The extent to which allochthonous material incorporated into food webs is less understood for many stream ecosystems (Marcarelli et al., 2011; Bartels et al., 2012; Collins et al., 2015; but see Wallace et al., 1999 for forest streams). Additionally, ecosystem metabolism is inherently linked to nutrient (N and/or P), and C-cycling; yet, given this fact, there are few studies which have coupled ecosystem metabolism to nutrient cycling (Hall and Tank, 2003; Webster et al., 2003; Hall et al., 2013; Hoellein et al., 2013), C-spiraling (Hall et al., 2016), or both nutrient and organic C egestion and assimilation (Hall et al., 2003).

Changes in environmental drivers, such as temperature or nutrient availability, can alter biodiversity and influence the transformation and fluxes of organic matter and nutrients in these ecosystems. Temperature has strong effects on growth rates and the physiology of phytoplankton (Eppley, 1972; Karentz and Smayda, 1984; Butterwick et al., 2005) and can also influence protist mean cell size (Atkinson et al., 2003; Forster et al., 2013), nutrient uptake rates (Senft et al., 2008), N metabolism and cell stoichiometry (Lomas and Glibert, 1999; Montagnes and Franklin, 2001; Litchman et al., 2010), and ER (Yvon-Durocher et al., 2012). Such effects on autotrophic and heterotrophic producers likely affect consumers directly. Thus, trophic interactions, food web structure and mutualistic networks can result in cascading effects on ecosystem metabolism or vice versa. Many studies take a biogeochemical approach (mainly in streams) focused on individual elements (e.g., Meyer and Likens, 1979; Triska et al., 1984; Mulholland et al., 2000) or on the effect of ratios on the flux of single elements (Dodds et al., 2004; Schade et al., 2011). Martiny et al. (2013) showed that strong latitudinal patterns exist in the elemental ratios of marine plankton and organic matter and others have examined the relationship between phytoplankton diversity and particulate ratios across biogeochemical gradients (Salter et al., 2014; Rembauville et al., 2015). In general, most studies from aquatic ecosystems focus on the cycling of N or P as these are the nutrients most likely to limit primary production. However, Elser et al. (2007) and Harpole et al. (2011) pointed towards the prevalence of multiple nutrient limitation to primary production in most aquatic and terrestrial habitats. Further, Boersma and Elser (2006) and Glibert et al. (2013) underscored the importance of nutrients not just at the limiting end of the availability spectrum, but across the continuum from limitation to excess. Combining biogeochemical models with ecological stoichiometry (ES), and thus using traceable mass balance relationships, can be a way to describe and understand the complex interactions and feedbacks more completely (Franklin et al., 2011).

Here, we discuss the many ways in which ES links food webs, ecosystem metabolism and biogeochemistry, thus influencing stocks and fluxes of key elements (cf. Glibert et al., 2011). The fate of multiple elements in ecosystems requires consideration of elemental stoichiometry for both biogeochemical and ecological research. Based on a literature search (**Table 1**), a large number of studies included any of the three terms—food webs, ecosystem metabolism, and biogeochemistry—together with ES, but only eight studies used ES in connection to all three terms. ES has the potential to be a concept unifying flux-oriented biogeochemistry, ecosystem metabolism, and population-oriented ecology, but so far only a few studies have achieved this (Reiners, 1986). For example, Hall et al. (2003) linked N production and demand, ecosystem metabolism, and snail production using ES. By assuming that net primary production was 50% of GPP, and based on the expected C:N ratio of 14:1 of C to N fixation, the authors estimated that these snails ingested 75% of daily GPP and that excretion of snails was estimated 65% of total NH_4 demand. The authors concluded that this invasive snail

TABLE 1 | Numbers of publications (Web of Science searching all databases, accessed March 2017) including key words for one of the research fields (metabolism, stoichiometry, food web, or biogeochemistry) and combinations of these key words.

Keyword	Number of publications
Metabolism	7,480,534
Stoichiometry	98,372
Food web	34,496
Biogeochemistry	11,441
Metabolism + stoichiometry	15,166
Metabolism + food web	4,950
Metabolism + biogeochemistry	1,620
Food web + stoichiometry	521
Food web + biogeochemistry	454
Biogeochemistry + stoichiometry	278
Metabolism + food web + biogeochemistry	79
Metabolism + food web + stoichiometry	111
Metabolism + biogeochemistry + stoichiometry	66
Food web + biogeochemistry + stoichiometry	39
Metabolism + food web + biogeochemistry + stoichiometry	8

dominated C and N fluxes, despite very high GPP and N demand. In this case, ES provided a quantitative framework for linking inorganic nutrients, stream metabolism, and secondary production.

Studies of ES have often focused on the elemental content of specific types of organisms rather than combining biological with physical and chemical drivers of element fluxes, including ecosystem metabolism. Changes in the diversity of key taxa can have major impacts on a range of biogeochemical transformations and overall fluxes. For example, both increased light and the introduction of the guppy (*Poecilia reticulata*) increased N fluxes to some invertebrate functional feeding groups (Collins et al., 2016). The advantage of combining these fields of expertise is that effects of multiple changes of more than one parameter can be investigated. For example, when considering multiple nutrient limitations, the flux of more than one element should be considered—a task that can be achieved by combining biogeochemical approaches using ES. Investigating the interactions of temperature and nutrients by combining ES (Sterner and Elser, 2002) and metabolic theory of ecology (Brown et al., 2004) will improve the understating of microbial and ecosystem ecology (Hall et al., 2010) on different levels of organization (individuals, populations, communities, food webs, ecosystem; see reviews by Cross et al., 2015; Vanni and McIntyre, 2016). Diet-induced metabolic plasticity contributes to variation in metabolic allometry, at least at small scales of body size due to the greater respiratory response of smaller species to altered diets (Jeyasingh, 2007). Moorthi et al. (2016) showed that unifying ES and metabolic theory allows us to predict production and trophic transfer in a marine planktonic food web. Changes in nutrient loading have become a major concern among all scales of organization and can have strong impacts on biogeochemical cycles (Falkowski et al., 2000). Results from Manning et al. (2016) indicate that changes in basal resource stoichiometry can occur due to effects on either autotrophic (e.g., biofilm) or heterotrophic microbial communities, resulting in diminished stream consumer biodiversity related to either heterotrophic or autotrophic food web pathways. Many environmental changes, such as climate warming, eutrophication, acidification, and CO_2 alter absolute nutrient supply and likely nutrient ratios (e.g., Boyd and Hutchins, 2012; Glibert et al., 2014). Therefore, a combined approach including metabolic theory and ES is valuable for assessing the possible effects of environmental changes (Hessen et al., 2013).

EMPIRICAL ASSESSMENTS

In the following section, we exemplify how food web interactions, ecosystem metabolism, and biogeochemistry can use ES theory to integrate from microbial to ecosystem-scale processes through a series of case studies. The examples are derived from a special session at the 2016 Association for the Sciences of Limnology and Oceanography (ASLO) meeting in Santa Fe, NM, United States, with the aim to merge the fields of biogeochemistry, food webs and ecosystem metabolism by using ES as a common theoretical framework. Using the following research highlights, we convey the depth and range of approaches which have been applied, that merge these disciplines, which are conceptualized in our model (**Figure 1**). In our first case study, ES links a general trait of metabolism (body mass dependence) to trophic interactions and biogeochemistry by demonstrating changes in resource transport and N:O ratios. Secondly, ES demonstrates the interactions between trophic dynamics of benthic aquatic invertebrates and two large-scale biogeochemical fluxes. Thirdly, the addition of trace elements to the traditional C:N:P ratios improves the understanding of altered trophic interactions and nutrient fluxes. And then in the subsequent two examples, the N:P loads shift over time, allowing for the proliferation of invasive species which further impact that quality of carbon and N:P availability. Furthermore, the sixth case study uses ES to demonstrate how changes to N:P alters ecosystem metabolism through enhanced microbial respiration rates and food web interactions. Finally, the interaction between biogeochemistry with regard to changing temperature is quantified using ES and the impact on ecosystem metabolism. The diversity of our examples illustrates the potential strength of this approach for understanding relationships among and across trophic levels, including biogeochemical interactions as well as direct and indirect effects.

A New Model to Explain the Body Mass Scaling of Diverse Biological Rates in Aquatic Invertebrates

Body size is a "master trait" that affects all vital rates, including feeding, reproduction, excretion and metabolism (Kleiber, 1932, 1961; Schmidt-Nielsen, 1984; Hirst et al., 2014). Understanding what drives the body mass dependence of such a wide diversity of rates is of fundamental biological importance, indeed, this has been a much-debated topic over the last century. Recent work has explored body mass scaling exponents of metabolic

rates within planktonic species (Hirst et al., 2014; Glazier et al., 2015) in order to better appreciate what controls these terms, and ultimately to better predict these rates for species and communities. These authors tested two groups of theories that predict the body-mass dependence of metabolism, those built upon internal transport networks (including the Metabolic Theory of Ecology; West et al., 1999; Savage et al., 2008; Banavar et al., 2010), and those based on a Surface Area model [a reapplication of Rubner's surface dependent model of heat exchange in endotherms (Rubner, 1883), but more broadly applied to the influx and efflux of materials and energy]. Importantly, many zooplankton change body shape as they grow, while also using significant proportions of their body surface for the exchange of materials. While the major geometric scaling theories produce rather similar predictions when shape does not change over ontogeny (i.e., they are isomorphic), the predictions from these two groups of theory diverge starkly when organisms increasingly flatten or elongate in shape over ontogeny. These shape changes result in a reduction in the predicted scaling exponents of many resource transport model, but increase the predicted scaling exponent for the Surface Area dependent model. While the mass-scaling of respiration has been shown to correlate with body surface enlargement in many pelagic invertebrates (Hirst et al., 2014; Glazier et al., 2015), Hirst et al. (2016) predicted that body-mass scaling exponents for rates of soluble N excretion (b_N) should also then relate to the degree of body-shape change during growth. They tested this hypothesis using literature data on b_N for pelagic invertebrates across five different phyla, and found that b_N is significantly positively correlated with predicted surface area enlargement, whilst also co-varying with the mass-scaling of respiration rate (b_R). Indeed, intraspecific differences between b_N and b_R values have revealed there are shifts in the ratio of O_2-consumed to N-excreted over ontogeny. This suggests that changes in the relative anabolism and catabolism of proteins and lipids over development, may cause these consumption-excretion ratios to change too. In conclusion, diverse pelagic invertebrates, that dominate vast open water ecosystems, therefore appear to falsify the predictions of general metabolic scaling theories built upon resource-transport networks, while supporting predictions of surface-area dependent theory. Furthermore, ontogenetic variation in ratios of O_2 consumed to N excreted of these species, may not only provide insight into the developmental metabolism, but also the stoichiometry of ecological systems, including, for example, seasonal changes in N-budgets that are linked to pelagic animal life cycles.

Enhancement of Carbon Dioxide, Methane, and Nitrous Oxide Flux by Invertebrates

Aquatic ecosystems can be sources of greenhouse gases (GHG), a process that is strongly controlled by the availability of C, N, and P, which can stimulate emission of nitrous oxide (N_2O), methane (CH_4), and carbon dioxide (CO_2) (Cao et al., 1996; Burgin et al., 2013; Nisbet et al., 2014; Deemer et al., 2016). However, mounting evidence suggests that benthic aquatic invertebrates such as midge larvae (Diptera: Chironomidae), snails (Gastropoda), and aquatic worms (Oligochaeta and Polychaeta) can enhance the emissions of GHG through high N excretion rates, by creating anoxic microenvironments within their guts, and through bioturbation and bioirrigation of surrounding sediments (Kristensen et al., 1991; Nielsen et al., 2004; Figueiredo-Barros et al., 2009; Stief et al., 2009; Heisterkamp et al., 2010; Nogaro and Burgin, 2014; Poulsen et al., 2014; Hölker et al., 2015; Mehring et al., 2017).

A large portion of the CH_4 produced in freshwater and marine sediments that is not released by ebullition is oxidized to CO_2 or assimilated by methanotrophic bacteria (Bastviken et al., 2008). Some species of midge larvae and zooplankton have been shown to assimilate methane-derived C through consumption of methanotrophic bacteria (Deines et al., 2007), as evidenced by exceptionally low stable isotopic ratios ($\delta^{13}C$ as low $-64‰$ for midge larvae; Jones et al., 2008). It is still unclear if differences in faunal isotopic ratios among aquatic ecosystems can be consistently linked to differences in ecosystem function, or if the effects of methanotroph consumption by invertebrates are substantial enough to influence emissions across the air–water interface of lakes and wetlands. For example, Kajan and Frenzel (1999) observed that both production and oxidation of CH_4 were enhanced in chironomid burrows in rice paddies, but there was no net effect on benthic CH_4 flux. The feeding activity of bacterivorous zooplankton such as Cladocera has been shown to suppress methanotrophic activity in laboratory mesocosms (Kankaala et al., 2007), but this has not yet been demonstrated to affect CH_4 fluxes at large scales. Conversely, bioturbation is a non-consumptive mechanism by which benthic fauna may influence CH_4 flux, which has been demonstrated in manipulative laboratory studies (Figueiredo-Barros et al., 2009) but has yet to be linked to differences in faunal stoichiometry.

While much work is needed to further elucidate the enhancement of microbial metabolic pathways and GHG flux by aquatic invertebrates, previous studies have demonstrated enhancement of GHG flux by invertebrates under highly controlled conditions in laboratories. An assessment of the effects of mixed assemblages (and likely resulting in a wide range of nutrient stoichiometry) under variable conditions is important to our understanding of faunal influence on GHG fluxes in aquatic ecosystems. Since taxa such as Tubificinae have been shown to enhance GHG flux (Nogaro and Burgin, 2014; Mehring et al., 2017) and also to reach high densities in eutrophic aquatic environments (Devine and Vanni, 2002), invertebrate enhancement of GHG emissions from aquatic ecosystems may be linked both to anthropogenically induced nutrient loading and resulting shifts in aquatic community structures. Given the variable environmental conditions in mixed biotic assemblages outside of controlled laboratory conditions, the degree to which the effects of invertebrates and their corresponding C:N:P can be detected relative to other drivers of GHG flux in field settings requires further investigation.

Including Trace Elements for a Holistic Stoichiometric Approach in Food Webs

ES is an important framework for examining paired biogeochemical processes; however, ES studies in both terrestrial and aquatic systems are biased toward C, N, and P while trace elements are often neglected (Sterner and Elser, 2002). Recently, Kaspari and Powers (2016) argued the importance of expanding traditional models of co-limitation to include all 25 of life's building elements. Including non-essential trace elements is also crucial to a holistic stoichiometric approach (MacNeill et al., 2016). Arsenic (As), mercury (Hg), selenium (Se) and other non-essential trace elements have been well studied individually (Boening, 2000; Farag et al., 2003; Schaller et al., 2010; Walters et al., 2015), but their pairings with other, more common elements have less frequently been evaluated (but see Wang et al., 2013). Integrating trace elements, their interactions with each other and their interactions with C, N, and P into studies of ES will provide a more complete picture of elemental cycling in ecosystems (Wang et al., 2013). The toxic trace element As can alter both ecosystem structure and function: In terms of ecosystem structure, As contamination decreases stream invertebrate abundance and diversity (Chaffin et al., 2005). Functionally, As affects cycling of common (N and P) stream nutrients (Lottig et al., 2007; Rodriguez Castro et al., 2015; MacNeill et al., 2016). In freshwaters, P is usually in the form of phosphate (PO_4^{3-}), which shares the same chemical structure as arsenate (AsO_4^{3-}), the most common form of As in oxygenated freshwaters (Button et al., 1973; Schaller et al., 2010). Consequently, As can be taken into bacterial, algal, and animal cells in place of P and decouple oxidative- and photo-phosphorylation, hindering energy production (Finnegan and Chen, 2012). Cells are less able to distinguish between As and P when P is low relative to As (Rodriguez Castro et al., 2015) and in particular when total P is less than ∼50 μg/L, as is the case in a majority of freshwaters (Villanueva et al., 2000; Binkley et al., 2004; Hall et al., 2013). Recently published research shows that As metabolism by the algae *Chlorella vulgaris* depends on the relative amount of P, which determines both uptake of P and the dominant metabolite excreted by cells (Baker and Wallschläger, 2016).

In addition to the interchangeability of As and P, the cycles of N and P are intimately linked (Cross et al., 2005; Schade et al., 2011). Because the cycles of N and P are so intertwined, it is likely that the As cycle is linked to the N cycle through P. Toxic effects of As tend to be greater in P limited environments (Rodriguez Castro et al., 2015) and P limitation depends on relative N availability (Tessier and Raynal, 2003; Schade et al., 2011; Rodriguez Castro et al., 2015). Therefore, linkages with N may explain why previous studies have not satisfactorily resolved how As affects P uptake (Pringle, 1991; Lottig et al., 2007; Hoellein et al., 2012). MacNeill et al. (2016) found evidence that ambient dissolved N:P, rather than P concentration alone or relative As:P, influences the amount of As removed from the water column by biofilm (assemblages of bacteria, algae, and fungi growing on rocks) uptake. The relative N:P dissolved in water as a driver of As uptake by biofilms has implications for the amount of As, metabolized by, retained in, and transferred through food webs. Therefore, expanding the framework of ES to include trace elements is important to understand their relationships with common elements and their effects on ecosystem functioning.

Applying Ecological Stoichiometry and Biogeochemistry Together to Understand Changes in Aquatic Food Webs and Invasive Species

ES, together with biogeochemistry has been applied to understanding invasive species and changes to aquatic food webs in the San Francisco Bay Delta (Glibert et al., 2011; Glibert, 2012). In this ecosystem, the food web has changed significantly over the past decades, from phytoplankton to fish. Using 30 years of records of nutrient loads and concentrations and abundances of phytoplankton, zooplankton, macroinvertebrates, and fish it was shown that changes in ratios of N and P, together with changes in N form, have been significant drivers of changes in the food web (**Figure 2**). Members of different trophic levels were found to have different correlations with N and P, as did taxa within trophic levels. These patterns were consistent with the premise that the fish community shifted to species that were proportionately more P-rich over time as N and P ratios increased due to substantial increases in N loading and reductions in P. The patterns were also consistent with increased importance of a benthic food web following reductions in P loading. Changes in external nutrient loads also drove changes in biogeochemical fluxes at the sediment water interface, leading to increasing abundance of macrophytes, clams, and of the toxic algae *Microcystis*, along with more omnivorous fish fueled by a benthic food web. The picture that has emerged of this ecosystem is one where changes in the food web are now understood to follow the conceptual model of stoichiometry, and not purely stochastic events. Previously considered one of the most heavily invaded estuaries in the world, it is now clear that environmental changes, including nutrient ratios and concentrations, interact with vectors of invasion to enhance their success.

The Role of Invasive Quagga Mussels in Affecting Dissolved Organic Matter in Lake Michigan

Invasive quagga mussels (*Dreissena rostriformis bugensis*) have caused unprecedented ecological and environmental changes in Lake Michigan. Declines in primary production, fish biomass, and turbidity as well as significant changes to food web structure, phytoplankton composition, and nutrient cycling pathways have all occurred as a result of the introduction of quagga mussels (Bunnell et al., 2006; Cuhel and Aguilar, 2013; Lin and Guo, 2016). As efficient ecosystem engineers, quagga mussels voraciously filter pelagic particulate matter and excrete/egest nutrients in the benthos resulting in significant alterations to water column and benthic chemistry (Schindler and Scheuerell, 2002; Madenjian et al., 2015). Specifically, nutrients and organic matter that served as an energy source for forage fish have been intercepted by quagga mussels and sequestered in the benthos. Therefore, quantifying the specific mechanisms and pathways

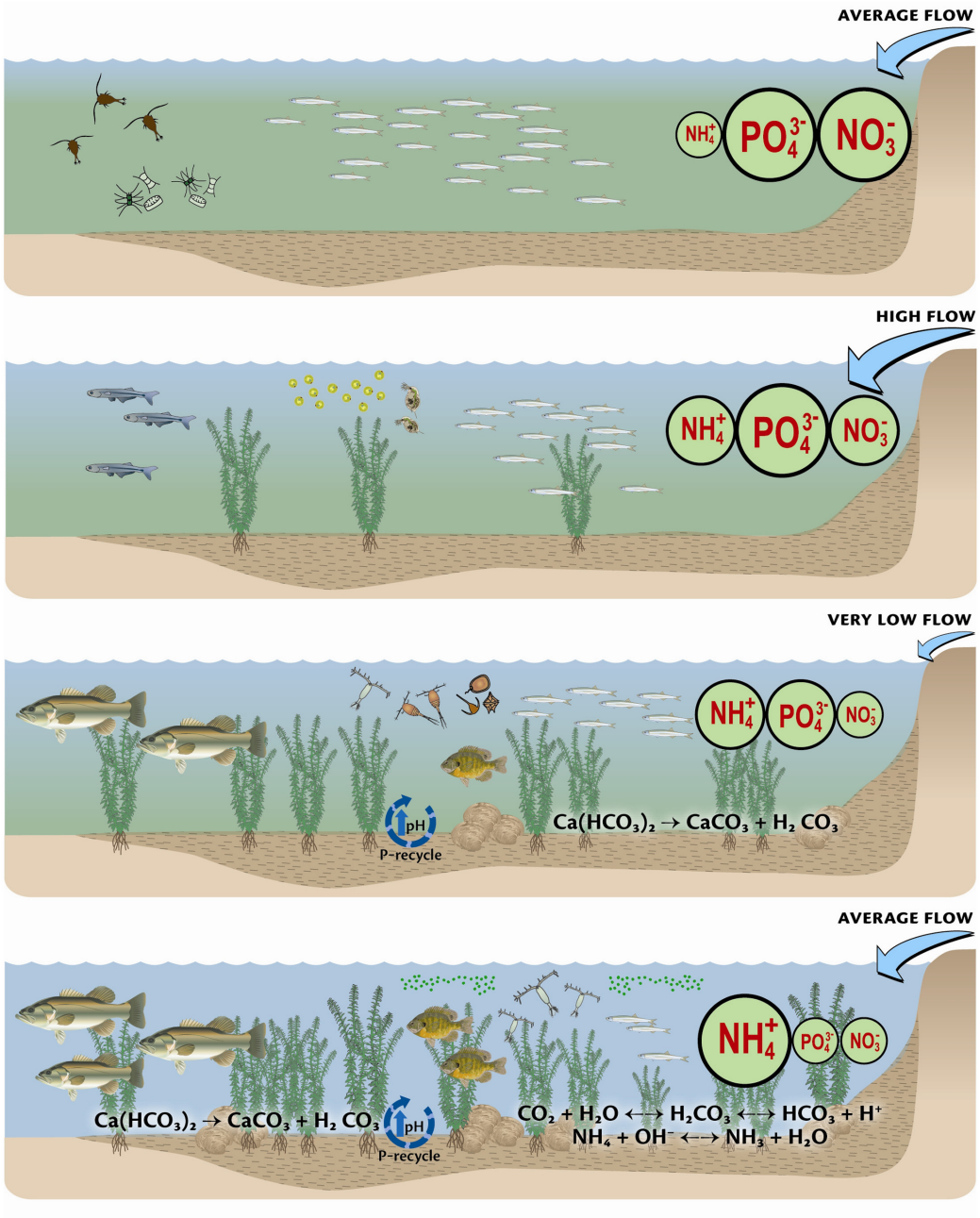

FIGURE 2 | Conceptual depiction of the change over time in major nutrients, flow, dominant biogeochemical processes, and the food web of the Bay Delta. The first panel represents the period from 1975 to ∼1982, when flow was low, and diatoms and *Eurytemora* were the dominant phytoplankton and zooplankton, respectively, and smelt were common. The second panel represents the period from ∼1982 to 1986 when flow was high, and NH_4^+ was increasing. During this period the food web began to change. Under very low flow conditions, depicted by the third panel, and representing ∼1987 to 1995, the NH_4^+ load was high but PO_4^{3-} began to decrease. The food web also began to change significantly, with changes in the dominant phytoplankton and zooplankton, increasing abundance of macrophytes, increased importance of sediment nutrient processes, and increase in piscivores. Finally, post 1995, NH_4^+ loads remain high, while PO_4^{3-} loads are proportionately low. Sediment biogeochemical processes are of increasing importance in nutrient processing, macrophyte production is important and omnivorous fish have increased. At the microbial level, *Microcystis* is more common and the zooplankton is dominated by cyclopoids, e.g., *Limnoithona*. Reproduced from Glibert (2012) with permission of the publisher.

by which invasive quagga mussels have altered organic C and nutrient cycling are needed to understand the response of the Lake Michigan ecosystem to these non-indigenous bivalves. In the absence of particulate organic matter, which has become scarce in the water column of Lake Michigan, quagga mussels have been shown to efficiently remove materials in the dissolved

and colloidal phase (DeVilbiss and Guo, 2017). For example, laboratory incubations have demonstrated the ability of quagga mussels to efficiently remove material as small as 0.5 μm, indicating their potential to directly uptake DOM in the water column. Quagga mussels also directly excrete DOM, with smaller mussels excreting at a significantly higher rate than larger mussels. Excreted DOM had unique chromophoric and fluorescent properties characteristic of protein-like materials, a colloidal size spectrum centered at 1–5 kDa, low TOC/TDN ratios (1.1 ± 0.1) but higher TDN/TDP ratios (33 ± 4) and was predominately composed of structural (refractory) polysaccharides. These results indicated that excreted DOM was chemically altered not only in regards to C molecules, but in N:P ratios as well. Based on initial estimations, only around 11% of consumed organic C was excreted/egested by quagga mussels, indicating that quagga mussels may be a potential sink for organic matter as well as a CO_2 source via metabolism.

Applying ES to Understand Effects of Added Nutrients on Microbial to Ecosystem-Scale Carbon Loss

Understanding effects of nutrient addition on microbial to ecosystem-scale metabolic processes is essential to expanding theoretical predictions of elemental limitation among ecosystems (Elser et al., 2007). Ecosystems that are autotrophic are generally co-limited by N and P (Elser et al., 2007), whereas donor-controlled, detritus-based ecosystems are dominated by heterotrophic consumers whose responses to added nutrients depend on the stoichiometry of detrital resources (Manning et al., 2015). Added N and P both accelerate C loss in detritus-based streams through enhanced organic matter breakdown and export (Benstead et al., 2009; Rosemond et al., 2015; Manning et al., 2016), as well as through substrate-specific and whole-stream ER (Suberkropp et al., 2010; Kominoski et al., 2017). Litter breakdown rates are constrained by microbial nutrient limitation (both N and P) at low-to-moderate concentrations through changes in litter C:N and C:P stoichiometry (Kominoski et al., 2015; Manning et al., 2015). These collective findings emphasize the importance of microbial processes on ecosystem C loss and the potential for long-term vulnerability to sustained C losses with sustained or increased N and P availability (Alexander and Smith, 2006), which ultimately can be linked to nutrient stoichiometry.

Long-term studies of nutrient enrichment in forest streams show declines in ecosystem-scale C. Studies of added N and P in streams of the Coweeta Long Term Ecological Research Program in the southern Appalachians, United States, illustrate that nutrients increase C loss through enhanced microbial respiration rates and invertebrate feeding activities (Benstead et al., 2009; Suberkropp et al., 2010). Increasing N and P concentrations while maintaining N:P ratios can accelerate in-stream biological process that result in up to a 50% reduction in residence time of terrestrial C (Rosemond et al., 2015). Declines in organic matter standing stocks and increases in associated respiration rates with nutrient enrichment, appear to be driven more by N than P. Nutrient enrichment can alter the relationships between N and P supply ratio and ecosystem-level processes. For example, prior to nutrient enrichment whole-stream ER in Coweeta streams was higher at lower N:P, but during enrichment ER increased with increasing N:P (Kominoski et al., 2017). Increased heterotrophy from microbial to ecosystem-scales can occur at concentrations of N and P that are now common among pristine and human-impacted ecosystems (Alexander and Smith, 2006).

Combining Metabolic Ecology and Ecological Stoichiometry to Develop a Mechanistic Understanding of How Temperature Influences Freshwater Metabolism

A central challenge for ecologists is to understand how climate warming will influence GPP and ER, due to the central role these processes play in structuring food web production and C and nutrient cycles (Peterson et al., 2001; Raymond et al., 2013; Hotchkiss et al., 2015). The combined frameworks of metabolic ecology and ES offer promise for developing a mechanistic understanding of how temperature influences freshwater metabolism (Sterner and Elser, 2002; Sibly et al., 2012). Yet, more explicit consideration of the coupling between metabolic theory and ES is required (Sterner, 2004; Cross et al., 2015). A growing literature suggests that temperature dependences of ecosystem processes may diverge strongly from predictions, particularly when temperature influences—or is associated with—changes in resource supply (Anderson-Teixeira et al., 2008; Valett et al., 2008; Yvon-Durocher et al., 2012; Huryn et al., 2014; Welter et al., 2015). A better mechanistic understanding of how temperature and nutrients interact to influence metabolism will likely improve these predictive models.

Model ecosystems, that are natural, can provide a powerful tool for quantifying these mechanisms at the ecosystem level. The Hengill geothermal area in Iceland represents one such natural laboratory for examining how temperature influences the structure and function of stream ecosystems (O'Gorman et al., 2012, 2014) by allowing a combination of field surveys, stream-side channel experiments, and whole-stream temperature manipulations. Recent experiments have discovered that temperature dependences (measured as apparent "activation energies"; Brown et al., 2004) for GPP and ER were 6.5- and 2.7-fold higher, respectively, than predicted by Metabolic Theory; interestingly, these relationships were similar to the temperature dependency of N_2-fixation (Welter et al., 2015), suggesting a strong interaction between temperature and nutrient supply. The stronger than expected temperature dependencies for GPP and ER likely resulted from N-limitation of production at low temperatures and release from N-limitation at warm temperatures by N_2-fixation and the addition of "new" N. In addition, these studies showed that N limitation was further alleviated by a temperature-induced increase in N use efficiency (Williamson et al., 2016). A similar increase in flux-based N use efficiency was found in a survey of natural geothermal streams, as well as a whole-stream warming experiment in this Icelandic catchment (Hood et al., unpublished data). Taken

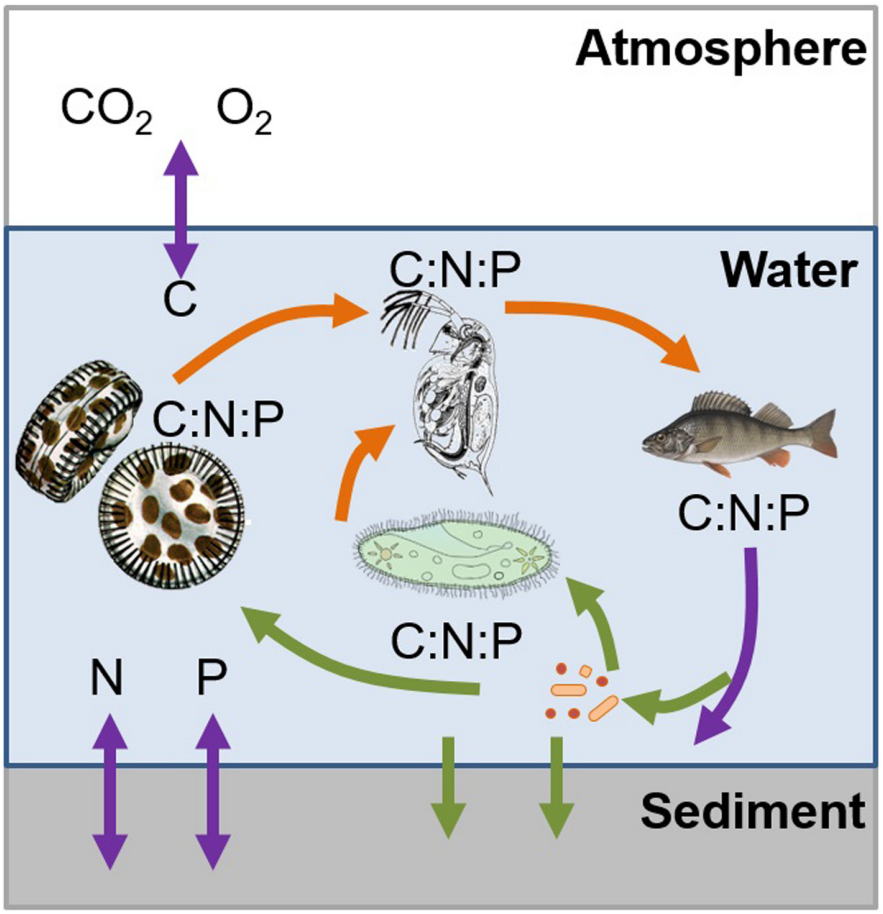

FIGURE 3 | Example demonstrating how ecological stoichiometry can be used to link food web interactions, ecosystem metabolism, and biogeochemistry in a system, as they are inherently linked by the transfer of carbon, nitrogen, and phosphorous through biotic and abiotic nutrient transformation and fluxes. The trophic interactions (orange arrows) are occurring based on the nutrient requirements which are limited by the available nutrients (green arrows) as they are transferred and transformed (purple arrows) between the atmosphere, water column, and sediment. The colors of arrows indicate the processes described in **Figure 1**.

together, these results promise that a better understanding of the interactive effects of temperature and nutrients on organisms and elemental fluxes can be used to develop a strong mechanistic understanding of how climate warming will influence river metabolism.

SUMMARY AND OUTLOOK

The examples described above demonstrate that ES can be a useful tool for linking food web interactions, ecosystem metabolism, and biogeochemistry (**Figure 1**). As demonstrated in the previous examples, altered nutrient concentrations, ratios or fluxes, either through anthropogenic or system-induced pathways, results in changes in ecosystem functioning (**Figure 3**). By increasing nutrient concentrations, organic matter decomposition increases and results in overall C loss in aquatic ecosystems. Furthermore, these increased nutrient concentrations may induce a shift toward favorable conditions for invasive species to persist (Glibert, 2015), or shifts toward community structures that enhance microbial metabolism and GHG emissions. Our examples show that it is not only the absolute nutrient concentrations that create these conditions; rather it is both, the concentrations and the ratio of the nutrients that can alter or drive one process over the other. Furthermore, organisms can alter the composition of chemical compounds (as illustrated by the quagga mussel example altering the DOM diversity in a lake), resulting in an overall change to the ecosystem. While we have begun to explore the role of macronutrients, the relative contribution of micronutrients, especially how they interact with other nutrients (as in the case of As and P), is less understood. Such interactions between macro- and micronutrients can potentially alter the stoichiometric balance and thus should be included in future studies. Temperature and nutrient turnover are inherently linked and the examples presented here point to the links between temperature and nutrient cycling and thus the effect of temperature on nutrient ratios.

Along with the above examples, we have demonstrated the current state-of-the-art approaches, which link food web

interactions, ecosystem metabolism, and biogeochemistry along the following concepts and processes (**Figure 1**):

1. Changing biogeochemistry affects trophic interactions and ecosystem processes by altering the elemental ratios of key species and assemblages.
   - The stoichiometry of biogeochemical processes links the biological turnover rates of major elements, such that changes in biodiversity result in changes in mineral nutrient ratios in biogeochemical pools and fluxes.
2. Changing trophic dynamics influences the transformation and fluxes of matter across environmental boundaries.
   - Through biogeochemical pathways, change in a focal group of organisms has propagating consequences on the functioning of other compartments and on the metabolism of aquatic ecosystems.
   - Trophic interactions, food web structure, and mutualistic networks will result in cascading effects on ecosystem metabolism or vice versa.
3. Changing ecosystem metabolism will alter the chemical diversity of the non-living environment.
   - The alteration of metabolic processes in aquatic ecosystems affects the transformation and fluxes of inorganic and organic matter.
   - The molecular diversity of non-living organic matter is functionally linked to the diversity of organisms. Chemical diversity influences and is influenced by shifts in biodiversity.

The future goal is to use the theory of ES as a common currency to connect food web interactions, ecosystem metabolism, and biogeochemistry as they are inherently linked by the transfer of C, N, and P through biotic and abiotic nutrient transformations and fluxes in order to improve our understanding of aquatic ecosystem functioning. Given the future projections of climate change for increasing temperature and anthropogenic nutrient loading, ES can be essential to understand and predict the links between food web interactions, biogeochemistry, and ecosystem metabolism and elucidate the controls which underpin the processes that ultimately drives nutrient and energy fluxes in aquatic ecosystems.

AUTHOR CONTRIBUTIONS

NW and MS contributed equally to this manuscript. NW, MS, and AU conceived the manuscript. All authors contributed substantially to the manuscript, revised it for important intellectual content, approved the final version, and agreed to be accountable for all aspects of the work.

FUNDING

Support to NW was provided through the Academy of Finland (grant number 258875: Mechanisms and atmospheric importance of nitrous oxide uptake in soils) for the preparation of this manuscript. MS was supported by the German Research Foundation SPP 1704 (STR 1383/1-1). HH was supported by the German Research Foundation Research Unit Jena Experiment (DFG HI 848/11-2).

ACKNOWLEDGMENTS

The authors would like to acknowledge the organizers of the 2016 ASLO Annual Meeting in Santa Fe, NM and all the participants in the session that resulted in this manuscript. The authors would like to acknowledge the reviewers and the editor for helpful comments that improved the manuscript. This is contribution number 5324 from the University of Maryland Center for Environmental Science.

REFERENCES

Alexander, R. B., and Smith, R. A. (2006). Trends in the nutrient enrichment of US rivers during the late 20th century and their relation to changes in probable stream trophic conditions. *Limnol. Oceanogr.* 51, 639–654. doi: 10.4319/lo.2006.51.1_part_2.0639

Anderson-Teixeira, K. J., Vitousek, P. M., and Brown, J. H. (2008). Amplified temperature dependence in ecosystems developing on the lava flows of Mauna Loa. Hawai'i. *Proc. Natl. Acad. Sci. U.S.A.* 105, 228–233. doi: 10.1073/pnas.0710214104

Atkinson, D., Ciotti, B. J., and Montagnes, D. J. (2003). Protists decrease in size linearly with temperature: ca. 2.5% C- 1. *Proc. R. Soc. Lond. B Biol. Sci.* 270, 2605–2611. doi: 10.1098/rspb.2003.2538

Baker, J., and Wallschläger, D. (2016). The role of phosphorus in the metabolism of arsenate by a freshwater green alga, *Chlorella vulgaris*. *J. Environ. Sci.* 49, 169–178. doi: 10.1016/j.jes.2016.10.002

Banavar, J. R., Moses, M. E., Brown, J. H., Damuth, J., Rinaldo, A., Sibly, R. M., et al. (2010). A general basis for quarter-power scaling in animals. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15816–15820. doi: 10.1073/pnas.1009974107

Bartels, P., Cucherousset, J., Steger, K., Eklöv, P., Tranvik, L. J., and Hillebrand, H. (2012). Reciprocal subsidies between freshwater and terrestrial ecosystems structure consumer resource dynamics. *Ecology* 93, 1173–1182. doi: 10.1890/11-1210.1

Bastviken, D., Cole, J. J., Pace, M. L., and Van de Bogert, M. C. (2008). Fates of methane from different lake habitats: connecting whole-lake budgets and CH4 emissions. *J. Geophys. Res. Biogeosci.* 113, G02024. doi: 10.1029/2007JG000608

Battin, T. J., Kaplan, L. A., Findlay, S., Hopkinson, C. S., Marti, E., Packman, A. I., et al. (2008). Biophysical controls on organic carbon fluxes in fluvial networks. *Nat. Geosci.* 1, 95–100. doi: 10.1038/ngeo101

Benstead, J. P., Rosemond, A. D., Cross, W. F., Wallace, J. B., Eggert, S. L., Suberkropp, K., et al. (2009). Nutrient enrichment alters storage and fluxes of detritus in a headwater stream ecosystem. *Ecology* 90, 2556–2566. doi: 10.1890/08-0862.1

Binkley, D., Ice, G. G., Kaye, J., and Williams, C. A. (2004). Nitrogen and phosphorus concentrations in forest streams of the United States. *J. Am. Water Resour. Assoc.* 40, 1277–1291. doi: 10.1111/j.1752-1688.2004.tb01586.x

Boening, D. W. (2000). Ecological effects, transport, and fate of mercury: a general review. *Chemosphere* 40, 1335–1351. doi: 10.1016/S0045-6535(99)00283-0

Boersma, M., and Elser, J. J. (2006). Too much of a good thing: on stoichiometrically balanced diets and maximal growth. *Ecology* 87, 1325–1330. doi: 10.1890/0012-9658(2006)87[1325:TMOAGT]2.0.CO;2

Boyd, P. W., and Hutchins, D. A. (2012). Understanding the responses of ocean biota to a complex matrix of cumulative anthropogenic change. *Mar. Ecol. Prog. Ser.* 470, 125–135. doi: 10.3354/meps10121

Brown, J. H., Gillooly, J. F., Allen, A. P., Savage, V. M., and West, G. B. (2004). Toward a metabolic theory of ecology. *Ecology* 85, 1771–1789. doi: 10.1890/03-9000

Bunnell, D. B., Madenjian, C. P., and Claramunt, R. M. (2006). Long-term changes of the Lake Michigan fish community following the reduction of exotic alewife (*Alosa pseudoharengus*). *Can. J. Fish. Aquat. Sci.* 63, 2434–2446. doi: 10.1139/f06-132

Burgin, A. J., Lazar, J. G., Groffman, P. M., Goldb, A. J., and Kellogg, D. Q. (2013). Balancing nitrogen retention ecosystem services and greenhouse gas disservices at the landscape scale. *Ecol. Eng.* 56, 26–35. doi: 10.1016/j.ecoleng.2012.05.003

Butterwick, C., Heaney, S. I., and Talling, J. F. (2005). Diversity in the influence of temperature on the growth rates of freshwater algae, and its ecological relevance. *Freshw. Biol.* 50, 291–300. doi: 10.1111/j.1365-2427.2004.01317.x

Button, D., Dunker, S. S., and Morse, M. (1973). Continuous culture of *Rhodotorula rubra*: kinetics of phosphate-arsenate uptake, inhibition, and phosphate-limited growth. *J. Bacteriol.* 113, 599–611.

Cai, Y., and Guo, L. (2009). Abundance and variation of colloidal organic phosphorus in riverine, estuarine, and coastal waters in the northern Gulf of Mexico. *Limnol. Oceanogr.* 54, 1393–1402. doi: 10.4319/lo.2009.54.4.1393

Cao, M., Marshall, S., and Gregson, K. (1996). Global carbon exchange and methane emissions from natural wetlands: application of a process-based model. *J. Geophys. Res. Atmos.* 101, 14399–14414. doi: 10.1029/96JD00219

Capps, K. A., and Flecker, A. S. (2013). Invasive fishes generate biogeochemical hotspots in a nutrient-limited system. *PLoS ONE* 8:e54093. doi: 10.1371/journal.pone.0054093

Chaffin, J. L., Valett, H. M., Webster, J. R., and Schreiber, M. E. (2005). Influence of elevated as on leaf breakdown in an Appalachian headwater stream. *J. North Am. Benthol. Soc.* 24, 553–568. doi: 10.1899/04-058.1

Collins, S. M., Kohler, T. J., Thomas, S. A., Fetzer, W. W., and Flecker, A. S. (2015). The importance of terrestrial subsidies in stream food webs varies along a stream size gradient. *Oikos* 125, 674–685. doi: 10.1111/oik.02713

Collins, S. M., Thomas, S. A., Heatherly, T. II, MacNeill, K. L., Leduc, A. O., López-Sepulcre, A., et al. (2016). Fish introductions and light modulate food web fluxes in tropical streams: a whole-ecosystem experimental approach. *Ecology* 97, 3154–3166. doi: 10.1002/ecy.1530

Cross, W. F., Benstead, J. P., Frost, P. C., and Thomas, S. A. (2005). Ecological stoichiometry in freshwater benthic systems: recent progress and perspectives. *Freshw. Biol.* 50, 1895–1912. doi: 10.1111/j.1365-2427.2005.01458.x

Cross, W. F., Hood, J. M., Benstead, J. P., Huryn, A. D., and Nelson, D. (2015). Interactions between temperature and nutrients across levels of ecological organization. *Glob. Change Biol.* 21, 1025–1040. doi: 10.1111/gcb.12809

Cuhel, R. L., and Aguilar, C. (2013). Ecosystem transformations of the Laurentian Great Lake Michigan by nonindigenous biological invaders. *Mar. Sci.* 5, 289–320. doi: 10.1146/annurev-marine-120710-100952

Deemer, B. R., Harrison, J. A., Li, S., Beaulieu, J. J., DelSontro, T., Barros, N., et al. (2016). Greenhouse gas emissions from reservoir water surfaces: a new global synthesis. *Bioscience* 66, 949–964. doi: 10.1093/biosci/biw117

Deines, P., Bodelier, P. L., and Eller, G. (2007). Methane-derived carbon flows through methane-oxidizing bacteria to higher trophic levels in aquatic systems. *Environ. Microbiol.* 9, 1126–1134. doi: 10.1111/j.1462-2920.2006.01235.x

DeVilbiss, S. E., and Guo, L. (2017). Excretion of organic matter and nutrients from invasive quagga mussels and potential impact on carbon dynamics in Lake Michigan. *J. Great Lakes Res.* 43, 79–89. doi: 10.1016/j.jglr.2017.03.002

Devine, J. A., and Vanni, M. J. (2002). Spatial and seasonal variation in nutrient excretion by benthic invertebrates in a eutrophic reservoir. *Freshw. Biol.* 47, 1107–1121. doi: 10.1046/j.1365-2427.2002.00843.x

Dodds, W. K., Gido, K., Whiles, M. R., Fritz, K. M., and Matthews, W. J. (2004). Life on the edge: the ecology of Great Plains prairie streams. *BioScience* 54, 205–216. doi: 10.1641/0006-3568(2004)054[0205:LOTETE]2.0.CO;2

Elser, J. J., Bracken, M. E., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Eppley, R. W. (1972). Temperature and phytoplankton growth in the sea. *Fish. Bull.* 70, 1063–1085.

Falkowski, P., Scholes, R. J., Boyle, E., Canadell, J., Canfield, D., Elser, J., et al. (2000). The global carbon cycle: a test of our knowledge of earth as a system. *Science* 290, 291–296. doi: 10.1126/science.290.5490.291

Farag, A. M., Skaar, D., Nimick, D. A., MacConnell, E., and Hogstrand, C. (2003). Characterizing aquatic health using salmonid mortality, physiology, and biomass estimates in streams with elevated concentrations of arsenic, cadmium, copper, lead, and zinc in the Boulder River watershed, Montana. *Trans. Am. Fish. Soc.* 132, 450–467. doi: 10.1577/1548-8659(2003)132<0450:CAHUSM>2.0.CO;2

Figueiredo-Barros, M. P., Caliman, A., Leal, J. J., Bozelli, R. L., Farjalla, V. F., and Esteves, F. A. (2009). Benthic bioturbator enhances CH4 fluxes among aquatic compartments and atmosphere in experimental microcosms. *Can. J. Fish. Aquat. Sci.* 66, 1649–1657. doi: 10.1139/F09-111

Findlay, S., Carlough, L., Crocker, M. T., Kay Gill, H., Meyer, J. L., and Smith, P. J. (1986). Bacterial growth on macrophyte leachate and fate of bacterial production. *Limnol. Oceanogr.* 31, 1335–1341. doi: 10.4319/lo.1986.31.6.1335

Finnegan, P. M., and Chen, W. (2012). Arsenic toxicity: the effects on plant metabolism. *Front. Physiol.* 3:182. doi: 10.3389/fphys.2012.00182

Forster, J., Hirst, A. G., and Esteban, G. F. (2013). Achieving temperature-size changes in a unicellular organism. *ISME J.* 7, 28–36. doi: 10.1038/ismej.2012.76

Franklin, O., Hall, E. K., Kaiser, C., Battin, T. J., and Richter, A. (2011). Optimization of biomass composition explains microbial growth-stoichiometry relationships. *Am. Nat.* 177, E29–E42. doi: 10.1086/657684

Glazier, D. S., Hirst, A. G., and Atkinson, D. (2015). Shape shifting predicts ontogenetic changes in metabolic scaling in diverse aquatic invertebrates. *Proc. R. Soc. Lond. B Biol. Sci.* 282:20142302. doi: 10.1098/rspb.2014.2302

Glibert, P. M. (2012). Ecological stoichiometry and its implications for aquatic ecosystem sustainability. *Curr. Opin. Environ. Sustain.* 4, 272–277. doi: 10.1016/j.cosust.2012.05.009

Glibert, P. M. (2015). More than propagule pressure: successful invading algae have physiological adaptations suitable to anthropogenically changing nutrient environments. *Aquat. Ecosyst. Health Manag.* 18, 334–341.

Glibert, P. M., Fullerton, D., Burkholder, J. M., Cornwell, J. C., and Kana, T. M. (2011). Ecological stoichiometry, biogeochemical cycling, invasive species, and aquatic food webs: San Francisco Estuary and comparative systems. *Rev. Fish. Sci.* 19, 358–417. doi: 10.1080/10641262.2011.611916

Glibert, P. M., Kana, T. M., and Brown, K. (2013). From limitation to excess: the consequences of substrate excess and stoichiometry for phytoplankton physiology, trophodynamics and biogeochemistry, and the implications for modeling. *J. Mar. Syst.* 125, 14–28. doi: 10.1016/j.jmarsys.2012.10.004

Glibert, P. M., Maranger, R., Sobota, D. J., and Bouwman, L. (2014). The Haber Bosch–harmful algal bloom (HB–HAB) link. *Environ. Res. Lett.* 9, 105001. doi: 10.1088/1748-9326/9/10/105001

Godwin, C. M., and Cotner, J. B. (2015). Aquatic heterotrophic bacteria have highly flexible phosphorus content and biomass stoichiometry. *ISME J.* 9, 2324–2327. doi: 10.1038/ismej.2015.34

Grossart, H., Engel, A., Arnosti, C., De La Rocha, C. L., Murray, A. E., and Passow, U. (2007). Microbial dynamics in autotrophic and heterotrophic seawater mesocosms. III. Organic matter fluxes. *Aquat. Microb. Ecol.* 49, 143–156. doi: 10.3354/ame01140

Hall, E. K., Singer, G. A., Kainz, M. J., and Lennon, J. T. (2010). Evidence for a temperature acclimation mechanism in bacteria: an empirical test of a membrane-mediated trade-off. *Funct. Ecol.* 24, 898–908. doi: 10.1111/j.1365-2435.2010.01707.x

Hall, R. O., Baker, M. A., Rosi-Marshall, E. J., Tank, J. L., and Newbold, J. D. (2013). Solute-specific scaling of inorganic nitrogen and phosphorus uptake in streams. *Biogeosciences* 10, 7323–7331. doi: 10.5194/bg-10-7323-2013

Hall, R. O., and Tank, J. L. (2003). Ecosystem metabolism controls nitrogen uptake in streams in Grand Teton National Park, Wyoming. *Limnol. Oceanogr.* 48, 1120–1128. doi: 10.4319/lo.2003.48.3.1120

Hall, R. O., Tank, J. L., Baker, M. A., Rosi-Marshall, E. J., and Hotchkiss, E. R. (2016). Metabolism, gas exchange, and carbon spiraling in rivers. *Ecosystems* 19, 73–86. doi: 10.1007/s10021-015-9918-1

Hall, R. O., Tank, J. L., and Dybdahl, M. F. (2003). Exotic snails dominate nitrogen and carbon cycling in a highly productive stream. *Front. Ecol. Environ.* 1:407–411. doi: 10.1890/1540-9295(2003)001[0407:esdnac]2.0.co;2

Harpole, W. S., Ngai, J. T., Cleland, E. E., Seabloom, E. W., Borer, E. T., Bracken, M. E., et al. (2011). Nutrient co-limitation of primary producer communities:

community co-limitation. *Ecol. Lett.* 14, 852–862. doi: 10.1111/j.1461-0248.2011.01651.x

Heisterkamp, I., Schramm, A., de Beer, D., and Stief, P. (2010). Nitrous oxide production associated with coastal marine invertebrates. *Mar. Ecol. Prog. Ser.* 415, 1–9. doi: 10.3354/meps08727

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Hirst, A. G., Glazier, D. S., and Atkinson, D. (2014). Body shape shifting during growth permits tests that distinguish between competing geometric theories of metabolic scaling. *Ecol. Lett.* 17, 1274–1281. doi: 10.1111/ele.12334

Hirst, A. G., Lilley, M. K. S., Glazier, D. S., and Atkinson, D. (2016). Ontogenetic body-mass scaling of nitrogen excretion relates to body surface area in diverse pelagic invertebrates: N-excretion in pelagic invertebrates. *Limnol. Oceanogr.* 62, 311–319. doi: 10.1002/lno.10396

Hoellein, T. J., Bruesewitz, D. A., and Hamilton, D. P. (2012). Are geothermal streams important sites of nutrient uptake in an agricultural and urbanising landscape (Rotorua, New Zealand)?: nutrient uptake and metabolism in geothermal streams. *Freshw. Biol.* 57, 116–128. doi: 10.1111/j.1365-2427.2011.02702.x

Hoellein, T. J., Bruesewitz, D. A., and Richardson, D. C. (2013). Revisiting Odum (1956): a synthesis of aquatic ecosystem metabolism. *Limnol. Oceanogr.* 58, 2089–2100. doi: 10.4319/lo.2013.58.6.2089

Hölker, F., Vanni, M. J., Kuiper, J. J., Meile, C., Grossart, H. P., Stief, P., et al. (2015). Tube-dwelling invertebrates: tiny ecosystem engineers have large effects in lake ecosystems. *Ecol. Monogr.* 85, 333–351. doi: 10.1890/14-1160.1

Hotchkiss, E., Hall, R. Jr., Sponseller, R., Butman, D., Klaminder, J., Laudon, H., et al. (2015). Sources of and processes controlling CO_2 emissions change with the size of streams and rivers. *Nat. Geosci.* 8, 696–699. doi: 10.1038/ngeo2507

Huryn, A. D., Benstead, J. P., and Parker, S. M. (2014). Seasonal changes in light availability modify the temperature dependence of ecosystem metabolism in an arctic stream. *Ecology* 95, 2826–2839. doi: 10.1890/13-1963.1

Jeyasingh, P. D. (2007). Plasticity in metabolic allometry: the role of dietary stoichiometry. *Ecol. Lett.* 10, 282–289. doi: 10.1111/j.1461-0248.2007.01023.x

Jones, R. I., Carter, C. E., Kelly, A., Ward, S., Kelly, D. J., and Grey, J. (2008). Widespread contribution of methane-cycle bacteria to the diets of lake profundal chironomid larvae. *Ecology* 89, 857–864. doi: 10.1890/06-2010.1

Kajan, R., and Frenzel, P. (1999). The effect of chironomid larvae on production, oxidation and fluxes of methane in a flooded rice soil. *FEMS Microbiol. Ecol.* 28, 121–129. doi: 10.1111/j.1574-6941.1999.tb00567.x

Kankaala, P., Eller, G., and Jones, R. I. (2007). Could bacterivorous zooplankton affect lake pelagic methanotrophic activity? *Fundam. Appl. Limnol.* 169, 203–209. doi: 10.1127/1863-9135/2007/0169-0203

Karentz, D., and Smayda, T. J. (1984). Temperature and seasonal occurrence patterns of 30 dominant phytoplankton species in Narragansett Bay over a 22-year period (1959–1980). *Mar. Ecol. Prog. Ser.* 18, 277–293. doi: 10.3354/meps018277

Kaspari, M., and Powers, J. S. (2016). Biogeochemistry and geographical ecology: embracing all twenty-five elements required to build organisms. *Am. Nat.* 188, S62–S73. doi: 10.1086/687576

Kleiber, M. (1932). Body size and metabolism. *Hilgardia* 6, 315–353. doi: 10.3733/hilg.v06n11p315

Kleiber, M. (1961). *The Fire of Life: An Introduction to Animal Energetics*. New York, NY: Wiley.

Kominoski, J. S., Rosemond, A. D., Benstead, J. P., Gulis, V., Maerz, J. C., and Manning, D. W. (2015). Low-to-moderate nitrogen and phosphorus concentrations accelerate microbially driven litter breakdown rates. *Ecol. Appl.* 25, 856–865. doi: 10.1890/14-1113.1

Kominoski, J. S., Rosemond, A. D., Benstead, J. P., Gulis, V., and Manning, D. W. P. (2017). Experimental nitrogen and phosphorus additions increase rates of stream ecosystem respiration and carbon loss. *Limnol. Oceanogr.* doi: 10.1002/lno.10610 [Epub ahead of print].

Kristensen, E., Hjorth Jensen, M., and Aller, R. C. (1991). Direct measurement of dissolved inorganic nitrogen exchange and denitrification in individual polychaete (*Nereis virens*) burrows. *J. Mar. Res.* 49, 355–377. doi: 10.1357/002224091784995855

Lennon, J., and Pfaff, L. (2005). Source and supply of terrestrial organic matter affects aquatic microbial metabolism. *Aquat. Microb. Ecol.* 39, 107–119. doi: 10.3354/ame039107

Lin, P., and Guo, L. (2016). Dynamic changes in the abundance and chemical speciation of dissolved and particulate phosphorus across the river-lake interface in southwest Lake Michigan. *Limnol. Oceanogr.* 61, 771–789. doi: 10.1002/lno.10254

Litchman, E., de Tezanos Pinto, P., and Klausmeier, C. A. (2010). Linking traits to species diversity and community structure in phytoplankton. *Hydrobiologia* 653, 15–28. doi: 10.1007/s10750-010-0341-5

Lomas, M. W., and Glibert, P. M. (1999). Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. *Limnol. Oceanogr.* 44, 556–572. doi: 10.4319/lo.1999.44.3.0556

Lottig, N. R., Maurice Valett, H., Schreiber, M. E., and Webster, J. R. (2007). Flooding and arsenic contamination: influences on ecosystem structure and function in an Appalachian headwater stream. *Limnol. Oceanogr.* 52, 1991–2001. doi: 10.4319/lo.2007.52.5.1991

Lovett, G. M., Cole, J. J., and Pace, M. L. (2006). Is net ecosystem production equal to ecosystem carbon accumulation? *Ecosystems* 9, 152–155. doi: 10.1007/s10021-005-0036-3

MacNeill, K. L., Collins, S. M., Encalada, A. C., Kohler, B. S., Thomas, S. A., Rosi-Marshall, E., et al. (2016). "Arsenic controls on stoichiometry and nutrient cycling in tropical streams," in *Proceedings of the ASLO Conference 2016* [Abstract ID:28047], Santa Fe, NM.

Madenjian, C. P., Bunnell, D. B., Warner, D. M., Pothoven, S. A., Fahnenstiel, G. L., Nalepa, T. H., et al. (2015). Changes in the Lake Michigan food web following dreissenid mussel invasions: a synthesis. *J. Great Lakes Res.* 41, 217–231. doi: 10.1016/j.jglr.2015.08.009

Manning, D. W., Rosemond, A. D., Gulis, V., Benstead, J. P., Kominoski, J. S., and Maerz, J. C. (2016). Convergence of detrital stoichiometry predicts thresholds of nutrient-stimulated breakdown in streams. *Ecol. Appl.* 26, 1745–1757. doi: 10.1890/15-1217.1

Manning, D. W., Rosemond, A. D., Kominoski, J. S., Gulis, V., Benstead, J. P., and Maerz, J. C. (2015). Detrital stoichiometry as a critical nexus for the effects of streamwater nutrients on leaf litter breakdown rates. *Ecology* 96, 2214–2224. doi: 10.1890/14-1582.1

Marcarelli, A. M., Baxter, C. V., Mineau, M. M., and Hall, R. O. (2011). Quantity and quality: unifying food web and ecosystem perspectives on the role of resource subsidies in freshwaters. *Ecology* 92, 1215–1225. doi: 10.1890/10-2240.1

Martiny, A. C., Pham, C. T. A., Primeau, F. W., Vrugt, I. A., Moore, J. K., Levin, S. A., et al. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci.* 6, 279–283. doi: 10.1038/ngeo1757

Mehring, A. S., Cook, P. L. M., Evrard, V., Grant, S. B., and Levin, L. A. (2017). Pollution-tolerant invertebrates enhance greenhouse gas flux in urban wetlands. *Ecol. Appl.* doi: 10.1002/eap.1572 [Epub ahead of print].

