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SANTA CRUZ

MOLECULAR BIOMARKERS FOR PHOSPHORUS STRESS IN THE MARINE CYANOBACTERIUM *CROCOSPHAERA*

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

OCEAN SCIENCES

by

Nicole A. Pereira

December 2017

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Abstract

MOLECULAR BIOMARKERS FOR PHOSPHORUS STRESS IN THE MARINE CYANOBACTERIUM *CROCOSPHAERA*
by
Nicole A. Pereira

The marine cyanobacterium *Crocosphaera* significantly contributes to nitrogen fixation and primary production in tropical and subtropical oceans. These regions are often characterized by low phosphorus (P) concentrations that may constrain growth and nitrogen fixation in *Crocosphaera*. Molecular markers are commonly used as diagnostic tools to detect when microorganisms are under P stress and to better identify the conditions influencing P limitation. Prior to this work, diagnostic biomarkers for P stress in *Crocosphaera* had yet to be characterized. To identify possible biomarkers, select gene transcripts were evaluated under P stress in cultures of the model strain, WH8501, a small-cell ecotype of *Crocosphaera*. Transcript levels of two genes were significantly higher: *pstS*, which codes for the high-affinity P-binding protein, and *arsB*, coding for the arsenite efflux protein. These genes were up-regulated at discrete stages of the P stress response, with *pstS* being an early indicator of P limitation and *arsB* transcripts detected through P starvation. Interestingly, *pstS* transcripts exhibited a diel expression pattern that was detected in P replete conditions. To further evaluate the efficacy of *pstS* as a biomarker for P stress in *Crocosphaera*, changes in its transcript levels were assessed in the presence of various phosphorus sources: inorganic (DIP) and organic (DOP), as well as changing light levels. While *pstS* transcripts were suppressed by all P sources tested, the response was more sensitive to DIP than DOP. Additionally, *pstS* was induced by

high light levels, independent of the P stress response. Although *pstS* is a commonly used indicator of P stress, these results suggest that it is not a reliable indicator for *Crocosphaera*. To evaluate P stress in natural assemblages, biomarkers were used to target environmental populations in the North Pacific. *pstS* and *arsB* identified P stress in *Crocosphaera*, and an alkaline phosphatase gene (*phoX*) targeted another nitrogen-fixing cyanobacterium, *Trichodesmium*. Detection of these biomarkers suggest that P is an important driver of *Crocosphaera* abundance, especially large cell ecotypes, while *Trichodesmium* abundance is not as negatively impacted by low P concentrations. Future studies with biomarkers differentiating between ecotypes of *Crocosphaera* will help determine the controls on P limitation between strains.

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I contributed to this chapter by conducting laboratory experiments on batch

cultures of *Crocosphaera* during which I collected and analyzed samples for

microscopy, photosynthetic efficiency, and molecular work. I developed

molecular probes specific for SYBR qPCR assays and conducted shipboard

incubations on a research cruise (KM101) where I collected and analyzed field

samples to test these probes. I also wrote the manuscript. Irina Shilova

supervised method development and research plans for all experiments and

provided editorial support for writing. Jonathan Zehr directed and supervised

the research which forms the basis for this dissertation.

INTRODUCTION

Cyanobacteria play a significant role in the marine environment as primary producers that fix carbon dioxide and are a part of the base of marine food webs. The growth and distribution of these organisms influences the overall productivity of marine ecosystems. Understanding what controls the abundance of cyanobacteria in the marine environment is an ongoing area of research for biological oceanographers (Palenik 2015). Depleted concentrations of nutrients such as nitrogen (N), phosphorus (P), and iron (Fe), that are required for cyanobacterial growth, can constrain primary production. Low-nutrient, or oligotrophic, areas are located in subtropical ocean gyres and are typically N-limited (Gruber 2008). Some species of cyanobacteria are diazotrophs capable of fixing atmospheric dinitrogen (N_2) gas into bioavailable N sources. Thus, they contribute to the biogeochemical cycling of both carbon (C) and N, as well as supporting productivity in oligotrophic regimes. Initially, the filamentous diazotroph *Trichodesmium* was thought to be the dominant N_2-fixing cyanobacterium. However, molecular methods of detection identified unicellular cyanobacteria, including *Crocosphaera watsonii*, as N_2-fixing microorganisms (Zehr et al. 1998). When present at high densities, N_2-fixation by unicellular diazotrophs can exceed that of *Trichodesmium* (Montoya et al. 2004). *Crocosphaera* can fix up to 3 times more N than is required for growth, suggesting that it may excrete the surplus into the environment for use as a readily available N source (Dron et al. 2012).

Crocosphaera watsonii was first isolated from the surface waters of the South Atlantic Ocean almost 30 years ago (Waterbury et al. 1988). This organism grows

optimally between 26°C and 34°C and is found at abundances of 10^3-10^4 liters in the upper water column of the tropical Pacific and Atlantic oceans, although recent research indicates a potential for expansion into other niches (Church et al. 2008, Langlois et al. 2008, Webb et al. 2009, Großkopf and La Roche 2012). There are ten known strains of this species that fall within two phylogenetically distinct groups: small (2-5μm) cell strains and large (3-6μm) cell strains (Webb et al. 2009). Large cell strains have higher rates of N_2-fixation and are viscous due to extracellular polysaccharides (EPS) produced during growth (Webb et al. 2009, Sohm et al. 2011). Despite there being phylogenetic differences between these two groups, low genetic variability exists among strains, both between those in culture and in the environment (Bench et al. 2011). The small cell strain *Crocosphaera watsonii* WH8501 is used as a model unicellular diazotroph for the majority of studies in culture.

In *Crocosphaera*, many cellular metabolic processes operate on a diel cycle (Pennebaker et al. 2010, Shi et al. 2010, Dron et al. 2012). Circadian control of these processes is mediated by topological changes in DNA that influence the regulation of gene expression (Pennebaker et al. 2010). Nitrogen fixation and photosynthesis are temporally segregated in *Crocosphaera* in order to avoid inactivation of nitrogenase, the key enzyme in N_2-fixation, by oxygen produced during photosynthesis (Waterbury et al. 1988). Since *Crocosphaera* has access to a large source of N through fixation, the growth of this organism is often controlled by other limiting nutrients, such as P.

P is an essential element for the cell: present in nucleic acids (DNA and

RNA), cell membrane components (phospholipids), energy transfer (ATP), and post-translational modification (phosphorylation). *Crocosphaera* must adapt to changing concentrations of dissolved inorganic phosphate (DIP) in its natural environment: ranging from 0.2-5nM in the upper water column (1-100m) in the North Atlantic and 10-100nM in the upper water column of the North Pacific (Wu et al. 2000, Cavender-Bares et al. 2001, Karl et al. 2001b). The dissolved organic phosphorus (DOP) pool can be 5-10 times larger than the DIP pool in the upper water column of oligotrophic regimes, although it remains largely uncharacterized (Orrett and Karl 1987, Bjorkman and Karl 2003). Orthophosphate (Pi) is the preferential form of P for cyanobacteria, since it can be taken up directly by the cell (Vershinina and Znamenskaya 2002). However, at limiting DIP concentrations, DOP can be converted to Pi by alkaline phosphatase and assimilated by cells (Rao and Torriani 1988). Karl et al. (Karl et al. 2001a) hypothesized that climate-driven changes in the North Pacific Subtropical Gyre (NPSG) would result in an increased selection of N_2-fixing organisms and a subsequent drawdown of bioavailable P. In cyanobacteria, physiological changes resulting from P deficiency lead to cell cycle arrest, decrease in photosynthetic efficiency and changes in cell size (Falkowski et al. 1992, Parpais et al. 1996, Vaulot et al. 1996). In diazotrophs, N_2-fixation is also limited by low P concentrations (Mills et al. 2004, Watkins-Brandt et al. 2011, Garcia et al. 2015).

