

ORIGINAL ARTICLE

Molecular insights into a dinoflagellate bloom

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In coastal waters worldwide, an increase in frequency and intensity of algal blooms has been attributed to eutrophication, with further increases predicted because of climate change. Yet, the cellular-level changes that occur in blooming algae remain largely unknown. Comparative metatranscriptomics was used to investigate the underlying molecular mechanisms associated with a dinoflagellate bloom in a eutrophied estuary. Here we show that under bloom conditions, there is increased expression of metabolic pathways indicative of rapidly growing cells, including energy production, carbon metabolism, transporters and synthesis of cellular membrane components. In addition, there is a prominence of highly expressed genes involved in the synthesis of membrane-associated molecules, including those for the production of glycosaminoglycans (GAGs), which may serve roles in nutrient acquisition and/or cell surface adhesion. Biotin and thiamine synthesis genes also increased expression along with several cobalamin biosynthesis-associated genes, suggesting processing of B₁₂ intermediates by dinoflagellates. The patterns in gene expression observed are consistent with bloom-forming dinoflagellates eliciting a cellular response to elevated nutrient demands and to promote interactions with their surrounding bacterial consortia, possibly in an effort to cultivate for enhancement of vitamin and nutrient exchanges and/or direct consumption. Our findings provide potential molecular targets for bloom characterization and management efforts.
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Introduction

Algal blooms have become a major nuisance in many estuarine and coastal marine systems worldwide (Hallegraeff, 1992; Anderson *et al.*, 2008). Toxins produced by some harmful algal bloom species may be detrimental to fish, birds, marine mammals and even humans (Anderson *et al.*, 2008). Just as important, blooms of nontoxic microalgae that result in bottom-water hypoxia are detrimental and potentially fatal to sessile macrofauna. Efforts made to manage nutrient inputs to estuaries and coastal waterways have achieved a limited degree of success in reducing bloom outbreaks. Most estuaries are under the influence of both nutrient-rich river runoff and climatic perturbations where sources of pollutants continue to steadily increase with growth in agriculture, industry and urbanization (Paerl, 2006). In addition to these sources, increased frequency and intensity of storms as a result of climate change have reduced the efficacy of both non-point and point-source nutrient reduction programs (Paerl, 2006).

The abundance and distribution of phytoplankton in shallow estuarine systems are greatly affected by physical, chemical and biological factors (Sellner *et al.*, 2003). Optimal temperature, salinity and light conditions may vary among bloom-forming algal species (Smayda, 1997; Litaker *et al.*, 2002). Similarly, nutrient supply rates, concentrations and ratios can play significant roles in algal bloom dynamics in coastal waters (Piehler *et al.*, 2004; Paerl, 2006). Heterotrophic bacteria may also facilitate the growth of phytoplankton through remineralization of nutrients and biosynthesis of essential growth factors such as vitamins (Cole, 1982). Some phytoplankton produce polysaccharides that render them 'sticky', especially under nutrient-limited conditions. This promotes bacterial–phytoplankton interactions by trapping bacteria close to their cell surface (Kiørboe *et al.*, 1990; Mykkestad, 1999). Many phytoplankton also have the ability to switch trophic modes of energy acquisition by transitioning from autotrophy to heterotrophy (Smayda, 1997). This phenomenon, known as mixotrophy, is common among certain groups of phytoplankton, especially the dinoflagellates.

A complicating factor to deciphering the triggers of algal blooms is our rather limited capacity to predict when and where they will occur, and a lack of knowledge of the underlying mechanisms involved in the promotion and propagation of algal blooms impedes our ability to formulate preventative

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measures. Also, recent realization of there being a high degree of inter- and intraspecific species interactions during rapidly changing bloom micro-environments contributes to the difficulty in identifying the physiological processes involved in bloom formation and longevity (Cole, 1982; Amin *et al.*, 2015; Lima-mendez *et al.*, 2015; Zhuang *et al.*, 2015). Primary methods for examining phytoplankton dynamics within water quality monitoring programs typically have used bulk plankton community analyses (for example, chlorophyll *a*, primary productivity, nutrient uptake rates, etc.). Sequencing of mRNA transcripts from the environment and subsequent analysis of gene expression patterns (commonly referred to as metatranscriptomics) has been shown to be a powerful tool for identifying the distinct cellular-level processes conducted by bloom-forming taxa within the background of all metabolic activities performed within a mixed plankton assemblage (Cooper *et al.*, 2014). Recent applications of metatranscriptomic approaches have provided new insights into microalgal processes in natural marine environments and include characterization of the phytoplankton response to iron enrichment (Marchetti *et al.*, 2012) and resource partitioning among distinct phytoplankton species in nutrient-rich coastal environments (Alexander *et al.*, 2015).

Here we present a comparative metatranscriptomic analysis of a dinoflagellate bloom that was opportunistically sampled and characterized in the Neuse River Estuary (NRE), North Carolina in the early fall of 2012. Sequences in combination with environmental data collected before, during and following the bloom event are used to examine the conditions that may have triggered the bloom and the organismal responses at the molecular level. Transcripts obtained from eukaryotic plankton within the bloom were compared with those from surrounding non-bloom environments. Normalization techniques provided us the ability to distinguish between potential shifts in taxonomic cell abundance and gene expression patterns. We focus on the changes in genes grouped in metabolic modules that were assigned to dinoflagellates as a means to elucidate the cellular-level shifts occurring during the bloom.

Materials and methods

Study area and sampling collection

Sampling for metatranscriptomic sequencing took place in 2012 in conjunction with the NRE Modeling and Monitoring (ModMon) Program (<http://www.unc.edu/ims/neuse/modmon>); we sampled on five occasions throughout the year at four Modmon stations: 20, 70, 120 and 180 (Figure 1a), resulting in 20 routine metatranscriptomic samples. On 24 September 2012, the ModMon biweekly sampling survey reported a significant algal bloom near station

30, associated with bottom-water hypoxia and fish kills (Supplementary Figure S1). On 26 September 2012, metatranscriptomic sampling of a dinoflagellate bloom located near station 50 along with two non-bloom surrounding stations (stations 20 and 70) was conducted between 0930 and noon. In conjunction with metatranscriptomic sampling, physical and chemical parameters obtained from vertical YSI (Yellow Springs Instrument) profiles and Lugol's preserved samples from the surface waters were also collected at each station (see Supplementary File 1). In this study, our primary analysis is focused on samples collected during this bloom event.

Metatranscriptomic sample preparation and sequencing

RNA extraction and polyadenosine tail (poly(A)⁺) selection were conducted as described in Supplementary File 1. Briefly, mRNA samples from multiple filters collected at each station were extracted and then pooled to achieve a minimum mRNA concentration of 80 ng and provided to the UNC-CH High-throughput Sequencing Facility for Illumina sequence library synthesis using the TruSeq mRNA Library Preparation Kit (San Diego, CA, USA). mRNA from each of the stations collected during the bloom period was bar-coded and pooled. The combined sample was then sequenced on one lane using the Illumina HiSeq2000 platform (San Diego, CA, USA), generating between 56 million and 91 million pairs 100 bp paired-end reads per sample.