Meyer, J. L., and Likens, G. E. (1979). Transport and transformation of phosphorus in a forest stream ecosystem. *Ecology* 60, 1255–1269. doi: 10.2307/1936971

Montagnes, D. J., and Franklin, M. (2001). Effect of temperature on diatom volume, growth rate, and carbon and nitrogen content: reconsidering some paradigms. *Limnol. Oceanogr.* 46, 2008–2018. doi: 10.4319/lo.2001.46.8.2008

Moorthi, S. D., Schmitt, J. A., Ryabov, A., Tsakalakis, I., Blasius, B., Prelle, L., et al. (2016). Unifying ecological stoichiometry and metabolic theory to predict production and trophic transfer in a marine planktonic food web. *Philos. Trans. R. Soc. B Biol. Sci.* 371:20150270. doi: 10.1098/rstb.2015.0270

Mulholland, P. J., Tank, J. L., Sanzone, D. M., Wollheim, W. M., Peterson, B. J., Webster, J. R., et al. (2000). Nitrogen cycling in a forest stream determined by a 15N tracer addition. *Ecol. Monogr.* 70, 471–493.

Murray, A. E., Arnosti, C., De La Rocha, C., Grossart, H. P., and Passow, U. (2007). Microbial dynamics in autotrophic and heterotrophic seawater mesocosms. II. Bacterioplankton community structure and hydrolytic enzyme activities. *Aquat. Microb. Ecol.* 49, 123–141. doi: 10.3354/ame01139

Nielsen, O. I., Gribsholt, B., Kristensen, E., and Revsbech, N. P. (2004). Microscale distribution of oxygen and nitrate in sediment inhabited by *Nereis diversicolor*: spatial patterns and estimated reaction rates. *Aquat. Microb. Ecol.* 34, 23–32. doi: 10.3354/ame034023

Nisbet, E. G., Dlugokencky, E. J., and Bousquet, P. (2014). Methane on the rise—again. *Science* 343, 493–495. doi: 10.1126/science.1247828

Nogaro, G., and Burgin, A. J. (2014). Influence of bioturbation on denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in freshwater sediments. *Biogeochemistry* 120, 279–294. doi: 10.1007/s10533-014-9995-9

O'Gorman, E. J., Benstead, J. P., Cross, W. F., Friberg, N., Hood, J. M., Johnson, P. W., et al. (2014). Climate change and geothermal ecosystems: natural laboratories, sentinel systems, and future refugia. *Glob. Change Biol.* 20, 3291–3299. doi: 10.1111/gcb.12602

O'Gorman, E. J., Pichler, D. E., Adams, G., et al. (2012). Impacts of warming on the structure and functioning of aquatic communities: individual-to ecosystem-level responses. *Adv. Ecol. Res.* 47, 81–176. doi: 10.1016/B978-0-12-398315-2.00002-8

Passow, U., De La Rocha, C. L., Arnosti, C., Grossart, H. P., Murray, A., and Engel, A. (2007). Microbial dynamics in autotrophic and heterotrophic seawater mesocosms. I. Effect of phytoplankton on the microbial loop. *Aquat. Microb. Ecol.* 49, 109–121. doi: 10.3354/ame01138

Pete, R., Davidson, K., Hart, M. C., Gutierreza, T., and Miller, A. E. J. (2010). Diatom derived dissolved organic matter as a driver of bacterial productivity: the role of nutrient limitation. *J. Exp. Mar. Biol. Ecol.* 391, 20–26. doi: 10.1016/j.jembe.2010.06.002

Peterson, B. J., Wollheim, W. M., Mulholland, P. J., Webster, J. R., Meyer, J. L., Tank, J. L., et al. (2001). Control of nitrogen export from watersheds by headwater streams. *Science* 292, 86–90. doi: 10.1126/science.1056874

Poulsen, M., Kofoed, M. V., Larsen, L. H., Schramm, A., and Stief, P. (2014). *Chironomus plumosus* larvae increase fluxes of denitrification products and diversity of nitrate-reducing bacteria in freshwater sediment. *Syst. Appl. Microbiol.* 37, 51–59. doi: 10.1016/j.syapm.2013.07.006

Pringle, C. M. (1991). Geothermally modified waters surface at La Selva Biological Station, Costa Rica: volcanic processes introduce chemical discontinuities into lowland tropical streams. *Biotropica* 23, 523–529. doi: 10.2307/2388390

Raymond, P. A., Hartmann, J., Lauerwald, R., Sobek, S., McDonald, C., Hoover, M., et al. (2013). Global carbon dioxide emissions from inland waters. *Nature* 503, 355–359. doi: 10.1038/nature12760

Reiners, W. A. (1986). Complementary models for ecosystems. *Am. Nat.* 127, 59–73. doi: 10.1086/284467

Rembauville, M., Blain, S., Armand, L., Quéguiner, B., and Salter, I. (2015). Export fluxes in a naturally iron-fertilized area of the Southern Ocean – Part 2: importance of diatom resting spores and faecal pellets for export. *Biogeosciences* 12, 3171–3195. doi: 10.5194/bg-12-3171-2015

Rodriguez Castro, M. C., Urrea, G., and Guasch, H. (2015). Influence of the interaction between phosphate and arsenate on periphyton's growth and its nutrient uptake capacity. *Sci. Total Environ.* 503–504, 122–132. doi: 10.1016/j.scitotenv.2014.06.094

Rosemond, A. D., Benstead, J. P., Bumpers, P. M., Gulis, V., Kominoski, J. S., Manning, D. W., et al. (2015). Experimental nutrient additions accelerate terrestrial carbon loss from stream ecosystems. *Science* 347, 1142–1145. doi: 10.1126/science.aaa1958

Rubner, M. (1883). Ueber den einfluss der korpergrosse auf stoffund kaftwechsel. *Z. Biol.* 19, 535–562.

Salter, I., Schiebel, R., Ziveri, P., Movellan, A., Lampitt, R., and Wolff, G. A. (2014). Carbonate counter pump stimulated by natural iron fertilization in the Polar Frontal Zone. *Nat. Geosci.* 7, 885–889. doi: 10.1038/ngeo2285

Santos, I. R., Burnett, W. C., Chanton, J., Mwashote, B., Suryaputra, I. G. N. A., and Dittmar, T. (2008). Nutrient biogeochemistry in a Gulf of Mexico subterranean estuary and groundwater-derived fluxes to the coastal ocean. *Limnol. Oceanogr.* 53, 705–718. doi: 10.4319/lo.2008.53.2.0705

Savage, V. M., Deeds, E. J., and Fontana, W. (2008). Sizing up allometric scaling theory. *PLoS Comput. Biol.* 4:e1000171. doi: 10.1371/journal.pcbi.1000171

Schade, J. D., MacNEILL, K., Thomas, S. A., Camille McNeely, F., Welter, J. R., Hood, J., et al. (2011). The stoichiometry of nitrogen and phosphorus spiralling in heterotrophic and autotrophic streams: stoichiometry of N and P spiralling in streams. *Freshw. Biol.* 56, 424–436. doi: 10.1111/j.1365-2427.2010.02509.x

Schaller, J., Weiske, A., Mkandawire, M., and Dudel, E. G. (2010). Invertebrates control metals and arsenic sequestration as ecosystem engineers. *Chemosphere* 79, 169–173. doi: 10.1016/j.chemosphere.2010.01.015

Schindler, D. E., and Scheuerell, M. D. (2002). Habitat coupling in lake ecosystems. *Oikos* 98, 177–189. doi: 10.1034/j.1600-0706.2002.980201.x

Schmidt-Nielsen, K. (1984). *Scaling: Why is Animal Size so Important?* Cambridge: Cambridge University Press. doi: 10.1017/CBO9781139167826

Scott, J. T., Cotner, J. B., and LaPara, T. M. (2012). Variable stoichiometry and homeostatic regulation of bacterial biomass elemental composition. *Front. Microbiol.* 3:42. doi: 10.3389/fmicb.2012.00042

Senft, W. H., Hunchberger, R. A., and Roberts, K. E. (2008). Temperature dependence of growth and phosphorus uptake in two species of *Volvox* (Volvovales. Chlorophyta). *J. Phycol.* 17, 323–329. doi: 10.1111/j.1529-8817.1981.tb00858.x

Sibly, R. M., Brown, J. H., and Kodric-Brown, A. (2012). *Metabolic Ecology: A Scaling Approach*. Hoboken, NJ: John Wiley & Sons. doi: 10.1002/9781119968535

Singer, G. A., Fasching, C., Wilhelm, L., Niggemann, J., Steier, P., Dittmar, T., et al. (2012). Biogeochemically diverse organic matter in Alpine glaciers and its downstream fate. *Nat. Geosci.* 5, 710–714. doi: 10.1038/ngeo1581

Sterner, R. W. (2004). A one-resource"stoichiometry"? *Ecology* 85, 1813–1816. doi: 10.1890/03-0724

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Stief, P., Poulsen, M., Nielsen, L. P., Brix, H., and Schramm, A. (2009). Nitrous oxide emission by aquatic macrofauna. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4296–4300. doi: 10.1073/pnas.0808228106

Suberkropp, K., Gulis, V., Rosemond, A. D., and Benstead, J. (2010). Ecosystem and physiological scales of microbial responses to nutrients in a detritus-based stream: results of a 5-year continuous enrichment. *Limnol. Oceanogr.* 55, 149–160. doi: 10.4319/lo.2010.55.1.0149

Tessier, J. T., and Raynal, D. J. (2003). Use of nitrogen to phosphorus ratios in plant tissue as an indicator of nutrient limitation and nitrogen saturation. *J. Appl. Ecol.* 40, 523–534. doi: 10.1046/j.1365-2664.2003.00820.x

Triska, F. J., Sedell, J. R., Cromack, K., Gregory, S. V., and Michael McCorison, F. (1984). Nitrogen budget for a small coniferous forest stream. *Ecol. Monogr.* 54, 119–140. doi: 10.2307/1942458

Valett, H. M., Thomas, S. A., Mulholland, P. J., Webster, J. R., Dahm, C. N., Fellows, C. S., et al. (2008). Endogenous and exogenous control of ecosystem function: N cycling in headwater streams. *Ecology* 89, 3515–3527. doi: 10.1890/07-1003.1

Vanni, M. J., and McIntyre, P. B. (2016). Predicting nutrient excretion of aquatic animals with metabolic ecology and ecological stoichiometry: a global synthesis. *Ecology* 97, 3460–3471. doi: 10.1002/ecy.1582

Vannote, R. L., Minshall, G. W., Cummins, K. W., Sedell, J. R., and Cushing, C. E. (1980). The river continuum concept. *Can. J. Fish. Aquat. Sci.* 37, 130–137. doi: 10.1139/f80-017

Villanueva, D., Queimaliños, C., Modenutti, B., and Ayala, J. (2000). Effects of fish farm effluents on the periphyton of an Andean stream. *Arch. Fish. Mar. Res.* 48, 283–294.

Wallace, J. B., Eggert, S., Meyer, J. L., and Webster, J. (1999). Effects of resource limitation on a detrital-based ecosystem. *Ecol. Monogr.* 69, 409–442. doi: 10.1890/0012-9615(1999)069[0409:EORLOA]2.0.CO;2

Walters, D. M., Rosi-Marshall, E., Kennedy, T. A., Cross, W. F., and Baxter, C. V. (2015). Mercury and selenium accumulation in the Colorado River food web, Grand Canyon, USA: Hg and Se in the Colorado River Food Web, Grand Canyon. *Environ. Toxicol. Chem.* 34, 2385–2394. doi: 10.1002/etc.3077

Wang, N. X., Li, Y., Deng, X. H., Miao, A. J., Ji, R., and Yang, L. Y. (2013). Toxicity and bioaccumulation kinetics of arsenate in two freshwater green algae under different phosphate regimes. *Water Res.* 47, 2497–2506. doi: 10.1016/j.watres.2013.02.034

Webster, J. R., Mulholland, P. J., Tank, J. L., Valett, H. M., Dodds, W. K., Peterson, B. J., et al. (2003). Factors affecting ammonium uptake in streams - an inter-biome perspective. *Freshw. Biol.* 48, 1329–1352. doi: 10.1046/j.1365-2427.2003.01094.x

Welter, J. R., Benstead, J. P., Cross, W. F., Hood, J. M., Huryn, A. D., Johnson, P. W., et al. (2015). Does N2 fixation amplify the temperature dependence of ecosystem metabolism? *Ecology* 96, 603–610.

West, G. B., Brown, J. H., and Enquist, B. J. (1999). The fourth dimension of life: fractal geometry and allometric scaling of organisms. *Science* 284, 1677–1679. doi: 10.1126/science.284.5420.1677

Williamson, T. J., Cross, W. F., Benstead, J. P., Gíslason, G. M., Hood, J. M., Huryn, A. D., et al. (2016). Warming alters coupled carbon and nutrient cycles in experimental streams. *Glob. Change Biol.* 22, 2152–2164. doi: 10.1111/gcb.13205

Yvon-Durocher, G., Caffrey, J. M., Cescatti, A., Dossena, M., del, Giorgio P, Gasol, J. M., et al. (2012). Reconciling the temperature dependence of respiration across timescales and ecosystem types. *Nature* 487, 472–476. doi: 10.1038/nature11205

Zhou, Z., Guo, L., and Minor, E. C. (2016). Characterization of bulk and chromophoric dissolved organic matter in the Laurentian Great Lakes during summer 2013. *J. Great Lakes Res.* 42, 789–801. doi: 10.1016/j.jglr.2016.04.006

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

REVIEW
published: 06 July 2017
doi: 10.3389/fevo.2017.00070

Impacts of Nitrogen and Phosphorus: From Genomes to Natural Ecosystems and Agriculture

*Maïté S. Guignard[1], Andrew R. Leitch[2], Claudia Acquisti[3], Christophe Eizaguirre[2], James J. Elser[4,5], Dag O. Hessen[6], Punidan D. Jeyasingh[7], Maurine Neiman[8], Alan E. Richardson[9], Pamela S. Soltis[10], Douglas E. Soltis[10,11], Carly J. Stevens[12], Mark Trimmer[2], Lawrence J. Weider[13], Guy Woodward[14] and Ilia J. Leitch[1]**

[1] Comparative Plant and Fungal Biology Department, Royal Botanic Gardens, Kew, Richmond, United Kingdom, [2] Organismal Biology, School of Biological and Chemical Sciences, Queen Mary University of London, London, United Kingdom, [3] Department of Biology, Institute for Evolution and Biodiversity, University of Münster, Münster, Germany, [4] School of Life Sciences, Arizona State University, Tempe, AZ, United States, [5] Flathead Lake Biological Station, University of Montana, Polson, MT, United States, [6] Centre of Ecological and Evolutionary Synthesis, University of Oslo, Oslo, Norway, [7] Department of Integrative Biology, Oklahoma State University, Stillwater, OK, United States, [8] Department of Biology, University of Iowa, Iowa City, IA, United States, [9] CSIRO Agriculture and Food, Canberra, ACT, Australia, [10] Florida Museum of Natural History, University of Florida, Gainesville, FL, United States, [11] Department of Biology, University of Florida, Gainesville, FL, United States, [12] Lancaster Environment Centre, University of Lancaster, Lancaster, United Kingdom, [13] Department of Biology, University of Oklahoma, Norman, OK, United States, [14] Comparative Plant and Fungal Biology Department, Royal Botanic Gardens, Kew, London, United Kingdom*

OPEN ACCESS

Edited by:
Peter Schausberger, University of Vienna, Austria

Reviewed by:
Valeria Souza, National Autonomous University of Mexico, Mexico
Botir Khaitov, Tashkent State Agrarian University, Uzbekistan

***Correspondence:**
Ilia J. Leitch
i.leitch@kew.org

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *03 March 2017*
Accepted: *19 June 2017*
Published: *06 July 2017*

Citation:
Guignard MS, Leitch AR, Acquisti C, Eizaguirre C, Elser JJ, Hessen DO, Jeyasingh PD, Neiman M, Richardson AE, Soltis PS, Soltis DE, Stevens CJ, Trimmer M, Weider LJ, Woodward G and Leitch IJ (2017) Impacts of Nitrogen and Phosphorus: From Genomes to Natural Ecosystems and Agriculture. Front. Ecol. Evol. 5:70. doi: 10.3389/fevo.2017.00070

Nitrogen (N) and/or phosphorus (P) availability can limit growth of primary producers across most of the world's aquatic and terrestrial ecosystems. These constraints are commonly overcome in agriculture by applying fertilizers to improve yields. However, excessive anthropogenic N and P inputs impact natural environments and have far-reaching ecological and evolutionary consequences, from individual species up to entire ecosystems. The extent to which global N and P cycles have been perturbed over the past century can be seen as a global fertilization experiment with significant redistribution of nutrients across different ecosystems. Here we explore the effects of N and P availability on stoichiometry and genomic traits of organisms, which, in turn, can influence: (i) plant and animal abundances; (ii) trophic interactions and population dynamics; and (iii) ecosystem dynamics and productivity of agricultural crops. We articulate research priorities for a deeper understanding of how bioavailable N and P move through the environment and exert their ultimate impacts on biodiversity and ecosystem services.

Keywords: crops, genome size, nitrogen, nutrients, phosphorus, polyploidy, stoichiometry

INTRODUCTION

Fertilizers are central to the "green revolution," which has seen about half of the world's land converted to agriculture (Kareiva et al., 2007). Nitrogen (N) and phosphorus (P) are the dominant rate-limiting nutrients in most natural systems and the major constituents of agrochemical fertilizers. The consequences of N and P losses from agricultural land, e.g., through runoff and leaching, can span multiple organizational levels and scales in time and space, threatening essential ecosystem services (Smith et al., 1999; Elser, 2012; Fowler et al., 2013). In nature excessive loadings

of N and P are extrinsic drivers that (i) often reduce biodiversity directly (Chapin et al., 2000; Erisman et al., 2008; Lambers et al., 2010), and have indirect effects through (ii) increased local extinction via dominance of a few competitive species, leading to (iii) altered plant community structure (Rohr et al., 2016), (iv) reduced functional trait diversity of communities and ecosystems (Tilman and Lehman, 2001; Díaz et al., 2006), and (v) ultimately reshaping ecosystem services (Millennium Ecosystem Assessment., 2005; **Figure 1**). Examples of such services include the provision of clean water for human needs and leisure, maintaining and regulating soil fertility, and supporting services such as nutrient cycling and the transfer of nutrients through trophic levels (Millennium Ecosystem Assessment., 2005; Bommarco et al., 2013; Harrison et al., 2014).

At the organism level, N and P availability is known to have powerful influences on functional traits and growth rates, but we are only just beginning to understand that genome structure (e.g., genome size, ploidal level) can also play an important role (e.g., Neiman et al., 2009, 2013b; Šmarda et al., 2013; Guignard et al., 2016). At the genomic level, environmental nutrient limitation may constrain cellular processes (e.g., photosynthesis, transcriptomes) and over time may result in divergence of genes and the proteins they encode (Acquisti et al., 2009a,b; Elser et al., 2011; Seward and Kelly, 2016; **Figure 1**).

Thus, the fluxes, feedbacks, and availability of N and P fundamentally impact biota at all levels from genes and genomes to ecosystems, reshaping ecological and ultimately ecosystem processes. It is therefore crucial to understand *how* environmental N and P impact all levels of biological organization, from genome dynamics and cell metabolism, to the structure and functioning of multispecies systems (**Figure 1**). Such research could dramatically improve both the efficacy of biodiversity conservation and the development of more sustainable farming systems with lower N and P demands.

This paper evaluates the roles of N and P (1) in the environment, (2) within organisms, (3) in multispecies systems, and (4) in meeting human needs in the context of rising to the dual challenges of increasing food production and maintaining functional biodiversity to underpin the delivery of essential ecosystem services. It also proposes research priorities in these areas.

(1) N and P in the Environment

Nitrogen and P both underpin photosynthetic processes, cell growth, metabolism, and protein synthesis (Chapin et al., 2011), but their natural sources and rates of supply are very different: in principle, N availability is unlimited as an atmospheric gas, whereas P comes from rock phosphate, renewed with the uplift of continental rock. N and P co-limitation is common across the Earth's ecosystems in all the major biomes (Elser et al., 2007a). Today the primary sources of N and P originate from the massive anthropogenic inputs of fertilizers onto agricultural land (Fowler et al., 2013; **Figure 2**). For instance, industrial N production via the Haber–Bosch process surpassed natural

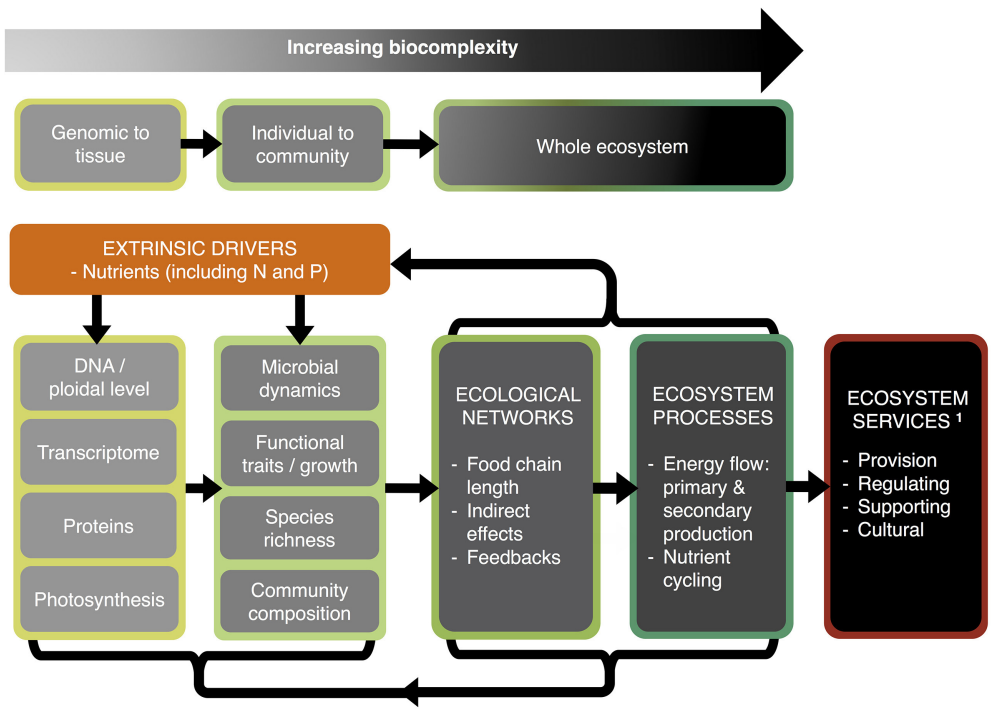

FIGURE 1 | Effects of N and P availability—from genomes to ecosystems. Inputs of nutrients, in particular nitrogen (N) and phosphorus (P), act as extrinsic drivers affecting biological dynamics at the genomic to the ecosystem level, and which in turn feed-back on these drivers. An essential component of the ecosystem is the cycling of nutrients through the food web and back to the atmosphere and soil in inorganic forms. Such ecosystems provide services of benefit to humans. [1]Services as defined by the Millennium Ecosystem Assessment. (2005).

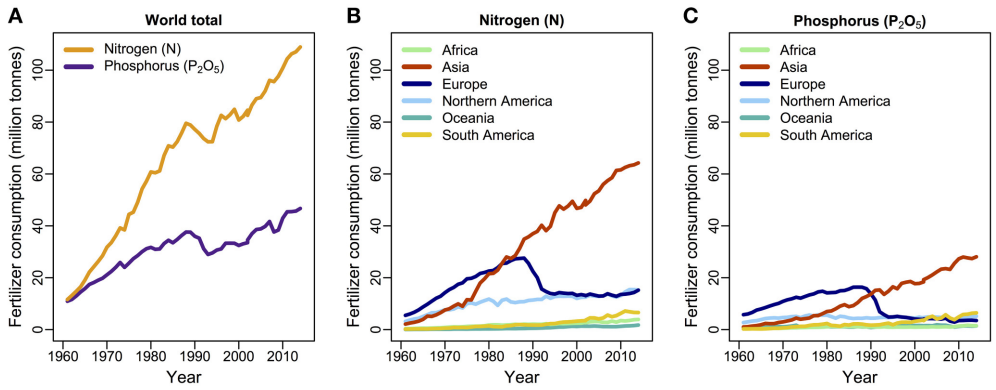

FIGURE 2 | Consumption of N and P fertilizer by year **(A)** globally, and **(B,C)** by world regions, from 1961 to 2014. Data are available from the Food and Agriculture Organization of the United Nations (FAO, 2016).

fixation of dinitrogen gas more than 50 years ago. It affects vast areas by increased deposition of oxidized and reduced N with increased runoff of N to freshwater and coastal areas (Galloway et al., 2013).

Anthropogenic N-deposition also originates from nitrogen oxides created during fossil fuel combustion, from nitrogen fixation of cultivated legumes (Ciais et al., 2014), and ammonia produced from animal wastes (Sutton et al., 2013). This elevated N-deposition may shift ecosystems from N to P limitation, with subsequent detrimental ecosystem impacts (Elser et al., 2009, 2010). Conversely, the deposition of P from P-rich dust from sand and agricultural soils may shift some ecosystems from P to N limitation (e.g., mountain lakes: Camarero and Catalan, 2012; Brahney et al., 2015).

In ecosystems, N and P are bound within waste organic products and dead organic matter (e.g., in nucleic acids). They must first be remineralized to release inorganic orthophosphates or dissolved and reduced to inorganic nitrate and ammonia before either element can be absorbed by primary producers (i.e., autotrophic bacteria, algae, plants). The mineralization of organic to inorganic forms of N and P is completed primarily by microorganisms as they metabolize carbon (Spohn and Kuzyakov, 2013). These microorganisms also require N investment to synthesize the alkaline phosphatase enzymes that release P. Because organic N concentrations are higher than those of P, this investment is balanced by N availability and the corresponding gain in P. In terrestrial systems, P cycling is closely linked to mycorrhizal fungi in plant roots that release extracellular phosphatases. If N is applied above a certain threshold, P cycles faster in response to greater N availability for producing phosphatase enzymes. This in turn results in faster rates of P removal and increased P limitation (Vitousek et al., 2010). This implies that elemental imbalances generated at the molecular level can influence elemental stoichiometry in the ecosystem.

In agriculture, nutrients are also lost from ecosystems when crop products are harvested, leaving less plant litter to decompose and fewer nutrients to be returned to the soil. Consequently, fertilizers are added to build and maintain soil fertility. When applied at excessive levels, however, N and P may be lost via leaching, runoff, and erosion (e.g., globally, an estimated 15 million tons of P are lost annually from crop fields due to erosion; Smil, 2000), and an estimated 8 million tons of P are lost in runoff from arable land annually (Cordell et al., 2009). Nitrogen is additionally removed from an ecosystem as N_2 and N_2O gases derived from microbial denitrification (when bacteria use nitrate as a source of oxygen) and anaerobic ammonium oxidation (or anammox, when the oxidation of ammonium is coupled with the reduction of nitrite). With entry into aquatic systems, N and P will trigger eutrophication before being cycled or buried as sediments.

Future Research Priorities

Frequently, the stores, fluxes, and cycling of N and P have been considered separately, partly because of the relative ease of tracing N cycles compared with P. A unified interdisciplinary approach is needed to fully understand macronutrient fate and transport through the ecosystem. It needs to incorporate both terrestrial and aquatic components of the landscape as well as how different macronutrient cycles interact (Grimm et al., 2003; Guenet et al., 2010; Soininen et al., 2015). A better understanding of how N:P supply ratios affect microbial activity (e.g., nitrogen-fixing bacteria and mycorrhizal fungi), and subsequently the flow of these nutrients to and between organisms (e.g., Cherif and Loreau, 2009), may be obtained via controlled experiments. This may help to develop a clearer understanding of the impact of fertilizers on soil and water health and a more informed and environmentally sensitive approach to how we use fertilizers. The urgent need for a more comprehensive understanding is highlighted by the rising concern of a potential global scarcity of P (Cordell et al., 2009).

(2) N and P Usage within Organisms and Genome Structure

Nucleic acids are approximately 39% N and nearly 9% P by mass (Sterner and Elser, 2002), making them among the most N- and P-demanding biomolecules of the cell. Accordingly, the nucleus of eukaryotes represents a substantial sink for N and P.

How this nucleic acid "sink" influences N and P stoichiometry remains unclear, yet these connections are potentially profound: genome size is one of the most variable of all organismal traits, varying 4-fold in mammals, 187-fold in insects, 378-fold in bony fishes, 460-fold in crustaceans (Gregory, 2016), and 2,400-fold in angiosperms (Pellicer et al., 2010). These differences in genome size reflect underlying genomic processes such as (retro)transposon amplification and deletion and whole-genome duplication (polyploidy). All of these processes can influence molecular evolution, gene expression, and organismal phenotype (Neiman et al., 2009, 2013a; Gerstein, 2013; Mayfield-Jones et al., 2013; Ramsey and Ramsey, 2014; Dodsworth et al., 2015; Selmecki et al., 2015; Soltis and Soltis, 2016). Polyploidy in particular has been implicated in the remarkably successful radiations of angiosperms (Soltis et al., 2009; Jiao et al., 2011; Albert et al., 2013; Tank et al., 2015; Van de Peer et al., 2017) and teleost fish (Van de Peer et al., 2009; Braasch and Postlethwait, 2012). Even so, many groups of eukaryotes are characterized by relatively small genomes, arising from genome-streamlining processes such as unequal and illegitimate recombination and chromosomal rearrangements triggered following polyploidy (Leitch and Bennett, 2004; Hessen et al., 2010; Dodsworth et al., 2016). Furthermore, the limited availability of environmental N and P and the expense of building and maintaining nucleic acids and associated proteins necessary to maintain a larger genome and hence cell may, under certain circumstances, act as a selection pressure driving the evolution of smaller genomes.

Selection on N and P use may also have an impact on genome composition. A comparative genomics approach on a set of animal and plant model organisms has shown N-conservation in the transcriptomes of wild plant taxa relative to both crop plants, which have a history of fertilizer application, and animals, which harvest N in organic form from other organisms (Acquisti et al., 2009a,b). Similarly, bias toward lower numbers of N atoms is reported in the highly expressed proteins of bacteria and yeast (Li et al., 2009), and in bacterial and eukaryotic parasites with low-N diets (Seward and Kelly, 2016).

The biochemical link between N and P in growth processes might underpin the broadly convergent ratios reported in many eukaryotic groups, such as the classic Redfield ratio, which states that marine plankton exhibit a mean C:N:P of 106:16:1 (Redfield, 1934; Klausmeier et al., 2004). Most eukaryotes, including microbes (Cleveland and Liptzin, 2007), maintain a certain degree of C, N, and P homeostasis. However, ratios vary with species growth rate (Hillebrand et al., 2013), trophic level, and environmental parameters. For example, N:P ratios range from 21:1 in broadleaved forests to 43:1 in tropical forests (McGroddy et al., 2004), while a more general ratio of 28:1 has been attributed to vascular plants (Chapin et al., 2011). Thus, while *general* ratios have been described, there is considerable variation due to fluctuations in environmental nutrient availability (Güsewell, 2004), phylogeny, and geography.

Biotic stoichiometric ratios are especially good reflections of nutrient availability at the base of the food web, where the scope for plasticity is greatest: proxy measures associated with latitudinal gradients have been described for both terrestrial plants (Reich and Oleksyn, 2004; Kerkhoff et al., 2006) and marine phytoplankton (Martiny et al., 2013). For example, older tropical soils are richer in N than P, whereas soils of recently glaciated regions at higher latitudes show the opposite pattern, implying reduced N:P ratios in colder areas (Reich and Oleksyn, 2004). Latitudinal and temperature-related changes in N:P likely reflect a higher demand for ribosomes (and thus P) to maintain sufficient protein synthesis at lower temperatures (Toseland et al., 2013; Thrane et al., 2017). This apparent link between temperature and stoichiometry in primary producers is perhaps unsurprising given that temperature drives the rates of many biological processes, including photosynthesis (C gain) and N and P uptake from the environment. At the genomic level, transcription rates in plants also increase with temperature (Sidaway-Lee et al., 2014). The higher demands for ribosomes at lower temperatures may occur to maintain protein synthesis or arise from the increased number of stored immature ribosomes in the nucleolus (Leitch et al., 1995), which can also translate into higher P-demands (and lower N:P) (Woods et al., 2003; Toseland et al., 2013; Thrane et al., 2017).

Future Research Priorities

At an organismal level, functional traits influence fitness via their effects on growth rate, reproduction, and survival (Violle et al., 2007). A stoichiometric perspective leads us to speculate that genome size and ploidal level are important but often ignored functional traits. For example, very large genomes in plants could be selected against in many ecosystems due to their higher demands for N and P. In animals, there may also be selection against larger genomes due to the reduced fitness associated with relatively low developmental rates and the relatively high demand for P invested in RNA (as much as 50–80% of cellular P) for protein synthesis (Hessen and Persson, 2009; Neiman et al., 2013a, but see Larkin et al., 2016). A definitive answer to the question of the extent to which larger genome sizes and higher ploidal levels translate into N and P costs will require characterization of organism-level consequences. For example, the increased N and P demands could be offset by the lower number of cells that are sometimes, but not always, associated with larger genomes (Neiman et al., 2017). Demands may also be offset by more efficient allocation of cellular P to RNA. Indeed polyploidy can rapidly induce a diversity of genetic and epigenetic responses, which can lead to highly variable total transcriptome volumes (Grover et al., 2012), a trait upon which selection can act.

Another key unanswered question is the nature of trade-offs between the genome, transcriptome, proteome, and metabolites for N and P usage, under differing N and P stress, at the cellular, tissue, and organismal levels and in organisms with different genome sizes. Controlled growth experiments under differing nutrient regimes, combined with biochemical, DNA/RNA, and genome analyses, are needed. Understanding the associations between C:N:P ratios and how these elements are partitioned for ribosomal synthesis vs., for example, histone synthesis may not only lead to important insights for organisms within ecosystems, but also provide novel medical insights into cancer dynamics (Elser et al., 2003, 2007b).

(3) The Roles of N and P and Genome Sizes in Species Assemblies

As we move beyond the organism and population levels, we can begin to elucidate some of the roles and consequences of these phenomena on multispecies systems such as communities, food webs, and entire ecosystems. Polyploidy is a genomic trait that may have cascading effects on food webs. For instance, in marine ecosystems, polyploidy may influence the composition of zooplankton communities. Polyploid zooplankton are more common in the Arctic, where a time constraint is imposed by the short growing season (Van Geest et al., 2010). The differential responses between taxa of different ploidal levels could shift the balance of power in both horizontal (competitive) and vertical (consumer-resource) interactions within ecological networks of interacting taxa.

The functional traits of species can influence energy and nutrient fluxes and the resilience of ecosystems to environmental disturbances. Recent evidence suggests that genome structure (i.e., genome size, ploidal level) and nutrient availability can influence plant distributions, community composition, and biomass production in grasslands (Šmarda et al., 2013; Guignard et al., 2016; Segraves, 2017). This possibility is bolstered by large-scale comparative analyses using the Plant DNA C-values database (Bennett and Leitch, 2012) which suggest that plants with large genomes are at greater risk of extinction and are less tolerant of polluted soils and extreme environmental conditions (Vinogradov, 2003; Knight et al., 2005; Greilhuber and Leitch, 2013). Although genome size effects can depend on ploidal level, these data clearly demonstrate that genome structure has ecological consequences that can shape the distribution and persistence of biodiversity.

Variation in DNA and RNA usage in primary producers may also cascade upwards to higher trophic levels. For example, higher ploidal-level representatives of aquatic animals fare better (biomass as well as amount of N and P in their tissues) than their lower ploidal-level congeners, when their diets (composed of primary producers) have relatively high nutrient content. The reverse holds true in low-nutrient conditions (Neiman et al., 2013b; Jeyasingh et al., 2015). Radiotracer assays have also revealed that polyploid *Daphnia* incorporated significantly more ^{33}P and excreted significantly less ^{33}P compared with diploids (Jeyasingh et al., 2015), indicating potentially strong effects of ploidal level on key population and, by extension, community parameters related to consumer-resource interactions. In addition to these "green pathways" that link autochthonous producers to herbivorous consumers, there is clearly the potential for the "brown pathways" that transfer energy and nutrients via detrital feeding links to also be affected by N and P and genome structure. For instance, terrestrial leaf litter fuels the base of many freshwater food webs and the main determinants of its consumption are its C:N:P stoichiometry, which is shaped by the taxonomic and functional attributes of the plants as well as environmental nutrient conditions (e.g., Hladyz et al., 2009; Woodward et al., 2012). Consequently, if the C:N:P stoichiometry of terrestrial plants is itself linked to genome size, genome attributes of these plants have clear potential to shape ecosystem-level processes and the trophic basis of production of the higher trophic levels in the food web. How, and to what extent, these influences are manifested in natural ecosystems also could depend on the extent of nutrient enrichment from agriculture within the surrounding landscape.

Future Research Priorities

Polyploids and taxa with larger genome sizes should be more common where nutrients are more abundant (Lewis, 1985; Leitch and Bennett, 2004; Leitch and Leitch, 2008; Hessen et al., 2010; Neiman et al., 2013a; Leitch et al., 2014). Support for these predictions has come from recent studies of freshwater snails (Neiman et al., 2013b) and angiosperms (Šmarda et al., 2013; Guignard et al., 2016). Further data collection on, and empirical tests of, the associations between N and P limitation, genome size, and ploidal-level variation in diverse habitat types and biomes are clearly needed to determine how far such predictions hold across different ecosystems and larger, continental scales. Such investigations may take advantage of geographical information systems and niche modeling approaches.

Organisms are frequently linked in stoichiometric feedback loops (Sterner, 1990; Gruner et al., 2008) within wider elemental cycles. Even so, and perhaps due to the sheer complexity of ecological systems, research is most often focused on top-down (consumer-directed) vs. bottom-up (resource-based) effects within only a small part of the food web. A broader system-level approach that can also include indirect effects or reciprocity is needed. Elemental availability can also play a large role in predator-prey interactions, including nutrient cycling by predators, which, in turn, influences what elements are available to prey (Grover, 2003; Andersen et al., 2004; Sardans et al., 2012). With respect to macro-organisms, plants form the base of the food web; the diversity of plant structures is hypothesized to influence multiple trophic levels via effects on differential nutrient requirements, intake, growth rates, and, thus, food quality for higher trophic levels, either as an autochthonous resource that is processed via the food web's green pathways or as detritus within the brown pathways.

Plants are affected by their environment but they can also modify this environment via shifts in microbial communities that have short- and long-term effects (Putten et al., 2013; Van Nuland et al., 2016). Such ecological feedbacks occur when interactions at one time determine the performance or interactions of organisms at another (Hendry, 2016). The impact of nutrient availability has already been demonstrated to alter eco-evolutionary dynamics in plants (Wooliver et al., 2016) and in fish due to increased eutrophication, resulting in changes to parasite load and individual feeding ecology (Anaya-Rojas et al., 2016; Brunner et al., 2017). Future research will thus not only have to focus on direct effects of N and P on species but also on how they impact the interactions between species at the ecosystem level.

(4) N and P and Genomes—toward Sustainable Agriculture

The world's consumption of N- and P-based fertilizers has increased substantially since the 1960's although that rise is now

largely driven by agriculture across Asia (FAO, 2016; **Figure 2**). As discussed above, N and P availability may influence the productivity of plant taxa differentially, depending on genome structure: when nutrients are in excess, polyploid plants tend to increase more in biomass production and competitiveness than diploids. This increased yield may be one reason why most crops are polyploids (Leitch and Leitch, 2008). The rapid rate of biomass production for which crops are typically selected is associated with high soil nutrient demands. Indeed this high demand for nutrients has essentially been "designed" into our current agricultural systems. That in turn has led to a high dependence for fertilizer inputs, whereby agricultural crops generally display high critical nutrient requirements (N and P) for optimal growth with high product removal. This is not only economically expensive but also biologically inefficient and environmentally destabilizing, with increased potential for collateral damage to aquatic ecosystems via eutrophication. For example, P inputs are two to five times greater than the amount exported in the final product (Simpson et al., 2011), and crops take up only 30–40% of applied N (Kant et al., 2011). Nutrient-use efficiency in crops can be improved by, for example, selection of morphological and physiological traits that maximize nutrient uptake (Richardson et al., 2011), optimizing traits that increase the efficiency of ribosomes (Kreps et al., 2002; Kant et al., 2011; Veneklaas et al., 2012), and reducing the carbon costs of nutrient uptake (Lynch and Ho, 2005). Crops are high in P content with a 15:1 N:P ratio (Veneklaas et al., 2012), in contrast to the 28:1 ratio across vascular plants as a whole. Moreover, high concentrations of P in grain crops are undesirable because P is predominantly stored as phytate, which is indigestible, and which reduces absorption of other nutrients in non-ruminant animals, including humans (Veneklaas et al., 2012). The indigestibility of this form of P means it ends up in our sewage and waterways, and one solution may be to reduce P uptake and/or P concentrations in seeds and grains using genomic approaches (Raboy, 2001; Yamaji et al., 2017).

Future Research Priorities

Many engineering approaches are being considered to help improve the management of N and P in the environment. One key goal is to apply less fertilizer while maintaining or even enhancing agricultural yields. A key part of this process will be to revisit our polyploid crops. To date, much plant breeding has exploited polyploids, where genic diversity is fixed and favorable characters can be selected (e.g., allopolyploids: wheat, cotton, tobacco, sugarcane; autopolyploids: strawberry, alfalfa, banana) (Udall and Wendel, 2006; Renny-Byfield and Wendel, 2014). Yet these crops have been developed in a context of high inputs of N, P, and other nutrients. By contrast, the wild relatives of these crop species typically grow in relatively infertile habitats. By targeting inbred introgressed lines and applying high-throughput sequencing in combination with marker-assisted or genomic selection approaches (Heffner et al., 2009; Xu et al., 2014; Jan et al., 2016; Lv et al., 2016), it may be possible to reduce nutrient requirements and/or improve nutrient-use efficiency (higher yield per unit used of fertilizer) of crop plants. There are significant commercial gains to be made from reducing our dependency on N- and P-containing fertilizers. For example, increasing N use efficiency by 1% alone could lead to estimated annual savings of $1.1 billion (Kant et al., 2011). Additional reductions in fertilizer use could also come from harnessing the microbiome in plant selection, especially under limiting N and P. Future avenues include the use of bacteria and fungi to increase a plant's uptake of nutrients, in particular P (reviewed in Owen et al., 2015), and to exploit new technologies aiming to inhibit P loss and increase fertilizer recovery (Withers et al., 2015).

CONCLUSION

In defining a "safe-operating space" for humanity in the Anthropocene, Rockström et al. (2009a,b) identified nine planetary boundaries and thresholds for anthropogenic activities to remain globally sustainable. They argued that some of these have already been surpassed, including a proposed boundary of 35 million tonnes (Tg) per year of N_2 removed from the atmosphere, far below the actual annual rate of 121 Tg (Rockström et al., 2009b). We are very close to the proposed boundary of 11 Tg per year of P flowing into oceans, currently at c. 9 Tg per year (Rockström et al., 2009b). However, if freshwater systems are also taken into account, we have also surpassed that P boundary (Carpenter and Bennett, 2011). Because N and P are linked to life systems ranging from global ecosystems (e.g., oceans) to genomes, a more complete understanding and an incorporation of stoichiometric analysis at all levels of biological organization are needed. This is indeed an urgent goal, as it will enable us to maintain or enhance agricultural productivity whilst simultaneously conserving and enriching the biodiversity that is essential for the continued provision of ecosystem services across the globe.

AUTHOR CONTRIBUTIONS

MG, AL, CA, CE, JE, DH, PJ, MN, AR, PS, DS, CS, MT, GW, LW and IL wrote, revised, and evaluated the manuscript, led by MG, AL, and IL.

FUNDING

This project received grants from the Research Council of Norway ("Genome" project, no. 196468), awarded to DH. MT and MG benefitted from funding by the Natural Environment Research Council (NE/J012106/1). CE acknowledges funding from the German Research Foundation (DFG, EIZ841/4-1, and EIZ841/6-1). LW was funded through the U.S. National Science Foundation (NSF-IOS-OEI) grant no. 1256881 during the manuscript preparation stage. The U.S. National Science Foundation, grant no. 1439461, provided support for graduate students and post-doctoral researchers to attend the conference. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

ACKNOWLEDGMENTS

This paper arose out of a Theo Murphy discussion meeting coordinated by AL, DH, PJ, MN, LW, and IL and held at the Kavli Royal Society Centre, Chicheley Hall, UK, on 1st and 2nd June 2015. The discussions are available online. We thank the Royal Society for sponsoring and hosting this meeting.

REFERENCES

Acquisti, C., Elser, J. J., and Kumar, S. (2009a). Ecological nitrogen limitation shapes the DNA composition of plant genomes. *Mol. Biol. Evol.* 26, 953–956. doi: 10.1093/molbev/msp038

Acquisti, C., Kumar, S., and Elser, J. J. (2009b). Signatures of nitrogen limitation in the elemental composition of the proteins involved in the metabolic apparatus. *Proc. R. Soc. London B Biol. Sci.* 276, 2605–2610. doi: 10.1098/rspb.2008.1960

Albert, V. A., Barbazuk, W. B., Der, J. P., Leebens-Mack, J., Ma, H., Palmer, J. D., et al. (2013). The *Amborella* genome and the evolution of flowering plants. *Science* 342:1241089. doi: 10.1126/science.1241089

Anaya-Rojas, J. M., Brunner, F. S., Sommer, N., Seehausen, O., Eizaguirre, C., and Matthews, B. (2016). The association of feeding behaviour with the resistance and tolerance to parasites in recently diverged sticklebacks. *J. Evol. Biol.* 29, 2157–2167. doi: 10.1111/jeb.12934

Andersen, T., Elser, J. J., and Hessen, D. O. (2004). Stoichiometry and population dynamics. *Ecol. Lett.* 7, 884–900. doi: 10.1111/j.1461-0248.2004.00646.x

Bennett, M. D., and Leitch, I. J. (2012). *Plant DNA C-Values Database (Release 6.0, Dec. 2012).* Available online at: http://www.kew.org/cvalues/

Bommarco, R., Kleijn, D., and Potts, S. G. (2013). Ecological intensification: harnessing ecosystem services for food security. *Trends Ecol. Evol.* 28, 230–238. doi: 10.1016/j.tree.2012.10.012

Braasch, I., and Postlethwait, J. H. (2012). "Polyploidy in fish and the teleost genome duplication," in *Polyploidy and Genome Evolution*, eds P. S. Soltis and D. E. Soltis (Berlin; Heidelberg: Springer), 341–383.

Brahney, J., Mahowald, N., Ward, D. S., Ballantyne, A. P., and Neff, J. C. (2015). Is atmospheric phosphorus pollution altering global alpine lake stoichiometry? *Global Biogeochem. Cycles* 29, 1369–1383. doi: 10.1002/2015GB005137

Brunner, F. S., Anaya-Rojas, J. M., Matthews, B., and Eizaguirre, C. (2017). Experimental evidence that parasites drive eco-evolutionary feedbacks. *Proc. Natl. Acad. Sci. U.S.A.* 114, 3678–3683. doi: 10.1073/pnas.1619147114

Camarero, L., and Catalan, J. (2012). Atmospheric phosphorus deposition may cause lakes to revert from phosphorus limitation back to nitrogen limitation. *Nat. Commun.* 3:1118. doi: 10.1038/ncomms2125

Carpenter, S. R., and Bennett, E. M. (2011). Reconsideration of the planetary boundary for phosphorus. *Environ. Res. Lett.* 6:14009. doi: 10.1088/1748-9326/6/1/014009

Chapin, F. S. III., Matson, P. A., and Vitousek, P. (2011). *Principles of Terrestrial Ecosystem Ecology*. New York, NY: Springer Science & Business Media.

Chapin, F. S. III., Zavaleta, E. S., Eviner, V. T., Naylor, R. L., Vitousek, P. M., Reynolds, H. L., et al. (2000). Consequences of changing biodiversity. *Nature* 405, 234–242. doi: 10.1038/35012241

Cherif, M., and Loreau, M. (2009). When microbes and consumers determine the limiting nutrient of autotrophs: a theoretical analysis. *Proc. R. Soc. London B Biol. Sci.* 276, 487–497. doi: 10.1098/rspb.2008.0560

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., et al. (2014). "Carbon and other biogeochemical cycles," in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (Cambridge; New York, NY: Cambridge University Press), 465–570.

Cleveland, C. C., and Liptzin, D. (2007). C: N: P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? *Biogeochemistry* 85, 235–252. doi: 10.1007/s10533-007-9132-0

Cordell, D., Drangert, J.-O., and White, S. (2009). The story of phosphorus: global food security and food for thought. *Glob. Environ. Chang.* 19, 292–305. doi: 10.1016/j.gloenvcha.2008.10.009

Díaz, S., Fargione, J., Chapin, F. S. III., and Tilman, D. (2006). Biodiversity loss threatens human well-being. *PLoS Biol.* 4:e277. doi: 10.1371/journal.pbio.0040277

Dodsworth, S., Chase, M. W., and Leitch, A. R. (2016). Is post-polyploidization diploidization the key to the evolutionary success of angiosperms? *Bot. J. Linn. Soc.* 180, 1–5. doi: 10.1111/boj.12357

Dodsworth, S., Leitch, A. R., and Leitch, I. J. (2015). Genome size diversity in angiosperms and its influence on gene space. *Curr. Opin. Genet. Dev.* 35, 73–78. doi: 10.1016/j.gde.2015.10.006

Elser, J. J. (2012). Phosphorus: a limiting nutrient for humanity? *Curr. Opin. Biotechnol.* 23, 833–838. doi: 10.1016/j.copbio.2012.03.001

Elser, J. J., Acquisti, C., and Kumar, S. (2011). Stoichiogenomics: the evolutionary ecology of macromolecular elemental composition. *Trends Ecol. Evol.* 26, 38–44. doi: 10.1016/j.tree.2010.10.006

Elser, J. J., Andersen, T., Baron, J. S., Bergström, A.-K., Jansson, M., Kyle, M., et al. (2009). Shifts in lake N:P stoichiometry and nutrient limitation driven by atmospheric nitrogen deposition. *Science* 326, 835–837. doi: 10.1126/science.1176199

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007a). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Elser, J. J., Kyle, M. M., Smith, M. S., and Nagy, J. D. (2007b). Biological stoichiometry in human cancer. *PLoS ONE* 2:e1028. doi: 10.1371/journal.pone.0001028

Elser, J. J., Nagy, J. D., and Kuang, Y. (2003). Biological stoichiometry: an ecological perspective on tumor dynamics. *Bioscience* 53, 1112–1120. doi: 10.1641/0006-3568(2003)053[1112:BSAEPO]2.0.CO;2

Elser, J. J., Peace, A. L., Kyle, M., Wojewodzic, M., McCrackin, M. L., Andersen, T., et al. (2010). Atmospheric nitrogen deposition is associated with elevated phosphorus limitation of lake zooplankton. *Ecol. Lett.* 13, 1256–1261. doi: 10.1111/j.1461-0248.2010.01519.x

Erisman, J. W., Sutton, M. A., Galloway, J., Klimont, Z., and Winiwarter, W. (2008). How a century of ammonia synthesis changed the world. *Nat. Geosci.* 1, 636–639. doi: 10.1038/ngeo325

FAO (2016). *FAOSTAT Statistics Database.* Food *and Agriculture Organization of the United Nations Statistics Database.* 453. Available online at: http://www.fao.org/faostat/en/#data/RF

Fowler, D., Coyle, M., Skiba, U., Sutton, M. A., Cape, J. N., Reis, S., et al. (2013). The global nitrogen cycle in the twenty-first century. *Philos. Trans R. Soc. Lond. B Biol. Sci.* 368:20130164. doi: 10.1098/rstb.2013.0164

Galloway, J. N., Leach, A. M., Bleeker, A., and Erisman, J. W. (2013). A chronology of human understanding of the nitrogen cycle. *Philos. Trans. R. Soc. B Biol. Sci.* 368:20130120. doi: 10.1098/rstb.2013.0120

Gerstein, A. C. (2013). Mutational effects depend on ploidy level: all else is not equal. *Biol. Lett.* 9:20120614. doi: 10.1098/rsbl.2012.0614

Gregory, T. R. (2016). *Animal Genome Size Database*. Available online at: http://www.genomesize.com

Greilhuber, J., and Leitch, I. J. (2013). "Genome size and the phenotype," in *Plant Genome Diversity Vol. 2: Physical Structure, Behaviour and Evolution of Plant Genomes*, eds I. Leitch, J. Greilhuber, J. Dolezel, and J. F. Wendel (Vienna: Springer), 323–344.

Grimm, N. B., Gergel, S. E., McDowell, W. H., Boyer, E. W., Dent, C. L., Groffman, P., et al. (2003). Merging aquatic and terrestrial perspectives of nutrient biogeochemistry. *Oecologia* 137, 485–501. doi: 10.1007/s00442-003-1382-5

Grover, C. E., Gallagher, J. P., Szadkowski, E. P., Yoo, M. J., Flagel, L. E., and Wendel, J. F. (2012). Homoeolog expression bias and expression level dominance in allopolyploids. *New Phytol.* 196, 966–971. doi: 10.1111/j.1469-8137.2012.04365.x

Grover, J. P. (2003). The impact of variable stoichiometry on predator-prey interactions: a multinutrient approach. *Am. Nat.* 162, 29–43. doi: 10.1086/376577

Gruner, D. S., Smith, J. E., Seabloom, E. W., Sandin, S. A., Ngai, J. T., Hillebrand, H., et al. (2008). A cross-system synthesis of consumer and nutrient resource control on producer biomass. *Ecol. Lett.* 11, 740–755. doi: 10.1111/j.1461-0248.2008.01192.x

Guenet, B., Danger, M., Abbadie, L., and Lacroix, G. (2010). Priming effect: bridging the gap between terrestrial and aquatic ecology. *Ecology* 91, 2850–2861. doi: 10.1890/09-1968.1

Guignard, M. S., Nichols, R. A., Knell, R. J., Macdonald, A., Romila, C., Trimmer, M., et al. (2016). Genome size and ploidy influence angiosperm species' biomass under nitrogen and phosphorus limitation. *New Phytol.* 210, 1195–1206. doi: 10.1111/nph.13881

Güsewell, S. (2004). N: P ratios in terrestrial plants: variation and functional significance. *New Phytol.* 164, 243–266. doi: 10.1111/j.1469-8137.2004.01192.x

Harrison, P. A., Berry, P. M., Simpson, G., Haslett, J. R., Blicharska, M., Bucur, M., et al. (2014). Linkages between biodiversity attributes and ecosystem services: a systematic review. *Ecosyst. Serv.* 9, 191–203. doi: 10.1016/j.ecoser.2014.05.006

Heffner, E. L., Sorrells, M. E., and Jannink, J.-L. (2009). Genomic selection for crop improvement. *Crop Sci.* 49, 1–12. doi: 10.2135/cropsci2008.08.0512

Hendry, A. P. (2016). *Eco-Evolutionary Dynamics*. Princeton; Oxford: Princeton University Press.

Hessen, D. O., Jeyasingh, P. D., Neiman, M., and Weider, L. J. (2010). Genome streamlining and the elemental costs of growth. *Trends Ecol. Evol.* 25, 75–80. doi: 10.1016/j.tree.2009.08.004

Hessen, D. O., and Persson, J. (2009). Genome size as a determinant of growth and life-history traits in crustaceans. *Biol. J. Linn. Soc.* 98, 393–399. doi: 10.1111/j.1095-8312.2009.01285.x

Hillebrand, H., Steinert, G., Boersma, M., Malzahn, A., Meunier, C. L., Plum, C., et al. (2013). Goldman revisited: faster-growing phytoplankton has lower N: P and lower stoichiometric flexibility. *Limnol. Ocean.* 58, 2076–2088. doi: 10.4319/lo.2013.58.6.2076

Hladyz, S., Gessner, M., Giller, P., Pozo, J., and Woodward, G. (2009). Resource quality and stoichiometric constraints on stream ecosystem functioning. *Freshw. Biol.* 54, 957–970. doi: 10.1111/j.1365-2427.2008.02138.x

Jan, H. U., Abbadi, A., Lücke, S., Nichols, R. A., and Snowdon, R. J. (2016). Genomic prediction of testcross performance in canola (*Brassica napus*). *PLoS ONE* 11:e0147769. doi: 10.1371/journal.pone.0147769

Jeyasingh, P. D., Chowdhury, P. R., Wojewodzic, M. W., Frisch, D., Hessen, D. O., and Weider, L. J. (2015). Phosphorus use and excretion varies with ploidy level in Daphnia. *J. Plankton Res.* 37, 1210–1217. doi: 10.1093/plankt/fbv095

Jiao, Y., Wickett, N. J., Ayyampalayam, S., Chanderbali, A. S., Landherr, L., Ralph, P. E., et al. (2011). Ancestral polyploidy in seed plants and angiosperms. *Nature* 473, 97–100. doi: 10.1038/nature09916

Kant, S., Bi, Y.-M., and Rothstein, S. J. (2011). Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. *J. Exp. Bot.* 62, 1499–1509. doi: 10.1093/jxb/erq297

Kareiva, P., Watts, S., McDonald, R., and Boucher, T. (2007). Domesticated nature: shaping landscapes and ecosystems for human welfare. *Science* 316, 1866–1869. doi: 10.1126/science.1140170

Kerkhoff, A. J., Fagan, W. F., Elser, J. J., and Enquist, B. J. (2006). Phylogenetic and growth form variation in the scaling of nitrogen and phosphorus in the seed plants. *Am. Nat.* 168, E103–E122. doi: 10.1086/507879

Klausmeier, C. A., Litchman, E., Daufresne, T., and Levin, S. A. (2004). Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. *Nature* 429, 171–174. doi: 10.1038/nature02454

Knight, C. A., Molinari, N. A., and Petrov, D. A. (2005). The large genome constraint hypothesis: evolution, ecology and phenotype. *Ann. Bot.* 95, 177–190. doi: 10.1093/aob/mci011

Kreps, J. A., Wu, Y., Chang, H.-S., Zhu, T., Wang, X., and Harper, J. F. (2002). Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol.* 130, 2129–2141. doi: 10.1104/pp.008532

Lambers, H., Brundrett, M. C., Raven, J. A., and Hopper, S. D. (2010). Plant mineral nutrition in ancient landscapes: high plant species diversity on infertile soils is linked to functional diversity for nutritional strategies. *Plant Soil* 334, 11–31. doi: 10.1007/s11104-010-0444-9

Larkin, K., Tucci, C., and Neiman, M. (2016). Effects of polyploidy and reproductive mode on life history trait expression. *Ecol. Evol.* 6, 765–778. doi: 10.1002/ece3.1934

Leitch, A. R., Glyn, M., Kingham, K., Aragon-Alcaide, A., Somasekaram, A., and Duckett, J. (1995). "The dynamic organization of interphase nuclei during cell differentiation and changing cell activity," in *Kew Chromosome Conference IV*, eds P. Brandham and M. Bennett (Kew: Royal Botanic Gardens), 83–94.