Cyanobacteria use a variety of strategies to adapt to low P environments, which are mediated by transcriptional activation of the Pho regulon. When extracellular Pi falls below a threshold concentration, it is sensed by a two-component

signal transduction system comprised of a histidine kinase (PhoR) and response regulator (PhoB). Under low Pi conditions, PhoR activates PhoB, which acts as a transcriptional regulator. PhoB then binds to target sequences (Pho box) of promoter regions, inducing transcription of Pho regulon genes (Wanner 1993). There is evidence of an alternative transcriptional activator under the control of the PhoBR system in cyanobacteria, suggesting that the response to P stress is more complex than originally thought (Ostrowski et al. 2010). Cellular strategies to mitigate P stress include the increased assimilation of Pi and the reduction in cellular P needs (La Roche et al. 1999). In order to increase P acquisition, gene products of the Pho regulon include proteins for high-affinity Pi-transport coupled with DOP hydrolysis. Pi is hydrolyzed from a variety of DOP compounds by alkaline phosphatases. C-O-P bonds are cleaved by phosphomonoesters (PhoA and PhoX) and phosphodiesters (PhoD), while C-P bonds are targeted by phosphonates (Phns) (Coleman 1992, Eder et al. 1996, White and Metcalf 2007, Wu et al. 2007). As Pi is released from organic compounds, a P-specific ABC transporter (Pst system) uses a periplasmic Pi-binding protein (PstS) to actively shuttle P into the cell (Surin et al. 1985). There is variability in the genes that make up the Pho regulons between cyanobacterial groups, thus their strategies for mitigating P limitation can differ. Unlike *Trichodesmium*, *Crocosphaera* strains lack *phn* genes for C-P hydrolysis and are unable to utilize phosphonate as a P source (Dyhrman and Haley 2006). Identifying which genes are responsive to P limitation in *Crocosphaera* will allow for a better understanding of when *Crocosphaera* is experiencing P stress and how it responds to changes in P

physiology.

The Pho regulon genes that respond to P limitation are often ideal diagnostic markers of cellular P status. While bulk seawater measurements of alkaline phosphatase activity are also used to indicate P stress in plankton communities, genetic markers can provide taxon-specific information. Molecular markers for P stress must be nutrient specific, induced by low external Pi concentrations, and repressible by bioavailable organic P sources (Scanlan and Wilson 1999). Both *pstS* and *phoA* are biomarkers predominately used to identify P limitation in marine cyanobacteria (Scanlan et al. 1997, Fuller et al. 2005, Munoz-Martin et al. 2011). These genes increase P acquisition as part of cellular acclimation to P stress, and are reversible when the stress is alleviated. Typically, the genes specific to nutrient stresses are transiently upregulated under limiting conditions, and decline during starvation (Ferenci 1999). However, the complexity and variability of the P stress response means that it is important to characterize the response of these markers in a controlled culture environment in order to interpret their detection in natural populations.

The overall objective of this dissertation was to identify and characterize potential genetic markers for P stress in cultivated *Crocosphaera* that could be used to better understand P physiology in natural populations. In chapter 1, I evaluated the transcriptional response to P limitation by targeting putative Pho regulon genes in

Crocosphaera. Genes that were strongly up-regulated in response to P deficiency were selected as potential markers, and their transcription pattern was characterized in cultures experiments throughout diel cycles. Additionally, I tested these markers in the environment via shipboard incubation experiments. In chapter 2, I further assessed a key biomarker for P stress, *pstS*, under various conditions in culture, including changes to light intensity and DOP additions. The experiments carried out in chapter 3 use the expression of biomarkers characterized in chapters 1 and 2 as markers for P limitation in natural populations of *Crocosphaera* in the NPSG. Additionally, another biomarker was used to target P limited *Trichodesmium* in order to compare the P status of these two diazotrophs under the same environmental conditions. By providing a detailed assessment of key P stress biomarkers in *Crocosphaera*, the results of this study allow from a more accurate interpretation of their detection in the field.

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CHAPTER 1: MOLECULAR MARKERS DEFINE PROGRESSING STAGES OF PHOSPHORUS LIMITATION IN THE NITROGEN-FIXING CYANOBACTERIUM, *CROCOSPHAERA*

Nicole A. Pereira, Irina N. Shilova and Jonathan P. Zehr (2016)

MOLECULAR MARKERS DEFINE PROGRESSING STAGES OF PHOSPHORUS LIMITATION IN THE NITROGEN-FIXING CYANOBACTERIUM, *CROCOSPHAERA*[1]

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Crocosphaera watsonii is a marine cyanobacterium that frequently inhabits low phosphate environments in oligotrophic oceans. While *C. watsonii* has the ability to fix atmospheric nitrogen, its growth may be limited by availability of phosphorus. Biomarkers that indicate cellular phosphorus status give insight into how P-limitation can affect the distribution of nitrogen-fixing cyanobacterial populations. However, adaptation to phosphorus stress is complex and one marker may not be sufficient to determine when an organism is P-limited. In this study, we characterized the transcription of key genes, activated during phosphorus stress in *C. watsonii* WH8501, to determine how transcription changed during the phosphorus stress response. Transcription of *pstS*, which encodes a high-affinity phosphate binding protein, was discovered to be quickly up-regulated in phosphorus-depleted cells as an immediate stress response; however, its transcription declined after a period of phosphorus starvation. In addition, diel regulation of *pstS* in *C. watsonii* WH8501 complicates the interpretation of this marker in field applications. Transcription of the gene coding for the arsenite efflux protein, *arsB*, was upregulated after *pstS* in phosphorus limited cells, but it remained upregulated at later stages of phosphorus limitation. These results demonstrate that a single molecular marker does not adequately represent the entire phosphorus stress response in *C. watsonii* WH8501. Using both markers, the variations in transcriptional response over a range of degrees of phosphorus limitation may be a better approach for defining cellular phosphorus status.

Key index words: biomarkers; *Crocosphaera*; cyanobacteria; nutrient stress; phosphorus

Abbreviations: DIP, dissolved inorganic phosphate; DOP, dissolved organic phosphate; FRRF, fast repetition rate fluorometer; MAGIC, magnesium induced co-precipitation

Marine diazotrophs are a unique and ecologically important group, contributing to primary productivity by providing fixed nitrogen and carbon to the oceans. These organisms play a critical role in the global cycling of these elements, since inputs of new nitrogen to the surface water can control export of organic matter to deeper water (Dugdale and Goering 1967, Eppley and Peterson 1979). When present at high densities, nitrogen fixation by unicellular diazotrophs can exceed that of larger organisms (Montoya et al. 2004). Thus, unicellular diazotrophic cyanobacteria, such as *Crocosphaera watsonii*, can be significant contributors to new production in the oligotrophic open ocean. Identifying the factors that constrain growth of diazotrophs will aid in our understanding of the controls on diazotrophic abundance and on nitrogen fixation.

Molecular diagnostic tools that target cell-specific responses to nutrient limitation have been developed to better understand how nutrients control the distributions and activities of nitrogen-fixing cyanobacteria (Webb et al. 2001, Dyhrman et al. 2002). *Crocosphaera watsonii* must deal with varying dissolved inorganic phosphate (DIP) availability in its natural environment: concentrations range from 0.2 to 5 nM in the North Atlantic (Wu et al. 2000, Cavender-Bares et al. 2001) and 10–100 nM in the North Pacific (Karl et al. 2001). Genes that are common markers for detecting phosphorus stress are usually part of the Pho regulon (Vershinina and Znamenskaya 2002) and are under the control of a two-component regulatory system, where the response regulator (PhoB) induces transcription under P-stress (Makino et al. 1989). Expression of the gene encoding a high-affinity phosphate-binding protein, *pstS*, is a common response to phosphorus deficiency because it increases the cellular efficiency of scavenging environmental DIP (Suzuki et al. 2004, Dyhrman and Haley 2006). Cells utilize alkaline phosphatase (*phoA*) to acquire phosphate from extracellular phosphomonoesters, another common mechanism for adapting to phosphorus stress (Ray et al. 1991). Various cyanobacteria also have the capability to hydrolyze phosphonates, facilitated by the *phn* gene cluster, as an extracellular dissolved organic phosphate (DOP) source (Moore et al. 2005). Interestingly, *C. watsonii*, in contrast to *Trichodesmium* (Dyhrman et al. 2006), is unable to use phosphonate as a phosphate source and no homolog of the phosphonate transport gene cluster (*phnCDE*) has been found in its gen-

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ome (Dyhrman and Haley 2006). Polyphosphates often serve as an intracellular phosphorus reservoir; thus, transcription of genes controlling polyphosphate synthesis (*ppK*) and hydrolysis (*ppX* and *ppA*) can be turned on in response to phosphorus starvation (Gomez-Garcia et al. 2003, Schwarz and Forchhammer 2005). Phytoplankton can also lower their cellular phosphorus requirement by substituting sulfolipid for phospholipid to mitigate phosphorus limitation in the marine environment (Van Mooy et al. 2009).

