CORAL REEFS IN THE ANTHROPOCENE

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PUBLISHED IN: Frontiers in Marine Science

2019 Spotlight Award Winner

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-418-7
DOI 10.3389/978-2-88963-418-7

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CORAL REEFS IN THE ANTHROPOCENE

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Citation: Sweet, M., Andradi-Brown, D. A., Voolstra, C. R., Head, C. E. I., Curnick, D., Frazer, T. K., Banaszak, A. T., eds. (2019). Coral Reefs in the Anthropocene. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-418-7

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frontiers in Marine Science

EDITORIAL
published: 12 June 2020
doi: 10.3389/fmars.2020.00364

Editorial: Coral Reefs in the Anthropocene – Reflecting on 20 Years of Reef Conservation UK

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Keywords: coral reef, reef conservation, United Kingdom, RCUK, trends, anthropocene

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *19 February 2020*
Accepted: *29 April 2020*
Published: *12 June 2020*

Citation:
Andradi-Brown DA, Banaszak AT, Frazer TK, Gilchrist H, Harborne AR, Head CEI, Koldewey HJ, Levy E, Richards K, Short R, Sweet M, Teleki K, Voolstra CR, Wilson B, Wood E, Yarlett RT and Curnick DJ (2020) Editorial: Coral Reefs in the Anthropocene – Reflecting on 20 Years of Reef Conservation UK. Front. Mar. Sci. 7:364. doi: 10.3389/fmars.2020.00364

Editorial on the Research Topic

Coral Reefs in the Anthropocene – Reflecting on 20 Years of Reef Conservation UK

INTRODUCTION

The term "Anthropocene" has been suggested as the current epoch (denoting the current geological age) and is viewed as the period where human-based activity is the dominant influence on climate and the environment (Lewis and Maslin, 2015). Arguably, one of the most prevalent and visible effects of this anthropogenic activity has manifested in the equatorial tropics—where coral reef ecosystems have suffered alarming declines (Pandolfi et al., 2003; Hughes et al., 2017). For example, recent increases in mass bleaching events brought about by prolonged periods of elevated sea surface temperatures highlight a worrying trend, with predictions that over half of reefs will experience annual severe bleaching before 2050 (van Hooidonk et al., 2016). For this reason, coral reefs have often been referred to as "canaries in the coal mine" for the marine biome. Yet reefs continue to be crucial sources of food, protection, livelihoods, and cultural identity for many people around the world (Teh et al., 2013; Hicks and Cinner, 2014; Lau et al., 2019). It is therefore critical that the link between healthy reefs, food security, and sustainable community livelihoods is maintained into the future.

This Research Topic represents the proceedings for the European Coral Reef Symposium (ECRS), which took place 13th–15th December, 2017 in Oxford, UK. ECRS was organised by the Reef Conservation United Kingdom (RCUK) committee, in association with the Zoological Society of London (ZSL), University of Oxford, and the International Coral Reef Society (ICRS). Over 550 coral reef scientists and conservationists joined the meeting for a series of talks, posters, and workshops. In addition to the papers in this Research Topic, ECRS provided a platform for many other coral reef-related events and outputs. For example, the symposium hosted the European

launch of the 2018 International Year of the Reef on the 13th December 2017, and several of the workshops produced published outputs (e.g., Turner et al., 2019).

In this editorial, we provide a brief history of RCUK—charting the course from inception in 1997 through to ECRS in 2017—including our sustainable conferencing efforts and commitments to diversity and inclusion. We strongly believe that all conferences should carefully consider sustainability, diversity, and inclusion and we hope our efforts will inspire and encourage other conference organisers to do the same. We then introduce the globally relevant coral reef science and conservation that has been presented at RCUK meetings and provide an overview of the papers submitted to this Research Topic. We close by highlighting our vision for RCUK into the future—and lay out how this can be applied to the upcoming Frontiers Spotlight Conference on Coral Reefs.

HISTORY OF RCUK

RCUK is an informal network for coral reef scientists, students, practitioners, educators, conservationists, aquarists, and policy makers. Though RCUK is UK-based, it is open to all, regardless of location. RCUK formed in response to the first International Year of the Reef (IYOR) in 1997. During that year, UK-based individuals and organisations came together to raise awareness about coral reefs, including a press event, networking meetings, development of communication materials, and education workshops. These activities brought together groups that had not previously communicated—leading to new collaborations and improved information exchange and networking, especially within the UK coral reef community. The original IYOR UK committee felt a conference would be a good way to build on this network for coral reef science and conservation in the UK but did not have the resources to deliver that in 1997. This led to the formation of the first RCUK coordinating committee, followed soon after by the first RCUK conference in 1998. The conference programme for the newly-formed RCUK stated: "*We hope that this is the beginning of a sustained and continued effort to ensure that the RCUK and the UK reef community maintain an active role in promoting conservation, public awareness and education about coral reefs, as well as ensuring that all reef-related activities are conducted in a responsible manner*" (RCUK, 1998).

Over 20 years later, with a conference held every year since, RCUK is firmly established as a major conference to attend for all things reef-based—and has become a cornerstone of the UK coral reef science and conservation community. RCUK meetings have more than doubled in size, from 100 delegates in the late 1990s (Teleki et al., 2001), to approximately 130 delegates in 2014 and 2015 (Andradi-Brown, 2015; Andradi-Brown et al., 2016a), and sell-out meetings with over 200 delegates (the ZSL venue capacity) in 2016 and 2018. In 2017, RCUK coordinated ECRS in Oxford, UK, with over 550 delegates attending. ECRS is a European coral reef conference initiated by ICRS, typically held every 4 years in a different European location. ECRS represents the European regional meeting counterpart to the ICRS-coordinated global International Coral Reef Symposium. Hosting ECRS marked the 20th annual conference of RCUK, and this Research Topic—representing ECRS conference proceedings—marks the important legacy of RCUK to the UK-based coral reef community.

RCUK meetings strive to be friendly conferences, welcome to anyone with an interest in reef studies or conservation regardless of background or career stage, and include an informal evening social event to encourage networking. RCUK meetings are also smaller and more affordable than most of the international coral reef conferences, and intentionally designed to cross academic hierarchies—with a high proportion of early career presenters and attendees. For example, 52% and 45% of attendees at RCUK in 2016 and 2015, respectively, were students. Additionally, for undergraduate and Master's students, RCUK is frequently their first experience attending or presenting at a conference. From 2015 onwards, RCUK has awarded prizes for the best student talk and poster presentations (Andradi-Brown et al., 2016a). RCUK presentations have been given by many students or early career conservationists who have gone on to become prominent and influential at the national or international level in academia, conservation organisations, or science communication. The original RCUK committee were all early career scientists and conservationists, including several Ph.D. students, with the majority still actively engaged in coral reefs today. While the meeting has expanded and committee members have invariably changed, RCUK continues to be led by a group of early career scientists and conservationists. RCUK has remained "*dedicated to the conservation and awareness of coral reefs*" (RCUK, 1998) over the past two decades—as envisaged when founded.

SUSTAINABLE AND INCLUSIVE CONFERENCING AT RCUK AND ECRS

ECRS was a chance to highlight the RCUK committee's values to the European and global coral reef community. Although predominantly a UK-based network, we seek for RCUK to have global relevance. When planning and hosting ECRS and our annual RCUK conference, we strive for them to be diverse, inclusive, and as environmentally sustainable as possible. Below are some steps we have taken on the journey toward this goal.

Diversity and Inclusion

We interpret diversity and inclusion in its broadest sense, with the aim of ensuring all potential attendees feel welcome and have equal access to the conference. Many of the actions we have taken and summarise below have previously been highlighted by researchers and are also being taken up by other conferences in conservation science (e.g., Sardelis et al., 2017; Smith et al., 2017) and by other organising societies (e.g., the Equity and Diversity Committee of the American Elasmobranch Society and the Marine Section Diversity Committee of the Society for Conservation Biology). Such approaches are also being formalised as codes of conduct (e.g., Favaro et al., 2016).

To reduce conscious and unconscious bias, we have conducted blind abstract reviews since 2015 at all RCUK

conferences. All submitted abstracts are initially reviewed and ranked blind to author names, institutions, and career stage. A minimum of five people review each abstract. Following review, we unblind all abstracts and have an open committee discussion to ensure that we accept the top ranked abstracts, but also ensure balance between institutions, types of institutions (university, non-governmental organisations, museums, aquariums, government, etc.), career stage of presenters (especially providing opportunities for students to present), gender, and geographical locations. For ECRS, we instigated a similar selection process, blinding the submitted abstracts before sending them to the symposium session organisers for initial review, and encouraging them to consider many of the issues above when making refinements to their sessions.

In terms of gender balance, 38% of presentations at RCUK between 1998 and 2016 had a woman as presenting author (**Figure 1**). However, as would be expected with a small conference, the percentage is highly variable year-to-year. There have been four occasions from 1998 to 2016 where the percentage of women as presenting author has been over 50%. Our analysis suggests that RCUK, and likely UK reef science more generally, still requires progress to address gender balance. While we do not have data available, we are aware there is a need for RCUK, as there is for marine science in general (e.g., Mauleón et al., 2013; Smith et al., 2017), to also improve representation of a broader range of ethnic groups and wider socio-economic backgrounds.

We introduced plenary speakers to RCUK meetings for the first time in 2015 (Andradi-Brown et al., 2016a), with these talks allocated more time than standard presentations. Prior to invitations being issued, the committee discussed using the plenary speaker presentations as a platform to showcase interesting and progressive reef science and conservation in more detail than is possible in shorter format talks, while also reflecting the same balance that we want in presenters. We were particularly keen to increase the number of women invited to give plenary talks, as this increases the visibility of women in coral reef science and can provide role models for early career women and students (Jones et al., 2014; Sardelis and Drew, 2016). From 2015 to 2017 (including ECRS) our plenary speakers included four women and two men. We also acknowledge that inequity applies to non-binary gender identities, those with disabilities, and other aspects of intersectional diversity, and are working to identify ways to address this moving forward to further increase inclusivity and diversity.

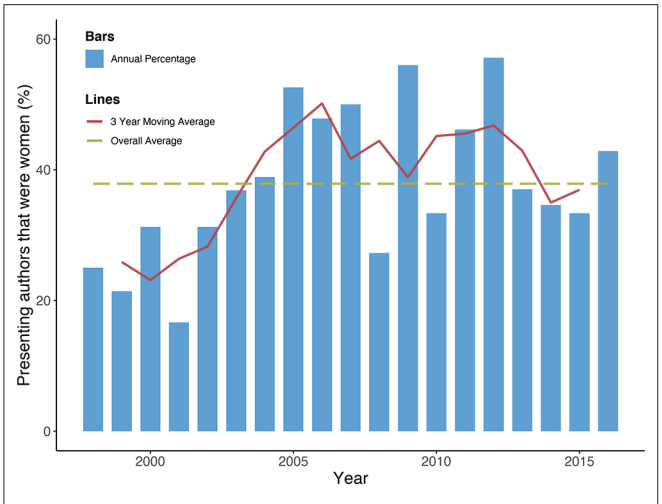

FIGURE 1 | Percentage of talks presented by women at RCUK conferences by year. The red line shows a three-year moving average (an average across the current year, the year prior, and the year following), while the green dashed line represents the mean percentage across the timeseries (38%).

RCUK was formed in the recognition that there was a considerable community of reef scientists and conservationists at UK institutions that would benefit from improved networking and coordination. As such, RCUK does not aim to be fully representative of global coral reef nations. We recognise, however, that bringing in more geographical representation is crucial to broaden perspectives—as the majority of tropical coral reefs are located far away from Europe. Since 2018, RCUK has fully funded a plenary speaker each year from a low- or lower-middle-income country that contains coral reefs. Our intention has been to hear about reef conservation projects from their home country, with a view to a shared learning between these applied reef conservation scientists or practitioners and UK-based researchers. We maximise the value of the trip for the speaker by providing financial support for a short placement with a UK-based conservation or academic partner relevant to their work, expanding on this shared learning. There are also many students from coral reef nations who study in the UK, and RCUK represents a valuable capacity-building and networking opportunity for them. We continue to seek opportunities to increase engagement and shared learning between the RCUK network and scientists and practitioners from tropical coral reef nations.

To increase financial accessibility of RCUK conferences and ECRS, we intentionally keep costs down and provide student travel grants. RCUK conferences are typically held at ZSL, as considerable administrative and in-kind support is provided. Furthermore, the use of ZSL facilities allows us to minimise costs. The first conference in 1998 charged a standard registration fee of £25, which had increased to £40 by 2016—though with an additional discount for students. We believe this represents excellent value for a full day conference that includes lunch, tea and coffee, and drinks and snacks at an evening networking reception surrounded by the coral reef exhibits in the ZSL Aquarium. For ECRS, we partnered with the University of Oxford to use university-owned conference facilities. These were substantially cheaper than professional conference centres, which allowed us to pass on savings directly to our attendees through reduced registration fees. RCUK also provides travel grants to students and recent graduates. These grants are intentionally not restricted on the basis of either age or country, but instead based on career stage. ECRS travel grants were supported by ICRS. During ECRS, free professional childcare was provided within the conference building, as childcare barriers are often prohibitive for parents to attend conferences (Sardelis et al., 2017).

Environmental Sustainability

As a coral reef science- and conservation-focused committee it is crucial we evaluate our environmental impact and maximise the sustainability of our conferences. At ECRS, we carbon offset all conference activities and all committee and plenary speaker travel required for planning and attendance. This commitment meant quantifying the venue's electricity usage during the conference and hosting the World Land Trust within the conference venue for delegates to join us in offsetting their conference travel. We avoided single-use plastic by providing water fountains around the venue and encouraging delegates to bring reusable water bottles. Paper drinking cones were provided on-request for anyone who did not have a reusable water bottle. No single-use plates, cutlery, or cups were used in serving catered food or drinks during lunches and coffee breaks, and delegates were provided with reusable bamboo coffee cups within their plastic-free conference bags. Name badges and lanyards for conference delegates also avoided plastic, instead using laser-cut, locally-sourced, wooden name badges and bamboo fibre lanyards. These have the advantage of being more durable than paper name badges without needing to be held in a plastic pouch, which was essential for a multi-day conference. The lanyards have also been used again for subsequent RCUK events. Our conference volunteer t-shirts were made from recycled plastic bottles and offcuts from the organic cotton trade and were ethically certified by Fair Wear. ECRS also strived to be a paper-free conference. The programme, schedule, and abstract book were provided through a free mobile phone application, or electronic document available on the ECRS website.

With ECRS catering for more than 550 people over three days, it was important to consider the environmental impact of the food we served. The conference was therefore fully vegetarian and used as much locally-produced food as possible—the first ICRS-sponsored event that has done this. We have continued this trend at annual RCUK meetings and are delighted that the 2021 International Coral Reef Symposium has followed our example and made their upcoming meeting fully vegetarian.

CHANGING PRIORITIES OF REEF SCIENCE AND CONSERVATION

RCUK was founded at a time when the drivers of global reef loss were still poorly recognised beyond the coral reef community, and most conservation attention was on local or regional threats. The International Coral Reef Initiative (ICRI)—a partnership between governments and other organisations to protect coral reefs (Dight and Scherl, 1997)—listed four key activities in its framework for action in 1995: (i) integrated management, (ii) capacity building, (iii) research and monitoring, and (iv) review/evidence synthesis (ICRI, 1995). Following a series of workshops, ICRI identified overfishing and pollution from sewage as the two main global threats to reefs, alongside destructive fishing and sedimentation as a consequence of poor land-use practices as additional inter-regional threats (Dight and Scherl, 1997). Indeed, widespread recognition of the effects of climate change on reefs was not apparent until the global mass coral bleaching event and mortality in 1998 (Wilkinson, 2000). For example, in the Status of Coral Reefs of the World: 2000, Wilkinson (2000) wrote: *"Many coral reef scientists and resource managers were considerably shocked and depressed during 1998 when there was massive coral bleaching and mortality of corals over large reef areas in many parts of the world. This caused a major paradigm shift in concepts about the degradation of coral reefs and mechanisms for management."* Since then, the role of climate change as a major global driver of coral reef degradation and loss has become well-established. For example, a Royal Society meeting produced a climate change and coral reef statement in 2009 ("*The coral reef crisis: The critical importance of <350 ppm* CO_2"; Veron et al., 2009), and ICRS issued a consensus statement in 2015 summarising the evidence base ("*Climate Change Threatens the Survival of Coral Reefs*"; Hoegh-Guldberg et al., 2015). Climate change was also explicitly mentioned throughout the 2018 third IYOR recommendation adopted by ICRI (ICRI, 2016). While reducing global carbon emissions is accepted as essential to support coral reef survival into the 21st century, there is much debate about the role of different local- and regional-level management interventions. This has led to recent advocacy efforts to avoid portraying an exclusively "doom and gloom" picture of the future of coral reefs (Abelson, 2020), and to instead promote ocean optimism (Knowlton, 2018). This can be achieved, for example, by highlighting appropriate management interventions (e.g., proper sewage treatment, effective fisheries management) that local decision-makers can take to enhance reef resilience and support reef conservation (Abelson, 2020). It is crucial to remember that reef stressors that can be addressed by local- or regional-scale management are still causing widespread reef loss in many locations globally (e.g., Häder et al., 2020). Many members of the RCUK network have played important roles in advancing coral reef conservation throughout these past two decades, through science, field conservation, capacity building, or policy.

Since 1998, many of these trends in broader reef science have been reflected in presentations at the RCUK annual meeting (**Figure 2**). Each year, when selecting abstracts for inclusion at the conference, the RCUK committee tries to select a balance across relevant disciplines, as well as representation of career levels and institutions. Therefore, the presentations selected each year can be considered a broad proxy for general reef science and conservation priorities at the time, with a bias toward work conducted by UK-based institutions. Across the 402 abstracts accepted for talks at the conference from 1998 to 2016, we counted the number of abstracts that included key words associated with reef threats and management or conservation interventions (**Table 1**).

Approximately two thirds (66%) of abstracts at RCUK conferences made reference to a "reef threat". Many of the threats identified by ICRI and IYOR are highlighted in the research presented (**Figure 2A**). Climate change was the single greatest reef threat presented at RCUK—included in 34% of abstracts. This was followed by fisheries-related issues (25%) and then sedimentation (12%). Disease and pollution were both mentioned in 9% of abstracts. Noteworthy is the fact that disease

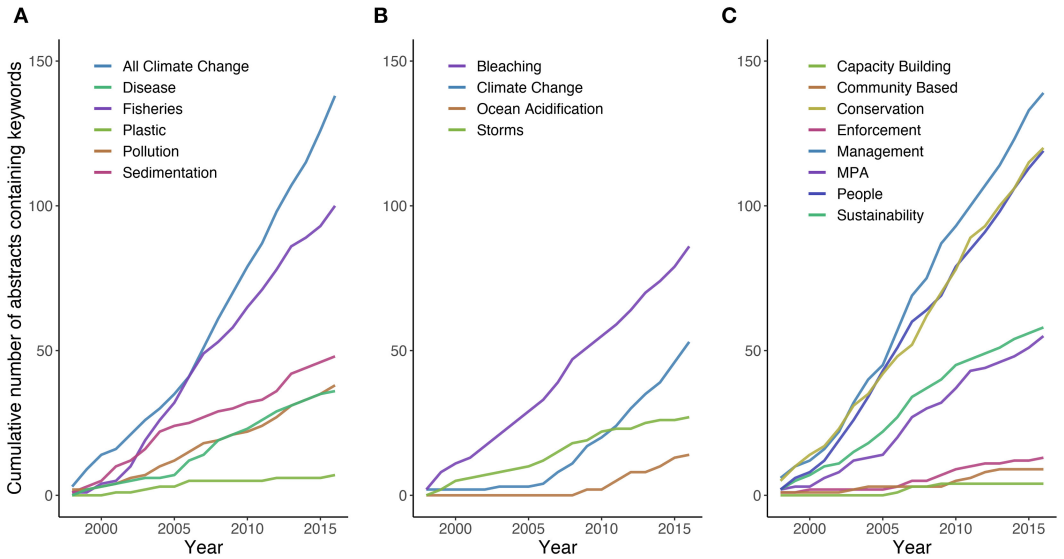

FIGURE 2 | Cumulative trends in the number of abstracts on key topics at RCUK from 1998 to 2016. Trends shown for **(A)** reef threats, **(B)** climate change related impacts, and **(C)** conservation and management actions. Results show the cumulative number of talk abstracts through time based on keyword analysis on the abstract contents (see **Table 1** for keywords included in each topic).

received little attention prior to 2005 (**Figure 2A**). The increased prominence of coral disease is likely associated with the increased profile of diseases emerging as a major scleractinian coral threat (Harvell et al., 1999; Sweet et al., 2012). Plastic pollution, despite a recent high profile (Villarrubia-Gómez et al., 2018; Stafford and Jones, 2019), has featured the least of all reef threats.

The most common climate change-related impact mentioned in 21% of RCUK presentations has been coral bleaching (**Figure 2B**). Unsurprisingly, as the first RCUK conference occurred in November 1998—toward the end of the first reported global mass coral bleaching event (Wilkinson, 2000)—presentations discussed preliminary results of the impact of bleaching on coral reefs. These first conference presentation titles included phrases such as "*catastrophic coral bleaching*" and "*can coral adapt to climate change*" (RCUK, 1998). More presentations refer to bleaching than climate change throughout the time series (**Figure 2B**), likely reflecting the fact that bleaching is a sign of coral stress directly observable by researchers and can also be caused by non-climate change related processes, e.g., disease. Presentations related to bleaching were a mainstay of meetings from 1998 to 2016, while climate change became much more prominent after 2006 (**Figure 2B**). Whilst storm impacts and ocean acidification were mentioned in 7 and 3% of abstracts, respectively, presentations relating to ocean acidification were completely absent prior to 2008 (**Figure 2B**).

From 1998 to 2016, over half (59%) of abstracts included reference to conservation or management. Given that the original aim of RCUK included "*promoting conservation...about coral reefs*" and "*ensuring that all reef related activities are conducted in a responsible manner*", it is encouraging to see a high number of abstracts either directly studying these issues or relating research to potential applied impact. Management issues (comprised of keywords "*management*", "*management capacity*", and "*effectiveness*") were the most commonly mentioned terms—present in 35% of abstracts (**Figure 2C**), followed by conservation and people (both 30% of accepted abstracts). Sustainability and Marine Protected Areas (MPAs) were each mentioned in 14% of abstracts, with MPAs being the single biggest named conservation intervention discussed at RCUK. However, the use of community-based conservation approaches and enforcement have been noticeably lacking in past RCUK conferences (**Figure 2C**). Overall, there has been little change through time in the discussion of conservation and management approaches.

RESEARCH TOPIC OVERVIEW

This Research Topic "Coral Reefs in the Anthropocene" marks the 20th anniversary of RCUK, and was assembled jointly as a conference proceedings volume for ECRS and an open call via the Frontiers in Marine Science journal website for additional submissions. This Research Topic contains 20 papers involving 104 authors and covers many issues at the cutting-edge of reef science and conservation. Papers span basic and applied science, such as the diversity of coral holobionts, coral disease, nutrient impacts, recovery potential for coral reefs, and support for MPA expansions. Here, we briefly summarise each contribution and highlight their importance to the study of coral reefs.

Coral reefs are built by stony corals that comprise metaorganisms, or so-called holobionts (Rohwer et al., 2002). For decades, the association between coral animals and their intracellular microalgal partners in the family *Symbiodiniaceae* has been the subject of intense research given that this symbiosis comprises the foundation of reef ecosystems (LaJeunesse et al., 2018). However, more recently, the role of bacteria in helping

TABLE 1 | Keywords used to identify changes in frequency of different topics presented.

Category	Group	Keywords
Reef Threats	Fisheries	Fishing; Fisheries
	Sedimentation	Sedimentation; Sediment
	Pollution	Pollution; Nutrient; Waste
	Plastic	Plastic
	Disease	Disease
	All climate change	Climate change; Bleaching; Global warming; Bleaching; Ocean acidification; Cyclone; Hurricane; Typhoon; Storm; Sea Surface Temperature; SST
Climate change	Climate change	Climate change; Global warming
	Bleaching	Bleaching; Sea surface temperature; SST
	Ocean acidification	Ocean acidification
	Storms	Cyclone; Hurricane; Typhoon; Storm
Conservation and management	Management	Management; Management capacity; Effectiveness
	MPA	Marine protected area; Marine reserve; MPA; Zoning
	Enforcement	Enforcement; Patrol
	Community based	Community based; Community-based
	Sustainability	Sustainab
	Capacity building	Capacity building
	People	Resource use; People; Human; Fisher; Well-being; Well-being; Social
	Conservation	Conservation

All talk abstracts (n = 402) presented at RCUK from 1998 to 2016 were analysed. The number of abstracts containing keywords associated with one or more of groups were identified for each year. Searches for keywords were not case-sensitive. Categories and groups align with ***Figure 2****.*

corals remain healthy and resilient has been acknowledged (Reshef et al., 2006; Bourne et al., 2016; Ziegler et al., 2017), but few studies investigated how they interact with other holobiont compartments (Robbins et al., 2019). In this Research Topic, several papers studied the coral holobiont to document microbial taxa present and how these differed between locations in the coral polyp or between different scleractinian coral species. For example, Engelen et al. looked at microbial communities associated with the surface mucus, tissue, and gastrovascular cavity of two azooxanthellate Caribbean cup corals. They found high similarity between microbial communities in both species in the surface mucus and tissue, but not in the gastrovascular cavity. Weiler et al. looked at bacterial communities associated with coral tissue and mucus in a deep-sea cold-water coral and found that many of these are likely to be involved in nitrogen cycling. Finally, van de Water et al. studied how seasonal environmental conditions, specifically changes in ultraviolet radiation, affected coral holobiont composition and coral functioning in *Acropora muricata*. Despite finding seasonal effects on the coral holobiont, they found little evidence that ultraviolet radiation was driving this. However, they found large seasonal effects on coral processes such as photosynthesis and calcification. Collectively, these studies demonstrate how much there is to learn about the microbiome, including the taxonomic diversity, their functional importance, and variation across temporal and biophysical gradients. While it is clear that a better understanding of the microbiome will be critical for understanding the impact of stressors on corals in the Anthropocene, these studies highlight the difficulty in predicting the response of corals under future scenarios of climate change.

A further benefit of understanding the microbiome is a better understanding of the causes of coral disease. Disease is a major threat to corals—particularly in places with high local anthropogenic impacts (Sweet and Brown, 2016), and was a key theme presented at ECRS. Three studies considered the effects of disease on either individual corals or on the reef systems as a whole. Rivera-Ortega and Thomé studied the properties of the surface mucus from three cnidarians, including a scleractinian coral and an anemone, finding that this had antibacterial properties. They also found corals with black band disease had diminished antibacterial capacity in the mucus layer. Walton et al. looked at the regional impact of an outbreak of white syndrome disease in Florida in 2014. The disease affected multiple coral species and led to a 30% loss of scleractinian coral density, and, in many cases, over 60% tissue loss from individual colonies. Also in Florida, Goergen et al. conducted a long-term study from 2008 to 2016 on two large *Acropora cervicornis* patches (each over 1 ha in extent). They found a range of factors that led to a >50% loss of *A. cervicornis* over the 8-years period—including diseases such as rapid tissue loss and white band disease, alongside storms and elevated sea surface temperatures. Overall, their conclusion was that the recovery time between disturbance events was not sufficient for this keystone Caribbean coral to recover and regrow. These papers show the importance of disease as a driver of declining coral cover, particularly when combined with other stressors, but also how little we know about their causes and epidemiology. Diseases, combined with storms and elevated SST can prevent keystone coral species from re-establishing, ultimately preventing reefs from recovering and regrowing. The recent coral mortality from stony coral tissue loss disease in the Caribbean (e.g., Precht et al., 2016) further reminds us of how important filling these research gaps will be in the Anthropocene, and how mitigating local stressors is still critical while simultaneously addressing global climate change.

While local stressors significantly impact some reefs, climate change clearly affects reefs throughout the world (Eakin et al., 2019). Elevated thermal stress is well-known to cause scleractinian coral bleaching, which can eventually lead to coral mortality if prolonged (Wilkinson, 2000; Hughes et al., 2017). However, less is known about sub-lethal effects of warming. Palmer considered the activity of key coral immune pathways and an antioxidant in response to coral tissue damage. When uninjured corals were exposed to warmer water (below the temperatures that induced bleaching) the background rate of production of immunity and antioxidant enzymes increased. At higher temperatures, however, the immune responses to tissue damage were significantly delayed. Considering coral bleaching, Wang et al. conducted a field experiment on *Agaricia* sp. colonies to investigate how elevated nitrogen and phosphorous levels affected bleaching and mortality. They found nitrogen in isolation prolonged bleaching and increased mortality. They

also found high prevalence of dark spot syndrome, but nutrient exposure did not increase the prevalence or severity of this disease. In a related study, Poquita-Du et al. tested how increased water temperature and sediment exposure affected gene expression in *Pocillopora acuta*. Whilst their results showed that sediment had little effect on gene expression, the combined treatments of elevated temperature and sediment resulted in a synergistic effect, with greater gene expression changes than would be predicted from the effects of either heat or sediment alone, including declines in symbiont density. It is clear that climate change will exert a major influence on all ecosystems, including coral reefs, during the Anthropocene. While coral bleaching is typically the focal impact of increasing sea surface temperatures, these papers demonstrate the need to also consider sub-lethal impacts, and this will be aided by new approaches such as epigenetics (Eirin-Lopez and Putnam, 2019). Poquita-Du et al. is also a timely reminder of the need to consider synergistic effects among reef stressors (Darling and Côté, 2008).

As coral reefs are highly diverse complex ecosystems, there are many different forms of ecological interactions occurring between species, and interactions between species and their abiotic environment. Rice et al. provided a review of corallivory—the predation of corals. Corallivory is an important feeding pathway for many reef organisms. This review, however, paints an unsettling picture of global corallivory, climate change, and local reef stressors potentially interacting to drive large-scale change on reefs. Our second ecological interaction study considered algal-coral-herbivore interactions on heavily impacted reefs in Singapore. Leong et al. found that the presence of macroalgae reduced coral settlement rate, while the loss of herbivores led to lower juvenile coral survival and increased sediment build up. Their results highlight the importance of interactions between species for maintaining healthy reef ecosystems. Bucher and Harrison studied the effects of elevated nutrient levels on growth rates of *Acropora longicyathus* and found that increased phosphate levels caused faster tissue growth rates and increased apical calcification. Phosphate exposure, however, also reduced coral surface mucus. Reduced mucus may reduce coral survival in polluted waters because of the important role it has in removing sediment from coral surfaces and in preventing disease. Bucher and Harrison also found corals exposed to higher nitrogen levels had reduced growth rates and reduced ability to heal tissue damage. In another paper in this Research Topic, Piñón-González and Banaszak investigated the effects of partial colony mortality on *Acropora palmata* in the Mexican Caribbean. They found there was no difference in growth rates between colonies that had experienced partial mortality versus those that had not. However, areas of the colony that had suffered mortality did not recover, and these colonies then also had decreased egg quality during reproduction. Studies such as these indicate that coral recovery is not straightforward, and under climate change conditions could be compromised even further. There has been significant progress on understanding the resilience of coral reefs (e.g., Mumby et al., 2007), and predicting the recovery of reefs after increasingly frequent disturbances in the Anthropocene will be critical. Better parameterisation of ecological processes and abiotic interactions are key for improving the model of reef benthic dynamics.

Understanding scleractinian coral reproductive patterns is crucial for effective reef restoration efforts. Yet much of the current global coral reef research effort does not align with the locations with the greatest coral reef threats or species richness (Fisher et al., 2011). In the Karimunjawa Archipelago, Indonesia, Wijayanti et al. investigated reproductive seasonality of 21 *Acropora* species over 5 years. They report that *Acropora* spp. exhibit a high degree of seasonality in their reproductive cycle and suggest that there could be some synchronicity in spawning in the region. Building on our inherent understanding of coral spawning in the Great Barrier Reef, Chan et al. hybridised two *Acropora* species pairs to investigate hybrid responses to elevated temperature and carbon dioxide. While hybrid responses were variable, some individuals exhibited greater survival under elevated temperature and carbon dioxide than the parental species. While much work is still required, these results support a growing evidence base that hybridisation can enhance climate resilience for scleractinian corals and hybrid production could become an important tool in coral restoration efforts.

Several papers in this Research Topic explored poorly-studied reef systems and include calls for urgent management—from MPA implementation to preventing oil and gas extraction. These papers contain highly valuable information for those at the front lines of conservation and have the potential to influence marine management decision-makers, alongside highlighting crucial knowledge gaps on neglected, human-influenced systems. First, Francini-Filho et al. provided an overview of the Great Amazon Reef system. While the existence of a reef system adjacent to the mouth of the Amazon River has been known to researchers since the 1970s (Collette and Rützler, 1977), it has been very poorly documented. In their paper Francini-Filho et al. report the first video surveys conducted that captured the structure of this system. Their paper shows that the reef system is likely larger than previously thought—extending both further along the coast and deeper—while also having greater habitat complexity and diversity than previously recorded. The authors also warn of the increasing threat to the reef from oil and gas extraction in the region, and call for a network of MPAs to be established to protect the Amazon reef. Their paper captured global attention, and currently is the most viewed article ever published by Frontiers in Marine Science.

This Research Topic also contained two complimentary papers from Garavelli et al. and Studivan and Voss investigating connectivity between mesophotic coral ecosystems (reefs from 30 to 150 m depth) in the Northwest Gulf of Mexico. Both of these papers were framed in response to proposals to expand the Flower Garden Banks National Marine Sanctuary with the potential to afford coral reef habitats in the Northwest Gulf of Mexico increased protection. While mesophotic coral ecosystems are historically poorly studied globally (Bridge et al., 2013), this has rapidly changed in recent years (Turner et al., 2017; Laverick et al., 2018), and there is now evidence they can be heavily impacted by anthropogenic activities (Andradi-Brown et al., 2016b). Garavelli et al. used larval dispersal modelling and

highlighted the high potential for scleractinian coral connectivity between all mesophotic offshore banks and also shallow reefs in the region. Studivan and Voss used molecular ecology methods to show high population connectivity for *Montastraea cavernosa* across the Northwest Gulf of Mexico. Taken together, these papers reach the conclusion that coral populations living in the region should be managed as a single unit, and advocate for the expansion of the national marine sanctuary. Finally, Gorospe et al. were interested in how reef fish recovery potential might inform marine spatial planning or MPA implementation. The authors constructed a series of Bayesian models to investigate the capacity for Hawaiian reefs to support fish biomass and compared these results to contemporary biomass levels. Their analysis showed surprising variation in the natural capacity for reefs to support herbivorous fish biomass and overall fish biomass, as well as the significant negative effect human population density has on fish biomass throughout Hawaii. As we move through the Anthropocene, inevitably research and conservation efforts will focus on well-studied shallow-water reefs, but this work reminds us of the need to continually search for and protect poorly documented reefal areas and consider potential conservation outcomes from protection.

The final paper in our Research Topic, by Chabanet et al. evaluated the impact of a coral reef education project in New Caledonia. The authors provide details of a fun and action-orientated toolbox of activities, including picture books, card games, and board games, for awareness-raising with children aged 5–11 years old titled: "*The Coral Reef in Our Hands.*" Students who went through this education activity had greater knowledge of reef biodiversity and awareness of connections between coral reefs and the wider environment.

It is axiomatic that coral reefs and the ecosystem services they provide are threatened by a wide range of stressors. While we can optimistically hope that some stressors can be mitigated, there is an increasing recognition that reefs in the Anthropocene will function differently and need managing accordingly (Rogers et al., 2015; Hughes et al., 2017; Bellwood et al., 2019). The basis of this new future will be a combination of science and conservation, international and interdisciplinary collaboration, and communication and outreach to the public and a wide range of stakeholders. Conferences such as RCUK aim to facilitate these efforts, and the papers included in this Research Topic demonstrate their value. Spanning topics from coral microbiomes to mesophotic reefs, the papers demonstrate the effects of a variety of stressors to corals and coral reefs—and once again underscore how little we currently know but also the urgency of more research and effective conservation to lead to better outcomes for people and nature.

RCUK INTO THE FUTURE

We continue to look for new ways to keep RCUK conferences fresh and engaging for the reef community we support. We are delighted that this Research Topic won the 2019 Frontiers Spotlight Award, as this will provide much needed funding and support to host a series of workshops on applied conservation science that will address the major issues facing coral reefs. We plan to invite the world's leading coral reef scientists, conservationists, and policy experts to produce several high-impact papers and policy briefs to chart the way forward for reefs in our rapidly changing world. We will be bringing our RCUK values to this event, with the resources provided by the Frontiers Spotlight Award providing an opportunity to progress our approaches to environmental sustainability, diversity, and inclusion.

Reflecting on the past 20 years of RCUK activities including ECRS, as a UK reef network and annual conference we feel that we have met and even exceeded the expectations of the original 1998 RCUK vision, while also continuing to progress and improve the mechanisms by which we deliver it. We will continue to build RCUK activities in the future and remain "*dedicated to the conservation and awareness of coral reefs*" (RCUK, 1998).

AUTHOR CONTRIBUTIONS

DC and DA-B conducted the analysis. DA-B wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

ACKNOWLEDGEMENTS

We thank ZSL for continued long-term support to RCUK. We also wish to thank ZSL, University of Oxford, and the International Coral Reef Society for support with ECRS. The RCUK committee expresses our heartfelt thanks to all coral reef enthusiasts who have participated in, volunteered at, or helped build the RCUK network and annual meetings since 1998. This is contribution #189 from the Coastlines and Oceans Division in the Institute for Environment at Florida International University.

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Conflict of Interest: KR was, at the time of writing, employed by the company Atmosphere Resorts. EW was a freelance consultant but participated on a voluntary basis in this manuscript.

The remaining 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

ORIGINAL RESEARCH
published: 28 March 2018
doi: 10.3389/fmars.2018.00101

Corals and Their Microbiomes Are Differentially Affected by Exposure to Elevated Nutrients and a Natural Thermal Anomaly

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OPEN ACCESS

Edited by:
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University of Derby, United Kingdom

Reviewed by:
Kimberly B. Ritchie,
University of South Carolina Beaufort, United States
Julie L. Meyer,
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: 24 January 2018
Accepted: 12 March 2018
Published: 28 March 2018

Citation:
Wang L, Shantz AA, Payet JP, Sharpton TJ, Foster A, Burkepile DE and Vega Thurber R (2018) Corals and Their Microbiomes Are Differentially Affected by Exposure to Elevated Nutrients and a Natural Thermal Anomaly. Front. Mar. Sci. 5:101. doi: 10.3389/fmars.2018.00101

Nutrient pollution can increase the prevalence and severity of coral disease and bleaching in ambient temperature conditions or during experimental thermal challenge. However, there have been few opportunities to study the effects of nutrient pollution during natural thermal anomalies. Here we present results from an experiment conducted during the 2014 bleaching event in the Florida Keys, USA, that exposed *Agaricia* sp. (Undaria) and *Siderastrea siderea* corals to 3 types of elevated nutrients: nitrogen alone, phosphorous alone, and the combination of nitrogen and phosphorus. Overall, bleaching prevalence and severity was high regardless of treatment, but nitrogen enrichment alone both prolonged bleaching and increased coral mortality in *Agaricia* corals. At the same time, the elevated temperatures increased the prevalence of Dark Spot Syndrome (DSS), a disease typically associated with cold temperatures in *Siderastrea siderea* corals. However, nutrient exposure alone did not increase the prevalence or severity of disease, suggesting that thermal stress overwhelms the effects of nutrient pollution on this disease during such an extreme thermal event. Analysis of 78 *Siderastrea siderea* microbial metagenomes also showed that the thermal event was correlated with significant shifts in the composition and function of the associated microbiomes, and corals with DSS had microbiomes distinct from apparently healthy corals. In particular, we identified shifts in viral, archaeal, and fungal families. These shifts were likely driven by the extreme temperatures or other environmental co-variates occurring during the 2014 bleaching event. However, no microbial taxa were correlated with signs of DSS. Furthermore, although nutrient exposure did not affect microbial alpha diversity, it did significantly affect microbiome beta-diversity, an effect that was independent of time. These results suggest that strong thermal anomalies and local nutrient pollution both interact and act independently to alter coral health in a variety of ways, that ultimately contribute to disease, bleaching, and mortality of reefs in the Florida Keys.

Keywords: bleaching, disease, corals, dark spot syndrome, viruses, fungi, reefs, bacteria

INTRODUCTION

Ocean warming and coastal pollution are two of the most widespread threats to coral reefs. Increases in sea surface temperatures of just a few degrees can exceed the thermal tolerance of many tropical corals, causing coral bleaching and warm-water associated epizootics that together threaten up to one-third of all coral species (Carpenter et al., 2008). Although it has been widely publicized that the frequency of coral bleaching will increase over the coming century (e.g., Magris et al., 2015; van Hooidonk et al., 2016), increasing frequency and severity of coral diseases may pose a greater threat to reefs than bleaching events (Maynard et al., 2015). At the same time, nutrient enrichment in nearshore waters is one of the major anthropogenic forces altering coastal ecosystems (Halpern et al., 2008) and can drive the increased prevalence of coral diseases and bleaching on reefs worldwide (Vega Thurber et al., 2014; Maynard et al., 2015). For example, field surveys suggest that the prevalence of coral disease is often correlated with nutrient concentrations (Haapkylä et al., 2011; Kaczmarsky and Richardson, 2011).

While the effects of coastal nutrient pollution on bleaching tolerance and disease have garnered a great deal of interest (e.g., Bruno et al., 2003; Wooldridge and Done, 2009; Wagner et al., 2010; Vega Thurber et al., 2014; Wooldridge, 2016), little is known about the interactions between nutrients, temperature stress, and coral diseases. Troublingly, large increases in coastal nitrogen loading are projected to occur alongside ocean warming as a result of climate change (Sinha et al., 2017), adding urgency to our need to understand the interactive effects of nutrients and temperature stress on coral health.

Nitrogen (primarily as nitrate) and phosphorus are two major nutrient pollutants in terrestrial run-off (Howarth, 2008), and the effects of each of these nutrients on coral physiology are distinct. Enrichment with nitrogen causes *Symbiodinium* to rapidly proliferate (Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1989; Marubini and Davies, 1996; Cunning and Baker, 2013), disrupting the translocation of nutrients between *Symbiodinium* and their coral hosts, and thus compromising the animal's energy budget (Shantz et al., 2016). Furthermore, nitrogen enrichment can result in limitation of other important nutrients. For example, nitrogen-induced phosphorus limitation is linked to reduced thermal tolerance in corals (Wiedenmann et al., 2013). Because the ability of corals to survive bleaching events is influenced by, among other things, a coral's energy reserves (Schoepf et al., 2015), nitrogen enrichment also reduces coral resilience in the face of bleaching events by jeopardizing coral energy budgets.

In contrast, surplus phosphorus increases stress tolerance in corals (e.g., Beraud et al., 2013; Wiedenmann et al., 2013). Under typical conditions, the impacts of phosphorus enrichment on coral physiology are small (Shantz and Burkepile, 2014; Ferrier-Pagès et al., 2016). However, under thermal stress, phosphorus uptake rates increase as phosphorus is required to maintain symbiont density, photosynthesis, and carbon translocation (Ezzat et al., 2016). Thus, while coastal pollution can impact coral physiology, the interactive effects of pollution and warming are likely mediated by the ratio of nitrogen:phosphorus delivered to the environment.

Less is known about the relative impacts of nitrogen and phosphorus on coral diseases, such as Dark Spot Syndrome (DSS) in scleractinian corals. DSS is one of the most common diseases of corals in the Florida Keys, representing 71% of all diseased corals and typically afflicting 26% of *Montastrea annularis* colonies and 8% of *Siderastrea siderea* colonies on most reefs (Porter et al., 2011). DSS is identified by darkened pigmentation of the coral tissue resulting in purple, black, or brown lesions that can either be circular or elongate (Weil, 2004; Gochfeld et al., 2006). A necrotizing disease, DSS can cause affected tissues to die at a rate of 4.0 cm/month in *S. siderea* corals (Cervino et al., 2001). Though it is often not obviously deleterious to whole coral colonies, it is a known marker for more aggressive diseases such as Black Band Disease and Yellow Band Disease (Richardson, 1998; Cervino et al., 2001). Additionally, DSS affected corals are more likely to bleach than their healthy counterparts (Brandt and McManus, 2009).

Nutrient loading increases the severity of coral diseases (e.g., Bruno et al., 2003; Voss and Richardson, 2006) and in some instances, may cause disease outbreaks. For example, Vega Thurber et al. (2014) showed that combined nitrogen and phosphorus enrichment increased both the severity and frequency of DSS in *S. siderea*, an abundant coral on reefs in Florida. Substantial evidence exists showing that nutrient enrichment drives changes in the microbial communities associated with corals (e.g., Thompson et al., 2015; Zaneveld et al., 2016; Shaver et al., 2017), and these changes are often associated with increases in pathogenic bacteria and the appearance of disease signs (for review see, McDevitt-Irwin et al., 2017). However, the relative role of nitrogen vs. phosphorus in shaping disease susceptibility and the coral microbiome is currently underexplored. To date, we are not aware of any studies that have investigated how phosphorus modifies the susceptibility of corals to diseases.

In the summer of 2014, an anomalous thermal event occurred in the Florida Keys, providing the opportunity to study how nutrient pollution interacted with thermal stress to impact coral bleaching and disease. We evaluated how increases in two nutrients (nitrate and phosphate) separately and in combination can exacerbate the effects of thermal stress on coral disease and bleaching. To test this question, we exposed individuals of two species of corals, *Siderastrea siderea,* and *Agaricia sp*. (Undaria) in the field to nitrogen alone, phosphorus alone, and the combination of each, in addition to control corals with no nutrients, for 6 months while following the visual health of corals throughout the experiment. Furthermore, we evaluated the microbial ecology of the control and exposed corals before, during, and after nutrient enrichment and thermal stress.

METHODS

Nutrient Enrichment Experimental Design

To evaluate the effects of nutrient enrichment on natural coral colonies, we conducted an *in situ* nutrient enrichment experiment at Pickles Reef (N24.99430, W80.40650) in the

Florida Keys from July 14th 2014 to January 12th 2015 (**Figure 1**). Along two 30 m transects, approximately 20 m apart, at a depth of ca. 5–6 m, we haphazardly selected 20 *Agaricia* sp. (Undaria) and 20 *Siderastrea siderea* colonies at least 10 cm^2 in area and visually deemed to be in good health. Individual coral colonies were randomly assigned to a nutrient treatment with either (1) nitrogen and phosphorus, (2) nitrogen, (3) phosphorus, or (4) left untreated to serve as controls, with five replicates per treatment.

To achieve our enrichments, we deployed nutrient diffusers constructed from PVC pipes with holes drilled throughout that were filled with either slow-release nitrate (150 g, 12% NO_3), phosphate (45 g, 40% PO_4), or both, and wrapped in mesh as

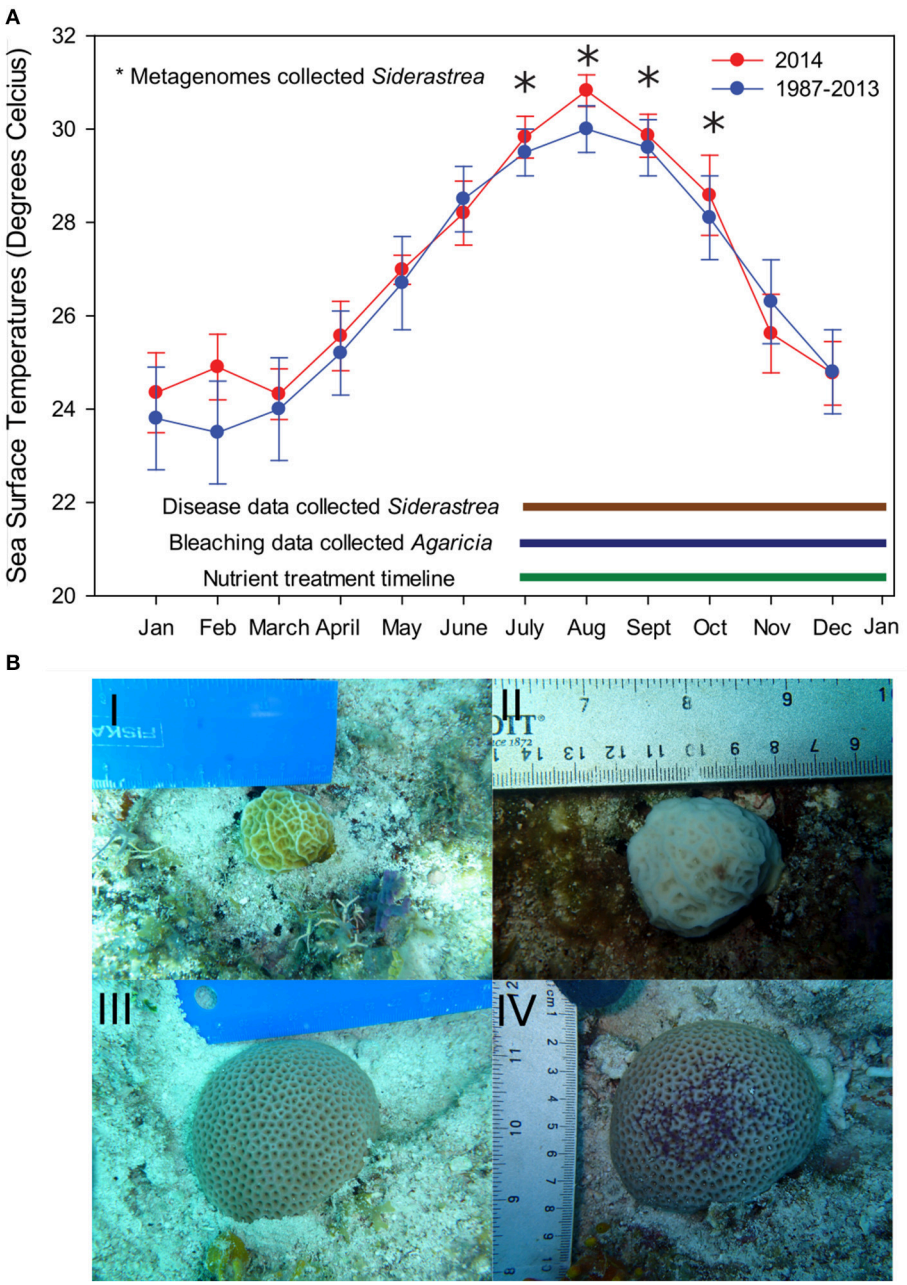

FIGURE 1 | Experimental design and temperature profile from field site. **(A)** Data from NOAA Molasses buoy show that mean monthly sea surface temperatures in 2014 were significantly elevated both in the winter and summer months (red lines) compared to the monthly means from 1987–2008 (blue lines). Error bars indicate the standard error of means of all temperature data available for that month. **(B)** During this summer, we monitored control and nutrient exposed *Agaricia* corals for bleaching prevalence, severity, and recovery as well as tissue loss from July (pretreatment) to January 2015. Photos I and II represent *Agaricia* corals from July and September, respectively. *Siderastrea* control and nutrient exposed corals were also monitored for disease prevalence and severity across the course of the experiment. Photos III and IV represent *Siderastrea* corals from July and September, respectively. *Siderastrea* mucus samples for metagenomes (indicated by the stars) were only collected at the pretreatment time point (July 14, 2014) and three post treatment months: August 13th, Sept 14, and October 14th.

described in Zaneveld et al. (2016, Supplementary Figure 1). We stationed each nutrient diffuser 10 cm from the target coral, and replaced the fertilizer monthly. We have successfully used this method in the past to enrich sections of the reef (e.g., Vega Thurber et al., 2014; Zaneveld et al., 2016). Water column NO_3 and PO_4 concentrations collected near the diffusers 24 h after deployment were ca. 4.5- and 2.4-fold higher in N and P respectively than concentrations at control sites (3.18 μM NO_3 and 0.34 μM PO_4 vs. 0.71 μM NO_3 and 0.14 μM PO_4). Per previous experiments, the nutrients from these apparatuses were shown to diffuse within approximately 1 m from the experimental area.

Disease and Bleaching Surveys

From July 2014 to January 2015, corals were surveyed monthly by SCUBA to track changes in their health throughout the course of the experiment (Supplementary Table 1). For each coral, divers recorded whether bleaching or disease symptoms were visually present and photographed the coral from a fixed position with an object of known length. Using ImageJ (v1.50), we analyzed photos from each monthly survey to estimate the total surface area of each coral, as well as the area of each colony afflicted by disease or bleaching (Abramoff et al., 2004). A portion of a coral was considered bleached when it no longer retained any pigmentation and the white coral skeleton was visible through the tissue. Bleached or diseased areas were divided by total surface area to calculate the percentage of the total colony surface afflicted as measures for disease and bleaching severity. In addition, tissue mortality was estimated from each coral by comparing the area of live tissue on each coral with the coral's initial live tissue area from our pre-treatment surveys. At the end of our final round of surveys in January, we also estimated bleaching recovery as the proportion of bleached tissue that had regained pigmentation. Although we recorded both bleaching and disease measurements for both coral species, here we only report measurements of DSS in *S. siderea* and bleaching in *Agaricia* sp.; only a single colony of *Agaricia* sp. ever showed disease, and only a single *S. siderea* colony showed signs of bleaching (data not shown).

Coral Mucus and Seawater Sampling

Coral mucus from each colony, as well as seawater samples, were collected by divers at four time points to generate microbial metagenomes. A pretreatment sample was collected in July of 2014, followed by monthly samples in August, September, and October. To investigate the role of microbes and viruses in the etiology of DSS, metagenomes were only made for the *Siderastrea siderea* corals. *Agaricia* sp. (Undaria) mucus samples were not explored using metagenomes due to the high mortality rate of our *Agarcia* specimen that ultimately resulted in low replication over time and treatment.

Surface coral mucus was collected by gently agitating the colony surface with a sterile syringe, as detailed in Zaneveld et al. (2016). Specifically, on all corals, we agitated the top of the animal, collecting mucus across the entire surface. We chose to sample mucus due to the benign effect of sampling upon the coral, and due to its role in providing a barrier for the coral from pathogens (Zaneveld et al., 2016). Mucus samples were brought back to the boat, where they were transferred into sterile 15 mL falcon tubes, immediately frozen on dry-ice for transport, and then stored at −80°C prior to nucleic acid extraction. Seawater samples were collected in duplicate 50 mL falcon tubes from 1 m above each transect and stored frozen as described above.

We also used mucus samples from a previous enrichment experiment (Zaneveld et al., 2016) to generate comparative *S. sideraea* metagenomes. In August of 2012, 25 apparently healthy and 25 DSS afflicted corals were selected from within control and nutrient enriched plots. In this enrichment experiment, only combined nitrogen and phosphorus was used to mimic nutrient pollution (for details see Vega Thurber et al., 2014). Mucus samples were collected in the same manner as described above and processed in the exact same manner for metagenome generation and analysis as described below.

Microbial Metagenome Library Generation and Sequencing

Thawed mucus and seawater samples were pre-filtered through 5.0 μm pore-size EMD Millipore Millex (Millipore) syringe filters to remove larger particles. Viral and microbial size-particles from resulting filtrates were further concentrated using the 30 kDa cutoff Amicon Ultra-15 centrifugal filter units (Millipore). DNA from microbial concentrates was then extracted using the MasterPure Complete DNA purification kit (Epicenter, Illumina). Purified DNA extracts served as input for the NexteraXT DNA library preparation (Illumina) to generate multiplexed metagenome libraries for high-throughput sequencing, following the manufacturer's recommendations. Multiplexed sample libraries were cleaned using AMPure XP magnetic beads (Agencourt) and checked for quality and size distribution on a Bioanalyzer 2100 (Agilent), prior to being pooled in equimolar concentrations for sequencing. Whole genome shotgun sequencing was conducted on the HiSeq2000 platform (Illumina) at the CGRB facility at the Oregon State University, yielding 2 × 100 bp long paired-end reads. This approach resulted in 86 metagenomes including 6 seawater samples, and 80 coral metagenomes that spanned 4 time points, 4 treatments, and 5 replicate colonies per treatment (Supplementary Table 2). Two metagenomes were removed from the analysis due to their low number of reads and one seawater sample from August and one seawater sample from September were lost during shipment. The resulting 84 metagenomes had an average of 4,753,686 reads, with about 77% of reads remaining after quality-control. These metagenomes are freely and publicly available online at the Sequence Read Archive (SRA; #SRP133535 for the 2014 metagenomes and #SRP133699 for the 2012 metagenomes) and our own websites: http://files.cgrb.oregonstate.edu/Thurber_Lab/NOAA_SSids/, and http://files.cgrb.oregonstate.edu/Thurber_Lab/DSS/.

Bioinformatic Analyses of Metagenomic Data

We used the program Shotcleaner (https://github.com/sharpton/shotcleaner) to filter out host and symbiont sequences and low-quality reads with quality scores below 25. This program also trimmed Illumina adapters and combined duplicate sequences.

Shotcleaner is a workflow program that integrates an ensemble of programs such as Trimmomatic v0.35 (Bolger et al., 2014), Bowtie2 v 2.3.2 (Langmead and Salzberg, 2012), and FastQC (Andrews, 2010). For this analysis, the coral, *Acropora digitifera* (RefSeq NW_015441057.1) was used as the reference host, as the *Siderastrea siderea* genome is not currently available. Sequences from the coral endosymbiont *Symbiodinium* were filtered out using the *Symbiodinium minutum* genome (GenBank DF242864.1). Host and symbiont genomic reads were filtered out using Bowtie2, which aligned the metagenome reads to the host and symbiont genomes. Bowtie2 was run using default "end-to-end" parameters set to "-sensitive." In short, both the read and its reverse complement were aligned end-to-end to the host and symbiont genomes. Mismatch penalties ranged from a minimum of 2 and a maximum of 6, depending on the quality value of the read character. A lower quality score would lead to a lower penalty to the overall alignment score in the case of a mismatch. Gap penalties were 5 to open a gap, and 3 for a gap extension, for both the read and reference sequences.

We used the program Kraken (v.0.10.5) to conduct taxonomic assignment of the filtered metagenomics reads (Wood and Salzberg, 2014). Paired-end reads were analyzed using the "--paired" option, which concatenates the pair and increases classification sensitivity (Wood and Salzberg, 2014). Then a custom MiniKraken database was built, comprising all Archaea, Bacteria, Fungi, Protozoans and Viruses RefSeq released genomes, using the k-mer length of 25. The resulting database contained all k-mers and the lowest common ancestor of genomes that possess any particular k-mer. Annotations were made by alignment of metagenomic reads to k-mers in the database. The Kraken output was then transformed into a taxonomy table using kraken-translate, with the option "--mpa-format."

We also used the program ShotMAP for functional annotations (Nayfach et al., 2015). ShotMAP utilizes Prodigal (https://doi.org/10.1186/1471-2105-11-119) to predict genes in unassembled shotgun reads *ab initio*, and compares the predicted protein coding sequences against a protein family database using alignment algorithms. For this analysis, KEGG (release 73.1) was used as the reference database (Kanehisa and Goto, 2000). ShotMAP was run using the option "–ags-method none," as the genome size estimation tool was not compatible with this dataset. ShotMAP outputs for all 85 metagenomes were combined using compare_shotmap_samples.pl.

ShotMAP outputs were sorted by KEGG identification numbers and grouped via KEGG BRITE functional hierarchies to level B (excluding the categories drug development, human diseases, and organismal systems). Given that KEGG ID numbers are often associated with multiple pathways, we calculated the ratio of the average count of unique KEGG IDs in a pathway and the sum of unique average counts for that KEGG ID in all pathways. The average count of unique KEGG IDs is the average count of all instances in each sample in which a pathway is associated with a unique KEGG ID. The original counts in the ShotMAP output count table were then replaced with the new unique average counts.

The unique averages were summed for each KEGG ID (added up unique averages of all pathways that are assigned to that KEGG ID). Then, the unique average of each pathway assigned to a KEGG ID was divided by the sum of unique averages of pathways in that KEGG ID. This determines which pathways are more abundant in the metagenomes, relative to all other pathways that also were assigned to that KEGG ID. This approach therefore results in a ratio that determines how well-represented a pathway is relative to other pathways associated with that KEGG ID in the sampling environment. The unique average count/sum of unique average count ratios for each KEGG pathway were used for all subsequent analysis and statistics.

Statistical Analyses for Environmental and Metagenomic Data

Comparisons of mean monthly temperature data from the NOAA Molasses Buoy (a station approximately 5 km from the experimental site) in the Upper Florida Keys were performed using a Kruskal-Wallis and Dunn's *post-hoc* test in SigmaPlot Version 11 (Supplementary Table 3). Differences in bleaching and disease prevalence were analyzed using generalized linear mixed models with a binomial distribution and logit link function in the lme4 package (Bates et al., 2015) in R (R Core Team, 2016). To assess significance, fitted models were tested against a null model that included only time and the random colony effect via likelihood ratio tests.

We analyzed the effects of enrichment on bleaching and disease severity using mixed-effects models. For these models, we used the logit-transformed severity scores as the response variable and included nitrogen, phosphorus, and date as interacting fixed factors and a random effect for coral colony. When significant effects were present, we conducted Tukey's *post-hoc* analyses using the glht() function in the multicomp package (Hothorn et al., 2008). Treatment and time were considered fixed effects, and a random effect was included for coral colonies. For analyses of bleaching and disease prevalence and severity, we excluded July data points, as July was the start of the experiment and corals were deliberately selected to have no signs of disease or bleaching. Additionally, we used data from our final surveys to test for differences in the recovery of bleached tissue and tissue mortality using two-factor ANOVA that included nitrogen and phosphorus enrichments as interacting factors. Both the recovery and mortality data were logit transformed to meet assumptions of parametric statistics.

Prior to statistical analysis, we first normalized metagenomic taxonomic raw results to relative abundance. Differences in taxonomic relative abundance between nutrient treatments and over time were tested using generalized linear mixed models using the lme4 package in R, with treatment and time as fixed effects, and individual corals as random effects (Bates et al., 2015). *Post-hoc* tests were conducted using the multicomp package in R (Hothorn et al., 2008). Statistical analysis comparing relative abundance of functional pathways found in the metagenomes over time and among treatments were also done as described above. All metagenomic data graphs were visualized using ggplot2 (Wickham, 2009).

We used the indicator species analysis function in Mothur v1.39.3 (Schloss et al., 2009) to generate microbial Operational Taxonomic Units (OTUs) indicative of the microbiome of apparently healthy or diseased *S. siderea*. We used the indicator() command with a shared OTU table and a design file containing the relevant metadata. An indicator value, ranging from 1 to 100, decides the indicator status of an OTU in a group of pre-determined sites or samples. The indicator value of an OTU is a calculation of its abundance and fidelity in a group of sites (how often the OTU is present in all sites of a group) (Dufrêne and Legendre, 1997). Ten thousand random permutation of sites among groups tests the statistical significance of an OTU's indicator species status. We used a cutoff indicator value of 30 to obtain the strongest indicators of any group. This threshold ensures that an OTU is present in over half of the samples in a group, and that its relative abundance in that group is at least 50% (Dufrêne and Legendre, 1997).

To check for effects of time and treatment on microbial diversity indices, alpha and beta-diversity were measured for both time and treatment separately and together using the Phyloseq package in R, with the estimate_richness() function for Chao1 calculations, and the distance() function on normalized data for Bray-Curtis indices (McMurdie and Holmes, 2013). Chao1 values were compared over time and between treatments using Kruskal-Wallis and Dunn's *post-hoc* test (Supplementary Table 4). Bray-Curtis data were further analyzed using the Adonis function in the Vegan package in R, and *post-hoc* testing was performed using the RVAideMemoire package using pairwise.perm.manova(), which conducts pairwise tests on matrix data using Adonis (Hervé, 2017; Oksanen et al., 2017).

RESULTS

Time and Treatment Variably Affect *Agaricia* sp. Bleaching

In 2013–2014, the Florida Keys experienced the warmest winter and summer on record up to that date (Manzello, 2015). These anomalously high temperatures were likely the main driver of the 2014 bleaching event as portions of the Florida Keys, including our study site, reached between 6 and 12 Degree Heating Weeks (NOAA and Coral Reef Watch, 2014; Barnes et al., 2015). Our experiment began on July 14th, 2014, just preceding the NOAA bleaching alert warning for the study area (**Figure 1**). The average hourly temperature on July 14th was 29.8°C. By August, the Upper Keys surpassed the thermal stress thresholds (max monthly mean sea surface temperature + 1°C) and significant bleaching occurred (Manzello, 2015). During this time, the maximum mean monthly temperature at our site was 30.8 ± 1.1°C in August, with the warmest time point within our experimental time period falling on August 15th at 31.9°C (**Figure 1**). The mean daily temperature in August was significantly higher than all other months during metagenome sampling (Kruskal Wallis, $p < 0.05$).

The thermal stress event in 2014 induced severe bleaching in our *Agaricia* sp. corals (**Figure 2**). While no corals showed signs of bleaching in July, all corals began to bleach in

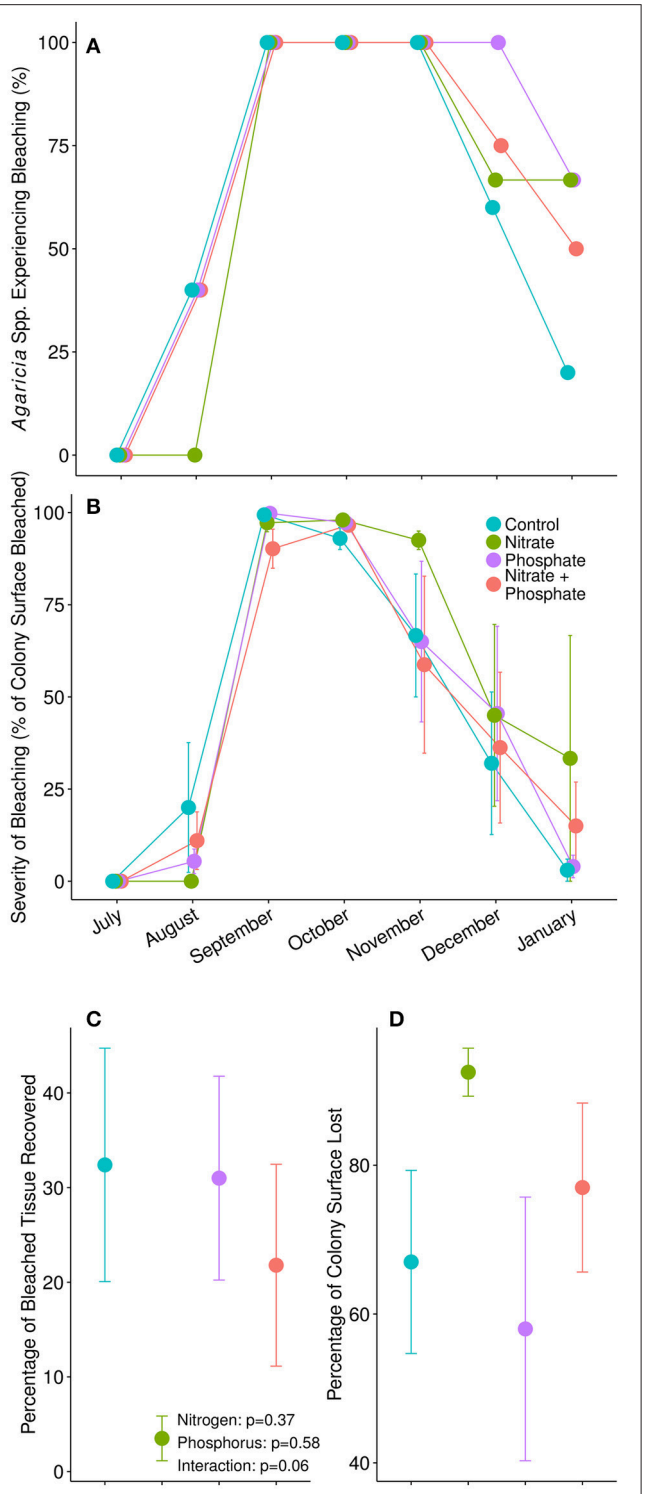

FIGURE 2 | (A) Proportion of *Agaricia* spp. in each treatment experiencing bleaching during our monthly surveys. **(B)** The average severity of bleaching, calculated as the percentage of colony surface area with no pigmentation, during each survey point. **(C)** The average percentage of previously bleached tissue in each colony that had regained pigment and recovered from bleaching by January, 2015. *P*-values are from a two-factor ANOVA. **(D)** The average percentage of each coral's surface area that died between pretreatment surveys in July, 2014, and final surveys in January, 2015.

August. By early September, 100% of the *Agaricia* sp. corals were bleached to some degree, regardless of nutrient treatment (**Figure 2A**). However, 4 months later, in January 2015, the control *Agaricia* sp. corals had mostly recovered with only 1 out of 5 control corals remaining bleached. In contrast, bleaching prevalence in surviving enriched corals remained between 50 and 66% depending on the treatment, however, this effect was not statistically significant [$\chi^2_{(3)} = 1.475$, $p = 0.692$] Similarly, we were unable to detect an impact of nutrients on bleaching severity in *Agaricia* sp., as upwards of 90% of the surface area of all colonies were bleached by September (**Figure 2B**). Interestingly, nitrogen tended to impede recovery of bleached coral tissue (**Figure 2C**), although differences were not statistically different from other nutrient treatments [$F_{(1, 15)} = 4.053$, $p = 0.06$]. Furthermore, reduced recovery in nitrogen alone enriched corals coincided with increased mortality in these corals. By January, mean tissue loss for the control *Agaricia* was 67 ± 12% vs. 92.5 ± 3.2% of tissue lost in nitrogen alone exposed *Agaricia* (**Figure 2D**).

Thermal Stress Associated With Dark Spot Syndrome in *Siderastrea siderea*

Across all treatments, the average prevalence of DSS in the experimental corals increased from 0% in July, to >40% in August, and peaked at >60% by September (**Figure 3A**). All of the nitrogen alone and combined nitrogen and phosphorus treated corals exhibited signs of disease by September. By October, half of the diseased corals in the control treatments had recovered (e.g., 80% in September to 40% in October) while more than 60% of the nutrient-enriched corals showed signs of DSS from October until January, suggesting that, like bleaching, nutrient exposure prolongs disease signs (**Figure 3A**). Overall, while a trend existed, we again did not detect a significant effect of enrichment on DSS prevalence [$\chi^2_{(3)} = 6.25$, $p = 0.09$].

Throughout the experiment, disease severity was lowest in August (3.9%, $p < 0.001$) and highest in November (14.9%) and December (14.8%) (**Figure 3B**). Disease severity in November and December were statistically higher than in August ($p < 0.001$), September ($p = 0.02$, $p = 0.005$), and October ($p < 0.001$). Amongst treatments, disease severity again tended to be higher in the nutrient exposed corals, with the controls exhibiting the lowest mean disease severity (5.4%) compared to nitrogen alone (7.3%), phosphorus alone (12.1%), and nitrogen and phosphorus combined (17.2%) diseased tissue levels. However, despite a visual trend there were no statistical differences in disease severity among nutrient types.

To track how time, treatment, or the interaction shifted overall diversity metrics of the microbiome, we generated shotgun metagenomes for all of the *S. siderea* and compared the community structure and function among the different coral microbiomes. There was a significant change in microbial alpha diversity metrics overtime (**Figure 4A**). *Post-hoc* tests showed that alpha diversity differed between August and September ($p < 0.01$), September and October ($p < 0.01$), and July and October ($p = 0.01$). September samples, which had the highest amount of DSS recorded, had the highest overall alpha diversity, with an average Chao1 OTU index of ∼5,277 ± 71.29 OTUs, compared to July (4,888 ± 203.44), August (4,420 ± 203.66), and October (4,130 ± 204.58) (**Figure 4A**). Nutrient exposure had no significant effect on alpha-diversity ($p > 0.01$, **Figure 4A**).

Although nutrients did not alter coral microbiome alpha diversity, nutrient enrichment did increase microbial beta-diversity, or sample to sample variation (**Figure 4B**; $p = 0.01$). As visualized on an ordination plot, control samples clustered together, while the phosphorus enriched samples were aligned along Axis 1, and the nitrogen enriched samples were aligned along Axis 2 (**Figure 4B**). *Post-hoc* tests showed significant differences between the combined nitrogen and phosphorus samples compared to control and nitrogen enriched samples ($p = 0.04$ & $p = 0.03$, respectively). Surprisingly there were no differences in beta-diversity over time (Adonis, $p = 0.11$), nor was there a significant interaction of time and nutrient treatments on beta-diversity (Adonis, $p = 0.96$).

Siderastrea siderea Microbiome Community Structure Shifts

In addition to alpha and beta-diversity analysis, we conducted metagenomics analysis to determine if different taxa, groups of taxa, or functions were differential affected by time, treatment, or the interaction. Hierarchical taxonomic and functional analysis showed clear effects of time but few effects of the nutrient additions on different individual or groups of microbial organisms (**Table 1**). Overall the mean number of microbial and viral annotations within the coral metagenomes were: 3.66% Archaea, 21.19% Bacteria, 1.14% Virus, and 61.24% Eukarya (excluding the host and symbiont). Archaea were composed of 82.90% Euryarchaeota, 10.35% Crenarcheota, and 6.06% Thaumarcheota. The Bacteria were composed primarily of Proteobacteria (36.10%), Firmicutes (28.52%), Bacteroidetes (16.15%), Actinobacteria (4.19%), and Cyanobacteria (2.84%). The top five viral families consisted of *Myoviridae* (25.98%), *Siphoviridae* (9.26%), *Mimiviridae* (7.89%), *Baculoviridae* (7.61%), and *Poxviridae* (5.81%). Approximately half of all the Eukaryotic reads (32.11%) were assigned as Fungi, consisting of the following top five phyla: Ascomycota (75.22%), Basidiomycota (17.75%), Microsporidia (1.56%), Chytridiomycota (0.64%), and Entomophthoromycota (0.04%). An average of 12.75% of the metagenome reads were unclassified. While time significantly affected the composition of the microbiome, (see below) there were no significant differences at any taxonomic level in relative abundance of different taxonomic groups among nutrient treatments ($p > 0.01$; Supplementary Figure 2).

Coral-Associated Viral Consortia Shift During Thermal Stress

Among the highest hierarchical categories, three taxonomic groups significantly changed with time: viruses, Archaea, and Fungi (**Table 2**). For example, viral annotations showed shifts

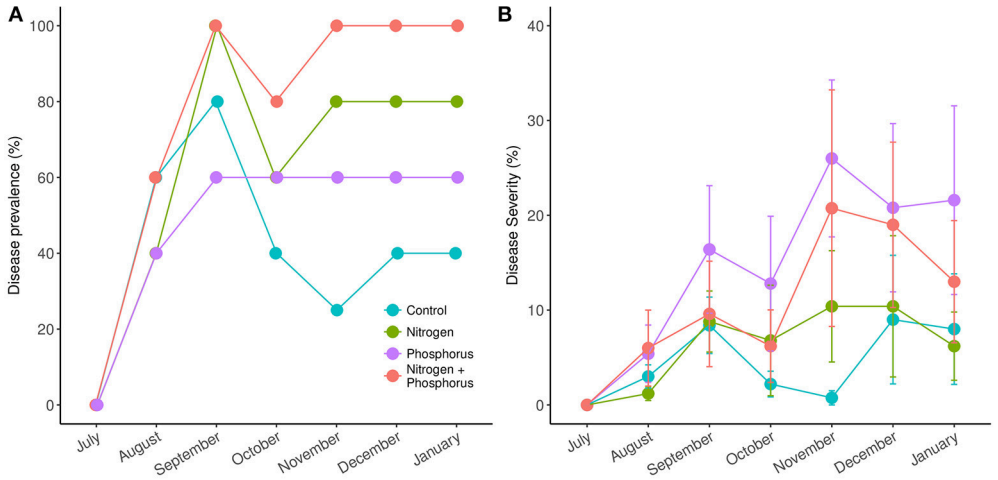

FIGURE 3 | Prevalence **(A)** and severity **(B)** of Dark Spot Syndrome in *Siderastrea siderea* corals during the course of study. Measurements taken in July were taken prior to nutrient treatment, while August, September, and October measurements were taken post treatment. Disease prevalence was calculated as the proportion of diseased individuals at each time point. Disease severity was calculated as the proportion of diseased tissue in each individual.

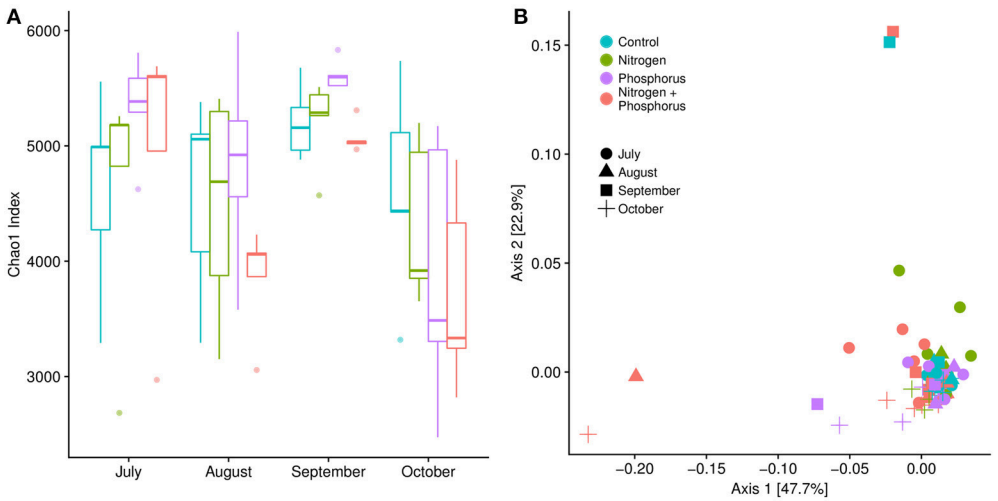

FIGURE 4 | (A) Alpha diversity of the microbial communities associated with *Siderastrea siderea* over time, calculated using the Chao1 index. Microbial alpha diversity in September was statistically different from alpha diversity metrics in August and October (Kruskal-Wallis test: $p < 0.01$; FDR-corrected Games-Howell *post-hoc* test: $p < 0.01$). **(B)** Beta diversity was calculated using a Bray-Curtis dissimilarity matrix and plotted on an MDS (multidimensional scaling) graph. Beta-diversity metrics were not different over time, but were instead significantly different between treatments (Adonis, $p = 0.01$). Pair-wise comparisons showed that the beta-diversity metrics were different between control sites and combined nutrient sites, and nitrogen and combined nutrient sites (Adonis, $p = 0.042$ and 0.03).

in the early part of the coral collections, with September corals consisting of a significantly higher relative abundance of viral annotations compared to July ($p = 0.01$) and August ($p = 0.006$) (**Figure 5**). The relative abundance of viral reads went from 1.07% in July, to 1.24% in September, declining again to 1.12% in October. A large part of this increase in viral annotations came from the order Caudovirales (dsDNA bacteriophages) which were significantly higher in September compared to July ($p = 0.005$) and August ($p = 0.03$) (**Table 2**). These September samples contained a higher abundance of annotations assigned to the family *Myoviridae* than July ($p = 0.002$) and August samples ($p = 0.007$) where they increased from ∼25% in July and August to 29.34% in September (**Table 2**). Around 70% of the *Myoviridae* annotations were unclassified, while 23.11% were classified as T4-like viruses. In October, *Myoviridae* annotations decreased back to 25.58%, similar to those in July (23.61%) and August (23.73%) samples (**Table 2**). Of the eukaryotic viral families, only the *Poxviridae* were found to change over time. Annotations to these nucleocytoplasmic large DNA viruses were highest in July, but then decreased in relative abundance in September ($p = 0.03$) and October ($p = 0.013$).

TABLE 1 | Mean relative taxonomic composition and standard error of the mean of different microbial groups identified from *Siderastrea siderea* coral metagenomes.

Month	Treatment	Archaea (%)	Bacteria (%)	Fungi (%)	Protozoan (%)	Virus (%)	Unclassified (%)
July	Control	3.61 ± 0.05	21.09 ± 0.24	32.16 ± 0.15	29.39 ± 0.17	1.1 ± 0.02	12.66 ± 0.25
July	Nitrogen	3.71 ± 0.09	21.89 ± 0.92	30.62 ± 0.46	29.97 ± 0.66	1.05 ± 0.01	12.76 ± 0.49
July	Nitrogen + Phosphorus	3.87 ± 0.04	22.48 ± 0.42	32.23 ± 0.42	28.32 ± 0.31	1.11 ± 0.02	11.99 ± 0.21
July	Phosphorus	3.57 ± 0.07	20.72 ± 0.52	31.99 ± 0.23	29.49 ± 0.42	1.07 ± 0.02	13.16 ± 0.35
July	Seawater	3.88 ± 0.1	33.76 ± 1.27	22.6 ± 0.56	24.19 ± 0.32	1.59 ± 0.03	13.99 ± 0.46
August	Control	3.62 ± 0.04	20.94 ± 0.23	31.94 ± 0.19	29.56 ± 0.21	1.11 ± 0.02	12.82 ± 0.31
August	Nitrogen	3.6 ± 0.08	20.82 ± 0.23	31.85 ± 0.48	29.44 ± 0.19	1.12 ± 0.02	13.17 ± 0.6
August	Nitrogen + Phosphorus	3.49 ± 0.04	21.08 ± 0.89	32.69 ± 0.62	28.5 ± 1.25	1.03 ± 0.04	13.21 ± 0.31
August	Phosphorus	3.58 ± 0.07	20.46 ± 0.29	32.08 ± 0.34	29.78 ± 0.22	1.06 ± 0.01	13.04 ± 0.46
August	Seawater	3.72 ± NA	31.67 ± NA	21.09 ± NA	25.61 ± NA	1.68 ± NA	16.23 ± NA
September	Control	3.8 ± 0.05	22.74 ± 1.64	30.8 ± 1.67	28.32 ± 0.93	1.56 ± 0.39	12.78 ± 0.54
September	Nitrogen	3.76 ± 0.06	21.14 ± 0.15	32.42 ± 0.15	29.23 ± 0.07	1.17 ± 0.02	12.28 ± 0.28
September	Nitrogen + Phosphorus	3.8 ± 0.05	22.9 ± 1.69	30.71 ± 1.87	28.16 ± 0.81	1.47 ± 0.29	12.97 ± 0.68
September	Phosphorus	3.67 ± 0.04	20.98 ± 0.19	32.84 ± 0.36	28.83 ± 0.38	1.14 ± 0.02	12.55 ± 0.16
September	Seawater	3.58 ± NA	20.32 ± NA	32.07 ± NA	29.75 ± NA	1.12 ± NA	13.16 ± NA
October	Control	3.64 ± 0.02	20.67 ± 0.13	32.41 ± 0.23	29.63 ± 0.2	1.14 ± 0.02	12.51 ± 0.2
October	Nitrogen	3.66 ± 0.08	20.65 ± 0.26	32.55 ± 0.31	29.37 ± 0.13	1.15 ± 0.02	12.63 ± 0.43
October	Nitrogen + Phosphorus	3.59 ± 0.11	21.01 ± 0.36	34.1 ± 1.07	28.15 ± 1.05	1.04 ± 0.06	12.1 ± 0.35
October	Phosphorus	3.64 ± 0.08	20.78 ± 0.31	32.97 ± 0.47	29.15 ± 0.37	1.1 ± 0.02	12.35 ± 0.45
October	Seawater	3.73 ± 0.01	27.24 ± 0.72	22.84 ± 0.36	26.45 ± 0.2	3.67 ± 0.24	16.07 ± 0.07

Data are separated by time (month of sampling) and treatment (control, nitrogen alone, phosphorus alone, and nitrogen and phosphorus combined).

TABLE 2 | Statistically significant shifts in taxonomic groups across time in *Siderastrea siderea* metagenomes as measured by generalized linear mixed models with Tukey's *post-hoc* test.

Taxonomic Shifts	July (%)	August (%)	September (%)	October (%)	Pairwise comparisons
ARCHAEA					
Thermoplasmata	2.78 ± 0.025	2.91 ± 0.053	2.85 ± 0.027	2.84 ± 0.069	*July < August
Thermococci	6.17 ± 0.094	6.45 ± 0.091	6.20 ± 0.093	6.68 ± 0.119	**July < October; *September < October
Desulfurococcaceae	1.38 ± 0.018	1.45 ± 0.022	1.39 ± 0.018	1.41 ± 0.022	*July < August
FUNGI					
Agaricales	3.27 ± 0.028	3.24 ± 0.027	3.33 ± 0.023	3.33 ± 0.025	**August < September; **August < October
Magnaporthales	1.25 ± 0.0084	1.25 ± 0.011	1.25 ± 0.0085	1.30 ± 0.013	*August < October; *September < October
Tremellales	1.59 ± 0.014	1.57 ± 0.017	1.60 ± 0.012	1.62 ± 0.019	**August < October
VIRUS					
Caudovirales	39.17 ± 0.27	40.05 ± 0.68	45.35 ± 2.43	40.35 ± 0.54	**July < September; *August < September
Myoviridae	23.67 ± 0.20	24.15 ± 0.35	28.99 ± 2.06	24.60 ± 0.46	**July < September; **August < September
Poxviridae	6.39 ± 0.15	5.82 ± 0.19	5.39 ± 0.22	5.71 ± 0.15	*September < July; *October < July

*$p < 0.05$; **$p < 0.01$.

Coral-Associated Archaea Shift in Abundance During Warming

Along with the viruses, there were shifts in the Archaea associated with the *Siderastraea* corals. At the class level, the Thermoplasmata had lower abundance in July (2.78%) than in August (2.91%; $p = 0.02$). In the case of Thermococci, the October metagenome contained higher relative abundance of 6.68% compared to 6.16% in July ($p < 0.001$) and 6.20% in September ($p = 0.02$). This class consisted solely of the order Thermococcales, and within that, the family Thermococcaceae. Additionally, the relative abundance of Desulfurococcaceae in July (1.38%) was lower than that in August (1.45%) ($p = 0.045$).

Coral-Associated Fungi Shift Across the Thermal Stress Event

Within the Eukaryotes there was a statistically significant change in fungal orders over time, with several orders of low relative abundance fungi becoming more abundant in the October metagenomes. Agaricales, a Basidiomycota, had a relative abundance of 3.24% in August and increased to

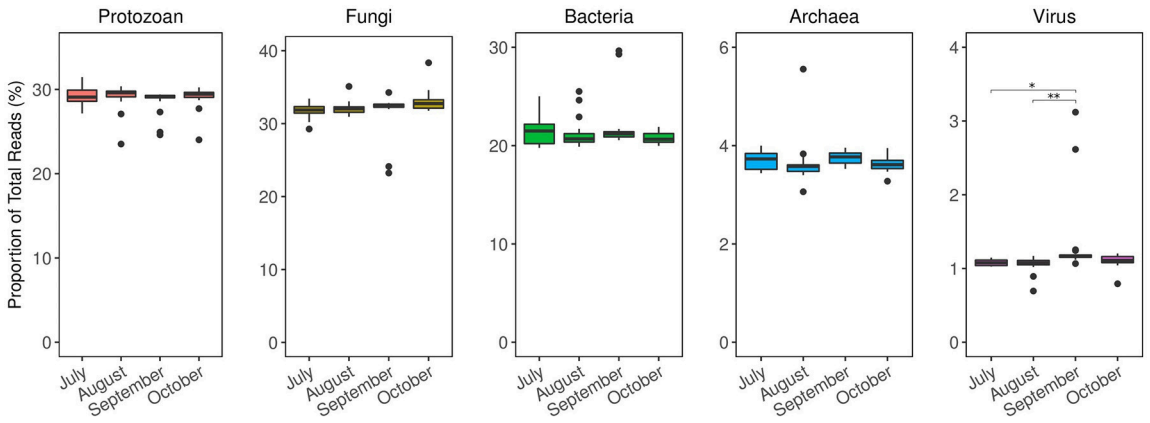

FIGURE 5 | Taxonomic distribution of *Siderastrea siderea* coral microbiomes from metagenome analysis over time. Results are normalized as the relative abundance of each taxa at every time point. The microbiome composition among domains remained relatively stable throughout time, except for viral annotations. The relative abundance of viral annotations was higher in September, compared to July ($p = 0.01$) and August ($p < 0.01$). *$p \leq 0.05$; **$p \leq 0.01$.

3.33% in September ($p < 0.001$) and October ($p = 0.008$). Another Basidiomycota, the Tremellales, also had higher relative abundance in October, 1.62%, compared to August (1.57%; $p = 0.001$). The Sordariomycetes order, Magnaporthales, had a higher relative abundance of 1.30% in October compared to 1.25% in August ($p = 0.03$), and 1.25% in September ($p = 0.01$).

Indicator Species of Healthy Coral Microbiomes

We conducted indicator species analysis on healthy and diseased *S. siderea* microbial metagenome samples to find the taxa most indicative of either healthy or diseased states. The indicator species of apparently healthy *S. siderea* included solely fungi and viruses. Fungal indicators include *Olpidium brassicae*, an unclassified Entomophthoromycete, *Polychytrium aggregatum* in the order Polychytriales, and *Pluteus saupei*, in the order Agaricales. Fourteen virus families also showed up as indicator taxa in healthy *S. siderea* microbiomes. These include Astroviridae, Baculoviridae, two OTUs within Betaflexiviridae, Bromovoridae, Circoviridae, Closteroviridae, two OTUs within Geminiviridae, Nyamiviridae, two OTUs in Polyomaviridae, Potyviridae, Secoviridae, two OTUs within Siphoviridae, Tombusviridae, and two OTUs within Totiviridae. Interestingly, we found no taxa indicative of diseased *S. siderea* microbiomes.

Coral Microbiome Function Is Altered During Thermal Stress

One advantage of metagenomes is the ability to quantify shifts in both the taxonomic structure of a microbiome as well as the functional potential of that community. Overall the functional potential of the coral microbiome showed the following distribution of classified functions: 9.77% Cellular Processes, 14.75% Environmental Information Processing, 24.23% Genetic Information Processing, and 51.25% Metabolism. We found that the functional potential of the coral microbiomes shifted across time but not with treatment (**Table 3**). Within the broadest hierarchical level, KEGG category 1, there was a higher abundance of genes for "genetic information processing" in October (24.75%) compared to July (23.91%; $p = 0.008$). This category houses the subcategories of "transcription," "translation," "folding," "sorting and degrading," and "replication and repair." Genes for "translation" were more abundant in October (9.51%) compared to July (9.29%) ($p = 0.02$), and genes for "replication and repair" were found to be lowest, at 6.76%, in July, compared to 6.92% in August ($p = 0.04$), 6.98% in September ($p < 0.001$), and 6.95% in October ($p < 0.001$).

In contrast, genes for "amino acid metabolism" were lower in October (9.05%) than July (9.66%) ($p = 0.03$) and September (9.89%) ($p = 0.04$) while genes for "metabolism of cofactors and vitamins" were also lower in October (6.62%) compared to July (6.84%) ($p = 0.02$). Lastly, genes for "xenobiotics biodegradation and metabolism" were more abundant in July (1.25%) and September (1.21%) compared to August (1.18%) ($p = 0.02$) and October (1.13%) ($p < 0.001$, $p = 0.01$).

Within the highest resolution KEGG categories, the subcategory "genetic information processing" showed that only a few genes increased in abundance over time while many were reduced. For example, genes for "homologous recombination" were higher in October (1.52%) than in July (1.45%; $p < 0.001$) while "nucleotide excision repair" genes were more abundant in September (1.49%) than in October (1.36%; $p = 0.007$). But genes assigned to functional subcategories within "environmental information processing, cellular processes, and metabolism" tended to be more abundant in July compared to later months with "ABC transporter" genes higher in July (4.89%) than September (4.77%; $p = 0.001$) and October (4.81%; $p = 0.04$), and genes for the "two-component system" also being elevated in July (3.03%) compared to September (2.86%; $p = 0.001$). "Photosynthesis" genes were more abundant in July (0.26%) than in August (0.23%; $p = 0.008$), and genes for "alanine, aspartate, and

TABLE 3 | Statistically significant shifts in functional assignments across time in *Siderastrea siderea* metagenomes as measured by generalized linear mixed models with Tukey's *post-hoc* test.

Functional Shifts	July (%)	August (%)	September (%)	October (%)	Pairwise comparisons
KEGG 1					
Genetic information processing	23.91 ± 0.22	24.34 ± 0.18	24.01 ± 0.28	24.75 ± 0.09	**July < October
KEGG 2					
Amino acid metabolism	9.66 ± 0.14	9.50 ± 0.16	9.89 ± 0.33	9.05 ± 0.10	*October < July; *October < September
Metabolism of cofactors and vitamins	6.84 ± 0.042	6.71 ± 0.051	6.87 ± 0.073	6.62 ± 0.055	*October < July
Replication and repair	6.76 ± 0.052	6.92 ± 0.050	6.98 ± 0.021	6.95 ± 0.036	*July < August; **July < September; **July < October
Translation	9.29 ± 0.089	9.42 ± 0.050	9.28 ± 0.050	9.51 ± 0.043	*July < October
Xenobiotics degradation and metabolism	1.25 ± 0.026	1.18 ± 0.019	1.21 ± 0.011	1.13 ± 0.027	*August < July; **October < July; *October < September
KEGG 3					
ABC transporters	4.89 ± 0.019	4.81 ± 0.024	4.77 ± 0.022	4.81 ± 0.030	**September < July; *October < July
Alanine, aspartate, and glutamate metabolism	1.43 ± 0.027	1.40 ± 0.036	1.46 ± 0.042	1.30 ± 0.026	**October < July; **October < September
Homologous recombination	1.45 ± 0.014	1.49 ± 0.009	1.49 ± 0.008	1.52 ± 0.019	**July < October
Nucleotide excision repair	1.43 ± 0.020	1.43 ± 0.017	1.49 ± 0.035	1.36 ± 0.019	** October < September
Two-component system	3.03 ± 0.058	2.90 ± 0.036	2.86 ± 0.023	2.92 ± 0.039	**September < July
Photosynthesis	0.26 ± 0.023	0.23 ± 0.017	0.26 ± 0.034	0.23 ± 0.015	**August < July

*$p < 0.05$; **$p < 0.01$.

glutamate metabolism" were higher in July (1.43%; $p = 0.007$) and September (1.46%; $p = 0.007$) compared to October (1.30%).

Thermal Stress Shifts the Microbiomes of DSS Afflicted Corals

Although there is no ascribed etiological agent responsible for DSS, by subdividing the data into corals with and without DSS, we found metagenomic evidence that corals experiencing DSS are unique microbiologically. Like nutrient exposure, DSS samples exhibited increased beta-diversity (Adonis, $p = 0.011$) compared to apparently healthy ones (**Figure 6A**). However, there was no significant difference in alpha diversity (Chao1 index) between DSS and non-DSS coral microbiomes (Welch's *T*-test, $p = 0.24$). To test if the thermal stress event altered the microbiomes, we compared only the August 2014 DSS ($n = 10$) and apparently healthy samples ($n = 8$) to another metagenomic dataset ($n = 42$) from coral mucus collected in August 2012. The 2012 samples (23 DSS and 19 apparently healthy) came from *S. siderea* corals that were either exposed to nutrient enrichment or control conditions. Compared to 2014, the 2012 samples were only experiencing moderate thermal stress (∼6 DHW) (Zaneveld et al., 2016). The 2012 DSS and apparently healthy corals had indistinguishable microbiomes regardless of treatment and disease, and they clustered separately from the 2014 microbiome samples (**Figure 6B**, Adonis, $p \leq 0.001$).

DISCUSSION

In 2014, corals in the Florida Keys experienced severe thermal stress of 6–12 degree heating weeks depending on location. We found that this thermal anomaly was associated with increased bleaching and disease alongside changes in the alpha diversity of the microbiome and distinct shifts in different groups of taxa associated with the corals, particularly fungi and viruses. Shifts in the function of the microbiomes were also correlated with time. Nutrient exposure, on the other hand, only caused clear shifts in beta-diversity of the microbiomes, a finding that was independent from time, and thus likely not a result of the thermal anomaly.

Nutrient Exposure May Prolong Temperature-Mediated Bleaching in Agaricia Corals

Nutrient exposed corals were more likely to remain bleached 5 months after thermal stress compared to control corals. Though all *Agaricia* spp. corals bleached after the thermal stress event in August, recovery trended in favor of the corals in ambient conditions (80% recovered), compared to the corals in nutrient-stressed conditions (less than 50% recovered) (**Figure 2A**). All corals experienced high bleaching severity after thermal stress in August, however, only control corals and corals exposed to phosphorus completely recovered by January; corals exposed to nitrogen alone, or nitrogen and phosphorus did not fully recover by the end of the experiment (**Figure 2B**). Though not

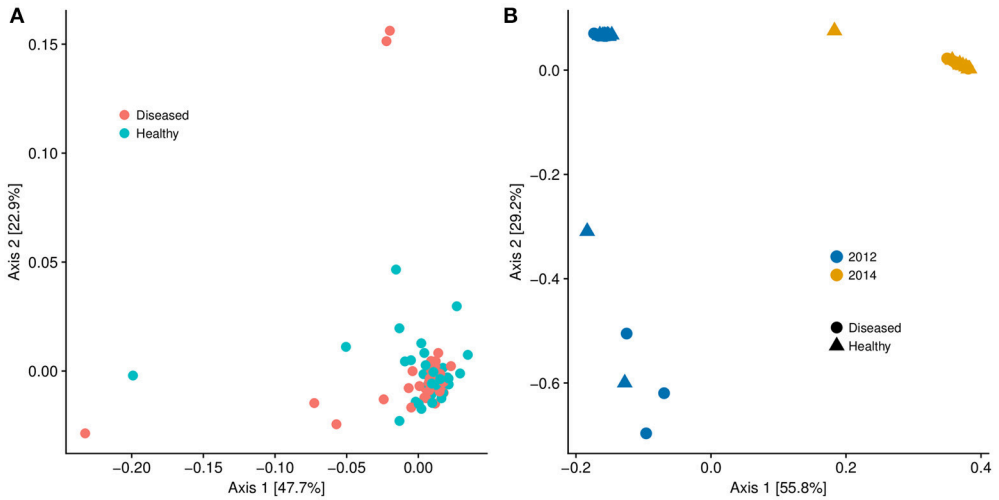

FIGURE 6 | (A) Multidimensional scaling graph of beta diversity of microbial communities of *Siderastrea siderea* samples broken into diseased and healthy groups. The microbiome of the diseased corals was statistically different from the microbiome of the healthy corals (Adonis, $p = 0.01$). **(B)** Multidimensional scaling graph of beta diversity of microbial communities of *Siderastrea siderea* corals. The 2012 data points were taken from *Siderastrea siderea* microbial metagenome data from a nutrient-enrichment experiment conducted in August of 2012. The 2014 data points were metagenomes from the current dataset sampled in August of 2014, which was subject to nutrient enrichment and high water temperatures. There was no statistical difference between the diseased and healthy microbiomes in 2012 and 2014, but there was a difference between the 2012 and 2014 samples (Adonis, $p < 0.01$).

of statistical significance, likely due to our low replication within each category, these trends suggest that nitrogen and phosphorus behave in different ways to influence susceptibility and resilience to bleaching.

Corals Disease Linked to Thermal Stress

Disease prevalence in all *S. sideraea* corals (**Figures 3A,B**) went from 0% in July 2014 to ∼80% within 2 months. DSS declined by October to 40% in the controls but remained steady at this level until January when we ended the experiment. This was a somewhat unexpected finding, as although a single study has also found that DSS prevalence can increases with higher water temperatures (Gil-Agudelo and Garzón-Ferreira, 2001), DSS generally peaks in the winter months, not the summer (Borger, 2005; Gochfeld et al., 2006). Interestingly, the prevalence of disease also remained above 50% from September to the conclusion of the experiment for all nutrient treatments, suggesting only in the presence of elevated nutrients do such thermal events reduce coral resilience by prolonging disease and/or preventing recovery.

Siderastrea siderea Disease and Microbial Diversity

Diversity within the *S. siderea* microbiome changed significantly across time with alpha diversity peaking in September across all treatments. Interestingly, the September alpha diversity metrics also had low variability compared to samples from other months. In contrast to microbial alpha diversity, the beta diversity of the metagenome samples varied with both nutrient treatment and disease status. In particular, we found that beta-diversity in the combined nitrogen and phosphorus enriched corals differed from both the control and the nitrogen-treated corals. Similarly, DSS-afflicted coral microbiomes clustered separately from healthy colonies, linking DSS with the coral microbiome, although it is unclear if this is a cause or an effect. Yet this increase and difference in beta diversity in the microbiomes of stressed and diseased corals aligns with the Anna Karenina principle, which states that the microbiomes of stressed animals are usually in an unstable dysbiosis, due the host being unable to regulate its microbial community (Zaneveld et al., 2017).

We used metagenomics instead of 16S analysis because we and others had previously found no correlation in microbial taxa shifts associated with DSS using 16S analysis. (Borger, 2005; Kellogg et al., 2014; Meyer et al., 2016). Using this approach, we again found no single taxon or groups of taxa that were associated with the disease. Yet in a study focusing on *Stephanocoenia intersepta*, the microbes of healthy and diseased patches of coral were characterized and found to differ among health states (Sweet et al., 2013). In DSS lesions, but absent in healthy tissue, four types of pathogenic bacteria were identified (*Corynebacterium*, *Acinetobacter*, *Parvularculaceae,* and *Oscillatoria*) along with the pathogenic fungi, *Rhytisma acerinum*, implicating that DSS in *S. intersepta* is caused not by a single pathogen but rather by a collection of taxonomically diverse microbes (Sweet et al., 2013). More recently, the transmission of DSS between *S. siderea* individuals was also experimentally tested, but there was no evidence of direct or indirect (water-borne) transmission of DSS symptoms, suggesting that DSS is not an infectious disease but rather a physiological one (Randall et al., 2016).

We have now extensively analyzed two metagenomic datasets of apparently healthy and DSS afflicted *S. siderea* from two different years (2012 and 2014) that were significantly different in terms of the ambient conditions present. Our indicator species analysis showed a plethora of viral and fungal taxa associated with

a healthy coral microbiome, but no indicator species was found for DSS-afflicted coral samples. This provides further evidence that there is likely no pathogen responsible for DSS, although these negative results could be due to the low power of our experiment design. However, given that we find increased beta-diversity in the DSS microbiomes, it is not surprising that we found no taxa or group of taxa that are exclusively associated or significantly elevated in DSS corals. These collective data contribute to the growing body of thought that the signs of this disease are likely manifestations of an alteration of host physiology as a response to severe temperatures and nutrient pollution and that one is manifested as increased instability of the microbiome.

Thermal Anomaly Associated With Taxonomic and Functional Microbiome Shifts

Although there was no significant shift in microbial taxa from nutrient exposure, we did find shifts in certain virus, Archaea, and Fungi over time. Because changes in time and temperature in this experiment were inherently connected, we hypothesize that these taxonomic shifts were directly related to changes in seawater temperatures or some covariate(s). We found a higher proportion of the virus order Caudovirales in September metagenomes compared to other months. Phages targeting bacteria and archaea are the most abundant viral types found in scleractinian corals (Vega Thurber et al., 2017). These phages are crucial in shaping the coral microbiome and controlling microbial populations. Phages serve as a lytic barrier against potential pathogens (Sweet and Bythell, 2017) and have been described as non-host-derived immunity (Barr et al., 2013). The viral order Caudivirales has consistently been found in coral viromes (Wood-Charlson et al., 2015; Vega Thurber et al., 2017; Weynberg et al., 2017), with its top three families being *Siphoviridae*, *Podoviridae*, and *Myoviridae*. Most of the Caudovirales reads from this experiment were assigned to the *Myoviridae* family, which consisted mainly of T4-like viruses. The abundance of these lytic phages suggests a high turnover of the microbial community, and may also have obscured any shifts in the bacterial community, including any potential pathogens.

The family *Poxviridae* had a higher relative abundance in July, the start of the experiment, compared to September and October, but there was no dominating viral genus within this family. Members of *Poxviridae* infect insects and terrestrial vertebrates such as humans and birds, but have also been found in dolphins, whales, and sea lions (Bracht et al., 2006). Marine *Poxviridae* often make up the top five viral families found in coral viromes (Vega Thurber et al., 2017; Weynberg et al., 2017). These eukaryotic viruses either infect the coral host or eukaryotic members of the microbiome, yet this taxon tends to be more abundant in healthy coral viromes compared to diseased or bleached viromes (Vega Thurber et al., 2017), which may explain the decline of the relative abundance of *Poxviridae* in *S. siderea* metagenomes as thermal stress increased and coral health declined. Interestingly, neither *Myoviridae* nor *Poxviridae* were identified as indicator species for a healthy *S. siderea* microbiome. However, the plethora of viral OTUs found to be indicative of the microbiome of a healthy coral host show the importance of viruses in shaping the coral-associated microbial community.

The Archaeal members of the *S. siderea* holobiont consisted mainly of Crenarchaeota and Euryarchaeota. While they are not known to form species-specific symbioses with their coral host, they are hypothesized to participate in nutrient cycling (Wegley et al., 2004). For example, it is hypothesized that the Crenarchaeota turn over nitrogen via ammonia oxidation (Siboni et al., 2008). In this study, we did not find any correlation between Archaeal communities and nutrient exposure. Instead, we found three Archaeal members of the microbiome to shift across time. Both the Euryarchaeota, Thermoplasmata, and the Crenarchaeota, Desulfurococcaceae, had higher relative abundance in August, when seawater temperature reached its peak.

Fungi, particularly endolithic fungi, have long been acknowledged as endemic members of the scleractinian coral holobiont (Bentis et al., 2000; Ainsworth et al., 2017). Though most marine fungi are thought to be opportunistic, with the exception of *Aspergillus sydowii*, the confirmed pathogen of Caribbean sea fans (Smith et al., 1996), the role of endolithic fungi in coral tissue has yet to be confirmed. These fungi are hypothesized to participate in nutrient cycling by participating in symbiotic relationships with nitrogen-fixing bacteria. One early metagenome study of the *Porites astreoides* holobiont found fungal reads to make up the majority of classified eukaryotic sequence sequences (Wegley et al., 2007). Most of these fungal reads consisted of Ascomycota, which are in many healthy coral holobionts (Wegley et al., 2007). Similarly, Ascomycota made up 75% of the fungal reads in this study. Ascomycetes also dominated the fungal community in another coral metagenome study of the *Porites compressa* holobiont (Vega Thurber et al., 2009), but in that study, nutrient enrichment did not affect the composition of the fungal community. Again, we saw the same result in this study, in which certain fungal orders shifted with time and temperature, but not with nutrient addition. Other hypothesized roles of these fungi include competition with algal members of the holobiont, contribution to coral resistance to disease and bleaching, and parasitism upon the coral host (Yarden, 2014; Ainsworth et al., 2017). In this study, we found an Entomophthoromycete, a Chytridiomycete, and an Agaricomycete as fungal indicator species of healthy *S. siderea*, showing that at least some fungal species exist in either a commensal or mutualistic relationship with the coral host.

Functional analysis of the *S. siderea* microbial metagenome showed several contrasts between the start of the nutrient enrichment experiment in July, and the end of metagenome sampling in October. Prior to the bleaching event there was a higher relative abundance of genes for metabolism of cofactors and vitamins, and metabolism of amino acids—in particular alanine, aspartate, and glutamate. Additionally, prior to the thermal stress there was a higher relative abundance of photosynthesis genes (compared to August), two-component system genes (compared to September), ABC transporter genes (compared to September and October), and genes for xenobiotics

biodegradation and metabolism (compared to August and October).

In contrast, microbial metagenomes of the latter months showed distinct functional potential. This manifested in the higher abundance of genes in October metagenomes for translation, replication and repair, homologous recombination, and a higher abundance of genes for nucleotide excision repair in September. The elevation of these genes categories could be interpreted as a shift in the community to more stress resistant taxa as a result of the thermal anomaly.

CONCLUSIONS

We conducted an *in situ* nutrient enrichment experiment in the Upper Florida Keys on *Agaricia* sp. and *Siderastrea siderea* corals in 2014, which coincided with a bleaching event due to a thermal anomaly. These unique environmental conditions allowed us to study the effects of high temperature and nutrient pollution on these corals. Elevated temperatures resulted in higher bleaching prevalence and severity of *Agaricia* sp. regardless of nutrient treatment and resulted in higher disease prevalence and severity in *Siderastrea siderea*. In the *Siderastrea siderea* metagenomes, there were several shifts in viral, archaeal, and fungal families across sampling time points, most notably a severe increase in the *Myoviridae* viruses associated with the aftermath of the thermal anomaly. Interestingly, we found no microbial taxa correlated with DSS.

Experimental Design Considerations and Future Work

Due to the low number of replicates in each coral category, there was a likelihood of Type II errors (false negatives). For example, many statistical tests failed to meet the standard *p*-value requirements after multiple corrections tests, especially since many animals died during the experiment. Trends in our data are thus likely suggestive of important patterns that should be tested and confirmed in the future. Repeat experiments with a higher number of replicates are suggested to provide better statistical power. Additionally, we acknowledge that many of our statistically significant results show shifts in the relative abundance of taxonomic or functional assignments of less than 3%. Whether these shifts are biologically significant and meaningful is debatable. However, for some groups, even small changes that occur in the background of host and symbiont genetic information is likely to be biologically important. In particular, viral genomes are typically many orders of magnitude shorter in length than bacterial or eukaryotic genomes, thus they make up a very small percentage of any host associated metagenome. Additionally, in other systems, it is well established that presence and absence of rare taxa have been found to be significant due to potentially high metabolic activity of low abundance bacteria and fungi members of microbial consortia (Kurm et al., 2017). Therefore, any statistically significant shift in the taxonomic composition of viruses could have meaningful consequences in the microbial community. Yet for shifts we discovered within fungi and Archaea, interpretations of such small shifts in the relative abundance of genes associated with these taxa should be tempered.

Lastly, to confirm the hypothesis that DSS is a physiological stress response, and not caused by a disease agent, we suggest future work to include transcriptome analysis of DSS-infected *S. siderea,* and the transcriptome analysis of the *Symbiodinium* associated with DSS-infected *S. siderea*. Studying differential gene expression in DSS-afflicted and healthy corals and *Symbiodinium* can provide answers for disease symptom initiation, progression, and restriction.

AUTHOR CONTRIBUTIONS

RV and DB designed the experiment. AS, DB, and JP conducted the fieldwork. JP and AF generated the metagenomes. LW, AS, TS, DB, and RV performed the analysis. LW, AS, JP, RV, and DB wrote the manuscript.

ACKNOWLEDGMENTS

We thank numerous volunteers for assistance with the experiment, and the Florida Keys National Marine Sanctuary for permits (FKNMS-2009-047 and FKNMS-2011-090). This research was funded by the NOAA Coral Reef Conservation Program Grant #NA14NOS4820090 to RV and DB with additional support from a NSF Biological Oceanography Grant (#OCE-1130786) to DB and RV.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00101/full#supplementary-material

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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

PERSPECTIVE
published: 23 April 2018
doi: 10.3389/fmars.2018.00142

Perspectives on the Great Amazon Reef: Extension, Biodiversity, and Threats

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Here we provide a broad overview of the Great Amazon Reef System (GARS) based on the first-ever video surveys of the region. This footage supports four major hypotheses: (1) the GARS area may be six times larger than previously suggested (up to 56,000 km^2); (2) the GARS may extend deeper than previously suggested (up to 220 m); (3) the GARS is composed of a greater complexity and diversity of habitats than previously recognized (e.g., reef platforms, reef walls, rhodolith beds, and sponge bottoms); and (4) the GARS represents a useful system to test whether a deep corridor connects the Caribbean Sea to the Southwest Atlantic Ocean. We also call attention to the urgent need to adopt precautionary conservation measures to protect the region in the face of increasing threats from extractive oil and gas practices. With less than 5% of the potential area of the GARS surveyed so far, more research will be required to inform a systematic conservation planning approach and determine how best to establish a network of marine protected areas. Such planning will be required to reconcile extractive activities with effective biodiversity conservation in the GARS.

Keywords: mesophotic coral ecosystem, calcareous algae, submersibles, systematic conservation planning, oil and gas fields

OPEN ACCESS

Edited by:
Christian Robert Voolstra, King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Aldo Cróquer, Simón Bolívar University, Venezuela
John Everett Parkinson, Oregon State University, United States

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *30 October 2017*
Accepted: *10 April 2018*
Published: *23 April 2018*

Citation:
Francini-Filho RB, Asp NE, Siegle E, Hocevar J, Lowyck K, D'Avila N, Vasconcelos AA, Baitelo R, Rezende CE, Omachi CY, Thompson CC and Thompson FL (2018) Perspectives on the Great Amazon Reef: Extension, Biodiversity, and Threats. Front. Mar. Sci. 5:142. doi: 10.3389/fmars.2018.00142

Unprecedented submersible video surveys disclose unique features of the Great Amazon Reef System (GARS). Previous surveys of the GARS were performed exclusively with indirect sampling (i.e., fishing and dredging) (Collette and Ruetzler, 1977; Cordeiro et al., 2015; Moura et al., 2016). The definition of "reef" used here is the same one given by Collette and Ruetzler (1977) in their seminal work about the GARS: i.e., complex hard bottoms created by living organisms.

The first images of the GARS and associated communities were obtained here by using a double Deep Worker submarine (Nuytco, Canada; Earle, 2010) and a drop camera system in depths between 70 and 250 m in January-February 2017. The examination of over 20 h footage obtained by means of submarine and 15 h footage obtained by the drop camera, together with previous data (Moura et al., 2016), allowed us to advance the knowledge put forward here. Each of the submarine dives ($n = 8$) lasted about 4 h and covered a total linear distance of about 1 km. Drop camera footages lasted ~30–40 min, and also covered a linear distance of about 1 km per dive. Main habitat types were visually recorded (**Figure 1**).

The occurrence of a reef system off the Amazon River mouth was first hypothesized by Collette and Ruetzler (1977). According to these authors: "from the behavior of the trawl and from fathometer readings, we conclude that hard bottoms are abundant in this region." In addition, Cordeiro et al. (2015), based on the analysis of museum material, have speculated on the occurrence of rhodolith beds in the GARS by stating that "Although our data were insufficient to confirm the existence of rhodolith banks offshore of the Amazon River, some of the sampled corals analyzed were adhered to coralline algae." Finally, Moura et al. (2016) performed extensive trawling and side scan sonar surveys, confirming the GARS' existence and estimating its size at 9,500 km^2. Results from the present survey suggest that the GARS is composed by typical mesophotic reefs (70–220 m depth) build mainly by living calcareous algae ("coralline algal frameworks," *cf.* Bosence, 1983), potentially covering an area of ∼56,000 km^2 (**Figure 2**). Similar calcareous platforms covered by living calcareous algae were recorded at depths between 80 and 120 m in tropical and subtropical Eastern Australia (Davies et al., 2004). Calcareous algae are also major reef builders elsewhere in the Atlantic, being the dominant element of the reef framework in the Rocas Atoll (Gherardi and Bosence, 2001; Villas-Bôas et al., 2005) and in coral reefs of the Abrolhos Bank (Francini-Filho et al., 2013). Beside calcareous algae, scleractinian corals were also recorded during our surveys, with *Madracis decactis* being by far the most abundant one. Only areas shallower than 70 m were devoid of consolidated substrata and dominated by fine sand and/or mud bottoms.

The GARS extends much deeper than previously anticipated, with a clear gradient from its deepest portion (∼220 m depth), where laterite outcrops alternate with areas with nearly 100% of live coverage (mainly sponges, octocorals and black corals), to its shallowest portion (∼70 m), which is nearly completely covered by sand (**Figures 1A–D**). At depths of 80–100 m, marine snow might also temporarily cover rhodolith beds and algal frameworks (**Figure 1B**). Rhodolith beds and biogenic calcareous platforms are the dominant features in depths between 70 and 180 m (**Figures 1C,E,H**). This is the deepest limit of the lower mesophotic zone recorded so far, as mesophotic reefs are believed to occur only down to about 150 m (Lesser et al., 2009). Dominant organisms of the lower mesophotic zone of the GARS (180–220 m) were typical of reef communities, such as black corals, barrel sponges (*Xetospongia muta*) and butterfly fishes (*Prognathodes* spp.) (Rosa et al., 2016). The areas deeper than 220 m recorded during our surveys were dominated by sediments.

High bottom complexity and a great diversity of habitats were recorded at the GARS, including algal frameworks, rhodolith beds, laterite bottoms, as well as sponge, soft coral and black coral gardens (**Figures 1A–D**). A large reef wall was recorded in the outer shelf of the central sector of the GARS, with an average height of 80 m (115–195 m depth) and a mapped linear extension of at least 12 km. Bordering the GARS on its shallowest portion, there were large sand wave fields (**Figure 1A**) which are indicative of strong currents and high hydrodynamic variations, with sand being eventually transported over the reef structure (**Figure 1B**). Thus, a combination of suspended load from the Amazon River and intense sediment transport in the middle continental shelf seems to determine the upper boundary of the GARS.

High spatial turnover of species was noted within the reef, as well as between the GARS and adjacent regions (i.e., Caribbean and N/NE Brazil). This pattern is plausibly explained by both habitat heterogeneity and the formation of an ecotone between the two biogeographical provinces, i.e., Brazil and the Caribbean, with a clear faunal overlap. An example is the record made in this study for the Blue chromis *Chromis cyanea*, which was previously known to occur only in the Caribbean, reinforcing the hypothesis of a connection between South America and the South Caribbean through the GARS (Rocha, 2003; Floeter et al., 2008). In fact, first evidence for the occurrence of a mesophotic corridor connecting Brazil and the Caribbean were obtained by Collette and Ruetzler (1977), which described a "typical reef fish fauna" composed by 45 species in the mouth of the Amazon River in depths between 48 and 73 m. Since then, several biogeographical studies have highlighted the existence of a biogeographical connection between Brazil and the Caribbean (Rocha, 2003; Floeter et al., 2008).

Several other interesting new observations of reef-associated organisms were made here. Aggregations of threatened and commercially important fishes (up to tens of individuals per dive), particularly large individuals (>50 cm Total Length, as measured by a laser scale) of *Lutjanus purpureus* and *Hyporthodus niveatus* (**Figure 1G**) were clearly associated with fractures and crevices on carbonate platforms and crevices created by complex bottoms of laterite rock. Most fish aggregations were associated with cleaning stations, with juveniles of Spotfin hogfish *Bodianus pulchelius* and the Peppermint shrimp *Lysmata grabhami* acting as cleaners (**Figure 1E**). Beside cleaning stations, nests of the Sand tilefish *Malacanthus plumieri*, which are formed by aggregations of rhodoliths, were also inhabited by several species of fish and invertebrates (**Figure 1F**). Two herbivorous fish were recorded foraging in depths between 100 and 140 m, the Agassiz's parrotfish *Sparisoma frondosum* and the Doctorfish *Acanthurus chirurgus*. Large barrens of sea urchins (unidentified Toxopneustidae) actively grazing macroalgae and leaving large paths of cleaned substrate, with thousands of meters in linear extension, were also recorded (**Figure 1H**).

Light that reaches the sea bottom in the GARS is dependent on the sediment laden Amazon plume and clear tropical waters of the North Brazil Current (NBC). Our estimates for the diffuse light attenuation coefficient for photosynthetically available radiation (Kd_{PAR}) for our sampling period (January-February 2017), based on MODIS aqua satellite images (Lee et al., 2002, 2005) ranged from 0.060 to 0.15 m^{-1} at diving/drop camera positions, with 0.01–19.3 $\mu E.m^{-2}.s^{-1}$ arriving at depths varying from 50 to 160 m. Healthy rhodoliths may be found in light environment varying from 0.0015 to 32 $\mu E.m^{-2}.s^{-1}$ (Littler et al., 1986; Riul et al., 2008; Figueiredo et al., 2012), indicating that even though turbidity is relatively high in the GARS, light is not a limiting factor for its existence. The underwater images obtained here show that living calcareous algae are prevalent in depths of up to 180 m (**Figures 1C,E,F,H**).

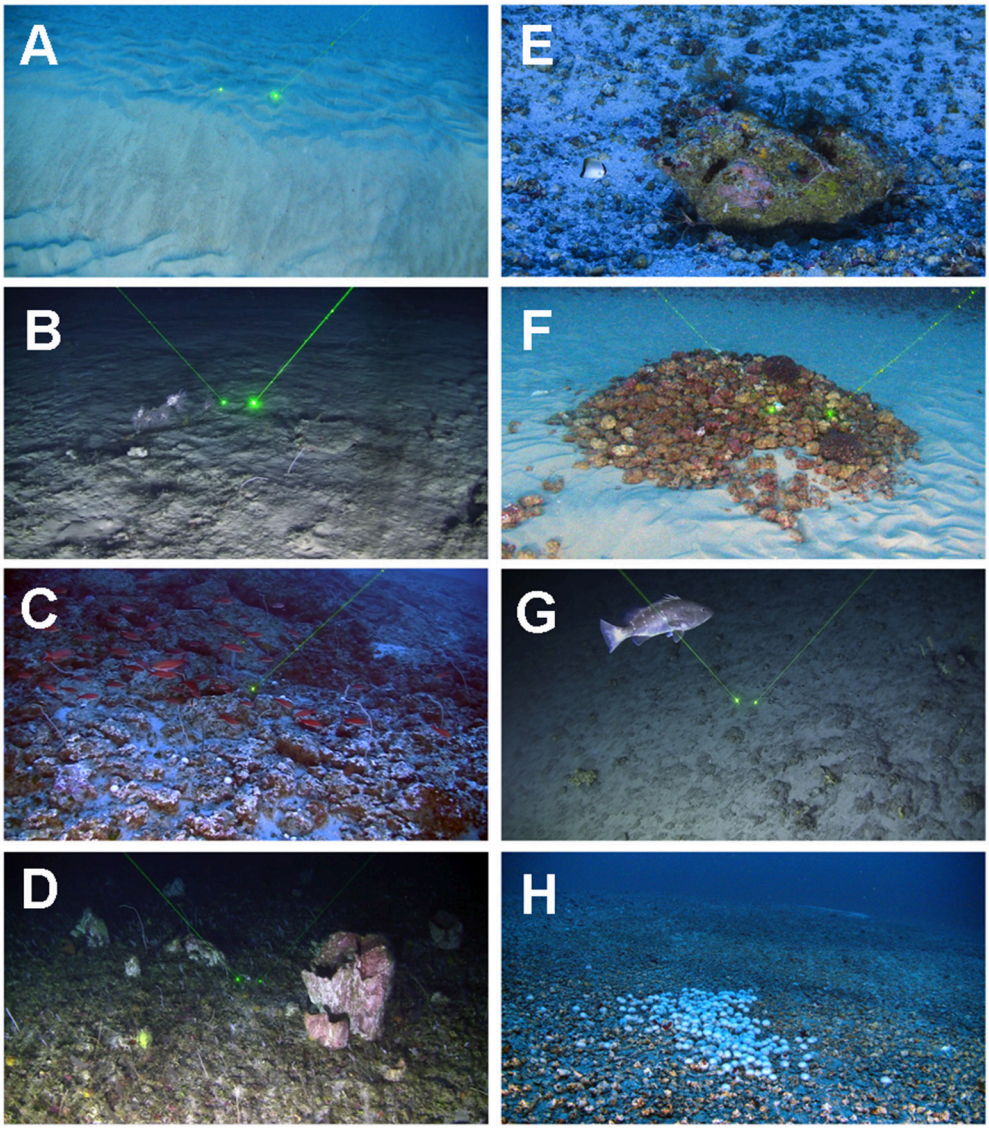

FIGURE 1 | Geodiversity and biodiversity of the GARS. Major structures along the inner and outer shelves. **(A)** Sand dunes in the shallowest portion of the reef (60–70 m), **(B)** Reef covered by sediments between 70 and 80 m depth, **(C)** Diverse reef community with schools of *Paranthias furcifer* and bottom dominated by live crustose calcareous algae and black corals at 130 m depth, **(D)** Deepest portion of the GARS (220 m) with nearly 100% of live benthic coverage (mostly sponges, octocorals and black corals), **(E)** A cleaning station of the Peppermint shrimp *Lysmata grabhami* at 110 m depth, **(F)** Rhodolith mound built by the Sand tilefish *Malacanthus plumieri* at 130 m depth, **(G)** A large individual (>60 cm Total Length) of the commercially important and threatened snowy grouper *Hyporthodus niveatus* at 190 m depth and **(H)** an urchin barren at 130 m depth. Laser scale: 20 cm.

Carbonate budget studies have demonstrated that accretion and erosion are highly variable in space and time, with many shallow reefs with small or zero net reef growth. In addition, large reef systems may show net accretion or erosion in different areas and/or periods depending on different biotic (e.g., bioerosion rates) and abiotic factors (e.g., wave intensity) (Grigg, 1998; Glynn and Manzello, 2015). Thus, additional studies are needed to understand carbonate budget dynamics within the GARS.

Despite our limited knowledge of the GARS (by our new approximation, less than 5% of the reef area has been surveyed so far), the region is coveted by large oil and gas companies (e.g., BHP-Billiton, Queiroz Galvão, Ecopetrol, Total, BP, and Petrobras) (see blocks in **Figure 2**). Oil exploration within the GARS poses serious threats to the biodiversity and sustainability of the region and the minimal data attained so far indicate that precaution is needed before starting any activity with great potential for reef degradation. For example, an oil spill in the Gulf of Mexico caused by the explosion of the BP-operated Deepwater Horizon (DWH) offshore oil rig led to a large-scale environmental catastrophe, largely impacting reefs and rhodolith beds (Goodbody-Gringley

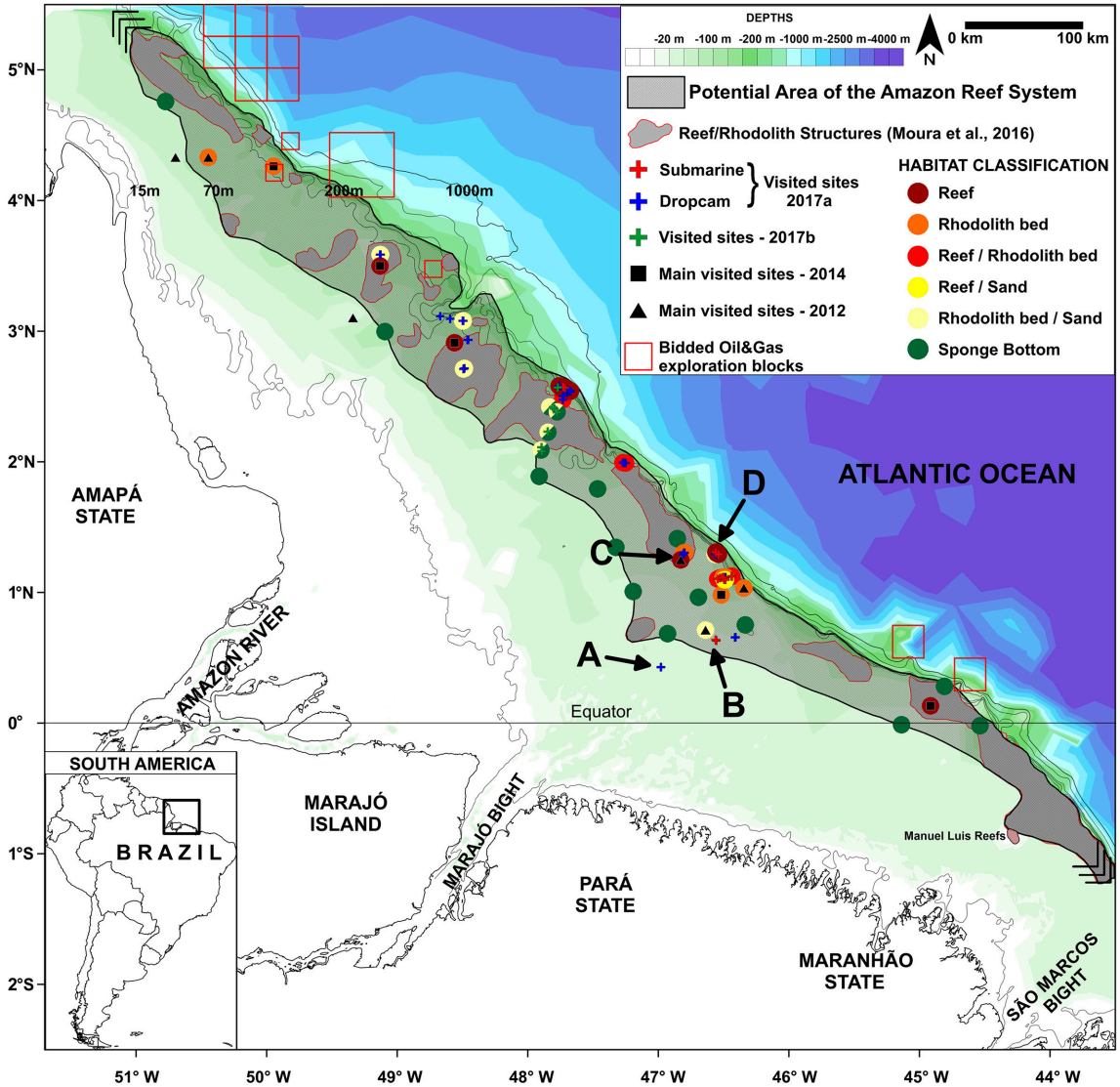

FIGURE 2 | Map of the Great Amazon Reef System (GARS) showing sampling sites. The gray area denotes the potential area covered by mesophotic reefs (56,000 km^2). Letters A-D correspond to sites in which images depicted in **Figure 1** (showing typical features across a 70–220 m depth gradient) are given.

et al., 2013; Krayesky-Self et al., 2017). The use of oil dispersant to prevent floating oil from reaching the shore caused even more damage to reef communities in the Gulf of Mexico, as the widely used dispersant (Corexit®) causes oil to sink, suffocating benthic communities, as well as decreased coral larvae settlement and survival (Goodbody-Gringley et al., 2013). Previous time-series studies performed in the GARS demonstrate current velocities below sea surface of ~0.3–1.5 m.s^{-1} close to the areas of oil exploration, indicating rapid spread may occur in the event of an oil spill (Geyer et al., 1991; Fontes et al., 2008).

