



Evaluation of Mixotrophy-Associated Gene Expression in Two Species of Polar Marine Algae

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Mixotrophic flagellates can comprise significant proportions of plankton biomass in marine ecosystems. Despite the growing recognition of the importance of this ecological strategy, and the identification of major environmental factors controlling phagotrophic behavior (light and nutrients), the physiological and molecular mechanisms underlying mixotrophic behavior are still unclear. In this study, we performed RNA-Seq transcriptomic analysis for two mixotrophic prasinophytes, *Micromonas polaris* and *Pyramimonas tychoetreta*, under dissolved nutrient regimes that altered their ingestion of bacteria prey. Though the strains examined were polar isolates, both belong to genera with widespread distribution. Our aim was to characterize the transcriptomes of these two non-model phytoflagellates, identify transcripts consistent with phagotrophic activity and assess their differential expression in response to nutrient stress. *De novo* assembly of the transcriptomes yielded large numbers of novel coding transcripts with no known match within public databases. A summary of the transcripts by Gene Ontology terms showed many expected expression patterns, including genes involved in photosynthetic pathways and enzymes implicated in nutrient uptake pathways. Searches of KEGG databases identified several genes associated with intra-cellular digestive pathways actively transcribed in both prasinophytes. Differential expression analysis showed a larger response in *P. tychoetreta*, where 23,373 genes were up-regulated and 1,752 were down-regulated in the low nutrient treatment when phagotrophy was enhanced. In contrast, in *M. polaris*, low nutrient treatments resulted in up-regulation of 314 transcripts while down-regulating 371. With respect to phagotrophic-related expression, 37 genes were co-expressed in both *P. tychoetreta* and *M. polaris*, and although the response was less pronounced in *M. polaris*, it is consistent with differences in observed ingestion behavior. This study presents the first genomic data for *Pyramimonas tychoetreta*, and also contributes to the limited available data for *Micromonas polaris*. Furthermore, it provides insight into the presence of genes associated with phagocytosis within the Prasinophyceae and contributes to the understanding of potential target genes required for the construction of a complete model of gene regulation of phagocytic behavior in algae.

Keywords: mixotrophy, *Pyramimonas*, *Micromonas*, RNA-Seq, transcriptomics, phagotrophy

INTRODUCTION

The traditional view of protistan species is that they can be defined in terms of a single trophic mode, either photo- or hetero-trophic (Azam et al., 1983). However, more recently, a greater recognition of the impact of photosynthetic protists with the ability to ingest prey has been growing (Pomeroy, 1974; Flynn et al., 2012). Ingestion of prey by phytoplankton is found to occur in both freshwater and marine environments, ranging from tropical to polar ecosystems (Boraas et al., 1988; Sanders, 1991; Pålsson and Granéli, 2004; Moorthi et al., 2009; Hartmann et al., 2013). This ecological strategy can account for significant impact with regard to the movement of nutrients and carbon, as well as through substantial reintroduction of immobilized carbon back into the food web (Boraas et al., 1988; Sanders, 2011; Unrein et al., 2014). Mixotrophic behavior has also been demonstrated to satisfy organismal requirements for macro- and micro-nutrient deficiencies, and ingestion behavior is often a response to these limiting conditions (Nygaard and Tobiesen, 1993; Maranger et al., 1998). Mixotrophic phytoflagellates can comprise a considerable proportion of planktonic communities and potentially graze large proportions of bacterial standing stock daily in both freshwater and marine planktonic food webs (Bird and Kalff, 1986; Sanders et al., 1989; Sanders, 2011), accounting for a majority of the bacterial mortality (Hitchman and Jones, 2000; Zubkov and Tarran, 2008; Sanders and Gast, 2012).

Despite the growing recognition of predatory behavior in photosynthetic protists, and the substantial collection of physiological measurements of mixotrophic behavior (e.g., predation rates and conversion efficiencies), the regulatory controls at the genetic level are still poorly understood. This is, in part, a result of the broad spectrum of mixotrophic organisms representing diverse evolutionary lineages, many of which lack established and well-curated model organisms for use in genetic and molecular studies (Sherr and Sherr, 2002). This area is of importance because phagotrophic behavior provides evidence of the mechanisms underlying the endosymbiotic theory, which unites all eukaryotic lineages, and all plastid containing organisms in particular (Burns et al., 2015). Some molecular targets involved in the process of cellular phagotrophy have been previously identified, including receptors associated with phagocytosis, actin filament remodeling and regulatory gene families (Yutin et al., 2009). Liu et al. (2016) also identified genes potentially associated with mixotrophy, however, the details in terms of regulation and biochemical controls need further study.

Within the Archeplastida, also referred to as the green algae, the Prasinophyta represent an ancient group of plastid containing eukaryotes that are widely distributed in both freshwater and marine environments, and have been found to make up significant portions of the standing biomass in polar microbial communities (Moro et al., 2002; Lovejoy et al., 2007; Li et al., 2009; Gast et al., 2014). Green algae were long thought to be a wholly autotrophic lineage of organisms, but recent observations have shown ingestion behavior in several green algal species, including several Prasinophytes (Maruyama and Kim, 2013; Raven, 2013; McKie-Krisberg and Sanders, 2014; McKie-Krisberg et al., 2015). Within the Prasinophyceae,

genomic data is available for a limited number of species, including *Ostreococcus tauri*, an isolate of *Micromonas pusilla* and *Cymbomonas tetramitiformis* (Derelle et al., 2006; Worden et al., 2009; Tirichine and Bowler, 2011; Burns et al., 2015). Of these prasinophytes, aside from *C. tetramitiformis*, none have been positively identified as mixotrophs. The recent advancements in genomic technologies, particularly RNA-Seq and related computational analysis, provide efficient, cost effective methods to gain a deeper understanding of the changes at the molecular level, particularly in non-model organisms.

In this study, we examine the transcriptional profiles of two polar mixotrophic prasinophytes representing different phytoplankton size fractions (pico- vs. nano-flagellates). We investigate the response at the molecular level, in terms of gene expression, to changes in nutrient availability previously shown to illicit changes in prey ingestion behavior in these two organisms. Our aims were to characterize the transcriptomes of these two non-model phytoflagellates, identify transcripts consistent with phagotrophic activity and assess their differential expression in response to stimulation of mixotrophy due to nutrient limitation. We hypothesize that nutrient availability is a major factor in driving ingestion behavior in polar mixotrophic algae, and that by using a comparative “-omics” approach, we can identify patterns of gene expression common among mixotrophic behavior in microbial eukaryotes. We obtained feeding activity and transcriptomic data simultaneously from an Arctic isolate of *Micromonas polaris*, and the Antarctic *Pyramimonas tychoetreta*, and identified changes in transcript abundance in response to a reduction in nutrients. By comparing the transcriptomes of the two prasinophytes, we also attempt to identify genes linked to phagotrophic behavior shared by both organisms, which could be potential targets for the evaluation of mixotrophic activity in environmental samples.