Leitch, A. R., and Leitch, I. J. (2008). Genomic plasticity and the diversity of polyploid plants. *Science* 320, 481–483. doi: 10.1126/science.1153585

Leitch, A. R., Leitch, I. J., Trimmer, M., Guignard, M. S., and Woodward, G. (2014). Impact of genomic diversity in river ecosystems. *Trends Plant Sci.* 19, 361–366. doi: 10.1016/j.tplants.2013.12.005

Leitch, I. J., and Bennett, M. D. (2004). Genome downsizing in polyploid plants. *Biol. J. Linn. Soc.* 82, 651–663. doi: 10.1111/j.1095-8312.2004.00349.x

Lewis, W. M. Jr. (1985). Nutrient scarcity as an evolutionary cause of haploidy. *Am. Nat.* 125, 692–701. doi: 10.1086/284372

Li, N., Lv, J., and Niu, D.-K. (2009). Low contents of carbon and nitrogen in highly abundant proteins: evidence of selection for the economy of atomic composition. *J. Mol. Evol.* 68, 248–255. doi: 10.1007/s00239-009-9199-4

Lv, Y., Liang, Z., Ge, M., Qi, W., Zhang, T., Lin, F., et al. (2016). Genome-wide identification and functional prediction of nitrogen-responsive intergenic and intronic long non-coding RNAs in maize (*Zea mays* L.). *BMC Genomics* 17:350. doi: 10.1186/s12864-016-2650-1

Lynch, J. P., and Ho, M. D. (2005). Rhizoeconomics: carbon costs of phosphorus acquisition. *Plant Soil* 269, 45–56. doi: 10.1007/s11104-004-1096-4

Martiny, A. C., Pham, C. T. A., Primeau, F. W., Vrugt, J. A., Moore, J. K., Levin, S. A., et al. (2013). Strong latitudinal patterns in the elemental ratios of marine plankton and organic matter. *Nat. Geosci.* 6, 279–283. doi: 10.1038/ngeo1757

Mayfield-Jones, D., Washburn, J. D., Arias, T., Edger, P. P., Pires, J. C., and Conant, G. C. (2013). Watching the grin fade: tracing the effects of polyploidy on different evolutionary time scales. *Semin. Cell Dev. Biol.* 24, 320–331. doi: 10.1016/j.semcdb.2013.02.002

McGroddy, M. E., Daufresne, T., and Hedin, L. O. (2004). Scaling of C: N: P stoichiometry in forests worldwide: implications of terrestrial redfield-type ratios. *Ecology* 85, 2390–2401. doi: 10.1890/03-0351

Millennium Ecosystem Assessment. (2005). *Ecosystems and Human Well-being: Synthesis*. Washington, DC: Island Press.

Neiman, M., Beaton, M. J., Hessen, D. O., Jeyasingh, P. D., and Weider, L. J. (2017). Endopolyploidy as a potential driver of animal ecology and evolution. *Biol. Rev.* 92, 234–247. doi: 10.1111/brv.12226

Neiman, M., Kay, A. D., and Krist, A. C. (2013a). Can resource costs of polyploidy provide an advantage to sex? *Heredity* 110, 152–159. doi: 10.1038/hdy.2012.78

Neiman, M., Kay, A. D., and Krist, A. C. (2013b). Sensitivity to phosphorus limitation increases with ploidy level in a New Zealand snail. *Evolution* 67, 1511–1517. doi: 10.1111/evo.12026

Neiman, M., Theisen, K. M., Mayry, M. E., and Kay, A. D. (2009). Can phosphorus limitation contribute to the maintenance of sex? A test of a key assumption. *J. Evol. Biol.* 22, 1359–1363. doi: 10.1111/j.1420-9101.2009.01748.x

Owen, D., Williams, A. P., Griffith, G. W., and Withers, P. J. A. (2015). Use of commercial bio-inoculants to increase agricultural production through improved phosphorus acquisition. *Agric. Ecosyst. Environ. Appl. Soil Ecol.* 86, 41–54. doi: 10.1016/j.apsoil.2014.09.012

Pellicer, J., Fay, M. F., and Leitch, I. J. (2010). The largest eukaryotic genome of them all? *Bot. J. Linn. Soc.* 164, 10–15. doi: 10.1111/j.1095-8339.2010.01072.x

Putten, W. H., Bardgett, R. D., Bever, J. D., Bezemer, T. M., Casper, B. B., Fukami, T., et al. (2013). Plant–soil feedbacks: the past, the present and future challenges. *J. Ecol.* 101, 265–276. doi: 10.1111/1365-2745.12054

Raboy, V. (2001). Seeds for a better future:"low phytate"grains help to overcome malnutrition and reduce pollution. *Trends Plant Sci.* 6, 458–462. doi: 10.1016/S1360-1385(01)02104-5

Ramsey, J., and Ramsey, T. S. (2014). Ecological studies of polyploidy in the 100 years following its discovery. *Philos. Trans. R. Soc. B Biol. Sci.* 369:20130352. doi: 10.1098/rstb.2013.0352

Redfield, A. C. (1934). *On the Proportions of Organic Derivatives in Sea Water and their Relation to the Composition of Plankton*. Liverpool: Liverpool University Press.

Reich, P. B., and Oleksyn, J. (2004). Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11001–11006. doi: 10.1073/pnas.0403588101

Renny-Byfield, S., and Wendel, J. F. (2014). Doubling down on genomes: polyploidy and crop plants. *Am. J. Bot.* 101, 1711–1725. doi: 10.3732/ajb.1400119

Richardson, A. E., Lynch, J. P., Ryan, P. R., Delhaize, E., Smith, F. A., Smith, S. E., et al. (2011). Plant and microbial strategies to improve the

phosphorus efficiency of agriculture. *Plant Soil* 349, 121–156. doi: 10.1007/s11104-011-0950-4

Rockström, J., Steffen, W. L., Noone, K., Persson, Å., Chapin, F. S. III., Lambin, E., et al. (2009a). Planetary boundaries: exploring the safe operating space for humanity. *Ecol. Soc.* 14:32.

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F. S., Lambin, E. F., et al. (2009b). A safe operating space for humanity. *Nature* 461, 472–475. doi: 10.1038/461472a

Rohr, R. P., Saavedra, S., Peralta, G., Frost, C. M., Bersier, L.-F., Bascompte, J., et al. (2016). Persist or produce: a community trade-off tuned by species evenness. *Am. Nat.* 188, 411–422. doi: 10.1086/688046

Sardans, J., Rivas-Ubach, A., and Penuelas, J. (2012). The elemental stoichiometry of aquatic and terrestrial ecosystems and its relationships with organismic lifestyle and ecosystem structure and function: a review and perspectives. *Biogeochemistry* 111, 1–39. doi: 10.1007/s10533-011-9640-9

Segraves, K. A. (2017). The effects of genome duplications in a community context. *New Phytol.* 215, 57–69. doi: 10.1111/nph.14564

Selmecki, A. M., Maruvka, Y. E., Richmond, P. A., Guillet, M., Shoresh, N., Sorenson, A. L., et al. (2015). Polyploidy can drive rapid adaptation in yeast. *Nature* 519, 349–352. doi: 10.1038/nature14187

Seward, E. A., and Kelly, S. (2016). Dietary nitrogen alters codon bias and genome composition in parasitic microorganisms. *Genome Biol.* 17:226. doi: 10.1186/s13059-016-1087-9

Sidaway-Lee, K., Costa, M. J., Rand, D. A., Finkenstadt, B., and Penfield, S. (2014). Direct measurement of transcription rates reveals multiple mechanisms for configuration of the *Arabidopsis* ambient temperature response. *Genome Biol.* 15:R45. doi: 10.1186/gb-2014-15-3-r45

Simpson, R. J., Oberson, A., Culvenor, R. A., Ryan, M. H., Veneklaas, E. J., Lambers, H., et al. (2011). Strategies and agronomic interventions to improve the phosphorus-use efficiency of farming systems. *Plant Soil* 349, 89–120. doi: 10.1007/s11104-011-0880-1

Šmarda, P., Hejcman, M., Brezinová, A., Horová, L., Steigerová, H., Zedek, F., et al. (2013). Effect of phosphorus availability on the selection of species with different ploidy levels and genome sizes in a long-term grassland fertilization experiment. *New Phytol.* 200, 911–921. doi: 10.1111/nph.12399

Smil, V. (2000). Phosphorus in the environment: natural flows and human interferences. *Annu. Rev. Energy Environ.* 25, 53–88. doi: 10.1146/annurev.energy.25.1.53

Smith, V. H., Tilman, G. D., and Nekola, J. C. (1999). Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environ. Pollut.* 100, 179–196. doi: 10.1016/S0269-7491(99)00091-3

Soininen, J., Bartels, P., Heino, J., Luoto, M., and Hillebrand, H. (2015). Toward more integrated ecosystem research in aquatic and terrestrial environments. *Bioscience* 65, 174–182. doi: 10.1093/biosci/biu216

Soltis, D. E., Albert, V. A., Leebens-Mack, J., Bell, C. D., Paterson, A. H., Zheng, C., et al. (2009). Polyploidy and angiosperm diversification. *Am. J. Bot.* 96, 336–348. doi: 10.3732/ajb.0800079

Soltis, P. S., and Soltis, D. E. (2016). Ancient WGD events as drivers of key innovations in angiosperms. *Curr. Opin. Plant Biol.* 30, 159–165. doi: 10.1016/j.pbi.2016.03.015

Spohn, M., and Kuzyakov, Y. (2013). Phosphorus mineralization can be driven by microbial need for carbon. *Soil Biol. Biochem.* 61, 69–75. doi: 10.1016/j.soilbio.2013.02.013

Sterner, R. W. (1990). The ratio of nitrogen to phosphorus resupplied by herbivores: zooplankton and the algal competitive arena. *Am. Nat.* 136, 209–229. doi: 10.1086/285092

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry. The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Sutton, M. A., Reis, S., Riddick, S. N., Dragosits, U., Nemitz, E., Theobald, M. R., et al. (2013). Towards a climate-dependent paradigm of ammonia emission and deposition. *Philos. Trans. R. Soc. B Biol. Sci.* 368:20130166. doi: 10.1098/rstb.2013.0166

Tank, D. C., Eastman, J. M., Pennell, M. W., Soltis, P. S., Soltis, D. E., Hinchliff, C. E., et al. (2015). Nested radiations and the pulse of angiosperm diversification: increased diversification rates often follow whole genome duplications. *New Phytol.* 207, 454–467. doi: 10.1111/nph.13491

Thrane, J., Hessen, D. O., and Andersen, T. (2017). Plasticity in algal stoichiometry: experimental evidence of a temperature-induced shift in optimal supply N: P ratio. *Limnol. Oceanogr.* doi: 10.1002/lno.10500. [Epub ahead of print].

Tilman, D., and Lehman, C. (2001). Human-caused environmental change: impacts on plant diversity and evolution. *Proc. Natl. Acad. Sci. U.S.A.* 98, 5433–5440. doi: 10.1073/pnas.091093198

Toseland, A., Daines, S. J., Clark, J. R., Kirkham, A., Strauss, J., Uhlig, C., et al. (2013). The impact of temperature on marine phytoplankton resource allocation and metabolism. *Nat. Clim. Chang.* 3, 979–984. doi: 10.1038/nclimate1989

Udall, J. A., and Wendel, J. F. (2006). Polyploidy and crop improvement. *Crop Sci.* 46, S-3. doi: 10.2135/cropsci2006.07.0489tpg

Van de Peer, Y., Maere, S., and Meyer, A. (2009). The evolutionary significance of ancient genome duplications. *Nat. Rev. Genet.* 10, 725–732. doi: 10.1038/nrg2600

Van de Peer, Y., Mizrachi, E., and Marchal, K. (2017). The evolutionary significance of polyploidy. *Nat. Rev. Genet.* 18, 411–424. doi: 10.1038/nrg.2017.26

Van Geest, G. J., Sachse, R., Brehm, M., Van Donk, E., and Hessen, D. O. (2010). Maximizing growth rate at low temperatures: RNA: DNA allocation strategies and life history traits of Arctic and temperate *Daphnia*. *Polar Biol.* 33, 1255–1262. doi: 10.1007/s00300-010-0814-z

Van Nuland, M. E., Wooliver, R. C., Pfennigwerth, A. A., Read, Q. D., Ware, I. M., Mueller, L., et al. (2016). Plant–soil feedbacks: connecting ecosystem ecology and evolution. *Funct. Ecol.* 30, 1032–1042. doi: 10.1111/1365-2435.12690

Veneklaas, E. J., Lambers, H., Bragg, J., Finnegan, P. M., Lovelock, C. E., Plaxton, W. C., et al. (2012). Opportunities for improving phosphorus-use efficiency in crop plants. *New Phytol.* 195, 306–320. doi: 10.1111/j.1469-8137.2012.04190.x

Vinogradov, A. E. (2003). Selfish DNA is maladaptive: evidence from the plant Red List. *Trends Genet.* 19, 609–614. doi: 10.1016/j.tig.2003.09.010

Violle, C., Navas, M., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., et al. (2007). Let the concept of trait be functional! *Oikos* 116, 882–892. doi: 10.1111/j.0030-1299.2007.15559.x

Vitousek, P. M., Porder, S., Houlton, B. Z., and Chadwick, O. A. (2010). Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen-phosphorus interactions. *Ecol. Appl.* 20, 5–15. doi: 10.1890/08-0127.1

Withers, P. J. A., Elser, J. J., Hilton, J., Ohtake, H., Schipper, W. J., and Van Dijk, K. C. (2015). Greening the global phosphorus cycle: how green chemistry can help achieve planetary P sustainability. *Green Chem.* 17, 2087–2099. doi: 10.1039/C4GC02445A

Woods, H. A., Makino, W., Cotner, J. B., Hobbie, S. E., Harrison, J. F., Acharya, K., et al. (2003). Temperature and the chemical composition of poikilothermic organisms. *Funct. Ecol.* 17, 237–245. doi: 10.1046/j.1365-2435.2003.00724.x

Woodward, G., Gessner, M. O., Giller, P. S., Gulis, V., Hladyz, S., Lecerf, A., et al. (2012). Continental-scale effects of nutrient pollution on stream ecosystem functioning. *Science* 336, 1438–1440. doi: 10.1126/science.1219534

Wooliver, R., Pfennigwerth, A. A., Bailey, J. K., and Schweitzer, J. A. (2016). Plant functional constraints guide macroevolutionary trade-offs in competitive and conservative growth responses to nitrogen. *Funct. Ecol.* 30, 1099–1108. doi: 10.1111/1365-2435.12648

Xu, Y., Wang, R., Tong, Y., Zhao, H., Xie, Q., Liu, D., et al. (2014). Mapping QTLs for yield and nitrogen-related traits in wheat: influence of nitrogen and phosphorus fertilization on QTL expression. *Theor. Appl. Genet.* 127, 59–72. doi: 10.1007/s00122-013-2201-y

Yamaji, N., Takemoto, Y., Miyaji, T., Mitani-Ueno, N., Yoshida, K. T., and Ma, J. F. (2017). Reducing phosphorus accumulation in rice grains with an impaired transporter in the node. *Nature* 541, 92–95. doi: 10.1038/nature20610

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 09 May 2017
doi: 10.3389/fmicb.2017.00814

Stoichiometry and Life-History Interact to Determine the Magnitude of Cross-Ecosystem Element and Biomass Fluxes

Thomas M. Luhring[1,2,3]*, *John P. DeLong*[1] *and Raymond D. Semlitsch*[2†]

[1] *Biological Sciences, University of Nebraska, Lincoln, NE, USA,* [2] *Biological Sciences, University of Missouri, Columbia, MO, USA,* [3] *Savannah River Ecology Laboratory, Ecology, University of Georgia, Aiken, SC, USA*

OPEN ACCESS

Edited by:
James Cotner, University of Minnesota, USA

Reviewed by:
Michael J. Vanni, Miami University, USA
Michael William Lomas, Bigelow Laboratory for Ocean Sciences, USA

***Correspondence:**
Thomas M. Luhring
tomluhring@gmail.com

[†] *Deceased: June 10, 2015.*

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 February 2017*
Accepted: *20 April 2017*
Published: *09 May 2017*

Citation:
Luhring TM, DeLong JP and Semlitsch RD (2017) Stoichiometry and Life-History Interact to Determine the Magnitude of Cross-Ecosystem Element and Biomass Fluxes. Front. Microbiol. 8:814. doi: 10.3389/fmicb.2017.00814

Ecosystems are linked through the transfer of materials and energy. Studies examining material fluxes across habitat boundaries frequently quantify unidirectional flows of nutrients and energy. However, material fluxes can be multidirectional, and we lack a conceptual framework to describe how their quantity and stoichiometry influence the net transfer of individual elements between ecosystems. Here we develop a zero net transfer isocline (ZNTI) framework that integrates the relative mass and stoichiometry of fluxes into and out of an ecosystem. We then use case studies with amphibians and salmon to elucidate how life history, ontogenetic shifts in stoichiometry, and trophic interactions shape relative fluxes of nutrients between aquatic and terrestrial ecosystems. Because they increase in both size and Ca content from ova to metamorphs, amphibian life histories strongly bias them toward net Ca export into the terrestrial environment. Because amphibian biomass, C, P, and Ca ZNTIs do not overlap, there is no value of survivorship where the net flux of biomass, C, P, and Ca are simultaneously balanced between terrestrial and aquatic habitats. The degree of iteroparity and semelparity in salmon strongly affects both the magnitude of net biomass and P flux between riverine and marine environments. While the net direction of biomass flux generally remains strongly biased toward import into the riverine system, net P flux can reach net export into the marine environment because of increasing adult breeding survival leading to reduced mass and %P of what they deposit in rivers (e.g., ova vs. whole carcasses). These examples highlight how ontogenetic shifts in body size and stoichiometry result in asymmetric fluxes of elements and biomass that can lead to simultaneous net imports and exports of different elements within the same system. Furthermore, they demonstrate how changes in life-history characteristics and stage-specific survivorship can lead to changes in net elemental transport between ecosystems.

Keywords: body size, calcium, carbon, nitrogen, nutrient cycling, phosphorus, reciprocal subsidy

INTRODUCTION

The movement of energy and nutrients between ecosystems has far-reaching impacts on the structure of food webs and their productivity (Polis et al., 1997). Both physical forces (e.g., wind, flooding) and motile organisms move nutrients and energy between ecosystem compartments (e.g., benthos and water column of a pond) and among ecosystems (DeAngelis, 1992; Vanni, 2002; Regester et al., 2006). The movement of organisms generates a unique type of material flux among ecosystems as its quantity and stoichiometry are subject to evolutionary, ecological, and physiological pressures and constraints. Furthermore, the movement of living biomass across ecosystem boundaries is governed by the movement ecology of the organisms themselves instead of being limited to only passive transport by physical forces.

Organisms move energy and nutrients among ecosystems through two main pathways, excreta and their individual bodies. Organisms with feeding migrations that move across ecosystem boundaries forage, excrete, and egest in different habitats, resulting in a strong directional flux out of the foraging environment and into the habitat where they deposit nutrients (e.g., sea birds; Anderson and Polis, 1999, hippopotomi; Subalusky et al., 2015). Inter-ecosystem fluxes of living biomass likewise have important effects on recipient systems (Helfield and Naimann, 2001, 2006; Marczak et al., 2007). Furthermore, several of these living energy and nutrient vectors have life cycles that obligatorily tie them to multiple habitats, creating reciprocal fluxes of biomass among those habitats (e.g., many insects, diadromous fishes, and amphibians). In these cases, anything that shapes the stoichiometry and or quantity of individuals moving between ecosystems may change the quantity and or quality of the flux itself.

Several factors can contribute to within species stoichiometric variation including local environmental differences and phenotypic variation (El-Sabaawi et al., 2012). In particular, when the relative contribution of body structures (e.g., phosphorus-rich bone) to whole body mass changes across ontogeny, so too does whole body stoichiometry (Sterner and Elser, 2002; Luhring, 2013; Boros et al., 2015; Stephens et al., 2016). Thus, organisms that simultaneously change in body composition and move between ecosystems change the stoichiometry of the biomass they bring with them. In these cases, even when the total biomass coming into and out of a system is equivalent, changes in the stoichiometry of the biomass coming in and going out of the system results in net import or export of individual elements (Luhring, 2013).

Though previous work has successfully cataloged the flow of materials among habitats and subsequent impacts on recipient ecosystems (e.g., Regester et al., 2006; Schriever et al., 2014; Capps et al., 2015), we still lack a conceptual framework to describe how the quantity and stoichiometry of organisms influence the net transfer of individual elements across habitat boundaries. Here we develop a framework integrating the quantity and stoichiometry of biomass movement between two systems.

We first develop the framework conceptually and then use four case studies to illustrate the use of this approach. In the first case study, we use this framework for amphibians with obligate aquatic and terrestrial life stages and show how changes to size and elemental composition between these stages alters the survival needed for net directional flux of biomass and different elements into or out of the aquatic system. In the second case study, we apply data from an amphibian mesocosm experiment to illustrate that the movement of individuals may cause biomass and different elements to vary in both the direction and magnitude of their net flux, and that trophic interactions that alter survivorship and life history alter the net influx and outflux of different elements. Third, we use Atlantic salmon to illustrate the effects of complete adult breeding mortality (semelparity) on biomass and P transfer between marine and riverine systems. Fourth, we develop the Atlantic salmon model to include partial adult breeding mortality (iteroparity) which allows for intermediate possibilities between the amphibian (breeding mortality absent) and semelparous salmon (100% breeding mortality) examples.

MATERIALS AND METHODS

The Zero Net Transfer Model

In our model there are two flux directions that occur between two ecosystems within a set time period (e.g., 1 lifecycle, 1 year). For simplicity, we will define terms in relation to a focal system and define fluxes as "in" (components moving into the focal system) and "out" (components moving out of the focal system). Because we are interested in the net direction of overall flux, we define the conditions needed for them to be equal and then explore conditions that cause net flux transfers to deviate from balanced conditions. Thus, we start with the assumption that Total Flux In = Total Flux Out, not because we expect the fluxes in to be equal to the fluxes out, as our results below will show, but because this is the most obvious reference point for assessing net import or export. We define the "in" flux as equal to the product of the number of components entering the system (N_{In}) and their per capita mass ($Mass_{In}$) (Equation 1). Likewise, the "out" flux is equal to the product of the number of components (N_{Out}) and their per capita mass ($Mass_{Out}$). Given an assumption that the flux in equals the flux out, we have:

$$N_{In} * Mass_{In} = N_{Out} * Mass_{Out} \quad (1)$$

If we rewrite Equation (1) with N terms on the same side, we get an equation describing the inverse relationship between the relative number of individuals moving in each direction and their relative masses (Equation 2).

$$\frac{N_{Out}}{N_{In}} = \frac{Mass_{In}}{Mass_{Out}} \quad (2)$$

This equation can then be solved for values of mass and number where both sides are equal to each other, and we can use this solution as a zero net transfer reference isocline (ZNTI) to graphically depict expected patterns of biomass transport given equal fluxes in and out. Any deviations from this isocline tell us if a system is moving toward net import (values below the isocline)

or export (values above the isocline) of biomass (see **Figure 1** for example ZNTI).

To specify relative amounts of elements moving in each direction, we can adjust the biomass isocline equation (Equation 2) to include element-specific terms. E_{In} and E_{Out} are the proportions of each flux composed of a specific element "*E*."

$$\frac{N_{Out}}{N_{In}} = \frac{Mass_{In} * E_{In}}{Mass_{Out} * E_{Out}} \quad (3)$$

We simplify Equation (3) by defining the relative per capita mass of the "in" flux to the "out" flux as $\mathrm{RM_{In/Out}}$ and the relative elemental composition of the "in" flux to the "out" flux as $\mathrm{RE_{In/Out}}$. Rewriting Equation (3) with these definitions, we get:

$$N_{Out}/N_{In} = RM_{In}/_{Out} * RE_{In}/_{Out} \quad (4)$$

Case Study 1: Shifts in Amphibian Stoichiometry across Ontogeny

Amphibians with complex life histories move biomass and nutrients from terrestrial into aquatic systems in the form of their ova and transport biomass and nutrients from aquatic systems into terrestrial systems in the form of their metamorphosing juveniles (hereafter "metamorphs"; Regester et al., 2006). We set the aquatic system as the focal system whereby ova are the "in" flux and metamorphs are the "out" flux. Substituting flux stages for "in" and "out" we get the biomass ZNTI for this system:

$$N_{Metamorphs}/N_{Ova} = RM_{Ova}/_{Metamorphs} * RE_{Ova}/_{Metamorphs} \quad (5)$$

The left hand side of Equation (5) describes the proportion of ova surviving to metamorphosis (larval survival); the RM term describes the relative size of an ovum to a metamorph; and the RE term describes the relative elemental composition of an ovum relative to a metamorph.

We used dry mass and elemental data collected for ova and metamorphs of six amphibian species to demonstrate how changes in body size and stoichiometry across ontogeny affect the net movement of biomass and elements between terrestrial and aquatic ecosystems (see Luhring, 2013 for details). Briefly, samples of each species were collected from Aiken, South Carolina and vacuum dried (Labconco Freezone vacuum dryer) prior to being homogenized for elemental analyses. C and N were analyzed at the MBL Stable Isotope Laboratory, Woods Hole, MA with a Europa ANCA-SL elemental analyzer. All other elements were analyzed at the University of Georgia's Soil, Plant, and Water Laboratory through microwave assisted digestion (EPA method 3052) followed by axially viewed ICP-AES (EPA method 6010b).

We calculated metamorph masses of the six species in two ways to account for differences in the magnitude of variation seen in the larger species. For the largest four species (*Ambystoma opacum, Ambystoma talpoideum, Lithobate catesbeianus, Lithobates sphenocephalus*), we use the midpoint between the largest and smallest individuals. For the smallest two species (*Anaxyrus terrestris* and *Scaphiopus holbrookii*), we used composite samples of 15–30 individuals to estimate an average body size (i.e., multiple individuals dried in groups, weighed, and total mass divided by number of individuals). Composite samples also were used to approximate individual ovum dry mass. Elemental data for ova were derived from two different composite samples. For the four amphibians metamorphosing at larger sizes we averaged elemental data from large individuals and composites of small individuals (two samples in total). Metamorph elemental data from the two smallest species are derived solely from composite samples.

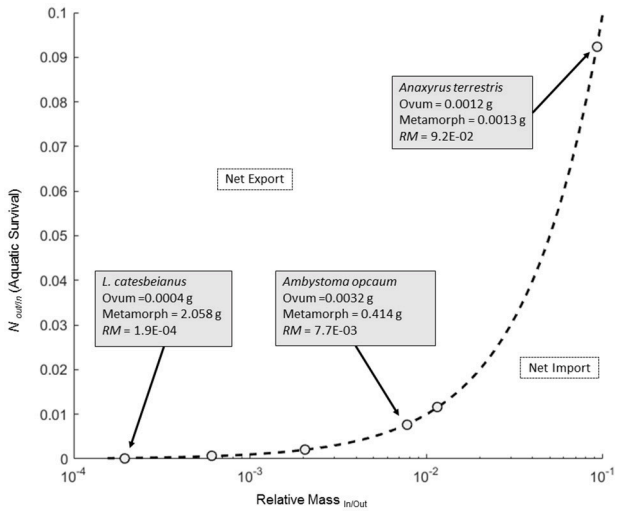

FIGURE 1 | Plot of the zero net transfer isocline (ZNTI; solid line) across a range of relative masses (*RM*) of ova and metamorphs for six amphibians. Open circles illustrate the location of six *RM* values for amphibians from **Table 1**. Details for three data points are shown in boxes with an arrow pointing to their corresponding location on the isocline. Space below the ZNTI correspond to values of *RM* and $N_{\mathrm{Out/In}}$ where there is a net biomass transfer into the aquatic system, whereas space above the ZNTI corresponds to net biomass transport out of the aquatic system.

These data were then used to calculate *RM*'s and *RE*'s for each species. For each species' *RM*, we solve for values of larval survival that results in a balanced transfer of biomass. We then use an average amphibian *RE* to create ZNTI's for three elements (P, C, and Ca) to illustrate how the locations of element-specific ZNTIs may not line up with the biomass ZNTI. What this shows is that any given level of survivorship will likely not balance the import and export of different elements or biomass at the same time.

Case Study 2: Applying Experimental Amphibian Data to Isoclines

Amphibian body size at metamorphosis, survival, and thus biomass export is affected by a myriad of factors (Semlitsch and Caldwell, 1982; Morin, 1986; Earl et al., 2011). We estimated *RM*'s and survivorship from a mesocosm experiment (see Luhring, 2013 for details) which manipulated light, competition, and predation on three anuran species representing a continuum of *RM*'s: *Anaxyrus americanus* (RM = 1/10), *Hyla versicolor* (1/150), *L. sphenocephalus* (1/185). Briefly, each species was raised in replicated 1000-L mesocosms either alone (single-species), with all three anuran species together (competition

TABLE 1 | Summary of ova and metamorph size and elemental composition for 6 amphibian species with larval survivorship values (boldfaced) where biomass flux (*RM*) and element-specific flux (*RM*RE*) are balanced between terrestrial and aquatic systems.

Species	Stage	N*	Mean mass		C**	N**	Ca***	P***	S***	C:Ca
Ambystoma opacum	Ova	695	0.0032		50.56	10.22	475	11,976	5429.5	1064.5
	Meta	15	0.414		45.80	10.80	27,342	17325.5	6675.5	16.7
		***RM* =**	**7.7E-03**	***RE* =**	1.10	0.95	0.02	0.69	0.81	
				***RM * RE* =**	**8.53E-03**	**7.31E-03**	**1.34E-04**	**5.34E-03**	**6.29E-03**	
Ambystoma talpoideum	Ova	2,670	0.0011		49.55	10.50	603	13048.5	5,818	821.7
	Meta	5	1.805		43.36	9.02	23204.5	15402	6449.5	18.7
		***RM* =**	**6.1E-04**	***RE* =**	1.14	1.16	0.03	0.85	0.90	
				***RM * RE* =**	**6.96E-04**	**7.10E-04**	**1.58E-05**	**5.16E-04**	**5.50E-04**	
Anaxyrus terrestris	Ova	3,444	0.0012		50.59	9.96	577	12445.5	6209.5	876.8
	Meta	306	0.013		37.31	9.73	25,691	16,477	5,863	14.5
		***RM* =**	**9.2E-02**	***RE* =**	1.36	1.02	0.02	0.76	1.06	
				***RM * RE* =**	**1.25E-01**	**9.45E-02**	**2.07E-03**	**6.97E-02**	**9.78E-02**	
Lithobates catesbeianus	Ova	31,806	0.0004		51.56	11.35	1,371	9332.5	7132.5	376.1
	Meta	2	2.058		45.76	9.95	32230.5	18544.5	5,467	14.2
		***RM* =**	**1.9E-04**	***RE* =**	1.13	1.14	0.04	0.50	1.30	
				***RM * RE* =**	**2.19E-04**	**2.22E-04**	**8.27E-06**	**9.78E-05**	**2.54E-04**	
Lithobates sphenocephalus	Ova	4,424	0.0013		51.17	10.16	556.5	11,079	6335.5	919.5
	Meta	4	0.638		41.89	9.72	25,501	14,211	5685.5	16.4
		***RM* =**	**2.0E-03**	***RE* =**	1.22	1.05	0.02	0.78	1.11	
				***RM * RE* =**	**2.49E-03**	**2.13E-03**	**4.45E-05**	**1.59E-03**	**2.27E-03**	
Schaphiopus holbrookii	Ova	2,400	0.0012		50.25	9.77	1,373	11949.5	6,376	366.0
	Meta	13	0.104		48.75	8.98	24,054	11,938	4,715	20.3
		***RM* =**	**1.2E-02**	***RE* =**	1.03	1.09	0.06	1.00	1.35	
				***RM * RE* =**	**1.19E-02**	**1.26E-02**	**6.59E-04**	**1.15E-02**	**1.56E-02**	

*RM, relative mass (ovum mass/metamorph mass). RE, Relative elemental composition (ovum element concentration/ metamorph elemental concentration). Mass is in g dry mass. *Total individuals in all composite samples. **Elemental content as percent of dry mass. ***Elemental content as mg kg^{-1} of dry mass.*

treatment), or all three anuran species and caudate predators (competition + predation treatment). All of these treatments were crossed with 2 light levels: high (77% of ambient) and low (27% of ambient) light. Anuran larvae were removed from the mesocosms upon metamorphosis, measured and weighed (wet mass in g). A subset from each species were subsequently measured for dry mass to create species-specific wet mass to dry mass conversion rates for the experiment (*A. americanus*: 0.113, *H. versicolor*: 0.143, and *L. sphenocephalus*: 0.149). For each species, we used individual ova dry mass from batches of ova for *L. sphenocephalus* (0.0013 g) or for congeneric species as necessary (*A. terrestris*: 0.0012 g, *H. cinerea*: 0.0006 g). Mesocosm average metamorph dry masses were derived by converting average metamorph wet mass to dry mass by their species- or genera-specific wet to dry mass conversion. We then use average mesocosm *RM* and realized larval survival rate to graphically depict the effects of light, competition and predation on net movement of biomass and Ca. Plots of biomass isoclines represent x-fold isoclines for each element where x is equal to the inverse of the product of *RE* and *RM*. For example, the ZNTI for biomass is a 1-fold export (where the ratio of biomass export to biomass import is 1), and a 10x NTI for biomass is a 10-fold export. For a species with an RE_{Ca} of 1/45, 1x and 10x biomass isoclines would be equal to 45-fold, and 450-fold net transfer isoclines of Ca, respectively.

Case Study 3: Complete Breeding Mortality in Semelparous Salmonids

Salmonids move massive amounts of biomass between marine and riverine systems every year and the resultant effects of this flux on recipient systems (e.g., Janetski et al., 2009) and the net flow of nutrients among them are of particular interest (e.g., Moore et al., 2007; Ebel et al., 2015). Semelparous salmonids flux biomass and nutrients from marine into riverine systems in the form of their entire body mass (including gonads) and flux biomass and nutrients from riverine systems into marine systems in the form of their juveniles (hereafter "smolts"; e.g., Gende et al., 2002; Schindler et al., 2003; Ebel et al., 2015). We set the river as the focal system whereby adult salmon are the "in" flux and smolt

are the "out" flux. Substituting flux stages for "in" and "out" we get:

$$N_{Smolts}/N_{Adults} = RM_{Adults}/_{Smolts} * RE_{Adults}/_{Smolts} \quad (6)$$

The left hand side in Equation (6) describes the per capita conversion of in-migrating adults into out-migrating smolts (per capita net recruitment), the RM term describes the relative size of an adult to a smolt, and the RE term describes the relative elemental composition of an adult relative to a smolt. We used wet mass and P content collected for Atlantic salmon from three river systems (Ebel et al., 2015) to demonstrate how size and elemental asymmetries between smolt and adult salmon of different rivers affect net biomass and P flux between marine and riverine systems. We solve for RM and RE_P using the same approach as described in the previous examples. This data is then used in a subsequent adaptation of our model for incomplete breeding mortality (see Case Study 4: Partial Breeding Mortality in Salmonids).

Case Study 4: Partial Breeding Mortality in Salmonids

Although many species of salmonids are semelparous (100% adult mortality after breeding), some are at least partially iteroparous and spend part of their post-breeding recovery time in the river in which they breed. Incomplete breeding mortality results in an additional life stage (hereafter "kelts") moving biomass and nutrients out of the river which is partially offset by what it deposits while in the river (gametes, egested, and excreted materials). Using the same source of data from Section Case Study 3: Complete Breeding Mortality in Semelparous Salmonids, we adapt Equation (1) to include partial mortality of adults and bi-directional movement of materials by overwintering kelts. Because the mass and stoichiometry of kelts leaving the river is different than that of adults entering the river, the $Mass_{\mathrm{In}}$ term has to account for changes in mass, elements, and the mortality of adults. The adult mass that enters the system and stays in is the fraction of those adults that die times the mass and elemental composition of adults ($D * M_A * E_A$), with terms subscripted A for adult. Likewise, the adult mass that leaves the system is the fraction of the adults that live times the mass and elemental composition of kelts $[(1-D) * (M_A * E_A - M_K * E_K)]$, with terms subscripted K for kelt. Substituting these expressions into Equation (1), and subscripting smolt terms with an S, we get:

$$N_A\big(D * M_A * E_A + (1-D) * (M_A * E_A - M_K * E_K)\big) = N_S * M_S * E_S \quad (7)$$

This simplifies to the elemental ZNTI for salmon systems:

$$N_S/N_A = D * RM_A/_S * RE_A/_S + (1-D) * \frac{(M_A * E_A - M_K * E_K)}{M_S * E_S} \quad (8)$$

that like the previous ZNTIs, relates the transformation of individuals while in the system to the changes in individual mass and elemental composition while in the system.

We use the same wet mass and P content collected for Atlantic salmon from the three river systems as in the previous example (Section Case Study 3: Complete Breeding Mortality in Semelparous Salmonids; Ebel et al., 2015) to demonstrate how different levels of adult breeding mortality (D) affect net biomass and P fluxes. This model (Equation 8) thus offers an intermediate between the amphibian example (Section Case Study 1: Shifts in Amphibian Stoichiometry Across Ontogeny, Equation 5) and the semelparous salmon example (Section Case Study 3: Complete Breeding Mortality in Semelparous Salmonids, Equation 6) which are derived assuming either 0 or 100% breeding mortality respectively. When D is 1, complete breeding mortality occurs and Equation (8) becomes identical to Equation (6). When D is 0, it effectively becomes Equation (5) wherein the elemental content of an adult coming in minus its elemental content going out is the contribution to the "in" habitat (e.g., ova).

RESULTS AND DISCUSSION

Case Study 1: Shifts in Amphibian Stoichiometry across Ontogeny

The amphibians we considered varied considerably in relative sizes of ova to metamorphs, with the $RM_{In/Out}$ ranging from 1/5145 to 1/11 (**Figure 1**; **Table 1**). Because a ZNTI is essentially a 1:1 line, these values stipulate the survival required to balance the transfer of mass into and out of the system. Although survivorship to metamorphosis is highly variable in natural amphibian populations, we can get an idea of where different species fall along the spectrum of export vs. import potential. Species with small ova and a large size at metamorphosis have the smallest $RM_{In/Out}$ and, subsequently, the lowest survival required for a zero net transfer of biomass. Conversely, species with the largest $RM_{In/Out}$ recorded in this study (*A. terrestris;* $RM_{In/Out} = 1/11$) had the smallest size at metamorphosis (0.001 g dry mass) and the highest survival required for zero net transfer of biomass (1 in 11 ova surviving to metamorphosis). Because both ovum size and size at metamorphosis determine $RM_{In/Out}$, species with similar $RM_{In/Out}$'s such as *S. holbrookii* and *A. opacum* (1/86.7 vs. 1/129.4) may differ both in ova size (2.6-fold difference) and size at metamorphosis (4-fold difference) yet still require the same survival to balance mass transfers (**Table 1**).

Whenever elemental composition changes with life history stages of individuals entering or leaving a focal system (RE $\neq$ 1), the net flux of biomass and that element cannot be equivalent. For our set of amphibians, there was considerable asymmetry in the transport of biomass and elements into and out of a pond. $RE_{In/Out}$ net direction and magnitude for each element was generally consistent across amphibian species, but varied widely across elements (**Table 1**). $RE_{In/Out}$ ranged from a high of 1.36/1 for $RC_{In/Out}$ (*A. terrestris*) to a low of 1/57.56 for $RCa_{In/Out}$ (*A. opacum*; **Table 1**). The six species in this study all had at least an order of magnitude decrease in C:Ca ratio from ova to metamorphosis driven by both a decrease in C and a 17- to 57-fold increase in Ca (**Table 1**). Of all the elements, Ca was the most strongly asymmetric between ova and metamorphs (average $RCa_{In/Out} = 1/37.9$). The combined strong asymmetries

in both *RCa* and *RM* of amphibians meant that most species were strongly biased toward exporting Ca even at levels with biomass or P import (**Figure 2**; RE*RM in **Table 1**).

Similar to how fluxes of elements with RE $\neq$ 1 cannot be simultaneously balanced with biomass fluxes, fluxes of elements with differing *RE*'s cannot be simultaneously balanced with each other. For any given element "Y," there are two scenarios when the incoming and outgoing fluxes have different "Y" values ($RY_{In/Out} \neq 1$): (1) $P_{0,Y}$ isocline is above the $P_{0,\text{Biomass}}$ isocline ($RY_{In/Out} > 1$; bias toward import), or (2) $P_{0,Y}$ isocline is below the $P_{0,\text{Biomass}}$ isocline ($RY_{In/Out} < 1$; bias toward export). When elemental P_0's are situated above and below the $P_{0Biomass}$ isocline (e.g., C and Ca in **Figure 2**), there exist several possible outcomes for element-specific transfer between systems. For example, any point along the $P_{0,\text{Biomass}}$ isocline in the amphibian example (**Figure 2**) would correspond with a net import of C and a simultaneous export of Ca. However, any point along the $P_{0,\text{Ca}}$ isocline where Ca movement between the systems is balanced would correspond to a strong import of biomass and C. Values not falling directly on any isoclines demonstrate simultaneous inequalities in net movement for all materials considered (i.e., biomass, C, P, and Ca in **Figure 2**).

Case Study 2: Applying Experimental Data to Isoclines

Changes in average body size at metamorphosis and larval survival resulting from predation readily moved mesocosms across biomass and elemental isoclines (**Figure 3**). The smallest species (by metamorph size) was most readily drawn below the biomass ZNTI through predation, but remained above the Ca ZNTI unless there was 0 survivorship (**Figure 3**). *A. americanus* failed to metamorphose from 6 of 12 predator mesocosms, in contrast to *H. versicolor* and *L. sphenocephalus* which both failed to metamorphose from 3 of 12 predator mesocosms. In these cases, our gape-limited predators appeared to have a relatively stronger ability to force the net flux of the smaller species into a state of total import (i.e., a complete biomass and nutrient sink).

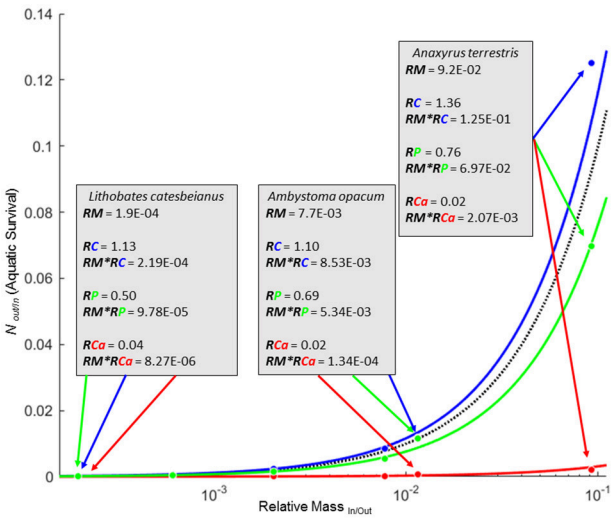

FIGURE 2 | Zero net transfer isoclines (ZNTI) for biomass (black), C (solid blue), P (solid green), and Ca (solid red) for 6 amphibian species where *P* corresponds to balanced flux between terrestrial and aquatic systems for biomass, C, or Ca. Isoclines for C and Ca depict the average *RE* for C and Ca for the six amphibians. Details for three species are shown in boxes with arrows pointing to their corresponding location on the isoclines. Regions between lines represent simultaneous import and export of two different materials. For example, the region between biomass and Ca isoclines represent values at which there would be an import of biomass and an export of Ca.

Although changes to either body size or survivorship would have predictable effects on net transfers of biomass, they are not always independent of each other. This is because body size at metamorphosis results from the interaction of a multitude of environmental and evolutionary factors (Werner, 1986). Links between body size at metamorphosis and aquatic survival thus change the rates at which changes in survival may shift systems between states of net import or export. The causes of linked $RM_{In/Out}$ (as a function of changing size at metamorphosis) and survival in our systems are well-known and provide an opportunity to understand how these phenomena affect the movement of biomass and nutrients across ecosystems.

The smallest species, *A. americanus*, demonstrates an Allee effect whereupon size at metamorphosis is highest at an intermediate density (our stocking density), but decreases with a decrease in population density (Wilbur, 1977). Our mesocosms demonstrated a similar pattern for *A. americanus* whereby predation lowered population density of *A. americanus* in our mesocosms and reduced the sizes of the few successfully metamorphosing juveniles. Predation thus appeared to accelerate *A. americanus* toward net import by simultaneously increasing the required survivorship for balanced flux (through increased $RM_{In/Out}$) while decreasing survivorship. Predation can also lead to a thinning effect whereby predators decrease population density and thus intra or interspecific competition, leading to larger sizes at metamorphosis when survival decreases (Wilbur et al., 1983). This pattern was most apparent in our largest species, which may be heavily predated at smaller sizes but escape gape-limited predators at larger sizes. In their case, the largest metamorphs and thus smallest mean $RM_{In/Out}$ values came from mesocosms with the lowest aquatic survival rates (**Figure 3**). Although predation decreases survivorship in the larger species, the ability of predators to draw the system into a net import is partially offset by a simultaneous decrease in *RM* caused by a larger size at metamorphosis.

Because elemental transfer isoclines are directly linked to biomass isoclines through Equation (5), any isocline that is a multiple of the biomass ZNTI can be readily interpreted for a given element by multiplying the biomass NTI by the inverse of the $RE_{In/Out}$ for the element of interest. This allows the use of additional biomass NTI's (10x NTI, 100x NTI, etc.) that can be used as reference contours to depict the relative magnitude of both biomass and elemental import or export. For example, a 10x NTI and 100x NTI indicate the 10- and 100-fold relative export of biomass (**Figure 3**). In the case of *L. sphenocephalus* where $RCa_{In/Out} = 1/45.82$ and $RC_{In/Out} = 1/0.82$ (**Table 1**), the

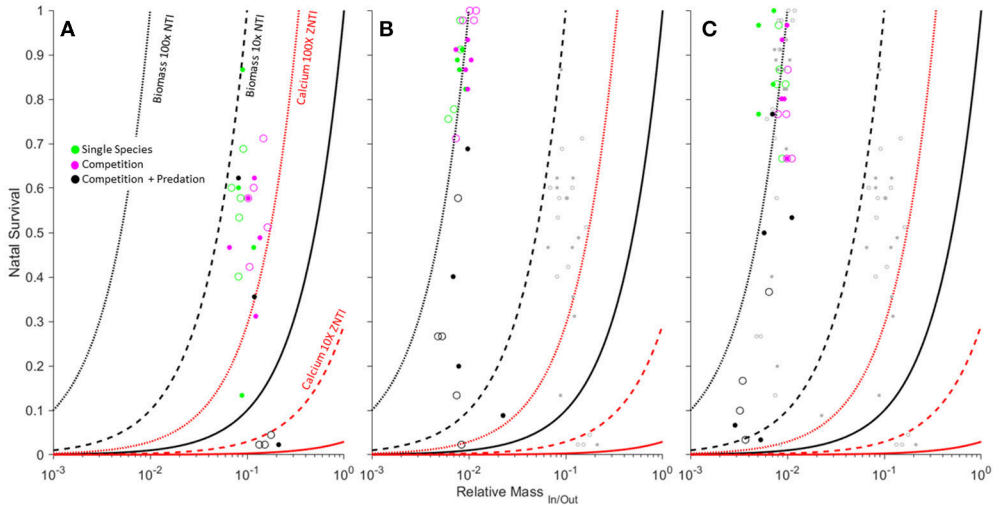

FIGURE 3 | Effects of competition, predation and light levels (low [open] vs. high [filled]) on biomass and calcium transfer magnitude for *Anaxyrus americanus* (A) ,*Hyla versicolor* **(B)**, and *Lithobates sphenocephalus* **(C)**. Data points represent mesocosm average relative mass of ova/metamorphs (*RM*) and larval survivorship ($N_{Out/In}$) from a previous study (Luhring, 2013). Zero net transfer isoclines for biomass (solid black), and Ca (solid red) and their 10x and 100x net transfer isoclines (dashed and dotted NTI's) are shown for reference. Each panel includes the data from panels to their left as gray dots for reference.

10x NTI would correspond to a 458.2x NTI for Ca, and a 8.2x NTI for C. The 100x NTI where many of the *L. sphenocephala* mesocosms are clustered (right panel of **Figure 3**) would thus correspond with a 4582x NTI for Ca and a 82x NTI for C.

Case Study 3: Complete Breeding Mortality in Semelparous Salmonids

As with amphibians, two life stages of salmon move biomass between ecosystems. However, semelparous salmon are different in two key ways. First, breeding adults deposit their entire body mass (body and gametes) in the "in" system (river) when they die. Second, while the conversion of $N_{In/Out}$ (larval survival) for amphibians has an upper limit of 1 (solid horizontal line in **Figure 4**), $N_{In/Out}$ for salmon is a measure of net recruitment of smolts per breeding adult and has a much higher ceiling. These combined factors extend the salmon biomass ZNTI farther to the right of the amphibians while still allowing for potential import or export (**Figure 4**).

Salmon in the three rivers examined had net recruitment rates ($N_{Out/In}$) of 11.90–26.15 (**Table 2**). If we assume that spawning adults in these rivers experience complete mortality (Equation 6), then net biomass flux would be into the river as all recruitment rates were below the ZNTI (**Figure 4**; **Table 2**). To investigate net P flux, we incorporate P composition for adults and smolts. Because in-migrating adults are lower in P than out-migating smolts ($RP_{In.Out} < 1$), they lower the P isocline relative to the biomass isocline (inset **Figure 4**). Thus, although adults represent a higher per capita biomass flux, their lower P content reduces the amount of relatively P-rich smolt biomass that has to exit the system in order for P flux to be balanced. None of the rivers had the net recruitment needed to reach their biomass ZNTIs ($N_{Out/In}/RM_{In/Out}$) or net recruitment need to reach their P ZNTIs ($N_{Out/In}/RM{*}RP$). However, one river (Conne; **Table 2**) reached 87% of the recruitment needed to reach its P ZNTI (**Table 2**). This case study demonstrates the outcomes of complete breeding mortality (semelparity) on cross-ecosystem fluxes of biomass and elements. However, for many diadromous fishes, some fraction of adults survive spawning events and exit the breeding system (e.g., iteroparous species). Below, we will see how these outcomes shift after incorporating partial breeding mortality in the same system.

Case Study 4: Partial Breeding Mortality in Salmonids

Many reciprocal fluxes resulting from linked life stages may fall between the extremes of complete breeding mortality (semelparity) and complete survival of breeding adults (e.g., an implicit assumption of the amphibian mesocosm example). Equation (8) describes the intermediate case of partial breeding mortality that simplifies into Equation (6) when $D = 1$ (semelparity) and Equation (4) when $D = 0$ (where eggs are deposited by females who then leave the system). The change from deposition of an adult carcass to partial deposition of adult biomass ("Adult-Kelt" in **Table 2**) results in a net per capita reduction of biomass deposited by adults. Increases in breeding mortality (D) increase the per capita contribution of adults to the "in" fluxes of biomass and raise the recruitment rate required to reach the biomass ZNTI. Decreases in D decrease the per capita contribution of adults to the "in" fluxes of biomass and lower recruitment rates required to reach the biomass ZNTI. Although adults are lower in P content than kelts, the partial contributions of adults to rivers resulting from breeding activity and overwintering are even lower in biomass and %P content ("Adult-Kelt" in **Table 2**). Because P in this partial deposition is lower than that of adult carcasses,

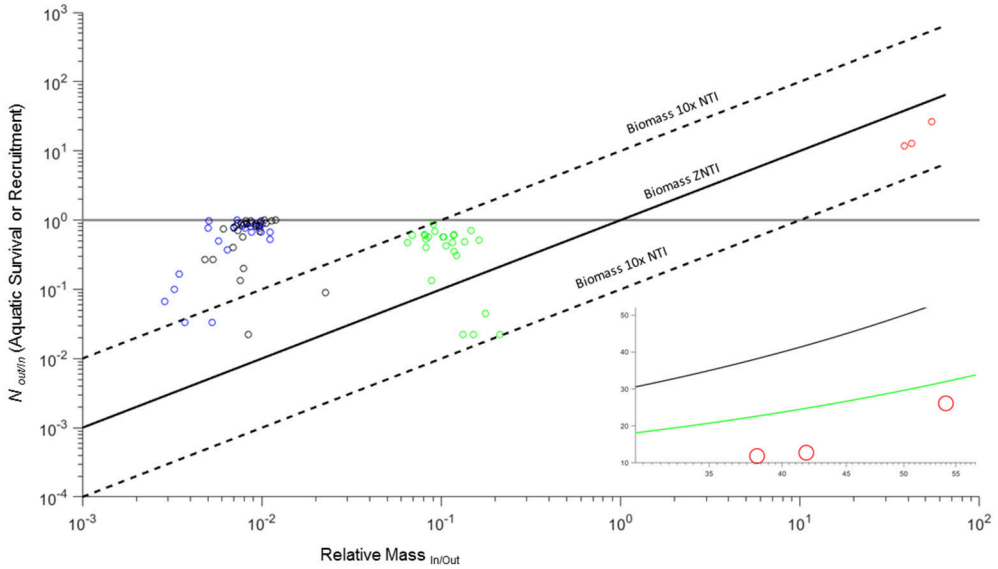

FIGURE 4 | Zero net transfer and 10x net transfer biomass isoclines for $RM_{In/Out}$ and $N_{Out/In}$ for amphibian mesocosms (blue, black, and green: *L. sphenocephalus, H. versicolor,* and *A. americanus*) and Atlantic salmon (red: assuming complete breeding mortality). Horizontal gray line indicates the upper limit of $N_{Out/In}$ for aquatic survival for amphibian larvae (cannot exceed 1). Inset image shows salmon data against the biomass ZNTI (black line) and P ZNTI (green line: derived from average smolt and adult P across three populations).

TABLE 2 | Summary of Atlantic salmon adult, smolt, and kelt size and P content by river from Ebel et al. (2015) with derived model parameters (boldface).

River	Stage	Wet mass (g)*	%P**	$RM_{In/Out}$	$RP_{In/Out}$	*RM * RP*	N_{out}	N_{in}	$N_{out/In}$
Cambellton	Adult	1,910	0.38	**38.2**	**0.66**	**25.03**	40,146	3,375	**11.90**
	Smolt	50	0.58						
	Kelt	1331.4	0.5				$N_{out/In}/RM_{In/Out}$		**0.31**
	Adult-Kelt	578.6	0.10				$N_{out/In}/(RM*RP)$		**0.48**
Conne	Adult	1,620	0.36	**54**	**0.55**	**29.91**	67,209	2,570	**26.15**
	Smolt	30	0.65						
	Kelt	730	0.53				$N_{out/In}/RM_{In/Out}$		**0.48**
	Adult-Kelt	890	0.22				$N_{out/In}/(RM*RP)$		**0.87**
Western Arm	Adult	2,090	0.37	**41.8**	**0.57**	**23.79**	15,756	1,234	**12.77**
	Smolt	50	0.65						
	Kelt	826.9	0.58				$N_{out/In}/RM_{In/Out}$		**0.31**
	Adult-Kelt	1263.1	0.23				$N_{out/In}/(RM*RP)$		**0.54**

*Adult-Kelt quantifies mass loss and the %P of that loss for adults surviving the breeding season. $N_{Out/In}/RM$ and $N_{Out/In}/RM$ *RP are the amount of recruitment observed relative to that needed for zero net transfer of biomass and P (respectively) between river and marine systems. *Adult mass is a weighted mean of wet mass (g) weighted by number of adults in two size classes. **%P is on a wet mass basis.*

the total amount of P deposited per adult decreases at a faster rate than biomass with increased adult breeding survival $(1 - D)$.

Shifting from complete breeding mortality to partial or complete survival of breeding adults changes the net biomass or P flux between rivers and marine systems. Scenarios where adult breeding mortality is 100% ($D = 1$; dotted black and green lines in **Figure 5**) are identical to that of the previous example (**Figure 4**; Section Case Study 3: Complete Breeding Mortality in Semelparous Salmonids) and serve as a reference. Even with 100% breeding survival ($D = 0$; solid lines in **Figure 5**), biomass flux generally remained biased toward import with only the Cambellton River showing a minor net export of biomass (**Figure 5** left panel). However, P flux among systems was more variable. As opposed to net biomass flux, P was much more likely to transition to a net export from rivers to marine systems with increasing levels of adult survival (**Figure 5**). All rivers showed net P export when 100% of adults survived and were close to or above P ZNTIs when adult survival was 50% (**Figure 5**). Thus, while biomass in the salmon examples are strongly biased toward

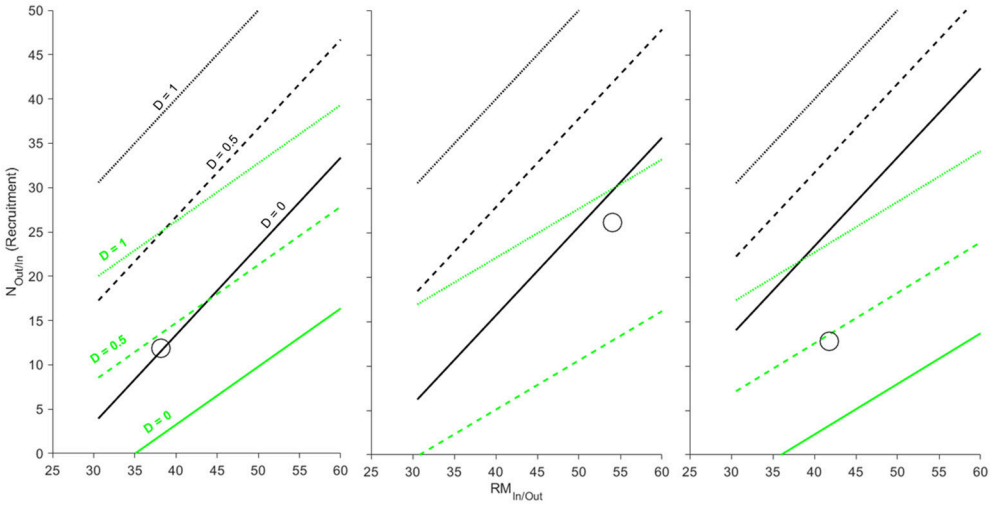

FIGURE 5 | Zero net transfer isoclines for biomass (black) and P (green) for three populations of Atlantic salmon (from left to right: Cambellton, Conne, and Western Arm; data from Ebel et al., 2015). ZNTI's are shown for varying levels of breeding mortality ($D = 0$, 0.5, 1) and are derived for population-specific differences in stage-specific size, %P, spawning run size (N_{In}) and smolt production (N_{Out}). Only when 100% of adults survive and become outmigrating kelts in Cambellton ($D = 0$) would a net biomass export into the marine environment occur (above black line). All populations show potential for P import or export based on varying levels of breeding mortality (between green lines for $D = 0$ and $D = 1$).

net import into rivers, partial breeding survival can transition these systems into simultaneous net fluxes of P out of and biomass into rivers.