Differential expression analysis

Reads from each sample were assembled and functionally and taxonomically annotated. Details on assembly and annotation procedures are provided in Supplementary File 1. Differential expression of dinoflagellate-associated genes from the bloom versus the two non-bloom samples was performed on all genes that were annotated as belonging to dinoflagellates based on sequence similarity. Taxonomic grouping of genes was performed to the class level for expression analysis because of the scarcity of genomic or transcriptomic sequences for the blooming genus and incomplete transcriptomes for other closely related dinoflagellate genera (for example, *Gyrodinium* and *Gymnodinium*). Genes were examined at the KEGG Orthology (KO) level and reads for each KO were normalized for differences in dinoflagellate-specific transcript abundance among sequence libraries using the 'TMM' normalization procedure in the edgeR Bioconductor package, which normalize reads by computing a scaling factor after excluding genes that have high average counts and/or have large expression differences between samples with the assumption that most of the genes are not differentially expressed (Robinson and Oshlack, 2010). edgeR was then used to detect differentially expressed KOs between bloom and

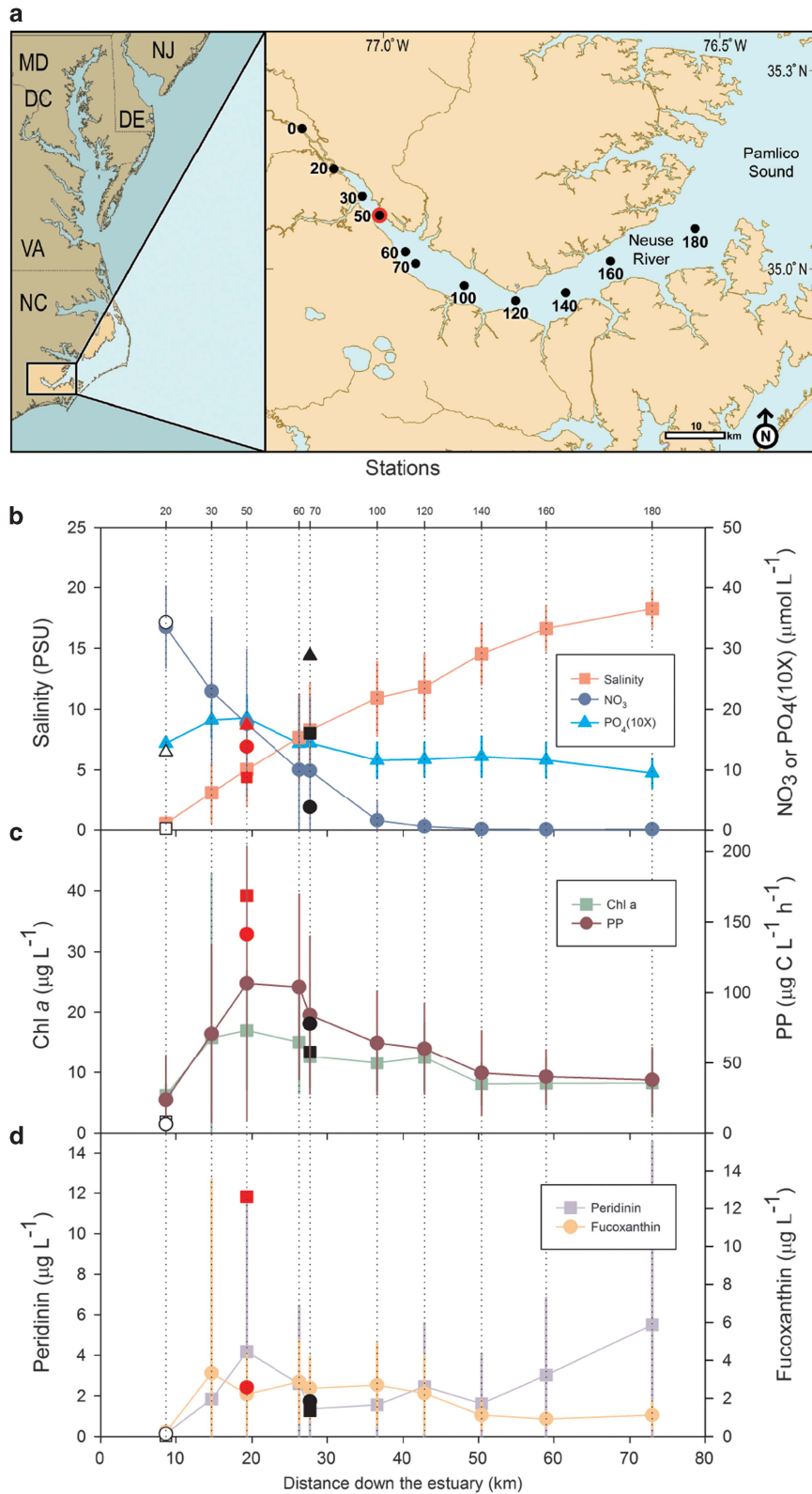


Figure 1 Map of the Neuse River estuary and water quality parameters measured in 2012. (a) Location of ModMon stations (20–180) in the NRE. The bloom station is indicated with a red circle. (b) Salinity, phosphate (PO_4) and nitrate (NO_3^-) concentrations, (c) chlorophyll a (Chl a) concentrations and primary productivity (PP) and (d) primary photopigment concentrations of dinoflagellates (peridinin) and diatoms (fucoxanthin). Plotted are the annual averages and associated standard deviations of surface samples. Also provided are measurements obtained during the dinoflagellate bloom event in September of 2012 where the bloom station (red symbols) and the non-bloom stations (white and black symbols) are indicated.

non-bloom samples as described in Marchetti *et al.* (2012), and significance of differentially expressed KOs were assigned with the 'exactTest' program in edgeR. Normalized reads for each KO were then grouped into modules using their KO-associated 'Module ID'. To measure whether each module is significantly enriched with over- or underrepresented KOs between the bloom sample and each of the non-bloom samples, a Mann–Whitney *U*-test was conducted for each module (Nielsen *et al.*, 2005). Fold change, counts per million (c.p.m.) and *P*-values for each module are provided in Data set 2.

Results and discussion

Bloom environmental conditions

The NRE is a tributary of North Carolina's Albemarle-Pamlico Estuarine System, the USA's largest lagoonal estuarine system (Figure 1a). The NRE is a partially mixed estuary, with riverine runoff and wind as the primary drivers of circulation. Strong vertical stratification is especially common in summer when the mild, cross-channel, southwest winds prevail (Luettich *et al.*, 2000). Over the past several decades, increasing agricultural and industrial activities along with coastal urbanization have accelerated nutrient inputs to the NRE (Paerl and Whitall, 1999; Whitall and Paerl, 2001). Combined with a long residence time and strong seasonal stratification, the NRE suffers from eutrophication (Paerl, 2006). Under the influence of both anthropogenic and climatic activities, algal blooms occur frequently (Paerl *et al.*, 1998, 2004).