MATERIALS AND METHODS

Culture Origin and Maintenance

Pyramimonas tychoetreta (I-9 Pyram) was isolated from the Ross Sea, Antarctica in 1999 and maintained at Woods Hole Oceanographic Institution and at Temple University (<http://www.whoi.edu/science/B/protists/>). *Micromonas* isolate (CCMP 2099, Provasoli-Guillard National Center for Marine Algae and Microbiota), recently identified as *M. polaris* (Simon et al., 2017), was originally isolated from an Arctic polyna. The prasinophytes in the present study differ in terms of cell size (1.2 μm *M. polaris* vs. 5–7 μm average diameter for *P. tychoetreta*). Experimental cultures of these algae were maintained at 4°C in 32 PSU f/2 + Si (Guillard, 1975; Caron, 1993). The cultures were non-axenic and grown under 14 h light/10 h dark irradiance from 50 W cool-white fluorescent bulbs at $\sim 50 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Experimental Setup

Previous studies have shown both *P. tychoetreta* and *M. polaris* to ingest particles, and that ingestion rates are influenced by nutrient availability in the culture media (McKie-Krisberg and Sanders, 2014; McKie-Krisberg et al., 2015). Replicate flasks of *P. tychoetreta* (2.5×10^3 cells ml^{-1}) and *M. polaris* ($1.44 \times$

10^5 cells ml^{-1}) were grown in either high- or reduced-nutrient conditions for 1 week for transcriptional analysis ($n = 4$ per treatment/species/time point; total 16 per species) and ingestion experiments ($n = 3$ per treatment/species/time point; total 12 per species) conducted at the time of whole cell collection for sequencing to confirm differences in feeding. The differences in cell abundances are reflective of the shorter doubling time and smaller size of *M. polaris* as compared to *P. tychochreta*. High-nutrient treatments were full strength $f/2 + \text{Si}$ (i.e., the maintenance media, $882 \mu\text{M N}$, $36.2 \mu\text{M PO}_4^{3-}$), while low-nutrient treatments were a 10-fold dilution of $f/2 + \text{Si}$ culture media ($88.2 \mu\text{M N}$, $3.62 \mu\text{M PO}_4^{3-}$) prepared with filter-sterilized seawater. The low-nutrient conditions were previously found to elicit increases in ingestion by these two species (McKie-Krisberg and Sanders, 2014; McKie-Krisberg et al., 2015) and the concentrations of nitrogen and phosphorous fall within the range observed in both the Arctic and Antarctic environments (Wheeler et al., 1997; Smith et al., 2003). Illumination and temperature conditions were the same as used for culture maintenance (14 h light/10 h dark irradiance at $\sim 50 \mu\text{mol m}^{-2} \text{s}^{-1}$).

RNA Extraction, Transcriptome Assembly and Differential Expression Analysis

Replicate cultures of *P. tychochreta* ($n = 4$) and *M. polaris* ($n = 4$) that had been incubated under high- and reduced-nutrient conditions known to affect ingestion were collected on 25 mm, $0.2 \mu\text{m}$ polycarbonate filters, frozen at -80°C , then extracted using the Qiagen Mini RNA kit, with confirmation of nucleic acid abundance and quality assessed by Bioanalyzer (Agilent Technologies, Santa Clara, CA). Replicate samples were sent to the Sulzberger Genome Center at Columbia University for poly-A selection, library construction and sequencing by Illumina Mi-Seq (150 bp paired-end reads, 30 million reads per sample). Raw reads have been deposited in the NCBI Sequence Read Archive under accession number SRP090401.

Overall quality of Illumina raw reads data was assessed using FastQC (Andrews, 2010). Poor quality reads and adapters were removed using Trimmomatic 0.32, with settings for removing seed mismatches = 2, palindrome clip threshold = 10, and a simple clip threshold = 30. Additional settings included removing leading and trailing bases below quality scores of 3 and excluding reads less than 36 bp (Bolger et al., 2014). Prior to assembly, FLASH (version 1.2.11) was used to find overlaps and merge read pairs to increase the quality of the transcript assembly (Magoc and Salzberg, 2011). Following these preparatory steps, all paired-end reads for each species were combined and assembled using Trinity (version 2.3.2), using default assembly settings (Grabherr et al., 2011) (Table 1). Following assembly, bacterial decontamination was performed by Deconseq (version 0.4.3) using the bacterial data base generated by the script designers (Schmieder and Edwards, 2011). Transcriptomes of each species were filtered through CD-hit to identify unique transcripts (Li and Godzik, 2006). This step clustered transcripts that were $\geq 90\%$ similar. In the absence of sequenced genomes and proper functional annotation of either of these prasinophytes, this was a conservative approach

TABLE 1 | Summary statistics from Trinity *de novo* transcriptome assembly for two polar mixotrophic algae *Micromonas polaris* and *Pyramimonas tychochreta*.

Species	<i>Micromonas polaris</i>	<i>Pyramimonas tychochreta</i>
Illumina paired-end reads	3.59×10^8	3.07×10^8
Number of bases assembled	2.48×10^7	7.89×10^7
Number of transcripts	12,368	111,043
Mean contig length	2005.4	710
N50	4,021	1,074
GC content (%)	59.53	54.72

that allowed for the occurrence of differential splicing of transcriptional products, but constrained computational artifacts from the *de novo* assembly that could not be validated. Transcript abundance from each replicate was determined by sequence-read alignment to transcriptomes with Bowtie2 (Langmead and Salzberg, 2012), followed by count estimation with eXpress (Roberts et al., 2011). Differential expression of *M. polaris* and *P. tychochreta* was determined using edgeR according to the experimental design, using the exact test based on quantile-adjusted conditional maximum likelihood (qCML) method (Robinson et al., 2010). Our threshold for differential regulation of Trinity “genes” was defined by transcript differences of at least 2 Log_2 -fold (4-fold) increase/decrease in expression. Transcript assembly, differential expression analysis and gene annotation were all performed using computational resources available via the Owlsnest Super-Computing Cluster at Temple University, and the CUNY High Performance Computing Center at the CUNY College of Staten Island.