CONCLUSIONS

How Stoichiometry Influences Net Fluxes of Elements between Ecosystems

When inter-ecosystem fluxes are carried out by different life stages of a single organism, predictable asymmetries in net biomass and elemental fluxes arise through changes in body size and ontogenetic changes in stoichiometry. For example, large shifts in both body size and C:Ca from ovum to metamorphosis create large size and elemental imbalances in amphibian derived biomass fluxes between aquatic and terrestrial ecosystems. Aquatic survival is generally low in amphibians (Herreid and Kinney, 1966; Calef, 1973; Shoop, 1974; Semlitsch, 1987; Regester et al., 2006), which may cause many species with smaller metamorphs relative to ova size (and thus larger RM's) to generally be net importers of biomass. However, because of the large increase in Ca from ova to metamorph, nearly all species of amphibians are also typically net exporters of Ca from wetlands, barring complete breeding failure. Less drastic changes to individual size and survivorship would be needed to switch elements with *RE*'s closer to 1 between net import and export.

Future Directions

We present a framework that can be adapted to include more complicated eco-evolutionary dynamics and their effects on inter-ecosystem fluxes. For example, relationships between survivorship and body size (e.g., thinning, Allee effects; **Figure 3**) and their subsequent effects on fluxes can be characterized by their effects on the slope of survivorship vs. relative mass. Furthermore, because stoichiometry changes with body size among and within species, the assumption that an *RE* value would be static across values of *RM* may not hold for all models. Although these initial models successfully describe the effects of asymmetries in body size and stoichiometry on reciprocal flux dynamics, additional modifications that reflect increasingly complicated dynamics (e.g., size and stoichiometry) may reveal additional insights.

The integration of the ZNTI approach into food web ecology would be a logical and important next step. While our models explicitly incorporate the effects of life stage asymmetries in size and stoichiometry on net transfer magnitude and direction, they do not yet integrate these asymmetries into food web dynamics (e.g., the merged ecosystem and food web approach advocated by Marcarelli et al., 2011). Relative composition, magnitude, and amount of available resources in incoming fluxes vs. existing resources determine the effects that incoming fluxes have on recipient ecosystems (Marczak et al., 2007; Marcarelli et al., 2011). Thus, the same stage-specific changes in size or stoichiometry in our models that determine net flux magnitude and direction will also determine their effects on recipient systems.

Generalities of the Isocline Approach

Our modeling and isocline approach is readily applicable for a variety of systems for determining the effects of processes (e.g., per capita recruitment, larval survival) and properties of the fluxes (e.g., relative sizes and elemental composition) in changing net flux direction and magnitude between ecosystems. Because conversion rates of in to out (larval survival, smolt recruitment per adult) and their relative sizes and elemental composition are readily estimable or available in the literature for many systems, initial models can be used to estimate the

relative sensitivities of flux direction and magnitude to changes in these processes and properties *a priori*. Gathering the data for individual flux components to estimate whether a system experiences a net import or export within or across seasons is highly time and resource intensive (e.g., Regester et al., 2006; Ebel et al., 2015). The details garnered from such studies are essential to understand variation in contributions of individual-level fluxes to between-ecosystem fluxes. However, a general model which can account for the wide disparity in processes that link reciprocal fluxes (e.g., larval survival, recruitment) to each other as well as their relative differences in size and elemental composition is required to begin synthesizing these processes across time and spatial scales.

ETHICS STATEMENT

This study was carried out in accordance with the University of Missouri's Animal Care and Use Committee under permit MU ACUC 6144, 7403.

AUTHOR CONTRIBUTIONS

TL, JD, and RS contributed to the conception of the work and revised various drafts of previous versions. TL and JD wrote the final version of the manuscript.

FUNDING

TL was funded by the University of Nebraska's Population Biology Program of Excellence. Postdoctoral Fellowship and the University of Missouri's Life Sciences and Trans-World Airlines Fellowships.

ACKNOWLEDGMENTS

We thank K Capps for her input into the generalization of the model. TL thanks the reviewers from an older review of a previous version of this manuscript for their helpful insight and suggestions.

REFERENCES

Anderson, W. B., and Polis, G. A. (1999). Nutrient fluxes from water to land: seabirds affect plant nutrient status on Gulf of California Islands. *Oecologia* 118, 324–332. doi: 10.1007/s004420050733

Boros, G., Sály, P., and Vanni, M. J. (2015). Ontogenetic variation in the body stoichiometry of two fish species. *Oecologia* 179, 329–341. doi: 10.1007/s00442-015-3349-8

Calef, G. W. (1973). Natural mortality of tadpoles in a population of Rana aurora. *Ecology* 54, 741–758. doi: 10.2307/1935670

Capps, K. A., Berven, K. A., and Tiegs, S. D. (2015). Modelling nutrient transport and transformation by pool-breeding amphibians in forested landscapes using a 21-year dataset. *Freshw. Biol.* 60, 500–511. doi: 10.1111/fwb.12470

DeAngelis, D. (1992). *Dynamics of Nutrient Cycling and Food Webs*. New York, NY: Chapman & Hall.

Earl, J. E., Luhring, T. M., Williams, B. K., and Semlitsch, R. D. (2011). Biomass export of salamanders and anurans from ponds is affected differentially by changes in canopy cover. *Freshw. Biol.* 56, 2473–2482. doi: 10.1111/j.1365-2427.2011.02672.x

Ebel, J. D., Leroux, S. J., Robertson, M. J., and Dempson, J. B. (2015). Ontogenetic differences in Atlantic salmon phosphorus concentration and its implications for cross ecosystem fluxes. *Ecosphere* 6, 136. doi: 10.1890/ES14-00516.1

El-Sabaawi, R. W., Zandonà, E. Z., Kohler, T. J., Marshall, M. C., Moslemi, J. M., Travis, J., et al. (2012). Widespread intraspecific organismal stoichiometry among populations of the Trinidadian guppy. *Funct. Ecol.* 26, 666–676. doi: 10.1111/j.1365-2435.2012.01974.x

Gende, S. M., Edwards, R. T., Willson, M. F., and Wipfli, M. S. (2002). Pacific salmon in aquatic and terrestrial ecosystems. *Bioscience* 52, 917–928. doi: 10.1641/0006-3568(2002)052[0917:PSIAAT]2.0.CO;2

Helfield, J. M., and Naimann, R. J. (2001). Effects of salmon-derived nitrogen on riparian forest growth and implications for stream productivity. *Ecology* 82, 2403–2409. doi: 10.1890/0012-9658(2001)082[2403:EOSDNO]2.0.CO;2

Helfield, J. M., and Naimann, R. J. (2006). Keystone interactions: salmon and bear in riparian forests of Alaska. *Ecosystems* 9, 167–180. doi: 10.1007/s10021-004-0063-5

Herreid, C. F., and Kinney, S. (1966). Survival of Alaskan wood frog (Rana sylvatica) larvae. *Ecology* 47, 1039–1041. doi: 10.2307/1935651

Janetski, D. J., Chaloner, D. T., Tiegs, S. D., and Lamberti, G. A. (2009). Pacific salmon effects on stream ecosystems: a quantitative synthesis. *Oecologia* 159, 583–595. doi: 10.1007/s00442-008-1249-x

Luhring, T. M. (2013). *Complex Life-Histories and Biogeochemical Cycles; Interactions between Amphibian Life-History Strategies and Elemental Cycling*. Dissertation, University of Missouri, Columbia, MO.

Marcarelli, A. M., Baxter, C. V., Mineau, M. M., and Hall, R. O. Jr. (2011). Quality and quality: unifying food web and ecosystem perspectives on the role of resource subsidies in freshwaters. *Ecology* 92, 1215–1225. doi: 10.1890/10-2240.1

Marczak, L. B., Thompson, R. M., and Richardson, J. S. (2007). Trophic level, habitat, and productivity shape the food web effects of resource subsidies. *Ecology* 88, 140–148. doi: 10.1890/0012-9658(2007)88[140:MTLHAP]2.0.CO;2

Moore, J. W., Schindler, D. E., Carter, J. L., Fox, J., Griffiths, J., and Holtgrieve, G. W. (2007). Biotic control of stream fluxes: spawning salmon drive nutrient and matter export. *Ecology* 88, 1278–1291. doi: 10.1890/06-0782

Morin, P. J. (1986). Interactions between intraspecific competition and predation in an amphibian predator-prey system. *Ecology* 67, 713–702. doi: 10.2307/1937694

Polis, G. A., Anderson, W. B., and Holt, R. D. (1997). Toward an integration of landscape and food web ecology: the dynamics of spatially subsidized food webs. *Annu. Rev. Ecol. Syst.* 28, 289–316. doi: 10.1146/annurev.ecolsys.28.1.289

Regester, K. J., Lips, K. R., and Whiles, M. R. (2006). Energy flow and subsidies associated with the complex life cycle of ambystomatid salamanders in ponds and adjacent forest in southern Illinois. *Oecologia* 147, 303–314. doi: 10.1007/s00442-005-0266-2

Schindler, D. E., Scheuerell, M. D., Moore, J. W., Gende, S. M., Francis, T. B., and Palen, W. J. (2003). Pacific salmon and the ecology of coastal ecosystems. *Front. Ecol. Environ.* 1, 31–37. doi: 10.1890/1540-9295(2003)001[0031:PSATEO]2.0.CO;2

Schriever, T. A., Cadotte, M. W., and Williams, D. D. (2014). How hydroperiod and species richness affect the balance of resource flows across aquatic-terrestrial habitats. *Aquat. Sci.* 76, 131–143. doi: 10.1007/s00027-013-0320-9

Semlitsch, R. D. (1987). Relationship of pond drying to the reproductive success of the salamander *Ambystoma talpoideum*. *Copeia* 1987, 61–69. doi: 10.2307/1446038

Semlitsch, R. D., and Caldwell, J. P. (1982). Effects of density of growth, metamorphosis, and survivorship in tadpoles of *Scaphiopus holbrookii*. *Ecology* 63, 905–911. doi: 10.2307/1937230

Shoop, C. R. (1974). Yearly variation in larval survival of *Ambystoma maculatum*. *Ecology* 55, 440–444. doi: 10.2307/1935233

Stephens, J. P., Stoler, A. B., Sckrabulis, J. P., Fetzer, A. J., Berven, K. A., Tiegs, S. D., et al. (2016). Ontogenetic changes in sensitivity to nutrient limitation of tadpole growth. *Oecologia* 183, 263–273. doi: 10.1007/s00442-016-3746-7

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: the Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Subalusky, A. L., Dutton, C. L., and Rosi-Marshall, E. J. (2015). The hippopotamus conveyor belt: vectors of carbon and nutrients from terrestrial grasslands to aquatic systems in sub-Saharan Africa. *Freshw. Biol.* 60, 512–525. doi: 10.1111/fwb.12474

Vanni, M. J. (2002). Nutrient cycling by animals in freshwater ecosystems. *Annu. Rev. Ecol. Syst.* 33, 341–370. doi: 10.1146/annurev.ecolsys.33.010802.150519

Werner, E. E. (1986). Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. *Am. Nat.* 128, 319–341. doi: 10.1086/284565

Wilbur, H. M. (1977). Density-dependent aspects of growth and metamorphosis in Bufo americanus. *Ecology* 58, 196–200. doi: 10.2307/1935122

Wilbur, H. M., Morin, P. J., and Harris, R. N. (1983). Salamander predation and the structure of experimental communities: anuran responses. *Ecology* 64, 1423–1429. doi: 10.2307/1937496

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

PERSPECTIVE
published: 15 December 2016
doi: 10.3389/fevo.2016.00138

Pollen Stoichiometry May Influence Detrital Terrestrial and Aquatic Food Webs

Michał Filipiak *

Ecosystem Ecology Group, Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland

Pollen rains may temporally mitigate nutritional limitations experienced by terrestrial and aquatic detritivores by supplying stoichiometrically balanced food during periods of detritivore growth and development (spring-summer). This may affect the functioning of food webs and thus influence fundamental processes, e.g., by enabling fungi to decompose nutritionally scarce litter. Nutritional limitation may be studied within the framework of ecological stoichiometry by comparing the stoichiometric mismatches experienced by organisms feeding on various foods. To this end, the elemental compositions of pine pollen, litter and detritivores (fungi, protozoans, worms, insects, mites, millipedes, isopods and slugs) were compared, as were the stoichiometric mismatches experienced by the detritivores feeding on litter and pollen. Additionally, the contribution of pollen to the nutrient flow from the land to aquatic ecosystems was estimated through a literature review. Compared to litter, pine pollen is a stoichiometrically well-balanced food source in terms of its *C:N:P* ratio but also because of its high concentrations of K, S, and Cu and its favorable *Zn:Fe* ratio. This characteristic is especially suitable to fungi, which may be responsible for the redistribution of pollen-derived nutrients in food webs, particularly aquatic ones. Pollen rains of various plant species act as temporal pulses of nutrients that are rapidly utilized and quickly introduced into the food web, so calculations of annual biomass input may be misleading. Pollen is an easily available, digestible and nutritious food for fungi, bacteria, protozoans, and various groups of invertebrates, which suggests that pollen plays an important role in within- and cross-ecosystem nutrient cycling.

Keywords: ecological stoichiometry, food chain, nutritional ecology, ecosystem ecology, trophic ecology, trophic interactions, nitrogen, phosphorous

OPEN ACCESS

Edited by:
James Joseph Elser,
University of Montana, USA

Reviewed by:
Shawn M. Wilder,
Oklahoma State University, USA
Jessica R. Corman,
University of Wisconsin-Madison, USA
Angelica L. Gonzalez,
Rutgers University, USA

***Correspondence:**
Michał Filipiak
michal0filipiak@gmail.com,
michal.filipiak@uj.edu.pl

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *10 August 2016*
Accepted: *30 November 2016*
Published: *15 December 2016*

Citation:
Filipiak M (2016) Pollen Stoichiometry May Influence Detrital Terrestrial and Aquatic Food Webs. Front. Ecol. Evol. 4:138. doi: 10.3389/fevo.2016.00138

BACKGROUND

For organisms feeding on plant materials, the proportion of non-C elements in their food may be more limiting than energy (Pokarzhevskii et al., 2003; Moe et al., 2005) and diet supplementation with high-quality resources may promote the development of animals feeding on dead plant matter (Filipiak and Weiner, 2014, 2016; Filipiak et al., 2016; Horvathova et al., 2016). Hence, it might be advantageous to include in the diet plant matter that is nutritive, easily available in considerable mass and relatively easily digestible, i.e., pollen. Indeed, it was suggested that, in detrital food webs, litter-decomposing fungi are colimited by the scarcity of N, P, and S in litter, which they mitigate by foraging on pollen and which allows them to complete litter decomposition

(Stark, 1972; Staaf and Berg, 1982; Hutchison and Barron, 1997). Forests may produce pollen in masses reaching 100 to 1000 kg/ha (Greenfield, 1996), and the bulk of this mass is deposited onto forest floors and in lakes (Richerson et al., 1970; Proctor et al., 1996; Shumilovskikh et al., 2015), thus increasing the productivity of the ecosystem (Graham et al., 2006; Masclaux et al., 2013). The present study considers the ecological stoichiometry framework to relate data on pollen nutritional quality to the role of pollen in nutrient cycling within and between terrestrial and aquatic ecosystems.

Pollen Consumption and Digestibility

The extracellular walls of pollen are difficult to destroy chemically (Brooks et al., 1970), but they can be destroyed through mechanical disruption (crushing by chewing) or osmotic shock, which does not require special adaptations, to make pollen digestible (but see Franchi et al., 1997; Greenfield, 1999; Roulston and Cane, 2000; Johnson and Nicolson, 2001). Bacteria and fungi, however, are able to chemically destroy pollen walls (Brooks et al., 1970; Bradley, 2015; Shumilovskikh et al., 2015) and increase the productivity of the ecosystem through the introduction of pollen-derived nutrients to the food web (Masclaux et al., 2011, 2013; Rösel et al., 2012). Pollen rapidly decomposes in both terrestrial and aquatic ecosystems, liberating large amounts of nutritionally rich matter soon after deposition (Greenfield, 1999; Cho et al., 2003; Webster et al., 2008; Rösel et al., 2012). Ponge (1991) observed pollen in the guts of potworms, earthworms and dipterans, and a wide array of other terrestrial and aquatic organisms has been reported to actively feed on pollen including detritivorous, herbivorous and predatory bacteria, fungi, plankton, insects, arachnids, worms, and gastropods (cf. Supplementary Table 1).

Pollen Deposition and Nutritional Quality

Generally, the amount of pollen deposited annually by various plants (grasses, trees, and herbs) in different ecosystems varies from several to hundreds of kg/ha, but the majority of studies are only concerned with pine pollen (Richerson et al., 1970; Stark, 1972; Doskey and Ugoagwu, 1989; Greenfield, 1996, 1999; Proctor et al., 1996; Lee et al., 1996a,b; Hicks, 1999; Perez-Moreno and Read, 2001; Cho et al., 2003; Lee and Booth, 2003; Graham et al., 2006; Shumilovskikh et al., 2015). Maggs (1985) showed that pollen constitutes 3.5% of the total mass of the yearly slash pine biomass fall (litterfall plus pollen rain) but accounts for up to 30% of the total amount of deposited N, P, and K, so one could compare the general patterns of mass and nutrient inputs from pollen and litter. In forests, the annual inputs of individual elements from pine pollen rain can reach approximately 0.3–0.5 kg/ha N; 0.04–0.07 kg/ha P; 0.1–0.2 kg/ha K; 0.02 kg/ha S; and 0.01 kg/ha Mg (Lee et al., 1996a; Cho et al., 2003; Lee and Booth, 2003). Read and Perez-Moreno (2003) estimated the annual N and P deposition from pollen in forests as 1.6 and 0.32 kg/ha, respectively, while Webster et al. (2008) estimated the yearly N input from various pollens into soils as 20 kg/ha. Ukonmaanaho et al. (2008) reported mean annual inputs of 9 elements through litterfall; in pine stands, the means (kg/ha) were N: 6.44–23.67, P: 0.19–1.92, K: 1.23–4.39, S: 0.47–0.98, and Mg: 0.56–1.61 and for spruce stands: N: 4.94–58.51, P: 0.55–5.25, K: 0.84–17.10, S: 0.42–4.66, and Mg: 0.51–5.35. The authors also suggested a range of 600–5000 kg/ha of yearly litter production in boreal forests. Bray and Gorham (1964) concluded that annual forest litter production is approximately 1 t/ha in arctic and alpine zones, 3.5–5.5 t/ha in temperate zones and 11 t/ha in equatorial zones. Thus, in forests, the contribution of the pollen mass to the total biomass fall is estimated to be 1–10%, but the contribution of pollen to the total fall of non-C elements should be several-fold higher and may reach 5–50%, because pollen is rich in non-C elements especially if considering P. The N content in the pollen of various plants may reach approximately 0.4–10%, most commonly 2–4%, while the P content may reach 0.05–0.7%, most commonly 0.2–0.5% (Todd and Bretherick, 1942; Nielsen et al., 1955; Stanley and Linskens, 1974; Roulston and Cane, 2000). These values are high compared to those of other plant tissues (e.g., Güsewell, 2004; Marshner, 2012) and especially high compared with those of plant litter (Berg and McClaugherty, 2014). Ignoring pollen outputs, the quality of the matter produced may be more important than its quantity in terms of the biomass flow that is relevant to consumers and ecosystems (Marcarelli et al., 2011; Sitters et al., 2015; Mehner et al., 2016). Due to the high concentrations of non-C elements, the pollen of various taxa may be hypothesized to be stoichiometrically well-balanced, i.e., good quality food for herbivores and detritivores. This would be important since pollen rains affect a wide array of habitats and are produced by a variety of plants, including trees (Betulaceae, Corylaceae, Fagaceae, Salicaceae, Ulmaceae, Oleaceae, Sapindaceae) and herbs, those of which make the greatest contribution to the pollen rain are grasses (Poaceae, also cereals), sedges (Cyperaceae), rushes (Juncaceae), plantains (Plantaginaceae), docks (Polygonaceae), goosefoots (Chenopodiaceae), nettles (Urticaceae), and Asteraceae (including ragweed) as well as many others (Proctor et al., 1996). The total annual pollen production of a ruderal ecosystem was estimated to be 45–590 kg/ha, including species that produce pollen rains (Denisow, 2011), but the amount of pollen that is deposited on the floor of such a habitat is unknown.

Although most pollen remains in the area where it was produced (Koski, 1970), a portion can be moved over distances of hundreds (Proctor et al., 1996; Sitters et al., 2015) or even thousands of kilometers (Campbell et al., 1999), translocating nutrients between ecosystems. The yearly deposition of pollen from a single species into lakes was estimated to range from several to hundreds of kilograms, while the translocation of P in pine pollen from the land to a lake may reach approximately 0.1–10 kg/ha per year, which accounts for half of the yearly external P input for a small oligotrophic lake (Doskey and Ugoagwu, 1989; Cole et al., 1990; Banks and Nighswander, 2000; Graham et al., 2006; Rösel et al., 2012). This input, even if insignificant in terms of annual mass, is short term, and pollen decomposes rapidly, releasing nutrients within a few days of incubation in lake water (Rösel et al., 2012). Therefore, pollen rain may act as a considerable, temporally limited pulse of nutrients. Pollen rains of various species supply food webs with nutritional elements from early spring to late summer, the time when high

amounts of these elements are needed to build the bodies of the developing biota (Roulston and Cane, 2000; Beckman and Hurd, 2003; Lundgren, 2009; Wilder, 2011; Eggs and Sanders, 2013). Thus, existing calculations of the nutritional supplementation of ecosystems provided by pollen rain annually may be misleading. Yet, there exist species producing pollen in fall (e.g., Anderson and Hill, 2002).

POLLEN IN DETRITAL FOOD WEBS

Pine pollen and pine litter have been studied in sufficient depth to provide data that are appropriate for a comparative analysis of their elemental compositions in various pine species distributed worldwide. To that end, to comprehensively determine how pollen stoichiometry influences the nutritional mismatches experienced by detritivores, the pine forest ecosystem was considered as an exemplary food web in the present study. To determine whether pollen flux may promote the development of detritivores, the elemental compositions and stoichiometry of pollen, litter and detritivores were compared. The available data on the elemental composition of pine litter and pollen of various pine species inhabiting different forests worldwide were collected (details in Supplementary Table 2).

A comparison of the elemental composition of pine pollen with that of litter (**Table 1**) demonstrated that the C concentration in pollen is similar to that in litter; the concentrations of N, P, S, K, Mg, and Cu are approximately 2- to 12-fold higher in pollen; and concentrations of Ca, Mn, and Fe are approximately 3- to 19-fold higher in litter. As a detritivore food source, pollen stoichiometry is advantageous compared with that of litter, particularly the *C:P* ratio (12-fold lower in pollen). P-rich pine pollen is deposited in the spring, when young detritivores develop and have the greatest need for P and the highest vulnerability to suboptimal diets during their ontogeny (Bullejos et al., 2014). Thus, feeding on pine pollen may benefit detritivore development. Other elements supplied to a high degree by pine pollen include K and S. Together, these three elements may be limiting for fungi exploiting pine litter (Staaf and Berg, 1982), so pollen might be a particularly important resource for these organisms. Indeed, the utilization of pollen by forest fungi has been reported previously (Stark, 1972; Hutchison and Barron, 1997; Perez-Moreno and Read, 2001).

Nutritionally scarce food is stoichiometrically unbalanced, which is reflected in a stoichiometric mismatch (differences in the concentrations of elements in the food and in the body of the consumer) that limits consumer growth and development (Sterner and Elser, 2002; Denno and Fagan, 2003; Fagan and Denno, 2004; Hessen et al., 2013). To detect potential stoichiometric mismatches and their negative consequences (development limitation), simple comparisons of element ratios are sufficient (Filipiak and Weiner, 2016), so the stoichiometric ratios in food and in the bodies of consumers ($C{:}X_{food}/C{:}X_{consumer}$, where C is the carbon concentration and X is the concentration of the other element) were calculated to comprehensively detect and compare the stoichiometric mismatches that reflect the variety of detritivores consuming either litter or pollen. Hereafter, these values will be referred to as trophic stoichiometric ratios (*TSRs*, cf. Filipiak and Weiner, 2016). To this end, data from the literature (means) and newly collected data on the elemental composition of detritivores inhabiting forest litters and soils (means) were used (details in Supplementary Tables 2–4).

Five elements (P, K, N, S, and Cu; **Figure 1**) were found to be the most limiting to detritivore development. The *TSRs* calculated for feeding on pine pollen were approximately 10-fold lower than those for feeding on pine litter for P, K, N, and S and 2-times lower for Cu; the mitigating effect was strongest for P and K. The pollen diet might be particularly advantageous for isopods, millipedes and fungi because these organisms have the greatest need for K, P, and S supplementation (**Figure 1**). Pine pollen might be an excellent source of nutrients for terrestrial detritivores, mitigating stoichiometric mismatches, thereby it might promote detritivore growth and development.

THE CONTRIBUTION OF POLLEN TO THE NUTRIENT FLOW FROM LAND TO AQUATIC ECOSYSTEMS

Pollen has been suggested to be an important source of nutrients for aquatic organisms and a factor that influences nutrient cycling in aquatic ecosystems (Masclaux et al., 2011; Rösel et al., 2012), and the stoichiometric characteristics of pollen are consistent with this suggestion. Pine pollen *C:N:P* stoichiometry is more similar to that of freshwater than terrestrial autotrophs; i.e., pollen is relatively more stoichiometrically balanced for aquatic consumers compared with other types of plant matter (Elser et al., 2000). Pine pollen has markedly lower *C:P* and *N:P* ratios compared with the other matter that flows in large masses from terrestrial to aquatic ecosystems, e.g., foliage (Elser et al., 2000). This may promote the growth of P-limited aquatic detritivores (Sterner and Hessen, 1994; Sterner and Elser, 2002; Sardans et al., 2012). It is possible that pollen does not act as a direct source of nutrients for aquatic herbivores but is first utilized by aquatic fungi, consequently increasing the productivity of the entire system (Masclaux et al., 2011, 2013).

In aquatic food webs, fungal action may be more important to the introduction of pollen-derived nutrients than in terrestrial ecosystems. Although pollen is nutritive and digestible, recent studies have shown that pollen alone is insufficient to promote zooplankton growth (Masclaux et al., 2011, 2013), suggesting that pollen walls are highly resistant to the action of zooplankton and that saprotrophic fungi are responsible for weakening and degrading these walls, thereby introducing pollen-derived nutrients into the food web and boosting ecosystem productivity (Goldstein, 1960; Masclaux et al., 2011, 2013; Rösel et al., 2012; Wurzbacher et al., 2014). Pine pollen is an attractive source of nutrients for fungi due to its *C:N:P* ratio, but it may also provide other limiting elements, especially K, S, and Cu (**Figure 1**).

Knowledge of the ecological stoichiometry of aquatic fungi is lacking, and specific predictions or comparisons with food stoichiometry are currently impossible (Danger et al., 2016). The limited data on elemental compositions are either for

TABLE 1 | Dry mass element concentrations and stoichiometry of pine pollen compared with pine litter.

		% of an element in dry mass							ppm of an element in dry mass				Atomic ratios			
		C	N	P	S	K	Mg	Ca	Fe	Zn	Mn	Cu		*C:N*	*C:P*	*N:P*
Pine litter	Mean	52.40	0.42	0.03	0.05	0.10	0.06	0.57	115.06	60.13	1328.06	2.78	**Mean**	145	4725	33
	Median	52.50	0.41	0.02	0.04	0.08	0.05	0.56	79.00	50.50	1335.00	2.60	**Lowest possible**	78	2026	9
	SD	2.26	0.10	0.01	0.02	0.05	0.03	0.16	93.49	28.62	605.93	1.27	**Highest possible**	260	10,258	115
	CV	0.04	0.23	0.37	0.37	0.56	0.42	0.28	0.81	0.48	0.46	0.46				
	Min	48.70	0.25	0.01	0.03	0.03	0.03	0.05	53.00	39.60	260.00	1.40				
	Max	55.69	0.73	0.06	0.10	0.24	0.15	0.87	300.00	144.12	3670.00	5.00				
	N	9	49	42	16	40	40	41	11	12	32	9				
Pine pollen	Mean	50.79	2.24	0.34	0.26	0.94	0.15	0.05	35.82	48.58	70.33	5.70	**Mean**	26	386	15
	Median	50.70	2.26	0.32	0.24	0.97	0.09	0.03	34.00	54.00	69.00	4.90	**Lowest possible**	17	246	5
	SD	1.42	0.46	0.10	0.14	0.43	0.18	0.08	17.63	16.34	23.56	1.77	**Highest possible**	53	795	44
	CV	0.03	0.20	0.29	0.56	0.45	1.24	1.57	0.49	0.34	0.33	0.31				
	Min	49.70	1.17	0.17	0.08	0.33	0.08	0.01	10.00	25.20	38.00	4.50				
	Max	53.16	3.43	0.52	0.78	2.52	0.85	0.33	65.00	75.00	100.00	10.00				
	N	5	37	24	17	29	18	14	11	9	9	9				
Mean pollen/litter ratio		**1**	**5**	**12**	**5**	**10**	**2**	**0**	**0**	**1**	**0**	**2**	**Mean litter/pollen ratio**	**5**	**12**	**2**

Various pine species inhabiting different forests worldwide were considered (details in Supplementary Table 2). The pollen/litter ratio indicates that the element concentration in pollen is x-fold higher than in litter, and the litter/pollen ratio indicates that the stoichiometric atomic ratio of litter is x-fold higher than that of pollen. The ranges in the atomic ratios were calculated using the minimum and maximum reported element concentrations as follows: lowest X:Y, minX/maxY; highest X:Y, maxX/minY. Compared with litter, pollen has high concentrations of P, K, S, and N. The atomic C:N:P ratios in pollen are relatively low, particularly the C:P ratio which is more than 10-fold lower in pollen than in litter.

terrestrial fungi consumed by humans and consider only a finite number of elements, excluding C (Rudawska and Leski, 2005; Dursun et al., 2006), or are related to fungal strains cultured in laboratories on artificial media, which are not relevant to natural situations (Mouginot et al., 2014). A study on the mineral requirements of aquatic fungi, which covered other elements besides C, N, and P (Schoenlein-Crusius et al., 1999), showed that aquatic hyphomycetes may be sensitive to the stoichiometry of nutrients, specifically the contents of Ca, S, K, Mg, Mn, Na, and Zn (positively correlated) and Fe and Al (negatively correlated). Among these elements, S and K are highly concentrated in pine pollen (**Table 1**), and the positive effect of Zn and the negative effect of Fe on fungi might be associated with competition among these elements for absorption sites, as the excess of one could induce a deficiency of the other. This reported effect occurred with food with a low *Zn:Fe* atomic ratio (approximately 0.01–0.007); the mean *Zn:Fe* atomic ratio in pine pollen is 1.2, thus neutral and 24-fold higher than that in pine litter and thus relatively favorable. Pine pollen is also relatively high in Cu, another element that is rich in fungal tissues.

UNDERSTANDING THE ROLE OF POLLEN IN THE FLOW OF NUTRITIONAL ELEMENTS WITHIN AND BETWEEN ECOSYSTEMS

Nutrients that are incorporated into the ecosystem with pollen rain might either be directly utilized by invertebrates, thus mitigating their stoichiometric mismatches (**Figure 1**), or introduced into the food web via microorganisms (mainly fungi). Pollen on the forest floor decomposes rapidly, losing approximately 20–70% of its initial mass in a month solely as a result of microbial action (Greenfield, 1999; Webster et al., 2008). The majority of the water-extractable macronutrients in pollen (more than 80%) can leach within a few hours in both land (Lee et al., 1996a) and water (Rösel et al., 2012) ecosystems. These nutrients might subsequently be incorporated, redistributed and recycled by microorganisms (Stark, 1972; Hutchison and Barron, 1997; Davidson et al., 1999; Perez-Moreno and Read, 2001; Van Mourik, 2003).

The contribution of pollen to litter decomposition is underrated. It was reported that the N, P, and S supplied by pine pollen to the forest floor enabled fungi to decompose nutritionally scarce pine litter (Stark, 1972; Staaf and Berg, 1982; Hutchison and Barron, 1997), but pine pollen may be even more important, supplying decomposers with sufficient amounts of K and Cu and having a desirable *Zn:Fe* atomic ratio.

Data on the elemental content of pollen are scarce. Surprisingly, data concerning the concentration of C in litter, pollen and detritivores are also scarce, so studies of particular food webs that utilize a set of elements and allow *C:X* ratios to be calculated are needed to understand the role of pollen in nutrient cycling. These studies should move beyond traditional *C:N:P* stoichiometry and incorporate other physiologically important elements, of which K, S, Zn, Fe, and Cu might be the most important.

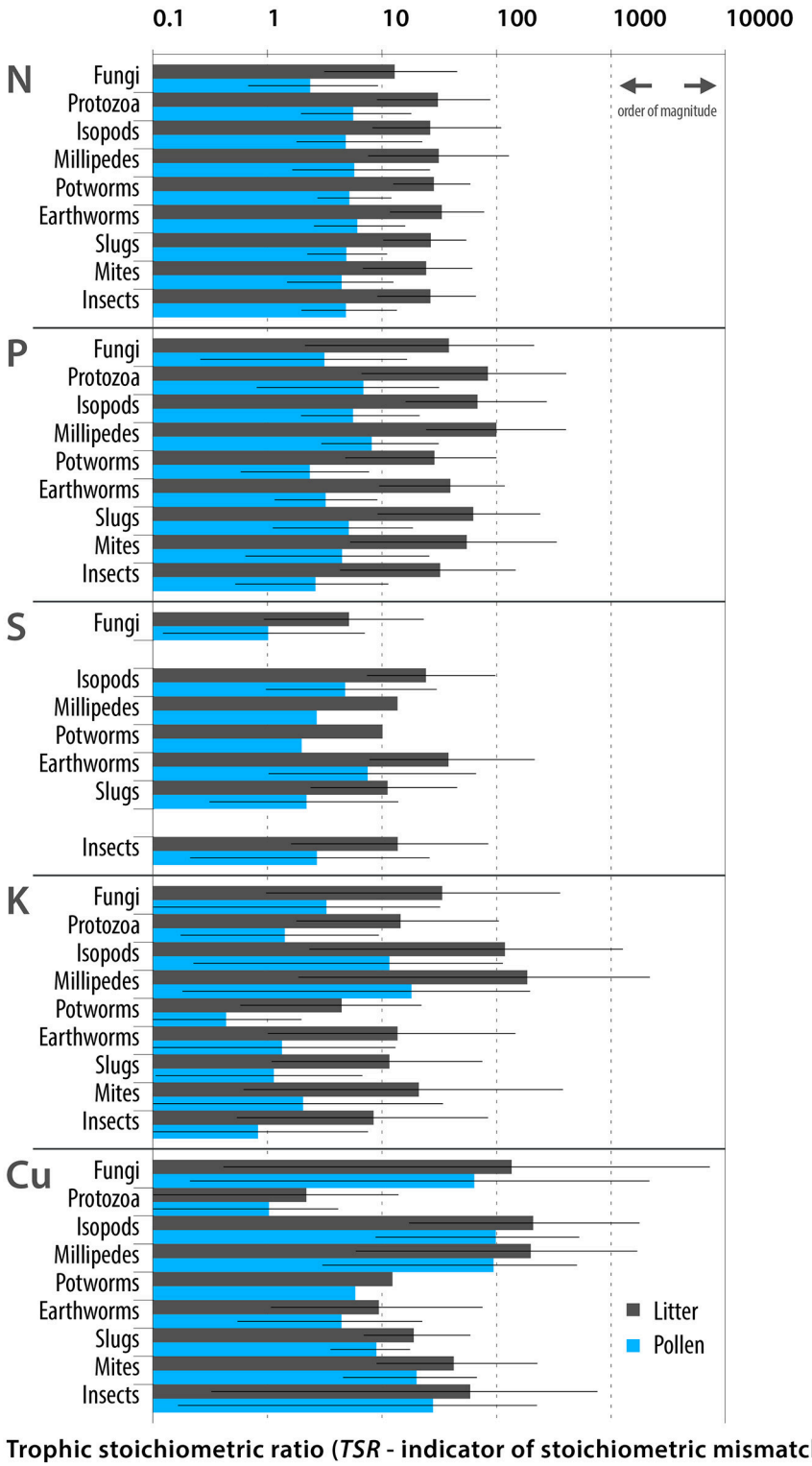

FIGURE 1 | Stoichiometric mismatches (*TSRs*) calculated for detritivorous soil and litter dwellers that may utilize pollen as a supplementary food. *TSR* values were calculated for the two sources of food: pine litter and pine pollen. Bars denote means; whiskers denote minima and maxima. Various pine species inhabiting different forests in diverse locations were considered. The scale of the Y-axis is logarithmic. After lowering any mismatches by approximately one order of magnitude, feeding on pollen mitigated N, P, S, and K nutritional mismatches in a variety of organisms.

Even if the annual contribution of pollen-derived nutrients is not significant in terms of the mass of the elements input to the ecosystem, pollen rains may act as a temporal pulse of nutrients. Pollen is cycled through ecosystems during short periods from early spring (primarily from trees) to late summer (primarily from herbs; Lee et al., 1996a; Proctor et al., 1996; Cho et al., 2003). Cole et al. (1990) claimed that summer pollen rains do not contribute to enriching aquatic ecosystems, but data show that spring pollen rains dominated by anemophilous trees relocate considerable amounts of nutrients from terrestrial to aquatic ecosystems (Graham et al., 2006; Rösel et al., 2012). Rösel et al. (2012) suggested that algal blooms may be connected with short-duration but massive pollen deposition from trees. The present study indicates that pine pollen stoichiometry and the ability of pollen rains to rapidly introduce nutrients into food webs makes pollen an ideal agent for triggering algal-blooms.

Future studies could (1) track the pathways of pollen-derived nutrients within and between ecosystems, (2) undertake feeding experiments that supplement the diets of detritivores with various amounts of pollen and evaluate their physiological responses and life history traits (e.g., growth rate, larval development time, size at maturity, assimilation rates of limiting elements, etc.), (3) experimentally manipulate natural food webs by preventing pollen deposition during pollen rain periods and observing the impact on ecosystem productivity. Also needed are studies of the seasonal variations in pollen stoichiometry and deposition that consider various pollen species in different ecosystems. In the case of aquatic ecosystems, the factors affecting the quantity of deposited pollen (e.g., surrounding flora, length of shoreline/lake area ratio, distance from the shore, etc.) should be acknowledged as well as the potential power of a pollen enrichment effect (e.g., trophic state of the pollen-receiving ecosystem). Further suggestions for merging ecological stoichiometry and cross-ecosystem material flows in a spatial context were presented by Sitters et al. (2015).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

FUNDING

This study was supported by grants from the Polish Ministry of Science and Higher Education (Grant No. DS/WBiNoZ/INoŚ/DS 756) and the National Science Centre (Grant No. DEC-2013/11/N/NZ8/00929).

ACKNOWLEDGMENTS

The author is indebted to Zuzanna Świątek and January Weiner for their constructive critical comments and thanks American Journal Experts (AJE) for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00138/full#supplementary-material

REFERENCES

Anderson, G. J., and Hill, J. D. (2002). Many to flower, few to fruit: the reproductive biology of *Hamamelis virginiana* (*Hamamelidaceae*). *Am. J. Bot.* 89, 67–78. doi: 10.3732/ajb.89.1.67

Banks, H. H., and Nighswander, J. E. (2000). "Relative contribution of hemlock pollen to the phosphorus loading of the Clear Lake Ecosystem near Minden, Ontario," in *Proceedings: Symposium on Sustainable Management of Hemlock Ecosystems in Eastern North America*, eds K. A. McManus, K. S. Shields, and D. Souto (Delaware, OH: U.S.D.A. Forest Service), 167–174.

Beckman, N., and Hurd, L. E. (2003). Pollen feeding and fitness in praying mantids: the vegetarian side of a tritrophic predator. *Environ. Entomol.* 32, 881–885. doi: 10.1603/0046-225X-32.4.881

Berg, B., and McClaugherty, C. (2014). *Plant Litter*. Berlin, Heidelberg: Springer.

Bradley, R. S. (ed.). (2015). "Pollen," in *Paleoclimatology* (Oxford: Elsevier), 405–451.

Bray, J. R., and Gorham, E. (1964). Litter production in forests of the world. *Adv. Ecol. Res.* 2, 101–157. doi: 10.1016/S0065-2504(08)60331-1

Brooks, J., Grant, P. R., and Muir, M., (eds.). (1970). "Sporopollenin," in *Proceedings of a Symposium Held at the Geology Department*, *Imperial College*, (London: Elsevier Ltd).

Bullejos, F. J., Carrillo, P., Gorokhova, E., Medina-Sánchez, J. M., Balseiro, E. G., and Villar-Argaiz, M. (2014). Shifts in food quality for herbivorous consumer growth: multiple golden means in the life history. *Ecology* 95, 1272–1284. doi: 10.1890/13-0410.1

Campbell, I. D., McDonald, K., Flannigan, M. D., and Kringayark, J. (1999). Long-distance transport of pollen into the Arctic. *Nature* 399, 29–30. doi: 10.1038/19891

Cho, Y.-J., Sung Kim, I., Kim, P., and Ju Lee, E. (2003). Deposition of airborne pine pollen in a temperate pine forest. *Grana* 42, 178–182. doi: 10.1080/00173130310016158

Cole, J. J., Caraco, N. F., and Likens, G. E. (1990). Short-range atmospheric transport: a significant source of phosphorus to an oligotrophic lake. *Limnol. Oceanogr.* 35, 1230–1237. doi: 10.4319/lo.1990.35.6.1230

Danger, M., Gessner, M. O., and Bärlocher, F. (2016). Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. *Fungal Ecol.* 19, 100–111. doi: 10.1016/j.funeco.2015.09.004

Davidson, D. A., Carter, S., Boag, B., Long, D., Tipping, R., and Tyler, A. (1999). Analysis of pollen in soils: processes of incorporation and redistribution of pollen in five soil profile types. *Soil Biol. Biochem.* 31, 643–653. doi: 10.1016/S0038-0717(98)00123-0

Denisow, B. (2011). *Pollen Production of Selected Ruderal Plant Species in the Lublin Area*. Lublin: University of Life Sciences in Lublin Press [Wydawnictwo Uniwersytetu Przyrodniczego].

Denno, R. F., and Fagan, W. F. (2003). Might nitrogen limitation promote omnivory among carnivorous arthropods? *Ecology* 84, 2522–2531. doi: 10.1890/02-0370

Doskey, P. V., and Ugoagwu, B. J. (1989). Atmospheric deposition of macronutrients by pollen at a semi-remote site in northern Wisconsin. *Atmos. Environ.* 23, 2761–2766. doi: 10.1016/0004-6981(89)90556-8

Dursun, N., Özcan, M. M., Kaşık, G., and Öztürk, C. (2006). Mineral contents of 34 species of edible mushrooms growing wild in Turkey. *J. Sci. Food Agric.* 86, 1087–1094. doi: 10.1002/jsfa.2462

Eggs, B., and Sanders, D. (2013). Herbivory in spiders: the importance of pollen for orb-weavers. *PLoS ONE* 8:e82637. doi: 10.1371/journal.pone.0082637

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580. doi: 10.1038/35046058

Fagan, W. F., and Denno, R. F. (2004). Stoichiometry of actual vs. potential predator-prey interactions: insights into nitrogen limitation for arthropod predators. *Ecol. Lett.* 7, 876–883. doi: 10.1111/j.1461-0248.2004.00641.x

Filipiak, M., Sobczyk, Ł., and Weiner, J. (2016). Fungal transformation of tree stumps into a suitable resource for xylophagous beetles via changes in elemental ratios. *Insects* 7:13. doi: 10.3390/insects7020013

Filipiak, M., and Weiner, J. (2014). How to make a beetle out of wood: multi-elemental stoichiometry of wood decay, xylophagy and fungivory. *PLoS ONE* 9:e115104. doi: 10.1371/journal.pone.0115104

Filipiak, M., and Weiner, J. (2016). Nutritional dynamics during the development of xylophagous beetles related to changes in the stoichiometry of 11 elements. *Physiol. Entomol.* doi: 10.1111/phen.12168. [Epub ahead of print].

Franchi, G. G., Franchi, G., Corti, P., and Pompella, A. (1997). Microspectrophotometric evaluation of digestibility of pollen grains. *Plant Foods Hum. Nutr.* 50, 115–126. doi: 10.1007/BF02436031

Goldstein, S. (1960). Degradation of pollen by phycomycetes. *Ecology* 41, 543–545. doi: 10.2307/1933329

Graham, M. D., Vinebrooke, R. D., and Turner, M. (2006). Coupling of boreal forests and lakes: effects of conifer pollen on littoral communities. *Limnol. Oceanogr.* 51, 1524–1529. doi: 10.4319/lo.2006.51.3.1524

Greenfield, L. (1996). Plant pollen production in selected tree species. *Canterbury Bot. Soc. J.* 31, 10–13.

Greenfield, L. (1999). Weight loss and release of mineral nitrogen from decomposing pollen. *Soil Biol. Biochem.* 31, 353–361. doi: 10.1016/S0038-0717(98)00134-5

Güsewell, S. (2004). N : p ratios in terrestrial plants: variation and functional significance. *New Phytol.* 164, 243–266. doi: 10.1111/j.1469-8137.2004.01192.x

Hessen, D. O., Elser, J. J., Sterner, R. W., and Urabe, J. (2013). Ecological stoichiometry: an elementary approach using basic principles. *Limnol. Oceanogr.* 58, 2219–2236. doi: 10.4319/lo.2013.58.6.2219

Hicks, S. (1999). The relationship between climate and annual pollen deposition at northern tree-lines. *Chemosph. Glob. Change Sci.* 1, 403–416. doi: 10.1016/S1465-9972(99)00043-4

Horvathova, T., Babik, W., and Bauchinger, U. (2016). Biofilm feeding: microbial colonization of food promotes the growth of a detritivorous arthropod. *Zookeys* 577, 25–41. doi: 10.3897/zookeys.577.6149

Hutchison, L. J., and Barron, G. L. (1997). Parasitism of pollen as a nutritional source for lignicolous Basidiomycota and other fungi. *Mycol. Res.* 101, 191–194. doi: 10.1017/S095375629600233X

Johnson, S. A., and Nicolson, S. W. (2001). Pollen digestion by flower-feeding Scarabaeidae: protea beetles (Cetoniini) and monkey beetles (Hopliini). *J. Insect Physiol.* 47, 725–733. doi: 10.1016/S0022-1910(00)00166-9

Koski, V. (1970). A study of pollen dispersal as a mechanism of gene flow in conifers. *Commun. Inst. For. Fenn.* 70, 1–78.

Lee, E. J., and Booth, T. (2003). Macronutrient input from pollen in two regenerating pine stands in southeast Korea. *Ecol. Res.* 18, 423–430. doi: 10.1046/j.1440-1703.2003.00566.x

Lee, E. J., Kenkel, N., and Booth, T. (1996a). Atmospheric deposition of macronutrients by pollen in the boreal forest. *Ecoscience* 3, 304–309. doi: 10.1080/11956860.1996.11682347

Lee, E. J., Kenkel, N., and Booth, T. (1996b). Pollen deposition in the boreal forest of west-central Canada. *Can. J. Bot.* 74, 1265–1272. doi: 10.1139/b96-153

Lundgren, J. G. (2009). *Relationships of Natural Enemies and Non-Prey Foods*. Dordrecht: Springer Netherlands.

Maggs, J. (1985). Litter fall and retranslocation of nutrients in a refertilized and prescribed burned Pinus elliottii plantation. *For. Ecol. Manage.* 12, 253–268. doi: 10.1016/0378-1127(85)90094-5

Marcarelli, A. M., Baxter, C. V., Mineau, M. M., and Hall, R. O. (2011). Quantity and quality: unifying food web and ecosystem perspectives on the role of resource subsidies in freshwaters. *Ecology* 92, 1215–1225. doi: 10.1890/10-2240.1

Marshner, P. (2012). *Marschner's Mineral Nutrition of Higher Plants. 3rd Edn.* Oxford: Elsevier Ltd.

Masclaux, H., Bec, A., Kagami, M., Perga, M., Sime-Ngando, T., Desvilettes, C., et al. (2011). Food quality of anemophilous plant pollen for zooplankton. *Limnol. Oceanogr.* 56, 939–946. doi: 10.4319/lo.2011.56.3.0939

Masclaux, H., Perga, M.-E., Kagami, M., Desvilettes, C., Bourdier, G., and Bec, A. (2013). How pollen organic matter enters freshwater food webs. *Limnol. Oceanogr.* 58, 1185–1195. doi: 10.4319/lo.2013.58.4.1185

Mehner, T., Attermeyer, K., Brauns, M., Brothers, S., Diekmann, J., Gaedke, U., et al. (2016). Weak response of animal allochthony and production to enhanced supply of terrestrial leaf litter in nutrient-rich lakes. *Ecosystems* 19, 311–325. doi: 10.1007/s10021-015-9933-2

Moe, S. J., Stelzer, R. S., Forman, M. R., Harpole, W. S., Daufresne, T., and Yoshida, T. (2005). Recent advances in ecological stoichiometry: insights for population and community ecology. *Oikos* 109, 29–39. doi: 10.1111/j.0030-1299.2005.14056.x

Mouginot, C., Kawamura, R., Matulich, K. L., Berlemont, R., Allison, S. D., Amend, A. S., et al. (2014). Elemental stoichiometry of fungi and bacteria strains from grassland leaf litter. *Soil Biol. Biochem.* 76, 278–285. doi: 10.1016/j.soilbio.2014.05.011

Nielsen, N., Grömmer, J., and Lunden, R. (1955). Investigations on the chemical composition of pollen from some plants. *Acta Chem. Scand.* 9, 1100–1106. doi: 10.3891/acta.chem.scand.09-1100

Perez-Moreno, J., and Read, D. J. (2001). Exploitation of pollen by mycorrhizal mycelial systems with special reference to nutrient recycling in boreal forests. *Proc. R. Soc. B Biol. Sci.* 268, 1329–1335. doi: 10.1098/rspb.2001.1681

Pokarzhevskii, A. D., van Straalen, N. M., Zaboev, D. P., and Zaitsev, A. S. (2003). Microbial links and element flows in nested detrital food-webs. *Pedobiologia* 47, 213–224. doi: 10.1078/0031-4056-00185

Ponge, J. F. (1991). Food resources and diets of soil animals in a small area of Scots pine litter. *Geoderma* 49, 33–62. doi: 10.1016/0016-7061(91)90090-G

Proctor, M., Yeo, P., and Lack, A. (1996). *The Natural History of Pollination*. London: Harper Collins New Naturalist.

Read, D. J., and Perez-Moreno, J. (2003). Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance? *New Phytol.* 157, 475–492. doi: 10.1046/j.1469-8137.2003.00704.x

Richerson, P. J., Moshiri, G. A., and Godshalk, G. L. (1970). Certain ecological aspects of pollen dispersion in Lake Tahoe (California–Nevada). *Limnol. Oceanogr.* 15, 149–153. doi: 10.4319/lo.1970.15.1.0149

Rösel, S., Rychła, A., Wurzbacher, C., and Grossart, H. (2012). Effects of pollen leaching and microbial degradation on organic carbon and nutrient availability in lake water. *Aquat. Sci.* 74, 87–99. doi: 10.1007/s00027-011-0198-3

Roulston, T., and Cane, J. (2000). Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* 222, 187–209. doi: 10.1007/BF00984102

Rudawska, M., and Leski, T. (2005). Macro- and microelement contents in fruiting bodies of wild mushrooms from the Notecka forest in west-central Poland. *Food Chem.* 92, 499–506. doi: 10.1016/j.foodchem.2004.08.017

Sardans, J., Rivas-Ubach, A., and Pe-uelas, J. (2012). The elemental stoichiometry of aquatic and terrestrial ecosystems and its relationships with organismic lifestyle and ecosystem structure and function: a review and perspectives. *Biogeochemistry* 111, 1–39. doi: 10.1007/s10533-011-9640-9

Schoenlein-Crusius, I. H., Pires-Zottarelli, C. L. A., Milanez, A. I., and Humphreys, R. D. (1999). Interaction between the mineral content and the occurrence number of aquatic fungi in leaves submerged in a stream in the Atlantic rainforest, São Paulo, Brazil. *Rev. Bras. Botânica* 22, 133–139. doi: 10.1590/S0100-84041999000200004

Shumilovskikh, L. S., Schlütz, F., Achterberg, I., Kvitkina, A., Bauerochse, A., and Leuschner, H. H. (2015). Pollen as nutrient source in Holocene ombrotrophic bogs. *Rev. Palaeobot. Palynol.* 221, 171–178. doi: 10.1016/j.revpalbo.2015.07.001

Sitters, J., Atkinson, C. L., Guelzow, N., Kelly, P., and Sullivan, L. L. (2015). Spatial stoichiometry: cross-ecosystem material flows and their impact on recipient ecosystems and organisms. *Oikos* 124, 920–930. doi: 10.1111/oik.02392

Staaf, H., and Berg, B. (1982). Accumulation and release of plant nutrients in decomposing Scots pine needle litter. Long-term decomposition in a Scots pine forest II. *Can. J. Bot.* 60, 1561–1568. doi: 10.1139/b82-199

Stanley, R. G., and Linskens, H. F. (1974). *Pollen Biology Biochemistry Management*. Berlin, Heidelberg: Springer.

Stark, N. (1972). Nutrient cycling pathways and litter fungi. *Bioscience* 22, 355–360. doi: 10.2307/1296341

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Sterner, R. W., and Hessen, D. O. (1994). Algal nutrient limitation and the nutrition of aquatic herbivores. *Annu. Rev. Ecol. Syst.* 25, 1–29. doi: 10.1146/annurev.es.25.110194.000245

Todd, F. E., and Bretherick, O. (1942). The composition of pollens. *J. Econ. Entomol.* 35, 312–317. doi: 10.1093/jee/35.3.312

Ukonmaanaho, L., Merilä, P., Nöjd, P., and Nieminen, T. M. (2008). Litterfall production and nutrient return to the forest floor in Scots pine and Norway spruce stands in Finland. *Boreal Environ. Res.* 13, 67–91.

Van Mourik, J. M. (2003). Life cycle of pollen grains in mormoder humus forms of young acid forest soils: a micromorphological approach. *CATENA* 54, 651–663. doi: 10.1016/S0341-8162(03)00116-4

Webster, E. A., Tilston, E. L., Chudek, J. A., and Hopkins, D. W. (2008). Decomposition in soil and chemical characteristics of pollen. *Eur. J. Soil Sci.* 59, 551–558. doi: 10.1111/j.1365-2389.2008.01022.x

Wilder, S. M. (2011). Spider nutrition: an integrative perspective. *Adv. Insect Physiol.* 40, 87–136. doi: 10.1016/B978-0-12-387668-3.00002-7

Wurzbacher, C., Rösel, S., Rychła, A., and Grossart, H.-P. (2014). Importance of Saprotrophic freshwater fungi for pollen degradation. *PLoS ONE* 9:e94643. doi: 10.1371/journal.pone.0094643

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 29 June 2017
doi: 10.3389/fmicb.2017.01184

Comparing the Ecological Stoichiometry in Green and Brown Food Webs – A Review and Meta-analysis of Freshwater Food Webs

Michelle A. Evans-White[1*†] *and Halvor M. Halvorson*[2†]

[1] *Department of Biological Sciences, University of Arkansas, Fayetteville, AR, United States,* [2] *Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS, United States*

OPEN ACCESS

Edited by:
Dedmer B. Van de Waal, Netherlands Institute of Ecology (NIOO-KNAW), Netherlands

Reviewed by:
Aleksandra M. Lewandowska, University of Oldenburg, Germany
Judith Sitters, Vrije Universiteit Brussel, Belgium

***Correspondence:**
Michelle A. Evans-White
mevanswh@gmail.com

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *02 March 2017*
Accepted: *12 June 2017*
Published: *29 June 2017*

Citation:
Evans-White MA and Halvorson HM (2017) Comparing the Ecological Stoichiometry in Green and Brown Food Webs – A Review and Meta-analysis of Freshwater Food Webs. Front. Microbiol. 8:1184. doi: 10.3389/fmicb.2017.01184

The framework of ecological stoichiometry was developed primarily within the context of "green" autotroph-based food webs. While stoichiometric principles also apply in "brown" detritus-based systems, these systems have been historically understudied and differ from green ones in several important aspects including carbon (C) quality and the nutrient [nitrogen (N) and phosphorus (P)] contents of food resources for consumers. In this paper, we review work over the last decade that has advanced the application of ecological stoichiometry from green to brown food webs, focusing on freshwater ecosystems. We first review three focal areas where green and brown food webs differ: (1) bottom–up controls by light and nutrient availability, (2) stoichiometric constraints on consumer growth and nutritional regulation, and (3) patterns in consumer-driven nutrient dynamics. Our review highlights the need for further study of how light and nutrient availability affect autotroph–heterotroph interactions on detritus and the subsequent effects on consumer feeding and growth. To complement this conceptual review, we formally quantified differences in stoichiometric principles between green and brown food webs using a meta-analysis across feeding studies of freshwater benthic invertebrates. From 257 datasets collated across 46 publications and several unpublished studies, we compared effect sizes (Pearson's r) of resource N:C and P:C on growth, consumption, excretion, and egestion between herbivorous and detritivorous consumers. The meta-analysis revealed that both herbivore and detritivore growth are limited by resource N:C and P:C contents, but effect sizes only among detritivores were significantly above zero. Consumption effect sizes were negative among herbivores but positive for detritivores in the case of both N:C and P:C, indicating distinct compensatory feeding responses across resource stoichiometry gradients. Herbivore P excretion rates responded significantly positively to resource P:C, whereas detritivore N and P excretion did not respond; detritivore N and P egestion responded positively to resource N:C and P:C, respectively. Our meta-analysis highlights resource N and P contents as broadly

limiting in brown and green benthic food webs, but indicates contrasting mechanisms of limitation owing to differing consumer regulation. We suggest that green and brown food webs share fundamental stoichiometric principles, while identifying specific differences toward applying ecological stoichiometry across ecosystems.

Keywords: food quality, detrital food webs, light nutrient hypothesis, growth, excretion, egestion

INTRODUCTION

Ecological stoichiometry was developed and has been considered extensively within the context of autotroph-based, or "green" food webs (Sterner and Elser, 2002) that conform nicely to the classic trophic level concept of primary producers and upper level consumers (Lindeman, 1942; Hairston et al., 1960). Although most energy and organic nutrients available to organisms are ultimately derived from autotrophs, the majority of energy [carbon (C)] fixed by primary producers enters the pool of detritus and becomes part of the "brown," detritus-based food web (Cebrian, 1999; Cebrian and Lartigue, 2004). Brown food webs remain comparatively under-studied by ecological stoichiometry theory, but the framework can provide insight into controls on brown trophic processes by examining the interplay of materials and energy between detritus, decomposer microbes, and detritivores (Moore et al., 2004). While there are shared stoichiometric constraints, there are still notable differences between green and brown food webs. For example, unlike green food webs in which herbivores directly ingest but do not themselves contribute organic C and organic nutrients to the autotroph pool, detrital organic carbon and nutrients are repackaged and consumed several times in brown food webs, resulting in a "microbial loop" or "detrital processing chain." This and other inherent differences may result in distinct stoichiometric principles throughout green versus brown food webs.