During 2012, mean annual salinity along the estuary increased gradually from close to 0 at the head of the NRE (station 20) to about 18 at the mouth (station 180; Figure 1b). Phosphate concentrations were relatively consistent throughout the estuary, whereas nitrate concentrations were more variable, but, in general, were high at the head of the estuary and decreased down-estuary from an annual mean of $34 \mu\text{mol l}^{-1}$ at station 20 to commonly being below the detection limit by station 120. High variability in nitrate along the transect, particularly at the mid-estuary stations, is primarily due to fluctuations in river flow (Paerl *et al.*, 2010). Chlorophyll *a* concentrations were consistently lower at station 20 and peaked at mid-estuary stations with an annual average of $25 \mu\text{g l}^{-1}$ before steadily declining down-estuary (Figure 1c). The low biomass at the head of the NRE likely results from short residence times in combination with the 'geochemical filter', where flocculation and aggregation of clay minerals and metal ions increase the turbidity within the water column (Sharp *et al.*, 1984; Peierls *et al.*, 2012). Thus, in combination with rapid flushing rates, light as well as nutrient supplies are likely limiting factors for phytoplankton growth in this section of the NRE (Peierls *et al.*, 2012). Down-estuary of station 30, the NRE widens, resulting in reduced flushing rates, a decrease in turbidity and subsequent increase in

primary production and phytoplankton biomass, which results in a rapid depletion of nutrients from the upper water column (Pinckney *et al.*, 1997). At station 70 onward, low N supplies in surface waters tend to limit primary productivity, resulting in reduced phytoplankton biomass (Twomey *et al.*, 2005).

During the bloom event, there were significant increases in chlorophyll *a* (Student's *t*-test, *P*-value < 0.001) at the bloom station relative to surrounding stations and the annual mean values (Figure 1c). Primary productivity was also slightly higher at the bloom station compared with the annual average, although not statistically significant (Student's *t*-test, *P*-value = 0.12). Concentrations of accessory pigments were used to distinguish the presence of different phytoplankton groups (Pinckney *et al.*, 2001; Peierls *et al.*, 2003). Among the accessory pigments measured, peridinin and fucoxanthin were used as proxies of dinoflagellate and diatom relative abundances, respectively. The peridinin concentration was significantly higher at the bloom station compared with other accessory pigments (Student's *t*-test, *P*-value < 0.001), indicating the blooming algae were primarily that of a single or multiple dinoflagellate species (Figure 1d). Consistent with the pigment data, cell densities of dinoflagellates at the bloom station were significantly higher compared with the surrounding non-bloom stations (one-way analysis of variance; Figure 2a). Blooming dinoflagellates were morphologically identified to be predominantly *Levanderina fissa* (formerly known as *Gyrodinium instriatum*; Moestrup *et al.*, 2014).

Transcriptome sequencing of the dinoflagellate bloom

One hundred and twelve million paired-end raw reads from the bloom station along with 182 million and 122 million paired-end raw reads from the two non-bloom samples located at stations 20 and 70, respectively, were sequenced (Supplementary Table S1). Assembly of reads yielded 1–1.6 million assembled contigs from each sample. For the bloom sample, 53% of contigs had sequence similarity to the reference sequences contained in the taxonomic sequence database, MarineRefII, and 39% of contigs had sequence similarity to genes in the functional KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Of these KEGG matches, 25% had KEGG module annotations and were used for differential expression analysis. From stations 20 and 70, 58% and 59% of contigs were assigned a taxonomic identification, with 44% and 46% of these being assigned a functional annotation, in which 23% and 26% had KEGG module annotations, respectively (Supplementary Table S1).

In the bloom sample, ~42% of the taxonomically annotated sequence reads were from dinoflagellates (Figure 2b), whereas from stations 20 and 70, only 22% and 17%, respectively, of these reads were from dinoflagellates. In all samples collected for sequencing in 2012, the average proportion of sequences assigned to dinoflagellates was 20% (Figure 2b). There

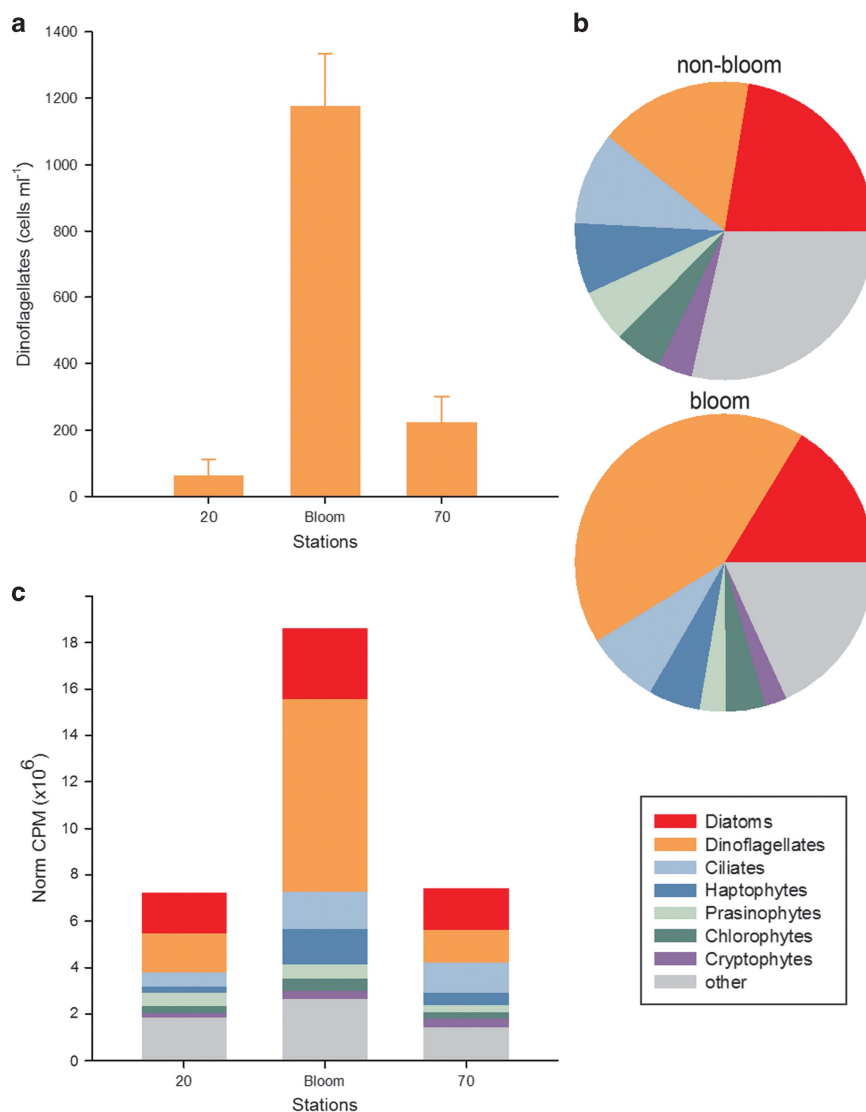


Figure 2 Comparison of dinoflagellate abundances between the bloom and non-bloom samples. (a) Dinoflagellate mean cell counts in the bloom sample and stations 20 and 70. Error bars represent standard deviations ($n=3$). (b) Taxonomic proportions of read counts in the bloom sample and the average of 22 other samples obtained in 2012. (c) Community-wide TMM normalized transcript counts (counts per million reads, c.p.m.) of major taxonomic groups in the bloom sample and stations 20 and 70 (Supplementary File 1).