Functional Annotation

Functional annotation of assembled transcripts was performed with Trinotate 2.0 (Haas et al., 2013). Searches of several genomic databases, including Uniprot databases (<http://www.uniprot.org/>) and *nr* and *nt* (<http://www.ncbi.nlm.nih.gov/>) were performed using BLAST utility and employing Transdecoder to identify predicted Open Reading Frames (ORFs). ORFs were also queried using hmmscan, and rnammer for predictions of conserved domains with homology to protein families and domains found in the Pfam database (Lagesen et al., 2007; Eddy, 2011; Finn et al., 2015). Gene Ontology (GO) associated with identified genes obtained from Interproscan searches were grouped according to “Biological Process” (BP), “Cellular Component” (CC) or “Molecular Function” (MF) (Jones et al., 2014). Whole transcriptome Gene Ontologies were summarized with the goSlim function of the GSEABase package, within the R statistical environment using the plant ontology (<http://www.plantontology.org/>) (Gentleman et al., 2004; Morgan et al., 2008; R Core Team, 2013). GO term information from transcript data was integrated into Gene Enrichment analysis. We obtained the ontology pathways that were enriched by the treatment conditions using the runTest function in the topGO package, performing a Fisher Exact test inputting normalized gene expression measurements along with differential expression analysis results (Alexa and Rahnenfuhrer, 2017). Assembled transcripts of both *M. polaris*

and *P. tychotreta* were submitted to the KAAS-KEGG Automatic Annotation Server provided by the Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/tools/kaas/>) for the identification of additional enzymes involved in primary nutrient metabolism (Carbon, Nitrogen), as well as phagotrophic related pathways, such as autophagy (Moriya et al., 2007). Additionally, genes found to be involved in phosphorus (P) limitation response in plants (Rouached et al., 2010) were identified by blast searches of *M. polaris* and *P. tychotreta* transcriptomes (**Supplementary Data Sheet 1**).

Ingestion Experiments

Both microspheres and fluorescently-labeled bacteria have been used to demonstrate ingestion behavior (Nygaard and Tobiesen, 1993), but the fluorescent particles employed in the present work were also used in previous studies of phagotrophy of both *P. tychotreta* and *M. polaris*, and therefore provide the most suitable comparison to the previous experimental work (McKie-Krisberg and Sanders, 2014; McKie-Krisberg et al., 2015). A total of 1×10^6 microspheres were added to each flask and the T_0 replicates (controls) were immediately fixed using the Lugol's/formaldehyde/ $\text{Na}_2\text{S}_2\text{O}_3$ method that prevents egestion (Sherr et al., 1993). The remaining replicates were incubated under the same light and temperature used for culture maintenance for 20 min (T_f), after which they were fixed as for the T_0 samples. Previous studies indicate that 20 min is within the period of time when there is a linear uptake of microspheres for both *P. tychotreta*, *M. polaris* (McKie-Krisberg et al., McKie-Krisberg and Sanders). Ten milliliter of fixed sample were filtered onto 25 mm Poretics polycarbonate membranes (0.8 μm pore size for *M. polaris* and 3 μm pore size for *P. tychotreta*, GE Osmonics, Minnetonka MN, USA) and stained/mounted using VectaShield[®] with DAPI (Vector Laboratories Inc., Burlingame, CA). Epifluorescence microscopy was used to examine at least 500 cells per filter for *P. tychotreta*, while 10 fields per filter were counted for *M. polaris*. Mixotrophic behavior was identified as the ingestion of beads after background correction with the T_0 samples.

RESULTS

Under high nutrient treatment conditions, a low level of particle ingestion was observed for *M. polaris*, and undetectable for *P. tychotreta*. However, in the low nutrient treatments, the percent of cells that ingested microspheres increased by 69% for *M. polaris* and 31% for *P. tychotreta*. These observations are consistent with previous studies where grazing in both species was affected by concentrations of nutrients in the culture media, with *P. tychotreta* showing a particularly distinct response (McKie-Krisberg and Sanders, 2014; McKie-Krisberg et al., 2015).

Transcriptome Assembly and Functional Annotation

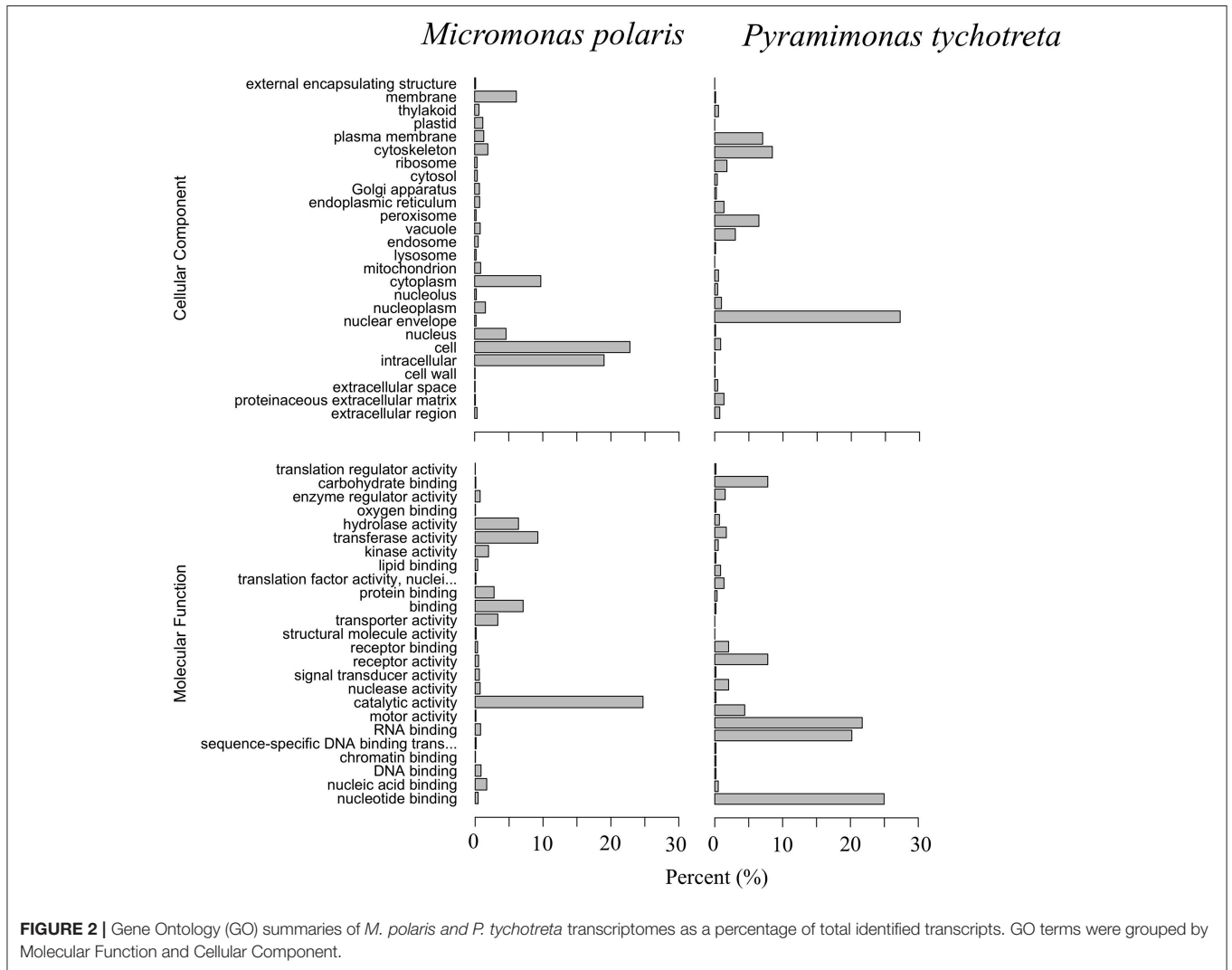
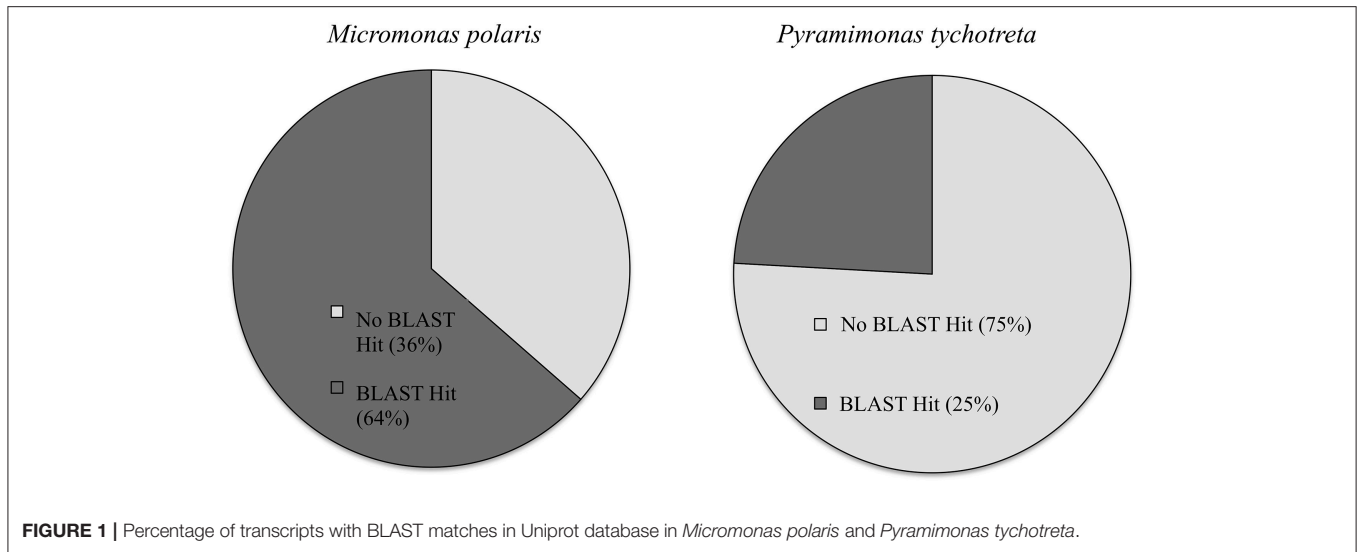
The *de novo* transcriptome assembly of RNA-Seq data identified $\sim 12,000$ and $\sim 110,000$ unique transcripts for *M. polaris* and *P. tychotreta*, respectively (**Table 1**). Current available genomic data for two isolates of *Micromonas* (CCMP1545 v3.0, and

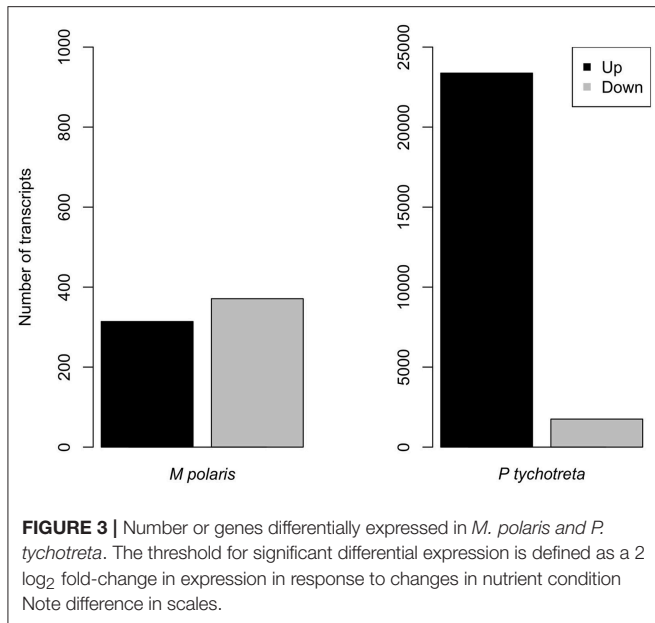
RCC299 v.3, Phytozome) include annotation of 10,660 and 10,103 genes, respectively (Goodstein et al., 2012). Therefore, even with our conservative approach, the transcriptome for *M. polaris* likely includes differential splicing products. The large transcriptome size of *P. tychotreta* was associated with a smaller N50 value, which may be due to a large portion of short length contigs (unique, consensus regions of RNA produced as a result of the *de novo* assembly) (**Table 1**). No genomic data is currently available for *P. tychotreta*, but annotation of the related prasinophyte, *Cymbomonas tetramitiformis* (Order Pyramimondales) was found to have almost 4 times the number of genes ($\sim 37,000$ genes) compared to the published *Micromonas pusilla* genomes (Burns et al., 2015).

The functional annotation of the transcriptomes for each species revealed a large number of novel transcripts that had no matches to previously identified genomic information in available databases. Functional annotation identified 64% of transcripts in *M. polaris*, while in *P. tychotreta*, only $\sim 24\%$ of transcripts matched previously identified proteins in the SwissProt database, indicating a large proportion of the transcripts represented potential novel sequences (**Figure 1**). Regardless of the relatively small percentage of identified transcripts, 2.7×10^3 transcripts were matched to existing accessions and functional annotation via the KEGG database. Looking in detail at differential expression results for both *M. polaris* and *P. tychotreta*, we observed changes in mechanisms associated with acquisition for both light and nutrient uptake pathways (**Supplementary Table 1**). Not surprisingly, proteins involved in photosynthesis represented a large number of the GO terms associated with Biological Processes in both *M. polaris* and *P. tychotreta* (**Figure 2**, **Supplementary Data Sheet 2**). These include Light Harvesting Complex proteins (LHC) and Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (RUBISCO) (**Supplementary Table 1**).

Overall, the effect of lower concentrations of dissolved nutrients was less pronounced in *M. polaris*, resulting in fewer changes in gene expression as compared to *P. tychotreta*. In cultures of *M. polaris*, lower nutrient concentrations resulted in 314 up-regulated and 371 down-regulated, at least a Log_2 -fold change of 2 (**Figure 2**). This response is less, but still comparable to, earlier studies of a tropical isolate of *Micromonas* (CCMP2709), which found up-regulation of 738 out of 960 differentially-expressed transcripts under the effect of phosphorus limitation (Whitney and Lomas, 2016). In low-nutrient treatments of *P. tychotreta*, we found 23,373 up-regulated and 1,752 down-regulated at a 2 Log_2 -fold change) (**Figure 3**). Reduction in nutrient availability resulted in up-regulation of genes related to carbon metabolism, and to some extent nitrogen metabolism, in both prasinophytes, although the response was more pronounced in cultures of *P. tychotreta* (**Figures 4,5**). Differential expression of genes associated with phosphorus limitation was greater in cultures of *P. tychotreta*, but *M. polaris* appears to constitutively express these genes, as they did not respond to the experimental conditions (**Figures 3–5**).

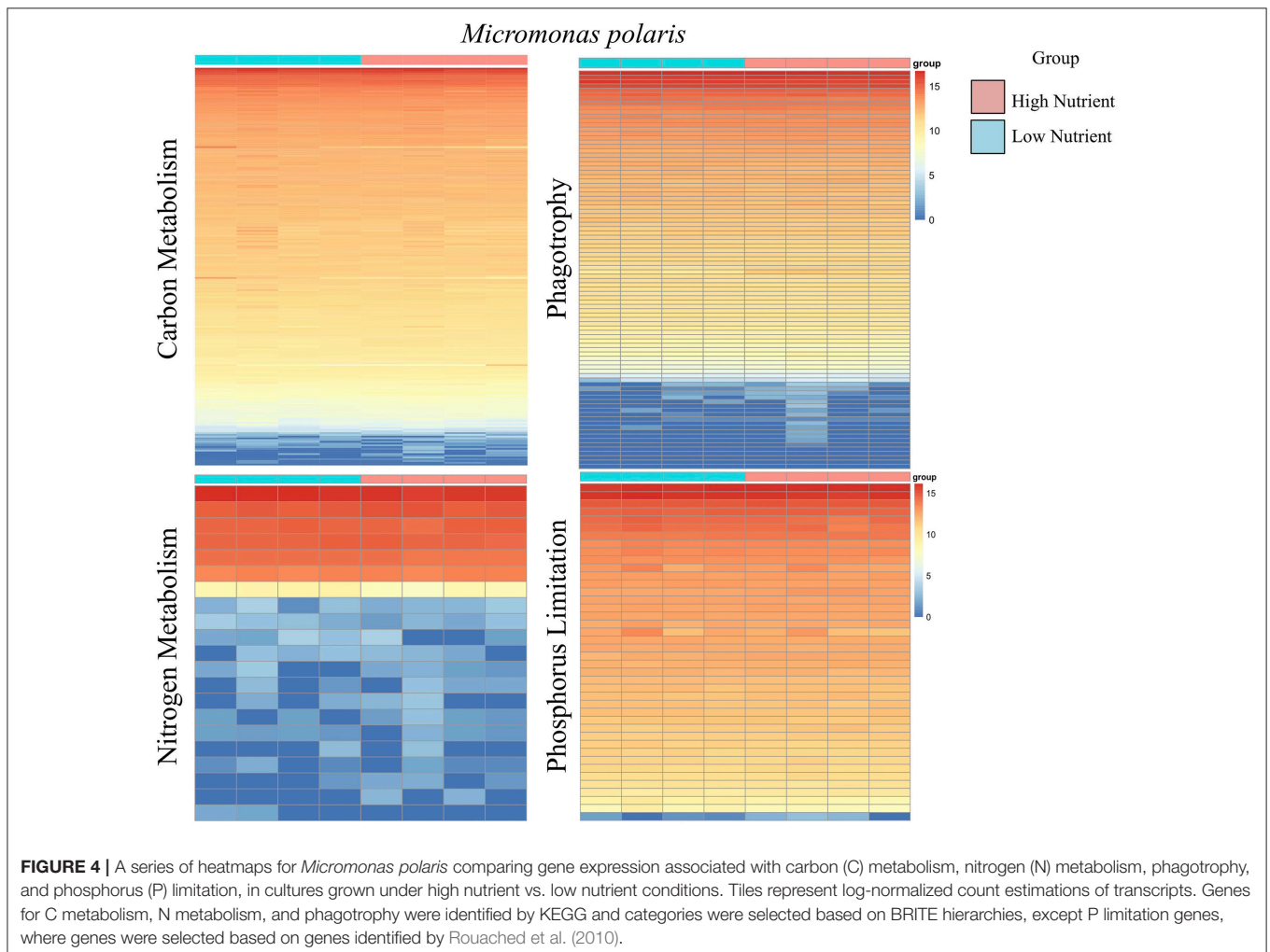
Specific to phagotrophy, low nutrient conditions resulted in up-regulation of 153 genes related to phagotrophic ingestion,

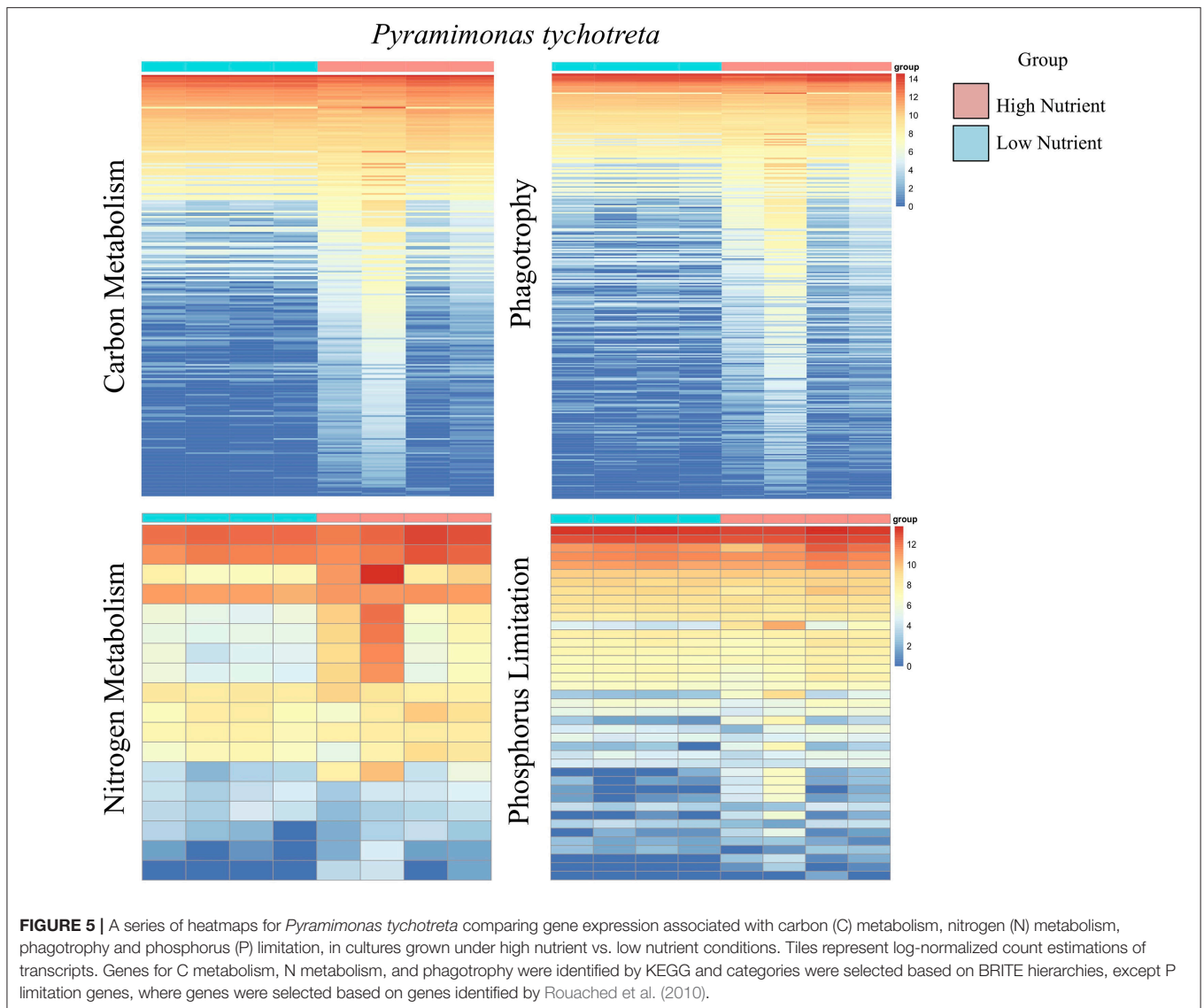




as defined by KEGG (Table 2). We also found 37 genes (in some cases, with multiple transcript matches) to be present in both *M. polaris* and *P. tychothreta*. These include genes that function in endocytosis (20), formation of phagosome (8), lysosome production (11), and regulation of autophagy (7) (Table 2, Supplementary Table 1). Several transcripts in the transcriptome of both *M. polaris* and *P. tychothreta* contained predicted Ankyrin domains (identified by KEGG), which have been suggested to play a role in phagocytosis (Moffat et al., 1996; Grolleau et al., 2003; Nguyen et al., 2014). Many of these genes appear to be found across eukaryotic taxa and not only among prasinophytes (Yutin et al., 2009).

Although genes related to phagotrophic processes, as identified by KEGG, were represented in *M. polaris*, none of these genes were found to be differentially expressed at the defined threshold rate (at least 2 \log_2 -fold change). The relatively constant level of expression of many of these genes may indicate the ubiquitous, though low, nature of ingestion behavior exhibited by *M. polaris*. Ingestion by this isolate has been found to be less responsive to media conditions than was the case for *P. tychothreta*, which was not phagotrophic in nutrient replete





media (McKie-Krisberg and Sanders, 2014; McKie-Krisberg et al., 2015) (**Figure 4, Supplementary Table 1**, Normalized Transcript count data).

DISCUSSION

Light and dissolved nutrients are often considered to be the most important factors affecting phagotrophy in mixotrophs, as these environmental factors represent two significant resource pools required by obligate phototrophs and also used by mixotrophs. The degree to which these factors may induce changes in trophic plasticity (i.e., phagotrophic activity) in a given mixotroph will vary (Stoecker, 1998; Sanders et al., 2001; Liu et al., 2011). Liu et al. (2016) modified light levels when comparing the transcriptomes of three temperate mixotrophs and found significant differences in patterns of gene expression in response to those changes that were consistent with the degree of mixotrophic behavior. We did not include dark treatment here

because previous work indicated that *P. tychotreta* populations decline in dark conditions, and that nutrient concentrations were the major driver of ingestion behavior in this organism (McKie-Krisberg et al., 2015). Both macro- and micro-nutrient concentrations have previously been shown to affect the ingestion rates of these and other mixotrophic phytoflagellates and, in some cases, can dictate whether flagellates will engage in phagotrophy at all (Nygaard and Tobiesen, 1993; Maranger et al., 1998; McKie-Krisberg and Sanders, 2014; McKie-Krisberg et al., 2015). Other factors can also affect ingestion in both mixotrophs and heterotrophs. For example, the differences in cell size between the prasinophytes in the present study (1.2 μm *M. polaris* vs. 5–7 μm average diameter for *P. tychotreta*) seems to constrain the number (and size) of bacteria that can be ingested, and prey size has been recognized as a factor limiting ingestion in various single-celled eukaryotes (Fenchel, 1986; Sanders and Gast, 2012; McKie-Krisberg and Sanders, 2014).

TABLE 2 | Functional annotation of transcripts encoding for enzymes associated with **(A)** Endocytosis, **(B)** Phagosome, **(C)** Lysozome, **(D)** Regulation of Autophagy, expressed in both *M. polaris* and *P. tychoetreta* that as identified by the Kyoto Encyclopedia of Genes and Genomes.

KO	Gene symbol	Description	EC
(A) ENDOCYTOSIS			
K00889	PIP5K	1-phosphatidylinositol-4-phosphate 5-kinase	2.7.1.68
K12492	ARFGAP1	ADP-ribosylation factor GTPase-activating protein 1	–
K12200	PDCD6IP, ALIX, RIM20	Programmed cell death 6-interacting protein	–
K11824	AP2A	AP-2 complex subunit alpha	–
K12489	ACAP	Arf-GAP with coiled-coil ANK repeat and PH domain-containing protein	–
K18442	ARFGEF, BIG	Brefeldin A-inhibited guanine nucleotide-exchange protein	–
K12483	EHD1	EH domain-containing protein 1	–
K07901	RAB8A, MEL	Ras-related protein Rab-8A	–
K07889	RAB5C	Ras-related protein Rab-5C	–
K12194	CHMP4, SNF7, VPS32	Charged multivesicular body protein 4	–
K18464	RTSC, SPG8	WASH complex subunit strumpellin	–
K12198	CHMP5, VPS60	Charged multivesicular body protein 5	–
K12562	AMPH	Amphiphysin	–
K04646	CLTC	Clathrin heavy chain	–
K12471	EPN	Epsin	–
K13649	FOLR	Folate receptor	–
K18443	GBF1	Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	–
K03283	HSPA1_8	Heat shock 70kDa protein 1/8	–
K17917	SNX1_2	Sorting nexin-1/2	–
K12486	SMAP	Stromal membrane-associated protein	–
(B) PHAGOSOME			
K03661	ATPeV0B ATP6F	V-type H ⁺ -transporting ATPase 21kDa proteolipid subunit	–
K10414	DYNC2H DNCH2	dynein heavy chain 2 cytosolic	–
K00921	PIKFYVE FAB1	1-phosphatidylinositol-3-phosphate 5-kinase	2.7.1.150
K08054	CANX	Calnexin	–
K08057	CALR	Calreticulin	–
K01365	CTSL	Cathepsin L	3.4.22.15
K10956	SEC61A	Protein transport protein SEC61 subunit alpha	–
K07374	TUBA	Tubulin alpha	–
(C) LYSOZOME			
K02154	ATPeV0A ATP6N	V-type H ⁺ -transporting ATPase subunit a	–
K12391	AP1G1	AP-1 complex subunit gamma-1	–
K12396	AP3D1	AP-3 complex subunit delta-1	–
K12400	AP4E1	AP-4 complex subunit epsilon-1	–
K02146	ATPeV0D ATP6D	V-type H ⁺ -transporting ATPase subunit d	–
K03661	ATPeV0B ATP6F	V-type H ⁺ -transporting ATPase 21kDa proteolipid subunit	–
K02155	ATPeV0C ATP6L	V-type H ⁺ -transporting ATPase 16kDa proteolipid subunit	–
K05656	ABCB9 TAPL	ATP-binding cassette subfamily B (MDR/TAP) member 9	–
K04646	CLTC	Clathrin heavy chain	–
K01074	PPT	Palmitoyl-protein thioesterase	3.1.2.22
K02144	ATPeV1H	V-type H ⁺ -transporting ATPase subunit H	–
(D) REGULATION OF AUTOPHAGY			
K08269	ULK1_2_3, ATG1	serine/threonine-protein kinase ULK/ATG1	2.7.11.1
K07198	PRKAA, AMPK	5'-AMP-activated protein kinase catalytic alpha subunit	2.7.11.11
K08333	PIK3R4, VPS15	phosphoinositide-3-kinase regulatory subunit 4	2.7.11.1
K08334	BECN1, VPS30, ATG6	Beclin 1	–
K00914	PIK3C3, VPS34	Phosphatidylinositol 3-kinase	2.7.1.137
K08339	ATG5	Autophagy-related protein 5	–
K08342	ATG4	Cysteine protease ATG4	3.4.22.-

KO, KEGG Orthology number; EC, Commission nomenclature number produced by the IUBMB/IUPAC Biochemical Nomenclature Committee.

M. pusilla (CCMP1545) has a relatively small genome (~20 Mbp, Joint Genome Institute), certainly much smaller than its green algal counterpart, *P. tychotreta*. Although a sequenced genome of *P. tychotreta* is not yet available, a recent study of the alga *C. tetramitiformis*, which is from the same Order, identified a genome size of ~250 Mbp, more than 10 times greater than that of *M. pusilla* (Worden et al., 2009). The contrasting size of the transcriptomes for *M. polaris* and *P. tychotreta* is likely a reflection of the diversity of genome size within the green algal clade. The extensive number of transcripts (over 100 K) found in *P. tychotreta* suggest additional functions present in this alga, compared to *M. polaris*, but it may also represent an increase in differential splicing, and/or alternative transcripts. These mechanisms have been shown to exist in other green algae such as *Chlamydomonas reinhardtii*, as well as in other algal groups including the cryptophytes and chlorarachniophyte algae (Curtis et al., 2012; Blaby et al., 2014). Understanding these types of regulatory mechanisms can only be resolved with additional genome sequencing and annotation efforts, creating a reference wherein the true number of genes and the relation of gene-to-transcript can be determined.