In this paper, we use a conceptual review and quantitative meta-analysis to summarize work over the last decade that has developed the application of ecological stoichiometry from green to brown food webs. We first identify three main areas where green and brown food webs differ, yet stoichiometric principles are shared and translate from green to brown systems. First, light is not a direct nutritional resource for heterotrophic organisms and the light and nutrient resource gradient that is recognized as an important control on the autotrophic community and primary production (Sterner et al., 1997) has received less attention within the context of the ecological stoichiometry of brown food webs than green ones. Second, detritivores have evolved with lower quality [<nutrient:C] food resources than herbivores (Frost et al., 2006) and their physiological responses to food resource enrichment may differ, having consequences for community structure and consumer-driven nutrient dynamics (CND). Third, the stoichiometry of CND in brown food webs has received much less consideration than that in green food webs (Moe et al., 2005; Halvorson et al., 2015a; Atkinson et al., 2016), and we highlight how CND may differ between the two trophic systems. We complement our review with a meta-analysis of existing studies from aquatic ecosystems, assessing how stoichiometric constraints on consumer growth, consumption, and waste production (egestion/excretion) compare between green and brown benthic food webs. The meta-analysis provides a focused, quantitative test of several predictions generated by our conceptual review. Throughout this paper we focus on plant litter as the basis of brown food webs, because it is a widespread form of detritus across inland ecosystem types.

LITERATURE REVIEW – COMPARING ECOLOGICAL STOICHIOMETRY OF GREEN AND BROWN FOOD WEBS IN THREE MAIN AREAS

Comparing Light and Nutrient Effects on the Resource Base of Green and Brown Food Webs

Autotroph stoichiometry varies widely across resource gradients (e.g., light and nutrients) due to their ability to store nutrients beyond what is needed for growth (Sterner et al., 1997; Persson et al., 2010). Autotrophs also tend to have lower N:C and P:C ratios than heterotrophs due to the presence of a cell wall and greater structural C material like cellulose and lignin (Sterner and Elser, 2002). Algal N:C and P:C tend to be lower than terrestrial plant tissue due to the presence of more structural material in plants (Elser et al., 2000), and the stoichiometry of different tissues varies across leaves, stems, wood, and roots (Sterner and Elser, 2002). Given this variation, plant litter that contributes regularly to detrital pools varies widely across species and biomes (Mcgroddy et al., 2004; Cornwell et al., 2008; Vergutz et al., 2012). Large particulate detritus tends to have even lower N:C and P:C than living plant tissue, due to resorption and leaching of soluble compounds after senescence. This resorption and leaching results in generally N- and P-deplete resources at the base of brown compared to green food webs (Lemoine et al., 2014).

One complication in examining basal food resource stoichiometry for macroconsumers in brown relative to green food webs is that detrital stoichiometry is derived from autotrophic as well as microbial heterotrophic decomposer tissue. Heterotrophic bacteria and fungi tend to have higher N:C and P:C ratios than leaf litter (Makino et al., 2003; Danger and Chauvet, 2013) and elemental imbalances between the microbial decomposers and the detritus can be alleviated at the organismal level by flexible stoichiometry or by changing physiological efficiencies (Manzoni et al., 2008, 2010; Kaiser et al., 2014; Manzoni, 2017). The limited data available for fungi suggest that they can have flexible nutrient:C ratios across

resource gradients and that their biomass can range more broadly in elemental composition than other heterotrophs (Danger and Chauvet, 2013; Danger et al., 2016); however, autotroph elemental composition still varies more broadly than heterotrophs (Sterner and Elser, 2002; Mcgroddy et al., 2004). Bacteria can also have variable P:C stoichiometry across strains (Scott et al., 2012) and some are more homeostatic than others (Cotner et al., 2006, 2010; Godwin and Cotner, 2014, 2015). Together, this variation across heterotrophic microbes results in a greater possible range of detrital stoichiometry, additional to that attributable to variation across plant tissues alone (Fanin et al., 2013).

Nutrients and light availability are key controls on resources in both green and brown food webs, because both factors can stimulate primary production, increasing the flux and changing the chemical quality of autotroph material that interacts with or enters the detrital pool (Gusewell and Gessner, 2009; Valera-Burgos et al., 2013; Liu et al., 2016). Nutrient enrichment often alleviates autotroph growth limitation, enhancing biomass (Elser et al., 2007) and increases the N:C and P:C of algal tissue and the nutrient:lignin and nutrient:C ratios of plants (Coulson and Butterfield, 1978; Aerts, 1997; Xu and Hirata, 2005). Further, nutrients may interact with light availability to determine the stoichiometry of autotrophs; the nutrient:light hypothesis (note we have switched numerator and denominator to provide consistency with our use of nutrient:C ratios) suggests that the balance between these two autotroph resources regulates autotroph nutrient:C ratios. Autotroph nutrient:C ratios should be positively related to the nutrient:light ratio (Sterner et al., 1997). Tests of the nutrient:light hypothesis have primarily focused on pelagic ecosystems and relationships between P:light and seston or autotroph P:C (Sterner et al., 1997). A few studies have applied the nutrient:light hypothesis to benthic aquatic algae (Hill and Fanta, 2008; Hill et al., 2009; Fanta et al., 2010), but few studies have extended it to terrestrial plants and they have primarily focused on plant:mycorrhizal interactions (Elliott and White, 1994; Treseder, 2004; Johnson et al., 2010). This extension will be key to understanding the broader applicability of this stoichiometric concept across interfaces of green and brown trophic systems, especially because terrestrial plant litter provides a major resource base for brown food webs.

As in green food webs, nutrient enrichment in brown food webs increases the P:C and N:C ratios of basal food resources because microbial decomposers on detritus are capable of assimilating dissolved N and P from the water column (Suberkropp and Chauvet, 1995; Cheever et al., 2013; Scott et al., 2013). Since heterotrophic bacteria and fungi can have weakly flexible N:C and P:C that are higher than the detrital substrate (Makino et al., 2003; Danger and Chauvet, 2013), their growth and nutrient storage can result in increased N and P contents of detritus during decomposition. Notably, increased microbial biomass also enhances the quality of detrital C, through accumulation of microbial lipids, soluble carbohydrates, and protein that are nutritionally valuable compared to plant polysaccharides like cellulose and lignin that dominate detrital substrate C and are resistant to breakdown and assimilation (Martin et al., 1980; Chung and Suberkropp, 2009a,b). As elevated nutrients stimulate microbial growth, increased decomposition rates often accompany nutrient enrichment (Ferreira et al., 2015; Kominoski et al., 2015; Manning et al., 2015, 2016), stimulating C loss from ecosystems (Benstead et al., 2009; Rosemond et al., 2015). In this way, nutrient enrichment increases the quality (nutrient:C) of basal food resources in both green and brown food webs. However, enrichment has contrasting effects on resource quantity because nutrients stimulate autotroph growth, enhancing resource quantity in green food webs, whereas nutrients increase decomposition rates and therefore reduce resource quantity in brown food webs (Rosemond et al., 2015).

The role of light availability in brown food webs is less clear than in green food webs, because microbial decomposers cannot directly use light as a resource and detritus is only affected directly by light through photolysis that stimulates breakdown (Wetzel et al., 1995). The role of light in decomposition has been largely neglected under the assumption that most decomposition occurs in low-light environments with minimal algal biomass (Fisher and Likens, 1973). However, sufficient light can occur in many aquatic settings, where light permits algal growth on detritus, changing the microbial assemblage and altering detrital stoichiometry and decomposition (Lagrue et al., 2011; Danger et al., 2013a; Kuehn et al., 2014). Notably, periphytic algae could reduce detrital P:C or N:C under low nutrient levels, but increase the maximum detrital P:C or N:C under high nutrient levels, because of autotrophs' greater stoichiometric flexibility and ability to store excess nutrients (Persson et al., 2010; Danger et al., 2013a; Halvorson et al., 2016a). A key indirect effect of light may also be to "prime" decomposition because algae exude fresh, labile C that may be used by fungi and bacteria to invest in growth or enzyme production, stimulating breakdown of recalcitrant detritus via the priming effect (Kuzyakov et al., 2000; Guenet et al., 2010; Kuehn et al., 2014). This coupling of periphytic autotrophs and heterotrophs may depend on the nutrient:light ratio, which influences autotroph nutrient:C ratios and algal C exudation rates that probably elicit the priming effect (Sterner et al., 1997; Guenet et al., 2010; Wyatt and Turetsky, 2015). Existing studies suggest high light and nutrient levels suppress decomposition through negative priming effects, whereas high light and low nutrient levels stimulate decomposition through positive priming effects (Danger et al., 2013a; Halvorson et al., 2016a). Further studies are clearly needed to address the interactive effects of light and nutrients on brown food webs, especially regarding variation in detrital stoichiometry and recalcitrance, the stoichiometry of algal-heterotroph interactions, and implications of periphytic algae for detrital food quality to detritivores (Guo et al., 2016).

Effects of Food Resource Nutrient Enrichment in Brown and Green Food Webs

Physiological responses to food resource elemental ratios are central to understanding organismal homeostasis, growth, fitness, and nutrient cycling (Sterner and Elser, 2002; Cross et al., 2005; Frost et al., 2005a; Sperfeld et al., 2017). Although the degree of stoichiometric homeostasis varies across metazoans

(Persson et al., 2010), often some degree of constraint on body elemental contents and ratios occurs due to biomolecular composition, body plans, and life history traits (Elser et al., 1996; Sterner and Elser, 2002). These constraints are common and shape stoichiometric principles throughout a diversity of food webs. Indeed, since the turn of the century, studies have shown herbivores and detritivores often have higher N:C and P:C ratios than their food resources (Elser et al., 2000; Lemoine et al., 2014) potentially leading to widespread nutrient limitation of growth. Understanding how resource stoichiometry affects consumer growth and physiology is key to comparing stoichiometric constraints in green and brown food webs, including under anthropogenic enrichment that broadly increases resource N:C and P:C (Cross et al., 2003; Peñuelas et al., 2013).

Herbivore and detritivore responses to nutrient enrichment will likely differ due to contrasting stoichiometry and C quality (recalcitrance and digestibility) of autotroph versus detrital food resources. Because organism nutrient:C ratios often decrease as one moves from unicellular autotrophs to land plants, terrestrial herbivores and detritivores, as well as aquatic detritivores, rely on food resources of lower nutrient contents compared to aquatic herbivores (Cebrian and Lartigue, 2004). These taxa have therefore likely faced greater elemental imbalances during their evolutionary history than aquatic herbivores, and may have evolved lower demands for nutrients in food resources (Frost et al., 2006). As autotroph nutrient:C ratios decrease, C quality also declines across the spectrum from unicellular autotrophs to vascular plants, leading to greater digestion resistance and constraining the proportion of resource C assimilated by consumers of vascular plant tissue (Sterner and Elser, 2002; Cebrian, 2004). The quality of C available further differs between living, actively growing plant material consumed by herbivores versus dead plant litter consumed by detritivores (Vergutz et al., 2012), setting an additional contrast between resources of the two trophic groups. These differences will shape consumers' response to nutrient enrichment because C assimilation constrains animals' ability to use ingested nutrients (DeMott et al., 2010; DeMott and Van Donk, 2013). Together, these trends support a general prediction that herbivores may be better-equipped to respond positively to resource nutrient enrichment, relative to detritivores.

The consumption response to resource stoichiometry is an important component of growth, but the direction (positive or negative) and magnitude in response to nutrient enrichment may differ between herbivores and detritivores. Detritivores targeting the acquisition of limiting resources may increase their consumption rates (Ott et al., 2012; Flores et al., 2014; Fuller et al., 2015) or selectively feed on food resources more rich in potentially limiting nutrients (Frainer et al., 2016). On the other hand, herbivores tend to exhibit reduced consumption on higher-nutrient diets (Plath and Boersma, 2001; Boersma and Elser, 2006; Fink and Von Elert, 2006) and detritivore consumption rates increase at a similar rate with the nutrient content and the production of their autotrophic and detrital food resources across terrestrial and aquatic ecosystems (Cebrian and Lartigue, 2004). Therefore, we may expect aquatic herbivores and detritivore consumption rates to increase similarly as their food resources become enriched although herbivore responses may be weaker than detritivores'.

The complexity of detritivore food resources (e.g., recalcitrant N bound to lignin; Chapin et al., 2002) and lower nutrient content (Cross et al., 2003) relative to living autotrophic tissue may result in lower detritivore assimilation efficiencies (AEs) and lower GGEs compared to herbivores. A meta-analysis found that detritivores tended to have a lower C GGE than herbivores (Frost et al., 2006), but other element-specific GGEs and AEs were not commonly available across feeding guilds, and it remains unclear how efficiently detritivores assimilate and convert nutrients into new growth. However, recent estimates for aquatic detritivore element-specific AEs and GGEs (Halvorson et al., 2015b, 2016b) suggest that N- and P-specific AE and GGE are lower than those estimated for aquatic herbivores (DeMott et al., 1998; Ferrão-Filho et al., 2007). Therefore, detritivores will likely excrete elements at lower and egest at higher rates than taxa in other feeding guilds (McManamay et al., 2011). This trend is likely to persist even with nutrient enrichment of food resources, because nutrient enrichment does not appear to improve detritivore AE or GGE, possibly because the recalcitrance of detrital C ultimately constrains detritivores' ability to invest energy or resources toward acquisition of added nutrients (Halvorson et al., 2015b, 2016b).

Bioenergetic models indicate that aquatic herbivores have a greater growth demand for P relative to C (i.e., higher P:C threshold elemental ratios) and greater C GGEs than do aquatic detritivores (Frost et al., 2006). A positive relationship between P demand and growth has been observed across broad taxa (Elser et al., 2003) and across aquatic taxa (Frost et al., 2006; Benstead et al., 2014) suggesting aquatic detritivores may have traded the ability to grow fast for the ability to utilize food resources with a low P:C (i.e., terrestrial detritus). Even within herbivorous zooplankton, species C- and P- specific growth rates are coupled and growth rate is an important predictor of taxa responses to P enrichment of food resources (Hood and Sterner, 2014). Aquatic herbivores may have evolved greater growth rates and may have a greater capacity for growth responses to nutrient enrichment of food resources compared to aquatic detritivores. We address many of these questions below in a meta-analysis comparing aquatic herbivore and detritivore responses to resource N:C and P:C in controlled feeding studies.

Comparing Consumer-Driven Nutrient Dynamics in Green and Brown Food Webs

Consumers can play important roles in ecosystem nutrient dynamics (Elser and Urabe, 1999; Vanni, 2002; Pastor et al., 2006), but these roles likely differ in green and brown food webs due to contrasting resource stoichiometry and recalcitrance, as well as differing processing of consumer wastes after release. Most studies of consumer-driven nutrient dynamics (CND) have focused on herbivores in pelagic green food webs, where the unidirectional flow of energy and nutrients and tight consumer-resource feedbacks may, in part, simplify CND (Elser and Urabe, 1999). While CND can be easily translated across systems, CND

is probably more complex in terrestrial systems and in aquatic brown food webs, because multiple forms of waste – including excreta, egesta, exuvia, and carcasses – must be considered as components of CND, with potential to affect nutrient availability and consumer-resource feedbacks (Vanni et al., 2013; Sitters et al., 2017). In particular, the iterative re-packaging and processing of detritus along a transfer chain may result in multiple steps and controls on the strength of CND in brown food webs (Heal and Maclean, 1975; Navel et al., 2011; Bundschuh and Mckie, 2016). Further understanding of CND in brown and green food webs will be important to quantify the broad roles of animals in ecosystem nutrient cycles (Vanni, 2002; Atkinson et al., 2016), including under prevalent "multichannel" feeding by omnivorous taxa (Wolkovich et al., 2014).

Ecological stoichiometry has historically focused on dissolved and bioavailable excreta rather than particulate wastes like egesta, because autotrophs are capable of directly assimilating excreta, forming direct consumer-resource nutrient feedbacks (Sterner, 1986; McNaughton et al., 1997b; Elser and Urabe, 1999; Evans-White and Lamberti, 2005). Moreover, dissolved excreta are often considered the dominant nutrient waste flux from the consumer pool (Zanotto et al., 1993; DeMott et al., 1998); these assumptions are directly tied to the natural history and community structure of green food webs (but see Higgins et al., 2006). However, brown food webs can also show a tight interplay between consumer wastes and heterotrophic activity, because microbial heterotrophs are capable of assimilating consumer excreta (Fornara and Du Toit, 2008; Cheever et al., 2012; Rugenski et al., 2012; Villanueva et al., 2012). In this way, consumer nutrient recycling is likely to promote biomass turnover of both autotrophic and heterotrophic microbes (Hill and Griffiths, 2017); however, in green food webs with plentiful light, this may come with minimal reductions in autotroph standing stocks (Hobbs, 1996; Knoll et al., 2009), whereas in brown food webs with limited detrital stocks, consumers will enhance decomposition both directly via consumption and indirectly via nutrient recycling that stimulates heterotrophy.

Studies from both green and brown food webs increasingly consider nutrient wastes released as egesta (Liess and Haglund, 2007; Hood et al., 2014; Halvorson et al., 2015a). Nutrient egestion rates by aquatic herbivores and detritivores can equal or exceed excretion rates (Hood et al., 2014; Liess, 2014; Halvorson et al., 2015a; Norlin et al., 2016). In terrestrial settings, both egesta and excreta are substantial, often concurrent nutrient subsidies of consumers to soils (McNaughton et al., 1997a; Clay et al., 2014; Sitters et al., 2014), and both forms of waste have historically been considered as important pathways of CND (Hobbs, 1996). The relative importance of egestion versus excretion as components of CND will likely vary with the resource N and P contents (Zanotto et al., 1993; Hobbs, 1996; Halvorson et al., 2015a) and the recalcitrance of ingested nutrients, including whether ingested nutrients are bound in living versus dead tissues. The recalcitrance of associated C may also set limits on assimilation and subsequent growth and storage of nutrients in animal tissues (Atkinson et al., 2016). Given greater recalcitrance of detrital C and nutrients compared to autotrophic C and nutrients, egestion is likely to play a relatively greater role in CND in brown food webs than in green food webs. However, the ecological importance of egestion versus excretion will also depend on environmental processing of each form of waste (Liess and Haglund, 2007; Sperfeld et al., 2016); egesta, in particular, can play diverse roles in nutrient dynamics because they are subject to microbial breakdown, direct ingestion by animals, and transport/deposition (Wotton and Malmqvist, 2001). Egested nutrients probably occur in recalcitrant forms that limit the rate and magnitude of nutrient release, slowing nutrient turnover relative to excretion (Liess and Haglund, 2007; Sperfeld et al., 2016). Furthermore, decomposing egesta may exhibit uptake of inorganic nutrients to support microbial growth, which would slow ecosystem-level nutrient turnover (Halvorson et al., 2017). As a subsidy of C and nutrients to depositional zones like soil or the aquatic hyporheos, egestion probably fuels ecosystem respiration and supports the subterranean food web (Navel et al., 2011). Overall, the fates of animal egesta versus excreta must be further studied to holistically understand CND, especially in brown food webs (Navel et al., 2011; Bundschuh and Mckie, 2016).

The lower nutrient content of detrital resources, compared to living plant matter, may indicate brown food webs to be more strongly nutrient-limited than green food webs, and therefore animals may be generally less-efficient recyclers of nutrients in brown food webs. This is consistent with evidence that aquatic detritivorous animals display lower N and P excretion rates than their herbivorous counterparts (McManamay et al., 2011), but comparisons from additional settings are clearly needed. Moreover, generalizations of bulk detritus as the stoichiometry of ingested resources are likely to underestimate excretion and egestion rates (Hood et al., 2014). This is because detritivorous animals selectively feed on nutrient-rich biofilms on detritus. Such selective feeding likely varies across animal species (Arsuffi and Suberkropp, 1989) and confounds predictions of aquatic CND across animals (Dodds et al., 2014). Predictions of CND could be aided by quantifying the degree of selectivity across species and identifying trends across coarse traits such as mouthpart morphology, trophic mode, or body size (Dodds et al., 2014), as done among large terrestrial herbivores (Pastor et al., 2006). This work is necessary to accurately place animals within ecosystem processes, including consumption, release, and storage of nutrients, and thereby understand how CND may depend on an ecosystem's trophic basis (Atkinson et al., 2016; Hill and Griffiths, 2017).

In many systems, CND may also provide a link between seemingly disparate nutrient dynamics in green and brown food webs (Cherif and Loreau, 2013; Zou et al., 2016). Because autotrophs and heterotrophs share the same pool of inorganic nutrients, inorganic wastes from consumers can easily interchange between detritus and autotrophs, resulting in complex interplay between trophic processes in each food web (Zou et al., 2016). Moreover, herbivores themselves produce organic wastes including egesta, and these wastes are subject to microbial and other breakdown processes within

the pool of detritus, but do not return to autotrophs until mineralization (Hawlena and Schmitz, 2010). The entanglement of CND between green and brown food webs challenges the traditional dichotomy between these energy flow channels, leading toward weaker consumer-resource nutrient feedbacks when a consumer's nutrient wastes are incorporated by a food resource inaccessible to that consumer (i.e., herbivore excreta are assimilated by heterotrophic decomposers; Fornara and Du Toit, 2008; Zou et al., 2016). The nutrient interchange between green and brown food webs also occurs when omnivores consume and subsequently recycle nutrients derived from both autotrophs and detritus (Polis and Strong, 1996; Wolkovich et al., 2014). In this way, CND provides a connection between green and brown food webs, but may not facilitate the tight feedbacks between consumers and their resources originally conceived by ecological stoichiometry theory (Elser and Urabe, 1999).

META-ANALYSIS OF FRESHWATER BENTHIC INVERTEBRATE FEEDING STUDIES TO QUANTITATIVELY COMPARE ECOLOGICAL STOICHIOMETRY IN BROWN AND GREEN FOOD WEBS

Methods

We sought to assess the current literature regarding stoichiometric constraints on organismal growth and stoichiometric regulation in green and brown food webs, because many existing studies remain limited to single or a handful of similar taxa, and there has been little synthesis across the breadth of studies, and few formal comparisons between green and brown food webs (but see Lemoine et al., 2014). We collected data on freshwater benthic invertebrate herbivore and detritivore taxa that had been fed food resources where nutrient:C ratios were controlled or manipulated. Published datasets were identified using the following search strings in Web of Science, searched on September 15, 2016 (TS means "topic search"; keywords): TS = (herbivor* OR graz* OR detritivor* OR invertebrate OR shredd* OR macroinvertebrate OR zooplankton) AND TS = (stoichiometr*). This search yielded 1,144 studies, from which we identified publications suitable for data extraction. Although we initially planned to include zooplankton, we narrowed our selection to benthic invertebrates to focus the meta-analysis. We supplemented the Web of Science search with a Google Scholar search of 2,000 additional hits for more recent literature and dissertations/theses (excluding any duplicate publications). From each study, we used figures (extraction using DataThief), tables, and appendices to collect the following variables where available: diet N:C and P:C, growth rates, consumption rates, and N and P excretion and egestion rates. We also noted sample sizes, consumer trophic mode (detritivore or herbivore), consumer and diet taxonomy, whether dietary gradients were monospecific or across multiple species, and temperature. To a total of 46 published studies ultimately included in the meta-analysis, we added eight unpublished studies of our own. Because many publications reported data from >2 experiments such as at multiple temperatures, contrasting diet types (e.g., litter or algal species) or from multiple consumer species, we treated each experiment as an independent dataset suitable for inclusion in the meta-analysis. Note our meta-analysis assumed independence of datasets among closely-related taxa and when datasets were from the same study or research group. Where studies used only two levels of resource N:C or P:C, we obtained raw data from the corresponding author to permit calculation of effect size. We also excluded datasets in which minimum and maximum mean resource N:C or P:C overlapped within 1 SD, ensuring a robust gradient of resource stoichiometry (Halvorson and Small, 2016). Altogether, 257 datasets were included in the meta-analysis.

From each dataset, we calculated effect sizes of resource P:C or N:C (Pearson's r) on each response variable (growth, consumption, excretion, or egestion), such that positive effects indicate a positive response to food resource nutrient enrichment (Persson et al., 2010). Pearson's r was transformed to Fisher's Z and weighted according to its variance as $[1/(n\text{-}3)]$ where n = sample size for a dataset (Rosenberg et al., 2013). We used a weighted mixed effects model to test differences in effect size between detritivorous and herbivorous taxa (Rosenberg, 2013). This model treated trophic mode (categories = herbivore or detritivore) as a fixed effect and dataset identity as a random effect. The use of random effects accounts for heterogeneity across studies due to variable factors including temperature, taxonomy, and diet. We assessed heterogeneity of effect sizes across studies using the I^2 statistic, which equates to the proportion of total heterogeneity attributable to between-study variance (**Table 1**) (Senior et al., 2016). Because of insufficient datasets regarding N and P egestion by herbivores, we decided to exclude herbivores from the meta-analysis of those effect sizes and focus only on detritivore datasets. I^2 and the random variance terms are calculated only for a global mean model (null hypothesis = effect size of zero) in those sets, accordingly. We also used one-sample weighted t-tests to determine if effect sizes differed from a null hypothesis of $Z = 0$ (no response to resource stoichiometry) for each trophic mode in each analysis. All statistics were conducted using R version 3.3.1 (R Core Team, 2013) and the R package 'weights' (Pasek, 2016).

Results

We report sample sizes, I^2, and random effects variance of the meta-analysis in **Table 1**. Further description of datasets and associated effect sizes and citations can be found in Supplementary Table 1.

Across feeding studies included in the meta-analysis, resource N:C and P:C contents spanned a wide range across all datasets (**Figure 1**). Although there was notable overlap in the overall range, herbivores' resources (autotrophs) were generally greater in N:C and P:C contents compared to detritivores' resources (detritus; **Figure 1**). The datasets spanned organisms from eight taxonomic orders, with most herbivore studies using Gastropoda and detritivore studies showing a broader diversity, but primarily using Trichoptera, Plecoptera, Amphipoda, and

TABLE 1 | Sample sizes, I^2, and random effects variance for each of eight variables in response to resource N:C or P:C manipulations in the met-analysis.

Response variable	Resource manipulation	# Datasets	I^2	Random effects variance
Growth	N:C	39	93.6%	0.486
Growth	P:C	54	96.5%	0.900
Consumption	N:C	44	96.2%	0.638
Consumption	P:C	34	96.6%	0.572
N excretion	N:C	15	87.5%	0.423
P excretion	P:C	23	96.5%	1.221
N egestion	N:C	21	92.8%	0.730
P egestion	P:C	27	91.0%	0.584

For a summary of all datasets and effect sizes, see Supplementary Table 1.

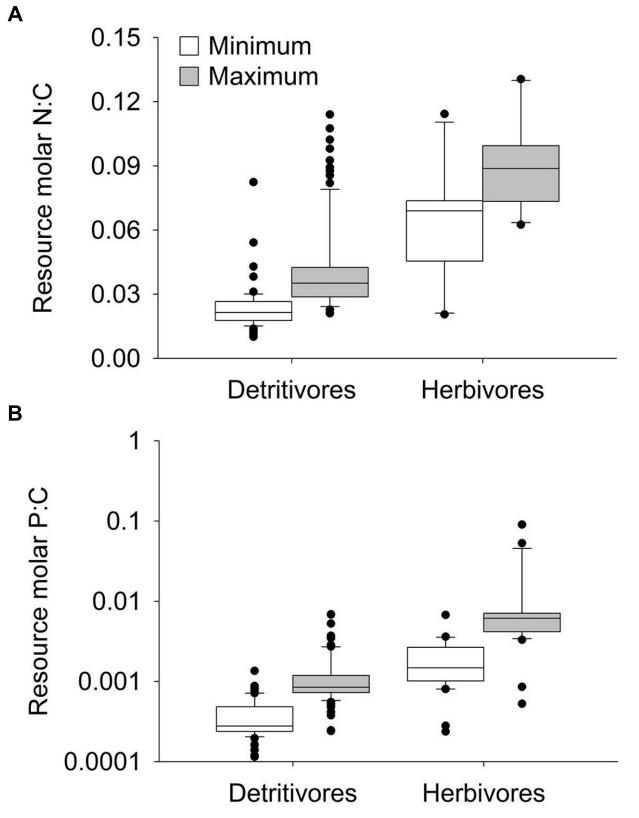

FIGURE 1 | Boxplot of minimum and maximum resource N:C contents **(A)** and P:C contents **(B)** in benthic detritivore and herbivore feeding studies included in the meta-analysis. Note the logarithmic Y-axis in **(B)**. The black horizontal lines indicate median values within each group.

Diptera (Supplementary Figure 1). Most studies used organisms from streams or rivers, followed by lakes and wetlands/ponds (Supplementary Figure 2).

Detritivore and herbivore growth responses to resource N:C contents were similar and positive, although only the detritivore response was significantly greater than zero ($t_{1,27} = 5.39$, $P < 0.001$; **Figure 2A**). The two trophic modes also did not differ in growth responses to resource P:C. Detritivorous taxa showed a positive P:C-growth response significantly greater than zero ($t_{1,33} = 3.14$; $P < 0.01$) whereas the herbivore response did not differ from zero (**Figure 2B**).

Effect sizes of resource N:C on herbivore consumption were significantly lower than effects on detritivore consumption ($P < 0.001$; **Figure 2C**). Herbivore consumption responded negatively to resource N:C, but mean effect size was not different from zero, whereas detritivore consumption rates responded significantly positively to resource N:C ($t_{1,37} = 3.31$, $P < 0.01$; **Figure 2C**). Similarly, herbivores and detritivores differed significantly in the effect size of P:C on consumption ($P < 0.001$; **Figure 2D**). The P:C-consumption effect size was significantly greater than zero for detritivores ($t_{1,29} = 2.75$, $P < 0.05$) whereas that of herbivores was below zero ($t_{1,3} = 3.22$; $P < 0.05$; **Figure 2D**).

The effects of resource N:C on N excretion did not differ between trophic modes, and neither mode exhibited effect sizes significantly different from zero (**Figure 2E**). In contrast, the effect size of P:C on P excretion differed between trophic modes, with herbivores displaying a higher, positive effect size significantly greater than zero ($t_{1,6} = 3.63$, $P < 0.05$) compared to an effect size indistinguishable from zero among detritivores (**Figure 2F**).

We limited our meta-analysis of N and P egestion to detritivores because we obtained only two herbivore datasets. The response of detritivore N egestion to resource N:C was significantly greater than zero ($t_{1,20} = 2.49$, $P < 0.02$; **Figure 2G**), as was the response of P egestion to resource P:C ($t_{1,26} = 5.31$, $P < 0.001$; **Figure 2H**).

Discussion

Our meta-analysis of feeding studies supports broad N and P growth limitation among both herbivorous and detritivorous freshwater invertebrates. However, only detritivores exhibited N and P growth effect sizes significantly different from zero (**Figures 2A,B**), suggesting that counter to our predictions in the review above, detritivores' growth responses to nutrient enrichment may actually be stronger than herbivores'. The greater strength and consistency of limitation among detritivores may be partly attributable to larger sample sizes from detritivorous taxa throughout the meta-analysis, highlighting a literature gap of feeding studies from benthic herbivores, especially among non-Gastropoda (Supplementary Figure 1). Despite limited sample sizes, the mechanisms of growth limitation also appear to differ between trophic modes, given contrasting responses of consumption and P excretion to food resource nutrient enrichment (**Figures 2C,D,F**). These results indicate distinct responses of brown versus green benthic food webs to nutrient enrichment, likely driven by inherent differences in the stoichiometry (**Figure 1**) and C quality of detrital versus autotrophic resources.

Compared to herbivores, detritivores may display stronger and less variable growth responses to elevated resource nutrients because elevated detrital N and P are accompanied by greater C quality in the form of increased microbial biomass (Gulis and Suberkropp, 2003; Manning et al., 2015), whereas autotrophic C quality may only weakly co-vary with N and P contents.

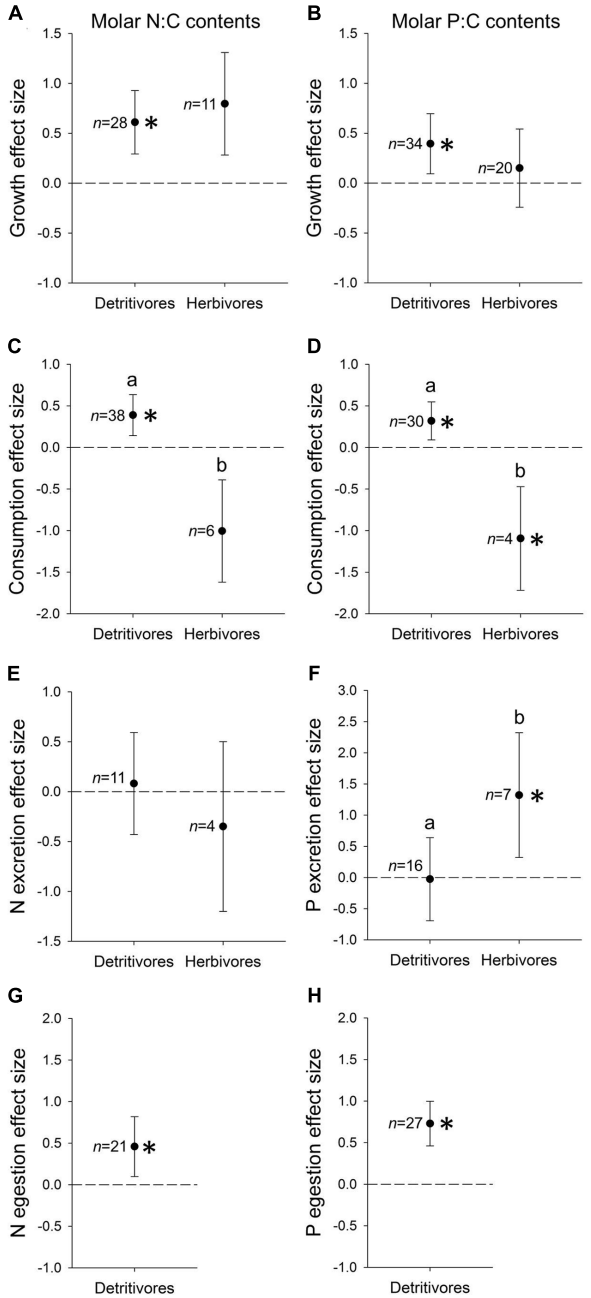

FIGURE 2 | Mean ± 95% CI effect sizes (weighted Z-scores) of resource molar N:C contents **(A,C,E,G)** and P:C contents **(B,D,F,H)** on rates of growth **(A,B)**, consumption **(C,D)**, N excretion **(E)**, P excretion **(F)**, N egestion **(G)**, and P egestion **(H)** of detritivorous and herbivorous benthic invertebrates. Sample sizes (*n*) are indicated to the left of each effect size. Asterisks indicate effect sizes different from zero (*t*-test, $P < 0.05$). Letters indicate effect sizes differ between trophic modes (ANOVA, $P < 0.05$). Only detritivores were included in **(G,H)** due to insufficient egestion datasets from herbivores ($n = 2$).

Given the importance of microbial C in supporting detritivore growth (Chung and Suberkropp, 2009a; Halvorson et al., 2016b), it is difficult to determine whether positive growth effect sizes are driven by elevated dietary microbial biomass or increased N and P availability. However, one feeding study explicitly manipulated detrital P content without changing fungal biomass and still found strong P-limitation of growth, suggesting P can limit detritivore growth, independent of microbial biomass (Danger et al., 2013b). In the case of autotrophs, increased N and P contents may not affect or may actually drive lower C quality, for example due to diminished eicosapentaenoic acid contents as cyanobacteria form a greater proportion of algal assemblages (Muller-Navarra et al., 2000), which could dampen the herbivore growth response to elevated autrotroph N and P contents. We also note that most herbivore feeding studies (69% of datasets) used resource gradients containing multiple species – especially periphyton composed of multi-species assemblages – which could have weakened or increased variation among herbivore effect sizes. This is in contrast to the majority of detritivore feeding studies (76% of datasets) that employed resource gradients using litter from only one plant species (Supplementary Figure 3). Although microbial taxa on detritus may shift with N or P availability (Lecerf and Chauvet, 2008), a consistent detrital substrate across resource stoichiometry gradients could reduce inter-individual variation and increase growth effect sizes within detritivore feeding studies. While the growth effect sizes are similar, we expect the underlying mechanisms of enhanced growth (e.g., altered consumption or assimilation) to differ between herbivorous and detritivorous taxa, due to inherent contrasts between autotrophic and detrital food resources (see above).

The contrasting consumption effect sizes suggest different bottom–up effects of nutrients on consumption in green versus brown food webs, given benthic detritivores and herbivores exhibit different compensatory feeding with increased resource nutrient content (**Figures 2C,D**). While herbivores may up-regulate consumption on low-nutrient resources, perhaps to increase intake of limiting nutrients (Fink and Von Elert, 2006; Liess, 2014), detritivores up-regulate consumption on high-nutrient resources. This is surprising in light of predictions that both herbivore and detritivore consumption increase positively with resource nutrient enrichment (see review above; Cebrian and Lartigue, 2004). We attribute this dichotomy to the lower nutrient content (**Figure 1**) and low C quality of detritus, relative to that of autotrophs. Detritivores fed low-nutrient resources probably slow their feeding rates to increase gut residence time and maximize assimilation of limiting C and nutrients (Golladay et al., 1983). Indeed, assimilation probably imposes strong limits on detritivore growth, owing to the recalcitrance of detrital C and nutrients that set low maximum assimilation efficiencies (Halvorson et al., 2015b, 2016b). In contrast, herbivores fed low-nutrient resources may retain comparatively high assimilation efficiencies and improve growth by increasing intake rates (Fink and Von Elert, 2006; Liess, 2014). In this way, our meta-analysis suggests herbivores and detritivores exhibit divergent strategies of handling low-nutrient diets and responding positively to nutrient enrichment. Notably, elevated detritivore consumption on high-nutrient litter would contribute to enhanced detritivore-mediated decomposition under nutrient enrichment (Manning et al., 2016), whereas reduced herbivore consumption on high-nutrient diets would alleviate grazing pressure, magnifying

the stimulatory bottom–up effects of dissolved nutrients on autotroph biomass (Dodds, 2007).

Although excretion may be an important means for consumers to regulate stoichiometric homeostasis as resources increase in nutrient contents (DeMott et al., 1998; Frost et al., 2005b), we observed no response of N excretion to resource N:C, and only herbivores elevated P excretion on high-P:C resources (**Figures 2E,F**). The small N excretion effect size suggests that in benthic systems, detrital and autotrophic resources may rarely reach a point of excess N contents relative to consumer demands, unlike higher resource P contents that can inhibit growth and are accompanied by elevated P excretion (Boersma and Elser, 2006; Morehouse et al., 2013). One factor shaping these excretion patterns is probably body stoichiometry, especially the stoichiometry of growth, which determines consumer stoichiometric demands (Vanni et al., 2002; Hood and Sterner, 2014; Halvorson et al., 2015b). We did not collect body stoichiometry data in our meta-analysis, but based on limited body stoichiometry data from field-collected benthic invertebrates, body N contents stay consistently high through development and may therefore dampen up-regulated N excretion on high-N:C resources, whereas body P contents often decline during development and could cause individuals to exhibit lower P growth demands and excrete excess P on high-P:C resources (Back and King, 2013). The lack of N or P excretion responses among benthic detritivores suggests other regulatory pathways of nutrient release – namely egestion – may increase when detritivores are fed high-nutrient litter. Indeed, we found detritivores consistently increase N and P egestion when fed high-N:C and high-P:C resources, respectively (**Figures 2G,H**). However, we were unable to assess egestion effect sizes among herbivores, and we reiterate calls for additional excretion and egestion data from diverse taxa, which will help resolve animal nutrient budgets and CND in aquatic ecosystems (McManamay et al., 2011; Vanni and Mcintyre, 2016). One key implication of our meta-analysis is that P enrichment may increase the strength of dissolved CND in green food webs, via increased P excretion, indicative of tight herbivore-autotroph links that we predict in our review. In contrast, brown food webs may exhibit little change in dissolved CND with P enrichment, indicative of weaker detritivore-heterotroph linkages in brown food webs. Instead, nutrient enrichment in brown food webs will elicit strong effects on particulate CND, affecting nutrient availability throughout particle processing chains (Halvorson et al., 2015a).

Our meta-analysis synthesizes current data regarding N and P limitation of freshwater benthic invertebrates, but it carries some weaknesses that limit inferences and should be addressed by future experiments and meta-analyses. First, we narrowed our data collection to controlled feeding studies, primarily from the laboratory, because field studies often have difficulty accurately characterizing resource stoichiometry, face many confounding factors across study sites, and typically have low sample sizes (Halvorson and Small, 2016). During our literature search, however, we found many studies across resource stoichiometry gradients in the field (e.g., Cross et al., 2006; Rothlisberger et al., 2008; McManamay et al., 2011), and a separate meta-analysis of these field studies is warranted to compare effect sizes from controlled studies (see Moody et al., 2015). Second, our meta-analysis addressed consumer limitation by resource N and P separately, but availability of these two elements was likely positively correlated in many studies, and therefore some responses may be driven by increases of N and P together. Among the 46 publications included in our meta-analysis, 27 (59%) manipulated both resource N and P contents. For this reason, we hesitate to explicitly compare effect sizes between the N and P datasets, and we suspect co-limitation by N and P may partly drive the effect sizes in our meta-analysis. Third, we note that herbivore feeding studies on average used higher temperatures (18.2°C) than detritivore feeding studies (10.8°C), which may partly drive different responses between trophic modes (Supplementary Figure 4), especially if the effects of nutrients depend on temperature (Kendrick and Benstead, 2013; Cross et al., 2015). While a temperature scaling coefficient could standardize metabolic rates across varying temperatures (e.g., Vanni and Mcintyre, 2016), such standardization would not affect our inferences because each effect size was calculated from individuals held at the same temperature. Many of the factors that differed across studies likely drove high heterogeneity (I^2) across effect sizes (**Table 1**), but this heterogeneity was accounted by using a mixed effects model and I^2 was similar to that reported across other meta-analyses in ecology (Senior et al., 2016). Finally, our classification of benthic invertebrates into herbivores versus detritivores was based solely on diets fed in experiments, and may not reflect feeding ecology or the stoichiometry of feeding in the field, where animals can feed selectively on nutrient-rich biofilms (Hood et al., 2014) or forage on multiple resource types and confound trophic classification (Wolkovich et al., 2014; Snyder et al., 2015; Stoler et al., 2016). Future studies should investigate consumer feeding behavior in the field to accurately quantify bottom–up constraints on consumer growth, consumption, and excretion/egestion in green and brown benthic food webs.

CONCLUSION

Our review and meta-analysis focusing on freshwater systems highlight current understanding of ecological stoichiometry in brown food webs, providing conceptual and quantitative comparison to green food webs. Although stoichiometric principles apply to both trophic systems, we suggest the nature of these principles differs in several important ways. Notably, inorganic nutrients and light availability can affect resource quantity and quality in both brown and green food webs, but in the former, both factors are likely to reduce detrital quantity via stimulated decomposition (Danger et al., 2013a; Rosemond et al., 2015) while enhancing detrital quality (Cross et al., 2003; Manning et al., 2015; Halvorson et al., 2016a), whereas in the latter, light and nutrients are likely to concurrently increase autotroph quantity while eliciting opposing effects on autotroph quality (nutrient:light hypothesis; Sterner et al., 1997). We suggest detrivorous and herbivorous consumers may respond differently to elevated resource nutrient contents, because herbivores have evolved to use resources of greater C quality

and nutrient contents compared to detritivores; underlying mechanisms of these responses are also likely to differ, owing to contrasting consumption responses and assimilation efficiencies between trophic modes (Cebrian, 2004; Frost et al., 2006). Patterns in consumer-driven nutrient dynamics (CND) are also likely to differ, with egestion playing a greater relative role than excretion in brown food webs due to the recalcitrance of detrital C and nutrients, but we note excretion connects detritivores and herbivores to a shared inorganic nutrient pool, weakening direct consumer-resource feedbacks and increasing nutrient exchange between green and brown food webs (Zou et al., 2016). In a meta-analysis across controlled feeding studies, we directly compared stoichiometric constraints on invertebrates in green versus brown benthic food webs. The meta-analysis shows that herbivore and detritivore growth rates often increase with greater resource N and P contents. However, we found contrasting responses of consumption and P excretion between trophic modes, reflecting distinct herbivore and detritivore regulatory responses to elevated nutrients, probably due to contrasting resource C quality and stoichiometry.

We see several directions for continued investigation of ecological stoichiometry in both autotroph- and detrital-based systems, especially at interfaces of autotrophic and detrital-heterotrophic biomass and activity. First, there is a need for further study of how light and inorganic nutrient availability affect autotroph–heterotroph interactions on submerged detritus (Kuehn et al., 2014; Halvorson et al., 2016a) and subsequent feeding and growth of consumers (Guo et al., 2016; Stoler et al., 2016). In both trophic systems, but particularly among brown food webs, it remains difficult to accurately characterize the stoichiometry of ingested resources relative to that of bulk resources (Hood et al., 2014) and studies must address selective feeding and other foraging behavior as a mechanism of stoichiometric regulation, especially when animals may actively choose nutrient-rich resources (Dodds et al., 2014; Snyder et al., 2015; Sperfeld et al., 2017). The role of selective feeding is especially important to understand roles of multichannel consumers that can feed on both autotrophs and detritus, blurring the distinction between green and brown food webs (Wolkovich et al., 2014). Finally, our meta-analysis documents a lack of feeding experiments measuring herbivore consumption, excretion, and (especially) egestion across resource stoichiometry gradients in benthic systems. This is important because there may be distinct top–down effects of consumers on nutrient dynamics in green versus brown food webs that remain poorly known, given the lack of data. Indeed, the understudied components of CND (e.g., egestion, storage, and mortality) could notably distinguish brown food webs from their green counterparts (Atkinson et al., 2016). These directions will help workers understand the interplay of energy flow and nutrient cycling between green and brown food webs, advancing understanding of bottom–up changes like nutrient enrichment and furthering the application of ecological stoichiometry to systems along the continuum between green or brown.

AUTHOR CONTRIBUTIONS

ME-W led the review. HH led the meta-analysis.

FUNDING

This manuscript was supported in part by National Science Foundation DEB 1701808 and DEB1020722 to HH and ME-W, respectively.

ACKNOWLEDGMENTS

We thank all authors who kindly provided raw data for the meta-analysis: Michael Danger, Michael Kendrick, Carrie Deans, Liliana García, Amy Krist, Maurine Neiman, Clifton Ruehl, and Kaven Dionne.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01184/full#supplementary-material

REFERENCES

Aerts, R. (1997). Climate, leaf litter chemistry and leaf litter decomposition in terrestrial ecosystems: a triangular relationship. *Oikos* 79, 439–449. doi: 10.2307/3546886

Arsuffi, T. L., and Suberkropp, K. (1989). Selective feeding by shredders on leaf-colonizing stream fungi - comparison of macroinvertebrate taxa. *Oecologia* 79, 30–37. doi: 10.1007/BF00378236

Atkinson, C. L., Capps, K. A., Rugenski, A. T., and Vanni, M. J. (2016). Consumer-driven nutrient dynamics in freshwater ecosystems: from individuals to ecosystems. *Biol. Rev.* doi: 10.1111/brv.12318 [Epub ahead of print].

Back, J. A., and King, R. S. (2013). Sex and size matter: ontogenetic patterns of nutrient content of aquatic insects. *Freshw. Sci.* 32, 837–848. doi: 10.1899/12-181.1

Benstead, J. P., Hood, J. M., Whelan, N. V., Kendrick, M. R., Nelson, D., Hanninen, A. F., et al. (2014). Coupling of dietary phosphorus and growth across diverse fish taxa: a meta-analysis of experimental aquaculture studies. *Ecology* 95, 2768–2777. doi: 10.1890/13-1859.1

Benstead, J. P., Rosemond, A. D., Cross, W. F., Wallace, J. B., Eggert, S. L., Suberkropp, K., et al. (2009). Nutrient enrichment alters storage and fluxes of detritus in a headwater stream ecosystem. *Ecology* 90, 2556–2566. doi: 10.1890/08-0862.1

Boersma, M., and Elser, J. J. (2006). Too much of a good thing: on stoichiometrically balanced diets and maximal growth. *Ecology* 87, 1325–1330. doi: 10.1890/0012-9658(2006)87[1325:TMOAGT]2.0.CO;2

Bundschuh, M., and Mckie, B. G. (2016). An ecological and ecotoxicological perspective on fine particulate organic matter in streams. *Freshw. Biol.* 61, 2063–2074. doi: 10.1111/fwb.12608

Cebrian, J. (1999). Patterns in the fate of production in plant communities. *Am. Natural.* 154, 449–468. doi: 10.1086/303244

Cebrian, J. (2004). Role of first-order consumers in ecosystem carbon flow. *Ecol. Lett.* 7, 232–240. doi: 10.1111/j.1461-0248.2004.00574.x

Cebrian, J., and Lartigue, J. (2004). Patterns of herbivory and decomposition in aquatic and terrestrial ecosystems. *Ecol. Monogr.* 74, 237–259. doi: 10.1890/03-4019

Chapin, F. S., Matson, P. A., and Vitousek, P. (2002). *Principles of Terrestrial Ecosystem Ecology*. New York, NY: Springer-Verlag.

Cheever, B. M., Kratzer, E. B., and Webster, J. R. (2012). Immobilization and mineralization of N and P by heterotrophic microbes during leaf decomposition. *Freshw. Sci.* 31, 133–147. doi: 10.1899/11-060.1

Cheever, B. M., Webster, J. R., Bilger, E. E., and Thomas, S. A. (2013). The relative importance of exogenous and substrate-derived nitrogen for microbial growth during leaf decomposition. *Ecology* 94, 1614–1625. doi: 10.1890/12-1339.1

Cherif, M., and Loreau, M. (2013). Plant - herbivore - decomposer stoichiometric mismatches and nutrient cycling in ecosystems. *Proc. Biol. Sci.* 280:20122453. doi: 10.1098/rspb.2012.2453

Chung, N., and Suberkropp, K. (2009a). Contribution of fungal biomass to the growth of the shredder, *Pycnopsyche gentilis* (Trichoptera: Limnephilidae). *Freshw. Biol.* 54, 2212–2224. doi: 10.1111/j.1365-2427.2009.02260.x

Chung, N., and Suberkropp, K. (2009b). Effects of aquatic fungi on feeding preferences and bioenergetics of *Pycnopsyche gentilis* (Trichoptera: Limnephilidae). *Hydrobiologia* 630, 257–269. doi: 10.1007/s10750-009-9820-y

Clay, N. A., Yanoviak, S. P., and Kaspari, M. (2014). Short-term sodium inputs attract microbi-detritivores and their predators. *Soil Biol. Biochem.* 75, 248–253. doi: 10.1016/j.soilbio.2014.04.021

Cornwell, W. K., Cornelissen, J. H. C., Amatangelo, K., Dorrepaal, E., Eviner, V. T., Godoy, O., et al. (2008). Plant species traits are the predominant control on litter decomposition rates within biomes worldwide. *Ecol. Lett.* 11, 1065–1071. doi: 10.1111/j.1461-0248.2008.01219.x

Cotner, J. B., Hall, E. K., Scott, J. T., and Heldal, M. (2010). Freshwater bacteria are stoichiometrically flexible with a nutrient composition similar to seston. *Front. Microbiol.* 1:132. doi: 10.3389/fmicb.2010.00132

Cotner, J. B., Makino, W., and Biddanda, B. A. (2006). Temperature affects stoichiometry and biochemical composition of *Escherichia coli*. *Microb. Ecol.* 52, 26–33. doi: 10.1007/s00248-006-9040-1

Coulson, J. C., and Butterfield, J. (1978). Investigation of biotic factors determining rates of plant decomposition on blanket bog. *J. Ecol.* 66, 631–650. doi: 10.2307/2259155

Cross, W. F., Benstead, J. P., Frost, P. C., and Thomas, S. A. (2005). Ecological stoichiometry in freshwater benthic systems: recent progress and perspectives. *Freshw. Biol.* 50, 1895–1912. doi: 10.1111/j.1365-2427.2005.01458.x

Cross, W. F., Benstead, J. P., Rosemond, A. D., and Wallace, J. B. (2003). Consumer-resource stoichiometry in detritus-based streams. *Ecol. Lett.* 6, 721–732. doi: 10.1046/j.1461-0248.2003.00481.x

Cross, W. F., Hood, J. M., Benstead, J. P., Huryn, A. D., and Nelson, D. (2015). Interactions between temperature and nutrients across levels of ecological organization. *Glob. Change Biol.* 21, 1025–1040. doi: 10.1111/gcb.12809

Cross, W. F., Wallace, J. B., Rosemond, A. D., and Eggert, S. L. (2006). Whole-system nutrient enrichment increases secondary production in a detritus-based ecosystem. *Ecology* 87, 1556–1565. doi: 10.1890/0012-9658(2006)87[1556:WNEISP]2.0.CO;2

Danger, M., and Chauvet, E. (2013). Elemental composition and degree of homeostasis of fungi: are aquatic hyphomycetes more like metazoans, bacteria or plants? *Fungal Ecol.* 6, 453–457. doi: 10.1016/j.funeco.2013.05.007

Danger, M., Cornut, J., Chauvet, E., Chavez, P., Elger, A., and Lecerf, A. (2013a). Benthic algae stimulate leaf litter decomposition in detritus-based headwater streams: a case of aquatic priming effect? *Ecology* 94, 1604–1613.

Danger, M., Funck, J. A., Devin, S., Heberle, J., and Felten, V. (2013b). Phosphorus content in detritus controls life-history traits of a detritivore. *Funct. Ecol.* 27, 807–815. doi: 10.1111/1365-2435.12079

Danger, M., Gessner, M. O., and Barlocher, F. (2016). Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. *Fungal Ecol.* 19, 100–111. doi: 10.1016/j.funeco.2015.09.004

DeMott, W. R., Gulati, R. D., and Siewertsen, K. (1998). Effects of phosphorus-deficient diets on the carbon and phosphorus balance of Daphnia magna. *Limnol. Oceanogr.* 43, 1147–1161. doi: 10.4319/lo.1998.43.6.1147

DeMott, W. R., Mckinney, E. N., and Tessier, A. J. (2010). Ontogeny of digestion in Daphnia: implications for the effectiveness of algal defenses. *Ecology* 91, 540–548. doi: 10.1890/08-2103.1

DeMott, W. R., and Van Donk, E. (2013). Strong interactions between stoichiometric constraints and algal defenses: evidence from population dynamics of Daphnia and algae in phosphorus-limited microcosms. *Oecologia* 171, 175–186. doi: 10.1007/s00442-012-2404-y

Dodds, W. K. (2007). Trophic state, eutrophication and nutrient criteria in streams. *Trends Ecol. Evol.* 22, 669–676. doi: 10.1016/j.tree.2007.07.010

Dodds, W. K., Collins, S. M., Hamilton, S. K., Tank, J. L., Johnson, S., Webster, J. R., et al. (2014). You are not always what we think you eat: selective assimilation across multiple whole-stream isotopic tracer studies. *Ecology* 95, 2757–2767. doi: 10.1890/13-2276.1

Elliott, K. J., and White, A. S. (1994). Effects of light, nitrogen, and phophorus on red pine seedling growth and nutrient use efficiency. *For. Sci.* 40, 47–58.

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* 6, 936–943. doi: 10.1046/j.1461-0248.2003.00518.x

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x

Elser, J. J., Dobberfuhl, D. R., Mackay, N. A., and Schampel, J. H. (1996). Organism size, life history, and N:P stoichiometry. *Bioscience* 46, 674–684. doi: 10.2307/1312897

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., et al. (2000). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580. doi: 10.1038/35046058

Elser, J. J., and Urabe, J. (1999). The stoichiometry of consumer-driven nutrient recycling: theory, observations, and consequences. *Ecology* 80, 735–751. doi: 10.1890/0012-9658(1999)080[0735:TSOCDN]2.0.CO;2

Evans-White, M. A., and Lamberti, G. A. (2005). Grazer species effects on epilithon nutrient composition. *Freshw. Biol.* 50, 1853–1863. doi: 10.1111/j.1365-2427.2005.01452.x

Fanin, N., Fromin, N., Buatois, B., and Hattenschwiler, S. (2013). An experimental test of the hypothesis of non-homeostatic consumer stoichiometry in a plant litter-microbe system. *Ecol. Lett.* 16, 764–772. doi: 10.1111/ele.12108

Fanta, S. E., Hill, W. R., Smith, T. B., and Roberts, B. J. (2010). Applying the light: nutrient hypothesis to stream periphyton. *Freshw. Biol.* 55, 931–940. doi: 10.1111/j.1365-2427.2009.02309.x

Ferrão-Filho, A. D., Tessier, A. J., and DeMott, W. R. (2007). Sensitivity of herbivorous zooplankton to phosphorus-deficient diets: testing stoichiometric theory and the growth rate hypothesis. *Limnol. Oceanogr.* 52, 407–415. doi: 10.4319/lo.2007.52.1.0407

Ferreira, V., Castagneyrol, B., Koricheva, J., Gulis, V., Chauvet, E., and Graça, M. A. S. (2015). A meta-analysis of the effects of nutrient enrichment on litter decomposition in streams. *Biol. Rev.* 90, 669–688. doi: 10.1111/brv.12125

Fink, P., and Von Elert, E. (2006). Physiological responses to stoichiometric constraints: nutrient limitation and compensatory feeding in a freshwater snail. *Oikos* 115, 484–494. doi: 10.1111/j.2006.0030-1299.14951.x

Fisher, S. G., and Likens, G. E. (1973). Energy flow in bear brook, new hampshire: an integrative approach to stream ecosystem metabolism. *Ecol. Monogr.* 43, 421–439. doi: 10.2307/1942301

Flores, L., Larranaga, A., and Elosegi, A. (2014). Compensatory feeding of a stream detritivore alleviates the effects of poor food quality when enough food is supplied. *Freshw. Sci.* 33, 134–141. doi: 10.1086/674578

Fornara, D. A., and Du Toit, J. T. (2008). Browsing-induced effects on leaf litter quality and decomposition in a southern african savanna. *Ecosystems* 11, 238–249. doi: 10.1007/s10021-007-9119-7

Frainer, A., Jabiol, J., Gessner, M. O., Bruder, A., Chauvet, E., and Mckie, B. G. (2016). Stoichiometric imbalances between detritus and detritivores are related to shifts in ecosystem functioning. *Oikos* 125, 861–871. doi: 10.1111/oik.02687

Frost, P. C., Benstead, J. P., Cross, W. F., Hillebrand, H., Larson, J. H., Xenopoulos, M. A., et al. (2006). Threshold elemental ratios of carbon and phosphorus

in aquatic consumers. *Ecol. Lett.* 9, 774–779. doi: 10.1111/j.1461-0248.2006.00919.x

Frost, P. C., Cross, W. F., and Benstead, J. P. (2005a). Ecological stoichiometry in freshwater benthic ecosystems: an introduction. *Freshw. Biol.* 50, 1781–1785. doi: 10.1111/j.1365-2427.2005.01457.x

Frost, P. C., Evans-White, M. A., Finkel, Z. V., Jensen, T. C., and Matzek, V. (2005b). Are you what you eat? Physiological constraints on organismal stoichiometry in an elementally imbalanced world. *Oikos* 109, 18–28. doi: 10.1111/j.0030-1299.2005.14049.x

Fuller, C. L., Evans-White, M. A., and Entrekin, S. A. (2015). Growth and stoichiometry of a common aquatic detritivore respond to changes in resource stoichiometry. *Oecologia* 177, 837–848. doi: 10.1007/s00442-014-3154-9

Godwin, C. M., and Cotner, J. B. (2014). Carbon:phosphorus homeostasis of aquatic bacterial assemblages is mediated by shifts in assemblage composition. *Aquat. Microb. Ecol.* 73, 245–258. doi: 10.3354/ame01719

Godwin, C. M., and Cotner, J. B. (2015). Aquatic heterotrophic bacteria have highly flexible phosphorus content and biomass stoichiometry. *ISME J.* 9, 2324–2327. doi: 10.1038/ismej.2015.34

Golladay, S. W., Webster, J. R., and Benfield, E. F. (1983). Factors affecting food utilization by a leaf shredding aquatic insect - leaf species and conditioning time. *Holarctic Ecol.* 6, 157–162. doi: 10.1111/j.1600-0587.1983.tb01077.x

Guenet, B., Danger, M., Abbadie, L., and Lacroix, G. (2010). Priming effect: bridging the gap between terrestrial and aquatic ecology. *Ecology* 91, 2850–2861. doi: 10.1890/09-1968.1

Gulis, V., and Suberkropp, K. (2003). Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. *Freshw. Biol.* 48, 123–134. doi: 10.1046/j.1365-2427.2003.00985.x

Guo, F., Kainz, M. J., Valdez, D., Sheldon, F., and Bunn, S. E. (2016). High-quality algae attached to leaf litter boost invertebrate shredder growth. *Freshw. Sci.* 35, 1213–1221. doi: 10.1086/688667

Gusewell, S., and Gessner, M. O. (2009). N: P ratios influence litter decomposition and colonization by fungi and bacteria in microcosms. *Funct. Ecol.* 23, 211–219. doi: 10.1111/j.1365-2435.2008.01478.x

Hairston, N. G., Smith, F. E., and Slobodkin, L. B. (1960). Community structure, population control, and competition. *Am. Natural.* 94, 421–425. doi: 10.1086/282146

Halvorson, H. M., Fuller, C., Entrekin, S. A., and Evans-White, M. A. (2015a). Dietary influences on production, stoichiometry and decomposition of particulate wastes from shredders. *Freshw. Biol.* 60, 466–478. doi: 10.1111/fwb.12462

Halvorson, H. M., Hall, D. J., and Evans-White, M. A. (2017). Long-term stoichiometry and fates highlight animal egestion as nutrient repackaging, not recycling, in aquatic ecosystems. *Funct. Ecol.* doi: 10.1111/1365-2435.12875 [Epub ahead of print].