Genes that respond to phosphorus deficiency, even if they are not directly involved in alleviating phosphorus stress, can also be used as molecular markers. For example, a gene coding for the arsenite efflux pump, *arsB*, is upregulated under phosphorus stress as a protective mechanism for the cell (Dyhrman and Haley 2011), since toxic arsenate (As) is a phosphate analog, and higher As:P correlates with increasing phosphorus stress. Genes used as molecular diagnostic tools should be stress-specific and their transcription should not be affected by other conditions (La Roche et al. 1999). Since cellular processes in cyanobacteria, including *C. watsonii*, follow strict circadian regulation and gene activity often exhibits a diel pattern (Shi et al. 2010), this creates an additional complication when interpreting influences on transcriptional changes, especially in natural populations. Thus, the transcription of these genes in response to increasing environmental stress must be empirically validated.

While the expression of molecular markers are often used as indicators of phosphorus status of cells, the response and regulation of nutrient utilization genes is complex. Examining the shifts in the transcript abundance of relevant genes during different phases of phosphorus limitation will help to better define the nutrient status of *C. watsonii* under variable conditions. Here, we analyzed the molecular responses to low availability of phosphorus in the unicellular diazotrophic cyanobacterium *C. watsonii* strain WH8501 to better understand how to apply and interpret well-characterized biomarkers for phosphorus stress in field applications. *C. watsonii* has two main phenotypes: large cells, which produce exopolysaccharides, and small cells. The decades-long cultured strain for *C. watsonii*, WH8501, is a small-cell type. It has a larger genome (6.2 Mb) than the genomes of five other strains (4.5 Mb) and contains a higher number of transposase genes and repeated genomic sequences (Bench et al. 2013). While *C. watsonii* WH8501 is not the best representative of the natural populations, the genes shared among strains have higher than 98% nucleotide identity (Bench et al. 2013). The high conservation of genes among strains will allow the analyzed markers to be used for the *C. watsonii* populations in environmental samples.

MATERIALS AND METHODS

Cell culturing. *Crocosphaera watsonii* WH8501 was grown at 27°C with 45 μmol photons · m^{-2} · s^{-2} in a 12:12 light:dark cycle. At this light level, *C. watsonii* WH8501 grew at a photosynthetic efficiency of Fv/Fm = 0.4, similar to other studies with small-celled *C. watsonii* (Fv/Fm = 0.35; Sohm et al. 2011). We found that growth at higher light intensities led to photoinhibition in this lab strain. For all experiments, triplicate cultures were grown in seawater based media (SO; pH 8.0, salinity 28), prepared without added nitrogen (Waterbury et al. 1986). Phosphate-replete (+P) cultures were grown in SO medium, prepared with a 0.2 μm-filtered North Pacific (Station ALOHA, HI) seawater base and 60 μM K$_2$HPO$_4$. Phosphate-deplete (-P) cultures were grown in SO medium without added phosphate. Residual phosphate in the seawater base used for SO medium was measured at 60 nM using (MAGIC; Karl and Tien 1992). Cells used as the inocula for all treatments were grown with replete phosphate and harvested with a 0.2 μm filter at mid-log phase from a single mother culture, then washed three times and re-suspended in medium without added phosphate to restrict carry-over. All cultures were inoculated at a starting density of 5 × 10^4 cells · mL^{-1}.

Microscopy and photosynthetic efficiency. In each culture experiment, samples were taken every 24 h to monitor cellular physiology. Cell growth and abundance of all cultures was monitored using epifluoresence microscopy with an Axioplan 2 Zeiss microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA). Aliquots of 1 mL taken from triplicate cultures were filtered using a vacuum pump directly on 25 mm black polycarbonate, 0.22 μm pore filters (Poretics, Osmonics Inc., Minnetonka, MN, USA). Each filter was mounted on a glass slide (Fischer Sci., Pittsburgh, PA, USA) and cells were counted using 4,000× magnification under blue excitation light. For each slide, cell counts were obtained by averaging counts from 10 fields of view. Specific growth rates (k) were calculated per day, using the formula: $k = \ln (N_2/N_1)/1$, where N_2 was the cell count 24 h after N_1. Growth curves derived from cell counts were verified using relative fluorescence with a TD-700 fluorometer (Turner Designs, Sunnyvale, CA, USA). Photosynthetic efficiency (F_v/F_m), which is the ratio of the maximum change in variable fluorescence ($F_v = F_m - F_o$) to the maximum fluorescence yield (F_m) was measured using a fast repetition rate fluorometer (FRRF) (Kolber et al. 1998). F_v/F_m is a rapid and highly sensitive method for detecting stress, including P-limitation, in primary producers (Beardall et al. 2001), and can be used as a proxy for cell health. Aliquots of 5 mL culture samples for FRRF analysis were taken daily at the peak of the light cycle (L6) and measured in triplicate.

RNA extraction and cDNA synthesis. RNA samples were collected by passing 10 mL of liquid culture through a 25 mm, 0.2 μm pore-sized Suporfilter (Pall Corporation, Port Washington, NY, USA) using gentle vacuum filtration. Filters were placed in bead beater tubes with β-mercaptoethanol and RLT buffer, then flash frozen in liquid nitrogen immediately following filtration, after which they were stored at −80°C. RNA extractions were carried out using a modified RNeasy Mini Kit protocol (Qiagen, Germantown, MD, USA). Samples were thawed on ice and subjected to 2 min of bead-beating. Filters were removed using sterile needles and the remaining buffer was placed on spin columns for automated extraction in a QIAcube (Qiagen). The extraction protocol included a DNase step, where each sample was treated with 10 μL RNase-Free DNase (Qiagen) to remove genomic DNA. Aliquots of 8 μL extracted RNA were converted to single stranded complementary DNA (cDNA) using the QuantiTect

Reverse Transcription Kit (Qiagen) with random hexamer priming according to manufacturer's guidelines. While generating cDNA, parallel reactions were run with no reverse transcriptase to check for any residual DNA contamination. All cDNA was subsequently stored at −20°C.

Quantitative PCR assays. Primers were designed to target 11 genes known to be involved in phosphorus metabolism in cyanobacteria (Table 1). Using BLAST, homologs of these genes were found in *C. watsonii* WH8501 and primers were designed to specifically target these sequences. Primer3 (Untergasser et al. 2012) was used to create primers with specifications for relative quantitative PCR (qPCR) (primer3.sourceforge.net/). Candidate primers were each evaluated for their probability to form hairpin loops, self-dimers and heterodimers using IDT Oligoanalyzer 3.1 web tool (Integrated DNA technologies, Coralville, IO, USA). We did a BLAST search with the selected primers against the National Center for Biotechnology Information (NCBI) nt database to verify that the primers did not match anything other than their specific target gene sequence. Since the expression of multiple genes was analyzed in this study, relative quantification was used to compare gene expression profiles. The comparative ($\Delta\Delta Ct$) method was used to normalize the expression of each gene. Transcription of each gene of interest (GOI) was normalized to *rnpB*, which encodes the RNA component of RNase P, and whose transcript abundance does not fluctuate with phosphorus stress (Gomez-Baena et al. 2009). This constitutively expressed housekeeping gene is not cell-cycle dependent in the marine cyanobacterium *Prochlorococcus* (Holtzendorff et al. 2001). In addition, *rnpB* transcript abundance has been shown to be stable under varying nutrient, light, and temperature conditions in another diazotrophic marine cyanobacterium, *Trichodesmium* (Chappell and Webb 2010). Finally, a diel transcriptome study in *C. watsonii* WH8501 provides evidence that transcription of the *rnpA* gene, a protein subunit of RNase P, does not exhibit diel variation (Shi et al. 2010). For each qPCR assay, the amplification efficiency was first tested by normalizing each primer set to *rnpB*. Amplification efficiencies of primer sets between 95% and 105% with an $r^2 \geq 0.98$ were determined to be acceptable.