was an approximate fivefold higher abundance of normalized transcripts assigned to dinoflagellates in the bloom sample relative to the non-bloom samples (Figure 2c), further confirming pigment and microscopic determinations of elevated dinoflagellate biomass and cell density at the bloom station. According to the taxonomic assignment of dinoflagellate sequences from the bloom sample, greatest sequence similarity was to transcriptomes of isolates from the genus *Karenia* (Supplementary Figure S2), a genus formerly part of *Gymnodinium* and closely related to *Levanderina* based on 18 S rDNA phylogeny (Daugbjerg et al., 2000; Heimann, 2012).

Of the total dinoflagellate reads, 20%, 22% and 15% from the bloom, station 20 and 70, respectively, obtained a KEGG module annotation. Sequences in the bloom sample were assigned to six major

functional gene categories. Generally, about three-quarters of annotated reads were assigned to metabolic pathways, including synthesis of carbohydrates, fatty acids, amino acids, proteins, cofactors and vitamins, nucleotides and secondary metabolites, carbon fixation and energy-related processes such as ATP synthesis and photosynthesis; about one-fifth were related to genetic information processing, including processes involved in RNA degradation and other genetic information processing; and <1% reflected intracellular responses to the environment, including regulatory systems and transportation of macro/micronutrients, carbohydrates and amino acids.

Dinoflagellate-associated genes were grouped into a total of 399 KEGG modules, of which 352, 378 and 314 modules were expressed in the bloom sample

and stations 20 and 70, respectively. Differential expression analysis was conducted at the module level to investigate the regulation of functional units within different biochemical processes. These modules represent a broad range of processes, including nutrient metabolism and transportation, carbohydrate production and transportation, and vitamin metabolism and transportation.

Differential expression analysis was performed on dinoflagellate-associated modules between the bloom sample versus station 20 and 70 to compare metabolic profiles under bloom conditions to those under non-bloom conditions. Modules were detected

as over- or underrepresented in the bloom sample according to their transcripts' fold change and relative abundance (c.p.m.) compared with the upstream and downstream non-bloom stations (Figure 3). Unless noted, the dinoflagellate-associated module expression patterns described herein are those that were differentially expressed between the bloom sample versus both stations 20 and 70.

Growth-related metabolic pathways during the dinoflagellate bloom

An algal bloom is often characterized by rapid growth of cells within a relatively short period of time (Pinckney *et al.*, 1997), although it is recognized that there are numerous alternative conditions conducive to algal blooms, including a release of grazing pressure (Litaker *et al.*, 2002). The relatively high concentrations of peridinin and abundance of dinoflagellates in the bloom sample is synonymous with high growth rates anticipated during the bloom event (Figure 2). Consistent with these biomass trends, putative genes encoding for a cell division transport system ATP-binding protein (*FtsE*; de Leeuw *et al.*, 1999; Arends *et al.*, 2009) as well as genes encompassing a two-component regulatory system for cell wall metabolism (*VicK-VicR* system; Bisicchia *et al.*, 2007) were identified in dinoflagellates and highly overrepresented in the bloom sample, suggesting high rates of cell division (Figure 3). Although *FtsE* and *VicK-VicR* genes were previously only thought to be present in bacteria, our