Halvorson, H. M., Scott, E. E., Entrekin, S. A., Evans-White, M. A., and Scott, J. T. (2016a). Light and dissolved phosphorus interactively affect microbial metabolism, stoichiometry and decomposition of leaf litter. *Freshw. Biol.* 61, 1006–1019. doi: 10.1111/fwb.12763

Halvorson, H. M., Scott, J. T., Sanders, A. J., and Evans-White, M. A. (2015b). A stream insect detritivore violates common assumptions of threshold elemental ratio bioenergetics models. *Freshw. Sci.* 34, 508–518. doi: 10.1086/680724

Halvorson, H. M., and Small, G. E. (2016). Observational field studies are not appropriate tests of consumer stoichiometric homeostasis. *Freshw. Sci.* 35, 1103–1116. doi: 10.1086/689212

Halvorson, H. M., White, G., Scott, J. T., and Evans-White, M. A. (2016b). Dietary and taxonomic controls on incorporation of microbial carbon and phosphorus by detritivorous caddisflies. *Oecologia* 180, 567–579.

Hawlena, D., and Schmitz, O. J. (2010). Herbivore physiological response to predation risk and implications for ecosystem nutrient dynamics. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15503–15507. doi: 10.1073/pnas.1009300107

Heal, O. W., and Maclean, S. (1975). "Comparative productivity in ecosystems–secondary productivity," in *The Unifying Concepts in Ecology*, ed. W. H. Van Dobben (Amsterdam: Elsevier), 89–108.

Higgins, K. A., Vanni, M. J., and Gonzalez, M. J. (2006). Detritivory and the stoichiometry of nutrient cycling by a dominant fish species in lakes of varying productivity. *Oikos* 114, 419–430. doi: 10.1111/j.2006.0030-1299.14745.x

Hill, W. R., and Fanta, S. E. (2008). Phosphorus and light colimit periphyton growth at subsaturating irradiances. *Freshw. Biol.* 53, 215–225.

Hill, W. R., Fanta, S. E., and Roberts, B. J. (2009). Quantifying phosphorus and light effects in stream algae. *Limnol. Oceanogr.* 54, 368–380. doi: 10.4319/lo.2009.54.1.0368

Hill, W. R., and Griffiths, N. A. (2017). Nitrogen processing by grazers in a headwater stream: riparian connections. *Freshw. Biol.* 62, 17–29. doi: 10.1111/fwb.12833

Hobbs, N. T. (1996). Modification of ecosystems by ungulates. *J. Wildlife Manag.* 60, 695–713. doi: 10.2307/3802368

Hood, J. M., Mcneely, C., Finlay, J. C., and Sterner, R. W. (2014). Selective feeding determines patterns of nutrient release by stream invertebrates. *Freshw. Sci.* 33, 1093–1107. doi: 10.1086/678693

Hood, J. M., and Sterner, R. W. (2014). Carbon and phosphorus linkages in Daphnia growth are determined by growth rate, not species or diet. *Funct. Ecol.* 28, 1156–1165. doi: 10.1111/1365-2435.12243

Johnson, N. C., Wilson, G. W. T., Bowker, M. A., Wilson, J. A., and Miller, R. M. (2010). Resource limitation is a driver of local adaptation in mycorrhizal symbioses. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2093–2098. doi: 10.1073/pnas.0906710107

Kaiser, C., Franklin, O., Dieckmann, U., and Richter, A. (2014). Microbial community dynamics alleviate stoichiometric constraints during litter decay. *Ecol. Lett.* 17, 680–690. doi: 10.1111/ele.12269

Kendrick, M. R., and Benstead, J. P. (2013). Temperature and nutrient availability interact to mediate growth and body stoichiometry in a detritivorous stream insect. *Freshw. Biol.* 58, 1820–1830. doi: 10.1111/fwb.12170

Knoll, L. B., Mcintyre, P. B., Vanni, M. J., and Flecker, A. S. (2009). Feedbacks of consumer nutrient recycling on producer biomass and stoichiometry: separating direct and indirect effects. *Oikos* 118, 1732–1742. doi: 10.1111/j.1600-0706.2009.17367.x

Kominoski, J. S., Rosemond, A. D., Benstead, J. P., Gulis, V., Maerz, J. C., and Manning, D. W. P. (2015). Low-to-moderate nitrogen and phosphorus concentrations accelerate microbially driven litter breakdown rates. *Ecol. Appl.* 25, 856–865. doi: 10.1890/14-1113.1

Kuehn, K. A., Francoeur, S. N., Findlay, R. H., and Neely, R. K. (2014). Priming in the microbial landscape: periphytic algal stimulation of litter-associated microbial decomposers. *Ecology* 95, 749–762. doi: 10.1890/13-0430.1

Kuzyakov, Y., Friedel, J. K., and Stahr, K. (2000). Review of mechanisms and quantification of priming effects. *Soil Biol. Biochem.* 32, 1485–1498. doi: 10.1016/S0038-0717(00)00084-5

Lagrue, C., Kominoski, J. S., Danger, M., Baudoin, J.-M., Lamothe, S., Lambrigot, D., et al. (2011). Experimental shading alters leaf litter breakdown in streams of contrasting riparian canopy cover. *Freshw. Biol.* 56, 2059–2069. doi: 10.1111/j.1365-2427.2011.02637.x

Lecerf, A., and Chauvet, E. (2008). Diversity and functions of leaf-decaying fungi in human-altered streams. *Freshw. Biol.* 53, 1658–1672. doi: 10.1111/j.1365-2427.2008.01986.x

Lemoine, N. P., Giery, S. T., and Burkepile, D. E. (2014). Differing nutritional constraints of consumers across ecosystems. *Oecologia* 174, 1367–1376. doi: 10.1007/s00442-013-2860-z

Liess, A. (2014). Compensatory feeding and low nutrient assimilation efficiencies lead to high nutrient turnover in nitrogen-limited snails. *Freshw. Sci.* 33, 425–434. doi: 10.1086/675533

Liess, A., and Haglund, A. L. (2007). Periphyton responds differentially to nutrients recycled in dissolved or faecal pellet form by the snail grazer *Theodoxus fluviatilis*. *Freshw. Biol.* 52, 1997–2008. doi: 10.1111/j.1365-2427.2007.01825.x

Lindeman, R. L. (1942). The trophic-dynamic aspect of ecology. *Ecology* 23, 399–418. doi: 10.2307/1930126

Liu, J., Wu, N. N., Wang, H., Sun, J. F., Peng, B., Jiang, P., et al. (2016). Nitrogen addition affects chemical compositions of plant tissues, litter and soil organic matter. *Ecology* 97, 1796–1806. doi: 10.1890/15-1683.1

Makino, W., Cotner, J. B., Sterner, R. W., and Elser, J. J. (2003). Are bacteria more like plants or animals? Growth rate and resource dependence of bacterial C: N: P stoichiometry. *Funct. Ecol.* 17, 121–130. doi: 10.1046/j.1365-2435.2003.00712.x

Manning, D. W. P., Rosemond, A. D., Gulis, V., Benstead, J. P., Kominoski, J. S., and Maerz, J. C. (2016). Convergence of detrital stoichiometry predicts thresholds of nutrient-stimulated breakdown in streams. *Ecol. Appl.* 26, 1745–1757. doi: 10.1890/15-1217.1

Manning, D. W. P., Rosemond, A. D., Kominoski, J. S., Gulis, V., Benstead, J. P., and Maerz, J. C. (2015). Detrital stoichiometry as a critical nexus for the effects of streamwater nutrients on leaf litter breakdown rates. *Ecology* 96, 2214–2224. doi: 10.1890/14-1582.1

Manzoni, S. (2017). Flexible carbon-use efficiency across litter types and during decomposition partly compensates nutrient imbalances-results from analytical stoichiometric models. *Front. Microbiol.* 8:661. doi: 10.3389/fmicb.2017.00661

Manzoni, S., Jackson, R. B., Trofymow, J. A., and Porporato, A. (2008). The global stoichiometry of litter nitrogen mineralization. *Science* 321, 684–686. doi: 10.1126/science.1159792

Manzoni, S., Trofymow, J. A., Jackson, R. B., and Porporato, A. (2010). Stoichiometric controls on carbon, nitrogen, and phosphorus dynamics in decomposing litter. *Ecol. Monogr.* 80, 89–106. doi: 10.1890/09-0179.1

Martin, M. M., Martin, J. S., Kukor, J. J., and Merritt, R. W. (1980). The digestion of protein and carbohydrate by the stream detritivore, Tipula abdominalis (Diptera, Tipulidae). *Oecologia* 46, 360–364. doi: 10.1007/BF00346265

Mcgroddy, M. E., Daufresne, T., and Hedin, L. O. (2004). Scaling of C: N: P stoichiometry in forests worldwide: implications of terrestrial redfield-type ratios. *Ecology* 85, 2390–2401. doi: 10.1890/03-0351

McManamay, R. A., Webster, J. R., Valett, H. M., and Dolloff, C. A. (2011). Does diet influence consumer nutrient cycling? Macroinvertebrate and fish excretion in streams. *J. N. Am. Benthol. Soc.* 30, 84–102. doi: 10.1899/09-152.1

McNaughton, S. J., Banyikwa, F. F., and Mcnaughton, M. M. (1997a). Promotion of the cycling of diet-enhancing nutrients by African grazers. *Science* 278, 1798–1800.

McNaughton, S. J., Zuniga, G., Mcnaughton, M. M., and Banyikwa, F. F. (1997b). Ecosystem catalysis: soil urease activity and grazing in the Serengeti ecosystem. *Oikos* 80, 467–469. doi: 10.2307/3546619

Moe, S. J., Stelzer, R. S., Forman, M. R., Harpole, W. S., Daufresne, T., and Yoshida, T. (2005). Recent advances in ecological stoichiometry: insights for population and community ecology. *Oikos* 109, 29–39. doi: 10.1111/j.0030-1299.2005.14056.x

Moody, E. K., Corman, J. R., Elser, J. J., and Sabo, J. L. (2015). Diet composition affects the rate and N:P ratio of fish excretion. *Freshw. Biol.* 60, 456–465. doi: 10.1111/fwb.12500

Moore, J. C., Berlow, E. L., Coleman, D. C., De Ruiter, P. C., Dong, Q., Hastings, A., et al. (2004). Detritus, trophic dynamics and biodiversity. *Ecol. Lett.* 7, 584–600. doi: 10.1111/j.1461-0248.2004.00606.x

Morehouse, R. L., Dzialowski, A. R., and Jeyasingh, P. D. (2013). Impacts of excessive dietary phosphorus on zebra mussels. *Hydrobiologia* 707, 73–80. doi: 10.1007/s10750-012-1407-3

Muller-Navarra, D. C., Brett, M. T., Liston, A. M., and Goldman, C. R. (2000). A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature* 403, 74–77.

Navel, S., Simon, L., Lecuyer, C., Fourel, F., and Mermillod-Blondin, F. (2011). The shredding activity of gammarids facilitates the processing of organic matter by the subterranean amphipod *Niphargus rhenorhodanensis*. *Freshwater Biology* 56, 481–490. doi: 10.1111/j.1365-2427.2010.02513.x

Norlin, L., Bystrom, P., Karlsson, J., Johansson, M., and Liess, A. (2016). Climate change will alter amphibian-mediated nutrient pathways: evidence from Rana temporaria tadpoles in experimental ponds. *Freshw. Biol.* 61, 472–485. doi: 10.1111/fwb.12720

Ott, D., Rall, B. C., and Brose, U. (2012). Climate change effects on macrofaunal litter decomposition: the interplay of temperature, body masses and stoichiometry. *Philos. Trans. R. Soc. B-Biol. Sci.* 367, 3025–3032. doi: 10.1098/rstb.2012.0240

Pasek, J. (2016). *Weights: Weighting and Weighted Statistics. R package version 0.85*. Available at: https://cran.r-project.org/web/packages/weights/weights.pdf

Pastor, J., Cohen, Y., and Hobbs, N. T. (2006). "The roles of large herbivores in ecosystem nutrient cycles," in *Large Herbivore Ecology, Ecosystem Dynamics and Conservation*, eds K. Danell, P. Duncan, R. Bergström, and J. Pastor (New York, NY: Cambridge University Press), 289–325. doi: 10.1017/CBO9780511617461.012

Peñuelas, J., Poulter, B., Sardans, J., Ciais, P., Van Der Velde, M., Bopp, L., et al. (2013). Human-induced nitrogen-phosphorus imbalances alter natural and managed ecosystems across the globe. *Nat. Commun.* 4:2934. doi: 10.1038/ncomms3934

Persson, J., Fink, P., Goto, A., Hood, J. M., Jonas, J., and Kato, S. (2010). To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos* 119, 741–751. doi: 10.1111/j.1600-0706.2009.18545.x

Plath, K., and Boersma, M. (2001). Mineral limitation of zooplankton: stoichiometric constraints and optimal foraging. *Ecology* 82, 1260–1269. doi: 10.1890/0012-9658(2001)082[1260:MLOZSC]2.0.CO;2

Polis, G. A., and Strong, D. R. (1996). Food web complexity and community dynamics. *Am. Natural.* 147, 813–846. doi: 10.1086/285880

R Core Team (2013). *R: A Language and Environmental for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rosemond, A. D., Benstead, J. P., Bumpers, P. M., Gulis, V., Kominoski, J. S., Manning, D. W. P., et al. (2015). Experimental nutrient additions accelerate terrestrial carbon loss from stream ecosystems. *Science* 347, 1142–1145. doi: 10.1126/science.aaa1958

Rosenberg, M. S. (2013). "Moment and least-squares based approaches to meta-analytic inference," in *Handbook of Meta-analysis in Ecology and Evolution*, eds J. Koricheva, J. Gurevitch, and K. Mengerson (Princeton, NJ: Princeton University Press), 108–124.

Rosenberg, M. S., Rothstein, H. R., and Gurevitch, J. (2013). "Effect sizes: conventional choice and calculations," in *Handbook of Meta-analysis in Ecology and Evolution*, eds J. Koricheva, J. Gurevitch, and K. Mengerson (Princeton, NJ: Princeton University Press), 61–71.

Rothlisberger, J. D., Baker, M. A., and Frost, P. C. (2008). Effects of periphyton stoichiometry on mayfly excretion rates and nutrient ratios. *J. N. Am. Benthol. Soc.* 27, 497–508. doi: 10.1899/07-145.1

Rugenski, A. T., Murria, C., and Whiles, M. R. (2012). Tadpoles enhance microbial activity and leaf decomposition in a neotropical headwater stream. *Freshw. Biol.* 57, 1904–1913. doi: 10.1111/j.1365-2427.2012.02853.x

Scott, E. E., Prater, C., Norman, E., Baker, B. C., Evans-White, M., and Scott, J. T. (2013). Leaf-litter stoichiometry is affected by streamwater phosphorus concentrations and litter type. *Freshw. Sci.* 32, 753–761. doi: 10.1899/12-215.1

Scott, J. T., Cotner, J. B., and Lapara, T. M. (2012). Variable stoichiometry and homeostatic regulation of bacterial biomass elemental composition. *Front. Microbiol.* 3:42. doi: 10.3389/fmicb.2012.00042

Senior, A. M., Grueber, C. E., Kamiya, T., Lagisz, M., O'dwyer, K., Santos, E. S. A., et al. (2016). Heterogeneity in ecological and evolutionary meta-analyses: its magnitude and implications. *Ecology* 97, 3293–3299. doi: 10.1002/ecy.1591

Sitters, J., Bakker, E. S., Veldhuis, M. P., Veen, G. F., Venterink, H. O., and Vanni, M. J. (2017). The stoichiometry of nutrient release by terrestrial herbivores and its ecosystem consequences. *Front. Earth Sci.* 5:32. doi: 10.3389/feart.2017.00032

Sitters, J., Maechler, M. J., Edwards, P. J., Suter, W., and Venterink, H. O. (2014). Interactions between C: N: P stoichiometry and soil macrofauna control dung decomposition of savanna herbivores. *Funct. Ecol.* 28, 776–786. doi: 10.1111/1365-2435.12213

Snyder, M. N., Small, G. E., and Pringle, C. M. (2015). Diet-switching by omnivorous freshwater shrimp diminishes differences in nutrient recycling rates and body stoichiometry across a food quality gradient. *Freshw. Biol.* 60, 526–536. doi: 10.1111/fwb.12481

Sperfeld, E., Halvorson, H. M., Malishev, M., Clissold, F. J., and Wagner, N. D. (2016). Woodstoich III: integrating tools of nutritional geometry and ecological stoichiometry to advance nutrient budgeting and the prediction of consumer-driven nutrient recycling. *Oikos* 125, 1539–1553. doi: 10.1111/oik.03529

Sperfeld, E., Wagner, N. D., Halvorson, H. M., Malishev, M., and Raubenheimer, D. (2017). Bridging ecological stoichiometry and nutritional geometry with homeostasis concepts and integrative models of organism nutrition. *Funct. Ecol.* 31, 286–296. doi: 10.1111/1365-2435.12707

Sterner, R. W. (1986). Herbivores direct and indirect effects on algal populations. *Science* 231, 605–607. doi: 10.1126/science.231.4738.605

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry the Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Sterner, R. W., Elser, J. J., Fee, E. J., Guildford, S. J., and Chrzanowski, T. H. (1997). The light:nutrient ratio in lakes: the balance of energy and materials affects ecosystem structure and process. *Am. Natural.* 150, 663–684. doi: 10.1086/286088

Stoler, A. B., Golembieski, M. N., Stephens, J. P., and Raffel, T. R. (2016). Differential consumption and assimilation of leaf litter by wetland herbivores:

alternative pathways for decomposition and trophic transfer. *Freshw. Sci.* 35, 178–187. doi: 10.1086/684205

Suberkropp, K., and Chauvet, E. (1995). Regulation of leaf breakdown by fungi in streams - influences of water chemistry. *Ecology* 76, 1433–1445. doi: 10.2307/1938146

Treseder, K. K. (2004). A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO2 in field studies. *New Phytol.* 164, 347–355. doi: 10.1111/j.1469-8137.2004.01159.x

Valera-Burgos, J., Zunzunegui, M., and Diaz-Barradas, M. C. (2013). Do leaf traits and nitrogen supply affect decomposability rates of three Mediterranean species growing under different competition levels? *Pedobiologia* 56, 113–119. doi: 10.1016/j.pedobi.2013.03.002

Vanni, M. J. (2002). Nutrient cycling by animals in freshwater ecosystems. *Ann. Rev. Ecol. Syst.* 33, 341–370. doi: 10.1146/annurev.ecolsys.33.010802.150519

Vanni, M. J., Boros, G., and Mcintyre, P. B. (2013). When are fish sources vs. sinks of nutrients in lake ecosystems?. *Ecology* 94, 2195–2206. doi: 10.1890/12-1559.1

Vanni, M. J., Flecker, A. S., Hood, J. M., and Headworth, J. L. (2002). Stoichiometry of nutrient recycling by vertebrates in a tropical stream: linking species identity and ecosystem processes. *Ecol. Lett.* 5, 285–293. doi: 10.1046/j.1461-0248.2002.00314.x

Vanni, M. J., and Mcintyre, P. B. (2016). Predicting nutrient excretion of aquatic animals with metabolic ecology and ecological stoichiometry: a global synthesis. *Ecology* 97, 3460–3471. doi: 10.1002/ecy.1582

Vergutz, L., Manzoni, S., Porporato, A., Novais, R. F., and Jackson, R. B. (2012). Global resorption efficiencies and concentrations of carbon and nutrients in leaves of terrestrial plants. *Ecol. Monogr.* 82, 205–220. doi: 10.1890/11-0416.1

Villanueva, V. D., Albarino, R., and Canhoto, C. (2012). Positive effect of shredders on microbial biomass and decomposition in stream microcosms. *Freshw. Biol.* 57, 2504–2513. doi: 10.1111/fwb.12023

Wetzel, R. G., Hatcher, P. G., and Bianchi, T. S. (1995). Natural photolysis by ultraviolet irradiance of recalcitrant dissolved organic matter to simple substrates for rapid bacterial metabolism. *Limnol. Oceanogr.* 40, 1369–1380. doi: 10.4319/lo.1995.40.8.1369

Wolkovich, E. M., Allesina, S., Cottingham, K. L., Moore, J. C., Sandin, S. A., and De Mazancourt, C. (2014). Linking the green and brown worlds: the prevalence and effect of multichannel feeding in food webs. *Ecology* 95, 3376–3386. doi: 10.1890/13-1721.1

Wotton, R. S., and Malmqvist, B. (2001). Feces in aquatic ecosystems. *Bioscience* 51, 537–544. doi: 10.1641/0006-3568(2001)051[0537:FIAE]2.0.CO;2

Wyatt, K. H., and Turetsky, M. R. (2015). Algae alleviate carbon limitation of heterotrophic bacteria in a boreal peatland. *J. Ecol.* 103, 1165–1171. doi: 10.1111/1365-2745.12455

Xu, X. N., and Hirata, E. J. (2005). Decomposition patterns of leaf litter of seven common canopy species in a subtropical forest: N and P dynamics. *Plant Soil* 273, 279–289. doi: 10.1007/s11104-004-8069-5

Zanotto, F. P., Simpson, S. J., and Raubenheimer, D. (1993). The regulation of growth by locusts through postingestive compensation for variation in the levels of dietary-protein and carbohydrate. *Physiol. Entomol.* 18, 425–434. doi: 10.1111/j.1365-3032.1993.tb00617.x

Zou, K. J., Thebault, E., Lacroix, G., and Barot, S. (2016). Interactions between the green and brown food web determine ecosystem functioning. *Funct. Ecol.* 30, 1454–1465. doi: 10.1111/1365-2435.12626

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 09 May 2017
doi: 10.3389/fmicb.2017.00826

The Importance of H in Particulate Organic Matter Stoichiometry, Export and Energy Flow

David M. Karl and Eric Grabowski*

Daniel K. Inouye Center for Microbial Oceanography: Research and Education, Department of Oceanography, School of Ocean and Earth Science and Technology, University of Hawaii, Honolulu, HI, USA

The discipline of marine ecological stoichiometry has progressed rapidly over the past two decades, and continues to be at the forefront of microbial oceanography. Most of this effort has been focused on the elements carbon (C) and nitrogen (N), and to a lesser extent phosphorus (P), with little consideration of hydrogen (H), or the redox state of the organic matter pools despite the fact that H is the most abundant, and possibly the most important, element in biogeochemistry. Obtaining accurate estimates of the H content of organic matter, either in suspended or sinking particles, is a major analytical challenge. While many aquatic science laboratories have access to commercial "C–H–N elemental analyzers," few investigators report H values due to analytical difficulties in obtaining accurate estimates of H. Because organic compounds vary considerably in their H:C ratio and therefore in their energy content, measurements of H combined with C-specific caloric estimates will ultimately be required for a more comprehensive understanding of ecosystem dynamics.

Keywords: hydrogen, energy, organic matter, particle flux, stoichiometry

OPEN ACCESS

Edited by:
James Joseph Elser, University of Montana, USA

Reviewed by:
James T. Hollibaugh, University of Georgia, USA
Hans-Peter Grossart, Leibniz Institute of Freshwater Ecology and Inland Fisheries (LG), Germany

****Correspondence:***
David M. Karl
dkarl@hawaii.edu

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *11 January 2017*
Accepted: *24 April 2017*
Published: *09 May 2017*

Citation:
Karl DM and Grabowski E (2017) The Importance of H in Particulate Organic Matter Stoichiometry, Export and Energy Flow. Front. Microbiol. 8:826. doi: 10.3389/fmicb.2017.00826

INTRODUCTION

More than one century ago, in his authoritative treatise on *Conditions of Life in the Sea*, James Johnstone stated that "chemical analysis shows that the animal and plant body is mainly built up from four elements: nitrogen (N), carbon (C), hydrogen (H), and oxygen (O). Added to these are the metals sodium, potassium, and iron, and the non-metals chlorine, sulfur (S), and phosphorus (P)" (Johnstone, 1908). He went on to conclude "that in an exhaustive study of the cycle of matter from the living to the non-living phases, and vice versa, we should have to trace the course of each."

Pioneering research conducted by Alfred C. Redfield on the composition of marine plankton, which first appeared in the James Johnstone Memorial Volume, established a quantitative relationship between dissolved nitrate and phosphate, and between dissolved nitrate and total carbon dioxide in water column profiles from the western North Atlantic Ocean. The mean molar changes with depth, expressed as $\Delta N{:}\Delta P$ and $\Delta C{:}\Delta N$ ratios, were 20:1 and 7:1, respectively, or a C:N:P molar ratio of approximately 140:20:1 (Redfield, 1934). Furthermore, the molar ratio of dissolved oxygen (O_2) decrease to dissolved nitrate increase was 6:1, suggesting that microbial decomposition of organic matter was the ultimate source of nitrate in the mesopelagic zone (400–1,000 m) of the ocean (Redfield, 1934). Furthermore, elementary analysis of naturally occurring plankton samples yielded proportions of C, N, and P that were "not greatly different" from those observed in oceanic waters. Redfield lamented that this exact balance of N and P, two major plant nutrients, "calls for some explanation" since it would appear that it was more than a coincidence (Redfield, 1934). Several potential mechanisms were presented to explain what Redfield termed

"a phenomenon of the greatest interest" thereby setting the stage for the ecological stoichiometry revolution that was to follow (Sterner and Elser, 2002).

Subsequent research by Redfield and many others led to a more comprehensive understanding of biological control of nutrient distributions in the sea, and the structure and pace of biogeochemical cycles. Redfield (1958) also laid the foundation for what was later dubbed the biological pump. The main components included: (1) photosynthetic production of organic matter in the upper 0–200 m of the water column, (2) loss of a small portion of the newly synthesized organic matter by the combined effects of passive sinking and active vertical migration, and (3) decomposition of organic matter at depth to regenerate inorganic nutrients. Both the net production and net remineralization of organic matter resulted in changes in the concentrations of dissolved inorganic C, N, and P, and the quantity of O_2 required for complete oxidation reflected both the elemental composition and the redox state of the major biogenic constituents. This quantitative analysis of photosynthesis and respiration, including restoration of the N:P ratio via N_2 fixation and extension to anaerobic sulfate reduction processes in selected regions where the resupply of O_2 was restricted, established the unifying principle that we now commonly refer to as "the Redfield–Ketchum–Richards (R–K–R) Ratio" (Redfield et al., 1963).

ELEMENTAL COMPOSITION OF MARINE PHYTOPLANKTON

The R–K–R model assumes that marine phytoplankton have an average molar composition of $C_{106}H_{263}O_{110}N_{16}P$, and further assumes that the complete oxidation of this model organic matter following cell death would consume 138 molecules of O_2. These values, or slight variations of this elemental stoichiometry (see below), have been used for nearly 50 years to model biogeochemical processes in the sea (Sarmiento and Gruber, 2006). However, the amount of O_2 consumed during complete microbial remineralization of organic matter depends critically on its molecular composition, specifically the bulk H:C ratio, the S content and the presence or absence of reduced storage materials. Furthermore, no previous study of ecological stoichiometry has considered the possible presence of reduced P (valence states $< +5$) which is now established as a ubiquitous constituent of marine organic matter (Karl, 2014; Van Mooy et al., 2015; Repeta et al., 2016). Finally, for mass balance consideration of ecological stoichiometry and reduction–oxidation processes, one also needs to consider dissolved organic matter (DOM), reduced gases (e.g., H_2, N_2O, CO, CH_4, dimethyl sulfide (DMS)/H_2S) and reduced inorganic compounds that may be produced and released during photosynthesis, and that ultimately contribute to the demand for O_2 during subsequent microbial oxidation. For example, during the process of N_2 fixation, H_2 is produced in stoichiometric proportion to N_2 reduced. A variable portion of this H_2 is lost from the cell, along with ammonia and dissolved organic N. These reduced compounds are in excess of those required for the synthesis of new plankton biomass and should be considered for an accurate accounting of ecological stoichiometry, biochemical O_2 demand and energy flow in marine systems.

All living organisms are comprised of basic macromolecules including carbohydrate, lipid, protein, and nucleic acid, each with a unique bulk elemental stoichiometry and energy content. Direct analysis of the macromolecular composition of sample material, a so-called proximate analysis, has been used to estimate the energy content of organic matter by the application of class-specific conversion factors (e.g., 9.45 calories mg^{-1} for lipid, 5.65 calories mg^{-1} for protein; Craig et al., 1978). This approach fails to account for all possible organic and reduced inorganic compounds, and is probably more accurately applied to pure cultures or tissues than to naturally occurring dissolved and particulate organic matter (POM). Since the H:C and O:C ratios among these macromolecular classes vary considerably (Laws, 1991), the theoretical amount of reducing potential required for biosynthesis and of O_2 consumed for complete oxidation following death (generally expressed as the $-O_2$:P ratio; see below) will also vary. Based on considerations of the proximate analysis of typical marine phytoplankton cells, Anderson (1995) concluded that the R–K–R elemental stoichiometry (i.e., $C_{106}H_{263}O_{110}N_{16}P$) was too high in both H and O, relative to P. He proposed a new formulation, $C_{106}H_{175 \pm 11}O_{42 \pm 16}N_{16}P$ with a ΔO_2: ΔP ratio of -150 ± 19, as a more accurate representation. The H and O contents for this revised elemental stoichiometry are 32–35% and 50–74%, respectively, lower than the R–K–R model plankton (Anderson, 1995).

Platt and Irwin (1972) reported a partial proximate analysis (i.e., carbohydrates, protein, and lipid, but did not include nucleic acids or storage products), the bulk C:N ratios and caloric content (measured directly using a Phillipson microbomb calorimeter; Phillipson, 1964) for "phytoplankton" samples (76–153 μm size fraction) collected during the spring bloom (April 1969) in St. Margaret's Bay, Nova Scotia. This comprehensive, 1-month field study revealed large day-to-day variations in percentage composition of major macromolecular classes [relative to total dry weight (dw)]. For example, the % carbohydrate:% protein ratios ranged from 0.56 to 2.45, and % carbohydrate:% lipid ratios ranged from 0.74 to 8.9, while bulk C:N molar ratios ranged from 5.5 to 17.2. Despite this time-variable composition, they observed a much smaller range in the C-specific caloric content from 10.07 to 13.66 calories mg^{-1} C. Platt and Irwin (1972) concluded that "percent carbon in dry phytoplankton should be a good predictor of its caloric value," and presented the following relationships for general use in marine ecology: calories (mg dw^{-1}) $= 0.632 + 0.086$ (%C), and if N content is also known: calories (mg dw^{-1}) $= -0.555 + 0.113$ (%C) $+ 0.054$ (C:N).

The approach proposed by Platt and Irwin (1972) was developed for the phytoplankton-enriched portion of the spring bloom where most of the POM pool was comprised of living cells. However, in most ecosystems the organic matter pool is dominated (>90% by mass) by DOM, not POM, and the large, chemically complex DOM pool may have a molecular composition and elemental stoichiometry that is different from living cells. For example, whereas the contributions of amino

acids, neutral sugars, and lipids together account for >80% of the organic C content of phytoplankton, they contribute <10% of the total C in DOM (Benner, 2002). Although the molecular composition of DOM is poorly known, significant progress has been made in recent years using several DOM extraction methods and a variety of high resolution analytical techniques (Repeta, 2015). For open ocean environments, the H:C and O:C ratios of DOM collected by solid-phase extraction (SPE) of DOM onto polymeric resins (e.g., Amberlite® XAD®), range from 0.5 to 1.7 and 0.2 to 0.8, respectively, and are mostly outside the range of proteins, carbohydrates, and lipids, suggesting that SPE-DOM is either extensively degraded relative to plankton or has some other non-marine source (Repeta, 2015). Humic substances, operationally defined as SPE-DOM that is retained on XAD-2 or XAD-8 resins under acidic conditions (Benner, 2002), are ubiquitous in marine environments and appear to be resistant to microbial decomposition. However, selected microorganisms can use humic substances as electron acceptors during anaerobic metabolism, thereby facilitating the degradation of organic matter (Lovley et al., 1996).

Furthermore, in most oligotrophic environments, a majority of the POM pool is non-living (Karl and Dobbs, 1998), so it too may be chemically distinct from the elemental composition of biomass. Lee et al. (2004) have shown that the preferential removal of certain components from POM leads to changes in the chemical composition of the residual material as particles sink through the water column. Compared to surface ocean plankton where ∼85% of total C can be chemically identified, the majority of POM collected in a sediment trap deployed at a 1,000 m reference depth could not be characterized. Consequently, models of the molecular composition of organic matter that are based on living cells may not accurately reflect organic matter that is present in natural ecosystems. Compared to a wealth of field data on particulate organic C:N:P, and a much smaller data base on dissolved organic C:N:P, there is a virtual absence (with very few notable exceptions; see below) of direct measurements of the H, O, S, or total caloric content of either the POM or DOM pools. This situation exists despite the central role of H in stoichiometry and energy flow in marine ecosystems.

REMINERALIZATION RATIOS IN THE OPEN SEA

Several field studies, beginning with Takahashi et al. (1985), have measured concentrations of phosphate, nitrate, total carbon dioxide, and O_2 along deep isopycnal horizons to constrain the elemental composition of the organic matter that supplied the deep sea with excess regenerated nutrients via aerobic microbial activity. For intermediate waters of the North Atlantic Ocean

TABLE 1 | Elemental analysis of sinking particulate organic matter collected in the North Pacific Subtropical Gyre.

Study location, water depth (m), and reference	Trap depth (m)	C flux (mg m^{-2} d^{-1})	H flux (mg m^{-2} d^{-1})	N flux (mg m^{-2} d^{-1})	H:C (molar)	C:N (molar)
15°21′N, 151°29′W,	378	3.56	0.53	0.46	1.79	9.00
5,792 m (Honjo, 1980)	978	0.55	0.07	0.07	1.53	9.17
	2,778	1.09	0.14	0.12	1.54	10.6
	4,280	0.88	0.11	0.10	1.50	10.3
	5,582	0.66	0.09	0.08	1.64	9.62
28°N, 155°W, 5,000 m	100*	38.5	4.05	6.41	1.26	6.99
(Martin et al., 1987)	200	19.6	2.20	2.97	1.35	7.70
	300	13.2	1.54	1.89	1.40	8.15
	500	8.04	0.97	1.07	1.45	8.77
	750	5.42	0.68	0.68	1.51	9.30
	1,000	4.10	0.53	0.50	1.55	9.52
	1,500	2.76	0.37	0.32	1.61	10.1
	2,000	2.09	0.29	0.23	1.67	10.6
22°45′N, 158°W,	100	43.8 ± 0.6	6.11 ± 0.04	7.10 ± 0.08	1.66	7.21
Station ALOHA,	110	46.8 ± 0.9	6.40 ± 0.08	7.42 ± 0.14	1.63	7.36
4,850 m (This study)	120	51.7 ± 1.6	6.93 ± 0.24	8.19 ± 0.50	1.60	7.37
	130	33.0 ± 0.9	4.24 ± 0.18	4.70 ± 0.29	1.53	8.19
	140	33.8 ± 0.2	4.22 ± 0.03	5.03 ± 0.10	1.49	7.83
	150	34.2 ± 1.1	4.25 ± 0.17	4.76 ± 0.27	1.48	8.40
	160	41.6 ± 2.1	5.17 ± 0.32	6.09 ± 0.45	1.48	7.97
	175	34.3 ± 0.3	4.19 ± 0.04	4.54 ± 0.08	1.46	8.81
	200	27.9 ± 0.5	3.31 ± 0.06	3.61 ± 0.05	1.42	9.00
	250	26.0 ± 0.7	3.10 ± 0.08	3.13 ± 0.19	1.42	9.69
	300	19.36 ± 1.0	2.25 ± 0.14	2.16 ± 0.13	1.38	10.5

*Values for the reference depths presented were calculated from the normalized power function [$F = F_{100}$ $(z/100)^b$] for the VERTEX 5 data set.

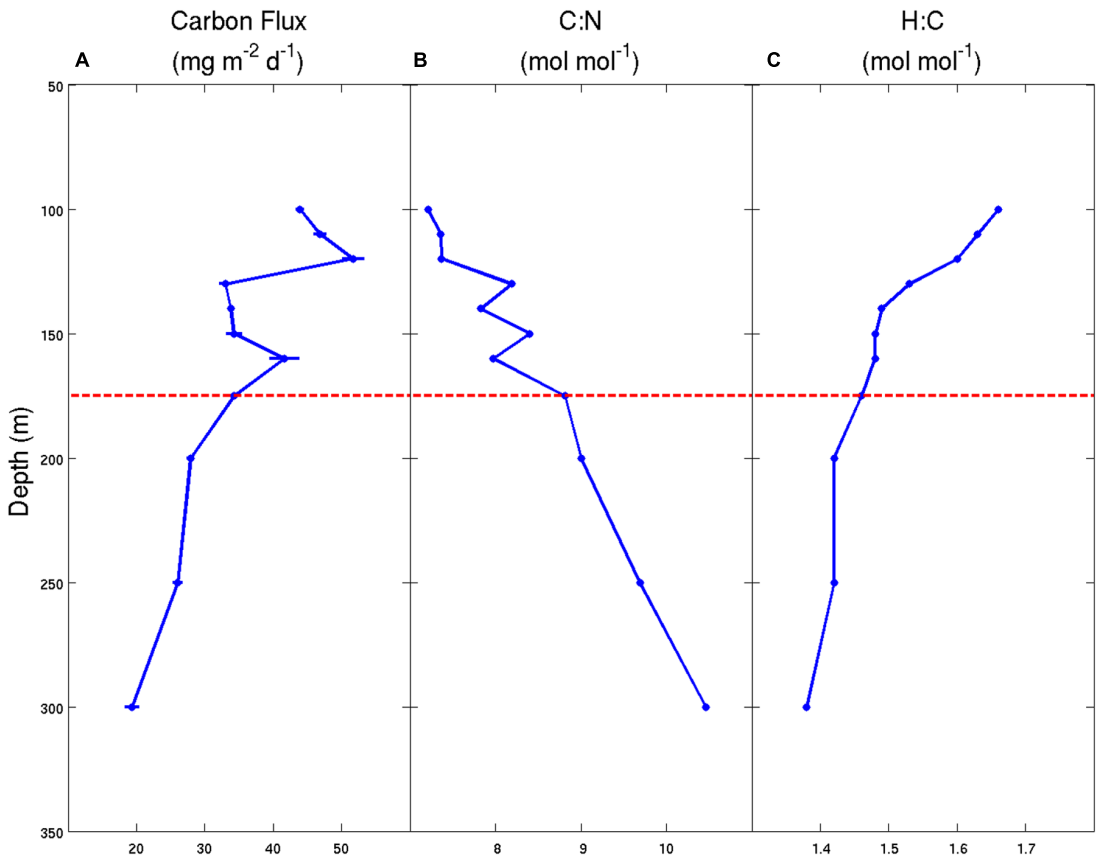

FIGURE 1 | Carbon flux and stoichiometry of particulate matter for samples collected using sediment traps deployed in the NPSG during the Hawaii Ocean Experiment-Dynamics of Light And Nutrients (HOE-DYLAN) IX expedition (August to September 2012). (A) Particulate C flux (mg C m^{-2} d^{-1}) ± 1 standard deviation of the mean (n = 3); **(B)** molar C:N ratios of the total particulate matter pool; **(C)** molar H:C ratios of the total particulate matter pool. The horizontal dashed line at 175 m represents the approximate location of the photosynthetic compensation depth where net photosynthesis is equal to 0 over a 24-h period.

(σ_θ = 27.0–27.2), their analysis indicated that the C:P and N:P ratios were not significantly different from the remineralization of model R–K–R plankton. However, the mean $-O_2$:P ratio of 171 ± 8 was much larger than that predicted from either the remineralization of R–K–R or Anderson (1995) model plankton (e.g., 138 and 150, respectively). They further concluded that the decomposing organic matter was more accurately represented by the chemical reaction, $C_2H_4 + 3O_2 \rightarrow 2CO_2 + 2H_2O$, rather than the oxidation of CH_2O. They emphasized the importance, but also acknowledged the absence, of quantitative knowledge of the H content of the organic matter (Takahashi et al., 1985). Similar results of higher than predicted $-O_2$:P ratios were also reported by Broecker et al. (1985), Minster and Boulahdid (1987), Peng and Broecker (1987), Boulahdid and Minster (1989), and Anderson and Sarmiento (1994), all in excess of −170:1. These extensive field results are consistent with the oxidation of organic matter that is on average more reduced than carbohydrate (CH_2O), the assumed oxidation state in R–K–R model plankton. Alternatively, yet unidentified reduced constituents contained in marine organic matter (e.g., reduced P; Karl, 2014) might also contribute to the demand for O_2 during microbial decomposition.

STATION ALOHA: A CASE STUDY

Station ALOHA (22°45′N, 158°W) was established in October 1988 as a strategically located oligotrophic ocean benchmark in the North Pacific Subtropical Gyre (NPSG) to improve our understanding of microbial and biogeochemical processes that control carbon and energy fluxes in the upper kilometer of the ocean (Karl and Lukas, 1996). On approximately monthly intervals since the establishment of Station ALOHA, samples have been collected for a host of physical, chemical, and biological measurements including several key features of the biological pump (Karl and Church, 2014, 2017). The C:N:P of both suspended (Hebel and Karl, 2001) and sinking (Karl et al., 1996) particulate matter pools, and the N:P of the DOM pool (Karl et al., 2001) have revealed complex, time-variable interactions between the dynamics of N and P, and unexpected temporal variability in stoichiometry, in addition to secular trends (also see Karl, 2014).

Li et al. (2000) developed a simple mixing model of two end-members to estimate the organic matter remineralization ratios at Station ALOHA. Based on their analysis of data collected during 1994, and assuming a H:C ratio of 1.65 for marine phytoplankton (Anderson, 1995), the molar formula

of remineralized organic matter was $C_{135}H_{280}O_{105}N_{13}P$ or $C_{25}(CH_2O)_{101}(CH_4)_9(NH_3)_{13}(H_3PO_4)$. Complete oxidation of this hypothetical organic matter would consume 170 O_2 (i.e., $-O_2$:P = −170), and yield 135 CO_2 + 132 H_2O + 13 NO_3^- + 1 $H_2PO_4^-$ + 14 H^+ (Li et al., 2000). These results were consistent with the Takahashi et al. (1985) observations and indicative of a more reduced organic matter source than either R–K–R or Anderson (1995) model plankton.

Two pioneering sediment trap experiments reported organic C, H, and N data for sinking particles collected in the NPSG (Honjo, 1980; Martin et al., 1987). We have recently refined the analytical procedures necessary for the routine estimation of the H in sinking particulate matter and, herein, add a third NPSG data set that we compare and contrast to the pioneering efforts of Honjo (1980) and Martin et al. (1987).

Honjo (1980) reported depth-variable molar H:C ratios for POM samples collected in an array of five bottom-moored sediment traps positioned at depths between 378 and 5,582 m (**Table 1**). Although the bulk molar H:C ratio was highest at the shallowest reference depth sampled (e.g., 1.79 at 378 m) and was lower at greater depths (molar H:C ranged from 1.50 to 1.64), there was no systematic change with depth despite a large decrease in the flux of total organic C from 3.56 mg C m^{-2} d^{-1} at 378 m to 0.66 mg C m^{-2} d^{-1} at 5,582 m (**Table 1**). These observations suggest that there are only relatively small changes in the mean redox state of organic matter as particles sink through the water column despite a loss of >80% of organic matter mass over this same depth interval. These results are more consistent with a physical (disaggregation/dissolution) model of particle attrition with increasing water depth than with biochemical (microbial degradation) control. The latter might be expected to selectively remove more reduced, energy-rich organics during the decomposition process. Alternatively, these results could be explained by the presence of recalcitrant (or semi-labile) reduced organic matter that buffers the bulk H:C molar ratio in sinking particles. Honjo (1980) also reported the elemental composition of different size fractions (>1 mm, 63 μm – 1 mm and <63 μm) of the collected organic matter. Although the percentage of the total organic matter collected in these various size fractions changed systematically with depth, with a shift toward smaller particles in deeper waters, the molar H:C ratios did not track the particle size distributions, and overall exhibited much greater variability than the values reported for the elemental composition of the "total" POM pool (presumably the mass-weighted mean value; Honjo, 1980). This comprehensive study by Honjo (1980) also included sediment trap data sets from sites in the Sargasso Sea (two reference depths) and the equatorial Atlantic Ocean (four reference depths). For samples collected in the equatorial Atlantic Ocean, the molar H:C ratios systematically increased from 1.44 at 389 m to 2.75 at 5,068 m. Honjo (1980) commented that these unusually high deep ocean H values, and depth-dependent increases in %H of the organic matter and molar H:C ratios "may be partly due to the evaporation of structural or pseudostructural water from opal and clay particles" and, therefore, may not accurately reflect the H contents of POM. Despite these limitations and uncertainties, the report by Honjo (1980) stands as a benchmark study of the elemental stoichiometry of exported particles even 35 years after it was published. Getting good H data in field samples continues to be a significant analytical challenge.

The second major study of bioelemental cycling in the NPSG was conducted as part of the decade-long VERtical Transport and EXchange (VERTEX) program that included observations at 28°, 155°W during summer 1983 (Martin et al., 1987). Measurements of C, H, and N in sinking POM were made at nine separate depths between 50 and 2,000 m using a free-drifting array of sediment traps. This experiment provided estimates of element-specific fluxes, as well as remineralization rates based on changes in element fluxes versus water depth (**Table 1**). The trap-derived flux data were analyzed using a log–log transformation and a normalized power function, $F_z = F_{100}\ (z/100)^b$, where F_z is the flux at any depth (z), F_{100} is the log–log intersect (also equivalent to the flux at 100 m), z is the depth in meters and the exponent b is the log–log slope of the flux versus depth relationship (also defines the flux attrition with depth). For samples collected in the NPSG, the attrition exponents (b) for C, H, and N were −0.973 ($r^2 = 0.95$), −0.883 ($r^2 = 0.91$), and −1.110 ($r^2 = 0.95$), respectively, suggesting that the average composition of sinking particles becomes N-depleted and H-enriched, relative to C, as particles sink/age. At the 2,000 m reference depth, the molar C:N and H:C ratios were 10.6 and 1.67 compared to 6.99 and 1.26 at the 100 m reference depth (see **Table 1**). These observations contradict the results of Honjo (1980), who failed to show any systematic change in either C:N or H:C as a function of water depth for sediment-trap collected particles in the NPSG (**Table 1**). The Martin et al. (1987) observations of an increase in the molar H:C ratio in bulk organic matter as a function of depth/age, might be explained by the presence of a pool of recalcitrant (or semi-labile), reduced organic matter that resists microbial decomposition, though we have no direct evidence for any such pool. Furthermore, this trend of increasing H:C with depth was not seen in the Honjo (1980) data, even though sinking particulate matter was sampled over a much greater depth range (378–5,582 m).

Similar to the sediment trap data sets of Honjo (1980) and Martin et al. (1987), our observations at Station ALOHA also reveal significant and systematic decreases in the fluxes of C, H, and N with increasing water depth (**Table 1**). Over a fairly narrow depth interval (100–300 m), the fluxes of all three bioelements decreased by 60–70% relative to the maximum fluxes observed in the 100–120 m depth stratum. This rapid loss of sinking particles is a manifestation of the combined effects of particle dissolution, disaggregation, consumption by metazoans and microbial decomposition. Though we lack quantitative information on the primary controlling mechanism(s), we hypothesize that the two latter processes are most important in the upper mesopelagic zone. Furthermore, our data suggest that sinking particles become more C-enriched, relative to N and H, with increasing water depth (**Table 1** and **Figure 1**). The molar H:C ratios decreased from 1.66 at 100 m to 1.38 at 300 m, indicating the selective removal of organic matter that had, on average, a molar H:C ratio of approximately 1.9 based on the ΔH:ΔC observed over this depth interval. This value

is nearly identical to the H:C ratio in monosaccharides (e.g., $C(H_2O)n$; H:C = 2.0) and to the C_2H_4 substrate hypothesized by Takahashi et al. (1985). Without direct estimates of the O or total caloric content of the sinking particles, we are not able to further characterize the mean particle redox state or energy content, respectively. However, it appears almost certain that particles become more oxidized, and have a lower C-specific energy content and a lower C-specific biochemical O_2 demand for complete oxidation as they sink/age. These lower energy particles are less desirable substrates for zooplankton and bacteria, and are able to escape the remineralization-intensive zone of the upper mesopelagic and continue their journey toward the abyss.

Most current models of C and energy flow through planktonic marine ecosystems do not include an explicit consideration of redox state or energy content of organic matter or how it varies during the decomposition process or as particles sink/age. Measurements of H help to constrain these processes and add a new dimension to ecosystem dynamics and the controls on the biological C pump. Consideration of the H in stoichiometry, in our opinion, is long overdue.

AUTHOR CONTRIBUTIONS

DK designed the study. EG provided data and interpretation. DK and EG wrote the paper.

FUNDING

This research was supported in part by funding from the National Science Foundation (EF-0424599), Gordon and Betty Moore Foundation (#3794), Simons Foundation (SCOPE #329108), and the Fondazione Internazionale Balzan.

REFERENCES

Anderson, L. A. (1995). On the hydrogen and oxygen content of marine phytoplankton. *Deep Sea Res. Part I* 42, 1675–1680. doi: 10.1016/0967-0637(95)00072-E

Anderson, L. A., and Sarmiento, J. L. (1994). Redfield ratios of remineralization determined by nutrient data analysis. *Global Biogeochem. Cycles* 8, 65–80. doi: 10.1029/93GB03318

Benner, R. (2002). "Chemical composition and reactivity," in *Biogeochemistry of Marine Dissolved Organic Matter*, eds D. A. Hansell and C. A. Carlson (San Diego, CA: Elsevier), 59–90. doi: 10.1016/B978-012323841-2/50005-1

Boulahdid, M., and Minster, J.-F. (1989). Oxygen consumption and nutrient regeneration ratios along isopycnal horizons in the Pacific Ocean. *Mar. Chem.* 26, 133–153. doi: 10.1016/0304-4203(89)90057-1

Broecker, W. S., Takahashi, T., and Takahashi, T. (1985). Sources and flow patterns of deep-ocean waters as deduced from potential temperature, salinity, and initial phosphate concentration. *J. Geophys. Res.* 90, 6925–6939. doi: 10.1029/JC090iC04p06925

Craig, J. F., Kenley, M. J., and Talling, J. F. (1978). Comparative estimations of the energy content of fish tissue from bomb calorimetry, wet oxidation and proximate analysis. *Freshwater Biol.* 8, 585–590. doi: 10.1111/j.1365-2427.1978.tb01480.x

Hebel, D. V., and Karl, D. M. (2001). Seasonal, interannual and decadal variations in particulate matter concentrations and composition in the subtropical North Pacific Ocean. *Deep Sea Res. Part II* 48, 1669–1696. doi: 10.1016/S0967-0645(00)00155-7

Honjo, S. (1980). Material fluxes and modes of sedimentation in the mesopelagic and bathypelagic zones. *J. Mar. Res.* 38, 53–97.

Johnstone, J. (1908). *Conditions of Life in the Sea: A Short Account of Quantitative Marine Biological Research*. Cambridge: University Press. doi: 10.5962/bhl.title.10734

Karl, D. M. (2014). Microbially mediated transformations of phosphorus in the sea: new views of an old cycle. *Annu. Rev. Mar. Sci.* 6, 279–337. doi: 10.1146/annurev-marine-010213-135046

Karl, D. M., Björkman, K. M., Dore, J. E., Fujieki, L., Hebel, D. V., Houlihan, T., et al. (2001). Ecological nitrogen-to-phosphorus stoichiometry at Station ALOHA. *Deep Sea Res. Part II* 48, 1529–1566. doi: 10.1016/S0967-0645(00)00152-1

Karl, D. M., Christian, J. R., Dore, J. E., Hebel, D. V., Letelier, R. M., Tupas, L. M., et al. (1996). Seasonal and interannual variability in primary production and particle flux at Station ALOHA. *Deep Sea Res. Part II* 43, 539–568. doi: 10.1016/0967-0645(96)00002-1

Karl, D. M., and Church, M. J. (2014). Microbial oceanography and the Hawaii Ocean Time-series programme. *Nat. Rev. Microbiol.* 12, 699–713. doi: 10.1038/nrmicro3333

Karl, D. M., and Church, M. J. (2017). Ecosystem structure and dynamics in the North Pacific Subtropical Gyre: new views of an old ocean. *Ecosystems* 20, 433–457. doi: 10.1007/s10021-017-0117-0

Karl, D. M., and Dobbs, F. C. (1998). "Molecular approaches to microbial biomass estimation in the sea," in *Molecular Approaches to the Study of the Ocean*, ed. K. E. Cooksey (London: Chapman & Hall), 29–89.

Karl, D. M., and Lukas, R. (1996). The Hawaii Ocean Time-series (HOT) program: background, rationale and field implementation. *Deep Sea Res. Part II* 43, 129–156. doi: 10.1016/0967-0645(96)00005-7

Laws, E. A. (1991). Photosynthetic quotients, new production and net community production in the open ocean. *Deep Sea Res. Part A* 38, 143–167. doi: 10.1016/0198-0149(91)90059-O

Lee, C., Wakeham, S., and Arnosti, C. (2004). Particulate organic matter in the sea: the composition conundrum. *Ambio* 33, 565–575. doi: 10.1579/0044-7447-33.8.565

Li, Y.-H., Karl, D. M., Winn, C. D., Mackenzie, F. T., and Gans, K. (2000). Remineralization ratios in the subtropical North Pacific gyre. *Aquat. Geochem.* 6, 65–86. doi: 10.1023/A:1009676300859

Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P., and Woodward, J. C. (1996). Humic substances as electron acceptors for microbial respiration. *Nature* 382, 445–448. doi: 10.1038/382445a0

Martin, J. H., Knauer, G. A., Karl, D. M., and Broenkow, W. W. (1987). VERTEX: carbon cycling in the Northeast Pacific. *Deep Sea Res.* 34, 267–285. doi: 10.1016/0198-0149(87)90086-0

Minster, J.-F., and Boulahdid, M. (1987). Redfield ratios along isopycnal surfaces–A complementary study. *Deep Sea Res.* 34, 1981–2003. doi: 10.1016/0198-0149(87)90094-X

Peng, T.-H., and Broecker, W. S. (1987). C/P ratios in marine detritus. *Global Biogeochem. Cycles* 1, 155–161. doi: 10.1029/GB001i002p00155

Phillipson, J. (1964). A miniature bomb calorimeter for small biological samples. *Oikos* 15, 130–139. doi: 10.2307/3564751

Platt, T., and Irwin, B. (1972). Caloric content of phytoplankton. *Limnol. Oceanogr.* 18, 306–310. doi: 10.4319/lo.1973.18.2.0306

Redfield, A. C. (1934). "On the proportions of organic derivatives in sea water and their relation to the composition of plankton," in *James Johnstone Memorial Volume*, ed. R. J. Daniel (Liverpool: University Press), 177–192.

Redfield, A. C. (1958). The biological control of chemical factors in the environment. *Am. Sci.* 46, 205–221.

Redfield, A. C., Ketchum, B. H., and Richards, F. A. (1963). "The influence of organisms on the composition of sea-water," in *The Sea: Ideas and Observations on Progress in the Study of the Seas*, Vol. 2, ed. M. N. Hill (New York, NY: John Wiley & Sons, Inc.), 26–77.

Repeta, D. J. (2015). "Chemical characterization and cycling of dissolved organic matter," in *Biogeochemistry of Marine Dissolved Organic Matter*, 2nd Edn, eds D. A. Hansell and C. A. Carlson (San Diego, CA: Elsevier), 21–63. doi: 10.1016/B978-0-12-405940-5.00002-9

Repeta, D. J., Ferrón, S., Sosa, O. A., Johnson, C. G., Repeta, L. D., Acker, M., et al. (2016). Marine methane paradox explained by bacterial degradation of dissolved organic matter. *Nat. Geosci.* 9, 884–887. doi: 10.1038/ngeo2837

Sarmiento, J. L., and Gruber, N. (2006). *Ocean Biogeochemical Dynamics*. Princeton, NJ: Princeton University Press.