Such as mesophotic reefs elsewhere, the GARS can be considered a biodiversity refuge in periods of climate changes that are extirpating shallow reefs (reseeding or deep reef refuge hypothesis; Bongaerts et al., 2010, 2017). Data from the present survey suggest that the reef size, contiguousness, and the biodiversity associated with the GARS could be far greater than realized. Our perspective is that broad baseline studies (i.e., geophysical, geological, physical, chemical, and biological oceanographic surveys) are urgently required for better understanding the GARS and for applying a systematic conservation planning approach for the creation of a network of Marine Protected Areas (MPAs). Precautionary conservation measures should be adopted to avoid drilling, mining, and unregulated fisheries in sensitive areas and a comprehensive baseline assessment is needed for future evaluations of impacts in the case of oil spills. In summary, broader studies and the creation of a network of MPAs may help to reconcile extractive activities

(mining, fishing) with effective biodiversity conservation in the GARS.

AUTHOR CONTRIBUTIONS

RF-F, NA, ES, and FT delineated the study. RF-F, NA, ES, JH, KL, AV, and FT collected the data. CO processed and analyzed the MODIS Ocean Color data. RF-F, NA, ES, JH, KL, ND, AV, RB, CR, CO, CT, and FT contributed with resources, analyzed the data and wrote the manuscript.

FUNDING

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) through its IODP/CAPES-Brasil program, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

ACKNOWLEDGMENTS

We thank the Greenpeace Esperanza and Nuytco crew for the help during the cruise and dives. RF-F, NA, ES, CR, CT, and FT are CNPq research fellows. CO was supported by an IODP/CAPES fellowship.

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Conflict of Interest Statement: The handling Editor declared a past co-authorship with one of the authors FT.

The other 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

ORIGINAL RESEARCH
published: 09 May 2018
doi: 10.3389/fmars.2018.00162

Local Biomass Baselines and the Recovery Potential for Hawaiian Coral Reef Fish Communities

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Understanding the influence of multiple ecosystem drivers, both natural and anthropogenic, and how they vary across space is critical to the spatial management of coral reef fisheries. In Hawaii, as elsewhere, there is uncertainty with regards to how areas should be selected for protection, and management efforts prioritized. One strategy is to prioritize efforts based on an area's biomass baseline, or natural capacity to support reef fish populations. Another strategy is to prioritize areas based on their recovery potential, or in other words, the potential increase in fish biomass from present-day state, should management be effective at restoring assemblages to something more like their baseline state. We used data from 717 fisheries-independent reef fish monitoring surveys from 2012 to 2015 around the main Hawaiian Islands as well as site-level data on benthic habitat, oceanographic conditions, and human population density, to develop a hierarchical, linear Bayesian model that explains spatial variation in: (1) herbivorous and (2) total reef fish biomass. We found that while human population density negatively affected fish assemblages at all surveyed areas, there was considerable variation in the natural capacity of different areas to support reef fish biomass. For example, some areas were predicted to have the capacity to support ten times as much herbivorous fish biomass as other areas. Overall, the model found human population density to have negatively impacted fish biomass throughout Hawaii, however the magnitude and uncertainty of these impacts varied locally. Results provide part of the basis for marine spatial planning and/or MPA-network design within Hawaii.

Keywords: coral reef fishery, population assessment, pristine biomass, hierarchical model, human impacts

OPEN ACCESS

Edited by:
Dominic A. Andradi-Brown, World Wildlife Fund, United States

Reviewed by:
Juan Pablo Quimbayo, Universidade Federal Fluminense, Brazil
Thiago Mendes, Universidade Federal do Rio de Janeiro, Brazil
Craig W. Osenberg, University of Georgia, United States

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *10 January 2018*
Accepted: *23 April 2018*
Published: *09 May 2018*

Citation:
Gorospe KD, Donahue MJ, Heenan A, Gove JM, Williams ID and Brainard RE (2018) Local Biomass Baselines and the Recovery Potential for Hawaiian Coral Reef Fish Communities. Front. Mar. Sci. 5:162. doi: 10.3389/fmars.2018.00162

INTRODUCTION

The fragility of coral reefs combined with the pervasiveness of human impacts threatens the long-term future of these ecosystems (Mora et al., 2016; Hughes et al., 2017). The continuing degradation of coral reefs in the Anthropocene era has hastened calls for scientists to provide information that enables environmental decision-making and effective prioritization of management efforts (McNie, 2007; Cvitanovic et al., 2015). One management strategy that could simultaneously address local stressors to coral reefs and increase their resilience to global climate

threats is marine spatial planning (MSP) (Pandolfi et al., 2011)—the systematic organization and zoning of human use of the marine environment into designated areas (Gilliland and Laffoley, 2008). Scientists can assist MSP efforts by providing spatially-explicit, locally-relevant benchmarks essential to the process (Day, 2008). This requires an understanding of how habitat and oceanographic conditions influence coral reef ecosystem state, as well as how those states have been influenced by human impacts (Crowder and Norse, 2008).

Multiple biotic (e.g., coral and algal cover) and abiotic (e.g., substrate complexity) factors contribute to the considerable natural variability among coral reef ecosystems. When considering the fish assemblages of these systems, habitat characteristics such as coral cover and substrate complexity greatly influence potential species richness and diversity (Chabanet et al., 1997). At larger scales, coral reef fish communities are also influenced by oceanographic factors such as oceanic productivity, temperature, and wave energy (Friedlander et al., 2003; Heenan and Williams, 2013; Williams et al., 2015). Furthermore, characteristics that relate to fishing pressure, such as distance to human population centers, have been shown to influence fish biomass at multiple scales (Brewer et al., 2009). Most coral reefs are subject to human impacts, but these impacts operate on top of background variation in environmental conditions (Williams et al., 2015). Given the range of ecosystem status and trends, there are a variety of options for managers to consider in addressing potential and ongoing stressors. By integrating multiple management objectives and benchmarks, MSP has the potential to effectively account for both the natural and anthropogenic heterogeneity that exists across different stretches of coasts and seascapes (Crowder and Norse, 2008).

Baselines, such as pristine reef fish biomass, can be one such benchmark for guiding MSP efforts. Estimates of baseline reef fish biomass (Nadon et al., 2012; MacNeil et al., 2015; Williams et al., 2015) provide a means for quantifying the extent and spatial variation of depletion (i.e., difference between baseline and present-day state). Areas that have a high baseline biomass (i.e., have a high natural capacity to support fish biomass) and whose present-day levels of fish biomass already closely matches their baseline could be highly valued, and thus prioritized for conservation purposes. On the other hand, were conservation planners and managers more concerned with restoring areas in most urgent need of attention, it would be useful to identify those areas that have experienced the most amount of depletion (i.e., have the greatest potential for recovery). Ultimately management objectives and the decision to protect the strong or the weak (Game et al., 2008) is a societal choice but here we present both baseline biomass and recovery potential (i.e., the difference between present-day and baseline biomass), as a useful framing to guide such decisions.

Knowing the baseline state of an ecosystem with certainty requires a time series of data, dating from prior to the onset of degradation. However, sufficiently long-term trends are exceedingly rare for coral reef ecosystems. Alternatively, this can be done spatio-temporally (e.g., using a chronosequence) whereby time since protection for different areas can be used to generate expectations about recovery (McClanahan et al., 2007, 2016; MacNeil et al., 2015). Finally, in the absence of such a chronosequence, baseline fish biomass can be estimated spatially, for example, by comparing (Friedlander and DeMartini, 2002) or modeling (Williams et al., 2015; D'agata et al., 2016) reefs along a gradient of human-induced impact. Here, we apply this spatial approach to estimating both baseline biomass and the recovery potential of coral reef fish assemblages around the main Hawaiian Islands.

This study is timely because, following unprecedented levels of coral bleaching and mortality observed throughout Hawaii between 2015 and 2016, Hawaii's Division of Aquatic Resources (DAR) became interested in developing management strategies to promote recovery of its coral reef communities, as well as resilience to likely future events (University of Hawaii Social Science Research Institute, 2017). Their systematic review of the literature and synthesis of expert opinion highlighted two proposed actions that addressed the management goal of promoting coral recovery (University of Hawaii Social Science Research Institute, 2017): (i) the establishment of a network of permanent no-take marine protected areas (MPAs) and (ii) the establishment of a network of herbivore management areas. MPAs are a widely-used conservation tool that function by protecting the diversity, density, and size of targeted species found within the reserve. By preserving ecosystem function, it is believed that MPAs create stability in community assemblages and increase resilience to future disturbance events (Mellin et al., 2016). Herbivorous fishes, in particular, are believed to play a disproportionately large role in ecosystem processes of coral reefs, with different herbivorous functional groups mediating different ecological processes. For example, by keeping algal communities in a cropped and productive state, browsers have been implicated in preventing the establishment of macroalgae, while grazers, scrapers, and excavators may facilitate the settlement, survival and growth of crustose coralline algae and coral (Hatcher and Larkum, 1983; Hay et al., 1983; Steneck, 1988; Bellwood and Choat, 1990; Green and Bellwood, 2009). Overall, by managing coral-algal dynamics, herbivores can enhance coral reef resilience to bleaching events by preventing algal overgrowth (Graham et al., 2015). Given the interest from local managers in the potential for both no-take MPAs (i.e., protection of all reef fishes) and herbivore management areas (i.e., protection of just herbivorous fishes) in coral reef resiliency planning (University of Hawaii Social Science Research Institute, 2017), here we focus on both total reef fish community biomass, as well as the herbivorous fish component of the assemblage.

Specifically, we use a large-scale dataset [NOAA's Pacific Reef Assessment and Monitoring Program (Pacific RAMP, Coral Reef Ecosystem Program: Pacific Islands Fisheries Science Center, 2007)] to characterize coral reef fish assemblages throughout the main Hawaiian Islands. We implement a Bayesian, hierarchical framework to: (i) account for the hierarchical nature of processes affecting coral reefs (MacNeil et al., 2009) as well as the hierarchical design of Pacific RAMP (i.e., sites nested within sectors nested within islands nested within region); (ii) model spatially nested effects such that broad-scale processes are allowed to vary among locations, allowing for prediction at local

scales relevant to management; and (iii) quantify uncertainty in our estimation of both baseline biomass and recovery potential (Ellison, 1996). We do this by first modeling herbivore and total reef fish biomass as response variables to multiple habitat, oceanographic, and human drivers. Then, by setting human population density to the minimum level found in our dataset, (i.e., minimizing the effect of humans on fish biomass), we estimate: (i) baseline biomass and (ii) percent recovery potential or the proportional increase from present-day to baseline biomass across the main Hawaiian Islands, while incorporating the uncertainty associated with the effect of humans.

METHODS

Data Collection

Fish surveys were conducted throughout the main Hawaiian Islands in 2012, 2013, and 2015 (Coral Reef Ecosystem Program: Pacific Islands Fisheries Science Center, 2007) using a stratified random design sampling 25 sub-island sectors (**Figure 1**; Maui-Hana was not analyzed because only one site was available here, and no data was available for Maui-Southeast). Refer to Heenan et al. (2017) for a more in-depth description of our data, including how the survey method used by this monitoring program compares with the more commonly used belt transect method for surveying fish. These 3 years of data were selected to represent a recent snapshot, i.e., what we refer to as "present-day" biomass in our analysis. Sector divisions were based on broad-scale categorizations (i.e., presumed fishing pressure, including shoreline accessibility, and coarse habitat type), and are currently being used as part of the survey design for NOAA's Pacific RAMP.

Each survey consisted of a pair of divers, simultaneously collecting data for adjacent survey areas (7.5 m radius cylinders) (Ayotte et al., 2011). Diver comparisons are published annually in our monitoring reports as quality control measures that assess whether any large diver-associated bias exists with regards to either the total biomass and/or species diversity being recorded; none were found in the datasets analyzed here (Heenan et al., 2012; McCoy et al., 2015). Site-level total and herbivorous reef fish biomasses (g m^{-2}) were calculated by using species-specific length-weight conversion parameters (Froese, and Pauly, 2016) and by averaging the two diver replicates. For our list of herbivore reef fish species, we follow the trophic classifications of Sandin and Williams (2010) (Supplementary Table S1). Finally, roving predators (e.g., sharks, large jacks, rays, barracudas, tunas) were excluded from all biomass calculations, because they are not well sampled by small-scale survey methods and because there is potential for bias due to behavioral differences of those species in relation to divers (i.e., diver-attracted and diver-avoiding behaviors) at different levels of fishing pressure and human presence (Gray et al., 2016). Other targeted species that may exhibit these behaviors are still included in our analysis which would tend to exaggerate differences between heavily-fished and remote locations. For Hawaii, bias from fish behavior appears to be limited to locations with the heaviest fishing pressure (i.e., Oahu). While this effect should certainly be controlled for

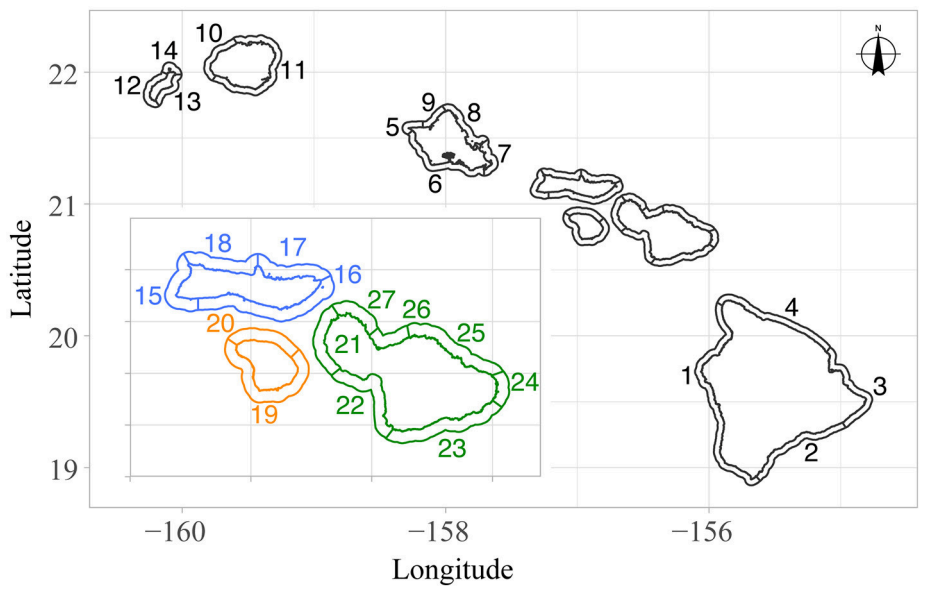

FIGURE 1 | Sub-island sectors of the main Hawaiian Islands, used in the NOAA Pacific Reef Assessment and Monitoring Program's survey design, as well as in our hierarchical analysis. Sector names, as they appear in the text, include: 1, Hawaii-Kona; 2, Hawaii-Southeast; 3, Hawaii-Puna; 4, Hawaii-Hamakua; 5, Oahu-Kaena; 6, Oahu-South; 7, Oahu-East; 8, Oahu-Northeast; 9, Oahu-Northwest; 10, Kauai-Na Pali; 11, Kauai-East; 12, Niihau-West; 13, Niihau-East; 14, Niihau-Lehua; 15, Molokai-West; 16, Molokai-South; 17, Molokai-Pali; 18, Molokai-Northwest; 19, Lanai-South; 20, Lanai-North; 21, Maui-Lahaina; 22, Maui-Kihei; 23, Maui-Southeast; 24, Maui-Hana; 25, Maui-Northeast; 26, Maui-Kahului; 27, Maui-Northwest. Note that sector widths are not to scale (i.e., the sampling domain for fish surveys only extends from the shoreline to 30 m depth).

in certain contexts (e.g., smaller scale, site-level comparisons), observed biomasses at broader scales such as the scale of this study are still large enough to allow for relative comparisons.

Based on the numerous studies that have utilized the Pacific RAMP reef fish dataset, we expect *a priori* the following broad categories to be important for our model: benthic cover (Williams et al., 2015; Heenan et al., 2016), physical characteristics of the habitat (Williams et al., 2015; Cinner et al., 2016; Heenan et al., 2016; Robinson et al., 2017), oceanographic environment (Williams et al., 2015; Cinner et al., 2016; Heenan et al., 2016; Robinson et al., 2017), and human population density (Williams et al., 2015; Heenan et al., 2016; Robinson et al., 2017). However, the relative strength of these different variables for different locations at a sub-island scale—crucial information for local management decisions—remained unclear. A list of all candidate variables can be found in **Table 1**.

To estimate benthic cover, a photo quadrat transect ($n = 30$ photos taken through the middle of the survey area) was taken at each fish survey site. Photos were then processed using point count software ($n = 10$ points per photo), either CPCe (2012–2014 data) or CoralNet (2015 data). At each site, divers also recorded *in situ* physical characteristics including depth, water clarity, and substrate complexity. Here, we consider underwater water clarity to be an environmental driver rather than a proxy for detectability—as surveys were not conducted when visibility was low. Divers assessed substrate complexity by estimating the proportion of the survey area that fell into five substrate height categories: 0–25; 25–50; 50–100; 100–150 cm; and >150 cm, later summarized as a weighted mean of each bin's midpoint. Other metrics of substrate complexity included the maximum substrate height and the standard deviation of the difference between each substrate bin and the overall weighted mean (i.e., a measure of substrate height variability).

Biophysical oceanographic variables were derived from remotely-sensed data to provide site-level estimates related to sea surface temperature, chlorophyll-*a* concentration, photosynthetically active radiation (i.e., irradiance), and wave power. For ocean color metrics (i.e., chlorophyll concentration and photosynthetically active radiation), a "quality control mask" (Gove et al., 2013; Wedding et al., 2017) is applied that removes data pixels known to be optically erroneous due to issues associated with shallow water bottom reflectance. Wave power, which incorporates both wave period and wave height and therefore represents a more realistic estimate of wave-induced stress on coral reefs, was obtained using University of Hawaii's high-resolution SWAN (Simulating WAves Nearshore) wave model (Li et al., 2016). All metrics were based on 2003–2014 time series data (other than wave energy, which was based on data through 2013), summarized by various standard temporal statistics (**Table 1**), and joined to our fish dataset at the site-level based on averaging the three nearest pixels to each fish survey site. In other words, all oceanographic metrics are summary statistics, for which temporal variation has been compressed.

We use human density as a coarse proxy for human impacts on the fish community including coral reef fishing catch and effort, as well as other human related stressors, such as the indirect effects of land-based sources of pollution. Site-level human-related impact was characterized as the number of people (United States Census Bureau, 2010) within a certain distance of each fish survey site. Two spatial scales were explored for this purpose: number of people within 20 km and within 200 km.

TABLE 1 | List of all candidate drivers for modeling total and herbivorous reef fish in the main Hawaiian Islands.

Driver categories	Full list of candidate drivers
Benthic habitat cover	% cover of: Hard Coral Macroalga Turf Alga Crustose Coralline Alga Sand
Physical habitat characteristics	Diver-collected site characterization including: Depth (m) Visually-estimated water-column clarity or visibility (m) Three separate measure of substrate complexity including: (1) Mean substrate height (2) Substrate height variability (3) Maximum substrate height
Oceanographic characteristics	Satellite-derived measures of: Sea surface temperature (SST; C) – 5 km resolution Chlorophyll-a concentration (mg m^{-3}) – 4 km resolution Photosynthetically active radiation (PAR; Einstein m^{-2} d^{-1}) – 4 km resolution Wave energy (kW m^{-1}) – 500 m resolution Summarized by their: Mean Standard deviation Maximum monthly climatological mean Mean of maximum annual anomalies Mean frequency of annual anomalies (presented as a fraction of a year)
Human density	Number of humans within 20 km Number of humans within 200 km

Model Construction

All site-level data and metadata needed for this analysis can be found in Supplementary Text S1A,B. A total of $N = 717$ fish surveys were used for this analysis (**Figure 2**; four sites had no herbivores and were not included for the herbivore analysis, as all data were log-transformed; Supplementary Figure S1A). We removed all sites that fell in areas where fishing was restricted or prohibited (Friedlander et al., 2014). We log-transformed our positive fish biomass densities to obtain normally distributed residual errors, and thus model the response as a normal distribution. All logs mentioned herein refer to the natural log. Furthermore, site-level maps of all covariate data were produced in order to verify the appropriate scale(s) at which they should enter the analysis. All (Supplementary Figures S1B–K) exhibited intra-sector variation (e.g., **Figure 3** coral cover), indicating that they could potentially be informative at this scale and thus, should enter the analysis at the site-level.

All covariates were first checked for correlation (Pearson's $r > 0.5$). Correlations were found within each suite of

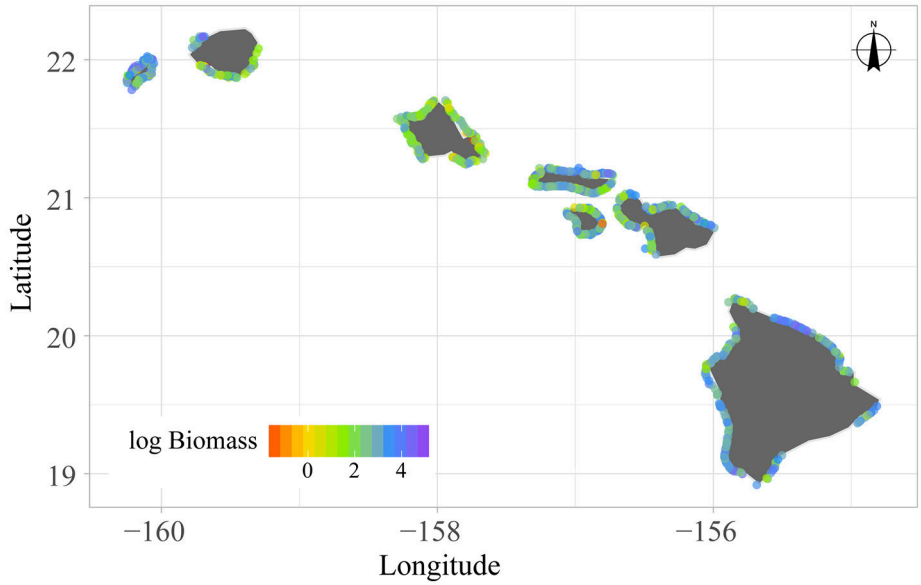

FIGURE 2 | Observed log biomass of total reef fish at 717 surveys conducted around the main Hawaiian Islands between 2012 and 2015.

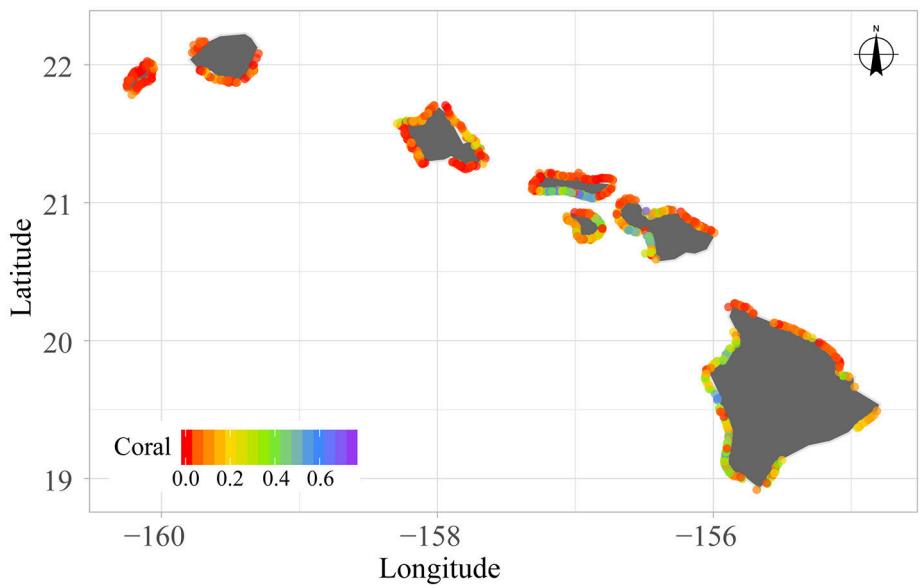

FIGURE 3 | *In situ* percent coral cover for 717 reef fish surveys conducted around the main Hawaiian Islands between 2012 and 2015.

oceanographic variables (e.g., mean SST was correlated with other temporally averaged metrics of SST, but not with any other oceanographic variable) as well as within the full suite of substrate complexity measures (Supplementary Table S2). While island-scale wave energy and SST have been shown to be strongly correlated at other spatial scales (Heenan et al., 2016), we did not find this pattern at the site-level. Retaining the most straightforward variable from each set of correlated variables resulted in the list of variables in the first column of Supplementary Table S3. In order to account for multi-collinearity, variance inflation factors (VIFs) were calculated for the remaining set while removing variables with the highest VIF in a stepwise manner until all VIFs < 5. We initially retained coral cover and turf algae cover despite their high negative correlation and allow the VIF calculation to decide which should be dropped first (Supplementary Table S3). The result was a set of 17 non-collinear variables retained for further consideration (last column of Supplementary Table S3 and first column of **Table 2**).

To address the non-linear relationship between human population density and reef fish biomass and following Nadon et al. (2012) and Williams et al. (2015), we used log(no. of humans) for our human population density variables. Furthermore, we include a squared term for coral cover and wave energy (i.e., coral + coral^2 and wave + wave^2) to capture the known non-linear relationship between those drivers and reef fish biomass (Friedlander et al., 2003; Williams et al., 2015; Heenan et al., 2016).

We model fish biomass, yi, at the site-level, using a hierarchical linear model. This allows us to account for the multi-scale, nested structure of our data observations as well as our model parameters. Specifically, our model structure has site i nested in sector j, nested in island k, nested in region, such that

$$\log(yi) \sim \text{normal}(\text{XiB}_{j[i]}, \sigma_y^2) \quad (1)$$

for i = 1,...,N sites, where X is the n x P matrix of P predictors including the intercept (i.e., the first column is a column of 1's), and Bj is a vector of regression coefficients, such that $\text{XiB}_{j[i]}$ is the linear regression model for site i in sector j.

We then nest $B_{j[i]}$ within the island-level such that

$$B_{j[i]} \sim \text{multivariate normal}(\text{MB}_{k[j]}, \Sigma B_{k[j]}) \quad (2)$$

for j = 1,...,J sectors, and k = 1,...,K islands, where B is the J x P matrix of regression coefficients and $B_{j[i]}$ is a vector of length P of regression coefficients for sector j; $\text{MB}_{k[j]}$ is a vector of length P corresponding to the means of the distribution of the intercept and slope of all sectors in island k; and $\Sigma B_{k[j]}$ is the P x P matrix of the covariances between the intercept and slopes for island k.

We then nest $\text{MB}_{k[j]}$ within the regional-level (i.e., the main Hawaiian Is- lands) such that

$$\text{MB}_{k[j]} \sim \text{multivariate normal}(\text{MB}, \Sigma B) \quad (3)$$

where ΣB is the P x P matrix of the regional-level (i.e., overall) covariances between the intercept and slopes.

Following Gelman and Hill (2007) and Barnard et al. (2000), we then model the covariance matrices using a scaled inverse-Wishart distribution, the over-all effect of which, is to set a uniform distribution between −1 and +1 on the individual correlation parameters of the covariance matrix (See Supplementary Text S2). Finally, because our regression coefficients are nested, we only have to give a prior to the regional level such that each regression coefficient in the vector MB is given a normal distribution with a mean of 0 and a variance of 10. All scaling parameters (see Supplementary Text S2) are given a uniform prior distribution between 0 and 1.

Model Fitting and Analysis

We first ran our model with the full list of non-collinear variables (first column of **Table 2**) using Markov Chain Monte Carlo (MCMC) algorithms in JAGS (Just Another Gibbs Sampler; Plummer, 2003) called from R (R Core Team, 2016) using the R package, rjags (Plummer, 2011). We ran three parallel chains of length 500,000 with a burn-in period of 400,000 and 1/10 thinning leaving a total of 10,000 samples from the MCMC history to be used in calculating Bayesian credible intervals for all parameters. We then followed Gelman and Hill (2007) as our framework in deciding which drivers to include in the final model. Briefly, we removed those drivers that did not have a significant effect on fish biomass (i.e., its 95% confidence intervals overlapped with zero for at least 80% or 20 out of the 25 analyzed sectors) and only retained those (**Table 2**) that had a clear effect.

TABLE 2 | List of all non-collinear variables, with those in bold retained in the final model, and their abbreviations in the manuscript.

Non-collinear variables	Abbreviations of retained variables
Crustose Coralline Alga	CCA
Hard Coral	Coral + Coral × Coral*
Macroalga	
Sand	Sand
Depth	Depth
Visually-estimated water-column clarity	Water clarity
Mean substrate height	Complexity
Chlorophyll-a: mean	
Chlorophyll-a: annual anomaly frequency	
PAR: mean	
PAR: maximum annual anomaly	
SST: mean	SST
SST: SD	
SST: annual anomaly frequency	
Wave energy: mean	Waves + Waves × Waves*
Wave energy: maximum annual anomaly	
Number of humans within 20 km	Human density
Number of humans within 200 km	

** In the final model, squared terms were included for both coral cover and wave energy.*

We then re-ran our final model with the final list of retained drivers (final column of **Table 2**) using the same MCMC specifications above. Convergence was assessed by: (i) inspecting traceplots of all estimated parameters and ensuring that all chains were well-mixed and stable and (ii) calculating Gelman-Rubin statistics (Gelman and Rubin, 1992)—all were close to 1, indicating that variance within and between chains were close to equal. Our JAGS code can be found in Supplementary Text S3. Posterior predictive checks were used to assess model fit (i.e., a step was added in each MCMC iteration to simulate data based on our model's posterior predictive distribution, which we then compare to our observed dataset).

Goodness of fit was evaluated using Bayesian *p*-values, which are based on comparing the discrepancies between observed and simulated data. Bayesian *p*-values for the mean ($p = 0.50$) and standard deviation ($p = 0.61$) were both close to 0.5, indicating that differences between observed and simulated data are likely due to chance. Furthermore, we checked full model residuals as well as individual covariate residuals against predicted values to verify they are normally-distributed, uncorrelated and homoscedastic.

Next, we checked predictive power at both the sector and site-level. At the site-level, the model appeared to show some bias

at the extremes (Supplementary Figures S2A,B), but the model's predictions of sector-level total (**Figure 4**) and herbivorous (Supplementary Figure S3) reef fish biomass (median: black dots; gray rectangles: 95% Bayesian credible intervals) agree with observed levels of fish biomass (red diamond and whiskers). We consider this model performance to be appropriate since we summarize our simulation of biomass baselines at the sector-level. For all other results, we report 66% Bayesian credible intervals to express "likely" outcomes, following the United Nations – Intergovernmental Panel on Climate Change's guidance for expressing uncertainty (Mastrandrea et al., 2010).

To estimate the impact of human population density on both total and herbivorous fish biomass throughout Hawaii, we first used our final model to estimate the effect of human population density—given the variation in fish biomass that is attributable to spatial differences in environmental habitat and oceanographic drivers—at each location. Then, we added a step in each MCMC iteration to simulate fish biomass baselines, by setting human population levels to its minimum value found in the main Hawaiian Islands (in order to keep our predictions within the range of our data). For the 2010 U.S. Census (United States Census Bureau, 2010) on which our human population data is based, the minimum population level within 20 km of a fish survey site was 117 humans, located in the Niihau-Lehua sector (**Figure 1**). Because this is done within the MCMC, these estimates of biomass baselines incorporate the model uncertainty in the effect of human population density on fish biomass. In addition, each MCMC iteration is coded to calculate the percentage increase between present-day and baseline levels of fish biomass—i.e., the percentage change from present-day fish biomass if human impacts were minimized (i.e., set to the minimum level within the current dataset)—and is termed "percent recovery potential" here.

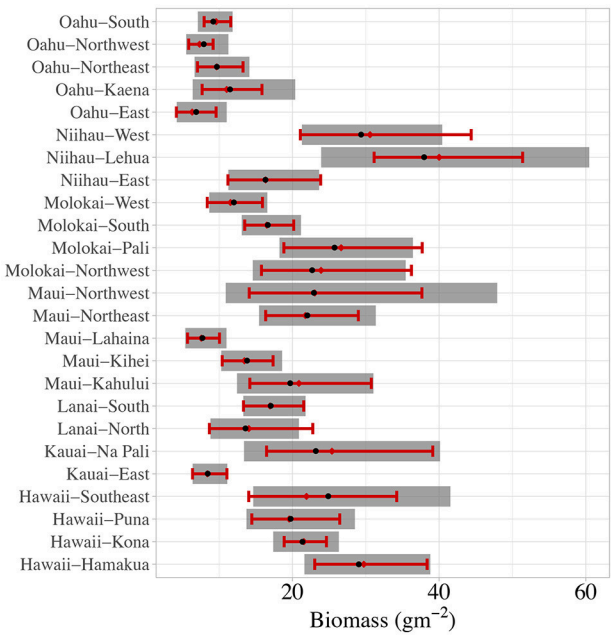

FIGURE 4 | Observed (red whiskers) vs. predicted (gray rectangles) 95% quantiles for sector-level total reef fish biomass.

RESULTS

A total of 11 drivers were found to have a significant effect and thus retained in the final model for both the herbivorous and total reef fish analyses (**Table 2** including abbreviations). Scatterplots of each variable vs. log total reef fish biomass can be found in Supplementary Figures S4A–I (and Supplementary Figures S5A–I for log herbivorous reef fish biomass). For each driver, and for each analysis (herbivorous and total reef fish biomass), the model provided estimates of driver coefficients for multiple levels: sector, island, and region. The regional-scale (i.e., overall) effect of all drivers were largely similar for total and herbivorous reef fish (**Figure 5**; Supplementary Table S4). The consistency of these results is not surprising given that the correlation between site-level total and herbivorous reef fish biomass was high (Pearson's $r = 0.81$); nevertheless because of interest from the local coral reef management community, we provide results from both analyses. Drivers with a positive effect on fish biomass were: CCA, Complexity, Depth, and Water Clarity. Drivers with a negative effect on fish biomass were: Human Density, Sand, and SST. Coral and Waves exhibited non-linear relationships with fish biomass (positive with Coral and Waves, negative with Coral x Coral and Waves x Waves). Finally, for the remainder of this article, we focus our results and discussion on our analysis of total reef fish biomass. All outputs for our herbivorous reef fish analysis can be found in the Supplementary Materials.

At lower, nested spatial scales (i.e., islands, sectors), spatial variation in driver effects was more apparent (**Figure 6**; Supplementary Figures S6A–K for total reef fish; Supplementary Figures S7A–L for herbivores). For example, the median sector-level effect of human density on total reef fish log biomass ranged from −0.18 in Hawaii-Kona to −0.46 in Niihau-Lehua (**Figure 6**). In contrast to human density, other drivers had relatively consistent effects. For example, the coefficient for wave energy (Supplementary Figure S6J) was relatively consistent across sectors and islands (only ranging between 0.22 and 0.25 despite site-level, mean wave energy ranging between 0.43 and 35.5 kW m^{-1}). The means of all island-level coefficient estimates are shown as light gray bars (See Supplementary Tables S5A,B for means and credible intervals).

Since the effect of human density is negative for all sectors (**Figure 6**), we see the model's median prediction of baseline biomass to always be greater than the median of present-day fish biomass for each sector (**Figure 7**; Supplementary Figure S8 for herbivores). Among sectors, however, there is considerable variation in the difference between the present-day and baseline biomass distributions, such that some sectors are considerably more different from baseline biomass than others. The proportional difference between these distributions is what we call "percent recovery potential" here (i.e., difference between present-day and baseline biomass as a proportion of present-day biomass; **Figure 8**; Supplementary Figure S9 for herbivores).

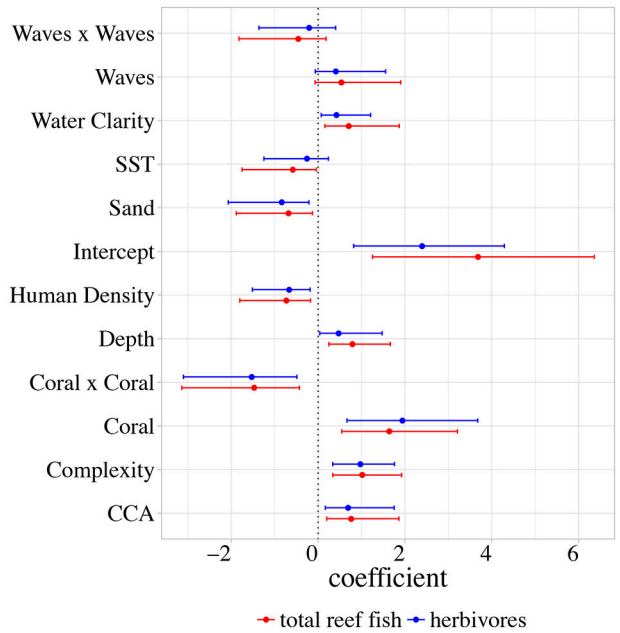

FIGURE 5 | Region-scale (i.e., overall) effect of all drivers on total (red) and herbivorous (blue) reef fish log biomass in our hierarchical model for the main Hawaiian Islands. Mean (circle) and 66% Bayesian Credible Intervals (whiskers) are shown for each driver.

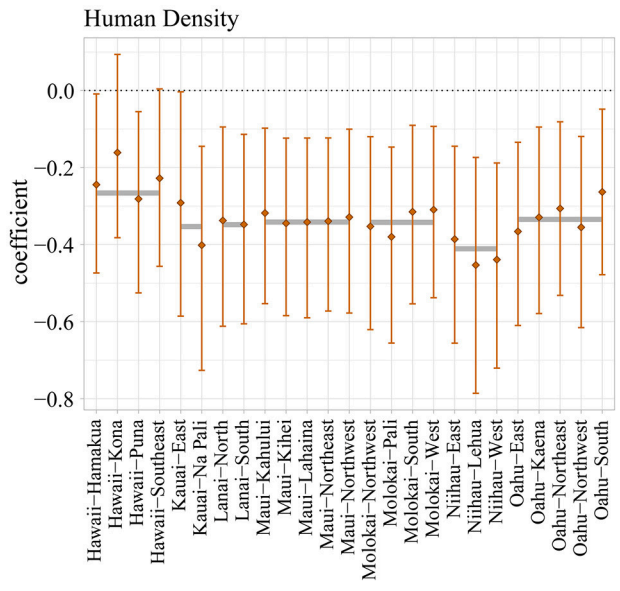

FIGURE 6 | Sector-level effect (vertical whiskers) of human density on total reef fish log biomass in our hierarchical model for the main Hawaiian Islands. The mean (diamond) effect and 66% Bayesian Credible Intervals (whiskers) are shown for each sector. Island-level mean effects are also shown (gray horizontal bars).

Estimated baseline biomass and percent recovery potential are also displayed spatially in **Figures 9**, **10** (and for herbivores in Supplementary Figures S10, S11). For those interested in absolute, rather than proportional change in fish biomass, bar graphs (Supplementary Figure S12) and maps (Supplementary Figure S13) for both total and herbivorous reef fish are also provided.

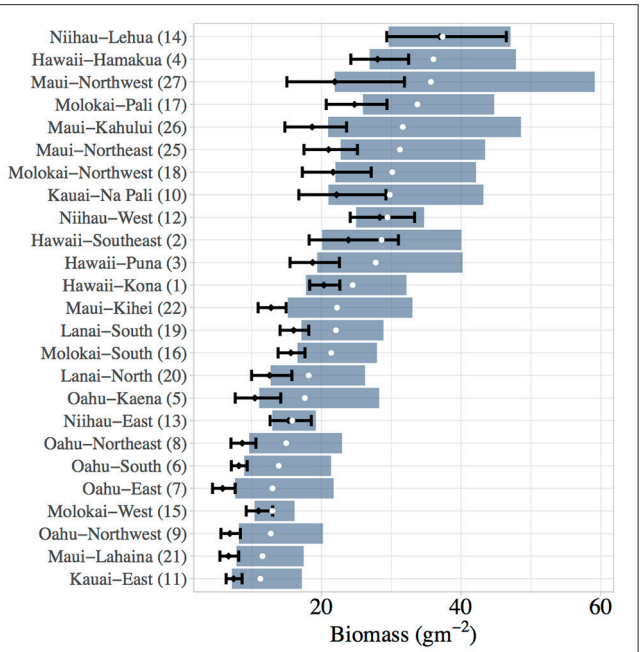

FIGURE 7 | Sector-level model predictions of present-day (black whiskers) vs. baseline (blue rectangles) biomass for total reef fish in the main Hawaiian Islands. Means and 66% Bayesian Credible Intervals are shown. Baseline biomass is calculated by setting human density to its present-day minimum across all fish-survey sites.

DISCUSSION

Overall, our analysis provides two potential lenses with which the heterogeneity of coral reef fishery systems in the main Hawaiian Island can be understood. Our biomass baseline estimates highlight areas that have the greatest capacity to support reef fish biomass, given multiple habitat and oceanographic drivers and after removing the effect of human population density. This approach reveals considerable spatial variability in the natural carrying capacity of reef fish throughout the archipelago. The sector with the greatest baseline biomass (Niihau-Lehua) could support more than three times as much total reef fish biomass as the sector with the lowest baseline biomass (Kauai-East) (**Figures 7**, **9**). And for herbivorous fish (Supplementary Figures S8, S10), this difference was even greater – the sector with the highest ability to support herbivorous reef fish (Maui-Northwest) could support ten times as much herbivorous biomass as the sector with the lowest baseline herbivorous fish biomass (Kauai-East). For total reef fish (**Figure 9**), the north coasts of Niihau, Maui, Molokai, and Hawaii have the greatest biomass baselines across all sectors. On the other hand, the four sectors with

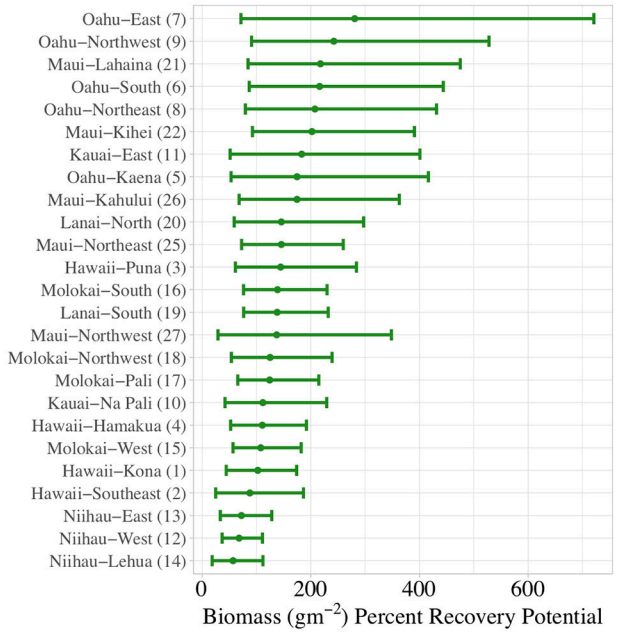

FIGURE 8 | Sector-level percent recovery potential (i.e., difference between model-predicted present-day and baseline biomass as a proportion of present-day biomass) for total reef fish in the main Hawaiian Islands. Means and 66% Bayesian Credible Intervals are shown.

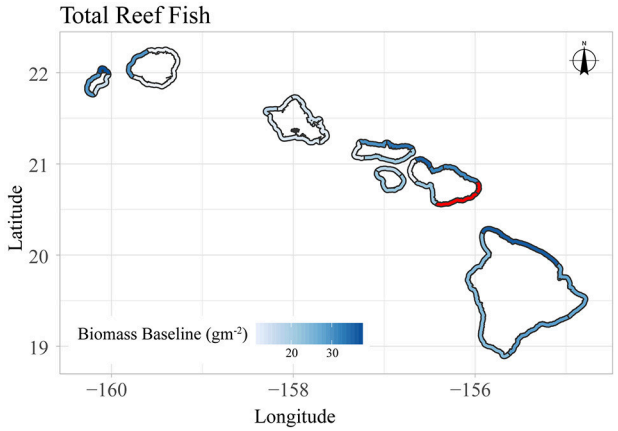

FIGURE 9 | Map of sector-level, mean baseline biomass for total reef fish in the main Hawaiian Islands. Sectors with limited data are shown in red.

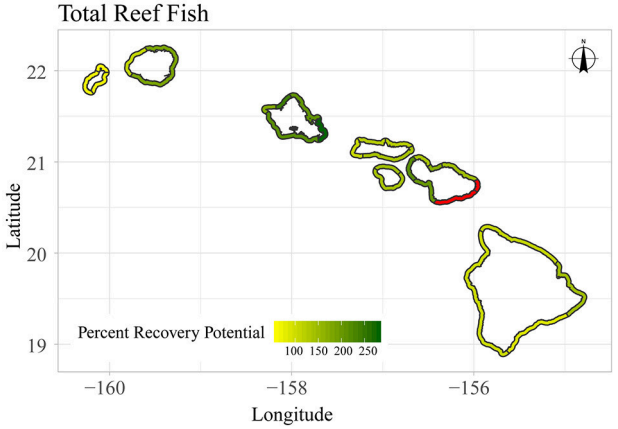

FIGURE 10 | Map of sector-level, mean percent recovery potential for total reef fish biomass in the main Hawaiian Islands. Sectors with limited data are shown in red.

the greatest herbivorous fish biomass baselines are found on Maui, with the north coasts of Molokai, Niihau, and Kauai also having appreciable capacities to support herbivorous fish (Supplementary Figure S10).

An alternate perspective with which the variation in reef fish assemblages can be assessed is to consider which areas have the greatest capacity for recovery. Here, we defined recovery potential as the proportional increase in fish biomass after minimizing the effects of human density (i.e., percent recovery potential). While our simulation of minimizing human population density shows an increase from present-day to baseline biomass across all sectors, some sectors appear to be more sensitive to this reduction than others. For example, total reef fish biomass (**Figures 8**, **10**) in Oahu-East was predicted to be able to experience a 280% increase from present-day levels if human impacts could be minimized. On the other hand, Niihau-Lehua was only predicted to have a 57% recovery potential. For herbivorous reef fish, the percent recovery potential was even greater (Supplementary Figures S9, S11), ranging from a minimum of 287% (Niihau-Lehua) to a maximum of 1764% (Oahu-Northwest). Overall, areas with the highest percent recovery potential for total reef fish are located throughout all of Oahu, as well as in the Maui-Lahaina and Maui-Kihei sectors (**Figure 10**). For herbivores (Supplementary Figure S11), the northern coast of Oahu as well as all of Maui island, especially the Maui-Kihei sector, had the greatest percent recovery potential.

The two perspectives we provide here, baseline biomass and recovery potential, however, do not have to be mutually exclusive criteria for designing an overall management plan for the main Hawaiian Islands. Through its creation of multi-use ocean zoning plans and the delineation of different marine zones for different uses, MSP has the ability to implement multiple management objectives across time and space (Crowder and Norse, 2008; Day, 2008). Environmental management objectives can be broadly divided into conservation (e.g., preservation of areas that are near-pristine) and restoration (e.g., revival of areas with high recovery potential) activities (Hobbs et al., 2009). Specifically, sectors such as Niihau-Lehua, could be highly valued (e.g., for tourism purposes or as source of spillover into adjacent areas) due to the fact that they have a high baseline biomass and because their present-day biomass already closely matches their baseline. Areas like this could be prioritized for conservation management strategies aimed at preventing human impacts that cause biotic and abiotic changes to the system. On the other hand, sectors with high recovery potential (e.g., Oahu-East, Northwest, South, and Northeast as well as Maui-Lahaina and Kihei) could be prioritized for restoration purposes.

In contrast to conservation-focused management activities, areas with high recovery potential would benefit instead from restoration management actions designed to reverse biotic and abiotic changes and promote recovery toward a previous state (Hobbs et al., 2009).

In the absence of a reliable time series that predates coral reef degradation, we estimated baseline biomass through the use of spatial gradients, along a spectrum of most to least impacted reef areas in the region. Such an approach is not without caveats. The ability for an ecosystem to rebound from the present-day state to a baseline, requires that several other assumptions be met. For example, if present-day environmental conditions are not able to fully account for the observed current state of an ecosystem due its historical trajectory (i.e., hysteresis) or if other processes such as larval recruitment patterns and successional dynamics are not fully understood, then the recovery pathway may not simply be the reverse of the decline pathway (Diaz and Rosenberg, 2008). Furthermore, if the ecosystem has been tipped past a threshold into an alternative stable state threshold, recovery to its original state may not even be possible (Hughes et al., 2017). In light of these caveats, our estimate of recovery potential should be considered as a robust estimate of current levels of depletion from baselines, but *actual* recovery trajectory remains uncertain. Although we could not address these issues related to ecosystem recovery, we did address at least one critical challenge by providing estimates at a scale that is relevant to local managers.

Our ability to bring previous island-level analyses (Williams et al., 2015) to the sub-island (sector) scale stems from our use of a hierarchical analytical framework, which considers the effects of drivers as being spatially-nested and operating on multiple scales. At the regional-scale, the strongest drivers of fish biomass in the main Hawaiian Islands were from coral cover and complexity (**Figure 5**). Coral had a negative non-linear relationship with fish biomass (positive for Coral and negative for Coral x Coral); other studies (Williams et al., 2015; Heenan et al., 2016) have indicated that intermediate levels of coral cover tend to have the highest levels of fish biomass. One possible explanation of this nonlinear effect of coral cover is that increasing coral cover and associated substrate complexity provide refugia for reef fish against predation (Beukers and Jones, 1998; Almany, 2004), but as coral cover increases to become the dominant benthic organism, this may eventually lead to the exclusion of other benthic organisms (e.g., turf, endolithic algae) that are important food sources for certain functional guilds (Wismer et al., 2009). Although increasing coral cover can help to build and maintain high complexity reef habitats, the two variables were not correlated at the site-level for our dataset and are likely mediating different dynamics for different groups of reef fish (e.g., changes in coral cover vs. complexity will likely have different effects on corallivores vs. other groups) (Emslie et al., 2014). Wave power produced a similar non-linear effect (positive for Waves and negative for Waves x Waves). This has also been demonstrated previously on this scale (Friedlander et al., 2003; Rodgers et al., 2010), and one potential mechanism for this may have to do with the availability of algae and accumulation of detritus in areas of intermediate wave forcing (Crossman et al., 2001).

In general, the model coefficients for human density tended be more variable among sectors than those for environmental drivers (**Figure 6** vs. Supplementary Figures S6A–K). In our analysis, we transformed both fish biomass and human density so that their relationship would be linear on the log-log scale (Supplementary Figures S4E, S5E), which means that their coefficients should be interpreted as elasticities, i.e., a human density coefficient of X means that a 10% increase in human density results in a X*10% decrease in fish biomass, regardless of the human density of the sector. In other words, a 10% increase of human density will have a larger effect on fish biomass on Niihau (4.1%) than on Oahu (3.3%; **Figure 6**). However, as Oahu's population is so large, the effect of minimizing human population density there corresponds to a large total effect on fish biomass. Therefore, in contrast to Niihau's sectors, the biomass baselines for Oahu are quite different from their present-day biomass levels (**Figure 7**). Overall, the linear, log-log relationship between fish biomass and human population density that we find in this study, and corresponding interpretation of the human density coefficient as elasticities, is consistent with other studies (Nadon et al., 2012; Heenan et al., 2016).

As is becoming increasingly recognized, the effects of local human populations are highly context- and scale-dependent, and in some cases other, related, metrics such as distance to markets are stronger drivers of coral reef fisheries conditions (Cinner and McClanahan, 2006; Brewer et al., 2009; Cinner et al., 2013). The exact mechanism by which human population density negatively affects standing reef fish biomass was not explicitly tested here, although others have suggested this to be related to a combination of fishing pressure and/or degraded water quality from land development (Mora et al., 2011). Thus, independently of any change in human populations in the main Hawaiian Islands, managing the human footprint as it relates to these ecosystem stressors will be crucial to ensuring the sustainability of coral reef fisheries.

In order to address the diversity of human activities that impact coral reef ecosystems, advocates of MSP suggest a hierarchical management approach, whereby larger (e.g., national) levels of management provide context for nested, lower (e.g., local) levels (Gilliland and Laffoley, 2008). Coral reefs, in turn, are hierarchically-structured ecosystems, lending themselves to hierarchical analyses (MacNeil et al., 2009); yet rarely has this analytical approach been explicitly applied toward guiding coral reef MSP efforts. Our study should highlight the applicability and utility of hierarchical analyses to providing management-relevant input to MSP. Specifically, our analytical framework allowed for the characterization of biophysical and human impact drivers operating at multiple levels, as well as the downscaling of coral reef ecosystem benchmarks to a scale relevant to local managers. This allowed for the identification of areas with the greatest scope for recovery (e.g., heavily impacted areas with high background oceanic productivity and high-quality habitat) and conversely, areas which, because of poor habitat quality and other factors, are not likely to be able to ever support high levels of fish biomass. Furthermore,

by taking a Bayesian approach, our estimates of baseline biomass and recovery potential incorporated the uncertainty of all modeled parameters, including the effect of humans. Uncertainty is a common denominator in resource management and conservation, with scientists asked to quantify it and managers asked to buffer against it. Being transparent about the uncertainty around modeled predictions is critical to effective collaboration between science and management.

MSP has the potential to reconcile the multiple economic, social, and environmental demands placed on coral reef ecosystems (Gilliland and Laffoley, 2008). Controlling for multiple habitat, oceanographic, and human factors in the way we have done makes it possible to reveal the natural heterogeneity of coral reef ecosystems as well as how they have been affected by human impacts. Our estimates of baseline biomass and recovery potential can guide MSP efforts as managers integrate multiple sources of information and begin to delineate management actions across heterogeneous stretches of coasts and seascapes. Ultimately the decision of which management objectives to prioritize is a societal choice, but these decisions should be informed by scientific input. By providing spatially-explicit, locally-relevant benchmarks (Crowder and Norse, 2008; Day, 2008), scientists can guide MSP efforts and enable managers to make informed decisions of how and where to prioritize their efforts.

AUTHOR CONTRIBUTIONS

KG, MD, AH, and IW: conceived the research, shaped the analyses, and interpreted the results; KG: analyzed the data; MD and KG: developed the model; KG: wrote the main manuscript, with MD, AH, and IW providing considerable edits. JG: provided oceanographic data; IW and RB: coordinated and fundraised for research cruises and data collection. All authors provided critical feedback and helped with finalizing the manuscript

FUNDING

Data collection and research were supported by NOAA's Coral Reef Conservation Program (http://coralreef.noaa.gov) and the NOAA Pacific Islands Fisheries Science Center.

ACKNOWLEDGMENTS

We thank the members of the Coral Reef Ecosystem Program's fish team for their tireless efforts in collecting reef fish monitoring data around the main Hawaiian Islands as well as the dedicated officers and crews of the NOAA Ships *Hi'ialakai* and *Oscar Elton Sette* for providing safe and productive platforms for our research teams. We also thank Tomoko Acoba for GIS support with this project, Amanda Dillon for graphics support, and Hal Koike for initial discussions on the framing of this analysis for Hawaii Division of Aquatic Resources. Finally, we thank the NOAA Coral Reef Conservation Program and the Pacific Islands Fisheries Science Center for supporting this research. The contents in this manuscript are solely the opinions of the authors and do not constitute a statement of policy, decision, or position on behalf of NOAA or the U.S. Government.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00162/full#supplementary-material

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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

ORIGINAL RESEARCH
published: 14 May 2018
doi: 10.3389/fmars.2018.00160

Interspecific Hybridization May Provide Novel Opportunities for Coral Reef Restoration

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Climate change and other anthropogenic disturbances have created an era characterized by the inability of most ecosystems to maintain their original, pristine states, the Anthropocene. Investigating new and innovative strategies that may facilitate ecosystem restoration is thus becoming increasingly important, particularly for coral reefs around the globe which are deteriorating at an alarming rate. The Great Barrier Reef (GBR) lost half its coral cover between 1985 and 2012, and experienced back-to-back heat-induced mass bleaching events and high coral mortality in 2016 and 2017. Here we investigate the efficacy of interspecific hybridization as a tool to develop coral stock with enhanced climate resilience. We crossed two *Acropora* species pairs from the GBR and examined several phenotypic traits over 28 weeks of exposure to ambient and elevated temperature and pCO_2. While elevated temperature and pCO_2 conditions negatively affected size and survival of both purebreds and hybrids, higher survival and larger recruit size were observed in some of the hybrid offspring groups under both ambient and elevated conditions. Further, interspecific hybrids had high fertilization rates, normal embryonic development, and similar *Symbiodinium* uptake and photochemical efficiency as purebred offspring. While the fitness of these hybrids in the field and their reproductive and backcrossing potential remain to be investigated, current findings provide proof-of-concept that interspecific hybridization may produce genotypes with enhanced climate resilience, and has the potential to increase the success of coral reef restoration initiatives.

Keywords: hybridization, restoration, coral reefs, climate change, *Acropora*, assisted evolution, genetic rescue, hybrid vigor

OPEN ACCESS

Edited by:
Anastazia T. Banaszak, Universidad Nacional Autónoma de México, Mexico

Reviewed by:
James R. Guest, Newcastle University, United Kingdom
Mathieu Pernice, University of Technology Sydney, Australia

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *19 January 2018*
Accepted: *23 April 2018*
Published: *14 May 2018*

Citation:
Chan WY, Peplow LM, Menéndez P, Hoffmann AA and van Oppen MJH (2018) Interspecific Hybridization May Provide Novel Opportunities for Coral Reef Restoration. Front. Mar. Sci. 5:160. doi: 10.3389/fmars.2018.00160

INTRODUCTION

The rapid increase in atmospheric CO_2 to levels not documented for millions of years (Hönisch et al., 2012) and associated ocean warming and acidification have profoundly transformed the marine realm (Pandolfi et al., 2011). Higher-than-usual seawater temperatures can cause coral bleaching, the breakdown of the symbiotic relationship between the coral host and its dinoflagellate endosymbionts (*Symbiodinium* spp.), and associated coral mortality (Hoegh-Guldberg, 1999). Ocean acidification is reducing carbonate ion availability in seawater and can depress calcification rates of calcifying organisms like corals (Langdon et al., 2000; Doney et al., 2009; Chan and Connolly, 2013). These global changes, coupled with local stressors such as pollution, overfishing, and outbreaks of crown-of-thorns starfish, have drastically

altered coral cover and community composition at a global scale. In the last three decades, multiple mass bleaching events have decimated coral reefs worldwide including in 1998, 2010, and 2014–2017 (Eakin et al., 2016; Heron et al., 2016; Hughes et al., 2017, 2018). The Great Barrier Reef (GBR) is no exception with 50–80% coral mortality recorded on many northern reefs following the 2016 mass bleaching event (Great Barrier Reef Marine Park Authority, 2017), followed by another high mortality mass bleaching event in 2017. Climate models predict a <5% chance of reaching the Paris agreement target of limiting the global temperature rise to <2°C compared to pre-industrial times by 2100 (Raftery et al., 2017), and most coral reefs are forecasted to experience annual severe bleaching before the end of the century (van Hooidonk et al., 2016). Several observations of an increase in tolerance of coral bleaching after successive bleaching events suggest that adaptation and/or acclimatization are possible under certain conditions (Maynard et al., 2008; Berkelmans, 2009; Guest et al., 2012; Penin et al., 2013). Nevertheless, over 50% of the world's coral reefs has been lost in the last three decades, with the Caribbean having lost over 80% of its coral cover (50 Reefs, 2017)[1], indicating that the rates of natural adaptation and acclimatization are overall insufficient to keep pace with the rate of environmental changes (van Oppen et al., 2017).

Active reef restoration is one way to assist the recovery of coral reefs that are degraded, damaged or destroyed. Reef restoration is still in its infancy and all of the few successful efforts so far occurred on a small spatial scale (e.g., Nakamura et al., 2011; Omori, 2011; Villanueva et al., 2012; Guest et al., 2014; dela Cruz and Harrison, 2017). Traditionally, locally sourced biological material is used for restoration based on the assumption that these populations are locally adapted and therefore most likely to survive (Breed et al., 2013). However, anthropogenic disturbances are rapidly changing the environment and shifting selection pressures (Becker et al., 2013), and locally sourced stock is therefore potentially mismatched with the altered environment. An effective restoration strategy should thus incorporate an understanding of present day ecological characteristics of species, characteristics of future available habitats, and adaptive potential of species (Becker et al., 2013). The use of non-local and climate resilient materials is controversial, but is gaining traction in wildland restoration (Jones and Monaco, 2009), revegetation (Sgrò et al., 2011; Breed et al., 2013), and coral reef restoration (Rau et al., 2012; van Oppen et al., 2017).

One possible way to improve the adaptive potential of species is via hybridization, which can increase genetic variation, break genetic correlations that constrain evolvability of parental lineages, and assist species to acquire adaptive traits (Hoffmann and Sgrò, 2011; Becker et al., 2013; Carlson et al., 2014; van Oppen et al., 2015; Hamilton and Miller, 2016; Meier et al., 2017). Hybridization can be conducted either via targeted crossing of individuals or species carrying desired phenotypic traits (e.g., high thermal tolerance) or via crossing between species with the goal of increasing genetic diversity and new variation for natural selection to act upon, and potentially generating hybrid vigor. The relative fitness of F1 hybrids (**Figure 1**) depends on whether there are additive (i.e., hybrids are of intermediate fitness between the parental species), dominant (i.e., hybrids are of equal fitness to the dominant parent species), over-dominant (i.e., hybrids are more fit than both parental species), under-dominant (i.e., hybrids are less fit than both parental species) gene effects, and/or maternal effects (i.e., hybrids are of equal fitness to their maternal parent species) (for review, see Lippman and Zamir, 2007; Li et al., 2008; Chen, 2013). Reciprocal hybrids are predicted to have equal fitness, except under maternal inheritance. With maternal effects, the fitness of the hybrids is directly affected by the fitness of the maternal parental species, regardless of the offspring's own genotype (Roach and Wulff, 1987; Bernardo, 1996). In the context of restoration, hybrid vigor which can be driven by dominant or over-dominant mechanisms, is a desirable outcome. The value of hybridization in enhancing fitness has been demonstrated in multiple cases. For instance, hybridization has provided genetic variance in morphology for adapting to changing environments in Darwin's finches (Grant and Grant, 2010), altered chemical defense of hybrid Brassicaceae plants and aided their survival through the Last Glacial Maximum (Becker et al., 2013), and facilitated extensive adaptive radiation in haplochromine cichlid fishes (Meier et al., 2017).

Hybridization is known to occur naturally in some scleractinian corals and has played an important role in the evolution and diversification of the genus *Acropora* (van Oppen et al., 2001; Willis et al., 2006). In the Caribbean, recent environmental degradation and massive population decline in *Acropora cervicornis* and *Acropora palmata* have favored hybridization and expansion of their F1 hybrid, *Acropora prolifera* (Fogarty, 2012). These hybrids either have equivalent or higher fitness relative to the parent species in most life history stages examined (Fogarty, 2012). In recent years, *A. prolifera* has been reported in increasingly high abundance in many reef locations (Fogarty, 2012; Japaud et al., 2014; Aguilar-Perera and Hernández-Landa, 2017) and the hybrid has expanded to marginal environments where parent species are absent (Fogarty, 2012).

Although interspecific hybridization is a potential tool to enhance restoration outcomes, it is often dismissed in restoration initiatives. Concerns raised include the possibility of outbreeding depression in later generations (i.e., F2, F3, backcross), and the loss of diversity through losing part of the parental species' genome (for review, see Hamilton and Miller, 2016). Most examples of outbreeding depression, however, are associated with the admixture of populations or species that are geographically distant, or when life history or phenological differences are large (Hwang et al., 2012; Whiteley et al., 2015). Outbreeding depression can also be transient and can be overcome by natural selection (Jones and Monaco, 2009; Aitken and Whitlock, 2013; Hamilton and Miller, 2016). Instead of reducing genetic diversity, hybridization may conserve diversity by protecting the parental genome from the risk of extinction, and can also increase genetic diversity by combining two divergent genomes within a single organism (Garnett et al., 2011). For example, hybridization has successfully enhanced genetic diversity, improved the population size and rescued the highly inbred, remnant population of Florida

[1]https://50reefs.org/ (Accessed April 3, 2018).

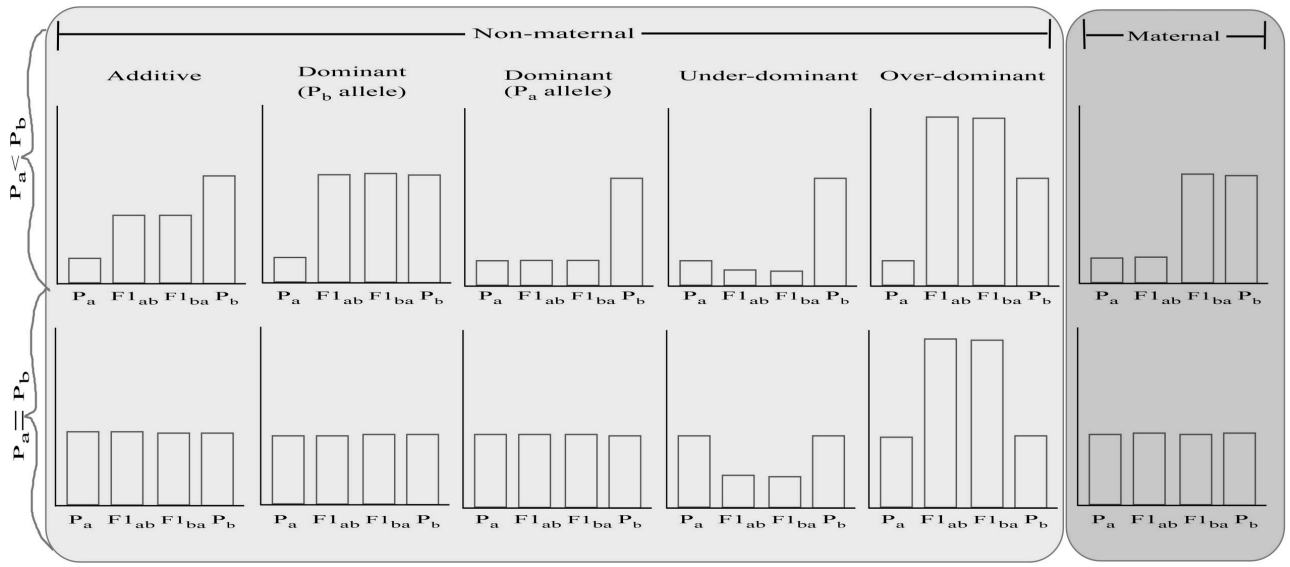

FIGURE 1 | Possible relative fitness of reciprocal F1 hybrids ($F1_{ab}$ and $F1_{ba}$) based on fitness of the parental species (P_a and P_b) and the driving mechanism. In the top graphs, parental species are assumed to differ in phenotype while the bottom graphs indicate a situation where parental species are similar. For further explanation, see text.

panther (Johnson et al., 2010) and the Mt. Buller mountain pygmy-possum (Weeks et al., 2017) from extinction (i.e., genetic rescue).

Here we investigate interspecific hybridization as a novel tool to increase genetic diversity and develop coral stock with increased climate resilience. Parental species were not chosen for their relative climate resilience, but based on our expert knowledge of the probability that they would cross-fertilize as well as their evolutionary relatedness. We examined the performance of hybrids from reciprocal crosses of two *Acropora* species pairs raised under ambient and elevated seawater temperature and pCO_2 conditions, and assessed (1) whether prezygotic barriers exist in interspecific hybrids of *Acropora* corals from the GBR, and (2) whether hybrids show enhanced fitness and resilience compared to the purebreds. Four phenotypic traits (i.e., survival, recruit size, *Symbiodinium* uptake, and photochemical efficiency) were measured in hybrid and purebred offspring as proxies for fitness. Surviving hybrids and purebreds at the end of the experiment were transplanted to long-term grow-out tank for rearing with the aim to allow future assessment of their reproductive and backcrossing potential when they reach sexual maturity at ∼4 years of age. We continued to monitor these survivors for survival and size during the grow-out period.

MATERIALS AND METHODS

Coral Spawning, *in Vitro* Fertilization, and Experimental Design

A detailed timeline of the experiment, sampling and measurement of each trait is shown in Figure S1. Parental colonies were collected from Trunk Reef, central GBR, prior to full moon on 22nd Nov 2015 and maintained in flow-through aquaria of the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS). When signs of imminent spawning were observed ("setting," i.e., where the sperm-egg bundles begin to protrude through the mouth of the polyps), colonies were isolated in individual tanks to avoid uncontrolled mixing of gametes prior to *in vitro* crossing. The five most profusely spawning colonies of each parental species were used for crossing to form (1) an *Acropora tenuis* × *Acropora loripes* cross, and (2) an *Acropora sarmentosa* × *Acropora florida* cross (**Figure 2**). These two species pairs were chosen to represent a phylogenetically divergent cross and a phylogenetically closely related cross. The phylogeny of *Acropora* spp. is divided into two distinct groups: the "early spawners" and the "late spawners," where the "late spawners" spawn about 1.5–3 h before the other group (Fukami et al., 2000; van Oppen et al., 2001; Márquez et al., 2002). *A. tenuis* (early spawner) and *A. loripes* (late spawner) are phylogenetically divergent, while *A. sarmentosa* and *A. florida* (both are "late spawners") are closely related and fall within the same phylogenetic clade (Fukami et al., 2000; van Oppen et al., 2001; Márquez et al., 2002). Little information is available from the literature about the relative resilience of these four parental species, but this has limited relevance for this study as our purpose was to increase genetic diversity (and thus adaptive potential) via hybridization, and not to conduct targeted breeding with species of known relative bleaching tolerance. Only two *A. florida* colonies spawned on the same night as *A. sarmentosa,* therefore, only two colonies were used for this species.

Egg-sperm bundles of individual colonies were collected and eggs and sperm were separated using a 100 μm filter. Eggs were washed three times with filtered seawater to remove any residual sperm and placed in a 3 L bowl until crosses were set up (within 3 h). Sperm concentration of every colony was

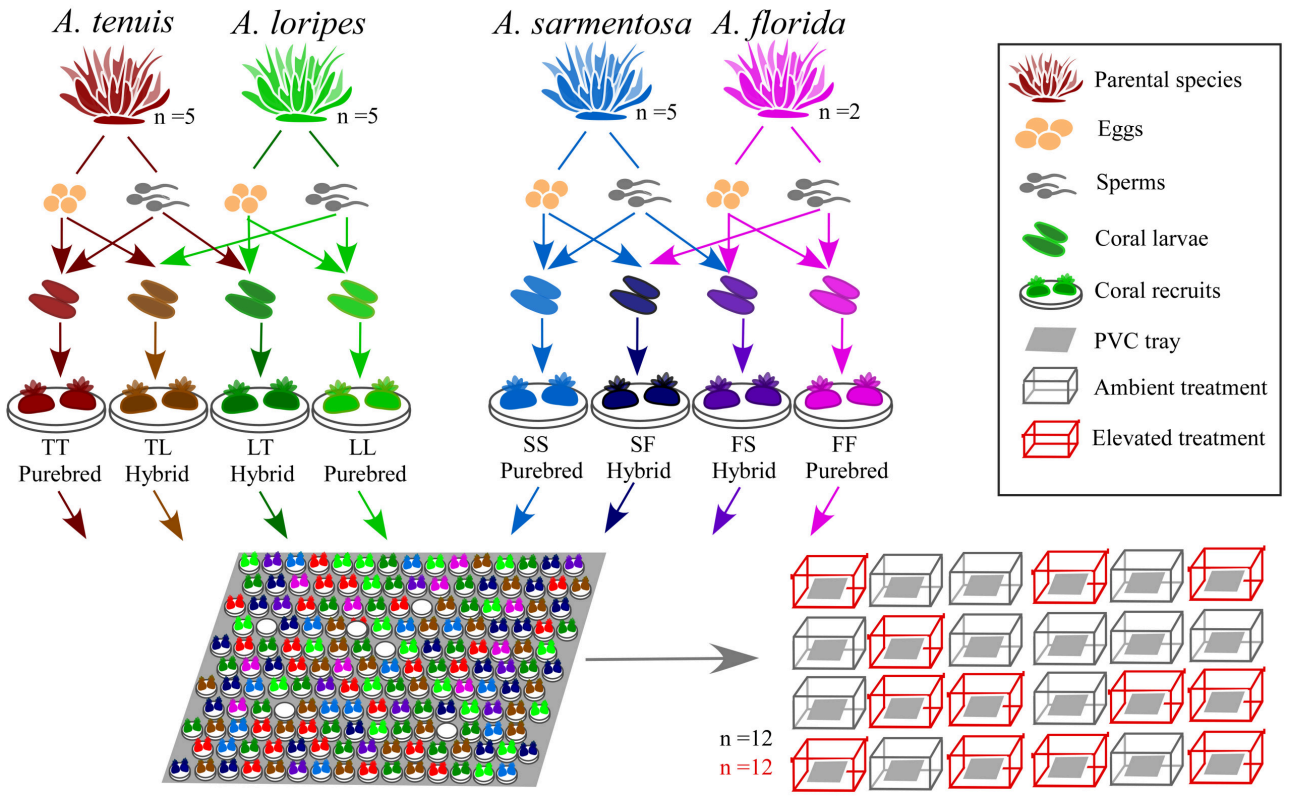

FIGURE 2 | Experimental set up showing the two interspecific crosses [i.e., *A. tenuis* (T) × *A. loripes* (L) and *A. sarmentosa* (S) × *A. florida* (F)], the four resultant offspring groups from each cross (TT, TL, LT, LL; and SS, SF, FS, FF, respectively), larval settlement, and comparison of hybrid and purebred fitness under ambient and elevated conditions. The abbreviation of the offspring groups throughout this paper is that the first letter represents the origin of the eggs and the second letter the origin of sperm [e.g., SF is a hybrid of *A. sarmentosa* (S) eggs with *A. florida* (F) sperm]. The different colors used for the offspring groups in the figure reflect differently colored settlement plugs used for each offspring group.

measured with a hemocytometer on a compound microscope with 40x magnification. Similar quantities of sperm from each conspecific colony were pooled to create a mixed sperm solution. For the hybrid crosses, the mixed sperm solution of the other parental species was added to the eggs of each interspecific colony. This method prevented intraspecific fertilization by possible remaining sperm that was not washed off the eggs (note that no self-fertilization was observed in any of the crosses performed). Fertilization was conducted under ambient conditions at a sperm concentration of 10^6 sperm mL^{-1}. Three samples of 100 eggs were collected for each species as a self-fertilization test and a "no sperm" control. Each species pair cross produced four offspring groups, two purebreds and two hybrids (**Figure 2**). Embryos of each offspring group were then placed in rearing tanks for development under ambient conditions.

Fertilization Rates and Embryonic Development

Fertilization rates were assessed at 3.5 h, and embryonic development at 9, 15, 21, 33, 45, 57, and 93 h after sperm was added to the eggs. All embryos had reached planula stage and were ready to settle by 93 h. Triplicate samples of 100 embryos of each offspring group were collected and fixed in 4% formaldehyde. Developmental stages were assessed and counted under a dissecting microscope based on the stages described in Randall and Szmant (2009).

Larval Settlement and *Symbiodinium* Uptake

Prior to coral spawning, ceramic plugs of eight different colors were preconditioned in the outdoor SeaSim flow-through aquaria under ambient conditions for 6 weeks to develop crustose coralline algae (CCA) and a microbial biofilm to provide a larval settlement cue. Five days after fertilization, planula larvae of the eight offspring groups were each settled onto one assigned color of the pre-conditioned plugs under ambient conditions. Plugs of eight different colors were used so that each offspring groups could easily be identified and randomized in the experimental PVC trays holding the plugs (**Figure 2**). During settlement, *Symbiodinium* (i.e., algal symbionts) isolated from the parent colonies were added to achieve a final density of 2×10^6 cells mL^{-1} in each settlement tank. Larvae of each offspring group only received *Symbiodinium* from their parental

species. To isolate the *Symbiodinium*, an ∼6 cm fragment with three branches was removed from each parental colony with a bone cutter. Soft tissues of the fragment were then removed using an airbrush. The mixed soft tissues/seawater solution was collected and centrifuged at 200 g for 5 min to pellet the *Symbiodinium*. The *Symbiodinium* cells were resuspended and washed three times with filtered seawater before being added to the larvae. *Symbiodinium* uptake was assessed under a dissecting microscope prior to exposure to elevated conditions. Recruits ($n = 20$ per offspring group) were categorized as either with or without *Symbiodinium*.

Settled recruits were randomized and evenly distributed on 24 tailor-made PVC trays to rear under (1) ambient conditions of 27°C, 415 ppm pCO_2, or (2) elevated conditions of ambient +1°C, 685 ppm pCO_2 (**Figure 2**). Recruits for the elevated conditions were ramped to the target temperature and pCO_2 from ambient at a rate of +0.2°C and + ∼50 ppm per day. There were 12 replicate tanks for each of the two treatment conditions and tank positions in the experiment room were randomized (**Figure 2**). Every tank held one PVC tray with 20 plugs of each offspring group, with the exception of *A. florida* purebred (FF) and hybrid (FS) which had only 10 plugs due to fewer larvae being available. To avoid sediments from accumulating on top of the recruits, the trays were placed at an approximately 45° angle. Experimental conditions followed Davies Reef (18.83°S, 147.63°E) diurnal and annual temperature variations, a reef in proximity to Trunk Reef were the adult corals used for spawning were collected. A mixed marine microalgae diet of *Isochrysis*, *Pavlova*, *Tetraselmis*, *Chaetocerous calcitrans*, *Thalassiosira weissflogii,* and *Thalassiosira pseudonana* was fed to the recruits twice a day at a final concentration of ∼5,000 cells mL^{-1} in the tank.

Survival and Recruit Size

Recruits from each tank were imaged using a high-resolution camera (Nikon D810) mounted on a quadpod with a waterproof case. Imaging was conducted fortnightly in the first 8 weeks of the experiment, thereafter every 4 weeks until 28 weeks. The numbers of surviving recruits were visually counted and recorded. Detailed images were taken at 28 weeks for size measurement. Recruit size was estimated as surface area of a circle from the measured recruit diameter since recruits were circular in shape and were not yet forming upright branches. Measurements were made using the software ImageJ and calibrated on the scale chart presented on every image. Recruits were maintained under the treatment conditions for 28 weeks. Surviving juveniles were thereafter relocated to long-term grow-out tanks to accommodate their larger size and maintained under ambient raw water (i.e., unfiltered seawater) to cater for higher feeding demand. Due to the small size of some recruits at 28 weeks and therefore difficulty to make comparisons, size was again measured at 1 year of age (i.e., about 5 months after all surviving recruits were moved to ambient raw water conditions) using the same measurement method. Furthermore, a set of photos of the median sized juvenile were taken at 2 years of age.

Photochemical Efficiency

Photochemical efficiency (i.e., dark adapted maximum photosystem II quantum yield, Fv/Fm) was measured at week 28 as a proxy for coral health. Measurements were made using Imaging- Pulse Amplitude Modulation (I-PAM) derived by the software ImagingWin (v2.40b). Recruits ($n = 15$ per offspring group per treatment) were dark adapted overnight, and remained submerged in the treatment seawater during imaging. A recruit would only be measured if: (1) it was not obscured by filamentous algae, and (2) its size was no smaller than the software's area of interest requirement.

Seawater Chemistry

Automated controls of seawater chemistry were provided by SeaSim via the SCADA (Supervisory Control and Data Acquisition) system. Experimental conditions are summarized in **Table 1**. Seawater temperature and pH were recorded every hour using resistance temperature detector (RTD) and a pH probe (Tophit CPS471D). pCO_2 was measured bi-weekly using a CO_2 equilibrator calibrated to a standard gas of 500 ppm. Total alkalinity (A_T) was measured using VINDTA calibrated to Dickson's Certified Reference Material. Salinity was measured weekly with an HACH IntelliCAL™ CDC401 Standard Conductivity Probe calibrated with IAPSO Standard Seawater. Seawater carbonate chemistry parameters, including Ω_{arag}, DIC (dissolved inorganic carbon), CO_3^{2-}, and HCO_3^- were calculated using the measured values of seawater A_T, pCO_2, temperature and salinity, with the program CO2SYS (Lewis and Wallace, 1998 as implemented in Microsoft Excel by Pierrot et al., 2006).

Statistical Analysis

Survival

Generalized linear mixed models (GLMM) (McCulloch and Neuhaus, 2013) for binomial data with logistic link functions were used to estimate the effects of treatment and offspring group on recruit survival at week 28. Analyses were conducted separately for the offspring groups of the *A. tenuis* × *A. loripes*

TABLE 1 | Experimental conditions of the ambient and elevated treatment.

Parameter*	Ambient Mean	Ambient *SD*	Elevated Mean	Elevated *SD*
Temperature (°C)	26.5	2.1	27.5	2.1
pCO_2^- (μatm)	399	5	666	36
pH_T	8.04	0.00	7.86	0.02
A_T (μmol kg^{-1})	2327	21	2327	21
Ω_{arag}	3.6	0.3	2.7	0.3
HCO_3^- (μmol kg^{-1})	1766	25	1915	21
CO_3^{2-} (μmol kg^{-1})	226	15	167	15
DIC (μmol kg^{-1})	2004	15	2100	12
Salinity (ppt)	35.5	0.8	35.5	0.8

Means and standard deviations (SDs) are given.

**pCO_2, partial pressure of CO_2 of air in equilibrium with seawater; pH_T, pH in total scale; A_T, total alkalinity; Ω_{arag}, aragonite saturation state; HCO_3^-, bicarbonate ion concentration; CO_3^{2-}, carbonate ion concentration; DIC, dissolved inorganic carbon.*

cross and the offspring groups of the *A. sarmentosa* × *A. florida* cross using R Core Team (2016) with packages *lme4* (Bates et al., 2014) and *multcomp* (Hothorn et al., 2008). In order to account for tank differences in the experimental design, a random tank effect was included in the models. Models were checked for overdispersion using a Chi-square test (Bolker et al., 2009) and goodness of fit using Akaike Information Criteria (Akaike, 1974). AIC of the GLMM of the *A. tenuis* × *A. loripes* cross was 397, the *A. sarmentosa* × *A. florida* cross was 331. Tukey's pairwise comparisons were then conducted and *p*-values were corrected using the Benjamini–Hochberg method (Benjamini and Hochberg, 1995). To obtain a visual overview of survival over time, longitudinal generalized linear models (GLM) for binomial data were used to estimate the survival for the offspring groups across all time points, and the combined hybrid offspring vs. purebred offspring. A summary table of the mean survival is provided in **Table 7**. Survival data were also analyzed with Cox proportional hazards regression as a comparison to GLMM. Results of the Cox regression were very similar to those of the GLMM and are shown in the Supplementary Methods and Results section (Table S1).

Size

Statistical analyses of size were conducted separately for the offspring groups of the *A. tenuis* × *A. loripes* cross and the offspring groups of the *A. sarmentosa* × *A. florida* cross at 28 weeks and at 1 year of age. For the 28-week time point, the absence of growth in a large number of samples under elevated conditions resulted in non-normality of the data, and non-parametric Kruskal–Wallis tests were undertaken followed by Dunn's pairwise comparisons (Dunn, 1964). *P*-values for the multiple pairwise comparisons were adjusted with the Benjamini–Hochberg method. For the 1 year time point, due to the absence of survivors in some offspring groups, not all size comparisons could be undertaken. Offspring groups with no or less than three survivors were excluded from the analyses. The remaining size data were normally distributed (tested using Shapiro–Wilk tests; Shapiro and Wilk, 1965) and variances were homogeneous (tested by Levene's tests; Levene, 1960) and five pairwise comparisons were undertaken using combined variance *t*-tests. For the *A. tenuis* × *A. loripes* cross, three *t*-tests were possible for offspring groups that were previously exposed to ambient conditions (note they have been relocated to long-term grow-out tank under raw ambient seawater after 28 weeks). The *p*-values of these comparisons were adjusted using the Benjamini–Hochberg method. The above analyses were run in R (version 3.3.1). A summary of the mean sizes of the recruits is shown is **Table 7**.

Symbiodinium Uptake and Photochemical Efficiency

Generalized linear models (GLM) (McCulloch and Neuhaus, 2013) were used to test the effect of offspring group on rates of *Symbiodinium* uptake, which was treated as a binomial distributed variable (i.e., *Symbiodinium* taken up/not taken up). Treatment was not included in this model as *Symbiodinium* uptake was assessed prior to the start of treatment. For photochemical efficiency (i.e., dark adapted yield, Fv/Fm), generalized linear models were also used to test the effects of offspring group and treatment on the response. Tukey pairwise comparisons were then used and the *p*-values were adjusted with the Benjamini–Hochberg method. These analyses were run with R packages *lme4* (Bates et al., 2014) and *multcomp* (Hothorn et al., 2008).

RESULTS

Spawning Time, Fertilization Rates, and Embryonic Development

The date and time of spawning of the *Acropora* spp. used in this study are summarized in **Table 2**. The *A. tenuis* × *A. loripes* cross was conducted on the 6th day after the full moon, where *A. tenuis* spawned at ∼19:00–19:30 and *A.* loripes at ∼21:45. The *A. sarmentosa* × *A. florida* cross was conducted on the 7th day after the full moon, where *A. sarmentosa* spawned at ∼20:30–20:45 and *A. florida* at ∼21:15–21:30. Fertilization rates of all but one hybrid offspring group were high (averaged 93%) (**Figure 3**). Hybrid LT had lower fertilization rates (averaged 79%) compared to all other offspring groups. No fertilization was observed in the "no-sperm" control and self-fertilization tests. Purebred and hybrid embryos developed normally and reached the planula stage 93 h after fertilization (Figure S2). The hybrid LT also had a slower initial embryonic development rate with the majority of the LT embryos being at the 2–4 cell stage at 3.5 h after fertilization, while embryos of all other offspring groups were at the 8–16 cell stage (Figure S2). From 9 h onwards, however, all offspring groups developed at similar rates.

Survival

Offspring Groups

Overall, maternal effects were observed in the hybrid offspring groups of the *A. tenuis* × *A. loripes* cross and over-dominance in the *A. sarmentosa* × *A. florida* cross, with some variations between treatment conditions (**Figure 4**). Offspring groups differed significantly for survival both in the *A. tenuis* × *A. loripes* cross (GLMM, $\chi^2 = 252.2$, df = 3, $p < 0.001$), and the *A. sarmentosa* × *A. florida* cross (GLMM, $\chi^2 = 32.2$, df = 3, $p < 0.001$). The values present below are mean survival and

TABLE 2 | Spawning date and time of the *Acropora* spp. from Trunk Reef, central GBR.

Date	Species	Days after full moon	Setting time	Spawning time
30/11/2015	*A. tenuis*	4	1,815	1900–1930
1/12/2015	*A. tenuis*	6	1,830	1,900
1/12/2015	*A. loripes*	6	2,000–2,045	2,145
2/12/2015	*A. loripes*	7	1,930	2,145
2/12/2015	*A. sarmentosa*	7	1,930	2,030–2,045
2/12/2015	*A. florida*	7	2,030	2,130
3/12/2015	*A. florida*	8	2,000	2,115

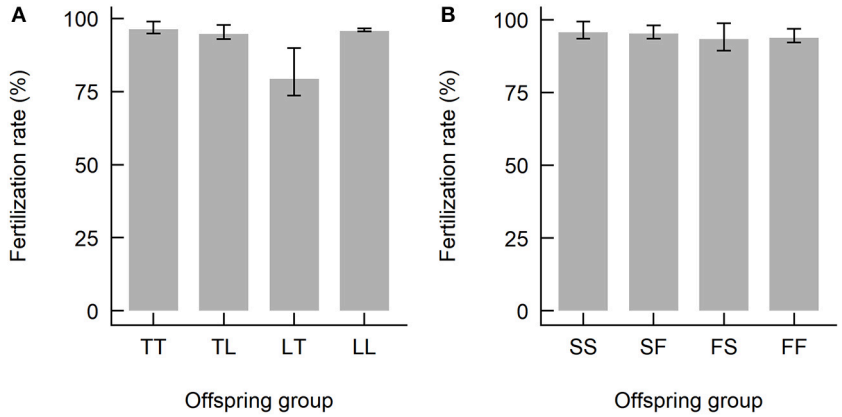

FIGURE 3 | Fertilization rates of the offspring groups from **(A)** the *Acropora tenuis* (T) × *Acropora loripes* (L) cross, and **(B)** the *Acropora sarmentosa* (S) × *Acropora florida* (F) cross. The abbreviation of the offspring groups is that the first letter represents the origin of the eggs and the second letter the origin of sperm, where TL, LT, SF, FS are hybrids and TT, LL, SS, FF are purebreds.Values are mean and error bars represent 95% CI calculated using the angular transformed data back-transformed into percentages.

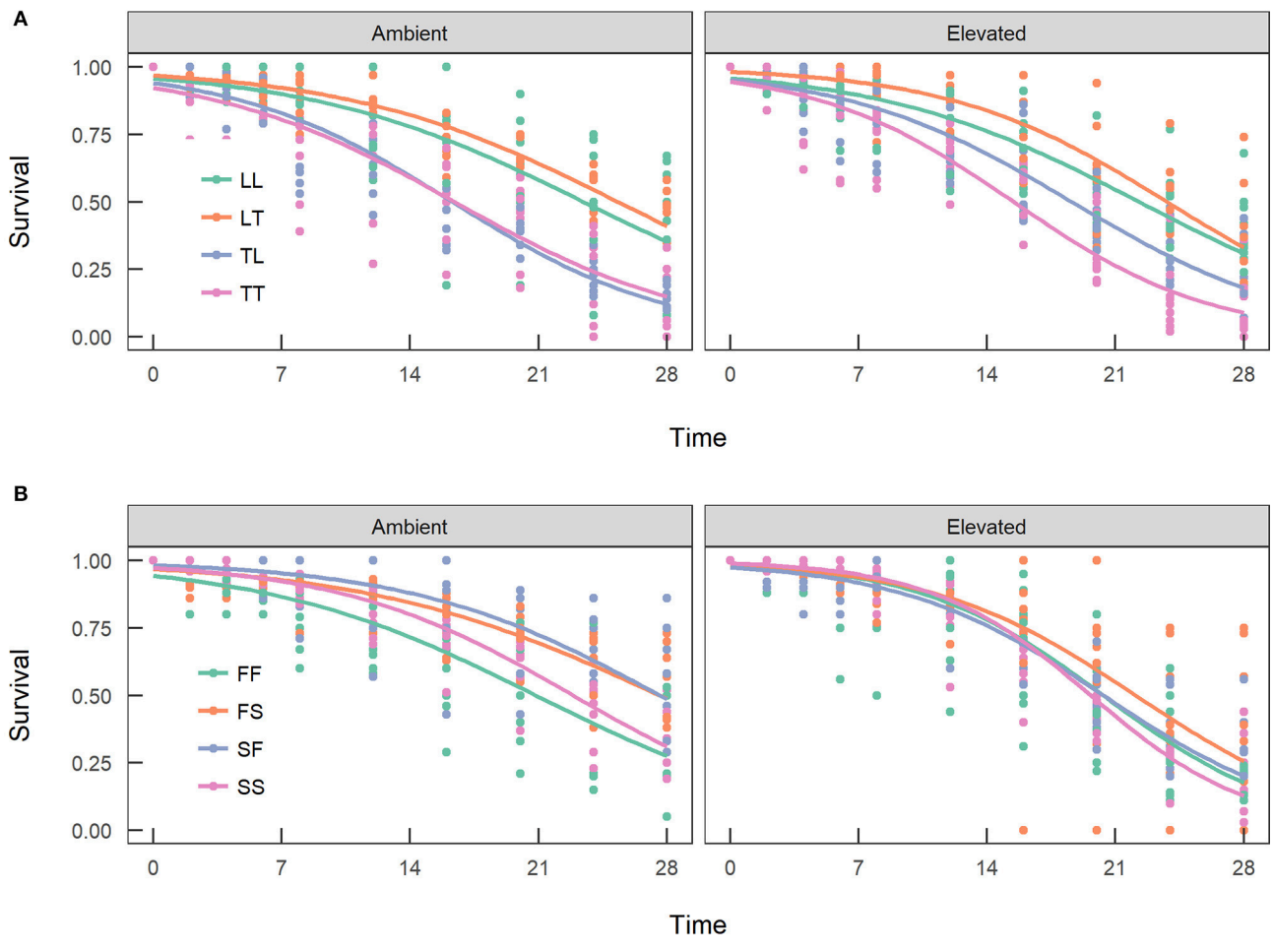

FIGURE 4 | Survival of the offspring groups of **(A)** the *Acropora tenuis* (T) × *Acropora loripes* (L) cross, and **(B)** the *Acropora sarmentosa* (S) × *Acropora florida* (F) cross under ambient and elevated conditions across 28 weeks. The abbreviation of the offspring groups is that the first letter represents the origin of the eggs and the second letter the origin of sperm. Lines represent the estimates of the longitudinal generalized linear models.

the associated 95% confidence intervals are shown in **Table 3**. For the *A. tenuis* × *A. loripes* cross, survival of hybrid LT (49%) and purebred LL (46%) was higher than that of TT (13%) and TL (16%) under ambient conditions ($p < 0.001$ for all) (**Tables 3**, **4**, **7**). Under elevated conditions, survival of hybrid LT (41%) and purebred LL (36%) was also higher than that of TT (7%) and TL (23%) (**Tables 3**, **4**, **7**). Survival of hybrids was similar to that of their maternal parental purebred offspring. Under elevated conditions, survival of hybrid TL (23%) was also higher than that of purebred TT (7%) ($p < 0.001$; **Tables 3**, **4**, **7**).

For the *A. sarmentosa* × *A. florida* cross, survival of both hybrid SF (51%) and FS (53%) was higher than that of the purebred FF (31%) ($p = 0.014$, $p = 0.006$, respectively) and SS (35%) ($p = 0.022$, $p = 0.007$, respectively) under ambient conditions (**Tables 3**, **4**, **7**). Under elevated conditions, only hybrid FS (32%) had higher survival than purebred SS (18%) ($p = 0.007$; **Tables 3**, **4**, **7**). When combining the data for all hybrid and purebred offspring groups for an overall comparison, hybrid offspring had a consistently higher survival than purebred offspring (Figure S3).

Treatments

Treatment had a significant effect on survival of both the *A. tenuis* × *A. loripes* cross (GLMM, $\chi^2 = 26.9$, df = 1, $p < 0.001$), and the *A. sarmentosa* × *A. florida* cross (GLMM, $\chi^2 = 13.6$, df = 1, $p < 0.001$). Tukey pairwise comparisons suggest that TT, SS, SF, and FS had lower survival under elevated conditions compared to ambient conditions at week 28 ($p = 0.025$, 0.002, 0.007, 0.015, respectively; **Table 5**).

TABLE 3 | Mean survival, SE, as well as lower and upper 95% CI of offspring groups from the *Acropora tenuis* (T) × *Acropora loripes* (L) cross and the *Acropora sarmentosa* (S) × *Acropora florida* (F) cross under ambient and elevated conditions.

Treatment	Offspring group	Effect	SE	Lower CI	Upper CI
Ambient	TT	0.13	0.23	0.09	0.19
	TL	0.16	0.21	0.11	0.22
	LT	0.49	0.20	0.39	0.58
	LL	0.46	0.23	0.35	0.57
Elevated	TT	0.07	0.23	0.04	0.10
	TL	0.23	0.18	0.17	0.29
	LT	0.41	0.17	0.33	0.49
	LL	0.36	0.19	0.28	0.45
Ambient	SS	0.35	0.17	0.28	0.43
	SF	0.51	0.26	0.39	0.64
	FS	0.53	0.23	0.41	0.64
	FF	0.31	0.24	0.22	0.41
Elevated	SS	0.18	0.19	0.13	0.24
	SF	0.26	0.24	0.18	0.37
	FS	0.32	0.22	0.23	0.42
	FF	0.20	0.25	0.13	0.29

The first letter of the abbreviation of the offspring group indicates the origin of the eggs and the second letter the origin of sperm.

Recruit Size

Twenty-eight Weeks

For the *A. tenuis* × *A. loripes* cross, treatment had a significant effect on recruit size (Kruskal–Wallis, $\chi^2 = 33.6$, df = 1, $p < 0.001$) but offspring group did not (Kruskal–Wallis, $\chi^2 = 6.9$, df = 3, $p = 0.096$; **Figure 5**). For the *A. sarmentosa* × *A. florida* cross, treatment also had a significant effect on recruit size (Kruskal–Wallis, $\chi^2 = 38.2$, df = 1, $p < 0.001$). Offspring group had a significant effect on size under ambient conditions (Kruskal–Wallis, $\chi^2 = 18.2$, df = 3, $p < 0.001$), but not under elevated conditions (Kruskal–Wallis, $\chi^2 = 1.0$, df = 3, $p = 0.793$). Under ambient conditions, the mean size of hybrids FS (41 mm^2) and SF (43 mm^2) was larger than that of the purebred SS (16 mm^2) ($z = 3.19$, $p = 0.003$; $z = 3.56$, $p = 0.001$, respectively), but not different in size from FF (56 mm^2; **Table 7**).

One Year

At the 1-year time point, several offspring groups no longer had survivors (**Figure 6**, **Table 7**). Note that the treatment condition

TABLE 4 | Tukey's pairwise comparisons of survival between the offspring groups from the *Acropora tenuis* (T) × *Acropora loripes* (L) cross and the *Acropora sarmentosa* (S) × *Acropora florida* (F) cross following generalized linear mixed models.

Treatment	Offspring group	Log odds ratio	SE	*z*-value	*p*-value	Odds ratio
Ambient	LT–LL	0.13	0.23	0.577	0.564	1.14
	TT–TL	−0.19	0.22	−0.831	0.437	0.83
	TT–LT*	−1.83	0.22	−8.426	<0.001	0.16
	TL–LT*	−1.64	0.20	−8.376	<0.001	0.19
	TT–LL*	−1.70	0.25	−6.786	<0.001	0.18
	TL–LL*	−1.51	0.23	−6.451	<0.001	0.22
Elevated	LT–LL	0.19	0.18	1.063	0.323	1.21
	TT–TL*	−1.41	0.22	−6.328	<0.001	0.24
	TT–LT*	−2.28	0.22	−10.523	<0.001	0.10
	TL–LT*	−0.87	0.17	−5.212	<0.001	0.42
	TT–LL*	−2.09	0.23	−8.887	<0.001	0.12
	TL–LL*	−0.68	0.19	−3.600	<0.001	0.51
Ambient	SF–FF*	0.88	0.32	2.772	0.014	2.42
	FS–FF*	0.93	0.29	3.160	0.006	2.54
	SS–FS*	−0.74	0.24	−3.064	0.007	0.48
	SS–SF*	−0.69	0.27	−2.546	0.022	0.50
	SF–FS	−0.05	0.31	−0.154	0.878	0.95
	SS–FF	0.19	0.25	0.776	0.533	1.21
Elevated	SF–FF	0.38	0.32	1.209	0.302	1.47
	FS–FF	0.65	0.30	2.148	0.052	1.91
	SS–FS*	−0.77	0.26	−3.033	0.007	0.46
	SS–SF	−0.51	0.27	−1.878	0.094	0.60
	SF–FS	−0.26	0.29	−0.903	0.466	0.77
	SS–FF	−0.13	0.28	−0.445	0.707	0.88

*The abbreviation of the offspring groups is that the first letter represents the origin of the eggs and the second letter the origin of sperm. An odds ratio of >1 indicates higher survival, and <1 indicates lower survival of the first cross in the comparison. *Indicates significant difference between this offspring group pair.*

in this section refers to the treatment conditions that the recruits were exposed to during the 28 week period following settlement, but that they were transferred to long-term grow-out tanks with ambient raw (i.e., unfiltered) seawater afterward. For recruits that were previously under ambient conditions, there were no survivors of purebreds TT and FF, while all hybrid groups had survivors. The mean size of LT (362 mm^2) and LL (366 mm^2) offspring was larger than that of TL offspring (47 mm^2) (*t*-test, $p = 0.008$, 0.015, respectively, **Tables 6**, **7**). The size of the LT hybrids was the same as that of the maternal parent species LL (i.e., maternal effect). The mean size of FS hybrids (304 mm^2) was larger than that of the pure breds SS (30 mm^2) (*t*-test, $p = 0.004$, **Tables 6**, **7**). The mean size of hybrid SF (245 mm^2, average of 2 recruits) was also relatively larger than SS (30 mm^2), however, statistical comparison was not possible due to the low sample size for SF ($n = 2$; **Table 7**). For recruits that were previously under elevated conditions, there were no survivors of purebreds TT and SS as well as hybrids TL and SF. The mean size of the hybrid LT recruits (326 mm^2) was the same as that of the maternal parent species LL (290 mm^2) (**Tables 6**, **7**). Median sized survivors of hybrid and purebred juveniles at 2 years of age and are shown in Figure S4.

TABLE 5 | Tukey's pairwise comparisons of treatment effect within an offspring group from the *Acropora tenuis* (T) × *Acropora loripes* (L) cross and the *Acropora sarmentosa* (S) × *Acropora florida* (F) cross following generalized linear mixed models.

Treatment	Offspring group	Log odds ratio	SE	*z*-value	*p*-value	Odds ratio
Elevated vs. Ambient	TT*	−0.77	0.32	−2.394	0.025	0.46
	TL	0.46	0.27	1.678	0.119	1.58
	LT	−0.32	0.26	−1.227	0.257	0.73
	LL	−0.38	0.30	−1.253	0.256	0.68
Elevated vs. Ambient	SS*	−0.90	0.26	−3.531	0.002	0.41
	SF*	−1.08	0.36	−3.033	0.007	0.34
	FS*	−0.87	0.32	−2.720	0.015	0.42
	FF	−0.59	0.35	−1.686	0.135	0.56

*The abbreviation of the offspring groups is that the first letter represents the origin of the eggs and the second letter the origin of sperm. An odds ratio of >1 indicates higher survival, and <1 indicates lower survival under elevated treatment. *Indicates significant differences in survival under different treatments in this offspring group.*

Symbiodinium Uptake and Photochemical Efficiency

There was no significant difference in *Symbiodinium* uptake between the offspring groups of the *A. tenuis* × *A. loripes* cross (GLM, $\chi^2 = 3.25$, df = 3, $p = 0.354$) or the offspring groups of the *A. sarmentosa* × *A. florida* cross (GLM, $\chi^2 = 5.35$, df = 3, $p = 0.148$; Figure S5, **Table 7**). For the *A. sarmentosa* × *A. florida* cross, neither treatment nor offspring groups had a significant effect on photochemical efficiency (Treatment: GLM, $\chi^2 = 0.51$, df = 1, $p = 0.477$; offspring group: GLM, $\chi^2 = 4.28$, df = 3, $p = 0.233$). For the *A. tenuis* × *A. loripes* cross, treatment had a significant effect on photochemical efficiency (GLM, $\chi^2 = 6.87$, df = 1, $p = 0.009$) but offspring groups did not (GLM, $\chi^2 = 2.43$, df = 3, $p = 0.488$; Figure S6). Tukey pairwise comparisons show that purebreds TT and LL under elevated conditions had lower photochemical efficiency than their counterparts under ambient conditions ($p = 0.035$, 0.002, respectively).

Summary Table

The results of the various traits measured are summarized in **Table 7**.

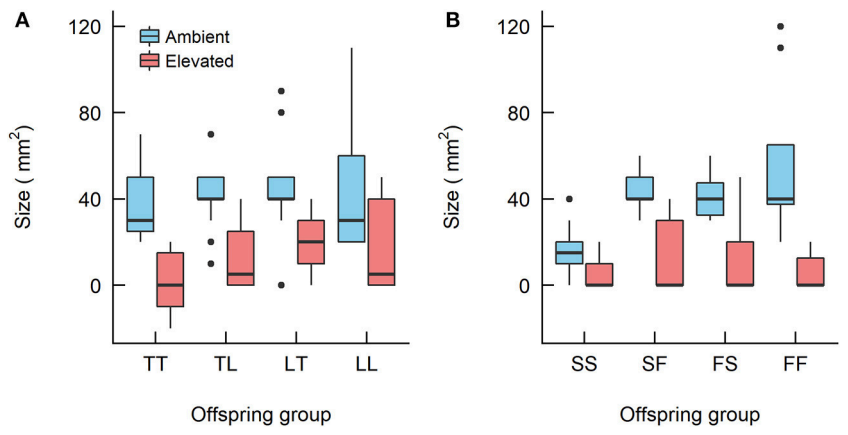

FIGURE 5 | Boxplots showing the size of the *Acropora* offspring groups at 28 weeks since treatment began from **(A)** the *Acropora tenuis* (T) × *Acropora loripes* (L) cross and **(B)** the *Acropora sarmentosa* (S) × *Acropora florida* (F) cross. The first letter of the offspring groups' abbreviation represents the origin of the eggs and the second letter the origin of sperm. The horizontal bars represent median values, box length represents the interquartile range, and the small circles denote unusual points.

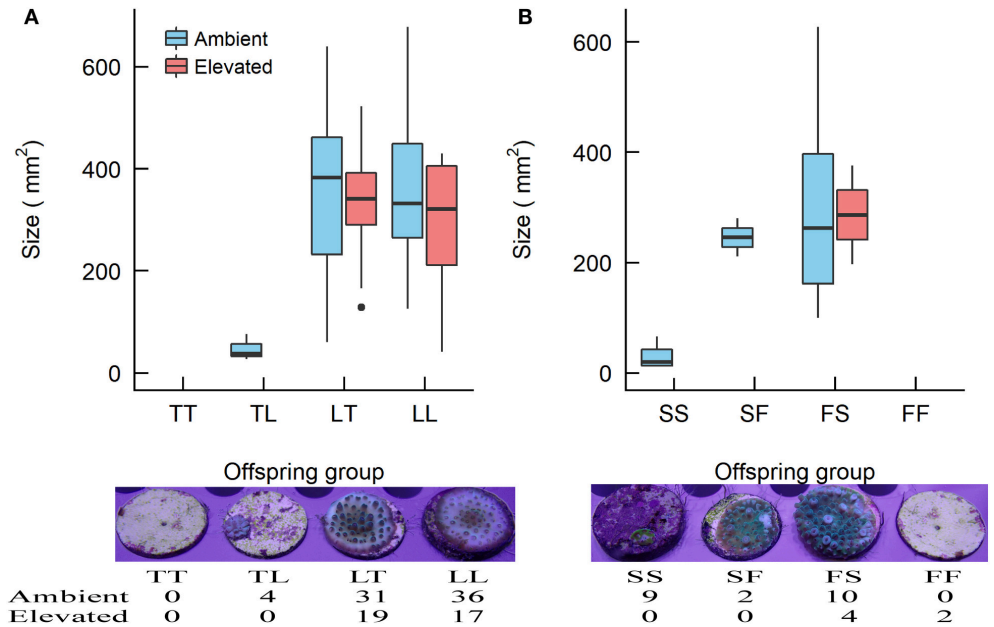

FIGURE 6 | Boxplots showing the size of the *Acropora* offspring groups at 1 year of age (i.e., ∼5 month since relocation to long-term grow-out tank under raw ambient seawater. **(A)** The *Acropora tenuis* (T) × *Acropora loripes* (L) cross and **(B)** the *Acropora sarmentosa* (S) × *Acropora florida* (F) cross. The first letter of the offspring groups' abbreviation represents the origin of the eggs and the second letter the origin of sperm. Where no data are presented there were no survivors in that offspring group. The horizontal bars indicate the medians, box length indicates the interquartile range, and the small circles indicate unusual points. Images below the graphs show examples of median size recruits of the offspring groups reared under ambient conditions in the experiment, and the number of survivors of each offspring group.

TABLE 6 | Results of *t*-tests comparing size at the 1-year time point for remaining offspring groups of the *Acropora tenuis* (T) × *Acropora loripes* (L) cross, and the *Acropora sarmentosa* (S) × *Acropora florida* (F) cross.

Treatment	Offspring group	*t*	df	*p*
Ambient	TL–LT*	3.204	17	0.008
	TL–LL*	2.911	8	0.015
	LT–LL	0.054	21	0.478
	SS–FS*	3.372	10	0.004
Elevated	LT–LL	−0.597	18	0.721

*The first letter of the offspring groups' abbreviation represents the origin of the eggs and the second letter the origin of sperm. *Indicates significant difference between this offspring group pair.*

DISCUSSION

Limited Prezygotic Barriers to Interspecific Hybridization in *Acropora* Corals

To understand the value of hybridization for coral reef restoration, it is important to establish whether prezygotic barriers exist. Interspecific hybridization among *Acropora* spp. has been shown to occur in experimental crosses, with varying degrees of prezygotic barriers (Willis et al., 1997; Van Oppen et al., 2002; Fogarty et al., 2012; Isomura et al., 2013). Among multiple pairs of *Acropora* spp. from the central GBR, crossing resulted in eight pairs with high fertilization (50–80%), seven pairs with moderate fertilization (10–50%) and three pairs with low fertilization (3–10%) (Willis et al., 1997; Van Oppen et al., 2002). The high fertilization rates and normal embryonic development of the interspecific hybrids produced in this study indicate prezygotic barriers are limited in these species pairs. This was unexpected in the case of the *A. tenuis* × *A. loripes* cross which involved an "early spawner" and a "late spawner." These "early spawners" and "late spawners" are believed to have diverged 6.6 Mya (Fukami et al., 2000). We hypothesize that our observations can be explained by the fact that the gametes of these species do not normally encounter one another in the field due to a 2 h difference in spawning times, and selection on prezygotic barriers has therefore been absent. Conversely, *A. sarmentosa* and *A. florida* are phylogenetically closely related, occur sympatrically and spawned ∼30 min to 1 h apart. Our results indicate a prezygotic barrier has not evolved to maintain reproductive isolation of these two species either, and that hybridization may occasionally occur in nature.

Lower fertilization in one direction in *Acropora* hybrid crosses, as was observed for hybrid LT, is not uncommon (Fogarty et al., 2012; Isomura et al., 2013). The likelihood of *A. palmata* eggs being fertilized by *A. cervicornis* sperm, for instance, is smaller than the likelihood of *A. cervicornis* eggs being fertilized by *A. palmata* sperm (Fogarty et al., 2012). The lower fertilization rate of the hybrid LT, however, did not affect recruit size or survival of this offspring group. The slight delay in embryonic development as observed in the hybrid LT was similar to observations for another *Acropora*

TABLE 7 | Summary of the traits measured in the two offspring groups.

Treatment	Cross	Survival-28 weeks (%)	Size-28 weeks (mm^2)	Size-1 year (mm^2)	Photochemical efficiency	Symbiodinium uptake^
Ambient	*A. tenuis* × *A. loripes*	TT: 13 TL: 16 LT: 49 LL: 46	No difference	TT: no survivor TL: 47 LT: 362 LL: 366	No difference[#]	No difference
Elevated	*A. tenuis* × *A. loripes*	TT: 7 TL: 23 LT: 41 LL: 36	No difference	TT: no survivor TL: no survivor LT: 326 LL: 290	No difference[#]	
Ambient	*A. sarmentosa* × *A. florida*	SS: 35 SF: 51 FS: 53 FF: 31	SS: 16 SF: 43 FS: 41 FF: 56	SS: 30 SF: 245[+] FS: 304 FF: no survivor	No difference	No difference
Elevated	*A. sarmentosa* × *A. florida*	SS: 18 SF: 26* FS: 32 FF: 20*	No difference	SS: no survivor SF: no survivor FS: 287[+] FF: 582[+]	No difference	

Values are provided when significant differences between offspring groups were detected.

**Values are not significantly different from other offspring groups of this set. Values are provided for information only.*

[+]*Statistical comparison was not possible due to low sample sizes (ambient SF: n = 2; elevated FS: n = 2, elevated FF n = 2). Values are provided for information only.*

[#]*There was no offspring group effect (i.e., no difference between offspring under the same treatment). However, there was a treatment effect, where purebreds TT and LL under elevated conditions had lower photochemical efficiency than their counterparts under ambient conditions.*

^*Symbiodinium uptake was assessed before treatment commenced, hence recruits were not under treatment conditions.*

cross in Japan (Isomura et al., 2013). This delay, however, was only limited to one early time point and no aberrant development was observed in the hybrids at any time point. The results of this and previous studies suggest that the degree of prezygotic barriers varies between *Acropora* species, and a range of species with limited prezygotic barriers can be used for hybridization with the aim to enhance climate resilience. Interspecific hybridization may also be applied to several other coral genera. Experimental crosses have successfully hybridized species within the genera *Montipora* and *Platygyra* (Willis et al., 1997), but were unsuccessful for species in the genus *Ctenactis* (Baird et al., 2013). Future studies to test the success of interspecific hybridization in additional coral genera will be valuable to determine the extent to which this approach for coral reef restoration can be applied.

Positive Effects of Hybridization Were Observed in Some F1 Hybrids

Given only limited prezygotic barriers exist, we explored whether hybrid offspring had increased resilience and may be used to enhance coral reef restoration efforts. If hybrids are comparatively resilient, interspecific hybridization may be combined with methods being developed for deploying coral larvae or recruits onto reefs requiring restoration (e.g., Nakamura et al., 2011; Omori, 2011; Villanueva et al., 2012; Guest et al., 2014; dela Cruz and Harrison, 2017). Overall, maternal effects were observed in hybrids of the *A. tenuis* × *A. loripes* cross and over-dominance in hybrids of the *A. sarmentosa* × *A. florida* cross, with some variations between traits and treatment conditions. Possible benefits of hybridization in enhancing reef restoration can be observed in both crosses (**Table 7**). In the *A. tenuis* × *A. loripes* cross, hybrids of both directions exhibited ∼16–34% higher survival than purebred *A. tenuis* under conditions with elevated temperature and pCO_2 (**Table 7**), suggesting hybrids have higher climate resilience than the purebred species. Both purebred species also showed reduced photochemical efficiency under elevated compared to ambient conditions while both hybrid species did not. Furthermore, purebred *A. tenuis* had no survivors at the 1-year time point, yet hybrids from both directions survived. For the *A. sarmentosa* × *A. florida* cross, hybrids of one or both directions showed ∼14–22% higher survival and were larger in size than both or one parental purebred species (**Table 7**). One hybrid offspring group (FS) had survivors in both ambient and elevated conditions at the age of 1 year, while both purebred species had no survivors in one of these conditions (**Table 7**). The FS hybrid was also 10 times larger in size than the only surviving purebred species (*A. sarmentosa*) at 1 year of age under ambient conditions (**Table 7**).

Across all traits measured, hybrids were either equivalent to or more fit than at least one parent, and none of the hybrids performed worse than both parents. These patterns are similar to those seen in some other comparisons. The natural hybrid *A. prolifera* in the Caribbean (Fogarty, 2012) and experimentally produced hybrids of *A. millepora* × *A. pulchra* (Willis et al., 2006) had equivalent or higher fitness compared to their parental species. Experimentally produced hybrids of *A. millepora* × *A. pulchra* grow larger in size than purebreds in the reef-flat environment (Willis et al., 2006). In this study, photochemical efficiency was the same between offspring groups in the same treatment, suggesting that (1) the observed differences in recruit size of the offspring groups were unlikely caused by carbon translocation from *Symbiodinium*, and (2) there was no coral host effect on photochemical efficiency of the *Symbiodinium*.

Hybrid Fitness and Its Relevance to Coral Reef Restoration

A comprehensive assessment of the value of interspecific hybridization to coral reef restoration requires multi-generation fitness examinations of hybrids and backcrosses. Such an assessment will require years given the long generations time of corals (3–7 years to reach reproductive maturity). The present study is one of the few studies that examines the long-term fitness of F1 hybrids and provides detailed assessments from fertilization to embryonic development, *Symbiodinium* uptake, photosynthesis efficiency, survival and size. The results provide evidence that hybridization may have value to reef restoration. From the restoration point of view, hybridization increases genetic variation which can potentially enhance adaptive capacity and release a population from adaptive limits (Hoffmann and Sgrò, 2011; Becker et al., 2013; Carlson et al., 2014; van Oppen et al., 2015; Hamilton and Miller, 2016; Meier et al., 2017). In this study, genetic diversity would have increased in the F1 hybrids and positive effects on survival and recruits size were observed in some cases. Furthermore, none of the hybrid offspring groups performed worse than the purebreds across all traits, suggesting that there was no negative effect of hybridization in the F1. Higher survival and larger recruit size as observed in some hybrids can enhance reef restoration initiatives by reducing post-settlement mortality. Moreover, reproductive maturity of a coral is related to its size (Soong and Lang, 1992; Smith et al., 2005). Corals that achieve a large size earlier can begin to reproduce sooner, which may further assist the recovery of degraded reef systems.

In the *A. tenuis* × *A. loripes* cross, maternal effects were observed in fitness. Hybrid survival and size were similar to that of the maternal purebred species, although it exceeded purebred values in some occasions/conditions. Maternal effects have previously been shown for survival of interspecific hybrid larvae from an *A. florida* × *A. intermedia* cross (Isomura et al., 2013), and for thermal tolerance and gene expression levels of intraspecific *A. millepora* hybrid larvae from a higher and lower latitude cross (Dixon et al., 2015). It is unclear whether the observed fitness for the F1 hybrids from our study is due to nuclear or cytoplasmic maternal effects. If survival and size are governed by nuclear maternal effects, F2 hybrids of both directions will have similar fitness to each other, which will be different from their maternal parent. If survival and size are controlled by cytoplasmic maternal effects, fitness of the F2 hybrids will follow maternal fitness (Roach and Wulff, 1987; Bernardo, 1996). In this case, species that are known to carry desirable traits under climate change may therefore be ideal candidates as a source of eggs for creating coral stock for restoration via interspecific hybridization.

When selecting species pairs for hybridization to facilitate reef restoration, both targeted crossing with species or individuals that carry phenotypic traits of value under climate change (e.g., high thermal tolerance) and non-targeted crossing between species could be considered. Species with high climate resilience (e.g., *A. loripes* in the present study) may be useful for targeted hybridization efforts. Alternatively, non-targeted crossing among related species could be used to generate hybrid vigor and increase genetic diversity for future adaptation.

Knowledge Gaps and Future Studies

This study provides the first steps toward the assessment of interspecific hybridization as an approach to create coral stock with augmented climate resilience. While our findings are supportive of this novel strategy, additional research is required. Three important outstanding questions are: (1) The fitness of hybrids vs. purebreds in the field. While laboratory studies are ideal to investigate the responses of corals to one or two specific stressor(s), corals in the wild are subjected to other selection pressures difficult to simulate in the laboratory. An important next stage of this research would involve outplanting the hybrids and purebreds to the field and monitoring their relative fitness. (2) The reproductive and backcrossing potential of F1 hybrids. Isomura et al. (2016) have shown that experimentally produced *A. intermedia* × *A. florida* F1 hybrids were fertile and able to produce F2 offspring with high fertilization rates. Transgressive segregation can happen in F2 hybrids, where segregating variation of parental species recombines in hybrids at multiple loci to produce extreme phenotypes and may result in some F2 hybrids with extremely high fitness (for review, see Hamilton and Miller, 2016). Conversely, outbreeding depression may become apparent in the F2 generations and result in hybrids with low fitness. F1 hybrids of the *A. intermedia* × *A. florida* cross were able to backcross with either both parent species or the maternal parent species only, depending on the direction of the hybrid cross (Isomura et al., 2016). In the Caribbean, molecular evidence has shown unidirectional gene flow from *A. palmata* into *A. cervicornis*, suggesting that their hybrid *A. prolifera* is fertile and able to backcross with at least one parental species (Vollmer and Palumbi, 2002, 2007). Current knowledge on fertility of F1 coral hybrids remains limited and future studies in the area will be invaluable. (3) The fitness of advanced generation hybrids and backcrosses. If F1 hybrids are fertile and sexual reproduction is successful, high fitness will have to be maintained in the F2, backcrosses and advanced generation hybrids for interspecific hybridization to be beneficial to reef recovery and resilience in the long-term.

F1 hybrids can theoretically propagate via asexual reproduction, and via sexual reproduction with other hybrids or the parental species. The likelihood of the latter depends on the spawning time of the F1 hybrids and the parental species. The *A. tenuis* × *A. loripes* cross in the present study had 2 h difference in spawning time and the *A. sarmentosa* × *A. florida* cross had 30 min to 1 h difference. While the spawning time of the hybrids remains unknown until they reach reproductive maturity, Isomura et al. (2016) showed that F1 *Acropora* hybrid spawned at the same time as their maternal parental species. This suggests that the F1 in the present study will likely be able to at least backcross with the maternal parent species for the *A. tenuis* × *A. loripes* cross, and potentially with both parental species for the *A. sarmentosa* × *A. florida* cross due to closer spawning

time. Asexual reproduction (i.e., fragmentation, polyp bail-out) is a common reproductive strategy of broadcast spawning scleractinian corals (Highsmith, 1982; Sammarco, 1982) and F1 hybrids may also persist in the wild via this method. *A. prolifera*, the natural F1 hybrid in the Caribbean for example, is known to persist and colonize large reef areas through asexual reproduction (Irwin et al., 2017).

In sum, it is likely that interspecific *Acropora* hybrids are able to propagate over extended periods of time, either sexually, asexually, or via both reproductive methods. Before hybrids can safely be used as stock for restoration, however, it must be demonstrated that the risk of this strategy is low by showing that the fitness of later generations remains equal or superior to that of the parental species in the wild.

AUTHOR CONTRIBUTIONS

WC, MvO, LP, and AH: designed the experiment; MvO: developed the concept for this study; WC and LP: conducted the experiment and collected the data; PM, WC, and AH: undertook statistical analyses; WC and MvO: wrote the manuscript and all authors contributed to the final edited version of the manuscript.

ACKNOWLEDGMENTS

This study was funded by the Paul G. Allen Family Foundation and the Australian Institute of Marine Science (AIMS). We thank staffs of the National Sea Simulator at AIMS for technical support. Thanks are also extended to M. Nordborg for her contribution to analysing the coral embryos, K. Damjanovic, R. Alfred, and L. Blackall for help with coral spawning work, and M. Shanahan for seawater alkalinity measurements. WC acknowledges receipt of the University of Melbourne International Research Scholarship and Fee Remission Scholarship.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00160/full#supplementary-material

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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

ORIGINAL RESEARCH
published: 17 May 2018
doi: 10.3389/fmars.2018.00152

Assessment of Mesophotic Coral Ecosystem Connectivity for Proposed Expansion of a Marine Sanctuary in the Northwest Gulf of Mexico: Population Genetics

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OPEN ACCESS

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Duke University, United States

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *17 February 2018*
Accepted: *13 April 2018*
Published: *17 May 2018*

Citation:
Studivan MS and Voss JD (2018) Assessment of Mesophotic Coral Ecosystem Connectivity for Proposed Expansion of a Marine Sanctuary in the Northwest Gulf of Mexico: Population Genetics. Front. Mar. Sci. 5:152. doi: 10.3389/fmars.2018.00152

While there are several areas containing shallow coral habitats in the Gulf of Mexico (GOM), the availability of suitable reef habitat at mesophotic depths (~30–150 m) along the continental shelf margin suggests the potential for ecologically connected coral populations across hundreds of kilometers in the northwest (NW) GOM. The NW GOM includes a relatively high proportion of mesophotic habitats, including salt diapirs in Flower Garden Banks National Marine Sanctuary (FGBNMS), Bright Bank, and McGrail Bank, the latter two being Habitat Areas of Particular Concern (HAPCs). In response to a proposed expansion plan for the sanctuary boundaries to include additional mesophotic banks in the NW GOM, we investigated the genetic connectivity of the depth-generalist coral *Montastraea cavernosa*, a ubiquitous member of scleractinian communities throughout the Tropical Western Atlantic. *Montastraea cavernosa* populations in the NW GOM demonstrated strong connectivity with relatively high levels of gene flow and no significant genetic differentiation occurring over banks up to 120 km apart. Historical migration models based on genetic data predicted panmixia of *M. cavernosa* across the NW GOM. The comparisons between genetic and biophysical models (see Garavelli et al., 2018) highlight not only the importance of incorporating multiple assessments of connectivity into management schemes, but also the potentially stochastic nature of oceanographic patterns in the NW GOM and their effect on migration estimates among coral habitats. These trends indicate that *M. cavernosa* populations have remained well-connected in the NW GOM and that coral populations on each bank have likely been receiving larval recruitment through time. Thus, *M. cavernosa* populations should be managed as a combined unit within the NW GOM, which supports the proposal to expand the NMS boundaries to include mesophotic habitats beyond West and East FGB.

Keywords: population genetics, mesophotic coral ecosystems, northwest Gulf of Mexico, Flower Garden Banks National Marine Sanctuary, *Montastraea cavernosa*, microsatellites, sanctuary expansion, marine spatial planning

INTRODUCTION

Understanding population dynamics in marine environments is especially challenging without prior knowledge of important habitats and larval characteristics (Palumbi, 2003; Cowen and Sponaugle, 2009; Weersing and Toonen, 2009). Identification and characterization of all possible habitats and knowledge of larval biology *in situ* is limited if not entirely undescribed for many sessile species in marine environments (Carr et al., 2003; Cowen et al., 2006; Jones et al., 2009). Methodologies to address population dynamics by measuring genetic differentiation across multiple markers are becoming more cost-effective through sample multiplexing and reduced sequencing costs (Hilbish, 1996; Manel et al., 2003). Patterns of gene flow and migration across generations can be estimated from population genetic data, allowing inferences to be made in regards to historical and current population dynamics (Palumbi, 2003). Therefore, population genetics can be an important approach to better understand regional dynamics of coral species, particularly on deeper reefs beyond traditional exploration limits (i.e., recreational SCUBA depths) or in geographically-isolated locations (Kahng et al., 2014).

Marine reserves aim to maintain biodiversity through the protection of population sources and habitats critical to larval dispersal and survival (Palumbi, 2001, 2003; Fogarty and Botsford, 2007). Conservation strategies to protect the biodiversity and persistence of coral reef ecosystems worldwide require knowledge of how populations interact and persist (Palumbi, 2003; Cowen et al., 2006; Cowen and Sponaugle, 2009). Understanding the larval biology and dispersal characteristics of all species in an ecosystem is a daunting task given the species diversity on coral reefs (Jones et al., 2009). However, suitable model or keystone species may provide sufficient information for management needs. As widely-dispersed ecosystem engineers, corals are useful candidate species to describe system-level population dynamics (Nunes et al., 2011).

The northwest Gulf of Mexico (NW GOM) is home to extensive high-latitude coral reef systems. Salt diapirs that rose during the Jurassic period now form dozens of banks along the continental shelf margin, some of which have carbonate caps (Hickerson et al., 2008; Precht et al., 2008; Schmahl et al., 2008). Most of these habitats are at mesophotic depths (∼30–150 m), but notably West and East Flower Garden Banks (FGB), approximately 180 km from the Texas coast, also have relatively shallow coral reef habitats from 17 to 30 m. While at higher latitudes than most other reefs in the Tropical Western Atlantic (TWA) and therefore spatially isolated, coral populations in the NW GOM are thought to persist due to thermal stability and relatively high larval dispersal potential from the Gulf of Mexico Loop Current (Oey et al., 2005; Atchison et al., 2008; Nunes et al., 2011; Precht et al., 2014; Rippe et al., 2017). Westward-moving eddy formations and coastal currents are also quite common in the NW GOM and may connect reef populations along the continental margin (Ohlmann and Niiler, 2005; Schmahl et al., 2008; Gough et al., 2017). Despite residing at sub-tropical latitudes, reefs in the NW GOM have relatively stable seasonal temperatures between 18 and 30°C due to persistence of currents, and major coral bleaching events are rare (Rezak et al., 1990; Schmahl et al., 2008). Coral diversity along these banks is relatively low, with 21 species of scleractinian corals compared to ∼82 species found throughout the TWA. However, coral cover on the shallow caps at West and East FGB is comparatively high (>50%) and the habitats are characterized as some of the most pristine in the TWA (Hickerson et al., 2008, 2012; Johnston et al., 2016).

The NW GOM is also one of the most active regions for offshore oil and gas exploration and production in the world; indeed, the proximity of petroleum industry to coral reef ecosystems led in part to protection of the Flower Garden Banks in 1974 by the Minerals Management Service (Aronson et al., 2005). West and East FGB were designated as part of Flower Garden Banks National Marine Sanctuary (FGBNMS) in 1992, and the nearby, but less coral-dominated, Stetson Bank was added in 1996 (Schmahl et al., 2008; Johnston et al., 2016). Long-term monitoring of the coral reef communities at FGBNMS since 1989 has documented relatively stable coral cover compared to degrading reefs elsewhere in the wider TWA (Gardner et al., 2003; Johnston et al., 2016). Some notable studies have included FGBNMS in regional population genetics analyses (Atchison et al., 2008; Goodbody-Gringley et al., 2012; Serrano et al., 2014; Rippe et al., 2017) and larval dispersal models (Davies et al., 2017; Garavelli et al., 2018), identifying the potential importance of shallow coral populations in the NW GOM to other regions beyond the Gulf of Mexico. Studies describing the geomorphology and reef communities inside the sanctuary (Aronson et al., 2005; Clark et al., 2014; Johnston et al., 2016) have been informative as well, but little research has been done on the primarily-mesophotic bank habitats outside the sanctuary boundaries (Rezak and Bright, 1985; Rezak et al., 1990; Sammarco et al., 2016), particularly regarding the relative roles of their coral populations in connectivity across the NW GOM.