Previous work with marine mixotrophs indicated shifts in gene expression related to both nutrient metabolism and limitation in response to changes in light and nutrient conditions (Koid et al., 2014; Liu et al., 2016; Caron et al., 2017). The results presented here indicate a comparable, though lesser, response noted for a tropical isolate of *Micromonas* (CCMP2709), which found up-regulation of 738 out of 960 differentially-expressed transcripts under the effect of phosphorus limitation (Whitney and Lomas, 2016). However, low phosphorus treatments in our present work are 4 times higher than the low nutrient treatments used by Whitney and Lomas ($0.5 \mu\text{M PO}_4^{3-}$ vs. $1.81 \mu\text{M PO}_4^{3-}$ in our study). Furthermore, Whitney and Lomas (2016) identified the isolate as *M. pusilla*, but the current nomenclature for this isolate via CCMP, designates CCMP2709 as *M. commoda*, and was found to be evolutionarily distinct from our *M. polaris*, which may also contribute to the relative differences in patterns of transcriptional response to changes in nutrients (Worden et al., 2009). In addition, *M. polaris* may respond to fluctuations in nutrient concentration by employing alternative acquisition strategies, like mixotrophy, which have not been observed in *Micromonas* sp. (CCMP 2709) to date, and may not be consistent across the genus. In the case of *P. tychotreta*, we observed significant up regulation of at least one transcript identified as nitrate reductase (NR, EC 1.7.1.1, EC 1.7.1.2, EC 1.7.1.3) as well as ferredoxin-nitrite reductase (EC 1.7.7.1). These results are consistent with previous results of nutrient limitation responses in several non-phagotrophic types of algae from diverse evolutionary lineages, suggesting a basic algal response that is still consistent with a mixotroph that is primarily photosynthetic (Allen, 2003; Berg et al., 2008; Wurch et al., 2011; Dyhrman et al., 2012).

Koid et al. (2014) used the EuKaryotic Orthologous Groups (KOG) tool to compare functional categories in four prymnesiophyte algae, including three known mixotrophs, and found that the overall distributions were very similar. However, when data from an additional 37 protistan species were added to

their analysis, the effects of both trophic mode and phylogenetic grouping were detected and non-alveolate mixotrophs formed a distinct cluster. The *Micromonas* strain CCMP2099 used in our study was also included in the broader analysis of Koid et al. (2014). The prasinophytes clustered together, but were distant from other known mixotrophs, so it may be that in this case the clustering more strongly reflected phylogeny than nutritional mode for the mixotrophs. Genome size may also have affected the analysis of Koid et al. (2014), as the three *Micromonas* strains and *Ostreococcus lucimarinus* that were included and clustered together are among the smallest eukaryotes with correspondingly small genomes.

Using the genes linked to phagotrophic behavior proposed by the work of Yutin et al. (2009), a recent genomic study of *Cymbomonas tetramitiformis* identified the presence of enzymes associated with phagotrophic pathways (Burns et al., 2015). Some of those phagotrophy-associated enzymes could also be identified in the current study. However, the majority of these enzymes were described in metazoan cell types, for example, in macrophages of the mouse, *Mus musculus*. Additionally, many of these associated genes may have multiple functions within a cell, which makes it difficult to determine the direct participation of a specific gene in cell phagocytosis based solely on the presence of the transcript coding for these enzymes. From an evolutionary perspective, dual function in genes creates the opportunity for conservation of these pathways among phyla containing phagotrophic and non-phagotrophic members, such as the Chlorophyceae. Conversely, it has been suggested that feeding behavior in algae may be associated with loss of biosynthetic pathways, which could drive mixotrophs to seek out prey to replace essential biomolecules—just as this loss is associated with obligatory phagotrophy (Swingley et al., 2007; Burns et al., 2015). Also, even for those genes able to be identified, the nucleotide sequence for a particular gene can vary among organisms from different evolutionary lineages. Depending on the limitations of genomic databases available for query, this variation in nucleotide sequence for a gene may limit reliable identification, even when genes are present in the subject organism.

The dearth of well-annotated reference genomes as well as transcriptomic data on a wide distribution of microbial eukaryotes, particularly within the Prasinophyceae, presents a challenge to interpreting these results. Despite this, the more pronounced response of ingestion-related genes in *P. tychotreta* as compared to *M. polaris* is consistent with previous work, which indicated that in *P. tychotreta* grazing activity is induced only under nutrient-limited conditions. In comparison, *M. polaris* grazing activity was found at low rates when nutrients were replete, and were only slightly further stimulated when nutrients were limited (McKie-Krisberg and Sanders, 2014; McKie-Krisberg et al., 2015). The results presented here also echo those of Liu et al. (2016), in which expression changes in phagotrophy-associated genes (specifically PEP carboxykinase and vacuolar ATPase) reflected the different ingestion behaviors of *Prymnesium parvum* and *Dinobryon*. Under dark conditions, the higher expression of PEP carboxykinase by *P. parvum* indicated that prey became

an important source of organic carbon. In contrast, *Dinobryon* did not increase PEP carboxykinase expression in the dark, and showed a decrease in vacuolar ATPase expression. This suggests that prey digestion is not active in the dark, and is also supported by the fact that *Dinobryon* prey ingestion dramatically decreased in the dark (Caron et al., 1993). An additional outcome of employing a comparative genomic approach in this study is the identification of 37 phagotrophy related genes that are shared, and expressed, between these two prasinophytes (Supplementary Information). These studies, and that of a mixotrophic oligotrich ciliate by Santoferrara et al. (2014), provide the basis for future multi-species comparative genomic studies of mixotrophic regulation at the genetic level. These efforts will yield greater understanding of whether the mechanisms regulating prey ingestion in mixotrophic protists are conserved across evolutionarily distinct groups.

The present work contributes to the limited, but growing, data set on the transcription dynamics of green algal lineages in contrasting nutrient regimes. We observed responses to reduction in nutrients consistent with predicted responses at the level of gene expression by photosynthetic marine microalgae. These results were obtained using RNA-Seq technology, and despite the presence of bacteria in these non-axenic conditions, we were able to obtain good quality transcriptomic data from target species using a sufficiently conservative bioinformatic analysis pipeline. In addition, we have identified genes in both organisms that are associated with phagotrophic activity, one of the main aims of the study, despite the fact that *M. polaris* and *P. tychothreta* are representatives of different phylogenetic Orders with a large difference in genome size. Additionally, several transcripts containing conserved domains associated

with phagotrophic ingestion were found in both transcriptomes, further supporting that these processes are operating within otherwise photosynthetic organisms.

AUTHOR CONTRIBUTIONS

RG conducted grazing experiments and RNA isolation for sequencing. RG and ZM-K conducted the bioinformatic analysis of RNA-seq data including, *de novo* assembly, functional annotation, and differential expression analysis. ZM-K was the primary writer of the manuscript. RG and RS provided critical revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00273/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.

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