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Takahashi, T., Broecker, W. S., and Langer, S. (1985). Redfield ratio based on chemical data from isopycnal surfaces. *J. Geophys. Res.* 90, 6907–6924. doi: 10.1029/JC090iC04p06907

Van Mooy, B. A. S., Krupke, A., Dyhrman, S. T., Fredricks, H. F., Frischkorn, K. R., Ossolinski, J. E., et al. (2015). Major role of planktonic phosphate reduction in the marine phosphorus redox cycle. *Science* 348, 783–785. doi: 10.1126/science.aaa8181

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

PERSPECTIVE
published: 25 April 2017
doi: 10.3389/fmicb.2017.00722

Ecological Stoichiometry beyond Redfield: An Ionomic Perspective on Elemental Homeostasis

Punidan D. Jeyasingh[1], Jared M. Goos[2], Seth K. Thompson[3], Casey M. Godwin[4] and James B. Cotner[5]*

[1] Department of Integrative Biology, Oklahoma State University, Stillwater, OK, USA, [2] Department of Biology, University of Texas at Arlington, Arlington, TX, USA, [3] Water Resources Science Program, University of Minnesota, St. Paul, MN, USA, [4] School of Natural Resources and Environment, University of Michigan, Ann Arbor, MI, USA, [5] Department of Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, MN, USA

Elemental homeostasis has been largely characterized using three important elements that were part of the Redfield ratio (i.e., carbon: nitrogen: phosphorus). These efforts have revealed substantial diversity in homeostasis among taxonomic groups and even within populations. Understanding the evolutionary basis, and ecological consequences of such diversity is a central challenge. Here, we propose that a more complete understanding of homeostasis necessitates the consideration of other elements beyond C, N, and P. Specifically, we posit that physiological complexity underlying maintenance of elemental homeostasis along a single elemental axis impacts processing of other elements, thus altering elemental homeostasis along other axes. Indeed, transcriptomic studies in a wide variety of organisms have found that individuals differentially express significant proportions of the genome in response to variability in supply stoichiometry in order to maintain varying levels of homeostasis. We review the literature from the emergent field of ionomics that has established the consequences of such physiological trade-offs on the content of the entire suite of elements in an individual. Further, we present experimental data on bacteria exhibiting divergent phosphorus homeostasis phenotypes demonstrating the fundamental interconnectedness among elemental quotas. These observations suggest that physiological adjustments can lead to unexpected patterns in biomass stoichiometry, such as correlated changes among suites of non-limiting microelements in response to limitation by macroelements. Including the entire suite of elements that comprise biomass will foster improved quantitative understanding of the links between chemical cycles and the physiology of organisms.

Keywords: elemental profiling, freshwater heterotrophic bacteria, ionome, ionomics, nutrient limitation, phosphorus supply

OPEN ACCESS

Edited by:
Télesphore Sime-Ngando, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Cedric Leo Meunier, Alfred Wegener Institute, Germany
Ivan Baxter, Agricultural Research Service (USDA), USA

****Correspondence:***
Punidan D. Jeyasingh
puni.jeyasingh@okstate.edu

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *01 February 2017*
Accepted: *07 April 2017*
Published: *25 April 2017*

Citation:
Jeyasingh PD, Goos JM, Thompson SK, Godwin CM and Cotner JB (2017) Ecological Stoichiometry beyond Redfield: An Ionomic Perspective on Elemental Homeostasis. Front. Microbiol. 8:722. doi: 10.3389/fmicb.2017.00722

INTRODUCTION

Ecological stoichiometry considers individuals as collections of chemical elements akin to a very large molecule. At the most fundamental level, ecological stoichiometry is the study of the sub-organismal mechanisms, and supra-organismal consequences of the principle of mass balance. It operates on the axiom that living entities are not a passive conduit of chemical supply, but rather

actively regulating their elemental stoichiometry, referred to as elemental homeostasis (Sterner and Elser, 2002). Elemental homeostasis is the fulcrum for most stoichiometric models predicting processes at the level of the individual- (e.g., Frost et al., 2005), population- (e.g., Andersen et al., 2004), community- (e.g., Elser and Urabe, 1999), ecosystem- (e.g., Sterner et al., 1992), and global (e.g., Doney et al., 2009; Galbraith and Martiny, 2015) levels of organization. Indeed, without elemental homeostasis "ecological stoichiometry would be a dull subject" (Sterner and Elser, 2002).

At least two different approaches have been used to quantify the degree of elemental homeostasis (Sterner and Elser, 2002; Meunier et al., 2014). We quantify it using the slope of the log-log relationship between resource and consumer stoichiometry (**Figure 1**). Most stoichiometric models assume osmotrophs at the base of food webs exhibit relaxed stoichiometric homeostasis compared to phagotrophs occupying higher trophic levels. While this assumption has been a subject of debate and found to be of negligible relevance to stoichiometric models in consumers (Wang et al., 2012) the great diversity in the degree of elemental homeostasis (e.g., Frost et al., 2005; Scott et al., 2012; Godwin and Cotner, 2014, 2015; Meunier et al., 2014) remains largely unexplained. Such diversity is surprising because nutrient supply environments can impose strong selection on elemental quotas and consumption, which may be linked to stoichiometry. For example, Godwin and Cotner (2015) found that P content of isolating medium selected for strains of heterotrophic bacteria differing in P homeostasis and elemental quotas. The eco-evolutionary processes that maintain such substantially divergent phenotypes in natural populations is a central frontier in ecological stoichiometry.

Variation in genomic architecture has a major effect on physiological responses of both autotrophs (e.g., *Arabidopsis;* Misson et al., 2005) and heterotrophs (e.g., *Daphnia;* Roy Chowdhury et al., 2014) to changes in supply stoichiometry with important implications for homeostasis and fitness (e.g., Jeyasingh et al., 2009). Although, several informative loci for P use have been identified in crop plants (recently reviewed in van de Wiel et al., 2016), it is important to note that different genes and physiological pathways can underlie similar homeostatic and fitness outcomes among genotypes in autotrophs (*Glycine max;* Li et al., 2016) and heterotrophs (*Daphnia pulicaria;* Sherman et al., in review). This raises the possibility that genotypes exhibiting differing degrees of elemental homeostasis can vary in other traits. At the simplest level, we can think of elements that share similar properties that can be replaced when supply of one is limiting (e.g., substituting P-lipids with S-lipids under P limitation; Van Mooy et al., 2009; Bellinger et al., 2014). It is possible that a homeostatic genotype can maintain P content via efficient use of P, while a flexible genotype decreases P content, but increases S content to maintain basic cellular functions (**Figure 1**). As such, we need to understand coupled elemental quotas (such as P and S), as well as selection operating on such pathways for a complete understanding of the processes maintaining phosphorus homeostasis in populations.

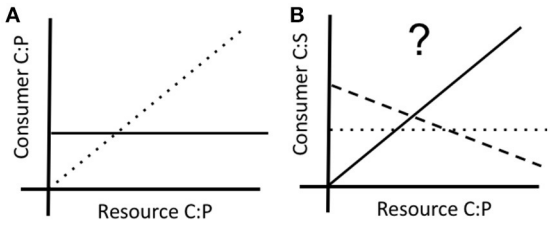

FIGURE 1 | (A) Schematic representation of carbon: phosphorus (C:P) homeostasis in relation to supply C:P in homeostatic (solid line) and non-homeostatic (dotted line) consumers. **(B)** Hypothesized correlated shifts in carbon: sulfur (C:S) stoichiometry in the same consumer, showing no effect of P on S (dotted line), decrease in S under P limitation (dashed line), and increase in S under P-limitation (solid line).

Potential for correlated changes in homeostasis along multiple elemental axes is perhaps more apparent when one considers the complex physiological adjustments organisms make to maintain net anabolism in limiting conditions of elemental supply. Seminal studies in *E. coli* revealed the complex nature of responses to P limitation (Van Bogelen et al., 1996), involving differential expression of ∼400 proteins orchestrating not only P use physiology, but also those involving other bulk and trace elements. Such complex physiological responses appear to be common. For example, studies in *Pseudovibrio* (Romano et al., 2015), *Saccharomyces* (Boer et al., 2010), *Chlamydomonas* (Moseley et al., 2006), *Arabidopsis* (Misson et al., 2005), and *Daphnia* (Jeyasingh et al., 2011), reveal that organisms differentially express a significant proportion of genes and metabolic pathways depending on P supply, often by several-fold. While several candidate P-stress response genes are up-regulated (e.g., P transporters, phosphatases; reviewed in Jeyasingh and Weider, 2007), so are several hundreds of other genes involved in a variety of pathways. Merchant and Helmann (2012) provided a comprehensive treatise on the diversity of microbial strategies to variation in elemental supply. An important message arising from this work is the fundamental interconnectedness of elements in biomass. In other words, acclimatory or adaptive responses to supply stoichiometry often involve changes in the physiological processing of many elements.

In this perspective, we ask whether such broad physiological changes in response to supply stoichiometry alters the entire suite of elements encompassing an individual. Defined as the mineral nutrient and trace element composition of an organism, the ionome represents all the elements of cellular and organismal systems (Salt et al., 2008). As such, the ionome is a dynamic network of elements that underlies the morphological, anatomical, and physiological state of an organism, which are ultimately controlled by the genome in response to the environment. We review evidence in the literature as well as analyze experimental data to illuminate the dynamic nature of the ionome, and discuss its implications for elemental homeostasis specifically, and the framework of ecological stoichiometry in general.

EVIDENCE IN THE LITERATURE:

Ionomics is a relatively new field that has focused primarily on plants (see Huang and Salt, 2016 for a recent review). Ionomics was first employed to better understand the genomic architecture underlying mineral and trace element use in *Arabidopsis,* because studying the use of one element resulted in an incomplete picture of the genotype-to-phenotype map (Lahner et al., 2003). This approach has since been used as a low-cost, multi-proxy diagnostic tool in agronomy (Baxter et al., 2014) as well as medicine (Malinouski et al., 2014). Ionomics connects genetic potential and evolutionary history to growth, physiology and fitness in contemporary ecological conditions. Studies on both *Saccharomyces cerevisiae* (Eide et al., 2005; Yu et al., 2012) and *Arabidopsis thaliana* (Baxter et al., 2008) clearly show that both genetics and supply stoichiometry alter ionomes. Specifically, Eide et al. (2005) characterized the ionomes of over 4,000 yeast strains and found considerable variation in all 13 elements quantified, with both strong positive (e.g., P-Co) and negative (e.g., P-S) correlations under optimal growth conditions. Furthermore, they found that genotypes with mutations in similar functional categories (e.g., vacuolar, mitochondrial) showed similar ionomic signatures. Yu et al. (2012) utilized gene deletion and open reading frame overexpression collections of yeast (∼5,000 strains) and found general patterns in the genomic basis of ionomic divergence. Mutations in genes involved in protein metabolism or transport had the largest impacts on the ionome, followed by changes in gene copy number. Baxter et al. (2008) found that *Arabidopsis* exhibited consistent ionomic patterns depending on supply stoichiometry such that the nature of nutrient stress could be predicted based on the ionome. While P content of leaves decreased under P limitation, there was considerable variation among genotypes, making P content a poor predictor of physiological status compared to a six element (As, B, Co, Cu, P, Zn) model, which included strong positive (e.g., P-Cu) and negative (e.g., P-Zn) correlations among elements. Considering combinations of elements as phenotypes, as opposed to considering single elements at a time, allows for greater sensitivity in identifying stoichiometric variation because of the fundamental interconnectedness among elements in biomass (Baxter, 2015).

An underappreciated component in understanding ionomes is likely to be transmembrane elemental transport systems, with transporters possessing multi-element specificity found to be more common than previously appreciated (Morrissey et al., 2009; Mitani-Ueno et al., 2011). As such, ionomic approaches are well suited to illuminate the complex physiological adjustments that organisms make in response to changes in supply stoichiometry. For example, P limitation increases the expression of several high-affinity phosphate transporters, which are also known to take up As (Muchhal et al., 1996) and thus explains increased As content in P-limited plants. Similarly, plants are known to scavenge metals such as Zn to minimize the formation of complexes with P (Misson et al., 2005) which could underlie the observed increase of Zn content under P limited conditions.

Evidence for ionome-wide shifts from the field are also available. *Synechococcus* cells collected from regions of the Sargasso Sea that vary in N and P supply exhibited several-fold cell quota differences in a variety of elements (e.g., Mn, Ni, Zn) (Twining et al., 2010). In addition, utilizing a global dataset, Loladze (2014) reported striking changes in the ionomes of C_3 plants from four continents in response to elevated atmospheric CO_2. In general, elevated CO_2 significantly decreased not only N and P content, but also several other elements, including K, Ca, S, Mg, Fe, Zn, Cu, and Mn. These observations clearly indicate that supply stoichiometry alters entire suites of elements, beyond the commonly studied Redfield elements. Understanding the dynamics and regulation of these minor elements is important not only in understanding the ecology and evolution of microbes and plants, but as discussed by Loladze (2014), the variation in the composition of trace elements plays an important role in the nutrition of consumers, including humans (Myers et al., 2014).

EXPERIMENTAL EVIDENCE

Are there patterns in ionomic architecture relevant to key parameters in stoichiometric theory such as homeostasis of Redfield elements? The staggering diversity of stoichiometric physiologies discovered among strains of heterotrophic bacteria inhabiting glacial lakes in northern U.S.A (Godwin and Cotner, 2015) provides an ideal testbed for answering such questions. We studied a subset of strains that were found to exhibit divergent homeostatic coefficients in terms of phosphorus (5 flexible, heterostoichs; 4 inflexible, homeostoichs) at two levels of P supply (C:P = 100 and 10,000; see Supplementary Material for methods). The nine strains used in this study represented three unique genera (*Brevundimonas, Flavobacterium,* and *Sphingomonas*) with three strains coming from each genus. All three of the *Brevundimonas* strains were characterized as flexible, whereas *Flavobacterium* and *Sphingomonas* each had two strains characterized as inflexible and one strain as flexible. Although we expected strong strain-specific responses, we generally predicted that homoestoichs should exhibit greater changes in other elements (e.g., S) between high and low P supply conditions compared to heterostoichs due to upregulation of compensating mechanisms. Each strain was originally isolated from lakes within Minnesota using either agar plates or dilution isolation as described previously (Godwin and Cotner, 2015).

A total of 25 elements were detected, of which nine (Co, Cr, K, Mg, Mn, Na, P, S, Zn) were present above detection limits in all samples and were used for further analyses. As expected, there was considerable strain-specific variation in biomass P content (**Figure 2**). Considerably more genotypic replicates are required to rigorously test for systematic differences among P homeostasis and correlated changes in the content of other elements.

Nevertheless, important trends were apparent in this dataset. We quantified the magnitude of elemental change in each strain and compared the differences between the two levels of homeostasis (hereto- vs. homeostoichs). Although considerable strain-specific responses preclude identification of any robust patterns, the response of the two homeostoich phenotypes appear to be distinct (**Figure 2**). Closer examination revealed that certain

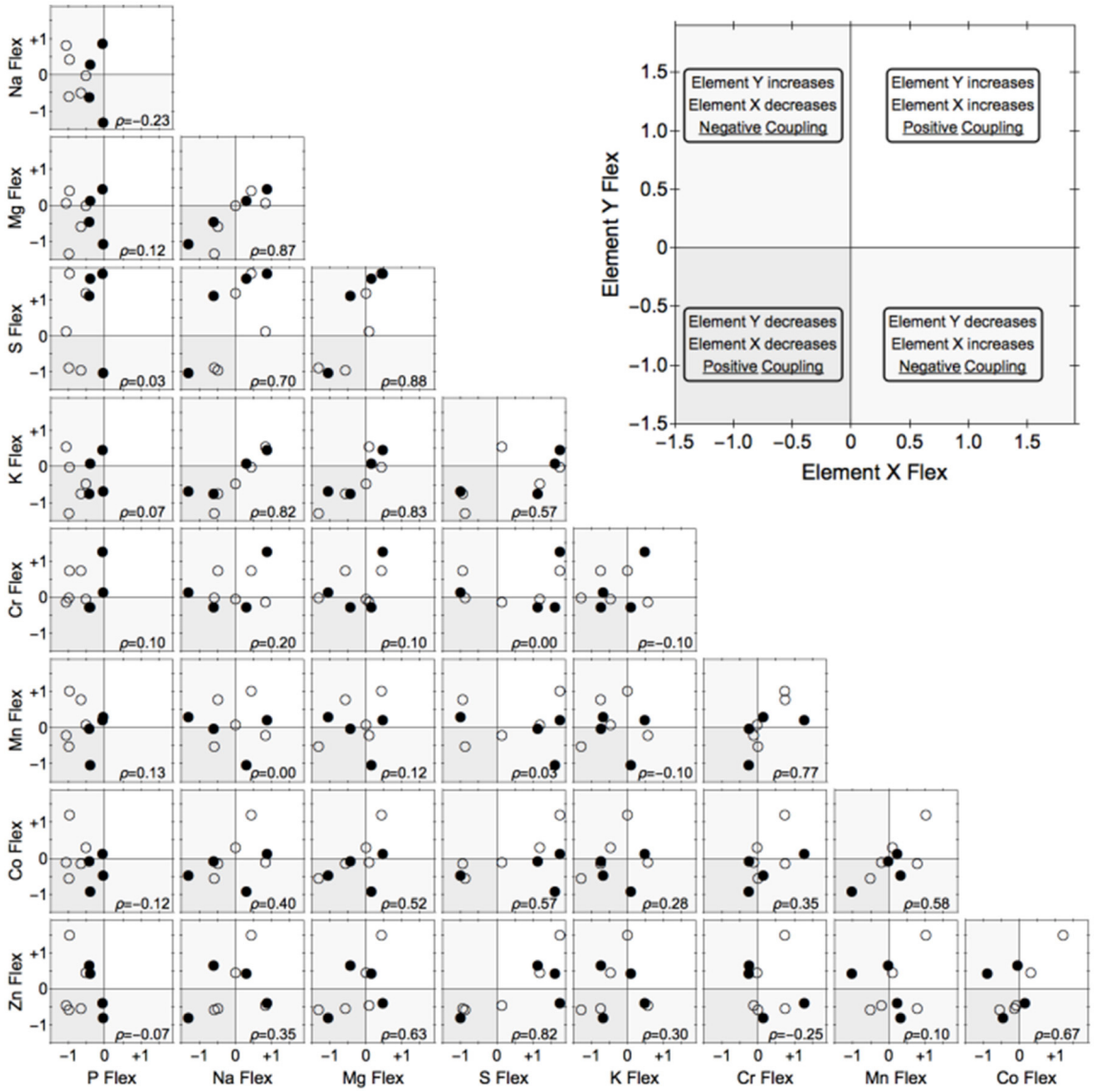

FIGURE 2 | Flexibility of nine elements in relation to each other in nine strains of freshwater heterotrophic bacteria. We define flexibility as the $\log_{10}$[elemental content (mass/mass) at C:P of 10,000:1/ elemental content at C:P of 100:1]. Negative values represent a reduction in element quota under C:P = 10,000, zero means homeostasis, and positive values indicate that the quota increased under P-limitation. Symbols denote strains that are relatively flexible (open circles) or inflexible (closed circles) in phosphorus content. ρ = Spearman's rank coefficient.

groups of elements were positively correlated with each other, namely Mg-Na-K, and to a lesser degree, Zn-S and Cr-Co-Mn (**Figure 2**). Of particular interest was how flexibility in P content related to flexibility of other elements (leftmost column in **Figure 2**). Consistent with previous studies (Godwin and Cotner, 2015), P content of all strains was lower under the C:P = 10,000 treatment. Interestingly, strains differed in the responses of other elements, with roughly half of the strains becoming more concentrated while the other half becoming less concentrated for the eight other elements. However, no systematic patterns with regard to the P homeostasis phenotype (flexible vs, inflexible) were apparent. We note that this preliminary result needs to be rigorously verified because the experiment had limitations (see Supplementary Material).

DISCUSSION

It is clear that ionomes are sensitive to both the external environment as well as the genomic composition. Whether such changes in the ionome are ecologically relevant is an important question worthy of attention by both empirical and theoretical practitioners of ecological stoichiometry. Studies with plants indicate that individual growth, even under strong P limitation is better predicted, not by P use efficiency alone,

but by the uptake of a few other elements as well (Baxter et al., 2008). We posit that the correlated nature of elements in biomass predisposes organisms to tradeoffs in maintaining homeostasis of a particular element. If these tradeoffs occur, being homeostatic along one axis should be associated with changes in homeostasis along other elemental axes. At this point, we do not understand ionomes sufficiently to make robust predictions about what the most relevant trade-offs are. An understanding of the role and relevance of these other elemental axes, and the costs associated with the trade-offs among axes certainly is important to understanding the stoichiometry of organisms and ecological systems. The nature of such changes will depend on the material demands of biochemical pathways utilized to maintain homeostasis. Such an inclusive perspective of elemental homeostasis is required for understanding the diverse stoichiometric physiologies observed in both osmotrophic and phagotrophic populations (e.g., Frost et al., 2005; Godwin and Cotner, 2014, 2015; Meunier et al., 2014), and reflects the current state of evolutionary biology wherein the multifarious nature of selection is a prerequisite for understanding trait evolution (e.g., Kaeuffer et al., 2012).

As our understanding of nutrient limitation shifts from single nutrient models, to more complex, multiple nutrient models predicting co-limitation (e.g., Saito et al., 2008; Harpole et al., 2011; Bracken et al., 2015), the importance of attention to ionomic patterns in natural systems is magnified. Although there is a paucity of ionomic data in natural ecosystems, such data should reveal important patterns that could illuminate the mechanisms underlying co-limitation, which is increasingly common and should replace the Leibig paradigm (Kaspari and Powers, 2016). As such, simultaneous limitation of multiple elements may be strong sources of selection structuring populations with important implications for contemporary nutrient budgets. Nevertheless, it is unlikely that all of the 25-odd elements represented in biology will impart the same magnitude of selection or ecological significance. Although an organism should acquire all elements from the environment, some elements (e.g., copper) can be recycled within the organism quite efficiently that it may not need to be constantly acquired from the environment (Nose et al., 2006), while others (e.g., phosphorus) are excreted as a byproduct of metabolic processes and need to be constantly acquired from the environment. However, if P and Cu are coupled, then the evolutionary and ecological importance of Cu is amplified. Thus, focusing on correlations among elements may be a particularly informative approach.

Ionomic data from both the literature and our experiment reveal several correlations, although the functional basis for such correlations appears to be more complex than what can be predicted by linkages based on cellular physiology of element processing. For example, sodium dependent phosphate uptake by cells is a well-established mechanism (discussed in the context of ecological stoichiometry in Jeyasingh and Weider, 2007), yet sodium and phosphorus do not appear to be correlated at the ionome level. Clearly, much more remains to be understood about the complex processes underlying such patterns. For example, Malinouski et al. (2014) studied HeLa cell lines to characterize mechanisms that regulate trace elements by performing a genome-wide siRNA/ionomics screen to identify the major pathways. They analyzed a total of 21,360 human gene knockdowns for changes in trace elements in HeLa cells and detected many known genes involved in transport and regulation of trace elements while also identifying several novel genes that regulate the processing of trace elements. As such, the mechanisms underlying correlations among elements in an ionome is difficult, and perhaps of little ecological relevance. However, general patterns in correlations among elements at the level of the ionome will have important ecological ramifications. Discovering such patterns and associated ecological implications should be viewed as a central challenge.

Genotype-specific effects common in ionomic studies discussed above are similar to discoveries about intraspecific variation in biomass C:N:P stoichiometries (e.g., Bertram et al., 2008; Goos et al., 2014; Downs et al., 2016) which are shaped by selection (e.g., El-Sabaawi et al., 2012; Tobler et al., 2016), and generate discernable patterns at higher levels of organization (e.g., Elser et al., 2000). While genetic recombination can produce endless varieties of biota, organismal evolution is bounded by principles of physics and chemistry (Williams and Frausto da Silva, 2006). Ecological stoichiometry, by virtue of abstracting such complexity, has unraveled general patterns linking elements such as phosphorus with fitness-relevant traits and subsequent ecological consequences. The focus on only three of the ∼25 elements represented in biology is limited, however, and perhaps has misrepresented both patterns and processes in ecological stoichiometry. Whether, and to what extent, predictions of stoichiometric models are enhanced, similar to those predicting individual growth (e.g., Baxter et al., 2008), by inclusion of the entire suite of elements remains to be seen. Advances in low-cost, high throughput elemental analyses already enable an ionomic view of ecological stoichiometry, and such data will be required to make sense of central parameters in stoichiometric models in light of genomic information, and perhaps also metagenomic data using meta-ionomics, for a more comprehensive genes-to-ecosystems picture of the biosphere.

AUTHOR CONTRIBUTIONS

All authors made equal, substantial contributions toward the creation of this manuscript, and approve its publication.

ACKNOWLEDGMENTS

This perspective was catalyzed by a group discussion at the Conference on Biological Stoichiometry in 2015, Trent University, Canada. Support from National Science Foundation grant #1256867 to PJ and #1257571 to JC facilitated this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00722/full#supplementary-material

REFERENCES

Andersen, T., Elser, J. J., and Hessen, D. O. (2004). Stoichiometry and population dynamics. *Ecol. Lett.* 7, 884–900. doi: 10.1111/j.1461-0248.2004.00646.x

Baxter, I. R. (2015). Should we treat the ionome as a combination of individual elements, or should we be deriving novel combined traits? *J. Exp. Bot.* 66, 2127–2131. doi: 10.1093/jxb/erv040

Baxter, I. R., Vitek, O., Lahner, B., Muthukumar, B., Borghi, M., Morrissey, J., et al. (2008). The leaf ionome as a multivariable system to detect a plant's physiological status. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12081–12086. doi: 10.1073/pnas.0804175105

Baxter, I. R., Ziegler, G., Lahner, B., Mickelbart, M. V., Foley, R., Danku, J., et al. (2014). Single-kernel ionomic profiles are highly heritable indicators of genetic and environmental influences on elemental accumulation in maize grain (*Zea mays*). *PLoS ONE* 9:e87628. doi: 10.1371/journal.pone.0087628

Bellinger, B. J., Van Mooy, B. A. S., Cotner, J. B., Fredricks, H. F., Benitez-Nelson, C. R., Thompson, J., et al. (2014). Physiological modifications of seston in response to physicochemical gradients within Lake Superior. *Limnol. Oceanogr.* 59, 1011–1026. doi: 10.4319/lo.2014.59.3.1011

Bertram, S. M., Bowen, M., Kyle, M., and Schade, J. D. (2008). Extensive natural intraspecific variation in stoichiometric (C : N : P) composition in two terrestrial insect species. *J. Insect Sci.* 8, 1–7. doi: 10.1673/031.008.2601

Boer, V. M., Crutchfield, C. A., Bradley, P. H., Botstein, D., and Rabinowitz, J. D. (2010). Growth-limiting intracellular metabolites in yeast growing under diverse nutrient limitations. *Mol. Biol. Cell* 21, 198–211. doi: 10.1091/mbc.E09-07-0597

Bracken, M. E. S., Hillebrand, H., Borer, E. T., Seabloom, E. W., Cebrian, J., Cleland, E. E., et al. (2015). Signatures of nutrient limitation and co-limitation: responses of autotroph internal nutrient concentrations to nitrogen and phosphorus additions. *Oikos* 124, 113–121. doi: 10.1111/oik.01215

Doney, S. C., Lima, I., Moore, J. K., Lindsay, K., Behrenfeld, M. J., Westberry, T. K., et al. (2009). Skill metrics for confronting global upper ocean ecosystem-biogeochemistry models against field and remote sensing data. *J. Mar. Syst.* 76, 95–112. doi: 10.1016/j.jmarsys.2008.05.015

Downs, K. N., Hayes, N. M., Rock, A. M., Vanni, M. J., and Gonzalez, M. J. (2016). Light and nutrient supply mediate intraspecific variation in the nutrient stoichiometry of juvenile fish. *Ecosphere* 7:e01452. doi: 10.1002/ecs2.1452

Eide, D. J., Clark, S., Nair, T. M., Gehl, M., Gribskov, M., Guerinot, M. L., et al. (2005). Characterization of the yeast ionome: a genome-wide analysis of nutrient mineral and trace element homeostasis in *Saccharomyces cerevisiae*. *Genome Biol.* 6:R77. doi: 10.1186/gb-2005-6-9-r77

El-Sabaawi, R., Kohler, T., Zandoná, E., Travis, J., Marshall, M., Thomas, S., et al. (2012). Environmental and organismal predictors of intraspecific variation in the stoichiometry of a neotropical freshwater fish. *PLoS ONE* 7:e32713. doi: 10.1371/journal.pone.0032713

Elser, J. J., O'Brien, W. J., Dobberfuhl, D. R., and Dowling, T. E. (2000). The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* 13, 845–853. doi: 10.1046/j.1420-9101.2000.00215.x

Elser, J., and Urabe, J. (1999). The stoichiometry of consumer-driven nutrient recycling: theory, observations, and consequences. *Ecology* 80, 735–751. doi: 10.2307/177013

Frost, P. C., Evans-White, M. A., Finkel, Z. V., Jensen, T. C., and Matzek, V. (2005). Are you what you eat? Physiological constraints on organismal stoichiometry in an elementally imbalanced world. *Oikos* 109, 18–28. doi: 10.1111/j.0030-1299.2005.14049.x

Galbraith, E. D., and Martiny, A. C. (2015). A simple nutrient-dependence mechanism for predicting the stoichiometry of marine ecosystems. *Proc. Natl. Acad. Sci. U.S.A.* 112, 8199–8204. doi: 10.1073/pnas.1423917112

Godwin, C. M., and Cotner, J. B. (2014). Carbon:phosphorus homeostasis of aquatic bacterial assemblages is mediated by shifts in assemblage composition. *Aquat. Microb. Ecol.* 73, 245–258. doi: 10.3354/ame01719

Godwin, C. M., and Cotner, J. B. (2015). Stoichiometric flexibility in diverse aquatic heterotrophic bacteria is coupled to differences in cellular phosphorus quotas. *Front. Microbiol.* 6:159. doi: 10.3389/fmicb.2015.00159

Goos, J. M., French, B. J., Relyea, R. A., Cothran, R. D., and Jeyasingh, P. D. (2014). Sex-specific plasticity in body phosphorus content of Hyalella amphipods. *Hydrobiologia* 722, 93–102. doi: 10.1007/s10750-013-1682-7

Harpole, W. S., Ngai, J. T., Cleland, E. E., Seabloom, E. W., Borer, E. T., Bracken, M. E. S., et al. (2011). Nutrient co-limitation of primary producer communities. *Ecol. Lett.* 14, 852–862. doi: 10.1111/j.1461-0248.2011.01651.x

Huang, X. Y., and Salt, D. E. (2016). Plant ionomics: from elemental profiling to environmental adaptation. *Mol. Plant* 9, 787–797. doi: 10.1016/j.molp.2016.05.003

Jeyasingh, P. D., and Weider, L. J. (2007). Fundamental links between genes and elements: evolutionary implications of ecological stoichiometry. *Mol. Ecol.* 16, 4649–4661. doi: 10.1111/j.1365-294X.2007.03558.x

Jeyasingh, P., Ragavendran, A., Paland, S., Lopez, J., Sterner, R., and Colbourne, J. (2011). How do consumers deal with stoichiometric constraints? Lessons from functional genomics using Daphnia pulex. *Mol. Ecol.* 20, 2341–2352. doi: 10.1111/j.1365-294X.2011.05102.x

Jeyasingh, P., Weider, L., and Sterner, R. (2009). Genetically-based trade-offs in response to stoichiometric food quality influence competition in a keystone aquatic herbivore. *Ecol. Lett.* 12, 1229–1237. doi: 10.1111/j.1461-0248.2009.01368.x

Kaeuffer, R., Peichel, C. L., Bolnick, D. I., and Hendry, A. P. (2012). Parallel and nonparallel aspects of ecological, phenotypic, and genetic divergence across replicate population pairs of lake and stream stickleback. *Evolution* 66, 402–418. doi: 10.1111/j.1558-5646.2011.01440.x

Kaspari, M., and Powers, J. S. (2016). Biogeochemistry and geographical ecology: embracing all twenty-five elements required to build organisms. *Am. Nat.* 188, S62–S73. doi: 10.1086/687576

Lahner, B., Gong, J. M., Mahmoudian, M., Smith, E. L., Abid, K. B., Rogers, E. E., et al. (2003). Genomic scale profiling of nutrient and trace elements in *Arabidopsis thaliana*. *Nat. Biotechnol.* 21, 1215–1221. doi: 10.1038/nbt865

Li, H. Y., Yang, Y. M., Zhang, H. Y., Chu, S. S., Zhang, X. G., Yin, D. M., et al. (2016). A genetic relationship between phosphorus efficiency and photosynthetic traits in soybean as revealed by QTL analysis using a high-density genetic map. *Front. Plant Sci.* 7:924. doi: 10.3389/fpls.2016.00924

Loladze, I. (2014). Hidden shift of the ionome of plants exposed to elevated CO2 depletes minerals at the base of human nutrition. *Elife* 3:e02245. doi: 10.7554/eLife.02245

Malinouski, M., Hasan, N. M., Zhang, Y., Seravalli, J., Lin, J., Avanesov, A., et al. (2014). Genome-wide RNAi ionomics screen reveals new genes and regulation of human trace element metabolism. *Nat. Commun.* 5:3301. doi: 10.1038/ncomms4301

Merchant, S., and Helmann, J. (2012). Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation. *Adv. Microb. Physiol.* 60, 91–210. doi: 10.1016/B978-0-12-398264-3.00002-4

Meunier, C. L., Malzahn, A. M., and Boersma, M. (2014). A new approach to homeostatic regulation: towards a unified view of physiological and ecological concepts. *PLoS ONE* 9:e107737. doi: 10.1371/journal.pone.0107737

Misson, J., Raghothama, K. G., Jain, A., Jouhet, J., Block, M. A., Bligny, R., et al. (2005). A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11934–11939. doi: 10.1073/pnas.0505266102

Mitani-Ueno, N., Yamaji, N., Zhao, F. J., and Ma, J. F. (2011). The aromatic/arginine selectivity filter of NIP aquaporins plays a critical role in substrate selectivity for silicon, boron, and arsenic. *J. Exp. Bot.* 62, 4391–4398. doi: 10.1093/jxb/err158

Morrissey, J., Baxter, I. R., Lee, J., Li, L. T., Lahner, B., Grotz, N., et al. (2009). The ferroportin metal efflux proteins function in iron and cobalt homeostasis in Arabidopsis. *Plant Cell* 21, 3326–3338. doi: 10.1105/tpc.109.069401

Moseley, J. L., Chang, C. W., and Grossman, A. R. (2006). Genome-based approaches to understanding phosphorus deprivation responses and PSR1 control in *Chlamydomonas reinhardtii*. *Eukaryot. Cell* 5, 26–44. doi: 10.1128/EC.5.1.26-44.2006

Muchhal, U. S., Pardo, J. M., and Raghothama, K. G. (1996). Phosphate transporters from the higher plant *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 93, 10519–10523. doi: 10.1073/pnas.93.19.10519

Myers, S. S., Zanobetti, A., Kloog, I., Huybers, P., Leakey, A., Bloom, A., et al. (2014). Increasing CO2 threatens human nutrition. *Nature* 510, 139–142. doi: 10.1038/nature13179

Nose, Y., Kim, B. E., and Thiele, D. J. (2006). Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. *Cell Metab.* 4, 235–244. doi: 10.1016/j.cmet.2006.08.009

Romano, S., Schulz-Vogt, H. N., Gonzalez, J. M., and Bondarev, V. (2015). Phosphate limitation induces drastic physiological changes, virulence-related gene expression, and secondary metabolite production in Pseudovibrio sp strain FO-BEG1. *Appl. Environ. Microbiol.* 81, 3518–3528. doi: 10.1128/AEM.04167-14

Roy Chowdhury, P., Lopez, J. A., Weider, L. J., Colbourne, J. K., and Jeyasingh, P. D. (2014). Functional genomics of intraspecific variation in carbon and phosphorus kinetics in daphnia. *J. Exp. Zool. A Ecol. Genet. Physiol.* 321, 387–398. doi: 10.1002/jez.1869

Saito, M. A., Goepfert, T. J., and Ritt, J. T. (2008). Some thoughts on the concept of colimitation: three definitions and the importance of bioavailability. *Limnol. Oceanogr.* 53, 276–290. doi: 10.4319/lo.2008.53.1.0276

Salt, D., Baxter, I., and Lahner, B. (2008). Ionomics and the study of the plant ionome. *Annu. Rev. Plant Biol.* 59, 709–733. doi: 10.1146/annurev.arplant.59.032607.092942

Scott, J. T., Cotner, J. B., and LaPara, T. M. (2012). Variable stoichiometry and homeostatic regulation of bacterial biomass elemental composition. *Front. Microbiol.* 3:42. doi: 10.3389/fmicb.2012.00042

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry : the Biology of Elements from Molecules to the Biosphere*. Princeton, NJ: Princeton University Press.

Sterner, R. W., Elser, J. J., and Hessen, D. O. (1992). Stoichiometric relationships among producers, consumers and nutrient cycling in pelagic ecosystems. *Biogeochemistry* 17, 49–67. doi: 10.1007/BF00002759

Tobler, M., Alba, D. M., Arias-Rodriguez, L., and Jeyasingh, P. D. (2016). Using replicated evolution in extremophile fish to understand diversification in elemental composition and nutrient excretion. *Freshw. Biol.* 61, 158–171. doi: 10.1111/fwb.12691

Twining, B., Nunez-Milland, D., Vogt, S., Johnson, R.S., and Sedwick, P.N. (2010). Variations in *Synechococcus* cell quotas of phosphorus, sulfur, manganese, iron, nickel, and zinc within mesoscale eddies in the Sargasso Sea. *Limnol. Oceanogr.* 55, 492–506. doi: 10.4319/lo.2010.55.2.0492

Van Bogelen, R. A., Olson, E. R., Wanner, B. L., and Neidhardt, F. C. (1996). Global analysis of proteins synthesized during phosphorus restriction in *Escherichia coli*. *J. Bacteriol.* 178, 4344–4366. doi: 10.1128/jb.178.15.4344-4366.1996

van de Wiel, C. C. M., van der Linden, C. G., and Scholten, O. E. (2016). Improving phosphorus use efficiency in agriculture: opportunities for breeding. *Euphytica* 207, 1–22. doi: 10.1007/s10681-015-1572-3

Van Mooy, B. A. S., Fredricks, H. F., Pedler, B. E., Dyhrman, S. T., Karl, D. M., Koblízek, M., et al. (2009). Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature* 458, 69–72. doi: 10.1038/nature07659

Wang, H., Sterner, R. W., and Elser, J. J. (2012). On the "strict homeostasis" assumption in ecological stoichiometry. *Ecol. Modell.* 243, 81–88. doi: 10.1016/j.ecolmodel.2012.06.003

Williams, R. J. P., and Frausto da Silva, J. J. (2006). *The Chemistry of Evolution: the Development of Our Ecosystem*. Amsterdam; Boston, MA: Elsevier.

Yu, D., Danku, J. M., Baxter, I., Kim, S., Vatamaniuk, O. K., Vitek, O., et al. (2012). High resolution genome-wide scan of genes, gene-networks and cellular systems impacting the yeast ionome. *BMC Genomics* 13:623. doi: 10.1186/1471-2164-13-623

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 January 2017
doi: 10.3389/fmicb.2016.02113

The Role of Microbes in the Nutrition of Detritivorous Invertebrates: A Stoichiometric Analysis

Thomas R. Anderson[1]*, *David W. Pond*[2] and *Daniel J. Mayor*[1]*

[1] National Oceanography Centre, Southampton, UK, [2] Scottish Association for Marine Science, Natural Environment Research Council, Oban, UK

Detritus represents an important pool in the global carbon cycle, providing a food source for detritivorous invertebrates that are conspicuous components of almost all ecosystems. Our knowledge of how these organisms meet their nutritional demands on a diet that is typically comprised of refractory, carbon-rich compounds nevertheless remains incomplete. "Trophic upgrading" of detritus by the attached microbial community (enhancement of zooplankton diet by the inclusion of heterotrophic protozoans) represents a potential source of nutrition for detritivores as both bacteria and their flagellated protistan predators are capable of biosynthesizing essential micronutrients such as polyunsaturated fatty acids (PUFAs). There is however a trade-off because although microbes enhance the substrate in terms of its micronutrient content, the quantity of organic carbon is diminished though metabolic losses as energy passes through the microbial food web. Here, we develop a simple stoichiometric model to examine this trade-off in the nutrition of detritivorous copepods inhabiting the mesopelagic zone of the ocean, focusing on their requirements for carbon and an essential PUFA, docosahexaenoic acid (DHA). Results indicate that feeding on microbes may be a highly favorable strategy for these invertebrates, although the potential for carbon to become limiting when consuming a microbial diet exists because of the inefficiencies of trophic transfer within the microbial food web. Our study highlights the need for improved knowledge at the detritus-microbe-metazoan interface, including interactions between the physiology and ecology of the associated organisms.

Keywords: detritus, microbial loop, stoichiometry, trophic upgrading, polyunsaturated fatty acids, mesopelagic zone

OPEN ACCESS

Edited by:
James Joseph Elser, University of Montana, USA

Reviewed by:
Andrew Decker Steen, University of Tennessee, USA
Tom Andersen, University of Oslo, Norway
Angela Peace, Texas Tech University, USA

***Correspondence:**
Thomas R. Anderson
tra@noc.ac.uk
Daniel J. Mayor
dan.mayor@noc.ac.uk

Specialty section:
This article was submitted to Aquatic Microbiology, a section of the journal Frontiers in Microbiology

Received: *07 November 2016*
Accepted: *14 December 2016*
Published: *04 January 2017*

Citation:
Anderson TR, Pond DW and Mayor DJ (2017) The Role of Microbes in the Nutrition of Detritivorous Invertebrates: A Stoichiometric Analysis. Front. Microbiol. 7:2113. doi: 10.3389/fmicb.2016.02113

INTRODUCTION

The production of dead and decaying particulate organic matter ("detritus" hereafter) may account for as much as 56% of primary production when averaged across a range of ecosystems (Cebrián and Duarte, 1995). This flux of detritus thereby constitutes a significant term in the global carbon cycle (Ciais et al., 2013) and is a major conduit through which organic matter is transported both within and between ecosystems (Bartels et al., 2012). It also provides sustenance to countless detritivorous invertebrates, which we loosely interpret as any animal that has a trophic association with dead organic matter, including organismal egesta. Detritus-detritivore interactions influence the potential for carbon sequestration in both terrestrial and aquatic environments. Understanding

the interface between living and dead organic matter is therefore a prerequisite to improving predictions of global biogeochemical cycles and climate (Burd et al., 2016; Luo et al., 2016).

Detritus is mainly composed of refractory compounds such as structural polysaccharides (Mann, 1988; Kiem and Kögel-Knabner, 2003), but is depleted in micronutrients such as amino acids and fatty acids (Cowie and Hedges, 1996; Pokarzhevskii et al., 1997; Mayor et al., 2011) that are considered essential for the growth of metazoan animals (Müller-Navarra et al., 2000; Anderson et al., 2004; Sampedro et al., 2006; Larsen et al., 2016). The nutritional challenge facing detritivores may, however, be mitigated by the presence of microorganisms that colonize the detrital substrate (Moran and Hodson, 1989; Turley and Mackie, 1994). Detritivores actively ingest this detritus-associated microbial community which, unlike the basal substrate, is readily absorbed and provides a rich source of micronutrients (Bärlocher and Kendrick, 1975; Phillips, 1984; Lawrence et al., 1993; Koski et al., 2005). Indeed, a key functional characteristic of many detritivorous invertebrates is their propensity to shred or fragment detritus (Anderson and Sedell, 1979; Iversen and Poulsen, 2007), an activity that has been proposed to stimulate the production of microbial biomass by increasing the surface area of the substrate, so-called "microbial gardening" (Fenchel, 1970; Mayor et al., 2014). The resulting uplift in the nutritional content of detritus represents a form of "trophic upgrading," a term which originates from the marine literature and refers to the enhancement of zooplankton growth by the inclusion of micronutrient-rich heterotrophic protozoans in an otherwise herbivorous diet (Klein Breteler et al., 1999). Relying on microbes as a primary source of nutrition does, however come at an energetic cost because their gross growth efficiencies are typically $<$30 % (Del Giorgio and Cole, 1998) and the majority of organic carbon in the detrital substrate is therefore lost during the trophic upgrading process. Detritivorous invertebrates thus face a trade-off between consuming a high quality, low quantity diet that is rich in microbes versus the low quality, high quantity detritus (Mayor et al., 2014).

Here, we use a simple stoichiometric model to examine the extent to which invertebrates maximize growth by incorporating microbes into their diet, using detritivorous zooplankton in the mesopelagic zone (MPZ) of the ocean as a case study. The MPZ extends from the base of the sunlit (euphotic) zone down to $\sim$1000 m and many of the resident organisms are primarily sustained by an estimated global detrital flux of 5–12 Gt C yr^{-1} (Henson et al., 2011). The depth at which organic matter is remineralized within the MPZ influences the residence time of carbon in the oceans and hence global climate (Kwon et al., 2009). Sinking detrital particles in the MPZ exhibit the characteristic poor nutritional status described above, having undergone stripping of the most desirable compounds by bacteria and/or multiple ingestion events by zooplankton (Podgórska and Mundryk, 2003; Wilson et al., 2008). The resulting substrate is thus largely devoid of essential micronutrients such as amino or fatty acids (Wakeham et al., 1997; Fileman et al., 1998; Schneider et al., 2003). We suggest that the problem of obtaining sufficient nutrition may be felt acutely by detritivorous zooplankton that permanently reside in the MPZ, e.g., copepods of the genus *Oithona* that are ubiquitous throughout the world ocean (Gallienne and Robins, 2001; Dahms et al., 2015). Members of this genus are well known to interact with detrital particles (Gonzalez and Smetacek, 1994; Iversen and Poulsen, 2007), particularly in the mesopelagic (Suzuki et al., 2003). Organisms inhabiting the MPZ experience high hydrostatic pressure and low temperatures, both of which negatively affect the functioning of cellular membranes (Hazel and Williams, 1990). Zooplankton overcome these difficulties by increasing the relative abundance of the essential polyunsaturated fatty acid, docosahexaenoic acid (DHA), in their membranes (Pond et al., 2014). Copepods and other highly motile zooplankton also possess myelin-like sheathes around their nerve axons to facilitate rapid escape responses (Raymont et al., 1974; Davis et al., 1999) and DHA has been suggested to be an important component of the associated sphingomyelin lipid pool (Scott et al., 2002). The model presented herein has C and DHA as currencies and is used to examine the trade-off for detritivorous zooplankton when consuming a high quantity, low DHA:C diet (detritus) versus a nutritionally-upgraded diet of microbial biomass present in low quantity, but with a high DHA:C ratio. Our analysis, which is underpinned by empirical data from a number of sources, highlights the need for improved understanding of food web processes in the mesopelagic, including the associated physiology of the resident organisms.

MODEL DESCRIPTION

Equations

The model is a steady-state flow analysis of the detrital food web in the MPZ of the ocean, including colonization of detritus by microbes (particle-attached bacteria and protistan bacterivores) and their consumption by detritivorous zooplankton (**Figure 1**; lists of model variables and parameters are provided in **Tables 1**, **2**). The main focus is the growth of zooplankton and its stoichiometric regulation by C and DHA. The baseline currency of the model is C from which flows are calculated throughout the food web as a whole. Zooplankton growth, on the other hand, is calculated from stoichiometric equations involving both C and DHA. Fixed ratios (model parameters) are specified for DHA:C in detritus, bacteria and bacterivores which, in conjunction with predicted C cycling throughout the food web, permits an assessment of the roles of C and DHA in limiting the growth of zooplankton (depending on the relative availability of each food type to their diet). It is thus possible to examine the potential trade-off between consuming a high quantity, low quality diet (detritus with a low DHA:C ratio) versus a low quantity, high quality diet (microbes with a high DHA:C ratio). In this context, it is useful to define the two end-members of the nutritional spectrum: a "detritivorous pathway" and a "microbial pathway." The former represents consumption of the non-living detrital substrate, whereas the microbial pathway consists of a diet solely of microbes. Our default assumption is that detritivorous zooplankton selectively ingest protistan bacterivores on the basis of their motility. The microbial pathway therefore represents a diet consisting solely

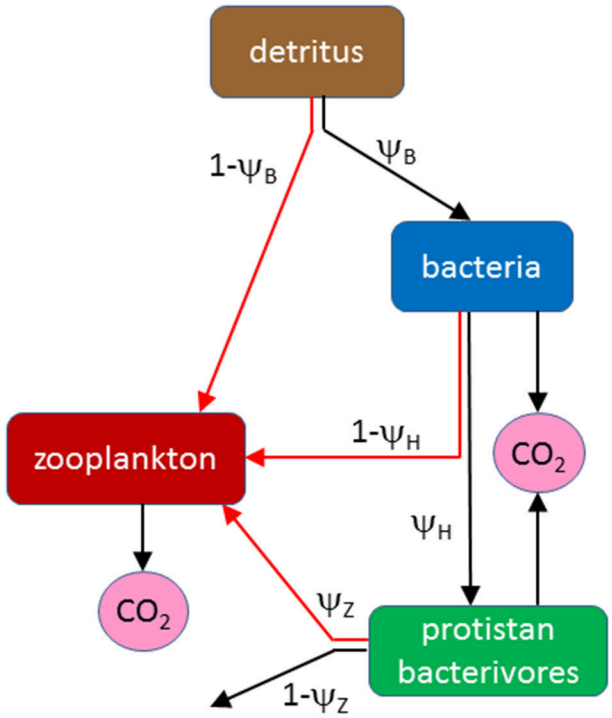

FIGURE 1 | Flow diagram of the model showing pathways of organic matter between detritus, bacteria, protistan bacterivores and zooplankton, as specified by parameters ψ_B, ψ_H, and ψ_Z. Black arrows represent C-only flows, red arrows involve both C and DHA (involving stoichiometric calculations).

TABLE 1 | Model variables.

Variable	Definition	Unit of measure
F_D	Entry flux of D into system	mol C m^{-3} d^{-1}
$A_{C,det}$	Absorption C: detrit. path	mol C m^{-3} d^{-1}
$A_{DHA,det}$	Absorption DHA: detrit. path	mmol DHA m^{-3} d^{-1}
$A_{C,mic}$	Absorption C: microb. path	mol C m^{-3} d^{-1}
$A_{DHA,mic}$	Absorption DHA: microb path	mmol DHA m^{-3} d^{-1}
G_B	Bacterial production	mol C m^{-3} d^{-1}
G_H	Bacterivore production	mol C m^{-3} d^{-1}
G_Z	Zooplankton production	mol C m^{-3} d^{-1}

of these organisms and excludes particle-attached bacteria. The sensitivity of predicted zooplankton growth to whether or not bacteria constitute a food source will nevertheless be investigated by including the possibility of ingesting bacteria in the model structure and parameterization.

The stoichiometric calculations of zooplankton growth assume that these animals are unable to synthesize DHA *de novo* (Bell et al., 2007) in which case this essential fatty acid can be treated in the same way as elements such as C, N and P when using theoretical stoichiometry to analyze limitation of growth (Anderson and Pond, 2000). Bacteria and bacterivores are, on the other hand capable of synthesizing essential acids, including DHA, *de novo* (Klein Breteler et al., 1999; Russell and Nichols, 1999; Fang et al., 2002) and so their growth is calculated assuming that limitation is by C.

TABLE 2 | Model parameters.

Parameter	Definition	Default value	Unit of measure
θ_D	DHA:C, detritus	0.21	mmol DHA mol C^{-1}
θ_Z	DHA:C, zooplankton	1.76	mmol DHA mol C^{-1}
θ_B	DHA:C, bacteria	0.08	mmol DHA mol C^{-1}
θ_H	DHA:C, bacterivores	1.40	mmol DHA mol C^{-1}
ω_B	bacteria GGE	0.12	dimensionless
β_H	AE, bacterivores on bacteria	0.72	dimensionless
k_H	max. NPE, bacterivores: C	0.44	dimensionless
β_{ZC}	AE, zooplankton on D: C	0.1	dimensionless
β_{ZDHA}	AE, zooplankton on D: DHA	0.1	dimensionless
β_{ZBH}	AE, zooplankton on B,H	0.72	dimensionless
k_{ZC}	max. NPE, zooplankton: C	0.36	dimensionless
k_{ZDHA}	max. NPE, zoopl.: DHA	0.9	dimensionless
ψ_B	partitioning D to bacteria	0.1	dimensionless
ψ_H	partitioning B to bacterivores	1.0	dimensionless
ψ_Z	partitioning H to zoopl.	0.8	dimensionless

Detritus provides the foundation of the mesopelagic food web, specified as an input flux to the model, F_D (mol C m^{-3} d^{-1}). The detrital substrate is acted on by either particle-attached bacteria (fraction ψ_B) or by zooplankton (fraction 1-ψ_B). The latter gives rise to the detritivorous pathway, which we consider first. Ingested C and DHA following this pathway, i.e., from direct consumption of non-living detritus by zooplankton, are subject to absorption efficiencies (AEs) β_{ZC} and β_{ZDHA} in which case quantities of absorbed C and DHA, $A_{C,det}$ and $A_{DHA,det}$, are:

$$A_{C,\mathrm{det}} = (1-\psi_B)\beta_{ZC}F_D \quad (1)$$

$$A_{DHA,\mathrm{det}} = (1-\psi_B)\beta_{ZDHA}\theta_D F_D \quad (2)$$

where θ_D is the DHA:C ratio in detritus (excluding microbes within the detrital matrix).

The alternative is for detritivores to obtain nutrition by consuming microbes, the "microbial pathway," which necessitates predicting the availability of bacteria and protistan bacterivores deriving from trophic transfer within the food web. Bacteria utilize detritus with growth efficiency ω_B, from which their growth, G_B, is:

$$G_B = \psi_B\omega_B F_D \quad (3)$$

The fate of bacteria in the model is either consumption by protistan bacterivores within the particle-attached food web (fraction ψ_H) or zooplankton (fraction 1-ψ_H); note that our default assumption is that of zero consumption by zooplankton, i.e., $\psi_H = 1$. The growth of the bacterivores, G_H, is calculated as the product of ingestion ($\psi_H G_B$), absorption efficiency (for C; parameter β_H) and net production efficiency (NPE; the fraction of absorbed C allocated to growth; parameter k_H):

$$G_H = \psi_H\beta_H k_H G_B \quad (4)$$

Total ingestion of C by zooplankton via the microbial pathway is the sum of that on bacteria, (1-ψ_H)G_B, and protistan bacterivores, $\psi_Z G_H$ (fraction ψ_Z of bacterivore production is utilized by zooplankton), with corresponding intake of DHA calculated from the DHA:C ratios of these food sources (θ_B and θ_H for bacteria and protistan bacterivores, respectively). The resulting quantities of absorbed C and DHA following the microbial pathway, $A_{C,mic}$ and $A_{DHA,mic}$, are then:

$$A_{C,mic} = \beta_{ZBH}((1-\psi_H)G_B + \psi_Z G_H) \quad (5)$$

$$A_{DHA,mic} = \beta_{ZBH}((1-\psi_H)\theta_B G_B + \psi_Z \theta_H G_H) \quad (6)$$

where β_{ZBH} is absorption efficiency for zooplankton on bacterivores (applied equally to C and DHA).

Zooplankton growth can now be calculated using established stoichiometric equations (e.g., Anderson and Hessen, 1995) that compare the relative availability of C and DHA in absorbed substrates, as supplied by both the detritivorous and microbial pathways. If C is limiting then growth, G_Z (mol C m^{-3} d^{-1}), is:

$$G_Z(C) = k_{ZC}(A_{C,\mathrm{det}} + A_{C,mic}) \quad (7)$$

where parameter k_{ZC} is the maximum NPE for C (maximum k_{ZC} occurs when C is limiting; realized k_{ZC} is lower when DHA is limiting growth because C is then in stoichiometric excess). The corresponding equation for G_Z when DHA is limiting is:

$$G_Z(DHA) = k_{ZDHA}(A_{DHA,\mathrm{det}} + A_{DHA,mic})/\theta_Z \quad (8)$$

where k_{ZDHA} is maximum net production efficiency for DHA and θ_Z is the DHA:C ratio in zooplankton biomass. Realized growth is then then the minimum of the calculated C- and DHA-limited rates:

$$G_Z = MIN[G_Z(C), G_Z(DHA)] \quad (9)$$

A threshold elemental ratio (TER) can be calculated, θ_A^*, which is the optimum ratio of DHA and C in absorbed substrates for growth:

$$\theta_A^* = \frac{k_{ZC}\theta_Z}{k_{ZDHA}} \quad (10)$$

With parameters as in **Table 2** (k_{ZC} = 0.36, k_{ZDHA} = 0.9 and θ_Z = 1.76), calculated θ_A^* is 0.70 meaning that optimal growth requires that each mol of absorbed C is accompanied by 0.70 mmol of absorbed DHA.

Parameterization

Model parameters fall into three categories: those specifying trophic transfer (growth efficiencies), those that define the fractionation of C between the different flow pathways in the model, and the four parameters that define DHA:C ratios in biomass. Starting with the first category, the absorption efficiency of C for zooplankton grazing on detritus, parameter β_{ZC}, was assigned a low value of 0.1 because of the refractory nature of the substrate (Bärlocher and Kendrick, 1975). The same absorption efficiency was applied to DHA, i.e., β_{ZDHA} = 0.1, thereby assuming that zooplankton are unable to selectively extract DHA from the detritus matrix; this parameter will be subject to sensitivity analysis. Living microbes are considerably more amenable to digestion by zooplankton and so the efficiencies with which ingested bacteria and protistan bacterivores are absorbed, parameter β_{ZBH} (applied equally to both groups), was assigned a value of 0.72 (Anderson and Tang, 2010). The net production efficiency with which absorbed C is used for growth is well below 1.0 because of the energetic costs of metabolism. We set k_{ZC} = 0.36 based on a mean gross growth efficiency (GGE) of 0.26 for copepods (Straile, 1997) from which NPE is calculated by dividing through by AE of 0.72 (GGE is the product of AE and NPE). The role of essential fatty acids such as DHA in metabolism is not well known. The simplest assumption is that they are not heavily involved in which case DHA may be utilized for growth with high NPE e.g., k_{ZDHA} = 0.9 (Anderson and Pond, 2000; Mayor et al., 2009).

Moving on to the microbial food web, a typical BGE for particle-attached bacteria is 0.24 (Anderson and Tang, 2010) but this does not take into account that as much as 50% of the substrate may be lost in dissolved form through solubilization by exoenzymes (Anderson and Tang, 2010; Mayor et al., 2014). The model here does not explicitly represent solubilization losses and therefore, in practical terms, the value of 0.24 should be halved, giving ω_B = 0.12. The magnitude of BGE is not well understood in marine systems and so this parameter, which sets the inflow of carbon to the microbial pathway, will be the subject of sensitivity testing. Protistan bacterivores graze on the particle-attached bacteria. As for the zooplankton, an absorption efficiency of 0.72 was applied, along with a NPE for C of 0.44 (derived from a GGE of 0.32 for flagellates: Straile, 1997), parameters β_H and k_H, respectively.

Parameters for the fractionation of C via the flow pathways in the food web, ψ_B, ψ_H, and ψ_Z, are not easy to estimate. The first of these, namely the partitioning of detritus usage between particle-attached bacteria (parameter ψ_B, leading to the microbial pathway) and detritivorous zooplankton (1-ψ_B; leading to the detritivorous pathway) was guesstimated at 0.75 by Anderson and Tang (2010) based on the data of Steinberg et al. (2008). An improved estimate of ψ_B = 0.5 was justified by Mayor et al. (2014), based on data from the North Atlantic. Most of our analysis of the model will focus on the two separate ends of the spectrum of this parameter, i.e., ψ_B = 0,1, in order to provide a theoretical comparison of the nutritional benefits of the detritivorous and microbial pathways in isolation to each other. Values of ψ_B that lead to optimal zooplankton nutrition are then calculated, which can be compared to the estimates above. The trophic linkages of the microbial food web on particles are not well known but it is reasonable to expect a tight coupling between bacteria and protistan bacterivores because of their close proximity (Grossart and Ploug, 2001), and thereby a high value of ψ_H. Moreover, it may be that the detritivorous zooplankton selectively ingest protistan bacteriovores on the basis of their motility (Kiørboe, 2011), leaving the bacteria untouched, in which case ψ_H = 1 (the default value used in our analysis). The fate of flagellate biomass is even less certain. We tentatively assume that, without other obvious predators, the majority of

the flagellate loss term is available to support the growth of zooplankton and set $\psi_Z = 0.8$.