Habitat and benthic community characterization of mesophotic reefs in the NW GOM has indicated that overall species diversity remains high despite lower scleractinian cover and diversity, and that these reefs serve as important fish habitats (Rezak and Bright, 1985; Rezak et al., 1990; Schmahl et al., 2008; Clark et al., 2014). The Bureau of Ocean Energy Management (BOEM) currently has "No Activity Zones" based on topographic complexity thresholds established for many of the habitats outside the sanctuary that preclude oil and gas exploration, while fewer banks include additional Habitat Area of Particular Concern (HAPC) or Coral HAPC protection that establishes fishing gear restrictions. Based on decades of exploration and characterization in the NW GOM, the FGBNMS Sanctuary Advisory Council recently proposed an expansion of sanctuary boundaries to include additional mesophotic habitats currently under varying degrees of existing protection. The preferred expansion plan (Preferred Alternative 3) includes an additional 15 reef habitats, bringing the area of protected habitat from ∼145 to 990 km^2 (Office of National Marine Sanctuaries, 2016). Perhaps most importantly, the expansion plan would co-manage the majority of coral habitats in the NW GOM as a single management unit within the NMS framework.

Most of this habitat supports mesophotic reef assemblages, primarily dominated by antipatharians, gorgonians, and crustose coralline algal communities (Rezak and Bright, 1985; Rezak et al., 1990; Schmahl et al., 2008; Sammarco et al., 2016). A few banks, notably Bright and McGrail Banks, have upper mesophotic habitat (45–60 m) dominated by scleractinian and macroalgal communities. In both cases, the extreme depth-generalist species *Montastraea cavernosa* is one of the most abundant scleractinians; it is also ubiquitous at West and East FGB (Voss et al., 2014). The population dynamics of coral species across the entire NW GOM are relatively unknown, including the potential importance of coral populations in the upper mesophotic zone of these additional banks for maintaining coral populations within the existing FGBNMS boundaries. The sanctuary expansion plan proposes managing all these habitats in the same management structure, making this study to assess population connectivity among discrete habitats within the NW GOM a timely endeavor.

To support the growing knowledge of coral population dynamics in the Gulf of Mexico (Atchison et al., 2008; Goodbody-Gringley et al., 2012; Serrano et al., 2014; Rippe et al., 2017), this study used a microsatellite approach to describe population connectivity across the NW GOM using the coral model species *M. cavernosa*. *Montastraea cavernosa* is typically one of the most common scleractinian species present across upper mesophotic reef habitats in the NW GOM. This species is also relatively ubiquitous throughout the TWA and has been consistently used in previous studies of population connectivity (Bak et al., 2005; Atchison et al., 2008; Lesser et al., 2009; Nunes et al., 2009, 2011; Goodbody-Gringley et al., 2012; Brazeau et al., 2013; Serrano et al., 2014). Studies combining genetic and oceanographic data are of growing interest within marine ecosystems (Galindo et al., 2006; Baltazar-Soares et al., 2018) and increase understanding of how spatially isolated coral reefs remain connected through time. In particular, the comparison of genetic and biophysical models (Garavelli et al., 2018) allows for more comprehensive investigations of populations with low genetic differentiation, and may provide empirical support for theoretical oceanographic patterns inferred by gene flow (Selkoe et al., 2008). The ultimate objectives of this study were to integrate genetic data into existing reef management strategies for Flower Garden Banks National Marine Sanctuary, as well as to provide critical data to inform the proposed expansion of NMS boundaries in the NW GOM.

MATERIALS AND METHODS

Coral Collection

Coral samples used to assess population structure in the NW GOM were collected from >1 m distant *M. cavernosa* colonies using SCUBA and remotely operated vehicle (ROV) and were preserved independently in either TRIzol reagent or salt-saturated DMSO. Sampling sites were chosen based on the availability of coral reef habitat including dominant presence of *M. cavernosa* populations determined from previous exploration missions (Hickerson et al., 2008; Schmahl et al., 2008; Voss et al., 2014). Corals were sampled at two banks currently within the sanctuary boundaries, and two HAPC reef habitats included in the sanctuary expansion plan (**Figure 1**). A total of 252 coral colonies were sampled across the four banks to ensure at least 30 individuals per population (Hale et al., 2012). Corals were sampled with sufficient replication at both shallow and mesophotic depth zones where continuous habitat was present (West and East FGB) to allow population comparisons within bank. From within the sanctuary, sampled populations included West Flower Garden Bank (WFGB) and East Flower Garden Bank (EFGB). Outside the sanctuary, Bright Bank (BRT) and McGrail Bank (MCG) were sampled along the scleractinian-dominated mesophotic caps (**Table 1**).

Microsatellite Amplification

DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit for DMSO samples or modified phenol-chloroform extraction for TRIzol samples (Chomczynski and Sacchi, 2006). Nine microsatellite loci (Serrano et al., 2014) were amplified in triplex reactions with the fluorescent primers 6FAM, VIC, and NED using a self-labeling technique (Schuelke, 2000) and the Qiagen Type-It Microsatellite PCR Kit according to a modified manufacturer protocol. Modifications from the kit protocol were made to forward primer concentrations (0.1 M forward primer, 0.1 M fluorescent tag) and to increase amplification to 35 cycles. Triplex groups were designed to minimize overlap of allele size ranges as follows: Plex 1 (MC29, MC41, MC49), Plex 2 (MC46, MC65, MC97), and Plex 3 (MC4, MC18, MC114). Amplified alleles were visualized via gel electrophoresis, diluted according to amplification efficiency, and sized on an ABI 3130xl genetic analyzer (Applied Biosystems) with ROX500 size standard. Alleles were scored using GeneMapper 3.7 (Applied Biosystems). Samples missing alleles from more than three loci were excluded from further analyses (**Table 1**).

Population Differentiation and Structure

Unique multi-locus genotypes (MLGs) were identified using GenAlEx 6.5 (Peakall and Smouse, 2006, 2012) and clonal genotypes were removed from the dataset. GenAlEx was used to conduct tests for Hardy-Weinberg Equilibrium (HWE), allele frequencies, and genetic differentiation with fixation index (F_{ST}). Linkage disequilibrium (LD) assumptions were tested with Arlequin 3.5 (Excoffier and Lischer, 2010). False discovery rate (FDR) corrections were calculated for HWE and LD *p*-values with the R package *FDRtool* (Strimmer, 2008). Deviations from HWE and LD were tested for null alleles with MicroChecker 2.2.3 and FreeNA (van Oosterhout et al., 2006; Chapuis and Estoup, 2007). Population-level F_{ST} values were calculated to compare pairwise population differentiation and tested with an analysis of molecular variance (AMOVA; 9999 model permutations, 9999 pairwise permutations) in GenAlEx (Peakall and Smouse, 2006, 2012). Population differentiation was also visualized with principal coordinates analysis using Nei genetic distance (D_A). Geographic isolation among banks were tested for effects on genetic variation with a Mantel isolation by distance test (9999 permutations) (Peakall and Smouse, 2006, 2012).

Structure 2.3.4 (Pritchard et al., 2000) was used to assess population structure and estimate the number of genetic clusters present in the NW GOM. Ten replicate simulations

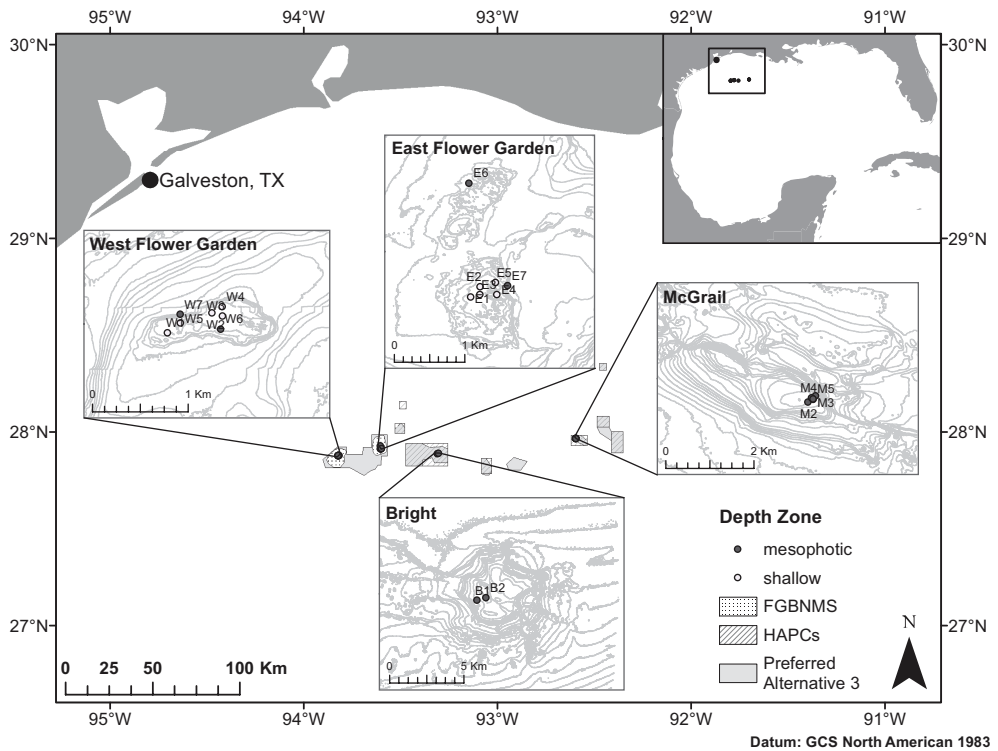

FIGURE 1 | Map of the NW GOM and sampling locations across four reef banks including West Flower Garden Bank (WFGB), East Flower Garden Bank (EFGB), Bright Bank (BRT), and McGrail Bank (MCG). FGBNMS boundaries indicated by spotted polygons, HAPCs by crosshatched polygons, and the Preferred Alternative 3 expansion boundaries shown as gray polygons. Sampling site names as in **Table 1**.

were run with values of hypothesized genetic clusters, or K, ranging between 1 and 9 using the following parameters: 10^3 burn-in iterations, 10^6 Markov Chain-Monte Carlo replicates, LOCPRIOR, assuming correlated allele frequencies and admixed populations. Additional simulations were run beyond the number of sampled populations (K = 7–9) to determine whether subpopulations were present within banks. Delta log likelihood values were compared across all model simulations to estimate the most likely value of K according to the Evanno method with Structure Harvester (Evanno et al., 2005; Earl and VonHoldt, 2012).

Estimation of Migration Rates

Migration rates were estimated among the banks across historical time scales (ancestral populations, $\sim 4N_e$ generations). Migration criteria were developed to specifically address horizontal migration patterns across banks that may influence management strategies. Shallow and mesophotic habitats within West and East FGB were combined to form a total of four populations in the NW GOM (WFGB, EFGB, BRT, MCG) due to software limitations and reduced inferential power with complex models. To assess historical gene flow, criteria were designed *a priori* and tested with Migrate 3.6 (Beerli, 2006; Beerli and Palczewski, 2010). Migrate uses coalescence theory to estimate effective population sizes and gene flow among populations relative to mutation rate. While generation times and microsatellite mutation rates are unknown for coral species, it was assumed since *M. cavernosa* is long-lived and repeatedly reproductively viable after a small size threshold that Migrate estimations represent migration patterns over hundreds to thousands of years prior (Szmant, 1991; Soong, 1993). Criteria developed to estimate horizontal connectivity in the NW GOM simulated four potential migration dynamics based on dominant currents in the region: (1) *Symm*: full model with symmetric migration across all banks, (2) *Eastward*: asymmetric migration from westward to eastward banks, (3) *Westward*: asymmetric migration from eastward to westward banks, and (4) *Pan*: panmixia. The final parameters used across all simulations were long-inc 100, long-sample 15000, 20 replicates, burn-in 20000, and four heated chains of 1, 1.5, 3, 10^5, and the prior distributions for theta and migration rate were set at 0–100 and 0–1000, respectively. Bezier log marginal likelihoods for each model were compared and ranked according to the thermodynamic integration method in Beerli and Palczewski (2010).

RESULTS

Data Validation and Sample Screening

Fifteen samples were removed due to amplification failure across more than three loci and eight clonal MLGs were removed,

TABLE 1 | *Montastraea cavernosa* genotyped samples ($n = 252$) collected across the northwest Gulf of Mexico, compared with the number of unique multi-locus genotypes (MLGs) shown as n_g used for the analyses ($n_g = 229$).

Reef	Population	Site name	Site in Map	Depth (m)	*n*	n_g	Latitude	Longitude
West Flower Garden Bank	WFGB-meso			∼45	42	40		
		West High Reef	W6	40	8	8	27.87371	−93.81655
		West Transplant Mesophotic	W7	44	34	32	27.8751	−93.82035
	WFGB-shallow			∼20	38	38		
		West High Relief	W1	21	6	6	27.87337	−93.82154
		West Cap 1	W2	21	7	7	27.87523	−93.81737
		West Cap 2	W3	21	7	7	27.87495	−93.81637
		West Cap 3	W4	21	4	4	27.87582	−93.8164
		West Transplant Shallow	W5	22	14	14	27.87429	−93.82033
East Flower Garden Bank	EFGB-meso			∼45	41	39		
		East High Reef	E6	40	10	10	27.9241	−93.6016
		East Transplant Mesophotic	E7	46	31	29	27.91102	−93.59668
	EFGB-shallow			∼20	42	40		
		East High Relief	E1	22	7	7	27.90956	−93.60139
		East Cap 1	E2	21	7	7	27.91085	−93.60018
		East Cap 2	E3	21	6	6	27.90987	−93.60021
		East Cap 3	E4	21	7	7	27.90987	−93.59804
		East Transplant Shallow	E5	21	15	13	27.9114	−93.59821
Bright Bank	BRT-meso			∼50	37	37		
		Bright Cap 1	B1	55	2	2	27.88467	−93.30712
		Bright Cap 2	B2	48	35	35	27.8862	−93.30174
McGrail Bank	MCG-meso			∼50	35	35		
		McGrail Cap 1	M1	54	1	1	27.96364	−92.59216
		McGrail Cap 2	M2	50	5	5	27.96235	−92.59369
		McGrail Cap 3	M3	49	6	6	27.96288	−92.59266
		McGrail Cap 4	M4	49	4	4	27.96321	−92.59295
		McGrail Cap 5	M5	49	19	19	27.96299	−92.59262

Geographic coordinates given as decimal degrees (WGS84).

resulting in 229 out of the 252 original coral samples used for downstream analyses. Final sample sizes for each population are shown as n_g in **Table 1**. Per locus missing rates were MC4 3.98%, MC18 2.62%, MC29 0.86%, MC41 8.30%, MC46 0%, MC49 3.98%, MC65 2.62%, MC97 3.98%, and MC114 0.86%. Assumption testing for Hardy-Weinberg Equilibrium (HWE), linkage disequilibrium (LD), and the presence of null alleles indicated no apparent pattern across populations in the NW GOM (**Table 2**).

Population Differentiation and Structure

The analysis of molecular variance indicated low differentiation across populations (AMOVA; 0.4%, df = 5,229, SS = 21.24, $p < 0.009$), while the majority of significant genotypic variation was at the individual level within populations (AMOVA; 6.8%, df = 223,229, SS = 755.86). Pairwise F_{ST} values in the NW GOM revealed a definitive lack of genetic differentiation across the region, including between shallow and mesophotic depth zones within banks (**Figure 2**). Only a single pair of banks were significantly differentiated (EFGB-meso vs. BRT-meso), indicating that the NW GOM is extremely well-mixed. Similarly, principal coordinates analysis mirrored a lack of clustering among banks, with the largest distance between the EFGB-meso and BRT-meso populations (**Figure 3**). The Mantel test indicated no significant correlation between geographic and genetic distance ($p = 0.51$, $R^2 = 0.0007$), indicating that physical distance between banks did not contribute any genetic variation among populations. Pairwise geographic distances between banks for the isolation by distance analysis ranged from 0.08 to 120.96 km.

Structure analysis suggested two genetic clusters (K = 2) using the Evanno method for the six sampled populations in the NW GOM (**Figure 4**). However, the model likelihood was higher when K = 1 than K = 2, suggesting a lack of significant genetic structure within the NW GOM. Given the limitations of the Evanno method for populations with weak genetic structure (see section Discussion), the model

TABLE 2 | Summary of genetic diversity statistics across loci and populations.

Population	Statistic	MC4	MC18	MC29	MC41	MC46	MC49	MC65	MC97	MC114
WFGB-meso	N	40	39	40	38	40	39	40	39	40
	Na	29	9	8	7	4	9	4	6	15
	Ho	0.900	0.744	0.800	0.737	0.300	0.692	0.300	0.538	0.850
	He	0.943	0.839	0.820	0.751	0.268	0.685	0.396	0.736	0.885
	*p*HWE	0.753	0.052	0.825	0.854	0.856	0.854	0.765	0.251	0.479
WFGB-shallow	N	35	36	38	34	38	37	37	36	38
	Na	20	9	9	6	4	12	4	6	14
	Ho	0.829	0.778	0.763	0.618	0.342	0.676	0.459	0.556	0.947
	He	0.918	0.848	0.788	0.710	0.341	0.816	0.409	0.619	0.878
	*p*HWE	0.818	0.793	0.830	**0.019**	0.703	**0.001**	0.825	0.815	0.834
EFGB-meso	N	38	38	39	35	39	38	38	38	38
	Na	29	9	8	6	4	13	4	6	14
	Ho	0.842	0.868	0.692	0.657	0.359	0.763	0.289	0.500	0.816
	He	0.937	0.833	0.781	0.713	0.313	0.845	0.260	0.633	0.873
	*p*HWE	0.104	**0.031**	0.840	0.806	0.850	**0.043**	0.857	0.785	0.853
EFGB-shallow	N	36	39	40	37	40	39	37	38	40
	Na	27	8	9	5	3	10	4	6	14
	Ho	0.917	0.744	0.750	0.568	0.400	0.641	0.324	0.711	0.925
	He	0.942	0.818	0.755	0.721	0.373	0.736	0.358	0.676	0.898
	*p*HWE	0.712	**0.046**	0.856	0.559	0.785	0.207	0.844	0.857	0.729
BRT-meso	N	37	36	37	37	37	37	37	36	36
	Na	26	9	9	8	5	9	5	6	17
	Ho	0.892	0.694	0.757	0.703	0.216	0.757	0.459	0.611	0.917
	He	0.940	0.816	0.772	0.740	0.199	0.755	0.552	0.629	0.905
	*p*HWE	0.673	0.746	0.469	0.546	0.859	0.854	0.364	0.703	0.643
MCG-meso	N	34	35	33	30	35	30	34	33	35
	Na	26	9	8	5	4	9	5	6	13
	Ho	0.824	0.743	0.606	0.633	0.371	0.700	0.382	0.576	0.914
	He	0.939	0.802	0.775	0.570	0.360	0.708	0.396	0.632	0.894
	*p*HWE	**0.019**	**0.001**	**0.013**	0.721	0.855	0.701	0.855	0.846	0.672

N, number of samples; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HWE}, FDR-corrected p-values for tests of Hardy Weinberg Equilibrium (HWE). Significant violations of HWE shown in bold.

with the highest likelihood was chosen as the most likely (K = 1).

Estimation of Migration Rates

Historical migration rates among banks indicated population panmixia over symmetrical or directional migration across genetically distinct populations (**Table 3**). The second most likely scenario was net eastward to westward migration, although model probabilities for all other migration patterns were near zero compared to panmixia.

DISCUSSION

Patterns of genetic variation across banks included in this study support that *M. cavernosa* are acting as a single population in the NW GOM. The lack of significant genetic differentiation across reef habitats up to 120 km apart indicates gene flow among depth zones and banks has been sustained at a relatively high level through time. Genetic connectivity within the region is likely maintained through strong and persistent currents in the Gulf of Mexico, dominated by the Loop Current that flows from the Yucatan Peninsula into the Straits of Florida through the NW GOM (Lugo-Fernández, 1998; Oey et al., 2005). The estimates of gene flow and population structure for *M. cavernosa* in the GOM are likely higher than for other coral species (Holstein et al., 2015; Serrano et al., 2016; Bongaerts et al., 2017; Rippe et al., 2017), given the abundance of this species across shallow and mesophotic reefs in the region (Bak et al., 2005; Lesser et al., 2009; Nunes et al., 2009) and its high dispersal potential due to its broadcast spawning reproduction (Nunes et al., 2011; Goodbody-Gringley et al., 2012; Brazeau et al., 2013).

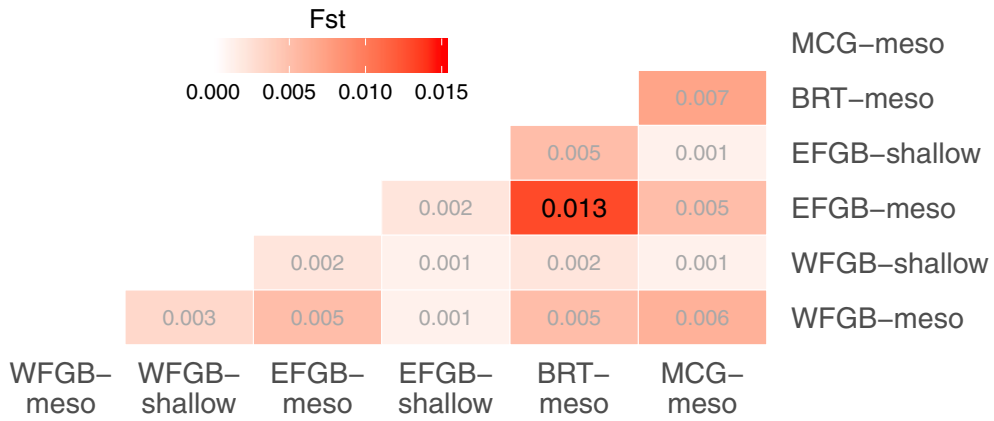

FIGURE 2 | Pairwise comparisons of population differentiation as measured by fixation index (F_{ST}). The level of differentiation among populations is shown in increasing intensities of red. Significantly differentiated F_{ST} estimates are shown in larger black font ($p < 0.05$ after FDR correction).

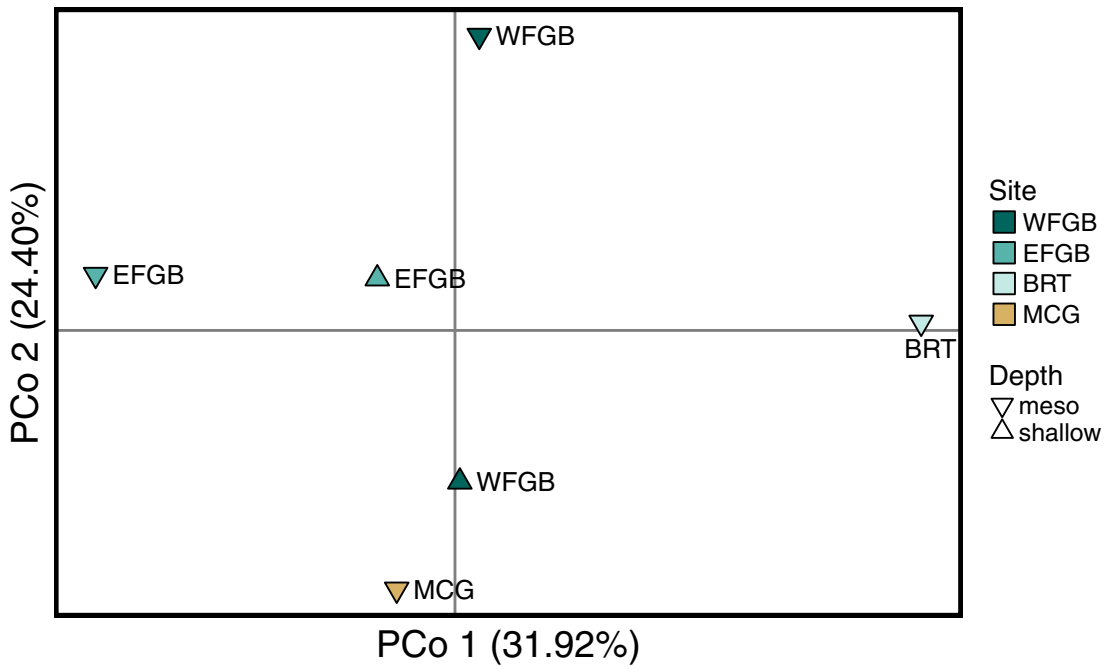

FIGURE 3 | Principal coordinate analysis of Nei genetic distance (D_A) matrix generated from pairwise population differentiation comparisons. Samples from each population were first compared to generate a single eigenvector, represented by a single point in three-dimensional space, and the relative distance between populations approximates the degree of genetic differentiation. The three axes have been flattened to a two-dimensional representation and explain 73.48% of the total genotypic variation (Coordinate 1 31.92%, Coordinate 2 24.40%, Coordinate 3 17.16%). Color of each point corresponds to discrete banks.

The results of the population structure analysis match the conclusions of the F_{ST} analysis that there is no genetic structure ($K = 1$) and that the NW GOM represents a single uniform *M. cavernosa* population. Despite the Evanno method identifying two genetic clusters, the model likelihood when $K = 1$ was indeed higher than when $K = 2$. This discrepancy is due to the manner in which the Evanno method calculates the stepwise change in model likelihoods (Delta K) between simulated values of K (Evanno et al., 2005). Therefore, this method cannot calculate Delta K when $K = 1$. This operationally precludes the selection of $K = 1$ as the most likely scenario despite the high model likelihood and represents a shortcoming of this statistical approach in systems with little population structure (Waples and Gaggiotti, 2006). Analyses using both population genetics (this study) and biophysical modeling approaches (Garavelli et al., 2018) predicted *M. cavernosa* in the NW GOM to come from the same population and identified connectivity across banks (via gene flow and larval dispersal, respectively).

As a result of the open gene flow within the NW GOM region, historical migration estimates across reefs were overwhelmingly

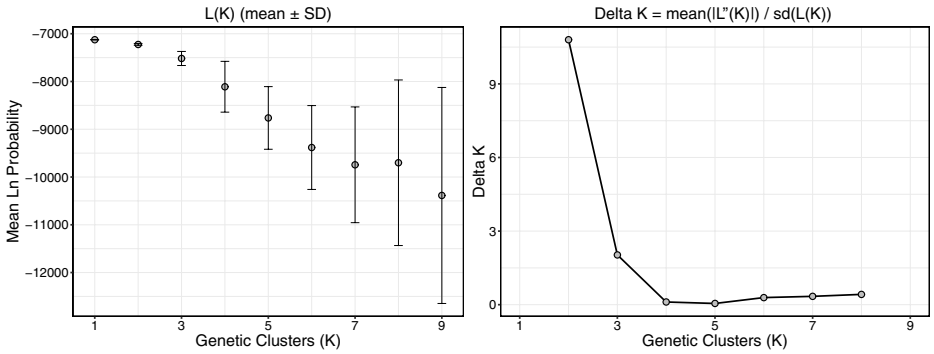

FIGURE 4 | Plots describing the process behind population cluster (K) selection in structure analysis. Ten replicate structure models were run across a range of K values from 1 to 9 and model log likelihoods were compared. The Evanno method was used to determine the most likely number of K by identifying the largest change in likelihood (L(K)) and by comparing model probabilities in conjunction with variance (Delta K). Error bars represent standard deviation of the mean.

in support of a panmictic population. The second most likely migration scenario, albeit with an extremely low model probability, was net migration westward along the continental shelf margin with more eastward populations contributing to the westward neighbors. If panmixia was excluded as a possible outcome and model probabilities were recalculated, however, westward migration became the most likely scenario with a probability of 1. Taken together with what is known about hydrodynamic patterns in the region, these results provide limited evidence that in the absence of panmixia, migration may be occurring in a counter-current direction to the prominent Loop Current that travels from a southwestern to northeastern direction in the NW GOM (Lugo-Fernández, 1998; Oey et al., 2005). This suggests that counter-current features in the region may be the dominant drivers of coral larval dispersal through ecological timescales rather than the Loop Current (Schmahl et al., 2008). Westward migration may be facilitated by Loop Current Eddies or coastal currents including the Louisiana Coastal Current (LCC), which transports Mississippi River outflow westward due to the Coriolis Effect (Jarosz and Murray, 2005; Oey et al., 2005; Ohlmann and Niiler, 2005).

The comparison of *M. cavernosa* migration patterns estimated from genetic data in this study to larval dispersal using a biophysical model in the associated study demonstrates that annual hydrodynamic variability can greatly affect connectivity predictions (Garavelli et al., 2018). The fact that the biophysical model predicted net eastward export of larvae from all banks, while the genetic model predicted panmixia (or net westward migration to a lesser extent), suggests that the inconsistent and ephemeral nature of currents in the NW GOM may be resulting in widespread dispersal across the region (Oey et al., 2005; Schmahl et al., 2008). For the 3 years of oceanographic data available for the biophysical model, eddies were not consistently present across the study region in the latter 2 years and would therefore not be significant drivers of larval migration. In the latter years, the Loop Current was likely the most influential factor affecting larval transportation, resulting in the export of larvae eastward outside the study area (Garavelli et al., 2018). Our observed panmictic migration pattern hypothesizes that multiple larval dispersal patterns may be effective to enable gene flow over time among all banks in the study.

TABLE 3 | Comparison of Bezier log marginal likelihood (lmL) differences, model probabilities, and rank for four different gene flow models (Symm, symmetric horizontal migration; Eastward, migration from westward to eastward banks; Westward, migration from eastward to westward banks; Pan, panmixia) within the NW GOM.

Model description	Model	lmL for model	Model prob.	Rank of model	θ (±95% CI)
Symmetric	Symm	−2440085	0	4	
Westward to eastward	Eastward	−1938001	0	3	
Eastward to westward	Westward	−1218566	0	2	
Panmixia	Pan	0	1	1	6.57 (3.27–9.27)

Reef banks with both shallow and mesophotic habitats (WFGB and EFGB) were combined to form a single population per bank. The mutation-scaled population size (θ) with 95% confidence intervals in parentheses is given for the most likely model.

The LCC experiences seasonal variability, and in rarer cases, eastward transport following impingement on the continental shelf (Jarosz and Murray, 2005; Gough et al., 2017). The LCC may reverse direction during summer months (June–August) and flow eastward at a weaker speed. Coral spawning has been observed at FGBNMS to occur 7–8 days after the full moon in late August (Vize, 2006), which may result in spawning in August or September when the LCC could potentially be moving either westward or eastward. The potential variability in LCC direction across spawning events may result in different larval dispersal patterns across years. In the case of the biophysical model by Garavelli et al. (2018), it appears the oceanographic data from 2013–2015 captured one such rare impingement events, causing dispersal predictions of net eastward movement of coral larvae. The variability of oceanographic patterns over short time scales and its potential effect on migration predictions from year to year emphasizes the periodic nature of successful coral recruitment events and suggests that eddies and coastal currents play an important, if not dominant, role in determining the destination

of coral larvae (Tang et al., 2006; Chérubin and Garavelli, 2016; Vaz et al., 2016; Gough et al., 2017; Pan et al., 2017). Naturally, this does not preclude the potential for occasional export of larvae beyond the NW GOM and successful gene flow to other reefs hundreds of kilometers away, as has been suggested by biophysical and genetic data (Davies et al., 2017; Rippe et al., 2017; Garavelli et al., 2018). Stepping-stone dynamics among other reef habitats in the NW GOM including scleractinian communities on oil and gas platforms (Sammarco et al., 2004, 2012, 2013, 2014) and mesophotic banks in the eastern GOM (Hine et al., 2008; Locker et al., 2010; Silva and MacDonald, 2017) may also influence coral population connectivity in the region.

The population genetics data presented in this study supports the combined management of mesophotic reef habitats across the NW GOM since *M. cavernosa* across four banks act as a single population and would likely benefit from integrated protection of all population source habitats within the region. Comparison of genetic and biophysical models demonstrated that multiple analysis methods can be integrated to more accurately represent relationships between oceanographic conditions across individual spawning events and multi-generational gene flow. This is particularly important in regions where management actions can be influenced by the model outcomes (Galindo et al., 2006; Selkoe et al., 2008; Baltazar-Soares et al., 2018). It has previously been suggested with several coral species that FGBNMS is a population refugia in the GOM (Atchison et al., 2008; Goodbody-Gringley et al., 2012; Davies et al., 2017; Rippe et al., 2017). We propose that additional banks with established coral communities including Bright and McGrail Banks likely represent additional refugia habitat important for population persistence within the NW GOM, and possibly for other reefs in the wider GOM. High gene flow among reefs, with evidence for occasional export of migrants to nearby reefs indicates that these habitats are self-sufficient but may still serve as population sources over ecological timescales (Davies et al., 2017; Garavelli et al., 2018). If these trends are similar across other taxa as they have been with corals, the NW GOM may provide important ecosystem services despite its relative spatial isolation.

While less diverse than other areas of the TWA in terms of coral communities, West and East FGB support biodiverse assemblages of reef organisms and fishes that have been relatively stable through time (Schmahl et al., 2008; Hickerson et al., 2012; Clark et al., 2014; Johnston et al., 2016). Mesophotic habitats outside the current sanctuary boundaries are less biodiverse from a scleractinian standpoint and likely represent smaller populations as compared to FGB (Hickerson et al., 2008; Schmahl et al., 2008; Sammarco et al., 2016). Nonetheless, the results presented here demonstrate that Bright and McGrail Banks may serve as important population sources for the rest of the NW GOM region as well and should not be excluded from larval dispersal models and management strategies. The development and implementation of conservation applications, including marine reserves, are frequently designed to protect known population sources (Palumbi, 2001, 2003; Fogarty and Botsford, 2007). Therefore, management actions are warranted to protect the coral populations at Bright and McGrail Banks identified in this study.

DATA AVAILABILITY STATEMENT

All protocols, including sample preparation, data analysis, and data templates are available in a GitHub repository (https://github.com/mstudiva/Mcav-microsats.git). The datasets generated for this study can be found in a Dryad repository (Studivan and Voss, 2018).

AUTHOR CONTRIBUTIONS

JV: designed and funded this research through the Cooperative Institute for Ocean Exploration, Research and Technology (CIOERT); MS and JV: conducted sample collections; MS: generated and analyzed the microsatellite data. All authors contributed to the final edited version of the manuscript.

FUNDING

This research was funded by the NOAA Office of Ocean Exploration and Research under awards NA09OAR4320073 and NA14OAR4320260 to the Cooperative Institute for Ocean Exploration, Research and Technology (CIOERT) at Florida Atlantic University-Harbor Branch Oceanographic Institute. Additional funding was provided by a donation from the Banbury Fund to Harbor Branch Oceanographic Institute and by graduate student fellowships and grants from Florida Atlantic University.

ACKNOWLEDGMENTS

We thank the staff of Flower Garden Banks National Marine Sanctuary, crews of the R/V Manta, L. Horn, and J. White from the University of North Carolina at Wilmington Undersea Vehicle Program, J. Beal, J. Emmert, R. Susen, C. Ledford, J. Polinski, D. Dodge, P. Gardner, M. McCallister, M. Ajemian, R. Christian, and M. Dickson for diving support. We also thank G. O'Corry-Crowe and T. Ferrer for capillary sequencing, and X. Serrano and A. Baker for assistance with pipeline optimization. This paper is Contribution Number 2147 from Harbor Branch Oceanographic Institute at Florida Atlantic University.

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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

ORIGINAL RESEARCH
published: 22 May 2018
doi: 10.3389/fmars.2018.00174

Assessment of Mesophotic Coral Ecosystem Connectivity for Proposed Expansion of a Marine Sanctuary in the Northwest Gulf of Mexico: Larval Dynamics

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In coral reef ecosystems, mesophotic coral habitat (>30 m to the end of the photic zone) are extensions of shallow reefs and contribute to the persistence of coral reef populations. In the North West Gulf of Mexico (NW GOM), the Flower Garden Banks National Marine Sanctuary (FGBNMS) is an isolated reef ecosystem comprising contiguous shallow and mesophotic reefs habitats on two central banks along the margin of the continental shelf. A future expansion of the sanctuary is proposed to include additional mesophotic banks and aims at building a network of protected areas in the NW GOM to ensure the persistence of the coral reef populations inhabiting the sanctuary. To evaluate the feasibility of this expansion and investigate the overall dynamics of coral species in the region, we studied the patterns of larval connectivity of *Montastraea cavernosa*, a common depth generalist coral species, using a larval dispersal modeling approach. Our results highlighted larval exports from the NW GOM banks to the northeastern and southwestern GOM, larval connectivity between all banks investigated in this study, and the potential for exporting larvae from mesophotic to shallower reefs. Our study associated with Studivan and Voss (2018; associate manuscript) demonstrates the relevance of combining modeling and genetic methods to consider both demographic and genetic timescales for the evaluation of the connectivity dynamics of marine populations. In the case of the NW GOM, both studies support the future management plan for expanding FGBNMS.

Keywords: mesophotic coral ecosystems, northwest Gulf of Mexico, Flower Garden Banks National Marine Sanctuary, *Montastraea cavernosa*, larval connectivity, sanctuary expansion, marine spatial planning

OPEN ACCESS

Edited by:
Michael Sweet,
University of Derby, United Kingdom

Reviewed by:
Yoichi Miyake,
The University of Tokyo, Japan
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University of the Philippines Diliman, Philippines

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *21 February 2018*
Accepted: *02 May 2018*
Published: *22 May 2018*

Citation:
Garavelli L, Studivan MS, Voss JD, Kuba A, Figueiredo J and Chérubin LM (2018) Assessment of Mesophotic Coral Ecosystem Connectivity for Proposed Expansion of a Marine Sanctuary in the Northwest Gulf of Mexico: Larval Dynamics. Front. Mar. Sci. 5:174. doi: 10.3389/fmars.2018.00174

INTRODUCTION

Coral reefs ecosystems face severe degradation from local and global threats including overfishing, nutrient and sediment run-off, and climate change (Hoegh-Guldberg et al., 2017; Hughes et al., 2018). Worldwide bleaching events have contributed to global loss of coral cover and consequent decline of coral reef ecosystems (Gardner et al., 2003; Bellwood et al., 2004; Hughes et al., 2017). Despite the socio-economic importance of coral reefs,

protection of these critical ecosystems remains limited. Most of the degradation has been documented in shallower reef ecosystems (<30 m), while deeper mesophotic coral ecosystems (MCEs) are projected to act as refugia and contribute to the persistence of coral reef populations (Glynn, 1996; Lesser et al., 2009; Bongaerts et al., 2010; Slattery et al., 2011; Van Oppen et al., 2011; Thomas et al., 2015). MCEs are light-dependent reef communities, standing between 30 m depth and the lower limit of the photic zone (Bongaerts et al., 2010; Hinderstein et al., 2010; Voss et al., 2014). In the Caribbean region, MCEs are hypothesized to be relatively less impacted by thermal stress and anthropogenic impacts as compared to shallower reefs (Bak et al., 2005; Menza et al., 2008).

In the northwest Gulf of Mexico (NW GOM), around 180 km off the coasts of Texas and Louisiana, coral reef ecosystems form dozen of banks mainly comprising mesophotic reefs along the margin of the continental shelf (Schmahl et al., 2008). Despite the extensive degradation of other ecosystems in the northern hemisphere, the coral cover in the NW GOM has remained relatively stable compared to other regions of the wider Caribbean (Aronson et al., 2005; Hickerson et al., 2012). This stability is mainly explained by low temperatures variations and high larval supply driven by the Gulf of Mexico Loop Current (Oey et al., 2005; Nunes et al., 2011; Rippe et al., 2017). Along the banks of the NW GOM, the coral diversity is relatively low. Among these banks, West and East Flower Garden Banks (FGB) include contiguous shallow and mesophotic reefs ecosystems (Schmahl et al., 2008). Reefs on West FGB and East FGB are described as some of the most pristine reef habitats and exhibit 50% to 80% coral cover, mainly massive brain and star corals (Hickerson et al., 2012; Johnston et al., 2016). Other banks such as Bright, Geyer, and McGrail Banks contain mesophotic habitats only and are mainly constituted of scleractinian and macroalgal communities.

In 1992, the FGB National Marine Sanctuary (FGBNMS) was created to protect the fish and benthic species living in this ecosystem. West FGB and East FGB were the first banks included in the sanctuary followed by Stetson Bank in 1996. Stetson Bank supports the least coral communities in the FGBNMS, with a coral cover of less than 8% (DeBose et al., 2013; Johnston et al., 2016). Following extensive research in the NW GOM, a future expansion of the sanctuary has been proposed to include additional mesophotic reef banks (Department of Commerce, National Oceanic and Atmospheric Administration, 2015). This future management plan aims to build a network of protected coral banks in the NW GOM to ensure the persistence of the sanctuary's coral and fish populations.

To ensure the persistence of marine species and develop efficient management strategies such as the expansion of the FGBNMS, the assessment of larval connectivity is essential (Hastings and Botsford, 2006; Gaines et al., 2010; Burgess et al., 2014). For benthic species with a pelagic larval stage such as corals, larval connectivity depends on both biotic and abiotic processes such as hydrodynamic current, availability of spawning and settlement habitat, fecundity, larval behavior, larval pre-competency period, and larval duration (Pineda et al., 2007). Using a modeling approach, the influence of spawning periodicity, fecundity, dispersal duration, and time to competency have been shown to influence larval connectivity of coral species (Holstein et al., 2014, 2015a; Kough and Paris, 2015). However, those biological processes are not always known and are often complicated to estimate. In a recent study, Davies et al. (2017) modeled the larval dispersal in the FGB of *Pseudodiploria strigosa*, a scleractinian coral species with a short planktonic larval duration (PLD; 3 to 20 days). Their model did not predict larval export outside the FGB for this species and larvae released from the West FGB and East FGB had limited dispersal. Davies et al. (2017) based their results on virtual larvae passive surface transport only and their recruitment method did not include a pre-competency period, during which the larvae cannot settle, which would tend to overestimate local retention and underestimate long-distance dispersal.

In this study, we focus on *Montastraea cavernosa*, a common depth generalist, broadcast-spawning coral species. *M. cavernosa* is one of the most abundant coral species in the West FGB, East FGB, and in other mesophotic reef habitats of the NW GOM (Pattengill-Semmens et al., 2000; Voss et al., 2014). Its larval connectivity in the NW GOM and, in particular between the FGBNMS and potential additional protected banks, is unknown. By developing a biophysical model of larval dispersal for *M. cavernosa* among mesophotic reef banks in the NW GOM, we predict patterns of connectivity that may influence population persistence in this region. The model accounts for critical biotic and abiotic processes driving *M. cavernosa* larval connectivity both horizontally across banks and vertically between shallow and mesophotic reefs. To understand the overall dynamics of *M. cavernosa* in the region, this study 1. Investigates the dispersal potential of *M. cavernosa* larvae in the GOM; 2. Determines the sustainability of *M. cavernosa* populations in the northwestern banks of the NW GOM by assessing its local connectivity patterns; and 3. Evaluates the larval exchange between mesophotic and shallow reefs by assessing the vertical connectivity in the West FGB and East FGB. The results are designed to serve as a basis for spatial management of *M. cavernosa* in the NW GOM.

MATERIAL AND METHODS

Study Area

Our study focuses on five banks located in the NW GOM, from west to east: West FGB, East FGB, Bright, Geyer, and McGrail (**Figure 1**). The former two are currently protected within FGBNMS and the later three are under consideration for protection in the pending FGBNMS expansion plan (Department of Commerce, National Oceanic and Atmospheric Administration, 2015). Stetson Bank (the third bank in FGBNMS) was not considered for this study due to its low coral cover (DeBose et al., 2013; Johnston et al., 2016). West FGB and East FGB are characterized by the presence of both shallow and mesophotic reefs habitat while Bright, Geyer, and McGrail Banks include mesophotic reefs only.

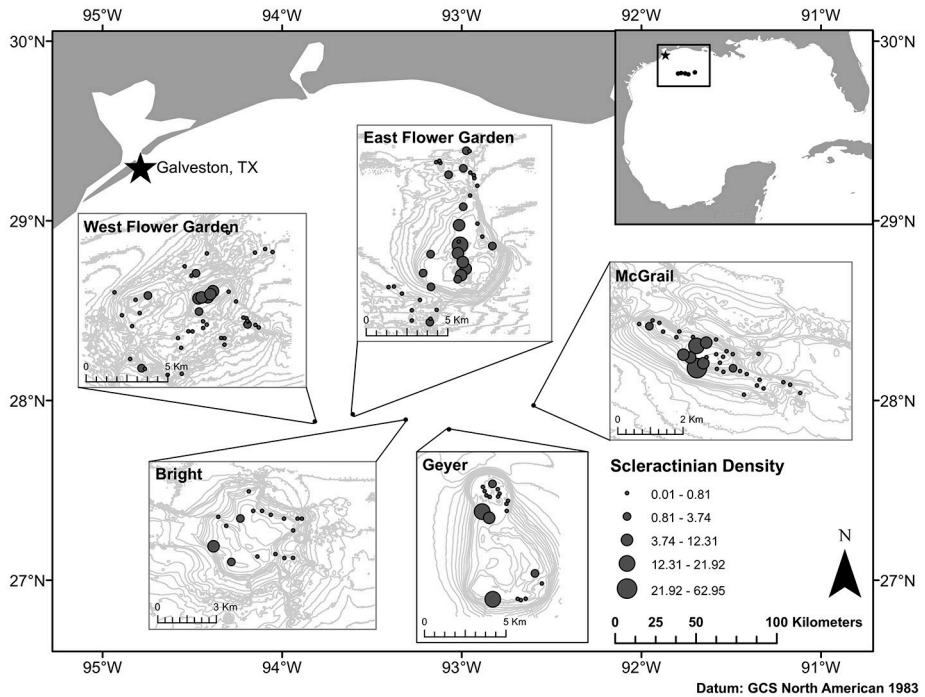

FIGURE 1 | Map of the northwest Gulf of Mexico showing the locations of five reef banks and the density of scleractinian (number of colonies by m^2) for each bank. Banks from west to east: West Flower Garden, East Flower Garden, Bright, Geyer, and Mc Grail. The inset map shows the location of the five banks in the entire Gulf of Mexico.

Hydrodynamic Model

The Naval Oceanographic Office (NAVOCEANO) operates regional ocean prediction systems based on the Navy Coastal Ocean Model (NCOM; Barron et al., 2006). The Naval Research Laboratory developed NCOM, which is based on the Princeton Ocean Model with time invariant hybrid (sigma over Z) vertical coordinates. NCOM AMSEAS is a regional NCOM simulation whose domain covers the Americas Seas region including the Gulf of Mexico and the Caribbean Sea at 1/36 degree (∼3 km) horizontal resolution and is discretized over 40 levels in the vertical. The model topography comes from Naval Research Laboratory Digital Bathymetry Data Base 2-min resolution (NRL DBDB2). The atmospheric forcing fields are provided over this domain by a 15 km application of the Navy's Coupled Ocean/Atmosphere Mesoscale Prediction System (COAMPS®) model. The AMSEAS ocean prediction system assimilates all quality-controlled observations in the region including satellite sea surface temperature and altimetry, as well as surface and profile temperature and salinity data using the NRL-developed Navy Coupled Ocean Data Assimilation (NCODA) system. Boundary conditions are applied from the NAVOCEANO operational 1/12 degree Global HYCOM (Chassignet et al., 2009). The model is forced by tides and discharges from 53 rivers in the region (Ko et al., 2003). NAVOCEANO distributes 3-h NetCDF files containing ocean temperature, salinity, eastward and northward currents, and elevation, along with the atmospheric forcing fields. The files are accessible from the National Centers for Environmental Information (NCEI). AMSEAS time aggregated dataset is available from 2010 to present. The AMSEAS model has been deployed in response to the Deepwater Horizon oil spill event in 2010 (Zaron et al., 2015). A simulation was conducted using a Lagrangian particle tracker with random walk diffusion of archived AMSEAS data, with a particular focus on pollution pulses that penetrate into the estuaries east of the Mississippi River. This modeling formulation was capable of reproducing the oil spill transport (Zaron et al., 2015). This model was also used to predict likely drift tracks of sea turtles carcasses in the north central Gulf of Mexico (Nero et al., 2013). Surface currents and wind forcing used in estimating leeway and subsequent carcass drift backtracks were obtained from AMSEAS. In a similar manner, we used the velocity and density fields from AMSEAS in a biophysical model to simulate the transport of *M. cavernosa* larvae.

Larval Dispersal Model

To model *M. cavernosa* larval dispersal in the NW GOM, the individual-based offline Lagrangian tool Ichthyop v3.1 was used (Lett et al., 2008). The virtual larvae were represented as particles in three dimensions and characterized by their latitude, longitude, and depth at each time step of the model (i.e., 1 h). In Ichthyop, the particles are advected by velocity fields generated by the NCOM AMSEAS hydrodynamic model. A forward-Euler advection was implemented in the model and horizontal diffusion was included following Peliz et al. (2007). The vertical diffusion coefficient was not included. Spawning and settlement habitat polygons were designed based on multibeam bathymetry

data assembled from datasets of the United States Geological Survey, University of New Hampshire. Depth distribution of scleractinian coral at FGB was used to select spawning and settlement depths (**Table 1**). Our study focuses on 3 years: 2013, 2014, and 2015. These 3 years correspond to the same years during which Studivan and Voss (2018; associate manuscript) collected the coral samples used to assess the genetic population structure of *M. cavernosa*. To answer our study's objectives, different simulations were performed. Those simulations are detailed below.

M. cavernosa Regional Larval Dispersal

The goal of the first set of simulations was to investigate the dispersal potential of *M. cavernosa* larvae in the GOM. We created 19 polygons of 16 km^2 representing the habitat of *M. cavernosa* in the NW GOM banks: 6 at West FGB; 7 at East FGB; 2 at Bright, Geyer, and McGrail Banks. Observations of spawning events in FGB showed that *M. cavernosa* spawning occurs around the first week after the full moon from July to September (Annual reports of coral spawning cruise, NOAA). The same features have been observed in the Caribbean and Bermudian reefs (Szmant, 1991). Over the polygons, 10,000 particles were randomly released in July, August, and September from 2013 to 2015. Releases occurred between 6 and 9 days after the full moon of each month (Acosta and Zea, 1997; Sammarco et al., 2004; Annual reports of coral spawning cruise, NOAA). In absence of data for the larval dispersal duration of *M. cavernosa*, we used data from another broadcasting scleractinian coral of the Caribbean region (*Orbicella faveolata*; Holstein et al., 2014, 2015a). The PLD was set to 20 days. (A sensitivity analysis was performed with 10 days of PLD and dispersal results were qualitatively similar). No data on larval behavior was available for *M. cavernosa*. We included a buoyancy scheme of the larvae in the model following Holstein et al. (2015a; Table S1.1). *M. cavernosa* larvae positions were recorded at the end of their modeled dispersal to estimate their putative destination in the GOM. The mean larval dispersal distances were calculated from their release to their destination locations.

M. cavernosa Local Larval Connectivity

The goal of the second set of simulation was to assess *M. cavernosa* local larval connectivity patterns in the banks of the NW GOM. The same modeling approach was used as for the first set of simulation. The main difference here is the incorporation of settlement habitat. The 19 polygons previously described (i.e., 6 at West FGB; 7 at East FGB; 2 at Bright, Geyer, and McGrail Banks) were used as both release and settlement areas for *M cavernosa* larvae. Biological parameters of *M. cavernosa* larvae are the same as in the first set of simulation. The main difference with the first set of simulation was the addition of a larval pre-competency period in the model. The duration of 3.97 days of pre-competency period was chosen based on larval experiments results (data obtained from laboratory experiment, Supporting Information S1, Figure S1.1). Over the 19 polygons representing release areas, 10,000 larvae were randomly released in July, August, and September from 2013 to 2015. The simulated larval dispersal lasted for 20 days (i.e., duration of PLD; A sensitivity analysis was performed with 10 days of PLD. Connectivity patterns were qualitatively similar and values were slightly higher with shorter PLD.). During their dispersal, larvae located in a settlement area and that were at least 3.97 days old were considered settled in the model.

To predict the larval connectivity patterns of the species between release and settlement areas of the NW GOM banks, the outputs of the model simulation were analyzed in connectivity matrices. Values of the connectivity matrix represent the larval transport success $C_{i,j}$ from release polygon i to settlement polygon j. In order to account for the coral fecundity in the model, we used density data of scleractinian coral species collected in each bank (see Voss et al., 2014 for methods; **Figure 1**). The coral density was then estimated in each habitat polygon of the model. The rows of the connectivity matrix (i.e., release areas) were multiplied by the estimated density of coral in each polygon. Monthly larval transport success was averaged between all the banks and compared for each year of simulation to assess the monthly and annual variability of *M. cavernosa* larval connectivity patterns. To calculate larval exchanges of *M. cavernosa* between potential subpopulations and identify approximately independent subpopulations or metapopulations (i.e., connected subpopulations), we applied a clustering method developed by Jacobi et al. (2012). The method consists of dividing the study area into a finite number of mutually exclusive subpopulations based on minimization of an objective function that calculates larval exchange between potential subpopulations using the connectivity matrix. The connectivity matrix used was the average per bank and over the 3 years of study (2013, 2014, and 2015).

TABLE 1 | Minimum and maximum spawning and settlement depths (i.e., depth range; in m) for each bank of the study area.

Banks	Depth range (m)
West FGB	18–112
East FGB	16–110
Bright	33–84
Geyer	50–90
McGrail	45–87

M. cavernosa Vertical Larval Connectivity in West FGB and East FGB

The goal of the third set of simulations was to assess the larval vertical connectivity between the shallow and mesophotic reefs of the West FGB and East FGB. Those two banks have the deepest mesophotic reefs in the region (112 m for the West FGB and 110 m for the East FGB). Coordinates of the polygons previously defined for the West FGB and East FGB were used. Polygons representing shallow reefs of the FGB were set between 18 and 30 m depth for the West FGB and between 16 and 30 m depth for the East FGB. Polygons representing mesophotic reefs were set between 31 and 112 m depth for the West FGB and between 31 and 110 m depth for the East FGB. Over these polygons

representing both shallow and mesophotic reefs in the West and East FGB, 10,000 larvae were randomly released in July, August, and September from 2013 to 2015 for a duration of 20 days (i.e., duration of PLD). During their dispersal, larvae located in one of the polygons and that were at least 3.97 days old were considered settled in the model. The biological parameters of *M. cavernosa* were similar to the previous detailed simulations.

To predict the larval vertical connectivity patterns of *M. cavernosa* between the shallow and mesophotic reefs of West FGB and East FGB, the outputs of the model simulation were analyzed in connectivity matrices. The results were averaged over the 3 years of simulation.

RESULTS

Regional Larval Dispersal in the NW GOM

For all 3 years of simulation, larval abundance was higher close to the NW GOM banks, i.e., all of the banks used in the simulation (**Figure 2**). Generally, larvae remain in the northern part of the GOM although none was transported north onto the continental shelf. However, direction of the larval transport differs depending on the year. While larvae are mainly exported southwest of the release banks in 2013 (**Figure 2A**; mean larval dispersal distance = 54.4 ± 46.3 km), they follow a northeastern path along the northern shelf of the GOM in 2014 and 2015 (**Figures 2B,C**; mean distance = 203.4 ± 133.5 km for 2014, 212.6 ± 146 km for 2015). In the last 2 years of simulation, larval trajectories split into two branches from around the Mississippi river delta (29° N, 89° W). The maximum distance of larval dispersal was 731.7 km in 2014 (it was 458.2 km in 2013 and 646.7 km in 2015).

Local Larval Connectivity Between Banks

The model predicts significant yearly and monthly variability in larval transport success (**Figure 3**). In 2013, larval transport success was overall 2.2% and higher than in the other years (0.7% in 2014; 0.94% in 2015). Monthly variability was mostly observed in 2013. Larval transport success was lower in August than in July and September (2.51% in July; 0.65% in August; 3.44% in September). This tendency was also noted for 2015 (1.01% in July; 0.69% in August; 1.11% in September). In 2014, transport success was higher in July compared to August and September (1.10% in July; 0.61% in August; 0.40% in September).

Larval connectivity patterns for *M. cavernosa* between banks exhibited interannual variability (**Figure 4**). The model predicted larval settlement in all the banks. The lowest larval transport success was toward the West FGB and the highest toward McGrail. As previously observed, a decrease in larval connectivity was noted in 2014 compared to the other years. East FGB (mainly

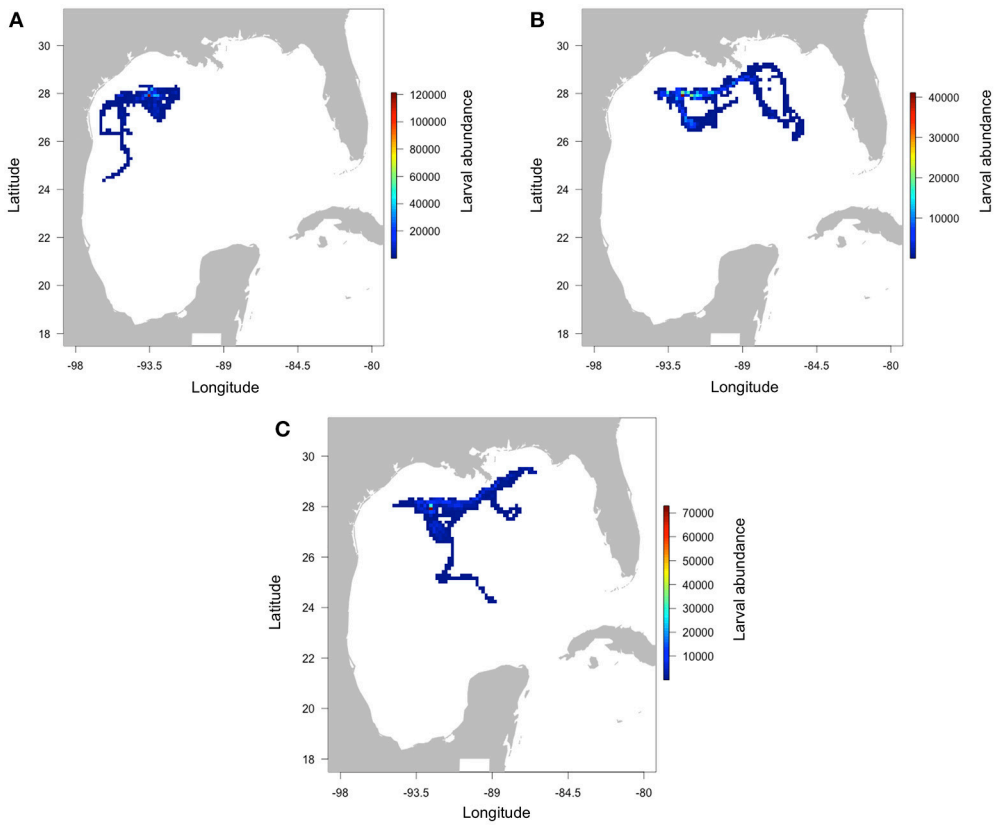

FIGURE 2 | Predicted larval abundance of *M. cavernosa* in the Gulf of Mexico after release from the banks (West FGB, East FGB, Bright, Geyer, Mc Grail; see **Figure 1** for the location of each bank) for the years 2013 **(A)**, 2014 **(B)**, and 2015 **(C)**. The number of larvae was summed inside a grid cell of 0.15 × 0.15°.

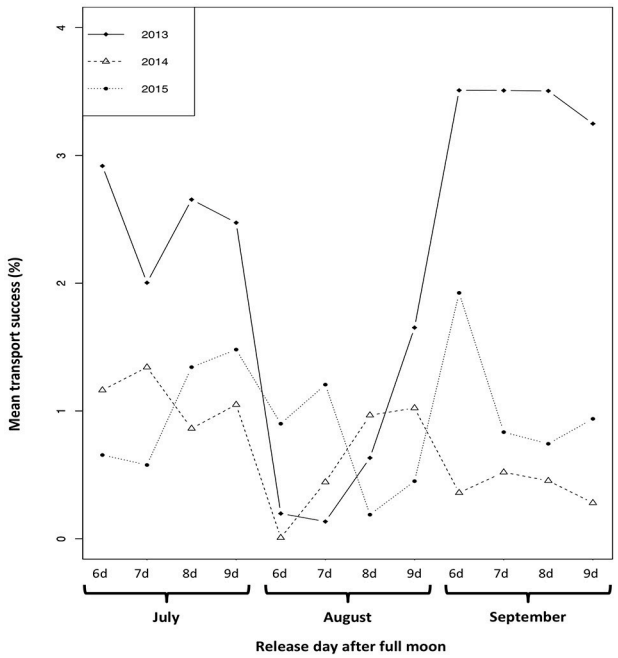

FIGURE 3 | Mean transport success (percentage) of *M. cavernosa* larvae per month and year of release averaged over West FGB, East FGB, Bright, Geyer, and Mc Grail.

in 2013) and McGrail are the banks where to larvae were the most transported, mostly coming from West FGB, and the West FGB, East FGB, and Bright, respectively. Local retention (larvae settling on the same bank where they were released) is higher in 2013 compared to 2014 and 2015 (0.72% in 2013; 0.17% in 2014; 0.009% in 2015). In 2013, local retention is higher in East FGB (1.15%) and McGrail (1.52%). It is null in both 2014 and 2015 in the West FGB and McGrail. The clustering method applied to our connectivity results suggests that all the banks belong to the same metapopulation.

Vertical Larval Connectivity in the West FGB and East FGB

Vertical connectivity of *M. cavernosa* was investigated for the 3 years of simulation in the West FGB and East FGB (**Figure 5**). Globally, more larvae settled in the mesophotic reefs than in the shallow reefs for both banks (mean transport success to mesophotic reefs = 3.84%; mean transport success to shallow reefs = 0.44%). Larval transport was also mostly unidirectional from the West FGB to the East FGB. The mesophotic reefs of the East FGB received the most larvae from both shallow and mesophotic reefs of the West FGB. Local larval retention was higher inside the East FGB (from shallow and mesophotic reefs toward mesophotic reefs; 1.15%) than inside the West FGB (0.32%) and higher in the mesophotic reefs for both banks (0.43% in mesophotic reefs vs. 0.02% in shallow reefs).

DISCUSSION

The biophysical larval dispersal model developed for *M. cavernosa* in the NW GOM highlighted larval exports from the NW GOM banks to the northeastern and southwestern GOM, larval connectivity between all banks investigated in this study, and the potential for exporting larvae from mesophotic to shallow reefs.

The larval dispersal model predicted large-scale larval dispersal of *M. cavernosa* outside the NW GOM banks. Larvae were transported from the northwestern to the northeastern GOM in 2014 and 2015. Predictive likelihood of habitat for mesophotic coral in the GOM shows potentially suitable depths and benthic composition for scleractinian coral along nearly all of the outer continental shelf (Kinlan et al., 2013). In 2014, we found that larvae could be transported as far as 731.7 km despite a relatively short PLD of 20 days. In the NW GOM, the banks investigated in this study could serve as larval sources for MCE that fall within the area of possible dispersal such as Pinnacles Reef Trend and Florida Middle Grounds (Locker et al., 2010).

M. cavernosa subpopulations in the NW GOM banks selected in this study were connected at least once between each other through larval dispersal. The overall patterns of *M. cavernosa* larval connectivity in the NW GOM banks can be described as a main transport from the western banks to the eastern banks with few dispersal events in the opposite direction. However, connectivity patterns were considerably variable intra and inter-annually. Year 2013 showed the highest local larval transport success in July and September, consequently the shortest range of larval transport, which is to the west of the banks. In contrast, years 2014 and 2015 show the longest transport range and in both years, it is eastward and south along the shelf break in 2015 or southeast from the banks in 2014.

The variability of *M. cavernosa* larval dynamics patterns could be explained by the variability in direction and magnitude of oceanic currents in the NW GOM. The surface circulation in the NW GOM is dominated by the northern extent of a persistent western boundary current that flows eastward along the continental shelf break. In addition, the impingement of Loop Current Eddies (LCEs), and their cyclonic counterpart is a major driver of the variability of the western boundary current (Vukovich and Crissman, 1986; Sutyrin et al., 2003). Shoreward of the western boundary current, over the broad Louisiana-Texas shelf (LaTex), the flow is relatively weak, generally cyclonic, and largely driven by wind stress. The annual variability between larval dispersal patterns obtained in 2013 and the other two years (2014 and 2015) is due to the lack of impingement of LCEs and cyclonic counterparts on the LaTex shelf break, as shown in **Figure 6**. In July 2013, FGB is filled with submesoscale features (**Figure 6A**) favorable to local larval transport, while in July 2014 and 2015, unbroken mesoscale vorticity filaments replaced these features and are associated with relatively strong along-shelf currents leading to longer larval dispersal distances (**Figures 6B,C**). LCEs (cyclones) impingement on the LaTex and Mexico-Texas shelves drives strong eastward (westward) flows along the LaTex shelf break that replaced the submesoscale features seen in summer 2013 (**Figure 6A**), enhancing remote

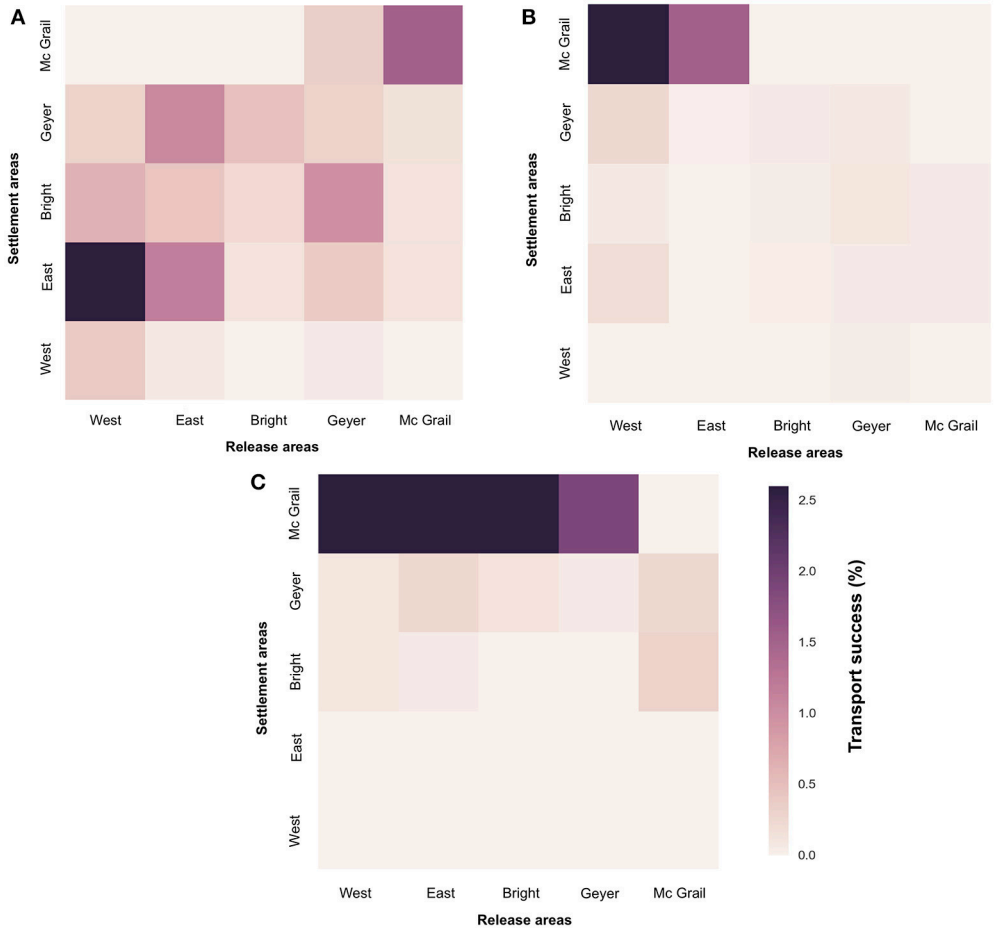

FIGURE 4 | Connectivity matrices representing the percentage of transport success of *M. cavernosa* larvae from release to settlement areas in the banks (West FGB, East FGB, Bright, Geyer, Mc Grail) for the years 2013 **(A)**, 2014 **(B)**, 2015 **(C)**.

along-shelf transport and cross-shelf transport in eddies. The 2013 transport pattern is characteristic of that region as revealed by climatological Lagrangian Coherent Structures (cLCS) in Gough et al. (2018). This pattern is likely to be driven by the sub-mesoscale features found near the shelf break (**Figure 6**). In 2014 and 2015, cross-shelf export is localized in the region of the hook-like cLCS pattern identified in Gough et al. (2018) and seems to be associated with significant eddy driven alongshore currents. Significant eddy impingement on the NW GOM is thus identified as one of the drivers of long-range dispersal of coral larvae from the NW GOM banks to the eastern GOM. Such eastward dispersal events have been previously described around the FGB in 1997 and 1998 (Lugo-Fernández et al., 2001). Persistent Lagrangian patterns in the NW GOM identified by Gough et al. (2018), who used 18-year average flow field data (1995-2012), suggest that short westward dispersal near FGB has been most likely to occur as observed in Davies et al. (2017).

The unidirectional larval transport from West FGB and East FGB to Bright, Geyer, and McGrail arises the question of sufficient larval supply inside West FGB and East FGB. For both West FGB and East FGB, larval dispersal from mesophotic reefs to shallow reefs was predicted, supporting the role of mesophotic reefs as larval sources for shallower reefs (Lesser et al., 2009; Slattery et al., 2011; Holstein et al., 2015a,b). However, contrasting spatial patterns of vertical larval connectivity between West FGB and East FGB were also observed. The most significant pattern of vertical larval exchanges was a cross-vertical connectivity from the West FGB toward the East FGB. Both shallow and mesophotic reefs of the West FGB were exporting larvae to the East FGB. The low larval connectivity toward the East FGB from the other banks considered in this study and previously observed may be counterbalanced by the local retention in the East FGB and the vertical larval exchanges from the West FGB, and be sufficient to sustain the populations of *M. cavernosa* in the area. Also, including other mesophotic reefs located southern of the area in the analysis could offset the low larval transport success toward West FGB.

Although banks of the NW GOM are relatively spatially close the ones from the others, local retention in the area and larval connectivity between banks were low. However, all the banks were predicted to belong to the same metapopulation. Therefore, recurrent larval exchanges between banks may be sufficient to allow the sustainability of *M. cavernosa* populations in this region, although it is isolated from other MCEs. Both the larval dispersal model from our study and genetic data from Studivan and Voss (2018; associate manuscript) suggest

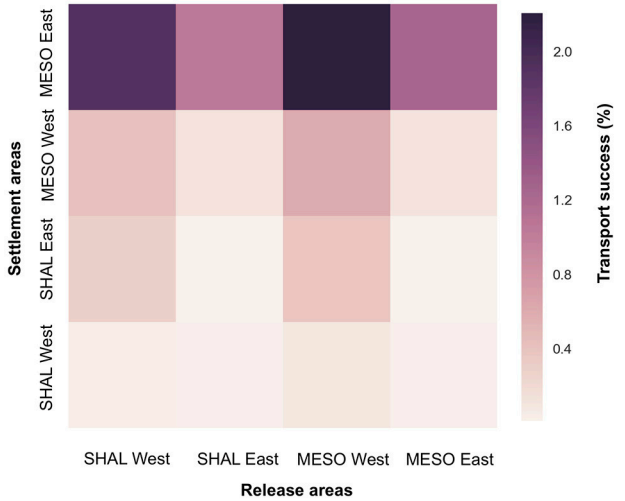

FIGURE 5 | Connectivity matrices representing the mean percentage of vertical transport success of *M. cavernosa* larvae between shallow (18–30 m depth for West FGB and 16–30 m depth for East FGB) and mesophotic (30–112 m depth for West FGB and 30–110 m depth for East FGB) reefs in the West and East FGB for the 3 years of simulation (2013, 2014, and 2015). SHAL, shallow; MESO, mesophotic.

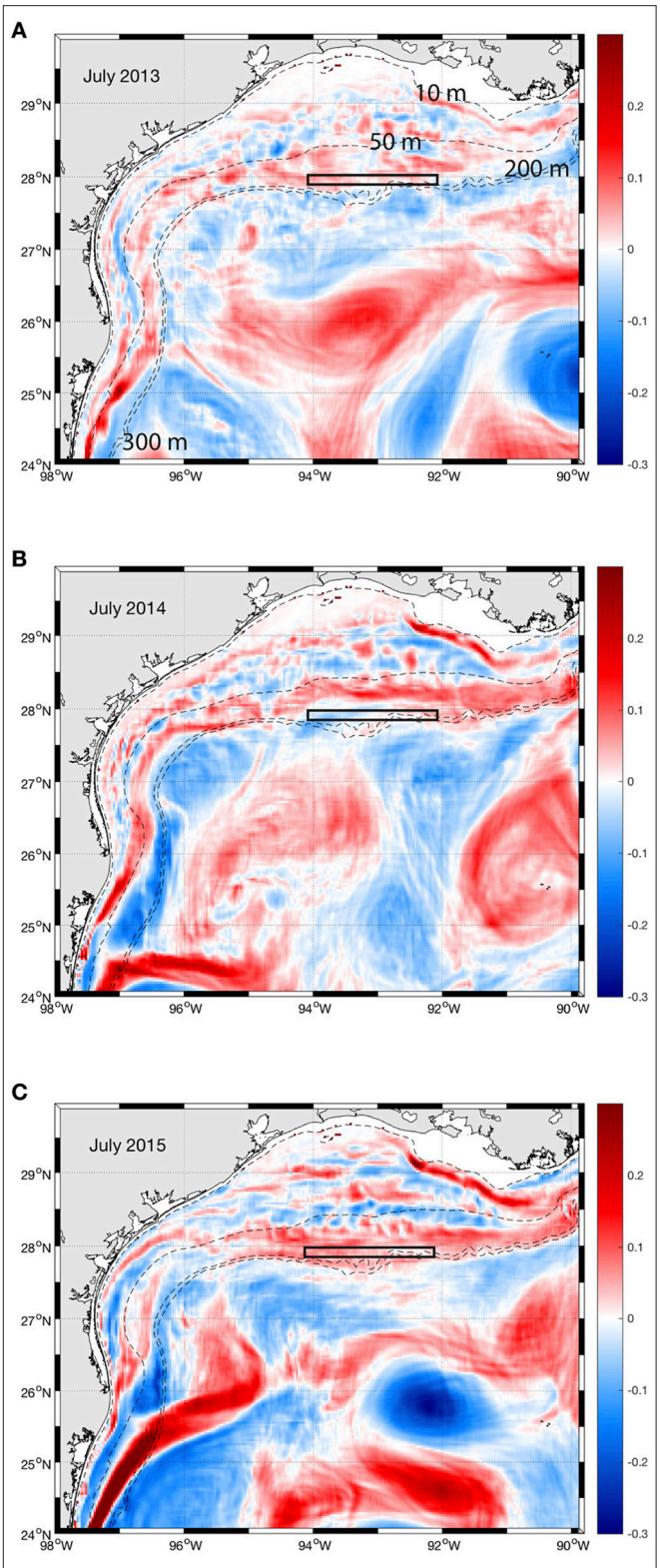

FIGURE 6 | Monthly mean normalized relative vorticity (ζ/f) at 10 m in the northwest Gulf of Mexico. ζ is the curl of the velocity and f is the Coriolis parameter. Numbers show the isobaths depth. **(A)** July 2013. **(B)** July 2014. **(C)** July 2015. The black rectangle shows the location of the banks (West FGB, East FGB, Bright, Geyer, Mc Grail).

that the NW GOM is well mixed. However, genetic data also showed a downstream to upstream net migration likely explained by the seasonal freshwater westward transport over geological timescales (Oey et al., 2005; Schmahl et al., 2008). The difference in findings between our study and Studivan and Voss (2018; associate manuscript) study is most likely due to the short integration time (i.e., demographic) vs. long (i.e., genetic) timescales, which happened to encompass 2 years over three of intense eddy impingement, which yielded the eastward larval transport. Given the possibility that individual coral colonies can live hundreds of years, a relatively rare larval dispersal event from one bank to the other is likely enough to observe low population differentiation between two banks. More importantly, the results from the demographic (this study), and genetic Studivan and Voss (2018; associate manuscript) connectivity suggest that the reduced demographic connectivity is sufficient to sustain high genetic connectivity between all banks.

Notwithstanding, our biophysical model was used only to provide a snapshot of the larval dynamics of *M. cavernosa* in some of the northwestern MCEs of the GOM. Considering what seem to be the recent yearly variability of the larval connectivity patterns and of the accrued eddy impingement in the region, eastward dispersal events similar to the ones described here may occur more often in the future. The singularity of our results suggests that the years selected were atypical, in particular 2014 and 2015. Our results' annual variability highlights the necessity to perform modeling studies over enough years, particularly in small-scale larval dispersal studies, in order to smooth the effects rare events (Holstein et al., 2015a; Kough and Paris, 2015; Chérubin and Garavelli, 2016; Davies et al., 2017). Furthermore, because of a lack of available larval biological

data on *M. cavernosa*, our modeling approach used PLD and buoyancy data from another scleratinian species of the Caribbean region (i.e., *O. faveolata*). Although these two species are closely related, more studies on the larval life cycle of *M. cavernosa* are needed to improve our knowledge on its larval dynamics.

Isolated mesophotic reefs, such as the northwestern banks of the GOM, have a role in the persistence of coral reefs population as well as in the supply of larvae toward remote MCEs (Thomas et al., 2015). Findings on connectivity of *M. cavernosa* among banks of the NW GOM from our study and Studivan and Voss (2018; associate manuscript) study support the future management plan for expanding FGBNMS by including Bright, Geyer, and McGrail Banks. Moreover, the large-scale dispersal patterns of *M. cavernosa* in the GOM observed in our study emphasizes the necessity of future connectivity research between all MCEs in the region using multiple species models to assess the possibility of establishing a regional conservation plan. Finally, our study associated with Studivan and Voss (2018; associate manuscript)'study show the relevance of combining modeling and genetic methods in investigating the connectivity patterns of marine populations for management purpose.

DATA AVAILABILITY STATEMENT

The data are available in the GitHub repository (https://github.com/lgaravelli/data-cavernosa-dispersal).

AUTHOR CONTRIBUTIONS

LG, MS, JV, and LC designed the study. MS and JV provided the coral density and depth distribution data. AK and JF performed the larval experiments in laboratory. LC provided the hydrodynamic model outputs. LG developed the larval dispersal model, performed the simulations, analyzed the data, and wrote the paper. All authors contributed to the final version of the manuscript.

FUNDING

This study was funded by the NOAA Office of Ocean Exploration and Research under awards NA09OAR4320073 and NA14OAR4320260 to the Cooperative Institute for Ocean Exploration, Research and Technology (CIOERT) at Florida Atlantic University-Harbor Branch Oceanographic Institute.

ACKNOWLEDGMENTS

We thank the NOAA/NOS National Centers for Coastal Ocean Science (NCCOS) for maps of predictable coral suitable habitat in the Gulf of Mexico and Emma Hickerson of FGBNMS for information on *Montastraea cavernosa* reproduction at East and West Flower Garden Banks. Samples of *M. cavernosa* for larval competency trials were collected under Broward County permit #DAN1410-037 and Florida Fish and Wildlife Conservation Commission permit #SAL-15-1645-SCRP. This is Contribution Number 2148 from Harbor Branch Oceanographic Institute at Florida Atlantic University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00174/full#supplementary-material

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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

ORIGINAL RESEARCH
published: 18 July 2018
doi: 10.3389/fmars.2018.00253

Warmer Water Affects Immunity of a Tolerant Reef Coral

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Corals are multipartite sedentary organisms, which have evolved complex, physiological networks in order to survive perturbations and environmental fluctuations. However, climate change is warming tropical waters, pushing the limits of coral tolerance and driving global declines. Coral susceptibility to thermal anomalies is variable among species and through time, and directly relates to constituent immunity. Constituent immunity refers to immune activities required to ensure homeostasis, whereas an immune response is acutely heightened immune activity to a perturbation. Understanding the mechanisms behind coral health, and sustained health through adverse conditions, is increasingly important for establishing effective reef conservation and restoration projects. However, most experimental studies of coral health use species that are highly susceptible to thermal events, potentially skewing our understanding. To determine the influence of warmer water on immunity, activities of key coral immune pathways and an antioxidant were compared under ambient (27°C) and warmer water (32°C), and between injured and uninjured (control) branches of the tolerant reef coral *Porites cylindrica*. Three types of phenoloxidase, mono-phenoloxidase, *ortho*-diphenoloxidase and *para*-diphenloxidase, indicative of two melanin synthesis pathways (the tyrosinase and laccase-type), and peroxidase were measured at 0 (control), 1, 6, 24, 48, and 168 h post-injury. All four enzymes demonstrated consistent levels of activity under ambient conditions (27°C), indicating constituent immunity. Upon injury at ambient temperature, all enzyme activities were significantly higher 1 h post-injury as compared to uninjured controls, demonstrating a comprehensive immune response to tissue disruption. Under warmer water, constituent immunity increased through time indicative of immune modulation to maintain homeostasis. However, warmer water, within the non-bleaching summer range, suppressed the immune response to injury, delaying it by 24 h. Therefore, upon the environmental cue of warmer water, the tolerant coral *P. cylindrica* may divert resources away from immune responses (immunosuppression) while enhancing constituent immunity (immune modulation) so as to maintain health through sub-optimal conditions. These changes in immunity with warmer water demonstrate that temperature affects coral immunity and, for this tolerant coral, triggers immune-modulation that may provide cross-tolerance to perturbations more frequent in summer months, such as bleaching and disease.

Keywords: coral, immunity, tolerance, immunosuppression, climate change, bleaching, immune modulation

OPEN ACCESS

Edited by:
Michael Sweet,
University of Derby, United Kingdom

Reviewed by:
Simon K. Davy,
Victoria University of Wellington, New Zealand
Melissa Susan Roth,
University of California, Berkeley, United States

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *29 April 2018*
Accepted: *29 June 2018*
Published: *18 July 2018*

Citation:
Palmer CV (2018) Warmer Water Affects Immunity of a Tolerant Reef Coral. Front. Mar. Sci. 5:253. doi: 10.3389/fmars.2018.00253

INTRODUCTION

Immunity underpins organism health and survival and determines tolerance (Sheldon and Verhulst, 1996; Palmer et al., 2010; Palmer, 2018). Accelerating climate change, due to global carbon emissions, is challenging the survival of reef corals, and therefore the persistence of functional coral reefs (Hughes et al., 2018a). Tropical waters are warming to temperatures beyond the range of normal diurnal and seasonal fluctuations historically experienced by reef corals. This shift is more frequently inducing breakdowns in the mutualism between the coral and microscopic algae, *Symbiodinium* spp., known as coral bleaching (Hughes et al., 2018b). Corals have differing tolerances to shifts in thermal regimes, as evidenced by the variable extent of bleaching among corals during extreme thermal events (Marshall and Baird, 2000; van Woesik et al., 2011; Hughes et al., 2018a). This variation suggests that coral holobiont immune systems, as proponents of survival and determinants of tolerance, are variably effective and are being increasingly pushed to and beyond their limits (Palmer et al., 2010, 2012).

Immunity is costly with energetic investment consequently being diverted away from other life history traits such as reproduction and growth (Sheldon and Verhulst, 1996). Immune system activity is therefore dynamic in the pursuit of optimal health, operating with finite resources under fluctuating biotic and abiotic conditions. Immunity also incurs the cost of self-harm, known as autoimmunity, and the additional resource costs required to mitigate or recover from it (Sadd and Schmid-Hempel, 2009). Therefore, at any given time, the manifestation of organism immunity, such as that of the coral holobiont, represents current physiological trade-offs operating under evolutionary constraints i.e., Life history trade-offs (Sadd and Schmid-Hempel, 2009; see Palmer, 2018).

There are two aspects of organism immunity. One is constituent immunity that maintains holobiont homeostasis under normal, fluctuating conditions. Constituent immunity provides surveillance and maintenance in the absence of an infection or acute perturbation—similar to our circulating white blood cells (Tauber, 2015). Levels of coral constituent immunity relates directly to both bleaching and disease susceptibility (Palmer et al., 2010). The second aspect of immunity is the immune response. This is acute, localized heightened immune activity in the presence of a threat so as to reestablish homeostasis (Pastori and Foyer, 2002; Tauber, 2015; Foyer et al., 2016). Of these, constituent immunity provides continual health maintenance at low cost, whereas an immune response provides acute protection incurring comparatively high cost (Sheldon and Verhulst, 1996; Sadd and Schmid-Hempel, 2009). Therefore, immune responses are used sparingly, are tightly controlled (Sheldon and Verhulst, 1996) and are a balanced consequence of signaling, organism condition and overall immune strategy (Lazzaro and Rolff, 2011; Palmer, 2018).

Corals are sedentary, multipartite organisms that have evolved complex, integrated physiological systems to survive fluctuations in local environmental conditions, predation and disease (e.g., Miller et al., 2007; Palmer and Traylor-Knowles, 2012; Mydlarz et al., 2016). As such, differing immune strategies have likely evolved among corals to maintain optimal fitness while mitigating the energetic and autoimmune costs in upregulating an immune response (Palmer et al., 2010, 2011c). Various coral immune strategies, and their efficacies, are therefore evident in comparative tolerances to perturbations (Palmer, 2018), such as thermal events (e.g., Hawkins et al., 2014, 2015; Hughes et al., 2018a). However, coral stress and health studies are predominantly conducted on fast growing, branching coral species e.g., *Acropora* spp. (Moya et al., 2012; van de Water et al., 2015a; Traylor-Knowles et al., 2017) and *Pocillopora* spp. (Sogin et al., 2016), with few exceptions (e.g., Fuess et al., 2017). The well-studied fast-growing coral genera have amongst the lowest levels of constituent immunity and are the most susceptible to disease and bleaching (Palmer et al., 2010, 2012). It is likely, therefore, that the immune strategy of these susceptible corals to perturbations differs from that of coral species with higher constituent immunity that are more tolerant of them (Palmer, 2018). By focusing research efforts on the immune responses of these susceptible corals, our information on coral tolerance, resistance and, ultimately, reef resilience as a whole may be both skewed and underestimated (Palmer, 2018).

Corals, as other invertebrates, use phenoloxidase cascades, also known as melanin synthesis pathways, as mainstays of immunity (Cerenius et al., 2010; Palmer and Traylor-Knowles, 2012). Several types of phenoloxidase cascades have been identified in multiple corals, as determined by enzymatic activity using different substrates (Palmer et al., 2012) and corroborated by gene searches within available coral genomes (Palmer, unpublished). Of the phenoloxidase pathways, the more cytotoxic tyrosinase type is the better understood within invertebrates in general, and involves both mono-phenoloxidase and *o*-diphenoloxidases to resist infection (Cerenius et al., 2010; Mydlarz and Palmer, 2011; Palmer et al., 2012). The laccase-type phenoloxidase pathway, indicated by *p*-diphenoloxidase activity, is likely involved in reinforcing damaged tissue and may have a sensory function (Mydlarz and Palmer, 2011; Palmer et al., 2012). Both pathways have the potential to deposit melanin—a redox-active brown/black pigment that absorbs light. Found within mobile coral cells, melanin may offer photoprotection of algal endosymbionts and mitigate bleaching (Palmer et al., 2010). The phenoloxidase pathways produce cytotoxic radicals, particularly the tyrosinase type, which must be tightly controlled to avoid autoimmunity (Cerenius et al., 2010). Immunity-induced oxidative stress and that experienced during warmer water and high light conditions, can be mitigated with increased antioxidant activity (Hawkins et al., 2015). Coral antioxidants include catalase, superoxide dismutase, peroxidase and fluorescent proteins, and may be used to maintain health by reducing autoimmunity and limiting self-harm (Palmer et al., 2009b; Mydlarz and Palmer, 2011; Hawkins et al., 2015).

With the intention to better elucidate how warmer water may affect coral immunity, constituent immunity and the immune responses of a tolerant Indo-Pacific reef-building coral species, *Porites cylindrica,* were investigated. The activities of three phenoloxidases (PO) as well as the antioxidant peroxidase were measured in response to injury at both ambient and elevated temperature.

METHODS

Sample Collection

Large, >50 cm diameter, and visibly healthy colonies of brown *Porites cylindrica* were located on the upper reef slope (∼1–2 m depth) in Pioneer Bay at Orpheus Island, Great Barrier Reef (GBR), Australia, and sampled in May 2008. From each of three colonies of *P. cylindrica*, 60 branches were removed (Tn = 180) and transported in 1 μm filtered seawater to the laboratory.

Experimental Set-Up

Six aquaria within temperature-controlled rooms at Orpheus Island Research Station were supplied with flow-through filtered seawater (1 μm) at ambient temperature (27°C), and with 12 h light/12 h dark light regimes using metal halide lights, with light levels maintained at 150–250 μmol quanta $m^{-2}s^{-1}$. Three aquaria were each designated to ambient or elevated temperature treatments, and the 180 coral branches were randomly allocated among them. Coral branches were held upright using labeled plastic clothes pegs. All six aquaria were maintained at ambient conditions for 3 days to allow branches to recover from sampling-wounds seal in this species in 48 h (Palmer et al., 2011b)- and to acclimate to aquaria conditions. On day four, three branches from each colony were randomly selected and placed into liquid nitrogen as pre-treatment controls (0 h), and stored at −30°C. Water temperatures in three of the aquaria were then increased by 1 to 1.5°C per day, for 3 days and stabilized at 32°C. After 2 days at 32°C, three branches from each colony were sampled randomly from both elevated and control temperature treatments. A wound was then created on half of the remaining branches using bone-cutters to score a ring of damage approximately 2.5 mm deep, 1 cm below the branch tip. Three injured branches and three non-injured (control) branches were sampled from each colony per temperature treatment at 1, 6, 24, 48, and 168 h (7 d) post-injury. All samples were immediately snap-frozen in liquid nitrogen and stored at −30°C. For the duration of the experiment no mortality occurred and all coral branches appeared healthy with no visible paling (loss of zooxanthellae) or infection.

Biochemical Analyses

A ∼6 polyp-wide band of tissue surrounding the lesion was removed, and from equivalent areas on control branches, using an airgun with 100 $mmol.l^{-1}$ phosphate buffer with 5 $mmol.l^{-1}$ 2-mercaptoethanol (Sigma-Aldrich M7522; Palmer et al., 2008). While 2-mercaptoethanol has some inhibitory effect on phenoloxidase activity, the low concentration and methods ensured this was minimal and consistent among samples, making them directly comparable. Tissue from branch tips and bases were avoided. Resulting tissue slurries were frozen in liquid nitrogen and stored at −30°C. Thawed samples were homogenized using a vortex with glass beads for 1 min and centrifuged for 5 min to remove tissue debris, glass beads and the zooxanthellae. Supernatants were carefully removed and aliquots stored at −30°C. All samples were treated the same, ensuring direct comparability among samples despite any inadvertent PO degradation during processing.

Phenoloxidase (PO) activities were quantified as change in absorbance (at 410 nm) over time using the substrates: tyramine (Fluka 93810) for mono-phenoloxidase activity, dopamine hydrochloride (Sigma-Aldrich H8502) for *o*-diphenoloxidase activity and hydroquinone (Sigma H9003) for *p*-diphenoloxidase activity (as per Palmer et al., 2012). For each of the PO assays, three 10 μl aliquots of each sample extract were placed in wells of a clear 96-well microtiter plate. To each well, 50 μl of 50 $mmol.l^{-1}$ phosphate buffer (pH 7.5) and 25 μl of deionized water (DI) were added. Plates were then covered and incubated at either 27°C or 32°C, depending on the experimental treatment regime of each sample, for 15 min in order to record the activity of enzymes under experimental treatment conditions. The appropriate substrate (30 μl of 50 $mmol.l^{-}$) was then added and the change in absorbance for each well, including blank controls, was measured at 410 nm for 45 min. Peroxidase activity was measured by adding 40 μl of guaiacol (25 $mmol.l^{-1}$) to 10 μl of sample and 35 μl of 50 $mmol.l^{-1}$ phosphate buffer (pH 6.0). The reaction was activated by the addition of 25 μl of H_2O_2 (20 $mmol.l^{-1}$) and measured at 470 nm for 45 min. For each sample, the change in absorbance was calculated for the linear portion of the reaction curve and standardized to total protein concentration, as determined by the Quick Start Bradford assay (Bio-Rad).

Protein activities were compared between injury treatments (uninjured or injured) and temperature treatments (27°C or 32°C) using two-way repeated measures ANOVAs, executed using log-transformed data in SPSS. Data satisfied assumptions of sphericity, as determined with Mauchley's test, and homogeneity of covariance as determined by Box's test.

RESULTS

Porites cylindrica demonstrated significantly higher mono-phenoloxidase activity in injured than uninjured samples at ambient seawater temperatures (27°C) (**Table 1** and **Figure 1A**). Heightened mono-phenoloxidase activity was particularly noticeable 1 h post-injury, when it was 4.5-fold greater than for uninjured branches, indicating the rapid activation of an immune response. Levels of mono-phenoloxidase activity were similar between injured and uninjured branches between 6 and 168 h, (**Table 1**). At elevated water temperature (**Figure 1B**), there was no difference in mono-phenoloxidase activity between injured and uninjured branches, at any time point (**Table 1**), indicating a lack of response of this enzyme to injury in warm waters. However, mean mono-phenoloxidase activity of uninjured branches increased by approximately 3-fold over time in the elevated temperature treatment (**Table 1**), which was absent in the ambient water temperature treatment (**Figure 1**). Injured branches demonstrated a peak of activity at 24 h post-injury with elevated water temperature (**Figure 1B**), which was similar in magnitude to the peak of activity at 24 h post-injury in the injured treatment at ambient water temperature. Consistently, mono-phenoloxidase activity of injured branches over time was dependent on temperature (temperature x time, **Table 2**).

TABLE 1 | A summary of the *F*- and *P*-values results of the two-way repeated measures ANOVAs examining the effects of injury (uninjured controls vs. injured samples) and time post-injury (1, 6, 24, 48, and 168 h), and their interaction on immunity protein activity for both ambient (27°C) and elevated (32°C) temperatures.

Immunity enzyme	Injury (df = 1, 10)				Time (df = 4, 10)				Injury × time (df = 4, 10)			
	Ambient		Elevated		Ambient		Elevated		Ambient		Elevated	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Mono-PO	5.4	**0.04**	0.01	0.93	3.1	0.69	6.8	**<0.01**	3.0	0.07	0.5	0.84
o-diPO	4.7	0.06	0.6	0.48	0.5	0.71	8.3	**0.03**	1.1	0.40	1.7	0.23
p-diPO	3.5	0.09	0.1	0.78	2.7	0.09	0.9	0.49	2.3	**<0.01**	4.5	**0.03**
Peroxidase	13.8	**0.04**	0.01	0.92	2.8	0.09	8.0	**<0.01**	1.6	0.25	2.0	0.18

P values <0.05 are in bold.

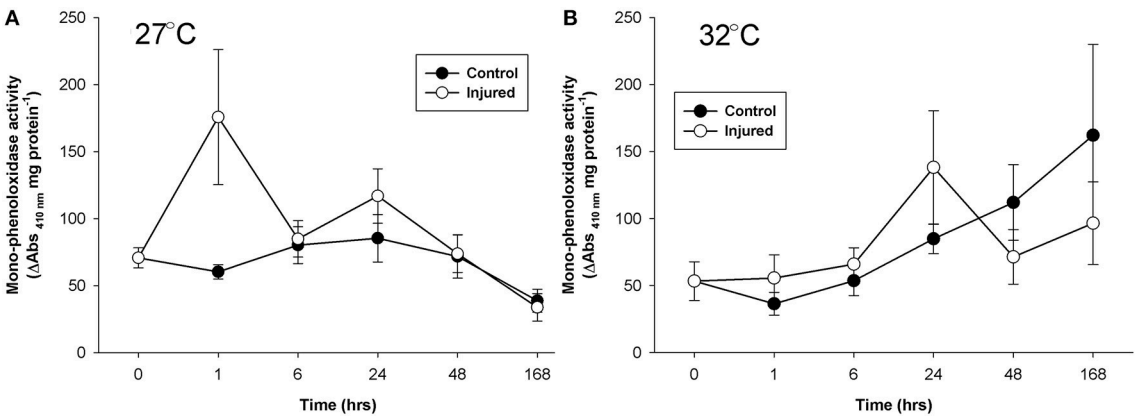

FIGURE 1 | Mean mono-phenoloxidase activity (± SE) for uninjured (control) and injured branches of *P. cylindrica* at: **(A)** ambient (27°C), and **(B)** elevated (32°C) water temperature over time post-injury.

Mean *o*-diphenoloxidase activity at ambient water temperature did not differ significantly between injury treatments, or over time (**Figure 2A**, **Table 1**). However, at both 1 and 24 h post-injury, *o*-diphenoloxidase activity was more than 2-fold higher in the injured treatment than the control treatment. This indicates a rapid response of *o*-diphenoloxidase activity to injury, which was sustained for 24 h. At elevated water temperature (**Figure 2B**), there was no difference in *o*-diphenoloxidase activity between injured and uninjured branches (**Table 1**), indicating no immediate response to injury. However, at elevated water temperature *o*-diphenoloxidase activity in uninjured samples at 24 h was equivalent to that of the injured ambient samples (**Figure 2B**), suggesting that the response of *o*-diphenoloxidase activity is delayed at higher temperatures. Additionally, and similar to the mono-phenoloxidase activity, control levels of *o*-diphenoloxidase activity significantly increased over time by approximately 4-fold at elevated water temperature (**Table 1**) and overall were higher than control levels at ambient water temperature (**Table 2**). This indicates a response of *o*-diphenoloxidase activity to warmer water.

Mean *p*-diphenoloxidase activity with injury depended upon the time post-injury at both ambient and elevated water temperature (injury × time, **Table 1**) and the timing of activity in response to injury significantly varied between the water temperature treatments (Time, **Table 2**). At ambient water temperature *p*-diphenoloxidase activity of injured samples at 1 and 24 h post-injury was approximately 3-fold and 2.5-fold higher than controls, respectively (**Figure 3A**). This up-regulation in activity indicates a direct response of laccase-type PO activity to injury. At elevated water temperature (**Figure 3B**), there was a trend of increasing *p*-diphenoloxidase activity over time in the control treatment, although this was not significant (Time, **Table 1**).

At ambient water temperature, mean peroxidase activity (**Figure 4A**) was significantly higher in the injured treatment as compared to the control treatment (Injury, **Table 1**). There was a 25-fold increase in peroxidase activity with injury at 1 h at ambient water temperature, which remained up-regulated, compared to controls, for 48 h, demonstrating a direct and sustained response of this antioxidant to injury. At elevated water temperature (**Figure 4B**), there was no significant difference in peroxidase activity between controls and injured samples (Injury, **Table 1**), although activity varied over time post-injury (Time, **Table 1**). Overall, peroxidase activity differed significantly with the temperature treatments over time (Temperature x time, **Table 2**), and control levels of activity were significantly higher at elevated water temperature as compared to ambient, likely driven

TABLE 2 | A summary of the *P*-values results of the two-way repeated measures ANOVAs examining the effects of temperature (ambient 27°C vs. elevated 32°C) and time post-injury (1, 6, 24, 48, and 168 h), and their interaction on immunity protein activity for both uninjured controls and injured samples.

Immunity enzyme	Temperature (df = 1, 10)				Time (df = 4, 10)				Temperature × time (df = 4, 10)			
	Control		Injured		Control		Injured		Control		Injured	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Mono-PO	0.1	0.73	1.5	0.25	2.1	0.15	0.6	0.70	2.8	0.08	5.5	**0.01**
o-diPO	5.5	**0.04**	0.01	0.83	1.0	0.45	2.4	0.12	1.4	0.31	5.5	**0.01**
p-diPO	4.7	0.06	0.05	0.83	1.4	0.30	3.8	**0.04**	1.6	0.26	1.1	0.41
Peroxidase	11.0	**<0.01**	7.5	**0.02**	1.0	0.44	4.5	**0.02**	0.8	0.53	6.0	**<0.01**

P values <0.05 are in bold.

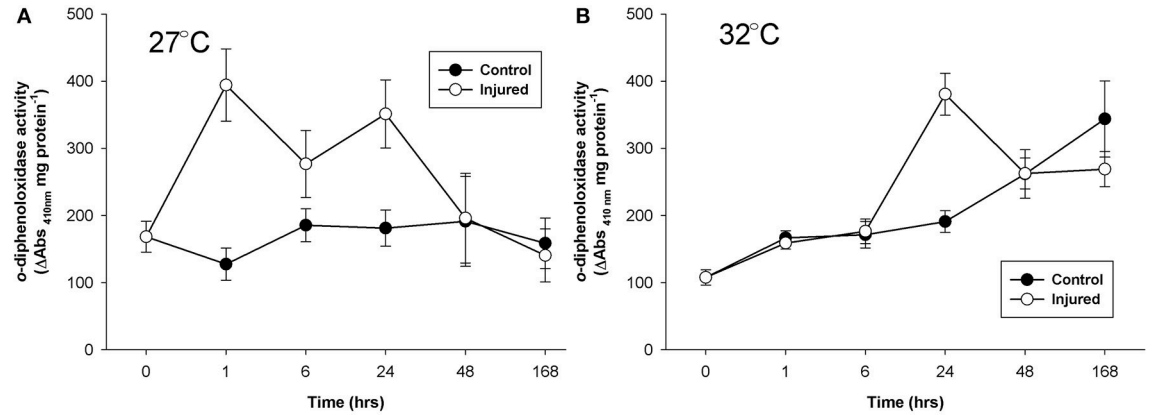

FIGURE 2 | Mean *o*-diphenoloxidase activity (± SE) for uninjured (control) and injured *P. cylindrica* at: **(A)** ambient (27°C) and, **(B)** elevated (32°C) water temperature over time post-injury.

by the 6 h time point, whereas the reverse was true for the injured samples (Temperature, **Table 2**). These results demonstrate that peroxidase activity increases in response to both elevated water temperature and injury, but is suppressed when these treatments are combined.

DISCUSSION

Porites cylindrica demonstrated consistent activity of all investigated enzymes in the absence of injury at 27°C, confirming the presence of constituent immunity and its stability under constant ambient conditions (Palmer et al., 2010; van de Water et al., 2016). An immune response occurred within 1 h of injury under ambient water temperature. Warmer water induced a gradual ramping of constituent immunity in control samples and the immune response to injury was altered as compared to ambient conditions. Elevated water temperature, within non-bleaching summer range, had an immuno-enhancing effect on constituent immunity but an immunosuppressive impact on the *P. cylindrica* immune response.

Immune Response Under Ambient Conditions

The constituent immunity levels of *P. cylindrica,* under ambient control conditions, remained approximately constant for the duration of the experiment, representing activities used to maintain homeostasis in the absence of a perturbation (Palmer et al., 2010; Tauber, 2015; Palmer, 2018). Of 18 Indo-Pacific hard coral species *P. cylindrica* has amongst the highest levels of constituent immunity (Palmer et al., 2012). Such investment into maintaining homeostasis fosters tolerance and promotes survival through both biotic and abiotic fluctuations and perturbations (Palmer, 2018).

The immune response of *P. cylindrica* to injury occurred within 1 h and was demonstrated by heightened activity of all four enzymes as compared to their constituent levels (Tauber, 2015). These results reconfirm the involvement of phenoloxidases and peroxidase in a *P. cylindrica* immune response (Palmer et al., 2008, 2011a) and are consistent with the timings of de-granulation of melanin-containing granular cells observed in *P. cylindrica* with injury (Palmer et al., 2011b). The tyrosinase-type phenoloxidase pathway (mono-phenoloxidase and *o*-diphenoloxidase) is highly cytotoxic, and therefore potentially plays a pathogen-killing or sterilization role at the wound site (van de Water et al., 2015b). The laccase-type pathway, indicated by *p*-diphenoloxidase activity, is less cytotoxic and likely used for structural reinforcement of injured coral tissue (Palmer et al., 2011b). Coincident with the increase in cytotoxic immune pathway activity was the corresponding heightened activity of the antioxidant peroxidase, a hydrogen peroxide-scavenging enzyme. Peroxidase activity, serving a protective

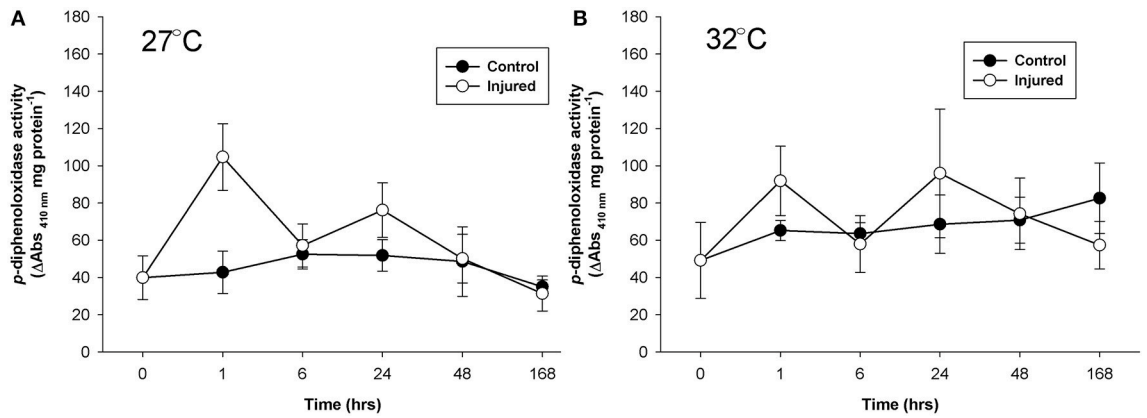

FIGURE 3 | Mean *p*-diphenoloxidase activity (± SE) for uninjured (control) and injured *P. cylindrica* at: **(A)** ambient (27°C) and **(B)** elevated (32°C) water temperature over time post-injury.

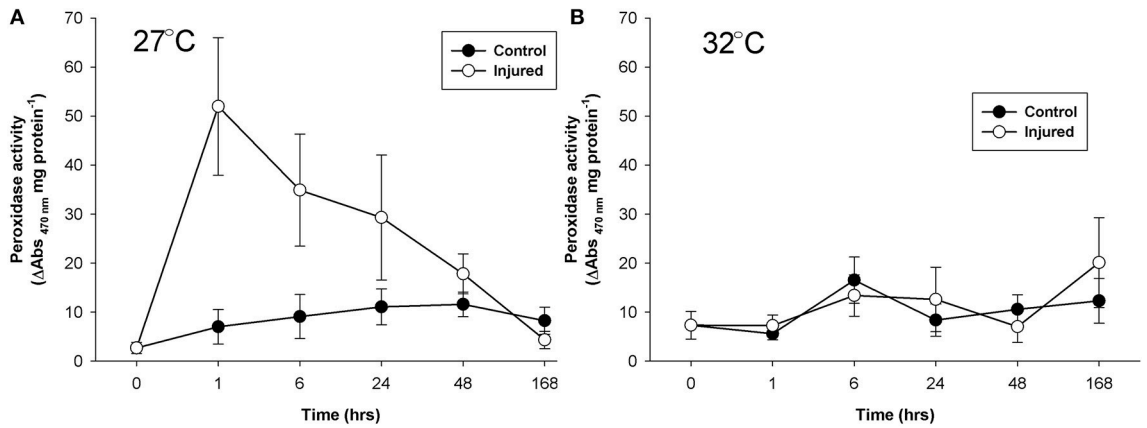

FIGURE 4 | Mean peroxidase activity (± SE) for control and injured *P. cylindrica* at **(A)** ambient (27°C) and **(B)** elevated (32°C) water temperature over time post-injury.

role, upon injury was approximately 25-fold higher than control levels, whereas phenoloxidase activities were just 2- to 4.5-fold higher, suggestive of their toxicity and tight regulation due to the potential for self-harm.

Inducing a rapid immune response to efficiently seal a wound restores homeostasis and reduces the likelihood of infection, and therefore promotes survival. Using proteolytic cascades enables rapid immune activation, by-passing transcription. Given the energetic cost and the autoimmune risk of inducing an immune response (Lee, 2006; Palmer, 2018) benefits of *P. cylindrica* mounting a response to injury must out-weigh the costs incurred or the risk of not doing so (Lazzaro and Rolff, 2011). Therefore, under ambient and healthy control conditions, *P. cylindrica* has the resources available to mount an effective immune response and to mitigate autoimmunity. However, decreased activity of enzymes after the immune response, as compared to constituent immunity levels, are suggestive of the cost of heightened immunity (Harvell, 1990). The immediacy of the coral immune response is characteristic of innate immunity (Palmer et al., 2008, 2011c) and highlights how easy it is to miss a coral immune peak. Caution should therefore be taken to avoid underestimating or misinterpreting a coral immune response in studies where sampling times are delayed (e.g., van de Water et al., 2015b; Wright et al., 2016).

Immune Trade-Offs With Warmer Water

Warmer water, within normal summer ranges, enhanced constituent immunity of *P. cylindrica* controls and suppressed the immune response of injured samples. These data, compared to those of ambient conditions, indicate a shift in immune strategy with warmer water. This provides further evidence that coral holobiont immune systems are responsive—phenotypically plastic—to environmental shifts (e.g., Mydlarz et al., 2008; Palmer et al., 2011c; Pinzón et al., 2015; Palmer, 2018). Such temperature-induced immune modulation and consequent shift in immune strategy is likely the result of trade-offs between maintaining optimal health and the costs incurred in doing so—both energetic and autoimmune (Sadd and Schmid-Hempel, 2009; Lazzaro and Rolff, 2011; Palmer, 2018).

The gradual investment into constituent immunity with warmer water may represent a relatively low-cost strategy to maintain homeostasis and minimize the necessity of costly

and dangerous immune responses (Lee, 2006; Palmer, 2018). Investment into constituent immunity with temperature change is supported by the direct relationship of high coral constituent immunity with low disease and bleaching susceptibility (Palmer et al., 2010, 2012). Such immuno-dynamics are suggestive of the cross-tolerance phenomenon, whereby exposure to one type of disturbance, e.g., adverse environmental conditions, enhances protection against concurrent disturbances, such as infection or injury (Foyer et al., 2016).

Elevating constituent immune activity with the environmental cue of warmer water seems an appropriate strategy since both disease and bleaching pose higher threats during summer months (e.g., Sato et al., 2009). Therefore, there is the potential that *P. cylindrica* has adapted to modulate immunity—i.e., enhance constituent immune function- in order to increase survival during warmer conditions (Palmer, 2018). However, the generalized protection afforded by increased investment into constituent immunity and consequent cross-tolerance will come at a cost to other biological functions (Harvell, 1990; Sheldon and Verhulst, 1996). Such costs could be manifested as an inhibition of an effective immune response to acute, localized perturbations, such as injury and as observed in this study. Alternatively, or additionally, costs of elevated constituent immunity may be exhibited by compromised reproductive output or growth. However, in this study it is impossible to determine the evolutionary-scale influences and trade-offs from the physiological ones (Harvell, 1990; Sadd and Schmid-Hempel, 2009).

With warmer water, the immune response to injury occurred at 24 h with both mono and *o*-diphenoloxidase activities. In this temperature-induced delayed immune response, heightened activity was present but limited for *p*-diphenoloxidase and absent for peroxidase. The lack of immune response within 1 h of injury under warmer water suggests that implementing an immune response would be too costly. While variable through time, activity of the antioxidant peroxidase did not demonstrate a clear response to either temperature or injury. This suggests that the temperature changes in this experiment did not induce oxidative stress conditions (Jin et al., 2016) and/or that an alternative strategy may have been used to mitigate autoimmunity—possibly relying on the redox nature of melanin systhesis (Cerenius et al., 2010). After the immune response peak of injured samples, immune activity levels fell below constituent immunity levels of the control samples, indicative of the high cost of an immune response. The immune response, albeit delayed, appears to come at the cost of constituent immunity and suggests that *P. cylindrica* may be more vulnerable to disease and bleaching after physical injury during warmer water conditions. Such immune-energetics have implications for coral reefs during the summer months, when water temperatures are higher and cyclone activity raises the potential for physical injury.

Having a thermal cue for increasing constituent immunity in a tolerant coral seems appropriate given that elevated water temperature on coral reefs is associated with high photosynthetically active radiation (PAR; Brown and Dunne, 2015) and increased pathogen load and virulence (Harvell et al., 1999, 2002; Mydlarz et al., 2006). Two, possibly interacting, hypotheses of how water temperature triggers immune modulation include; (1) that during periods of elevated water temperature coral cells release "danger" components, such as nitric oxide and uric acid (Hawkins et al., 2014), which modulate immunity (Gallucci et al., 1999; Palmer, 2018) and (2) that the increase in water temperature is the cue in itself detected, potentially, by an anthozoan endocrine system yet-to-be thoroughly explored (Tarrant, 2015), which signals the immunity network. Regardless of the mechanism, inducing melanin synthesis pathways with warmer water may be particularly helpful as melanin is a photoprotective, radical-scavenging, brown to black pigment (Meredith et al., 2006) that is located within mobile coral cells (Palmer et al., 2008). Melanin synthesis pathway activity therefore has the potential to mitigate coral bleaching by affording photoprotection of *Symbiodinium* spp. (Palmer et al., 2010).

Consistent with a role in mitigating coral bleaching, increased prophenoloxidase activity (the zymogen form of phenoloxidase) has been documented in bleached *Montastraea faveolata* as compared to controls (Mydlarz et al., 2009). However, results of studies of other coral species at various stages of thermal stress are highly variable (e.g., Palmer et al., 2011a,c; Pinzón et al., 2015; van de Water et al., 2016). Variability in phenoloxidase activities both among and within coral species during thermal stress is likely the result of the physiological, ecological and historical context of the coral, leading to different immune strategies and energetic trade-offs (Sadd and Schmid-Hempel, 2009; Palmer, 2018).

Coral immunity is intrinsically linked to ecological-scale patterns of coral health and ultimately, therefore, coral reef resilience (Palmer, 2018). This study demonstrates that *P. cylindrica* is able to make physiological adjustments in response to environmental cues in order to promote survival. The potential for corals to demonstrate cross-tolerance provides a tantalizing avenue of exploration as we seek to effectively conserve, restore and manage coral reefs.

AUTHOR CONTRIBUTIONS

CP conceived, designed, implemented, conducted the research and wrote the paper.

FUNDING

Research funding was provided by a Graduate Research School Grant, James Cook University, awarded to CP and a Natural Environment Research Council (NERC) UK grant and studentship to J. Bythell and CP, respectively.

ACKNOWLEDGMENTS

I would like to thank John Bythell and Bette Willis for comments on early drafts and Allison Paley for field assistance. A version of this research first appeared in CV Palmer's PhD thesis (Palmer, 2010).

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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 Marine Science

ORIGINAL RESEARCH
published: 10 August 2018
doi: 10.3389/fmars.2018.00275

Ultra-Violet Radiation Has a Limited Impact on Seasonal Differences in the Acropora Muricata Holobiont

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *03 April 2018*
Accepted: *20 July 2018*
Published: *10 August 2018*

Citation:
van de Water JAJM, Courtial L, Houlbrèque F, Jacquet S and Ferrier-Pagès C (2018) Ultra-Violet Radiation Has a Limited Impact on Seasonal Differences in the Acropora Muricata Holobiont. Front. Mar. Sci. 5:275. doi: 10.3389/fmars.2018.00275

Environmental conditions are known to influence corals and their associated communities of microorganisms. However, our insights into the impacts of seasonal changes in ultraviolet radiation (UVR) on both coral physiology and microbiome remain very limited. To address this challenge, we maintained the coral *Acropora muricata* shaded from UVR or under ambient UVR levels during two contrasting seasons, i.e. summer and winter, and assessed the impact of UVR on the coral holobiont at each season. To this end, we analyzed the physiology (e.g., calcification, protein content, photosynthesis-related parameters) and coral microbiota composition, as well as the abundance and composition of the microbial communities and organic matter contents of the surrounding seawater. Our results show major seasonal effects on coral phenotype: (1) a lower host biomass and photosynthesizing, but fast calcifying phenotype in summer, and (2) a higher host biomass and photosynthesizing, but slow calcifying phenotype in winter. UVR had only a significant impact on *Symbiodinium* functioning. Specifically, high UVR levels reduced photosynthesis efficiency in summer, but an increase in chlorophyll *a* content may have compensated for this effect. The coral microbiota, which was variable but generally dominated by *Endozoicomonas*, was not affected by UVR, but its composition differed between seasons. In contrast, UVR had a major, but differential impact on the seawater microbial communities at both seasons. Particularly in summer, bacteria from the Alteromonadaceae were significantly more abundant (15-fold; up to 75%) in seawater under ambient UVR levels. Overall, our study suggests that UVR has only a limited impact on coral holobiont composition and functioning, despite major fluctuations in the surrounding seawater microbiome; seasonal changes in the holobiont are thus mostly driven by other environmental factors.

Keywords: coral, holobiont, symbiosis, ultra-violet radiation, seasonality, bacteria, symbiodinium, physiology

INTRODUCTION

Population dynamics and reproductive cycles of many species are influenced by seasonal variations in environmental factors (Clarke, 1988; Gooday et al., 1990; Coma et al., 2000), leading to a long-standing interest in the way organisms react to the changing of seasons. Although tropical corals live in a relatively stable environment, they experience seasonal fluctuations in environmental

conditions, such as seawater temperatures (up to 9°C, Dandan et al., 2015) or photosynthetically active radiation (PAR; up to 5 times, Browne et al., 2015). Nutrient levels and water quality can also vary by a factor 5 between seasons, through for example, sediment loading, or metal and nutrient pollution (Browne et al., 2015; Watson et al., 2017). Seasonality is reflected both in the coral physiology (Crossland, 1984; Warner et al., 2002; Ulstrup et al., 2008) and in the composition of coral-associated microbial communities (Brown et al., 1999; Fitt et al., 2000; Littman et al., 2010; Li et al., 2014). The symbiotic association of the coral animal with its diverse assemblages of microorganisms consisting in bacteria, archaea, fungi, viruses, and protists, including the dinoflagellate algae *Symbiodinium* (Kelly et al., 2014; Apprill et al., 2016), is termed the coral holobiont (Rohwer et al., 2002).

Most studies, which investigated seasonal changes in the physiology and composition of the coral holobiont, have focused on the effect of PAR intensities on *Symbiodinium* density (Fitt et al., 2000) and photosynthetic capacities (Edmunds and Davies, 1986; Warner et al., 2002; Ulstrup et al., 2008). The effect of seawater temperature variations on coral physiology has also received a lot of attention; in particular because episodes of temperature extremes can induce coral bleaching (loss in symbionts and photosynthetic pigments), resulting in decreased photosynthesis and calcification rates (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2017; Hughes et al., 2017; Wolff et al., 2018), as well as shifts in the composition of coral-associated microbial communities (Littman et al., 2010, 2011; Ziegler et al., 2017). However, little is known about the response of coral holobionts to seasonal changes in ultraviolet radiation (UVR) levels. UVR is an important component of the sunlight received by corals in shallow waters, where high intensities prevail (Madronich et al., 1998). UVR levels actually show depth and seasonal variations (Smith and Baker, 1981; Torres et al., 2007; Zepp et al., 2008), which may contribute to the seasonal changes that have been observed in coral physiology. For example, variations in tissue content of mycosporine-like amino acids (MAAs), which protect corals from the deleterious effects of UVR (Shick et al., 1996; Shick and Dunlap, 2002) has been attributed to changes in UV exposure (Lesser and Lewis, 1996; Lesser, 2000), and severe bleaching has been linked to the concomitant increase in seawater temperature and UVR levels (Lesser et al., 1990). Despite these few studies, the seasonal changes in UVR levels on *Symbiodinium* functioning, coral calcification, organic matter release and coral microbiota need to be further studied. UVR also affects the growth, metabolism, and viability of seawater microorganisms (Korbee et al., 2010; Llabrés et al., 2010; Carrillo et al., 2015), but the seasonal effect of UVR exposure on reef bacterioplankton has been poorly studied (Lyons et al., 1998; Conan et al., 2008; Häder et al., 2015). Changes in the composition of bacterioplankton surrounding coral colonies can affect the composition of coral-associated bacterial communities (Wear and Thurber, 2015 and references therein); the contrary may be true as well, as corals release microbes into the environment, for example via mucus excretion (Allers et al., 2008; Garren and Azam, 2012; Nelson et al., 2013; Taniguchi et al., 2015).

To better understand the impact of UV radiation on coral holobiont functioning, we investigated the physiology and microbiota of the holobiont of the scleractinian coral *Acropora muricata* and its surrounding seawater during two seasons (summer and winter) under ambient and reduced UVR levels. *A. muricata* is a dominant coral species in New Caledonia, whose populations have severely suffered from a mass bleaching events in 2016. Such knowledge provides novel insights into how environmental conditions influence the status of the coral holobiont and its interactions with seawater microbial communities.

EXPERIMENTAL PROCEDURES

Coral Collection and Experimental Setup

Twenty 3 cm-long nubbins of *Acropora muricata* were collected from the 10 different parent colonies at 3 m depth on the reef of Îlot Maître in the Nouméa lagoon (22°19′S, 166°24′E) in January 2016 (Austral summer - before the mass bleaching event of February 2016) (permit number: APA-NCPS-2016-001). In June 2016 (Austral winter), nubbins were collected from the same parental colonies. At both seasons, nubbins were evenly distributed in four 100 L outdoor experimental tanks continuously supplied with lagoon water at a rate of 72 L h^{-1}. Corals were fed once a week with *Artemia salina* nauplii. Photosynthetically active radiation (PAR) in the experimental tanks was maintained at the same level as *in situ* using shade cloths, and measured using a LI-1000 data logger connected to a LI-193 spherical quantum sensor (LI-COR, Lincoln, NE, USA). UVR levels were measured using an ILT1400 portable radiometer connected to SEL033/UVA/TD UVA and SEL240/UVB-1/TD UVB detectors (International Light Technologies, Peabody, MA, USA). In summer, the maximal irradiance obtained at midday on a sunny day was ∼900 μmole quanta m^{-2} s^{-1} PAR, ∼20 W m^{-2} ultraviolet A (UVA, 315–400 nm) and ∼1.2 W m^{-2} of ultraviolet B (UVB, 280–315 nm) radiation. In winter, irradiance levels on a sunny day at mid-day were ∼500 μmole quanta m^{-2} s^{-1} of PAR, ∼9 W m^2 of UVA, ∼0.7 W m^2 of UVB. Two out of the four tanks were shielded from UVR (condition hereafter called "no UV") using specific UVR filters (226 Lee U.V. filters), while the two other tanks received the ambient dose of UVR (condition hereafter called "UV"). Following collection, coral nubbins were maintained under experimental UVR conditions for 1.5 months prior to the assay incubations (described below) in summer and winter. At both seasonal time points, five nubbins from each condition (UV and no UV), were randomly sampled from the two tanks per condition, and used to assess tissue parameters (symbiont density, chlorophyll and protein concentrations), calcification rates and photosynthetic efficiency (assay details described below). Following experimental incubations, nubbins were frozen at −20°C until further analyses. The remaining five nubbins per condition were used to assess coral-associated bacterial communities (see details below).

All incubations were performed under the same temperature (29.3 ± 0.9°C in summer and 23.1 ± 0.6°C in winter) and light regime as the experimental tanks. Symbiont density, chlorophyll a and protein content, net and gross photosynthesis, respiration

and calcification rates were normalized to the nubbin surface area (cm^2) measured using the wax technique (Stimson and Kinzie, 1991).

Photochemical Efficiency

Five nubbins per condition were incubated in the dark for 10 min before the relative electron transport rate (rETR) versus irradiance, or rapid light curves, were generated using a Pulse Amplitude Modulation (PAM) fluorometer (Diving-PAM, Walz, Germany) according to Ralph and Gademann (2005). For this purpose, nubbins were illuminated for 10 s with seven different light intensities (from 0 to 900 μmole quanta m^{-2} s^{-1}) and the $rETR_{max}$ was deduced from the rapid light curves.

Calcification Rates

Nubbins were incubated 2 h in individual 100 mL beakers filled with 0.45 μm filtered seawater, continuously stirred with stirring bars and hermetically closed to avoid any oxygen exchange with the ambient air. The incubation seawater in each beaker was filtered through 0.2 μm and stored at 4°C for the subsequent determination of the calcification rates using the alkalinity anomaly method (Smith and Key, 1975). The measurement was performed using a Titralab TIM865 titration manager (Hach, Loveland, CO, USA). Data were expressed in μmol $CaCO_3$ cm^{-2} d^{-1} and corrected against a blank (filtered seawater incubated for the same period without nubbin).

Organic Matter and Planktonic Microbe Concentrations in Seawater

Nubbins were incubated for 2 h in 200 mL of 0.45 μm filtered seawater under each experimental condition. The total organic carbon (TOC) and total nitrogen (TN) concentration was assessed by sampling 20 mL seawater in triplicate from each beaker with sterile syringes at the end of the incubation. Samples were transferred to pre-combusted (450°C, 5 h) glass vials, acidified with phosphoric acid (20%, 250 μL) and kept frozen until subsequent analysis. For dissolved organic carbon (DOC) and dissolved nitrogen (DN) concentration, 20 mL subsamples were taken from each beaker in triplicate and filtered through 0.22 μm pore size filter (Millipore, Burlington, MA, USA). Samples were analyzed using a TOC-L analyzer (Shimadzu, Japan) and data were expressed in μg L^{-1}. To determine the concentrations of planktonic microbes, 4.8 mL seawater were sampled at the end of the incubation, fixed with 0.2 mL of glutaraldehyde (25%) for 30 min in the dark and then stored at −80°C. Samples were analyzed by flow cytometry as described by Jacquet et al. (2013).

Symbiont, Chlorophyll *a,* and Protein Content

Five coral nubbins maintained under each experimental condition were incubated in 1L of 0.45 μm filtered seawater, continuously stirred with stirring bars, for 2 h, as described above. After the incubations, nubbins were frozen at −20°C for the subsequent determination of tissue parameters. Nubbin tissue was airbrushed in filtrated seawater and homogenized with a Potter-Elvejhem tissue grinder. A sub-sample was taken for the determination of the symbiont density of each sample using a Neubauer cell, on five replicated counts. A second sub-sample was used to assess the protein content according to Hoogenboom et al. (2010), using BCA assay kit (Interchim Protein Quantification Kit). For chl *a* measurements, another subsample was centrifuged at 5,000 × g for 10 min at 4°C to separate the symbionts (in the pellet) from the host tissue. The pellet was then re-suspended in 10 mL acetone and kept in the dark at 4°C for 24 h prior to measurements. Samples were then centrifuged for 15 min at 10,000 × g and the absorbance was measured at 630, 663, and 750 nm using an EVOLUTION 201 UV-Visible spectrophotometer (Thermo Fisher Scientific). Chlorophyll concentrations were computed using the equations of Jeffrey and Humphrey's (1975).

Statistical Analysis

Effects of UVR and season on coral tissue parameters, *Symbiodinium* photophysiology, and seawater organic matter content and microbial abundances were assessed with two-way ANOVAs using UVR and season as factors. Normality of the residuals and variance homoscedasticity were tested using Shapiro and Bartlett tests, respectively. When needed, data were log transformed in order to fulfill those criteria. A Tukey's High Significance Difference post hoc test was performed when results of the ANOVAs were significant, with the *a priori* decision to determine only the effects of UVR in winter and summer, as well as the effects of season under ambient conditions (i.e., under UVR).

Bacterial Community Analysis

To assess the bacterial diversity, seawater from each experimental condition was filtered sequentially through 10, 3, and 0.2 μm Whatman Nuclepore Track-Etched filters (Sigma-Aldrich) and the last filter was kept in RNA*later* (ThermoFisher Scientific) at −20°C. Five nubbins from each tank condition were preserved in RNA*later* at 4°C for 24 h before being stored at −20°C. Reference samples of seawater and coral preserved directly after fragment collection from the lagoon had not been collected, and it should therefore be taken into account that the microbiota in aquaria may differ slightly from the natural conditions as previously observed (Kooperman et al., 2007; Mohamed et al., 2008; Pratte et al., 2015; Röthig et al., 2017). RNAlater-preserved coral nubbins were crushed frozen in liquid nitrogen. DNA was extracted from crushed coral and from the filters using the Genomic DNA Buffer Set and Genomic-tip 20/G columns (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. DNA concentrations were determined for each sample and samples were sent to Molecular Research Laboratory (MR DNA, Shallowater, Texas, USA) for 16S rRNA gene amplicon library generation using the 515F/806R primer pair, which targets the V4 region of the 16S rRNA gene, and paired-end (2 × 300 bp) sequencing on the Illumina MiSeq platform.

16S rRNA Gene Amplicon Data Analysis

The quality (.qual) and reads (.fasta) files were obtained from MR DNA and contained a total of 5,163,731 reads. The QIIME v1.9 pipeline (Caporaso et al., 2010) was used to process the

data as detailed in van de Water et al. (2018b). Briefly, low quality (Phred <20) sequences, and reads <200 bp in length were removed and each read was assigned to its respective sample using the split_libraries.py script. Chimeric sequences were identified and removed using the UCHIME algorithm (Edgar et al., 2011) and the SILVA v123 database (Quast et al., 2013) as reference. The quality filtered sequence file contained 4,582,530 reads, with an average of 114.563 reads per sample (range 55,679–170,361). Operational Taxonomic Units (OTUs) were defined at the level of 97% similarity. Taxonomic identities were assigned using the UCLUST algorithm (Edgar, 2010), implemented in the assign_taxonomy.py script, against the SILVA reference database (version 123). Singletons, and OTUs classified as chloroplast or mitochondria were subsequently removed from the dataset, resulting in an average of 98,189 reads per sample (range 43,353–158,135). Alpha and beta diversity metrics were generated from OTU tables rarified to 43.353 reads per sample using the QIIME pipeline. OTU tables, sample metadata and representative sequences of each OTU are provided in the **Supplementary Data**. Raw sequences were deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA408048.

The phyloseq package (McMurdie and Holmes, 2013) integrated in R was used to generate statistically relevant graphical presentations of the microbiome data obtained, including (1) abundance plots of relevant taxa, and (2) a non-metric dimensional scaling (nMDS) on Bray-Curtis dissimilarity matrices, to visualize differences in the beta diversity of seawater and coral-associated bacterial communities under the different experimental conditions. Permutational Analysis of Variance (permANOVA) performed under Type III partial sums of squares and 9,999 permutations under the reduced model was used to statistically assess differences in bacterial community diversity using PRIMER 6 & PERMANOVA+ (PRIMER-E Ltd, Auckland, New Zealand) (Clarke and Gorley, 2006; Anderson et al., 2008). Negative binomial modeling (likelihood ratio tests for the effect of experimental factors and Wald tests for pair-wise comparisons) implemented in the DESeq2 package in R (Love et al., 2014), was used for differential abundance analysis followed by Benjamini-Hochberg False Discovery Rate corrections to test which bacterial OTUs were impacted by UV radiation and/or season. Overall effects of UV radiation and season on the seawater and coral-associated bacterial communities, and potential differences in dispersion among samples within treatment groups, were investigated using the ADONIS and betadisper functions in the R-package vegan, respectively (Oksanen et al., 2011).