For each biological replicate, triplicate samples were run using SYBR Green Mastermix (Life Technologies, Carlsbad, CA, USA) with reactions as follows: 8 µL sterile water, 10 µL SYBR Mastermix, 250 nM forward and reverse primers, and 1 µL cDNA. The qPCR reactions were prepared in 96-well plates (Applied Biosystems, Foster City, CA, USA) and run on an ABI 7500 Real-time PCR System (Applied Biosystems). Thermocycler conditions were as follows: 50°C for 2 min, 95°C for 10 min, and then 45 cycles of 95°C for 15 s and 60°C for 1 min. Melt curves were run with every reaction to detect the occurrence of any nonspecific amplification. No template controls were run in triplicate for each qPCR assay on each plate. A single T_0 time point sample was used as a calibrator for calculating fold changes in each experiment. Fold changes were calculated using Relative Quantification software (Applied Biosystems).

Shipboard incubations. Whole water incubations were conducted at St. ALOHA (22° 45′ N, 158° 00′ W) in August 2010 during KM1016. Triplicate 4 L flasks were incubated with P, Fe and P+Fe treatments, alongside controls (no addition). About 1 µM of K_2HPO_4 and 2 nM of $FeCl_3$ were added to P and Fe treatments, respectively. Along with an initial (T0) sample at the start of the incubation, RNA was taken at two additional sampling points: 24 and 36 h after T0. At these two timepoints, 2 L of seawater from each bottle was pre-filtered through 10 µM filter and then a 2 µM filter, which would later be extracted for RNA. Genes shown to have significant response to P-stress in previously conducted culture

TABLE 1. *Crocosphaera watsonii* WH8501 specific primers for phosphate metabolism genes.

Gene	Association	QwaDRAFT	NCBI GI	Forward primer (5'→3')	Reverse primer (5'→3')	Amplification efficiency[a]
pstS	Phosphate substrate binding protein	4928	67921475	TTGTTGCAACTCAACACAGCA	TTCGGATCATTCCAGTTGGT	105%
sphX	Phosphate substrate binding protein	5160	67921474	CAGCAACACCAAGATAAACTCAAAG	GAAACAAAGGACGGGGATAAAGG	105%
arsB	Arsenate resistance protein	5214	67921397	GTYGTGGCCGAGACAATTAGACG	TTCGTCGTTGCCACTGGTTG	101%
sqdB	Sulfolipid biosynthesis protein	5287	67920767	CGAGGCCACTGATCTAAACCAAGG	CGCCGTACCAAAGACAACCATC	102%
phoH	Unknown	4435	67922063	TCCGAAACCCTGCAATTACC	GACCAAGTTAGCTCCCGTATGAC	106%
phoU	Negative regulator	5911	67920826	TCGGATGATGCTTACGAGGAAC	GCGTGAATCAGCCATTCTTTC	100%
phoA	Alkaline phosphatase	1549	67924612	CACCGCTGATCGCTAACTTG	ATTCGTACCCGCTTCTTGTTCC	105%
phoB	Transcriptional regulator	2775	67923615	AACCGCTCGGATGTTCGTATTCG	TCCAAACCGATAACGCAAAGC	101%
ppK	Polyphosphate kinase	6491	67920515	GTYGGTGGTTCGTGATCGTG	AGGTGTTTGGTCGTCGTTCCTTG	101%
ppX	Exopolyphosphate	1948	67924239	CGTTCCCTAGCTTCTTTTACGAC	GTCGATCGCCAACTTCGTCGCTTG	106%
ppA	Inorganic pyrophosphatase	2235	67924135	CGGGTTTGTGTCATTGCAG	AGCGTGGGATCCTTCATCAGG	95%
rnpB[b]	RNase	4794	67856398	GACTCCCGAAAGATCCAGACTTG	GTTTACCGAGGAGCAGTMCGCTCAC	100%

[a] All primers had an amplification efficiency of ± 6%.
[b] *rnpB* is used as a housekeeping gene.

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starvation experiments were screened in each sample. The field products were not sequenced in this study.

RESULTS

Growth of P-stressed cultures. In batch culture experiments, *C. watsonii* WH8501 cells were exposed to P-replete and P-deficient conditions. Twenty-four h after transferring cells into low-P seawater medium, the specific growth rates for day 1 in the two treatments were comparable: 0.34 ± 0.02 d^{-1} in P-replete and 0.31 ± 0.03 d^{-1} in P-deficient cultures. Control cultures grew with an Fv/Fm ~0.4, while cells subjected to low-P had a 20% decline in Fv/Fm within 24 h (Fig. 1B). As the experiment continued, growth rates of cells in P-replete cultures surpassed that of P-deficient cultures, which entered stationary phase. The Fv/Fm in P-deficient cells steadily declined and was 16% of P-replete cells by the end of the experiment (96 h). While both cultures started at a biomass of 5×10^4 cells · mL^{-1}, the final biomass in P-replete cultures was 2.4 times higher than in P-deficient cultures (Fig. 1A).

Select genetic response to P-limitation. We tested transcription of 11 genes affiliated with mediating the P-stress response, such as those involved in gene regulation, cellular protection, P acquisition and substitution (Table 1). PCR amplification efficiencies for each primer set were within ±6% of *rnpB* (Table 1), signifying that every GOI amplified approximately equally with the endogenous control. The transcription of each GOI in P-deficient cultures relative to P-replete cultures, or fold change, was analyzed.

Sampling from the middle of a P-starvation batch culture experiment showed 2 genes were significantly up-regulated with a fold change >2, while all other genes responded with a fold change <2 (Fig. 2). The gene transcription of both *pstS* and *arsB* rose significantly in P-deficient cultures, 5-fold and 11-fold, respectively. Other GOI's were not up-regulated with a fold change >2 at any other time point sampled during the P-stress experiments (data not shown).

Characterizing biomarkers of P-status. While *pstS* and *arsB* were shown to respond to P-stress, we wanted to further characterize this response to evaluate their efficacy as biomarkers in *C. watsonii*. High-frequency sampling was conducted to get a more accurate resolution of the sensitivity and variability in each gene's response during batch culture P-starvation experiments. *pstS* responded quickly to initial phosphate stress; a significant difference was detected as early as 1 h after cells were transferred to low-P media (Fig. 3). In contrast, transcription of *arsB* was up-regulated only after 9 h and transcription peaked 33 h after the initial transfer (Fig. 3). High-resolution sampling revealed that *pstS* exhibited strong diel regulation in both P-replete and P-deficient cultures. Despite this, it maintained elevated transcription due to P-stress and *pstS* transcript abundance peaked at 29 h. Although *pstS* was not significantly up-regulated in P-deficient versus P-replete cultures after 71 h, *arsB* continued to be up-regulated well after *pstS* transcription declined.

Diel regulation of pstS. *pstS* transcript abundance had a cyclic pattern of transcription in healthy

Fig. 1. Growth and photosynthetic efficiency of *Crocosphaera watsonii* WH8501. Cell cultures grown in phosphate replete (60 μM; closed symbols) and phosphate depleted (60 nM; open symbols) media. Cell counts (A) taken for cultures starting at T0 and photosynthetic efficiency (Fv/Fm) (B) measured during the same experiment. All error bars indicate standard deviation of triplicate biological replicates.