Data Sources

Studies that concurrently present data on the C and DHA content of marine seston and/or organisms are scarce, and almost non-existent for the MPZ. Parameter values for the DHA:C values in seston biomass, $\theta_D = 0.21$ mmol mol^{-1} (detritus), $\theta_B = 0.08$ (bacteria), $\theta_H = 1.4$ (protistan bacterivores) and $\theta_Z = 1.76$ (zooplankton) were therefore obtained from a variety of representative sources.

The DHA:C content of detritus ($\theta_D = 0.21$ mmol mol^{-1}) is for seston collected on a pre-combusted GF/F filter (0.7 μm) at a depth of 215 m in the Bellingshausen Sea, Antarctica (Fileman et al., 1998). This likely represents an upper-estimate of this parameter because the sample came from the upper MPZ and the collection method made no attempt to distinguish between non-living detritus and (DHA-rich) organismal biomass. The DHA:C content of particle-attached bacteria ($\theta_B = 0.08$ mmol mol^{-1}) represents an average value derived from various culture studies on deep-sea microbes ($\theta_B = 0.11, 0.11, 0.03$; Fang et al., 2002, 2003, 2004, respectively). The DHA:C content of protistan bacterivores ($\theta_H = 1.4$ mmol mol^{-1}) is an average value for the heterotrophic dinoflagellate, *Oxyrrhis marina*, reared on the algae *Rhodomonas* sp. ($\theta_H = 1.54$) and *Dunaliella* sp. ($\theta_H = 1.32$) (Klein Breteler et al., 1999). An average value for the DHA:C content of zooplankton ($\theta_Z = 1.76$ mmol mol^{-1}) was used based on published data for female copepods of the species *Oithona similis*, collected from between 400 m depth and the surface in Antarctic waters (Pond and Ward, 2011). Interested readers are guided to the relevant citations for further details of individual sample collection and analysis.

RESULTS

The main focus of the analysis presented herein is a theoretical examination of the two ends of the nutritional spectrum, namely the detritivorous pathway ($\psi_B = 0$; zooplankton diet of non-living detritus) and the microbial pathway ($\psi_B = 1$; diet consisting solely of protistan bacterivores). This provides the most effective means of examining the trade-off between consuming a high quantity, low quality diet (detritus with a low DHA:C ratio) versus a low quantity, high quality diet (microbes with a high DHA:C ratio). The growth of zooplankton on a mixed diet incorporating both detritus and microbes will be investigated thereafter.

The utilization of C and DHA by zooplankton for growth, via ingestion and absorption, is compared for the detritivorous and microbial pathways in **Figure 2** (parameters as in **Table 2**). The detritus flux into the system, F_D, was nominally set at 1 mol C m^{-3} d^{-1}, facilitating ease of analysis (everything is normalized to an input of 1; there is no need to use an observed value of F_D in order to compare the relative merits of the detritivorous and microbial pathways as a source of nutrition for zooplankton). The supply of C via the detritivorous pathway is plentiful whereas ingestion of C via the microbial pathway is reduced by 97% because of C losses in trophic transfer associated with the growth efficiencies of bacteria and bacterivores (**Figure 2A**). Perhaps surprisingly, detritus is also predicted to be the most plentiful source of DHA, with intake of 0.21 mmol m^{-3} d^{-1} compared to 0.043 mmol m^{-3} d^{-1} via the microbial pathway (**Figure 2A**). This is again a consequence of the much diminished stocks of bacterivore biomass compared to detritus and occurs despite the DHA:C ratio being more than six times higher in bacterivores (1.4 in bacterivores versus 0.21 mmol mol^{-1} in detritus). Microbial biomass is, however, absorbed with much higher efficiency than detritus ($\beta_{ZBH} = 0.72$ versus $\beta_{ZC} = \beta_{ZDHA} = 0.1$) and so the difference in substrate supply between the two pathways is diminished post-absorption (**Figure 2B**). The absorbed quantity of DHA is greatest following the microbial pathway (0.031 vs. 0.021 mmol m^{-3} d^{-1}) whereas the amount of absorbed C remains considerably lower than in the detritivorous pathway (0.022 vs. 0.1 mol C m^{-3} d^{-1}).

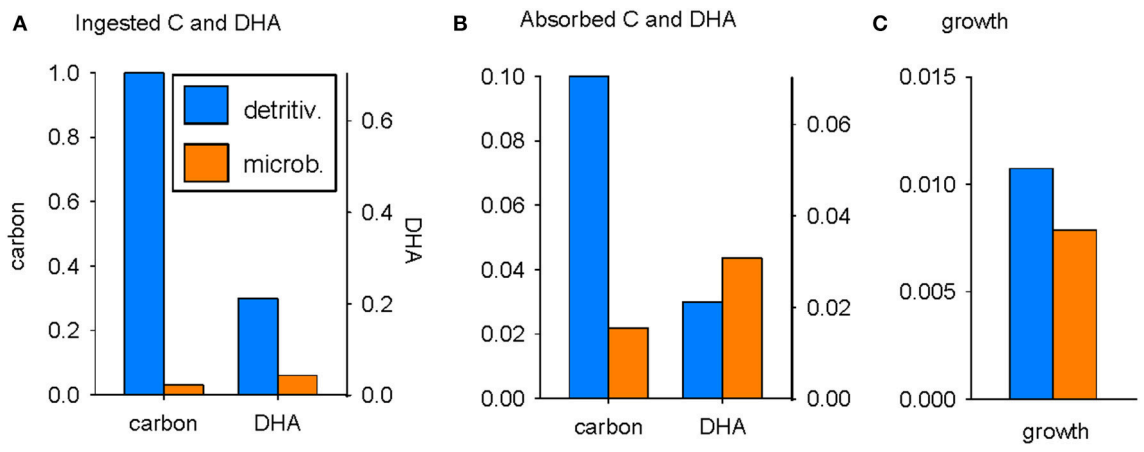

FIGURE 2 | Utilization of C and DHA by zooplankton following the detritivorous ($\psi_B = 0$; blue) and microbial ($\psi_B = 1$; orange) pathways: (A) ingestion, **(B)** absorption, **(C)** growth. $F_D = 1$ mol C m^{-3} d^{-1}; units of ingestion and absorption of C, and growth, are mol C m^{-3} d^{-1}; units for ingestion and absorption of DHA are mmol m^{-3} d^{-1}. DHA is scaled to the optimum absorption ratio (Equation 10: see text).

The growth of zooplankton depends not only on quantities of absorbed substrates, but also on the net production efficiencies for DHA and C, k_{ZDHA} and k_{ZC} respectively, as well as the DHA:C ratio in biomass, θ_Z (Equations 7, 8). Note that the DHA axes in **Figure 2** are scaled to the optimal DHA:C ratio in absorbed substrates ($\theta_A^* = 0.70$; Equation 10) so that the potential for growth limitation by C or DHA can be determined by visual comparison of the bar heights for a given trophic pathway. It can be seen that predicted zooplankton growth following the detritivorous pathway is limited by DHA (the blue bar for DHA is lower than that for C in **Figure 2B**) whereas growth following the microbial pathway is limited by C (the orange bar for C is lower than that for DHA). Overall, the assembled parameter set indicates that growth is greatest following the detritivorous pathway, although the margin is small (0.011 vs. 0.008 mol C m^{-3} d^{-1}; **Figure 2C**).

We used parameter sensitivity analysis to investigate the circumstances under which predicted zooplankton growth is greatest following the microbial pathway. **Figures 3A,B** illustrate how chosen parameter values for zooplankton net production

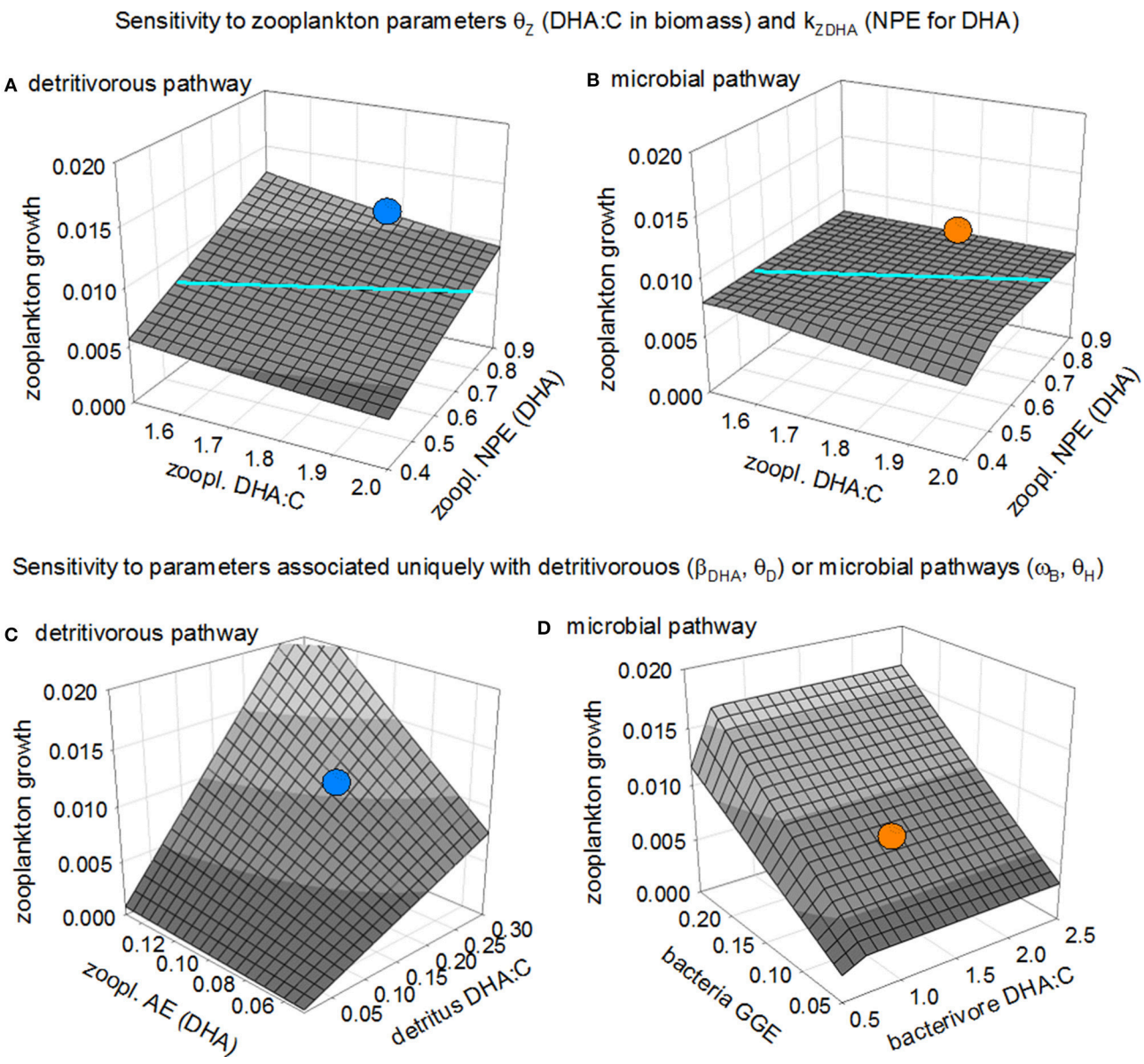

FIGURE 3 | Sensitivity of predicted zooplankton growth to parameters θ_Z (zooplankton DHA:C ratio; mmol mol^{-1}) and k_{ZDHA} (zooplankton NPE of DHA) for the detritivorous and microbial pathways (panels A and B; the colored lines demarcate where the two planes intersect) and sensitivity to key parameters associated with the two pathways: (C) detritivorous pathway, parameters θ_D (detritus DHA:C ratio) and β_{ZDHA} (zooplankton absorption efficiency for DHA in detritus) and **(D)** microbial pathway, parameters θ_H (bacterivore DHA:C ratio) and ω_B (B GGE). The two blue points indicate predicted growth following the detritivorous pathway as shown in **Figure 2**, and the two orange points the corresponding predicted growth following the microbial pathway.

efficiency for DHA (k_{ZDHA}) and the DHA:C in zooplankton biomass (θ_Z) influence growth following the two pathways. Zooplankton are DHA-limited in the detritivorous pathway throughout the parameter domain (**Figure 3A**). Recent work has shown that a range of aquatic invertebrates, including marine zooplankton, catabolize essential PUFAs at high rates (Mezek et al., 2010; Mayor et al., 2011, 2015; Maity et al., 2012) in which case our default zooplankton NPE for DHA of 0.9 (Anderson and Pond, 2000; Mayor et al., 2009) may be too high. Reducing the value of this parameter results in a proportional lowering of predicted zooplankton growth, to the extent that the detritivorous pathway becomes an inferior source of nutrition relative to the microbial pathway (in areas of the plane shown in **Figure 3A** that are lower than those of the corresponding parameter space shown in **Figure 3B**). Increasing the DHA:C ratio in the biomass of zooplankton, thereby increasing the demand for DHA, likewise causes a decrease in predicted growth following the detritivorous pathway. Growth following the microbial pathway is, in contrast, relatively insensitive to changing either k_{ZDHA} or θ_Z throughout most of the parameter space because limitation is by C (**Figure 3B**).

Figures 3C,D show the sensitivity of zooplankton growth to the absorption efficiency for DHA (β_{ZDHA}) and the detritus DHA:C ratio (θ_D) for the detritivorous pathway, and bacterial gross growth efficiency (ω_B) and DHA:C ratio in protistan bacterivores (θ_H) for the microbial pathway. Predicted growth following the detritivorous pathway is limited by DHA and so declines as this micronutrient becomes less available, either due to decreased absorption efficiency and/or reduced availability in detritus (**Figure 3C**). Our default value for the DHA:C of detritus ($\theta_D = 0.21$ mmol DHA mol C^{-1}) is likely too high because the samples upon which it is based were from a relatively shallow depth and did not exclude microbes from the detrital matter (see "Data sources" section), leading to overestimated growth following the detritivorous pathway. We assumed that C and DHA within detritus are absorbed by zooplankton with the same efficiency ($\beta_{ZC} = \beta_{ZDHA} = 0.1$), i.e., these animals are unable to selectively extract DHA from the detritus matrix. If they were able to do so, which is achieved in the model by increasing parameter β_{ZDHA} while keeping β_{ZC} at 0.1, the detritivorous pathway then becomes more profitable as a source of nutrition (**Figure 3C**). Growth of zooplankton following the microbial pathway shows no sensitivity to the DHA:C ratio in protistan bacterivores, except when this ratio is very low (<0.7; **Figure 3D**) because, although the bacterivores are a plentiful supply of DHA, limitation is by C. Growth does, however, increase with increasing bacterial growth efficiency because this results in more C being incorporated into the microbial food web.

In summary, the sensitivity analysis presented in **Figure 3** confirms the findings of **Figure 2**, showing the basic trade-off facing detritivorous zooplankton: a choice between consuming high quantity, low quality detritus via the detritivorous pathway which leads to limitation by DHA, or a low quantity, high quality protistan diet via the microbial pathway, with limitation by C. The analysis of **Figure 2** showed that, with the default parameter set, the growth of zooplankton was greatest following the detritivorous pathway. The trade-off choice of opting for DHA-rich microbes (the microbial pathway) was less favorable in this instance because the losses of C due to trophic transfer in the microbial food web overrode the gains in greater DHA availability. The sensitivity analysis showed that this situation can easily be reversed by alteration of various parameter values, leading to the microbial pathway being the superior source of nutrition for zooplankton: predicted growth via the detritivorous pathway decreased when the net production efficiency for DHA (k_{ZDHA}) or the DHA:C in detritus (θ_D) are lowered, or when the DHA:C of zooplankton biomass (θ_Z) was increased. Increasing bacterial gross growth efficiency (ω_B), which promotes protistan growth, also reduced the relative effectiveness of the detrital pathway. On the other hand, the detritivorous pathway became a better source of nutrition if zooplankton were assumed to selectively absorb DHA from detritus (increase in β_{ZDHA} relative to β_{ZC}). We conclude that, given uncertainty associated with these various parameters, it is currently impossible to say with any certainty that either pathway will necessarily provide the best source of nutrition for detritivorous zooplankton in the MPZ of the ocean. The analysis has nevertheless highlighted that the microbial pathway, i.e., trophic upgrading, has the potential to be the best source of nutrition in many instances, based on results for the combinations of parameters investigated in the sensitivity analysis.

The analysis of the microbial pathway has thus far assumed that 100% of bacterial losses are due to grazing by protistan bacterivores ($\psi_H = 1$) and that bacteria do not therefore contribute to the diet of detritivorous zooplankton. Decreasing this parameter short-circuits the microbial food chain as fraction (1-ψ_H) of bacteria are then consumed directly by zooplankton. Taken to the extreme ($\psi_H = 0$), all bacteria go to zooplankton. The effects of increasing the proportion of bacteria directly ingested by zooplankton ($0 \leq \psi_H \leq 1$) on predicted ingestion of C and DHA following the microbial pathway, and the resulting zooplankton growth, are shown in **Figure 4**. Bacteria constitute the base of the microbial food web and so direct access to this food source (low values of ψ_H), rather than the bacterivores one trophic level above, increases the C available to zooplankton (**Figure 4A**). On the other hand, bacterial biomass has a low DHA:C ratio and so the quantity of ingested DHA decreases as the proportion of bacteria ingested by zooplankton increases (low ψ_H; **Figure 4B**). A point is reached, $\psi_H = 0.78$, where the supply of C and DHA is optimal and growth is maximized (**Figure 4C**). Growth is limited by C for $\psi_H > 0.78$ and by DHA for $\psi_H < 0.78$, respectively. Increasing bacterial gross growth efficiency (parameter ω_B) supplies extra DHA and C via the microbial pathway but does not influence the ratio of bacterial growth to bacterivore growth in the microbial food web and therefore has no effect on the optimum dietary intake of bacterial biomass (ψ_H). Overall, the analysis of **Figure 4** shows that C-limitation of zooplankton growth via the microbial pathway can be alleviated if these animals are able to access bacteria directly as a food source.

We conclude our analysis of the model by moving away from examining the detritivorous and microbial pathways in isolation from each other, and look at zooplankton growth when the two pathways are utilized simultaneously. In other words, rather than examining the two end members, the detrital pathway ($\psi_B = 0$)

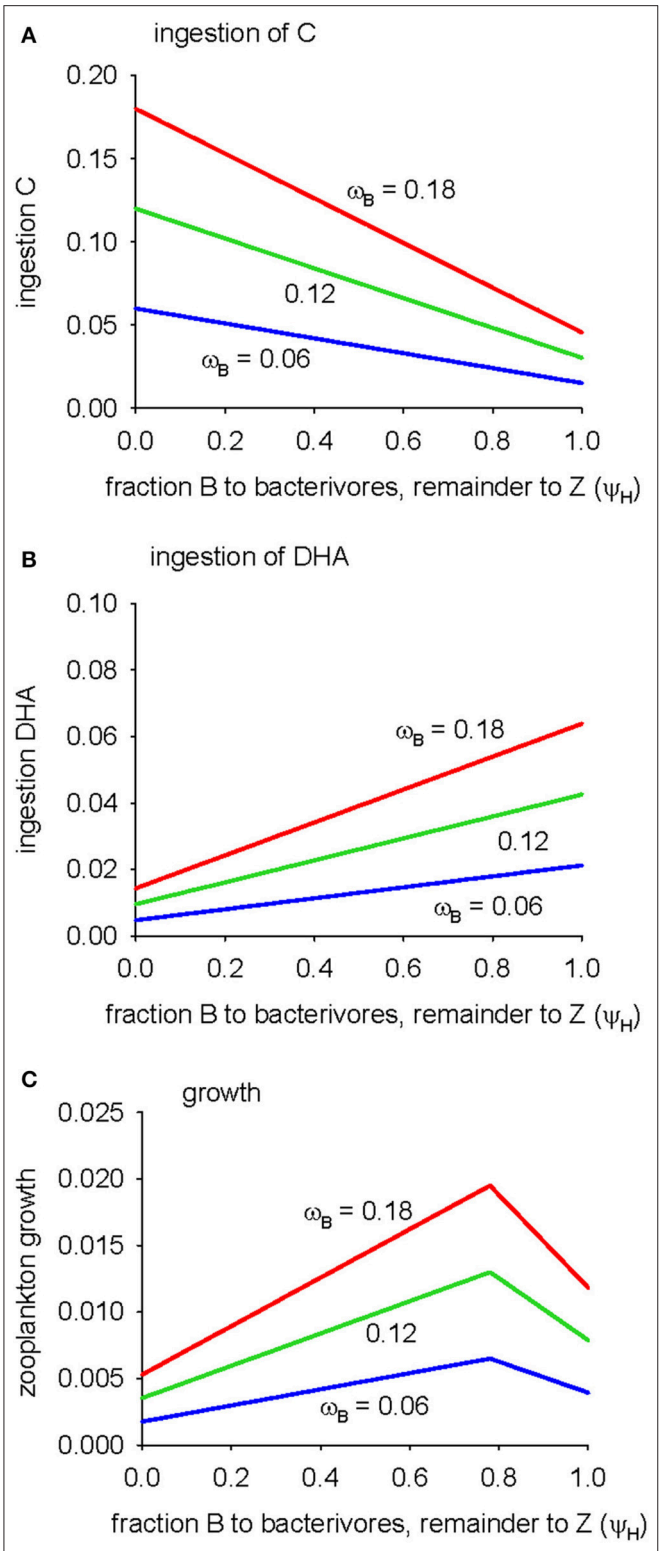

FIGURE 4 | Sensitivity of zooplankton growth via the microbial pathway to parameter ψ_H (the fate of bacteria: fraction ψ_H to flagellates and fraction 1-ψ_H to zooplankton; standard value (Table 2) is $\psi_H = 1$), for B GGE (parameter ω_B) = 0.06, 0.12, 0.18: (A) ingestion of C, **(B)** ingestion of DHA, **(C)** growth.

and microbial pathway ($\psi_B = 1$), growth is now shown for the full range, $0 \leq \psi_B \leq 1$ (**Figure 5**). The growth of zooplankton is maximized when the diet consists of a mix of detritus and protistan bacterivores, irrespective of the bacterivore DHA:C ratio (θ_H). The growth of these copepods is limited by C to the right of the optimum because of C losses in the microbial food web, whereas limitation is by DHA to the left because of the low DHA content in detritus. Increasing the bacterivore DHA:C ratio offsets DHA limitation and thus increases the requirement for C in detritus in order to achieve optimal nutrition (and so the optimum ψ_B shifts to the left). Assuming that the DHA:C ratio in protistan bacterivores (θ_H) is 1.4 (**Table 2**), growth is maximized when ψ_B is 0.76, indicating that the optimal diet is primarily microbial.

DISCUSSION

A new model is presented and used herein to investigate the nutrition of metazoan detritivores, specifically the trade-off between consuming a diet of high-quantity, low-quality detritus versus a low-quantity, high quality diet that is rich in nutritious microbial biomass. The study focuses on the MPZ of the open ocean and involves a stoichiometric analysis of the growth of metazoan zooplankton with model currencies of C, because of its role in structural biomass and energy provisioning, and DHA, which is central to physiological adaptations to the cold temperatures and high pressures typical of the MPZ (Hazel and Williams, 1990). The model extends our previous C-only flow analysis (Mayor et al., 2014) that examined the potential gains that mesopelagic zooplankton stand to make from promoting and subsequently harvesting microbial growth via the fragmentation of large detrital particles, so-called "microbial gardening" (Fenchel, 1970). The model here was first used to compare the growth of zooplankton when consuming a diet consisting solely of non-living detritus (the "detritivorous pathway") versus growth when consuming a

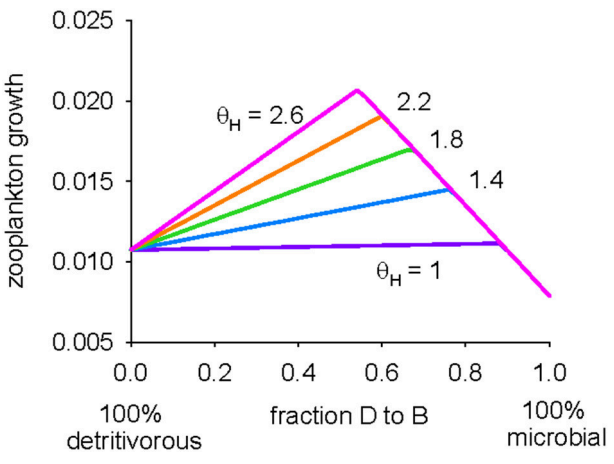

FIGURE 5 | Predicted zooplankton growth for 0 (pure detritivorous) ≤ ψ_B ≤ 1 (pure microbial pathway) and θ_H (DHA:C ratio in protistan bacterivores) between 1.0 and 2.6 mmol mol^{-1}.

purely microbial diet (the "microbial pathway"). The microbial pathway represents "trophic upgrading" (Klein Breteler et al., 1999) of the non-living detrital substrate, i.e., consumption of the community of micronutrient-rich protistan bacterivores that colonize detritus, but which are present in low biomass because of losses in trophic transfer within the microbial food web. The conditions which maximize the growth of zooplankton were subsequently examined, where both detritus and microbes are utilized simultaneously in a mixed diet.

Our initial comparison of the two pathways, detritivorous and microbial, showed that predicted zooplankton growth could, at least in theory, be higher on the former (**Figure 2**). The nutritional benefits of consuming microbes were offset by the increased potential for zooplankton to be limited by food quantity (C). We assumed that zooplankton only had access to the protistan bacterivores in our baseline calculations, with no consumption of bacteria. The movements of motile protists, such as the myriad flagellates that colonize sinking marine detritus (Patterson et al., 1993; Turner, 2002), indicate that they should be readily detected by mechanoreceptors that are typical to copepods (Kiørboe, 2011). If zooplankton consume a diet consisting of protistan bacterivores, much of the detrital C is lost to bacterial and protistan respiration within the particle-attached microbial loop (Azam et al., 1983). This facet of the model underscores the need to understand the dynamics of microbial food webs and their interaction with higher trophic levels.

The limitation of zooplankton growth by food quantity (C) following the microbial pathway can be alleviated if direct ingestion of bacteria is possible. This short-circuits the microbial loop, removing losses of C through protistan respiration, but also lowering the DHA content of the ingested ration because the DHA:C content of bacterial biomass is considerably lower than that of their protistan predators (see Data Sources section). The potential for limitation by DHA therefore becomes more acute under this scenario, although the optimum ratio between the size of copepods of their prey (18:1; Hansen et al., 1994) suggests that direct and deliberate ingestion of bacteria by zooplankton (0.1-1 mm) is unlikely. Another possible short circuit of the microbial pathway occurs if the protists in our model are allowed to directly consume detritus (e.g., Poulsen et al., 2011). This shortening of the food chain between detritus and zooplankton via the microbial pathway is more favorable for zooplankton growth, relative to the bacteria short circuit, because the protists are rich in DHA. It follows that understanding the efficiency and structure of the microbial loop, and the trophic level at which detritivorous consumers interact with this food web, are both crucial for the development of quantitative models to explore the biogeochemistry of detrital ecosystems.

Further exploration of the model involving parameter sensitivity analysis highlighted a range of conditions where the microbial pathway is more favorable than the detritivorous pathway as a source of zooplankton nutrition. Increasing bacterial growth efficiency beyond its standard value of 0.12 is perhaps the most obvious way to achieve this, thereby directly increasing the flow of C into the microbial food web. Reported BGEs are highly variable and often very low (Steinberg et al., 2008). The stoichiometric prediction of zooplankton growth also depends heavily on the DHA:C ratios in seston used in the calculation. These are not well known for the MPZ. Our default value for the ratio in detritus may be somewhat high because the underlying data were derived from measurements in the upper MPZ using methods that did not distinguish between detritus and the associated detrital community (see Data Sources section). Decreasing this ratio, or increasing the DHA:C ratio in zooplankton biomass, both lead to the microbial pathway becoming more favorable than the detritivorous pathway. A further assumption in the model parameterization is that zooplankton can utilize DHA with high efficiency ($k_{ZDHA} = 0.9$; **Table 2**), i.e., this essential micronutrient is solely required for physiological adaptations and is not used for energy generation (Anderson and Pond, 2000; Mayor et al., 2009). Recent observations suggest, however, that at least some marine copepods have high metabolic demands for DHA and other PUFAs (Mayor et al., 2011, 2015) and thus utilize these compounds with relatively low efficiency. Lowering the assumed efficiency with which DHA is utilized increases the demand for this essential fatty acid and so is another way of increasing the potential for the microbial pathway to be a superior source of nutrition to the detritivorous pathway. We are unaware of any data that specifically relates to the demands for DHA or other micronutrients in mesopelagic copepods and call for observations and experiments that may generate such information.

The idea that microbes support the growth of higher trophic levels is not new. An early study found that a detritus-consuming amphipod, *Parhyalella whelpleyi*, obtains its nutrition from the associated microbial communities, the non-living plant residue passing undigested through the gut (Fenchel, 1970). Stream invertebrates have also been observed to preferentially feed on leaves that have been colonized and "conditioned" by microorganisms (Kaushik and Hynes, 1971; Bärlocher and Kendrick, 1975). The nutritional environment facing detritivores has been likened to humans eating peanut butter and crackers (Cummins, 1974), microbial biomass being akin to the nutritious peanut butter spread on the indigestible crackers. Following on from this early work, a number of studies have since shown microbial biomass to be a potentially important source of nutrition for invertebrates in a range of systems including deposit-feeding mayflies (Edwards and Meyer, 1990; Hall and Meyer, 1998), leaf shredders (Connolly and Pearson, 2013), benthic polychaetes (Gontikaki et al., 2011), earthworms (Larsen et al., 2016) and other soil animals including collembolans, mites, woodlice and centipedes (Pollierer et al., 2012; Lemanski and Scheu, 2014). Recent observations have even revealed potentially important trophic linkages between detritus-associated microbes and vertebrates such as fish (e.g., Choy et al., 2015). Given the global importance of heterotrophic protists in the MPZ of the ocean (Pernice et al., 2015) and their role in biosynthesizing essential micronutrients such as DHA (Zhukova and Kharlamenko, 1999), we suggest that these organisms are highly likely to feature in the diets of metazoans that reside in this habitat.

Analysis of zooplankton ingesting a mixture of pure detritus and protistan biomass (**Figure 5**) showed that it may be that

the optimal diet involves utilization of both the detritivorous and microbial pathways in combination, with C supplied by the former balanced by DHA from the microbes. The predicted optimal diet using the standard parameter set (**Table 2**) contained a strong microbial component (the detritivorous and microbial pathways contributed 24 and 76% respectively to nutrition; $\psi_B = 0.76$). The analysis thus demonstrates the potential for protistan biomass to be the primary, if not the sole, part of the diet of metazoan zooplankton (Mayor et al., 2014), although this result is of course subject to the uncertainties in predicted growth highlighted by the parameter sensitivity analyses shown in **Figures 3**, **4**. Both our study and that of Mayor et al. (2014) achieve this result, at least in part, because they are underpinned by the assumption that energy and nutrients within detritus are absorbed with much lower efficiencies than those in microbial biomass, i.e., flagellates and other soft bodied protists are more easily digested than detrital particles consisting of refractory compounds such as cellulose and chitin. We are unaware of any empirical data to directly verify this assumption, but it is supported by the conspicuous absence of flagellate remains in the guts and feces of zooplankton (reviewed by Turner, 2002), despite their long-since acknowledged significance as prey items (Stoecker and Capuzzo, 1990). We further reason that it is likely harder for zooplankton to digest and absorb detrital material, particularly as particles sink deeper into the oceans interior, because it is continuously reworked and repackaged by heterotrophic organisms that strip out anything of energetic or nutritional value (Podgórska and Mundryk, 2003; Wilson et al., 2008). The effects of this stripping are manifest as declining particulate concentrations of nitrogen and micronutrients such as fatty acids and amino acids with increasing water depth (Wakeham et al., 1997; Fileman et al., 1998; Schneider et al., 2003). An improved knowledge of the efficiencies with which mesopelagic zooplankton process different food items is required in order to further our quantitative understanding of the flows of energy and organic matter in detrital food webs. This is a particularly challenging task, potentially requiring the need for *in situ* experiments that determine absorption efficiencies and food preferences for a range of detritivorous invertebrates.

Evolving the means for internal digestion of recalcitrant organic compounds represents a stark alternative to encouraging, or even allowing, microbial growth on external particles of detritus. Recent work on terrestrial detritivores has highlighted a plethora of intricate relationships between invertebrates and their microbiome that facilitate the internal digestion of lignocellulose and other refractory molecules (König and Varma, 2006). In termites, for example, digestion of refractory material is achieved through symbiotic relationships with both bacteria and flagellates (Bignell et al., 2011; Brune, 2014). Relationships of this kind typically require the presence of one or more enlarged gut compartments to house specific microbial communities that carry out fermentation under anoxic conditions (Plante et al., 1990), such as the voluminous hindgut paunch observed in termites (Brune and Dietrich, 2015). The apparent absence of specialized gut structures in copepods commonly found in the mesopelagic, e.g., *Oithona* spp. and *Oncaea* spp., and their small size ($\leq$1 mm) relative to typical detritivorous invertebrates on land (>10 mm), suggest that internal digestive symbioses are not particularly prevalent in midwater crustaceans. Indeed, the conspicuous difference in size between detritivorous invertebrates in terrestrial and mesopelagic ecosystems may arise because the evolutionary pressures to remain small (Kiørboe, 2011) outweigh the need for internal microbially-mediated fermentation in particle-collecting marine zooplankton. More effort is required to identify the internal microbiome of mesopelagic copepods and understand its physiological roles.

Marine detritivorous zooplankton, including *Oithona*, contain significant levels of DHA (Kattner et al., 2003; Pond and Ward, 2011) and numerous studies have highlighted the physiological roles of unsaturated fatty acids in adaptations to temperature and pressure (Cossins and Macdonald, 1989; Hazel and Williams, 1990; Pond et al., 2014). It was assumed that detritivorous invertebrates in our model have physiological requirements for DHA that cannot be met by endogenous biosynthesis, either by the copepods or their internal microbiome, i.e., DHA is an essential micronutrient. The potential for endogenous DHA biosynthesis in detritivorous copepods, by contrast, remains equivocal. Work on benthic copepods suggests that these animals may be capable of elongating shorter-chain PUFA [e.g., 18:3(n-3)] into DHA (Norsker and Støttrup, 1994; Nanton and Castell, 1998; De Troch et al., 2012), but this is not the case for epipelagic zooplankton (Bell et al., 2007). Terrestrial invertebrates are reported to obtain essential micronutrients such as amino acids and fatty acids via their biosynthesis by gut microbes (e.g., Sampedro et al., 2006; Brune, 2014) but the extent to which this occurs in marine invertebrates remains unclear (Plante et al., 1990; Harris, 1993). The guts of marine copepods are known to harbor bacteria (Sochard et al., 1979), some of which show potential for PUFA biosynthesis (Jøstensen and Landfald, 1997), but their actual role(s) within these organisms remains poorly understood. Indeed, we can find no clear evidence that marine copepods are capable of endogenous DHA biosynthesis in the absence of pre-cursor PUFAs, as we propose would be necessary for mesopelagic copepods consuming refractory detritus alone. New information on the source(s) of DHA and other micronutrients in mesopelagic detritivores will provide useful insight into the ecology and biogeochemistry of their habitat. Advances in this area may arise from examining the isotopic signatures of specific micronutrient compounds in detritivores and comparing these to the values found in autotrophic producers and mesopelagic detritus. Improved understanding of the biosynthetic capabilities of animals from the mesopelagic and the significance of internal microorganisms, potentially arising through the application of genomic, transcriptomic and metabolomic techniques, will further help resolve this knowledge gap.

In conclusion, our results indicate that ingesting nutrient-rich microbial biomass potentially represents a beneficial strategy relative to consuming refractory detritus, despite the considerable losses of C due to the inefficiency of the microbial loop. Overall, our work has highlighted how little we know about the physiology of the organisms within detritivorous food webs and hence how and why they interact with organic matter and the wider ecosystem. "Despite their global distribution and essential

roles in nutrient cycling, microbial decomposers are among the least known organisms in terms of elemental concentrations and stoichiometric relationships" (Danger et al., 2016). We suggest that better understanding the ecology and physiology of organisms in the mesopelagic is urgently required if we are to develop mechanistic biogeochemical models of this important ecosystem.

AUTHOR CONTRIBUTIONS

TA led the work, developing the model and generating the results, with a major contribution from DM in terms of advising on parameterizations, data, and in the analysis and writing of the manuscript. DP provided additional advice, especially regarding the fatty acid work described in the manuscript.

ACKNOWLEDGMENTS

TA, DP, and DM are funded by the Natural Environment Research Council (NERC), UK. This work contributes to the NERC-funded programme "Controls over Ocean Mesopelagic Interior Carbon Storage" (COMICS), NE/M020835/1 and the "Culture Collection of Algae and Protozoa" (CCAP) National Capability. We wish to thank three reviewers for their constructive critique of the manuscript.

REFERENCES

Anderson, N. H., and Sedell, J. R. (1979). Detritus processing by macroinvertebrates in stream ecosystems. *Ann. Rev. Entomol.* 24, 351–377. doi: 10.1146/annurev.en.24.010179.002031

Anderson, T. R., Boersma, M., and Raubenheimer, D. (2004). Stoichiometry: linking elements to biochemicals. *Ecology* 85, 1193–1202. doi: 10.1890/02-0252

Anderson, T. R., and Hessen, D. O. (1995). Carbon or nitrogen limitation of marine copepods? *J. Plankton Res.* 19, 317–331. doi: 10.1093/plankt/17.2.317

Anderson, T. R., and Pond, D. W. (2000). Stoichiometric theory extended to micronutrients: comparison of the roles of essential fatty acids, carbon, and nitrogen in the nutrition of marine copepods. *Limnol. Oceanogr.* 45, 1162–1167. doi: 10.4319/lo.2000.45.5.1162

Anderson, T. R., and Tang, K. W. (2010). Carbon cycling and POC turnover in the mesopelagic zone of the ocean: insights from a simple model. *Deep-Sea Res. Part II Topic. Stud. Oceanogr.* 57, 1581–1592. doi: 10.1016/j.dsr2.2010.02.024

Azam, F., Fenchel, T., Field, J. G., Gray, J. S., Meyer-Reil, L. A., and Thingstad, F. (1983). The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* 10, 257–263. doi: 10.3354/meps010257

Bärlocher, F., and Kendrick, B. (1975). Leaf-conditioning by microorganisms. *Oecologia* 20, 359–362. doi: 10.1007/BF00345526

Bartels, P., Cucherousset, J., Steger, K., Eklöv, P., Tranvik, L. J., and Hillebrand, H. (2012). Reciprocal subsidies between freshwater and terrestrial ecosystems structure consumer resource dynamics. *Ecology* 93, 1173–1182. doi: 10.1890/11-1210.1

Bell, M. V., Dick, J. R., Anderson, T. R., and Pond, D. W. (2007). Application of liposome and stable isotope tracer techniques to study polyunsaturated fatty acid biosynthesis in marine zooplankton. *J. Plankton Res.* 29, 417–422. doi: 10.1093/plankt/fbm025

Bignell, D. E., Roisin,Y., and Lo, N. (eds.). (2011). *Biology of Termites: A Modern Synthesis*. Dordrecht: Springer.

Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nat. Rev.* 12, 168–180. doi: 10.1038/nrmicro3182

Brune, A., and Dietrich, C. (2015). The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. *Annu. Rev. Microbiol.* 69, 145–166. doi: 10.1146/annurev-micro-092412-155715

Burd, A. B., Frey, S., Cabre, A., Ito, T., Levine, N. M., Lønborg, C., et al. (2016). Terrestrial and marine perspectives on modeling organic matter degradation pathways. *Glob. Change Biol.* 22, 121–136. doi: 10.1111/gcb.12987

Cebrián, J., and Duarte, C. M. (1995). Plant growth-rate dependence of detrital carbon storage in ecosystems. *Science* 268, 1606–1608. doi: 10.1126/science.268.5217.1606

Choy, C. A., Popp, B. N., Hannides, C. C. S., and Drazen, J. C. (2015). Trophic structure and food resources of epipelagic and mesopelagic fishes in the North Pacific Subtropical Gyre ecosystem inferred from nitrogen isotopic compositions. *Limnol. Oceanogr.* 60, 1156–1171. doi: 10.1002/lno.10085

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., et al. (2013). "Carbon and other biogeochemical cycles," in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change,* eds T. F. Stocker, D. Qin, G. K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex, and P. M. Midgley (Cambridge: Cambridge University Press).

Connolly, N. M., and Pearson, R. G. (2013). Nutrient enrichment of a heterotrophic stream alters leaf litter nutritional quality and shredder physiological condition via the microbial pathway. *Hydrobiologia* 718, 85–92. doi: 10.1007/s10750-013-1605-7

Cossins, A. R., and Macdonald, A. G. (1989). The adaptation of biological membranes to temperature and pressure: fish from the deep and cold. *J. Bioenerg. Biomembr.* 21, 115–135. doi: 10.1007/BF00762215

Cowie, G. L., and Hedges, J. I. (1996). Digestion and alteration of the biochemical constituents of a diatom (*Thalassiosira weissflogii*) ingested by an herbivorous zooplankton (*Calanus pacificus*). *Limnol. Oceanogr.* 41, 581–594. doi: 10.4319/lo.1996.41.4.0581

Cummins, K. W. (1974). Structure and function of stream ecosystems. *BioScience* 24, 631–641. doi: 10.2307/1296676

Dahms, H.-U., Tseng, L. C., and Hwang, J. S. (2015). Biogeographic distribution of the cyclopoid copepod genus Oithona - from mesoscales to global scales. *J. Exp. Mar. Biol. Ecol.* 467, 26–32. doi: 10.1016/j.jembe.2015.02.009

Danger, M., Gessner, M. O., and Bärlocher, F. (2016). Ecological stoichiometry of aquatic fungi: current knowledge and perspectives. *Fungal Ecol.* 19, 100–111. doi: 10.1016/j.funeco.2015.09.004

Davis, A. D., Weatherby, T. M., Hartline, D. K., and Lenz, P. H. (1999). Myelin-like sheaths in copepod axons. *Nature* 398:571. doi: 10.1038/19212

Del Giorgio, P. A., and Cole, J. J. (1998). Bacterial growth efficiency in natural aquatic systems. *Annu. Rev. Ecol. Syst.* 29, 503–541. doi: 10.1146/annurev.ecolsys.29.1.503

De Troch, M., Boeckx, P., Cnudde, C., Van Gansbeke, D., Vanreusel, A., Vincx, M., and Caramujo, M. J. (2012). Bioconversion of fatty acids at the basis of marine food webs: insights from a compound-specific stable isotope analysis. *Mar. Ecol. Prog. Ser.* 465, 53–67. doi: 10.3354/meps09920

Edwards, R. T., and Meyer, J. L. (1990). Bacterivory by deposit-feeding may fly larvae (*Stenonema* spp.). *Freshwater Biol.* 24, 453–462. doi: 10.1111/j.1365-2427.1990.tb00724.x

Fang, J., Barcelona, M. J., Abrajano, T., Nogi, Y., and Kato, C. (2002). Isotopic composition of fatty acids of extremely piezophilic bacteria from the Mariana Trench at 11,000 m. *Mar. Chem.* 80, 1–9. doi: 10.1016/S0304-4203(02)00069-5

Fang, J., Chan, O., Kato, C., Sato, T., Peeples, T., and Niggemeyer, K. (2003). Phospholipid FA of piezophilic bacteria from the Deep Sea. *Lipids* 38, 885–887. doi: 10.1007/s11745-003-1140-7

Fang, J., Kato, C., Sato, T., Chan, O., and McKay, D. (2004). Biosynthesis and dietary uptake of polyunsaturated fatty acids by piezophilic bacteria. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 137, 455–461. doi: 10.1016/j.cbpc.2004.01.009

Fenchel, T. (1970). Studies on the decomposition of organic detritus derived from the turtle grass *Thalassia testudinum*. *Limnol. Oceanogr.* 15, 14–20. doi: 10.4319/lo.1970.15.1.0014

Fileman, T. W., Pond, D. W., Barlow, R. G., and Mantoura, R. F. C. (1998). Vertical profiles of pigments, fatty acids and amino acids: evidence for

undegraded diatomaceaous material sedimenting to the deep ocean in the Bellingshausen Sea, Antarctica. *Deep Sea Res. I Oceanogra. Res. Papers* 45, 333–346. doi: 10.1016/S0967-0637(97)00824-8

Gallienne, C. P., and Robins, D. B. (2001). Is Oithona the most important copepod in the world's oceans? *J. Plankton Res.* 23, 1421–1432. doi: 10.1093/plankt/23.12.1421

Gontikaki, E., van Oevelen, D., Soetaert, K., and Witte, U. (2011). Food web flows through a sub-arctic deep-sea benthic community. *Prog. Oceanogr.* 91, 245–259. doi: 10.1016/j.pocean.2010.12.014

Gonzalez, H. E., and Smetacek, V. (1994). The possible role of the cyclopoid copepod Oithona in retarding vertical flux of zooplankton faecal material. *Mar. Ecol. Prog. Ser.* 113, 233–246. doi: 10.3354/meps113233

Grossart, H.-P., and Ploug, H. (2001). Microbial degradation of organic carbon and nitrogen on diatom aggregates. *Limnol. Oceanogr.* 46, 267–277. doi: 10.4319/lo.2001.46.2.0267

Hall, R. O. Jr., and Meyer, J. L. (1998). The trophic significance of bacteria in a detritus-based stream food web. *Ecology* 79, 1995–2012. doi: 10.1890/0012-9658(1998)079[1995:TTSOBI]2.0.CO;2

Hansen, B., Bjørnsen, P. K., and Hansen, P. J. (1994). The size ratio between planktonic predators and their prey. *Limnol. Oceanogr.* 39, 395–403. doi: 10.4319/lo.1994.39.2.0395

Harris, J. M. (1993). The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. *Microb. Ecol.* 25, 195–231. doi: 10.1007/BF00171889

Hazel, J. R., and Williams, E. E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res.* 29, 167–227. doi: 10.1016/0163-7827(90)90002-3

Henson, S. A., Sanders, R., Madsen, E., Morris, P. J., Le Moigne, F., and Quartly, G. D. (2011). A reduced estimate of the strength of the ocean's biological carbon pump. *Geophys. Res. Lett.* 38:L04606. doi: 10.1029/2011GL046735

Iversen, M. H., and Poulsen, L. (2007). Coprorhexy, coprophagy, and coprochaly in the copepods *Calanus helgolandicus*, *Pseudocalanus elongatus*, and *Oithona similis*. *Mar. Ecol. Prog. Ser.* 350, 79–89. doi: 10.3354/meps07095

Jøstensen, J. P., and Landfald, B. (1997). High prevelance of polyunstaurated fatty acid producing bacteria in Arctic invertebrates. *FEMS Microbiol. Lett.* 151, 95–101. doi: 10.1016/S0378-1097(97)00148-1

Kattner, G., Albers, C., Graeve, M., and Schnack-Schiel, S. B. (2003). Fatty acid and alcohol composition of the small polar copepods, *Oithona* and *Oncaea*: indication on feeding modes. *Polar Biol.* 26, 666–671. doi: 10.1007/s00300-003-0540-x

Kaushik, N. K., and Hynes, H. B. N. (1971). The fate of dead leaves that fall into streams. *Arch. Hydrobiol.* 68, 465–515.

Kiem, R., and Kögel-Knabner, I. (2003). Contribution of lignin and polysaccharides to the refractory carbon pool in C-depleted arable soils. *Soil Biol. Biochem.* 35, 101–118. doi: 10.1016/S0038-0717(02)00242-0

Kiørboe, T. (2011). How zooplankton feed: mechanisms, traits and tradeoffs. *Biol. Rev.* 86, 311–340. doi: 10.1111/j.1469-185X.2010.00148.x

Klein Breteler, W. C. M., Schogt, N., Baas, M., Schouten, S., and Kraay, G. W. (1999). Dynamics of bacterial community composition during degradation of copepod fecal pellets. *Mar. Biol.* 135, 191–198. doi: 10.1007/s002270050616

König, H., and Varma, A. (eds.). (2006). *Intestinal Microorganisms of Termites and Other Invertebrates.* NewYork, NY: S*pringer.*

Koski, M., Kiørboe, T., and Takahashi, K. (2005). Benthic life in the pelagic: aggregate encounter and degradation rates by pelagic harpacticoid copepods. *Limnol. Oceanogr.* 50, 1254–1263. doi: 10.4319/lo.2005.50.4.1254

Kwon, E. Y., Primeau, F., and Sarmiento, J. L. (2009). The impact of remineralization depth on the air-sea carbon balance. *Nat. Geosci.* 2, 630–635. doi: 10.1038/ngeo612

Larsen, T., Pollierer, M. M., Holmstrup, M., D'Annibale, A., Maraldo, K., Andersen, N., et al. (2016). Substantial nutritional contribution of bacterial amino acids to earthworms and enchytraeids: a case study from organic grasslands. *Soil Biol. Biochem.* 99, 21–27. doi: 10.1016/j.soilbio.2016.03.018

Lawrence, S. G., Ahmad, A., and Azam, F. (1993). Fate of particle bound bacteria ingested by Calanus pacificus. *Mar. Ecol. Prog. Ser.* 97, 299–307. doi: 10.3354/meps097299

Lemanski, K., and Scheu, S. (2014). Fertilizer addition lessens the flux of microbial carbon to higher trophic levels in soil food webs of grassland. *Oecologia* 176, 487–496. doi: 10.1007/s00442-014-3037-0

Luo, Y., Ahlstrom, A., Allison, S. D., Batjes, N. H., Brovkin, V., Carvalhais, N., et al. (2016). Toward more realistic projections of soil carbon dynamics by Earth system models. *Global Biogeochem. Cycles* 30, 40–56. doi: 10.1002/2015GB005239

Maity, S., Jannasch, A., Adamec, J., Gribskov, M., Nalepa, T., Höök, T. O., et al. (2012). Metabolite profiles in starved *Diporeia* spp. using liquid chromatography-mass spectrometry (LC-MS) based metabolomics. *J. Crustacean Biol.* 32, 239–248. doi: 10.1163/193724011X615578

Mann, K. H. (1988). Production and use of detritus in various freshwater, estuarine, and coastal marine systems. *Limnol. Oceanogr.* 33, 910–930.

Mayor, D. J., Anderson, T. R., Pond, D. W., and Irigoien, X. (2009). Limitation of egg production in Calanus finmarchicus in the field: a stoichiometric analysis. *J. Mar. Syst.* 78, 511–517. doi: 10.1016/j.jmarsys.2008.12.020

Mayor, D. J., Cook, K., Thornton, B., Walsham, P., Witte, U. F. M., Zuur, A. F., et al. (2011). Absorption efficiencies and basal turnover of C, N and fatty acids in a marine Calanoid copepod. *Funct. Ecol.* 25, 509–518. doi: 10.1111/j.1365-2435.2010.01791.x

Mayor, D. J., Sanders, R., Giering, S. L. C., and Anderson, T. R. (2014). Microbial gardening in the ocean's twilight zone: detritivorous metazoans benefit from fragmenting, rather than ingesting, sinking detritus. *BioEssays* 36, 1132–1137. doi: 10.1002/bies.201400100

Mayor, D. J., Sommer, U., Cook, K. B., and Viant, M. R. (2015). The metabolic response of marine copepods to environmental warming and ocean acidification in the absence of food. *Nat. Sci. Rep.* 5:13690. doi: 10.1038/srep13690

Mezek, T., Simčič, T., Arts, M. T., and Brancelj, A. (2010). Effect of fasting on hypogean (*Niphargus stygius*) and epigean (*Gammarus fossarum*) amphipods: a laboratory study. *Aquat. Ecol.* 44, 397–408. doi: 10.1007/s10452-009-9299-7

Moran, M. A., and Hodson, R. E. (1989). Bacterial secondary production on vascular plant detritus: relationships to detritus composition and degradation rate. *Appl. Environ. Microbiol.* 55, 2178–2189.

Müller-Navarra, D. C., Brett, M. T., Liston, A. M., and Goldman, C. R. (2000). A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature* 403, 74–77. doi: 10.1038/47469

Nanton, D. A., and Castell, J. D. (1998). The effect of dietary fatty acids on the fatty acid composition of the harpacticoid copepod, *Tisbe* sp. for use as a live food for marine fish larvae. *Aquaculture* 163, 251–261. doi: 10.1016/S0044-8486(98)00236-1

Norsker, N.-H., and Støttrup, J. G. (1994). The importance of dietary HUFAs for fecundity and HUFA content in the harpacticoid, *Tisbe holothuriae* Humes. *Aquaculture* 125, 155–166. doi: 10.1016/0044-8486(94)90292-5

Patterson, D. J., Nygaard, K., Steinberg, G., and Turley, C. M. (1993). Heterotrophic flagellates and other protists associated with oceanic detritus throughout the water column in the mid North Atlantic. *J. Mar. Biol. Assoc. U.K.* 73, 67–95. doi: 10.1017/S0025315400032653

Pernice, M. C., Forn, I., Gomes, A., Lara, E., Alonso-Saez, L., Arrieta, J. M., et al. (2015). Global abundance of planktonic heterotrophic protists in the deep ocean. *ISME J.* 9, 782–792. doi: 10.1038/ismej.2014.168

Phillips, N. W. (1984). Role of different microbes and substrates as potential supplies of specific, essential nutrients to marine detritivores. *Bull. Mar. Sci.* 35, 283–298.

Plante, C. J., Jumars, P. A., and Baross, J. A. (1990). Digestive associations between marine detritivores and bacteria. *Annu. Rev. Ecol. Syst.* 21, 93–127. doi: 10.1146/annurev.es.21.110190.000521

Podgórska, B., and Mundryk, Z. J. (2003). Distribution and enzymatic activity of heterotrophic bacteria decomposing selected macromolecular compounds in a Baltic Sea sandy beach. *Est. Coast. Shelf Sci.* 56, 539–546. doi: 10.1016/S0272-7714(02)00204-4

Pokarzhevskii, A. D., Zaboyev, D. P., Ganin, G. N., and Gordienko, S. A. (1997). Amino acids in earthworms: are earthworms ecosystemivorous. *Soil Biol. Biochem.* 29, 559–567. doi: 10.1016/S0038-0717(96)00180-0

Pollierer, M. M., Dyckmans, J., Scheu, S., and Haubert, D. (2012). Carbon flux through fungi and bacteria into the forest soil animal food web as indicated by compound-specific 13C fatty acid analysis. *Funct. Ecol.* 26, 978–990. doi: 10.1111/j.1365-2435.2012.02005.x

Pond, D. W., Tarling, G. A., and Mayor, D. J. (2014). Hydrostatic pressure and temperature effects on the membranes of a seasonally migrating marine copepod. *PLoS ONE* 9:e111043. doi: 10.1371/journal.pone.0111043

Pond, D. W., and Ward, P. (2011). Importance of diatoms for *Oithona* in Antarctic waters. *J. Plankton Res.* 33, 105–1181. doi: 10.1093/plankt/fbq089

Poulsen, L. K., Moldrup, M., Berge, T., and Hansen, P. J. (2011). Feeding on copepod fecal pellets: a new trophic role of dinoflagellates as detritivores. *Mar. Ecol. Prog. Ser.* 441, 65–78. doi: 10.3354/meps09357

Raymont, J. E. G., Krishnaswamy, S., Woodhouse, M. A., and Griffin, R. L. (1974). Studies on the fine structure of Copepoda: observations on *Calanus finmarchicus* (Gunnerus). *Proc. R. Soc. Lond. B* 185, 409–424. doi: 10.1098/rspb.1974.0026

Russell, N. J., and Nichols, D. S. (1999). Polyunsaturated fatty acids in marine bacteria – a dogma rewritten. *Microbiology* 145, 767–779. doi: 10.1099/13500872-145-4-767

Sampedro, L., Jeannotte, R., and Whalen, J. K. (2006). Trophic transfer of fatty acids from gut microbiota to the earthworm *Lumbricus terrestris* L. *Soil Biol. Biochem.* 38, 2188–2198. doi: 10.1016/j.soilbio.2006.02.001

Schneider, B., Schlitzer, R., Fischer, G., and Nöthig, E.-M. (2003). Depth-dependent elemental compositions of particulate organic matter (POM) in the ocean. *Global Biogeochem. Cycles* 2:1032. doi: 10.1029/2002gb001871

Scott, C. L., Kwasniewski, S., Falk-Petersen, S., and Sargent, J. R. (2002). Species differences, origins and functions of fatty alcohols and fatty acids in the wax esters and phospholipids of *Calanus hyperboreus*, *C. glacialis* and *C. finmarchicus* from Arctic waters. *Mar. Ecol. Prog. Ser.* 235, 127–134. doi: 10.3354/meps235127

Sochard, M. R., Wilson, D. F., Austin, B., and Colwell, R. R. (1979). Bacteria associated with the surface and gut of marine copepods. *Appl. Environ. Microbiol.* 37, 570–579.

Steinberg, D. K., Van Mooy, B. A. S., Buesseler, K. O., Boyd, P. W., Kobari, T., and Karl, D. M. (2008). Bacterial vs. zooplankton control of sinking particle flux in the ocean's twilight zone. *Limnol. Oceanogr.* 53, 1327–1338. doi: 10.4319/lo.2008.53.4.1327

Stoecker, D. K., and Capuzzo, J. M. (1990). Predation on protozoa: its importance to zooplankton. *J. Plankton Res.* 12, 891–908. doi: 10.1093/plankt/12.5.891

Straile, D. (1997). Gross growth efficiencies of protozoan and metazoan zooplankton and their dependence on food concentration, predator-prey weight ratio, and taxonomic group. *Limnol. Oceanogr.* 42, 1375–1385. doi: 10.4319/lo.1997.42.6.1375

Suzuki, H., Sasaki, H., and Fukuchi, M. (2003). Loss processes of sinking fecal pellets of zooplankton in the mesopelagic layers of the Antarctic marginal ice zone. *J. Oceanogr.* 59, 809–818. doi: 10.1023/B:JOCE.0000009572.08048.0d

Turley, C. M., and Mackie, P. J. (1994). Biogeochemical significance of attached and free-living bacteria and the flux of particles in the NE Atlantic Ocean. *Mar. Ecol. Prog. Ser.* 115, 191–203. doi: 10.3354/meps115191

Turner, J. T. (2002). Zooplankton fecal pellets, marine snow and sinking phytoplankton blooms. *Aquat. Microb. Ecol.* 27, 57–102. doi: 10.3354/ame027057

Wakeham, S. G., Hedges, J. I., Lee, C., Peterson, M. L., and Hernes, P. J. (1997). Compositions and transport of lipid biomarkers through the water column and surficial sediments of the equatorial Pacific ocean. *Deep-Sea Res. Part II Topic. Stud. Oceanogr.* 44, 2131–2162. doi: 10.1016/S0967-0645(97)00035-0

Wilson, S. E., Steinberg, D. K., and Buesseler, K. O. (2008). Changes in fecal pellet characteristics with depth as indicators of zooplankton repackaging of particles in the mesopelagic zone of the subtropical and subarctic North Pacific Ocean. *Deep-Sea Res. Part II* 55. 1636–1647. doi: 10.1016/j.dsr2.2008.04.019

Zhukova, N. V., and Kharlamenko, V. I. (1999). Source of essential fatty acids in the marine microbial loop. *Aquat. Microb. Ecol.* 17, 153–157. doi: 10.3354/ame017153

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership