Differential gene expression supports a resource-intensive, defensive role for colony production in the bloom-forming haptophyte, *Phaeocystis globosa*

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Abstract

Phaeocystis globosa forms dense, monospecific blooms in temperate, northern waters. Blooms are usually dominated by the colonial morphotype—non-flagellated cells embedded in a secreted mucilaginous mass. Colonial *Phaeocystis* blooms significantly affect food-web structure and function and negatively impact fisheries and aquaculture, but factors initiating colony production remain enigmatic. Destructive *P. globosa* blooms have been reported in tropical and subtropical regions more recently and warm-water blooms could become more common with continued climate change and coastal eutrophication. We therefore assessed genetic pathways associated with colony production by investigating differential gene expression between colonial and solitary cells in a warm-water *Phaeocystis globosa* strain. Our results illustrate a transcriptional shift in colonial cells with most of the differentially expressed genes downregulated, supporting a reallocation of resources associated with colony production. Dimethyl sulfide and acrylate production and pathogen interaction pathways were upregulated in colonial cells, suggesting a defensive role for colony production. We identify several protein kinase signaling pathways that may influence the transition between morphotypes, providing targets for future research into factors triggering colony production. This study provides novel insights into genetic mechanisms involved in *Phaeocystis* colony formation and provides new evidence supporting a defensive role for *Phaeocystis* colonies.

Keywords
Plankton; phytoplankton; DMS; DMSP; algal bloom; algae bloom; RNA-seq; transcriptome; transcriptomics; colonial morphotype

Introduction

Phaeocystis is a cosmopolitan bloom-forming haptophyte genus encompassing 6 species (Schoemann et al 2005, Andersen et al. 2015). Most *Phaeocystis* species (*P. globosa, P. antarctica, P. pouchetii*, and *P. jahnii*) exhibit a polymorphic life-cycle, alternating between colonial and free-living morphotypes. *Phaeocystis* blooms are usually dominated by the colonial morphotype and are typically very dense, produce large biomasses, and impact food-web structure and function (Schoemann et al. 2005). *Phaeocystis* is a major contributor to Dimethylsulfoniopropionate (DMSP) and dimethyl sulfide (DMS) production globally (Liss et

al. 1994) and regional peaks in DMS production are closely correlated with colonial *Phaeocystis* blooms (Van Duyl et al. 1998). DMS produced in the surface ocean is aerosolized and its oxidation products promote cloud formation, increase albedo, and affect global climate (Charlson et al. 1987). In algal cells, DMSP and its cleavage products, DMS and acrylate, contribute to osmotic balance, neutralize reactive oxygen species, and deter grazing (Sunda et al. 2002, Noordkamp et al. 2000). Despite the ecological importance of colony formation in *Phaeocystis*, triggers for transition to the colonial morphotype remain enigmatic, and the functional role of colony formation in the *Phaeocystis* life-cycle is not clearly delineated (Peperzak & Gabler-Schwarz 2012).

Myriad factors have been studied in regard to their roles in instigating colony formation in *Phaeocystis* species, including nutrient and light availability (Cariou et al. 1994, Wang et al. 2011, Bender et al. 2018), temperature (Verity and Medlin 2003), mechanical stress (Cariou et al. 1994), grazing cues (Tang 2003, Long et al. 2007, Wang et al. 2015), and viral infection (Brussaard et al. 2005, Brussaard et al. 2007). However, these studies used different *Phaeocystis* species, strains, and morphotypes with a range of experimental conditions, which yielded variable and sometimes contradictory results. Nonetheless, several lines of evidence suggest that colony formation serves a defensive role. First, while viruses can cause 30-100% cell lysis in solitary *Phaeocystis,* viruses rarely infect colonial cells, which lyse primarily due to nutrient limitation (Brussaard et al. 2005, 2007). Second, ciliates and other microzooplankton that graze solitary *Phaeocystis* are unable to graze on colonies (Tang et al. 2001) and chemical cues from these grazers induce colony formation and promote increased colony size (Tang 2003, Long et al. 2007). Third, acrylate, which is produced with DMS when DMSP is cleaved, accumulates within colonies and may further deter macro- and micro-grazers and heterotrophic bacteria (Noordkamp et al. 2000, Hamm et al. 2000). However, while cellular growth rate increases in colonial cells relative to solitary cells if colonies are induced in nutrient rich conditions, it decreases when colonies are induced under nutrient limiting conditions (Wang et al. 2015). Thus, colony formation can defend against pathogens and grazers, but it is costly (Wang et al. 2015), suggesting that colony formation is likely a complex response to interacting biotic and abiotic factors (Long et al. 2007).

Colony formation may also play a fundamental role in *Phaeocystis* reproduction. *Phaeocystis* has one of the most complex and polymorphic life cycles among phytoplankton genera, and despite extensive study, it remains largely unresolved in most species. Studies have implicated at least 6 different life stages and up to 15 functional components to the life-cycle (Gaebler-Schwarz et al. 2010). In *P. globosa,* four morphotypes are believed to exist: diploid colonial cells devoid of scales and flagella, diploid scale-free flagellates arising from mechanically disrupted colonies, and two types of small, scaled, haploid flagellates—those that produce vesicles containing star-shaped alpha-chitin filaments and those that do not (Rousseau et al. 2007). Haploid flagellates may fuse (syngamy) to produce diploid colony-forming cells, which in turn undergo meiosis and produce haploid flagellates (Rousseau et al. 2013). Haploid flagellates are often observed swarming inside colonies, suggesting that colonial bloom formation may contribute to successful sexual reproduction in *Phaeocystis* (Peperzak et al. 2000, Rousseau et al. 2013). However, neither syngamy nor meiosis have been directly observed in *Phaeocystis* spp. (Peperzak and Gabler-Schwarz 2012), even though both events have been documented in several other

haptophyte genera (Houdan et al. 2003). If colonial *Phaeocystis* blooms are necessary for sexual reproduction, it would further justify the resource costs associated with colony formation.

Historically, colonial *Phaeocystis* blooms have been restricted to cold, high-latitude waters—*P. globosa* blooms in the English Channel and North Sea, *P. pouchetii* in the North Atlantic and Arctic, and *P. antarctica* in the Southern Ocean (reviewed in Schoemann et al. 2005). In the last two decades, however, blooms have increasingly been reported in tropical and subtropical regions, including the subtropical N. Atlantic (Long et al. 2007) and the subtropical and tropical South China Sea (Chen et al. 2002, Doan-Nhu et al. 2010, Liu et al. 2015). Decaying colonial biomass sinks and produces anoxic conditions, making *Phaeocystis* blooms detrimental to benthic fisheries and aquaculture (Desroy & Denis 2008, Peperzak & Poelman 2008, Spilmont et al. 2009). In warmer waters, *Phaeocystis globosa* blooms have been especially catastrophic to local aquaculture (Chen et al. 2002, Doan-Nhu et al. 2010), possibly because the hemolytic activity of *P. globosa* liposaccharides increases with temperature (Peng et al. 2005). Global climate change and increasing nutrient pollution in coastal regions may mean harmful *Phaeocystis* blooms will continue to increase in range and frequency. Given the ecological impact of colonial *Phaeocystis* blooms and their complex and enigmatic initiating triggers, particularly in warm waters, it is imperative to better understand the regulation of colony formation.

Transcriptional approaches have become an exceptionally useful tool to illuminate physiological responses to environmental cues and genes associated with specific life-stages in algae and other protists (Caron et al. 2017). In this study, we investigated genetic regulation of colony formation by analyzing gene expression in colonial and flagellated morphotypes of a warm-water *Phaeocystis globosa* strain. Since *Phaeocystis* is an important marine producer of DMSP and DMS—and because these molecules may be associated with colonial defense, we queried our dataset for algal genes involved in DMSP production (*DSYB*, Curson et al. 2018) and its cleavage to DMS and acrylate (*Alma1*, Alcolombri et al. 2015). *DSYB* and *Alma1* are the only algal genes that encode proteins experimentally proven to catalyze DMSP, DMS, and acrylate production, but neither has been identified in *P. globosa* previously. Overall, our results demonstrate a dramatic transcriptional shift in colonial *P. globosa*, with the vast majority of differentially expressed genes downregulated in colonial cells. Such a strong transcriptional shift supports an allocation of resources toward colony formation and away from other cellular processes such as translation, cell growth, and cell division. Genes associated with DMSP production (*DSYB*-like) were not differentially expressed, but an *Alma* family-like gene was upregulated in colonies, suggesting colonies may produce more DMS and acrylate than do solitary cells. Results further indicate that pathogen interaction affects colony formation, further supporting a defensive role for colonies, and implicating several cell-signaling pathways as important to colony formation. This study provides new insights into the functional role of *Phaeocystis* colonies and physiological processes associated with colony formation. These insights will guide future investigations into triggers that initiate colony formation in harmful *Phaeocystis* blooms.

Materials and Methods:

Culture strain and maintenance

138
139 Starter cultures of *Phaeocystis globosa* CCMP1528, a warm-water, colony forming *P. globosa*
140 strain (Wang et al. 2011), were purchased from the National Center for Marine Algae and
141 Microbiota (NCMA, Maine, U.S.A.) in January 2016. CCMP1528 did not initially form colonies
142 in our culture conditions, but it is known that *P. globosa* sometimes stops forming colonies in
143 culture (Janse et al. 1996). We maintained non-colonial cultures in replicate 150 mL Erlenmeyer
144 flasks with 100 mL L1-Si media in ambient light and temperature on a gently rotating twist
145 mixer (TM-300, AS ONE, Osaka, Japan, speed setting 1). Culture media was prepared by
146 enriching 34 ppt autoclaved artificial seawater prepared from milliQ water and sea salts (Marine
147 Art SF-1) with the NCMA L1 media kit (-Si) and filtering through sterile 0.22 μm pore-size
148 filters. Cultures were diluted biweekly with freshly prepared media. When one culture replicate
149 began producing colonies in January 2017, experimental culture conditions were promptly
150 initiated.
151
152 ***Experimental culture conditions***
153
154 We prepared four biological replicates each of colony-forming and non-colonial *P. globosa*
155 CCMP1528 by inoculating 45 mL of sterile L1 media with 1 mL stock culture in 50 mL
156 Erlenmeyer flasks. Replicates were placed on a gently rotating twist mixer in a plant growth
157 chamber with cool white fluorescent lamps (CLE-305, TOMY, Tokyo, Japan) set to 22°C with
158 light level 4 and a 12:12 day:night ratio. A HOBO temperature and light logger (Onset, MA,
159 U.S.A.) was kept in the growth chamber during the experiment. The daytime temperature was
160 21°C with about 1900-2000 Lux (~30 μmol m^{-2} s^{-1}) light intensity, and the nighttime temperature
161 was 22°C. Positions of replicates were rotated daily to prevent position in the chamber from
162 systematically affecting replicates. On days 1, 3, and 4, chlorophyll fluorescence was measured
163 at the middle of the light period by transferring 200 μL aliquots to a black 96-well plate
164 (ThermoFisher, MA, U.S.A) and recording fluorescence (excitation: 440 nm, emission: 685 nm)
165 with a Tecan Ultra Evolution microplate reader (Tecan, Switzerland). Exponential growth phase
166 was determined by comparing measured fluorescence to a growth curve for *Phaeocystis globosa*
167 CCMP 1528 grown under identical conditions. On day 4 of the first round, 1 mL of each
168 replicate was transferred to 45 mL of sterile L1 media and the experimental setup was repeated,
169 which allowed for adaptation to experimental culture conditions. Algal cells were harvested for
170 RNA extraction on day 4 of the second experimental culture round, when replicates were in
171 middle to late exponential growth phase (Supplemental Figure S1).
172
173 Prior to RNA extraction, each culture replicate was imaged with light microscopy (Olympus
174 CKX53) to ensure that colony-forming replicates were indeed producing colonies and that non-
175 colonial replicates were not (Supplemental Figure S2). Flagellates present in the colonial and
176 non-colonial replicates were actively swimming, suggesting that flagellates in this study were
177 scaled haploid flagellates, rather than scale-free diploid flagellates originating from disrupted
178 colonies, but neither flow-cytometry nor electron microscopy were performed (Rousseau et al.
179 2007). Colony-forming culture replicates were filtered through polytetrafluoroethylene (PTFE)
180 filters (pore size, 10 μm) (Millipore) under gentle vacuum. Colonies were visible by eye on the
181 filter surface and swimming flagellates were observed in the flow-through when viewed with
182 light microscopy. The non-colonial culture replicates were first filtered through 50 μm nylon
183 mesh to remove culture debris, and then filtered through 1.0 μm pore-size PTFE filters. No

flagellates were visible when the filtrates were viewed with light microscopy. Filters were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA Extraction, Library Preparation and Sequencing

Total RNA was extracted from filters by following the manufacturer's protocols for the MoBio PowerWater RNA extraction kit (Qiagen), including the optional initial heating step. Following extraction, we assessed RNA quality and concentration. RNA extracts were diluted so that 10 ng of RNA were used for each sample with the SMART-seq v4 Ultra Low Input RNA Kit (Clonetech) along with 2 μL of a 1:10,000 dilution of External RNA Controls Consortium (ERCC) spike-in mix 1 (Ambion), an internal quality control. The SMART-seq kit employs poly-A priming to target eukaryotic mRNA and to reduce the amount of ribosomal and bacterial RNA present in sequencing libraries. The quality and concentration of the resulting cDNA was assessed before continuing with the manufacturer's protocols for the Nextera XT DNA Library Prep Kit (Illumina). Finally, we checked cDNA fragment size before submitting libraries to the Okinawa Institute of Science and Technology (OIST) DNA Sequencing Section for paired-end 150x150 bp sequencing across 8 lanes of an Illumina Hiseq4000 flow-cell.

Bioinformatic Processing and Quality Control

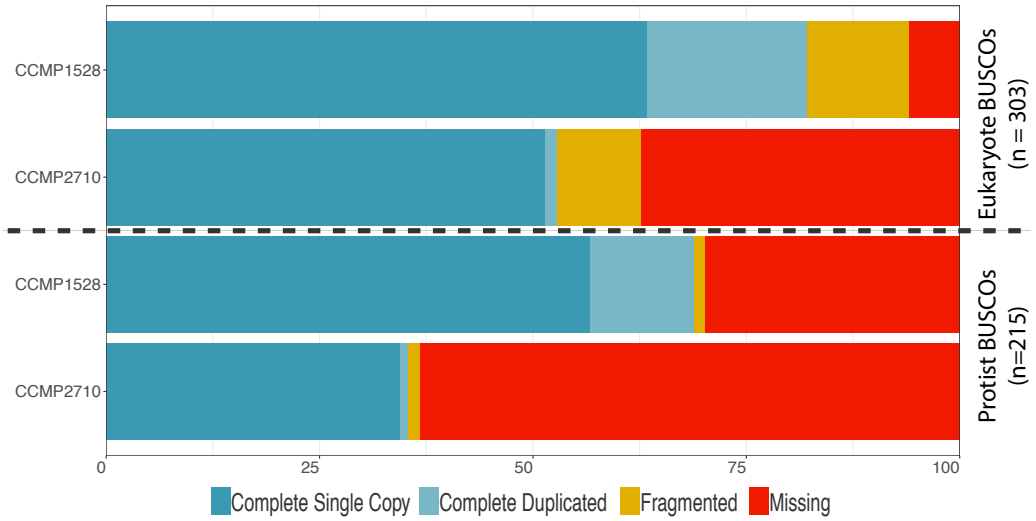
Sequencing reads were processed with Trimmomatic software to remove adapter sequences and to filter low-quality sequences (Bolger et al. 2014). Read quality was checked with FastQC before and after trimming to ensure that adapters were removed (Andrews 2010). Remaining reads were mapped to the ERCC reference sequences (Cronin et al. 2004) and mapped reads were counted with RSEM software (Li & Collin, 2011). Counts were further analyzed in the R statistical environment (R Core Team, 2013). Reads mapping to the ERCC reference sequences were then removed from each sample with SAMtools (Li et al. 2009) and BEDTools (Quinlan & Hall, 2010).

Transcriptome Assembly, Assessment, and Functional Annotation

The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP, Keeling et al. 2014) assembled a transcriptome for *Phaeocystis sp.* CCMP2710, which groups with the *Phaeocystis globosa* species complex in phylogenetic analyses (Supplemental Figure S3). Only 25% of our reads, however, mapped to this reference transcriptome. We therefore assembled a *de novo* transcriptome for *Phaeocystis globosa* CCMP1528 to serve as a reference for read mapping in this study. We used Trinity software for transcriptome assembly (Grabherr et al. 2013) and dereplicated the transcriptome by removing reads with 95% similarity using CD-HIT-EST (Fu et al. 2012). Bacterial contamination was removed by performing a blastn query against the NCBI nucleotide database (downloaded March 2018, ncbi-blast v2.6.0+, Camacho et al. 2009) and parsing results to identify and remove bacterial contigs. The final assembly was assessed for completeness with Benchmarking Universal Single-Copy Orthologs (BUSCO v3, Simao et al. 2015) and results were compared with those for the MMETSP *Phaeocystis sp.* CCMP2710 transcriptome.

229 We annotated the CCMP1528 transcriptome using two different databases, Pfam (Finn et al.
230 2010) and KEGG (Kanehisa et al. 2016). Pfam annotation was performed with the dammit
231 software (Scott 2018), which wraps Transdecoder to translate transcriptome contigs to the
232 longest possible amino acid sequence (Haas et al. 2013), and HMMER to assign protein
233 homologs to sequences (Eddy 2011). After discarding annotations with e-values greater than 1E-
234 5, the annotation with the lowest e-value was selected for each contig. Gene Ontology (GO)
235 terms were assigned to Pfam annotations using the Gene Ontology Consortium's Pfam2GO
236 mapping (geneontology.org/external2go/pfam2go, version 07/14/2018, Mitchell et al. 2015).
237 KEGG annotation was performed with the GhostKOALA tool and translated amino acid
238 sequences (kegg.jp/ghostkoala, 05/21/2018, Kanehisa et al. 2016). Annotated K numbers were
239 then used to assign KEGG pathways by accessing the KEGG API
240 (kegg.jp/kegg/rest/keggapi.html, July 2018).
241
242 ***Differential Gene Expression Analysis***
243
244 Quality filtered sequences from each sample were mapped to the assembled *P. globosa*
245 CCMP1528 transcriptome and counted with RSEM software. Counts for each sample were
246 imported into the R statistical environment, where differential gene expression between colonial
247 and solitary culture replicates was tested with the DESeq function in the Bioconductor package
248 DESeq2 (Love et al. 2014). Genes that were differentially expressed were considered statistically
249 significant if the False Discovery Rate (FDR) adjusted p-value (padj) was less than 0.05.
250
251 ***Gene Set Enrichment Testing***
252
253 We identified GO terms enriched among significantly upregulated and downregulated genes by
254 applying a hypergeometric test in the R package GOstats (Falcon and Gentleman 2007). GOstats
255 accommodates user-defined GO annotations, which are necessary when studying non-model
256 organisms like *Phaeocystis*. Likewise, a hypergeometric test for significant enrichment of KEGG
257 pathways was applied using the enricher function from the R package ClusterProfiler (Yu et al.
258 2012). Because of the lower annotation rate, KEGG pathway enrichment was further investigated
259 by additionally applying linear model analysis with the kegga function in the R package edgeR
260 (Robinson et al. 2010). GO terms and KEGG pathways were considered significantly enriched
261 when the statistical test returned a p-value less than 0.05.
262
263 ***Genes associated with DMSP and DMS production***
264
265 Because *Phaeocystis* is a profusive producer of DMSP and DMS, we specifically queried our
266 dataset for recently discovered algal genes involved in DMSP production (DSYB) and its
267 cleavage to DMS and acrylate (Alma family genes). We performed blastp queries with curated
268 DSYB protein sequences (provided by Curson et al. 2018) and *E. huxleyi* and *Symbiodinium*
269 Alma family protein sequences downloaded from UniProt (July 2018) against *Phaeocystis*
270 *globosa* CCMP1528 amino acid sequences. Expression levels of putative *Phaeocystis globosa*
271 *DSYB* and *Alma* family genes were then checked in colonial and solitary culture replicates.
272
273 **Results**
274

275 ***Bioinformatic Processing and Quality Control***
276
277 Sequencing for this project produced over 1.9 billion read pairs with 159-383 million read pairs
278 per sample. Following quality filtering with Trimmomatic, 1.7 billion read pairs remained, with
279 140-341 million read pairs per sample (Supplemental Table S1). After mapping reads from each
280 sample to ERCC reference sequences, we plotted the Log_2 FPKM for each sequence against the
281 Log_2 of its concentration in the standard mix. A simple linear regression was fitted for each
282 sample and R^2 values ranged from 0.93-0.937 for each sample (Supplementary Figure S4). The
283 strong correlation between observed FPKM and initial concentration for ERCC sequences
284 indicates that minimal bias was introduced during PCR amplification, library preparation, and
285 sequencing.
286
287 ***Transcriptome Assembly, Assessment, and Functional Annotation***
288
289 The final assembly of the *Phaeocystis globosa* CCMP1528 transcriptome included 69,528
290 contigs and a total of 43.9 Mbp. The CCMP1528 transcriptome was about 3 times larger than the
291 MMETSP CCMP2710 transcriptome, but the minimum, maximum, and mean contig lengths
292 were about the same for both (Supplemental Table S2). When Transrate was used to align the
293 two transcriptomes, only 18% of CCMP1528 contigs aligned to the CCMP2710 transcriptome,
294 but 55% of the CCMP2710 contigs aligned to the CCMP1528 transcriptome. BUSCO software
295 was utilized to assess completeness of the *P. globosa* CCMP1528 transcriptome. It included
296 more complete eukaryote and protist BUSCOs than the MMETSP *Phaeocystis sp.* CCMP2710
297 transcriptome (Figure 1). Together, these results demonstrate that the transcriptome generated in
298 this study is more complete than the MMETSP transcriptome and is a better reference for this
299 study. The results also indicate that CCMP2710 and CCMP1528 are more genetically distant
300 than was expected based upon ribosomal RNA gene sequences (Supplemental Figure S3).
301



302
303 **Figure 1. Percent of eukaryote and protist Benchmarking Universal Single Copy Orthologs (BUSCOs)**
304 **complete, fragmented, or missing in *Phaeocystis globosa* CCMP1528 and *Phaeocystis sp.* CCMP2710**
305 **transcriptomes.** BUSCO software was used to determine the percent of eukaryotic and protistan BUSCOs
306 represented by complete single copies, complete but duplicated copies, copies that were fragmented or missing in
307 the *Phaeocystis globosa* CCMP1528 transcriptome assembled for this study and the *Phaeocystis sp.* CCMP2710
308 transcriptome assembled for the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP). More

eukaryotic and protistan BUSCOs were represented in the *Phaeocystis globosa* CCMP1528 transcriptome than the *Phaeocystis sp.* CCMP2710 transcriptome. The plot was rendered with the R package ggplot2.

Annotation was possible for relatively few of the contigs in the *P. globosa* CCMP1528 transcriptome assembly, but more genes were annotated with the Pfam and GO annotation pipeline (26%) than with the KEGG pipeline (14%). Additionally, both annotation methods annotated the significantly differentially expressed (DE) genes at a higher rate than the whole transcriptome (Table 1).

	Pfam / GO	**KEGG**
Total genes annotated	17,826 (26%)	9,967 (14%)
Total w. pathway or GO	8,962 (13%)	5,585 (8%)
DE genes annotated	5,180 (66%)	3,305 (43%)
DE genes w. pathway or GO	2,764 (35%)	1,883 (24%)

Table 1. Pfam, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation statistics for the *Phaeocystis globosa* CCMP1528 transcriptome assembly and differentially expressed (DE) genes. Pfam annotations were assigned with dammit software and corresponding GO terms were determined with Pfam2GO mapping provided by the GO Consortium. KEGG K numbers were assigned with GhostKOALA tool and KEGG pathways were determined by accessing the KEGG API.

Differential Gene Expression Analysis

Gene expression patterns in colonial and solitary replicates were explored with a principal component analysis (PCA) and an expression heatmap. Initial data exploration revealed the colonial replicate 'C2' as an outlier to other colonial replicates and solitary replicates (Supplemental Figures S5A, B) and this sample was excluded from further analyses. The remaining 3 colonial replicates clustered separately from the 4 solitary replicates in a PCA plot (Figure 2A). Differential expression analysis identified 535 genes as significantly upregulated and 7,234 genes as significantly downregulated in colonial replicates. An expression heatmap of the most differentially expressed genes sorted by FDR adjusted p-value (padj) clearly illustrates the overall expression pattern—the majority of significantly differentially expressed genes are downregulated in colonial replicates (Figure 2B).

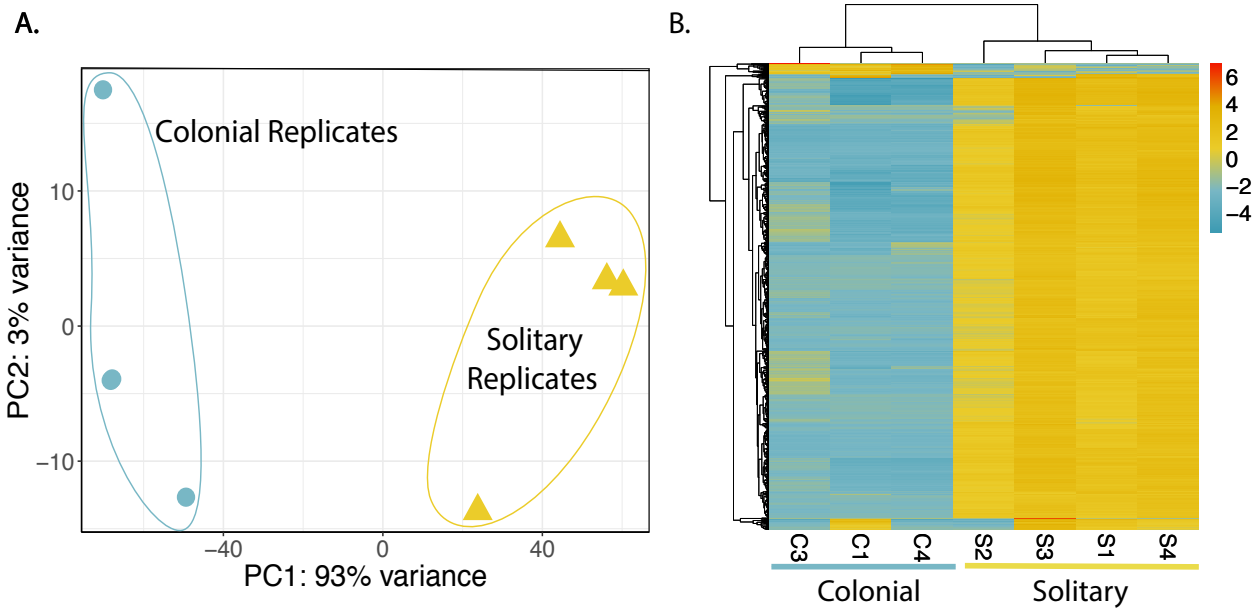


Figure 2. Principal component analysis (PCA) and heatmap demonstrating gene expression patterns in colonial and solitary *Phaeocystis globosa*. A. PCA performed on distances between samples derived from regularized log transformed counts. Colonial and solitary replicates cluster separately, and the majority of variance is between sample type rather than within replicates. Results plotted with R package ggplot2. **B.** Heatmap includes the 1000 significantly differentially expressed genes with the lowest FDR adjusted p-values. Heatmap color represents difference from the mean regularized log transformed count for each contig in each sample. The majority of differentially expressed genes are downregulated in colonial replicates, and replicates cluster by sample type. Results plotted with R package pheatmap.

Gene Set Enrichment Analysis

In order to identify Biological Process (BP) GO terms over-represented in significantly up- and downregulated gene sets, we applied a hypergeometric test with a significance cut-off of $p < 0.05$. Twenty BP GO term were over-represented among significantly upregulated genes and were primarily involved in cell signal transduction in response to external stimuli (Figure 3, Supplemental Table S3). Notably, GO terms involving arabinose, a component of the colonial matrix, were also enriched among upregulated genes. In the downregulated gene set, 48 BP GO terms were enriched, including several involved in cation transport, response to oxygen-containing compounds, translation and protein transport, and vacuolar transport and exocytosis (Figure 3, Supplemental Table S4). REVIGO software was used to remove redundant GO terms from lists of enriched terms and to visualize results in a Multidimensional Scaling (MDS) plot (Figure 3) based on GO term semantic similarities (Supek et al. 2011) as determined by shared ancestry (Pesquita et al. 2009).

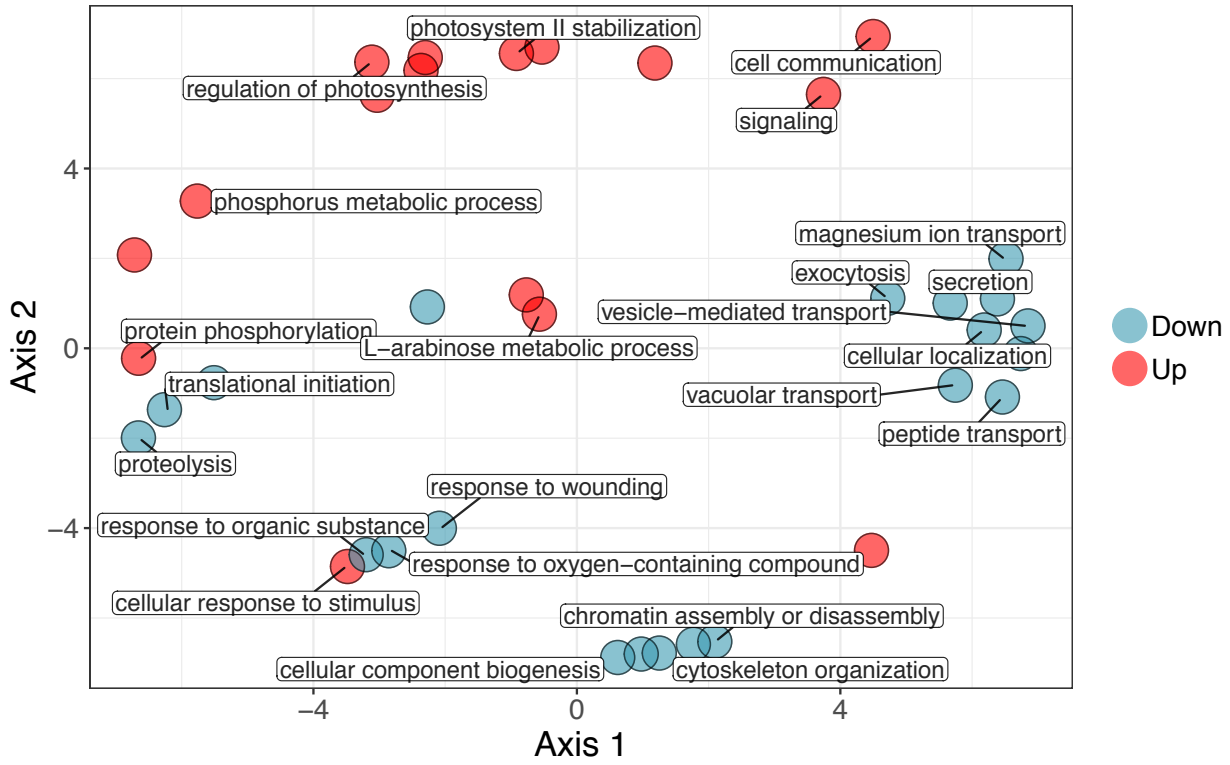


Figure 3. Multidimensional scaling plot of semantic similarities between non-redundant GO terms over-represented in significantly up- and downregulated gene sets. Analysis performed using the REVIGO tool (http://revigo.irb.hr/) with the allowed similarity set to 0.7 (to remove redundant GO terms) and the SimRel metric selected to calculate similarities. REVIGO results were exported to the R statistical environment and plotted with ggplot2. Representative GO terms were manually selected and labeled on the plot. To view all GO term labels, an interactive version of the plot made with R package ggplotly is available at: https://brisbin.shinyapps.io/shinycolsol/.

When we applied hypergeometric testing to KEGG pathways, only the cGMP-PKG signaling pathway (cyclic guanosine monophosphate-protein kinase G pathway) was enriched in the upregulated gene set ($p<0.05$) (Supplemental Table S5). Five pathways were enriched among downregulated genes: Lysosome, Autophagy, MAPK signaling pathway (mitogen activated protein kinase signaling pathway), AMPK signaling pathway (adenosine monophosphate-activated protein kinase signaling pathway), and Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor resistance (Supplemental Table S6). The lower annotation rate for KEGG pathways compared with GO terms contributed to the difference in enrichment testing results. We therefore also applied a linear model test for KEGG pathway enrichment, which identified several additional pathways as being significantly enriched in the up- (6) and downregulated (7) gene sets. With this additional test, the PI3K-Akt signaling (phosphoinositide 3-kinase-protein kinase B signaling pathway), Glycosphingolipid biosynthesis, Ferroptosis, Plant-pathogen interaction, Circadian rhythm, Viral carcinogenesis pathways were also enriched among upregulated genes (Supplemental Table S7). The Protein processing in endoplasmic reticulum, Oxidative phosphorylation, Ras signaling pathway, Sphingolipid metabolism, Steroid biosynthesis, Fatty acid degradation, and Taste transduction pathways were additionally identified as enriched in downregulated genes (Supplemental Table S8).

390
391 ***Genes associated with DMSP and DMS production***
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393 A blastp query against curated DSYB protein sequences from Curson et al. (2018) and Alma
394 family protein sequences from Alcolombri et al. (2015) identified 4 *Phaeocystis globosa* contigs
395 as putative *DSYB* or *Alma* family genes (Table 2). The *P. globosa* amino acid (AA) sequence
396 translated from Transcript_30752 aligned with the sequence for *Prymnesium parvum*
397 CCAP946/1B DSYB protein, which is experimentally proven to be highly active. It is therefore
398 likely that this gene is actively involved in DMSP biosynthesis in *Phaeocystis globosa*. A second
399 *P. globosa* AA sequence, from Transcript_31221, aligned with the *Pseudonitzchia fraudulenta*
400 DYSB protein sequence, making it also a possible *DSYB* gene. The *Pseudonitzchia* DSYB
401 protein has not been experimentally proven to be active, but its sequence is phylogenetically
402 close to the *Fragillariopsis* DSYB, which has been proven to be active. Neither putative *P.*
403 *globosa DSYB* genes were differentially expressed between solitary and colonial culture
404 replicates in this study, but both were expressed at relatively high levels in both sample types
405 (Figure 4).
406
407 Two *Alma* family-like genes were identified in the *Phaeocystis globosa* transcriptome. One *P.*
408 *globosa* AA sequence, from Transcript_36000, aligned with *Emiliania huxleyi* Alma7. All 4
409 *Alma* homologs identified from the MMETSP *Phaeocystis antarctica* transcriptome are
410 phylogenetically closest to the *E. huxleyi Alma7*, but *E. huxleyi* Alma7 has not been proven to
411 have DMSP-lyase activity. Another *P. globosa* AA sequence, from Transcript_68879, aligned
412 with *E. huxleyi* Alma4, which also has not been experimentally proven active. Both putative *P.*
413 *globosa Alma* family genes were expressed at lower rates than *DSYB*-like genes. The *P. globosa*
414 *Alma4*-like gene was not differentially expressed in solitary and colonial culture replicates in this
415 study (Figure 4). The *P. globosa Alma7*-like gene, however, was significantly upregulated in
416 colonial replicates (padj = 1.44E-12, logFC = 3.55), suggesting that DMSP biosynthesis is
417 occurring in both colonial and solitary cells, but colonial cells may be cleaving DMSP to DMS
418 and acrylate more actively than solitary cells.
419

	Database Sequence	%ID	Alignment Length (AAs)	Gaps	E-value	Bit Score
Transcript_30752	*Prymnesium parvum* CCAP946/1B DSYB	74	314	2	1.15E-170	472
Transcript_31221	*Pseudonitzschia fraudulenta* WWA7 DSYB	45	263	8	1.92E-65	210
Transcript_36000	*Emiliania huxleyi* Alma7	61	272	4	3.70E-119	336
Transcript_68879	*Emiliania huxleyi* Alma4	40	253	4	2.64E-48	166

420 **Table 2. Top *Phaeocystis globosa* CCMP1528 blastp results against *DSYB* and *Alma* family reference**
421 **sequences.** The *Phaeocystis globosa* CCMP1528 transcriptome assembly was queried against curated DSYB
422 reference sequences (Curson et al. 2018) and Alma family reference sequences from *Emiliania huxleyi* and
423 *Symbiodinium* (Alcolombri et al. 2015). Blastp hits with e-values < 1E-30 are included in the table.

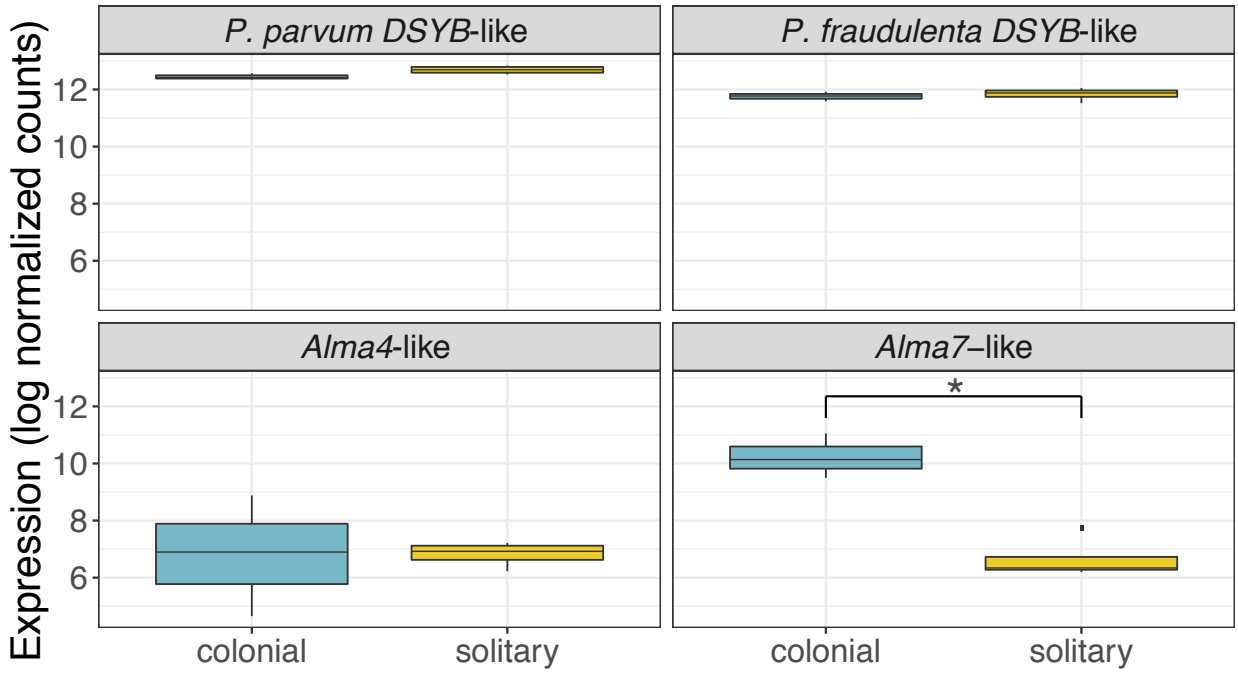

Figure 4. Normalized expression levels of *DSYB*-like and *Alma*-like genes in *Phaeocystis globosa* colonial and solitary cell cultures. Box plots show the range, quartiles and median of the log normalized counts for each gene in colonial and solitary culture replicates. Only the *E. huxleyi Alma7*-like gene was significantly differentially expressed and was upregulated in colonial samples (padj = 1.44E-12, log fold-change = 3.55) and is represented with an asterisk (*) on the plot. Plots made with R package ggplot2.

Discussion

Colonial *Phaeocystis* blooms widely impact ecosystem function and can be extremely detrimental in some systems, particularly in subtropical and tropical coastal regions. Although many hypotheses exist, factors initiating colonial blooms and the ecological function of colonial formation remain enigmatic. We investigated gene expression associated with colony formation in a warm-water colony-forming strain of *Phaeocystis globosa* to identify cellular processes associated with colony formation and potentially provide clues for what initiates colony formation and the functional role of colonies in the *Phaeocystis* life-cycle. Overall, we observed a transcriptional shift in colonial cultures compared to solitary cell cultures, with vastly more genes significantly downregulated in colonial cells than upregulated (Figure 2). This shift suggests that there are trade-offs associated with colony production and resources must be diverted to construct and maintain the colonial matrix. A relatively small number of genes are upregulated to produce colonies, but the low annotation rate of these genes, and the transcriptome overall, make it challenging to fully interpret the results (Table 1). Gene set enrichment analyses inherently relies on how many and which genes are annotated and systematic biases likely in gene annotation will influence results (Haynes et al. 2018). However, these analyses still assist in identifying pathways and functions that may be important to the question at hand and indicate genes and pathways that should be followed up in future studies. The results presented here highlight genes involved in constructing the colonial matrix, changes

451 in cellular morphology, responding to external stimuli, cellular proliferation, and producing
452 DMSP, DMS, and acrylate. Results from this study support a defensive role for colony formation
453 in *Phaeocystis globosa*.
454
455 ***Colony matrix carbohydrates and colonial cell morphology***
456
457 Differential expression of genes associated with clearly observable changes between treatment
458 groups can serve to "ground-truth" results from RNA-seq experiments and therefore increase
459 confidence in expression changes detected for genes for less observable traits. In this study,
460 changes in cellular morphology and colony formation itself are clearly observable differences for
461 which several associated genes are differentially expressed. The observed expression patterns for
462 these genes can additionally provide new insight into the construction of the colonial matrix and
463 pathways associated with morphological changes in colonial *Phaeocystis* cells. Many different
464 polysaccharides are recognized as contributors to the matrix structure of *Phaeocystis globosa*
465 colonies, including arabinose, rhamnose, xylose, mannose, galactose, glucose, gluconuronate,
466 and O-methylated pentose sugars (Janse et al. 1996). *Phaeocystis* isolated from different
467 locations tends to have distinct matrix carbohydrate fingerprints, which may be due to genetic
468 attributes of different strains or which could arise from different environmental conditions, such
469 as light or nutrient availability. For example, arabinose is the most abundant matric carbohydrate
470 in *P. globosa* sampled from the North Sea (Janse et al. 1996). In this study, the GO term for
471 Arabinose metabolic process was enriched among upregulated genes in colonial cells (Figure 3).
472 These results indicate that arabinose is likely the dominant matrix polysaccharide in *P. globosa*
473 CCMP1528 and that arabinose production is specifically associated with colony formation in this
474 strain. The colonial matrix also contains nitrogen (Hamm 2000), which is likely included in
475 amino sugars (Solomon et al. 2003). Our results, however, did not indicate that amino sugar
476 biosynthesis or metabolism was upregulated in colonial cells.
477
478 Divalent cations, particularly Mg^{2+} or Ca^{2+}, are required for colonial polymers to gel and
479 contribute to the stability of the colonial matrix (van Boekel 1992). GO terms for Divalent
480 Inorganic cation transport, Magnesium ion transport, and Divalent metal ion transport, however
481 were enriched in downregulated genes in colonial replicates (Figure 3). Similarly, Bender et al.
482 (2018) found that *Phaeocystis antarctica* produces more calcium-binding proteins when iron
483 limitation decreases colony formation. These results may be due to the importance of divalent
484 cations for flagellate motility. Actively swimming *Phaeocystis globosa* flagellates, as observed
485 in this study, may require continuous transport of divalent cations to the point of masking their
486 shared importance in colony formation. Calcium signaling also induces secretion of vesicles
487 containing gel forming polymers (Chin et al. 2004), which further confounds the observed
488 downregulation of divalent cation transport genes in colonies. However, flagellates also secrete
489 vesicles, but instead of gel polymers they contain star-shaped structures composed of chitinous
490 filaments (Chretiennot-Dinet et al. 1997). The exact function of these structures is unknown, but
491 they may be involved in mating or defense (Dutz and Koski 2006). In addition to divalent cation
492 transport, a number of other GO-terms enriched in downregulated genes may be involved in
493 secreting these structures, such as Exocytosis, Secretion, Vesicle mediated transport, and
494 Vacuolar transport (Figure 3). Alternatively, these GO terms may be involved in scale formation
495 and secretion (Taylor et al. 2007), as scales are only observed on *Phaeocystis globosa* flagellates
496 and not colonial cells (Rousseau et al. 2007).

497
498 ***A defensive role for colony formation: resource allocation, pathogen interaction, and***
499 ***DMS/acrylate production***
500
501 Out of 7,769 genes that were significantly differentially expressed between colonial and solitary
502 replicates, 7,234 genes were downregulated in colonial cells. This dramatic transcriptional shift
503 in colonial cells supports a high resource cost associated with producing colonies (Wang et al.
504 2015). Specifically, our results indicate that resources are being diverted from protein translation
505 and transport and cell division in order to produce the colonial matrix. Several GO terms
506 involved in the synthesis of larger nitrogenous compounds and their transport, including
507 Translation initiation, Protein metabolic process, Protein N-linked glycosylation, and Protein
508 transport were significantly enriched in downregulated genes in colonial cells (Figure 3,
509 Supplemental Table S4). Similarly, the KEGG pathway, Protein processing in endoplasmic
510 reticulum, was also enriched in downregulated genes in colonies (Supplemental Table S7).
511 Likewise, several mitosis-associated GO terms (Chromatin assembly and disassembly,
512 Cytoskeleton organization, Cellular component biogenesis) were also enriched among
513 downregulated genes in colonial cells (Figure 3, Supplemental Table S4). However, the
514 downregulation of mitosis-associated genes in colonial cells conflicts with observations in
515 previous studies. Veldhius et al. (2005) observed that colonial cells divide at a higher rate than
516 solitary cells and proposed that in addition to experiencing less grazing and viral lysis, colonial
517 cells may dominate blooms because they outgrow solitary cells. We believe the difference in our
518 results may be due to the type of solitary cells observed—the solitary cells in previous studies
519 could have been diploid flagellates, especially if they were derived from disrupted colonies,
520 whereas solitary cells in our study are likely haploid flagellates, which have been reported to
521 divide extremely rapidly (Rousseau et al. 2007).
522
523 There were also several signaling pathways represented in the results suggesting that colonial
524 cells are exposed to fewer general stressors, but may be responding to more strongly to specific
525 pathogens. In plants, the MAPK (mitogen activated protein kinase) pathway primarily transduces
526 signals from extracellular stressors to the nucleus or cytoplasm and initiates an appropriate
527 response (Taj et al. 2010). In our results, the MAPK signaling pathway was significantly
528 enriched in genes downregulated in colonies. The downregulated genes in this pathway encode
529 MAP3Ks, MAP2Ks, and MAPKs, which are activated in response to pathogen attack and
530 infection, phytohormones, cold and salt stress, and reactive oxygen species (Taj et al. 2010).
531 Downregulation of genes associated with stress response is also evidenced by related GO terms
532 enriched among downregulated genes, specifically Response to wounding and Response to
533 oxygen-containing compounds. These results support the hypothesis that colony formation
534 serves a defensive purpose. Defense responses regulated by the MAPK pathway are unneeded
535 because the colony skin is protecting cells from these stressors. However, the Plant-pathogen
536 interaction pathway was enriched in upregulated genes in colonial cells, indicating that specific
537 pathogens may penetrate the colonial fortress or that pathogen interaction may play a role in
538 stimulating colony formation. Genes upregulated in this pathway were for calcium-dependent
539 protein kinases and calcium-binding protein CML (calmodulin-like protein), immune response
540 genes that are activated following recognition of specific pathogen-associated molecular patterns
541 (Cheval et al. 2013). Specific bacterial interactions are known to influence transitions between
542 life-cycle stages in several other protists: specific bacteria stimulate growth in marine diatoms

543 (Amin et al. 2015) and specific bacterial signaling molecules are responsible for inducing both
544 colony formation (Woznica et al. 2016) and sexual reproduction (Woznica et al. 2017) in
545 choanoflagellates, another single-celled colony-forming marine plankton. In *Phaeocystis*, axenic
546 cultures exhibit decreased growth rates (Solomon et al. 2003), but the effects of specific bacteria
547 on colony formation have not yet been investigated.
548
549 Phaeocystis is a copious producer of DMSP and its cleavage products, DMS and acrylate. DMS
550 and acrylate have been indicated as grazer-deterrents and antimicrobials (Wolfe and Steinke
551 1996, Hamm 2000, Noordkamp et al. 2000). While algal genes associated with DMSP
552 biosynthesis (*DSYB*, Curson et al. 2018) and cleavage (*Alma* family genes, Alcolombri et al.
553 2015) have been identified in many algal transcriptomes, including *Phaeocystis antarctica*, this
554 study is the first to identify these genes for *Phaeocystis globosa*. We found that colonial and
555 solitary *P. globosa* expressed *DSYB*-like genes at the similar levels, suggesting that the two cell
556 types produce similar amounts of DMSP. *DSYB* expression in *Prymnesium parvum*, the
557 haptophyte in which DSYB was discovered, is affected only by salinity, potentially indicating
558 that DMSP production functions primarily in osmoregulation rather than as a defensive or stress
559 response (Curson et al. 2018). Similar *DSYB*-like gene expression levels in colonial and solitary
560 *P. globosa* cells support a basic, shared function for DMSP in the two cell types. Contrastingly,
561 an *Alma* family gene was upregulated in colonial cells. Acrylate accumulates in *Phaeocystis*
562 colonies and may serve to deter grazers and pathogens from disrupting the colonial matrix
563 (Noordkamp et al. 2000). While acrylate may accumulate in colonies simply because it cannot
564 escape through the colonial skin, the upregulation of an *Alma*-like gene in colonies suggests that
565 colonial *Phaeocystis* cells may actively produce excess acrylate and DMS than solitary cells,
566 further supporting a defensive role for colony production in *P. globosa*.
567
568 ***Role of colonies in* Phaeocystis *reproduction***
569
570 Colony formation is believed to be involved in sexual reproduction in *Phaeocystis* since
571 swarming flagellates have been observed within senescent colonies (Peperzak et al. 2000,
572 Rousseau et al. 2013). However, we did not find meiosis or sexual reproduction GO terms or
573 KEGG pathways enriched in up- (or down-) regulated genes in this study. These results may
574 arise from RNA being extracted during mid- to late exponential growth phase. Previous
575 observations suggest that colonies produce flagellates during bloom decay, so we might have
576 found meiosis genes upregulated in colonial cells if we had sampled toward late stationary phase
577 instead of exponential phase.
578
579 ***Signaling pathways associated with colony formation***
580
581 Processes and pathways involved in cell-signaling, cell communication, and response to stimuli
582 that are enriched in upregulated-genes are particularly interesting because they shed some light
583 on factors stimulating colony formation in *Phaeocystis globosa*. The cGMP-PKG signaling
584 pathway was the only KEGG pathway significantly enriched among upregulated genes when a
585 hypergeometric test was used. Three genes in this pathway were upregulated: 1) cGMP-
586 dependent protein kinase, which phosphorylates biologically important targets, has been
587 implicated in cell division and nucleic acid synthesis, and reduces cytoplasmic Ca^{2+}
588 concentrations (Lincoln et al. 2001); 2) cAMP-dependent protein kinase regulator; and 3) a

589 cAMP-responsive element-binding protein (CREB), which binds to DNA to increase or decrease
590 transcription and is associated with increased cell survival (Chrivia et al. 1993). The PI3K-Akt
591 signaling pathway was also significantly enriched in upregulated genes when the additional
592 linear model test was used. Within this pathway, two Extracellular Matrix (ECM) focal adhesion
593 genes, for Tenascin (a glycoprotein) and Type IV collagen, were significantly upregulated. Focal
594 adhesion proteins connect cells to extracellular matrices both literally and figuratively, by
595 holding cells in place and by initiating cellular responses to external conditions (Wozniak et al.
596 2004). Bender et al. (2018) also found focal adhesion proteins, specifically glycoproteins,
597 upregulated in colonial *Phaeocystis antarctica*. It is therefore likely that these proteins have an
598 important function in structurally maintaining cell positions in the colonial matrix and signaling
599 between colonial cells. Focal adhesion proteins may be mediating interactions with protein
600 kinases in colonial cells, which go on to promote cell proliferation and differentiation into the
601 colonial morphotype. These signaling pathways represent important candidates for continued
602 study of molecular mechanisms regulating colony formation.
603
604 ***Conclusions and Future Directions***
605
606 This study investigated gene expression associated with colony formation in *Phaeocystis globosa*
607 for the first time and discovered a large transcriptional shift associated with colony production.
608 Differentially expressed genes were mostly downregulated in colonies, providing evidence for
609 extensive resource allocation toward colony formation. Together, activation of pathogen
610 interaction pathways, reduced expression of stress-response pathways, and increased expression
611 of a DMSP-lyase, which produces DMS and acrylate, supporting a defensive role for colony
612 formation. Future studies may extend this work by investigating *P. globosa* gene expression in
613 colonial and solitary cells in a time course study through the waxing and waning of a bloom and
614 under different nutrient and grazing regimes, potentially by using mesocosms or
615 metatranscriptomic methods in natural communities. While our ability to fully interpret the
616 results was inhibited by an overall lack of annotated genomes and transcriptomes for diverse
617 protist lineages, this study represents a step in the right direction by contributing a new and
618 deeply sequenced transcriptome for *Phaeocystis globosa*. Identification of *DSYB* and *Alma*
619 family-like genes in this transcriptome will additionally allow for further investigation into *P.*
620 *globosa* DMSP and DMS production in the oceans. We were also able to identify several protein
621 kinase signaling pathways that are potentially important for regulating colony formation and
622 should be experimentally investigated in follow-up studies. The results presented here will guide
623 and facilitate continued efforts to unravel the complex factors responsible for triggering harmful
624 colonial *Phaeocystis* blooms, which will likely increase with continued climate change and
625 nutrient pollution in the future.
626
627 **Data Availability**
628
629 Sequence data is available in the NCBI Sequence Read Archive (SRA) with accession numbers
630 SRR7811979–SRR7811986. The *Phaeocystis globosa* CCMP1528 *de novo* transcriptome
631 produced and used in this study, data files, and data analysis scripts can be accessed at
632 https://doi.org/10.5281/zenodo.147649.
633
634 **Acknowledgements**

This work was supported by funding from the Marine Biophysics Unit of the Okinawa Institute of Science and Technology Graduate University. MMB is supported by a JSPS DC1 graduate student fellowship. We thank Angela Ares Pita for valuable discussion and suggestions and for helpful feedback on manuscript drafts. Hiroki Goto and the OIST sequencing section provided important guidance for RNA library preparation and other sequencing considerations. Steven D. Aird edited the manuscript and provided helpful comments.

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