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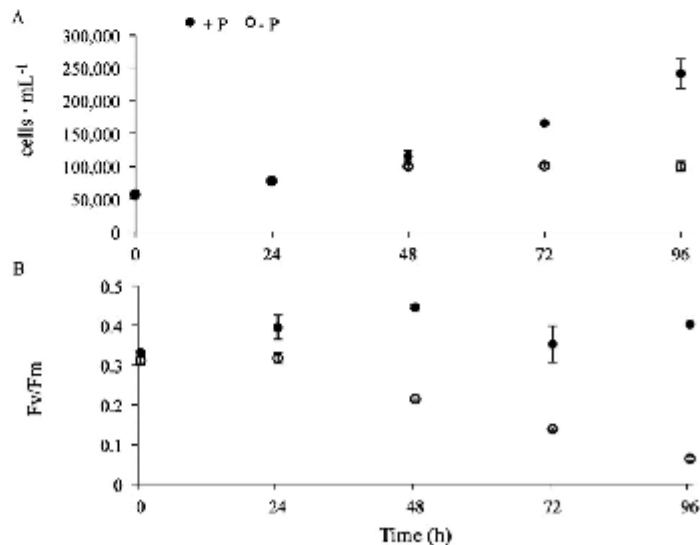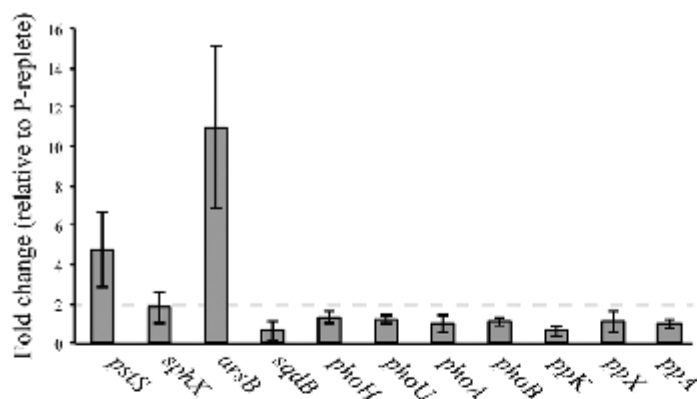
Fig. 2. Transcription of six genes relative to reference gene *rnpB* and secondarily normalized to P-replete cultures. Genes with a fold change between P-deplete/P-replete >2 were considered significantly upregulated. Error bars indicate standard error of triplicate biological replicates.
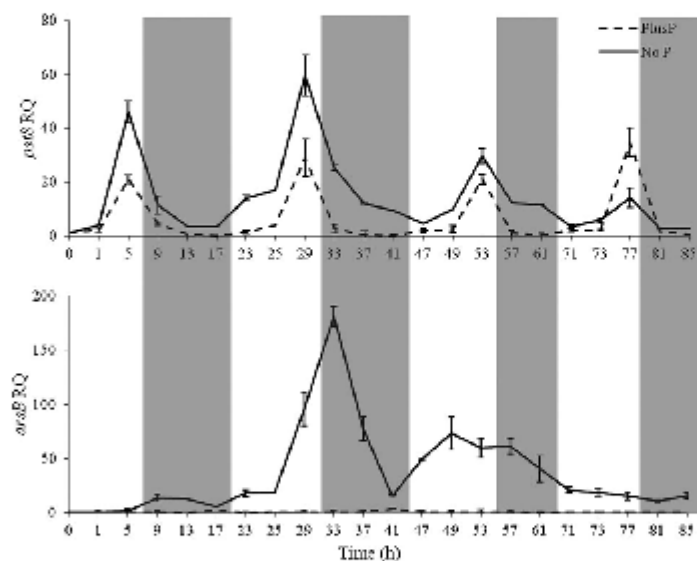
Fig. 3. Transcription profiles of *pstS* and *arsB* during a batch culture experiment where phosphorus is limited. *Crocosphaera watsonii* WH8501 cultures grown over 85 h and sampled approximately every 4 h during the light and dark (shaded area) cycles. Relative Quantification (RQ) of each gene is normalized to a reference gene, *rnpB*, and time zero time point (T0). Error bars indicate the standard deviation of triplicate biological replicates.

P-replete cells, which was enhanced in cells growing under P-deficiency. Since daily expression of *pstS* peaked at late-light phase (L11) in both the control and treatment cultures (Fig. 4), the resulting fold change (−P/+P) was lowest (~2) at that time. The highest fold change (~100) was displayed at late dark phase (D11), when *pstS* transcription was elevated in P-deficient cultures, but low in P-replete cultures. Overall, we found that fold change was higher in the dark period (D3 – D7) than in the light period (L5 – L11), but varied greatly depending on relative transcription in control cultures.

Biomarkers in natural populations of Crocosphaera. Using incubation experiments at St. ALOHA, natural populations of *C. watsonii* were screened for *pstS* and *arsB* transcription. After 24 h, *pstS/arsB* >1 in all treatments: Plus P, Plus Fe, Plus P + Fe, and the no addition control (Fig. 5). At this time, transcription of *pstS* was elevated in all bottles, while transcription of *arsB* was not detected. 39 h later, *arsB* transcript abundance was detected at a significantly higher level than *pstS* in both the control and Plus Fe bottles, presumably where P concentrations were reduced. However, *pstS/arsB* remained >1 in Plus P and Plus P + Fe treatments.
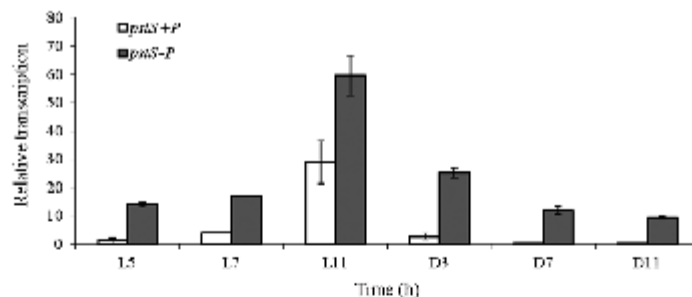
Fig. 4. Daily gene expression of *pstS* in a batch culture experiment. *Crocosphaera watsonii* WH8501 cultures grown in P-replete and P-depleted media. Relative transcription of each gene indicates the fold change between the sample expression and the zero time point, normalized to housekeeping gene *rnpB*. Samples were taken at intervals during light (L; 5, 7 11 h) and dark (D; 3, 7, 11 h) phases within a 12:12-h light-dark cycle. All error bars represent standard deviation of triplicate biological replicates.
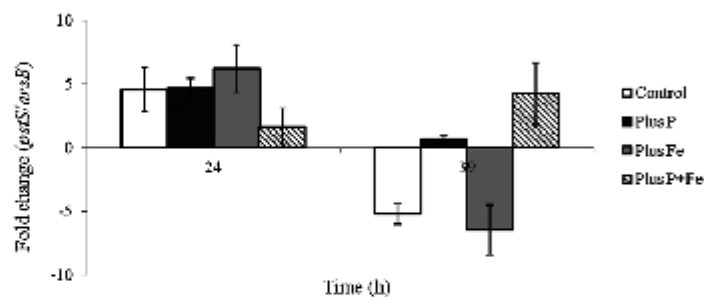
Fig. 5. Natural samples of *C. watsonii* during an incubation experiment at St. ALOHA. Gene expression showing the fold change between *pstS* and *arsB* in control (no addition) and treatment bottles: Plus P (1 µM K₂HPO₄), Plus Fe (2 nM FeCl₃), and Plus P+Fe. All error bars represent standard deviation of triplicate biological replicates.

DISCUSSION

When cells progress toward nutrient-limited growth, they respond with adaptations that allow them to maintain growth under stressful conditions. In this study, the transcription levels of genes known to be involved in phosphorus metabolism in cyanobacteria were assayed for responses in *C. watsonii* WH8501. Genes involved in phosphorus acquisition and transport, regulation, alternative metabolism, and substitution were all tested in P-starvation experiments using qPCR (Table 1). In these experiments, the decline in photosynthetic efficiency (Fv/Fm) demonstrates that *C. watsonii* is sensitive to decreasing P concentrations (Fig. 1B).

Interestingly, transcription of the genes for DOP metabolism, both intracellularly (*ppK*, *ppX* and *ppA*) and extracellularly (*phoA*) was not significantly upregulated during phosphorus limitation, at least for the duration of this study. Intracellular cycling of polyphosphates is an adaptation used by other marine cyanobacteria, such as *Synechococcus* (Gomez-Garcia et al. 2003) and *Trichodesmium* (Orchard et al. 2010) under low-P conditions. The increasing

activity of phosphatases that enable cells to use alternative extracellular DOP sources in response to P-stress, indicated by the transcription of *phoA* or its protein product, has been extensively used as a biomarker (Moore et al. 2005, Orchard et al. 2009, Munoz-Martin et al. 2011). Transcription of the *phoA* and *sphX* genes also did not increase under P-limitation in another study with *C. watsonii* WH8501 (Dyhrman and Haley 2006). It is possible that the internal phosphorous reserves prevented the cells from being under strong P-stress because the cells were grown with replete DIP before being transferred into the medium with no added DIP.

Another commonly used adaptation to phosphorus stress in cyanobacteria is the substitution of sulfolipids for phospholipids. Multiple species of *Synechococcus*, *Prochlorococcus*, and *Trichodesmium* were found to have a higher sulfolipid to phospholipid ratio in phosphorus-limited cultures, however the ratio was not significantly different in *C. watsonii* (Van Mooy et al. 2009). In parallel to these results, we did not see a significant response in the gene regulating the sulfolipid biosynthesis protein (*sqdB*) (Fig. 2). This inconsistent response between *C. watsonii* and other cyanobacteria to P-stress suggests

heterogeneity in the strategies of organisms that compete for bioavailable phosphorus.

Of the genes that were screened, there were two that exhibited a significant response to DIP depletion: *pstS* and *arsB* (Fig. 2). Similar to many marine cyanobacteria (Scanlan et al. 2009), the genome of *C.watsonii* WH8501 has three copies of the *pstS* gene: CwatDRAFT_4928 (*pstS*), CwatDRAFT_5160 (*sphX*), and CwatDRAFT_6534 (*pstS2*). The genes share little similarity among them (21%–24% amino acid identity): (Bench et al. 2013). Transcription of *sphX* did not significantly change under P-stress for the duration of this study (Table 1). The *sphX* gene is located 334 bp upstream of the *pstS* gene in the opposite orientation, and the lack of transcription regulation by DIP availability indicates that regulatory regions in the *pstS* promoter are not used for regulation of *sphX* transcription. Transcription of the *pstS2* gene was not analyzed in this study. The *pstS* gene (CwatDRAFT_4928), with observed significantly up-regulated transcription in response to P-deficiency, is part of the only complete *pstSCAB* operon (Dyhrman and Haley 2006) which encodes the genes for the high-affinity phosphate transport system. Notably, *Crocosphaera* genomes of the small-cell type (like WH8501) have 3–4 copies of the *pstS* gene, while genomes of the large-cell types have 5–7 copies. It is possible that in the large-cell types, transcription of other *pstS* genes is also regulated based on the DIP availability.

Transcription of both *pstS* and *arsB* in *C. watsonii* was characterized with high-resolution sampling during phosphorus starvation experiments (Fig. 3). High-affinity phosphate transport may be an early response used by *C. watsonii*, since increased transcription of *pstS* was detected within 1 h of cells exposed to low-P (Fig. 3). This highly sensitive response to changing extracellular P is detected prior to physiological changes within the cell; photosynthetic efficiency declined after 24 h and growth rates decreased after 48 h (Fig. 1). Natural populations of *C. watsonii* may temporarily express *pstS* as a response to intermitant changes in environmental [P], without experiencing changes in growth rate or photosynthetic efficiency. The peak of *arsB* transcription under P-stress is slightly shifted from that of *pstS*, possibly in response to the influx of arsenate due to a lower P:As ratio. While *C. watsonii arsB* can also be directly upregulated by [As] >30 nM (Dyhrman and Haley 2011), this condition does not occur in the marine environment, where [As] ranges from 10 to 20 nM (Cutter et al. 2001, Cutter and Cutter 2006); however, the internal cellular [As] is unknown. Neither marker singularly captures the entire phosphorus stress response; at late P-limitation, the fold change in *pstS* declines, even as stress increases, while transcription of *arsB* is up-regulated after a period of exposure to low-P. Yet, used in tandem, these markers can effectively evaluate the P-status of *C. watsonii*. Both *pstS* (Cwat-

DRAFT_4928) and *arsB* genes are highly conserved (99%–100% nt identity) among *C. watsonii* strains, at least among the six strains isolated from multiple oceanic regions and with the genomes sequenced. Thus, the qPCR primers designed in this study can be used for examining the cellular P-status in natural *C. watsonii* populations.

We also found that regulation of *pstS* is different in *C. watsonii* compared to other cyanobacteria (Scanlan et al. 1997), where increasing levels of *pstS* reflects increasing stress. Although *pstS* is up-regulated immediately when external DIP falls below a threshold concentration, *pstS* transcript abundance does not consistently correlate with increasing phosphorus stress, rather it exhibits a strong diel pattern. The peak of *pstS* expression corresponds with the end of the light cycle before S-phase, which occurs in the dark in *C. watsonii* (Dron et al. 2012) and may reflect an increasing cellular requirement for phosphorus during replication. There are other instances where P-stress response is uncoupled from exogenous DIP supply. In *Synechocystis*, phosphorus starvation response can be triggered under high light, when growth rate surpasses the rate of phosphorus assimilation (Bhaya et al. 2000). Basal *pstS* transcription raises the question of alternative regulation of this gene outside of the P-stress response (Esteban et al. 2008). The variable background levels of *pstS* in *C. watsonii* make it difficult to identify whether this organism is responding to phosphorus stress from *pstS* expression alone; *pstS* transcripts may be detected in cells that are not under phosphorus stress, depending on the time of day. Since the detection of *pstS* is a common tool for predicting phosphorus stress in natural populations of picocyanobacteria (Scanlan et al. 1997, Fuller et al. 2005, Hung et al. 2013), this finding has implications for the use of *pstS* as an environmental biomarker for *Crocosphaera*. When working with natural populations, late dark phase (D7–D11) is the ideal time to sample for *pstS*, since diel expression is lowest at that time (Fig. 4).

Shipboard incubation experiments were conducted to test transcription of *pstS* and *arsB* genes in the environment. Since Fe and P are the major limiting nutrients for nitrogen-fixing microorganisms (Mills et al. 2004), the experiments were conducted with both Fe and P additions. We found that in natural populations of *C. watsonii*, using the combination of *pstS* and *arsB* gene expression differentiates between early and late P-limitation. As expected, the ratio of *pstS/arsB* transcript abundance >1 after 24 h in all treatments, even in bottles treated with phosphate (+P; +P+Fe), due to basal transcription. Fe was added to shipboard incubations to facilitate the depletion of DIP in treatment bottles. In bottles without added P (control; +Fe), basal transcription of *pstS* makes it difficult to differentiate any increased mRNA synthesis that cells may produce due to early P-stress. However, after 39 h,

the *pstS*/*arsB* <1 in bottles without added P, suggests a transition of cells to elevated levels of P-stress. This corresponds to an increase in *pstS* transcription seen in cultures of *C. watsonii* initially exposed to low-P to a shift of increasing *arsB* transcription at a later stage of P-stress (Fig. 3). This shift does not occur in bottles treated with P (Fig. 5). This illustrates consistency between the response of environmental and cultured *C. watsonii* to P-limitation and provides evidence of how *pstS* and *arsB* can be used to interrogate the P-status of natural populations.

Often, a single biomarker is used to identify stress in an organism. Here, we show the need for a more comprehensive approach, with the use of multiple biomarkers that lead to a more accurate analysis of P-status. The transcriptional response of *pstS* and *arsB* indicate different phases of phosphorus stress. In the environment, detecting *arsB* transcripts would suggest that cells are experiencing a higher level of phosphorus stress. Although it is widely used as a biomarker, cyclic basal regulation of *pstS* in *C. watsonii* indicate possible alternative regulation and make it unreliable as a sole indicator of P-stress in this open ocean diazotrophic organism. When using these markers to interrogate field populations, we recommend sampling at night (6 h before sunrise) if possible, to coincide with the lowest level of *pstS* basal transcription. During other sampling times, *pstS* diel transcription will need to be considered. Future experiments that define how rapidly *pstS* responds to added phosphorus could be used to infer P-status in environmental populations of *C. watsonii* by assessing changes in transcript abundance. While our study highlights potential markers, further experiments would be required to show that their transcription is not also affected by conditions other than P-deficiency. In *Synechocystis* sp. PCC 6803, *pstS* had no significant change in response to Fe deficiency or reconstitution (Singh et al. 2003), however no comparative study has been done in *Crocosphaera*. In addition, studies using semi-continuous cultures would provide useful information about the response of these genes under long-term P-stress. The results of this paper provide one step toward the full evaluation of both genes as markers for use in the environment and emphasize the complexity of gene transcription under stress.

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CHAPTER 2: THE USE OF THE PHOSPHORUS-REGULATED GENE, *PSTS*, AS AN INDICATOR FOR PHOSPHORUS STRESS IN THE MARINE DIAZOTROPH *CROCOSPHAERA WATSONII*

Abstract

The marine diazotroph *Crocosphaera watsonii* provides fixed carbon (C) and nitrogen (N) to open ocean regimes, where nutrient deficiency controls productivity. The growth of *Crocosphaera* itself can be limited by low phosphorus (P) concentrations in these oligotrophic environments. Biomarkers such as the phosphate transfer gene, *pstS*, are commonly used to monitor when such organisms are under P stress, however transcriptional regulation of these markers is often complex and not well-understood. In this study, we have interrogated changes in *pstS* transcript levels in *Crocosphaera watsonii* cells under P starvation, and in response to added dissolved inorganic phosphorus (DIP), dissolved organic phosphorus (DOP), and changing light levels. We found that *pstS* transcript levels in *C. watsonii* WH8501 were elevated at DIP concentrations less than 60 nmol L^{-1}. Transcript levels were suppressed by both inorganic and bioavailable organic phosphorus, however the P stress response was more sensitive to DIP than DOP sources. Increasing light intensity induced *pstS* transcription independently of low external P, and seemed to exacerbate the physiological effects of P stress. The variable response to different phosphorus compounds and independent influence of light on *pstS* transcription suggests that it is not a reliable predictor of P limitation in *Crocosphaera*.

1. Introduction

Diazotrophic cyanobacteria that fix dinitrogen gas (N_2) are important in areas where nitrogen (N) limits primary productivity, since they supply fixed bioavailable N to these regions (Montoya et al. 2004). *Crocosphaera watsonii* is a unicellular diazotroph that inhabits oligotrophic regions (Webb et al., 2009), and was first cultivated from tropical Atlantic Ocean surface water over 20 years ago (Waterbury et al. 1988). Because diazotrophs such as *Crocosphaera* can use an abundant form of fixed inorganic N, N_2, their growth is limited by the availability of other nutrients. Iron (Fe) and phosphorus (P) are essential nutrients required for the growth of marine microorganisms and known to be important for diazotrophs, however the complex factors controlling N_2-fixing populations in the environment remain poorly understood (Sanudo-Wilhelmy et al. 2001, Mills et al. 2004, Dekaezemacker and Bonnet 2011). Acquisition of P is critical for growth of organisms in nutrient-limited regions since it is a major element in biomolecules such as lipids and nucleotides (Karl, 2014). Dissolved inorganic phosphorus (DIP) is the most readily accessible source of P for cyanobacteria, but DIP concentrations are highly variable in the marine environment (Bjorkman and Karl 2003), ranging from 0.2-100 nmol L^{-1} in surface waters of the North Atlantic and Pacific Oceans (Wu et al. 2000, Cavender-Bares et al. 2001, Karl 2014). Dissolved organic P (DOP) can also serve as a source of P for microorganisms, but much of the marine DOP pool has yet to be fully characterized (Young and Ingall 2010). Unlike other cyanobacteria, *Crocosphaera* is unable to utilize phosphonates as an organic P source, which often comprise up to

25% of the marine DOP pool (Clark et al. 1998). However, *Crocosphaera* can grow on phosphomonoesters, and its genome contains genes for alkaline phosphatase (Dyhrman and Haley, 2006; Bench et al., 2013).

Cyanobacteria in oligotrophic marine environments have a variety of strategies that allow them to mitigate the effects of P limitation. For example, marine cyanobacteria, including the non-N_2-fixing picocyanobacterial groups *Prochlorococcus* and *Synechococcus*, as well as the larger filamentous diazotroph *Trichodesmium*, substitute sulfolipids for phospholipids under P stress, which reduces their cellular P demand (Van Mooy et al. 2006, Van Mooy et al. 2009). Interestingly, there is evidence that *Crocosphaera* does not use this strategy to reduce its cellular P requirement (Van Mooy et al., 2009; Pereira et al., 2016). In addition to reducing the cellular P demand, microorganisms use multiple mechanisms to assimilate DIP. When extracellular P is in excess, *Crocosphaera* uses the constitutively expressed phosphate inorganic transport (PIT) system to transport P into the cell (Dyhrman and Haley, 2006). Under P-limiting conditions, the phosphate-specific transport (PST) system uses ATP-mediated transport and contains a high-affinity binding protein (PstS) to meet P needs. Environmental populations of diazotrophs must increasingly compete for low ambient P, based on the predicted shift toward a decline in P supply in future open oceans (Karl et al. 2001, Talarmin et al. 2016). The ability to rapidly respond to small changes in P fluxes by synthesizing high affinity Pi uptake systems provides an ecologically competitive edge to microorganisms in these oligotrophic marine environments (Degerholm et al. 2006). Increasing uptake affinity for external

DIP via PstS is a key strategy used by *Crocosphaera* under P stress, since it does not utilize phosphonates or sulfolipids as do other cyanobacteria. Interestingly, some *Crocosphaera* strains have up to seven copies of the *pstS* gene (Bench et al. 2013).

Commonly used as a biomarker for P stress in natural microbial populations (Fuller et al. 2005, Hung et al. 2013), *pstS* transcript abundance is considered to be a sensitive metric of deficiency and adaptive responses to P availability. Molecular biomarkers such as *pstS* have an advantage over bulk measurements of nutrient stress, since they can be used to determine when and where P availability limits the growth of specific N_2-fixing microorganisms, such as *Crocosphaera*. *pstS* transcription in a model freshwater cyanobacterium is regulated by P availability, and is activated when orthophosphate (Pi) falls below a threshold concentration that varies between organisms (Aiba and Mizuno 1994). It has been previously shown that transcription of *pstS* in *Crocosphaera* is up-regulated within 1 h of exposure to low external Pi (Pereira et al. 2016). However, *pstS* transcript levels have a diel cycle in *Crocosphaera*, which results in elevated transcript levels that do not necessarily quantitatively reflect a decrease in external P availability (Shi et al. 2010, Pereira et al. 2016). The peak of basal *pstS* transcript levels occurs at the end of the light cycle, just prior to DNA replication in *Crocosphaera* (Dron et al., 2012; Pereira et al., 2016). In *Prochlorococcus*, *pstS* has high basal transcript levels even in P-replete conditions, suggesting that *pstS* may be important for uptake even during periods of higher P availability (Reistetter et al. 2013). Thus, it is necessary to understand the effects of time of day, as well as external factors in order to accurately use *pstS*

transcript levels as a biomarker. Apart from low external Pi, abiotic factors such as DOP availability, light intensity, and pH can influence *pstS* expression (Bhaya et al., 2000; Harke et al., 2012; Fischer et al., 2006). Such indirect influences on *pstS* regulation can complicate its use as a biomarker for P stress.