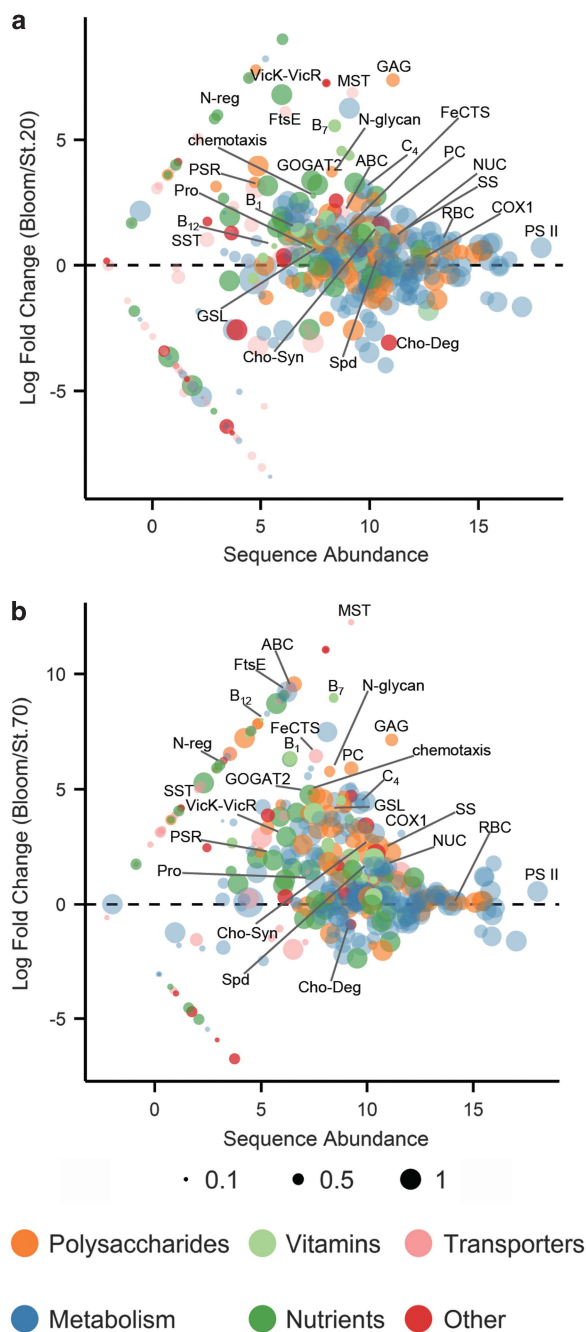


Figure 3 Metabolic pathway color-coded scatter plot of log fold change in transcript abundance of dinoflagellates between the bloom sample and the non-bloom sample for (a) bloom versus station 20, and (b) bloom versus station 70. Plotted are the log fold-change of module transcripts over average sequence abundance (log c.p.m.). Modules are grouped into different categories based on their metabolic functions. Circle size indicates the percentage of enzymes/proteins in each KEGG module with reads mapping to the underlying KEGG genes in dinoflagellates. The largest circles are 100% complete modules, whereas the smallest have at least a single gene mapped. The circle size legend indicates the proportion of genes with mapped reads for a given module. Both axes are in log scale (base 2). Selected modules are annotated as follows: ABC, putative ABC transport system (M00211); B₁, thiamin biosynthesis (M00127); B₇, biotin biosynthesis (M00123); B₁₂, cobalamin biosynthesis (M00122); C₄, C₄ carbon fixation (M00172); Cho-Syn, cholesterol biosynthesis (M00101); Cho-Deg, cholesterol degradation (M00104); COX1, cytochrome *c* oxidase (M00155); FeCTS, iron complex transport system (M00240); *FtsE*, cell division transport system (M00256); GAG, glycosaminoglycan biosynthesis (M00059); GOGAT2, glutamate synthase(ferredoxin) (K00284); GSL, glycosphingolipid biosynthesis (M00068); MST, putative multiple sugar transporters (M00207); N-reg, GlnL-GlnG two-component regulatory system (M00497); NUC, nucleotide sugar biosynthesis (M00361); PC, phosphatidylcholine biosynthesis (M00090); Pro, proline biosynthesis (M00015); PS II, photosystem II (M00161); PSR, phosphate starvation response (M00434); SS, sphingosine biosynthesis (M00099); SST, putative simple sugar transport system (M00221); RBC, RuBisCo (W00008); Spd, spermidine biosynthesis (M00133); *VicK-VicR*, cell wall metabolism (M00459).

taxonomic analyses as well as evidence from other studies (For example, genomes of *Thalassiosira pseudonana*, *Phaeodactylum tricorutum*, *Grateloupia taiwanensis* and *Paramecium tetraurelia*) demonstrates their common presence in many eukaryotic phytoplankton.

Transcripts for components of C₄/CAM carbon fixation were highly expressed in the bloom sample (Figures 3 and 4). C₄ and CAM are CO₂ concentrating mechanisms acquired by algae and plants to cope with the higher atmospheric O₂ concentrations relative to CO₂ (Reinfelder *et al.*, 2000; Giordano *et al.*, 2005). Transcripts for both pyruvate orthophosphate dikinase, which converts pyruvate to phosphoenolpyruvate, and NADP-malate decarboxylase, which catalyzes the decarboxylation of malate to pyruvate, releasing CO₂ in the process, were overrepresented in the bloom sample compared with the non-bloom stations (Figure 4). Interestingly, transcripts for phosphoenolpyruvate carboxylase, which has the important role of catalyzing bicarbonate attachment to phosphoenolpyruvate, forming the four-carbon compound oxaloacetate, were underrepresented

in the bloom sample relative to station 20 (Figure 4). Thus, although the high expression of genes encoding pyruvate orthophosphate dikinase and NADP-malate decarboxylase may be in response to higher O₂ and lower CO₂ concentrations and/or increased energy demands, the lack of corresponding overrepresentation of phosphoenolpyruvate carboxylase transcripts draws into question as to whether dinoflagellates use the full C₄/CO₂ concentrating mechanism pathway and how this pathway is regulated under bloom conditions (Haimovich-Dayan *et al.*, 2013).

Dinoflagellate-associated genes involved in N metabolism, including nitrate/ammonium transporters, nitrate/nitrite reductases, urease and glutamine and glutamate synthases were expressed in all sequence libraries, indicating that dinoflagellates are likely using multiple N sources (Zhuang *et al.*, 2015; Figure 4). However, expression patterns revealed discrepancies in N metabolism between the bloom and the non-bloom samples. Ammonium transporters, ferredoxin-dependent glutamate synthase (GOGAT2) and several components of the urea cycle were overrepresented relative to both non-bloom stations, whereas nitrite

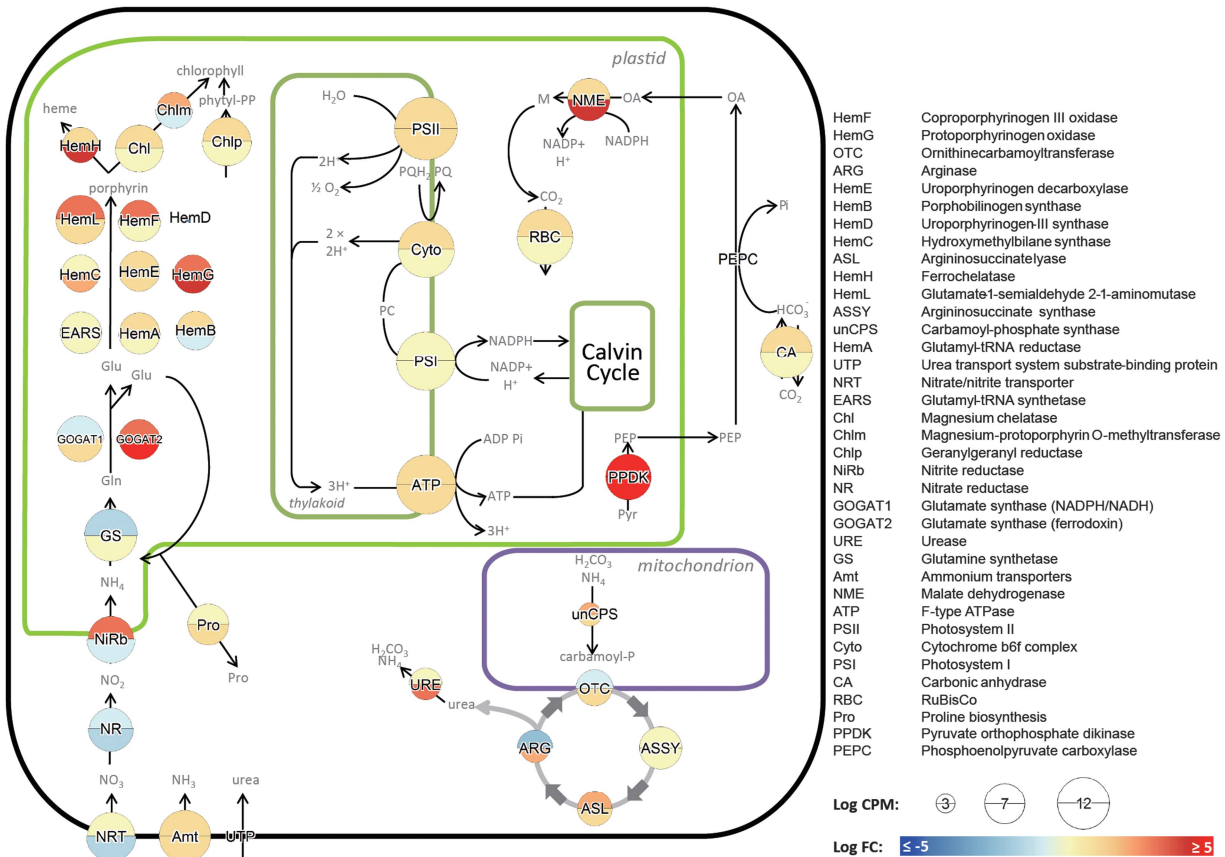


Figure 4 Schematic plot of select components in photosynthesis and carbon fixation pathways and the predicted flow of nitrogen into select dinoflagellate biosynthetic compounds. Metabolic reactions are illustrated as pie charts. Within each pie chart, the upper half represents log fold change (log FC) of transcripts between the bloom and station 20 samples, and the bottom half represents log FC between the bloom and station 70 samples, with radius indicative of average sequence abundance (log c.p.m.). Pie chart color gradients: cooler colors indicate underrepresented in bloom sequence library versus stations 20 or 70; warmer colors, indicate overrepresented in bloom sequence library versus stations 20 or 70; and gray, no change between samples. C₄ pathway is incomplete and shown is the predicted localization of proteins based on Giordano *et al.* (2005).

reductase was overrepresented relative to station 20 (Figure 4). GOGAT2 has a significant role in ammonium uptake and assimilation (Okuhara *et al.*, 1999). Carbamoyl-phosphate synthase, argininosuccinate synthase and argininosuccinate lyase are three (out of five) key enzymes in the urea cycle and were also overrepresented in the bloom sample. Overrepresentation of genes in urea cycle along with those of genes involved in other nitrogen metabolisms infer high N compound cycling by blooming dinoflagellates because of high N demands (Coschigano *et al.*, 1998; Okuhara *et al.*, 1999; Maheswari *et al.*, 2010; Allen *et al.*, 2011; Kang *et al.*, 2015). Genes involved in proline (Pro), glutamate (GOGAT) and spermidine (Spd) were also overrepresented (Figures 3 and 4). These N-rich metabolites are critical components for protein biosynthesis and cell wall formation, as well as being influential in regulating cellular N status (Allen *et al.*, 2011). Spermidine synthesis has also been found to increase under rapid growth (Nishibori and Nishio, 1997; Nishibori and Nishijima, 2004) consistent with high growth rates in the bloom sample. In addition, a regulatory system for N (GlnL-GlnG two-component regulatory system), where expression increases under N limitation (Reitzer, 2003), was overrepresented in the bloom sample (Figure 3). Noticeably, porphyrin and chlorophyll metabolism (HemA-HemH, HemL, EARS, Chl, Chlp, Chlm), which are downstream biochemical processes of glutamate biosynthesis, were also primarily overrepresented in the bloom condition (Figure 4). The high production of chlorophyll is indicative of high growth rates and probably higher photosynthetic activities (Bollivar, 2006). Taken together, despite the steep gradients in NO₃ concentrations and variations in N sources observed during our sampling effort, the biological processes in N-requiring metabolic pathways that are overrepresented in the bloom sample suggest a higher metabolic demand for N likely stemming from rapid cell growth within an increasingly N-depleted environment. Another differential expression analysis was conducted between station 20 and station 70 samples. Station 70 was characterized with module expression profiles consistent with low nitrogen availability (Figure 1, Data set 2). Overrepresentation of nitrogen assimilation modules at the bloom station compared with both non-bloom samples suggests that blooming dinoflagellates had higher nitrogen demand/nitrogen stress compared with that at station 70 where a high nitrogen demand was also inferred by changes in expression of nitrogen-associated modules relative to station 20.

Signals of higher metabolic demands for phosphorous (P) and iron (Fe) were also detected. Transcripts for a phosphate starvation response system and a Fe complex transport system were overrepresented in the bloom sample (Figure 3). Phosphorous is a major component of nucleic acids and phospholipids (Van Mooy *et al.*, 2006). The high expression of the phosphate starvation response suggests an increased P demand of dinoflagellates, which was possibly caused by higher growth rates.

Iron serves as an electron carrier in various protein complexes and has a central role in photosynthesis, cellular respiration and N metabolism, as well as other biological reactions (Marchetti and Maldonado, 2016). Expression of Fe complex transport system has been found to increase under Fe starvation conditions (Kato *et al.*, 2001; Yuan *et al.*, 2005, 2008). Thus, the increased expression of genes involved in this system can also serve as a signal of increased Fe demands in dinoflagellates during the bloom. Thus, our study highlights how cellular nutrient demands associated with rapid growth in specific members of a phytoplankton community may elicit nutrient limitation-like responses regardless of the external nutrient concentrations.

Under the bloom condition, production and transportation of various polysaccharides were overrepresented, including metabolism of N-glycans, GAGs, glycosphingolipids, sphingosines and phosphatidylcholines (PCs), as well as putative single/multiple sugar transport systems (SST/MST; Figure 3). The highly represented polysaccharides are structural components within cell membranes and surface-bound proteins, involved in cell interactions, nutrient acquisition, growth factors and acquisition of numerous other substances from the surrounding environment (Sarrazin *et al.*, 2011; Delbarre-Ladrat *et al.*, 2014). With higher metabolic rates and growth rates, dinoflagellates would likely need to produce more polysaccharides to meet the increased demand for cell membrane formation and other polysaccharide-involved biological processes.

A putative multiple sugar transporter system and another polysaccharide-associated ABC transporter system (ABC) were overrepresented (Figure 3), both of which have been shown to export polysaccharides outside of cells (Delbarre-Ladrat *et al.*, 2014). Secretion of polysaccharides has previously been observed in blooming phytoplankton in response to elevated nutrient demands (Myklestad, 1999; Decho, 2000). Exopolysaccharides are made of a large number of exported polysaccharides that attach to cellular membranes or are dispersed into the environment. They may have important roles in relieving environmental stress, mostly through trapping nutrients to alleviate nutrient shortages and enhancing cell membrane integrity under harsh physical environments (Delbarre-Ladrat *et al.*, 2014). In particular, genes involved in the synthesis of GAGs, a major group of mucopolysaccharides present in almost all domains of life, were overrepresented in the bloom sample (Figure 3), suggesting their elevated production under bloom conditions. Although GAGs have multiple suspected roles, they are often involved in cell adhesion processes. In the planktonic diatom *Bacteriastrium jadrinum*, the formation of GAG-like exopolysaccharide particles forms a fibrillar network that can extend large distances from the cell surface, facilitating colony formation and other interactions with their surrounding environment (Bosak *et al.*, 2012).

Cholesterol biosynthesis in dinoflagellates was overrepresented, whereas cholesterol degradation was underrepresented in the bloom sample relative to the surrounding non-bloom stations (Figure 3). Dinoflagellate sterols (also referred to as dinosterols) have been shown to increase during resting cyst formation, possibly for use as long-term organic carbon reserves (Amo *et al.*, 2010). Chemically reduced carbon allocated to the synthesis of cholesterol would then be used to support later growth when environmental conditions are more favorable to proliferate. Because of the high biomass of dinoflagellates along with an increased nutrient demand within an environment containing a reduced nutrient supply, the inferred accumulation of dinosterols in dinoflagellates at the peak of the bloom could be a response to the suboptimal environmental conditions where both intra- and interspecies competition for limiting growth resources is intense.

Vitamin synthesis

Phytoplankton require multiple B vitamins, including vitamin B₁₂ (cobalamin), vitamin B₁ (thiamine) and vitamin B₇ (biotin), as growth factors that have been shown to be in varying supply in marine environments (Sañudo-Wilhelmy *et al.*, 2014). Combinations of these B vitamins are required for different phytoplankton (Croft *et al.*, 2006). B vitamin auxotrophy is present in several unrelated phyla, suggesting that it has arisen independently in the evolution of phytoplankton, and variability in B vitamin requirements is observed at even the species level (Croft *et al.*, 2006). For auxotrophic eukaryotic phytoplankton, bacteria have pivotal roles in vitamin supply to support phytoplankton growth (Croft *et al.*, 2006). Expression of biotin and thiamine biosynthesis genes was detected in all of our sequence libraries (Figure 3), indicating the ability of at least some of the dinoflagellates in our samples to biosynthesize both of these vitamins. Although eukaryotic phytoplankton are thought to be unable to synthesize cobalamin (Croft *et al.*, 2005), unexpectedly, several dinoflagellate-associated genes encoding enzymes involved in the biosynthesis of cobalamin were detected in our sequence libraries, as discussed below.

Biotin is a cofactor for several fundamental carboxylase enzymes and is involved in central pathways in both prokaryotic and eukaryotic cell metabolisms (Streit and Entcheva, 2003). Four enzymes, BioF, BioA, BioD and BioB are required to convert pimeloyl-CoA, which is the precursor for biotin biosynthesis, to biotin (Croft *et al.*, 2006). Expression of BioF, BioA and BioB were detected in both the bloom and non-bloom samples, whereas BioD was not detected in either sample. However, the absence of BioD transcripts does not necessarily negate dinoflagellates' ability to synthesize BioD, as BioD is not present in the genomes of many biotin-producing phytoplankton and plant species

(for example, *Thalassiosira pseudonana* and *Arabidopsis thaliana*). The absence of BioD suggests that the function of BioD might be carried out by another unidentified enzyme (Croft *et al.*, 2006). Under the bloom condition, genes for biotin biosynthesis were overrepresented, consistent with higher biotin production corresponding with the high metabolic rates of the blooming dinoflagellates.

Thiamine is another important cofactor for carbohydrate and branched-chain amino-acid metabolisms. Biosynthesis of thiamine requires the coupling of thiazole and pyrimidine to form thiamine monophosphate, which is then catalyzed by thiamine pyrophosphokinase to form thiamine pyrophosphate, the active form of thiamine (Croft *et al.*, 2006). Similar to biotin, genes involved in the biosynthesis of thiamine were also overrepresented in the bloom condition. All enzymes involved in pyrimidine metabolism in dinoflagellates were detected in each sample in addition to genes encoding enzymes for catalyzing the coupling of pyrimidine and thiazole. However, genes involved in thiazole metabolism were not detected in the bloom sample nor was thiamine pyrophosphokinase (Data set 2, M00127). The absence of genes encoding for some of these essential enzymes in thiamine biosynthesis may be a result of our current incomplete understanding of this pathway in phytoplankton. However, the high expression of most of the thiamine biosynthesis genes in the blooming dinoflagellates suggests an increased production of thiamine.

Cobalamin is a cofactor catalyzing either rearrangement–reduction reactions or methyl transfer reactions, and is essential for cobalamin-dependent enzymes (Croft *et al.*, 2006). Some phytoplankton require cobalamin for cobalamin-dependent methionine synthase (MetH), whereas an alternative form of methionine synthase (MetE) exists in some microalgae that do not require B₁₂, alleviating them of most of their B₁₂ requirement (Helliwell *et al.*, 2011; Bertrand *et al.*, 2012). Eukaryotic phytoplankton must obtain B₁₂ from their environment and/or close bacterial associations (Croft *et al.*, 2005). Surprisingly, two putative genes assigned to dinoflagellates, *CobC1* and *CobC2*, encoding for part of the cobalamin biosynthesis pathway were detected in the bloom sample (*CobC2* only) as well as station 20 (both genes; Supplementary Figure S3). α -Ribazole phosphatase (*CobC2*) is an enzyme that transforms α -ribazole 5'-phosphate to α -ribazole, and threonine-phosphate decarboxylase (*CobC1*) catalyzes a step in the pathway of adenosylcobalamin biosynthesis, both of which are necessary steps in the formation of the final product, cobalamin coenzyme (Scott and Roessner, 2002). Besides *CobC1* and *CobC2*, other putative genes involved in B₁₂ biosynthesis were also detected in NRE samples collected at different time points (data not shown); however, we did not detect the expression of the entire suite of genes required for B₁₂ synthesis in a single sample of our eukaryotic

sequence libraries. The high expression of genes for putative dinoflagellate-associated *CobC2* in the bloom sample is consistent with dinoflagellates being able to at least partially biosynthesize vitamin B₁₂ following the acquisition of B₁₂ intermediates. MetH was highly expressed at station 20 but was not detected at the bloom station, suggesting low B₁₂ availability during the bloom (Data set 4; Bertrand *et al.*, 2013). Given bacteria are major B₁₂ suppliers to dinoflagellates, we thus hypothesize that bacterially derived B₁₂ intermediates may be acquired by dinoflagellates and processed to biosynthesize B₁₂. Similar to our findings, Zhang *et al.* (2013) detected the expression of the cobalamin biosynthesis gene, *CobW*, in dinoflagellates, before which there was only limited support for full or partial cobalamin synthesis within any algal lineage.

Implications for dinoflagellate-bacterial interactions

Phytoplankton secrete dissolved organic matter, especially when nutrient stressed (Wear *et al.*, 2015). Similarly, previous studies have demonstrated that under bloom conditions, phytoplankton can release large amounts of extracellular polysaccharides into their surrounding environment, forming large sticky aggregates (Mykkestad, 1995). The mucilage material coagulates both phytoplankton and surrounding bacteria cells, possibly serving as protection for phytoplankton against predation and a means to deal with turbulence within the water column (Smayda, 2002). Some of the mucilage secreted by phytoplankton can also be used as an organic carbon source for heterotrophic bacteria (Decho, 2000; Croft *et al.*, 2005). Production and transportation of simple sugars (ABC, SST), which are readily assimilated by heterotrophic bacteria, were also overrepresented during the bloom (Figure 3, Data set 2). Correspondingly, previous studies have shown that organic carbon uptake increases in bacteria during algal blooms, as well as adhesiveness of bacterial cell surfaces that may also be associated with phytoplankton mucilage formation (Rinta-Kanto *et al.*, 2012). The inferred increased production of both simple sugar and polysaccharide exudates suggests that dinoflagellates may actively promote the growth and interaction with their surrounding heterotrophic bacteria community. Notably, bacterial communities have also been shown to have higher biomass and more efficient remineralization of organic materials when in aggregates, producing more inorganic nutrients that could then be made available to phytoplankton (Müller-MNiklas *et al.*, 1994; Decho, 2000; Cooper and Smith, 2015).

Croft *et al.* (2005) also found that coculturing with phytoplankton significantly increases bacteria's B₁₂ production. Similarly, increased biosynthesis and secretion of biotin and thiamine by dinoflagellates would support further bacterial growth, as there are numerous bacteria that do not possess the metabolic

pathways for synthesis of these vitamins (Mykkestad, 2000; Sañudo-Wilhelmy *et al.*, 2014). In addition to roles in nutrient trapping and cell adhesion, polysaccharide exudates can bind chemokines, which are used to facilitate cell-to-cell communications (Sarrazin *et al.*, 2011). Remarkably, a two-component regulatory system for chemotaxis was overrepresented in dinoflagellates under bloom conditions (Figure 3). The expression of chemotaxis was shown to be a response of a marine heterotrophic bacterial assemblages to higher nutrient concentrations (Shi *et al.*, 2012). Taken together, we hypothesize that under bloom conditions, dinoflagellates produce excess polysaccharides and vitamins in efforts to cultivate bacteria so that they may acquire essential macro- and micronutrients (N, P, B₁₂, etc.) produced by bacteria to prolong the bloom. Alternatively, if the blooming dinoflagellates are mixotrophic, which many of them are (Jeong *et al.*, 2010), bacterial cultivation may provide a source of organic matter when inorganic nutrients are depleted to levels where autotrophy is no longer favored.

Potential molecular indicators for bloom development

Characterized by rapid growth, blooming phytoplankton have high metabolic activity rates. Overrepresented transcripts in the nucleotide sugar biosynthesis and other DNA/RNA processing systems are consistent with the blooming phytoplankton experiencing high rates of energy-demanding transcription and translation (Figure 3). To meet this increase in energy demands, high proportions of transcripts were devoted to ATP synthesis, carbon fixation and photosynthesis in the bloom sample (Figures 3 and 5). As organic carbon synthesis and energy production are fundamental to phytoplankton growth, increased expression of photosynthesis and carbon fixation are crucial for the formation and propagation of an algal bloom. Thus, molecular activities of these processes could be used as markers for assessing bloom potential. However, their increased expression alone does not guarantee a bloom will develop. In addition to these metabolic processes, carbohydrate biosynthesis and transportation may also serve as molecular proxies for bloom formation. Increased production of carbohydrate exudates is common in intense algal blooms, which are consistent with the overrepresentation of transcripts for single/polysaccharide transportation and biosynthesis activities in our bloom sample (Figure 3). Lastly, as increased sterol synthesis may be associated with resting cysts, the accumulation of dinosterols may be indicative of bloom termination, providing a useful indicator of the bloom entering senescent phase. Although more work is needed, we suggest such genes as potential targets that can be used in conjunction with advancing probe technologies for bloom characterization and management efforts.

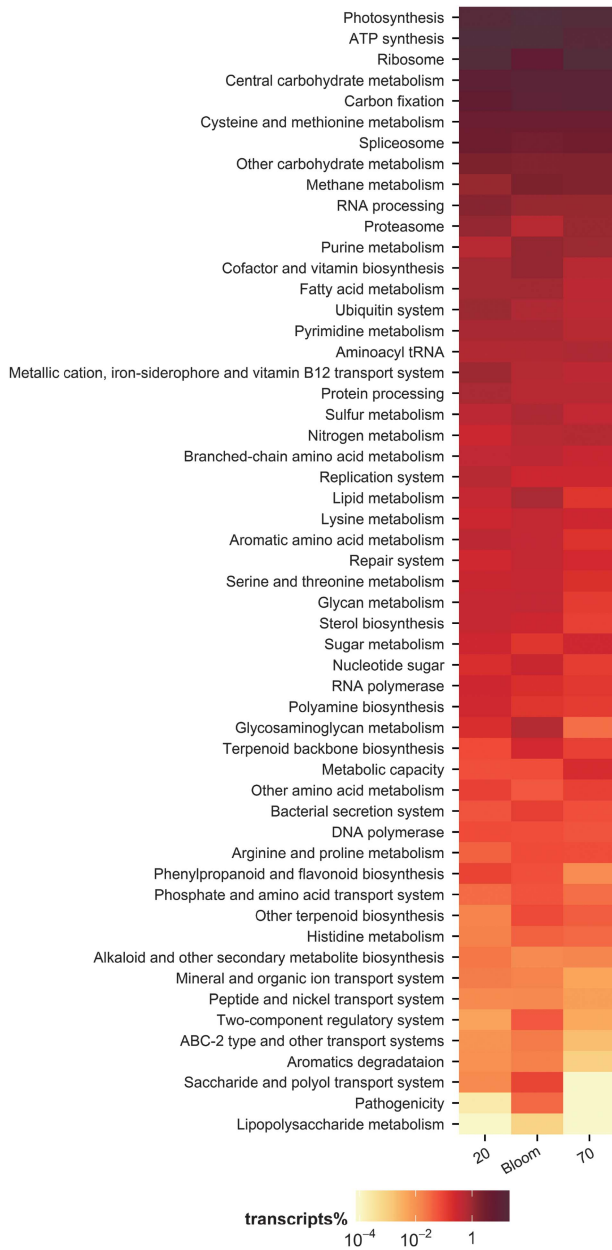


Figure 5 Heatmap of transcript proportions in KEGG categories (Class 3) of sequenced samples collected from stations 20, bloom and 70. Color gradient indicates the percentage of transcripts mapping to each KEGG category of the total KEGG annotated reads.

Summary

Metatranscriptomic analysis has provided a comprehensive examination into the molecular underpinnings of blooming dinoflagellates within a natural setting. When combined with environmental data, our study provides a unique and in-depth approach to coupling the transcriptome-level responses by dinoflagellates to changes in their surrounding environment under bloom-favorable conditions. Our findings indicate that dinoflagellates increased many components of their cellular metabolism and growth, including a possible reliance on various

components of CO₂-concentrating mechanisms. In response to increased cellular demands, dinoflagellates increased expression levels of genes involved in N, P and Fe acquisition, as well as the biosynthesis of B vitamins. Despite these efforts, a decreasing N supply in combination with gene expression patterns indicative of high N demand suggests that the blooming dinoflagellates were possibly experiencing the onset of N limitation. We speculate dinoflagellates were producing and exporting sugars, exopolysaccharides and vitamins to facilitate bacterial interactions, either to increase remineralization of nutrients by bacteria and promote nutrient acquisition or for their direct consumption. Thus, we speculate increased interactions between dinoflagellates and bacteria may be a common phenomenon that facilitates the propagation of dinoflagellate blooms. Our study is based on transcriptomes. Dinoflagellates have been shown to undergo widespread trans-splicing, which may complicate interpretations based on gene transcripts (Lidie and van Dolah, 2007; Lin *et al.*, 2010). Thus, further research would be beneficial to assess whether changes in the gene module expression we have discussed are reflective of protein expression and activity. In addition, further investigation of our hypothesized phytoplankton and bacterial interactions under bloom conditions is warranted.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

AM and HP designed research; WG, AM and JB performed research, WG and DMS contributed new analytical tools; WG, AM and NH analyzed data; and WG and AM wrote the paper.

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