RESULTS

Impact of Season and UVR on Coral Holobiont Physiology

Under ambient UVR exposure (reef conditions), there was no difference in symbiont density and chlorophyll *a* content in the tissues of *A. muricata* between seasons (**Table S1**, **Figures 1A,B,C**), but the maximal relative electron transport rate ($rETR_{max}$), which is a proxy for the photosynthetic efficiency of the symbionts, was 54% lower in summer compared to winter (**Figure 1D**). UVR shading significantly decreased the $rETR_{max}$ by 29% in winter (**Table S1**); however, it was increased by 48% in summer, despite a lower chl *a* content in the tissues and per *Symbiodinium* cell.

Protein content of coral tissue was 32% lower in summer compared to winter, while calcification rate was 57% higher (**Figures 1E,F**, **Table S1**). UVR did not have an effect on these coral physiological parameters at either season.

Impact of Season and UVR on Seawater Organic Matter

Significant differences in the concentrations of total organic carbon (TOC) and total nitrogen (TN) as well as dissolved organic carbon (DOC) and dissolved nitrogen (DN) were found between summer and winter regardless of UVR level (**Figure 2**; **Table S1**). Under ambient UVR levels, TOC and DOC were 33 and 43% higher in winter compared to summer, respectively. Contrary, levels of TN were 62% higher in summer compared to winter, while DN levels were 65% higher.

Impact of Season and UVR on Coral-Associated Bacterial Communities

The bacterial communities associated with *Acropora muricata* were highly distinct from those present in the surrounding seawater ($p < 0.001$; **Figure 3**, **File S1**). The coral microbiota was consistently dominated by members of the Hahellaceae genus *Endozoicomonas* and to a lesser extent by Alteromonadaceae and Flammeovirgaceae (**Figure 4**). These assemblages were not impacted by UVR, but did change between the seasons (**Figure 3**; **Table S2**; $p = 0.0079$). Alpha diversity and richness were similarly impacted, but the evenness remained the same between summer and winter (**Table 1**; **Table S3**; $p = 0.0142$). The relative contribution of Rhodospirillaceae was high in a few winter samples, while members of the Rhodobacteraceae were increased in a few summer samples (**Figure 4**), which caused a reduction in *Endozoicomonas* abundances in these samples. Despite the apparent variability in microbiota composition between samples, no differences in sample dispersion were observed between the UVR and no UVR conditions at either season. To assess which OTUs were primarily responsible for the seasonal shift in microbiome composition, differential abundance analysis was performed (output can be found in **File S1**). Results showed significant effects on various bacteria, particularly 25 OTUs varied significantly in abundance between summer and winter (**Figure 5**, **Table S4**, and **Figure S1**). Overall, the abundance of four *Endozoicomonas* OTUs, one *Thalassospira* (Rhodospirillaceae) OTU, one cyanobacterium OTU and two Unassigned OTUs were significantly reduced in summer compared to winter. In contrast, four Cyanobacterium OTUs and two *Thalassotalea* (Alteromonadales) OTUs along with OTUs belonging to a range of bacterial taxa [e.g., Candidatus *Amoebophilus* (Flammeovirgaceae), LWSR-14 (Rickettsiales), *Paracoccus* (Rhodobacteraceae)] were significantly more abundant in summer.

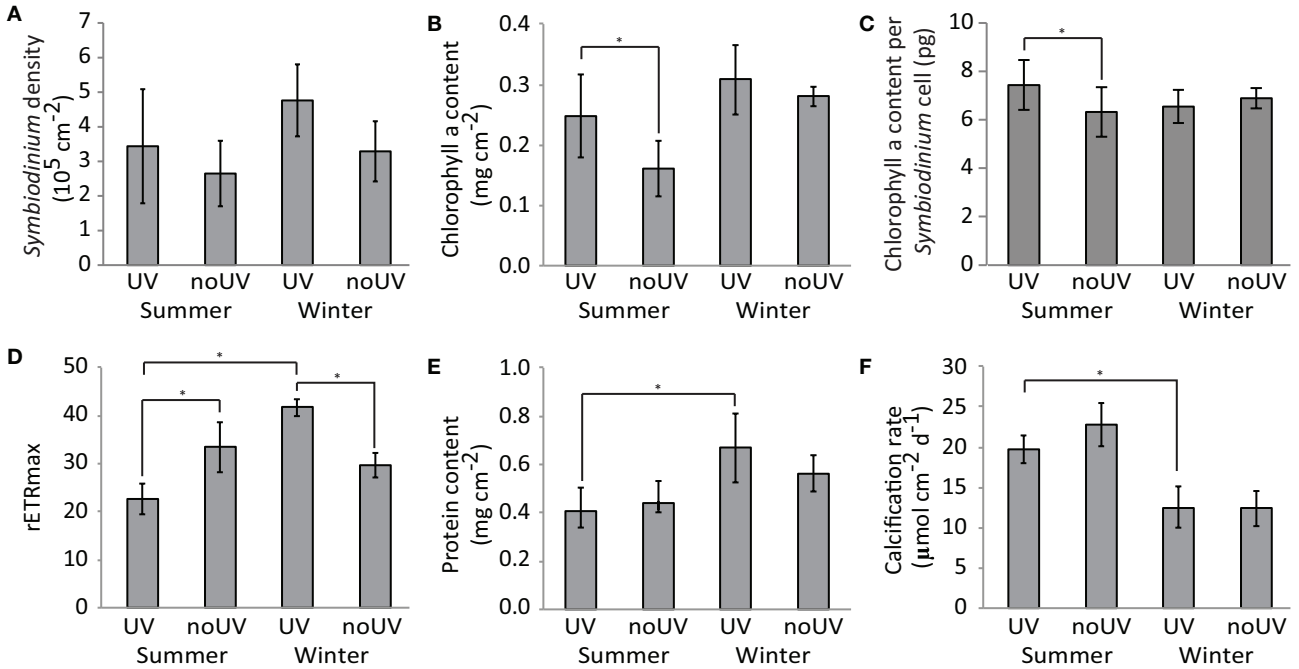

FIGURE 1 | Changes in the physiological parameters of the *A. muricata* holobiont [coral and *Symbiodinium*: **(A)** Sybiodinium density, **(B)** Chlorophyll a content, **(C)** Chlorophyll content per Symbiodinium cell, **(D)** maximum relative Electron Transport Rate, **(E)** Protein content, and **(F)** Calcification rate] under ambient or no UV radiation at two contrasting seasons (summer and winter). Data are the mean and standard deviations of five biological replicates. Comparisons indicated with *are significantly different (Tukey's Honest Significant Difference-corrected $p < 0.05$).

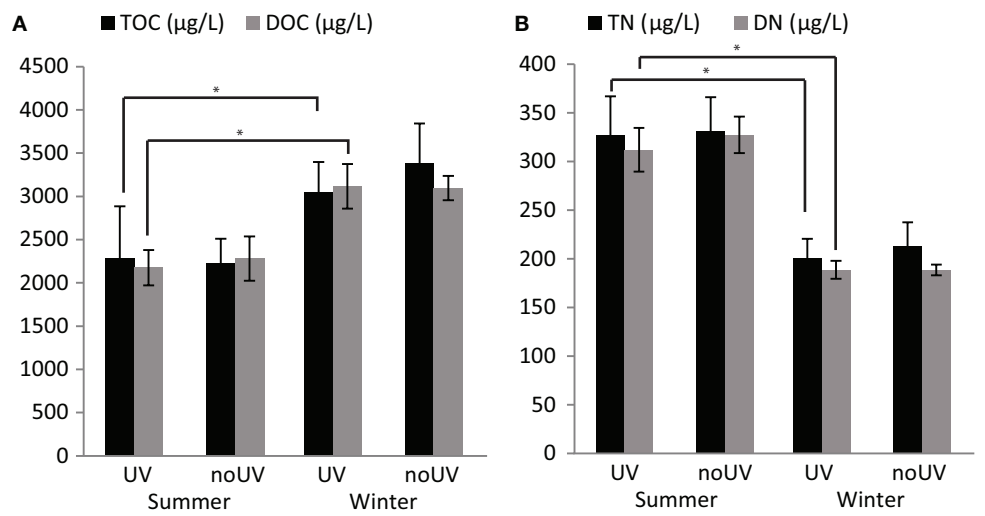

FIGURE 2 | Concentrations of organic matter in seawater. Differences in the concentrations of **(A)** total and dissolved organic carbon (TOC and DOC, respectively), and **(B)** total and dissolved nitrogen (TN and DN, respectively) in the seawater. Data are the mean and standard deviations of five biological replicates. Comparisons indicated with *are significantly different (Tukey's Honest Significant Difference-corrected *p*-value < 0.05).

Impact of Season and UVR on Seawater Microbial Communities

Flow cytometry analyses of the seawater surrounding the coral fragments revealed that the abundances of algae and virus-like particles (VLP) were significantly increased in summer compared to winter, but no effect of UVR level was observed (**Figures 6A,B**; **Table S1**). Bacterial abundances showed a seasonal trend, increasing in summer, but our condition comparisons did not show this effect under ambient UVR conditions (**Figure 6C**; **Table S1**). The ratio between VLPs and bacteria was approximately 2.7-fold higher in summer (**Figure 6D**; **Table S1**).

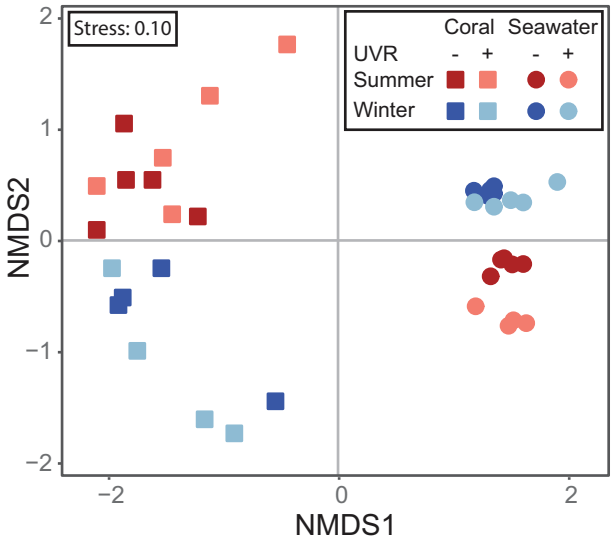

FIGURE 3 | Non-Metric Dimensional Scaling (nMDS) plot showing the (1) seasonal differences in the beta diversity of the coral microbiota, (2) seasonal changes in the beta diversity in seawater microbial communities, and (3) the effect of UVR on the beta diversity in seawater microbial communities at both seasons.

The diversity of the seawater microbial communities (**Table 1** – alpha diversity metrics), however, was highly influenced by both season and UVR level, showing interactive effects of these two factors (**Figure 3**; **Table S5**—beta diversity: $p = 0.0001$; **Table S6**—alpha diversity: $p = 0.0001$). Interestingly, alpha diversity (richness, evenness and diversity indices) was significantly reduced by UVR in summer (**Table S6**; $p = 0.0017$), but increased in winter (**Table S6**; $p = 0.0002$). Similarly, the alpha diversity under natural UVR conditions was reduced in summer compared to winter (**Table S6**; $p = 0.0001$). No significant differences in the dispersion of samples within treatment groups were observed between the UVR and no UVR conditions at either season. Several bacterial taxa made up the vast majority of the community in both seasons and under UVR treatments (**Figure 4**), but obvious changes were observed. Differential abundance analysis was used to assess which taxa contributed significantly to these differences in seawater community structure between (1) UVR and no UVR exposure in winter, (2) UVR and no UVR exposure in summer, and (3) UVR exposure in winter and summer (output can be found in **Table S7** and **File S1**). In winter, UVR exposure increased members of the Flavobacteriales and SAR11 clade, while reducing Bdellovibrionales, Alteromonadales, Caulobacterales, Cytophagales and DB1-14 (**Figure 4**; **Figure S2**). Interestingly, the abundance of various Oceanospirillales, Rhodospirillales, Rhodobacterales, and Rickettsiales OTUs were inversely impacted by UVR [i.e., the abundance of some OTUs was reduced under UVR, resulting in an increase in relative abundance of other OTUs (**Figure S2**)]. In summer, members of the order Alteromonadales (particularly genera *Marinobacter* and *Alteromonas*) were highly abundant under UVR, which resulted in a significant reduction in the relative abundance of SAR11, Rhodobacterales, Pseudomonadales, Flavobacteriales, Cytophagales, Phycisphaerales, and Oceanospirillales (**Figure 4**; **Figure S3**). Differences between seawater bacterial communities under natural UVR levels in summer (high UVR) and winter (low UVR) (**Figure 4**; **Figure S4**), showed highly similar patterns as ambient UVR and no UVR exposure treatments, respectively (**Figure 4**; **Figures S2, S3**).

DISCUSSION

In this study, we assessed the impact of UV radiation on the physiology and the microbiome of the scleractinian coral *A. muricata* at two contrasting seasons. Our findings show that seasonality in the coral holobiont composition and functioning is to some extend driven by UV radiation but primarily by other environmental factors. On the contrary, large seasonal and UVR-induced variations were observed in the seawater microbial communities, which was overall dominated by bacterial taxa known to degrade and/or grow on organic matter.

Seasonality Has Higher Impact on Coral Holobiont Than UV Radiation

In the *Acropora muricata* holobiont, the coral host and associated bacterial communities were only affected by seasonal changes, whereas the algal endosymbiont *Symbiodinium* was also impacted by UVR exposure. The effect of UVR exposure on *Symbiodinium* was, however, in part seasonally dependent and limited to changes in the cellular concentration of chlorophyll *a* and in the photosynthetic efficiency ($rETR_{max}$). In winter, exposure to naturally moderate levels of UVR enhanced the $rETR_{max}$ of *A. muricata*. Utilization of moderate doses of UVR (mostly UV-A) as a source of energy for photosynthesis has been previously observed in phytoplankton species (Gao et al., 2007; Wu et al., 2009), and our results suggest this may also be applicable to *Symbiodinium* in corals. On the contrary, in summer, exposure to naturally high UVR levels led to a significant reduction in *Symbiodinium* photosynthetic efficiency, which may have been compensated for through an increase in chlorophyll content, as previously observed (Cardini et al., 2015). Previous studies have also shown that the lowest photosynthetic yield (F_v/F_m) or $rETR_{max}$ values of diverse scleractinian coral species coincide with the highest irradiance and temperature levels on the reef, and are caused by damage to the photosystems (Fitt et al., 2000; Warner et al., 2002; Winter et al., 2016). The impairment of the photosynthetic capacities of *Symbiodinium*, and thereby energy acquisition by *A. muricata* in summer, may explain the lower coral tissue biomass (i.e., protein concentration) observed compared to winter. However, this effect may also have been exacerbated by enhanced respiratory metabolism at high temperatures as previously observed in various coral species (Fitt et al., 2000).

Despite having reduced energy reserves, calcification rates were enhanced in *A. muricata* in summer, which corresponds with reports on a positive linear relation between coral calcification and seawater temperature between 23° and 28°C

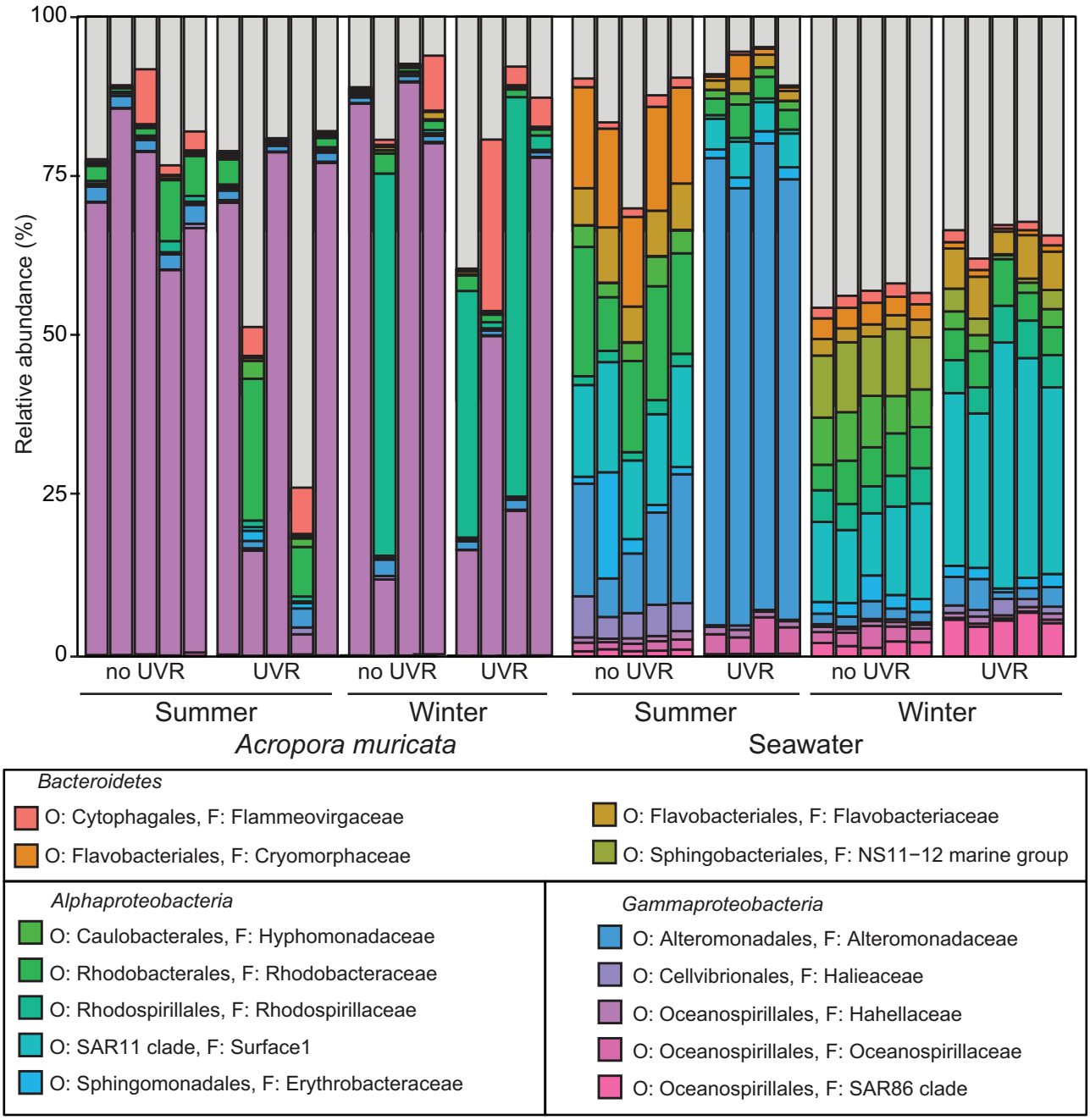

FIGURE 4 | Relative abundance of the major bacterial families present in the *A. muricata* holobiont and the surrounding seawater (>1% of the total community) under ambient or no UV radiation at two contrasting seasons (summer and winter).

(Lough and Barnes, 2000; Silverman et al., 2007). Although UVR has been shown to decrease calcification rates in several coral species (Jokiel and York, 1982; Gleason and Wellington, 1993; Torres-Pérez and Armstrong, 2012), calcification rates of *Acropora muricata* in this study as well as *Acropora validata* and *Porites compressa* in previous studies (Glynn et al., 1993; Kuffner, 2002) were not impacted by UVR. The different effects of UVR on coral skeletal growth may be linked to the amount of energy acquired by the coral via its algal endosymbiont *Symbiodinium*, and dedicated to calcification (Courtial et al., 2017). Overall, our data suggest that *A. muricata* has two seasonally driven phenotypes: thin tissue holobionts with faster calcification rates in summer, and thicker tissue but slower calcifying holobionts in winter.

Seasonal effects were also observed in the coral-associated bacterial communities, but these were not impacted by UVR

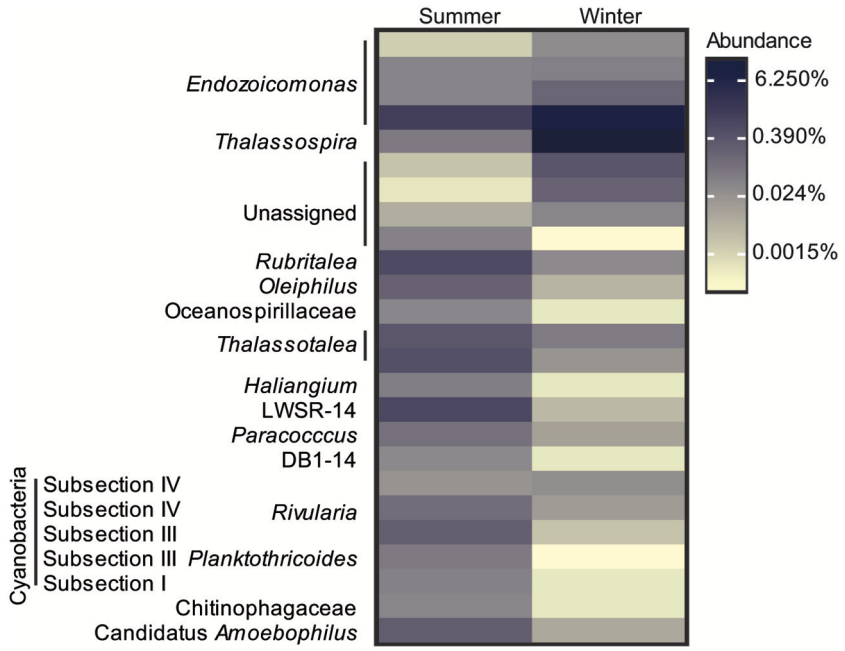

FIGURE 5 | Heatmap overview of bacterial OTUs associated with A. muricata that are differentially abundant between the summer and winter season under ambient UVR conditions.

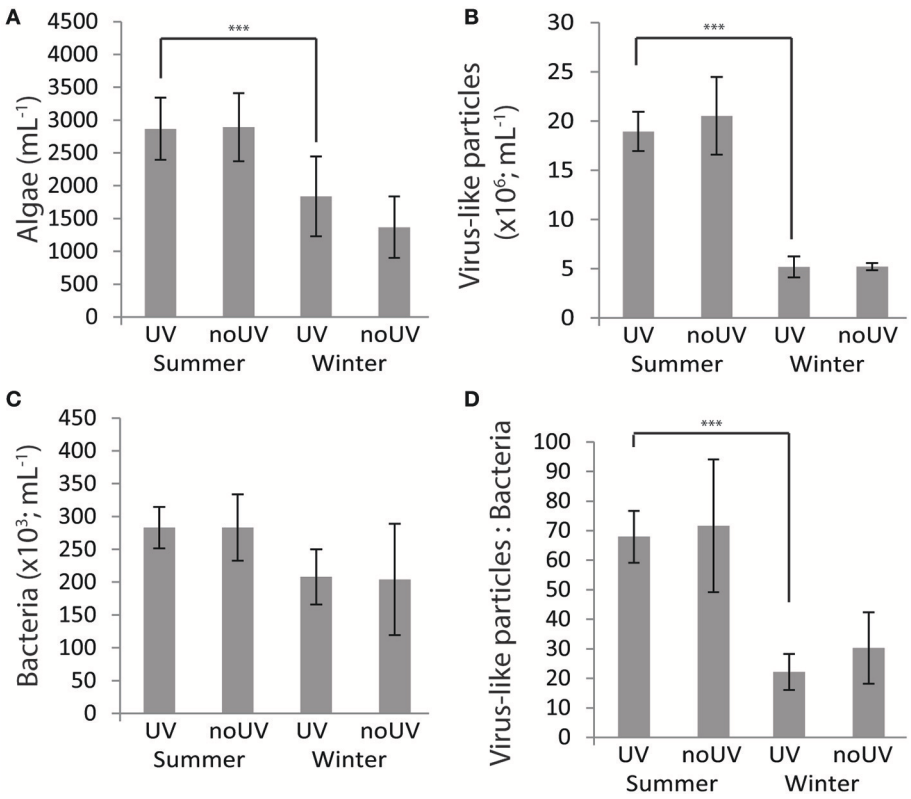

FIGURE 6 | Concentrations of **(A)** algae, **(B)** viruse-like particles (VLP) and **(C)** bacteria and **(D)** the ratio VLP:bacteria in the seawater under ambient and no UVR conditions at two contrasting seasons (summer and winter). Data are the mean and standard deviations of five biological replicates. Comparisons indicated with ***are significantly different ($p < 0.05$).

TABLE 1 | Overview of alpha diversity metrics and indices for richness, evenness, and diversity.

Richness			**Observed OTUs**	**Margalef**	**Menhinick**
A muricata	Summer	no UVR	1,242 ± 113	117.63 ± 10.61	6.04 ± 0.54
		UVR	1,206 ± 114	114.73 ± 10.69	5.89 ± 0.55
	Winter	no UVR	969 ± 61	91.83 ± 5.81	4.71 ± 0.30
		UVR	776 ± 62	73.78 ± 5.74	3.79 ± 0.29
Seawater	Summer	no UVR	1,488 ± 43	139.87 ± 4.08	7.18 ± 0.21
		UVR	1,130 ± 20	106.42 ± 1.86	5.46 ± 0.10
	Winter	no UVR	1,790 ± 16	168.96 ± 1.37	8.67 ± 0.07
		UVR	2,254 ± 64	212.94 ± 6.17	10.92 ± 0.32
Evenness			**Gini index**	**Simpson evenness**	**McIntosh evenness**
A muricata	Summer	no UVR	0.995 ± 0.001	0.003 ± 0.000	0.53 ± 0.03
		UVR	0.993 ± 0.001	0.013 ± 0.006	0.39 ± 0.09
	Winter	no UVR	0.997 ± 0.000	0.004 ± 0.001	0.54 ± 0.03
		UVR	0.998 ± 0.000	0.005 ± 0.001	0.54 ± 0.07
Seawater	Summer	no UVR	0.994 ± 0.000	0.010 ± 0.000	0.27 ± 0.01
		UVR	0.996 ± 0.000	0.006 ± 0.000	0.41 ± 0.00
	Winter	no UVR	0.987 ± 0.000	0.022 ± 0.001	0.17 ± 0.00
		UVR	0.984 ± 0.001	0.008 ± 0.001	0.25 ± 0.02
Diversity			**Shannon-wiener *H***	**Simpson index**	**Fisher's alpha**
A muricata	Summer	no UVR	2.53 ± 0.19	0.73 ± 0.03	240.12 ± 26.97
		UVR	3.30 ± 0.59	0.82 ± 0.07	231.66 ± 27.13
	Winter	no UVR	2.31 ± 0.08	0.71 ± 0.03	176.18 ± 13.58
		UVR	2.26 ± 0.28	0.69 ± 0.07	135.16 ± 12.90
Seawater	Summer	no UVR	3.73 , 0.04	0.93 ± 0.00	298.73 ± 10.70
		UVR	2.80 ± 0.04	0.84 ± 0.00	212.16 ± 4.60
	Winter	no UVR	4.87 ± 0.01	0.97 ± 0.00	377.75 ± 4.19
		UVR	4.64 ± 0.15	0.94 ± 0.01	505.53 ± 18.51

Analyses were performed on an OTU table rarefied to 43,353 reads per sample.

exposure. The presence of UVR-filtering "sunscreen" molecules (e.g., mycosporine-like amino acids (MAAs), fluorescent proteins) in coral tissue and/or mucus (Shick and Dunlap, 2002) may protect the microbial community from the damaging effects of UVR. However, we cannot exclude that the bacteria were affected in their physiology, as observed in the algal symbiont *Symbiodinium*. Few studies have assessed seasonal changes in coral-associated microbial communities thus far, but have generally reported highly different profiles between winter and summer in scleractinian corals (Hong et al., 2009; Ceh et al., 2011; Chen et al., 2011; Kimes et al., 2013; Sharp et al., 2017; Cai et al., 2018), but not in octocorals (van de Water et al., 2018a). Changes in the coral microbiota may be caused by fluctuations in seawater temperature, rainfall and sunlight intensity (Chen et al., 2011) as well as nutrient availability in seawater (Hernández-Zulueta et al., 2016). A recent study that investigated the effects of temperature, nutrient pollution and algal cover on the microbiomes of three coral species, however, found that temperature variation explained differences in microbial community structure over time better than other measured seasonal parameters (Zaneveld et al., 2016). In summer, *A. muricata* harbored a significantly higher abundance of several Cyanobacterium OTUs, which might play a role in providing new nitrogen to the coral holobiont (Bednarz et al., 2017), since many cyanobacteria are capable of fixing nitrogen (i.e., diazotrophs). Fixation of dinitrogen by these bacteria is an important functional process for corals thriving in oligotrophic tropical environments as they can provide up to 15% of new nitrogen to corals (Bednarz et al., 2017). Increases in the abundance and diversity of nitrogen-fixing bacteria in coral tissue has been observed previously under elevated temperatures (Santos et al., 2014; Cardini et al., 2015, 2016) and between winter and summer (Cai et al., 2018). We also observed a higher abundance of a *Candidatus* Amoebophilus bacterium in summer compared to winter. Previously, bacteria belonging to this taxon were reported to be part of the core microbiome of 3 coral species (Apprill et al., 2016). This bacterium is a *Bacteroidetes*-affiliated intracellular symbionts of amoebae and may be an endosymbiont of a protist host within coral tissues (Apprill et al., 2016).

In summer, the coral-associated microbial community also contained a higher abundance of bacteria belonging to the genus *Thalassotalea* (previously *Thalassomonas*), which have previously been found in increased numbers in diseased and stressed corals (Sunagawa et al., 2009; Weynberg et al., 2016). In fact, *T. loyana* has been identified as the causative agent of a white plague-like disease (Thompson, 2006). Such a shift toward a potentially

more pathogenic coral microbiota in summer may affect coral health and increase the susceptibility of the host to disease (Bourne and Munn, 2005; Ritchie, 2006; Bourne et al., 2008). Higher temperatures may directly affect bacterial growth and metabolism, or indirectly alter bacterial antibiotic production and bacteria–bacteria interactions (Rypien et al., 2010). Elevated temperatures may also impair a coral's defense capability (van de Water et al., 2015), leading to a reduced ability of the coral host to regulate its microbiome, or require the coral to invest in its innate immune system (van de Water et al., 2018a) and protective surface mucosal layer (Pratte and Richardson, 2014) to maintain a healthy microbiome, thereby potentially depleting its energy stores. As the immunocompetence of an animal largely depends on its energy reserves, the higher occurrence of potentially pathogenic OTUs in *A. muricata* in summer may be linked to a reduced fitness of the holobiont, as evidenced by the lower tissue biomass (proteins), and *Symbiodinium* photosynthetic efficiency compared to winter. In summer, we also observed a lower abundance of *Thalassospira*, known as potential symbionts of ctenophores (Hao, 2014), and four OTUs of *Endozoicomonas*, a genus commonly found in healthy corals (Bayer et al., 2013a,b; Neave et al., 2017; van de Water et al., 2017, 2018b), as well as in a wide range of other marine invertebrates (Forget and Juniper, 2013; Fiore et al., 2015; Katharios et al., 2015). Although their exact functions are unknown, both *Thalassospira* and *Endozoicomonas* may be involved in host health through nutrient acquisition and provision, and in structuring of the host microbial community (Hao, 2014; reviewed by Neave et al., 2016). Our results on the abundance of *Endozoicomonas,* and the observed changes in host physiology between summer and winter, however, do not appear to provide additional insights into the potential role of *Endozoicomonas* in corals.

Seawater Bacterial Communities Are Impacted by Both Season and UVR Exposure

Most of the organic carbon and nitrogen in seawater of Nouméa lagoon was in the dissolved form, attesting the oligotrophic nature of these tropical waters, with few large size suspended particles in the water (Biddanda et al., 2001). However, increased rainfall as well as higher diazotroph abundance and activity (Berthelot et al., 2015) may have been responsible for the higher amount of dissolved nitrogen in seawater in summer. Combined with the increased seawater temperatures, this may have driven the increased microbial abundances observed in summer (Moriarty et al., 1985). For example, when more dissolved inorganic nitrogen (DIN) is available, autotrophs tend to retain the photosynthetically-acquired carbon for their growth and proliferation, rather than releasing it in seawater as organic carbon (Naumann et al., 2010; Mueller et al., 2016). However, these DIN-repleted autotrophs may have released excess nitrogen (Naumann et al., 2010; Wymore et al., 2015). In addition, high temperatures and microbial abundances may have increased microbial respiration rates, leading to lower DOC levels in the water in summer. Taken together, these observations may explain the observed inverse relationship between DOC and DN in seawater. Only the abundances of bacteria didn't significantly change between summer and winter. As the number of VLPs, and concomitantly the ratio VLP:bacteria, was also higher in summer and most viruses in seawater are bacteriophages, our results suggest ongoing lytic infections of bacterioplankton and likely higher temporal dynamics within seawater microbial communities in summer (Parikka et al., 2017). Higher levels of UVR and seawater temperatures have previously been found to induce VLP production (42 and 33% of the cases examined, respectively) (Jiang and Paul, 1996). As we did not find an effect of UVR on VLP abundances or the ratio VLP:bacteria, this suggests that the seasonal differences observed here are likely related to seawater temperatures. VLPs have indeed been coupled to the dynamics of the coral reef bacterioplankton community on spatial scales (Seymour et al., 2005) and viral predation has been shown to remove 24–367% of the bacterial standing stock on coral reefs every day, releasing up to 62 micrograms of organic carbon per liter of seawater (Payet et al., 2014). As such, viral lytic infections may also explain the lower organic carbon content of the seawater in summer, and reflects the high abundant and dynamic microbial community within coral reefs.

Seawater bacterial communities were dominated by bacterial taxa known to degrade and/or grow on dissolved and particulate organic matter, or transparent exopolymeric particles released by phytoplankton, including *Flavobacteriales* and *Rhodobacterales* (Taylor et al., 2014), as well as SAR11, Alteromonadales and Oceanospirillales (Bergauer et al., 2018). It should, however, be noted that SAR11 may have been underrepresented in our dataset because of the 806r reverse primer used to construct the sequencing libraries, rather than the 806rb primer which better reflects SAR11 abundances (Apprill et al., 2015). The higher nitrogen levels and temperatures in summer may have in part driven the differences in seawater bacterial community diversity between the two seasons. Interestingly, however, the effect of UVR level was different between the seasons. This observation suggests that seawater bacterioplankton communities is very dynamic and may change within a few hours depending on the environmental conditions. Overall, UVR significantly reduced the bacterial community diversity, possibly due to UVR-induced inhibition of bacterial growth (Llabrés and Agustí, 2010; Korbee et al., 2012; Carrillo et al., 2015) linked to DNA photodamage (Jeffrey et al., 2000; Alonso-Sáez et al., 2006), However, patterns appeared to be relatively consistent, showing various bacterial taxa consistently increase or decrease in relative abundance under UVR exposure conditions at both seasons as well as between summer (high UVR levels) and winter (low UVR levels). Although the abundance of most of the main bacterial groups decreased in response to UVR, members of the order Alteromonadales (genera *Marinobacter* and *Alteromonas*) were significantly higher, particularly in summer, which may explain the reduced community diversity and suggests that these bacteria may be more resistant to the high summer UVR levels on the reef than other species. UVR resistance of Altermonadales bacteria has indeed been shown in experimental sterilization trails, showing rapid recolonization of the seawater and surfaces by bacteria from this order (particularly *Marinobacter*) after UVR sterilization treatment (Hess-Erga et al., 2010). As such,

this may explain why Altermonadales dominated the seawater microbial community under natural summer UVR levels, while other main groups such as Flavobacteriales, Rhodobacterales and SAR11, increased in abundance, and likely outcompeted Alteromonadales, when UVR levels were reduced experimentally or in winter. Alphaproteobacteria (Rhodobacterales and SAR11) are indeed known to be relatively sensitive to UVR, even in temperate environments (Alonso-Sáez et al., 2006) where UVR levels are lower than in tropical environments. In winter, however, the abundance of SAR11 was higher under ambient UVR conditions. This additional light in winter may be required for the activity of the ATP-generating proton pump proteorhodopsin in SAR11 (Lami and Kirchman, 2014), and may provide these bacteria an energetic advantage under moderate/low UVR levels. In general, our results confirm significant seasonal differences in the abundances and composition of the bacterioplankton communities of seawater, and that both UVR and organic matter concentrations are important determinants of the community structure.

CONCLUDING REMARKS

In this study, we provide an overview of the seasonal impact of UVR on the physiology and microbiome of the scleractinian coral *Acropora muricata*. In general, we observed two seasonal coral holobiont phenotypes: (1) a fast calcifying, but lower photosynthesizing and animal tissue biomass phenotype in summer, and (2) a slow calcifying, but higher photosynthesizing and biomass phenotype in winter. The impact of ambient UVR levels on the coral holobiont was limited, however, and affected only the photosynthetic process in *Symbiodinium*, particularly in summer. Although the coral microbiota showed seasonal differences, it was shielded from UVR impacts, unlike the microbial communities in the seawater surrounding the coral. Overall, our study suggests that UVR has a limited impact on coral holobiont composition and functioning, and that seasonal changes in the holobiont are thus mostly driven by other environmental factors.

AUTHOR CONTRIBUTIONS

LC, FH, and CF-P designed the experiment. LC and FH conducted the experiment. LC processed the samples and performed physiological analyses. SJ performed the flow cytometry analyses. JvdW analyzed the bacterial community data. All authors contributed to the writing and editing of the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank R. Rodolfo Metalpa and C. Rottier for laboratory assistance, C. Payri, director of the ENTROPIE UMR, for scientific support and Mr. R. Farman and the technical staff of the Aquarium des lagons (Nouméa, New Caledonia), for hosting the tanks and providing technical help during the experimental setup. We also would like to thank N. Wabete and M. Le Gall (Institut Français de Recherche pour l'Exploitation de la Mer) for their collaboration in lab analysis. JvdW would like to thank the Paul Hamel Foundation for financial support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00275/full#supplementary-material

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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

ORIGINAL RESEARCH
published: 13 September 2018
doi: 10.3389/fmars.2018.00323

Impacts of a Regional, Multi-Year, Multi-Species Coral Disease Outbreak in Southeast Florida

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Globally coral reefs have been declining at alarming rates as a result of anthropogenic stressors, leading to increased frequency and severity of widespread bleaching and disease events. These events are often associated with increased water temperatures due to climate change as well as regional and local stress from nutrient enrichment through runoff and sedimentation from coastal development. In late 2014, a white syndrome disease outbreak was reported off the coast of southeast Florida and was subsequently documented spreading throughout the region. This study examined the regional impacts of the disease event on the southeast Florida stony coral population utilizing stony coral demographic data from the Southeast Florida Coral Reef Evaluation and Monitoring Project (SECREMP). SECREMP is a long-term monitoring project examining 22 sites distributed from Miami-Dade County north to Martin County, Florida. The results revealed significant region-wide declines in stony coral diversity, density, and live tissue area corresponding with increased disease prevalence, which reached its maximum for the study period in 2016. Regional declines in coral density approached 30% loss and live tissue was upward of 60% as a result of the disease outbreak. Additionally, multiple species were severely impacted, especially the reef building, complexity-contributing species *Montastraea cavernosa*, *Meandrina meandrites, and Siderastrea siderea.* The disease outbreak resulted in acute mortality and altered the ecosystem function to a point such that recovery is uncertain. This multiyear, region-wide disease outbreak has been indiscriminate relative to coral species impacted and was arguably the most devastating disturbance event documented on the Southeast Florida Reef Tract.

Keywords: white syndrome, Florida Reef Tract, coral mortality, density, diversity, cover, long-term monitoring

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *31 May 2018*
Accepted: *23 August 2018*
Published: *13 September 2018*

Citation:
Walton CJ, Hayes NK and Gilliam DS (2018) Impacts of a Regional, Multi-Year, Multi-Species Coral Disease Outbreak in Southeast Florida. Front. Mar. Sci. 5:323. doi: 10.3389/fmars.2018.00323

INTRODUCTION

Disease outbreaks on coral reefs are emerging as significant causes of coral mortality altering ecosystem function and are predicted to become more frequent and severe globally (Harvell et al., 2002; Maynard et al., 2015). Evidence continues to build coupling increased water temperatures with increased coral bleaching and disease prevalence (Croquer and Weil, 2009; Miller et al., 2009; Ruiz-Moreno et al., 2012; Randall and van Woesik, 2015). In addition to thermal stress, other anthropogenically influenced stressors linked to increased coral disease prevalence and mortality include reduced water quality (Bruno et al., 2003) and clarity (van Woesik and McCaffrey, 2017),

nutrient enrichment (Vega Thurber et al., 2013), dredging associated sedimentation (Pollock et al., 2014; Miller et al., 2016), and plastic pollution (Lamb et al., 2018). As anthropogenic related stressors continue to drive disturbance events such as disease outbreaks, it is imperative to document how these events are affecting coral reef ecosystems.

Coral disease, especially in the Caribbean, was once typically described as more prevalent during summer months affecting less than 5% of the population and only a few species (Rutzler et al., 1983; Edmunds, 1991; Bruno et al., 2007; Weil and Croquer, 2009; Muller and van Woesik, 2012; Ruiz-Moreno et al., 2012). These typical conditions have now changed. The pathogens, vectors, or other causative agents of most coral diseases remain unknown. Sufficient information on background disease prevalence in coral populations or what factors contribute to prevalence levels increasing to a height such that major coral community changing disease outbreaks occur is not known. It is becoming increasingly important to understand the interactions between climate-related stress and local environmental factors that are likely to contribute to increased disease prevalence, whether through changes in pathogen abundance or virulence, or coral susceptibility to disease.

In this study, we use a 5-year data set collected as part of a long-term monitoring project to examine the impacts of a multi-year, widespread disease outbreak on stony coral populations in the northern portion of the Florida Reef Tract (FRT). The FRT is the third largest barrier reef system in the world extending approximately 577 km north from the Dry Tortugas. The northern extension of the FRT, the Southeast Florida Reef Tract (SEFRT), runs parallel to the heavily developed and densely populated mainland coast of southeast Florida, which includes Miami-Dade, Broward, Palm Beach, and Martin counties. Southeast Florida is home to more than six million residents and visited by millions of tourists each year. Existing within 3 km of this highly urbanized area, the SEFRT ecosystem is greatly influenced by commercial and recreational fishing and diving, major shipping port activities including dredging, wastewater and sewer outfalls, canal discharges, ship groundings, and other marine construction activities. The cumulative impact of these local stressors on the SEFRT is likely even more damaging through the interaction with increasingly frequent and severe thermal stress associated with global climate change.

Reports of numerous diseased corals in the southern portion (Miami-Dade County) of the SEFRT began in the fall of 2014 and by fall 2015 reports increased, identifying an apparent widespread disease outbreak affecting numerous species in the south and central portions of the SEFRT[1] (Precht et al., 2016). Increased ocean warming (Manzello, 2015) followed by coral bleaching were reported in late summer and fall 2014 in the FRT and continued through 2015 (Eakin et al., 2016; van Woesik and McCaffrey, 2017). With reports continuing through 2016, this regional disease outbreak appeared to be more severe, widespread, and prolonged compared to previous disease outbreaks documented along the FRT.

Historically, the Caribbean has been considered a "hotspot" for coral disease and the FRT has not been an exception. Disease outbreaks affecting multiple coral species were reported in the FRT in the 1970s (Dustan, 1977) and 1990s (Richardson et al., 1998a,b). The visual signs and tissue loss patterns affecting the corals in these events led to the disease being termed white plague (Dustan and Halas, 1987; Richardson et al., 1998a,b). In the Caribbean, white plague is now considered to be one of the most serious of the coral diseases (Croquer et al., 2003; Weil, 2004; Richardson and Voss, 2005; Miller et al., 2009). In addition to the previous observations of white plague along the FRT, black band disease (Carlton and Richardson, 1995; Kuta and Richardson, 1996) and Acroporid only affecting diseases, white-band (Gladfelter, 1982; Aronson and Precht, 2001), white-pox (Sutherland and Ritchie, 2004), and rapid tissue loss (Williams and Miller, 2005) have also been documented (Gilliam et al., 2017). The disease presentation in the current study commonly consisted of tissue loss from disease lesions or boundaries of denuded skeleton adjacent to apparently healthy tissue. As the outbreak has been studied and discussed among research partners multiple names have been used including, white plague, white blotch, and white-plague like. Given the current unknown information regarding the outbreak, the accepted identification of the white plague pathogen and that this study did not attempt to identify the pathogen, the disease is referred to as white syndrome (WS). This is supported by ongoing work of others trying to identify the pathogen[2,3] (Walczak, personal communication, 2018). Additionally, the term WS is commonly used in the Pacific for diseases with similar presentation and unknown etiologies (Pollock et al., 2011, 2017).

Here, we utilized Southeast Florida Coral Reef Evaluation and Monitoring Project (SECREMP) data collected between 2012 and 2016 to examine disease outbreak impacts on the stony coral community. SECREMP is a long-term monitoring project that includes 22 sites distributed along the SEFRT from Miami-Dade County north to Martin County (Gilliam et al., 2017). The specific objectives of this study were to determine the temporal and spatial extent of the outbreak and to quantify region-wide and species specific impacts to the stony coral community. The data presented here clearly show significant declines in coral diversity, density, and live tissue area (LTA) associated with an unprecedented disease outbreak. Additionally, capturing these declines demonstrates the value of continued region-wide, long-term monitoring, and the importance of these projects for determining the success of management actions that will be required in response to this disease outbreak.

MATERIALS AND METHODS

Coral Monitoring Surveys

To evaluate the impacts of the major coral disease outbreak offshore southeast Florida, this study utilized stony coral

[1]https://floridadep.gov/fco/coral/content/florida-reef-tract-coral-disease-outbreak

[2]https://futurism.com/white-syndrome-coral-reef-florida/

[3]https://www.nrdc.org/stories/scientists-are-battling-mysterious-pathogen-destroying-coral-reefs-floridas-coast

demographic data from SECREMP. SECREMP was established as a long-term monitoring project in 2003 to provide resource managers annual status updates on the SEFRT (Gilliam et al., 2017). In 2012, SECREMP began collecting detailed stony coral demographic data at 16 previously established permanent monitoring sites and in 2013 six additional sites were added. The 22 sites were distributed north from Miami-Dade County to Martin County (**Figure 1** and **Table 1**) with multiple reef habitats within each county (Walker, 2012) represented. Throughout the project, sites have been defined by the county followed by a site number (MC = Martin County, BC = Broward County, PB = Palm Beach County, and DC = Dade County). Each site consists of four stations, 1 m × 22 m, demarcated by stainless steel stakes permanently placed in the substrate. Annual monitoring surveys at all sites occurred in summer months (May-September) from 2012 to 2016. Using SCUBA, divers conducted 1 m ×22 m belt transects guided by measuring tapes deployed between the permanent stakes. Within each transect all stony corals ≥ 4 cm diameter were identified to species and colony maximum diameter and height, perpendicular to the plane of growth, were measured. Colonies were visually assessed for signs of disease, bleaching, and other conditions (i.e., predation, damselfish gardens, boring sponges, etc.). For the current study, diseases were classified as non-white syndrome or WS based on disease presentation and the unknown etiology. Non-white syndrome included black band disease, yellow band disease, white band disease (Acroporids only), and dark spot disease. Bleached colonies were differentiated from partially bleached colonies in the field, but data presented in this study combined bleached and partially bleached colonies. Percent colony tissue mortality was documented and estimated as either recent or old mortality. Recent mortality was defined as tissue loss areas having clearly distinguishable corallite structure and minimal overgrowth by algae or other fouling organisms. Any areas of colony mortality not meeting these criteria were considered old.

In addition to the coral surveys, SECREMP has been collecting benthic temperature data at all sites since 2007. Throughout the course of the project, three models of temperature loggers were utilized, StowAway TidbiT^{TM}, Hobo Pendant Temperature Data Logger, and Hobo Water Temp Pro v2 (Onset). Two loggers were deployed at each site with a sampling interval of every 2 h and were exchanged annually during the monitoring events. Data from one logger per site was used for analysis unless there were gaps in the data in which case data from the second logger was used to fill gaps.

Statistical Analysis

Temporal trends in regional monthly minimum, maximum, and mean temperatures were examined using a linear regression model in R (version 3.3.3) (R Core Team, 2017). Regional values were calculated using data from all sites and subregional values were calculated using the sites within each respective county.

To examine the extent of the disease outbreak, differences in bleaching and disease prevalence, coral community diversity [species richness (S), Shannon index (H′), and Inverse Simpson's index (D)], coral density, and coral LTA were analyzed at the regional level. As coral diseases often cause colony partial mortality, LTA is a useful metric because it takes partial mortality into account and is therefore better able to detect changes in the community whereas diversity and density require full colony mortality for changes to be detected. Coral colony surface areas were calculated using the following modified version of the Knud Thomsen approximation[4] for the surface area of an ellipsoid (Klamkin, 1971, 1976):

$$SA = 2\pi\left(\frac{a^p\left(\frac{1}{2}b\right)^p + a^p\left(\frac{1}{2}b\right)^p + \left(\frac{1}{2}b\right)^p\left(\frac{1}{2}b\right)^p}{3}\right)^{\frac{1}{p}}$$

The original equation was multiplied by 1/2 to estimate the surface area of a coral as the equivalent of the top half of an ellipsoid. In the equation, a = maximum colony height, b = maximum colony diameter, and p = 1.6075, a constant yielding a relative error of at most ± 1.061%, determined by Knud Thomsen based on Klamkin's (1971) work. Following the calculation of the surface area, the resulting value and the colony mortality were used to calculate LTA via the following formula:

$$\text{LTA} = \text{SA}\left(1 - \left(\frac{\%\text{ old Mortality} + \%\text{Recent Mortality}}{100}\right)\right)$$

Disease prevalence, species diversity, colony density, and colony LTA were also analyzed at the site level to detect any localized effects of the disease outbreak. Regional and site level differences were analyzed using linear mixed-effect models in the nlme package (Pinheiro et al., 2017) in R. For these models disease prevalence, diversity, density, or LTA were used as the response variables with year as the fixed effect. For the regional models, station was nested within site as a random effect and for site models station was used as a random effect.

To investigate species-specific effects of the disease outbreak disease prevalence, density, and LTA were examined for each coral species documented throughout the 5 years of monitoring. Species-level disease prevalence was tested for trends over time using the Mann-Kendall trend test in the Kendall package (McLeod, 2011) in R. Linear mixed-effects models were used with species density or LTA as the response variable and year as the fixed effect and a random effect for station nested within site.

For all models, if significant effects were found a Tukey's *post hoc* analysis was performed using the glht() (general linear hypothesis) function in the multcomp package (Hothorn et al., 2008). Additionally, all figures were generated using the ggplot2 package (Wickham, 2009) in R and all data are presented as mean ± standard error (SE) unless noted otherwise.

It should be noted that prior to 2013 SECREMP grouped *Orbicella* (formerly known as *Montastraea) annularis*, *Orbicella faveolata*, and *Orbicella franksii* as the *Orbicella annularis* complex and they are therefore grouped for this study. Additionally, *Agaricia* spp. are grouped as a result of highly variable inter-observer identifications of these species.

[4]http://www.numericana.com/answer/ellipsoid.htm

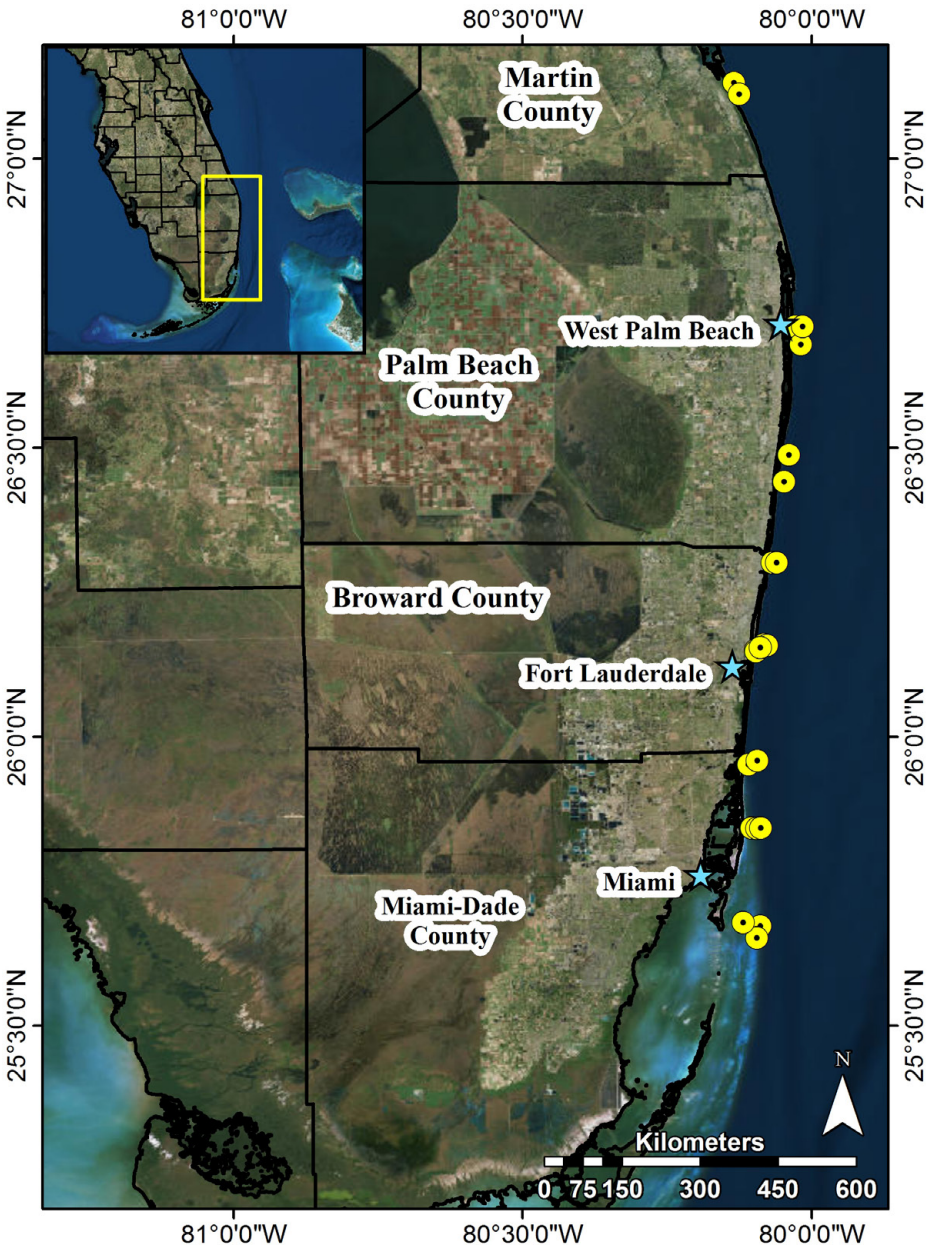

FIGURE 1 | Map of southeast Florida and locations of 22 permanent SECREMP monitoring sites. Each yellow point represents sites, stars name major metropolitan areas, and black outlines represent county boundaries. Site names and descriptive data are included in **Table 1**.

Additionally, species-level differences are for these complexes not individual species.

RESULTS

Increased Thermal Stress and Coral Bleaching and Disease

Benthic temperatures at the SECREMP sites exhibited continued increases from 2007 to 2016, suggesting increased thermal stress throughout the southeast Florida region, which is in agreement with sea surface temperature reports from southeast Florida as well as the Florida Keys (Manzello, 2015; NOAA Coral Reef Watch, 2016). In years with corresponding stony coral demographic data (2012–2016), 2015 was the warmest year documented by SECREMP (**Figure 2**), based on annual means, and had the most days with maximum temperatures exceeding a documented bleaching threshold of 30.5°C (Manzello et al., 2007), especially in Miami-Dade County (**Supplementary Table S1**). Regionally, increasing trends were observed for monthly maximum, minimum, and mean temperatures, although only the monthly minimum trend was

TABLE 1 | SECREMP monitoring site characteristics and locations.

Site Name	Reef Type	Depth (m)	Latitude (N)	Longitude (W)
MC1	NRC	4.6	27° 07.900′	80° 08.042′
MC2	NRC	4.6	27° 06.722′	80° 07.525′
PB1	NRC	7.6	26° 42.583′	80° 01.714′
PB2	Outer	16.8	26° 40.710′	80° 01.095′
PB3	Outer	16.8	26° 42.626′	80° 00.949′
PB4	Outer	16.8	26° 29.268′	80° 02.345′
PB5	Outer	16.8	26° 26.504′	80° 02.854′
BC1	NRC	7.6	26° 08.872′	80° 05.758′
BC2	Middle	12.2	26° 09.597′	80° 04.950′
BC3	Outer	16.8	26° 09.518′	80° 04.641′
BC4*	Inner	9.1	26° 08.963′	80° 05.364′
BC5*	Middle	13.7	26° 18.100′	80° 04.095′
BC6*	Outer	16.8	26° 18.067′	80° 03.634′
BCA	NRC	7.6	26° 08.985′	80° 05.810′
DC1	Inner	7.6	25°50.530′	80° 06.242′
DC2	Middle	13.7	25° 50.520′	80° 05.704′
DC3	Outer	16.8	25° 50.526′	80° 05.286′
DC4	Outer	12.5	25° 40.357′	80° 05.301′
DC5	Inner	7.3	25° 39.112′	80° 05.676′
DC6*	NRC	4.6	25° 57.099′	80° 06.534′
DC7*	Middle	16.8	25° 57.530′	80° 05.639′
DC8*	NRC	4.6	25° 40.707′	80° 07.111′

An asterisk (*) next to a site represents sites that were not surveyed in 2012. NRC = Nearshore Ridge Complex, MC = Martin County, PB = Palm Beach County, BC = Broward County, DC = Miami-Dade County.

significant ($p = 0.094$; $p = 0.031$; $p = 0.100$, respectively). As benthic temperatures increased in the region, increases in coral bleaching and disease prevalence were also observed. Mean bleaching (bleached + partially bleached) prevalence changed significantly over the 5 years of monitoring ($p = 0.005$) and 2014 ($6.32 \pm 0.88\%$) and 2016 ($7.55 \pm 1.19\%$) were significantly greater than 2012 ($2.01 \pm 0.51\%$, $p = 0.047$ and $p = 0.002$, respectively) (**Table 2** and **Supplementary Figure S1**).

Not only was the maximum coral bleaching prevalence for the study period observed in 2016, but the same was also seen for disease prevalence. Mean disease prevalence in 2016 ($3.29 \pm 0.60\%$) more than doubled compared to 2015 ($1.49 \pm 0.35\%$) and was significantly greater than all previous years ($p < 0.01$) (**Figure 3A**). The increase in disease prevalence was primarily due to a region-wide outbreak of WS, which was over 2.6 times more prevalent in 2016 ($2.48 \pm 0.57\%$) compared to all other study years ($p < 0.05$) (**Table 2**). Non-white syndrome diseases were prevalent in all study years, but the maximum prevalence for non-white syndrome never exceeded 1% (**Table 2**). Additionally, non-white syndrome disease prevalence was driven by dark spot disease. While WS prevalence was only significantly greater in 2016, the outbreak was documented prior to 2016 (**Figure 3B**). White syndrome was first documented in the SECREMP demographic data at two sites in 2013 (BC4 and DC8). Over the next 3 years, WS was documented in at least one site in all four counties, and each year all habitats had at least one site with WS (**Supplementary Table S2**). Additionally, the number of sites impacted progressively increased from four sites in 2014 to eight sites in 2015 and 13 sites in 2016. Seven sites had significant increases in WS prevalence ($p < 0.05$) between 2012 and 2016 with the greatest increase and highest mean occurring at BC1 in 2016 ($14.39 \pm 2.95\%$) (**Supplementary Table S2**).

In addition to being geographically widespread, the WS outbreak appeared indiscriminate, affecting 11 of the 24 coral species recorded throughout the study period (**Figure 4** and **Supplementary Table S3**). WS prevalence increased in terms of number of species affected as well as within species from 2013 to 2016, although no significant trends within species were found (Mann-Kendall, $p > 0.05$). In 2013, only two species, *Dichocoenia stokesii* and *Porites astreoides*, were affected by WS. This increased to six species in 2014, nine in 2015, and seven in 2016. Over the four years in which WS was observed, species prevalence ranged from 0.17% for *P. astreoides* in 2015 to 25% for *Eusmilia fastigiata* in 2016 (**Supplementary Table S3**). Additionally, the highest prevalence per species was observed in 2015 and 2016. In 2015, *D. stokesii* (10.91%) and *O. annularis* complex (8.33%) prevalence exceeded 5%. Prevalence was even greater in 2016 with four species having prevalence > 10%: *E. fastigiata* (25%), *Montastraea cavernosa* (15.32%), *Solenastrea bournoni* (13.16%), and *O. annularis* complex (12.5%).

White Syndrome Outbreak Coincides With Coral Community Declines

The wide geographical impact and the multiple host species susceptible to WS corresponded with changes to the coral communities of southeast Florida, which was evident by examination of community diversity. At the regional level, all three measures were significantly lower in 2016 (S: 4.92 ± 0.19, H': 1.24 ± 0.04, D: 3.08 ± 0.10) compared to all other years ($p < 0.001$) (**Supplementary Figure S2**). At the site level, significant changes were also observed for all three measures ($p < 0.05$), with one site that significantly increased, PB1 (S: $p < 0.01$; H': $p < 0.05$; D: $p < 0.001$). PB1 is a nearshore ridge complex site in Palm Beach County that has been impacted by sand movement. PB1 was buried by sediment for several years, but has become more exposed since 2013. For species richness nine sites had significantly fewer species in 2016 compared to other years ($p < 0.05$). Significant decreases were also observed for Shannon's index and Inverse Simpson's index at ten and seven sites, respectively ($p < 0.05$) (**Supplementary Table S4**).

In an effort to determine whole colony mortality related changes at the population-level, coral density was analyzed. Prior to 2016, annual mean stony coral density throughout southeast Florida was slightly increasing. Mean density significantly ($p < 0.001$) decreased from 1.29 ± 0.10 colonies/m^2 in 2015 to 1.07 ± 0.09 colonies/m^2 in 2016. Coral density in 2016 was also significantly lower than 2012 (1.15 ± 0.10 colonies/m^2, p 0.01), 2013 (1.21 ± 0.09 colonies/m^2, $p < 0.01$), and 2014 (1.26 ± 0.10 colonies/m^2, $p < 0.001$) (**Figure 5**). For the 22 sites monitored, 18 sites had declines in coral density, six of which were significant ($p < 0.05$). The declines ranged from 10 to 52% and were not localized to one subregion or habitat, but were distributed between all habitats in Palm Beach, Broward, and Miami-Dade counties (**Figure 6**).

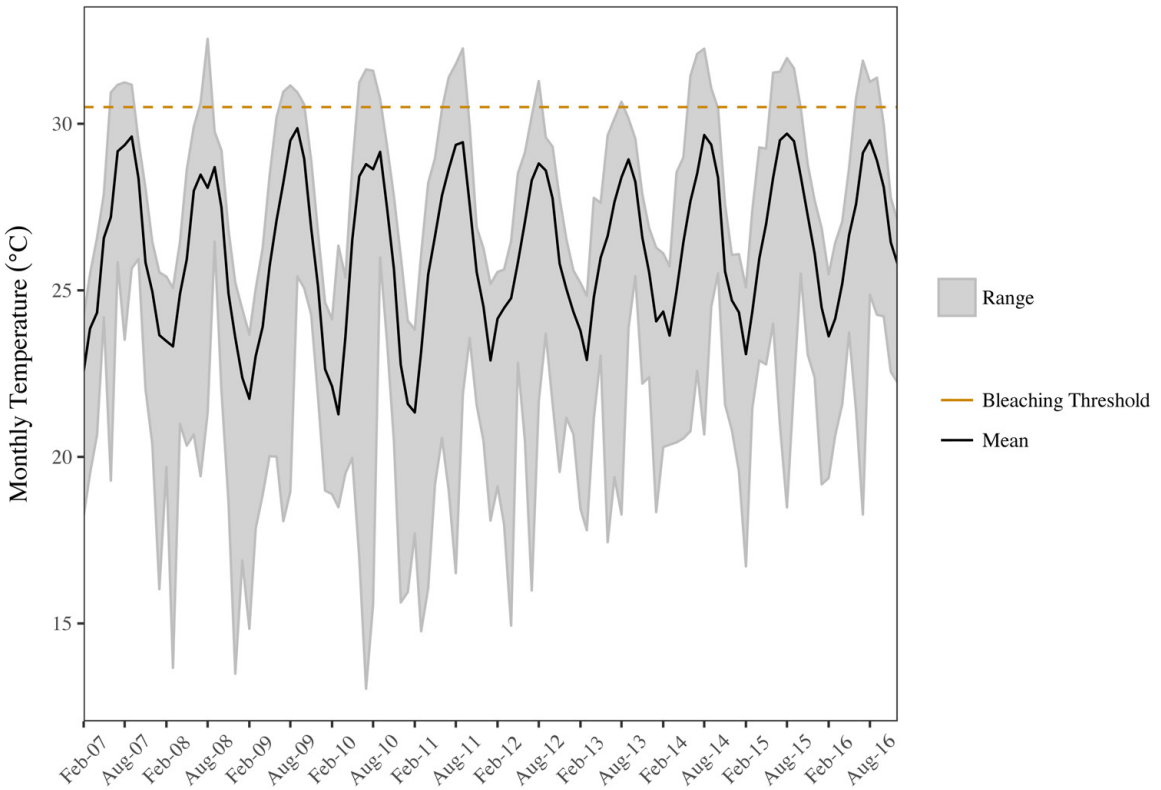

FIGURE 2 | Minimum, mean, and maximum monthly benthic temperatures for the SECREMP region. The black line indicates the mean monthly temperature, the gray ribbon is the range between the monthly minimum and maximum, and the dashed orange line indicates a documented bleaching threshold for the Florida Reef Tract (30.5°C; Manzello et al., 2007).

TABLE 2 | Mean (±SE) annual bleaching and disease prevalence.

Year	Bleaching (%)	All diseases (%)	White syndrome (%)	Non-white syndrome (%)
2012	2.01 ± 0.51	0.26 ± 0.11	0.00 ± 0.00	0.26 ± 0.11
2013	4.39 ± 1.27	0.51 ± 0.23	0.08 ± 0.05	0.43 ± 0.22
2014	6.32 ± 0.88	1.24 ± 0.34	0.82 ± 0.32	0.41 ± 0.14
2015	5.19 ± 0.80	1.49 ± 0.35	0.95 ± 0.30	0.54 ± 0.19
2016	7.55 ± 1.19	**3.29 ± 0.60**	**2.48 ± 0.57**	0.81 ± 0.23

Percent bleaching included bleached and partially bleached colonies and all disease prevalence included all corals exhibiting signs of disease. Values in bold are significantly different from all other years.

Species-level density was analyzed for changes indicating whole colony mortality. Four species had densities in 2016 that were significantly lower than they had been in previous years ($p < 0.05$) and five additional species had their lowest densities, although not significant ($p > 0.05$). *M. cavernosa*, *D. stokesii*, and *Meandrina meandrites*, all species with elevated WS prevalence, had significant declines in density (**Figure 5**; $p < 0.0001$). Additionally, significant decreases in density were also observed for *Siderastrea siderea* ($p < 0.0001$) and *Acropora cervicornis* ($p < 0.05$). The *A. cervicornis* decline is not due to WS as this disease has not been documented affecting *A. cervicornis*. *D. stokesii* mean density declined by 90% from 0.04 ± 0.01 colonies/m^2 (2014) to 0.004 ± 0.002 colonies/m^2 (2016). *Meandrina meandrites* density declined 95% (2013: 0.06 ± 0.01 colonies/m^2, 2016: 0.003 ± 0.001 colonies/m^2), while *M. cavernosa* (2012: 0.26 ± 0.05 colonies/m^2, 2016: 0.13 ± 0.02 colonies/m^2) and *S. siderea* (2012: 0.25 ± 0.02 colonies/m^2, 2016: 0.17 ± 0.02 colonies/m^2) declined 50 and 32%, respectively. Additionally, when raw abundance values were examined they revealed *D. stokesii* counts decreased from 78 colonies in 2014 to 55 colonies in 2015, and eight colonies in 2016. *M. meandrites* showed similar declines from 118 colonies in 2014 to 85 colonies in 2015 and five colonies in 2016. While *M. cavernosa* abundance decreased as well, the major decline was not observed over multiple years, but occurred between 2015 (457 colonies) and 2016 (248 colonies), which is a 45% colony loss (**Supplementary Table S3**).

Changes in diversity and density are dependent on losses of whole colonies and corals exhibit colony partial mortality; therefore, LTA was used to examine population and species level changes related to partial mortality. Mean regional LTA in 2016 (1.05 ± 0.20 m^2) was significantly less than 2012 (1.68 ± 0.42 m^2, $p < 0.0001$), 2013 (1.53 ± 0.25 m^2, $p = 0.0001$), 2014 (1.59 ± 0.28 m^2, $p < 0.0001$), and 2015 (1.62 ± 0.31 m^2, $p < 0.0001$) (**Figure 7A**). At the site level, LTA decreased at 18 of the 22 sites between 2015 and 2016, 16 of which resulted in the lowest LTA values documented at the respective

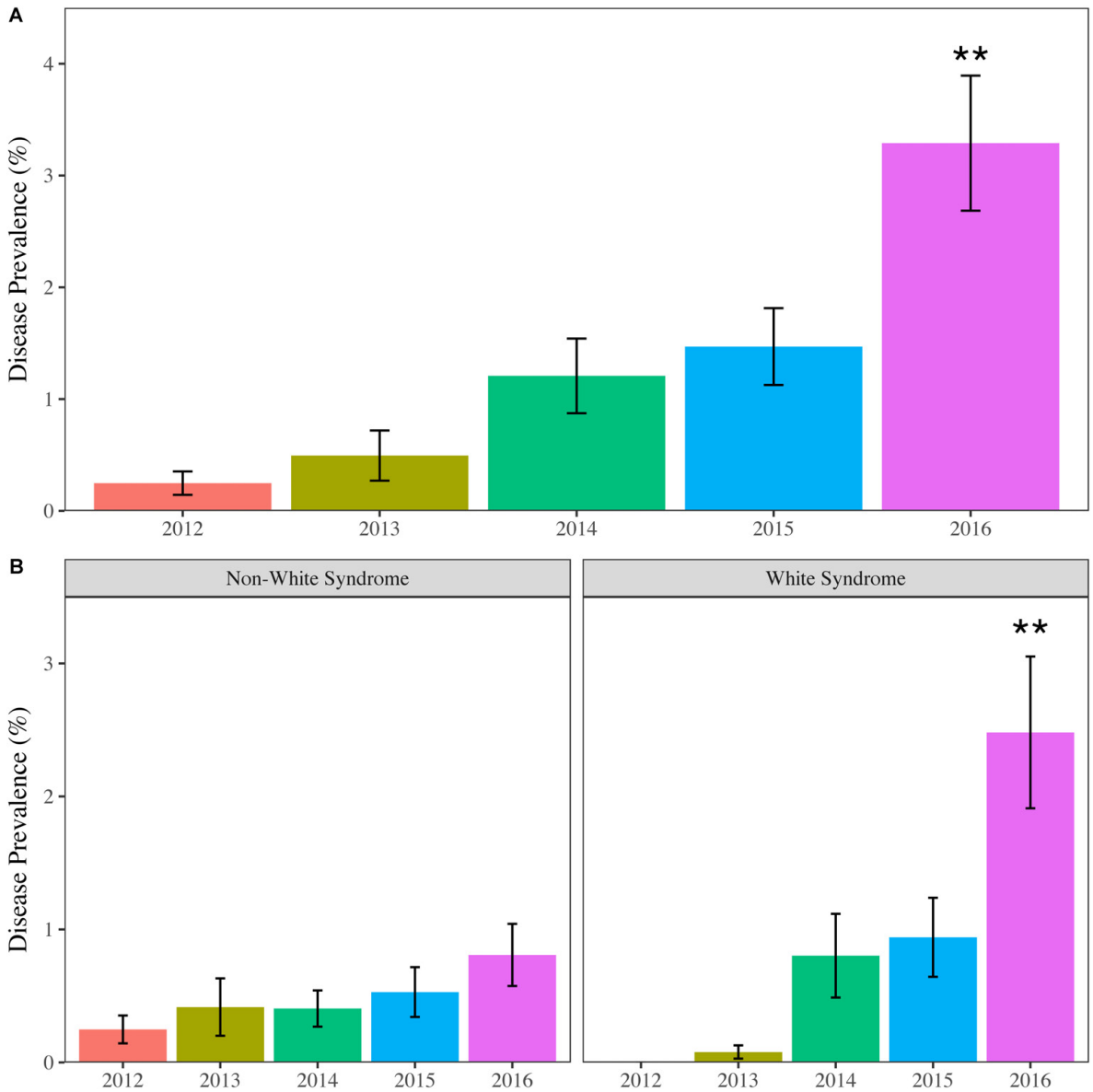

FIGURE 3 | Mean (±SE) annual regional coral disease prevalence for **(A)** all diseases combined and **(B)** Non-white syndrome and white syndrome. Asterisks indicate years that are significantly different. $^{**}p < 0.01$.

sites. Seven sites (PB2, PB4, and PB5; BC4 and BC5; DC1 and DC4) had significant decreases in LTA between 2012 and 2016 ($p < 0.05$) (**Supplementary Figure S3**). All habitats within each county were represented by sites with decreased LTA (**Supplementary Figure S3**). Broward County 1, a site historically dominated by *M. cavernosa*, did not exhibit a significant change overall ($p = 0.0564$), but examination of pairwise annual changes revealed a significant decline in LTA between 2015 (12.98 ± 2.06 m^2) and 2016 (8.06 ± 1.57 m^2, $p = 0.022$). The significant decline observed at BC1 corresponded with a significant regional decrease in *M. cavernosa* mean LTA, resulting in 2016 (0.66 ± 0.23 m^2) being significantly lower than 2015 (1.04 ± 0.32 m^2, $p = 0.0015$), 2014 (1.01 ± 0.30 m^2, $p = 0.0017$), and 2012 (1.35 ± 0.51 m^2, $p < 0.01$). In addition to *M. cavernosa*, three other species exhibited significant changes in LTA, *P. astreoides* ($p < 0.05$), *S. siderea* ($p < 0.05$), and *D. stokesii* ($p < 0.01$) between 2012 and 2016. *Siderastrea siderea* and *D. stokesii* had significant declines, whereas *P. astreoides* LTA increased, with mean LTA in 2015 (0.17 ± 0.03 m^2, $p < 0.05$) and 2016 (0.17 ± 0.02 m^2, $p < 0.05$) being significantly greater than 2012 (0.11 ± 0.02 m^2). For *S. siderea* mean LTA declined annually from 2013 (0.09 ± 0.02 m^2) to 2016 (0.07 ± 0.01 m^2), but only 2016 ($p < 0.05$) was significantly different from 2013. Similarly, *D. stokesii* mean LTA has steadily declined from 2013 to 2016, with 2015 (0.03 ± 0.02 m^2) and 2016 (0.02 ± 0.01 m^2) being significantly lower than 2013

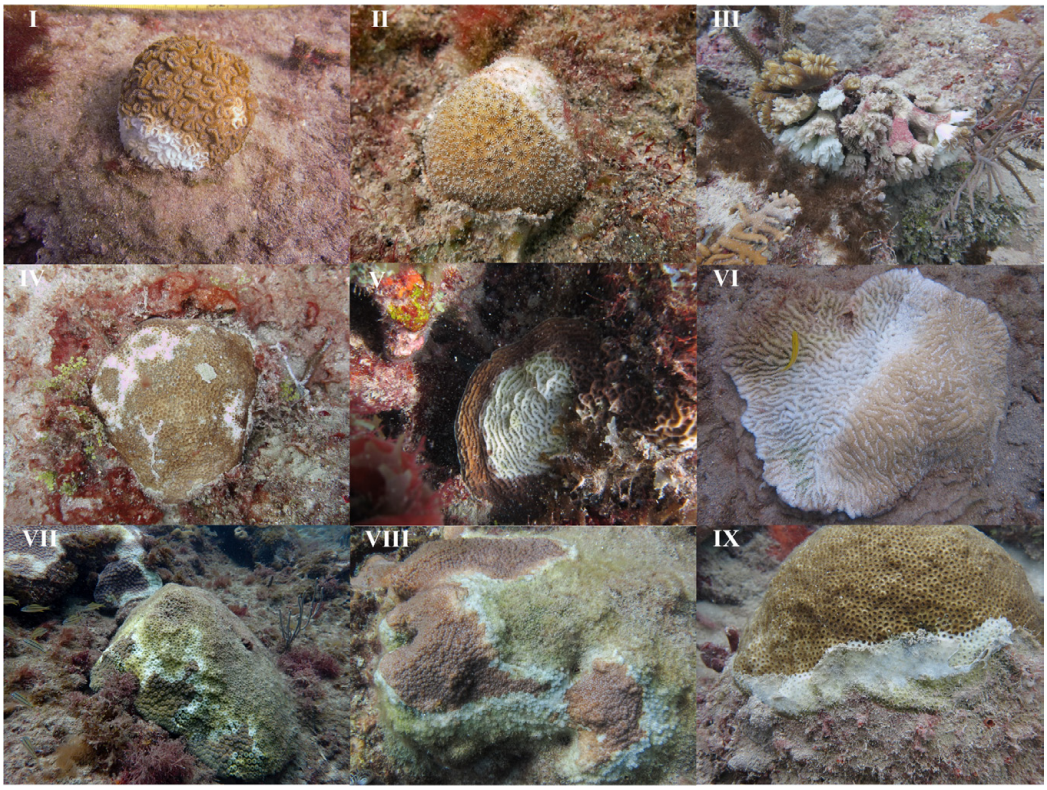

FIGURE 4 | Images of 9 of the 11 species affected by white syndrome during the outbreak. **(I)** *Dichocoenia stokesii*, **(II)** *Stephanocoenia intersepta*, **(III)** *Eusmilia fastigiata*, **(IV)** *Siderastrea siderea*, **(V)** *Agaricia* sp., **(VI)** *Meandrina meandrites*, **(VII)** *Montastraea cavernosa*, **(VIII)** *Orbicella* sp., **(IX)** *Solenastrea bournoni*.

(0.06 ± 0.02 m^2, $p < 0.05$). *M. meandrites* also exhibited a drastic decline with 2016 (0.004 ± 0.000) being significantly lower than 2012 (0.22 ± 0.09, $p < 0.01$), 2013 (0.22 ± 0.06, $p < 0.001$), and 2014 (0.19 ± 0.05, $p < 0.001$). This decline corresponded to the loss of all but five colonies in the region. In total, 15 species had declines in mean LTA between 2015 and 2016, leading to 10 total species having their lowest mean LTA in 2016 (**Figure 7B**). Additionally, of the 11 species exhibiting signs of WS during the study period, 10 had declines in LTA. *Porites porites* was the only species affected by WS that did not decline in LTA during the study period.

DISCUSSION

Our analysis of 5-years of SECREMP stony coral demographic data clearly shows that the Southeast Florida Reef Tract (SEFRT) was severely impacted by an ecosystem-altering disease outbreak that was unprecedented in terms of temporal scale (3 + years), geographic extent (nearly 100 km between affected sites), number of species affected, and severity (colony mortality). We demonstrate that the outbreak began as early as summer 2014 and continued through 2016. During this period, the outbreak was a region-wide, multi-habitat event corresponding with significant declines in stony coral diversity, colony density, and LTA. Region-wide, 11 of 24-recorded species were documented with WS, four of which had significant regional declines in density and LTA. Increased disease prevalence coincided with the three warmest years on record (NOAA Coral Reef Watch, 2016), thus, thermal stress was very likely a major, but not sole contributor to the outbreak. Although not specifically addressed in this study, the cumulative impacts of multiple local stressors related to the close proximity of the SEFRT to a highly urbanized coast likely contributed to the unprecedented nature of this outbreak.

The disease outbreak documented here was a multi-year event impacting the SEFRT coral community. Disease prevalence in Miami-Dade and Broward counties in 2013 were similar to previously documented background levels (Muller and van Woesik, 2012; Ruiz-Moreno et al., 2012). In 2014, disease prevalence increased in West Palm Beach, Broward, and Miami-Dade counties. Precht et al. (2016) reported the outbreak as white plague, near Virginia Key, Florida (Miami-Dade County) adjacent to major Port of Miami dredging activities in September 2014. Our data suggests the outbreak likely began earlier in 2014 as SECREMP sampling at all sites occurred prior to September 2014. Additionally, as early as 2014 the outbreak appeared to be more widespread than just in Miami-Dade County as Precht et al. (2016) reported: two SECREMP sites (BC5 and BC6) in northern Broward County in 2014 had WS prevalence values of 4 and 13%, respectively. White syndrome was also recorded in Palm Beach County in 2014, although at or below a previously documented background

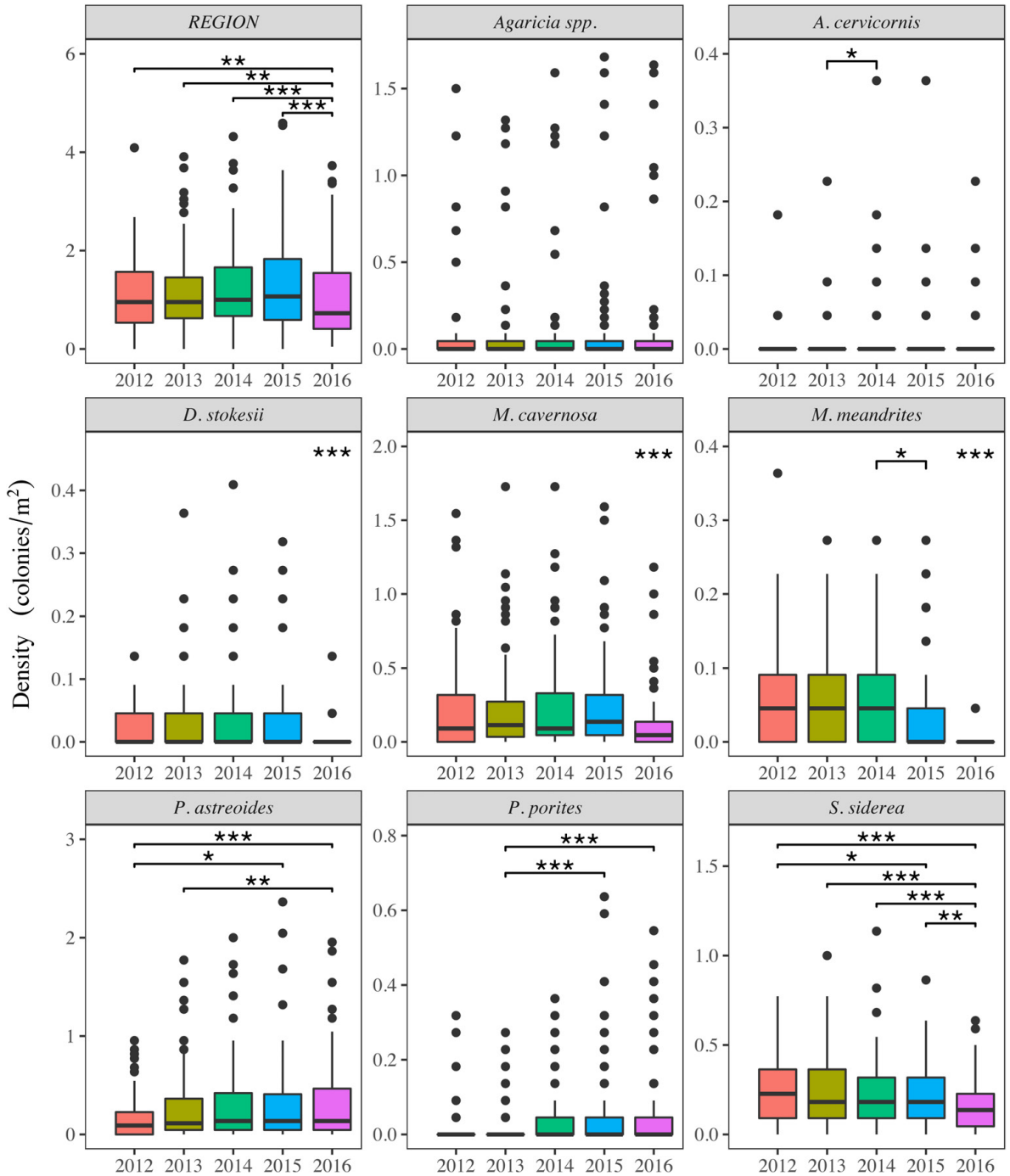

FIGURE 5 | Annual colony density for the region **(top left)** and coral species with significant changes from 2012 to 2016 ($p < 0.05$). The middle bars of the boxplots represent the median values, the box length represents the interquartile range (IQR), the whiskers extend from the upper and lower IQR to the maximum and minimum values, and the dots indicate possible outliers. During this time period *P. astreoides* and *P. porites* had significant increases, *Agaricia* spp. had a significant overall change, but none of the pairwise comparisons were significant. All other species showed significant declines. Asterisks represent significant pairwise differences and the bars below the asterisks indicate the pairwise comparisons. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

level (Muller and van Woesik, 2012; Ruiz-Moreno et al., 2012). White syndrome prevalence continued to increase reaching its maximum for the study period in 2016 and was documented in 13 of 22 sites distributed in multiple habitats ranging from 3 to 20 m depths in Palm Beach, Broward, and Miami-Dade counties. While non-white syndrome diseases were documented throughout the study, their prevalence remained < 1% (**Table 2**). Additionally, dark spot disease was the

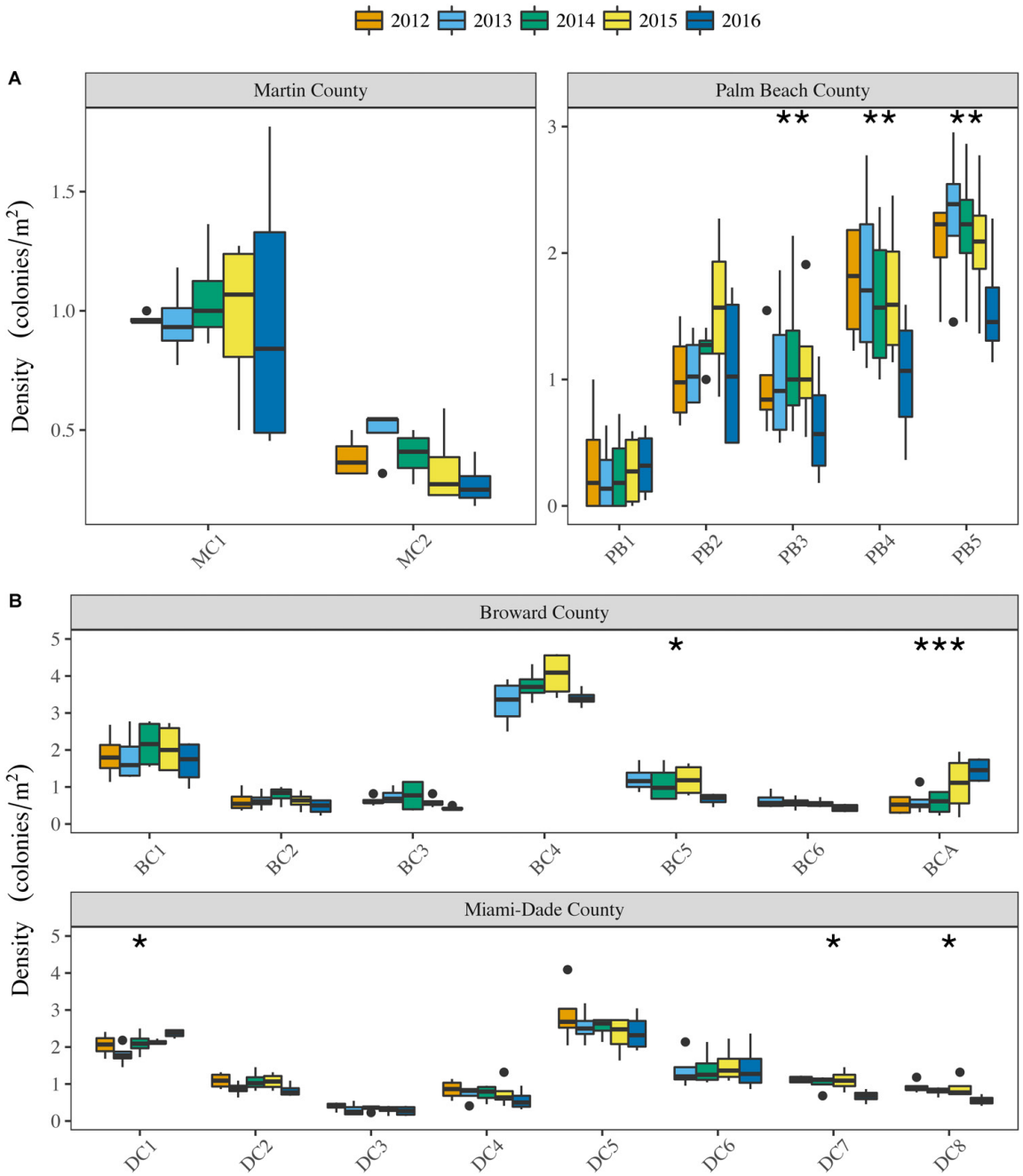

FIGURE 6 | Annual colony density per site in **(A)** Martin, Palm Beach, **(B)** Broward, and Miami-Dade counties. The middle bars of the boxplots represent the median values, the box length represents the interquartile range (IQR), the whiskers extend from the upper and lower IQR to the maximum and minimum values, and the dots indicate possible outliers. Asterisks indicate sites with significant differences between years. *Post hoc* tests for sites with significant differences showed: 2016 density was significantly less than all other years for PB3, PB4, PB5, BC5, DC7, and DC8; BCA density in 2016 was significantly greater than 2012-2014 and 2015 was significantly greater than 2012 and 2014; DC1 density in 2016 was significantly greater than 2013. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

primary contributor to non-white syndrome diseases, which most often does not result in mortality (Borger, 2003, 2005). Our prevalence data showed the wide geographic extent and longevity of the outbreak even in light of the challenges of capturing this type of data during annual monitoring events. Our annual data most certainly underestimated the extremely high disease prevalence such as those reported by Precht et al. (2016).

Coinciding with the outbreak, significant declines in coral species diversity and colony density were observed, both of which have been suggested as indicators of reef resilience (Maynard et al., 2017; van Woesik, 2017). White syndrome

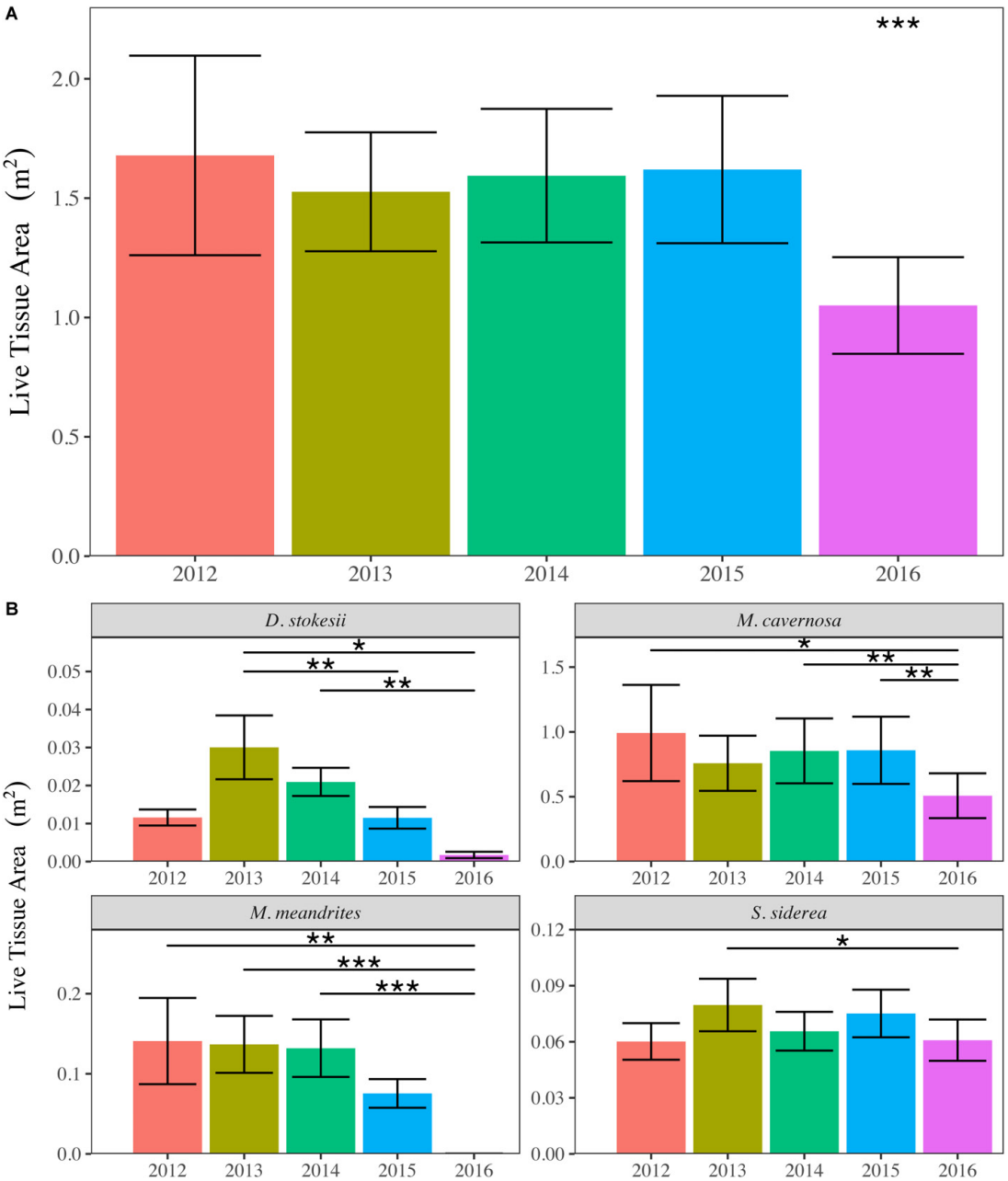

FIGURE 7 | Annual mean (±SE) stony coral live tissue area (LTA) for the SECREMP **(A)** region and **(B)** coral species with significant declines due to white syndrome. Regional LTA in 2016 was significantly less than all other years. Asterisks indicated significant differences between years and the bars below the asterisks indicate the pairwise comparisons. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

outbreak related declines in coral diversity and density are cause for concern for the already severely stressed SEFRT. Coral diversity declines have been associated with shifts in ecosystem function and stability, increased susceptibility to selective pressures, and reduced resilience and adaptability (van Woesik, 2002, 2017). Additionally, declines in diversity and density contribute to the loss of reef architecture, which can lead to decreased biodiversity of the reef system, specifically with respect to fish communities (Roberts et al., 2002; Alvarez-Filip et al., 2009). The loss of architecture is becoming evident as reef building, complexity-contributing species like *M. cavernosa*, *M. meandrina*, and *S. siderea* exhibited significant declines. The declines in these occurred as increases in more "weedy" species like *P. astreoides*, *P. porites*, and *Agaricia* spp. (**Figure 5**) were also documented. These species typically grow as small, flat colonies resulting in reduced reef complexity (Knowlton, 2001; Precht and Miller, 2007; Green et al., 2008).

Similar to reduced diversity and density, reductions in LTA can alter individual colony contributions to ecosystem function and reef habitat quality (Cote et al., 2005; Bruno and Selig, 2007). Regional LTA significantly decreased in 2016 (**Figure 7A**), particularly as a result of major losses in specific species affected by WS. Additionally when considering LTA as equivalent to cover, reduced LTA can affect a colony's reproductive potential, reducing the likelihood of recovery following disturbance events. Hartmann et al. (2017) showed high coral cover populations produced more larvae per square centimeter of tissue leading to higher larval outputs per square meter of reef. Based on this, reductions in LTA diminish the potential for larval production and output yielding decreased possibilities for successful sexual reproduction. Beyond the decline of LTA as a measure of benthic cover, decreased LTA is also indicative of reduced tissue per individual colony. Previously determined relationships between colony size and fecundity suggest that our observed decreases in LTA could lead to reductions in fecundity (Connell, 1973; Szmant-Froelich, 1985; Ward, 1995; Tsounis et al., 2006). As a result of the probable decreases in fecundity and reproductive potential of many of the remaining SEFRT corals, the likelihood of recovery is greatly reduced.

The 2014 to 2016 disease outbreak was arguably the most devastating disturbance event yet documented in the SEFRT, and has altered ecosystem function to a point where recovery is greatly challenged. Our data estimates that regionally as much as 30% of coral colony density and 60% of LTA was lost. Globally, ocean warming related coral bleaching events coupled with disease outbreaks are predicted to become more frequent and severe (Maynard et al., 2015). As thermal stress event frequency increases, time between events decreases, greatly limiting recovery potential, and this scenario is suggested to occur at even the most remote reefs (Hughes et al., 2018). The SEFRT is not remote. In addition to being a high-latitude system near the environmental threshold for significant coral reef growth, the SEFRT is offshore of a highly urbanized area driving increased nutrient loading from urban runoff and defining the ever-increasing need for marine construction projects (beach nourishment, port dredging, etc.) to support coastal development.

Global climate change related stress is challenging for local resource managers to address; however, with a greater understanding of the relative importance of local anthropogenic stressors contributing to increased coral disease prevalence, local management strategies can be developed to reduce disease-related impacts and facilitate recovery (Beeden et al., 2012). The irreplaceable economic and recreational benefits of the SEFRT are threatened because the system is under varied and chronic stress. Local resource management strategies need to include stronger regulations to reduce local drivers of stress such as development-related sedimentation (Pollock et al., 2014; Miller et al., 2016; Fourney and Figueiredo, 2017) and nutrient loading (Vega Thurber et al., 2013). Credible information is required to inform management, and SECREMP captured dramatic impacts to the SEFRT related to a major coral disease outbreak. Long-term monitoring provides annual status data on reef health and condition from which spatial and temporal changes to the system can be determined. Long-term monitoring will be required to document the success of management actions in response to this disease outbreak and further inform management decision-making. The SEFRT may be a harbinger for declines in other reef areas around the world subjected to similar cumulative global and local stresses. As population increases and coastal development intensifies, coral reef resiliency will depend upon our thorough understanding of the cumulative impacts local and global stressors driving declines and limiting recovery.

DATA AVAILABILITY STATEMENT

All data used in this manuscript are available from the Florida Fish and Wildlife Conservation Commission. Requests for data should be emailed to Corals@MyFWC.com and Francisco.Pagan@FloridaDep.gov.

AUTHOR CONTRIBUTIONS

CW, NH, and DG conducted the fieldwork and wrote and edited the manuscript. CW performed the statistical analysis.

FUNDING

This work was funded pursuant to Florida Department of Environmental Protection (FDEP) Grant Nos. RM085 and RM143 to the Florida Fish and Wildlife Conservation Commission (FWC) and then contracted to Nova Southeastern University. Though funded in part by a grant agreement from FDEP through National Oceanic and Atmospheric Administration (NOAA) Award Nos. NA13NOS4820015 and NA15NOS4820036 to FDEP, the views, statement, findings, conclusions, and recommendations expressed herein are those of the author(s) and do not necessarily reflect the views of the State of Florida or NOAA or any of their subagencies.

ACKNOWLEDGMENTS

We thank the numerous members of the NSU Coral Reef Restoration, Assessment and Monitoring Lab for their tireless efforts in the field and lab throughout the 14 years of SECREMP. We also thank our FRT monitoring partners in the Florida Fish and Wildlife Conservation Commission Coral Program, especially Rob Ruzicka, Mike Colella, Vanessa Brinkhuis, and Jeff Beal as well as the Florida Department of Environmental Protection Coral Reef Conservation Program, especially Joanna Walczak and Francisco Pagan.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00323/full#supplementary-material

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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

ORIGINAL RESEARCH
published: 26 September 2018
doi: 10.3389/fmars.2018.00340

Impact of the Use of a Teaching Toolbox in an Awareness Campaign on Children's Representations of Coral Reefs

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Environmental education for children is one of the fundamental tools required to reverse the degradation of our environment and the biodiversity erosion. Currently coral reefs are part of the vulnerable ecosystems which are most threatened by human activities and climate change. Responding to these pressures demands decisions at multiple scales, based on solid knowledge of coral reefs but also on strengthened awareness to build adaptive management solutions. Here we evaluate the impact of an environmental awareness campaign for children using a teaching toolbox developed by scientists (MARECO "The Coral Reef In Our Hands"). To assess this impact before and after using the toolbox, we analyzed the evolution of children's representations of coral reefs through drawings. This study was carried out in New Caledonia, focusing on five elementary schools in different social and cultural contexts (urban, rural and coastal). Two hundred and forty-eight drawings were made by children. The drawings were analyzed quantitatively using multivariate statistical analyses which reveals a diversity of representations in children with diverse sociocultural profiles, but also between schools, emphasizing that relationships with nature and marine environment vary according to direct and indirect experiences related to reefs. Furthermore, our results pointed out relevant differences in coral reef representations before and after the use of MARECO, particularly regarding their knowledge of reef biodiversity associated with multicolored organisms and the connection of coral reef with environment, the number of colors being used as a proxy of this holistic vision developed by children. These results point out the performance of MARECO as a playful tool to transfer scientific knowledge to children. Coral reef conservation is intimately linked to an awareness in young generations of the environmental challenges of tomorrow. To be agents of change in a sustainable world, children must be engaged in a fun, rigorous, action-oriented and socially responsible learning process such as the ones developed in participatory approaches.

Keywords: coral reefs, biodiversity, interdisciplinarity, environmental education, children drawings, representation, color variable, New Caledonia

OPEN ACCESS

Edited by:
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University of Derby, United Kingdom

Reviewed by:
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Kathleen M. Morrow,
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *04 May 2018*
Accepted: *04 September 2018*
Published: *26 September 2018*

Citation:
Chabanet P, Stoica G, Carrière SM, Sabinot C, Bedrossian C and Ferraris J (2018) Impact of the Use of a Teaching Toolbox in an Awareness Campaign on Children's Representations of Coral Reefs. Front. Mar. Sci. 5:340. doi: 10.3389/fmars.2018.00340

INTRODUCTION

The Anthropocene accentuated the rift between humankind and nature, accelerating the degradation of our environment and the biodiversity crisis (Steffen et al., 2011) without there being any major solutions at the present time (Carrière et al., 2013). Restoring better balance to the relationship between humans and nature firstly requires improving knowledge of nature and the links connecting living beings to their environment. For this, environmental education is one of the fundamental tools required to reverse the current trends in biodiversity loss but whose role remains underestimated and, as a consequence, still currently underused (Feinsinger, 1987; Brewer, 2002; Ballouard et al., 2011). In 2015, the international community recognized, through the 2030 Agenda for Sustainable Development, that education (SDG 4: ensure inclusive and equitable quality education) was essential for the success of all 17 of its goals, including SDG 14 (conserve and sustainably use the oceans, seas and marine resources for sustainable development) which includes the sustainable development of coral reefs.

Coral reefs are the most diverse marine ecosystem on Earth (Veron, 1995; Bellwood and Hughes, 2001; Paulay, 2017) and their biological, ecological, cultural and economic benefits are widely recognized (Moberg and Folke, 1999). They are charismatic ecosystems, with their esthetic value being a strong argument for conservation, a lever of communication, a factor of human well-being and an opportunity to reconnect humans to their environment (Tribot, 2017). Although some remote unpopulated wilderness areas remain in very good condition (Graham and McClanahan, 2013; Chabanet et al., 2015), an estimated 19% of the world's coral reefs have been lost and a further 35% are seriously threatened (Wilkinson, 2008; Veron et al., 2009; Burke et al., 2012). As a result, one-third of all reef-building corals are considered to be at risk of extinction (Carpenter et al., 2008). One of the main causes of coral reef degradation over the past decades is worldwide demographic growth and socio-economic development, which are often accompanied by overfishing, sedimentation from urban development, eutrophication, marine pollution, and coral diseases (Hughes et al., 2003; Hoegh-Guldberg et al., 2007; Newton et al., 2007; Veron et al., 2009; Johnson et al., 2013; Séré et al., 2015), but also the increase of recreational users in coastal areas (Gonson et al., 2016, 2017). Anthropogenic disturbances on coral reefs make them much more susceptible to current and future climate change (Hughes et al., 2017), particularly with the increase of mass bleaching events (Spalding and Brown, 2015; Heron et al., 2016; Hughes et al., 2018) and ocean acidification (Schönberg et al., 2017; Eyre et al., 2018; Wu et al., 2018). These changes have a significant impact on the welfare and livelihoods of over 500 million people worldwide, mainly in the Southern countries, where they depend directly on these ecosystems and the goods and services they provide (Moberg and Folke, 1999). The management of coral reef ecosystems is therefore critical, both ecologically and socially. Sustainability of coral reef ecosystems involves multi-level decision-making processes based on solid scientific knowledge of coral reefs' socio-ecological systems as well as empirical local ecological knowledge and observation within the environment that constitute pertinent indicators of socio-environmental changes (Sabinot and Lescureux, 2018). It must also involve education of local populations including children to enable them to understand critical issues associated with the importance of coral reefs, so they can become enlightened citizens engaged in the sustainability of the latter.

In 2010, recognizing the lack of links between science and society, a team of women (coral reef scientists, an educationalist, a nature artist, a graphic designer and an elementary-school teacher) developed the teaching toolbox MARECO, "The Coral Reef In Our Hands," which includes educational games. Created with the goal of transferring the results of research on coral reefs, the toolbox aims first and foremost at raising awareness and educating younger generations about reef biodiversity, the disturbance of coral ecosystems and the need for management through cooperation between actors (co-management). Childhood (5–11 years) was targeted as the key period for introducing environmental education effectively owing to the strength and lasting quality of an early relationship formed between children and the natural world (Caro et al., 1994; Rivas and Owens, 1999). The toolbox has been used in some schools in the Indo-Pacific (Vanuatu, New Caledonia, Reunion, Mayotte and Madagascar).

In 2014, an interdisciplinary team of researchers (ethno-ecologists, environmental anthropologists, and biologists) engaged a new research program in social and natural sciences around coral reef issues. This program combined an awareness campaign using the MARECO toolbox and an innovative approach based on children's drawings to document children's ecological knowledge. It has been conducted in four French territories (New Caledonia, Reunion, Mayotte, Southern France) targeting twenty elementary schools (1st and 2nd grades) in contrasted social and cultural contexts. Based on ethno-ecological and anthropological approaches (Berlin et al., 1973; Toledo, 1992; Ellen, 1993; Ingold, 2004), combined with social representation theory (Moscovici, 2003) applied to environmental sciences, the overall aim of the scientific program was to study children's representations and knowledge, and uses of coral reefs through drawings. Like Doise and Garnier (2002), we consider that social representation theory enables the exploration of the interactions and positions that social groups have (here, children in different schools) relative to social objects of varying complexity (here, coastal marine ecosystems, and more particularly the coral reef). The social representations correspond to opinions specific to a culture, a social class or a group relative to social environment objects (Moliner et al., 2002). As drawings are connected to the so-called "visual realism" when children "draw what they know" (Luquet, 1927), we seek to study through drawings how children from different sites and cultural environments perceive and live in their "nature" (Pagezy et al., 2010; Calandra, 2013; Sabinot and Carrière, 2015; Carrière et al., 2017), and how their experience, knowledge, perceptions and beliefs are shaping these ways of conceiving and dwelling with their "nature" (Ingold, 2004).

Here we evaluate the effect of an environmental awareness campaign on children through the changes in representations after using the coral reef teaching toolbox. More precisely, we seek to assess how and to what extent this specific campaign in

school influences the drawing representations of the coral reef, particularly in terms of marine biodiversity, and interactions of species in the ecosystem, including with humans.

Three hypotheses will be tested:

1- The groups of children in the different schools (with contrasting natural and socio-cultural environments) have specific representations of the marine ecosystems (sea or coral reef).
2- The number of colors used by the child for the drawings can be used as an indicator that the child has developed a holistic perception of the sea or coral reef.
3- The impact of MARECO is assessed on the basis that the child has developed a holistic representation of the coral reef, combining (i) diversity of colors (perceived diversity of species) to a healthy reef, (ii) human activities to the coral reef ecosystem; and (iii) coral reefs to their environment.

This paper presents the findings obtained from our field site in New Caledonia, a UNESCO World Heritage site, and one of the most well-known French coral reefs.

MATERIALS AND METHODS

Study Sites

New Caledonia is a French overseas collectivity located in the southwest Pacific Ocean, approximately 1,500 km off Eastern Australia (**Figure 1**). The archipelago as a whole has a land area of 18,600 km^2 for a population of 269,000 inhabitants (INSEE, 2014). The population of New Caledonia is young, in a country where the GDP (Gross Domestic Product) is one of the highest in the Pacific region but with major socio-economic disparities according to the various urban and rural zones (IEOM, 2008), in particular regarding the educational system and the employment market. New Caledonia is also characterized by high cultural diversity: for more than one, sometimes two centuries, European, Oceanian and Asian people have been living with Kanaks who represent 39% of the population today (INSEE, 2014). This leads to inequalities in terms of way of life, choice of jobs, access to the lagoon, manner of "dwelling in" the environment, politics, etc. The island is surrounded by a coral barrier reef (see geomorphology in Andréfouët et al., 2009) of 1,600 km in length, the world's second-largest barrier reef system, just behind Australia's Great Barrier Reef (Cuif et al., 2014). Its outstanding biodiversity (8,783 species inventoried, Payri and Richer de Forges, 2006) and its natural ecological habitats that are important for biodiversity conservation (coral reefs, mangroves, seagrass meadows, rocky coastlines, silty bay bottoms) generated growing interest from the 1980s onwards, culminating in 2008 when 15,000 km^2 of reef zones were given UNESCO World Heritage status, thus conferring international recognition of the exceptional value of these coral reefs.

The program involved elementary schools located in urban, coastal and rural areas. Five schools were selected (**Figure 1**): Paul Boyer (Nouméa, socially advantaged urban neighborhood), Isidore Noell (Nouméa, less advantaged urban neighborhood), Thio (close to a marine protected area), Banian (Yaté, within a large fishing community) and Coula Gondé (Houaïlou municipality, rural environment, at about 30 km from the sea, without regular public transport). This selection enabled coverage of populations that varied geographically, environmentally, economically and socially, thus potentially shaping the diversity of children's representations, knowledge and uses of coral reefs and their threats. The same criteria were used for all schools participating in the program, integrating recommendations from the educational authorities involved. The sampling is based on voluntary participation in the program by the heads of the selected schools, and more specifically teachers interested in the MARECO toolbox. A scientific protocol using this toolbox was implemented in each of these schools, in order to provide the same foundation of messages about the coral reefs conveyed by the educational games to all children. 88 children (55% male, 45% female) took part in the research program. They were distributed between classes of 1st Grade (15%) and 2nd Grade (85%), and their average age was 7.2 years (**Table 1**). Children of around 7 years old where targeted because at this age playing is a central mechanism in learning and they are old enough to be able to draw and explain their drawings.

The Teaching Toolbox Mareco

The teaching toolbox MARECO[1] is bilingual (French-English), a vernacular language having been added to the picture book. The toolbox comprises three educational games focusing on the importance and vulnerability of coral reef ecosystems and their management. The games in MARECO aim to convey key knowledge and concepts.

1- The picture book *The Colors of the Reef* focuses on the diversity and vulnerability of the coral ecosystem in the face of natural and human-induced disturbances;
2- The card game *Coral Reef Happy Families* focuses on biodiversity and interactions between the coral reef's species;
3- The board game *See You At The Reef* focuses on the place of humans within the coral ecosystem, and the necessity of cooperation between ocean users, stakeholders and the population to ensure the sustainability of the interactions between coral reefs and humans.

A teacher's handbook presents these games, together with suggestions for teaching activities.

The main messages of the MARECO educational tool kit are (**Table 2**).

1- The coral is a living animal, the polyp, represented by a tiny soft animal called Poly, who leads the players through all three games;
2- The reef is a place of high biodiversity (card game);
3- The reef is an ecosystem; species have important interactions with each other and the environment, including humans, and cannot exist without all the other species (card game, picture book);

[1] see http://umr-entropie.ird.nc/index.php/portfolio/projet-reso-ecorail and link with a film, "The Coral Reefs in Our Hands." Trade protection and an operating license by a games publisher is currently under process for the MARECO toolbox.

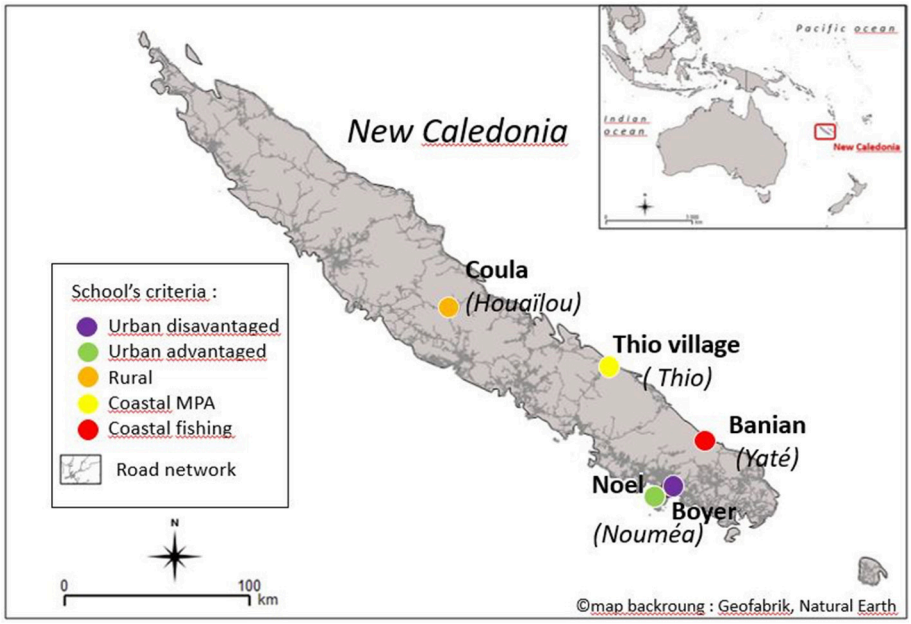


FIGURE 1 | Map of New Caledonia and location of the schools.

TABLE 1 | Distribution of children in classes by municipality and school according to the school level (G1,1st Grade; G2, 2nd Grade), age and gender (F, female; M, male).

Municipalities (schools)	G1 (%)	G2 (%)	F (%)	M (%)	Age	∑
Nouméa (Boyer)		100	36	64	7.3	22
Nouméa (Noell)		100	61	39	7.6	23
Thio	56	44	56	44	6.8	16
Yaté (Banian)		100	44	56	7.4	16
Houailou (Coula)	36	64	18	82	6.7	11
TOTAL	15	85	45	55	7.2	88

Boyer, socially advantaged urban neighborhood; Noell, less advantaged urban neighborhood; Thio, coastal area close to a marine reserve; Banian, coastal area within a large fishing community; Coula, rural environment.

4- Humans are part of the system; they interact with other species, and break, repair, protect the coral reef ecosystem (picture book, board game).

Data Collection

The data on children's representation of coral ecosystems were collected via a three-phase protocol implemented in each of the schools (**Table 2**):

- First phase. The children were asked to make two drawings following these instructions: first "Draw me the sea" and then "Draw me the coral reef," the children were given 20 min for each drawing, one after another. When the drawings were finished, each child was interviewed in order to gather information, name the elements that were drawn, and also acquire explanations about the possible direct and indirect experiences of children with the coral reef.

TABLE 2 | Different steps of games during the protocol before the teaching toolbox MARECO is left with the classes; and the messages associated with the games (1: The coral is alive; 2: The reef is a place of high biodiversity; 3: The reef is a whole; species interact with each other and the environment, including humans; 4: Humans are part of the system; they interact with others species, and enjoy, break, repair and protect the coral reef ecosystem that provide many services.

	Step 1	Step 2	Step 3
Card game (1, 2, 3)	Discovering the reef's inhabitants	Interaction between species (food chain)	Interactions between reef inhabitants and humans (food from the reef)
Picture book (1, 3, 4)	Discovering the coral ecosystem	The colors of a healthy reef	Pollution on the reef
Board game (4)	Destroying the reef without users' collaboration	The impact of collaboration on reef users	Roleplays and the importance of co-management

- Second phase. Nine activities with the three games were conducted by scientists and calibrated: for a duration of 30 min each, each game was used three times over three successive days with a view to giving the children the same messages and to familiarizing them with the games (see details in **Table 2**).

The teaching toolbox MARECO was then given to the teacher for a duration of 2 months. The teacher carried on with the toolbox activities in accordance with the class's teaching program in order not to interfere with the program.

- Third phase. The children made a last drawing of the coral reef 2 months after using the toolbox with the scientists. When the drawings were finished, the children were interviewed to gather information about the elements of the drawing.

Before each drawing session, the same boxes of colored pencils, containing 12 colors, were distributed to the children, together with half sheets of white drawing-paper. A total of 248 drawings were collected.

This study was carried out as part of the school curriculum in accordance with the recommendations of the National Education Directorate of New Caledonia that approved it (agreement n° CS16-3700-132, 20 April 2016).

Data Analysis

The items recorded in the drawings of each child were transformed into qualitative and quantitative data transcribed in a matrix with 16 quantitative variables belonging to three categories, seven for living natural items, four for seascape (non-living) items and five for human-related items (**Table 3**). The textual information, such as the list of the types of animal or plant items which the child had named during the census of items (belonging to the marine or terrestrial ecosystems), was saved in a database, together with the scans of the drawings needed for the interpretations of the results.

Data analysis was conducted in three stages in order to analyze the differences in coral reef representations before and after the awareness campaign using MARECO.

An initial stage describing the children's representations of marine ecosystems (sea or coral reef) consists in an exploratory multivariate analysis aiming at describing the structure of the data (individuals, i.e., the drawings, and variables) over the whole of the dataset (Sea-Reef1-Reef2). This analysis will notably enable the positioning of the drawings of the reef (before and after MARECO) compared to those of the sea, in order to analyze the children's relationships with nature as seen through their drawings. A Principal Component Analysis (PCA) is performed on the matrix describing the 248 drawings according to the three themes (sea and reef) and school (Thio, Noell, Boyer, Coula, Banian), from the log-transformed data of the 16 variables (**Table 3**), so as to make it conform more closely to normal distributions and obtain a better synthesis of the data on the factorial axes. With the technique of supplementary variables, the gravity centers of individuals (drawings) clustered in relation to explanatory variables (theme of the drawing—Sea-Reef1-Reef2—or school) are indicated in the first factorial plane with an ellipse bounding 95% of points or based on the calculation of a confidence interval of 95% around the gravity centers. Finally in order to take into account the non-independence of the samples and longitudinal data (the same children being involved in the program's framework for the three drawings), each child is compared with himself/herself to analyze the evolution of the

TABLE 3 | List of drawing descriptors (categories, variables) used to assess the representations of sea or coral reef.

Categories	Variables (codes)	Description
Living natural items	Number of flora types *(Nb_flora_type)*	Count of upper taxonomic levels including terrestrial and marine (tree, seagrass, seaweed …)
	Number of marine or terrestrial flora organisms *(Nb_flora_ind)*	Count of individuals
	Number of terrestrial fauna organisms *(Nb_terrestrial_ind)*	Count of individuals (including aerial organisms such as birds)
	Number of types of marine organisms *(Nb_fauna_type)*	Count of upper taxonomic levels (fish, mollusc, turtle, mammal, etc.…)
	Number of marine phenotypes *(Nb_ fauna_phenotype)*	Count of lower taxonomic levels, based on the change in form or color used to draw the marine organisms
	Number of marine organisms *(Nb_fauna_ind)*	Count of individuals
	Presence/absence of biological life-cycles *(Bio_cycle)*	Binary variable to pinpoint the presence or not of the biological life-cycle processes (predation, reproduction, migration, …)
Seascape items	Number of environmental items *(Nb_environment)*	Count of non-living environmental items (sea, land, sand, cloud, sun, …)
	Presence/absence of continental items *(Continent)*	Binary variable to pinpoint the presence or not of the continent (beach, island, mainland, …)
	Presence/absence of infrastructural items *(Infrastructure)*	Binary variable to pinpoint the presence or not of infrastructures built by humans (bridge, house, wharf, road, …)
	Number of colors *(Nb_color)*	Count of colors used in the drawing from a range of 8 colors (yellow, orange, red, pink/purple, blue, green, brown and black)
Anthropogenic items	Number of humans *(Nb_human)*	Count of men, women or children
	Number of marine users *(Nb_user)*	Count of users of the sea (sailor, fisher, swimmer, diver, …)
	Number of type of marine activities *(Nb_activity)*	Count of types of human activities linked with the sea (boating, on the beach, management, …)
	Number of technology items *(Nb_technology)*	Count of technology items built by humans, linked or not with the sea (motorboat, fishing gear, anchor, beach umbrella, beach chair, bottle, flag, …)
	Number of types of boats *(Nb_boat_type)*	Count of different boat types (sailboat, motorboat, fishing boat, transport vessel, …)

*A short description is given for each variable. A code (in italics) is associated with each variable (used in **Table 4** and for statistical analyses).*

drawings between Sea and Reef1, and between Reef1 and Reef2, via a *t*-test for paired data. The relations between qualitative variables (e.g., age vs. school) are tested by a Pearson's Chi-squared test.

A second stage aims to test the pertinence of the "number of colors" as a relevant indicator of representations through drawings. The hypothesis that the number of colors is an indicator of the other variables describing the sea or coral reef drawings is tested by statistical analysis. Because the samples Sea, Reef1 and Reef2 are non-independent as seen above, the means of the indicator "number of colors" in the drawings are compared between Sea and Reef1 and Reef1 and Reef2 via a *t*-test based on paired data. As the variable "marine phenotype" is built on the basis of the count of the individuals represented by a different shape or color, and is thus a direct function of the number of colors, only two variables of marine fauna biodiversity are used to avoid too much collinearity in the model: the number of marine types and the number of individuals. Data normality is tested by a Kolmogorov-Smirnov test in order to verify the conditions required for the statistical tests used in the following analyzes: multiple regressions to explain the number of colors by all other quantitative variables and multifactorial variance analyses to test the effect of schools or the drawings' themes on the number of colors, followed by a Tukey-test of multiple comparison of means. Owing to the fact that the samples are not independent and quantitative variables not normal, the validation phase of the model's outputs is an essential stage in the statistical procedure (see discussion). To this end, an analysis is conducted of the normality of residuals, their homoscedasticity (homogeneity of variance of residuals according to the estimated variable) and their independence (absence of apparent correlation). Moreover, the residuals are checked against explanatory factors of the variable to be explained ("number of colors"), i.e., the theme of the drawings, school, class level, and gender.

Finally, the third stage of data analysis takes into account the impact of MARECO more directly via analysis of the indicator variable "number of colors" on a reduced dataset (Reef1 and Reef2, 170 drawings) to better understand the factors explaining the change or shift in representation in the coral reef drawings after the use of MARECO. This analysis is conducted via a multifactorial variance analysis in which the explanatory factors are: school criteria (socially-advantaged urban neighborhood, disadvantaged urban neighborhood, close to a marine protected area, fishing environment or rural environment), the drawing's theme (Reef1, Reef2), class level (two grades: first year of elementary school, second year of elementary school) and gender (male and female).

RESULTS

Analysis of the Evolution of the Representation of Marine Ecosystems

The PCA conducted on the 248 drawings, including the three themes (Sea—Reef1—Reef2), the five schools (**Figure 2**) and described by the 16 quantitative variables (**Table 3**), explains 56.8% of the variability of the observations on the first three factorial axes (24.9, 17, and 14.9%; axes 1, 2, and 3, respectively).

The first factorial plane (axis 1 vs. axis 2) shows three patterns. The first axis separates marine fauna biodiversity from human activities (and, to a lesser degree, presence-absence of

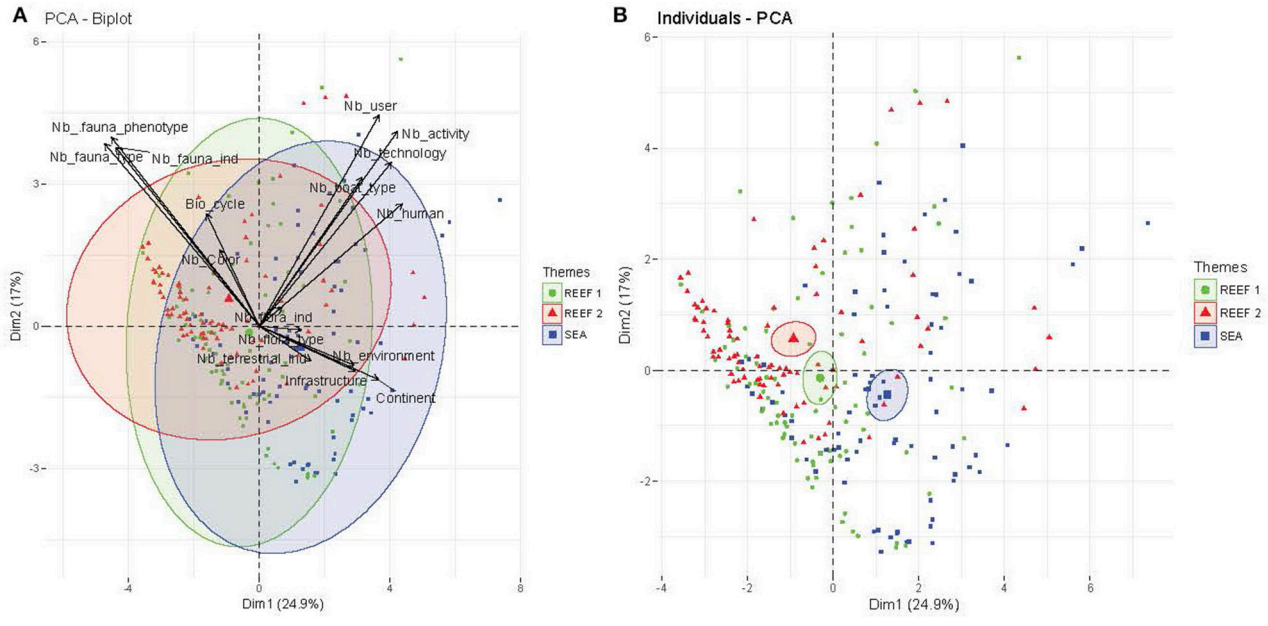


FIGURE 2 | Analysis of 248 drawings and 16 quantitative variables with Principal Component Analysis (PCA) (see **Table 3** for the listing of the variables). **(A)** Individuals (drawings) vs. variables that explain the two first axes; the ellipses delimit 95% of the individuals of each drawing's theme: Sea (blue), Reef1 (green, before MARECO), and Reef2 (red, after MARECO). **(B)** Individuals (drawings) and barycenter of the clouds of points with their confidence interval (confidence ellipse).

infrastructure, continent and non-living environment linked to the negative part of axis 2), which are both linked with the positive part of the second axis (**Figure 2A**). The variable "colors" is related to "marine fauna" but also with "flora," "terrestrial fauna," or "environment" on the third axis (not presented here). The cloud of individuals relating to the group Reef2 shows greater homogeneity of the samples of drawings (the ellipse containing 95% of the points is circular), whilst Sea and Reef1 demonstrate great variability in the drawings, with three trends: (i) drawings that are poor in information, whether pertaining to living organisms or human activities (negative part of axis 2); (ii) drawings rich in biodiversity (negative part of axis 1 and positive part of axis 2); (iii) drawings with representations of humans (positive part of axes 1 and 2). The gravity centers of the five schools reveals that they are slightly separated on the first factorial plane, but with an overlapping of the point clouds (**Figure 3A**).

The gravity centers of the groups (theme of the drawing or school) are positioned on the factorial plane with confidence ellipses corresponding to the confidence interval at 95% of the mean of the coordinates of each drawing of the group (**Figures 2B**, **3B**). Analysis of the confidence ellipse around the gravity centers shows in **Figure 2B** that axis 1 differentiates the theme "Sea" from the theme "Reef" (Reef1-Reef2), whilst axis 2 differentiates the drawings made before and after the use of MARECO (Sea-Reef1 vs. Reef2). In **Figure 3B**, axes 1 and 2 differentiates Coula and Boyer from the other schools, owing to the elements in the drawings relating to, respectively, the most frequently represented humans or marine biodiversity. A gradient is observed between Thio < Noell < Banian < Boyer on axis 2, moving from the negative part to the positive part of the axis, and therefore from the least rich to the richest regarding marine biodiversity and/or human-related items.

The comparison of paired data between Sea-Reef1 and Reef1-Reef2 of the various quantitative variables shows highly significant differences ($p < 0.001$) between (i) Sea and Reef1 for seven variables (Number of flora types, Number of terrestrial fauna organisms, Number of types of marine organisms, Number of marine phenotypes, Number of marine organisms, Number of environmental items, and Presence/absence of continental items), and (ii) Reef1 and Reef2 for five variables (Number of colors, Number of flora types, Number of types of marine organisms, Number of marine phenotypes, Number of marine organisms) (**Table 4**). An average of 4.6 marine phenotypes are represented per drawing (all types of drawing), with a distinct increase in the number of phenotypes in the drawings of the Sea, Reef1 and Reef2 (2.3, 4, and 7.6 phenotypes, respectively). This trend is also observed for the number of colors with the majority of the children used 5, 6, or 7 colors with a mean of 5.7 colors from the proposed color palette (8 from a box of 12 colored pencils) (**Figure 4**).

Although the initial variables are not normal (raw or log-transformed data), the deviations Sea-Reef1 ($\Delta_{SR1(i)}$) and Reef1-Reef2 ($\Delta_{R1R2(i)}$) are normal (Kolmogorov-Smirnov test, $p > 0.05$) and independent, thus enabling testing of the "school" effect and the link between the "color" variable and the other variables, whilst respecting conditions for applying parametric statistical tests.

Analysis of the Variable "Number of Colors"

The multiple regression model between the number of colors and quantitative variables established from the raw data and the three samples of drawings (Sea, Reef1, Reef2) accounts for 27.6% of the variability of colors (highly significant model, $p < 0.001$). The residuals are normal (Kolmogorov's $D = 0.146$, $p = 0.09$), with values showing a slight deviation from zero and from the estimated values at the extremes of the color palette (**Figure 5**). The residuals, checked against the variable "child's age," show that the number of colors is slightly underestimated (negative residuals) for the oldest children (9 years of age), who therefore use the most colors in their drawings. The model's

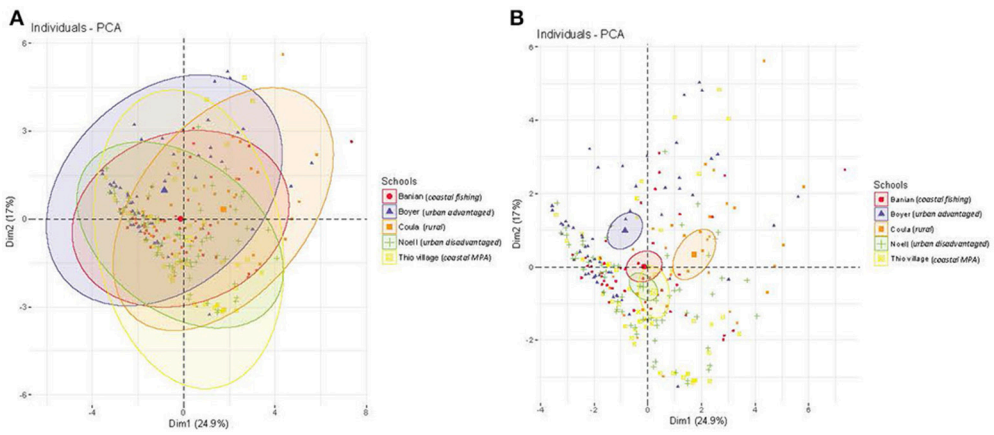


FIGURE 3 | Analysis of the 248 drawings and 16 quantitative variables with Principal Component Analysis (PCA) (see **Table 3** for the listing of the variables). **(A)** Individuals (drawings) with the ellipses delimiting 95% of the individuals of each drawing's school: Banian (coastal area within a large fishing community, in red), Boyer (socially advantaged urban neighborhood, in purple), Coula (rural environment, in orange), Noell (less advantaged urban neighborhood, in green) and Thio village (coastal area close to a marine reserve, in yellow). **(B)** Individuals (drawings) and barycenter of the clouds of points with their confidence interval (confidence ellipse).

TABLE 4 | Deviation for each variable between Sea/Reef1 and Reef1/Reef2: Mean of deviation and *t*-test of paired data with significance (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Variables (i)	$\Delta_{SR1(i)}$ = Sea-Reef1	$\Delta_{R1R2(i)}$ = Reef1-Reef2	Mean ± sd SEA	Mean ± sd Reef 1	Mean ± sd Reef 2
Nb_colour	0.43*	−1.43***	5.5 ± 1.30	5.1 ± 1.56	6.6 ± 1.26
Nb_flora_type	0.54***	−0.47***	1.0 ± 0.88	0.46 ± 0.61	0.96 ± 0.73
Nb_flora_ind	1.15	−3.12**	2.8 ± 6.02	1.7 ± 5.14	4.6 ± 8.71
Nb_terrestrial_ind	2.82***	−1.44	6 ± 6.35	3.2 ± 5.25	4.8 ± 6.31
Nb_fauna_type	−0.92***	−1.99***	1.3 ± 1.51	2.2 ± 1.65	4.3 ± 2.49
Nb_ fauna_phenotype	−1.73***	−3.67***	2.3 ± 2.96	4.0 ± 2.89	7.6 ± 4.11
Nb_fauna_ind	−5.34***	−3.82***	5.0 ± 5.86	10.0 ± 9.90	14.0 ± 9.28
Nb_human	0.66*	0.46	1.9 ± 2.25	1.2 ± 2.72	0.82 ± 1.93
Nb_user	0.07	0.21	0.57 ± 1.16	0.49 ± 1.93	0.33 ± 0.70
Nb_environment	0.96***	−0.14	3.8 ± 1.17	2.8 ± 1.23	3.0 ± 1.39
Nb_activity	0.20*	0.03	0.51 ± 0.75	0.3 ± 0.56	0.3 ± 0.64
Nb_technology	0.33	0.22	0.88 ± 1.61	0.54 ± 1.21	0.35 ± 0.75
Nb_boat_type	0.20**	−0.05	0.36 ± 0.63	0.16 ± 0.37	0.23 ± 0.53
Bio_cycle	−0.11*	−0.04	0.072 ± 0.27	0.18 ± 0.39	0.21 ± 0.41
Infrastructure	0.15**	0.03	0.34 ± 0.48	0.19 ± 0.40	0.17 ± 0.40
Continent	0.23***	−0.05	0.65 ± 0.47	0.42 ± 0.50	0.47 ± 0.50

*See **Table 3** for variable descriptions. If $\Delta_{MR1(i)} > 0$, then Sea > Reef1 or Reef1 > Reef2.If $\Delta_{RR2(i)} < 0$, then Reef1 > Sea or Reef2 > Reef1. Means and standard deviation are given for each variable by theme of drawings.*

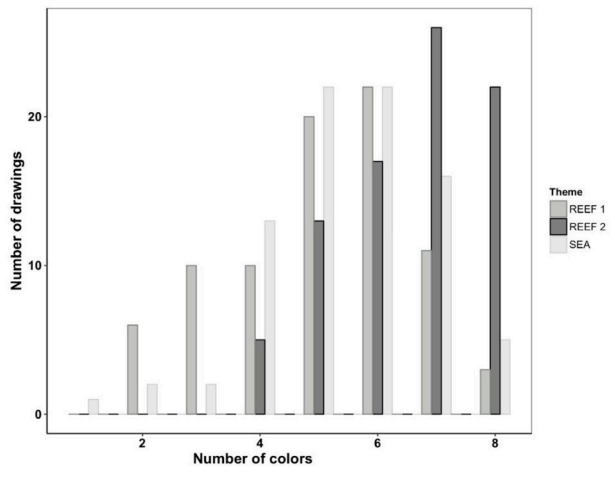


FIGURE 4 | Evolution in the number of colors used according to the themes (Sea, Reef1 and Reef2). Axes: number of colors vs. number of drawings.

estimated parameters together with their explanatory power are indicated in **Figure 5**. The number of colors is explained by the numbers of types of marine fauna ($p < 0.001$) and flora ($p < 0.01$), the number of marine organisms ($p < 0.01$), the number of elements of non-living environment ($p < 0.01$) and, at the limit of significance threshold ($0.05 < p < 0.1$), the number of terrestrial organisms and the number of users of the sea. Residual structure shows no significant relationships with the schools, themes, gender or class level: no variable accounts for the slight heteroscedasticity and particular structure of the residuals (point alignment). Although the model is significant, there remains approximately 73% of the variability of number of colors in the residuals: this residual variability is due to the heterogeneity between children but also between the schools and the themes of the drawings. Because of the non-respect of independence of observations, four other multiple models are established, by separating the samples (one model for each drawing theme) and by working on the deviations between Sea-Reef1 and Reef1-Reef2. In all four cases, the residuals of the models are normal, always featuring the same phenomenon: a deviation at the extremes of the number of colors, aligned in decreasing order.

When the analysis is conducted according to schools, the median values and the quartiles per school show a significant difference in the use of colors between the schools (**Figure 6**). Sea and Reef1 do not show a marked difference, but the drawings often present fewer colors in Reef1, with the exception of Boyer school, and the mean of differences based on paired data is at the limit of the significance threshold at 5% ($\Delta = 0.43$; $p = 0.045$, **Table 4**). For Reef2, after the use of MARECO, Boyer, Coula, Banian, and Thio schools present drawings with more colors than for Reef1. The difference on paired data (mean of deviation) is highly significant ($\Delta = -1.43$, $p < 0.001$, **Table 4**).

The number of colors (raw, log-transformed or square root data) do not correspond to a normal distribution. Despite the non-independence of the samples and the non-normality of the variable "color," and considering the robustness of the variance analysis model, the effect of the factors "theme" and "school" is tested, followed by a multiple comparison test. The validation of the model (results not pictured) shows that the residuals on raw variables are normal (Kolmogorov-Smirnov test and a right-skewed Normal Q-Q plot on standardized residuals). The model accounts for 27.2% of total variability (inter-group variability/total variability) with the "theme" factor emerging as highly significant ($p < 0.001$), the "school" factor as significant

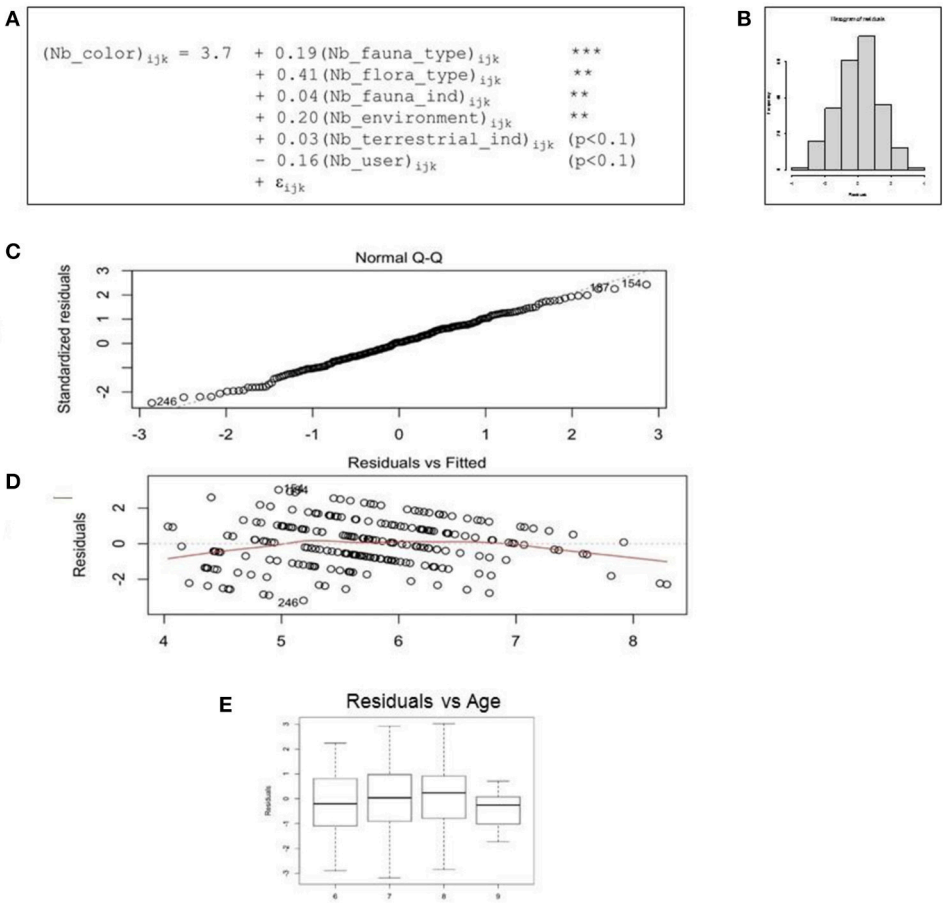


FIGURE 5 | Validation of the multiple regression model established between the number of colors and 14 quantitative variables describing the drawings (**Table 3** without number of marine phenotype). **(A)** regression model used with the most significant variables (***$p < 0.001$, **$p < 0.01$, and $0.05 < p < 0.1$), **(B)** histogram of residuals, **(C)** quantile-quantile plot of the standardized residuals, **(D)** standardized residuals vs. fitted values, **(E)** box plot of standardized residuals vs. children's age.

($p < 0.05$), and the interaction between "theme" and "school" as highly significant ($p < 0.001$). The residual plot according to estimated values shows no particular structure, and the residual mean is equal to zero with homogeneous variance. The model is therefore valid, despite the non-respect of application conditions. Considering the relationship between "school" and "class," it is important to interpret the results while taking into account the fact that Coula and Thio each have a mixed 1st/2nd grade class, compared to the three other schools whose classes are only 2nd grade (**Table 1**).

In order to confirm the above results, a new variance analysis is established from the data deviations (**Table 4**) according to the "school," "age," and "gender" factors, in order to take the non-independence of the samples into account (results not pictured). The results confirm the fact that the "school" factor is very significant ($p < 0.01$) between Sea and Reef1, and significant ($p < 0.05$) between Reef1 and Reef2. The significant effect of the "school" factor is due to the difference between Boyer and Coula (Tukey's test, $p < 0.05$), and to a lesser degree between Banian and Coula (Tukey's test, $p = 0.06$). Nonetheless, the indicator variable ("number of colors") shows larger differences between the themes Reef1 and Reef2 ($p < 0.001$) than between Sea and Reef1 ($p < 0.05$) (paired t-test, **Table 4**). Indeed, the deviation in the number of colors is positive between Reef1 and Reef2 ($\Delta_{SR(i)} = 0.43$), and negative between Sea and Reef1 ($\Delta_{RR2(i)} = -1.43$). These results show that (i) the drawings of Reef1 (mean = 5.1 ± 1.56) are less colorful than those of Sea (mean = 5.5 ± 1.30), and (ii) the drawings of Reef2 (mean = 6.6 ± 1.23) are more colorful than those of Reef1. Nonetheless, this effect needs to be moderated according to the schools.

It remains that the variance analysis model brings to light the fact that it is *via* the interaction between the themes and the schools that the differences can be understood (**Figure 6** and result of multiple regression). The "school" effect is therefore more marked for the drawings between Sea and Reef1 or Reef2 than between Reef1 and Reef2. This completes the result found by the PCA, i.e., that the content of the drawings is more homogeneous after the use of MARECO and that the children in certain schools demonstrate knowledge of the coral reef in the first drawing.

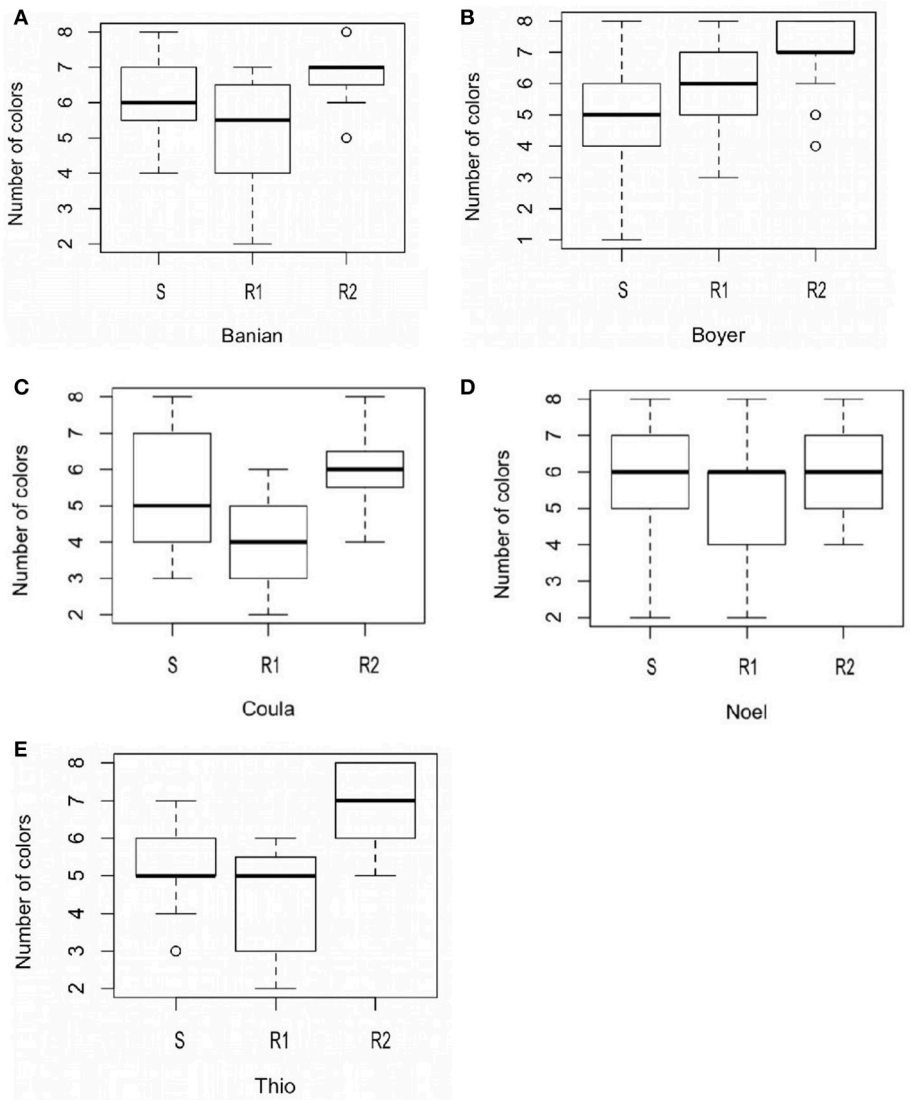


FIGURE 6 | Quantile's box plot of the number of colors used in the drawings between Sea, Reef1 and Reef2 per school. **(A)** Banian (coastal area within a large fishing community), **(B)** Boyer (socially advantaged urban neighborhood), **(C)** Coula (rural environment), **(D)** Noell (l less advantaged urban neighborhood), **(E)** Thio (coastal area close to a marine reserve).

MARECO Effect

The model from the multifactorial variance analysis to test the MARECO effect is totally validated: residual normality, variance homoscedasticity and absence of correlation structure in the residuals according to the variable number of colors to be estimated (**Figure 7**). With respect to the total variability, the model explains 35.4% of the variability of the number of colors. The class level, and therefore the children's age, gives rise to a significant effect ($p < 0.05$), with the theme of the drawing being highly significant ($p < 0.001$) and the school very significant ($p < 0.01$).

The results of the three phases of the analysis procedure enabled the various working hypotheses to be tested:

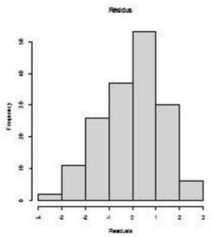
Hypothesis 1 verified. The groups of children in the different schools (with contrasting natural and socio-cultural environments) have specific representations of the marine ecosystems.

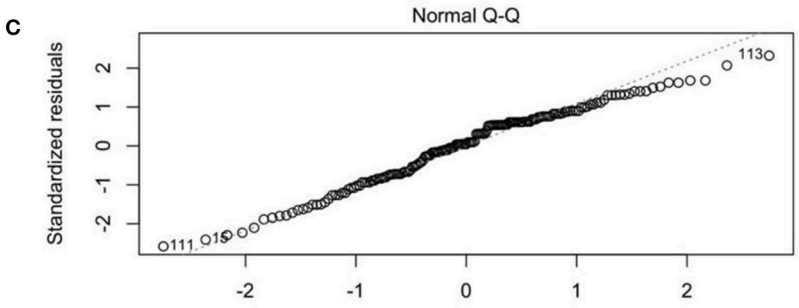
Hypothesis 2 verified. The numbers of colors used by the child for the drawings can be used as an indicator that the child has developed a holistic perception of coastal sea or coral reef.

Hypothesis 3. The impact of MARECO is assessed on the basis that the child has developed a holistic perception of the coral reef combining (a) biodiversity, (b) human activities, and (c) environment. Regarding the three points set out in this hypothesis, Point (a) and to a lesser extent Point (c) are verified. Conversely, Point (b) is rejected for the majority of children.

A

Facteur	DF	Sum Sq	Mean Sq	F value	Pr(>F)
Gender	1	3.21	3.21	1.85	0.1752
School's level	1	11.17	11.17	6.45	0.0121 *
Theme	1	91.64	91.64	52.87	$1.72\ 10^{-11}$ ***
School	4	26.67	6.67	3.85	0.0052 **
Theme*School	4	12.67	3.17	1.83	0.1264
Residuals	153	265.19	1.73		





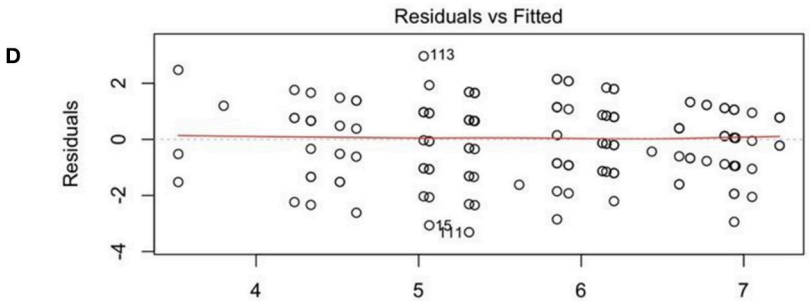


FIGURE 7 | Validation of multifactorial analysis of variance on the number of colors according to the explanatory factors of the representations: gender, class level, theme and school. **(A)** Table of variance, **(B)** histogram of residuals, **(C)** quantile-quantile plot of the standardized residuals, **(D)** standardized residuals vs. fitted values.

The discussion will address these hypotheses, together with the research methodology and MARECO's contributions as an educational tool for increasing awareness of coral environments.

DISCUSSION

Research Methodology

An interdisciplinary approach between social, ecological and biological sciences is now encouraged and practiced, but communication beyond the research methodologies is rare and leads to poor understanding of the interactions registered within the socio-ecological system (Chaboud et al., 2011; Stoica, 2012; Fabinyi et al., 2014). In this study, interdisciplinary skills (anthropology, ethnoecology, marine biology, biostatistics) were involved in order to develop the research methodology and improve data collection and interpretation. "Drawing to see" (Causey, 2016) and coding qualitative data to quantitative data for data analysis of the drawings are the heart of this interdisciplinary research program. Taking into consideration all the cases studied and the overall research process, methodological limits are referred to: (i) a continuous evolution of the research methodology that might be considered simultaneously a strength and a weakness; and (ii) the practice of interdisciplinarity "in the field" but also during "brainstorming" activities where the different steps of the research were discussed, modified and approved. Practical examples will illustrate each of the two points. For the first point, one of the major difficulties during the research was the "drawing task" and the instructions that were given in the classroom in order to make the "concept" of *coral reef* understandable to children. While the first drawing of the "sea" posed no problem, the second one relating to the "coral reef" provoked different reactions in the children. During the research process, the "drawing task" was constantly improved by asking one of the children who knew what a coral reef is to explain briefly what it represents. By choosing this strategy, the research team tried not to interfere or to orient children in their drawing representation. The second refers to the practice of interdisciplinarity and the "divergent points" of view related to each discipline. As an example, the exchanges and discussions developed during the selection and definition of criteria to be used to summarize and "read" the items depicted in the drawings were different and had different "understandings" for the disciplines involved in the project. Words such as *life cycle*, *protocol, replicate, homogeneity* may have different meanings in biology and anthropology or not usual for one discipline. A long and lively discussion was also had in reference to the term "ethnospecies," which is usually used in relation to folk taxonomy and to how local people name the

identified species they perceive, but which may not correspond to the biological one (Linnaean classification, for example) (Ellen, 1993). This term has been finally named "phenotypes" in this paper. These methodological limits drove us on the one hand to be aware of the difficulties of exchanging between disciplines, but on the other hand pushed us to revisit and reconsider our viewpoints and improve our strategy through a double process of critical reflection and understanding of the "problematic" issues that were encountered during the research process.

Once the data acquisition method has been validated and the data acquired, coding is an essential point in the analysis procedure (Thomas, 2006) as it orients the results obtained, but may also be a source of bias owing to errors made by the coders. The solidity of this point was reinforced by a double coding carried out by two people simultaneously. Finally a particular emphasis was placed on the validation of the statistical procedure which is based on the combining of exploratory and confirmatory approaches. Apart from PCA, a descriptive multivariate analysis enabling a global analysis of the dataset and the identification of the main sources of variability, the validating of the statistical models used were made respecting the recommended steps for data exploration and general linear model (Zuur, 2012). Exploring the models' residuals (e.g., **Figures 5**, **7**) enables the statistical model to be validated even if the application conditions are not completely verified: the main issue we faced was the non-independence of the observations, owing to the fact that the same children were involved in all three data samples (drawings for Sea, Reef1, and Reef2). This problem was countered in two ways: (i) cross-validation of the results obtained by conducting various analyses, notably paired data analysis, and (ii) validation of the model via analysis of the residual variability unexplained by the model. For instance, residual analysis of the regression model established on the number of colors vs. all the other quantitative variables enables the model to be validated whilst positing one hypothesis to explain why there are residuals slightly different from zero at the extremes of the palette of eight colors (**Figure 5D**). If a child uses more colors that the model predicts in view of the elements drawn, this might be explained by the fact that the child like colors, and therefore by its artistic side (e.g., rainbow). The tendency to see negative residues for older children (but not significant, **Figure 5E**) can also be explained by the degree of development of children and their ability to draw compared to younger ones.

Whatever the case may be, opting for the number of colors as an indicator for studying the MARECO effect is ultimately is finally a subtle choice, for it touches on behavioral aspects regarding the act of drawing that are largely beyond the scope of this paper, with notably a potential relationship with the child's degree of development, and thus their age but also with aspects related to the creativity of the child (Goodenough, 1926). As the research procedure is based on analyzing and comparing the groups, we restricted ourselves to the analysis of intra- and inter-group variability in order to tackle the issue of social representations without engaging in considerations of individual representation (Doise et al., 1992; Abric, 2003).

Children's Representations of Nature and Ecological Knowledge

Since children will be the adults of the future, it is important to understand their representations of nature in a changing world (Eriksen, 2016). These representations and knowledge are based on their interactions with other humans about the living environment, education received and children's direct experience with this aspect of nature (Battesti, 2007; Dounias, 2007; Calandra, 2013; Gallois et al., 2017). These interactions with nature might be indirect (viewing nature through a window at work or at home), incidental (spending time outside at work), or intentional (time spent in recreational activities). The groups of children who took part in this study were not chosen randomly but belong to a same classroom in different areas that are socioeconomically and ecologically contrasted, and vary in age. Despite these common criteria, the children each have their own lives, identities, beliefs and experiences, and thus their perception of nature is likely to appear in their drawings (Carrière et al., 2017). Nevertheless, social interactions between the children, their parents and teachers will mutually influence the representations of coral reef ecosystems in each child's group and thus similarly influence their encompassing perception of nature. It is therefore more likely that children, who may be considered as a social group, from the same class in a specific school, share the main representations of their environment, as a social group of adults (Ellen, 2006). This is confirmed by our first hypothesis, which establishes that the groups of children in the different schools have specific representations of the marine ecosystems, both of the sea and the coral reef, while children in the different schools show disparities in their drawings. Our results highlight differences between schools and differentiated three main patterns before the awareness campaign (**Figure 8**): (i) schoolchildren in less-advantaged urban environments and in coastal areas close to a marine reserve, but with a natural environment impacted by human-related activities, have drawings that are poorer in biodiversity or human activities; (ii) schoolchildren in socially advantaged urban environments or fishing environments in coastal areas have coral reef drawings that are richer in biodiversity compared to other schools, and (iii) schoolchildren in rural environments at a distance from the sea have drawings mostly characterized by the presence of humans, but with a low representation of marine biodiversity.

Greater marine biodiversity in drawings before the MARECO protocol by schoolchildren in the socially-advantaged urban environment can be explained by the richness of the environment offered to them within the educational system (Kopnina, 2012). The majority of urban people have weaker or non-existent traditional ecological knowledge compared to rural or fishing societies (Hurrell and Pochettino, 2014). This ecological knowledge acquired through direct and ancient experience with nature is transmitted orally by members of the same society, more specifically when people depend directly on the exploitation of nature to live (Balick and Cox, 1996; Hurrell and Pochettino, 2014). In spite of this deficit of local knowledge, the multicultural context of the city, the role played by the mass media (Huston

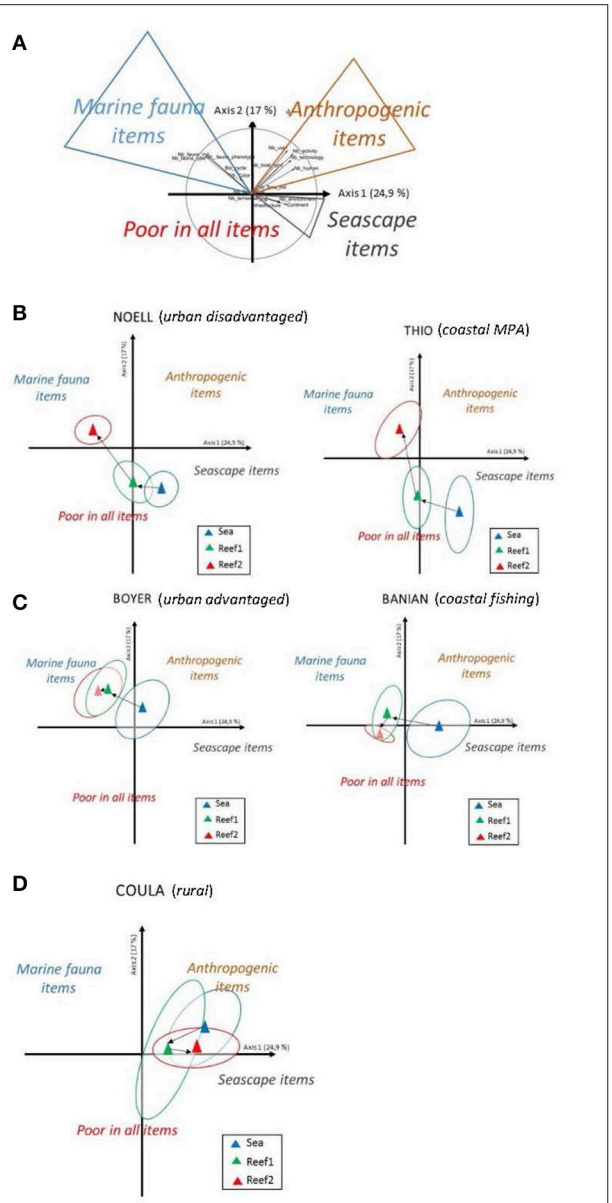


FIGURE 8 | Projection of the centers of gravity and confidence ellipses of the groups Themes*Schools on the first factorial plane of the PCA (blue: Sea; green: Reef1; red: Reef2). **(A)** Explanatory variables of the factorial axes 1 and 2. **(B)** Noell (disadvantaged school in urban neighborhood) and Thio (coastal school close to a marine reserve), **(C)** Boyer (socially-advantaged school in urban neighborhood) and Banian (coastal school near within a large fishing community), **(D)** Coula (rural school).

et al., 1999), the education programs in an advantaged school (Wagner, 2008) and the regular experience with coral reef of children whose family have recreational activities linked with the lagoon (Gonson et al., 2016, 2017), allows urban schoolchildren to acquire a lot of so-called scientific knowledge (McClatchey, 2005). Children in a advantaged urban environment show some knowledge of marine biodiversity in their drawings of the sea, and more than 50% of them drew human activities (mainly with boats). In a less-advantaged urban neighborhood, schoolchildren have drawings which are poorer in biodiversity or human activities linked with marine ecosystems, which could be reflecting their socio-economic context and the fact that they live beyond walking distance from the sea. This traditional knowledge is well-developed in a fishing community where schoolchildren demonstrate good marine ecological knowledge before the MARECO protocol, a trend observed in New Caledonia but also in coastal villages in Madagascar where the same protocol has been developed with Vezo communities (Ferraris et al., 2015; Stoica, 2016). This may be explained by classical ethno-ecological processes of knowledge acquisition (Ellen, 2006) in connection with close, longstanding and regular contacts of the children with the marine ecosystem through their family and the direct link between fishing activities and consumption. These regular contacts have the effect of tightening, increasing and homogenizing their ecological knowledge in a fairly extensive hybridization with information provided by the mass media and the school (Hurrell and Pochettino, 2014). Thus it seems surprising at first sight that schoolchildren in a coastal school near an MPA might make drawings which are poorer in biodiversity or human activities associated with the marine environment. This result can be explained by the fact that Thio is a mining village with adults working mainly in the nickel mines and where the marine protected area is recent and not connected directly to the village. In addition, the exploitation of nickel impacts the terrestrial environment with significant land erosion, making the coastal seawater turbid and leading to degradation of coral reefs (Chabanet et al., 2010), and unattractive for human activities. Finally, children in the rural school, located for our case study in the mountains, are characterized through their drawings by representations of the sea and the reef as seen from above, with people representative of the local community. They also make drawings where marine species are globally less represented, as they probably have trouble representing a marine ecosystem, mainly because they do not know it. This situation may explain the fact that in several drawings there are species such as whale or shark, more known through the media as emblematic species. In addition, local context or time circumstances may exacerbate a strong fascination, fear and imagination about the species, in particular, in our case, following an accident caused by a shark just before our intervention in the school and relayed in the media. This phenomenon was observed several times in Reunion in different classes involved in the research program where children drew sharks, the island having been impacted recently by several successive shark accidents (Lagabrielle et al., 2018).

The Impact of MARECO on the Children's Representations of Coral Reefs

Through the use of the MARECO protocol, the children developed a holistic perception that allows them to represent a healthy multi-colored coral reef (reflection of perceived biodiversity) and to connect it to their environment as demonstrated in our results. The colors of the reef come largely from corals that build the complex underwater seascape, in particular from pigments included in their algal symbionts

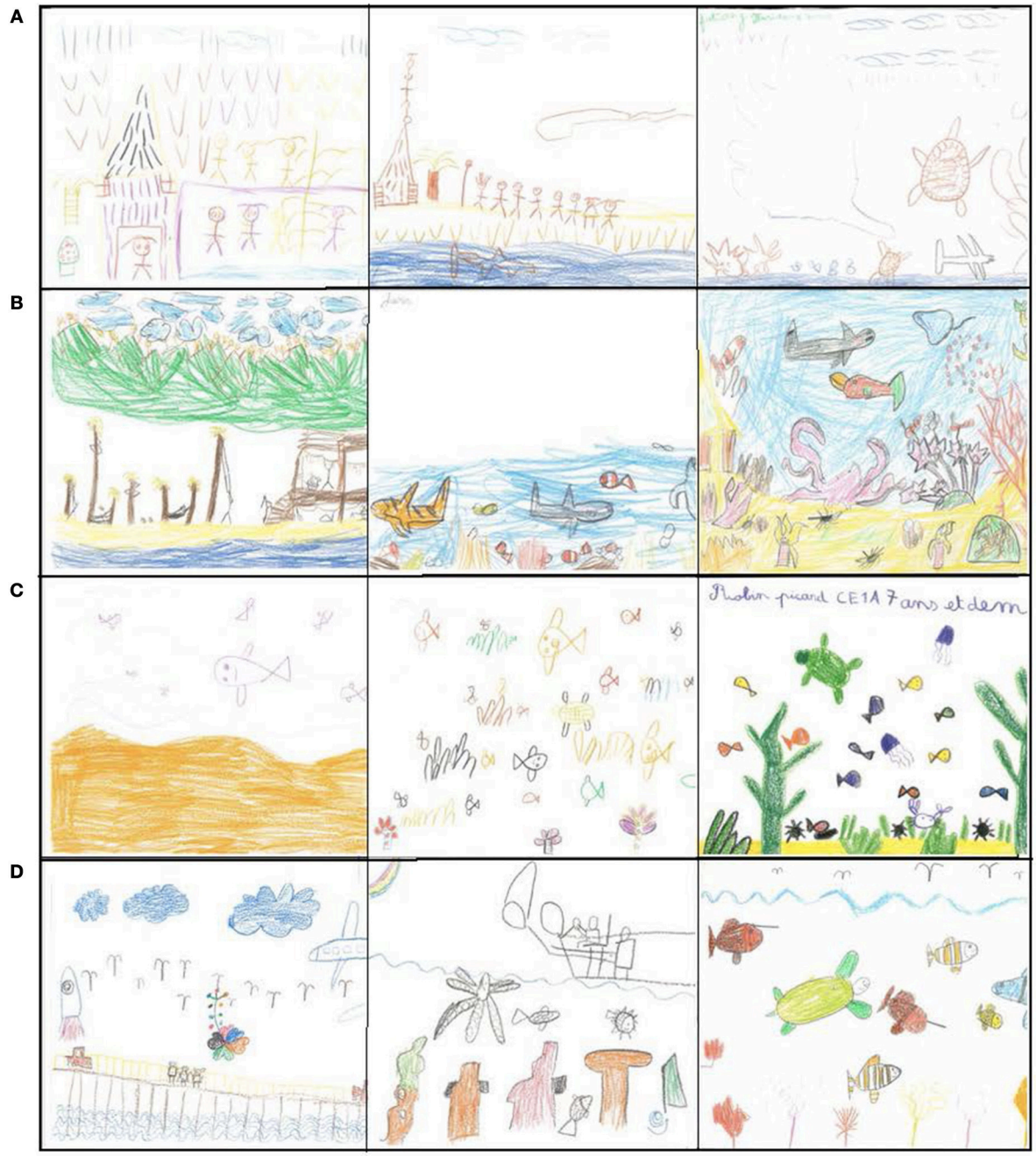


FIGURE 9 | Sequences of 4 Sea-Reef1-Reef2 drawings for 4 children (first name) who drew interesting features to highlight and scool indicated in parentheses. From up to below **(A)** Folialki (Noell), **(B)** Jaurès (Noell), **(C)** Robin (Boyer), **(D)** Yansi (Banian). Boyer: socially advantaged urban neighborhood, Noell: less advantaged urban neighborhood, Thio: coastal area close to a marine reserve, Banians: coastal area within a large fishing community, Coula: rural environment.

(zooxanthellae). When the concentration of pigments declines drastically (loss or expulsion of a major portion of zooxanthellae), the coral becomes pale and bleached due to the low concentration of pigments and the increased visibility of the coral's white skeleton. If corals do not regain their zooxanthellae, they die and are rapidly colonized by algae. Coral bleaching is a stress response usually associated with anthropogenic and natural disturbances (Glynn, 1991) which, when repeated though time, cause a "phase-shift" from coral dominance to algal dominance (Done, 1992; Bellwood et al., 2004). Degradation of coral reefs causes a reduction of coral cover, which is replaced by algae, but also of coral and fish diversity (Chabanet et al., 1997), with this reduction in biodiversity contributing to the loss of colors or their homogenization on coral reefs. Then the number of colors represented in the drawings is a relevant proxy of the marine biodiversity perceived by children after the MARECO

protocol (**Figure 9**). Furthermore, the number of colors used by the children is also significantly related to "seascape items" (living and non-living environment variables), with the marine ecosystem often being represented in relation to its external environment (continental or atmospheric, sky, clouds, sun, rainbow, island, continent, etc.) with characteristics specific to the landscapes where schools are located (e.g., mountainous for Coula or Noell located on the heights of Nouméa). The presence of brilliant colors, which are generally factors of attractiveness (Reber et al., 2004), is undoubtedly part of the interest aroused by the picture book and its appropriation by children.

Finally, the use of MARECO has led to an overall decrease in human activities (mainly fishing and diving) in the children's representation of the environment, while their link with the coral reef was one of the messages conveyed by the games (mainly the board game). This decrease in human activities could be related to the impact of the awareness campaign, with the reef being associated with management (e.g., establishment of marine protected areas) and protection to decrease the impact of human activities. A majority of children adopted an underwater vision to draw the coral reef after the use of the toolbox (**Figure 9**). This can be explained by the visual influence of the images of the picture book, mainly by the poster included with it that the child could take home, but also by a focus brought by the teacher (poster display in class) or by the fact that the coral reef is a pictorial object that is rich and difficult to draw. Globally MARECO has a positive effect in all schools, but compared to each other, the impact is stronger in the less-advantaged urban school and the coastal school near an MPA where children have very poor knowledge or experience of coral reefs before the awareness campaign. Nonetheless, the cultural and socio-economic factors related to MARECO impact need to be investigated further in social sciences including all countries where the program has been performed to obtain more replications by school type for statistical analysis. Another general trend of our study points out a greater homogeneity in Reef2 drawings compared to Sea and Reef1 which show greater interpersonal variability in perception of marine ecosystems within or between classes. This result illustrates the impact of the awareness campaign or education on children's learning, leading to a "normalization" of the representation of coral reefs by children.

Specificities of MARECO

Playing games as a vehicle for learning is not a new concept (Annetta et al., 2009). If games are used in class, it is necessary for the teachers to assess them from an educational perspective to determine whether they can be integrated into their teaching practices (Britain and Liber, 2000). Some studies on the impact of environmental education campaigns exist with the use of questionnaires passed to children in schools (e.g., Lindemann-Matthies, 2002; Ballouard et al., 2011). Nevertheless, these studies are related to field practice, observation or investigation (especially *via* the internet) but not to the impact of games in schools. The originality of the MARECO toolbox comes from the evaluation of the impact of the awareness campaign through drawings made by young schoolchildren, but also from its development by scientists in association with an elementary-school teacher who created a booklet including activities for each of the three games. Therefore the concepts developed in the games are part of the school curriculum in life sciences and thus integrated naturally into the schoolchildren's learning process. According to our results based on the analysis of drawings characterized by quantitative variables, it appears that the effect of the toolbox is more pronounced for the picture book and card game, and more limited for the board game, even though children particularly appreciated the latter. This must be checked against the analysis of the interviews made in parallel with the children and teachers, but also with the commentaries made by the children about their drawings. Moreover, the timescale of the research program does not enable analysis of the real longer-term assimilation of the messages conveyed *via* the teaching toolbox, nor its impact on effective changes in representation, linked to knowledge or behavior. Nevertheless, one of the teachers taking part in the program in a disadvantaged urban neighborhood had children in fifth grade make drawings. These children had been initiated to MARECO in 2013, when they were in second grade. In the meantime, there had been no new intervention or teaching regarding the coral reef within the framework of the school curriculum. For the entirety of their drawings, there is clear persistence of the knowledge acquired 3 years previously. The diversity of the colors, wealth of the organisms drawn, knowledge of their names (brain coral, tabular coral, branch coral, crown-of-thorns starfish, etc.) show an evolution in representations with conclusive and lasting assimilation. Therefore MARECO has retained a positive impact on children over time, but this result has only been verified for seven children.

Another specificity of the awareness campaign is linked to the teachers' appropriation of the MARECO toolbox as an educational tool. Some of the teachers focused on the scientific learning it enables (initiation to the scientific approach, learning of new vocabulary, the acquiring of knowledge of ecosystems, raising awareness of environmental problems, pollution, climate change, etc.). Others leant on the toolbox as an aid for productions both scientific and artistic intended for the parents (paintings, sculptures, etc.), which also enabled the parents to join in learning the games *via* their children. In Mayotte, for instance, a teacher emphasized that when the picture book was taken home, this was the first time the parents and the children had ever done something together (Surugue, 2017). The teachers participating in the project proved to be proactive, independently developing tools to optimize the experiment, as much from their own experience as by elaborating a reflexive analysis on their way of using it. A case in point is how, for the board game, a teacher enhanced the playful side to the game by giving more weight to the role of the marine-reserve manager. Taking on the role of mediator, the pupil acting as the reef's "warden" assumed their responsibility in order to defuse conflicts, generating very constructive, enriching discussions for the pupils. Teachers' appropriation of the toolbox was also observed in other cultural contexts such as Madagascar, in coastal villages with limited means and electricity (Stoica, 2016).

This point underscores another interesting aspect of the toolbox, which is its transferability to southern countries owing

to its simplicity of use (no need for electricity, a computer or the internet). This feature may be a brake for more developed countries where children tend to be drawn to more sophisticated games (video, internet network access, etc.) but this simplicity was intentional from the outset in view of the target for which MARECO's three educational games were developed, i.e., local coastal communities in southern countries in coral reef environments.

The MARECO toolbox thus appears to have fulfilled its objective of raising awareness of the coral reefs, with the caveat that humans are often, in this case study, absent from the representation of the ecosystem. This may be the reflection of the alarmist messages, unfortunately justified, of how humans disturb the reef, and which lend credence to the standpoint of nature protectionists to the detriment of the perspective of social conservationists (Caveen et al., 2015).

CONCLUSION

Our study demonstrates the performance of MARECO as a playful tool to transfer scientific knowledge to children. The results showed statistically that the drawings' representations of coral reefs before and after the awareness campaign are different, and that the numbers of colors used by the child for the drawings can be used as an indicator that the child has developed a more accurate holistic perception of the coral reef ecosystem, including marine biodiversity and seascape description. It appears necessary, however, to reinforce future awareness campaigns with messages boosting understanding of the place of humans in the ecosystem and how they can act for improved management of their activities (e.g., through marine protected areas). From a methodological viewpoint, it would be interesting in the future to develop an indicator related to ecosystem functions using the qualitative information collected on the drawings, e.g., the food chain, the number of interactions observed between species or the spatial representation of organisms in their environment.

In a context of Anthropocene where the degradation of coral reefs is accelerated in response to numerous anthropogenic drivers and climate change, the global challenge is to maintain the biological functions of the ecosystem and involve civil society in its management, including the young generation. As stated by Hughes et al. (2017), successful navigation of this transition will require radical changes in the science, management and governance of coral reefs. To strengthen the link between children and nature (in this instance, coral reefs), it is essential that children develop direct experience in order to develop an understanding of the biological world and implicitly in our case of the richness of coral reefs in terms of biodiversity but also interactions. To increase the effect of the MARECO teaching toolbox, it would be necessary for the children to see the reef in "real life" in order to integrate the notions acquired in a learning process at school. This gap could be at least be bridged by using movies and videos (e.g., https://www.chasingcoral.com) and interactive photography (e.g., https://www.google.com/streetview/#oceans). To be agents of change in a sustainable world, children must be engaged in a fun, rigorous, action-oriented and socially responsible learning process. Participatory approaches to learning for sustainability are recommended such as those developed in marine educational areas or Eco-Schools[2], which provide an integrated system for the environmental management of schools and involve children, teachers, managers and multi-level decision-makers.

AUTHOR CONTRIBUTIONS

JF, PC, GS, CS, and SC conceived the ideas and designed the methodology. GS, JF, CS, and PC collected the data. JF and CB performed the statistical analyses. All co-authors contributed to the manuscript originally written by PC, JF, and GS.

FUNDING

The research RESO-ECORAIL was funded by Fondation de France *Quels littoraux pour demain?* EU, France and Region Reunion brought a financial support for the edition of the MARECO toolboxes used during the research program.

ACKNOWLEDGMENTS

The authors would like to thank the school teachers for participating in this project and their availability: Valérie Boussemart, François Lahitete, Marie-Stéphanie Maperi, Jean-Charles Meyi, and Jean-Baptiste Welta. Our thanks to all the school children who participated enthusiastically as well as to the parents who took part in the exercise with the MARECO toolbox. Thanks to the New Caledonian Directorate of Education, particularly Vanessa Montagnat, and the school directors who helped us to intervene in the schools. Thanks to Marlène Dégremont and Claire Levacher who took part in the program RESO-ECORAIL in New Caledonia and provided the codes of the drawings. Special thanks also to Claire Garrigue for her help and availability during the implementation of the MARECO protocol and to Celia Northam for her valuable support in translating into English and proofreading. We also extend our thanks to the three referees who provided useful comments on the manuscript.

[2]http://www.ecoschools.global/

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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

ORIGINAL RESEARCH
published: 18 October 2018
doi: 10.3389/fmars.2018.00378

Bacterial Communities in Tissues and Surficial Mucus of the Cold-Water Coral *Paragorgia arborea*

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Coral bacterial associates can play important functional roles for the holobiont, such as nitrogen cycling, nutrient processing, and supporting immunity. While bacteria found within the microbiome of corals may benefit the host, they can also be linked to pathogenesis. In the deep-sea, cold-water corals, like their warm shallow-water counterparts, host bacterial communities, but have received little attention due to logistical constraints in sampling. In particular, bacteria associated with surficial mucus of cold-water corals have not yet been investigated. Here, tissue and mucus samples of *Paragorgia arborea* were collected from three submarine canyons along the continental slope of the Gulf of Maine. Bacterial DNA was extracted from tissue and mucus samples and sequencing of the V6–V8 hypervariable region of the 16S rRNA gene was performed using Illumina MiSeq. The bacterial communities associated with *P. arborea* compartments (tissue and mucus) and sampling locations (canyon) differed significantly in composition. Proteobacteria, Tenericutes, and Spirochaetes were the dominant phyla across the majority of coral tissue samples, with Gammaproteobacteria and Alphaproteobacteria identified as the largest Proteobacteria contributors across all samples. Operational taxonomic units (OTUs) belonging to the taxa *Spirochaeta, Mycoplasma*, Flavobacteriaceae, Terasakiellaceae, Campylobacterales, and Rickettsiales were identified as biomarkers (bacterial taxa significantly more abundant in a specific coral microhabitat) of *P. arborea* tissues, while *Paracoccus* was a biomarker of *P. arborea* mucus. Many of the recovered biomarker taxa may be involved in nitrogen cycling. Representatives from several bacterial families (Vibrionaceae, Campylobacteraceae, Rhodobacteraceae, Flavobacteriaceae, and Burkholderiaceae) previously reported in diseased scleractinians, were present in *P. arborea* as rare bacterial taxa. Characterizing the bacterial associates present in visibly healthy coral colonies provides a benchmark of dominant and rare bacterial groups present in the cold-water coral holobiont. This is the first characterization of bacterial groups associated with *P. arborea*, examining both tissue- and mucus-specific communities.

Keywords: cold-water corals, Alcyonacea, bacterial diversity, microbiome, mucus, high-throughput sequencing, 16S rRNA

OPEN ACCESS

Edited by:
Michael Sweet,
University of Derby, United Kingdom

Reviewed by:
Aldo Cróquer,
Simón Bolívar University, Venezuela
Bryan Wilson,
University of Bergen, Norway

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *30 April 2018*
Accepted: *27 September 2018*
Published: *18 October 2018*

Citation:
Weiler BA, Verhoeven JTP and Dufour SC (2018) Bacterial Communities in Tissues and Surficial Mucus of the Cold-Water Coral Paragorgia arborea. Front. Mar. Sci. 5:378. doi: 10.3389/fmars.2018.00378

INTRODUCTION

Corals host a wide range of microbial associates, including bacteria, eukaryotes, archaea, and viruses (Ainsworth et al., 2017). Of these, coral-associated bacteria (herein referred to colloquially as the "microbiome") may play important roles in host nitrogen metabolism (Grover et al., 2014; Rädecker et al., 2015; Kellogg et al., 2016; Lawler et al., 2016), nutrient cycling (Wild et al., 2004; Naumann et al., 2009), and antibacterial mechanisms (Kelman et al., 1998; Sutherland et al., 2004; Ritchie, 2006). The microbiome typically helps maintain coral health and provides a defense system against disease (Krediet et al., 2013). However, it can also harbor low abundances of pathogens that may become dominant when subjected to environmental stressors; such dysbiosis may lead to coral disease and/or death (Mouchka et al., 2010; Egan and Gardiner, 2016). The coral microbiome likely consists of a combination of commensals, transients, and long-term, stable partners selected by the host (Ainsworth et al., 2015), and is distributed among several anatomical compartments: the skeletal tissue, polyp tissue, and the external surface mucopolysaccharide layer (SML) (Bourne and Munn, 2005; Brown and Bythell, 2005; Sweet et al., 2011; Krediet et al., 2013; Ainsworth et al., 2015).

The SML has been reported as a first line of defense to protect the coral host against pathogens from the surrounding water column (Bythell and Wild, 2011; Sweet et al., 2011; Glasl et al., 2016). Additionally, the mucus is important for particulate feeding and can act as an energy carrier/provider and as a particle trap, as it is sloughed-off into the surrounding waters (Coffroth, 1991; Wild et al., 2008; Bythell and Wild, 2011). In shallow-water scleractinian corals, mucus is subject to diurnal or hourly replacement cycles, and its bacterial biodiversity could be changing with these cycles (Ainsworth et al., 2010; Sweet et al., 2011). The contrast between the more stable tissue/skeletal regions and the frequently renewing mucus compartment can explain differences in bacterial compositions between those coral microhabitats (Bourne and Munn, 2005; Sweet et al., 2011).

Like their warm, shallow-water counterparts, cold-water corals found in the deep-sea (beyond the photic zone to over 2000 m depth) host diverse microbial communities. However, they are difficult to access due to logistical and financial constraints (Kellogg et al., 2016) and therefore little is known about them, and even less about their microbiomes (Holm and Heidelberg, 2016). In particular, the bacterial communities colonizing the SML of corals from deep-sea regions remain unexplored and may differ from those of shallow-water corals due to expected discrepancies in physical conditions within the mucus layer (e.g., the absence of zooxanthellae-linked diel oxygen fluctuations in the SML of corals from deep habitats) and functions (e.g., a greater importance of particulate feeding in the deep sea). This study aims to characterize the bacterial associates of the cold-water alcyonacean coral, *Paragorgia arborea* (Linnaeus, 1758). *P. arborea* has been suggested to be a brooding azooxanthellate coral (Roberts et al., 2006; Lacharité and Metaxas, 2013) and is widely distributed in the Northwestern and Northeastern Atlantic, from the Gulf of Maine northward along the eastern coasts of Canada and the Davis Strait, to the continental slopes of Greenland, along the Reykjanes Ridge of Iceland, to the shelf of Norway (Cairns and Bayer, 2005; Mortensen and Buhl-Mortensen, 2005; Buhl-Mortensen et al., 2015; Brooke et al., 2017). *P. arborea* occupies a wide range of depth (200–1200 m) where temperatures range between 3 and 8°C, serving as a foundation species across a broad geographic area (Buhl-Mortensen et al., 2015). The arborescent morphology of *P. arborea* provides niches for prey refuge and creates habitats for facultative and obligate deep-sea symbionts (Buhl-Mortensen and Mortensen, 2004; Lacharité and Metaxas, 2013).

The bacterial communities of tropical, cold water, and deep-sea Alcyonacea and gorgonians are typically dominated by Proteobacteria, specifically Gammaproteobacteria (Penn et al., 2006; Bayer et al., 2013; Correa et al., 2013; La Rivière et al., 2013, 2015; Vezzulli et al., 2013; Ransome et al., 2014; Kellogg et al., 2016; Robertson et al., 2016). Additionally, Alphaproteobacteria were identified as notable contributors to the microbial consortia across multiple studies on alcyonaceans and gorgonians (Gray et al., 2011; Bayer et al., 2013; Correa et al., 2013; La Rivière et al., 2013, 2015; Vezzulli et al., 2013; Ransome et al., 2014; Holm and Heidelberg, 2016; Kellogg et al., 2016; Robertson et al., 2016). While Proteobacteria remain largely dominant across alcyonacean microbiomes, in some cases Spirochaetes (Holm and Heidelberg, 2016; Lawler et al., 2016; van de Water et al., 2016) and Tenericutes (Gray et al., 2011; Holm and Heidelberg, 2016) were the most abundant bacterial associates.

In deep-sea ecosystems, food sources are limited and variable and therefore alternate mechanisms are required by benthic invertebrates for metabolic processes (Mueller et al., 2014; Middelburg et al., 2015). Such mechanisms include bacteria-mediated nitrogen cycling, which has been documented in shallow-water corals (Wegley et al., 2007; Rädecker et al., 2015), and more recently in the cold-water scleractinian coral *Lophelia pertusa* (Middelburg et al., 2015), and postulated for the octocoral *Paramuricea placomus* (Kellogg et al., 2016) and Anthothelidae corals (Lawler et al., 2016). In these studies, several bacterial species are thought to facilitate nitrogen metabolism, with *Spirochaeta* implicated in nitrogen fixation (Lawler et al., 2016), Pirellulaceae in nitrification (Kellogg et al., 2016), Kiloniellales and *Bacillus* spp. in denitrification (Verbaendert et al., 2011; Kellogg et al., 2016; Lawler et al., 2016), *Propionibacterium* and Oceanospirillales in nitrogen reduction (Kellogg et al., 2016; Lawler et al., 2016), and Campylobacterales in nitrate/nitrite ammonification in a deep-sea octocoral (Kellogg et al., 2016). *P. arborea* has been observed to occupy the same benthic distribution and topography as *L. pertusa* (Buhl-Mortensen et al., 2015) and *P. placomus* (Buhl-Mortensen and Buhl-Mortensen, 2014), and may show similar host–bacterial interactions.

In the deep-sea, corals play a similar role to their shallow water counterparts, providing prey refuge and rugose habitats to many organisms (Hixon and Beets, 1993; Syms and Jones, 2000; Stone, 2006). However, coral ecosystems are threatened by several anthropogenic stressors and there is increasing concern that cold-water corals located along continental shelves are at risk from disturbances such as bottom trawling and oil exploration (Bavestrello et al., 1997; Fosså et al., 2002; Husebø et al., 2002; Roberts et al., 2006; Cordes et al.,

2016). As only a few cold-water corals have been studied, there is a knowledge gap regarding these organisms' bacterial communities and their functional roles in the host. Previous studies have reported variability in bacterial compositions between octocoral genera (Brück et al., 2007), between congeners (Holm and Heidelberg, 2016; Kellogg et al., 2016), between coral microhabitats (Weinbauer et al., 2012), within species and between sampling locations (Gray et al., 2011). In this study, we characterized the bacterial associates of *P. arborea* across two compartments: (1) skeletal and polyp tissue, and (2) surface mucus, and examined the degree of similarity in bacterial composition across three study locations in the Gulf of Maine (ranging in depth between 411 and 700 m) to explore the effects of relative sampling location proximity and depth on bacterial composition. Due to biological traits observed in *P. arborea* (i.e., abundant mucus production; Etnoyer et al., 2006, and brooding; Lacharité and Metaxas, 2013), and the previously observed differences in bacterial compositions in tissues and mucus in other coral species, we expect to see between-compartment variation as well as variability among sampling locations. We compared common/dominant bacterial associates, searched for taxa that could be markers of either tissue or mucus due to significant differences in relative abundance, and identified rare bacterial taxa of particular interest. To our knowledge, this work represents the first description of the bacterial associates recovered from tissue and mucus samples of the alcyonacean coral *P. arborea*.

MATERIALS AND METHODS

Sample Collection and Study Sites

Paragorgia arborea colony fragments were collected from multiple submarine canyons: Nygren-Heezen Intercanyon (N40°51.96′, W66°32.74′, depth 700 m); Corsair Canyon (N41°21.26′, W66°5.39′, depth 411 m); and Georges Canyon (N41°16.48′, W66°11.59′, depth 423 m), on the continental slope of the Gulf of Maine during a research cruise aboard the National Oceanic and Atmospheric Association (NOAA) Ship *Henry B. Bigelow* from June 8th to 22nd, 2017. Coral colonies were located and sampled using CSSF-ROPOS (Canadian Scientific Submersible Facility, Remotely Operated Platform for Ocean Sciences), with individual fragments held in separate water-filled chambers until surfacing. Tissue fragments were then dissected from the coral stalk and placed in individual, sterile cryovials. Mucus samples were collected by gently rolling a sterile cotton swab over the exterior of the specimen where mucus was visible. Three tissue replicates, and one mucus swab were collected from individual colonies and frozen (−20°C) until further analysis. At each dive location, a reference water sample was collected from approximately 1 m from the bottom, within proximity of the coral colonies, using a remotely triggered Niskin bottle attached to the ROV. On board, 50 mL aliquots of each water sample were passed through individual 0.22 μm syringe filters (MilliporeSigma, Canada). The syringe filters were frozen at −20°C until further analysis.

Nucleic Acid Extraction

Total DNA was extracted from *P. arborea* mucus and tissue samples, following the protocol described by Sunagawa et al. (2010) with modifications. Samples of coral tissue and skeleton (between 0.100 and 0.250 g total weight) were washed in 600 μL phosphate-buffered saline (PBS) three times to remove mucus and loosely associated bacteria. Once washed, the samples were flash frozen in liquid nitrogen and crushed using a sterile mortar and pestle. Crushed samples were transferred to tubes included with the PowerViral Environmental RNA/DNA extraction kit (Mo Bio, Carlsbad, CA, United States) containing 0.1 mm glass beads. DNA was extracted from water filters by adding 600 μL PV1 lysis buffer (Mo Bio) into the filter, incubating for 2 min at room temperature, and subsequently purging the filter cartridge using a syringe to collect all lysis buffer. The process was repeated for the reverse side of the syringe filter and once more for the original starting side to ensure maximum PV1 based lysis and recovery. Mucus swabs were placed in microfuge tubes and vortexed with PV1 lysis buffer (Mo Bio) for 10 min. After initial lysis, DNA was extracted according to the manufacturer's protocol. Prior to high throughput sequencing, the presence of bacterial DNA was confirmed by PCR amplification of 16S rRNA genes using the universal primers ECO8F (Edwards et al., 1989) and 1492R (Stackebrandt and Liesack, 1993), DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, United States), and thermal cycled: 30 cycles of 95°C for 5 min, 95°C for 30 s, 45°C for 30 s, 72°C for 1 min, and final extension of 72°C for 2 min. Extracted DNA was checked for quality and quantity using NanoDrop^{TM} 1000 (Thermo Fisher Scientific).

16S rRNA Gene Sequencing and Processing

Coral DNA extracts were outsourced for sequencing at the Centre for Comparative Genomics and Evolutionary Bioinformatics (Dalhousie University, Halifax, Canada). 2 × 300-bp paired-end sequencing of the 16S rRNA gene was performed using Illumina MiSeq v3, with all samples amplified using previously published primers targeting the V6–V8 regions (B969F/B1406R): V6 forward: 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG and V8 reverse: 5′- CCTATCCCCTGTGTGCCTTGGCAGTCTCAG (Comeau et al., 2011).

Sequence data were processed using the in-house developed SPONS-2 pipeline, as described in Verhoeven and Dufour (2017), with a few modifications. In short, sequences were trimmed to remove both low-quality bases using Trimmomatic version 0.38 (20-base sliding window with a minimum average quality of 15 per base) and short reads (<100 bases) (Bolger et al., 2014). Reads that passed the initial quality check were merged using PEAR version 0.9 (Zhang et al., 2014). Primers were trimmed from merged reads using CutAdapt (maximum error rate 0.2), filtering out reads that lack forward or reverse primers (Martin, 2011). Reads with an average Phred score below 20 were removed as a final quality check prior to defining operational taxonomic units (OTUs) using SWARM version 2.2 (Mahé et al., 2015). The step wherein Swarm defines OTUs was modified so the

maximum difference between amplicons (d) was increased from 1 to 3 (decreasing the potential for overestimating defined OTUs). Defined OTUs were analyzed using the RDP naïve Bayesian classifier (Wang et al., 2007) for taxonomic assignment, using the SILVA SSU database (release 132; Quast et al., 2013) with a 51% minimum bootstrap confidence estimate when assigning taxonomy.

Bioinformatics and Statistical Analyses

Microbiome high-throughput sequence (HTS) datasets are compositional (Gloor et al., 2017), and recent concerns have been raised regarding current microbiome analysis methodologies (i.e., normalizing HTS count data by rarefaction or other subsampling methods) (Fernandes et al., 2014; McMurdie and Holmes, 2014). Here, we used a compositional analysis approach to compare the bacterial communities from coral and seawater samples.

Operational taxonomic unit count and taxonomic data were imported in R for analyses (R Development Core Team, 2008). First, filtering was conducted to remove OTUs not classified as bacteria at the Kingdom level. Bacterial alpha diversity in each coral and water sample was then examined through the Hill's series of diversity indices. Sample count data were square-root transformed, and using the vegan package (Oksanen et al., 2016), we calculated the Hill's diversity series: the raw number of OTUs (H_0), the exponent of Shannon diversity (H_1), and the reciprocal of the Simpson's index (H_2). To test for significant differences between sample diversity index values across geographic locations and between anatomical compartments, *t*-tests were performed in PAST (Hammer et al., 2001).

Before performing beta diversity analysis to compare bacterial community composition across samples, low abundance OTUs were filtered out (minimum proportional abundance: 0.5%) and zero counts were replaced with non-zero calculated values using the *count zero multiplicative* method from the R packages CoDaSeq and zCompositions (Martín-Fernández et al., 2015; Palarea-Albaladejo and Martín-Fernández, 2015). The zero-adjusted data were then centered log-ratio (clr) transformed using the CoDaSeq package (Gloor and Reid, 2016; Gloor et al., 2017). The clr is scale-invariant, meaning that the same ratio is expected from samples with different read counts (Gloor et al., 2017). A principal component analysis (PCA) was performed by plotting a singular value decomposition of clr transformed values to visualize beta diversity. We also performed hierarchical clustering using the "hclust" command, using a Euclidian distance matrix and agglomeration method "Ward. D2". Bacterial community composition bar graphs representing phyla contribution (≥1%) and class contribution (≥3%) per sample were produced to visualize trends in the hierarchical cluster analysis dendrogram.

Quantitative analyses were conducted to examine the meta-data factors contributing toward the observed variance in bacterial community structure using the vegan R package "adonis" command for permutational multivariate analysis of variance (PERMANOVA) using a Euclidian distance matrix. A two-way design was used for the PERMANOVA, where location (canyons) and compartment (mucus and tissue) were both considered as fixed factors, and the interaction between the two factors was tested. Lastly, pairwise quantitative [ANOVA-like differential expression (ALDEx), Fernandes et al., 2013] analysis was performed to identify compartment-specific OTUs, or biomarkers. For this, clr-transformed count data were analyzed using differential relative abundance tests generated by 128 Monte Carlo samples sourced from a Dirichlet distribution. This ALDEx analysis was performed using the ALDEx2 package in R (Fernandes et al., 2013, 2014) to generate a list of OTUs (classified according to lowest available taxonomic rank) that possessed a significant association with either tissue or mucus. Here, we consider an OTU to be positively associated with tissue if the effect size is ≥ 1, and positively associated with mucus if the effect size is < -1.

RESULTS

Alpha and Beta Diversity in Coral Bacterial Assemblages

Rarefaction curve analysis showed that all samples were sequenced sufficiently (i.e., to a read depth considered representative of each sample's total microbial diversity), as indicated by the plateauing of the OTU count curves (aside from the Corsair Canyon seawater sample, which was omitted from further analysis; **Figure 1**). Sequence data were deposited in the NCBI short read archive linked to BioProject accession number PRJNA490387. The mucus swabs were amongst the lowest in sequencing depth, and reference seawater samples were among the highest (**Table 1**). Alpha and beta diversity measures were used to compare diversity trends and similarities between bacterial communities. The reference seawater samples were significantly higher than coral samples in all three Hill's series diversity indices for bacterial communities [H_0, H_1, H_2, two-sample *t*-test between coral samples ($n = 12$) and seawater ($n = 2$), $p < 0.001$ for each metric]. There were no significant differences between coral compartments or among geographic locations for any of the Hill's series diversity indices [H_0, H_1, H_2, two-sample *t*-test between coral tissue ($n = 9$) and mucus ($n = 3$), $p > 0.05$ for each metric].

Due to the significant differences in bacterial alpha diversity between seawater and coral samples, we chose to produce a PCA ordination using Euclidian distance to further visualize similarities in bacterial composition across samples (**Figure 2**). The PCA representation of *P. arborea* tissue, mucus, and surrounding seawater bacterial communities showed some separation between coral samples and the reference seawater along the primary PCA axis (PC1 = 46.2% of the variation in the dataset) (**Figure 2**). The majority of coral samples clustered together, with some outliers. Differences between coral mucus samples were largely along the secondary PCA axis (PC2 = 16.2% of the variation) (**Figure 2**). PERMANOVA analysis uncovered a significant difference in bacterial composition between compartments (tissue and mucus) in *P. arborea* ($p = 0.001$). Furthermore, sample geographic location (canyon) was a significant explanatory factor for observed differences within

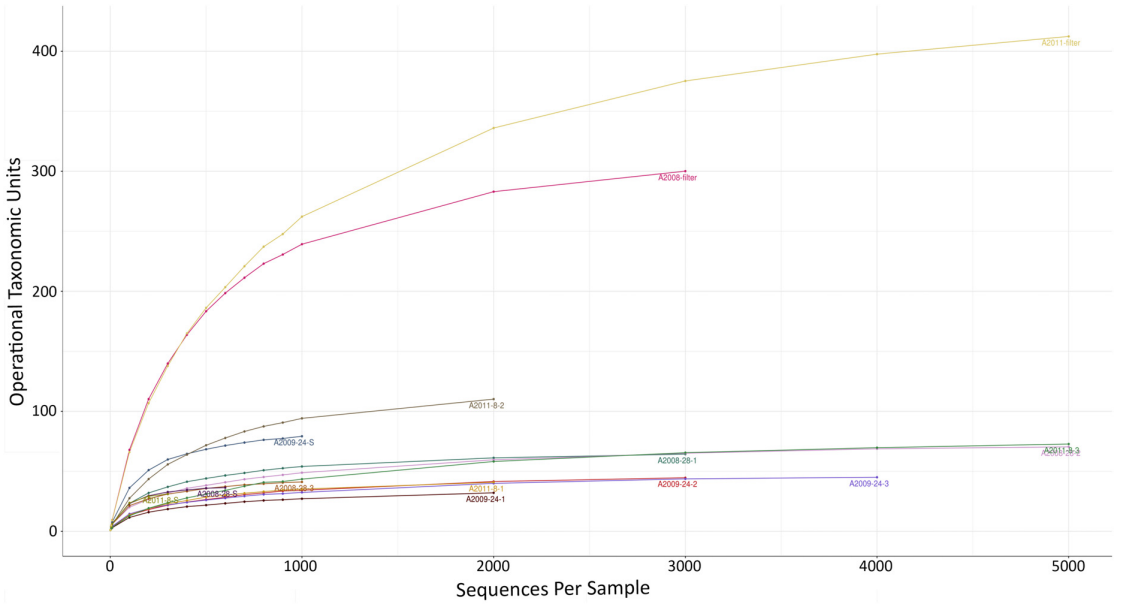

FIGURE 1 | Rarefaction curve showing the number of observed bacterial OTUs as a function of the number of sequences. *Paragorgia arborea* tissue and mucus samples (n = 12) and seawater samples (n = 2). Sample names as in **Table 1**.

P. arborea bacterial composition (p = 0.014). The PERMANOVA analysis showed no significant interaction between compartment and canyon. The PERMANOVA results were cross-checked using beta dispersion analysis, wherein significant results were validated by the PERMANOVA's assumption of homogeneity of dispersion.

Bacterial Community Composition Within Coral Samples

The phyla shown in **Figure 3A** accounted for ∼98% of the bacteria found within the samples. Proteobacteria were present in all coral and seawater samples and were dominant in both seawater samples, in two tissue samples from Nygren-Heezen Intercanyon (PA-N28-T1/T3), and in two mucus samples (PA-G08-MU & PA-N08-MU) (**Figure 3A**). The third mucus sample (PA-C24-MU) had similar proportions of Verrucomicrobia (∼37%), Proteobacteria (∼27%), and Bacteroidetes (∼26%). Tenericutes dominated (∼68%) all coral tissue samples from Corsair Canyon and Georges Canyon, with Proteobacteria (∼15%) and Spirochaetes (∼7%) as notable contributors. Furthermore, Tenericutes were observed in every coral sample with the exception of one mucus sample (PA-C24-MU) and were absent in the seawater sample. The Nygren-Heezen Intercanyon coral tissue samples appeared more variable in composition and contrasted with samples from Corsair Canyon and Georges Canyon, which showed similar bacterial community composition across tissue samples. Cyanobacteria were present in coral samples, with the exception of two tissue samples (PA-C24-T2 and PA-N28-T2). Actinobacteria were observed in all samples recovered from Nygren-Heezen Intercanyon (including seawater) and were a dominant contributor in one tissue sample from Nygren-Heezen Intercanyon, in all mucus samples, and in the Georges Canyon seawater sample (WA-G11). Lastly, phylum and class Chlamydiae were only present in coral tissue samples from Georges Canyon (∼8%), while Firmicutes were only found in coral mucus from Georges Canyon (∼3%).

At the class level, bacterial compositions were nearly identical in coral tissue samples from Corsair Canyon, where samples were made up of Mollicutes (∼70%), Alphaproteobacteria (∼11%), Spirochaetes (∼9%), and Gammaproteobacteria (∼6%) (**Figure 4**). Coral tissue samples from Georges Canyon were also similar in composition, with dominant contributors being Mollicutes (∼65%), Gammaproteobacteria (∼9%), Oxyphotobacteria (∼6%), Chlamydiae (∼5%), and Spirochaetes (∼5%). There was less consistency among tissue samples from the Nygren-Heezen Intercanyon, mainly due to the variation in sample PA-N28-T2. Excluding sample PA-N28-T2, dominated by Actinobacteria (68%), the other samples were very similar in composition, made up of Spirochaetes (∼32%), Gammaproteobacteria (∼28%), Alphaproteobacteria (∼18%), Mollicutes (∼10%), and Bacteroidia (∼6%). Among mucus samples, the one from Corsair Canyon differed from those from Nygren-Heezen Intercanyon and Georges Canyon. Mucus recovered from Corsair Canyon showed a large contribution from Verrucomicrobia (∼37%), and further composed of Bacteroidia (∼26%), Gammaproteobacteria (∼23%), and Alphaproteobacteria (∼4%). The remaining mucus samples were relatively consistent in composition, containing mostly Gammaproteobacteria (∼45%), Oxyphotobacteria (∼24%), Alphaproteobacteria (∼10%), Mollicutes (∼7%), and Bacteroidia (∼7%).

The trends in bacterial composition were observed across all samples in the produced hierarchical dendrogram (**Figure 3B**).

TABLE 1 | Sample collection, description, number of reads from processed data, and Hill's series diversity summary statistics of bacterial communities determined by analysis of 16S rRNA sequence libraries.

Specimen ID	Species	Submarine canyon	Description	Depth (m)	Number of reads	$Hill_0$	$Hill_1$	$Hill_2$
PA-N28-T1	*P. arborea*	Nygren-Heezen	Tissue replicate 1	700	3392	69	44.94	29.95
PA-N28-T2	*P. arborea*	Nygren-Heezen	Tissue replicate 2	700	6060	77	43.25	26.22
PA-N28-T3	*P. arborea*	Nygren-Heezen	Tissue replicate 3	700	1929	48	32.91	23.32
PA-N28-MU	*P. arborea*	Nygren-Heezen	Mucus	700	696	39	30.72	23.97
PA-C24-T1	*P. arborea*	Corsair	Tissue replicate 1	411	2227	37	19.17	9.5
PA-C24-T2	*P. arborea*	Corsair	Tissue replicate 2	411	3274	49	26.02	14.16
PA-C24-T3	*P. arborea*	Corsair	Tissue replicate 3	411	4038	49	25.88	14.25
PA-C24-MU	*P. arborea*	Corsair	Mucus	411	1459	87	69.53	54.21
PA-G08-T1	*P. arborea*	Georges	Tissue replicate 1	423	2744	46	25.6	12.72
PA-G08-T2	*P. arborea*	Georges	Tissue replicate 2	423	2553	121	85.73	48.14
PA-G08-T3	*P. arborea*	Georges	Tissue replicate 3	423	5220	81	39.9	15.86
PA-G08-MU	*P. arborea*	Georges	Mucus	423	363	35	28.66	22.36
WA-N08	Seawater	Nygren-Heezen	Water	837	3997	322	276.94	230.82
WA-G11	Seawater	Georges	Water	606	6076	446	361.32	276.65

Coral and seawater samples were observed to branch apart at 30% similarity, with the two seawater samples showing similar compositions (∼85% similarity). Coral tissue samples predominantly formed clusters, with groupings of Corsair Canyon samples (∼87% similar) and Georges Canyon samples (∼83% similar) most evident. Two mucus samples (PA-G08-MU and PA-N28-MU) contained relatively similar compositions (∼74%). Coral tissue samples from all three canyons converged at ∼51% similarity.

Bacterial Biomarkers Within Coral Anatomical Compartments

To observe whether any specific bacterial OTUs (biomarkers) were significantly more abundant in either compartment of *P. arborea* (tissue or mucus), samples were analyzed using an ALDEx analysis using the R package ALDEx2 (Fernandes et al., 2013, 2014). Three OTUS with genus-level assignment were recovered as biomarkers in *P. arborea*, with two recovered in the tissue and one in the surficial mucus (**Figure 5**). Two OTUs recovered as significant within the tissues belonged to the genus *Spirochaeta* of the phylum Spirochaetes and *Mycoplasma* of the phylum Tenericutes (effect sizes = 4.02 and 2.37, respectively) and were observed as relatively large contributors to the associated bacterial assemblage, particularly in samples from Nygren-Heezen Intercanyon (**Supplementary Table S1**). The mucus biomarker was identified as *Paracoccus marcusii* from the phylum Proteobacteria (effect size = −1.47). This biomarker was only recovered in two tissue samples, but was noted in the three mucus samples, comprising ∼1.2–2.4% of the bacterial assemblage (**Supplementary Table S1**). Four additional OTUs were recovered as significantly associated with tissues, but had no genus level assignment. Those four OTUs belonged to the families Flavobacteriaceae (phylum Bacteroidetes), and Terasakiellaceae (phylum Proteobacteria), and the orders Campylobacterales (phylum Epsilonbacteraeota) and Rickettsiales (phylum Proteobacteria) (effect sizes = 1.72, 1.41, 1.84, and 2.34, respectively). These four OTUs were rarely encountered in mucus and were most abundant in Nygren-Heezen Intercanyon tissue samples (**Supplementary Table S1**).

DISCUSSION

This is the first described characterization of the bacterial associates for *Paragorgia arborea,* a widely distributed cold-water alcyonacean coral that was collected from three submarine canyons off the Gulf of Maine (Nygren-Heezen Intercanyon, Georges Canyon, and Corsair Canyon). Sequencing of a fragment of the 16S rRNA gene allowed us to observe differences in bacterial composition between coral sample locations, and between compartments (tissue and mucus microhabitats). The numbers of reads obtained from extracts of *P. arborea* tissue samples were much higher than in mucus swabs, suggesting that our mucus sampling methodology could be improved. The low read depth of the water samples was likely due to the small aliquots (50 ml) of seawater at depth available to us on board the research cruise. Coral bacterial associates likely consist of a combination of commensals, long-term, stable partners selected by the host (including potentially intracellular symbionts), bacteria recently ingested and present in the gastro-vascular cavity, and, for mucus samples, either passively adhering bacteria or host-specific taxa (Ainsworth et al., 2015). While we are unable to confidently discriminate among those associate categories in this analysis, we draw upon our comparative analyses and on coral microbiomes described in the literature to suggest some bacterial taxa as potentially important members of the bacterial microbiome of those deep-sea, cold-water corals.

Alpha and Beta Measures of Bacterial Diversity

As observed in similar studies, the corals examined hosted bacterial communities with significantly lower bacterial

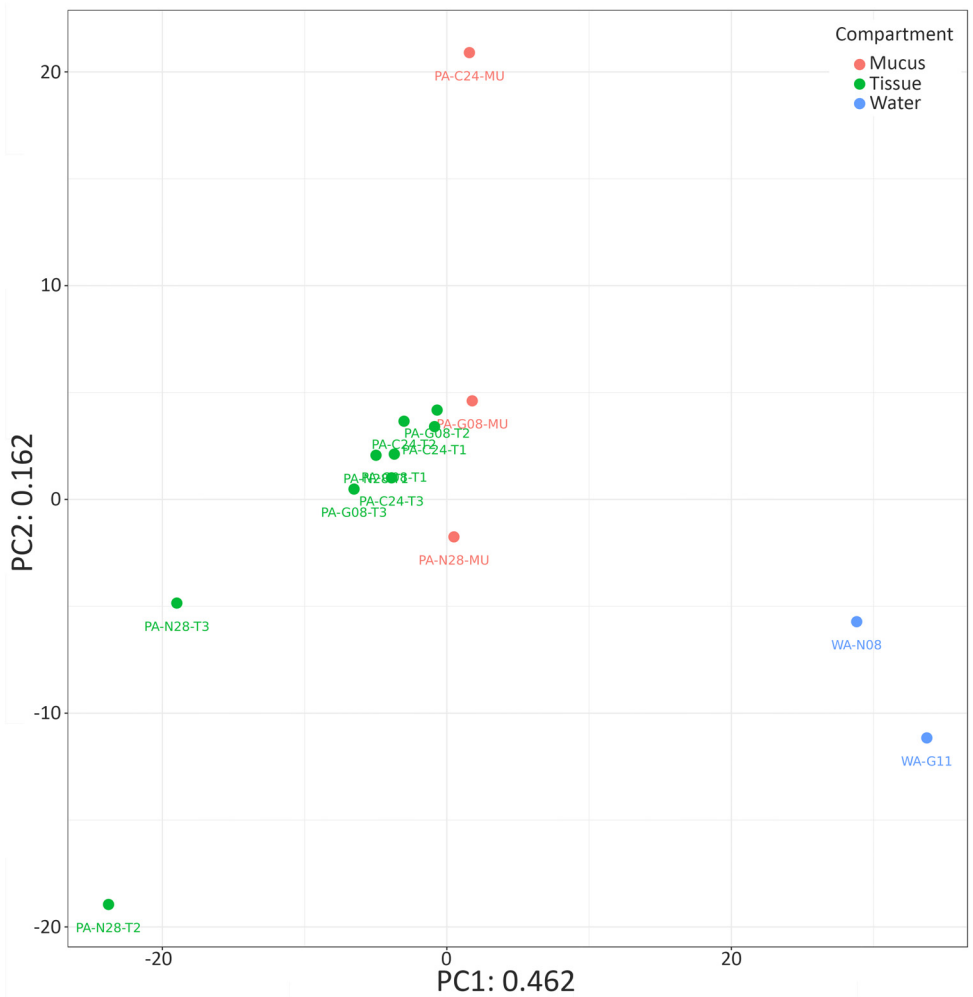

FIGURE 2 | Bacterial community similarity for *Paragorgia arborea* and reference seawater samples. Principal component analysis (PCA) ordination of bacterial communities in *P. arborea* (tissue samples in green and mucus samples in orange) and seawater samples (blue), developed from a singular value decomposition of centered log-ratio (clr)-transformed compositional data. Sample names as in **Table 1**.

biodiversity than those in surrounding seawater (Bayer et al., 2013; Holm and Heidelberg, 2016; van de Water et al., 2016). Within *P. arborea*, there was no significant difference in diversity measures between anatomical compartments (tissue and mucus) and among geographic locations (submarine canyons), suggesting spatial stability in the diversity (as characterized by Hill's indices) of bacterial communities associated with *P. arborea*.

The bacterial communities of seawater were ∼70% different in composition (according to hierarchical clustering) from those associated with *P. arborea*; therefore, we consider that the coral microbiome was predominantly comprised of taxa that were uncommon in surrounding seawater and that may show some specificity for this particular host. Other studies on corals provide evidence for bacterial host-specificity: for example, distinct bacterial populations were observed in three octocorals from the coast of Florida, *Leptogorgia minimata, Iciligorgia schrammi,* and *Swiftia exertia* (Brück et al., 2007). Similarly, two octocorals from the Eastern Pacific of the genus *Muricea* had distinct microbial assemblages, despite co-occurring habitats and similar morphological structure (Holm and Heidelberg, 2016). Even the alcyonacean congeners *P. placomus* and *Paramuricea clavata* showed negligible bacterial community similarities (Kellogg et al., 2016). However, another study reported a lack of significant variation between microbiomes in two species of Anthothelidae (Lawler et al., 2016). Based on our study, the bacterial communities associated with *P. arborea* show some species specificity (given the ∼50% assemblage similarity among samples from different locations) and are dominated by many of the same taxa reported in other cold-water corals. We note the presence of phototrophic bacteria among the recovered taxa (phylum Cyanobacteria and class Oxyphotobacteria), particularly within mucus samples. These bacteria were likely among phytoplankton and other surficial detritus transported to the deep-sea along the canyons and captured by the corals.

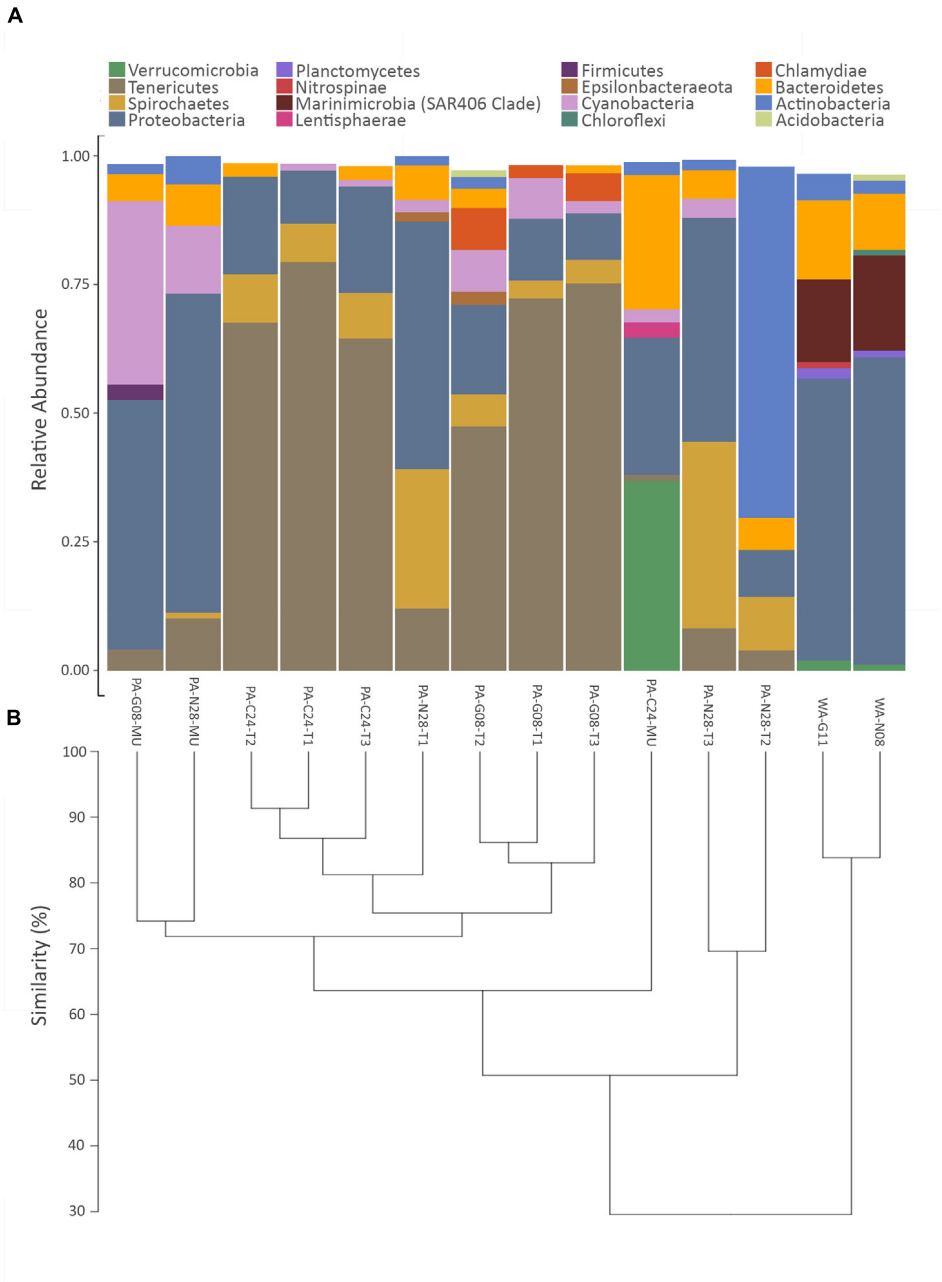

FIGURE 3 | Relative abundance of bacterial phyla and hierarchical dendrogram. **(A)** *X*-axis represents samples of *Paragorgia arborea* tissue and mucus, and seawater. *Y*-axis represents the most dominant bacterial phyla found within samples, with taxa contributing <1% relative abundance excluded; and **(B)** hierarchical clustering dendrogram for *P. arborea* and reference seawater samples, developed from a Euclidian distance matrix produced from centered log-ratio (clr)-transformed compositional data. Sample names as in **Table 1**.

The bacterial assemblages in *P. arborea* samples differed significantly in composition across geographic locations (submarine canyons); substantial variability in bacterial community composition across colonies of a particular coral species has been reported previously (see Hernandez-Agreda et al., 2017). Considering only tissue samples, those from Corsair Canyon (depth = 411 m) and Georges Canyon (depth = 423 m) were roughly 75% similar in bacterial community composition (**Figure 3B**); although those sites are within relative proximity to one another, they may differ in environmental and biotic factors, which may play a role in the diversity of available microbial associates and explain observed differences. The bacterial communities in coral tissue samples from the more distant and deeper (700 m) Nygren-Heezen Intercanyon showed greater compositional dissimilarity to the Corsair and Georges Canyon samples. Geographical differences in those

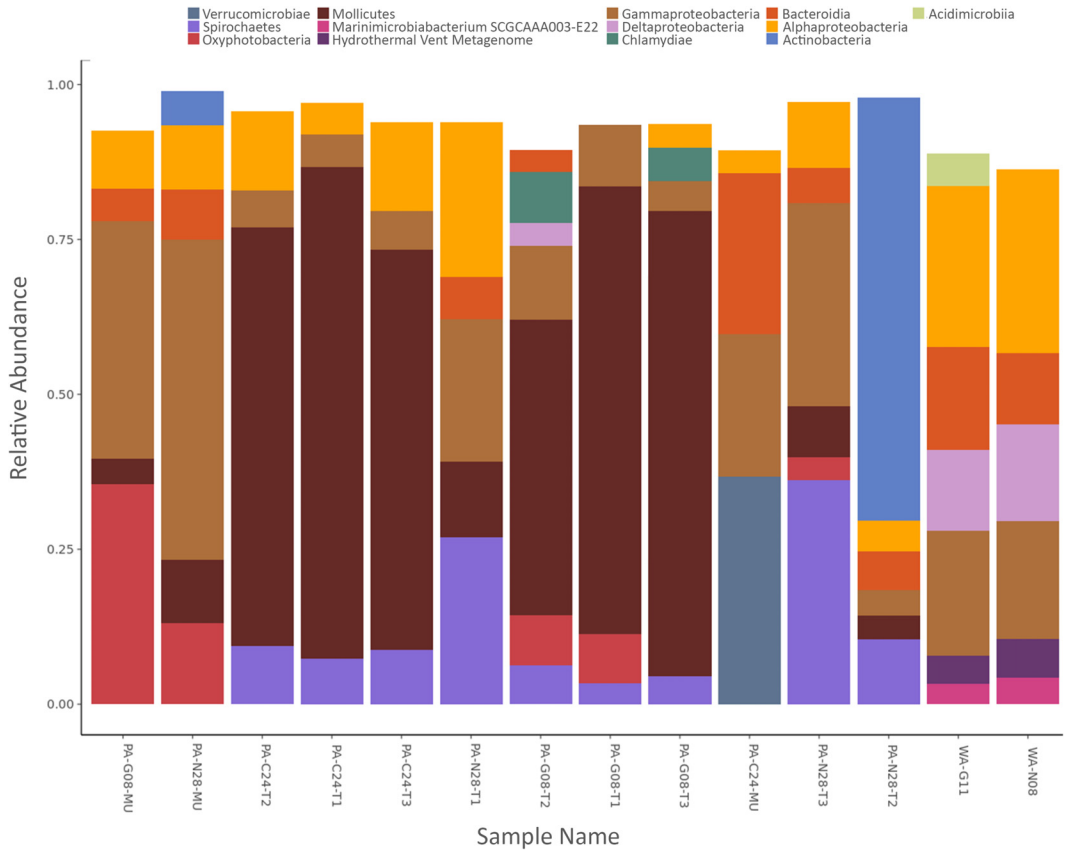

FIGURE 4 | Relative abundance of bacterial classes. *X*-axis represents samples of *Paragorgia arborea* tissue and mucus, and seawater. *Y*-axis represents the most dominant bacterial classes found within samples, with taxa contributing <3% relative abundance excluded.

bacterial assemblages may be driven mostly by commensals and/or more loosely associated or transient bacteria, but could also be influenced by the reproductive behavior of the host. *P. arborea* has been suggested to be a brooding coral, with fertilization occurring inside or at the surface of the female colony and the larval offspring settling nearby the parent colony (Lacharité and Metaxas, 2013). Some bacteria may be transmitted vertically in both broadcast and brooding corals (Apprill and Rappe, 2011; Ceh et al., 2013), and therefore the greater similarity in bacterial community composition between *P. arborea* colonies from nearby canyons could be partly linked to the relatively small larval dispersal distances in this coral species. Further research on vertical bacterial transmission in coral microbial communities is needed to shed light on this matter.

The surface mucus layer (SML) of a coral performs multiple functions and hosts a diverse assemblage of microbes (Bythell and Wild, 2011; Sweet et al., 2011). We observed a significant difference in bacterial composition between anatomical compartments of *P. arborea* and noted compositional differences between mucus samples within a host species. The mucus of shallow-water scleractinian corals is subject to diurnal or hourly replacement cycles, and therefore its bacterial diversity and richness could be constantly changing (Ainsworth et al., 2010; Sweet et al., 2011). In the deep-water scleractinian *Lophelia pertusa*, bacterial communities within the tissues were more stable than those in mucus, which is continuously being replenished following release in the water column (Wild et al., 2008; Weinbauer et al., 2012). It has been previously documented that *P. arborea* produces abundant mucus (Etnoyer et al., 2006). We also observed a large quantity of mucus following *P. arborea* sample collection, and its SML may constitute a perpetually changing microhabitat, as in *Lophelia pertusa* (Wild et al., 2008). Despite the variation in depth and associated physical parameters between shallow and deep-water habitats, we note that the mucus-derived bacterial composition of *P. arborea* shares the high diversity and variability of tropical corals.

Community Composition

Based on previous microbiome studies of alcyonaceans, certain bacterial taxa were expected to dominate the bacterial assemblage of *P. arborea*. In most alcyonaceans examined, Proteobacteria (especially Gammaproteobacteria and Alphaproteobacteria) dominate the bacterial assemblage (Penn et al., 2006; Gray et al., 2011; Bayer et al., 2013; Correa et al., 2013; La Rivière et al., 2013, 2015; Vezzulli et al., 2013; Ransome et al., 2014; Kellogg et al., 2016; Lawler et al., 2016; Robertson et al., 2016). Our results partly agree with these studies: Gammaproteobacteria

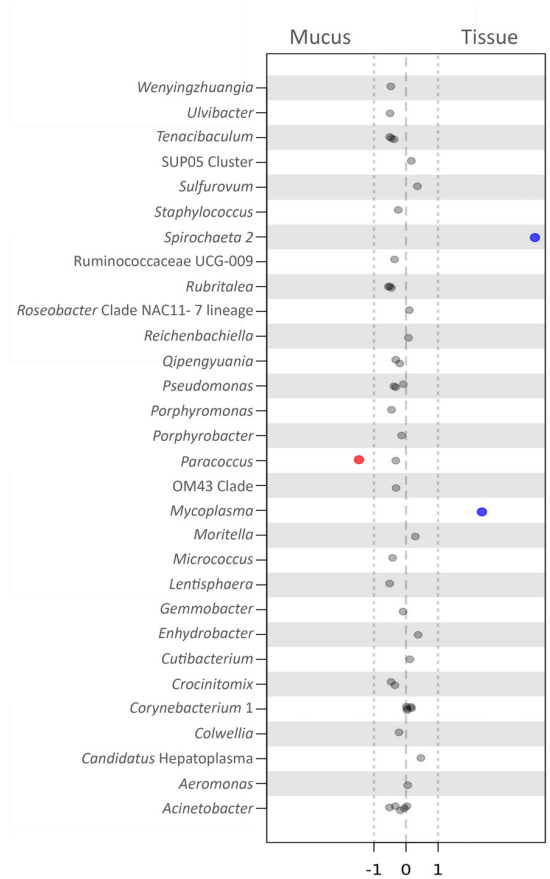

FIGURE 5 | Biomarker analysis of bacteria in *Paragorgia arborea* compartments. Effect size scores for bacterial OTUs, classified at the genus level, defined as biomarkers through the use of ANOVA-Like Differential Expression (ALDEx) analysis (R package ALDEx2). Significance is determined by effect size, comparing tissue to mucus: values >1 represent significant, positive associations with tissue (blue), values <–1 show a negative association with tissue and a significant, positive association with mucus (red), and values between –1 and 1 are close to but not significant. Each dot represents an OTU (within the specified genus) tested as a biomarker. The 30 OTUs (having assigned genera) with effect sizes closest to significance are shown here.

and Alphaproteobacteria were present in all *P. arborea* samples, but were not the largest contributor in most samples. In contrast, Tenericutes, or more specifically, Mollicutes were the most dominant across *P. arborea* tissue samples from two canyons (Corsair and Georges Canyon) and were present in all *P. arborea* samples, with the exception of mucus sample PA-C24-MU. Tenericutes were reported to be dominant in a few coral species (Kellogg et al., 2009; Gray et al., 2011; Holm and Heidelberg, 2016). The Tenericutes OTUs observed in *P. arborea* are of the orders Entomoplasmatales and Mycoplasmatales, the latter having been observed in three species of the alcyonacean coral *Muricea* (Ranzer et al., 2007; Holm and Heidelberg, 2016), in two additional alcyonacean species, *Plumarella superba* and *Cryogorgia koolsae* (Gray et al., 2011) and in the scleractinian cold-water coral, *Lophelia pertusa* (Kellogg et al., 2009). Spirochaetes are dominant bacterial contributors in various alcyonaceans (Holm and Heidelberg, 2016; Lawler et al., 2016; van de Water et al., 2016). Spirochaetes were present in all *P. arborea* samples, in some cases as major contributors, and have been described as chemoheterotrophic bacteria that thrive in a wide variety of environments (Ludwig et al., 2010). Spirochaetes dominated some *Anthothela* samples, and were suggested as potential nitrogen fixers (Lawler et al., 2016).

Many of the taxa present at lower abundance may serve crucial roles in coral–bacterial interactions (Ainsworth et al., 2015). Several members of the phylum Firmicutes (orders Bacillales, and Lactobacillales) were observed in extremely low abundance in *P. arborea*. However, Firmicutes were reported in other alcyonacean microbiome studies (Penn et al., 2006; Brück et al., 2007; Kellogg et al., 2009, 2016; Correa et al., 2013; Lawler et al., 2016). Closek et al. (2014) observed a higher abundance of Firmicutes such as Clostridia in yellow band diseased samples of the scleractinian *Orbicella faveolata*; our samples showed no evidence of disease. Verrucomicrobia were observed in one *P. arborea* mucus sample (PA-C24-MU) and in the seawater samples, and were found in low numbers in other alcyonacean microbiome studies (Gray et al., 2011; Correa et al., 2013; Ransome et al., 2014; Kellogg et al., 2016; Lawler et al., 2016). Verrucomicrobiales from our samples are associated with the family Rubritaleaceae. Bacteria from the Rubritaleaceae are carotenoid pigment producers (Rosenberg, 2014), that give a red coloration to colonies; it is uncertain why these bacteria were highly abundant in PA-C24-MU but absent from all other coral samples. Chlamydiae (taxonomic order Chlamydiales) were only observed in tissue samples from Georges Canyon; the role of Chlamydiales in invertebrates is not known. Campylobacterales were found within all *P. arborea* tissue samples in low abundance, absent in all mucus samples and were part of the Anthothelidae core microbiome (Lawler et al., 2016). Some members of the order Campylobacterales were suggested to contribute to the nitrogen metabolism cycle for nitrate/nitrite ammonification and denitrification (Tiedje, 1988; Hoffmann et al., 1998; Verbaendert et al., 2011); the Campylobacterales observed here belong to the families Sulfurovaceae, and Thiovulaceae.

Comparing rare taxa found in our study to previously described etiological agents in diseased corals and gorgonians, we found *Vibrio* spp. in one sample of *P. arborea* (PA-G08-T2) in very low relative abundance (data not shown). Various *Vibrio* strains may be etiological agents in diseased *P. clavata* colonies (Bally and Garrabou, 2007; Vezzulli et al., 2013). Additionally, Daniels et al. (2015) found high abundances of mRNA sequences from Vibrionaceae, Campylobacteraceae, Rhodobacteraceae, Flavobacteriaceae, and Burkholderiaceae in diseased *O. faveolata* coral samples. Representatives from the same bacterial families were found in low abundances in our visually healthy coral samples (data not shown). Further research regarding these bacterial groups is required to understand their functional roles in coral microbiomes, as rare bacterial taxa may be as important as dominant taxa and are typically overlooked (Ainsworth et al., 2017).

Bacterial Biomarkers Within *Paragorgia arborea* Anatomical Compartments

As previous studies have shown that bacteria found within specific microhabitats may serve particular roles for the host (Bythell and Wild, 2011; Sweet et al., 2011; Glasl et al., 2016), we explored whether any bacterial OTUs were considered statistically significant biomarkers within *P. arborea* tissues or mucus. Within *P. arborea*, OTUs from the genera *Spirochaeta* and *Mycoplasma,* as well as from the orders Campylobacterales and Rickettsiales and the families Flavobacteriaceae and Terasakiellaceae were biomarkers of tissue, while the genus *Paracoccus* was a biomarker of mucus. While these OTUs have been identified significantly as biomarkers, their abundance can vary markedly across samples and geographic locations and interpretations of key functional roles for these bacteria remain highly speculative.

The genus *Spirochaeta* has been observed to dominate *Anthothela* coral samples and was suggested to be a nitrogen fixer (Lawler et al., 2016). Interestingly, the *Spirochaeta* 2 OTU identified here as a tissue biomarker comprised up to 36% of the identified bacterial assemblage in a tissue sample from Nygren-Heezen Intercanyon. *Mycoplasma* strains have been described as pathogens and/or parasites (Rottem, 2003) and were observed in *Lophelia pertusa* (Kellogg et al., 2009). *Mycoplasma* were abundant in *Muricea* coral samples, with specific strains associated with bleached and unbleached coral samples (Ranzer et al., 2007; Holm and Heidelberg, 2016); although *P. arborea* is azooxanthellate (Roberts et al., 2006), *Mycoplasma* may nonetheless play some role in *P. arborea* health. In previous studies, Campylobacterales were suggested to play a role in denitrification (Verbaendert et al., 2011) and nitrate/nitrite ammonification (Hoffmann et al., 1998). Members of the family Terasakiellaceae and certain *Paracoccus* species were documented to be nitrogen fixers and denitrifiers, respectively, and may be important for alcyonacean nitrogen metabolism (Tiedje, 1988). A microbially mediated nitrogen cycle has been uncovered in the cold-water coral *Lophelia pertusa* (Middelburg et al., 2015), and may also be present in alcyonacean corals, such as *P. arborea,* which occupy similar, nutrient-limited environments (Buhl-Mortensen et al., 2015). Notably, we observed the highest proportions of *Spirochaeta*, Campylobacterales, Terasakiellaceae and *Paracoccus*, the biomarkers that might be involved in nitrogen cycling, in Nygren-Heezen Intercanyon, the deepest of our three sampling locations. This could signal a greater importance for bacterial associates involved in nitrogen cycling where food limitation could be more pronounced. Further investigations would be required to characterize any bacterial functions and their impact on the coral host.

CONCLUSION

This study provides the first characterization of bacterial associates for *Paragorgia arborea*, detailing the microbiome of this deep-sea cold-water coral during a visibly healthy state. While the bacterial communities of this species did not differ significantly in terms of diversity indices between compartments (mucus and tissue) or sampling location (canyon), there were significant compositional differences found among the bacterial assemblages from different compartments and sampling locations. Bacterial communities appeared more stable across colonies in *P. arborea* tissues than in mucus, and the relative abundance of the more common taxonomic groups tended to fluctuate across samples. In general, the bacterial microbiome of *P. arborea* was dominated by Tenericutes (orders Entomoplasmatales and Mycoplasmatales), with Spirochaetes, Gammaproteobacteria, and Alphaproteobacteria also making notable contributions to the bacterial assemblages. Bacteria from taxa known to contribute to nitrogen recycling and metabolism were identified as tissue and mucus biomarkers in *P. arborea*. Representatives of bacterial families previously found in higher abundance in diseased scleractinians (Vibrionaceae, Campylobacteraceae, Rhodobacteraceae, Flavobacteraceae, and Burkholderiaceae; Daniels et al., 2015) were present (but rare) in our coral samples. The work presented here provides baseline microbiome data for *P. arborea*, a common habitat-forming cold-water coral taxon. Additional research on deep-sea and cold-water coral health and susceptibility to stress is urgently needed for more informed conservation and marine policy planning.

AUTHOR CONTRIBUTIONS

BW, JV, and SD designed the experiments. JV collected the samples during the research cruise. BW and JV did the extractions and analyzed the data and prepared the figures. BW wrote the paper. JV and SD edited and reviewed drafts of the paper.

FUNDING

This project was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Ship Time Grant 501171-2017 (applicant: A. Metaxas; co-applicants P. Snelgrove, SD, and M. Kienast) and NSERC Discovery Grant RGPIN-2015-06548 to SD. The research cruise was part of a Northeast Regional Deep-Sea Coral Initiative, funded in part by NOAA National Marine Fisheries Service, NOAA Office of Marine and Aviation Operations, and NOAA's Deep-Sea Coral Research and Technology Program.

ACKNOWLEDGMENTS

The authors thank chief scientists A. Metaxas (Dalhousie University) and M. Nizinski (National Systematics Laboratory, National Museum of Natural History, Smithsonian Institution), the captain and crew of the NOAA Ship *Henry B. Bigelow*, and the CSSF-ROPOS team.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00378/full#supplementary-material

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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

ORIGINAL RESEARCH
published: 23 October 2018
doi: 10.3389/fmars.2018.00390

Changes in Radial Polyp Tissues of Acropora Longicyathus After Long-Term Exposure to Experimentally Elevated Nutrient Concentrations

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Coral tissues control growth and calcification processes that ultimately build coral reefs but relatively little information is available on the effects of nutrients on polyp tissues. The structure and organization of coral tissues were investigated using thin (0.5–1 μm) sections of young (<3 months) radial polyps of the reef-building coral *Acropora longicyathus* that had been exposed to elevated concentrations of ammonium ('N') and/or phosphate ('P') in the ENCORE experiment at One Tree Island, southern Great Barrier Reef. Young polyps of N-treated corals had similar porosity but significantly decreased length of calicoblastic body wall per cross-section of the septotheca compared with controls. Other studies using older skeletons of the same corals found they were significantly less porous than controls and their branches had reduced extension rates and reduced lesion-healing ability, indicating that increased calcification occurred in the infilling process rather than during apical extension. The free body wall epidermal tissues of P-treated polyps were significantly thicker than corals in control conditions and their calyx walls had significantly greater length of calicoblastic body wall per cross-section despite similar porosity to controls. This suggests that phosphate stimulated tissue growth and apical calcification. Although other studies of the chlorophyll content of older tissue found it was significantly increased by phosphate treatments, more rapid extension of the branches kept Symbiodiniacean densities in the younger polyps similar to controls. We recorded a reduction in the density of mucous bodies in P-treated corals, which is potentially significant for the survival of corals in polluted water because of the important roles of mucus in facilitating removal of sediment from the coral surface and as a barrier to pathogen infection.

Keywords: ammonium, phosphate, porosity, mucus, coral tissue growth, symbiodiniaceae

OPEN ACCESS

Edited by:
Michael Sweet,
University of Derby, United Kingdom

Reviewed by:
Susana Enríquez,
Universidad Nacional Autónoma de México, Mexico
Charles Alan Jacoby,
St. Johns River Water Management District, United States

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *30 May 2018*
Accepted: *04 October 2018*
Published: *23 October 2018*

Citation:
Bucher DJ and Harrison PL (2018) Changes in Radial Polyp Tissues of Acropora Longicyathus After Long-Term Exposure to Experimentally Elevated Nutrient Concentrations. Front. Mar. Sci. 5:390. doi: 10.3389/fmars.2018.00390

INTRODUCTION

The role of nutrification (elevated concentrations of inorganic nitrogen and phospate) in the demise of coral reef communities has been the subject of much research, particularly following sudden shifts from coral-dominated to algal-dominated communities were documented for reefs including Kaneohe Bay, Hawaii (Smith et al., 1981), and elsewhere (Pastorok and Bilyard, 1985;

Tomascik and Sander, 1987; Szmant, 2002). One mechanism by which nutrients were hypothesized to exert an effect was through direct negative impacts on coral physiology and health. However, while such impacts have been demonstrated in laboratory and field experiments, their magnitude is rarely sufficient to explain death or overgrowth of corals by other biota (Szmant, 2002).

One of the more ambitious experiments to manipulate nutrients in an intact coral reef system was the ENCORE project (Larkum and Steven, 1994). The project used 12 patch reefs in One Tree Reef Lagoon on the southern Great Barrier Reef. During 1995 concentrations of ammonium, phosphate, or a combination of both, were elevated by 10–20 times average lagoonal concentrations for the duration of each low tide. A number of studies of corals translocated into the ENCORE patch reefs have demonstrated significant impacts on reproduction and lipid levels (Ward, 1997; Ward and Harrison, 1997, 2000), photosynthetic processes (Takabayashi, 1996), growth and skeletal density (Hoegh-Guldberg et al., 1997; Bucher, 2000; Bucher and Harrison, 2000), and lesion-healing ability (Bucher, 2000) that support the hypothesis that nutrients may reduce coral resistance and/or resiliance to other stressors such as warm-water bleaching and physical damage. These results were subsequently questioned by Szmant (2002) who considered that the low abundance of acroporid corals in the micro-lagoons may have indicated that they did not provide a suitable natural habitat for these corals, and that nutrient effects on this genus may have been the result of some patch reefs being less suitable than others. However, the ENCORE design was replicated with three micro-lagoons for each treatment, and we had the opportunity to revisit the ENCORE reefs in November 2017 and noted that many of the transplanted colonies continue to thrive in the micro-lagoons some 20 years after the experiment. In particular, the smaller reefs to the western end of the lagoon are now almost impassable because of the high density of acroporid corals. We are therefore confident that the previous paucity of acroporid corals in these habitats was not due to poor environmental conditions. Moreover, the transplanted corals did not suffer from effects of crowding as the micro-lagoons have since supported a far higher density than was present during the experiment.

As a fast-growing, diverse taxon with a wide biogeographic distribution, the genus *Acropora* has been one of the groups on which nutrient studies have been focussed (Shantz and Burkepile, 2014). The gross anatomy of *Acropora* has been described by Constantz (1989), Veron (1986), and Wallace (1999). At the apex of each branch is a single apical polyp. Polyps that bud from the apical polyp, or which arise from the coenosarc, to line the sides of the branch, are termed radial polyps. The apical polyp of a rapidly extending branch, generally has very few microalgal dinoflagellates (family Symbiodiniaceae) in its tissues, is larger than the radial polyps around it, and its skeletal structure is extremely porous (Oliver, 1984; Fang et al., 1989).

The coelenteron of the axial polyp extends deep within the branch, occupying a canal along the central axis. The coelentera of the radial polyps also join the axial canal, and it has been suggested that excess photosynthate produced by the abundant symbiotic dinoflagellates in the radial polyps may be transported along the canal to the pale branch tip (Pearse and Muscatine, 1971; Oliver et al., 1983; Fang et al., 1989). Inorganic carbon produced by respiration of the deeper tissues may also be transported to the rapidly calcifying tip by ciliary currents, along with calcium ions. The unique anatomy of the *Acropora* colony therefore provides apical polyps with the supply of materials and energy they need for the rapid linear extension that is characteristic of the genus (Wallace, 1999).

The need for translocation of materials from deeper tissue implies that Symbiodiniaceae-free apical polyps probably require a critical mass of dinflagellate-rich tissue beneath them in order to maintain maximum extension rates. It therefore seems likely that a rapidly growing apical polyp may suppress initiation of new branches that would compete for translocated materials until there is a sufficient biomass of photosynthetic tissue to supply a second apical polyp. This may provide a simple model to explain the more frequent branching observed when colonies are transplanted into shallow water (e.g., Oliver et al., 1983), where the higher light levels may allow an apical polyp to be supplied by a smaller mass of dinflagellate-rich tissue. Supplies of molecules needed for organic growth could potentially have a similar effect. Suppression of branch initiation by the apical polyp would also explain the rapid initiation of new branches that occurs when an apical polyp is damaged. For this reason, we prefer the use of large transplanted fragments of colonies for growth experiments rather than juveniles, nubbins or branch tips that are commonly used (Takabayashi, 1996; Renegar and Riegl, 2005).

In 'bottlebrush' species such as *Acropora longicyathus,* there is a continuum of radial polyp shapes from sessile semicircular cups to elongate cylinders (**Figure 1**). The calices of all *Acropora* polyps are porous structures, consisting of skeletal elements that are parallel (septa) and perpendicular (synapticulae) to the polyp axis (**Figure 1**). Branches of the coelenteron fill the voids between these skeletal elements. For as long as they are in contact with the calicoblastic tissues of the coelenteron wall the skeletal elements will continue to thicken, presumably until the voids are no longer sufficiently interconnected to support the metabolic requirements of coral tissue. By this process, the basal portions

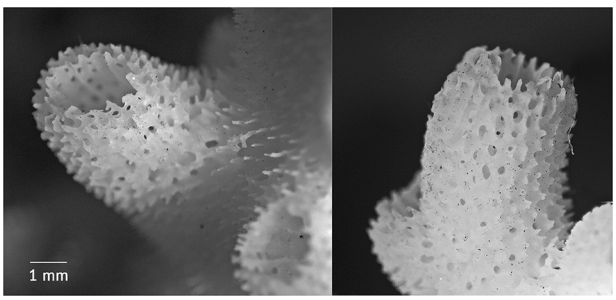

FIGURE 1 | Chemically bleached calices of individual radial polyps of *Acropora longicyathus*, showing the shape and the porous nature of the calyx walls (septotheca).

of a large *Acropora* skeleton may become the least porous of any coral skeleton (Hughes, 1987).

The general features of the endoderm (gastrodermis), mesogloea and ectoderm (epidermis and calicoblastic tissue) of corals have been described by Barnes (1973), and the finer structure of the tissues of *Acropora muricata* have been described by Harrison (1980). Processes in living corals that result in changes in the balance between calcification and skeletal extension (Bucher, 2000; Bucher and Harrison, 2000), or which alter the ability of the coral to grow and maintain itself may be investigated further by examining the tissues of the polyps. Of particular importance are tissues associated with the response to irritants (mucous cells in the free body wall), skeleton production (the calicoblastic body wall) and the energy supply of the polyp (Symbiodiniacea).

Many studies have used light microscopy or transmission electron microscopy to examine coral tissues for descriptive or taxonomic purposes (see review by Chapman, 1974), or to examine the process of skeletogenesis (Johnston, 1977, 1979; Le Tissier, 1991). Others have described cellular changes associated with bleaching (Glynn et al., 1985) or infection by pathogens (Peters, 1984; Glynn et al., 1989). Very few studies have used histological techniques to examine subtle sub-lethal changes in parameters such as cell densities (other than Symbiodiniacean cells) or tissue thicknesses in a manipulative experimental setting, although Renegar et al. (2008) examined tissue and cell ultrastructure around areas of lesion repair in *Montastrea cavernosa* and *Porites astreoides* and identified differences in Symbiodiniaceae distribution and condition in regenerating tissue exposed to elevated nutrients, but this gives little guide to impacts on undamaged tissue.

Harrison et al. (1990) examined changes in *Acropora muricata* tissues when exposed to oil and oil dispersants. They described the sequence of stress responses of coral tissue to a range of exposure times, ranging from excessive mucus secretion, through symbiotic dinoflagellate loss, to thinning and eventual disintegration of the tissues. In this study, we compare histological cross-sections of recently formed radial polyps of *A. longicyathus* grown under conditions of elevated ammonium, phosphate, and a combination of both nutrients, with those grown in ambient conditions during the high-dose phase of the ENCORE experiment (Larkum and Steven, 1994; Koop et al., 2001).

MATERIALS AND METHODS

The experimental design of ENCORE has been described in detail elsewhere (e.g., Larkum and Steven, 1994) and is only briefly outlined here. Automated nutrient dispensing units were used to add concentrated nutrient solution to micro-lagoons within patch reefs in the main lagoon of One Tree Reef, Great Barrier Reef (Lat. 23°30' Long. 152°06'). Three patch reefs remained unaltered as controls, three received ammonium ('N-only'), three received phosphate ('P-only'), and both nutrients were added to a further three patch reefs ('N + P'). Data presented here were collected during the 'high-dose' treatment period (see Koop et al., 2001), when nutrient concentrations were elevated to approximately 20 times background levels (20 μM NH_4^+, 4 μM PO_3^{2+}) three times every low tide between January 1995 and February 1996.

Sixty colonies of the branching reef coral *A. longicyathus* from around the main lagoon were used to provide sub-colonies for transplant into the micro-lagoons. Five colonies were randomly assigned to each patch reef of which three were used for histological study. Transplanted sub-colonies were supported on racks made from PVC floor tiles raised on short (~10 cm) lengths of PVC pipe. All data sets were analyzed with nested analyses of variance (ANOVA) and Tukey's honestly significant difference (HSD) multiple comparison of means. Results were initially analyzed using a two-way orthogonal model in which the treatments involving elevated ammonium ('+N': N-only and N + P) are compared with those with ambient ammonium ('-N': Controls and P-only), P-only and N + P ('+P') are compared with controls and N-only ('-P'), and the interaction between the two nutrient factors is assessed. Where there was a significant interaction between ammonium and phosphate treatments a model was applied in which each of the four treatments (Controls, N-only, P-only and N + P) were compared individually, with the emphasis placed on comparisons of elevated nutrient treatments with ambient controls. No significant interaction in the orthogonal model indicates that the effect of elevating one nutrient is independent of whether or not the other nutrient is also elevated and renders redundant the less-powerful linear model and *post-hoc* tests of multiple means.

Individual cylindrical radial polyps of *A. longicyathus* were removed with a knife blade from within 1.5–2 cm of the tip of the uppermost branches of each colony during June 1995. Linear extension rates (Bucher and Harrison, 2000) suggest that these polyps were no more than 5–6 months old, and had therefore been formed during the ENCORE high-dose nutrient treatment period (Koop et al., 2001).

All polyps were fixed immediately in cold 2.5% glutaraldehyde for 2–3 h in Millipore-filtered seawater, buffered with 0.1 M sodium cacodylate, and adjusted to a pH of 7.2 (after Harrison, 1980). After three rinses in the cacodylate-buffered seawater, the polyps were stored in the final rinse for transport. The polyps were post-fixed for 1 h in 1% OsO_4 in cacodylate-buffered seawater, rinsed three time in cacodylate-buffered seawater, and stored refrigerated in the final rinse solution. Prior to embedding, the polyps were decalcified in ascorbic acid according to the method of Dietrich and Fontaine (1975) and were embedded in Spurr's resin (Spurr, 1969) for sectioning. Thin (0.5–1.0 μm) sections were cut using glass knives on a Reichert OMU3 ultramicrotome. Sections were cut approximately 1 mm from the top of the calyx wall, perpendicular to the longitudinal axis. The sections were mounted on glass slides, stained with toluidine blue in borax (after Harrison, 1980) and photographed with a Panasonic CCTV digital video camera mounted on an Orion BM-LUX-2 compound microscope.

Digital images were captured using a 5 megapixal digital camera mounted on an Olympus compound microscope. The images were printed at a resolution of 144 dpi, giving a printed image size of 11 cm × 8.5 cm. All lengths and areas were

measured from the printed images using a Wacom ArtZ II 12" × 12" digitizing tablet. Scaling factors were obtained by photographing and digitizing 0.1 mm square haemocytometer grids at the same magnifications as the coral polyps. Images were obtained at three random locations on each of two polyps per colony. At each location three images were taken for analysis as shown in (**Figure 2**).

An image at x40 magnification enabled a cross-sectional view of a segment of the polyp from the outer body wall to near the inner margin of the mesenteries. The area of each image occupied by skeleton ($A_{skeleton}$) and the total area of calyx wall (A_{total}) in the image were digitized. Porosity of the calyx wall was then calculated as follows:

$$\text{Porosity} = 100^{*}(A_{total} - A_{skeleton})/A_{total}$$

On the x100 image, the number of Symbiodiniacean cells inside gastrodermal cells of the calicoblastic body wall were counted, and the length of calicoblastic body wall in the image was measured along the mesogloea.

At x400 magnification (**Figure 3**) the thickness of the epidermis and the total thickness of the outer body wall could be measured. The numbers of Symbiodiniacean cells in the gastrodermis of the free body wall, and the numbers of mucous bodies in the epidermis were counted and expressed as the number of mucus bodies per length of free body wall (measured along the mesogloea). We have followed the nomenclature of the symbiotic dinoflagellates recommended by LaJeunesse et al., 2018).

RESULTS

Tables 1, **2** summarize the means and results of analyses of variance for tissue variables measured from digital images of

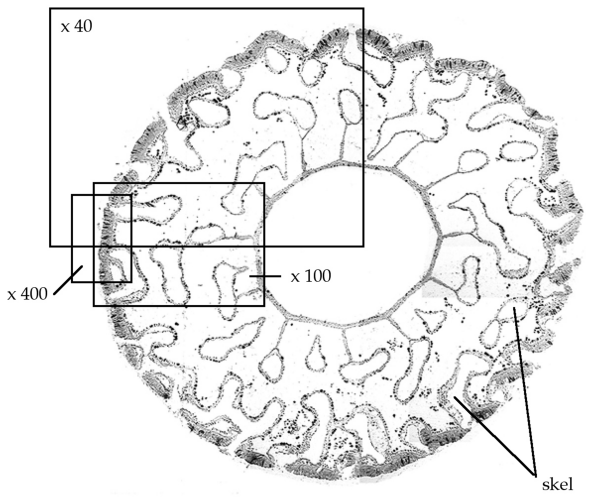

FIGURE 2 | Photo-mosaic cross-sections of typical polyps of *A. longicyathus* showing examples of image locations at x40, x100, and x400 magnifications. ***Skel***, areas of decalcified skeleton.

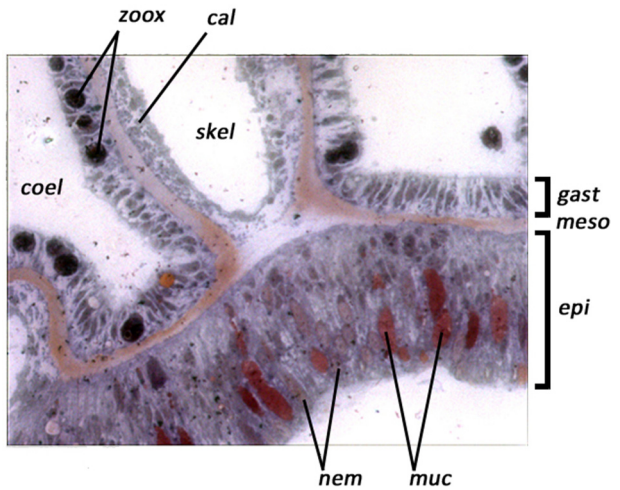

FIGURE 3 | Example of digital video images of a polyp cross section of *Acropora longicyathus* at x400 magnification, showing the tissue layers. Lining the coelenteron (***coel***) is the gastrodermis (***gast***) containing symbiotic dinoflagellates (***zoox***). The calicoblastic epithelium (***cal***) lines areas from which the skeleton has been decalcified (***skel***) and the epithelium (***epi***) of the free body wall contains mucous bodies (***muc***) and nematocysts (***nem***). Between the gastrodermis and epithelium lies the thin acellular mesogloea (***meso***).

A. longicyathus polyps. Three parameters produced significant treatment effects in the two-way orthogonal analyses of variance (**Table 2**). Elevated ammonium was associated with significantly increased Symbiodiniaceae densities in the calicoblastic body wall ('*CBW Zoox.*') and a significantly reduced length of calicoblastic body wall ('*CBW Length*'). Elevated phosphate concentrations were associated with significantly reduced densities of mucous bodies in the free body wall. Significant N × P interaction terms occurred in three analyses ('*Porosity*,' '*FBW Zoox.*' and '*FBW Width*') in which the responses to the P-only treatment produced highest values but the N + P treament produced values more similar to controls than any other nutrient treatment. Inter-reefal differences (within treatments) were detected in all parameters except '*FBW Width.*' The dispersion of treatments in different patch reefs spread across the lagoon reduced the likelihood of reef differences being responsible for observed treatment effects. Significant colony effects (within reef) were detected in '*Porosity*,' '*CBW Length*,' '*Mucous Bodies*,' and '*FBW Width.*' These significant effects were due to relatively small differences between colonies combined with low variation between polyps of the same colony.

Nested one-way analyses of variance and multiple comparisons of means (Tukey's HSD) showed that there were significant differences between treatments and control corals in porosity of the calyx wall ('*Porosity*'), the density of Symbiodiniaceae per unit length of calicoblastic body wall ('*CBW Zoox*'), width of the free body wall ('*FBW Width*' – due mostly to changes in ectodermal thickness), and the density of mucous bodies in the ectoderm of the free body wall ('*Mucous Bodies*'). P-only treated corals differed significantly from controls in regard to *Porosity* (increased relative to controls), *FBW Width*

TABLE 1 | Summary of tissue variables of *A. longicyathus* measured in images of cross-sections of polyps.

	Porosity (%)	CBW Length (mm)	CBW Zoox (mm^{-1})	FBW Zoox (mm^{-1})
-N (C, P)	71.2 ± 7.3 (99)	21.9 ± 4.5 (105)	18.7 ± 8.1 (104)	9.7 ± 11.6 (89)
+N (N, N+P)	70.3 ± 7.8 (104)	18.9 ± 4.5 (105)	22.9 ± 10.8 (105)	9.4 ± 13.3 (89)
-P(C, N)	70.6 ± 7.7 (107)	20.1 ± 4.8 (108)	21.1 ± 10.6 (108)	9.5 ± 14.2 (95)
+P (P, N+P)	70.9 ± 7.4 (96)	20.8 ± 4.6 (102)	20.5 ± 8.7 (101)	9.6 ± 10.3 (83)
Control	69.3 ± 7.3 (54)	21.7 ± 4.6 (54)	18.6 ± 8.3 (54)	7.3 ± 12.3 (49)
N-only (N)	71.3 ± 8.0 (53)	18.5 ± 4.6 (54)	23.6 ± 12.1 (54)	11.9 ± 15.7 (46)
P-only (P)	73.3 ± 6.9 (45)	22.1 ± 4.6 (51)	18.8 ± 7.9 (50)	12.7 ± 10.2 (40)
N + P	68.3 ± 7.3 (51)	19.4 ± 4.4 (51)	22.2 ± 9.2 (51)	6.7 ± 9.6 (43)
All treatments	67.4 ± 7.5 (203)	20.4 ± 4.7 (210)	20.8 ± 9.3 (209)	9.6 ± 12.5 (178)
	Mucous bodies (mm^{-1})	**FBW width (cm on image)**		
-N (C, P)	43.8 ± 22.5 (88)	8.4 ± 1.4 (90)		
+N (N, N+P)	44.2 ± 24.2 (88)	8.2 ± 0.9 (53)		
-P(C, N)	50.8 ± 23.9 (93)	8.0 ± 1.1 (68)		
+P (P, N+P)	36.4 ± 20.2 (83)	8.6 ± 1.3 (75)		
Control	50.5 ± 22.1 (47)	7.9 ± 1.2 (50)		
N-only (N)	51.0 ± 25.8 (46)	8.4 ± 0.8 (18)		
P-only (P)	36.0 ± 20.6 (41)	9.1 ± 1.4 (40)		
N + P	36.7 ± 20.0 (42)	8.1 ± 1.0 (35)		
All treatments	44.0 ± 23.3 (176)	8.4 ± 1.2 (143)		

*The number of replicates (n) varies between treatments as some cross-sections were damaged during collection, fixation or mounting, preventing accurate measurement of lengths or areas. '**Porosity**' is the % of total polyp area in an image at x40 magnification occupied by tissue or coelenteron. '**CBW Length**' is the length of calicoblastic body wall in each image at x100 magnification. '**CBW Zoox.**' is the density of symbiotic dinoflagellates per mm of calicoblastic body wall. '**FBW Zoox.**' is the density of symbiotic dinoflagellates per mm of free body wall. '**Mucous Bodies**' is the density of mucous bodies per mm of free body wall. '**FBW Width**' is the total width of the free body wall.*

TABLE 2 | Summary of analyses of variance for tissue variables of *A. longicyathus* measured in digital images of histological cross-sections of polyps collected in June 1995.

	Porosity (%)	CBW length (mm)	CBW zoox. (cells/mm)	FBW zoox. (cells/mm)	Mucous bodies (per mm of FBW)	FBW width (cm on image)
Orthogonal ANOVA model						
Main effects						
+N vs. -N	...	♦↓	♦↑	...	...	...
+P vs. -P	...	...	...	...	♦↓	...
Interactions						
NxP	♦	...	...	♦	...	♦
Nested terms						
Reef	♦	♦	♦	♦	♦	...
Colony	♦	♦	...	...	♦	♦
Polyp	...	...	...	...	...	...
Linear ANOVA model						
Highest	P] *	P]	N] *	P]	N]	P] *
	N]]	C]]	N + P]]	N]	C]	N]]
	C]	N + P]]	P]]	C]	N + P] *	N + P]
lowest	N + P]	N]	C]	N + P]	P] *	C]

*In the results of the orthogonal model indicates significant effects at $p < 0.05$ and arrows indicate the direction of the effects of elevated ammonium (+N) or phosphate (+P) compared with ambient concentrations (−N or −P). In the results of the linear ANOVA model, bars link treatment means that were not significantly different (Tukeys HSD test, $p < 0.05$) and * emphasizes treatments that were significantly different from controls.*

(increased relative to controls) and *Mucous Bodies* (decreased relative to controls). The N-only treatment differed significantly from controls in regard to '*CBW Zoox.*' (increased relative to controls) and had significantly lower '*CBW Length*' relative to P-only treated corals, but neither differed significantly from controls. The combined (N + P) treatment differed from controls only in regard to the reduced density of mucous bodies in the free body wall.

DISCUSSION

Although elevated phosphate produced a significantly higher porosity of the calyx wall than controls, there was a significant interaction with ammonium because when the two were elevated simultaneously the result was similar to controls. A similar pattern occurred with free body wall thickness. This pattern indicates that the ratio of nutrients may be more important than the absolute concentrations in determining the outcomes in these parameters. It has been demonstrated that excess nitrogen can cause symptoms of phosphate starvation (D'Angelo and Wiedenmann, 2014), which can be offset when phosphate is simultaneously elevated. A similar effect of elevated phosphate alone may be possible but a mechanism has yet to be demonstrated.

The significant increase in porosity of the calyx wall in phosphate treatments reported here is consistent with the reduction of skeletal bulk density reported by Bucher and Harrison (2000) for whole branches of the same colonies in elevated phosphate. In elevated ammonium treatments, a high annual rate of colony calcification and low rates of linear extension (Bucher and Harrison, 2000) in large fragments of the same colonies led to reduced porosity of branches, but in the young polyps used in the present study (those near the growing tip of the branches), this was not evident and calyx wall porosities were similar to controls. The difference in response to elevated ammonium between the two studies may be because there were seasonal fluctuations of ambient nitrogen in the lagoon causing differences in the effect of elevated nitrogen on colony calcification (Bucher and Harrison, 2000). The young polyps in this study may only show the effects of the rapid summer-autumn growth prior to sampling when calcification was similar between nitrogen-treated corals and controls (Bucher and Harrison, 2000).

Symbiodiniaceae densities in recently formed polyps did not correspond with the appearance and chlorophyll content of the older branches of the same *A. longicyathus* colonies (Bucher and Harrison, 2000), in which the P-only treatment produced significantly higher values and controls the lowest. Low density and high variability of Symbiodiniaceae in histological sections of the free body wall of *A. longicyathus* meant that no nutrient treatment produced densities that were significantly different from controls in this study. Unlike the polyps of *Acropora muricata* (Harrison, 1980), the Symbiodiniaceae of recently formed polyps in *A. longicyathus* were more numerous in the gastrodermis of the calicoblastic body wall than in the free body wall. Ammonium-treated corals had significantly higher densities of Symbiodiniaceae in the calicoblastic body wall. However, significant differences in skeletal geometry between treatments meant that in ammonium-treated corals there was less area of calicoblastic body wall per volume of calyx wall. This difference offset the higher density of Symbiodiniaceae and produced almost identical counts per cross-sectional area of the calyx wall in all treatments. While exposure to elevated inorganic nitrogen, resulting in phosphate starvation, can increase physiological symptoms of high-light and high-temperature stress in the coral-Symbiodiniaceae symbiont, which can potentially lead to increased susceptibility to bleaching and mortality (Wiedenmann et al., 2013; Higuchi et al., 2015), the effect would be further exacerbated by the nitrogen-exposed corals having less tissue to house the population of symbiotic algae.

There was a significant increase in total free body wall thickness in elevated phosphate treatments, suggesting a greater amount of tissue per surface area of the colony. In high-light, oligotrophic conditions Symbiodiniaceae produce excess organic carbon compounds that they cannot use for growth because of a shortage of either nitrogen or phosphorus (Dubinsky and Jokiel, 1994). These energy-rich compounds are translocated to the host coral to fuel respiration of the animal tissue. The more photosynthate available to the coral, the more tissue it can support. However, to make more tissue the coral needs compounds containing nitrogen and phosphate. The animal tissue cannot assimilate inorganic nutrients, so it must obtain them as organic compounds such as dissolved amino acids, or living or dead particulate matter (Anthony, 2000). In nutrient-enriched waters the symbiotic algae are apparently able to utilize a greater proportion of the photosynthate for their own growth and reproduction, leaving a smaller proportion to be translocated to the coral (Dubinsky and Jokiel, 1994). In some circumstances, the lower rate of translocation per algal cell can be compensated for by higher Symbiodiniacean densities (McGuire and Szmant, 1997), although this is not always the case (Scheufen et al., 2017). In the field conditions of this experiment, the higher rates of tissue production generally corresponded with higher Symbiodiniaceae densities.

If sufficient organic matter is available in the water column (as prey items, dissolved or particulate matter), then heterotrophic uptake may allow the coral to make use of any increased translocation of photosynthate for increased production of animal biomass (Muscatine et al., 1989). Growth of coral tissue could therefore increase when the concentration of the limiting nutrient is elevated, possibly after an initial depression of growth during the phase of rapid increase in the Symbiodiniaceae population. *Porites furcata* exposed to elevated nutrients from resident fish schools had significantly more tissue per unit surface area than colonies without fish schools (Muscatine et al., 1985). No significant differences in the ratio of tissue weight:skeletal weight were observed in *Stylophora pistillata* in the ENCORE project (Hoegh-Guldberg et al., 1997), although an examination of the three data sets presented in that paper suggests a possible increase in this ratio in the phosphate-only treatment over time, relative to the other three treatments. In the present study, increased tissue thickness of the free body wall in the phosphate treatment is a possible indication of greater tissue production. Ward (1997) demonstrated that the same colonies of *A. longicyathus* used in this experiment from the ENCORE phosphate treatments also had significantly higher concentrations of lipids than corals at ambient phosphate concentrations, suggesting that the phosphate-treated corals had higher energy reserves to support growth, metabolic activity and reproduction (Ward and Harrison, 2000). Increased tissue production requires construction of new skeletal structures to support the new tissue. Conversely, new skeletal elements require new tissue to cover them, whereas infilling of existing structures

reduces the space available for living tissue. Significantly higher skeletal linear extension and slightly faster growth in colony volume of fragments of the same coral colonies used in this study (Bucher, 2000; Bucher and Harrison, 2000), together with greater skeletal porosity in the phosphate treatment support the conclusion of faster coral tissue production.

The reduction of mucous bodies in phosphate-treated corals is a potentially important finding from the present study because it may indicate a mechanism for synergistic effects of sediment, pathogens and nutrients and may have resulted from either increased frequency of mucus release or decreased rates of production. One use of mucus is to slough away sediment which settles on the coral surface (Stafford-Smith, 1993; Humanes et al., 2017). A coral may be able to remove sediment when in nutrient-poor water or it may survive high nutrients in otherwise clear water. However, a coral may not be able to deal with a combination of sediment and nutrients if nutrients reduce the coral's ability to remove the sediment. Mucus production has been suggested as a means by which the coral releases carbon-rich organic matter that has been translocated by symbiotic dinoflagellates in excess of the respiratory needs of the coral (Crossland et al., 1980). If elevated phosphate allowed for a greater proportion of photosynthate to be directed toward growth of algal and coral biomass, it would leave less excess carbon to be secreted as mucus. Alternatively, the higher phosphate concentration may have acted as an irritant to the coral tissue, triggering mucus release more often than in controls. Either process would have resulted in a reduction of mucous bodies within the tissue. Mucus may also act as a barrier to pathogenic infection. Bruno et al. (2003) and Vega Thurber et al. (2014) have shown that corals in areas with artificially elevated nutrients had a higher incidence of disease than corals from control reef areas, although whether this was due to direct physiological impacts on the coral animal or alterations to the composition of the microbiome or a combination of both is not known.

The changes in the tissues of *A. longicyathus* revealed in this study indicate further mechanisms by which superficially healthy corals exposed to elevated ammonium or phosphate may be more sensitive to other environmental impacts. When nutrients remain the limiting factor in coral physiology, the ratio of nutrients may be more important than the absolute concentration for some symptoms of nutrient stress, but for others, such as reduction of mucous bodies in the presence of elevated phosphate or altered skeletal architecture in elevated nitrogen, the effects are independent of the presence of other nutrients.

AUTHOR CONTRIBUTIONS

PH conceived of the research idea and obtained initial funding. DB and PH equally shared in the experimental design and writing of this manuscript. DB conducted the primary field and laboratory work and statistical analyses, and PH contributed to field and laboratory work.

FUNDING

The field work for this study was funded by a grant from the Great Barrier Reef Marine Park Authority through the ENCORE program to PH, and several internal research grants from Southern Cross University to DB and PH.

ACKNOWLEDGMENTS

The two reviewers provided helpful comments that improved the final manuscript.

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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

ORIGINAL RESEARCH
published: 26 October 2018
doi: 10.3389/fmars.2018.00385

Effect of Coral-Algal Interactions on Early Life History Processes in *Pocillopora acuta* in a Highly Disturbed Coral Reef System

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OPEN ACCESS

Edited by:
Michael Sweet, University of Derby, United Kingdom

Reviewed by:
Deron Burkepile, University of California, Santa Barbara, United States
Craig W. Osenberg, University of Georgia, United States

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *31 May 2018*
Accepted: *01 October 2018*
Published: *26 October 2018*

Citation:
Leong RC, Marzinelli EM, Low J, Bauman AG, Lim EWX, Lim CY, Steinberg PD and Guest JR (2018) Effect of Coral-Algal Interactions on Early Life History Processes in Pocillopora acuta in a Highly Disturbed Coral Reef System. Front. Mar. Sci. 5:385. doi: 10.3389/fmars.2018.00385

Scleractinian corals are vulnerable to a range of environmental disturbances, but generally suffer the highest rates of mortality during early life-history stages, i.e., from larval settlement until a few months post-settlement. Variations in survival rates of corals during this period play a key role in structuring adult coral populations. Many coral reefs have experienced reductions in herbivory rates due to overfishing and consequent increases in macroalgae, however, the effect of increased coral-algal interactions may vary between coral life-history stages and among locations. Therefore understanding the relative importance of different drivers of mortality across early life-history stages, under a range of environmental conditions, is essential to effectively manage and restore coral reefs. To date, however, relatively few studies have (a) examined coral-algal interactions across several early life-history stages (i.e., from planulae to juvenile colonies) and (b) done so in highly disturbed reefs close to large urban centers. We investigated the effect of algal-coral-herbivore interactions on early life history stages in the coral *Pocillopora acuta* on coral reefs off mainland Singapore, a heavily disturbed "urbanized reef environment". Larval settlement rates were estimated in the presence of six macroalgal species *ex situ*. The effect of direct interaction with two macroalgal species on newly settled spat was examined *in situ* and the effect of reduced herbivory was tested with exclusion cages on naturally settled 9-month-old juveniles *in situ*. We found significant reductions in *P. acuta* settlement in the presence of four macroalgal species. Newly settled spat of *P. acuta* had significantly lower survivorship when in contact with *Sargassum* sp. on the reef crest but not with *Bryopsis* sp. on the reef flat. Herbivore exclusion reduced survivorship of juvenile corals, which was associated with increased sediment accumulation, but not with algal biomass. Our results suggest coral recruitment on heavily disturbed reefs can be impacted by species-specific macroalgal

effects via reduced settlement on ephemeral substrata and reduced survivorship when in direct contact with *Sargassum* sp. Furthermore, recruitment may be negatively impacted by reductions in herbivory, possibly via increased abundance of epilithic algal matrix leading to sediment trapping.

Keywords: coral recruitment, settlement, post-settlement mortality, sedimentation, urban reefs, *Pocillopora acuta*

INTRODUCTION

The world's coral reefs are under sustained pressure from multiple human impacts such as climate change, overfishing and coastal land-use changes (Hughes, 1994). In response to these anthropogenic stressors, coral cover, and structural complexity have declined dramatically on many reefs in recent decades (De'ath et al., 2012; Hughes et al., 2018). Relatively isolated reefs can recover rapidly following natural disturbances if ecological processes such as coral recruitment and herbivory have not been compromised by previous human disturbances (e.g., Gilmour et al., 2013). Much less is known about the recovery potential of corals subjected to chronic disturbances found on inshore, highly urbanized coral reefs (Cleary et al., 2014; Guest et al., 2016a) such as turbidity, sedimentation, and pollution (Fabricius, 2005).

Recruitment is a key ecological process in the recovery and persistence of coral reef communities. The early life history of reef-building corals involves a series of critical stages, all of which need to be successful for recruitment to occur (Vermeij and Sandin, 2008; Ritson-Williams et al., 2009; Doropoulos et al., 2016). These stages include a pelagic larval phase; a benthic "searching" phase, for larvae to identify suitable substratum for settlement and metamorphosis; and a period of somatic growth and sexual maturation (Babcock and Mundy, 1996; Heyward and Negri, 1999; Raimondi and Morse, 2000). Survivorship of early life history stages for corals has been likened to "running the gauntlet" (Arnold et al., 2010) because coral juveniles are vulnerable to high mortality rates (i.e., type III survivorship, *sensu* Deevey, 1947) during this period. Newly settled and juvenile corals are also more vulnerable to removal, overgrowth, sediment burial, shading and abrasion compared to adults due to their smaller size and lack of height (Zilberberg and Edmunds, 2001; Raymundo and Maypa, 2004; Birrell et al., 2005; Box and Mumby, 2007; Tebben et al., 2014). Thus, environmental perturbations may have a greater effect on the survival of juvenile corals compared to their adult counterparts and hence may strongly influence the structuring of adult coral populations.

Interactions with macroalgae can be particularly damaging to corals during the early life history stages. Reductions in successful settlement can occur due to macroalgae occupying potential settlement space (Olsen et al., 2015), direct inhibition of settlement via allelochemicals (Paul et al., 2011; Dixson et al., 2014), reductions in settlement surface due to increased sediment trapping by algae (Birrell et al., 2005), reductions in light availability (Strader et al., 2015), and direct physical abrasion of newly settled and juvenile corals (Box and Mumby, 2007; Venera-Ponton et al., 2011).

The extent of macroalgal herbivory on a reef can also govern coral recruitment success (Hughes et al., 2007). When herbivory is markedly reduced by overfishing, reef resilience is reduced and there is a greater chance of a reef undergoing a shift towards macroalgal dominance following acute disturbances (Hughes, 1994; Edwards et al., 2014). Paradoxically, fish herbivory may also contribute to early coral mortality via accidental removal of newly settled corals and coral juveniles (Brock, 1979; Baria et al., 2010; Venera-Ponton et al., 2011). Therefore, understanding the effects of macroalgae on recruitment success requires consideration of other ecological processes such as fish herbivory.

Tropical coastal zones are experiencing unprecedented rates of change, driven by human population growth and marked coastal development (Dsikowitzky et al., 2016). As a result, an increasing number of coral reefs will experience the effects of "urbanization" in the Anthropocene. Urban coral reefs are considered a distinctive group of marginal reefs characterized by extremely low water quality, high abundance of macroalgae, low herbivory and depth restricted coral communities composed of stress tolerant taxa (reviewed by Heery et al., 2018). Despite the extreme abiotic conditions, coral communities on some urbanized reefs can actually be quite resilient to acute disturbances (e.g., Guest et al., 2012, 2016a), suggesting that these reefs may have significant ecological and conservation value. Unfortunately, there are still relatively few studies examining coral-macroalgal-herbivore interactions from highly disturbed inshore-reefs, with studies limited to Kenya (Mwachireya et al., 2017) and India (Manikandan and Ravindran, 2017). Our lack of knowledge of key ecological processes such as recruitment and herbivory on these important reefs hinders our ability to effectively manage them.

In this study, we examined the effect of macroalgae and herbivory on *Pocillopora acuta*'s likelihood of successfully recruiting in Singapore reefs, a highly disturbed coral reef system. To achieve this, we (1) compared coral larval settlement rates in the presence of several macroalgal species, (2) examined the effect of direct interaction between macroalgae and newly settled spat, and (3) investigated the effect of reduced herbivory on juvenile coral growth and survival.

MATERIALS AND METHODS

Study Site and Species

Singapore's coral reefs have experienced multiple human disturbances over the last 200 years (Corlett, 1992), and are currently subject to high rates of sedimentation, eutrophication and other stressors (Todd et al., 2004; Van Maren et al., 2014).

Despite being severely disturbed, diverse coral communities (>250 coral species) still persist on the fringing reefs surrounding Singapore's southern islands (Huang et al., 2009). Hard corals are largely restricted to ∼8 m depth due to very high light attenuation and communities are mostly composed of stress tolerant and generalist taxa (Browne et al., 2015; Guest et al., 2016a). The mean cover of coral in Singapore at 3–4 m and at 6–7 m depth is ∼36 and ∼21%, respectively (Guest et al., 2016a). Meanwhile, macroalgae, particularly *Sargassum* spp., is highly abundant on shallow reef flats (0–2 m depth) (Low, 2015) but lower in cover ∼10% and ∼3% at 3–4 m and 6–7 m depths, respectively (Guest et al., 2016a). Singapore reefs also generally exhibit relatively low herbivory rates; herbivore biomass on reefs is dominated by browsers and small non-obligate herbivores with a very low abundance of scrapers and excavators (Guest et al., 2016b; Bauman et al., 2017). The sites used in the present study were two fringing reefs adjacent to Kusu Island (1°13′26″N, 103°51′39″E) and Sister's Island (1°12′55.4″N 103°50′09.3″E). The brooding species *P. acuta* was chosen as the study species due to its relatively high abundance at the study sites (Huang et al., 2009; Poquita-Du et al., 2017).

Algal-Coral Interactions

The Effects of Macroalgae on Settlement of Coral Larvae

Ten *P. acuta* colonies (15–20 cm diameter) were collected from the fringing reef of Kusu Island 2–3 days before the new moon in May and June 2014. Colonies were kept in flow-through, artificially lit indoor, seawater tanks. Larvae released in tanks were collected through outflow tubes fitted with 800 μm mesh nets each morning. Larvae were pooled together, cleaned with 0.2 μm filtered seawater (FSW), and kept in aerated tanks prior to experiments. Six locally abundant macroalgal species (Lee et al., 2009; Goh and Lim, 2015) were collected from Kusu and maintained in aquaria 2–3 days prior to experiments. The algal species collected were *Bryopsis pennata* var. *secunda*, *Hypnea pannosa*, *Halymenia durvillei*, *Lobophora variegata*, *Sargassum siliquosum* and *Padina* sp., (the latter could not be reliably identified to species; refer to **Supplementary Data 1B** for a detailed description on algal species identification).

To test for effects of algae on coral settlement, larval settlement rates in the presence and absence of each algal species was estimated. We used 15 replicate sterile petri dishes per species (90 mm × 11 mm). Each replicate contained 30 ml of 0.2 μm filtered seawater (FSW), a 2 cm × 2 cm × 0.5 cm conditioned ceramic tile to induce planula settlement (Heyward and Negri, 1999), 15 coral planulae and 0.5 ± 0.1 g of wet algal mass following Vermeij et al. (2009). The control treatment replicates contained conditioned tiles but no macroalgae. Ceramic tiles were biologically conditioned for several weeks in a flow-through seawater tank at Tropical Marine Science Institute at St. John's Island prior to the study. Tiles contained at least 50% cover of crustose coralline algae (CCA) of a multi species assemblage. CCA derived from these tanks have been used in several previous coral larval experiments and are therefore known to be settlement inducers (e.g., Tebben et al., 2014, 2015). In all treatment replicates, the tiles and macroalgae pieces were positioned haphazardly in the petri dishes but no more than 1 cm apart *in situ* (Vermeij et al., 2011). Replicates were covered with transparent covers to prevent evaporation and drying out of CCA, planulae, and macroalgae, but not fully sealed to allow gaseous exchange and prevent hypoxia for both macroalgae and larvae. Algal mimics were not included in the experiment as the aim of the study was to test for changes in settlement rates in the presence of macroalgae, and not to determine the mechanisms driving these changes (e.g., physical vs. allelochemical or microbial).

Replicates were placed into 29°C-water baths, with temperatures comparable to mean ambient outdoor sea surface temperature (28–31°C) and left for 24 h. The proportions of successfully settled spat were quantified under a dissecting microscope. Successful settlement is defined here as settlement and metamorphosis of a spat on any substrate (Babcock et al., 2003). Settlement proportions were measured in two categories; (1) on all surfaces (either on the tile, petri dish surface or algae) and (2) on surfaces excluding algal surfaces. We excluded settlement counts on algal surfaces in (2) in order to discriminate between absolute settlement rates on all surfaces and settlement on unsuitable and ephemeral substrata such as algal surfaces *sensu* Nugues and Szmant (2006).

We performed a one-way ANOVA to test for differences in the proportion of larval settlement among algal treatments. Separate ANOVAs were performed for settlement proportions in (1) and (2), followed by respective Tukey *post hoc* tests with a Bonferroni correction for multiple testing. Datasets were not transformed as they met homoscedasticity and normality assumptions prior to ANOVA. Analyses were performed with the R statistical software (R Core Team, 2014; version, 3.1.1).

Effects of Macroalgae on Early Post-settlement Mortality of Corals *in situ*

Sixty unglazed terracotta tiles (10 cm × 10 cm × 1 cm), each fabricated with a 1 cm-diameter hole in the center, were preconditioned in tanks fitted with flow-through of 0.2 μm filtered FSW for 3 months to develop biofilms that facilitate coral larval settlement (Harrison and Wallace, 1990). Conditioned tiles, only containing biofilm were placed at the bottom of settlement trays in 0.2 μm filtered FSW. Collected larvae were added to the trays and allowed to settle on the upper surface of the tiles for 24 h. The numbers of settled and living spat on each tile were counted under a dissecting microscope and only tiles containing between 8 and 65 non-fused settled spat were used for the study. A total of 38 tiles were labeled and placed in flow-through tanks 3 days before they were deployed on the reef.

Individuals of *Sargassum* sp. and *Bryopsis* sp. were used for this experiment as these algae are locally abundant and have been shown to reduce post-settlement survivorship in previous studies (Lee et al., 2012; Goh and Lim, 2015). The study was conducted on the fringing reef crest and flat of Sister's Island where *Sargassum* sp. and *Bryopsis* sp. were relatively abundant on the reef crest and flat, respectively (≥10 individuals per site). *Sargassum* sp. was uncommon on the reef flat whereas *Bryopsis* sp. were largely absent on the reef crest, preventing us

from carrying out a transplant or an algal exclusion experiment involving both species in both habitats. Ten *Sargassum* sp. individuals, nine *Bryopsis* sp. individuals and their equivalent number of control plots (i.e., plots of absent of algal individuals) were selected and tagged. Tiles containing settled spats were assigned to either algal or control patches and carefully attached to the reef substratum with stainless steel nails and bolts. For the algal treatment, the attached tiles were placed within 5 cm of individual alga to maximize physical contact. In each reef habitat, all control and treatment tiles were placed within a meter from each other, placed at the same depth, and on similar substrate composition to account for similarity in environmental conditions (e.g., light irradiance and wave exposure) and to minimize the possibility of micro-site differences. Treatments in each habitat (i.e., four levels in total) had a comparable number of recruits per tile at the start of the study (ANOVA, $F_{3,34} = 0.792$, $p = 0.508$).

Tiles were left for 3 weeks and photographed at the start, and at the end of the study. Tiles were subsequently removed, placed into individual, sealable plastic bags. All live spat were counted within 2 h of retrieval. The number of surviving spat was counted for each tile by the end of the study. Wet sediment on each tile was brushed off into re-sealable plastic bags, decanted using 45 μm pre-weighed filter sheets, dried (at 100°C) and weighed to obtain dry sediment mass. The fouling community cover on each tile was quantified from photographs using Coral Point Count with Excel extensions [CPCe] (2006) software (version 3.2) using a random-25 point grid. The benthic categories quantified on each tile included: epilithic algal matrix (EAM), CCA, macroalgae, bare space (absence of fouling organisms) and other living organisms (e.g., bryozoans, keelworms, sponges, etc.).

Generalized linear models were used to measure effect of macroalgae i.e., "treatment type" [control vs. macroalgae presence (*Sargassum* sp. and *Bryopsis* sp.)] on the survivorship of the newly settled spats. The "habitat type" (crest vs. flat), and its interaction with "treatment type" were included in the model to account for habitat effects. *Post hoc* Tukey analyses were carried out for the significant categorical covariates. A separate, generalized linear model was also used to compare survivorship of spat across continuous covariates (i.e., dry sediment biomass, dry algal biomass and benthic cover percentages). This was due to the presence of collinearity between several categorical and continuous variables (e.g., turf cover was significantly higher in the *Sargassum* sp. compared to *Bryopsis* sp. treatment; two sample *T*-test; $t = 7.707$, $df = 36$, $p < 0.001$). The response variable in the models, survivorship of spat, assumed a binomial distribution with a logit link function (i.e., "live" vs. "dead" for each spat). Initial spat count density was found to be positively associated to density-dependent survivorship (*sensu* Vermeij et al., 2009; Doropoulos et al., 2017) (see **Supplementary Data 2A,C**) and hence were included into both models as a continuous predictor variable. Sediment dry mass data were log (x + 1) transformed while all benthic cover percentages were square-root transformed prior to the analysis to reduce skewness. All continuous covariates were normalized across their respective means and standard deviations prior to analyses. Prior to all analyses, we performed data exploration as suggested by Zuur et al. (2009) and model validation using plots of residuals vs. fitted values as per Zuur and Ieno (2016). Generalized linear models (functions "generalized linear model" followed by "Anova" from "car" package; Fox and Sanford, 2011) and Tukey analyses ("glht" function from "multcomp" package; Hothorn et al., 2008) were performed with R (version, 3.1.1) (R Core Team, 2014).

The Effect of *in situ* Herbivore Exclusion on Survival of Juvenile Corals

For the exclusion experiment, 120 fabricated terracotta tiles, with identical specifications to tiles used in the experiment above (Section B), were deployed at ∼1 m intervals along the reef crest at Kusu Island at 3–4 m depth in November 2012. After 7 months, 41 tiles, each containing 1–3 naturally settled live *P. acuta* juveniles on the upper surface were located *in situ* with the aid of a Nightsea FL-1 fluorescent dive light, and sides of each tile were tagged. The geometric mean diameter (GMD) of each coral was measured with Vernier calipers and locations of each coral on tiles were mapped. Tiles were randomly divided into three treatments: full cage ($n = 13$ tiles), partial cage ($n = 13$ tiles) and no cage ($n = 15$ tiles) with comparable mean GMDs across all treatments (controls = 6.0 ± 2.7 mm, partial cages = 6.4 ± 3.7 mm, full cages = 6.6 ± 3.1 mm; ANOVA, $F_{2,54} = 0.204$, $p = 0.816$).

Full cages were constructed of stainless steel wire mesh (1.2 mm thickness and 10 mm mesh size) with each side measuring 30 cm. Partial cages had the same dimensions but consisted of two open sides and a closed top. Partial cages acted as procedural controls to physical artifacts of the full cage treatments (e.g., potential shading and reduced water flow). All cages were attached to the substrate surrounding individual tiles, monitored at intervals of 1, 3, 6, and 8 weeks and cleaned after monitoring to avoid excessive fouling. Damaged cages were immediately replaced with new cages. Newly settled corals were recorded but not monitored. Fouling EAM and macroalgae on each tile were not disturbed throughout the experimental period. Differences in incident light intensity (lux) across treatments were measured during monitoring surveys, with Hobo Pendant loggers and were not statistically significant between treatments (10 randomly sampled plots per treatment (2–3 per treatment per session), 1 h per treatment, approximately 1200 to 1300 h; ANOVA, $F_{2,38} = 0.826$, $p = 0.445$).

Fish bite rates on partially caged tiles and controls were measured using underwater video cameras (GoPro Hero 3) mounted on aluminum angle iron bars ∼20 cm away from each tile. One partial and one control tile were randomly chosen for monitoring each time for approximately 2 h between 1200 and 1400 which resulted in 21.7 and 20.6 h of footage, respectively. Each fish bite on tiles were counted and bite rates were calculated as bites h^{-1}. Where possible, fish were identified to species.

At the end of the experiment, sediments on each tile were brushed off into re-sealable plastic bags, decanted using 45 μm filter sheets, dried (at 100°C) and weighed. To estimate dry algal biomass, all algae (EAM and macroalgae) on each tile were

scraped off and dried at 65°C. Photographs of tiles were taken at the start (week 0), middle (week 3) and end (week 8) of the experiment and were used to quantify change in benthic community cover with CPCe (version 3.2) using the same calculation method for benthic groups from Section B.

Generalized linear models were used to examine the effects of caging treatments on the survivorship of juvenile corals. Initial juvenile count per tile did not contribute to density-dependent mortality (Vermeij et al., 2009; Doropoulos et al., 2017) (see **Supplementary Data 3A**) therefore was excluded in the models. *Post hoc* Tukey test was used to compare the 8-week survivorships amongst all caging treatments. Survivorship of the corals was also modeled as a function of all continuous covariates using a generalized linear model. Dry sediment and algal mass data were log (x + 1) transformed prior to the model analyses. Similar to Section B's model analyses, data exploration and model validation *sensu* Zuur et al. (2009) and Zuur and Ieno (2016) were also carried out. Bite rates were found to be heteroskedastic and non-normal, even after the transformation. Therefore, the non-parametric Mann–Whitney *U*-test was used to compare differences in bite rates between control and partially caged tiles throughout the experiment duration. Changes in each benthic category cover were also compared across treatments with ANOVA tests with additional Tukey tests when necessary. ANOVAs, generalized linear models, and Tukey tests were carried out with the same R software and packages as listed in Section B. Additional information on all analyses for the caging experiment can be found in **Supplementary Data 3**.

RESULTS

The Effects of Macroalgae on Settlement of Coral Larvae

Settlement of *P. acuta* larvae differed significantly among algal treatments on all surfaces (ANOVA, $F_{6,105} = 3.656$, $p < 0.001$) and on surfaces excluding settlement on macroalgae (ANOVA, $F_{6,105} = 5.646$, $p < 0.001$). Excluding direct settlement on macroalgae, settlement was significantly higher in the control treatment (77.7 ± SE 4.1%) compared to larvae exposed to *B. pennata* (47.7 ± SE 6.5%), *L. variegata* (45.4 ± SE 3.6%), *Padina* sp. (45.0 ± SE 5.1%) and *S. siliquosum* (48.4 ± SE 7.0%) (*post hoc* Tukey HSD tests, $p < 0.05$, see **Figure 1** and **Supplementary Data 1A**). With the inclusion of settlement on macroalgae, settlement on *Sargassum* sp. increased by 25.4 to 73.7 ± SE 5.1% and was comparable to controls. The total settlement for *B. pennata*, *L. variegata,* and *Padina* sp. was still significantly lower than on controls even with the addition of larvae settling directly on macroalgae.

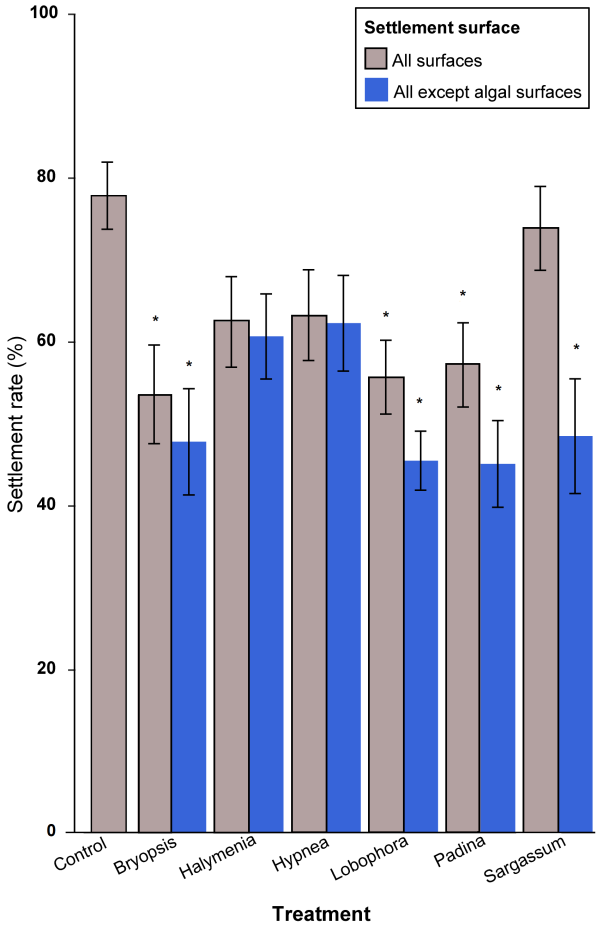

FIGURE 1 | Mean percentage larval settlement across all treatment groups for all algae substrate treatments. All macroalgae species labels in this figure are simplified to their respective genus names. The symbol * denotes significant differences in settlement percentages in comparison to the control treatment (*post hoc* Tukey HSD; *p*-value < 0.05) in all surfaces (gray bars) and settlement excluding leaf blade surfaces (blue bars), respectively. Error bars represent standard errors (±SE).

Effects of Macroalgae on Early Post-settlement Mortality of Corals *in situ*

There was a significant interaction between habitat types and treatments (LRT $\chi^2 = 10.318$, $df = 1$, $p < 0.001$, **Supplementary Data 2A-ii**). *Post hoc* Tukey tests (**Figure 2** and **Supplementary Data 2B**) revealed differences in mortality rates between spat interacting with *Sargassum* sp. (20.0 ± SE 6.7%) and spat in control treatments on the reef crest (50.0 ± SE 8.6%). Survivorship of spat was not significantly different between *Bryopsis* sp. (14.3 ± SE 5.6%) and control treatments on the reef flat (16.6 ± SE 3.9%). There were significant differences in survival rates between habitats for control treatments (i.e., reef crest vs. flat; 50.0 ± SE 8.6% vs. 16.6 ± SE 3.9%; $p < 0.001$) but not for algal treatments (20.0 ± SE 6.7% at reef crest vs. 14.3 ± SE 5.6% at reef flat; $p = 0.988$). Among continuous variables, only CCA (slope = 0.304, $p < 0.001$) and other living organisms covers were positively correlated with survivorship of coral spat (slope = 0.224, $p = 0.002$). There was no significant relationship between EAM cover and survivorship of spat (slope = −0.165, $p = 0.215$) (see **Supplementary Data 2C** for generalized linear model analysis with continuous variables).

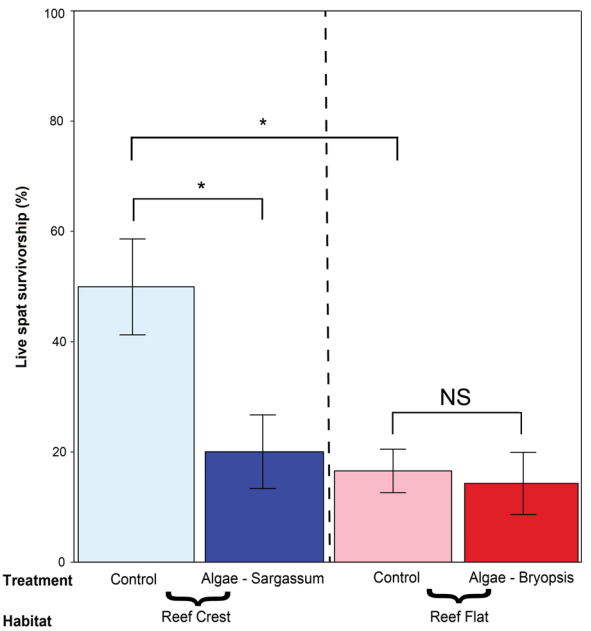

FIGURE 2 | Mean percentage of survivorship of spat across habitat types (Reef Crest vs. Rest Flat) and algal treatments (*Bryposis* sp. or *Sargassum* sp. vs. control). Error bars represent standard errors (±SE). The symbol '*' and NS denote significant and non-significant differences, respectively, across pair-wise, *post hoc* Tukey comparison of variable levels at Bonferroni corrected ($\alpha = 0.05/4$).

The Effect of *in situ* Herbivore Exclusion on Survival of Juvenile Corals

Caging had a significant effect on juvenile coral survivorship (LRT $\chi^2 = 6.690$, $df = 1$, $p = 0.031$) with significantly lower survivorship in the full cages (*F*; 30.8%) compared to the uncaged controls (C; 80.0%) (*Post hoc* Tukey HSD; F ≠ C, Z = −2.396, $p = 0.044$, **Supplementary Data 3B** and **Figure 3**). There were no differences in survivorship between partially caged plots (*P*; 65.4%) and uncaged controls (P = C, $p = 0.709$), or between caged and partial caged plots (F = P, $p = 0.185$) (**Figure 3**). Juvenile corals were not affected by density dependence through the initial spat number ($Z = -1.018$, $p = 0.309$, **Supplementary Data 3A**). Fish bite rates, however, were significantly higher on control tiles in comparison to partial caged tiles (4.6 ± SE 5.1 vs. 0.5 ± SE 0.7 bites h^{-1}; Mann–Whitney-*U*-test, $W = 117$, $p = 0.031$, **Figure 4B**). The fish grazers observed consisted of three species: *Halichoeres* sp., an invertivorous wrasse, was responsible for 90% of total bites (91 bites); *Pomancentrus littoralis*, a herbivorous damselfish, which took 9% of total bites (9 bites); and *Chaetodontoplus mesoleucus*, an omnivorous angelfish, which took 1% (1 bite) of the total bites.

Among the continuous covariates, there was an association between juvenile coral mortality and dry sediment mass (slope = −1.183, $p = 0.030$, **Figure 4A** and **Supplementary Data 3C**), but no association with algal biomass (slope = −0.079, $p = 0.876$), EAM (slope = −0.348, $p = 0.471$), macroalgal cover (slope = −0.611, $p = 0.264$) or change in cover of other living organisms (slope = −0.218, $p = 0.606$). There were no significant differences across the caging treatments for either dry sediment mass (ANOVA, $F_{2,38} = 0.564$, $p = 0.573$) or dry algal biomass (ANOVA, $F_{2,38} = 1.479$, $p = 0.241$). There were also no significant differences in change of benthic cover across all treatments and benthic categories (refer to analyses and results in **Supplementary Data 3C**).

DISCUSSION

With increasing human impact through the Anthropocene era, more coral reefs will be subjected to the types of disturbances found in Singapore and in other urbanized reefs (Heery et al., 2018). It is therefore essential to better understand the factors affecting coral recruitment success in these systems across multiple life history phases to mitigate reef degradation and manage restoration. Our study demonstrates that a combination of direct settlement inhibition and increased availability of unsuitable settlement substratum may contribute to significant reductions in successful coral settlement in the presence of macroalgae. Survival rates of newly settled coral spat were significantly lower when in physical contact with *Sargassum* sp. on the reef crest, but not with *Bryopsis* sp. on the reef flat, although overall mortality rates were higher on the reef flat. Exclusion of grazers led to higher mortality rates of juvenile corals *in situ* despite relatively low numbers of fish bite recorded, suggesting that fish grazing may still play an important role in coral recruitment success even in areas with relatively low biomass of herbivores (Guest et al., 2016b; Bauman et al., 2017). Our study, therefore, implies the need to monitor changes in the abundance of macroalgae in Singapore's reefs and other urbanized reefs, and potentially place mitigation measures to control the proliferation of certain taxa (e.g., *Sargassum*).

Lower larval settlement in the presence of macroalgae corroborate previous studies showing that interaction with macroalgae can inhibit settlement (Kuffner et al., 2006; Vermeij et al., 2009; Paul et al., 2011; Dixson et al., 2014). *Bryopsis*, *Lobophora*, *Padina,* and *Sargassum* spp. have all been shown to impact settlement and metamorphosis of larvae of several coral species including *Isopora palifera*, *Stylophora pistillata* (Baird and Morse, 2004), *Porites astreoides* (Kuffner et al., 2006), *Platygyra daedalea* (Diaz-Pulido et al., 2010), *Pocillopora damicornis* (Maypa and Raymundo, 2004), and *P. acuta* (Lee et al., 2012) to varying degrees either in the field or *ex situ*. Nevertheless, in these studies, direct settlement on algal fronds was either not quantified or was negligible. Therefore, macroalgae such as *Sargassum* may have two types of effects on coral recruitment: firstly, they reduce the probability of settlement; and secondly, they can serve as a substrate for settlement. However, given the fact that algae is not a stable substratum, the settled coral is unlikely to survive to adulthood (Nugues and Szmant, 2006; Olsen et al., 2016). Therefore, even if overall settlement rates are not reduced, recruitment may be negatively impacted. Clearly, further studies are needed to confirm the extent that corals settle directly on *Sargassum* sp. by quantifying *in situ*, natural rates of settlement and post-settlement survival of corals on algal fronds.

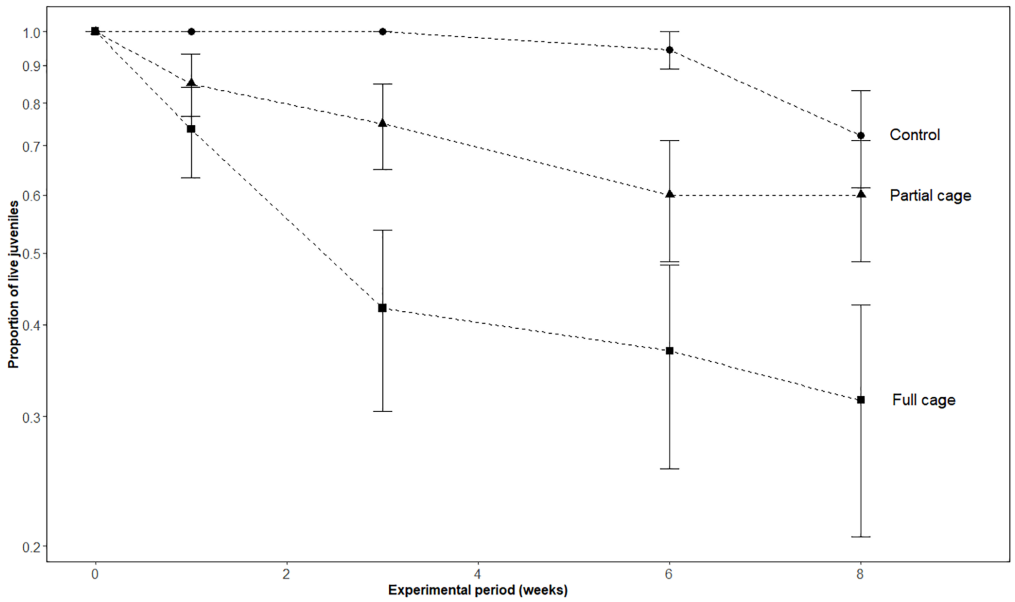

FIGURE 3 | Proportion of live coral juveniles across caging treatments during the experimental period. Points and error bars correspond to mean proportion and standard errors (± SE), respectively, at each monitoring period.

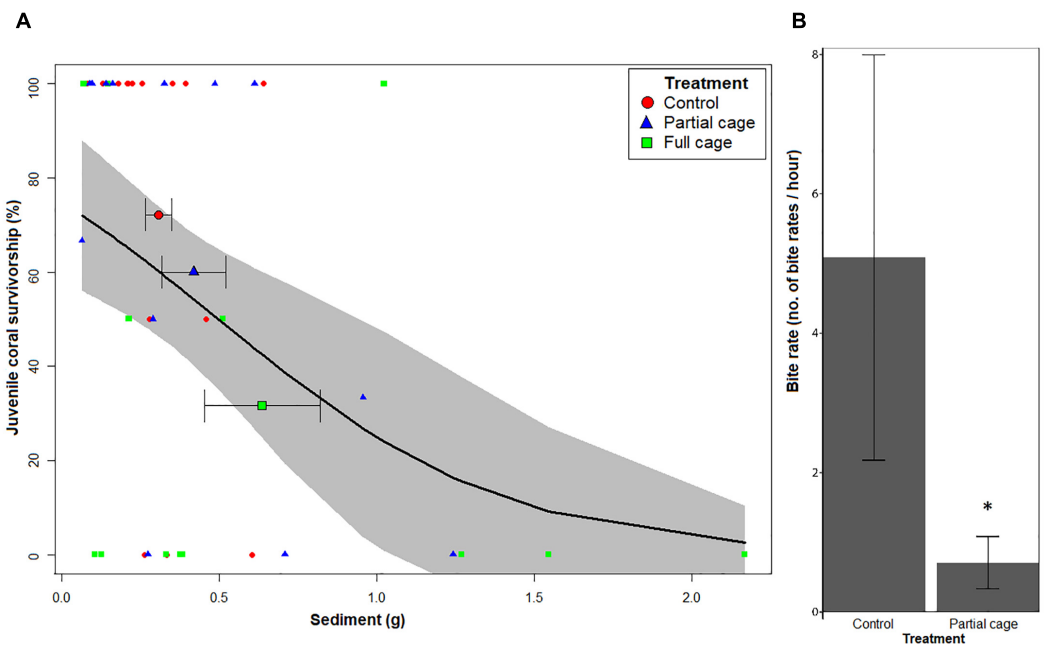

FIGURE 4 | (A) Survivorship of juvenile corals against dry sediment mass in the fitted generalized linear model. Bold line and shaded region represent the fitted generalized linear model and its 95% confidence interval (CI). Red circles, blue triangles and green squares represent the raw sediment mass and coral survivorship on each tile according to control, partial cage and full cage treatments, respectively. Enlarged bold points for each treatment represent the mean survivorship treatment and sediment mass. Horizontal error bars represent standard errors (± SE) for corresponding mean sediment mass. The symbol '*' denotes variable showing a significant association to coral survivorship at $\alpha = 0.05$ in the model. **(B)** Mean bite rates across caging treatment levels. The symbol '*' denotes significant differences in bite rates in partial cages in comparison to control tiles (Mann–Whitney-*U*-test; *p*-value < 0.05). Error bars are ± SE.

Larvae from brooding corals are often competent to settle soon after release (Nozawa and Harrison, 2005) and may be less discriminating in terms of settlement preferences compared to larvae from broadcast spawners (Olsen et al., 2016; Ritson-Williams et al., 2016). Therefore, our study can offer important insights into potential mechanisms determining recruitment

success for brooding corals on highly disturbed reefs. Further studies with larvae from broadcast spawning corals are required to test the generality of these responses. In Singapore, the reduced settlement rates due to interaction with *Sargassum* sp. has important implications for coral recruitment success as *Sargassum* spp. are the most abundant macroalgal taxa on these reefs (Low, 2015). Indeed, reefs with the lowest abundance of *Sargassum* were found to have the highest natural settlement rates and adult abundance of *P. acuta* in Singapore (Bauman et al., 2015, 2017) suggesting a negative role for *Sargassum* sp. in recruitment success for this coral species (Maypa and Raymundo, 2004).

A number of manipulative field experiments have demonstrated that physical contact with macroalgae can negatively impact juvenile and adult coral health (McCook et al., 2001; Ritson-Williams et al., 2009; Rasher and Hay, 2010a,b). For example, survivorship of newly settled *Acropora millepora* spat was reduced by almost 80% in 9 months in caged plots dominated by *Sargassum* sp. (Webster et al., 2015). Mechanisms by which macroalgae can damage corals are less well understood, but include physical damage due to abrasion, allelopathy (McCook, 2001) and indirectly through changes in microbial activity or composition (Smith et al., 2006; Nelson et al., 2013) upon algal-coral contact (Nugues et al., 2004). In our study, *Sargassum* sp. effect on newly settled spat on the reef crest may have been due to water-flow assisted abrasion as this taxa has leathery and cortified fronds capable of physically damaging newly settled corals (Maypa and Raymundo, 2004; Birrell et al., 2008). While we cannot rule out the possibility of chemical effects on newly settled spats, a number of studies have failed to find allelopathic effects by *Sargassum* sp. on corals (Rasher and Hay, 2010b; Rasher et al., 2011; Bonaldo and Hay, 2014), suggesting an important role of physical contact by *Sargassum* sp. to the survivorship newly settled corals. Control tiles were placed in areas devoid of algae but similar in environmental conditions as treatment tiles. We cannot, however, rule out the possibility that macroalgae may grow in specific micro-sites that are unfavorable to coral spat survival and this contributed to the differences in survival. Our results also demonstrate the importance of habitat in determining survivorship of newly settled spat. For example, there was a clear reduction (30%) in survivorship of spat between controls and treatments at the reef crest; however, the same was not true for control and treatment tiles on the reef flat. Although differences may also be due to different algal species, the clear difference in survivorship of spat between control tiles at the reef flat (14.3 $\pm$ SE 5.6%) and the reef crest (50.0 $\pm$ SE 8.6%) strongly suggest that local habitat processes in each habitat are equally important in determining survival rates of newly settled spat.

Removal of benthic algae by herbivores encourages coral post-settlement survival (Jompa and McCook, 2002) and there is a demonstrable positive relationship between herbivore biomass and recovery rates on many coral reefs (Gilmour et al., 2013; Graham et al., 2015). Paradoxically, grazing by some fishes may actively remove new coral spat (Raimondi and Morse, 2000; Rotjan and Lewis, 2008; Penin et al., 2010). Several studies have shown that excluding herbivores actually improves the survival of newly settled spat (Gleason, 1996; Baria et al., 2010; White and O'Donnell, 2010). Our study found that fish exclusion increased juvenile coral mortality by almost 50%, suggesting an imporatnt role for fish grazing in juvenile coral survival in the study system. Algal cover and biomass in caged plots increased in comparison to non-caged plots, however, the differences were not significant. Furthermore, algal abundance did not emerge as a significant explanatory variable, which suggests that macroalgae alone was not the main driver of increased juvenile coral mortality. It is possible that a non-significant increase in dry algal mass in caged plots might have an effect on the amount of trapped sediments, which in turn may reduce survivorship of juvenile corals (Birrell et al., 2005).

Contrasting results, in terms of juvenile coral survival, among herbivore exclusion studies most likely result from differences in coral age classes, life histories and the types and abundance of grazers in each study location. In studies that have shown positive effects of caging on spat survival (e.g., Baria et al., 2010), either spat were newly settled, making them more vulnerable to removal, or on coral reefs where large scraping or excavating parrotfish were abundant (e.g., Trapon et al., 2013). In our study, corals already had an average diameter of 6.3 mm at the start of the study, and therefore are likely to have exceeded a size vulnerable to grazers (Doropoulos et al., 2012). In addition, Singapore's reefs have low abundances of sea urchins, large scaping parrotfish and relatively low grazing rates (Guest et al., 2016b). In the present study 90% of bites on tiles were carried out by *Halichoeres* sp.. Coupled with the absence of graze marks (pers. obs), our results suggest these fishes were opportunistically picking epiphytes off the tiles. Nevertheless, the results of our caging experiment suggest that even low rates of "grazing" by non-obligate herbivores has a positive effect on juvenile coral survival, likely through cascading effects from predation of invertebrates (Dulvy et al., 2004) and occasional removal of sediment and EAM surrounding corals (Birrell et al., 2005; Goatley and Bellwood, 2010). Our results highlight the importance of maintaining diverse fish populations on heavily impacted reefs. The precise role of herbivores, specifically, small non-obligate species such as damselfishes, in controlling algal turfs in turbid environments needs to be better understood and potentially these species may need to be considered for protection alongside larger browsing species (Bauman et al., 2017).

AUTHOR CONTRIBUTIONS

JG, PS, and RL contributed to the experimental design. CL, AB, EL, JL, and RL did the field and laboratory work. EM and RL analyzed the data. RL and JG did the drafting. All authors revised the manuscript.

FUNDING

Rehabilitation of Singapore's reefs using sexually reared corals.

ACKNOWLEDGMENTS

All research was carried out with a research grant from National Parks Board, Singapore and under Permit no. NP/RP11-073. We are thankful to Aaron and Juward Ho from South East (S.E.A) Aquarium of Resorts World Sentosa for the provision of aquaria and assistance with the aquaria setup. We are grateful for logistics and administration assistance from Melvin Lee (Dolphin Explorer) and Ricky Lim from Advanced Environmental Biotechnology Center. We would also like to thank Dr. Adriana Vergés (University of New South Wales) and Dr. Alexandra Campbell (University of the Sunshine Coast) for valuable advice about experimental design.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00385/full#supplementary-material

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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

ORIGINAL RESEARCH
published: 30 October 2018
doi: 10.3389/fmars.2018.00392

Contrasting Antibacterial Capabilities of the Surface Mucus Layer From Three Symbiotic Cnidarians

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Coral reefs are affected by the deterioration of the oceans due to global warming and other anthropogenic perturbations, increasing the frequency, and severity of bleaching and disease. To overcome some of these conditions, reef corals and other cnidarians rely on a mucus layer housing a diverse community of beneficial microorganisms and mechanisms of innate immune response. The antimicrobial defense has been associated with the bacterial community in these organisms, but the potential antimicrobial activity of the mucus layer itself has not been explored fully. We hypothesized that the bacteria-free mucus layer of different cnidarians would show differential and specific antimicrobial and immunological responses when challenged with two potentially pathogenic bacteria. We evaluated this capability through antimicrobial properties, immune response and biochemical composition of the mucus. Results clearly showed that the mucus of healthy cnidarians has the capability of inhibiting the growth of *Serratia marcescens* and *Aurantimonas* sp. in a species-specific way that includes differences in the potency of the response. The anemone *Exaiptasia pallida* was particularly potent against *Aurantimonas* sp. while the coral *Pseudodiploria strigosa* and the medusa *Cassiopea xamachana* had similar capabilities against both bacterial strains. In coral affected by black band disease, this antibacterial capability diminished in the mucus layer, but the associated bacteria remained potent. Results showed that hydroxyproline and phenoloxidase increased in the transition zone of diseased corals, although melanin was not detected in any of the animals tested. Bleaching of anemones and medusas also diminished the antibacterial capability of the surface mucus layer, but in anemones, the associated bacteria did not show a significant reduction in their ability to inhibit the growth of the bacterial strains. The mucus of bleached medusas showed an increased inhibitory activity against *Aurantimonas* sp. that may be associated with a specific bacterial strain we isolated. Mucus collected from bleached anemones and medusas did not show a significant immune response.

OPEN ACCESS

Edited by:
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University of Derby, United Kingdom

Reviewed by:
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Simón Bolívar University, Venezuela
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *01 June 2018*
Accepted: *09 October 2018*
Published: *30 October 2018*

Citation:
Rivera-Ortega J and Thomé PE (2018) Contrasting Antibacterial Capabilities of the Surface Mucus Layer From Three Symbiotic Cnidarians. Front. Mar. Sci. 5:392. doi: 10.3389/fmars.2018.00392

In this work, we show that the surface mucus layer itself has antibacterial properties not associated with the bacteria this layer houses; such properties diminished due to disease or bleaching, while immunological responses increased in the mucus of diseased animals.

Keywords: antimicrobial capability, coral mucus, immunological defense, innate immunity, bacteria-free mucus, coral immunity, *Serratia marcescens*, *Aurantimonas*

INTRODUCTION

As many aquatic invertebrates, reef cnidarians possess a complex surface layer (SML) of mucopolysaccharides that functions as the interface between the animal tissue and the external environment, constituting a physicochemical and physiological barrier (Brown and Bythell, 2005; Wahl et al., 2012). This layer houses a microbial community mostly composed of bacteria, that varies depending on the animal species, geographic location, physiological status, nutrition, and health (Rohwer et al., 2002; Ainsworth et al., 2006; Koren and Rosenberg, 2006; Longford et al., 2007; Wahl et al., 2012; Thompson et al., 2015). Nevertheless, a recent study on the bacterial community of the model organism *Exaiptasia pallida* strongly suggests that this bacterial community may be more related to environmental conditions than to geographic location, animal species or symbiont type (Brown et al., 2017). Studies that have sequenced this community show that it differs from bacterial communities in the adjacent water column and the sediments, suggesting some degree of specificity (Rohwer et al., 2002; Brown and Bythell, 2005). Further, it has been demonstrated that the bacterial community that colonizes newly settled coral polyps has a composition apparently related to the host identity (Sharp et al., 2010). In addition, microbes in the SML alter the immune system by stimulating specific responses, and the immune system influences the microbial composition in return (Wahl et al., 2012; Shöder and Bosch, 2016).

The surface mucus layer represents a biofilm that provides various substrates for a complex microbial community. This layer also contains bioactive molecules with antimicrobial activity (some produced by resident bacteria). Then, the SML represents the first defense against potential pathogens, contributing to the function and composition of the microbial community it supports. As an important observation, microscopic examination of the coral epidermis beneath the mucus layer reveals a nearly sterile environment (Johnston and Rohwer, 2007). Further, the presence of the SML reduces fouling or settlement of unwanted microbes on cnidarian surfaces. The mucosal surface in other invertebrates has a role in chemical defense as part of the innate immune system (Abbas et al., 2007), defending the host organism with diverse substances that lyse potentially pathogenic bacterial cells, sequester microbial nutrients or act as decoy to bind and trap microorganisms (Fleming, 1922; Cole et al., 1999).

Mucosal surfaces appeared for the first time in animal evolution in Cnidaria (Bosch and McFall-Ngai, 2011). In *Hydra*, several studies have determined that different species house different microbial communities, strongly suggesting a specific interaction of the SML with potential microbial commensals and the environment (Fraune and Bosch, 2007; Franzenburg et al., 2013). Actually, the study of immunology in cnidarians initiated with studies of *Hydra* and later of *Nematostella*, that identified a lack of specific immune cells, thus implicating other mechanisms for their defense (reviewed in Augustin and Bosch, 2010; Ocampo and Cadavid, 2015). Additionally, cnidarians generate antimicrobial peptides (AMPs) like aurelin in the medusa *Aurelia aurita* (Ovchinnikova et al., 2006), hydramycin in *Hydra* (Jung, 2009) or damicornin in the coral *Pocillopora damicornis* (Vidal-Dupiol et al., 2011). They also have bacterial recognition receptors, in particular, Nod-like receptors that activate cell death, so that apoptosis seems to be important in the innate immune response (Augustin and Bosch, 2010). Other responses include melanin encapsulation of microbial cells catalyzed by phenoloxidase (Petes et al., 2003; Mydlarz et al., 2008), coagulation, and immune cell activation after disease and injuries, all of which have been defined as key components of this defense (Mydlarz et al., 2010; Palmer and Taylor-Knowles, 2012; Ocampo and Cadavid, 2015). All these immunological responses are located within live tissues, but there have been no assessments of immunological activity of the mucus layer in Cnidarians. However, a recent study of untreated mucus collected from the reef zoanthid *Palythoa caribaeorum* indicated the presence of bioactive compounds like lectins and proteolytic enzymes (Camargo Guarnieri et al., 2018). Further, scleractinian corals release some antibacterial compounds upon mechanical stress (Greffen and Rosenberg, 2005).

Environmental and anthropogenic perturbations have important consequences on coral health, because they modify the associated microbial community (e.g., increase under nutrient enrichment and decrease after bleaching). Although these responses suggest a dynamic community, such variations may allow for the infection by pathogens, compromising the health of the animals due to the loss of the protective qualities of the SML (Ritchie, 2006; Sekar et al., 2006; Bourne et al., 2007; Vega Thurber et al., 2009; Augustin and Bosch, 2010; Mydlarz et al., 2010; Thompson et al., 2015). Interestingly, no diseases have been reported for the model organisms *Cassiopea xamachana* or *E. pallida*. It is possible that these symbiotic cnidarians do get diseased, but soon die or are eaten by other animals.

The susceptibility of corals to disease and bleaching has been inversely correlated with the investment in protection when assessed by variables commonly associated with invertebrate immunity, such as the size of melanin-containing granular cells, phenoloxidase activity, and the concentration of fluorescent proteins (Palmer et al., 2010). The investment of energy in immune mechanisms can translate to the species being more resistant to perturbations such as high temperature. In addition,

thickness of the tissue and the mucus layer have been found to relate to the temperature tolerance of corals and to disease susceptibility (Glynn and D'Croz, 1990; Fitt et al., 2000, 2009; Green et al., 2008). Further, reproductive patterns and colony morphology in closely related coral species can vary the levels invested in immunity (Pinzón et al., 2014b); and in terms of evolutionary history, older coral lineages have a lower number of diseases and disease prevalence than more recently diverged lineages (Pinzón et al., 2014a). Within the Cnidaria, different taxonomic groups may, therefore, show important differences in the defensive capabilities of their SML, in addition to species-specific differences in resources allocated to immune defense, as well as variations in the composition of their microbial communities. Our hypothesis is that there is a differential, species-specific capability of the SML to help fight pathogens, not related to the bacterial community it houses. With this in mind, we included distant relatives in our experimental approach.

Although the antimicrobial function of the SML has been usually assigned to the microbial community housed by this layer (Gil-Turnes and Fenical, 1992; Castillo et al., 2001; Rypien et al., 2010), the potential antimicrobial activity of this layer *per se*, has not been explored fully (Ritchie, 2006). Therefore, the purpose of this study was to experimentally evaluate possible differences in the capabilities of the SML associated with different taxonomic groups within the Cnidaria when challenged by two potentially pathogenic bacterial strains. We hypothesize that the three cnidarians we selected, a coral (*P. strigosa*), an anemone (*E. pallida*) and a medusa (*C. xamachana*), will demonstrate different and specific protective capabilities of the SML. The bacterial pathogens we tested were identified as causative of diseases in certain coral species (Richardson et al., 1998; Patterson et al., 2002; Denner et al., 2003), but not associated with black band disease or bleaching. Our experimental approach included assays employing the surface mucus layer complex (MC), mucus layer free of bacteria (SML), and isolated bacteria from the CM (BAM), against two tester bacterial strains. We also looked at some of the innate immune activity by measuring hydroxyproline (Hyp), phenoloxidase activity (PO), and melanin. We collected mucus from three symbiotic cnidarians, healthy and affected by disease or bleaching, to contrast the responses.

MATERIALS AND METHODS

Animals and Sampling Conditions

Medium size (25 cm minimum diameter, 25 cm minimum high) coral colonies of *P. strigosa*, healthy or visibly affected with black band disease (BBD), were sampled from the back reef at Petempich location in the Puerto Morelos Reef National Park, México. Colonies from 3 to 5 m deep were sampled between October and November 2017. Mucus was collected from 6 colonies each, in triplicate samples, with sterile syringes after softly disturbing the surface layer with a tool (similar to a microbiological spreader) and stored in an ice chest to be processed in the lab. For diseased colonies, two areas were sampled: the transition zone (2–4 cm from the lesion band) and the apparently healthy-looking tissue (15 cm away from the band). *E. pallida* anemones were collected from the water pipes of the aquaria system at our department facilities. Bleaching of anemones was produced after cold shocks (Muscatine et al., 1991; Estes et al., 2003). Bleached anemones were kept in the dark for 2–3 weeks before being sampled. The Xcaret Park donated healthy and bleached *C. xamachana* medusas. Mucus from these animals was collected after rinsing the animals in sterile filtered seawater; after 10–20 min the liberated mucus was collected with a syringe.

Mucus Treatments

All mucus samples were centrifuged twice (20 min in a clinical centrifuge, 5 min in a microcentrifuge) at maximum speed to remove excess seawater, with a short vortex agitation in between. Mucus collected from the different animals was experimentally processed to compare the antibacterial activity of the mucus layer itself and that of the associated bacteria. Each sample was distributed in three microtubes for the following treatments: (1) Sterilization by irradiating for 20 min under UV-light (λ = 254 nm; Krediet et al., 2009), named SML. (2) Bacteria that were cultured after inoculating 1 mL of marine broth with 10 μL of mucus, grown overnight at 27°C and used in further tests, named MAB. (3) Mucus without treatment named MC. Sterilization of the mucus (treatment SML) was corroborated by incubating an aliquot on marine-agar at 27°C, overnight, and checking for the absence of bacterial growth.

Antibacterial Activity

We employed a swarming assay to evaluate the antimicrobial activity of the three mucus treatments. We first used a marine-agar layer (15 g L^{-1} of agar) which was left 12 h to settle. Glass fiber discs embedded in 10 μL of mucus or 7 μL of cultured bacteria were placed over this layer. Next, a second cooler (48°C), softer marine-agar layer (7 g L^{-1}) with an inoculum (100 μL of overnight culture) of each tester bacteria, was poured over and left to settle for 30 min. Plates were incubated at 27°C in a controlled culture chamber. Growth measurements were taken after the growth of the tester bacteria had formed a confluent lawn on the medium which was about 16 h for *Serratia marcescens* and 48h for *Aurantimonas* sp. We scored the presence/absence of an inhibition zone formed around each filter. As a negative control we used embedded filters in filtered seawater and for positive control, embedded filters in one of two wide-spectrum antibiotics, ciprofloxacin or ceftriaxone (at 100 mg mL^{-1}). Assays were done in triplicate.

Immune Response Assessment

The immune response was assessed by measuring the content of hydroxyproline and melanin, and the activity of phenoloxidase. The presence of collagen was evaluated by quantification of the unique amino acid Hyp following the protocol described in Hofman et al., 2011. We processed 10 μL of each mucus sample, with three technical replicates, to be read in a Biospectrometer (Eppendorf, United States) at 540 nm. Calculations were performed against the standard Trans-4-hydroxy-L-proline. For melanin quantification, we followed a protocol described in Mydlarz and Palmer, 2011 using 10 μL

of mucus for each sample, with three technical replicates. Readings in the spectrophotometer were at 410 and 490 nm to distinguish between two forms of melanin, employing pure melanin (SIGMA) as a standard. Phenoloxidase (PO) activity was measured according to Mydlarz and Palmer, 2011, using 10 μL of mucus from each sample, with three technical replicates; data are presented as change in absorbance A_{490} mg protein^{-1}min^{-1}.

Biochemical Determinations

A basic biochemical determination of the mucosal surface included the total content of protein, carbohydrates, and lipids. Total protein in mucus from each sample (10 μL) was estimated by the Bradford method against a standard curve with BSA, according to the manufacturer (SIGMA). For the estimation of total lipids, we followed the colorimetric method of sulfo-phospho-vanillin as described (Mishra et al., 2014), using 10 μL of sample and readings at 530 nm after processing. Total carbohydrates were estimated by a colorimetric sulfuric-phenol assay (Dubois et al., 1951) with 10 μL of mucus sample. Readings were done at 490 nm and normalized to a standard curve prepared with glucose. All assays had three technical replicates.

Statistical Analyses

Antibacterial activity of mucus samples against tester bacteria and among the condition of animals (diseased or bleached) was analyzed by the exact Fisher test, due to the qualitative nature of the results (nominal variables) and the sample size used ($n = 6$). To compare the different treatments of the mucus layer, we employed the Cochran Q analysis, using $Q_0 > \chi^2 = 5.99$ of significance. For the comparison of the immunological responses -hydroxyproline content and phenoloxidase activity- as well as the biochemical composition of the mucus samples, we employed a Wilcoxon test. This type of analysis was applied since the data were not normally distributed as evaluated by a graphical test with the software R Project. In all tests, statistical significance was established at $p < 0.05$.

RESULTS

Assessment of Protective Capabilities of the Mucus Layer in the Coral *P. strigosa*

To evaluate the antibacterial capability of the mucus layer in corals, anemones, and medusas, we conducted swarming assay experiments employing three differentially treated mucus samples: (1) mucus layer complex without treatment, or CM; (2) UV-sterilized mucus layer without bacteria, or SML; and (3) bacteria isolated and cultured from the mucus layer, or MAB. We tested this capability against the growth of two potentially pathogenic bacterial strains. In coral colonies, we sampled the mucus layer as follows: (1) on healthy colonies, and on diseased colonies in two positions, (2) close to the lesion (transition zone), and (3) away from the lesion (healthy-looking). Results showed that in healthy *P. strigosa* colonies, only coral mucus free of bacteria (SML) inhibited significantly the growth of *S. marcescens*, while the same assay against *Aurantimonas* sp. showed an inhibitory effect that was similar for all the differentially treated mucus (**Table 1**). The inhibitory capability in the mucus collected from the transition zone in diseased colonies was lost in the SML, but the MAB and CM treatments showed a positive inhibition of *S. marcescens*. However, only MAB treated mucus showed some inhibition against *Aurantimonas* sp. (**Table 1**). Mucus collected from healthy-looking tissues in diseased corals had a similar inhibitory effect on the growth of *S. marcescens* for all mucus treatments, but against *Aurantimonas* sp. the mucus only fraction (SML) apparently lost its inhibitory effect, although this was not statistically significant (**Table 1**). As controls, filtered seawater had no effect against the growth of both tester bacteria while the antibiotics ciprofloxacin and ceftriaxone inhibited completely the growth of both tester bacteria (data not shown).

To assess the immune response of the mucus surface layer in all three animals, we measured the relative concentration of Hyp and the activity of PO in this layer. We also measured melanin content, but values were below the limit of detection of the assay (results not shown). Mucus collected from healthy coral colonies had a lower concentration of Hyp than mucus collected from the transition zone of diseased colonies, while mucus collected away from the affected area in diseased colonies had intermediate levels of Hyp (**Figure 1A**). No statistically significant differences were detected among the three sampled tissues, probably due to the dispersion of the data, in particular in mucus sampled from the transition zone. The results for the activity of phenoloxidase in mucus from healthy and diseased coral colonies were similar to these results (**Figure 1B**).

We evaluated if the antibacterial and immunological responses of the different animals considered could be associated with the composition of the mucus layer. With this in mind, we compared the biochemical composition of the mucus surface layer from healthy to diseased coral colonies. Results showed that the total content of proteins, lipids, and carbohydrates was not significantly different in corals under three different conditions: healthy, close (transition zone) and away (healthy-looking) from the lesion (**Table 2**).

Assessment of Protective Capabilities of the Mucus Layer in the Medusa *C. xamachana* and the Anemone *E. pallida*

We also evaluated the antibacterial capability of the mucus layer from medusas and anemones, healthy and bleached. The mucus free of bacteria (SML) of healthy medusa showed some inhibitory effect against the growth of both tester strains (**Table 3**). In bleached medusas, the only positive, significant inhibitory activity was observed for the untreated mucus fraction (MC) against *Aurantimonas* sp. The mucus collected from healthy anemone *E. pallida* (**Table 3**) showed the highest inhibitory effect against both tester bacteria, except for mucus free of bacteria (SML) against *S. marcescens*, which showed no inhibitory effect on its growth. In mucus collected from bleached anemone, results were similar to healthy animals, except for a significantly higher inhibitory effect in the mucus complex (MC) against *Aurantimonas* sp.

TABLE 1 | Frequency for the presence of inhibition growth zone of treated mucus collected from healthy or diseased *Pseudodiploria strigosa* (coral). Treated mucus was offered to plated tester strains *Serratia marcescens* and *Aurantimonas* sp., their growth inhibition scored after 16 and 48 h, respectively.

Animal	Condition	*Serratia marcescens*				*Aurantimonas* sp.			
		SML	MAB	MC	Q_0	SLM	MAB	MC	Q_0
Pseudodiploria strigosa	H	3/6*	0/6	0/6	6*	1/5	4/5	2/5	4.6
	TZ	0/6*	3/6	3/6	15*	0/5	2/5	0/5	4
	HL	1/6	2/6	2/6	1.5	0/5	3/5	3/5	4.5
p		0.25	0.27	0.27		1	0.77	0.25	

H, Healthy; TZ, Transition zone (2–4 cm from diseased tissue); HL, Healthy-looking tissue (15 cm from diseased tissue); SML, Superficial Mucus Layer (bacteria-free); MAB, Mucus Associated Bacteria; MC, Mucus Complex (mucus + bacteria). Numbers for inhibited growth over total scores calculated from 5 to 6 biological replicates with 3 technical replicates each. Statistically significant different samples are denoted by an asterisk (*) for $p < 0.05$ in healthy vs. diseased animals, and for $Q > 5.99$ for mucus treatments.

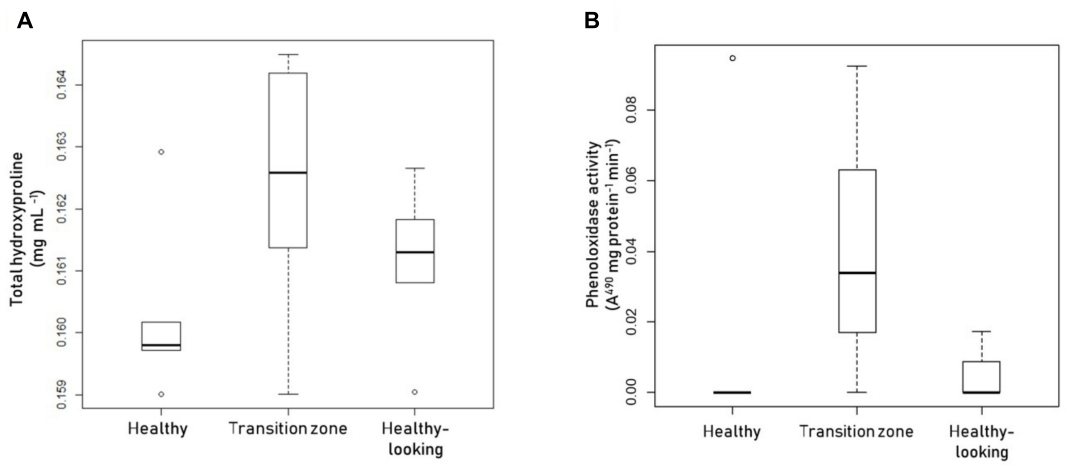

FIGURE 1 | Assessment of the immunological response of the mucus complex in coral colonies. Boxplots showing **(A)** total hydroxyproline or **(B)** phenoloxidase activity levels in mucus samples collected from healthy *Pseudodiploria strigosa* colonies (left), and colonies affected by black band disease, collected above the transition zone (center), or above healthy-looking tissues (right). Dots represent atypical data. Data from 5 independent determinations, with three technical replicates each.

The results of controls for medusa and anemone assays were identical to the controls used in coral assays (data not shown).

The immunological response of the mucus layer in medusas and anemones, healthy and bleached, was also evaluated. In mucus collected from medusas, the concentration of Hyp was not significantly different between healthy and bleached animals (**Table 4**). The activity of PO in healthy medusa was higher and the data more dispersed than in bleached medusa, although not statistically different (**Table 4**). In anemones, we did not measure significantly different levels of Hyp or PO between healthy and bleached animals, although average values for Hyp in bleached animals were higher (**Table 4**), as in bleached medusa.

The composition of the mucus layer from medusas and anemones indicated that in mucus collected from medusa, total protein and lipid content were significantly lower in bleached animals (**Table 5**). By contrast, in anemones, we measured significantly lower levels of protein and carbohydrates but similar lipid levels in bleached compared to healthy animals (**Table 5**).

DISCUSSION

The central role of the surface mucus layer in corals and reef cnidarians is the protection of live tissue, not only as a physical barrier but also as a chemical one (Brown and Bythell, 2005; Ritchie, 2006; Wahl et al., 2012). In addition, the SML has antimicrobial compounds and other molecules that can fight potential pathogenic microorganisms (Abbas et al., 2007; Mydlarz et al., 2010). We evaluated the antimicrobial capability of the SML *per se*, by irradiating collected mucus with UV light. We found that this layer has antimicrobial activity against tester strains *S. marcescens* and *Aurantimonas* sp. However, as we hypothesized, results differed among the mucus collected from the different animals we considered. In healthy animals, only anemones did not show an inhibitory effect against *S. marcescens*, associated exclusively with the surface mucus layer. On the other hand, the mucus layer of healthy corals and medusas was not as effective to inhibit the growth of *Aurantimonas* sp. as that of anemones. Our results agree with the concept of the SML as a chemical barrier with antimicrobial capabilities, containing molecules that contend potential pathogens, related

TABLE 2 | Total protein, lipid and carbohydrates in mucus samples collected from *P. strigosa*, healthy and affected by black band disease, on the transition zone and on healthy looking tissues.

Parameters	Proteins (mg ml^{-1})			Lipids (mg ml^{-1})			Carbohydrates (mg ml^{-1})		
	Healthy	TZ	HL	Healthy	TZ	HL	Healthy	TZ	HL
Mean*	0.210 ± 0.00	0.210 ± 0.00	0.211 ± 0.001	0.061 ± 0.001	0.061 ± 0.000	0.060 ± 0.000	0.041 ± 0.001	0.073 ± 0.012	0.039 ± 0.001
Median	0.210	0.210	0.210	0.060	0.061	0.060	0.040	0.069	0.038
Max. data	0.211	0.211	0.215	0.062	0.061	0.061	0.045	0.106	0.041
Min. data	0.209	0.210	0.210	0.060	0.061	0.059	0.039	0.049	0.038
n	6	6	6	4	4	4	4	4	4
		p	0.415		*p*	0.368		*p*	0.392

*H, Healthy; TZ, Transition zone (2–4 cm from diseased tissue); HL, Healthy-looking tissue (15 cm from diseased tissue). Kruskal-Wallis test did not show statistically significant differences among samples. *Data presented as the mean ± SE.*

to the phylogenetic position of these animals and perhaps to their particular life history.

A single publication on the antibacterial properties and the innate immune response of corals has analyzed these properties in the surface mucus layer (Ritchie, 2006). In this work, the author evaluated UV irradiated mucus collected from the reef coral *Acropora palmata* and found that it has antimicrobial activity, suggesting its role as a biochemical defense. In addition, some of the bacteria isolated and cultured from this same layer showed antimicrobial activity (Ritchie, 2006). In that same work, UV irradiated mucus collected from colonies that had experienced increased temperature also was evaluated and found that it had lost its antimicrobial capability. We found that, although the SML showed diminished inhibitory activity in diseased colonies, the antimicrobial capability of mucus-associated bacteria rather improved against *S. marcescens*. The antimicrobial capabilities of the mucus layer that we assessed in healthy *P. strigosa* colonies over two tester strains, demonstrated a differential response: the inhibitory activity against *S. marcescens* was associated with the bacteria-free mucus, while the inhibitory activity against *Aurantimonas* sp. was mostly associated with the bacteria cultured from the SML. These results suggest that the antimicrobial activity of the SML has some kind of species-specific effect, also indicating that *S. marcescens* may be a common potential pathogen.

Previous work has indicated that corals affected by a disease have reduced defensive capabilities (Palmer et al., 2010; Pinzón et al., 2014a). Consistent with these reports, diseased coral colonies of *P. strigosa* showed a diminished inhibitory effect against *Aurantimonas* sp. in mucus over the transition zone, for all mucus treatments. However, this capacity was not different in healthy colonies when compared to the mucus over healthy-looking tissue of diseased colonies. It is interesting that against *S. marcescens*, the SML antibacterial activity in diseased colonies was reduced, but the bacteria associated with this layer showed positive, inhibitory activity in mucus from diseased tissue, suggesting that SML associated bacteria may contribute significantly to protect diseased corals. Black band disease is characterized as a dark band, formed by a microbial consortium dominated by cyanobacteria, which migrates across living tissues degrading and killing entire colonies over a period of several months (Garret and Ducklow, 1975; Rützler and Santavy, 1983). Various studies have reported that the microbial community in different species of corals affected by BBD, increases in diversity, including some specific genera like *Desulfobacteraceae* and toxin-producing cyanobacteria (Frias-Lopez et al., 2004; Sekar et al., 2006). In *P. strigosa* colonies affected by BBD, the mucus layer showed the presence of hydroxyproline, suggesting that the immune response in these colonies is active and producing collagen to encapsulate potential pathogens (Mydlarz et al., 2010; Palmer and Taylor-Knowles, 2012; Ocampo and Cadavid, 2015). Although no melanin was detected in mucus from any of the animals studied, phenoloxidase activity behaved similarly to hydroxyproline in coral diseased colonies. These results are in agreement with studies on *Acropora millepora* affected with white syndrome that detected more phenoloxidase activity near the lesion (Palmer et al., 2011). In addition, the healthy-looking

TABLE 3 | Frequency for the presence of inhibition growth zone of treated mucus collected from healthy or bleached *Exaiptasia pallida* (anemone) and *Cassiopea xamachana* (medusa).

Animal	Condition	*Serratia marcescens*				*Aurantimonas* sp.			
		SML	MAB	MC	Q_0	SLM	MAB	MC	Q_0
Cassiopea xamachana	H	2/6	0/6	0/6	4	2/6	0/6	0/6	4
	B	0/6	0/6	0/6	0	0/6	0/6	5/6*	12*
p	0.45	1	1		1	0.77	0.25		
Exaiptasia pallida	H	0/6*	5/6	5/6	10*	6/6	6/6	6/6	0
	B	0/6*	5/6	5/6	10*	3/6	4/6	2/6*	1.5
p	1	1	1		0.18	0.45	0.06*		

Treated mucus was offered to plated tester strains S. marcescens and Aurantimonas sp., their growth inhibition scored after 16 and 48 h, respectively. H, Healthy; B, bleached; SML, Superficial Mucus Layer (bacteria-free); MAB, Mucus Associated Bacteria; MC, Mucus Complex (mucus + bacteria). Numbers for inhibited growth over total scores calculated from 6 biological replicates with 3 technical replicates each. Statistically significant different samples are denoted by an asterisk (*) for $p < 0.05$ in healthy vs. bleached animals, and for $Q > 5.99$ for mucus treatments.

TABLE 4 | Total hydroxyproline and phenoloxidase (PO) activity levels of the mucus complex samples collected from healthy and bleached *C. xamachana* and *E. pallida.*

Organism	Parameters	Hydroxyproline (mg ml^{-1})		PO activity (A_{490} mg $protein^{-1} min^{-2}$)	
		Healthy	Bleached	Healthy	Bleached
Cassiopea xamachana	Mean*	0.160 ± 0.001	0.164 ± 0.003	0.278 ± 0.137	0.055 ± 0.025
	Median	0.159	0.161	0.177	0.046
	Max. data	0.164	0.179	0.876	0.153
	Min. data	0.158	0.161	0	0
	n	6	6	6	6
		p	0.077	*p*	0.289
Exaiptasia pallida	Mean*	0.160 ± 0.001	0.164 ± 0.003	0.249 ± 0.044	0.453 ± 0.252
	Median	0.156	0.161	0.233	0.220
	Max. data	0.163	0.179	0.233	1.683
	Min. data	0.158	0.160	0.133	0.003
	n	6	6	6	6
		p	0.173	*p*	0.937

*Wilcoxon test did not show statistically significant differences among healthy and bleached mucus samples. *Data presented as the mean ± SE.*

TABLE 5 | Total protein, lipid and carbohydrates in mucus samples collected from healthy and bleached *C. xamachana* and *E. pallida.*

Organism	Parameters	Proteins (mg ml^{-1})		Lipids (mg ml^{-1})		Carbohydrates (mg ml^{-1})	
		Healthy	Bleached	Healthy	Bleached	Healthy	Bleached
Cassiopea xamachana	Mean*	0.182 ± 0.043	0.050 ± 0.000	0.065 ± 0.001	0.060 ± 0.000	0.038 ± 0.000	0.034 ± 0.007
	Median	0.207	0.050	0.066	0.060	0.038	0.035
	Max. data	0.278	0.051	0.066	0.061	0.039	0.055
	Min. data	0.061	0.050	0.063	0.059	0.037	0.010
	n	6	6	6	6	6	6
		p	**0.002**	***p***	**0.002**	*p*	1.00
Exaiptasia pallida	Mean*	0.271 ± 0.001	0.124 ± 0.022	0.058 ± 0.000	0.059 ± 0.000	0.040 ± 0.000	0.038 ± 0.000
	Median	0.271	0.146	0.058	0.059	0.040	0.038
	Max. data	0.276	0.149	0.059	0.062	0.041	0.040
	Min. data	0.265	0.015	0.057	0.058	0.039	0.037
	n	6	6	6	5	6	5
		p	**0.002**	*p*	0.329	***p***	**0.017**

**Data presented as the mean ± SE. Significant differences indicated with bold letters at $p < 0.05$ among samples (healthy vs. bleached) by Wilcoxon test.*

tissue of diseased colonies of *Orbicella faveolata* affected by yellow band disease was shown to have an active immune response (Weil et al., 2009; Morgan et al., 2015). Concurring with previous reports (Fleming, 1922; Ritchie, 2006; Abbas et al., 2007), we were able to detect an innate immune activity in the mucus layer, suggesting that corals expel molecules with such activity from their tissues.

It seems relevant to comment that there is a lack of reports on diseases in *Exaiptasia* anemones or *Cassiopea* medusas in the field. Our results suggest that main antibacterial capacity of these organisms seems to be located in different components of the superficial mucus layer, being specific to the tested bacteria: against *S. marcescens* the mucus of anemones seemed to depend on the associated bacteria, while inhibitory effects in medusas associated with the bacteria-free mucus layer. Against *Aurantimonas* sp., the anemones had the same inhibitory effect independent of the component of the mucus complex, which diminished in bleached animals. But in medusas, only the bacteria-free mucus layer demonstrated inhibition of the growth of *Aurantimonas* sp., which was also lost in bleached animals. Apparently, *E. pallida* does not have a characteristic core bacterial community (Brown et al., 2017; Herrera et al., 2017). This community varies according to the environmental conditions and does so rapidly (Herrera et al., 2017). This characteristic could explain the high resilience of this anemone to stress that we corroborated in bleached animals, as they did not show a diminished capacity to inhibit the growth of *S. marcescens* and, although lower, still showed an appreciable inhibitory effect against *Aurantimonas* sp. *S. marcescens* is a ubiquitous, opportunistic terrestrial pathogen, often infecting humans. In the ocean, it could be linked to municipal discharges. This bacterium has been found to cause white pox disease in the coral *A. palmata* (Patterson et al., 2002). Interestingly, the response in *E. pallida* could implicate a previous contact with this pathogen, contrasting with *P. strigosa* and *C. xamachana*, which were less successful in inhibiting its growth. As for *Aurantimonas* sp., it may be a more common inhabitant of the sea, which would explain why the mucus complex of all three cnidarians showed inhibitory activity against it.

Increased temperatures commonly cause bleaching in symbiotic reef cnidarians (Brown, 1997; Hoegh-Guldberg, 1999). After bleaching, there is a diminished translocation of carbon (Muscatine et al., 1991; McGill and Pomeroy, 2008) and an essential loss of energy for their health (Iglesias-Prieto et al., 1992; Grottoli et al., 2006; reviewed in Mydlarz et al., 2010). As a consequence, bleached corals have been found to be more susceptible to disease (Miller et al., 2006; Bruno et al., 2007). Our results showed that the mucus extracted from bleached anemones did not have a reduced effect against *S. marcescens* but the inhibitory effect against *Aurantimonas* sp. was reduced. Although bleaching of the medusa reduced the inhibitory effect of the SML, interestingly the mucus complex exhibited an increased inhibition over the growth of *Aurantimonas* sp.

On the other hand, bleaching did not change the levels of hydroxyproline or phenoloxidase in the SML of anemones or medusas, suggesting that the loss of symbionts is not enough to stimulate immunological activities that may reach the SML. However, the loss of symbionts leads to a loss of translocated carbon (Muscatine et al., 1991; McGill and Pomeroy, 2008). In anemones, this reflected directly in the levels of carbohydrates of the surface mucus layer. But in medusas, the loss of potentially translocated carbon was detected in lipid levels. This difference may relate to the time these animals had been without symbionts. *E. pallida* were bleached by cold shocks and maintained for 2–3 weeks before the experimental measures were taken, while *C. xamachana* were bleached at low light over a 2 months period with daily feeding, but their lipid reserves may have been significantly reduced. In any case, the loss of symbionts probably changes the composition of the surface mucus layer; but this change did not affect the antibacterial properties of this layer in anemones but did so in medusas. Although we did not find antibacterial and immunological responses to be associated with the composition of the mucus layer in any of the animals considered, it is interesting that the values for total proteins, carbohydrates, and lipids among healthy animals were similar, implying a balanced C-budget.

Disease and bleaching in tropical symbiotic cnidarians have increased severely in the past years due to the deterioration of the reef environment. However, the defensive responses these organisms possess may help them in a species-specific way. In coral colonies of *P. strigosa* affected by BBD, our results indicate that even though the superficial mucus layer and the associated bacterial community have antibacterial capabilities, and some innate immune response was measured, their response may not be strong enough to fight this disease successfully. Bleached animals did not show significant changes in their immune response. The techniques that were used to bleach anemones and medusas in this work did not involve high temperature, which may have resulted in less substantial changes in the associated bacterial community. Diminished carbohydrates or lipids in the mucus of bleached animals and the presence of immune activity, may have reduced the antimicrobial capability of the mucus layer, except for the response against *Aurantimonas* sp. in medusas, which was improved. The activity may have been due to a specific bacterium with antimicrobial capabilities that was isolated from bleached medusas. We are currently working on its identification.

AUTHOR CONTRIBUTIONS

JR-O and PT designed the experiments. JR-O conducted the experiments, undertook the statistical analyses, and prepared the figures and tables. PT wrote the manuscript. All authors contributed to the final version of the manuscript.

FUNDING

This work was supported by DGAPA-PAPIIT-UNAM Grant IN204318 to PT.

ACKNOWLEDGMENTS

JR-O wishes to recognize a scholarship for her master studies by CONACyT México and UNAM-DGAPA-PAPIIT through grant IN204318. Medusas were made kindly available by Xcaret Park, México. We thank Yislem Beltrán Díaz for fieldwork. We are grateful to two reviewers who helped to improve this manuscript. We also want to thank the Academic Writing Workshop CEP-UNAM for suggestions to the manuscript. Coral mucus was collected with a permit from INAPESCA number PPF/DGOPA-110/16.

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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

ORIGINAL RESEARCH
published: 05 November 2018
doi: 10.3389/fmars.2018.00396

Effects of Partial Mortality on Growth, Reproduction and Total Lipid Content in the Elkhorn Coral *Acropora palmata*

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Partial mortality (PM) is increasingly common in the Elkhorn coral *Acropora palmata* and, depending on the causative agent, is potentially lethal. The effects of PM on growth, reproduction and total lipid content in *A. palmata* were studied by sampling apparently healthy (AH) colonies in comparison with colonies showing signs of PM. Branch growth rates and lesion regeneration rates were estimated using monthly photographs over a four-month period prior to the summer spawning season. No differences were found in the growth rates of colonies with PM compared to AH colonies. The areas affected by PM did not regenerate during the period of the study. Colonization of the lesions by competing species and sediment cover were documented and did not show major changes. During the spawning season, percent fertilization, egg volume and embryonic development were evaluated for comparison between AH colonies and those with PM. Total lipids were also quantified in tissues from three branches per colony. Percentage fertilization was similar in both AH colonies and those with PM. Embryonic development was normal, regardless of proximity to the lesion borders. However, egg volume was significantly lower in PM colonies than in AH colonies. Lower lipid concentrations were found at the edges of the lesions and similar to those found at the growing edges of the branches. The lack of regeneration may be explained by the low lipid concentration, because the polyps adjacent to the lesion do not have an adequate energy budget as a result of the damage. This would also affect their ability to compete against organisms that colonize the site of the lesion, a distinct situation to the rapid regeneration rates characteristic of lesions due to physical injury of the colony. Therefore, we conclude that partial mortality in *A. palmata* affects the colony, inducing energetic stress due to both competition and decreased egg quality.

Keywords: Elkhorn coral, branch growth, developmental anomalies, lipid content, sexual reproduction, spawning

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *31 May 2018*
Accepted: *09 October 2018*
Published: *05 November 2018*

Citation:
Piñón-González VM and Banaszak AT (2018) Effects of Partial Mortality on Growth, Reproduction and Total Lipid Content in the Elkhorn Coral Acropora palmata. Front. Mar. Sci. 5:396. doi: 10.3389/fmars.2018.00396

INTRODUCTION

Coral reefs are complex formations created by the accumulation of calcium carbonate deposited by animals and algae. They are considered to be the marine ecosystems with the widest biological diversity on the planet (Bellwood and Hughes, 2001; Hughes et al., 2017). However, because they need strict conditions for their formation (Kleypas et al., 1999; Muir et al., 2015) they are restricted

to less than 0.1% of the ocean's surface (Spalding and Grenfell, 1997). Despite this, they provide important ecological and economic services (Bell et al., 2013; Cooper et al., 2014; Mitra and Zaman, 2015; Spalding et al., 2017). Hermatypic corals are primarily responsible for the formation of coral reefs (Cairns et al., 2009). For Caribbean reefs, *Acropora* spp., specifically *Acropora palmata* is of great importance due to their ability to withstand high energy conditions, high growth rates, ability to produce fragments and regenerate as well as their three-dimensional architecture, which provides habitat for many species (Lirman, 2002).

Acropora palmata, like most hermatypic corals, lives in a symbiotic relationship with a dinoflagellate alga called *Symbiodinium* that contributes to its host coral products that are derived from photosynthesis, in exchange for protection from external conditions and a constant supply of inorganic nutrients from the efficient recycling of the coral's metabolic waste (Davy et al., 2012; Pernice et al., 2012). The contribution of energy by the algae is more than the coral's daily requirement, so the surplus carbon, mainly glucose, is transformed into triglycerides and waxes to form large energy reserves (Oku et al., 2003). These energy reserves allow the coral to maintain its high productivity and sustain metabolic processes such as growth and sexual reproduction (Edmunds and Davies, 1986; Grottoli et al., 2004).

Lipids are key energetic biomolecules for hermatypic corals in particular, representing up to 40% of their dry weight (Harland et al., 1993; Oku et al., 2003; Imbs, 2013), although this percentage can vary in terms of environmental conditions such as light intensity and water temperature, as well as the coral's nutritional and health status (Harland et al., 1992; Oku et al., 2002). Under normal circumstances there is a balance between the energy that is produced and that which is consumed (Grottoli et al., 2004), however, under stressful conditions this balance can be broken, because the energy reserves, in addition to supporting growth and reproduction, are also the source of energy to combat the consequences of the stress (Seemann et al., 2013). Lipids represent up to 40% of coral tissue dry weight and, as such, are the primary energy reserve, therefore decreases or increases in the concentrations of total lipids are useful indicators of the use or accumulation, respectively, of these reserves (Stimson, 1987; Ward, 1995; Oku et al., 2002, 2003; Grottoli et al., 2004; Imbs, 2013; Lin et al., 2013; Conlan et al., 2017, 2018). For this reason, lipid concentrations are considered to be a good indicator of coral stress.

Corals can suffer injuries or lesions that are caused by events such as non-lethal diseases, predation, high or prolonged sedimentation or as a result of physical damage caused by various types of disturbances such as hurricanes or tropical storms (Lewis, 1997; Meesters et al., 1997; Wesseling et al., 2001; Cantin and Lough, 2014). Coral bleaching, whereby the symbiotic algae leave or are expelled from the coral tissue, can also result in lesions on the colony, especially if the bleaching event is prolonged (Gleason, 1993; Jones, 2008). Corals have mechanisms that respond immediately to an injury, which initiate the regeneration of the skeleton and tissue in the damaged area. The regeneration process consumes large amounts of energy, involving all the adjacent polyps up to 15 cm around the lesion (Oren et al., 2001; Klingenberg, 2008, 2014; Cocito and Sgorbini, 2014). On occasion, the regeneration process cannot cover the entire lesion, probably due to various factors such as the origin, shape, and size of the lesion relative to colony size, environmental conditions, and the high energy cost involved (Bak and Steward-Van Es, 1980; Oren et al., 1997, 2001; Denis et al., 2013). Consequently, the area becomes covered by colonizing organisms or sediment and the lesions become permanent, becoming what is known as partial mortality.

Corals are modular organisms and can survive partial tissue loss (Oren et al., 2001). However, because recovery from partial mortality represents a major expenditure of energy for the coral, in an effort to maintain colony integrity, it may interrupt other vital processes that require energy reserves such as reproduction and growth (Oren et al., 1997, 2001). Many studies have determined the effects of partial mortality on coral energy reserves focusing on reproduction (Kojis and Quinn, 1985; Rinkevich and Loya, 1989; Van Veghel and Bak, 1994; Oren et al., 2001; Graham and van Woesik, 2013; Riegl and Purkis, 2015), and growth (Bak, 1983; Fang et al., 1989; Guzmán et al., 1994; Meesters et al., 1994; Lirman, 2000; Alvarado and Acosta, 2009) while regeneration is active. To our knowledge, no studies have documented the effects, if any, once regeneration has stopped and partial mortality is permanent. This is relevant to the coral's energy budget if these lesions represent an area of high energy demand, i.e., the amount of lipids near the lesion is less than that in areas at a distance from the lesion. In this case, areas of partial mortality could continue to be a drain on the coral's energy and negatively affect other processes, such as growth and reproduction. Therefore, the objective of this contribution is to evaluate if there is a prolonged effect of partial mortality on the energy budget and its possible implications on branch growth and egg size, as well as embryonic development in the coral *A. palmata*, by comparing the growing edge of apparently healthy colonies to the borders of the lesions of colonies with partial mortality.

MATERIALS AND METHODS

Study Area

Coral tissue samples were collected from three sites, all located within the Puerto Morelos Reef National Park, Mexico (**Figure 1**). Growth measurements were also conducted in the same three sites whereas gamete bundles were collected only from Limones Reef. The northern site (20° 52′ 25.02″ N, 86° 51′ 4.67″ W) was located in Limones Reef, the central site (20° 55′ 4.01″ N, 86° 49′ 46.91″ W) was located in Cuevones Reef, and the southern site (20° 59′ 18.74″ N, 86° 47′ 26.66″ W) was located at "La Bocana" Reef. The sites were chosen based on the abundance of *A. palmata* and the presence of partial mortality (Banaszak and Álvarez-Filip, 2014).

The study was carried out prior to and during the *A. palmata* spawning season from March to August 2016 and again during the spawning season in 2017. A total of 16 colonies were used in the study: three colonies of apparently healthy *A. palmata* (AH) and three colonies with partial mortality (PM) were selected from

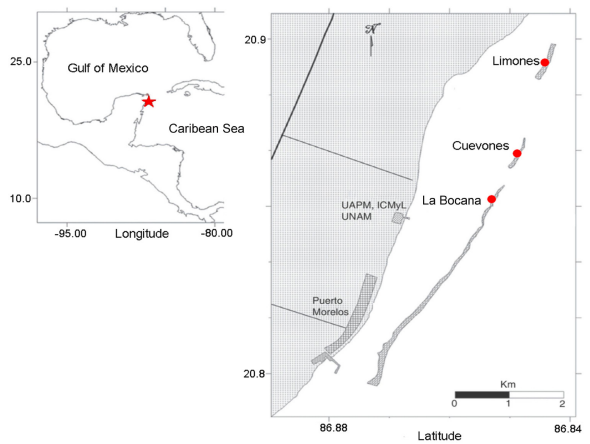

FIGURE 1 | The study area located in the Western Atlantic at Puerto Morelos Reef National Park, Mexico. The star denotes the location of the National Park and the circles show the location of the three study sties.

the Cuevones and Limones sites and two of each category from the La Bocana site, due to the smaller number of healthy colonies at that site. All colonies were tagged and ranged between 80 and 100 cm maximum diameter to ensure that the colonies were of reproductive size (Bruckner and Hourigan, 2000).

Growth Measurements

Three branches from each of the 16 *A. palmata* colonies were selected for the measurement of surface growth rates (defined as changes in surface area). A 1 cm diameter PVC disk was glued onto each of the branches at 5 cm from the apical limit to serve as a reference point (**Figure 2**). To document changes in surface area, ten photographs of each branch were taken at monthly intervals for four consecutive months using a Nikon Coolpix AW130 digital camera attached to an aluminum support that fitted over the disk. These photographs were used to calculate changes in the surface areas, from the disk to the growth edge, in branches unaffected by partial mortality as well as branches affected by partial mortality. Benthic composition and cover by organisms colonizing the lesion space were also documented from the photographs. The latter was based on the Caribbean Coastal Marine Productivity method (CARICOMP, 2001).

Developmental Anomalies

Gamete samples were collected only at Limones Reef due to logistical reasons. Specially designed collecting nets, approximately 1.5 m high and 90 cm in maximum diameter, were used to obtain gamete bundles. The bundles were transported to the laboratory where cross-fertilizations were carried out with the first samples taken 1 h after the first division was observed to calculate fertilization rates. Egg size was determined using Adobe Photoshop CS5 Extended v12.0 on digital photographs taken using an optical microscope (Leica DM500) at 100 times magnification. Further samples were taken every hour during the 4 h following the first embryonic division and then every 12 h until the planula larva stage was reached. The samples were photographed under an optical microscope (Leica DM500) at 100 times magnification. The anomalies present during development were evaluated and cataloged as malformations or delays in embryo development (López et al., 2012) at the four-cell stage.

Total Lipid Extraction and Determination

To determine the concentration of lipids in coral tissue, three samples of approximately 2 cm^2 each were taken. The first sample was taken from the branch edge and subsequent samples were taken at 1.5 cm intervals toward the branch base in AH individuals of *A. palmata*. For the colonies with PM, the first sample was taken at the edge of the lesion and at 1.5 cm intervals toward the edge of the branch. All samples were taken 11 days before the *A. palmata* spawning event. The samples were frozen immediately in liquid nitrogen and stored at −80°C for further analysis.

Total lipid extraction was carried out using the methods proposed by Harland et al. (1991) and modified by Harland et al. (1993) and Grottoli-Everett and Kuffner (1995) using the coral tissue samples stored at −80°C. The samples were measured,

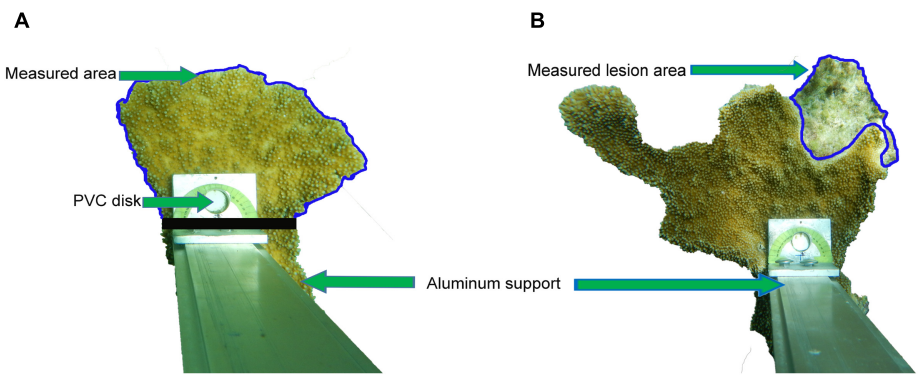

FIGURE 2 | The technique for the measurement of surface growth rates of *Acropora palmata* branches is shown. A camera was attached to an aluminum support that was fixed to a PVC disk. The blue outline indicates the area measured in an apparently healthy colony **(A)** and in **(B)** the blue outline indicates the measured area of a lesion in a colony with old partial mortality. The measured area of healthy tissue in colonies with old partial mortality were measured as in **(A)**.

photographed, and weighed before starting the extraction protocol. All the glassware used was washed two times with tap water, then with Milli-Q water and finally with chloroform before each extraction. A blank reference was run with each extraction cycle.

Data Analysis

Changes in surface area of each branch as well as the evaluation of anomalies and malformations in embryos were estimated using Adobe Photoshop CS5 Extended v12.0. All of the data were processed in R version 3.3.3 and normality and homoscedasticity tests were carried out to determine if the distribution of the data was normal. To compare monthly growth rates between AH and PM colonies a repeated measures ANOVA with Welch's correction was performed to compare the growth rates of both conditions (AH and PM) for each month using a repeated measures ANOVA test. This is a robust test for heteroscedastic data without the need to apply transformations (Derrick et al., 2016). We calculated growth rates of the individual branches rather than using an average value per colony to avoid over- or under-estimation (Weisburd and Britt, 2014) and thus obtain reliable growth values. Differences in the measured areas between the first (March–April) and the last month (June–July) of measurements, differences in the percentage of anomalies, changes in colonizing organism percent cover and branch growth, both together and separated by site, were analyzed using the nonparametric Wilcoxon test.

The percentage fertilization rates and comparison of egg volumes of eggs produced by AH and PM corals were analyzed using the Student's *t*-test with Welch's correction. A Wilcoxon test was performed to evaluate differences in the percentage of anomalies in developing embryos. Total lipid results for the three sites were grouped due to low sample numbers, to increase statistical power even though it was likely that variability would be higher due to differences between sites. The Student's *t*-test was used to analyze for differences in lipid concentrations between AH and PM colonies at each distance sampled (0–1 cm, 2.5–3.5 cm, and 5–6 cm). An ANOVA test with Welch's correction was used to compare lipid concentrations between distances for each condition. The data are expressed as median ± median absolute deviation (MAD) due to their wide variability. The data, including outliers, are shown in box and whisker plots where the horizontal lines within the boxes indicate the median, the boxes indicate the second and third quartiles, and the whiskers denote the first and fourth quartiles.

RESULTS

Area Occupied by Partial Mortality

The analysis of the 24 lesions on *A. palm*ata colonies showed that they were maintained over time, with no significant differences between the first (March–April) and last (June–July) month of sampling ($p = 0.190$, power test = 95%). The total area of partial mortality per colony branch was 49.2 cm^2 ± 4.45 in March–April 2016, which reduced to 38.8 cm^2 ± 12.6 by June–July of the same year, however, the difference was not statistically significant ($p = 0.190$). The variability in the areas occupied by lesions was greater in June than in March.

Colonization of Lesions

The composition of colonizing organisms that occupied the areas of partial mortality in the *A. palmata* branches changed (**Figure 3**), although there were no statistically significant differences in percent cover ($p > 0.05$, power test ≥ 75%) over the four-month sampling period. The percent cover occupied by the dominant algal group presented trends that were not statistically significant. Turf algae decreased from 50.5% ± 10.5 to 39.3% ± 5.25 with a concurrent increase in fleshy macroalgae from 10% ± 7.5 to 20.25% ± 18.7. Percent cover occupied by sand did not change over the four-month period.

Branch Growth Rates

The variations in the surface growth rates of the branches were high at all sites (**Figure 4**). The lowest positive daily growth rate

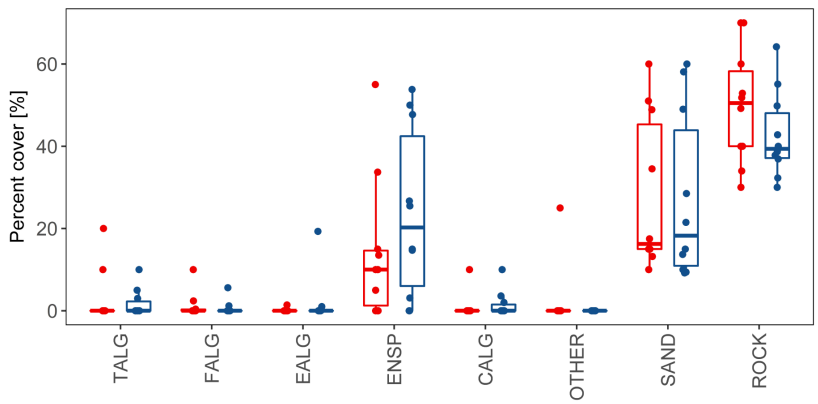

FIGURE 3 | Percent cover of the categories of organisms that colonized or non-biological material that covered the areas of partial mortality in branches of the coral *A. palmata* during the months of March (red bars) and June (blue bars), 2016. Groupings: TALG, Turf algae; EALG, Encrusting algae; ENSP, Encrusting sponges; CALG, Calcareous algae; FALG, Fleshy macroalgae; SAND, Sand; Substrates: ROCK, Rock; OTHER, Other.

recorded during the four-month period was 0.01 cm^2 day^{-1}, registered in Limones Reef in May–June, whereas the highest was daily growth rate was seventy times higher at 0.7 cm^2 day^{-1} in Limones Reef in April–May. Negative growth rates are due to the breakage of the branch during the previous month. Due to these large variations, we considered it more appropriate to analyse growth rates of the individual branches rather than using an average value per colony.

Analysis of the growth rates at each site (**Figures 5A–C**) indicates that there were no significant differences between the AH branches and the branches with PM, for the four-month period ($p > 0.05$) at any of the sites. However, significant monthly variation was found in the growth rates for both AH and PM colonies ($p < 0.05$, power test $\geq$ 95%) in Limones Reef between the months of April–May (0.14 cm^2 day^{-1} $\pm$ 0.4) when compared with March–April (0.31 cm^2 day^{-1} $\pm$ 0.8) for AH colonies, as well as for the months of March–April (0.43 cm^2 day^{-1} $\pm$ 0.13) compared with June–July (0.16 cm^2 day^{-1} $\pm$ 0.17) and April–May (0.08 cm^2 day^{-1} $\pm$ 0.12). For colonies with PM, differences were found ($P < 0.05$) between May–June (0.32 cm^2 day^{-1} $\pm$ 0.09) and April–May (0.08 cm^2 day^{-1} $\pm$ 0.12). At Cuevones Reef, only the AH branches showed differences ($p = 0.006$) between the months of June–July (0.11 cm^2 day^{-1} $\pm$ 0.02) when compared to May–June (0.32 cm^2 day^{-1} $\pm$ 0.09). At La Bocana Reef, over the four-month period, there were no statistically significant changes detected. Finally, there were no significant differences in growth rates of AH branches relative to the branches with PM for any site. However, there is high variation in the growth rates of the branches over time.

Gamete Quality

The first two-celled embryos were observed at 2.5 h after fertilization of the gametes collected from both AH colonies and colonies with PM. Fertilization rates were not significantly different ($p > 0.05$, power test = 89%) for samples collected from colonies with PM (median of 80.75% $\pm$ 0.54) when compared to samples from AH colonies (median of 71.45% $\pm$ 6.46). The fertilization percentage of AH corals shows a much wider variability in comparison to that observed in corals with PM (**Figure 6A**). The percentage of anomalies (**Figure 6B**), were variable and did not present significant differences ($p > 0.05$, power test = 35%) between the eggs of AH colonies (median of 14.98% $\pm$ 6.04) and those from colonies with PM (median of 15.55% $\pm$ 7.63). In terms of egg volume, the median volume of the eggs produced by AH colonies was 0.026 mm^3 $\pm$ 0.005, while those produced by colonies with PM was 0.021 mm^3 $\pm$ 0.005 (**Figure 7**). The eggs from AH colonies were significantly larger than those from colonies with PM ($p < 0.05$, power test = 99%).

Lipids in Tissues

The results of the total lipid analysis (**Figure 8**) showed that there were no significant differences in the amount of lipids between the branches with PM and the AH branches ($p > 0.05$, power test $\geq$ 30%). Of the colonies that were sampled, there are no significant differences in the amount of lipids at the lesion borders in colonies with PM when compared with the growth edges of AH colonies. This pattern holds for at least a 5 cm distance from the lesions in colonies with PM. On the other hand, the amount of lipids did vary significantly between sampling distances, both in the AH branches ($p = 0.013$, power test = 99%) and in the branches with PM ($p = 0.01$, power test = 99%). The *post hoc* multi-comparison test, after the ANOVA with Welch's correction, showed that the coral tissue at distances of 2.5–3.5 cm and 5–6 cm from the edge did not contain significant differences ($p > 0.05$) in the amounts of total lipids. This held true for both conditions of colonies: AH and PM. However, at a distance of 0–1 cm

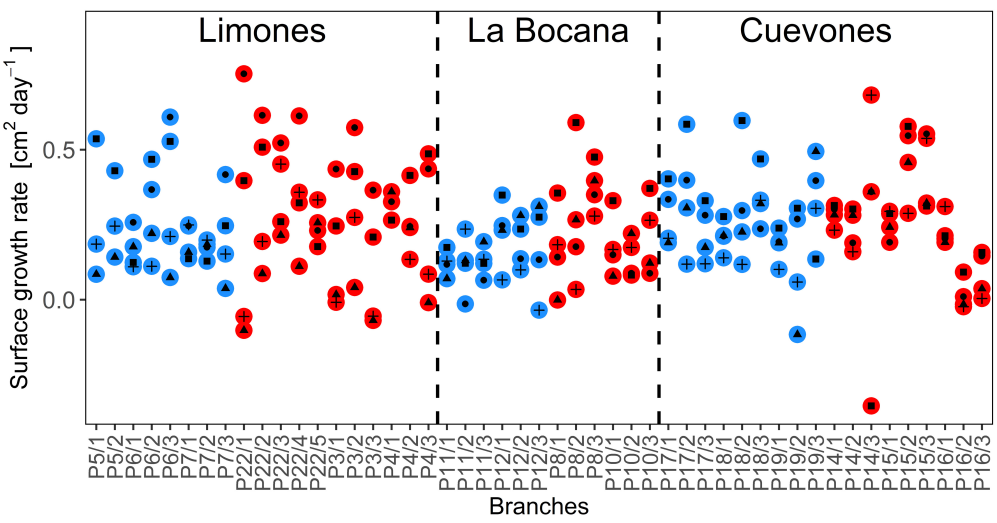

FIGURE 4 | The surface growth rates (cm^2 day^{-1}) of each branch that was measured at the three sampling sites during the 4 months of the study, for apparently healthy (AH, blue circles) colonies and those with partial mortality (PM, red circles). Within the colored circles, black circles represent the rates for March, triangles for April, squares for May, and plus signs for June.

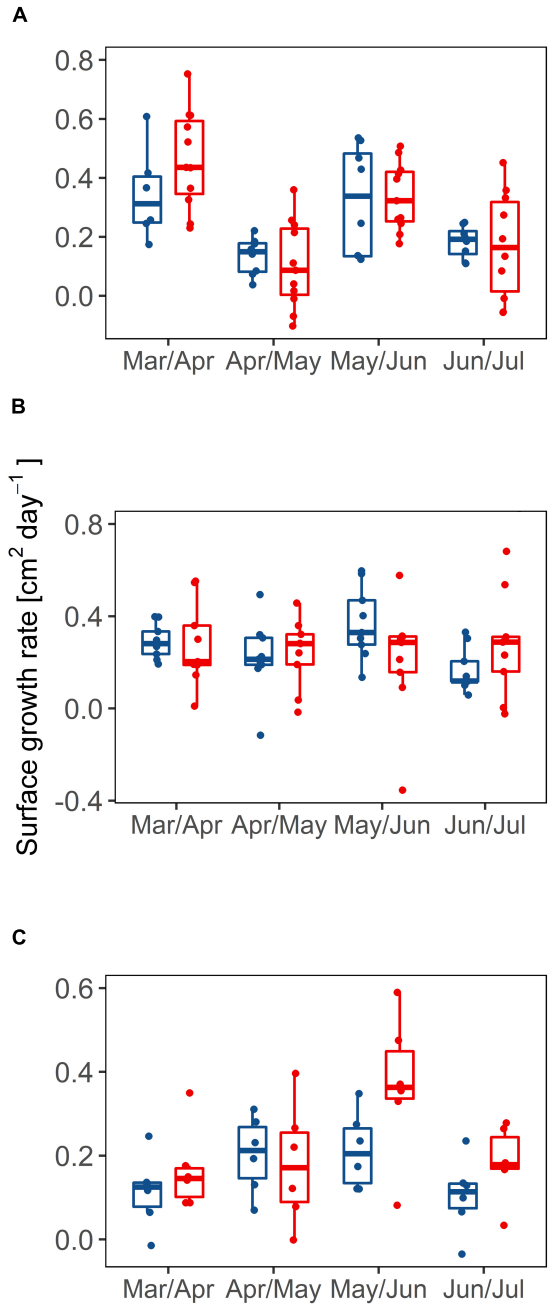

FIGURE 5 | Surface growth rates (cm^2 day^{-1}) for branches measured in Limones Reef **(A)**, Cuevones Reef **(B)** and La Bocana Reef **(C)**. Shown are growth rates of branches with partial mortality (PM, red bars) vs. apparently healthy branches (AH, blue bars) throughout the months of March to June, 2016.

from the growth edge in AH colonies and the lesion border for colonies with PM, there are significantly lower concentrations of lipids with respect to the other two distances ($p < 0.05$). The results show clear evidence of a low energy zone at the growth edge for AH colonies and at the lesion border for colonies with PM. From at least 2.5 to 3.5 cm distance from the growth edge of AH colonies and lesion border of colonies with PM, lipid concentrations increase.

DISCUSSION

Partial mortality in *A. palmata* colonies has a negative effect on their energy budget as shown by the results obtained in this study. The negative effects were detected in colonies with old partial mortality, where the lesions were completely colonized by other species or covered in sediment. Certain aspects of the reproduction biology of *A. palmata* were affected by the presence of old partial mortality, whereas colony branch growth appeared to be unaffected.

All studies to date, on the effects of partial mortality on reproduction and growth have only focused on the first stages of the injury or lesion when regeneration tends to be accelerated. Reported results differ widely ranging from a reduction in reproductive activity in *Stylophora pistillata* (Rinkevich and Loya, 1989) and massive *Porites* spp. (Welsh et al., 2015) and fecundity in *Goniastrea favulus* (Kojis and Quinn, 1985), to cases where the effects are localized and only affected up to a 15 cm radius around injured areas in *Favia favus* (Oren et al., 2001). Negative effects also vary depending on the morphotype of colonies of *Montastrea annularis* (Van Veghel and Bak, 1994). For *A. palmata* it has been reported that injured colonies completely stop gamete production (Lirman, 2000).

The area occupied by partial mortality in individuals of *A. palmata* did not change during the period of study, designed to coincide with the months leading up to the summer spawning season. No significant changes were found between the months of March–April and June–July (**Figures 5A–C**). This is likely explained by the fact that regeneration is a gradual process that decreases with time reaching the point where it stops, even when the injury has not completely regenerated. The most accepted explanation for this fact is that regeneration is stopped in order to not continue with the excessive energy expenditure caused by this process (Oren et al., 1997, 2001). However, the lipid results obtained in this study contradict this explanation because they show that even after the regeneration process was stopped, the lesions continue to represent areas of high energy demand, as reflected by the significantly lower lipid concentrations at the borders of the lesions (**Figure 8**).

Furthermore, our results show that the amount of lipids at the borders of the lesions does not differ from the lipid concentrations at the edges of maximum growth of the *A. palmata* branches. This would indicate that large amounts of energy are being consumed near the borders of the partial mortality, which is similar to the expense that supports the accelerated growth in the tips of the branches in *A. palmata*. Despite the possibility that there are variations in physico-chemical properties between the three sites, the edges of the zones with partial mortality consistently exhibited lower lipid concentrations (**Figure 8**) even when the data are separated by site.

Based on our results, we infer that the explanation for the arrest or reduction of regeneration and the low amount of lipids

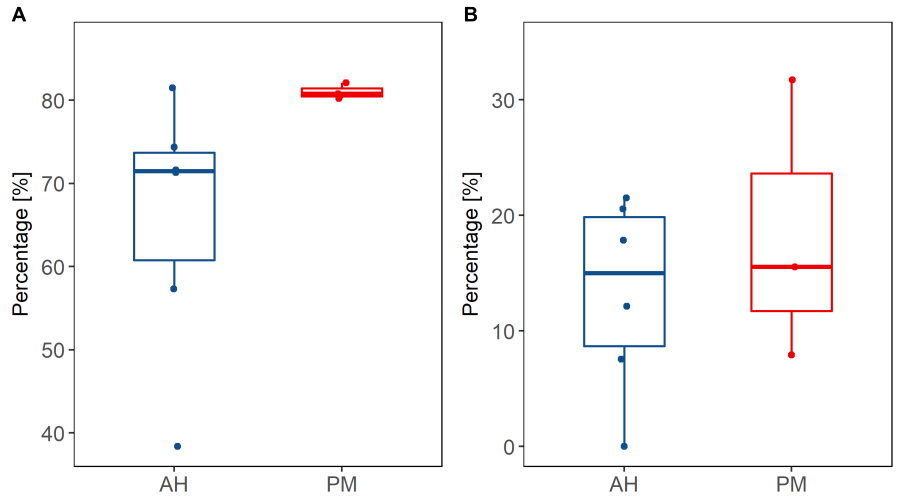

FIGURE 6 | Percentage fertilization **(A)** and percentage of anomalies **(B)** of gametes collected from apparently healthy (blue bars) colonies vs. those obtained from colonies with partial mortality (red bars), collected from the Limones site.

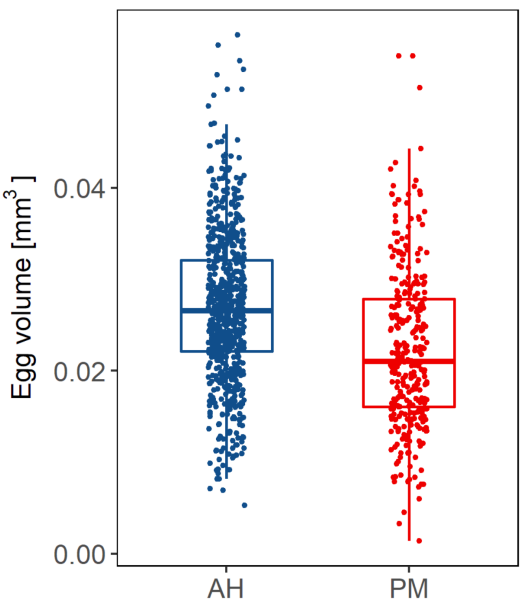

FIGURE 7 | Volume of eggs produced by apparently healthy (blue bars) colonies vs. colonies with partial mortality (red bars) from the Limones site, collected during the spawning season in 2017.

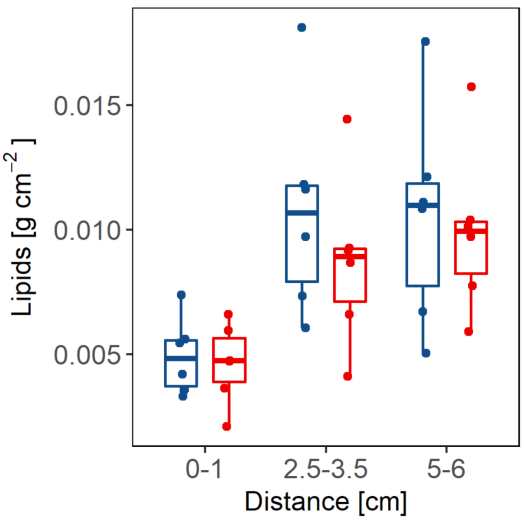

FIGURE 8 | The concentration of lipids (g) per tissue area (cm^2) is shown at different distances (cm) from the border of the lesion in branches with partial mortality (red bars) and growth edge in apparently healthy (blue bars) branches.

at the edges of the lesions (**Figure 8**) is produced by a possible competitive relationship induced by the organisms that colonize the injured areas, rather than a mechanism to stop energy expenditure. The space exposed by the lesions, as analyzed in this study, was dominated by filamentous algae (38%) and fleshy algae (20%), which are organisms that are characterized by engaging in competitive relationships with coral species by activating chemical mechanisms that allow them to maintain the space they have gained and, if possible, to overgrow the coral. Their very presence represents a physical obstacle that prevents the adequate regeneration of injured areas (Titlyanov et al., 2005, 2006) and promotes the accumulation of organic and inorganic sediment that in turn provides nutrients and support for algal development (Nugues and Roberts, 2003; Titlyanov and Titlyanova, 2008). This coincides with our results, because sediment, in addition to the algae, is the substrate that dominates the lesions with a median of around 20% (**Figure 3**). Therefore, the coral needs to activate defensive mechanisms, which require significant amounts of energy, and which would explain the results of the low amount of lipids at the borders of the lesions.

On the other hand, Oren et al. (1997, 2001) also mentions that excessive energy expenditure such as that which occurs when regeneration is active can affect other processes such as reproduction and growth since they depend on the same reserves. This energy expenditure has been estimated at about 33% of the total available energy in the coral *Porites porites* (Edmunds and Davies, 1986). Such a sizeable energy expenditure would affect processes such as growth and reproduction. Therefore, taking into account our results where it is shown that old lesions continue to be large energy sinks, one would expect that these negative effects would continue even after the regeneration stopped. However, our results showed that growth is not affected in corals with partial mortality in any of the three sites sampled (**Figure 4**), which is unusual and differs from previous studies. For example, Meesters et al. (1994) found that lesions in *Montastrea annularis* reduced the growth capacity of the coral even after regeneration was stopped. Bak (1983) found that branches of *A. palmata* that presented lesions that were within 25 cm of the growth edge of the branch had considerably lower linear extension rates. However, if the lesion was located at more than 25 cm from the growth edge, there was no negative effect on its growth (Bak, 1983). This is a plausible explanation for the results obtained in this work since the position of the lesions analyzed were greater than 25 cm from the growth edge.

As seen from the results presented here, the presence of old partial mortality did affect some aspects of the reproductive process in *A. palmata* colonies. These colonies were capable of producing and releasing gametes. However, the volume of the eggs released in gamete bundles by corals suffering from partial mortality was significantly lower than eggs released by corals that were apparently healthy (p = 8.52e−15). The eggs were almost 20% lower in terms of volume. This result could be due to the energy imbalance produced by partial mortality on the coral such that the eggs from colonies suffering from old injuries have lower lipid contents. These eggs with lower energy reserves would be at a disadvantage during embryonic development and long-distance dispersal in comparison to eggs produced by apparently healthy colonies with larger reserves. Decreased energy reserves have also been found in adult corals that have previously bleached (Jones and Berkelmans, 2011). Bleached colonies produce fewer eggs with lower lipid levels compared to healthy colonies (Ward et al., 2000; Sudek et al., 2012).

The difference in egg size did not affect the ability to be fertilized (**Figure 6A**). In fact, fertilization percentage was on average higher and much less variable in colonies with signs of partial mortality than the fertilization percentage from apparently healthy colonies. Another process that was not affected by the volume of the eggs was abnormalities in the developing embryos (**Figure 6B**). This would indicate then, that while smaller, the eggs produced by colonies suffering from partial mortality were of equivalent quality to the eggs produced by apparently healthy colonies. However, small egg size may have post-developmental repercussions such as reduced dispersal distances, ability to settle and colony growth.

With the results of this work we can conclude that partial mortality does affect the coral *A. palmata* and the negative effect is observed mainly as a constant energy expenditure, due to the process of competition against the colonizing organisms occupying the injured area, which in turn causes a decrease in the common energy reserves for other processes. Although the results show no negative effects on growth, we did detect a significant effect on the reproductive capacity of corals, specifically in the volume of the eggs, however, due to the low sample size used here and the large variability of this process more studies are needed to confirm these results. Finally, it is important to bear in mind that partial mortality is a condition that remains over time, consuming large amounts of energy and possibly weakening the coral, so that it is very likely that its resistance will decrease in the face of future stress events.

AUTHOR CONTRIBUTIONS

VP-G and AB developed the concept for this study and wrote the manuscript. VP-G conducted the field work, collected the data, and undertook statistical analyses.

FUNDING

This study was funded by project number 608 from the Instituto de Ciencias del Mar y Limnología, Universidad National Autónoma de México as well as project numbers 153260 and 247765 from the Consejo Nacional de Ciencia y Tecnología (Mexico) to AB. VP-G was supported by a postgraduate fellowship from the Consejo Nacional de Ciencia y Tecnología (Mexico).

ACKNOWLEDGMENTS

We thank Dr. Andrea Grottoli for providing helpful advice and protocols on lipid extractions.

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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

ORIGINAL RESEARCH
published: 06 November 2018
doi: 10.3389/fmars.2018.00391

Host Differentiation and Compartmentalization of Microbial Communities in the Azooxanthellate Cupcorals *Tubastrea coccinea* and *Rhizopsammia goesi* in the Caribbean

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Edited by:
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: 01 June 2018
Accepted: 08 October 2018
Published: 06 November 2018

Citation:
Engelen AH, Aires T, Vermeij MJA, Herndl GJ, Serrão EA and Frade PR (2018) Host Differentiation and Compartmentalization of Microbial Communities in the Azooxanthellate Cupcorals Tubastrea coccinea and Rhizopsammia goesi in the Caribbean. Front. Mar. Sci. 5:391. doi: 10.3389/fmars.2018.00391

We investigated the microbial communities associated with surface mucus layer, tissue, and gastrovascular cavity of two azooxanthellate Caribbean cup corals (*Tubastrea coccinea* and *Rhizopsammia goesi*) to explore potential differences in microbial community composition within and among these azooxanthellate scleractinian corals. Using next-generation sequencing of the V3–V6 region of the 16S rRNA gene we found that while alpha-diversity was overall very similar, the relative abundance of microbial taxa differed between host species and among locations within a polyp (i.e., compartments). The interspecific differentiation of microbial assemblages is only challenged by the relatively high similarity among mucus samples of both species. This suggests a stronger signal of the surrounding environment and weaker host control over the mucus compartment compared with the tissue and gastrovascular cavity. *T. coccinea* harbored four indicator OTUs (including a *Pseudoalteromonas* species, an unidentified *Gammaproteobacteria*, an unidentified OTU in the family *Comamonadaceae* and one in the genus *Burkholderia*). The single indicator for *R. goesi* was another undetermined OTU in the *Comamonadaceae*. The microbial communities of the gastrovascular cavity and the mucus overlapped substantially in indicator OTUs. None of these were exclusive of the gastrovascular cavity or mucus, while an OTU of the order *Thiohalorhabdales* occurred uniquely in the tissue. In contrast to the gastrovascular cavity and mucus, the tissue of both coral species was rich in chloroplasts of different algal taxa (mainly *Ulvophyceae* and *Stramenopiles*), and an OTU of the genus *Roseivirga* (family *Flammeovirgaceae*). The two coral species shared most indicator OTUs for microbial communities residing in their mucus and tissue, but not in their gastrovascular cavities. However, *Endozoicomonadaceae* occurred in the tissue of both coral species.

The genus *Pseudomonas* was found in *R. goesi* but was virtually absent in *T. coccinea*. This study demonstrates the influence of coral compartments and species identities on the composition of microbial communities associated with azooxanthellate cup corals and emphasizes the important effects of within-polyp microhabitats in structuring the coral microbiome.

Keywords: *Tubastrea coccinea*, microbiome, mucus, gastrovascular cavity, tissue, *Rhizopsammia goesi*, Curaçao

INTRODUCTION

As foundation organisms, corals form the ecological and constructive base of coral reefs, which are amongst the most productive marine ecosystems on the planet. The biology of corals has been progressively better understood from a holobiont perspective (Rohwer et al., 2002), that considers their associated microorganisms as integral determinants of the health and survival of coral colonies (Bourne et al., 2016). Historically, most research on microorganisms residing in corals was focused on endosymbiotic dinoflagellates of the family Symbiodiniaceae (zooxanthellae). More recently, the role of prokaryotic members of the coral microbiome has been increasingly studied. Members of the Alpha- and Gammaproteobacteria, Actinobacteria, Bacteroidetes, and Cyanobacteria appear to dominate coral prokaryotic microbiomes (Ainsworth et al., 2015; Bourne et al., 2016). In general, the microbial communities of corals are commonly host species-specific but also exhibit variation across biogeographic regions and environmental conditions (Hernandez-Agreda et al., 2016; Glasl et al., 2017). While at the scale of the colony, coral microbiomes are generally stable over time in the absence of major environmental change, this does not imply that their composition is uniformly distributed within individual coral polyps.

The mucus, tissue, gastrovascular cavity, and skeleton all form distinct specific micro-habitats, that can harbor different microbial communities providing various benefits to their coral host (Agostini et al., 2012; Ainsworth et al., 2015; Marcelino and Verbruggen, 2016). For example, microbes residing in the surface mucus layer of corals (e.g., Photobacterium, Endozoicimonaceae, and Firmicutes) can provide nutritional benefits to their hosts (Wild et al., 2004), protect them against pathogens through the production of antibiotics and/or occupation of specific niches (Ritchie, 2006; Glasl et al., 2016) and reduce the damaging effects of ultraviolet radiation (Ravindran et al., 2013). Members of the microbial Actinobacteria, Ralstonia, and Endozoicomonas are commonly found in coral tissue (Bayer et al., 2013; Ainsworth et al., 2015; Neave et al., 2016, 2017). Similar to the human gut, the microbiome in the gastrovascular cavity of a coral is thought to catabolize ingested organic matter, recycle nutrients and provide vitamins to their hosts (Agostini et al., 2012). Coral skeletons contain endolithic microalgae (Shashar et al., 1997; Marcelino and Verbruggen, 2016), bacteria (Yuen et al., 2013) and fungi (Bentis et al., 2000), of which some are considered beneficial to the coral host. For example, bleached corals can benefit from the presence of the endolithic alga *Ostreobium* that provides food to corals in the form of photosynthates after the algal endosymbionts have become expelled from the corals in response to elevated water temperatures (Fine and Loya, 2002).

Reef-building scleractinian corals and their microbiomes have been fairly well studied, particularly the endosymbiotic photoautotrophic community of eukaryotic microbes in the family Symbiodiniaceae (e.g., Kirk and Weis, 2016). Only rare studies, however, have focused on the microbiomes of azooxanthellate scleractinian corals, even though these corals account for one-third of all contemporary scleractinian species (Cairns, 2007; Roberts et al., 2009). Because azooxanthellate corals are the most basal extant scleractinians (Kitahara et al., 2010) the relationships they establish with microbes could be of interest to provide insights into the evolution of coral holobiont composition and functioning. Azooxanthellate corals obtain their energy exclusively through heterotrophic feeding and likely this has consequences for the lifestyle of their associated prokaryotic communities (Littman et al., 2010; Brener-Raffalli et al., 2018). Compared to zooxanthellate corals, azooxanthellate corals are characterized by larger distribution ranges, being found in all oceans from Antarctica (Cairns, 1982) to the Arctic (Roberts et al., 2009). Azooxanthellate corals also inhabit a wide spectrum of habitats, from shallow tropical waters to depths beyond 6,300 m (Keller, 1976), and they can even thrive under temperatures as low as −1°C (Vaughan and Wells, 1943). Due to their wide environmental tolerances as compared with zooxanthellate corals, azooxanthellate corals are capable of invading new areas relatively easily as exemplified by the relatively recent invasion of the Southwestern Atlantic by the Pacific species *Tubastraea coccinea* and *T. tagusensis* (Cairns, 2000; Creed et al., 2017).

In this study, we test the hypothesis that different polyp compartments (i.e., different functional areas or microhabitats within a coral polyp) of two azooxanthellate coral species harbor different microbial communities and that these compartment differences are pervasive across host species. To test this hypothesis, samples were collected from the surface mucus layer, tissue, and gastrovascular cavity of two common azooxanthellate sun corals in the Caribbean (*T. coccinea* and *Rhizopsammia goesi*) that co-occur on the fringing reefs around the island of Curaçao in the Southern Caribbean. *T. coccinea* is an invasive species whose expansion throughout the Caribbean has been well documented (Cairns, 2000; Creed et al., 2017), whereas *R. goesi*, a species invading more exposed areas on the reef, is an Atlantic species that is currently increasing in abundance

around the island of Curaçao (Vermeij and Engelen pers. observ.).

MATERIALS AND METHODS

Field Collections

Samples of *T. coccinea* (Lesson, 1830) and *R. goesi* (Lindström, 1877) (**Supplementary Figure S1**) were collected from approximately 20 m depth between February 1st and 8th in 2014 from the reef near the Holiday Beach Hotel in Curaçao (12.106582°, −68.948753°). In total thirty specimens were sampled from an area of ca. 50 m × 50 m. For each specimen three different compartments (within a polyp) were sampled *in situ* using different techniques; (1) the surface mucus layer was sampled by gently rolling a sterile cotton swab over the polyp's surface; (2) the contents of the gastrovascular cavity were sampled using a 1 ml syringe with blunt needle to avoid perforation of the epithelia resulting in a sample volume between 200 and 900 μL, which likely also included some surrounding seawater; finally, (3) the whole polyp was removed with a spatula to sample the microbiome associated with the cupcoral tissue and for host identification (via corallite characteristics and DNA barcoding). Reference sediment samples (n = 6) and ambient seawater (n = 2) were taken from the vicinity of the sampled corals. Sediment was collected from the superficial layer into 2 mL tubes whereas each seawater sample consisted of 2 L collected in Plexiglas cylinders. Whole polyps were then rinsed in the laboratory with 0.22-μm-filtered seawater to remove loosely attached microbes. Coral and sediment samples were flash-frozen in liquid nitrogen within 30 min after collection and stored at −80°C until further processing. Seawater samples were filtered onto 0.22-μm polycarbonate filters (GTTP, Millipore) and flash-frozen within 2 h after collection. When polyps were thawed (preceding DNA extraction) they were rinsed with milliQ to remove as much mucus as possible and reduce carry over of mucus microbes into the tissue samples. Research on Curaçao was performed under the annual research permit (48584) issued by the Curaçao Ministry of Health, Environment and Nature (GMN) to the CARMABI Foundation.

DNA Extraction and Sequencing

DNA was extracted from each individual sample using the FastDNA SPIN Kit for Soil (MP Biomedicals) following the manufacturer's protocol. To sample the microbiome of the tissue compartment, a small portion of the coenosarc of each sample was removed with a scalpel blade and used for DNA extractions, avoiding the inclusion of possible mucus residue remaining on the sample. To identify the sampled cup corals to species level, their 28S rRNA gene was sequenced after amplification using the primers C1′ (5′–ACC CGC TGA ATT TAA GCA T–3′) and D2MAD (5′–GAC GAT CGA TTT GCA CGT CA–3′) and following PCR conditions as in Cuif et al. (2003). This method produced 861-bp fragments, blasted against the NCBI database and submitted there under accession numbers[1]: X. *R. goesi* could not be identified molecularly due to lack of sequence data in public databases. We therefor rely on the visually identification by Bert Hoeksema (Naturalis Biodiversity Center, Netherlands; see Hoeksema and ten Hove, 2017).

To identify the microbiome of aforementioned samples, DNA extracts of 22 samples (i.e., n = 3 per compartment per host species, plus n = 2 for each reference habitat: sediment and seawater) were sent to the IMGM laboratories (Planegg, Germany) for sequencing. 728-bp fragments with variable regions V3–V6 of the archaeal and bacterial 16S rRNA gene were sequenced using 454 GL FLX+ technology (Roche) after PCR amplification using primers U341F (5′- CCT ACG GRA GGC AGC AG. -3′) and U1053R (5′- CTG ACG RCR GCC ATG C -3′) adapted by IMGM laboratories from Wang and Qian (2009). Each 25 μL of PCR reaction consisted of 1 × Taq buffer, 3 mM $MgCl_2$, 2 mM (of each) dNTP, 0.2 mg mL^{-1} BSA, 0.25 μM of each primer, 0.03U μL^{-1} of Taq DNA Polymerase (Fermentas) and 1–2 μL of template DNA. Cycling conditions consisted of 4 min at 95°C for initial denaturation, followed by 30 cycles performed at 95°C for 30 s, 65°C for 45 s and 72°C for 90 s. A final elongation step at 72°C for 10 min completed each reaction. PCR products were evaluated through electrophoresis on 1.5% agarose gels. The 22 samples sent for sequencing included two sediment and two seawater samples, plus 18 cup coral samples, representing three individual specimens for each of the two cup coral species comprising the three host compartments within each specimen.

Microbiome Analyses

Sequencing reads were demultiplexed and denoised in QIIME (version 1.9.0; Caporaso et al., 2010a) using a sliding window test of quality scores and a minimum Phred score of 25, after which primers were removed. Chimeric sequences were also removed using usearch61 (Edgar, 2017) with both *de novo* and reference-based detection as implemented in QIIME. A total of 135,524 reads with an average length of 620-bp passed all quality filtering steps and were successfully clustered into OTUs using a closed reference OTU picking method based on a 97% similarity cut-off value excluding singletons. Representative OTUs were picked and aligned with PyNAST (version 1.2.2; Caporaso et al., 2010b) against the Greengenes database (version 13.5), and taxonomy assigned using uclust as implemented in QIIME. Rare OTUs (comprising less than 0.005% of all sequences) were removed because they are often associated with spurious sequencing reads (Bokulich et al., 2013).

Analyses of alpha- and beta-diversity of retrieved microbial communities were performed on relative abundance data at the OTU level using the R packages "vegan," "labdsv," "MASS," "cluster," "indicspecies," "permute," "dplyr," "tidyr," "ggplot2," and "RColorBrewer" (R Development Core Team, 2013). In order to correct for varying sequencing efforts, data were rarefied to the minimum number of reads: 789 sequences. Samples had on average 6558 reads (range: 789–16,105). OTU richness and Shannon-Weaver diversity were compared amongst samples using Analysis of Variance (ANOVA). Non-metric Multidimensional Scaling ordination (nMDS, after 9999 permutations) was used to visualize the variation in community

[1]https://figshare.com/s/ffa8955c44ad93ce2023

structure amongst habitats, host species, and host compartments, based on Bray-Curtis dissimilarities among samples. The null hypothesis of no difference in the microbial community structure among sampling groups was tested using Permutational Multivariate Analysis of Variance (PERMANOVA; 9999 permutations), after the homogeneity of multivariate dispersion was confirmed with PERMDISP, a betadispersion measure. These were computed, respectively, with the adonis and betadisp functions in the "vegan" R package. Indicator Values analysis (IndVal) was used to identify the OTUs significantly associated with the different habitats, hosts and compartments, or groups thereof, based on their specificity and fidelity to particular sampling group(s) (De Cáceres and Legendre, 2009). Specificity is the probability that the OTU belongs to that particular sampling group and not to other groups, whereas fidelity is the probability of finding the OTU in the different samples of that sampling group. OTUs are considered significantly associated ($p < 0.05$) when both specificity and fidelity have probabilities above 0.5. This approach has in the past been used to identify microbial indicators of particular coral compartments (Li et al., 2014), particular depth habitats on a coral reef slope (Glasl et al., 2017) or particular coral health states after environmental disturbance (Glasl et al., 2016).

All demultiplexed 16S rRNA gene raw reads and sampling metadata are available in the NCBI Sequence Read Archive database[2] under BioProject accession number PRJNA494429.

RESULTS

A total of 131,173 reads clustering into 1040 non-spurious OTUs were included in our statistical analyses. Two of the three samples originating from the gastrovascular cavity of *T. coccinea* had only one and ten reads, respectively, and were therefore removed from the analyses. Although sample size precluded making statistical comparisons that include the sediment and seawater samples ($n = 2$ for each), there were no obvious differences in OTU richness among sediment (121–124 OTUs), seawater (122–125 OTUs) and the coral samples (mean: 127 OTUs, SD: 38). OTU richness was similar between the two cup coral species (OTUs; $F_{(14,1)} = 0.699$, $p = 0.417$) and compartments (OTUs; $F_{(13,2)} = 0.053$, $p = 0.949$). Shannon-Weaver diversity of all samples followed the same pattern, with values of 3.39–3.55 for sediment and 3.49–3.51 for seawater, and 3.59 ± 0.56 for the coral samples. Diversity did not differ between species ($F_{(14,1)} = 1.408$, $p = 0.255$) or among host compartments ($F_{(13,2)} = 0.503$, $p = 0.616$). Therefore, the number of OTUs identified within each sample cannot explain potential differences in beta-diversity (addressed below).

Microbial community structure differed between both coral species (PERMANOVA, pseudo $F_{(12,1)} = 1.694$, $p = 0.040$) and among compartments (pseudo $F_{(12,2)} = 1.557$, $p = 0.032$, **Figure 1**). Interaction between host species and compartment was not significant ($F_{(10,2)} = 1.486$, $p = 0.076$). Multivariate dispersions were homogeneous between host species (PERMDISP $F_{(14,1)} = 2.962$, $p = 0.107$), and among compartments ($F_{(13,2)} = 0.840$, $p = 0.454$). Microbial community structure of the mucus partly overlapped with both the gastrovascular and tissue community (**Figure 1**), however, the latter two compartments were more dissimilar from one another than they were from mucus. The gastrovascular cavity of *T. coccinea* appears to possess a unique microbiome, though this is based on only one sample (**Figure 1**), making it impossible to perform statistical comparisons including this sampling group. Samples from the gastrovascular cavity of *T. coccinea* consistently yielded low number of reads, suggesting that a low microbial biomass in this compartment could have resulted in the observed unsuccessful sequencing of its microbiome. Community analysis using presence/absence data also showed differentiation among the cup corals and compartments as described above based on abundance distribution data.

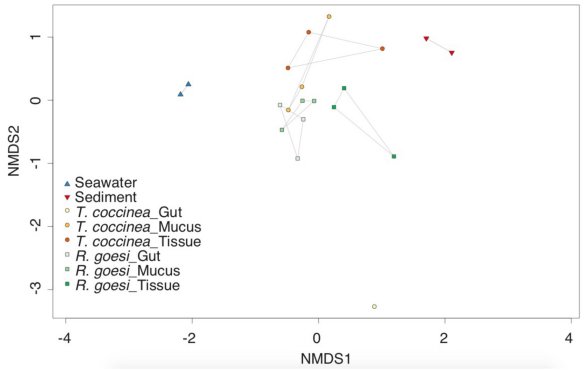

FIGURE 1 | Two-dimensional non-metric multidimensional scaling (nMDS) ordination depicting variation in microbial community structure among the cup corals *Rhizopsammia goesi* and *Tubastrea coccinea* across polyp compartment: surface mucus layer, tissue, and gastrovascular cavity (or "gut"). Seawater and sediment samples added as reference to all plots. Polygons delimitate the distribution of each sampling group within the nMDS.

Microbiomes were very diverse in each species vs. compartment combination and the 20 most abundant OTUs accounted for about half of the reads across all samples (44% on average; **Figure 2**). Seawater was dominated by a range of distinct OTUs belonging to the family *Pelagibacteraceae*, as well as the genera *Synechococcus* and *Prochlorococcus* (family *Synechococcaceae*). Chloroplast DNA-derived and Cyanobacteria dominated sediment microbiomes, whereas cup coral samples were characterized by several OTUs of the genus *Ralstonia*, one OTU of the genus *Cupriavidus* (both in the family *Oxalobacteraceae*) and several OTUs from the family *Comamonadaceae*. The single sample of the gastrovascular cavity of *T. coccinea* had a quite unique microbiome, which was dominated by many evenly abundant OTUs (**Figure 2B**) from the genus *Hymenobacter* (family *Cytophagaceae*), the families *Syntrophobacteracea* and *Rhyzobiaceae*, and an unidentified *Acidobacteria*. Compared with *R. goesi, T. coccinea* harbored, in general, more Gammaproteobacteria (including an abundant *Pseudoalteromonas* OTU, **Figure 2A**). Chloroplasts of different algal taxa (*Ulvophyceae* and *Stramenopiles*) and an OTU of the

[2]http://www.ncbi.nlm.nih.gov/sra/

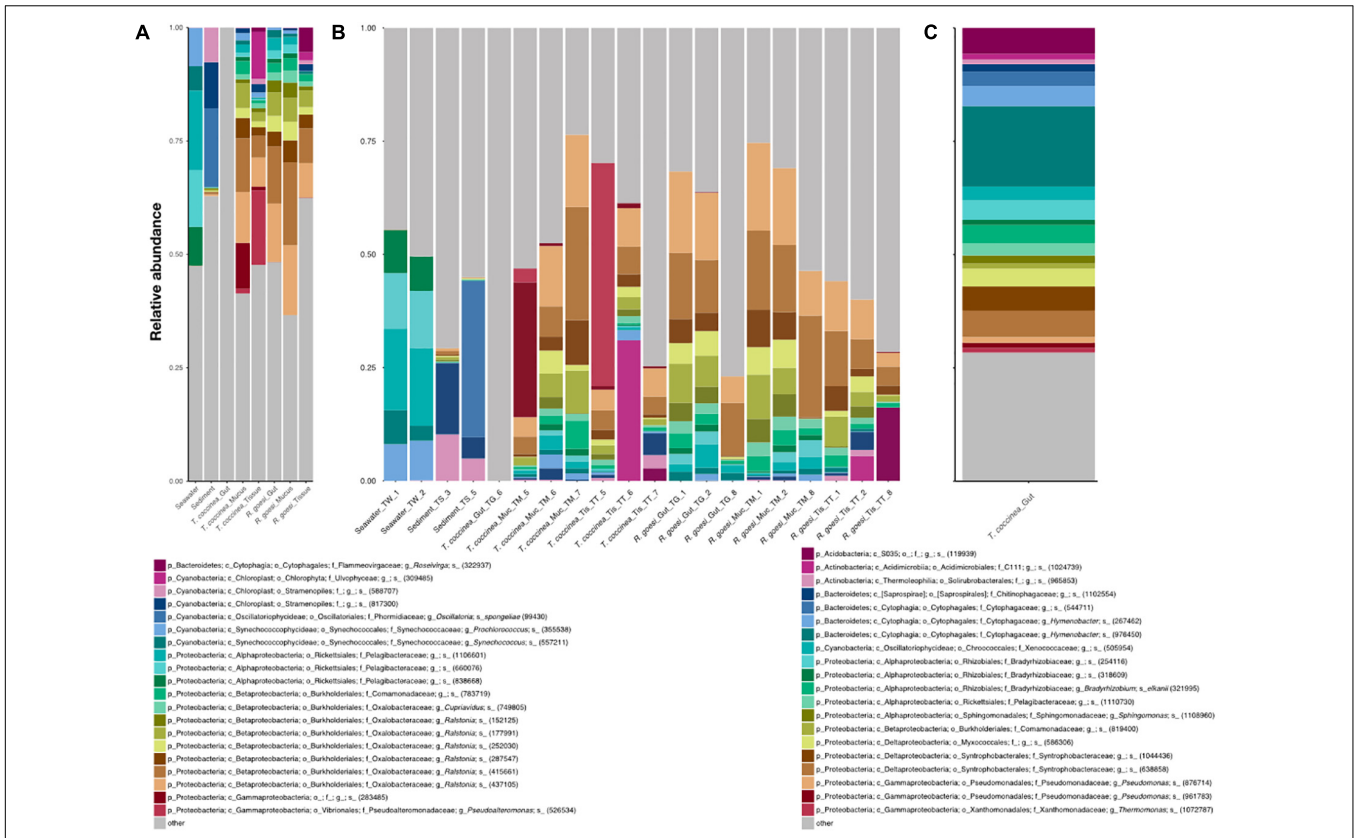

FIGURE 2 | Microbial community composition (relative abundance) resolved for **(A)** the seawater, sediment, and the cup corals *R. goesi* and *T. coccinea* across their surface mucus layer, tissue, and gastrovascular cavity (or "gut"), based on partial 16S rRNA amplicon sequencing. Only the 20 most abundant operational taxonomic units (OTUs) are depicted and the remainder pooled into "others"; **(B)** shows the same results but for each individual sample; **(C)** shows the particular community composition for the gastrovascular cavity of *T. coccinea*, which did not share any of the 20 most abundant OTUs with the other sampling groups. Affiliation of each OTU shows its lowest identified taxonomic rank: (p) phylum, (c) class, (o) order, (f) family, (g) genus, and s (species), plus an OTU code (within brackets).

genus *Roseivirga* (family *Flammeovirgaceae*) were largely absent in the gastrovascular or mucus compartments ($\leq$1% of reads) but dominated the tissue of both cup coral species (with a combined average of 10 and 14% of the reads for *R. goesi* and *T. coccinea*, respectively). *Archaea* (e.g., phylum *Euryarchaeota*) comprised less than 1% of the total reads from all three coral compartments and accounted on average for 3.1 and 0.9% of all reads from seawater and sediment samples, respectively.

Based on indicator value analysis, five OTUs emerged as indicators of both cupcoral species (as compared to the environmental samples): four distinct OTUs of the genus *Ralstonia* and one of the genus *Cupriavidus* (**Figure 3A**). Some of these OTUs were also present in the sediment samples but are not characteristic for this habitat as they did not occur in both samples. None of the microbial indicator OTUs of the cup coral species were present in the seawateryl. The analysis recognized four OTUs as indicators for *T. coccinea*: a *Pseudoalteromonas* species, an undetermined *Gammaproteobacteria*, an undetermined OTU of the family *Comamonadaceae* and one of the genus *Burkholderia*. The single indicator OTU recognized for *R. goesi* was an undetermined member from the family *Comamonadaceae*. Irrespective of cup coral species, the gastrovascular and mucus microbiomes exhibited substantial overlap in indicator OTUs (**Figure 3B**), including OTUs from the family *Pelagibacteraceae*, which were also typical indicators for the sampled seawater microbiome, and from the genus *Ralstonia*, which were also indicators of the sampled sediment microbiome (**Figure 3B**). While there were no exclusive microbial indicators identified for either the gastrovascular cavity or the mucus compartment (**Figure 3B**), the microbiome in the tissue compartments (of both coral species) contained an unique OTU affiliated to the order *Thiohalorhabdales*.

The two species of coral shared most of the indicator OTUs for the mucus compartment (**Figure 3C**) but not for the two other compartments. This was particularly evident for the gastrovascular cavity, where the community associated with *T. coccinea* greatly differed from the community in *R. goesi*, as well as from the microbiomes associated with all other species vs. compartment combinations. Since we only had a single sample from the gastrovascular cavity of *T. coccinea*, it becomes irrelevant to identify exclusive indicators for this sampling group. However, *Endozoicomonadaceae* proved to be significant indicators of the tissue of both coral species and

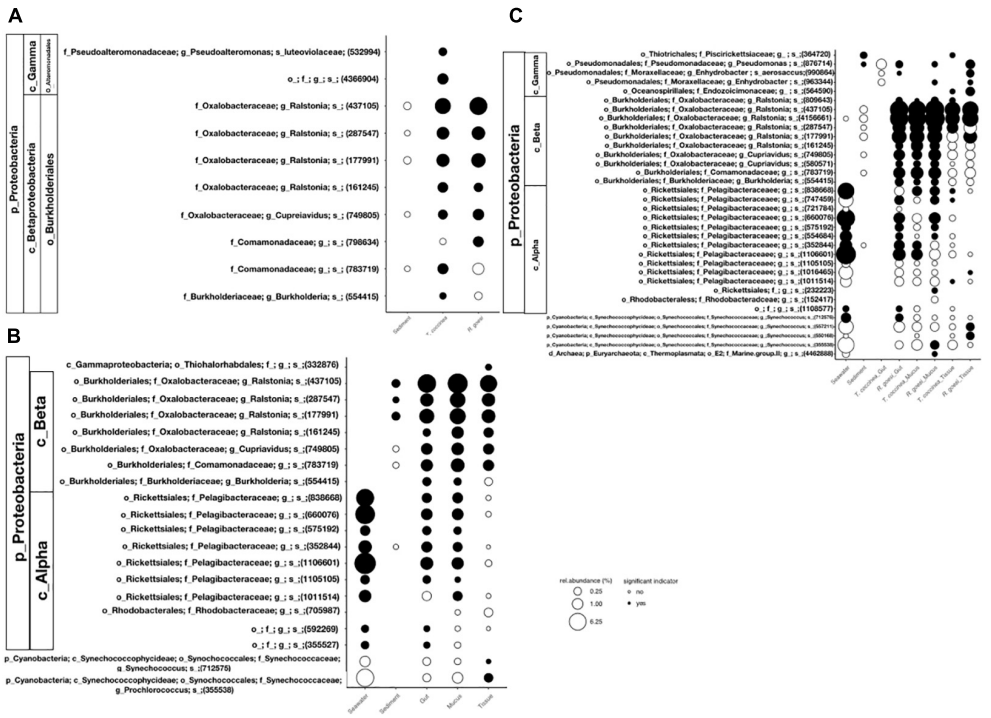

FIGURE 3 | Mean relative abundance of microbial indicator OTUs associated with **(A)** the cup corals *R. goesi* and *T. coccinea*, **(B)** the polyp compartment: surface mucus layer, tissue, and gastrovascular cavity (or "gut"), and **(C)** each species vs. polyp compartment combination. Seawater and sediment indicator OTUs are also shown when shared with any of the other sampling groups. Indicator taxa were identified with Indicator Value Analysis to be significantly associated with a certain sampling host or polyp compartment (indicated by black filled-in circles). Exclusive indicators for seawater, sediment or the gut of *T. coccinea* (in **C**) are not show. Affiliation of each OTU shows its lowest identified taxonomic rank: (p) phylum, (c) class, (o) order, (f) family, (g) genus, and s (species), plus an OTU code (between brackets).

the genus *Pseudomonas* was an indicator of all compartments of *R. goesi*, but rarely found in *T. coccinea* (except in the one gastrovascular sample successfully sequenced).

DISCUSSION

In this study, we reveal drivers of microbiome variation in azooxanthellate, heterotrophic scleractinian corals, by showing consistent differences in the microbiomes associated with the microhabitats formed by three distinct polyp compartments and for two different coral species: *T. coccinea* and *R. goesi*. Such intra-polyp microhabitat differences in microbial community structure had never been shown for tropical azooxanthellate cup corals. Our results showing that the microbial communities associated with these two tropical cup corals differed by coral species and polyp compartment are similar to patterns described for tropical and non-tropical reef-building scleractinian corals (e.g., Agostini et al., 2012). Some bacterial genera present in the microbial communities of *T. coccinea* and *R. goesi*, such as *Endozoicomonas* and *Ralstonia*, have been reported as important members of coral-associated bacterial communities (discussed below). However, further conclusions are restricted due to missing replicates, as only one of our three samples of the *T. coccinea* gastrovascular cavity provided reads.

The associated microbial communities differ between the two cup coral species, but those differences were smaller than those found between microbial communities residing in the sediment and seawater at the same location. Differences between environmental and macro-organism associated bacterial communities (e.g., Sunagawa et al., 2010; Carlos et al., 2013; Polónia et al., 2014) as well as between different host species (e.g., Aires et al., 2016) are commonly observed in the marine environment (e.g., Sunagawa et al., 2010; Carlos et al., 2013; Polónia et al., 2014). The azooxanthellate cold-water corals *Madrepora oculata* and *Lophelia pertusa* from a single location, for example, showed species-specificity of their associated bacterial communities but with interspecific distinct spatial and temporal variation patterns (Meistertzheim et al., 2016).

Our findings furthermore add to the growing body of evidence that different host compartments are also occupied by different microbial communities (e.g., Sweet et al., 2010; Apprill et al., 2016). To the best of our knowledge, this is the first time that such intra-polyp level differences in microbial community structure are shown for tropical azooxanthellate cup corals. The cup coral *T. coccinea* has been included in coral microbiome studies in the past. In Brazil, the overall mucus microbiome community of *T. coccinea* did not differ from that of the scleractinian corals *Madracis decactis* and *Mussismilia hispida*. However, *T. coccinea* did harbor three low abundance species-specific

OTUs belonging to the genera *Tenacibaculum* and *Haliagium* and a member of the *Alphaproteobacteria* (Carlos et al., 2013). Tissue of *T. coccinea* from the East China Sea was dominated by Betaproteobacteria (81%), Gammaproteobacteria (6%) and Cyanobacteria (5%) (Yang et al., 2013). Bacteria potentially active as denitrifiers and ammonium oxidizers were present, suggesting bacteria mediated nitrogen cycling in *T. coccinea* (Yang et al., 2013). The mucus microbiome of the Caribbean *Porites astreoides* (a mucus shedding species) showed shifts in prokaryotic community composition with aging and cycling of the mucus layer (Glasl et al., 2016). To what extent the mucus associated community of cupcorals changes over time remains to be explored.

The family *Endozoicomonadaceae* contains members of the genus *Endozoicomonas* which are known as symbionts of a wide variety of benthic marine macro-organisms and have been isolated, for example, from sponges, corals, bivalves, gastropods, and ascidians (Kurahashi and Yokota, 2007; Hyun et al., 2014; Appolinario et al., 2016; Schreiber et al., 2016; Sheu et al., 2017). Despite the fact that *Endozoicomonas* bacteria are associated with a wide variety of corals across oceanic basins, certain *Endozoicomonas* OTUs only occur in a single host species (Neave et al., 2016). This highly tuned host-microbe association has been proposed to have evolved through co-evolution (Neave et al., 2016). In our study, one out of the 10 *Endozoicomonaceae* OTUs was unique for *T. coccinea* tissue and three for *R. goesi* tissue, none were unique within a single species or environmental sample, but an additional three were only detected in the corals. This lack of host species specificity suggests that *Endozoicomonas* members in sun corals are perhaps less tightly associated with their coral host. Furthermore, their presence in these azooxanthellate corals might still be surprising since Bayer et al. (2013) showed *Endozoicomonas* cells live in close proximity to zooxanthellae cells within the coral tissue. In our and other studies, *Endozoicomonas* bacteria were found in azooxanthellate hosts, including the deep cold-water *M. oculata* (Hansson et al., 2009; Meistertzheim et al., 2016). While their functional role is still unclear, *Endozoicomonas* might be involved in nutrient acquisition and provision, the structuring of the host microbiome and the health status of the host holobiont (Jessen et al., 2013; Vezzulli et al., 2013; Appolinario et al., 2016; Neave et al., 2016, 2017). Although our *Endozoicomonadaceae* reads most closely matched *Endozoicomonas* members, the similarities ranging from 91.9 to 100% or matching 98–99% of an uncultured bacteria detected on *P. lutea* (Séré et al., 2013) suggest they might be undetermined non-*Endozoicomonas* members of the *Endozoicomonadaceae*.

The genus *Ralstonia* was represented by 17 OTUs in our dataset. Eight of the *Ralstonia* OTUs were highly abundant in both corals across all compartments, but were not exclusively found associated with the corals as they occurred in the sediment as well. Five *Ralstonia* OTUs were unique to the two coral species, but not species- or compartment-specific and four very low abundance OTUs were coral host-specific. *Ralstonia* is very common in zooxanthellate corals in the Red Sea (Lee et al., 2012), but also occurs in low abundances in certain coral species from the Caribbean (Sunagawa et al., 2010), the Coral Sea and Hawaii (Ainsworth et al., 2015). Similar to *Endozoicomonas*, *Ralstonia* bacteria seem to cooccur intracellularly with zooxanthellae-containing coral cells (Ainsworth et al., 2015). In addition, *Ralstonia* sp. accounted for 65 and 53% of genes associated with transport and amino acid metabolism, respectively, in *Acropora granulosa*, suggesting an active interaction with the coral host (Ainsworth et al., 2015). Our finding of *Ralstonia* in azooxanthellate, non-photosynthetic sun corals indicates they do not associate exclusively with photosynthetic organisms.

Various coral microbial studies have been performed around the island of Curaçao, none of them including cupcorals, but they suggest microbial communities of corals differ spatially across different scales. Corals with narrower depth distribution ranges (depth-specialists: *Agaricia grahamae* and *Madracis pharensis*) were associated with a stable prokaryotic community, whereas corals with a broader niche range (depth-generalists: *Stephanocoenia intersepta*) revealed a higher variability in their prokaryotic community (Glasl et al., 2017). The bulk of the microbial community of *P. astreoides* consisting of rare members, differed among locations around Curaçao (Rodriguez-Lanetty et al., 2013). Environmental conditions seem to contribute considerably to these community differences as the largest difference was associated with samples taken closest to the water outlet of the waterfactory of the island. Whether microbial communities of cupcorals exhibit similar patterns remains to be explored.

The rather unique microbiome composition in the gastrovascular cavity of *T. coccinea* shows high similarity to the gut microbiome of crustacean fed Atlantic cup corals (in the genus *Caryophyllia*), which contain a panoply of known anaerobes and facultative anaerobes (P. Frade, unpublished). This suggests that *T. coccinea* may heavily prey on zooplankton, but we cannot make any conclusive remark due to limitations in our sample size. In contrast, the similarity of microbial communities of mucus and gastrovascular cavity in *R. goesi* could indicate that this species relies more on mucus ingesting than *T. coccinea*. However, we suggest it is also possible that these differences are caused by the cavity content or time since feeding as the chemical and microbial characteristics in the coral gastrovascular cavity might undergo considerable temporal fluctuation depending on feeding regime (Agostini et al., 2012). This temporal variability and prey dependence of the gastrovascular cavity of corals certainly deserves further research.

CONCLUSION

In conclusion, in this study we show species and compartment differentiation of microbial communities in two tropical azooxanthellate cup corals. We hope this study will stimulate more holistic studies on these corals considering our limited knowledge, their evolutionary important position, their invasive characteristics and the practical advantages provided by their rather large polyps for manipulative studies.

AUTHOR CONTRIBUTIONS

AE, MV, and PF designed the study and performed the fieldwork. PF performed the laboratory work. TA, AE, and PF performed the analyses. All authors contributed to interpretations, manuscript writing, editing, and approved the submitted version.

FUNDING

This study was financially supported by Fundação para a Ciência e a Tecnologia (FCT, Portugal) in the form of scholarships SFRH/BPD/107878/2015 to AE and SFRH/BPD/110285/2015 to PF, and UID/Multi/04326/2013 to CCMAR. Fieldwork and lab consumables were covered by Marie Curie fellowship FP7-299320 to PF and the sequencing costs by the Austrian Science Fund (FWF) project P28781-B21 to GH.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00391/full#supplementary-material

FIGURE S1 | The studied sun coral species *Rhizopsammia goesi* (right hand side) and *Tubastrea coccinea* (left hand side) **(A)** *in situ* on the same very shallow reef on Curaçao, Dutch Caribbean during daytime and **(B)** at night time, showing differences in polyp size.

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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.

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REVIEW
published: 11 January 2019
doi: 10.3389/fmars.2018.00525

Corallivory in the Anthropocene: Interactive Effects of Anthropogenic Stressors and Corallivory on Coral Reefs

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Corallivory is the predation of coral mucus, tissue, and skeleton by fishes and invertebrates, and a source of chronic stress for many reef-building coral species. Corallivores often prey on corals repeatedly, and this predation induces wounds that require extensive cellular resources to heal. The effects of corallivory on coral growth, reproduction, and community dynamics are well-documented, and often result in reduced growth rates and fitness. Given the degree of anthropogenic pressures that threaten coral reefs, it is now imperative to focus on understanding how corallivory interacts with anthropogenic forces to alter coral health and community dynamics. For example, coral bleaching events that stem from global climate change often reduce preferred corals species for many corallivorous fishes. These reductions in preferred prey may result in declines in populations of more specialized corallivores while more generalist corallivores may increase. Corallivory may also make corals more susceptible to thermal stress and exacerbate bleaching. At local scales, overfishing depletes corallivorous fish stocks, reducing fish corallivory and bioerosion, whilst removing invertivorous fishes and allowing population increases in invertebrate corallivores (e.g., urchins, *Drupella* spp.). Interactive effects of local stressors, such as overfishing and nutrient pollution, can alter the effect of corallivory by increasing coral-algal competition and destabilizing the coral microbiome, subsequently leading to coral disease and mortality. Here, we synthesize recent literature of how global climate change and local stressors affect corallivore populations and shape the patterns and effect of corallivory. Our review indicates that the combined effects of corallivory and anthropogenic pressures may be underappreciated and that these interactions often drive changes in coral reefs on scales from ecosystems to microbes. Understanding the ecology of coral reefs in the Anthropocene will require an increased focus on how anthropogenic forcing alters biotic interactions, such as corallivory, and the resulting cascading effects on corals and reef ecosystems.

Keywords: corallivory, overfishing, nutrient pollution, global climate change, parrotfishes, butterflyfishes, *Acanthaster* spp.

OPEN ACCESS

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *31 May 2018*
Accepted: *22 December 2018*
Published: *11 January 2019*

Citation:
Rice MM, Ezzat L and Burkepile DE (2019) Corallivory in the Anthropocene: Interactive Effects of Anthropogenic Stressors and Corallivory on Coral Reefs. Front. Mar. Sci. 5:525. doi: 10.3389/fmars.2018.00525

INTRODUCTION

Reef-building corals are declining at an unprecedented rate worldwide as a result of anthropogenic forcing (Hughes et al., 2017a). From local impacts including overfishing and terrestrial runoff to global-scale phenomena such as ocean acidification and rising sea surface temperatures (Fabricius, 2011; Mora et al., 2011; Hoegh-Guldberg et al., 2017; Hughes et al., 2017a, 2018), anthropogenic stressors cause coral mortality and alter coral reef communities (Jackson et al., 2014; Hughes et al., 2017b). These shifts in coral density and reef community compromise both the resilience of coral reefs (Hughes et al., 2003; Putnam et al., 2017) and the ecological goods and services they provide (Moberg and Folke, 1999). The effects of individual human stressors on corals, such as warming-induced bleaching, are often well-documented (Fabricius, 2005; Mumby and Steneck, 2008; Mora et al., 2011; Hoegh-Guldberg et al., 2017; Hughes et al., 2018). However, it is also critical to understand how anthropogenic stressors alter important biotic interactions, such as predation by coral consumers (e.g., corallivory), that further impact coral growth and fitness and shape coral community dynamics.

Predation on tropical reef corals is common, with corallivores consuming coral mucus, tissue, and/or skeleton. Corallivores encompass a wide range of taxa including fishes, echinoids, crustaceans, mollusks, and annelids. The diversity of corallivore taxa is reflected in the diverse foraging behaviors and strategies (**Figure 1**), including: (1) mucus feeders, such as *Trapezia* and *Tetralia* crabs, that use specialized feeding appendages to consume mostly coral mucus along with some coral tissue while leaving the skeleton intact, (2) browsers that remove only coral tissue (e.g., butterflyfishes and *Acanthaster* spp.), (3) scrapers such as parrotfishes, that scrape live tissue from the surface of corals while also removing a thin layer of skeleton, and (4) excavators (bioeroders) that remove large amounts of coral skeleton while removing coral tissue (e.g., pufferfishes and some parrotfishes) [reviewed by Rotjan and Lewis (2008a)].

Corallivory is an important determinant of health and fitness for individual corals and can impact coral community dynamics. Predation on corals can inhibit coral growth and sexual reproduction through tissue and/or skeleton removal (Henry and Hart, 2005) and can even cause widespread mortality of corals (Turner, 1994; Kayal et al., 2012; Saponari et al., 2018). For example, predation by crown-of-thorns starfish (*Acanthaster* spp.) was identified as the second largest contributor of coral mortality on the Great Barrier Reef between 1985 and 2012 (De'ath et al., 2012). Yet, corallivores play an integral role in coral communities since they can mediate coral-coral and coral-algal competition (Cox, 1986; Miller and Hay, 1998; Kayal et al., 2011; Johnston and Miller, 2014), foster asexual reproduction in corals (Enochs and Glynn, 2017), and even deter more efficient coral predators (Glynn, 1976). Given that the impacts of corallivory on corals and community dynamics can be diverse in direction and magnitude of effect (Mumby, 2009), there is a critical need to understand how, when, and to what extent predation on corals may interact with the changing biotic and abiotic template of reefs in the Anthropocene.

A number of reviews have documented corallivore feeding behavior and their impacts on coral growth, fitness, and community dynamics (e.g., Robertson, 1970; Cole et al., 2008; Rotjan and Lewis, 2008a; Enochs and Glynn, 2017; Rotjan and Bonaldo, 2018). However, our goal is to synthesize more recent work examining how anthropogenic stressors shape the consequences of corallivory. This topic is timely due to pervasive anthropogenic effects on both the abundance of corallivores and how corals respond to predation. Specifically, we synthesize recent information about how global (i.e., increasing sea surface temperatures and ocean acidification) and local stressors (i.e., overfishing, terrestrial runoff, nutrient pollution) alter the relationship between corals and their predators. These impacts span scales ranging from coral communities down to coral microbiomes. Additionally, we identify key gaps in our knowledge that may help guide future work on corallivory in the Anthropocene.

EFFECTS OF CORALLIVORY ON THE INDIVIDUAL

The impact of corallivory on the wound healing, growth, and fitness of individual corals is well-documented (Henry and Hart, 2005; Cole et al., 2008; Rotjan and Lewis, 2008a). Here, we briefly discuss the effects of corallivory on individual corals in order to set the stage for the interactions between corallivory and anthropogenic forcing. We focus on more recent research to highlight current advances where possible.

Wound Healing Mechanisms

Corallivores can inflict damage to coral that initiate the host's immune responses involved in wound healing. Upon injury, several immune pathways are activated: (1) the TOLL-like pathway to fight pathogens and recolonize commensal bacteria, (2) melanin synthesis for tissue regeneration, (3) the complement system resulting in apoptosis of damaged cells and tissue regeneration, and (4) cell activation to move amoebocytes to the wound (reviewed by Toledo-Hernandez and Ruiz-Diaz, 2014). Amoebocytes, cells responsible for phagocytosis, are involved in cnidarian tissue regeneration (Toledo-Hernandez and Ruiz-Diaz, 2014) and melanin synthesis (i.e., production of new tissue) (Palmer and Traylor-Knowles, 2012). Tissue regeneration and its associated immune pathways require extensive cellular resources (e.g., carbon products and amoebocytes) to be translocated to the wound (Henry and Hart, 2005).

Corals that are better able to transfer these resources from healthy tissue to lesions will fare better in response to predation. For example, wound healing for imperforate corals (e.g., pocilloporids) is restrained to polyps adjacent to the wound (Henry and Hart, 2005). Yet, perforate corals with deep tissue (e.g., poritids, fungids, and acroporids) can translocate resources across the colony to wounds (Jokiel et al., 1993; Edmunds, 2008). In either case, polyps bordering wounds are essential to translocating cellular resources for tissue regeneration (Henry and Hart, 2005). Yet this can drain neighboring polyps

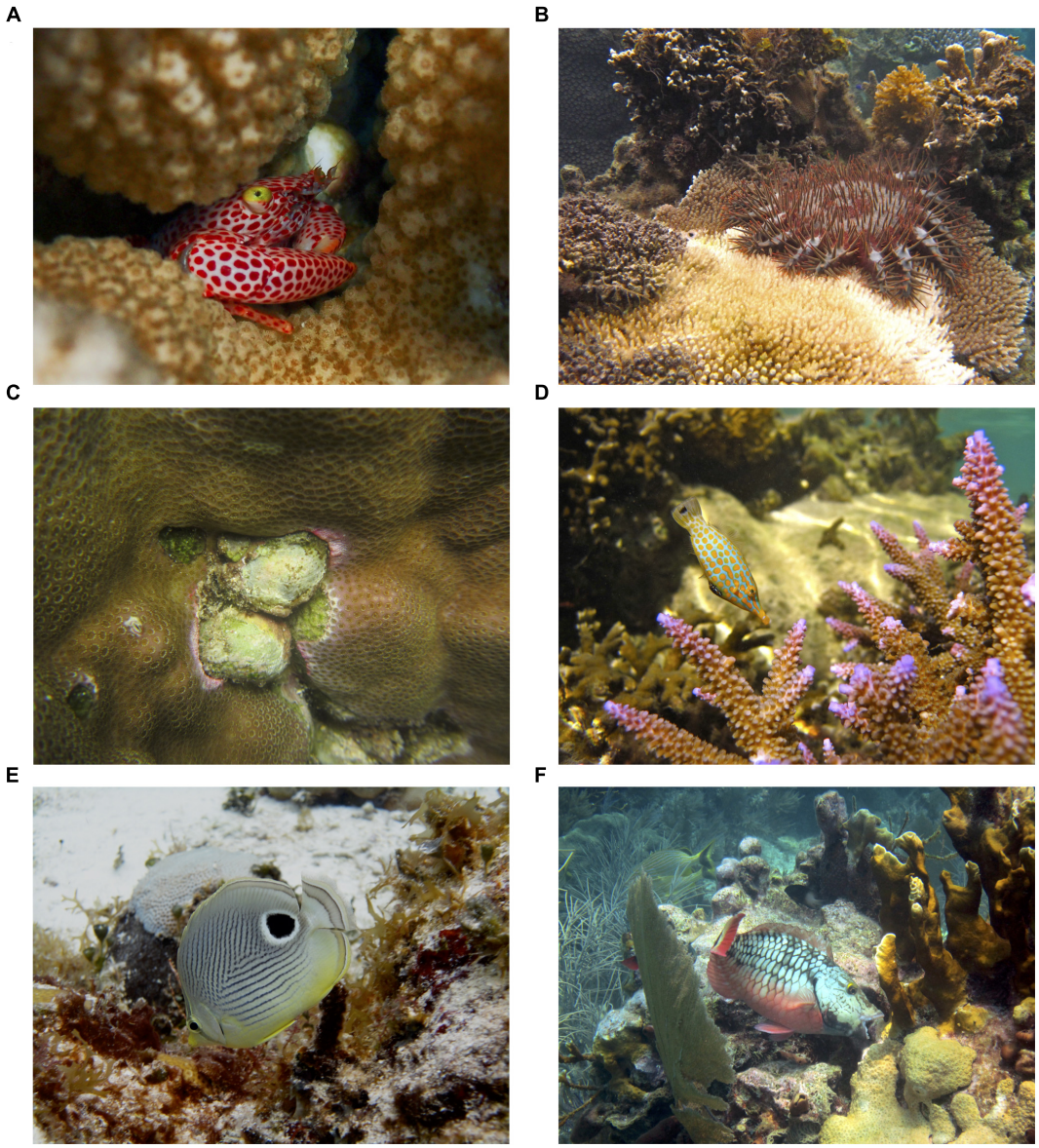

FIGURE 1 | Common corallivores that exhibit a broad range of foraging behaviors. **(A)** *Trapezia* crab within the branches of a *Pocillopora* coral feeding on coral mucus and tissue (image by Corinne Fuchs). **(B)** Substantial tissue removal by *Acanthaster* spp. on an *Acropora* colony in Fiji (image by Cody Clements). **(C)** Corallivorous snail *Coralliophila violacea* removing massive *Porites* tissue and inducing an immune response (note the pink margin on the feeding scar) (image by Cody Clements). **(D)** The filefish *Oxymonacanthus longirostris* removing coral mucus and tissue from an *Acropora* sp. (image by Cody Clements). **(E)** The facultative butterflyfish *Chaetodon capistratus* consuming coral polyps (image by Cody Clements). **(F)** An excavating terminal phase *Sparisoma viride* removes chunks of coral skeleton in the Florida Keys (image by Corinne Fuchs).

of metabolites and amoebocytes, directly affecting growth and reproduction.

Growth

The extent of damage often mediates the effects of corallivory on coral growth. Cameron and Edmunds (2014) observed that massive *Porites* and *Pocillopora meandrina* growth rates declined with more damaging modes of simulated fish corallivory (growth rates: browsed > scraped > excavated). Yet protection from corallivores with various foraging strategies can increase coral growth (Cox, 1986; Lenihan et al., 2011; Shantz et al., 2011), suggesting that even browsing corallivory has observable effects on coral growth (Shaver et al., 2017; Clements and Hay, 2018; Hamman, 2018). In fact, the gastropod *Coralliophila violacea* creates energy sinks as neighboring polyps translocate carbon products to the site of injury, allowing these snails to feed for extended periods of time (Oren et al., 1998). These energy sinks decrease coral growth rates as *C. violacea* size (Clements and Hay, 2018) and density increases (Hamman, 2018). Furthermore, mucus and tissue feeding *Trapezia* ectosymbionts can reduce

coral calcification (Doo et al., 2018), potentially decreasing coral growth. Thus, different corallivore foraging strategies can directly alter coral growth, yet the degree of damage can mediate these effects.

Reproduction

Corals use the same finite cellular resources for both tissue regeneration and reproduction (Henry and Hart, 2005), creating a tradeoff between these metabolic processes (Rinkevich, 1996). Immediately following damage, polyps neighboring lesions experience a limited supply of cellular resources, reducing local reproductive output within the colony as resources are shunted toward wound healing (Kojis and Quinn, 1981; Van Veghel and Bak, 1994; Rotjan and Lewis, 2008b). Neighboring polyps likely exhaust resources on wound healing, ultimately reducing their reproductive potential and creating within-colony heterogeneity in reproductive output.

Yet the effects of corallivory on coral reproductive potential may depend on corals reproductive methods (i.e., asexual vs. sexual reproduction). For example, the pufferfish *Arothron meleagris* feeds intensively on *Pocillopora damicornis*, often resulting in fragmented branches (Guzman and Lopez, 1991; Palacios et al., 2014) that may result in propagules that disperse across the reef and grow into new colonies via asexual reproduction (Enochs and Glynn, 2017). However, fragmentation by corallivores can also have detrimental effects on coral sexual reproduction. For instance, ∼55% of regenerating fragmented *Stylophora pistillata* colonies resembling pufferfish predation were sterile during the reproductive season and had an order magnitude less larvae released compared to intact colonies (Rinkevich and Loya, 1989). Moreover, parrotfish in Belize selectively preyed on *Montastrea annularis* polyps containing more gonads, which reduced coral reproductive effort (Rotjan and Lewis, 2008b). Thus, the mode of reproduction mediates the influence of corallivory on coral reproductive output.

GLOBAL STRESSORS AND THEIR INTERACTIONS WITH CORALLIVORY

Rising Sea Surface Temperatures

Global ocean warming has been occurring the past >40 years due to increased human activities (Allen et al., 2014). As a consequence, the frequency and severity of regional-scale mass bleaching events has been increasing the past several decades (Hughes et al., 2018). These thermal anomalies have dire consequences for reef communities as it induces thermal stress and can result in massive coral mortality (Hughes et al., 2018). Thermal stress can drain coral energy reserves (Schoepf et al., 2015), likely compromising corals' ability to meet the energy demands required to heal corallivory wounds. However, there are no clear patterns in how elevated temperatures affect tissue regeneration in scleractinians. For instance, warmer temperatures have little effect on tissue regeneration in *Porites* and *Pocillopora* (Edmunds and Lenihan, 2010; Lenihan and Edmunds, 2010; Traylor-Knowles, 2016) while increasing wound healing rates in cold water corals (Burmester et al., 2017). Yet elevated temperatures reduce wound healing in Indo-Pacific *Acropora* spp. (Denis et al., 2013; Bonesso et al., 2017) and mounding Caribbean corals (Meesters and Bak, 1993). However, the mechanisms behind why there appear to be taxa-specific impacts of temperature stress on wound healing are currently unclear.

The depletion of cellular resources due to chronic corallivory wounds may also affect corals' ability to withstand or recover from thermal stress. In Caribbean brain corals, corallivorous snails increased coral bleaching severity as compared to corals without snails, with the densest snail aggregations causing corals to bleach completely (Shaver et al., 2018). Further, the highest densities of snails also led to less recovery from bleaching and complete colony mortality in many corals. Similarly, chronic corallivory by parrotfishes hindered the recovery of *Symbiodinium* populations in *Orbicella* spp. following bleaching events and also resulted in a change in the community composition of *Symbiodinium* within the corals (Rotjan et al., 2006). Moreover, some corallivores may transmit the bacterium *Vibrio shiloi*, which causes bleaching in a narrow range of coral taxa (Sussman et al., 2003; Moreira et al., 2014). Thus, chronic predation may exacerbate stress from thermal anomalies and reduce the resilience to and recovery from coral bleaching.

Coral bleaching also affects corallivore ecology with the response to bleaching depending on the degree of dietary specialization of the corallivore. If preferred corals become rare then specialized corallivores (e.g., butterflyfishes) often increase feeding on remaining healthy conspecifics (Cole et al., 2009). Conversely, generalist predators (e.g., wrasses and muricid snails) tend to prey more intensely on bleached colonies (Cole et al., 2009; Tsang and Ang, 2014) or other healthy coral species (Pratchett et al., 2004; Hoeksema et al., 2013; Zambre and Arthur, 2018). These alterations to feeding behavior have indirect effects on corallivorous fish populations. For instance, specialized butterflyfishes are projected to disappear from reefs as preferred prey species decline in response to bleaching whilst generalist butterflyfish populations are expected to remain stable (Berumen and Pratchett, 2006; Graham, 2007; Graham et al., 2009; Emslie et al., 2011; Wilson et al., 2014). However, diet plasticity in some specialized corallivores (e.g., *Chaetodon trifasciatus*) in response to bleaching may allow local populations to persist (Zambre and Arthur, 2018). Altogether, these data suggest that bleaching events can reduce population sizes of specialized corallivorous fishes but also concentrate corallivory on the remaining corals after a bleaching event, potentially slowing coral recovery.

Ocean Acidification

Ocean acidification (OA) reduces coral calcification rates, and consequently, coral growth (Kroeker et al., 2013). In fact, decreasing pH has already slowed coral growth on some reefs (Albright et al., 2018). To maintain growth rates under low pH conditions, corals can decrease skeletal density, which can promote destruction by bioeroders, like parrotfishes, that favor lower-density substrates (Hoegh-Guldberg et al., 2007). Corals prone to bioerosion will experience increased energy expenditures to repair skeletal damage, which is energetically costlier under low pH conditions (Doney et al., 2009). Yet this

response may depend on coral life history strategies and the degree of damage. For example, end-of-century pH conditions (∼7.6) compromise wound healing rates for some mounding and branching corals more than faster-growing corals (Renegar et al., 2008; Horwitz and Fine, 2014; Hall et al., 2015; Edmunds and Yarid, 2017), although these effects appear variable depending on taxa. As pH continues to decline, there might be clear "losers" and "winners" for coral species under future OA conditions and concurrent corallivory (Renegar et al., 2008; Horwitz and Fine, 2014; Hall et al., 2015; Edmunds and Yarid, 2017). Research investigating the interactive effects of low pH and the different modes of corallivory (i.e., browser, scraper, and excavator) on corals with varying life history strategies would be especially valuable given that one would expect OA to compromise recovery from scraping and excavating corallivores much more than for browsing corallivores.

Some corallivores may also be subject to the effects of OA. Recent work suggests that the effects of OA on *Acanthaster* spp. may differ across life history stages. Low pH conditions (∼7.6) have dire effects during the early life history stages by hindering *Acanthaster* spp. fertilization, larval development, and settlement rates (Uthicke et al., 2013). However, juvenile *Acanthaster* spp. may maintain growth under projected OA scenarios, which is likely due to increased grazing rates on crustose coralline algae (Kamya et al., 2016, 2017). Thus, the magnitude of *Acanthaster* predation under future ocean conditions is likely to be governed by this taxa's response during the developmental and juvenile stages.

LOCAL STRESSORS AND THEIR INTERACTIONS WITH CORALLIVORY

Overfishing

Coral reef fishes play essential roles in reef community dynamics as predators, herbivores, and recyclers of nutrients (Sale, 2013). However, the overexploitation of reef fishes can significantly affect these ecological processes. For example, overfishing directly affects corallivory by reducing the biomass and species richness of corallivorous fishes (Guillemot et al., 2014), particularly larger parrotfish species (Bellwood et al., 2011). Parrotfishes are key herbivores, corallivores, and bioeroders on coral reefs worldwide (Hoey and Bonaldo, 2018). Yet, they are also a prime target of fisheries with some populations clearly threatened according to the IUCN Red List (i.e., *Bolbometopon muricatum*: vulnerable, *Scarus guacamaia*: near threatened, and *Scarus trispinosus*: endangered) (Chan and Donaldson, 2012; Choat et al., 2012; Padovani-Ferreira et al., 2012). The removal of these critically important fishes substantially reduces bioerosion and corallivory on fished reefs (Hoey and Bellwood, 2008; Bellwood et al., 2011). In fact, even at low densities *B. muricatum* is responsible for ∼88% of the corallivory and bioerosion on outer-shelf reefs in the Great Barrier Reef (Hoey and Bellwood, 2008). However, it disappears from reefs even at relatively low human densities and modest fishing pressure (**Figure 2**), drastically reducing bioerosion and corallivory rates (Dulvy and Polunin, 2004; Bellwood et al., 2011). Despite this dramatic example, the effect of overfishing corallivorous fishes on coral communities has received little attention (Bellwood et al., 2011).

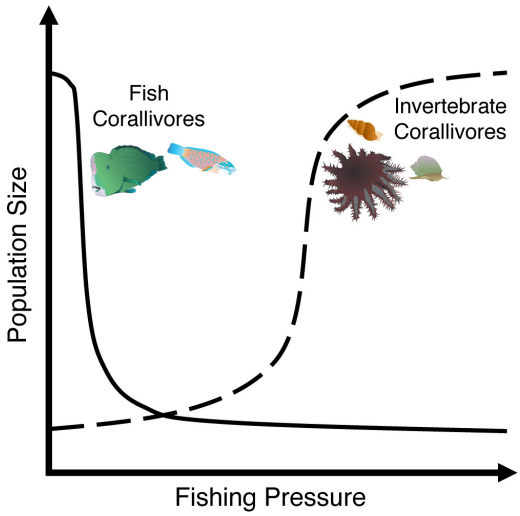

FIGURE 2 | As fishing pressure increases, the population of large corallivorous fishes (e.g., parrotfishes) often show a steep initial decline followed by marginal decreases in population size. Conversely, fishing pressure may release invertebrate corallivores from predation allowing these corallivores to increase logistically up to fivefold on fished reefs.

In addition, the depletion of predatory fishes alters corallivory indirectly by releasing invertebrate corallivores from top-down control, resulting in increased invertebrate corallivore populations that cause widespread coral mortality (**Figure 2**; McClanahan, 1994; Dulvy et al., 2004; Sweatman, 2008). For example, densities of the gastropods *Drupella cornus* and *Coralliophila violacea*, as well as the crown-of-thorns sea star *Acanthaster* spp. and the sea urchin *Echinometra mathaei*, were greater on heavily fished reefs with low predator densities compared to marine protected areas (MPAs) with higher predator densities (McClanahan and Muthiga, 1989; McClanahan, 1994; Sweatman, 2008; Clements and Hay, 2017). Similarly, densities of *Acanthaster* spp. throughout Fiji's Lau Islands were lower on islands with minimal fishing pressure (Dulvy et al., 2004). However, warmer temperatures and nutrient pollution that increase phytoplankton concentrations likely also facilitate *Acanthaster* outbreaks by benefitting larval and juvenile success (Pratchett et al., 2014; Uthicke et al., 2015; Brodie et al., 2017; Kamya et al., 2018). Thus, overfishing and nutrient pollution may work in concert to facilitate *Acanthaster* outbreaks. Indirect effects of overfishing on coral-corallivore interactions can also lead to changes in corallivore behavior. In Fiji, small, coral-dominated MPAs had ∼2–3.4 times greater densities of *Acanthaster* spp. than adjacent fished reefs dominated by macroalgae – likely due to migration of *Acanthaster* spp. from fished reefs into MPAs (Clements and Hay, 2017).

One consequence of overfishing on reefs is the rise of macroalgae as large herbivorous fishes are removed (Hughes et al., 2007; Mumby and Steneck, 2008; Holbrook et al., 2016). Competition with macroalgae may modify the effect of

corallivory in corals several ways. In some cases, macroalgae can exacerbate predation. For example, the green calcareous alga, *Halimeda opuntia*, can facilitate corallivory by the fireworm, *Hermodice carunculata* (Wolf and Nugues, 2013). The alga creates sites of mortality on corals that attracts *H. carunculata*, which may also vector coral disease and further exacerbate coral mortality (Wolf and Nugues, 2013).

Conversely, macroalgae may benefit corals by providing an associational refuge from corallivorous invertebrates (Clements and Hay, 2015) and fishes (Venera-Ponton et al., 2011; Bulleri et al., 2013; Brooker et al., 2016, 2017). For instance, dense stands of the brown alga *Sargassum polycystum* significantly reduced growth of *Montipora hispida,* but also hindered attacks by crown-of-thorns sea star (Clements and Hay, 2015). Additionally, coral-feeding butterflyfishes and the obligate corallivore, *Oxymonacanthus longirostris*, avoided preying on corals that had been in physical contact with macroalgae (Brooker et al., 2013, 2017). Thus, some coral-algal interactions create a trade-off where corals are protected against predation yet may experience reduced growth as a consequence (Venera-Ponton et al., 2011; Clements and Hay, 2015). Given the rise of algae on many reefs due to anthropogenic forcing, it is increasingly important to examine how these algae will modify existing interactions between corals and corallivores.

Nutrient Pollution

The global use of phosphate- and nitrogen-rich fertilizers has increased by over 300 and 800%, respectively, since 1961 (Food and Agriculture Organization of the United Nations, 2014), resulting in a drastic increase of nutrient inputs into coral reefs and other coastal ecosystems (Fabricius, 2005). Increased nitrogen and phosphorus (P) loading has direct and indirect negative effects on coral fitness and physiology from suppressing growth rates to increasing susceptibility to bleaching and disease (D'Angelo and Wiedenmann, 2014; Shantz and Burkepile, 2014). Some of these effects may compound the negative effects of corallivory. For instance, corals exposed to nutrients from river discharge have more porous skeletons (Mwachireya et al., 2016), likely due to P enrichment increasing coral skeleton porosity (Koop et al., 2001; Dunn et al., 2012) and weakening the skeletal matrix (Caroselli et al., 2011). These effects on coral skeletons could exacerbate the effects of corallivores as elevated nutrients magnify the damage of microborers and parrotfishes on calcium carbonate blocks, possibly due to weakened skeletal structure (Chazottes et al., 2017). By extension, corals subjected to nutrient enrichment may be more susceptible to mechanical damage from predation and may experience reduced wound healing as a consequence (Koop et al., 2001; Renegar et al., 2008). However, these questions have received little attention so far.

The interaction between corallivory and nutrients may depend on the type of corallivore and the degree of damage to the coral. For example, Zaneveld et al. (2016) showed that corallivory by scraping and excavating parrotfish interacted with nutrient exposure to increase *Porites* mortality by 62% as compared to corals that were preyed on under nutrient poor conditions. Changes in the coral microbiome may have driven this increase in mortality as opportunistic Proteobacteria increased on corals bitten under nutrient replete conditions as compared to corals bitten under nutrient poor conditions (**Figure 3**). Conversely, predation by the snail, *Coralliophila abbreviata*, resulted in ∼33% mortality in *Acropora cervicornis* while nutrient exposure did not affect mortality but did increase the abundance of opportunistic bacteria in the coral microbiome (Shaver et al., 2017). Yet, the interaction between predation and nutrient exposure did not affect *A. cervicornis* mortality rates or coral microbiomes (**Figure 3**; Shaver et al., 2017). These results suggest that bioeroding corallivory may interact with nutrients in fundamentally different ways than browsing corallivory. Bioeroders remove significant amounts of skeletal material that may provide more area for colonization by opportunistic bacteria. More direct comparisons investigating the response of corals to various modes of corallivory (i.e., tissue removal vs. skeletal damage) and the potential interactions with nutrient exposure are needed to better understand the effects on coral condition and associated microbial communities.

Sedimentation

Coastal development, agriculture, and dredging are some of the leading contributors of sediment accumulation on reefs (Rogers, 1990; Fabricius, 2005). Sediment increases turbidity, resulting in suppressed photosynthesis and growth in corals that ultimately results in lower fecundity and survivorship

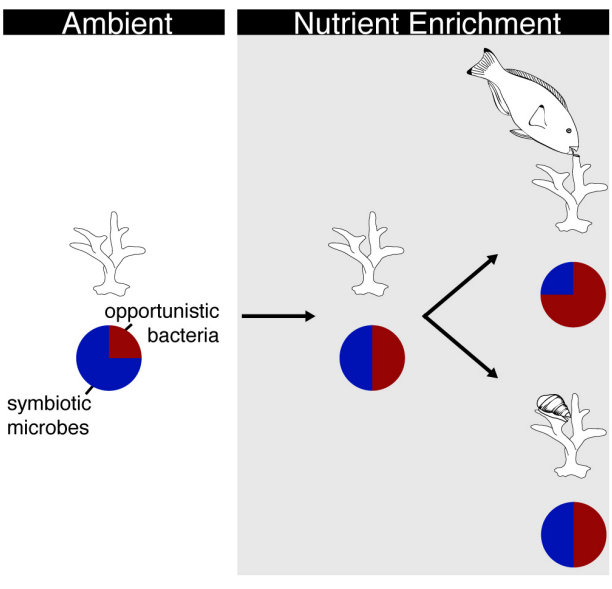

FIGURE 3 | Schematic of how different modes of corallivory interact with nutrient enrichment to affect the coral microbiome. Under ambient nutrient conditions, the microbiome of healthy corals is dominated by symbiotic microbes (blue portion of pie chart) with few opportunistic bacteria (red portion of pie chart). Upon nutrient enrichment (gray box), more opportunistic bacteria colonize the microbial community of corals. In this scenario, host corals have less stable microbiomes and are more prone to concurrent stressors. The microbiome of corals experiencing nutrient enrichment and simultaneous parrotfish corallivory becomes dominated by opportunistic bacteria. Conversely, there is no shift in the microbial community of corals exposed to nutrients and snail corallivory.

(Rogers, 1990; Fabricius, 2005). Sediment accumulation also depletes lipid and energy reserves in corals due to the energy expended to shed particles via mucus production (Sheridan et al., 2014). These energetically compromised corals may be more susceptible to other stressors, such as corallivory. In fact, wounds caused by corallivorous fishes healed slower at sites characterized by high sediment compared to low sediment sites (Cróquer et al., 2002). Importantly, sedimentation and high turbidity suppressed the recovery of simulated parrotfish bites more than simulated butterflyfish corallivory (Cróquer et al., 2002). Corallivore feeding mode and the degree of structural damage induced by corallivores, in this case excavated vs. browsed bites, appears to determine the extent to which sedimentation hinders wound healing.

Some small invertebrate corallivores benefit corals by removing sediments from their host corals. *Acropora* and *Pocillopora* corals housing corallivorous ectosymbionts that feed on coral tissue and mucus (i.e., trapeziid crabs and alpheid shrimps) are less likely to be smothered by sediment and have higher survival rates (Stewart et al., 2006, 2013; Stier et al., 2012). In fact, housing *Trapezia* crabs makes juvenile *Pocillopora* corals five times less likely to be smothered by sediment and increases juvenile coral survival by ∼35% at sites with high sedimentation (Stewart et al., 2013). Moreover, trapeziid crabs and alpheid shrimps also help coral hosts maintain growth rates when hosts are covered with vermetid gastropod mucus nets (Stier et al., 2010) and protect hosts from *Acanthaster* spp. and *Drupella* predation (Glynn, 1976; Samsuri et al., 2018). Although these small corallivores do feed directly on coral tissue, these symbionts can increase coral growth (Stewart et al., 2006), demonstrating a net overall benefit to corals hosting these corallivorous ectosymbionts.

One area of research that has received little attention is how sediment type (i.e., fine vs. coarse-grained sediments) may mediate both the response of corals to predation and corallivore feeding behavior. Terrigenous fine sediments composed of clay and silt have higher organic content and smaller grain sizes, resulting in higher turbidity and greater reduction in light availability that invokes more physiological stress in corals compared to larger, coarser sediments (Fabricius, 2005; Weber et al., 2006; Erftemeijer et al., 2012). Although corals are able to shed smaller grain sizes (Stewart et al., 2006), doing so drastically depletes energy reserves (Sheridan et al., 2014), likely leaving corals ill-equipped to heal wounds following corallivory. Thus, corals' response to corallivory may be fundamentally different in the presence of finer vs. coarser sediments.

In regard to the relationship between sediment type and corallivore feeding behavior, we know relatively little. Coarse sediments reduce herbivory rates for *Scarus rivulatus*, a facultative corallivore, more than fine sediments (Gordon et al., 2016). Similar trends may be observed for corallivory rates, but this has not been tested. Conversely, it seems plausible that finer sediments may deter invertebrate predators because smaller grain sizes may interfere with the proboscis feeding structure used by muricid gastropods. For example, the freshwater gastropod *Potamopyrgus antipodarum* is nearly four times more likely to be found on clean surfaces than surfaces with fine sediment (Suren, 2005). The same may hold true for reef gastropods but no data currently exist on this topic. The effects of different sediment types on corallivory, corallivore behavior, and coral condition is clearly an important area of research that needs attention.

Sewage Output

Human sewage is the largest contributor of global coastal pollution with most wastewater entering tropical coastal waters without prior treatment (Islam and Tanaka, 2004; Wear and Thurber, 2015). Sewage contains high concentrations of inorganic compounds, toxins, heavy metals, and pathogens, all of which can increase coral bleaching and mortality rates, reduce fecundity, and induce coral disease (Pantsar-Kallio et al., 1999). Pathogens from human sewage can destabilize the coral holobiont, providing invasive microbes the opportunity to colonize and potentially facilitate the vectoring of pathogens to corals by corallivores (Sutherland et al., 2010, 2011).

Sutherland et al. (2010) found that the enterobacterium strain *Serratia marcescens* PDR60, which is found in sewage, is also found in the obligate corallivorous snail *Coralliophila abbreviata*. Anthropogenic sources of sewage wastewater input this strain into the Florida Keys reef tract (Sutherland et al., 2011), infecting *Acropora palmata* with white pox disease. Moreover, *C. abbreviata* is a reservoir for *S. marcescens* because it preferentially consumes *A. palmata* (Miller, 2001) and can transmit the strain, and thus coral disease, to uninfected *Acropora* colonies (Sutherland et al., 2010). The propagation of this disease decreases live coral tissue and concentrates snail predators on remaining live coral, creating a positive feedback loop between muricid snail density and *Acropora* spp. predation (see Figure 6 in Williams and Miller, 2005). The interactions among sewage, corallivores, and coral disease is likely not exclusive to the Florida Keys as many tropical regions have little to no treatment of sewage and many species of corallivores may serve as vectors for pathogens that propagate disease. Examining how widespread these interactions are could be critical for understanding the full effects of sewage on coral reef communities.

FUTURE DIRECTIONS FOR CORALLIVORY RESEARCH

How Does Anthropogenic Forcing Affect Coral Dynamics?

Much of the existing literature on the interactions of corallivory and anthropogenic stressors focuses on the impact on individual corals. There has been much less focus on how these stressors may change the impact of corallivory at the level of reef communities. For example, there has been a strong effort to understand how MPAs help maintain herbivory and the direct effects on algae and indirect effects on corals (Mumby et al., 2006, 2007; MacNeil et al., 2015), yet there has been little focus on the consequences to corallivory. We know the processes that parrotfishes strongly influence (e.g., bioerosion and corallivory) decline with increasing fishing pressure in Indo-Pacific reefs

(Bellwood et al., 2011). Yet it is unclear if these patterns hold true in other regions where fewer corallivore species occur (i.e., Eastern Pacific, Caribbean). Conversely, we also know relatively little about how robust populations of these large corallivores impact coral populations and communities, especially on reefs where corals are declining. For example, parrotfishes in the Florida Keys, United States switch to preying on less favored coral species as preferred prey species become rare, which ultimately increases corallivory rates overall when coral cover is low and parrotfish density is high (Burkepile, 2012). This trend is a concerning one and understanding if it is a general phenomenon is important.

There is also limited knowledge on how corallivory interacts with local and/or global stressors to alter benthic community composition on reefs. The presence of select corallivores can reverse the competitive dominance amongst corals (Cox, 1986; Colgan, 1987; Kayal et al., 2011), altering community composition. For instance, when the corallivore *Chaetodon unimaculatus* is present, the competitive dominance shifts from *Montipora verrucosa* to *Porites compressa* because *M. verrucosa* is preferentially preyed upon (Cox, 1986). This reversed competitive hierarchy may be reinforced under different anthropogenic regimes. For example, *Acropora* and *Pocillopora* corals are preferred prey in the Indo-Pacific (Cole et al., 2008) and compete for space on the benthos. Under future ocean acidification scenarios, the competitive dominance shifts from *Pocillopora* to *Acropora* (Horwitz et al., 2017). Rapid *Acropora* growth rates (Anderson et al., 2017) may allow *Acropora* to outcompete *Pocillopora*, further reinforcing the reversed competitive hierarchy under low pH. Understanding these complex feedbacks will require both time series data of coral community dynamics as well as targeted experimentation to understand the mechanisms of interspecific interactions and predation patterns.

What Are the Interactions of Different Corallivore Feeding Modes and Anthropogenic Forcing?

The effects of corallivory can differ in magnitude depending on anthropogenic stressors. For example, browsing corallivory (e.g., tissue removal) does not exacerbate the effects of nutrient enrichment or sedimentation while excavating corallivory clearly does (Cróquer et al., 2002; Zaneveld et al., 2016; Shaver et al., 2017). Conversely, prudent gastropods that exert chronic predation and act as photosynthate energy sinks (e.g., *Coralliophila* spp.) (Oren et al., 1998) may exacerbate thermal stress, bleaching, and post-bleaching mortality (Shaver et al., 2018) more so than excavating corallivores that likely have more ephemeral effects on individual colonies (e.g., spot biting). Given that corallivore feeding mode has contrasting effects on corals under different anthropogenic templates, we need to better understand how these foraging strategies affect coral survivorship and energy budgets under both present-day conditions and future ocean scenarios. This question is especially important as corallivores with different feeding modes may have fundamentally different population trajectories under increasing anthropogenic forcing (**Figure 2**), potentially resulting in very different corallivore regimes in future oceans.

Both corallivory and anthropogenic stressors often have independent negative effects on coral fecundity (Van Veghel and Bak, 1994; Rotjan and Lewis, 2008b; Jones et al., 2015). However, how these forces interact to modulate reproductive potential in corals remains largely unknown. Tissue regeneration occurs at the expense of somatic growth and gametogenesis because these processes compete for the same cellular resources (Henry and Hart, 2005). Thus, the suppression of fitness likely scales with the degree of corallivory and the need for tissue regeneration. Yet it is unclear if anthropogenic stressors act additively or synergistically with various modes of corallivory to further reduce coral fitness. These interactions could serve as crucial negative effects on coral reproduction that lower the resilience of reefs in the face of increasing disturbances.

What Are the Effects of Corallivory at Molecular and Microbial Scales?

Anthropogenic pressures (e.g., sedimentation and seawater warming) often cause corals to upregulate immune response pathways, suggesting that corals may be robust to extrinsic stressors (Sheridan et al., 2014; van de Water et al., 2015b) and maintain the ability to regenerate tissue during thermal stress (van de Water et al., 2015a,c; Bonesso et al., 2017). However, research investigating the cellular mechanisms that govern tissue regeneration under other anthropogenic regimes (e.g., nutrient pollution) is surprisingly lacking. Studies that quantify the upregulation of enzymes and genes involved in immune response pathways are necessary to better understand host defense in response to these extrinsic factors and assess if local and global stressors act additively or synergistically.

Beyond affecting corals through mechanical damage, corallivores provide entry points for opportunistic bacteria (e.g., coral diseases) and can aid in parasite transmission on the reef. For instance, the coral-feeding butterflyfish *Chaetodon multicinctus* plays an implicit role in the dynamics of trematodiasis infection in some *Porites* spp., by serving as a final host in the life cycle of the trematode *Podocotyloides stenometra* (Aeby, 1998, 2002). Similarly, some corallivores can transmit disease pathogens to corals, and four invertebrate corallivores have been empirically linked to coral disease transmission (see Table 1 in Nicolet et al., 2018a). It is likely that this mechanism of disease transmission may be more common than currently appreciated. Similarly, butterflyfishes prey on diseased coral tissue (Aeby and Santavy, 2006; Chong-Seng et al., 2011; Nicolet et al., 2013, 2018b) with coral disease correlating positively with butterflyfish abundance (Raymundo et al., 2009). Corallivorous fishes may transmit disease pathogens orally or through feces (Aeby and Santavy, 2006), however, more research is needed to demonstrate if there is a causal link between corallivorous fishes (e.g., butterflyfishes and parrotfishes) and coral disease transmission (Nicolet et al., 2018a). Fish acting as vectors may be an important driver structuring coral-microbial communities, particularly for excavating corallivores, like parrotfishes, that create larger wounds in corals. Thus, there is a significant need

for studies that examine the causal link between corallivores, coral microbiome dynamics, and coral disease propagation.

In the context of anthropogenic forcing, corallivory may act synergistically to destabilize the coral microbiome, leading to compromised host immunity (Zaneveld et al., 2017) and increased mortality (Zaneveld et al., 2016). Moreover, compared to MPAs, overfished reefs have a higher prevalence of coral disease (Raymundo et al., 2009) and larger populations of invertebrate corallivores capable of transmitting opportunistic bacteria (McClanahan, 1994; Dulvy et al., 2004; Sweatman, 2008; Nicolet et al., 2018a). These local processes may further fuel coral disease propagation via corallivory. Understanding how anthropogenic pressures might shape the effects of corallivores on coral microbiomes will be a fruitful and important area of research.

CONCLUSION

In the Anthropocene, the convergence of multiple interacting stressors is altering the natural interactions among corals and corallivores. Although a small suite of corallivores have beneficial effects on corals, the negative effects of most corallivores appear to be exacerbated by anthropogenic stressors. As anthropogenic forcing strengthens, the rich biodiversity characterized by pristine reefs will decline (**Figure 4A**). Global stressors, such as ocean warming, will continue to induce bleaching and mortality in thermally sensitive coral species while local stressors, such as nutrient pollution and sedimentation, exacerbate coral disease, fundamentally altering coral community composition. Concurrently, local stressors such as overfishing is removing both large corallivorous fishes that impact coral community composition as well as the predators of corallivorous invertebrates, relaxing top-down control on these corallivores (**Figure 4B**).

As the pressure from anthropogenic forcing accumulates, thermally sensitive coral species will likely disappear from reefs along with their specialized corallivores (**Figure 4C**). The corals that remain will likely have compromised wound healing, growth, and fecundity due to chronic thermal stress and the increasingly unfavorable energetics under declining ocean pH. The cumulative effects of local stressors, such as overfishing and nutrient pollution, will lower corallivorous fish biomass on reefs, reduce average parrotfish size, and fuel invertebrate corallivore population growth that may prevent coral recovery. The increase in nutrient pollution, sedimentation, and algal abundance on these future reefs will likely contribute to corallivore-mediated propagation of coral diseases.

However, the mechanisms underlying many of the interactions between corallivory and anthropogenic stressors are still unclear. As coral cover continues to decline globally, the effect of corallivory on coral health and reef community dynamics will become more imperative to understand. Given that ∼60% of global coral reef management agencies are controlling corallivore populations to some extent (Shaver et al., 2018), investigating the feedbacks between corallivory and increasing stressors is a high priority for informing coral reef conservation and management. Moving forward, we need to improve our understanding of how anthropogenic forcing changes coral-corallivore trophic interactions across scales from coral community dynamics down to coral microbiomes.

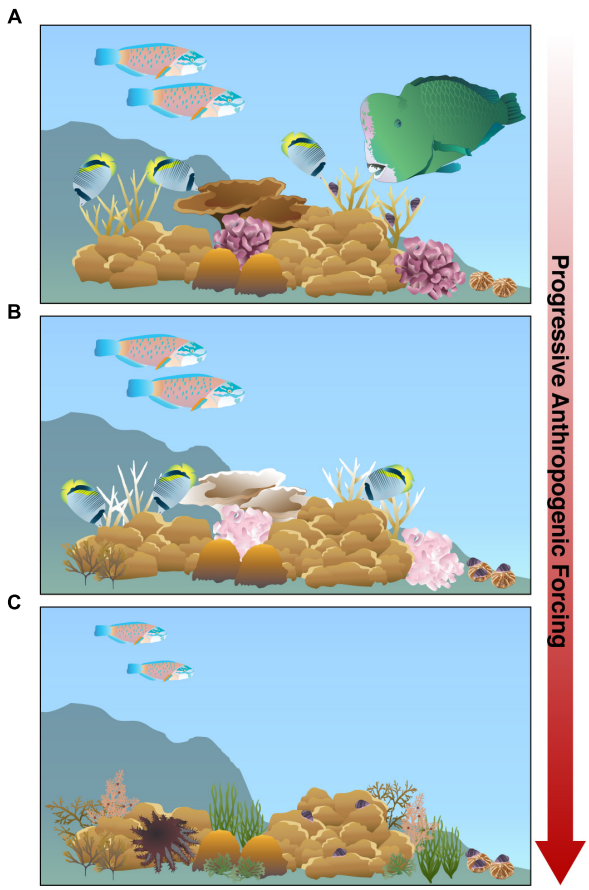

FIGURE 4 | Modern scleractinian reefs with progressive anthropogenic pressure will experience a shift from **(A)** pristine reefs with a high biodiversity of reef-building corals and fishes to degraded reefs. **(B)** Under moderate anthropogenic forcing, temperature-sensitive corals (e.g., *Acropora* and *Pocillopora*) experience bleaching, inducing obligate butterflyfishes and corallivorous gastropods, *Drupella* spp., to shift prey preferences to massive *Porites* and fungiid corals, respectively. The large excavating parrotfish *Bolbometopon muricatum* is removed due to fishing pressure, reducing the overall corallivorous fish biomass on the reef. **(C)** As anthropogenic pressures increase, coral composition shifts to more tolerant coral species, like massive *Porites*. Specialized, obligate corallivorous fishes disappear from the reef because preferred coral species die out. Overfishing removes larger fishes, including invertivores and terminal phase parrotfishes, resulting in smaller average sized parrotfishes and giving way for increased macroalgae colonization. Moreover, overfishing may release invertebrate corallivores from predation pressure, increasing corallivorous gastropod and *Acanthaster* spp. populations that may hinder remaining coral recovery and compromise reef resilience to these synergistic stressors.

AUTHOR CONTRIBUTIONS

All authors contributed to manuscript writing and revision.

FUNDING

Funding was provided by a National Science Foundation Graduate Research Fellowship to MR (#1650114) and a Swiss National Science Foundation Fellowship (#P2SKP3171764) to LE. This work was also supported by a National Science Foundation CAREER Grant OCE-1547952 to DB.

ACKNOWLEDGMENTS

We thank two reviewers for their valuable comments that helped to improve our manuscript. We thank the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/imagelibrary/) for the use of images for the conceptual figures. We also thank Corinne Fuchs for insightful comments on the manuscript.

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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

ORIGINAL RESEARCH
published: 12 February 2019
doi: 10.3389/fmars.2019.00036

Identifying Causes of Temporal Changes in *Acropora cervicornis* Populations and the Potential for Recovery

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Corals, specifically the Atlantic staghorn coral, *Acropora cervicornis*, are under major threat as disturbance events such as storms and disease and predation outbreaks increase in frequency. Since its population declines due to a wide spread disease event in the early 1980s, limited long-term monitoring studies describing the impact of current threats and potential recovery have been completed. The aim of this study was to document the impacts of environmental (tropical storms, increased wind) and biological (disease and predation) threats on *A. cervicornis* to further understand its population dynamics and potential for recovery. Two high-density *A. cervicornis* patches (greater than 1 hectare each) were surveyed tri-annually (winter, summer, fall) from 2008–2016. *A. cervicornis* percent cover, canopy height, census of individuals, and prevalence and occurrence of disease, predation, and bleaching were evaluated within permanent 3.5 m radial plots (n = 27 and 31). Temporal variability was observed in mean percent live cover at both patches and showed an overall loss of tissue. Frequent disturbances such as tropical storms, hurricanes, and disease events, caused increased, prolonged, and widespread mortality. Periods void of disturbance allowed for recovery and growth. Prevalence and occurrence of disease and predation were highly variable between monitoring events. They were also found to be significantly higher on masses (individuals $\geq$ 1.5 m) than on colonies and during summer surveys (June–August). These data indicate that substantial length of time between major disturbance events is necessary for recovery and growth of this species. The implication of these results is that given the current rates of growth, recruitment, and storm frequency, natural species recovery is unlikely unless large scale issues like climate change and ocean warming, which affect the intensity and frequency of disease and predation, are addressed.

Keywords: demographic monitoring, time series, disease, fireworm, long-term monitoring, Florida

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Edited by:
Michael Sweet,
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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *30 April 2018*
Accepted: *22 January 2019*
Published: *12 February 2019*

Citation:
Goergen EA, Moulding AL, Walker BK and Gilliam DS (2019) Identifying Causes of Temporal Changes in Acropora cervicornis Populations and the Potential for Recovery. Front. Mar. Sci. 6:36. doi: 10.3389/fmars.2019.00036

INTRODUCTION

Acropora cervicornis is a fragile, vulnerable, and dynamic species that has been known to change in abundance and/or cover quickly (e.g., weeks to years) in response to disease outbreaks, tropical disturbances, or climatic events (Shinn, 1976; Antonius, 1977; Davis, 1982; Knowlton et al., 1990; Schopmeyer et al., 2012; Miller et al., 2014a) and was frequently found lining the fore reef of many Caribbean, Florida, and Gulf of Mexico coral reefs. Its fast growth rate and ability to reproduce

asexually allow it to propagate quickly across a site, forming mainly monotypic stands referred to as thickets, fields, stands, or patches (Davis, 1982; Bruckner, 2002; Acropora Biological Review Team, 2005). *A. cervicornis* plays a significant role in the coral reef community by contributing to reef complexity and habitat framework (Goreau, 1959; Goreau and Goreau, 1973; Adey and Burke, 1977; Neigell and Avise, 1983). The habitat diversity and ecological benefits provided by the structure of *A. cervicornis* colonies are virtually irreplaceable within the natural marine community.

Acropora cervicornis populations became spatially and regionally isolated following a multi-decadal white band disease outbreak starting in the 1970s which left the surviving populations most commonly distributed as individual colonies or much smaller patches (Gladfelter, 1982; Bythell et al., 1989, 1993; Aronson and Precht, 2001; Acropora Biological Review Team, 2005). This dramatic decline leads to its listing as threatened under the United States Endangered Species Act (US ESA; National Marine Fisheries Service, 2006) and as critically endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Aronson et al., 2008). Since this dramatic decline, recovery has been limited with few known high cover populations remaining throughout the species' range (Vargas-Ángel et al., 2003; Keck et al., 2005; Grober-Dunsmore et al., 2006; Lirman et al., 2010; Walker et al., 2012; Busch et al., 2016). One region where numerous large patches of *A. cervicornis* exist today is within the Southeast Florida Coral Reef Ecosystem Conservation Area and more specifically in Broward County, FL, at the northern-most extent of this species' range (Vargas-Ángel et al., 2003; Walker et al., 2012; D'Antonio et al., 2016).

However, few data exist on the temporal and spatial variability of the demography and ecology of *A. cervicornis* (Mercado-Molina et al., 2015; Goergen et al., unpublished), and we are only beginning to define the impact disease and predation have on the persistence of this species outside of large scale catastrophic events (Williams and Miller, 2006; Miller et al., 2014a,b; Goergen et al., unpublished). To fully understand the population dynamics of this threatened species and to further inform restoration and conservation efforts, these data need to be evaluated over the long-term.

To address these questions, two semi-continuous patches of *A. cervicornis* (>1 hectare each) were used to evaluate temporal patterns in species abundance, percent cover, and the presence, prevalence, and occurrence of disease, predation, and bleaching. These analyses will be the first of its kind for this species, which further our understanding of the dynamics of the threats affecting remaining, future, and restored populations.

MATERIALS AND METHODS

Two large semi-continuous patches of *A. cervicornis,* BCA and Scooter, formally known as Dave and Oakland I patches, respectively (Vargas-Ángel et al., 2003), were surveyed three times per year during Winter [(WS) February/March], Summer [(SM) June–August], and Fall [(F) October/November] from Summer 2008 through Fall 2016. These monitoring periods will be referenced by the season followed by the last two digits of the year throughout the rest of this paper (e.g., SM09 is Summer 2009). An additional survey was completed 10 September 2012, following the passing of Tropical Storm Isaac (TSI12) on 26 August 2012. Prior to the initial survey (June 2008), the boundary of each patch was mapped using a handheld GPS. Plots were installed in a grid with spacing appropriate to cover the patch and the surrounding area to account for possible patch growth or movement (Walker et al., 2012). Thirty-two plots each separated by 30 m were installed at BCA, and 31 plots were installed at Scooter with 23 m separation.

Monitoring methodologies were modified from a previously developed *Acropora* spp. demographic monitoring protocol (Williams et al., 2006). Radial plots 7 m in diameter (38.48 m^2), marked by a permanent center pin and tag designated the survey area. Temporary transect lines, 7 m in length were laid perpendicular to each other across the center of each plot defining the survey area during monitoring events. Condition characteristics and a species census were completed in all plots. Condition characteristic data included: (1) estimates of percent cover of live *A. cervicornis*, (2) the presence and relative ranking of tissue loss caused by white band disease, rapid tissue loss (Williams and Miller, 2005), and predation by the bearded fireworm (*Hermodice carunculata*), three-spot damselfish (*Stegastes planifrons*), and the corallivorous snail (*Coralliophila abbreviata*), and (3) presence and severity of bleaching. Maximum *A. cervicornis* canopy height was measured within the plot boundary. During the species census, all *A. cervicornis* individuals were counted and categorized as either a loose fragment, colony [well defined boundary of continuous skeleton (dead or alive), attached, <1.5 m diameter] or a mass (difficult to define boundary, typically > 1.5 m in diameter). Beginning in F10, individuals that showed signs of disease were quantified to obtain disease prevalence of colonies and masses. Presence of disease was not quantified on loose fragments because the cause of recent mortality on fragments could not be identified confidently. All individual areas (occurrences) of recent mortality within the plot boundary were counted based on cause (rapid tissue loss, white band disease, fireworm, and snail); recently dead areas separated by living tissue were counted as separate occurrences. The occurrence of damselfish predation and bleaching were not recorded because of the difficulty in defining and enumerating individual gardens and areas of bleaching.

Meteorological data were obtained from multiple online resources to better describe the conditions during tropical disturbances and aiding in identifying other causal events. Storm track, wind swath data, and individual storm reports were downloaded from the National Hurricane Center[1]. Wind data for 2008 were collected from the National Centers for Environmental Information Fort Lauderdale Airport station[2], which is located approximately 3 km inshore and 10 km from the study sites; however, in 2009, a closer station was established on the ocean

[1]www.ncdc.noaa.gov

[2]www.nhc.noaa.gov

approximately 6.5 km south of the study sites. Therefore, 2009–2016 were downloaded from the National Data Buoy Center station PVGF1-Port Everglades Channel, FL[3]. Rainfall data were obtained from the South Florida Water Management Districts Hollywood Station (2008–Oct 2014) and S36-RR Station (Nov. 2014–2016[4]). Temperature (°C) was recorded every 2 h using Onset Hobo Pendant® Temperature/Light loggers or TidbiT® v2 Temperature loggers attached to a permanent pin at each patch. Data were recorded from June 2008 at BCA and February 2010 at Scooter until the end of the study. Loggers were exchanged every 3–6 months. Unfortunately, a series of faulty loggers at Scooter resulted in missing data from 27 February 2014 to 10 August 2016.

Data Analysis

Plots in which *A. cervicornis* were never recorded during the duration of the study were not included in the analysis (n = 5 for BCA). Data collected within each monitoring event by patch providing event means. For annual analyses, the three monitoring events completed during that calendar year were used; the TSI12 event was included in 2012 for a fourth event for that year. For the seasonal analyses, all years were used within each season; monitoring event TSI12 was included in the summer season (Winter n = 8 events, Summer n = 10, and Fall n = 8).

Percent cover was estimated for each plot during each event and was used to calculate mean cover by patch. Trends in mean percent cover of living (PCL) *A. cervicornis* were analyzed using Time Series Analyses followed by decomposing the components and analyzing the decomposed trend component with a linear regression (R Core Team, 2017). Simple linear regressions were used to analyze the annual trend observed in PCL. One-way analysis of variance (ANOVA) was used to assess the differences in PCL between seasons. *Post hoc* comparisons were performed using Tukey's HSD tests. Kruskal–Wallis test by ranks was used to explore absolute change in PCL. When significant, multiple comparisons two-tailed *post hoc* tests were performed to determine significance between factor levels.

The total abundance of fragments, colonies, and masses in each individual plot were averaged by patch for each event, year, and season. The trends in mean abundance of fragments, colonies, and masses were analyzed using a Poisson regression for both between monitoring events and years. In addition, to determine differences in mean abundance and absolute change in abundance between seasons, Kruskal–Wallis test by ranks followed multiple comparisons two-tailed *post hoc* tests were used.

The presence of disease and predation was analyzed through the prevalence of plots with each condition. During each event, researchers documented the presence or absence of white disease, fireworm predation, damselfish predation, snail predation, and bleaching. A sum of the total number of plots with each condition was divided by the total number of plots with living *A. cervicornis* surveyed providing a prevalence for each condition for each event. Mean prevalence of plots with each condition was calculated annually and seasonally. These data indicate how widespread each condition was at each patch. Prevalence of white disease was also calculated per plot by dividing the number of colonies or masses with disease by the total number of colonies or masses in each plot. Disease prevalence was analyzed using binomial (plot prevalence) and quasi-binomial (colony and mass prevalence) generalized linear models between monitoring event, years, and seasons. When the model identified significant factors, *post hoc* multiple comparisons with a Bonferroni correction were employed to define specific contrasts of factor levels.

The occurrence of white disease, fireworm predation, snail predation, and bleaching were summed by their occurrence on colonies or masses per plot. Mean number of occurrences of each condition per plot was calculated per monitoring event, year, and season. These data were analyzed using Kruskal–Wallis ANOVAs, and when significant, multiple comparisons two-tailed *post hoc* tests with a Bonferonni correction were performed.

Individual plot canopy height was used to calculate a mean canopy height per patch for each event. These data were analyzed across all events using linear regression analyses for both between monitoring events and annual changes.

RESULTS

Across the 8 years of the study, abundance and health of *A. cervicornis* were surveyed within 27 plots at BCA and 31 plots at Scooter, five plots at BCA never had *A. cervicornis* so were not included in the analysis. The center pin was not located for two plots at Scooter following the SM15 and F15 events because of *A. cervicornis* overgrowth and were not included following these events.

Disturbance Events

Tropical storm force winds, identified by the predicted area of the wind swath published by NOAA[5], impacted southeast Florida during six named storm events (**Table 1**). Each storm had different conditions (temperature change, wave height, rainfall, and wind speed) and relate to the range of impacts at each patch. The passing of all storms, except Hurricane Sandy at BCA, resulted in mean PCL losses ranging from 1.5–50% for both patches. The largest PCL losses by area were 78 m^2 for BCA during monitoring event WS09 and 116 m^2 for Scooter during TSI12. Besides named storms, additional high energy periods during the study were identified by elevated mean daily wind speeds greater than the average sustained wind produced by a tropical storm, 12.78 kts (**Table 2**). Additional losses of >20% relative mean cover per patch were observed between events not associated with named storms at at least one patch during: WS10, F11, SM15, F15, WS16, and SM16.

Cover Characteristics

Percent cover of living decreased for both patches during the study, although only BCA had a significant, decreasing linear trend [r^2 = 0.5013, $F(3,20)$ = 6.702, $p < 0.001$; Scooter:

[3]www.ndbc.noaa.gov

[4]my.sfwmd.gov

[5]http://www.nhc.noaa.gov

TABLE 1 | Tropical storm metadata for southeast Florida including days impacted (based on elevated wind levels), monthly average wind speed, rainfall, and wave height (calculated using the Beaufort Scale) for the month the storm occurred, overall mean sustained wind, rainfall and wave height across impact days, maximum daily average wind and wave height, maximum speed of wind gusts, rainfall, and wave height, and the decrease in *in situ* water temperatures during and following the storm and the duration of the change.

					Wind (kt)			Rain (cm/day)			Wave height (m)			Temperature	
Storm name	**Date**	**Approximate distance from storm (km)**	**Category**	**Impact days**	**Monthly average**	**Storm sustained average (max)**	**Max storm gusts**	**Monthly average**	**Storm average**	**Storm max**	**Monthly average**	**Storm average (max)**	**Storm max**	**Degree decrease (°C)**	**Duration of deviation (days)**
Fay	19 Aug 2008	135	TS	4	7.2	12.3 (16.5)	27.8**	1.05	3.8	9.1	0.6	1 (2)	3.5	3.21	10
Bonnie	24 July 2010	75	TS	5	7.37	12.1 (16.4)	31.8	0.94	0.7	2.8	0.6	1 (2)	4.5	nc	na
Irene	25 Aug 2011	294	H	3	6*	10.5 (11.4)	34.8	0.63	1.2	3.5	0.2	0.6 (1)	5.5	nc	na
Isaac	26 Aug 2012	287	TS	6	7.4	13.8 (20.8)	46.3	0.65	3.3	10.2	0.6	1.5(2.5)	7	1.1	10
Sandy	26 Oct 2012	309	H	9	9.9	15.7 (20.3)	45.7	0.81	0.3	1.6	1	1.5(2.5)	7	1.8	10
Matthew	6 Oct 2016	127	H	3	12.1	12.3 (14.2)	38.7	0.40**	0.8	1.7	1	1 (1.5)	6.5	nc	na

*7 days were missing from dataset.

**Max gust were not recorded, so fastest 2 min speed was used.

nc = no change observed; na = not applicable. Approximate distance from storm is from the site to the central path of the storm.

TABLE 2 | Number of days between monitoring events in which the wind speed was greater than the average wind speed (12.78 kts) observed in southeast Florida for the six tropical storms occurring during the study.

	2008	2009	2010	2011	2012	2013	2014	2015	2016
Fall to winter	na	19	13	6	10	0	11	9	19
Winter to summer	na	na	8	3	5	0	3	3	3
Summer to fall	na	4	4	14	18	6	3	7	18

na = not enough data were available or no surveys were conducted (i.e., Winter and Fall, 2008).

r^2 = 0.07924, $F(3,20)$ = 0.5738, $p > 0.05$; **Figure 1**). PCL within individual plots at BCA ranged from 0 to 70%, with an overall study mean of 8.6 ± 0.38% (±SE). PCL was greater at Scooter with an overall mean of 16.0 ± 0.48% but had a similar range from 0 to 75%. Fluctuation in cover was observed at both patches between monitoring events, years, and seasons with the greatest increases for both patches in 2013 and during the summer (**Figures 1**, **2**). However, these gains were not enough to outweigh the total losses, and by area BCA experienced a net loss of 144 m^2 of living *A. cervicornis* and Scooter 173 m^2. The greatest PCL losses occurred following system-wide disturbance events such as tropical storms, hurricanes, or disease events (**Table 1** and **Figure 2**).

The absolute change in mean PCL varied between monitoring events at both patches (X^2 = 106.88 and 174.28, df = 24, $p < 0.001$ BCA and Scooter, respectively; **Figure 2**). The largest increases were observed from the winter to summer monitoring events where average increases in percent cover per plot were 1.4 and 1.9% at BCA and Scooter, respectively (**Figure 2**, yellow bars). When all years were pooled, 74 (BCA) and 68% (Scooter) of the plots had a mean PCL increase during the summer monitoring events. A negative percent change in mean PCL was observed for a majority of the fall to winter and summer to fall monitoring periods. The magnitude of change was larger at Scooter for 60% of the monitoring events, and BCA and Scooter differed in gain or loss of tissue during six monitoring events (**Figure 2**).

Mean canopy height at BCA ranged from 38 to 55 cm and had an overall mean of 45.6 ± 0.74 cm (±SE). Mean canopy height at Scooter ranged from 32 to 48 cm and had an overall mean of 43.2 ± 0.50 cm. Monitoring event had a significant effect on the absolute change in canopy height [BCA-r^2 = 0.1069, $F(16,408)$ = 3.051, $p < 0.001$; Scooter-r^2 = 0.1907, $F(16,470)$ = 6.923, $p < 0.001$]. Canopy height varied across the study increasing during summer events and decreasing toward the end of the study, as indicated by a large portion of the plots having negative change in height for the final events (**Figure 3**).

Species Census

A total of 4,692 colonies were counted at BCA (density of 0.18 ± 0.01 col/m^2) and 11,894 at Scooter (0.40 ± 0.01 col/m^2)

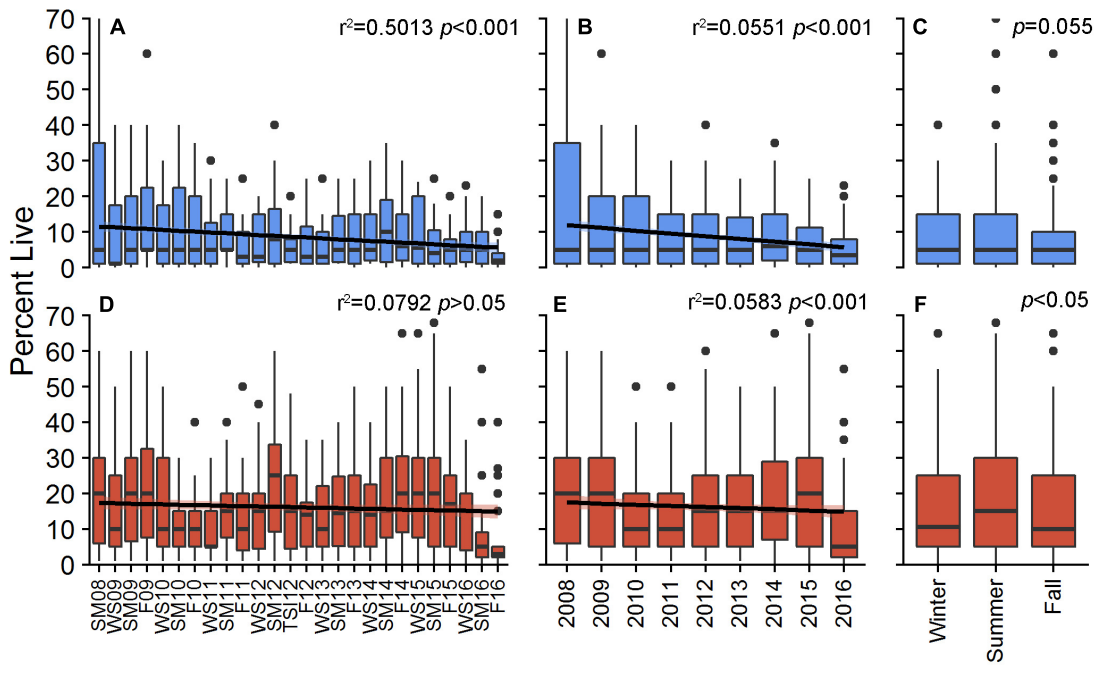

FIGURE 1 | Percent live cover of *Acropora cervicornis* per plot for BCA (blue) and Scooter (red) across monitoring periods **(A,D)**, annually **(B,E)**, and seasonally **(C,F)**. Boxes represent the 25 and 75% quartiles with whiskers extending 1.5 * interquartile range, the line represents the median, and dots are considered outliers.

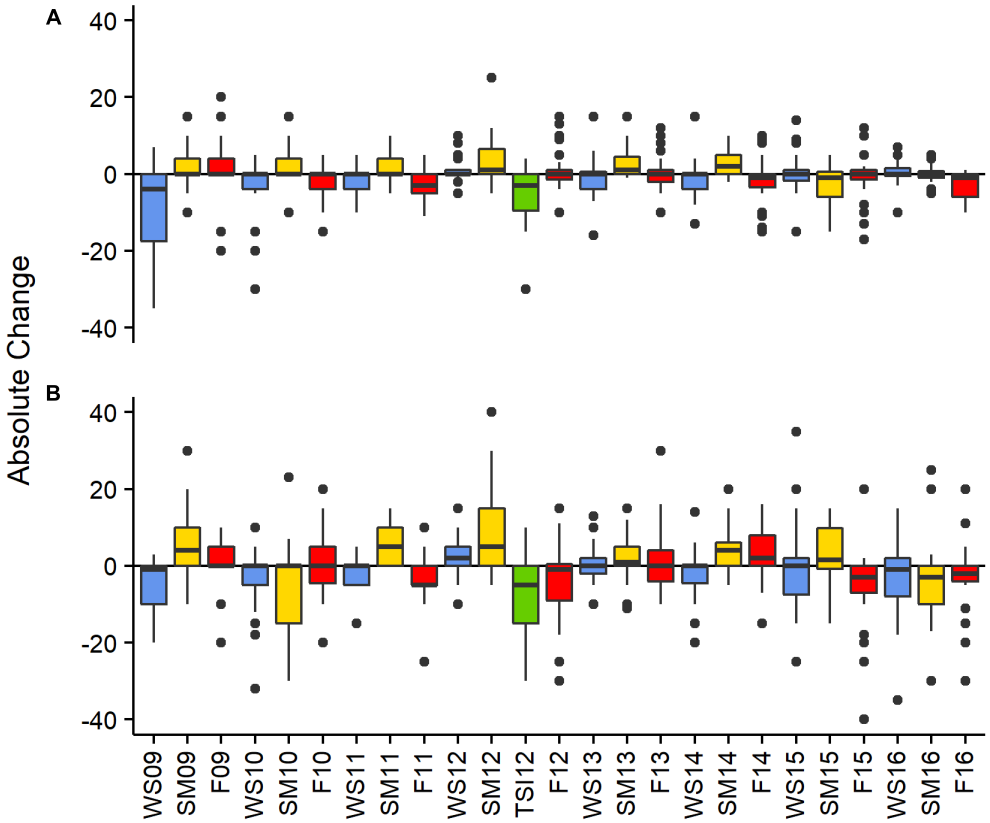

FIGURE 2 | Absolute change in percent cover between monitoring events by site BCA **(A)**, Scooter **(B)**. Colors represent seasonal changes from Fall to Winter—blue, Winter to Summer—yellow, and Summer to Fall—red, the green bar represents change in cover between the SM12 and the TSI12 monitoring events. Boxes represent the 25 and 75% quartiles with whiskers extending 1.5 * interquartile range, the line represents the median, and dots are considered outliers.

across the entire project. Mean colony abundance at Scooter exhibited a significant decreasing trend ($p < 0.001$), with moderate but significant seasonal variation ($X^2 = 17.097$, $p < 0.001$) whereas BCA remained relatively stable with only a few monitoring events having significant deviations from the mean ($p < 0.001$), but had no significant seasonal change ($X^2 = 1.5795$, $p > 0.05$; **Figure 4**). On average, 70 colonies were lost at Scooter and 20 at BCA between each summer and fall monitoring event. Significant increases in mean colony abundance were observed in the summer at Scooter and the winter events at both patches ($p < 0.05$). The mean number of masses per plot for both patches was less than 4 (**Figure 4**). Counts of masses increased at Scooter from 2008 to 2010 and then remained stable. The most masses counted during one monitoring event were at Scooter with 119 masses during SM11. Significant seasonal changes in the abundance of masses were observed at Scooter, with greatest changes observed in the summer ($p < 0.05$).

Nearly 18,000 fragments were counted at the patches during the study. Total fragments counted per monitoring event ranged from 15 to 359 at BCA (plot average = 6.3 ± 0.31 fragments) and 80 to 1,313 at Scooter (17.6 ± 0.73 fragments). Two major fragmentation events occurred at Scooter, WS10 and WS15, where total fragment counts were over 1,000. Four additional events (TSI12, F15, WS16, and F16) had counts 30% over the patch mean. Fragment counts at BCA were highest during TSI12 and WS16 where total fragment counts were over 300. Differences were found between the annual means of fragment counts, with 2010, 2015, and 2016 as high years at Scooter and 2012 and 2016 at BCA. Mean fragment counts differed significantly between seasons (**Figure 4**); on average, there were 88 and 243 fewer fragments counted in the summer than in the previous winter at BCA and Scooter, respectively ($p < 0.05$).

Condition Characteristics

The most prevalent condition recorded for both patches, when all seasons and years were pooled, was fireworm predation followed by white disease, damselfish predation, snail predation, then bleaching. Two white diseases were observed at both patches, rapid tissue loss and white band disease, but because the distinction between them is uncertain, they were pooled as white disease for analyses. Similar annual and seasonal patterns were found between patches for all conditions, although prevalence rates were higher at Scooter for all conditions besides damselfish predation and bleaching. Overall mean prevalence of plots at BCA and Scooter, respectively, was 44.1 ± 1.88 SE% and 72.0 ± 1.6% for fireworm predation, 44.3 ± 1.88 and 66.6 ± 1.68% for disease, 38.8 ± 1.84 and 33.2 ± 1.67% for

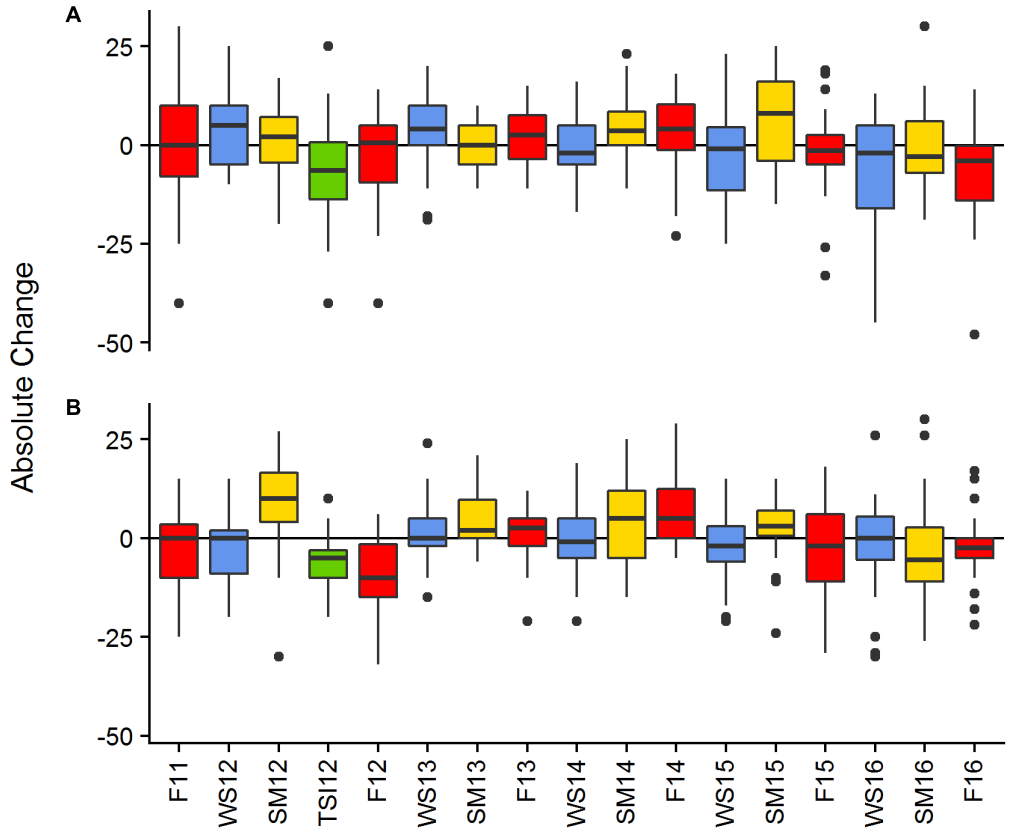

FIGURE 3 | Absolute change in canopy height between monitoring events of living *Acropora cervicornis* by site BCA **(A)**, Scooter **(B)**. Colors represent seasonal changes from Fall to Winter—blue, Winter to Summer—yellow, and Summer to Fall—red, the green bar represents change in cover between the SM12 and the TSI12 monitoring events. Boxes represent the 25 and 75% quartiles with whiskers extending 1.5 * interquartile range, the line represents the median, and dots are considered outliers.

damselfish predation, 6.6 ± 0.94 and 17.9 ± 1.36% for snail predation, and 6.2 ± 0.91 and 3.4 ± 0.65% for bleaching.

Disease prevalence oscillated during the study, resulting in monitoring event, year, and season as significant factors in explaining prevalence of disease (glm, <0.001; **Figures 5**, **6**). The presence of disease increased at times when water temperatures were warmer and following disturbance events. The highest (or near highest) disease prevalence was observed during TSI12, and highest number of occurrences was during SM15 (**Figures 5**, **6**). The year 2013 had the lowest mean maximum temperatures and significantly lower disease prevalence (Tukey, $p < 0.01$) and occurrence counts. Disease was more widespread (present in more plots) at Scooter than BCA (**Figures 5**, **6**), and when present, it was recorded as the primary cause of recent mortality 58 ± 5 and 57 ± 4% of the time at BCA and Scooter, respectively. Overall mean prevalence of disease was higher on masses 36 ± 2.5 and 41 ± 1.9% than on colonies 8 ± 0.8 and 7 ± 0.5% for BCA ($X^2 = 37.525$, $p < 0.001$) and Scooter ($X^2 = 88.801$, $p < 0.001$), respectively. Nearly three times the occurrence counts occurred on masses than colonies (BCA: $X^2 = 58.352$, $p < 0.01$; Scooter: $X^2 = 121.4$, $p < 0.001$).

Fireworm predation affected 40–90% of the plots at Scooter with mean occurrence counts ranging from 1–10 recently preyed upon branch tips on colonies and 1–44 tips on masses per plot. Prevalence of plots with fireworm predation was lower at BCA, affecting fewer than 70% of the plots during any monitoring event. However, BCA had similar mean occurrence counts on colonies (1–8 tips) as Scooter, but much fewer on masses (2–14 tips). When present, it was recorded as the primary cause of mortality in 30 ± 5 and 41 ± 4% plots on average for BCA and Scooter, respectively. Prevalence of fireworm predation was significantly higher in 2015 at both BCA and Scooter (Tukey, $p < 0.001$) and significantly lower in 2013 at Scooter (Tukey, $p < 0.001$). Summer prevalence at Scooter was significantly higher than fall and spring (Tukey, $p < 0.001$), and occurrence counts were the lowest in the fall on both colonies and masses (**Figures 5**, **6**).

Snail predation was not observed at every monitoring event and increased significantly in prevalence toward the end of the study (2013–2016) at Scooter (glm, $p < 0.01$). Prevalence was between 0 and 40% of plots at BCA and 0–60% at Scooter. Although snail predation was affecting close to half of the plots when present, mean occurrence counts were less than three per plot, affecting masses significantly more than colonies (Kruskal–Wallis, $p < 0.001$), and when present, was only the primary cause of mortality

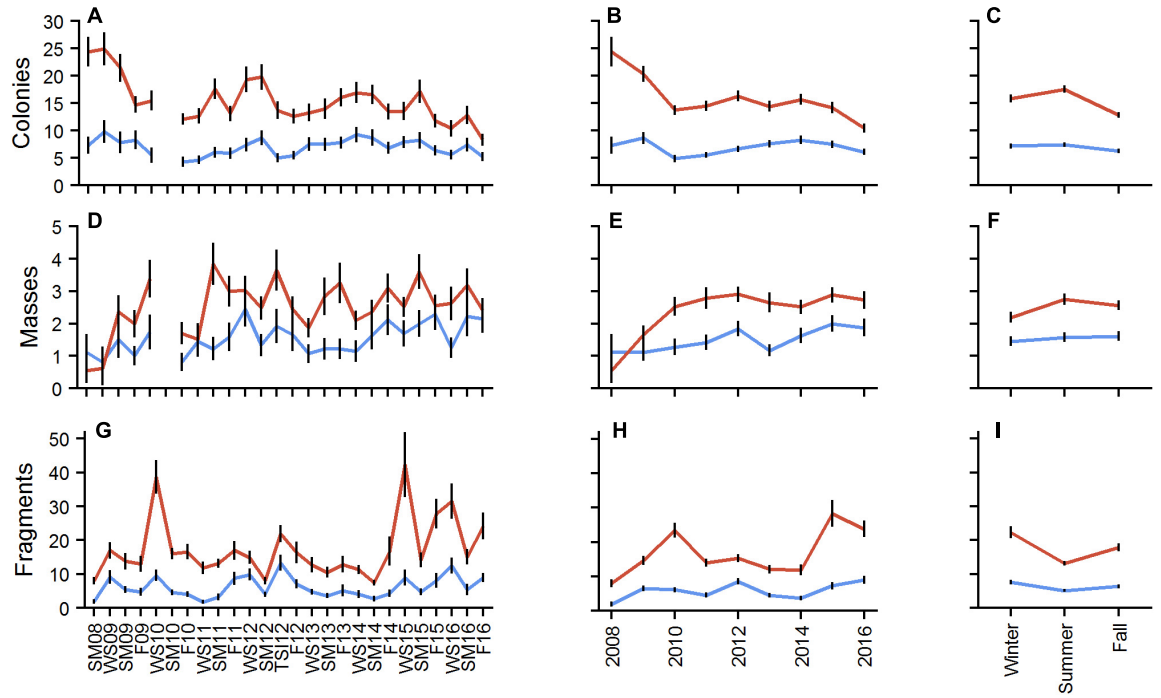

FIGURE 4 | Mean number of colonies **(A–C)**, masses **(D–F)**, and fragments **(G–I)** by plot for BCA (blue) and Scooter (red) by monitoring event **(A,D,G)**, year **(B,E,H)**, and season **(C,F,I)**. Error bars indicate ± 1 SE.

in 3 ± 2 and 12 ± 4% of plots on average at BCA and Scooter, respectively.

Damselfish predation was present during all events and was more wide-spread at BCA than Scooter. It was the primary condition when present in 48 ± 5 and 34 ± 5% of the plots on average at BCA and Scooter, respectively. Damselfish predation significantly increased during the study for Scooter (glm, $p < 0.05$). No seasonal trends were detected in the prevalence of damselfish predation.

Bleaching was not present during all events and was significantly higher during the fall for BCA (Tukey, $p < 0.001$) and summer for Scooter (Tukey, $p < 0.01$). Bleaching was more prevalent at BCA than Scooter, affecting up to 70% of the plots (**Figures 5**, **6**). Masses were more affected by bleaching than colonies.

Temperature

Monthly mean temperature increased during the study (**Figure 7**). The maximum monthly mean ranged between 29.2 and 30.8°C. The warmest month was August for all years except 2008 when July was the warmest. Mean daily temperatures were above 31°C for 1 day in 2010, 5 days in 2011, 10 days in 2014, and 11 days in 2015. Minimum monthly mean increased during the study, ranging from a low in 2009 of 21.5°C to a high in 2014 of 23.9°C. From January 2012 through 2016, only 5 days fell below 22°C, whereas from January 2009 through December 2011 there were 83 days below 22°C.

DISCUSSION

Presented here is a portion of the one of the longest continuous demographic-based monitoring datasets, specifically targeting long-term monitoring of the threatened coral *A. cervicornis*. Published studies on the demography of this species are either sporadic across many years, missing short-term temporal changes and drivers of mortality and recovery, or cover only a few years, missing long-term trends and factors affecting life history characteristics such as impacts from destructive events that may not occur during the time frame of the study. This study included 8 years of observations of two high density populations and documented temporal variation in: PCL, fragment, colony, and mass abundance, and prevalence and occurrence of disease and predation. Environmental disturbances and disease caused significant decreases in PCL and total abundance of colonies. Disease was constantly present and increased during the summer, following Tropical Storms, and on masses. Predation by fireworms, snails, and damselfish caused minimal mortality when compared to disease, but their chronic presence is concerning for species growth, reproduction, and possible transmitter of disease. Unfortunately, the overall health of the two patches deteriorated significantly over the 8 years of this study. Mean cover of living *A. cervicornis* decreased by over 50% at both patches (17–3% BCA; 26–7% Scooter) due to the increasing prevalence of predation and disease and the high frequency of disturbances such as tropical storms, hurricanes, high energy events, and a widespread disease event affecting the Florida Reef Tract (Precht et al., 2016).

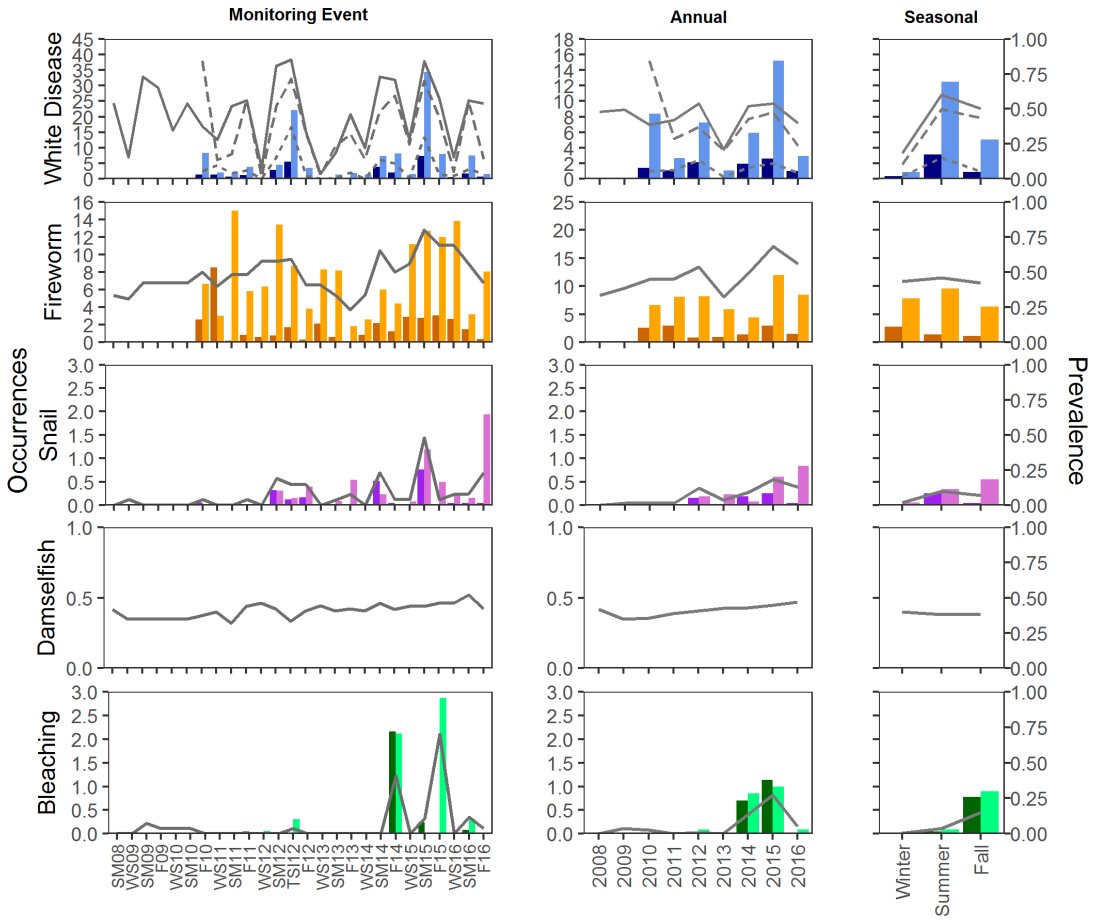

FIGURE 5 | Mean prevalence and occurrence of disease, predation, and bleaching by plot at BCA. Prevalence is indicated by the lines on each graph; solid lines represent prevalence of plots with condition. For disease, dotted and dashed lines represent prevalence on colonies and masses, respectively. Occurrences of each condition were counted on colonies (dark bars) and masses (light bars). Occurrences were not counted for Damselfish predation.

Disturbances during the study disrupted the demography of *A. cervicornis*. During these periods, we documented an increase in disease and predation (typically during the summer) and an increase in fragmentation (during the fall and winter). In fact, the two largest fragmentation events were subsequently followed by an increase in disease prevalence. Exposed skeleton from fragmentation could increase disease susceptibility (Knowlton et al., 1981). In the best-case scenario, we would have expected to see a shift from fragment to colonies and eventually to masses across the study. However, our data indicate that fragment survival and attachment rate may be very low, but similar to what has been previously reported (Highsmith et al., 1980; Knowlton et al., 1981, 1990; Heyward and Collins, 1985; Dollar and Tribble, 1993; Miller et al., 2016a). These rates were not enough to replace the loss of tissue caused by disturbance events. The frequency of disturbance events varied between years; however, during years of few or no disturbances such as 2013, both patches exhibited signs of recovery with increased PCL. This relatively mild year, with lowest maximum mean water temperatures, average wind speeds, and above average rainfall, resulted in the lowest prevalence of both disease and fireworm predation at both patches. This year could be a model year for conditions that allowed for population recovery.

Coral diseases are known to peak when there have been significant or prolonged changes in water temperature, sedimentation, pollution, predator lesions, or for unexplainable reasons (Harvell et al., 1999, 2007). Our data indicate that the diseases affecting *A. cervicornis,* while continuously observed in background levels, may also be exacerbated by increased water temperatures and disturbance events. It is also likely that fireworms and snails may be acting as vectors or reservoirs for pathogens as there is a relationship between the prevalence of disease and predation at both sites (Williams and Miller, 2005; Gignoux-Wolfsohn et al., 2012; Miller et al., 2014b; Bright et al., 2015). Above average air temperatures from May through mid-October 2009 caused SST to remain high through October, resulting in over 80 days at or above 30°C. This increased duration of warmer waters preceded one of the highest prevalence of disease (70–94% of plots) and predation (80–90% of plots) recorded for this study, and prevalence remained high for the next two monitoring events, leading to a major decrease in live tissue at Scooter (-121 m^2). Live tissue at BCA at this time

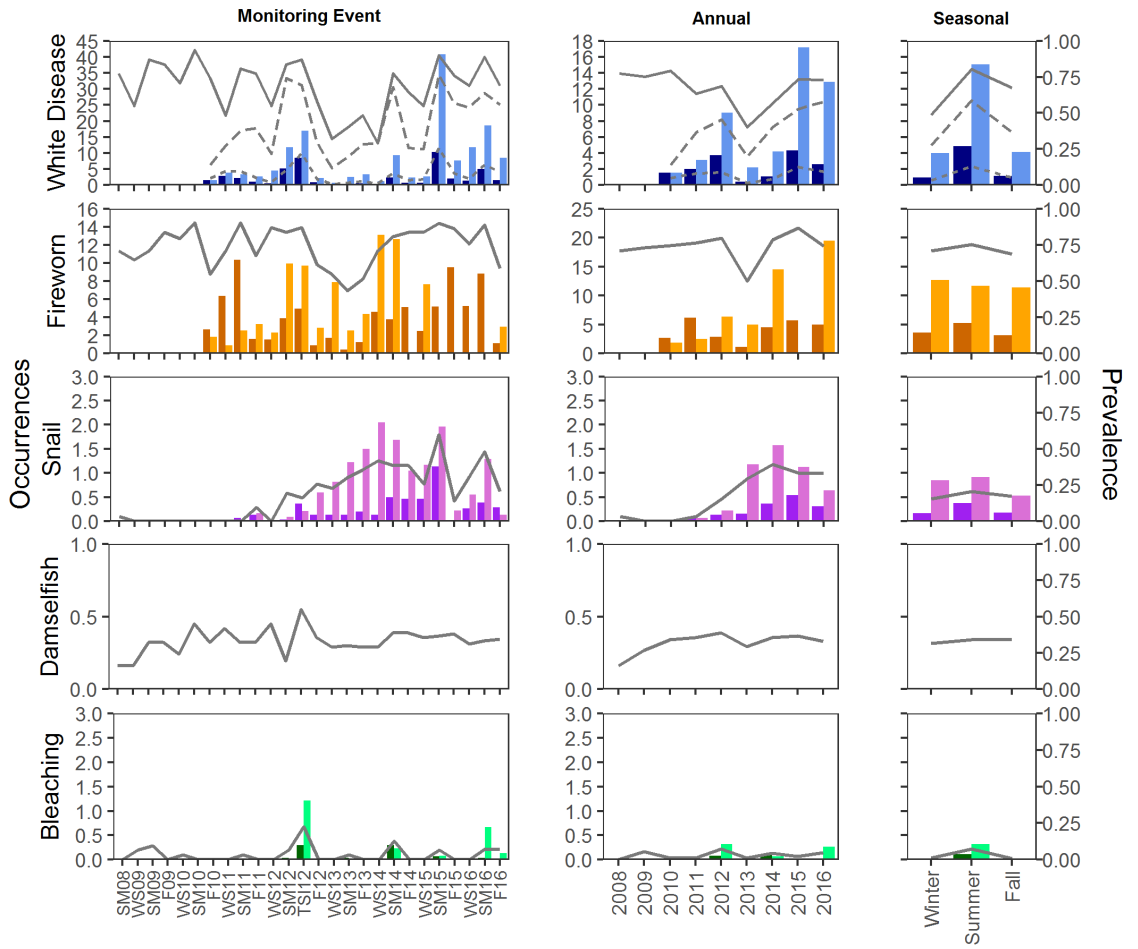

FIGURE 6 | Mean prevalence and occurrence of disease, predation, and bleaching by plot at Scooter. Prevalence is indicated by the lines on each graph; solid lines represent prevalence of plots with condition. For disease, dotted and dashed lines represent prevalence on colonies and masses, respectively. Occurrences of each condition were counted on colonies (dark bars) and masses (light bars). Occurrences were not counted for Damselfish predation.

also decreased but only slightly (-20 m^2), and the prevalence of disease and predation were elevated but lower than Scooter.

The occurrence of disease was significantly higher in 2015 during a widespread disease event affecting the entire Florida Reef Tract (Miller et al., 2016a; Precht et al., 2016). These two patches of *A. cervicornis* were not spared from this outbreak, but were affected on different time scales. Increased presence of disease was maintained at Scooter into the following year, and while there was a decrease in occurrences, prevalence indicated that disease was still present across the entire patch at greater than average prevalence rates. BCA however, had a slight reprieve from disease and a small increase in percent cover, until Hurricane Matthew passed by in October 2016, further reducing PCL at both sites. Prevalence of disease may have been lower at BCA simply due to the sparseness of tissue remaining.

Predation by fireworms and snails varied radically during the study by years, seasons, and sites. The variability was similar to what Miller et al. (2014b) reported across 2 years at multiple sites. While prevalence levels were chronic, the mean tissue lost per colony has been described as 3% (Goergen et al., unpublished). Fireworms typically feed on the live branch tips of colonies, removing the growing end, and stunting branch growth. Regrowth and repair over the consumed area is unlikely (Berkle, 2004; Miller et al., 2014b). Increased occurrence and prevalence of fireworm and snail predation toward the end of the study could be severely damaging for the future growth of the species because predation may become more focused due to the lack of tissue available, leading to the removal of more growth tips from the same colonies. Moreover, fireworms have been a proven vector of a bleaching pathogen (Sussman et al., 2003), which is of great concern because colonies with predation lesions may be more likely to become diseased (Miller et al., 2014b) and both fireworms and snails have been associated with increased disease prevalence (Knowlton et al., 1990; Miller et al., 2014b; Bright et al., 2016). Therefore, it may be advantageous to manage both snail and fireworm populations to increase the health and growth of *A. cervicornis*.

Not only do the presence of disease and predation have a spatial and temporal component, they were also variable

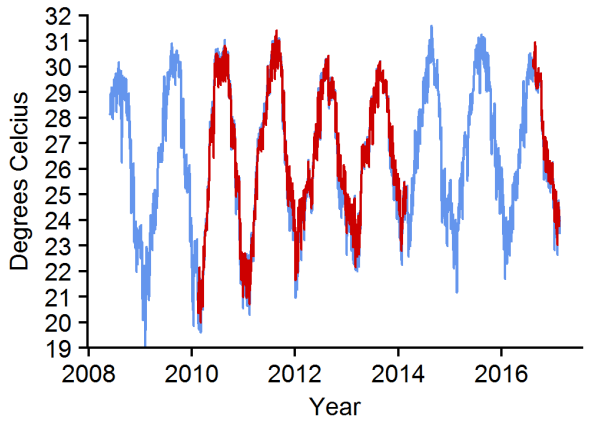

FIGURE 7 | Daily mean water temperature at BCA—blue and Scooter—red. Missing data for Scooter from 2014 to 2016 are due to faulty loggers.

across different life history stages, affecting masses more than colonies. The prevalence of disease for this study ranged from 0 to 37% on colonies (mean approximately 7%) which was similar to previous reports across the species' range (Lirman et al., 2010; Miller et al., 2014a; Goergen et al., unpublished). However, on masses (what others may consider large colonies, thickets, or patches) prevalence was higher, with a range of 2–84% (mean 38%), than previously reported (Vargas-Ángel et al., 2003; Ladd et al., 2016; Goergen and Gilliam, 2018). Because of this discrepancy, high density patches may not be able to persist long-term under modern day conditions. While healthy populations do still exist (Walker, 2017), the loss of cover may be a cyclical event linked to population growth (density) and age. While we were unable to age the patches anecdotal observations of patch structure and successional stages such as height and extant of old dead structure and abundance and size of *Agaricia* spp. colonies on dead structure, indicate that BCA is older and experienced cover decline prior to Scooter. Therefore, as populations grow and potentially expand into high density patches, disease and predation are likely to increase after some time, causing substantial mortality subsequently weakening the skeleton and increasing the likelihood of fragmentation. This process could be detrimental to the persistence of the dense patches unless the fragmentation of a patch can shift to an alternate stable state such as isolated colonies; however, we found very low reattachment success of loose fragments. On the other hand, signs of recovery were present in this study in 2013 when predation and disease prevalence were minimal, maximum water temperatures were lower, and there were only a few days of elevated winds. Unfortunately, reducing water temperatures and wind speeds is out of our direct control; however, active management of predators may be a feasible task. This may be even more prudent in high density areas where disease is more prevalent. Because we still do not know the etiology and transmission mechanisms of these diseases (spreading could be occurring through water movement or fish) by abating disease where it is most abundant will benefit the rest of the marine community.

Extreme changes in cover may not indicate a total loss of *A. cervicornis* tissue at the site. Its high frequency of fragmentation and dislodgement (Goergen et al., unpublished) and fast growth rate allow for fast propagation across sites if conditions are conducive (Highsmith et al., 1980). In previous research, we have shown that the centroids of the densest portions of these patches are indeed shifting (Walker et al., 2012). This shift in live cover is evident in the monitoring plots surrounding the high density areas in the direction of the centroid shift. However, increases in cover in these plots is very minimal (less than 5%) and is in no way equivalent to what was lost in the other plots. In addition, it was most common for plots to decrease in live cover and simultaneously increase in dead skeleton, indicating high mortality and not extreme movement that could support the notion that the population is just shifting spatially. However, there is evidence that propagation is still occurring through colony fragmentation and dislodgement. Propagation through fragmentation has the potential to support the existence of this species in low levels but gains do not keep up with the mortality observed. Despite there being evidence of reef recovery from the propagation of *A. cervicornis* through fragmentation in the Florida Keys in the 1970s (Gilmore and Hall, 1976; Shinn, 1976), current ocean conditions and the increase in frequency of disturbance events will make it difficult for *A. cervicornis* to recover naturally. Population enhancement by way of outplanting colonies in low density aggregations from nurseries could have a positive effect on this species' long-term sustainability while larger environmental issues are tackled (Miller et al., 2016b; Goergen and Gilliam, 2018; Hughes et al., 2018).

Overall, our results confirm that *A. cervicornis* is greatly affected by extreme environmental conditions, disease, and predation. Unfortunately, our data also indicate that prevalence of disease, predation, and fragmentation are increasing and having an even greater detrimental effect on the long-term persistence of this species. As oceans continue to warm (Hughes et al., 2018), warm water driven factors such as bleaching, disease, and predation will increase in frequency and likely intensity. Without time for recovery and growth between these major events, this species will not recover naturally. Of concern is the relationship between disease and predation prevalence and occurrences on masses, which is implying density driven mortality and indicating a cyclical component to the existence of the species. As populations become denser and age, disease and predation become more widespread causing populations to decline to remnant patches of isolated colonies. Furthermore, under modern day reef condition and the frequent occurrences of storms and elevated winds, paired with seasonal and sometimes chronic disease and chronic predation, the ability for a population to grow into these large patches may be difficult. However, these populations are of upmost importance to the continued existence of the species providing an abundance of larvae during spawning and through fragmentation these populations are likely a source to local expansion through propagation of fragments. Therefore, we suggest specific management actions such as the management of predator populations; this may not only lead to improved growth of colonies by reducing the number of damaged growth ends, but could also lead to a reduction in

disease due to their abilities to be vectors of pathogens. This may be even more prudent in high density areas where disease is more prevalent, because the etiology of these diseases is still unknown, and they could also be spreading by water movement or fish, by abating disease where it is most abundant would benefit the rest of the marine community. Furthermore, supporting population enhancement by advising practitioners to outplant at lower densities would also improve the health and longevity of *A. cervicornis*. While colonies may eventually grow together, outplanting them further apart provides more time for growth and healthy colonies to spread across the reef.

AUTHOR CONTRIBUTIONS

EG, DG, AM, and BW conceived and designed the research, performed the experiments, and edited the manuscript. EG analyzed the data and wrote the manuscript.

FUNDING

This work was made possible through the support of the National Coral Reef Institute by an award made to Nova Southeastern University from National Oceanic and Atmospheric Administration Coastal Ocean Program Award Number NA03NOS4260046, National Oceanic and Atmospheric Administration Award Number NA10NMF4720029 to the Florida Fish and Wildlife Conservation Commission, and Nova Southeastern University Presidents Faculty Research and Development Grant.

ACKNOWLEDGMENTS

We thank all the past, present, and honorary students of the Nova Southeastern University Coral Reef Restoration, Assessment, and Monitoring Lab for assisting in data collection. A special thanks goes to Dale Goergen for his never ending help and determination in designing and perfecting the figures in R and E.A. Goergen's Ph.D. committee for their thoughtful comments and critiques during project planning and dissertation reviews. This manuscript was submitted in partial fulfilment for E.A. Goergen's degree of Doctorate of Philosophy at Nova Southeastern University Halmos College of Natural Sciences and Oceanography.

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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

ORIGINAL RESEARCH
published: 19 March 2019
doi: 10.3389/fmars.2019.00121

Short Term Exposure to Heat and Sediment Triggers Changes in Coral Gene Expression and Photo-Physiological Performance

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OPEN ACCESS

Edited by:
Catherine Ellen Ivy Head, University of Oxford, United Kingdom

Reviewed by:
Eslam O. Osman, University of Essex, United Kingdom
Michael Sweet, University of Derby, United Kingdom
Bryan Wilson, University of Oxford, United Kingdom

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *28 April 2018*
Accepted: *27 February 2019*
Published: *19 March 2019*

Citation:
Poquita-Du RC, Huang D, Chou LM, Mrinalini and Todd PA (2019) Short Term Exposure to Heat and Sediment Triggers Changes in Coral Gene Expression and Photo-Physiological Performance. Front. Mar. Sci. 6:121. doi: 10.3389/fmars.2019.00121

Corals, together with their endosymbiotic Symbiodiniaceae, are known to exhibit a suite of mechanisms for survival under stressful conditions. However, exactly how the host animal responds to varying environmental conditions remains unclear. In this study, we tested two relevant environmental factors that can have deleterious effects on corals: heat and sediment. We examined among-genotype responses of *Pocillopora acuta* to these factors with RNA-Seq in concert with widely-used tools for assessing the physiological conditions of corals. Heat and sediment treatments were applied in a 2 × 2 crossed experimental design: (1) ∼30°C without sediment (control, "C"), (2) ∼30°C with sediment (sediment-only, "S"), (3) ∼32°C without sediment (heat-only, "H"), (4) ∼32°C with sediment (heat + sediment, "H+S") over four consecutive days (3-h daily exposure) in *ex situ* aquarium conditions. A clear differentiation in gene expression patterns was observed in corals exposed to heat alone and to heat with sediment, relative to the control treatment. Few transcripts (∼3) were found to be differentially expressed for corals exposed to sediment only. The greater impact of heat was supported by photo-physiological measurements that showed significant effects on maximum quantum yield and average symbiont density among genotypes of *P. acuta*. The combined effect of heat and sediment caused a greater reduction in average symbiont density than the effect of sediment alone. Furthermore, "H+S" disrupted the ability of corals to maintain processes involving assembly and disassembly of cilium which suggests a synergistic effect between the two factors. We also found that host-specific genes which were expressed differentially may not be related to their interactions with algal symbionts. Rather, these genes are involved in a variety of biological functions including, but not limited to, cilium biogenesis and degradation, cilia motility, innate and adaptive immune responses, cell adhesion and bone mineralization, and processes involved in the cell

cycle. These results reflect the complex response of the host alone. Overall, our findings indicate that acute heat stress in tandem with sediment can depress photo-physiological performance and trigger considerable changes in host gene expression.

Keywords: ***Pocillopora acuta*, *ex situ* experiment, transcriptome-wide, RNA-Seq, Symbiodiniaceae, genotypes**

INTRODUCTION

Coral communities situated in nearshore coastal waters are often exposed to stressful conditions generally linked to adjacent land-based activities. For example, land reclamation and port construction, which generally involve dredging, can cause elevated levels of turbidity and increased sedimentation rates in inshore reefs (Erftemeijer et al., 2012). As corals are generally sessile, they are unable to move away from such adverse conditions. Elevated levels of sediment can affect their photosynthetic performance due to light reduction when colony surfaces are covered with sediments or because of reduced light penetration caused by suspended solids (Falkowski et al., 1990; Anthony and Fabricius, 2000; Anthony and Connolly, 2004). Smothered corals expend energy clearing sediment off their tissues and their feeding activity can also be impaired (Rogers, 1990; Fabricius, 2005; Erftemeijer et al., 2012). Disturbances caused by sediment exposure have caused coral loss in numerous locations such as Bahrain (Zainal et al., 1993), Hong Kong (Hodgson, 1994), Thailand (Brown et al., 1990; Chansang et al., 1992), and Singapore (Hilton and Manning, 1995; Chou, 2006).

Large-scale climate change-related processes such as ocean warming can amplify local anthropogenic stressors, including high sedimentation and turbidity levels, resulting in greater environmental pressure on coral communities (Hoegh-Guldberg et al., 2007). Both heat- and sediment-related impacts can occur at various temporal scales, from long term (chronic) to brief but severe (acute) events. Acute heat stress due to anomalous sea surface temperature can cause corals to bleach and result in mass mortality (Baker et al., 2008; DeCarlo et al., 2017). Bleaching is an obvious manifestation of a stress response in corals due to the dissociation of symbiotic algae (Buddemeier and Fautin, 1993; Glynn, 1993). The global pattern of coral bleaching events associated with thermal anomalies shows more frequent recurrence with increasingly shorter time intervals for recovery of affected coral reefs (Hughes et al., 2018). Moreover, acute sediment stress events characterized by short-term high turbidity and/or high levels of downwelling sediments incur high energetic costs (Brown and Bythell, 2005; Browne et al., 2014), with the ultimate consequence of reduced energy for coral growth and reproduction (Fabricius, 2005). Recurrent coral bleaching events on top of local sediment impact raise a major concern regarding the capacity of existing coral assemblages to withstand multiple stressors.

It is well understood that survival mechanisms of corals in response to different stressors can vary considerably both among and within species due to differences in their life history strategies, along with other biological attributes (Yap et al., 1992; Todd et al., 2001; Darling et al., 2012; Lui et al., 2012). Previous studies on the physiological responses of corals to stress have highlighted the acclimatization capacity of the coral host, such as induction of heat shock proteins and regulation of enzymatic activity (Brown, 1997). Maintenance of protein turnover is a known acclimatization mechanism in several coral species (Sharp et al., 1994; Black et al., 1995; Fang et al., 1997). Symbiotic corals, in particular, can also acquire essential amino acids through translocated nutrients from their algal symbionts (Hoegh-Guldberg, 1999). The close relationship between coral hosts and their photosynthetic algal symbionts (currently referred to as Symbiodiniaceae; LaJeunesse et al., 2018) adds to the complexity of coral responses and survival strategies. Both the host coral and algal symbionts have inherent physiological attributes that enable them to survive under varying environmental conditions (Roth, 2014). However, due to the obligate nature of the symbiosis, exactly how the host animal responds to varying environmental conditions remains unclear.

Today, rapidly evolving technologies such as high-throughput sequencing of DNA have revolutionized the way coral scientists study responses of organisms to environmental changes (reviewed in Sweet and Brown, 2016). Highly sensitive and accurate genomic approaches have provided tools not only for biomedical sciences to identify causes of diseases, but also for ecological studies such as those attempting to identify responses of corals to various stressors. For instance, RNA sequencing, or RNA-Seq, is a relatively recent high-throughput sequencing approach that generates data for transcriptome-wide profiling suitable for non-model organisms such as corals (Kenkel and Matz, 2016). This approach offers advantages compared to previous first-generation approaches (e.g., quantitative polymerase chain reaction and microarray) as it produces very low background signal and a large range of transcript expression levels with high accuracy and reproducibility (Wang et al., 2010), and is therefore more cost-effective. The availability of genomic and transcriptomic data for both coral host and symbionts also allows independent examinations of these separate components. Indeed, transcriptome-wide profiling has changed the way coral scientists approach important biological questions as it provides information on the entire suite of transcripts that are regulated across different environmental conditions. This approach is particularly useful for examination of the physiological state of organisms (Evans and Hofmann, 2012) and their capacity to survive under different levels of stress.

Here, we employ RNA-Seq to characterize the gene expression patterns of corals collected from the highly-urbanized reefs of Singapore. The long history of coastal modification in this tropical island nation since the 1960s has resulted in a ∼60% loss of coral reefs (Hilton and Manning, 1995). Contemporary levels of turbidity and sediment accumulation on local reefs can reach up to 150 mg l^{-1} and 37 mg cm^{-2} day^{-1} respectively (Browne et al., 2015). Even the reefs furthest

from coastal development experience sediment exposure that is considered beyond "normal" levels (i.e., >10 mg cm^{-2} day^{-1}; see Rogers, 1990). This level of sediment pressure can severely affect the physiology and survival of reef corals. For instance, Browne et al. (2014) quantified the effects of 2-h acute sediment stress events on three coral species common in Singapore (*Merulina ampliata*, *Pachyseris speciosa,* and *Platygyra sinensis*) and reported a significant reduction in net energy production for all three species examined. This was attributed to a decrease in photosynthesis caused by high turbidity and low light availability. Furthermore, warmer periods in 1998 and 2010 coinciding with El Niño events where sea surface temperature level rose ≥ 1°C above the maximum monthly mean in Singapore (29.86°C) (National Oceanic and Atmospheric Administration Coral Reef Watch, 2000) caused mass coral bleaching on Singapore's reefs (Guest et al., 2012, 2016), adding to the suite of pressures they experience. These episodes are becoming more frequent in recent years and have affected a wide range of coral taxa (Chou et al., 2016).

In this study, we profile transcriptome-wide gene expression patterns and photo-physiological responses of a common coral, *Pocillopora acuta*, following acute exposure to the two major environmental factors associated with coral stress in Singapore: heat and sediment. Specifically, we: (1) test the separate and combined effects of heat and sediment exposure on photo-physiological performance and gene expression patterns in *P. acuta*; (2) characterize the functional profiles of the differentially-expressed genes; and (3) determine whether the responses vary among coral genotypes.

MATERIALS AND METHODS

Study Species and Sampling Sites

Pocillopora acuta Lamarck, 1816 can be found on most reefs in Singapore (Poquita-Du et al., 2017). Owing to its weedy life-history strategy (Darling et al., 2012), it is believed to allocate more energy toward growth than survival responses to environmental changes (Pinzon et al., 2014). The genus *Pocillopora* was previously categorized as one of the most susceptible to thermal stress (Loya et al., 2001; McClanahan et al., 2004), however, recent observations during coral bleaching events in Singapore identify *Pocillopora* as one of the least affected genera (Guest et al., 2012; Chou et al., 2016; Poquita-Du et al., 2019).

TABLE 1 | Colonies of *Pocillopora acuta* collected from different sites in the southern islands of Singapore.

Collection site	Location	No. of colonies collected	Genotype (G) ID
Raffles Lighthouse	1.1602°N 103.7403°E	2	G1, G4
Kusu Island	1.2257°N 103.8602°E	3	G2, G5, G7
St. John's Island	1.2236°N 103.8452°E	3	G3, G6, G8

A total of eight adult colonies (called genotypes hereafter) of *P. acuta* (∼25 cm maximum diameter) were collected from three reef sites (∼4 m depth) in Singapore's southern islands (**Table 1**). The colonies were immediately transported to the outdoor aquarium facilities at St. John's Island National Marine Laboratory and kept in a flow-through system for 5 days of acclimation. Each colony was cut into 12 fragments (∼6 cm maximum diameter) and attached individually on cement tiles using epoxy putty (Alteco EpoPutty). The coral fragments (N = 96) were placed in a holding tank for another round of acclimation (5 days) to recover from any fragmentation stress.

Ex situ Experiment

Heat and sediment treatments were applied in a 2 × 2 crossed design (n = 3): (1) ∼30°C without sediment (control, "C"), (2) ∼30°C with sediment (sediment-only, "S"), (3) ∼32°C without sediment (heat-only, "H"), and (4) ∼32°C with sediment (heat + sediment, "H+S"). Mini-vortex resuspension tanks (mVorts; **Figure 1A**) with a motor-driven paddle combined with an air-lift system (described in detail by Browne et al., 2014), were used to create the sediment regime. The mVorts were placed in two separate water baths (six in each water bath), one maintained at ∼30°C and the other subjected to heat (∼32°C) using aquarium heaters (Eheim Jager) (**Figure 1B**). Heat exposure was adjusted in a stepwise manner (increment of 1°C h^{-1}) until the water temperature reached 32°C and was then maintained for 3 h [from 12:00 PM – 3:00 PM; **Supplementary Figure (SF) 1A**]. The duration of the experiment was based on previous hourly water temperature records (unpublished data) during warmer periods where temperature tended to be higher only around noon. In a pilot experiment, *P. acuta* coral fragments were subjected to continuous exposure at 32°C but the corals bleached the following day. Therefore the 'treatment' conditions were designed to investigate how these organisms deal with non-lethal thermal anomalies. Sediment was added to three mVorts at each of the two temperatures. The procedure for sediment exposure was adapted from Browne et al. (2014) which used silicon carbide (Si_2C) as artificial sediment to avoid any confounding factors from using natural sediments (e.g., microbial organisms). Browne et al. (2014) developed a mixture of different grit sizes of Si_2C which matched Singapore's reef sediment profile. The present study used the same mixture and amount (∼25 g) placed in each mVort. To simulate acute sediment stress events, sediment was forced up the central hollow tube of the mVort by means of full air bursts at 30-min intervals. The environmental conditions (temperature and light levels) inside the mVorts were continuously monitored for the entire duration of the experiment using HOBO pendant temperature/light loggers (Onset HOBO UA-002-08) set to log every 5 min. Average light levels inside the mVorts exposed to a sediment event dropped from ∼70 to ∼21 μmol photons m^{-2} s^{-1} (**Supplementary Figure 1B**) immediately after the sediment event. As sediment started to settle, the light levels gradually increased until the next event was triggered.

Due to the limited number of mVorts (12), the experiment was conducted in three batches. Each batch was run for four consecutive days and the entire experiment was completed

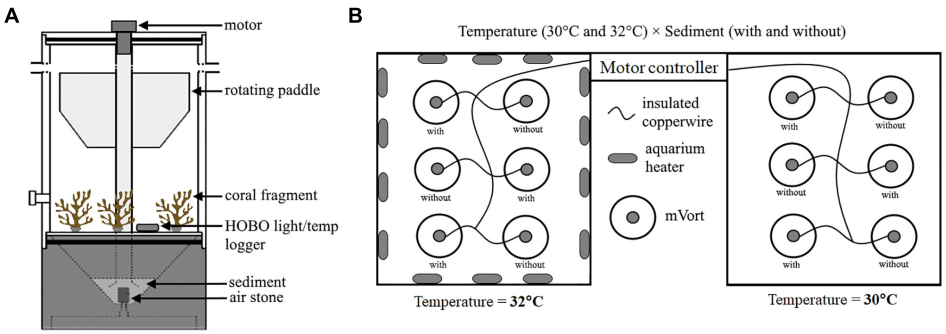

FIGURE 1 | Experimental setup showing **(A)** mini-Vortex resuspension tank (mVort) which was used to simulate sediment stress event and contained three *P. acuta* fragments from different genotypes; and **(B)** Two separate water baths containing the mVorts which were held at two temperature levels (30 and 32°C). In each water bath, three mVorts were exposed to sediment (i.e., with sediment) while the other 3 were not (i.e., without sediment).

in approximately 3 weeks. Three fragments from different genotypes were placed in each mVort, so 36 fragments were used per batch of the experiment. Within each batch, three genotypes were represented in each of the three treatments and control; beyond this caveat, all fragments were assigned to mVorts randomly.

Analyses of Coral Photo-Physiological Performance (F_v/F_m, Symbiodiniaceae Density and Chlorophyll (chl) *a* Concentration)

A portable diving PAM fluorometer (Walz) was used to measure maximum quantum yield [$F_v/F_m = (F_m - F_o)/ F_m$] (Beer et al., 1998). Before the start of the experiment, rapid light curves (RLCs) were performed for all coral fragments to assess their photosynthetic performance over a wide range of ambient light intensities. All of the coral fragments were able to maintain high photosynthetic performance over the course of RLC, suggesting the corals were in good condition. To determine the effects of heat and sediment exposure on coral photosynthetic efficiency, maximum quantum yield was measured at the end of experiment. The corals were placed in the dark (in glass beakers covered with black cloth) for 20 min prior to measurement to relax the photosynthetic reaction centers. Five measurements from different branches (upward pointing terminal branch) of each coral fragment were taken, keeping a constant distance of 5 mm between the optical probe and coral surface. The minimum fluorescence (F_o) was measured by applying a weak measuring beam of < 1 μmol photons m^{-2} s^{-1}, followed by a saturating pulse of light > 1000 μmol photons m^{-2} s^{-1} to measure maximal fluorescence (F_m). The five measurements were then averaged for each sample coral fragment.

Coral nubbins (∼2 cm) were clipped from each coral fragment at the end of the experiment for measurements of Symbiodiniaceae density and chl *a* concentration. All samples were individually wrapped in foil and kept on ice before being frozen at −80°C in the laboratory. Symbiodiniaceae were extracted following Ben-Haim et al. (2003). Coral tissues were stripped from their skeleton using a Waterpik water flosser with UV-filtered seawater (UV-fsw). The resultant slurries were filtered through 50 and 15 μm mesh to remove mucus and minimize clumping of cells. The slurries were centrifuged at 20°C for 30 min at 3220 × *g* to obtain an algal pellet which was then resuspended in 5 mL UV-fsw. The samples were mixed thoroughly by vortexing the tubes before taking aliquots of 1 mL for enumeration of Symbiodiniaceae. The remaining 4 mL was kept for subsequent chl *a* analysis. To reduce clumping of cells and for ease of counting, the aliquots were passed through a 27G × 0.5-inch syringe until no visible clumps were seen. Neubauer Improved Haemocytometer was used to count the number of Symbiodiniaceae cells inside the five squares under a compound microscope, performing eight replicate counts for each sample aliquot. Symbiodiniaceae density was calculated and normalized to coral surface area which was measured using the image analysis software, CPCe V4.1 (Kohler and Gill, 2006).

Following enumeration of Symbiodiniaceae, sample tubes containing the remaining 4 mL algal suspension were centrifuged to obtain algal pellets. The supernatant was discarded, and pellets were resuspended in 100% analytical grade acetone. The sample tubes were wrapped in aluminum foil and left to stand overnight at 4°C for chl *a* extraction (Siebeck et al., 2006). Pigment absorbance readings were measured at 630, 663, and 750 nm using a spectrophotometer (UV Visible Spectrophotometer UVmini-1240, Shimadzu, Japan). The concentrations of chl *a* per sample was calculated using the equation from Jeffrey and Humphrey (1975) and normalized to coral surface area.

To examine the effects on coral photo-physiological performance, the data were analyzed using mixed effects models in R statistical software (R Core Team, 2014). Both F_v/F_m and chl *a* concentration (continuous data) were fitted to linear mixed-effects models, while count data for Symbiodiniaceae density were fitted to a generalized linear mixed-effects model using Poisson error distribution. The models included fixed effects (genotype, temperature, and sediment) and random effects (batch and mVort). Stepwise model simplification and selection followed the Akaike Information Criterion to obtain the final model that best explained the variation.

RNA Isolation, cDNA Library Preparation and Sequencing

Immediately after the first batch of the experiment, ∼2 cm pieces were removed from all 36 coral fragments [three genotypes (G1, G2, G3) × four treatment/control conditions × three replicates] and placed in individual sample tubes pre-filled with RNAlater (Thermo Fisher) for stabilization. The sample tubes were inverted to mix for ∼30 s and kept at 4°C overnight to allow complete penetration of RNAlater into the coral tissues. The tubes were subsequently stored at −30°C until RNA extraction. Total RNA isolation was performed using TRIzol (Life Technologies), following the manufacturer's protocol with modifications based on Barshis et al. (2013). Quality of RNA was examined using gel electrophoresis, yield quantified using Qubit RNA (Broad-Range) assay kit, and quality assessed based on good RNA integrity number (RIN ≥ 8). Illumina-based cDNA library preparation was carried out for each sample. All 36 cDNA libraries were pooled and sequenced across three separate lanes (i.e., 36:36:36) on the Illumina HiSeq 2500 platform for 250-bp paired-end sequencing under the rapid run mode.

De novo Transcriptome Assembly and Open Reading Frame (ORF) Prediction

To allow consolidation of the entire set of transcripts for each genotype, all sequence reads from the same genotype (regardless of experimental treatments) were combined and assembled, *de novo*, using Trinity version 2.2.0 (Grabherr et al., 2011; Haas et al., 2013). Quality trimming (removal of poor-quality reads with Phred quality score < 20, and adapter sequences) and normalization of reads were performed using Trimmomatic, a built-in tool in Trinity. Gene expression profiles are known to vary among coral individuals (Seneca et al., 2010), and unaccounted biological variation can be mistaken as differences in gene expression profiles due to treatments (Hansen et al., 2011). Therefore, the approach of constructing an individual reference assembly (*de novo)* for each genotype was employed to account for inherent biological variability. It is important to note that in this study we are taking a functional approach with respect to the responding gene families. Therefore, identification of exact gene copies involved in the responses is not necessary (Rittschof and Robinson, 2016).

Transdecoder (V 3.0.0) was used to identify open reading frames (ORFs) in the assemblies to aid in gene prediction. Redundant transcript sequences were removed using the CD-HIT tool[1] through clustering analysis of transcripts that showed 100% nucleotide sequence similarities for both strands. To identify transcripts which were putatively coral in origin, de-duplicated assemblies (i.e., containing only unique transcripts) were aligned to predicted protein assemblies from existing genome and transcriptome sequences of 20 hard coral species (data source[2]) using *blastp*, retaining only matches with expected value (*e*-value) of $\leq 1e^{-5}$. To avoid taxonomic mis-assignment of ORFs, sequences which may have belonged to algal symbionts were filtered by mapping the obtained assemblies from the above step to available genomic resources of three Symbiodiniaceae taxa [*Breviolum minutum* (Shoguchi et al., 2013), *Symbiodinium microadriaticum* (Aranda et al., 2016), and *Fugacium kawagutii* (Lin et al., 2015)]. Subsequently, ribosomal RNA (rRNA) contamination was removed based on nucleotide sequence similarity (*blastn*, evalue $\leq 1e^{-5}$) to curated rRNA data[3] (Lagesen et al., 2007). The remaining sequences after the filtering steps were designated as final 'coral-only' reference assemblies.

Gene Expression Analysis and Functional Profiling

Differential gene expression (DGE) analysis was performed using the RNA-Seq Analysis tool in CLC Genomics Workbench (V 10.0.1) which employed empirical analysis of DGE (EDGE) (Robinson and Smyth, 2008; Robinson et al., 2010). Individual sample reads were mapped to their corresponding reference assemblies to obtain expression counts (mapping parameters: mismatch cost = 2; insertion cost = 3; length fraction = 0.8 and similarity fraction = 0.8). The test performed three pairwise comparisons to examine the effects of experimental treatment on gene expression relative to the control. The pairwise comparisons included: (1) "C" vs. "S" (2) "C" vs. "H" and (3) "C" vs. "H+S". The test used raw expression counts and implemented normalization of these counts to account for sequencing depth and differences of transcript lengths. Gene expression profiles of different experimental conditions for each of the genotype were visualized with principal component analysis (PCA) plots and heat maps in CLC. Differentially expressed transcripts (DETs) were analyzed based on the assumption that count data followed a negative binomial distribution. False discovery rate (FDR) was used to correct for multiple comparisons and was controlled at 5% (proportion of false positives). All DETs from each pairwise comparison for each genotype were aligned to known protein sequences curated in the Uniprot Swissprot protein database using *blastx* with a threshold *e*-value of $\leq 1e^{-5}$. Translated sequences that matched the same protein were assumed to be identical genes performing the same function and therefore originated from the same gene locus (**Supplementary Table 1**). Only annotated DETs that were present in all genotypes were presumed to be the core genes involved in the response mechanisms, and thus designated here as DEGs. The available information from all significant *blastx* matches (i.e., protein accession id) was then used to annotate the DEGs with likely functional profiles [i.e., Gene Ontology (GO) terms] based on UniProtKB[4].

RESULTS

Photo-Physiological Analyses

Heat significantly affected both maximum quantum yield (F_v/F_m) (DF = 31, *F*-value = 21.857, *p*-value = 0.0001) and average symbiont density (estimate = −1.168, se = 0.236,

[1] http://weizhongli-lab.org/cd-hit/ref.php

[2] http://comparative.reefgenomics.org/datasets.html

[3] http://www.cbs.dtu.dk/services/RNAmmer/

Pr (> |z|) = $7.56e^{-07}$), for which responses varied depending on the genotype (**Tables 2**, **3** and **Figures 2A,C**). Heat did not produce any significant effect on chl *a* concentration.

The main effect of sediment alone on all of the photophysiological parameters examined was not significant. However, there were some genotype-dependent significant effects of sediment on average symbiont density (**Table 3** and **Figure 2C**). A combined effect of heat and sediment was observed for chl *a* concentration (*F*-value = 6.119, *p*-value = 0.0193, **Figure 2B**) and responses of corals to these combined factors varied in terms of average symbiont density.

TABLE 2 | Results from linear mixed-effects model on the effects of different treatments on F_v/F_m and chl *a* concentration, using control as the reference category (intercept).

Parameter	**Maximum quantum yield (F_v/F_m)**			**chl *a* concentration**		
Final Model	F_v/F_m ~ Genotype + Temperature + Sediment + Genotype × Temperature			chl *a* ~ Genotype × Temperature × Sediment		
Effects	**DF**	***F*-value**	***p*-value**	**DF**	***F*-value**	***p*-value**
(Intercept)	46	2068	**0.000**	30	63.884	**0.000**
Genotype	46	5.877	**0.000**	29	1.619	0.169
Heat	31	21.857	**0.000**	30	0.4678	0.499
Sediment	31	1.603	0.215	30	0.6091	0.441
Genotype × Temperature	46	3.629	**0.003**	29	0.6168	0.737
Genotype × Sediment	–	–	–	29	0.6147	0.739
Temperature × Sediment	–	–	–	30	6.119	**0.019**
Genotype × Temperature × Sediment	–	–	–	29	1.551	0.190

Significant values are in bold.

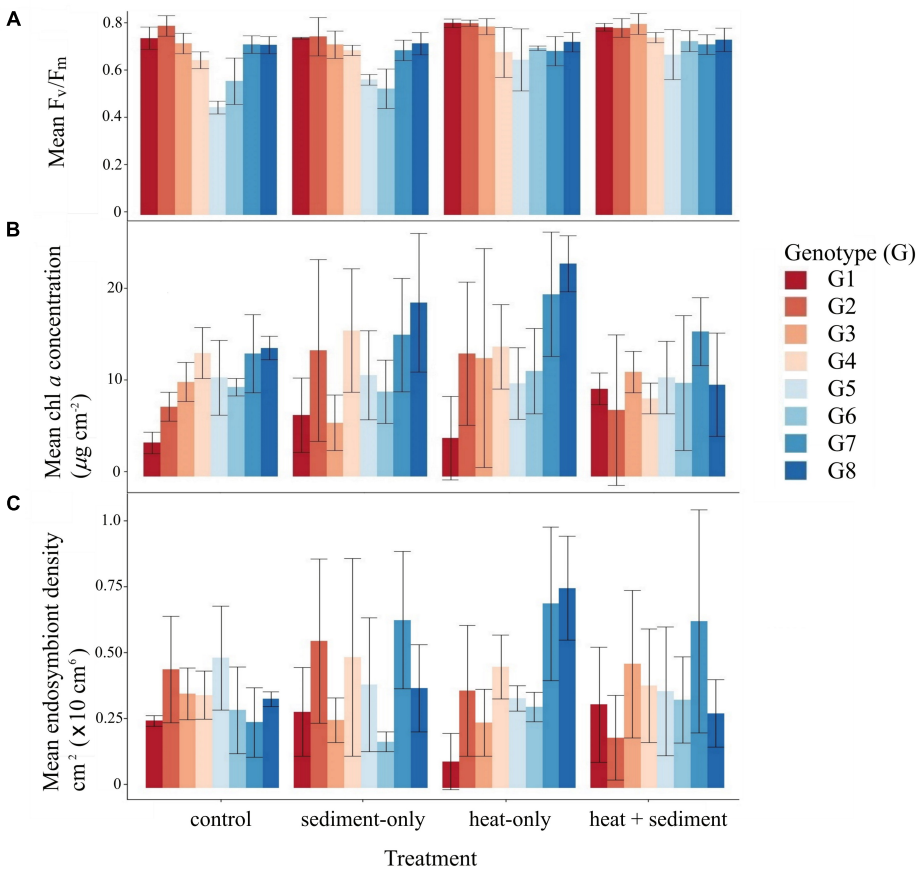

FIGURE 2 | Average levels of maximum quantum yield (F_v/F_m) **(A)**, chl *a* concentration **(B)** and, symbiont density **(C)** for all genotypes and different treatments. Box plots show median values (solid horizontal line), interquartile range (box outline) and highest and lowest extremes (whiskers).

TABLE 3 | Results from generalized linear mixed-effects model on the effects of different treatments on average symbiont density, using control as the reference category (intercept).

Parameter	**Symbiont density cm^2 (×10^6)**		
Final Model	symbiont density ~ Genotype × Temperature × Sediment		
Effects	**Estimate**	**SE**	**Pr(> |z|)**
(Intercept)	12.421	0.167	**<2e^{-16}**
Genotype 2	0.567	0.001	**<2e^{-16}**
Genotype 3	0.338	0.001	**<2e^{-16}**
Genotype 4	0.330	0.237	0.165
Genotype 5	0.665	0.237	**0.005**
Genotype 6	0.153	0.237	0.519
Genotype 7	−0.016	0.236	0.944
Genotype 8	0.2854	0.236	0.2265
Heat	−1.168	0.236	**7.56e^{-07}**
Heat × with sediment	0.365	0.237	0.124
With sediment	0.083	0.236	0.727
Genotype 2 × heat	0.936	0.003	**<2e^{-16}**
Genotype 3 × heat	0.50.067	0.003	**<2e^{-16}**
Genotype 4 × heat	1.442	0.335	**1.70 e^{-05}**
Genotype 5 × heat	0.806	0.335	**0.016**
Genotype 6 × heat	1.217	0.335	**0.000**
Genotype 7 × heat	2.214	0.333	**2.95 e^{-11}**
Genotype 8 × heat	1.993	0.333	**2.17 e^{-09}**
Genotype 2 × heat × with sediment	−1.277	0002	**<2e^{-16}**
Genotype 3 × heat × with sediment	−0.147	0.002	**<2e^{-16}**
Genotype 4 × heat × with sediment	−0.316	0.337	0.347
Genotype 5 × heat × with sediment	−0.707	0.337	**0.036**
Genotype 6 × heat × with sediment	−0.291	0.337	0.388
Genotype 7 × heat × with sediment	0.531	0.334	0.112
Genotype 8 × heat × with sediment	−0.573	0.334	0.086
Genotype 2 × with sediment	0.089	0.002	**<2e^{-16}**
Genotype 3 × with sediment	−0.456	0.002	**<2e^{-16}**
Genotype 4 × with sediment	0.253	0.334	0.448
Genotype 5 × with sediment	−0.317	0.334	0.343
Genotype 6 × with sediment	−0.607	0.334	0.069
Genotype 7 × with sediment	0.831	0.335	**0.013**
Genotype 8 × with sediment	0.009	0.335	0.979

Significant values are in bold.

Sequencing Yield and *de novo* Transcriptome Assembly Statistics

Combined raw reads from 3 lanes for each cDNA library (×36 libraries) ranged from 14.4 million (~3.63 Gb) to 57.1 million reads (~14.32 Gb) [**Supplementary Table 2**]. After quality trimming, we obtained an average of ~24 million high-quality reads per cDNA library (NCBI BioProject: PRJNA435468). *De novo* transcriptome assembly for each genotype produced a total of 613,378–733,169 transcripts with N50 of ~1449–1659 and an average of ~590 megabase pairs (Mbp) assembled (**Table 4**). An average of ~38% of the total assembled transcripts for each genotype were predicted to be protein-coding genes. After filtering redundant transcripts and sequences that matched rRNAs and Symbiodiniaceae, an average of ~31,501 transcripts were identified as putatively coral in origin (designated here as final 'coral-only' reference assembly), which is comparable to published coral transcriptome resources in Traylor-Knowles et al. (2011); Barshis et al. (2013), and Shinzato et al. (2014).

Differential Gene Expression Analysis and Functional Profiling

Principal component analysis and heat maps show that corals subjected to heat (i.e., "H" and "H+S") had similar gene expression profiles while samples maintained at ambient temperature (i.e., "C" and "S") responded similarly as well (**Supplementary Figures 2**, **3**). EDGE analysis resulted in 824 significant DETs (significance based on 5% FDR correction) from all genotypes (**Supplementary Table 3**). Results from pairwise comparisons showed significant differences in transcript expression levels for corals exposed to both treatments "H" and "H+S." Only a few DETs (3) were found for corals in the treatment "S". Of the total number of DETs (824), 306 were shared between pairwise treatment comparisons: "C" vs. "H" and "C" vs. "H+S," while a number of DETs were specific to a treatment comparison. The largest number of unique DETs was found in the comparison between "C" vs. "H" (423), followed by "C" vs. "H+S" (91) and lastly, "C" versus "S" (3) (**Figure 3A** and **Supplementary Table 4**).

Alignment of DETs to Uniprot protein sequences resulted in 174 unique genes for all treatment comparison and genotypes. A substantial number of DETs (212) did not match to any protein sequence in Uniprot database and therefore were excluded from downstream analyses as their functions were difficult to predict. From the total number of unique genes (174), 58 were shared between treatment comparisons: "C" vs. "H" and "C" vs. "H+S" while only a single gene was shared between "C" vs. "H" and "C" vs. "S." There were 112 genes specific for treatment comparison: "C" vs. "H," while only 2 genes and a single gene were specific for "C" vs. "S" and "C" vs. "H+S," respectively (**Figure 3B**).

Overall, 60 genes present in all genotypes were found to be core genes (DEGs, hereon) involved in the responses observed. From the 60 DEGs identified, only 40 genes had known GO terms for drawing biological inferences. We obtained 23 GO categories

TABLE 4 | Summary statistics of *de novo* assemblies.

***De novo* assembly (unfiltered)**	**Genotype 1**	**Genotype 2**	**Genotype 3**
Total number of transcripts	657481	733169	613378
Percent GC	44	43.64	43.64
Contig N50	1571	1449	1659
Average contig	910	826	925
Total assembled bases (Mbp)	598	605	567
Coral-only assembly (filtered)			
Total number of transcripts	31150	34090	29264
Percent GC	47.94	47.70	47.84
Contig N50	489	477	492
Average contig	555	538	559
Total assembled bases (Mbp)	17	18	16

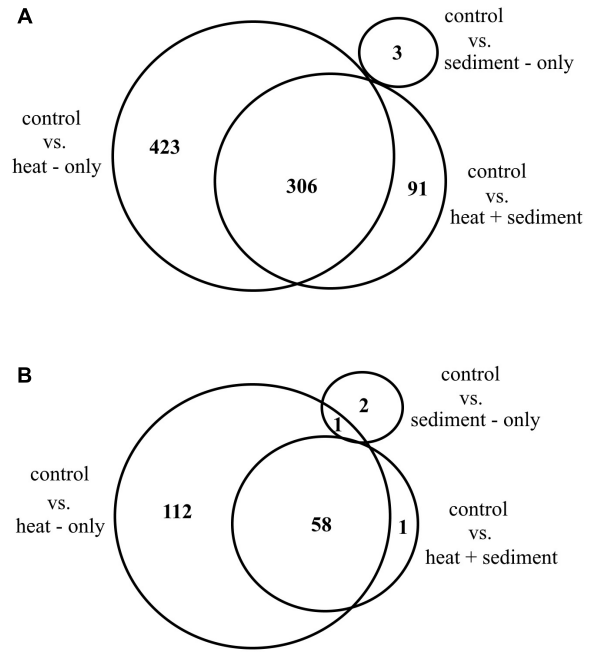

FIGURE 3 | Venn diagram showing overall number of differentially expressed transcripts (DETs) from RNA-Seq analysis which are shared and specific to three different treatment comparisons: control vs. heat-only, control vs. heat + sediment and, control vs. sediment-only **(A)**; and number of DETs with unique protein matches that are shared and unique for each of the treatment comparison **(B)**.

associated with various biological processes based on UniProtKB that were represented by mostly one gene for each category and two or more genes for some categories such as cell cycle, cilia motility, cilium biogenesis/degradation, stress responses, spermatogenesis, innate immune response and apoptosis. All DEGs had average fold change (FC) ranging from 2.18 to 46.54 for up-regulated genes (red) and −33.77 to −1.89 (blue) for down-regulated genes (**Figure 4** and **Supplementary Table 5**). While the genes specific to "C" vs. "S" were not common in all genotypes, results are shown in **Figure 4** for comparisons with other treatments.

Patterns of expression (up- or down-regulated) for most DEGs were relatively similar among genotypes. However, it is notable that the expression of *SVEP1* (associated to cell adhesion) in response to "H" showed positive regulation for G2 while it was highly down-regulated (FC: -33.78) for G3. On the other hand, G1 only expressed *SVEP1* when exposed to "H+S" and even showed an up-regulated expression (FC: 4.07) (**Supplementary Table 5**). There was also variation in the expression pattern observed for the gene *DYDC1* (associated with chromatin silencing at telomere) which was up-regulated for G1 under "H+S." In contrast, *DYDC1* was down-regulated for G2 and G3 for both treatment comparisons. Interestingly, *DNAJB13* was only specific for the treatment comparison "C" vs. "H+S" whereby all three genotypes showed down-regulation of this gene (FC range: −4.80 – −3.44).

Furthermore, there were genes that showed similar patterns of expression among genotypes, but the magnitudes of expression varied. For instance, the gene *NFKB1* (associated to innate immune response) was highly up-regulated for genotype G2 (FC range: 40.42 – 46.54) but less up-regulated for genotypes G1 (FC range: 3.44 – 6.29) and G3 (FC: 2.18). Another gene that showed different levels of up-regulation was *Gpsm1* (associated with cell differentiation) which was highly up-regulated particularly for genotypes G2 and G3 (FC range: 23.41 – 32.47) but less up-regulated (FC range: 3.11 – 5.04) for genotype G1.

DISCUSSION

Our experiment tested how different genotypes of *Pocillopora acuta* respond to heat and sediment, as individual stressors and in combination. Both traditional photo-physiological metrics and RNA-Seq data were used to determine responses of *P. acuta* to these stressors. We showed that heat was the main factor driving the changes in photo-physiological conditions and gene expression patterns among genotypes. The effects of exposure to sediment alone were much weaker. Notably, when sediment was combined with heat, significant responses were observed that suggest a synergistic effect of the two factors.

Photo-Physiological Performance of *P. acuta* Following Exposure to Heat and Sediment

The most apparent effect on photo-physiological performance was due to heat exposure alone. Thermal anomalies cause changes in photosynthetic functions such as degradation of Photosystem II (PSII; Warner et al., 1999), a light-harvesting complex crucial for photosynthesis. One of the most informative measurements for the photo-biology of the coral-Symbiodiniaceae relationship is the maximum quantum yield (F_v/F_m), which represents the maximum capacity of PSII (Roth, 2014). In our study, the significant effect of heat in F_v/F_m is in line with the elevated abundance of Symbiodiniaceae (**Tables 2**, **3** and **Figures 2A,C**). The significant increase in the average density of symbionts in corals under heat stress is likely an adaptive response. It is well known that Symbiodiniaceae abundance in corals is controlled in part by the coral host, which optimizes symbiont abundance to match its environment (Cunning, 2013), thereby controlling the net benefit of their symbiosis (Hoogenboom et al., 2010). While heat is considered a strong driver of coral bleaching (Brown, 1997; Woolridge, 2013), an increase in temperature can drive nitrogen fixation which, in turn, can stimulate cell division in the endosymbionts (Rädecker et al., 2017). Therefore, it is conceivable that the active regulation of symbiont density which resulted in a significant effect in photosynthetic yield is associated with the acclimatization capacity of *P. acuta* under suboptimal conditions.

Reduced light penetration due to sediment exposure was previously shown to affect coral photo-physiology (reviewed in Erftemeijer et al., 2012). In this study, exposure to sediment did not cause major changes in the photo-physiological performance of the corals except for significant changes in average symbiont

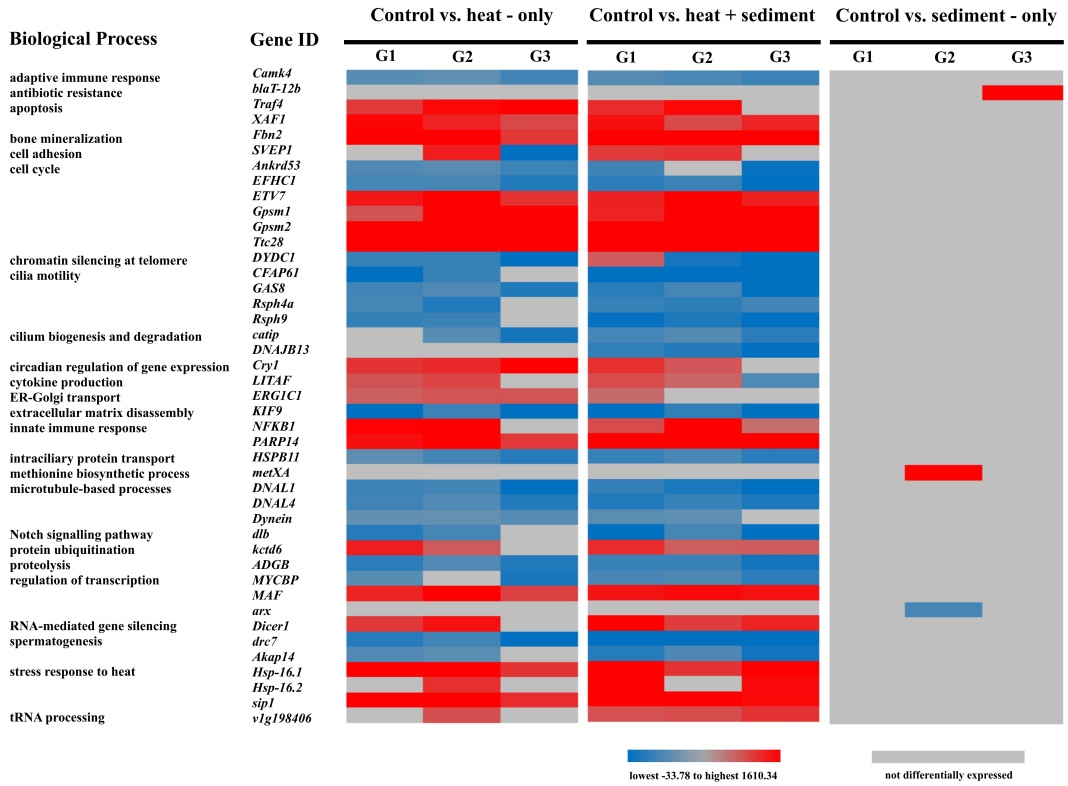

FIGURE 4 | Heat map showing functional categories based on UniProtKB, gene IDs and expression patterns [lowest fold change (FC) in blue to highest FC in red] of differentially expressed genes (DEGs) across genotypes – G1, G2, and G3; and across treatment comparisons: "C" vs. "H" and "C" vs. "H+S." While there was no DEG found for the comparison: control vs. sediment-only which is common across three genotypes, genes specific to this treatment and genotypes are included here for comparative purposes.

density for some genotypes (**Table 3** and **Figure 2C**). When sediment and heat were combined, there were significant effects on the performance of corals, but these were less obvious than those caused by heat alone. The decrease in average symbiont densities in corals exposed to "S," and "H+S," was likely due to the reduction of photosynthetically fixed carbon in low light conditions, which can affect the maintenance and growth of algal cells (Davy et al., 2012). Although reduction in symbiont density under low light might be considered a negative consequence, this can also represent an adaptive strategy to alleviate self-shading of cells to optimize light capture (McCloskey and Muscatine, 1984). Maximum quantum yield for corals exposed to sediment did not change significantly (**Table 2** and **Figure 2A**), suggesting that reduction in symbiont density observed here is an optimization strategy of *P. acuta* to maximize benefit from symbiosis in response to environmental change.

Functional Profile of DEGs

While photo-physiological measurements are useful for examining coral responses to stress, these are only proxy measurements derived from the algal symbionts and may not necessarily represent the condition of the coral host. Studies are now uncovering mechanisms of responses by both the coral host and its symbionts through transcriptome-level approaches (Mayfield et al., 2014; Shinzato et al., 2014). Changes in environmental conditions can affect an organism's transcriptional state whereby gene expression levels are orchestrated to match the surrounding environment (Kenkel and Matz, 2016). In general, results of the RNA-Seq analysis aligned well with the photo-physiological results. Significant DEGs were found for all coral genotypes exposed to "H" and "H+S," but very few for corals exposed to "S" (and only for G2 and G3). Below, we discuss the main genes (DEGs) involved in the response of *P. acuta* to acute heat and sediment exposure. These genes are from different functional categories associated with various biological processes that are well conserved and are critical for the survival of various metazoans including corals.

Shared Responses to Heat and Combined Heat and Sediment

Thermal stress is known to up-regulate heat-shock protein production (Advani et al., 2016; Maor-Landaw and Levy, 2016). We observed increased gene expression (FC range: 4.281 – 18.560) of small heat shock proteins (sHsps), heat shock proteins (Hsps) *Hsp16.1* and *Hsp16.2* which are believed to respond specifically to heat stress (Gates and Edmunds, 1999; Ding and Candido, 2000). The gene *sip1*, which encodes a stress-induced

protein as a result of a heat stimulus, was also up-regulated (FC range: 4.69 – 9.64). These genes help alleviate the negative impact of high temperature levels by means of reducing the number of structurally non-native proteins, believed to be produced by heat-stressed symbionts (Lesser, 1997), and refolding reversibly heat-damaged proteins. While several studies have shown Hsps to be involved in coral heat stress (Maor-Landaw and Levy, 2016), responses among Hsps can be variable. For instance, in a previous gene expression study by Barshis et al. (2013) on *Acropora hyacinthus* from two different populations, characterized as 'thermally tolerant' and more 'sensitive population,' the authors noted the absence of Hsps in the shared-response genes to thermal exposure. The canonical heat stress genes including *Hsp70* and sHsps (*Hsp23*/*HSPB1* and *Hsp16.2*) were found to have lower reactivity to heat stress particularly for the thermally tolerant population. For *Hsp70*, it was implicated in frontloading (i.e., higher expression under ambient conditions), which may prepare a coral individual for frequent stress. Conversely, the reduced transcription of sHsps was likely due to lower levels of physiological stress experienced by an individual. Our results, nevertheless, correspond to specific genes previously highlighted to respond to heat stress, including sHsps (Kenkel et al., 2011, 2014). The varying response of Hsps further underscores differential responses to thermal exposure among coral species.

Beyond the canonical Hsps, a set of cilia-related genes were significantly down-regulated. These genes are associated with cilium biogenesis and degradation, as well as cilia motility. Coral surfaces are densely covered by cilia, particularly in the pharynx of the polyps (Westbroek et al., 1980). Cilia-related activities include cleaning trapped sediment particles, which may contain pathogenic bacteria, off surfaces of coral colonies (Erftemeijer et al., 2012). More recently, ciliary structures in corals have been reported to actively enhance nutrient exchange with their surrounding environments through ciliary beating (Shapiro et al., 2014). It has been shown that ciliary beating is very tightly correlated with temperature which can cause defects in ciliary functions (Clary-Meinesz et al., 1992). The observed down-regulation of genes associated with cilia motility, i.e., growth arrest-specific protein 8 (*GAS8*), radial spoke head protein homologs 4a and 9 (*Rsph4a* and *Rsph9*) and cilia- and flagella-associated protein 69 (*CFAP69*), suggest a shutdown in feeding mechanisms of corals to divert resources to a stress response (Traylor-Knowles et al., 2017). As a mechanism of sensory signal adaptation, cilium exhibits adjustment to its morphology in response to changes in environmental conditions (Malicki and Johnson, 2017). Therefore, it is reasonable that the regulation of genes associated with cilium biogenesis and degradation constitutes part of the stress response.

Corals, like most animals, rely heavily on their immune response for protection from invading pathogenic organisms. In this experiment, we found DEGs associated with both adaptive and innate immune responses. Innate immunity is characterized as the non-specific first line of defense mechanism of organisms against any foreign bodies which may be harmful to them. Conversely, adaptive immunity involves more specific responses and is believed to be more complex than innate immunity as it involves a "memory" that facilitates future responses against specific stressors (Janeway et al., 2001). While cnidarians do not possess specialized immune cells, they are known to exhibit specific allorecognition characteristics believed to protect colonial cnidarians from fusion with genetically different individuals and from pathogenic organisms (Francis, 1973; Sebens, 1984; Miller et al., 2007). Interestingly, a recent report on the genome of *Pocillopora damicornis* has shown that immune strategies in this species have adaptive roles (Cunning et al., 2018). The immunity-related genes identified in this study included calcium/calmodulin-dependent protein kinase type IV (*CAMK4*) associated with adaptive immune response and two innate immunity genes such as nuclear factor NF-kappa-B p105 subunit (*NFKB1*) and poly (ADP-ribose) polymerase 14 (*PARP14*). The genes *NFKB1* and *CAMK4* are part of transduction signaling pathways that are activated following the detection of stress by toll-like receptors (van de Water et al., 2015). These downstream signaling pathways are essential for regulating expression of target genes involved in immunity and cell survival. Here, exposure of corals to heat triggered up-regulation of *NFKB1* (FC range: 2.178 – 243.889) and a downregulation of *CAMK4* (FC range: −6.548 – −2.369). The latter gene is a serine/threonine-specific protein kinase, which is regulated by the presence of $Ca^{2+/}$ calmodulin complex believed to be a mediator for memory associated with the adaptive immune response and crucial for various cellular processes (De Salvo et al., 2008). Almost all types of stressors have a common oxidative stress phase which triggers an increase in calcium concentration eventually leading to disruption in calcium homeostasis in the cell. The down-regulation of *CAMK4* observed here suggests a disruption in Ca^{2+} homeostasis which signals cell death, or apoptosis (Maor-Landaw and Levy, 2016). This is corroborated by significant increases in expression levels (FC range: 4.675 – 15.107) of two known regulators of apoptosis, TNF receptor-associated factor 4 (*TRAF4*) and XIAP-associated factor 1 (*XAF1*). Interestingly, the gene *PARP14* which is associated with anti-apoptotic pathway showed significant up-regulation (FC range: 2.12 – 99.56). These results suggest a complex orchestration of the immune system in corals when exposed to stressful conditions.

Furthermore, a number of genes that are part of signaling pathways associated with the control of cell proliferation (Davy et al., 2012) were detected. On the one hand, *Ttc28* and *Gpsm2* were highly expressed in terms of the number of transcripts with 131 and 28 DETs respectively (**Supplementary Table 4**). *Ttc28* is involved in checkpoint-mediated cell-cycle while *Gpsm2* modulates activation of chemoreceptor G-protein. On the other hand, two other genes, *Ankrd53* and *EFHC1*, were down-regulated. *Ankrd53* is known to be involved in normal regulation of cell division while *EFHC1* is a microtubule-associated protein involved in enhancing calcium influx and may stimulate apoptosis. Cell-cycle arrest and apoptosis due to DNA damage are part of a conserved pathway in multicellular organisms (Gartner et al., 2000). It is possible that these genes, as checkpoint controls, could mediate detrimental effects of stressful conditions on corals that are essential for their survival. To ensure genomic stability of organisms, surveillance mechanisms (i.e., checkpoint controls) are essential to interrupt

cell cycle progression that can be damaging to the genome (Gartner et al., 2000).

The coordinated cell cycle and growth for multicellular organisms is necessary for their development (Goranov et al., 2009). In our study, the suppression in the cell cycle is corroborated by down-regulation of the gene *KIF9* which encodes for a kinesin-like protein linked to extracellular matrix disassembly. It is well known that the physiological process associated with coral growth, i.e., calcification, is regulated by exogenous factors such as light and temperature (Lough and Barnes, 2000; Lough and Cooper, 2011). Calcification takes place directly within the extracellular calcifying matrix, or ECM (Cohen and McConnaughey, 2003; Allemand et al., 2011). In a previous study by Helman et al. (2008), coral cells were reported to maintain the ability to precipitate calcium carbonate on an ECM *in vitro*. For reef-building corals such as *P. acuta,* growth is a key strategy, hence, regulation in their calcification process is crucial to their development.

Treatment-Specific Gene Expression

A significant down-regulation of *DNAJB13*, a gene associated with cilium biogenesis and degradation (**Figure 4** and **Supplementary Table 5**), was observed to respond specifically to treatment "H+S". Ciliary movement in corals for self-cleaning and manipulation of sediments that settled on them can incur a sub-lethal energetic cost (Pechenik, 2006), and may have direct impacts on ciliary structures. Considering the detrimental effects of heat on ciliary functions (Clary-Meinesz et al., 1992), combining it with sediment here led to a synergistic effect on corals' ability to assemble and disassemble their ciliary structures. As cilia perform important functions for corals (e.g., for sediment rejection and feeding), a reduction in their capacity to continuously maintain their ciliary structures would therefore affect their survival.

Interestingly, exposure to "S" induced up-regulation (FC: 1610.339) of the gene *blaT-12b*, which encodes the protein beta-lactamase TEM-12 in antibiotic resistance. Although presence of this gene was only detected in G3, it has implications on corals' ability to fight antibiotic resistance against invading bacteria. A similar study by Sheridan et al. (2014) that used sterile sediments for acute sediment exposure on corals showed significant up-regulation of the melanin synthesis pathway– a key response to immune challenges in invertebrates (Cerenius and Söderhäll, 2004). A cascade of events in this pathway provides an antimicrobial defense to the organism, from production of melanin precursors and metabolites to formation of the melanin barrier which isolate the compromised tissue or physical lesions of the organism from pathogens or any other sources of infection (Cotter and Wilson, 2002; Cerenius et al., 2010; Palmer et al., 2010). Such observations can be linked to mechanical damage due to sediment abrasion, which may elevate the risk of pathogen exposure (Lamb et al., 2016). Apart from the mechanical effects of sediment exposure, reduction in light penetration can lower photosynthetic yield (Weber et al., 2012). The capacity of corals to meet their metabolic needs was previously shown by Fitzgerald and Szmant (1997) to involve methionine heavily. This may explain the observed up-regulation of *metXA* (FC: 72.238)—associated with methionine biosynthesis—in G2 when exposed to sediment.

Genotype-Specific Gene Expression Within-Treatment

While the levels of gene expression were relatively similar across genotypes for many different genes, some genes exhibited different patterns. For instance, the gene *SVEP1* which is associated with cell adhesion was highly down-regulated in response to "H" for G3 (FC: −33.78) but up-regulated for both G1 (FC: 4.07) and G2 (FC: 5.20). This gene is classified as active in the extracellular region based on the 'Cellular Component' GO category. For maintenance of multicellularity in cnidarians (including corals), ECM is produced to facilitate cell-cell adhesion and cell-substrate organization (Helman et al., 2008; Magie and Martindale, 2008). It is likely that down-regulation of *SVEP1* for G3 is related to the disruption of ECM production, which may have an effect on the calcification process. While *Fbn2*—associated with positive regulation in bone mineralization—is up-regulated in G3 in response to "H" (FC: 4.27) and "H+S" (FC: 7.58), the magnitude of up-regulation is not as high compared to G1 (FC: 23.29 and 15.20) and G2 (FC: 17.34 and 15.40). This suggests a reduced capacity of G3 to regulate skeletal calcification under stressful conditions as compared to other genotypes.

CONCLUSION

Our findings provide important biological insight into the capacity of *P. acuta* to respond to short-term disturbances by means of adjustments in their photo-physiological performances and gene expression. This could help explain a shift in the bleaching pattern of *P. acuta* (i.e., to being less susceptible) during recent coral bleaching events in Singapore. The functional DEGs discussed here provide opportunities for future validation studies to examine specific genes in concert with direct measurements of the associated phenotypes. Considering that the responses observed in this study occur at the level of gene regulation, it is also important to measure the actual turnover of protein products which are subject to further regulation. This will deepen our understanding of the cellular behavior of corals under stressful conditions.

Heat has been the most widely studied environmental stressor on corals, however, its effects in combination with other variables are not well understood. The present study helps to fill this knowledge gap. Our results show that heat was a strong driver of the observed responses, but we also found synergistic effects of heat and sediment on the ability of corals to maintain their ciliary structures (i.e., cilium biogenesis and degradation), an essential component for coral survival. Sustained periods of high temperatures, particularly during the summer, can potentially magnify the impacts of other environmental stressors (chronic or acute) and can be lethal for corals, especially in inshore shallow waters. The fate of coral reefs in the Anthropocene will depend largely on the capacity of reef-building corals to acclimatize to a

rapidly changing environment. Only genotypes that exhibit this ability are likely to survive.

DATA AVAILABILITY

The raw reads used for this study can be found in the NCBI BioProject ID PRJNA435468 (https://www.ncbi.nlm.nih.gov/bioproject/435468). All supplementary data can be found in https://doi.org/10.6084/m9.figshare.6163916.

AUTHOR CONTRIBUTIONS

All authors contributed conception and design of the study. RCP-D carried out the experiments, organized the data, and performed the statistical analysis. RCP-D and M contributed in the RNA-Seq analysis. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

This work was supported by the National Research Foundation, Prime Minister's Office, Singapore under its Marine Science Research and Development Program (Award Nos. MSRDP-P03 and MSRDP-P05). Additional support was provided by the Southeast Asian Biodiversity Genomics Center (R-154-000-648-646), and the Maritime and Port Authority (MPA) of Singapore (R-347-000-215-490).

ACKNOWLEDGMENTS

We would like to acknowledge the St. John's Island National Marine Laboratory for providing the facility necessary for conducting the research. The Laboratory is a National Research Infrastructure under the National Research Foundation Singapore. Many thanks to the members of Experimental Marine Ecology Laboratory and Reef Ecology Laboratory at the Department of Biological Sciences and the Marine Biology and Ecology Laboratory at Tropical Marine Science Institute, NUS, for fieldwork and laboratory support. Thank you also to Dr. David Suggett for allowing us to visit his laboratory at University of Technology, Sydney to observe their daily operations. All fieldwork was conducted under the National Parks research permits NP/RP13-082 and NP/RP15-009a with permission from MPA. High-throughput sequencing work was performed by the Singapore Centre for Environmental Life Sciences Engineering sequencing facility (http://www.scelse.sg/). The computational work for this study was partially performed on resources of the National Supercomputing Center, Singapore (https://www.nscc.sg). Comments from Drs. Eslam Osman, Michael Sweet, Bryan Wilson and an reviewer greatly improved this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00121/full#supplementary-material

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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 BW declared a past co-authorship with several of the authors, LC, PT, and DH, to the handling Editor.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 18 April 2019
doi: 10.3389/fmars.2019.00195

Reproductive Seasonality of Coral Assemblages in the Karimunjawa Archipelago, Indonesia

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OPEN ACCESS

Edited by:
Christian Robert Voolstra, King Abdullah University of Science and Technology, Saudi Arabia

Reviewed by:
Jessica Bouwmeester, Smithsonian Institution, United States
Till Röthig, King Abdullah University of Science and Technology, Saudi Arabia

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Specialty section:
This article was submitted to Coral Reef Research, a section of the journal Frontiers in Marine Science

Received: *09 July 2018*
Accepted: *28 March 2019*
Published: *18 April 2019*

Citation:
Wijayanti DP, Indrayanti E, Wirasatriya A, Haryanto A, Haryanti D, Sembiring A, Fajrianzah TA and Bhagooli R (2019) Reproductive Seasonality of Coral Assemblages in the Karimunjawa Archipelago, Indonesia. Front. Mar. Sci. 6:195. doi: 10.3389/fmars.2019.00195

Equatorial corals were previously thought not to spawn synchronously at the assemblage level. However, recent studies have reported multi-specific coral spawning events in equatorial regions. Here, we report the reproductive activity of 21 *Acropora* species in the Karimunjawa Archipelago over five consecutive years (2008–2012). We also infer the month of spawning for *Acropora humilis*, *Acropora gomezi*, and *Acropora muricata* from the presence of mature oocytes. We found that *Acropora* assemblages exhibit a high degree of inter-specific reproductive seasonality. The highest proportion of colonies with mature oocytes was observed in March 2011 (65%, $n = 80$). Oocytes likely developed during June–March, 6 to 10 months before spermatogenesis. Spermatocytes were observed in samples collected during March; however, the onset of spermatogenesis could not be precisely determined as samples were not collected in January and February. This was because of weather constraints and difficulty in detecting the early stages of spermatogenesis. Multi-specific spawning events were observed during the first transition period (March–April) and the second transition period (September–October) between monsoons. The number of species containing mature oocytes was higher during March–April (12 species) and September–October (8 species). Spawning patterns likely follow the lunar cycle. However, two distinct spawning events coincided with two periods of higher temperature (March–April and September–October). Daily temperature records indicate that spawning occurred on days where temperature dropped before the expected spawning time during the warming period. During the period of rising temperature, wind speeds were lower, which might serve as a signal leading to the multi-specific spawning of corals in the tropics, at least in the Karimunjawa Archipelago of Indonesia.

Keywords: coral reefs, Acroporidae, Karimunjawa Archipelago, multi-specific spawning, biannual spawning

INTRODUCTION

Knowledge on the timing of spawning within coral assemblages has developed rapidly in recent years, and this phenomenon has now been documented at more than 25 locations globally (Baird et al., 2009; Kongjandtre et al., 2010; Bouwmeester et al., 2011; Chelliah et al., 2015). This phenomenon has been documented over a wide geographic area, including remote regions, such as New Caledonia (Baird et al., 2010), the Persian Gulf (Bauman et al., 2011), Yemen (Baird et al., 2014), and the southernmost reef of Lord Howe Island, Australia (Harrison, 2008; Baird et al., 2015).

The reproductive patterns of corals vary widely during spawning, with wide variations existing in the intensity of spawning, extent of reproductive synchrony, and duration of the spawning period, both within and among coral populations at different locations (Harrison, 2011). However, the reproductive mode of most coral species is generally consistent and conserved. For example, most acroporids are hermaphrodite broadcast spawners throughout their distribution range (Baird et al., 2009).

It has been previously suggested that multi-specific spawning events only occur in the Great Barrier Reef (GBR), Australia. Existing studies suggest that populations that do not experience large environmental fluctuations, such as corals at low latitudes, exhibit less synchronous spawning (Shlesinger and Loya, 1985; Oliver and Willis, 1987; Oliver et al., 1988; Harrison and Wallace, 1990; Richmond and Hunter, 1990). However, multi-specific spawning has been observed in the Solomon Islands (Baird et al., 2002), Singapore (Guest et al., 2002, 2005a), the Java Sea (Edinger et al., 1996; Permata et al., 2012) and Western Australia (Gilmour J. et al., 2016). Thus, equatorial coral assemblages might exhibit a higher degree of coral spawning than previously assumed. Lower reproductive synchrony in corals at low latitudes has been recorded in the coral reef communities of Kenya, where most corals exhibit a greatly extended reproductive period (Mangubhai and Harrison, 2008). This finding triggered debate over using latitude to define variation in "mass spawning events" and "multi-specific species spawning" (Baird and Guest, 2009). In particular, a recent report suggests that corals in East Africa, a low latitude area, exhibit synchronous spawning periods (Sola et al., 2016).

Synchronous spawning within coral species is required to facilitate cross-fertilization. Sperm dilution represents a major constraint that decreases fertilization success when small colonies spawn synchronously (Levitan et al., 2004). As a result, many broadcaster corals spawn at predictable times every year (Willis et al., 1985). This phenomenon maximizes the fertilization rates of gametes in the water column (Guest et al., 2005b).

Various environmental factors might contribute to the timing of reproduction in corals, the control of the reproductive activity, at both proximate and ultimate levels (Harrison and Wallace, 1990). Babcock et al. (1986) suggested that environmental factors operate at progressively finer scales to regulate the time of year, the night, and the hour of spawning. Sea surface temperature (SST) might serve as the major seasonal cue to synchronize the maturation of most corals during the reproductive cycle. Broadcast spawning corals spawn their gametes during periods when water temperature is rapidly changing (Indo-Pacific region) or is close to its annual maximum (Western Atlantic region) (Harrison and Wallace, 1990; van Woesik et al., 2006; Keith et al., 2016). For example, coral populations at inshore and offshore reefs in the central region of the GBR spawn 1 month apart, corresponding to the rise in SST that occurs 1 month earlier in inshore reefs (Harrison et al., 1984; Willis et al., 1985; Babcock et al., 1986). However, other relationships between SST and spawning time are less well understood (Baird et al., 2009). For example, while most corals in the Indo-Pacific region spawn in spring during periods of rapidly increasing temperatures, some corals in western Australia spawn during fall when temperatures are rapidly decreasing (Rosser, 2013; Keith et al., 2016). While the peak in coral spawning might be best predicted by rapid seasonal changes in sea temperature, other factors might control the timing of coral spawning, such as insolation and wind speed (Mendes and Woodley, 2002; Penland et al., 2004; van Woesik et al., 2006; Baird et al., 2009).

In tropical and equatorial regions, the role of environmental factors in the reproductive scheduling of tropical corals remains unclear. Multi-specific spawning has been observed in these regions, despite a lack of strong fluctuations in the environment. In Mozambique, coral assemblages spawn during periods of rising SST and low wind and rainfall (Sola et al., 2016). In Singapore, spawning of the coral *Platygyra pini* follows periods of increasing monthly SST (Guest et al., 2012). Similarly, in Kenya, spawning peaks during periods of rapidly rising SST (Mangubhai and Harrison, 2008). Mendes and Woodley (2002) suggested that the spawning timing of *Orbicella annularis* in the Western Atlantic is controlled by a combination of temperature and monthly rainfall. These studies suggest that spawning synchrony does not break down at low latitudes.

Despite lying at the center of coral biodiversity (Veron et al., 2009) and harboring a high coral diversity of 580 scleractinian species (Burke et al., 2002), very few studies have focused on coral reproduction in Indonesia. In October 1995, Edinger et al. (1996) first documented 22 scleractinian species spawning over a three-night period following the full moon in the Karimunjawa Archipelago. Permata et al. (2012) observed biannual multi-specific spawning at the same location. Furthermore, Bachtiar (2001) reported spawning of *Acropora cytherea* and *A. nobilis* around Lombok between January and February after full moon, whereas *Hydnophora rigida* released gametes biannually in March and October after full moon. Baird et al. (2005) reported that *Acropora* species from Aceh sampled in March were gravid, as were *Acropora* assemblages from Seribu Island, Jakarta Province, and Nusa Lembongan, Bali. Thus, these corals probably spawned in March and April. A later study by Baird et al. (2009) found that *Acropora* species from Makassar and Manado contained mature oocytes in October.

In the current study, we document the extent of reproductive activity of *Acropora* assemblages from Karimunjawa Archipelago in the Java Sea of Indonesia, over a 5-year period (2008–2012). Gametogenesis was examined during the first year (2008), and the spawning activity of *Acropora* assemblages was monitored over the subsequent years (2009–2012). Potential environmental

predictors of the timing of coral spawning, such as SST and rainfall, were examined (Mendes and Woodley, 2002).

MATERIALS AND METHODS

Sampling and Survey Location

All surveys and sampling in this study were conducted on a cluster of three islands (Sambangan, Seruni, and Genting Islands) in the Karimunjawa Archipelago, which is located about 80 km northwest of Jepara, Central Java (**Figure 1**). Samples were collected for subsequent histological analysis at a depth of approximately 2–7 m on Sambangan Island, specifically at Taka Fadelan, which is a reef lagoon (05°50′ 42.5″ S, 110° 35′ 17.7″ E), and at Ocean Base, which is an outer reef (05°50′ 40.1″ S, 110° 34′ 48.5″ E). Surveys of reproduction were conducted at the same two sites, as well as at a site on Seruni Island (05°51′ 57.5″ S, 110° 35′ 24.8″ E) and another site on Genting Island (05°51′ 17.6″ S, 110° 36′ 37.9″ E). All surveys and sampling on Seruni and Genting Islands were approved by Karimunjawa National Park under permit number 889/BTNKJ-1.6/SIMAKSI/2008. The research permit for Sambangan Island was obtained from PT Pura Baruna Lestari, which is an organization that privately manages the island.

Histological Sampling and Observation of Gamete Maturity

Samples were collected from all three islands for subsequent histological analysis to document gametogenesis in *Acropora* species and to determine spawning times. Sampling was conducted once a month from May 2008 to March 2009 at Taka Fadelan and Ocean Base, which were both near Sambangan Island. However, due to weather and logistical constraints, sampling was not conducted in November 2008 and January and February 2009. The branches of three species (*Acropora gomezi, A. hyacinthus,* and *A. humilis*) were used as samples, and were cut using pliers. Five to 10 colonies of more than 20 cm diameter for each species were tagged at each study site. Branches were sampled from the center of the colony. The branches were broken 2–3 cm below the tip in corymbose and tabular colonies and 8–10 cm below the tip in arborescent colonies. This sampling approach was used to avoid sterile zones (i.e., areas of the colony with no gametes) that generally occur at the perimeter of colonies and the tips of branches, which tend to represent areas of recent growth (Wallace, 1985).

Fixation, Histological Procedures, and Measurements

Within 30 min of collection, branch segments were fixed in 10% formaldehyde in seawater for 24 h. The fixed branches were decalcified in a 1:1 mixture of 10% acetic acid and 10% formaldehyde in tap water for approximately 2 weeks. Tissue was dissected and cut into 1 cm × 1 cm pieces which were then dehydrated in a graded series of ethanol and cleaned using xylene. Tissues were embedded in paraffin and 6–8 μm sections were cut and stained with Delafield's hematoxylin and eosin

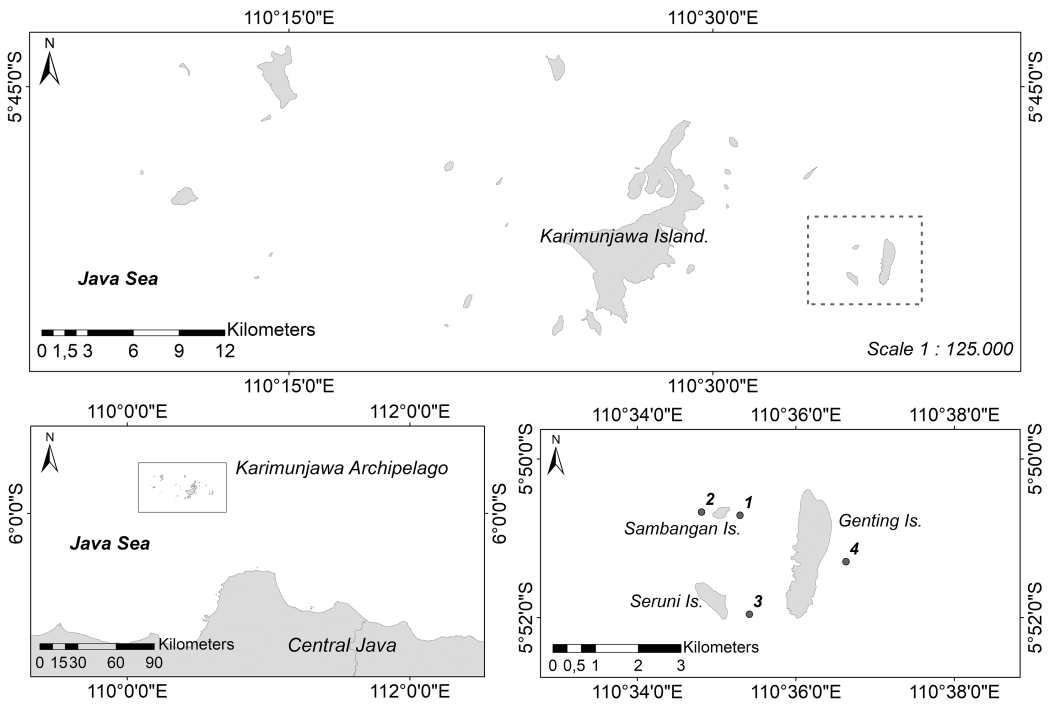

FIGURE 1 | Study sites. Sambangan Island, Genting Island, and Seruni Island, cluster of three islands about 15 km from main Karimunjawa Island and the study location of sampling sites; 1, Taka Fadelan; 2, Ocean Base; 3 Reef Lagoon of Seruni Island; 4 Outer Reef of Genting Island.

(Permata et al., 2000). Histological sections were observed under a light microscope (Nikon Labophot-2, New York, United States). Each colony was then classified into one of four stages of oogenesis or spermatogenesis (Harrison and Wallace, 1990; Vargas-Ángel et al., 2006 and **Supplementary File S1**). Oocytes of >300–800 μm in size were classified as the mature stage. Mature oocytes are packed with yolk granules, stain deeply, and have an indented nucleus. By comparison, spermaries are packed with mature spermatozoa that have developed tails and are approximately 80 μm in size (Vargas-Ángel et al., 2006). To measure the size of oocytes and testes, sections that contained the maximum planar area of the oocytes and testes, respectively, were selected and photographed under the microscope using an Olympus A80 digital camera. The diameter of oocytes and testes was measured using Adobe Photoshop version3.0 software. Oocytes and testes were measured using a size index, with a geometric mean of the short and long axes of the section of oocytes and testes (Permata et al., 2000).

Observations of Reproductive Conditions

Monsoons create four distinct seasons in the Karimunjawa Archipelago (Tomascik et al., 1997): southwest (SW) monsoon (wet season, November–February); northeast (NE) monsoon (dry season, May–August); first transition period (March–April); and second transition period (September–October). To determine the reproductive condition of *Acropora* corals, surveys were carried out during the first transition period before the southeast monsoon (wet season) from February or March in 2009–2012, and during the second transition period before the northeast monsoon (dry season) from September or October in 2011 and 2012. Sampling was conducted 1 to 3 weeks before the expected spawning date, which occurs between 1 and 8 days before full moon. Sampling periods are listed in **Table 1**.

During each sampling period, two scuba divers conducted three surveys each by swimming parallel to the reefs for a distance of approximately 100 m. Samples were only taken from colonies greater than 10 cm in diameter to increase the likelihood that the colonies were mature enough to produce gametes. Colonies were tagged before branches were collected, to avoid re-sampling in a given sampling period. To avoid sampling clones, sampled colonies were located at least 1 m apart. Some *Acropora* species were identified *in situ*, while unidentified species were further analyzed following bleaching in the laboratory and observation under a microscope to detect species-specific skeletal patterns, as per Wallace (1999). The reproductive condition – was established by breaking coral branches using cutting pliers and exposing the developing oocytes, as described by Harrison et al. (1984). Three branches were broken off from each colony to reveal the status of oocyte development based on the pigmentation of visible oocytes. It was assumed that mature oocytes would release gametes within 1 month and immature oocytes would release gametes within 2 months. In comparison, the absence of visible oocytes indicated that they had already spawned or that the oocytes were too small to be detected with the naked eye and, thus, would not spawn for at least 2 months (Baird et al., 2001, 2011; Guest et al., 2005a; Gilmour J.P. et al., 2016). The four sampling sites were surveyed during each sampling period. Data were pooled for each spawning season during the first and the second transition periods. Colonies containing mature or immature eggs were collected from the study sites and maintained in aquaria during each of the transition periods from 2009 to 2012 to determine the timing of spawning. The aquaria were checked every morning for gametes that were released overnight. If a colony had spawned its gametes in the aquaria or if a "fishy" smell was detected at sea, tagged colonies in the field were visited within 24 h and intensely observed during and before spawning hours. The tagged colonies were visited again the next day. In 2010, spawning could not be directly observed owing to weather and transportation constraints. Instead, spawning was inferred from slicks [the corals-egg-sperm bundles that are released during multi-specific coral spawning events (Oliver and Willis, 1987; Heyward and Negri, 1999)] deposited on the beaches.

Environmental Parameters During Spawning

To monitor SST, temperature loggers (HOBO Water Temp Pro v2, United States) were deployed at a depth of 5–6 m in April 2013 to April 2014 at two sites, Taka Fadelan reef (No. 1 in **Figure 1**) and Ocean Base (No. 2 in **Figure 1**). During logger deployment, the occurrence of multi-specific spawning was recorded and

TABLE 1 | Survey dates.

Year of observation	Length of observation during the first transition period (date of sample collection)	Time of spawning	Length of observation during the second transition period (date of sample collection)	Time of spawning
2009	20 March – 5 April (17 March)	3 April		
2010	20 February – 30 April (23 – 24 Feb; 29 – 30 March; 29 – 30 April)	25 March		
2011	3 March – 22 March (5–6 March)	17–19 March	3 March – 17 September (5 September)	14 September
2012	24 March – 13 April (26 – 27 March)	1, 2, 3, and 8 April	14 September – 7 October (16 September)	3 October
2013 (additional observation)	15 March – 20 April (16 March)	20, 22 March	5 September – 27 October (15 September and 12 October)	20 September, 20 October

Surveys were conducted at four sites. One site at Genting Island, one site at Seruni island and two sites at nearby Sambangan Island (see section "Materials and Methods" for details).

the daily amplitude of temperature variation was plotted. For 2012, SST was estimated by modeling variation in diurnal SST (DSST). The amplitude of DSST at a depth of about 1 m (ΔSST) was estimated from solar radiation (SR), wind speed (WS), and latent heat (LH), following the parametric model of Kawai and Kawamura (2003). The ΔSST generated from this model was validated against buoy measurements for the tropics. The root-mean-square error and bias was less than 0.3°C and + 0.1°C, respectively (Kawai and Kawamura, 2003).

$$\Delta SST = a(MS + H_l + e)^2 + b[\ln(U)] + c(MS + H_l + e)^2[\ln(U)] + d$$

where MS is the daily mean of SR (Wm^{-2}); U is the daily mean of WS (ms^{-1}); – H_l is the daily mean of latent heat flux (upward is negative, Wm^{-2}); a, b, c, d are the regression coefficients; and e is a constant value of 300 Wm^{-2}. MS and U data were obtained from NASA near real-time global radiation and meteorology web services. Solar energy flux and daily wind speed data with a grid resolution of 1 × 1[1] were acquired using this resource. H_l data were downloaded from OAFLUX[2] each day with a grid resolution of 1 × 1. The data were collected from 2009 to 2012 for the coordinates of 5.84°S and 110.58°E.

Rainfall data were obtained from the Indonesian Agency for Meteorology, Climatology and Geophysics (BMKG) Semarang Office and were recorded at the closest climate and meteorology station (6°58'S and 110°25'E). Wind-field data were extracted at 10 m above the water surface. A threshold of 6 ms^{-1} for wind velocity was chosen to allow comparison with the findings of van Woesik (2010), as significant waves begin to form when wind velocity is greater than 6 ms^{-1}.

RESULTS

Sexual Patterns, Gonadal Arrangement, and Gametogenic Cycles

Acropora hyacinthus, *A. humilis,* and *A. gomezi* are simultaneous hermaphrodites. They produce both types of gametes (oocytes and spermatocytes) within the same polyp. Only one gonad develops in each mesentery. The development of oocytes is illustrated in **Figure 2**. Mesenteries bearing stage I oocytes were found. Within the mesentery bearing oocytes, one vertically aligned row of oocytes was observed. Stage I oocytes appeared in June in all three species. Stage I oocytes ranged from 5 to 50 μm. Typically, stage I oocytes were irregularly shaped and round-edged. The cytoplasm was small and appeared dense and coarsely granular in all three species. The nucleus was similar in color to the cytoplasm, with a round, bright, and pink nucleolus. The nucleus membrane was difficult to distinguish (**Figure 2A**). In contrast, stage II oocytes were oval to round, ranging from 50 to 150 μm in diameter and exhibited an increase in cytoplasm volume (**Figure 2B**). Stage II oocytes were typically stained pink. The cytoplasm was finely or coarsely granular as lipid vesicles began to accumulate, indicating the onset of vitellogenesis. During stage III, oocyte size increased progressively, with diameters ranging from approximately 200 to 400 μm (**Figure 2C**). At this stage, the yolk was stained dark pink, with some staining purple. The yolk was loosely or coarsely granular and filled with lipid vesicles as vitellogenesis progressed. The nucleus was smooth and stained rose or pink, with a bright pink nucleolus. At this stage, the nucleus was positioned mostly in the middle of the cytoplasm. In some mid- and late- stage III oocytes, the nucleus began to shift position, moving from the center toward the periphery. During stage IV, the size of oocytes increased noticeably, with diameters ranging from approximately 400 to 800 μm (**Figure 2D**). Oocytes were scored as stage IV when the nucleus was positioned against, or adjacent to the vitelline membrane. The yolk was fairly homogeneous, and the nucleus was round to dome-shaped. The nucleolus, which was observed less often in sections, migrated within the nucleus, to reach a peripheral position. As stage IV progressed, the nucleus became more irregularly shaped (saddle to crescent-shaped) and indented (**Figure 2D**). A mature spermatocyte was observed as a "flower bouquet" arrangement, with light pink or lavender-tails aggregated in a generally uniform direction (Szmant-Froelich et al., 1985; Permata et al., 2000; Vargas-Ángel et al., 2006; **Figure 2E**).

From July to March, *A. gomezi* contained mature oocytes, whereas mature oocytes were observed in *A. hyacinthus* from December to March. *A. humilis* only had mature oocytes in March (**Figure 3**). The onset of gametogenesis was similar in all three species. The development of oocytes in all three species occurred from June to March, around 6–10 months earlier than spermatogenesis (**Figure 3**). It was difficult to determine when spermatogenesis began in the three species, as samples were not collected from January to February due to weather constraints. In December, no spermatocytes were observed in any of the three species. However, by March all samples contained mature spermatocytes (**Figure 3**). In the male mesentery, a mixture of spermatocytes of different stages were found, suggesting that spermatogenesis occurred before March.

Reproductive Status of Acropora

Acropora assemblages at Karimunjawa Archipelago showed a high degree of inter-specific reproductive seasonality. Mature oocytes were present during both biannual surveys of the first and the second periods, each year from 2009 to 2012.

In total, 21 *Acropora* species were sampled across the 4 years of surveys (including *A. hyacinthus*, *A. humilis*, and *A. gomezi*) and were subject to histological analysis. A low number of colonies per species was encountered, because many species formed a large single-species thicket that could grow to more than 10 m in length.

A. aspera, A. florida, A. muricata, A. gemmifera, A. gomezi, A. humilis, A. hyacinthus, A. jacquelineae, A. loripes, A. millepora, A. nasuta, and *A. tenuis* contained mature eggs in March–April. In comparison, *A. austera, A. carduus, A. caroliniana, A. muricata, A. humilis, A. kimbeensis, A. millepora*, and *A. tenuis* contained mature eggs during September–October (**Table 2**).

[1]http://power.larc.nasa.gov/

[2]http://oaflux.whoi.edu/data.html

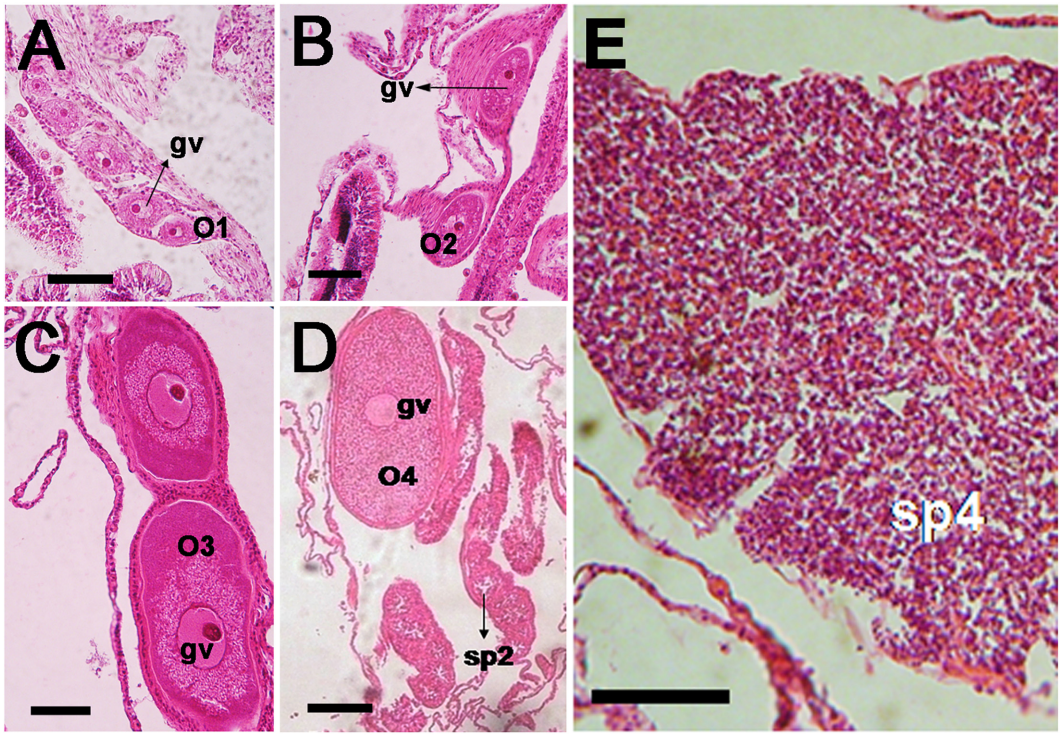

FIGURE 2 | Photomicrographs of histological sections of *Acropora humilis* and *A. hyacinthus* tissues illustrating the gonadal arrangement in both species. **(A)** *A. humilis*. Stage I oocytes developed in a mesentery. One vertically aligned row of oocytes was observed. Stage I oocytes ranged between 5 and 50 μm. **(B)** *A. humilis*. Stage II oocytes were oval to round, ranging from 50 and 150 μm in diameter and exhibited an increase in cytoplasm volume. They typically stained pink. The cytoplasm appeared finely or coarsely granular as lipid vesicles began to accumulate, indicating the onset of vitellogenesis. **(C)** *A. humilis*. Stage III oocyte the yolk stained darker pink, with some staining mauve. The yolk was loosely or coarsely granular, and filled with lipid vesicles, as vitellogenesis progressed. The nucleus was smooth, and stained rose or pink, with a bright pink nucleolus. **(D)** *A. humilis*. Stage IV oocyte size increased dramatically, with the diameter ranging from approximately 400 to 800 μm. The yolk was fairly homogeneous and the nucleus was round to dome-shaped. The nucleolus, which was less often observed in sections, also migrated within the nucleus, reaching a peripheral position. **(E)** Spermaries of *A. hyacinthus* filled with spermatids and surrounded by a thin spermatogonial wall while stage IV spermary was characterized by a pink or lavender color of a tail. Bouquet arrangement was observed in a late stage of spermary.

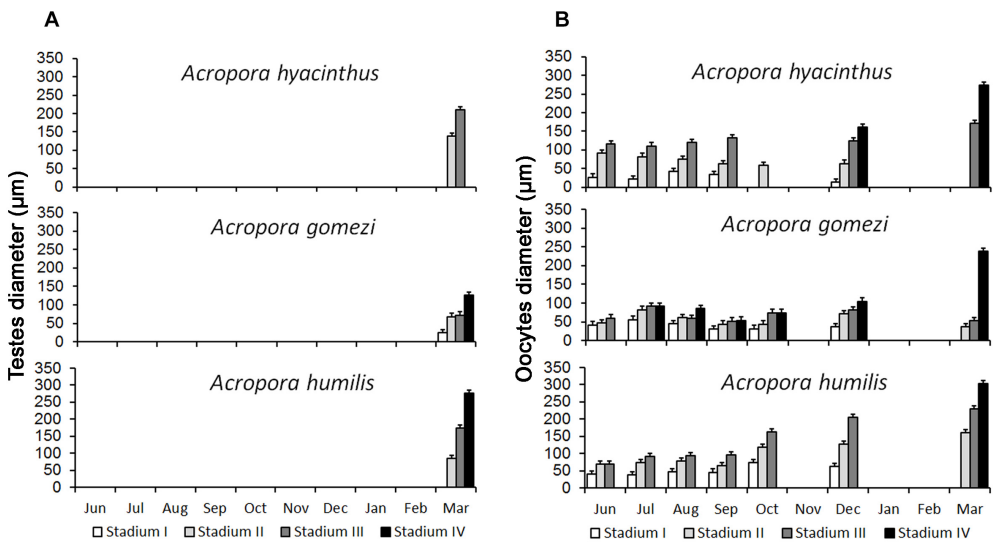

FIGURE 3 | Development of spermatocytes and oocytes in *Acropora*. Spermatocytes were present in samples collected in March **(A)** Oogenesis started in May and lasted until March. Peak maturation was observed in March and April prior to the spawning event **(B)** (10 – 20 colonies were sampled for each species).

TABLE 2 | Overview of observed coral colonies.

Species	The first transition period												The second transition period					
	17 March 2009			23–24 Feb 2010			5–6 March 2011			26–27 March 2012			5 Sept 2011			16 Sept 2012		
	M	I	N	M	I	N	M	I	N	M	I	N	M	I	N	M	I	N
A. aspera (58)	0	100	3	10	90	33	0	100	2	20	0	6	0	0	5	0	56	9
A. abrolhosensis (9)	NS	NS	NS	NS	NS	NS	NS	NS	NS	0	0	3	0	0	4	0	0	2
A. austera (12)	0	56	9	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	33	67	3
A. batunai (6)	NS	NS	NS	0	33	6	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
A. carduus (30)	NS	NS	NS	NS	NS	NS	NS	NS	NS	0	0	5	75	8	14	55	45	11
A. caroliniana (8)	NS	NS	NS	NS	NS	NS	NS	NS	NS	0	0	2	67	33	3	0	100	3
A. digitifera (28)	0	100	3	0	25	12	100	0	4	0	100	4	0	0	5	NS	NS	NS
A. florida (6)	NS	NS	NS	100	0	4	NS	NS	NS	100	0	2	NS	NS	NS	NS	NS	NS
A. muricata (121)	50	0	18	3	22	36	44	56	9	53	47	19	0	0	3	83	17	36
A. gemmifera (12)	67	33	9	NS	NS	NS	100	0	3	NS	NS	NS	NS	NS	NS	NS	NS	NS
A. gomezi (67)	55	45	20	20	80	15	18	82	11	38	62	13	0	0	8	NS	NS	NS
A. humilis (60)	42	58	26	0	17	6	0	100	5	50	50	2	0	0	6	20	26	15
A. horida (7)	NS	NS	NS	NS	NS	NS	NS	NS	NS	0	0	2	0	0	3	0	0	2
A. hyacinthus (105)	10	0	21	6	25	51	0	100	2	50	50	20	0	0	11	NS	NS	NS
A. jacquelineae (2)	NS	NS	NS	NS	NS	NS	50	50	2	NS	NS	NS	NS	NS	NS	NS	NS	NS
A. kimbeensis (24)	NS	NS	NS	NS	NS	NS	NS	NS	NS	0	0	3	60	13	8	46	38	13
A. loripes (12)	67	33	9	NS	NS	NS	100	0	1	100	0	2	NS	NS	NS	NS	NS	NS
A. millepora (45)	NS	NS	NS	NS	NS	NS	86	14	14	33	44	9	45	55	11	27	73	11
A. nasuta (12)	NS	NS	NS	NS	NS	NS	100	0	4	50	50	8	NS	NS	NS	NS	NS	NS
A. tenuis (73)	57	0	14	11	78	9	88	12	18	73	27	11	20	80	5	50	19	16
A. valida (2)	NS	NS	NS	NS	NS	NS	NS	NS	NS	0	50	2	NS	NS	NS	NS	NS	NS
Total number of colonies	54	43	132	15	76	172	47	23	75	46	44	113	19	13	86	57	41	121
Proportion of mature colonies of each sampling occasion			40%			7.5%			65%			39%			26.7%			47%

The proportion of Acropora colonies determined to be either mature or immature in the first Transition Period (March/April) and/or the second Transition Period (September/October) collected between 2009 and 2012 at Sambangan Island, Karimunjawa; M, colonies with mature oocytes (%); I, colonies with immature oocytes (%); N, number of samples including colonies without visible eggs; NS, no encountered colonies during sampling; number in bracket represents number colonies surveyed.

During each sampling period, 5–10 species contained mature eggs, with 16 to 71 colonies containing mature eggs (**Table 2**). *A. muricata*, *A. humilis*, *A. millepora*, and *A. tenuis* had mature colonies in March–April and September–October (**Table 2**). Brown colonies of *A. millepora* contained mature oocytes in October, while pink, blue, and green colonies contained mature oocytes in March. Two species, *A. abrolhosensis* and *A. horrida*, were encountered during sampling, but neither contained mature or immature eggs. *A. muricata* and *A. gomezi* were also encountered and always contained at least one mature colony during the first transition period in each year for all surveys. *A. tenuis* was the only species that contained mature colonies in March–April and September–October in surveys across all years (**Table 2**).

Timing of Spawning

Direct observations of the reefs showed that most of the corals spawned gametes several hours after dusk (**Table 3**). Gametes were released over several hours. Slicks started to disperse within 1 to 2 h of the onset of spawning. *A. gomezi* spawned gametes between 19:00 to 22:00, while *A. austera, A. gemmifera*, and *A. tenuis* spawned gametes at around 22:00. In March–April, spawning occurred between 3 and 9 days before the full moon. In comparison, in September–October, coral populations released gametes 1 day before or after the full moon. There was no difference in the time of the day of spawning between the first transition period (March–April) and the second transition period (September–October) (**Table 3**).

Gamete release within a given month lasted between one and 7 days; however, the colonies did not necessarily spawn on consecutive days. Indeed, spawning was sometimes split over several days or weeks, specifically March–April 2009 and 2012, and September–October 2012 (**Table 3**). Therefore, spawning might have occurred on additional days. For example, in 2009, spawning was detected 13 days after initial spawning. In 2012, the first spawning event that occurred on 1–3 April was followed by another multi-specific spawning event on April 8, which was detected from the slicks that were deposited on the beach. Split spawning was also observed in October 2012. The first spawning event was observed on October 3, while the second spawning event occurred 27 days later, when the slicks were found (**Table 3**).

TABLE 3 | Dates and times of spawning slicks at Sambangan Island, Karimunjawa between 2009 and 2012 with respect to the full moon and time of spawning; NBF, nights before full moon; NAF, nights after full moon; see Section "Materials and Methods" for details of observation.

Date of spawning	Full moon	NAF or NBF	Time of spawning (spawned species)
20 March 2009	11 March 2009	9 NAF	19:40 – 22:15 (19:40, *Acropora gomezi*; 19:50, *A. muricata*; 20:15, slick was dispersed; *A. aspera, A. gemmifera, A. humilis, A. hyacinthus, A. rosaria*, and *A. tenuis* had already spawned)
3 – 4 April 2009	9 April 2009	6 NBF	19:40 – 22:00 (19:40, *A. gomezi*; 19:50, *A. muricata*; 20:00, slick was observed; 22:00, *A. austera, A. gemmifera* were still spawning)
25 March 2010	31 March 2010	7 NBF	Slicks were deposited on the beach
17–19 March 2011	20 March 2011	3 NBF	20:35 dispersed slicks were observed; (*A. aspera, A. digitifera, A. muricata, A. gomezi, A. millepora, A. nasuta* and *A. tenuis* had already spawned)
14 September 2011	13 September 2011	1NAF	Slicks were deposited on the beach
1–3 April 2012	7 April 2012	7 NBF	1 April; 20:15 – 22:00 (20:15, *A. gomezi*; 20:30, *A. muricata, A. tenuis, A. nasuta*: 22:00, slick was observed) 2 April (19:15, *A. gomezi*; followed by *A. tenuis, A. humilis, A. hyacinthus* and *A. muricata*; 22:30, slicks were dispersed after 22:30) 3 April, slicks were seen around 20:00 and dispersed after 22:00.
8 April 2012	7 April 2012	1 NBF	21:00, slicks were seen around the beach
3 October 2012	1 October 2012	2 NAF	19:50, *A. carduus*; 20:00, *A. millepora*; 22:00, *A. tenuis* were seen still releasing their gametes.
30 October 2012	31 October 2012	1 NBF	Slicks were deposited on the beach

Environmental Conditions During Spawning

Sea surface temperature – Data from the temperature loggers deployed on Taka Fadelan and Ocean Base showed two periods of temperature rise. The first period occurred in September–November 2013 and the second period occurred in March–April 2014. The daily amplitude in temperature during the first period at Taka Fadelan and Ocean Base was 2.22°C and 2.65°C, respectively. During the second period, it ranged from 2.22 and 2.70°C, respectively (**Figure 4A**). Multi-specific spawning events at Sambangan Island occurred between March 26, 2013, and May 20, 2014, at 28.54–30.14°C. On September 20, 2013, spawning occurred at 28.54–28.68°C. On October 20, 2013, spawning occurred at 29.86–29.89°C. The multi-specific spawning event that was observed on April 19, 2014, occurred at 29.81–30.14°C (**Figure 4B**).

Several hours before spawning (19:00–22:00) on September 20, 2013, when a multi-specific spawning event occurred, the temperature decreased at Taka Fadelan, and a smaller decrease in temperature at Ocean Base (**Figure 5**). On the expected spawning day of October 20, 2013, a rise in temperature was observed. Temperature showed an upward trend from October 18 to 21, 2013, but a pronounced temperature rise was only observed on October 20, 2013. Variation in DSST before multi-specific spawning on April 19, 2014, included a distinct drop in temperature at Ocean Base after exposure to a fairly noticeable rise in temperature from 14:00 to 15:00. The same pattern of temperature change was observed at Taka Fadelan on this date, but no pronounced fluctuations were recorded (**Figure 5**).

A model of variation in DSST showed that variation in ΔSST followed variability in MS, the daily mean of SR and U, and the daily mean of WS. Higher MS and lower U resulted in higher ΔSST. Latent heat was not plotted, as it did not have a pronounced influence on variation in ΔSST. **Figure 6** shows that corals always spawned during or immediately after a period of high ΔSST (>0.5°C). A strong signal was found for the spawning periods on March 20, 2009, April 3, 2009, October 25, 2010, March 17–19, 2011, April 1–3, 2012, and October 3, 2012, when ΔSST reached at least 1.0°C (with October 2011 showing a less pronounced link).

Total monthly rainfall –SST, and to a lesser extent rainfall, showed a distinct seasonal pattern at our study site (**Supplementary File S2**). The pattern of rainfall was seasonal in three of the 4 years of our study. In 2010, rainfall exhibited a less distinct seasonal pattern than SST (**Supplementary File S2**).

DISCUSSION

Gametogenic Cycles and Spawning Synchrony

The histological observations showed that the gametogenic cycle of *Acropora hyacinthus*, *A. humilis,* and *A. gomezi* followed a similar timing. All three *Acropora* species contained oocytes of various stages from June 2008 to March 2009, with maturing oocytes occurring in March. All three *Acropora* species probably had a single gametogenic cycle. *A. gomezi* colonies with mature oocytes were found monthly from July 2008 to March 2009 of the following year. In comparison, *A. hyacinthus* had some stage III oocytes in September, but only stage II oocytes in October; thus, mature oocytes might have been released during that time (**Figure 3**). Colonies of *A. humilis* contained mature oocytes on September and every month thereafter (**Figure 3**). However, spermatocytes were not found in December 2008, or earlier, in any of the three species, and were first observed in samples collected during March 2009 (**Figure 3**). Spermatogenesis might have started earlier than March in all three species. However, before the second transition period (December to February), a strong current passed through Karimunjawa waters, preventing the collection of samples.

The synchrony of gamete maturation in the *Acropora* assemblages of Karimunjawa Archipelago seems to be moderate.

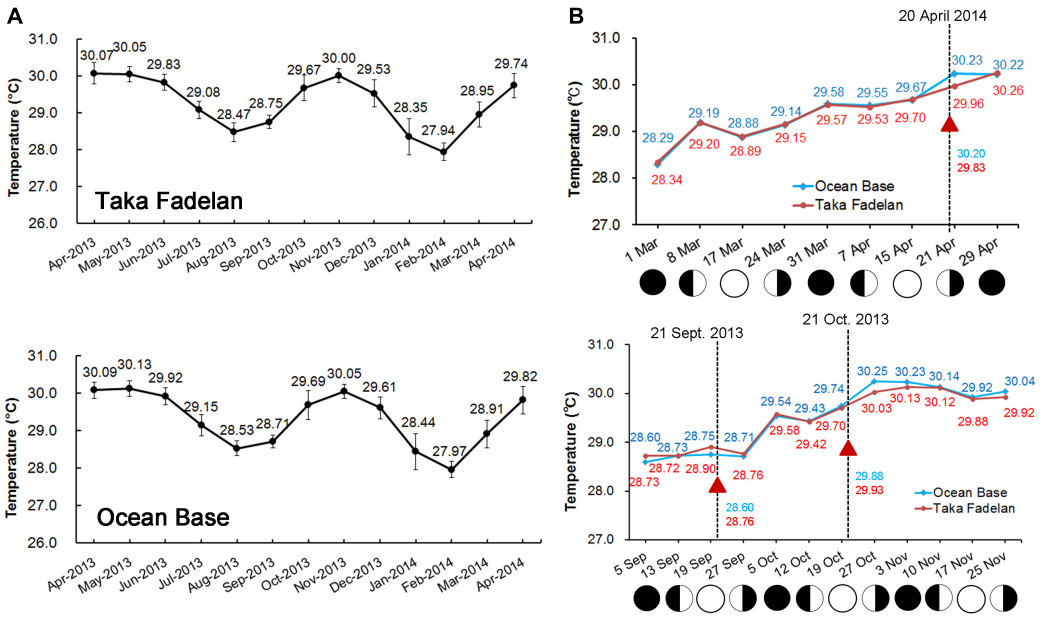

FIGURE 4 | Full year temperature profile at Karimunjawa Archipelago obtained from temperature loggers that were deployed at Taka Fadelan (05°50′ 42.5′′ S, 110° 35′ 17.7′′ E) and Ocean Base (05°50′ 40.1′′ S, 110° 34′ 48.5′′ E). Two periods of increasing temperature were observed, during the first transition period (March – April) and the second period (September – October) **(A)**. Spawning occurred during increasing SST and appeared to follow the lunar cycle; the triangle represents the spawning event **(B)**.

The proportion of mature colonies was comparable between the first (March–April) and second (September–October) transition periods, ranging from 0.08 to 65% and from 26.7 to 47%, respectively. *A. aspera, A. batunai, A. digitifera, A. gomezi, A. humilis, A, muricata, A. hyacinthus,* and *A. tenuis* showed a low proportion of mature gametes during the sampling period in February. Likely the sampling time was early thus only immature oocytes were present.

Field surveys indicated that 12 *Acropora* species (namely, *A. aspera, A. florida, A. muricata, A. gemmifera, A. gomezi, A. humilis, A. hyacinthus, A. jacquelineae, A. loripes, A. millepora, A. nasuta*, and *A. tenuis*) contained mature eggs in March–April. In comparison, 8 *Acropora* species (*A. austera, A. carduus, A. caroliniana, A. muricata, A. humilis, A. kimbeensis, A. millepora,* and *A. tenuis*) contained mature eggs during September–October (**Table 2**). Compared to surveys in the central GBR (Baird et al., 2009), the proportion of mature colonies during both spawning periods was lower. However, the proportion of colonies breeding in March 2011 was slightly higher (65%) compared to the northern GBR (63%) (Baird et al., 2002). The proportion of mature colonies was about 40%, which was similar to that obtained for reefs in Singapore (Guest et al., 2005a). Within species, a high synchrony in oocyte maturation was observed for *Acropora gomezi, A. muricata, A. millepora*, and *A. tenuis* (**Table 2**). Guest et al. (2005a) reported that, in April 2003, 12% of 8 *A. tenuis* colonies in reefs in Singapore contained mature oocytes. In comparison, 17% (n = 6) to 88% (n = 17) of *A. tenuis* in the Karimunjawa Archipelago contained mature oocytes in our study. *A. muricata* also showed high synchrony of gamete maturity during both sampling periods, with the highest proportion of mature colonies in September 2012 (83%; n = 36). This result differs to that of other species. In particular, *A. humilis* and *A. aspera* both contained high proportions of immature oocytes (**Table 2**) and might have spawned in different months. In Singapore (Guest et al., 2005a), 86% (n = 7) of *A. humilis* showed synchrony in gamete maturation, while, in New Caledonia (Baird et al., 2010), many *A. humilis* colonies lacked visible eggs. The lack of oocytes arose because colonies directed their energy toward growth likely counteracting environmental stressful conditions (Baird et al., 2010). In the present study, *A. carduus*, *A. kimbeensis*, and *A. millepora* only exhibited a high proportion of mature eggs during the second transition period.

A. hyacinthus, *A. muricata*, *A. millepora*, and *A. tenuis* contained mature and immature oocytes in March–April and September–October (**Table 2**). Edinger et al. (1996) reported that 22 species of scleractinian corals spawned in October in the Karimunjawa Archipelago, including *A. hyacinthus*. Biannual multi-specific spawning at the same location has been reported for coral assemblages that spawn their gametes in March–April and October–November (Permata et al., 2012). The oocytes of different morphotypes of *A. millepora* matured at different periods. Pink, blue, and green colonies had mature oocytes in March, while brown colonies had mature eggs in October. These findings correspond well with the literature. Rosser (2013) found that *A. millepora* in the waters off western Australia spawn in different seasons. In Singapore, two distinct periods of coral spawning have been reported for *A. humilis* containing mature eggs in October and November, and again in April (Guest et al., 2005a). Furthermore, in the GBR, *Montipora* corals

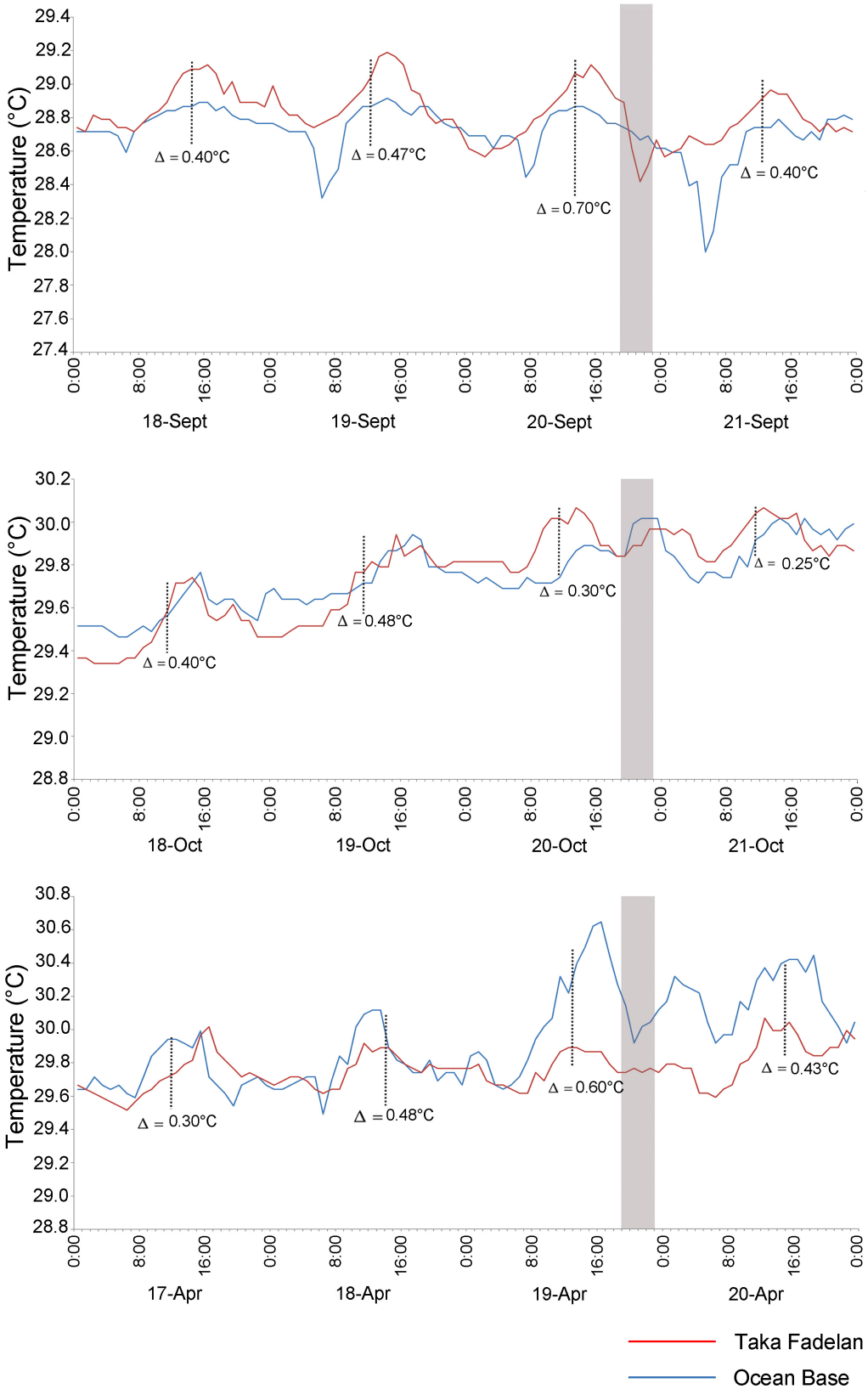

FIGURE 5 | Diurnal temperature variations prior to multi-specific spawning on 20 September, 20 October 2013, and 19 April 2014. The gray area represents the expected spawning time. Blue line represent Ocean Base, Red line represent Taka Fadelan.

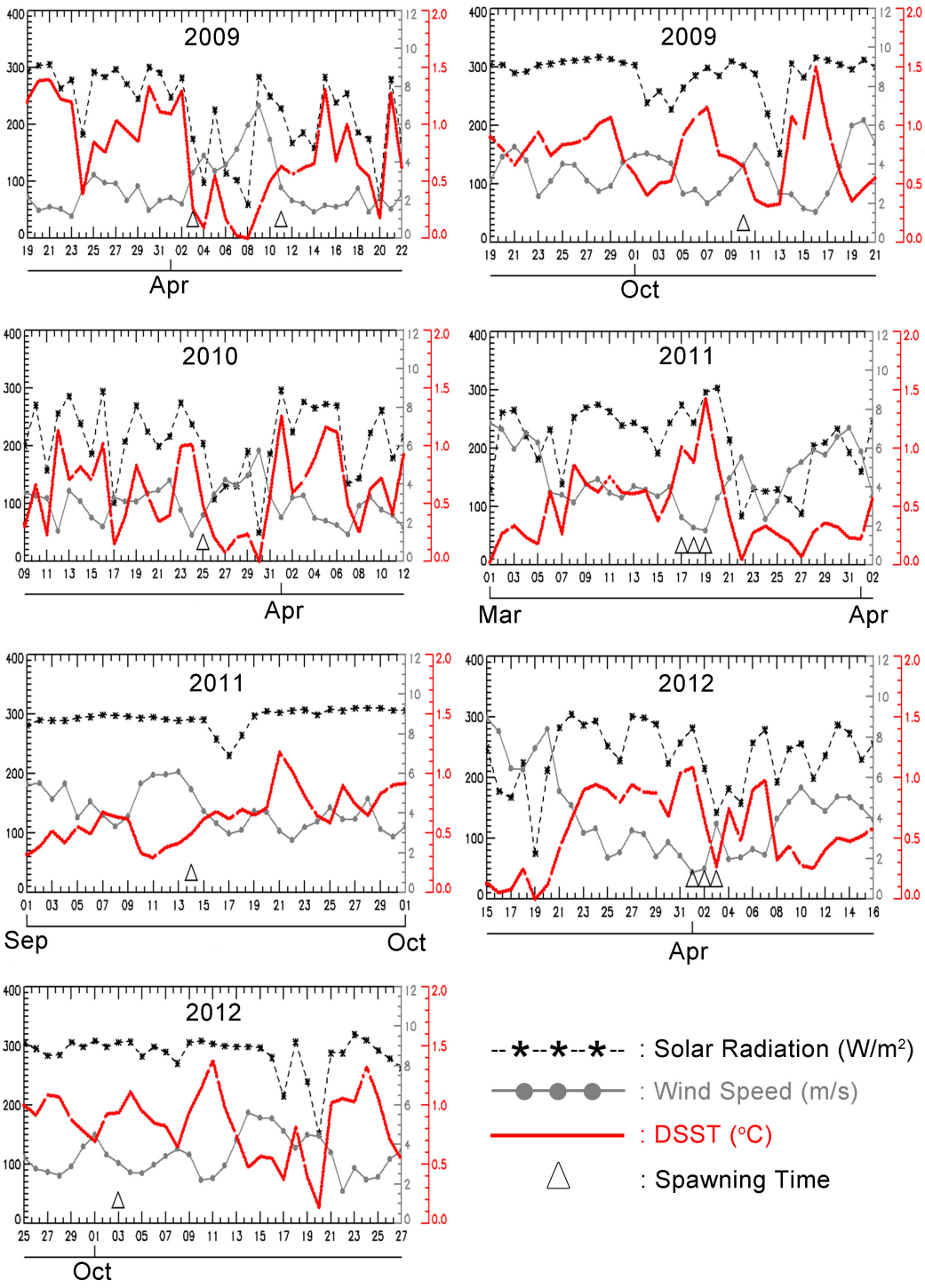

FIGURE 6 | Truncated time series of a daily average of solar radiation (W/m2/day), wind speed (m/sec), and amplitude of DSST (°C) obtained from equation (1) at 5.840S,110.580E, with the corresponding spawning time. The dates of multi-specific spawning are in March–April, and September – October, and are indicated by the triangle mark.

exhibit biannual spawning, in spring and fall, with some colonies undergoing two gametogenic cycles (Stobart et al., 1992). Coral populations in north-western Australia also spawn during spring and fall. Of seven species studied over two spawning events, five only spawned during fall and one released its gametes in spring (Gilmour J. et al., 2016).

In the northern region of Western Australia, coral species spawn during spring at Ashmore Reef, whereas *A. millepora* spawns in fall at Ningaloo Reef (Rosser, 2013). Favorable conditions (Harrison and Wallace, 1990), a genetic legacy (Babcock et al., 1994), and natural selection (Rosser, 2013) might explain why certain coral spawn in spring or fall, or why some species have a second gametogenic cycle.

Long-term research on corals has advanced our understanding of the environmental cues that dictate the month, day, and hour of spawning (Babcock et al., 1986; Hunter, 1988) and provides further cues about split spawning. For instance, the spawning patterns of corals in the Karimunjawa Archipelago appear to follow the lunar cycle (**Table 3**). Many corals species exhibit synchronous spawning according to lunar phases (Harrison and Wallace, 1990). Corals have extraordinarily sensitive photoreception, in terms of their ability to sense the blue region of the lunar spectrum; thus, corals are able to detect the intensity of moonlight (Gorbunov and Falkowski, 2002). However, in some spawning events observed in the present study (March 2009; April 2012; October 2012), split spawning occurred over an extended period, ranging from 8 days before full moon to 9 days after full moon in March 2009 and 7 days before full moon and 1 day after full moon in April 2012. In comparison, spawning occurred 2 days after full moon followed by the second spawning event 1 day before full moon in October 2012 (**Table 3**). One possible explanation for this variation is that when coral populations divide spawning over two consecutive months, the annual reproductive rhythm is affected by the number of lunar months, either 12 or 13 (Baird et al., 2009). There were 13 lunar months in both 2009 and 2012. Indeed, split spawning might be necessary in years with 13 lunar months to maintain an optimal environmental window for coral reproduction (Baird et al., 2009; Foster et al., 2018). Nozawa et al. (2006) suggested that the shift in the timing of spawning with respect to lunar months is related to the history of seawater temperature immediately before spawning.

In terms of the hour of spawning, during the 4 years of our study, all tagged colonies released gametes 1–2 h after dusk during the first and second transition period. For broadcast spawning corals, most species spawn after the onset of darkness (Babcock et al., 1986; Hunter, 1988). Similar behavior was observed in southern Japan and Palau, where *Acropora* species spawn at around 19:00–20:00 (Fukami et al., 2003; Levitan et al., 2004).

Empty colonies (i.e., colonies with no visible gametes) were observed in all 4 years of sampling. These colonies might have spawned earlier, or they might be sterile (Baird et al., 2010). Alternatively, these colonies might breed at other times of the year, outside of the observed annual gametogenic cycle.

Environmental Conditions During Spawning

Synchronization might be maintained by similar responses to a range of environmental signals, such as sea temperature patterns, lunar and tidal cycles, and diurnal changes to solar insolation, which work in concert to regulate the maturation and spawning of gametes (Babcock et al., 1986). Global comparisons between coral spawning times and SSTs are not always consistent. Coral spawning in Western Australia does not coincide with the maximum annual water temperature (Babcock et al., 1994). Penland et al. (2004) have also questioned the relationship between coral spawning and tropical water temperatures in Palau. Similarly, in a meta-analysis testing the relationship between solar insolation cycles and the timing of synchronous coral spawning, van Woesik et al. (2006) found a positive correlation. Insolation might be a more effective predictor of the timing of spawning than SSTs in the western Pacific Ocean (Penland et al., 2004) and western Atlantic Ocean (van Woesik et al., 2006). However, a recent study assimilating data on reproduction conditions in reefs throughout the Indo-Pacific region showed that the best predictor for the peak month of spawning in *Acropora* assemblages is a rapid change in SST (Keith et al., 2016).

Our study also documented two distinct spawning seasons in 2013 and 2014, which might correlate with two periods of rising temperature in September–November 2013 and March–April 2014 (**Figure 4**). Temperature might control the timing of spawning by initiating the development of testes, with coral populations in inshore reefs being likely to spawn earlier, coinciding with an earlier and more rapid rise in temperature (Babcock et al., 1986). In addition, our histological observations confirmed the presence of spermatocytes in March only (**Figure 3**), during a period of rising temperature.

We recorded a significant decrease in temperature several hours before multi-specific spawning. This phenomenon occurred on the expected date of multi-specific spawning in both March–April and September October (**Figure 5**). The modeled DSST (**Figure 6**) showed that daily solar radiation coupled with wind speed affected the amplitude of DSST. During the first (March–April) and second (September–October) transition period, the wind speed was lower, while the temperature increased (**Figure 6**). The same pattern was observed in data collected from temperature loggers around Genting Island, which is adjacent to Sambangan Island. This pattern commonly occurs in the Java Sea, as observed by Wirasatriya et al. (2018). Put differently, transition periods in the Java Sea are characterized by low wind speed, which leads to an increase in SST. During the transition periods, the daily temperature was probably affected by wind speed, daily insolation, and rainfall. A rise in temperature was followed by lower wind speed and an increase in the Insolation Clearness Index (Arfiyan and Munasik, 2016). Lower wind speed coupled with a significant decrease in temperature before the spawning date during a rising temperature period might provide the ultimate cue for the timing of multi-specific spawning among the coral population in the Karimunjawa Archipelago. These findings support those reported by van Woesik et al. (2006) and van Woesik (2010), who documented that spawning events occur during periods of low wind speed and high insolation. Our results also support the concept that these conditions generate high ΔSST, which might act as the natural trigger for corals to release gametes. Thus, ΔSST might be a better predictor for coral spawning than SST,

as SST displays inconsistencies with the timing of spawning in corals at some sites (Keith et al., 2016). However, the findings of this study require validation, as the data used here had low spatial resolution (1° × 1), and ΔSST was predicted at a depth of just 1 m. Therefore, more *in-situ* data of ΔSST, or a higher resolution SST dataset, is needed in future studies to understand how ΔSST influences coral spawning events. Wirasatriya et al. (2019) showed that high resolution SST datasets (i.e., 1 km × 1 km) can capture an amplitude of ΔSST of more than 5°C, even in higher latitude areas, i.e., around 40°N. Furthermore, laboratory experiments could be used to identify the ΔSST at which coral spawning is triggered more accurately.

In the present study, when the timing of spawning was connected with a period of rainfall, most coral species spawned just after the period of heaviest rainfall during the first transition period and before the heaviest rainfalls during the second transition period in all 4 years (**Supplementary File S2**). Mendes and Woodley (2002) suggested that spawning outside a period of heavy rainfall increases the fertilization rate. During periods with the heaviest rainfall, the level of nutrient influx from river run-off increases, which might reduce the successful development and settlement of coral larvae. Moreover, Hédouin et al. (2015) showed that rainfall on the night of coral spawning kills 70% of gametes released when salinity decreases to 26%. The present study reported similar patterns to those reported by Guest et al. (2012) in Singapore; specifically, coral spawning occurred before the period of heaviest rainfall.

The present study demonstrates that spawning in equatorial and tropical regions might be controlled by a drop in temperature several hours before spawning begins during periods when temperatures are rising. These results support the findings of Keith et al. (2016), with the rate of change in SST potentially representing a better predictor of multi-specific spawning throughout the Indo-Pacific region. The change in SST during periods of temperature rise coupled with lower winds might provide favorable conditions for multi-specific coral spawning. In addition, a drop in temperature during the period of rising temperature might help corals to adapt the timing of reproduction to the future effects of climate change. Corals that have experienced such shifts in phenology might have benefits compared with those that rely on certain absolute environmental cues for reproductive timing (Parmesan and Yohe, 2003). Additional research is required to investigate the relationship between drop in temperature during periods of rising temperature and the timing of multi-specific coral spawning in the tropics in more detail, as well as the potential involvement of other proximate cues.

ETHICS STATEMENT

The study was conducted at a cluster of island resort managed by PT Pura Baruna Lestari. Most corals that were sampled were collected for further laboratory observation. We cut small branches for studying the gametogenesis and reproductive status of the corals.

AUTHOR CONTRIBUTIONS

DW and EI designed the study. AW modeled the **Figure 6**. AH and DH led the fieldwork with Marine Diving Club members. AS conducted the histological study. TF analyzed the temperature data obtained from the loggers. RB helped to design the study. DW conducted the analyses and wrote the manuscript with assistance from EI, DH, and RB.

ACKNOWLEDGMENTS

We thank Dr. C Starger and Dr. Y Nozawa for reading an earlier version of the manuscript. We thank Dr. AH Baird for his suggestion on sample collection. We thank Professor Peter Harrison for his valuable correction throughout the manuscript. Special thanks go to Andhika Prabowo, Ertha Ankgusuma and the member of Marine Diving Club, Diponegoro University for assistance with fieldwork. We thank PT. Pura Baruna Wisata, Coral Unit for logistic support during sampling in Sambangan Island. We also thank NASA Climatology Resource Near real-time Global radiation and Meteorology (http://power.larc.nasa.gov/) and WHOI OAFlux project (http://oaflux.whoi.edu) funded by the NOAA Climate Observations and Monitoring (COM) program for providing the atmospheric and radiation data. This study was funded by Competition Grant provided by Indonesia Directorate General of Higher Education No. 381/SP2H/PP/DP2M/VI/2010 (year 2010) and No. 353/SP2H/PL/Dit.Litabmas/IV/2011 (year 2011), Diponegoro University Research Grant on International Publication No. 186-02/UN7.5.1/PG/2016 (year 2016) and No. SP DIPA-042.01.2.4.400898/2016 (year 2016).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00195/full#supplementary-material

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