This study evaluates the effectiveness of *pstS* as an indicator of P stress in *Crocosphaera watsonii*. Understanding how these factors affect transcription will help provide a framework to interpret the detection of *pstS* in the field under a variety of environmental conditions. Effective biomarkers for P stress must be P-specific, highly sensitive to bioavailable forms of P, and exhibit stable expression (Scanlan and Wilson 1999). We performed experiments designed to determine whether this marker is sensitive and specific to changes of bioavailable P sources and to the effect of light intensity. Our findings indicate that *pstS* is sensitive to changes in DIP and DOP concentrations, but the response varied for each P source. High light intensity also induced *pstS* transcription, suggesting that the regulation of this gene in *Crocosphaera* is not specific to low P conditions. The non-specific and variable transcriptional response of *pstS* under P stress indicates that this gene would not be a reliable indicator of *Crocosphaera* P status.

2. Methods:

2.1 Cell culturing

Crocosphaera watsonii WH8501 was grown in seawater-based media (SO; pH 8.0, salinity 28) prepared with 0.2 µm-filtered Sargasso Sea seawater, but without

added N (Waterbury et al. 1986). P-replete cultures were grown in SO medium with 60 μM K_2HPO_4, while P-deficient cultures were grown in SO medium without added Pi. Residual soluble reactive phosphate (SRP) in the seawater base used for SO medium was measured at 60 nmol L^{-1} using Magnesium Induced Co-Precipitation (MAGIC) (Karl and Tien 1992). Prior to experiments, *Crocosphaera* was grown at 27°C with 55 μmol quanta $m^{-2} s^{-2}$ in a 12:12 light/dark cycle. Cells used as inoculum for all treatments were grown with replete Pi and harvested with a 0.2 μm filter at mid-log phase from a single mother culture, then washed three times and re-suspended in medium without added Pi to limit P carryover.

Batch experiments were conducted with *Crocosphaera watsonii* WH8501 cultures under P-limited growth, re-fed with DOP, and at varying light intensities. In order to assess cultures under P stress, various metrics were measured over 5-6 day experiments: cell counts, [DIP], photosynthetic efficiency, and *pstS* transcript levels. Cells were initially inoculated into P-replete and P-deficient treatments. During the DOP re-feed experiment, replicate P-deficient cultures were re-fed with either glucose-6-phosphate (G6P; Sigma-Aldrich, Saint Louis, MO, USA) or DL-α-glycerol phosphate (GP; Sigma-Aldrich, Saint Louis, MO, USA) to a concentration of 50 μM. P (SRP) in the organic phosphorus reagents was found to be negligible (4 nmol L^{-1}) using MAGIC. During the light intensity experiment, six replicate cultures of P-replete and P-deficient treatments were initially grown in low light (LL) at 55 μmol quanta $m^{-2} s^{-2}$. After three days, duplicate cultures from each treatment were exposed to medium light (ML) at 180 μmol quanta $m^{-2} s^{-2}$ or high light (HL) at 450 μmol

quanta m^{-2} s^{-2} for the duration of the experiment.

2.2 Microscopy and photosynthetic efficiency

In each experiment, growth rates were determined using cell counts. Replicate samples of 1 mL aliquots from each treatment were filtered using a vacuum pump directly on 25 mm black polycarbonate, 0.22 µm pore filters (Poretics, Osmonics Inc., Minnetonka, MN, USA). Filters were mounted on glass slides (Fischer Sci., Pittsburgh, PA, USA) and cells were viewed with an Axioplan 2 Zeiss microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) at 4,000× magnification under blue excitation light. For each slide, cell counts were obtained by averaging counts from ten fields of view. During the light intensity experiment, cell size was additionally monitored. ImageJ software was used to estimate the diameter of at least 100 cells for each treatment. Photosynthetic efficiency (F_v/F_m) was also measured daily during every experiment. Aliquots of 5 mL from each treatment were assessed using a fast repetition rate fluorometer (FRRF) (Kolber et al. 1998). F_v/F_m is often used as a proxy for cellular health in primary producers (Beardall et al. 2001).

2.3 Alkaline phosphatase activity (APA)

Samples for APA were measured fluorometrically during the DOP re-feed experiment, as an additional proxy for phosphorus stress. Aliquots of 6 mL were filtered through 2.0 µm pore filters (Poretics, Osmonics Inc., Minnetonka, MN, USA) and resuspended in 2 mL artificial seawater medium (van Waasbergen et al., 1993).

To assay cell-associated APA, a modified version of the 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP, Life Technologies, Carlsbad, CA, USA) protocol was used (Dyhrman and Ruttenberg 2006). Briefly, 20 µL 1 mmol L^{-1} DiFMUP was added to the re-suspended sample, and was mixed by pipetting. Hydrolysis of DiFMUP to 6,8-difluro-7-hydroxy-4-methylcoumarin (DiFMU) was measured on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA), with seven readings taken on each subsample. Readings were initiated immediately after DiFMUP was added (T0) until around 120 minutes after addition, based on the activity of the samples. Activity was determined using a standard curve from 0 to 600 nmol L^{-1} DiFMU (Life Technologies, Carlsbad, CA, USA) in artificial seawater that was read simultaneously with the subsamples. Since volumetric APA is dependent on biomass (Karl et al., 2014), final APA measurements (nmol L^{-1} P/h/cell) were normalized to the number of cells in each treatment based on daily cell counts.

2.4 RNA extraction and quantitative PCR (qPCR)

RNA samples were taken from each experiment for relative quantitative PCR (qPCR) analysis. For each RNA time point, aliquots of 10 mL of each culture treatment were filtered through a 25 mm, 0.2 µm pore-sized Suporfilter (Pall Corporation, Port Washington, NY, USA) using gentle vacuum filtration. Filters were immediately placed in bead-beater tubes with β-mercaptoethanol and RLT buffer, then flash frozen in liquid nitrogen and subsequently stored at -80°C until extraction.

RNA extractions were carried out using a modified RNeasy Mini Kit protocol (Qiagen, Germantown, MD, USA) and converted to single stranded complementary DNA (cDNA) using the QuantiTect Reverse Transcription Kit (Qiagen) as previously outlined (Pereira et al. 2016). All cDNA was subsequently stored at -20°C.

qPCR primers for the *pstS* gene that have been previously characterized in *Crocosphaera watsonii* WH8501 were used (Pereira et al., 2016). For each biological replicate, duplicate samples were run using SYBR Green Mastermix (Life Technologies, Carlsbad, CA, USA) with reactions as follows: 8 µl sterile water, 10 µl SYBR Mastermix, 250 nmol L^{-1} forward and reverse primers, and 1 µl cDNA. The qPCR reactions were prepared in 96-well plates (Applied Biosystems, Foster City, CA, USA) and run on an ABI 7500 Real-time PCR System (Applied Biosystems) with a previously outlined run cycle (Pereira et al., 2016). The comparative ($\Delta\Delta Ct$) method was used to normalize the expression of *pstS* to a housekeeping gene (*rnpB*) previously used in *Crocosphaera*. A single T_0 time point sample was used as a calibrator for calculating fold changes in each experiment. Fold changes were calculated using Relative Quantification software (Applied Biosystems).

3. Results:

3.1 Characterization of P-limited growth

In P-deficient cultures, cell abundances decreased by 12% in comparison to P-replete cultures at 72 h when cells were entering into stationary phase (Fig. 1a). External DIP concentrations in P-deficient treatments were reduced to 20 nmol L^{-1} by

48 h and fell below the detection limit (15 nmol L^{-1}) by 96 h. Photosynthetic efficiency in P-replete cultures remained constant with an Fv/Fm of ~0.5 (Fig. 1b). There was a decrease of Fv/Fm in P-deficient treatments at 48 h, when photosynthetic efficiency was 12% lower in comparison to the control. Fv/Fm continued to decline in cells grown without further addition of P and reached lowest values (0.15) by 96 h. Initially (T0), *pstS* transcript levels were low and comparable to basal levels in both P-deficient and P-replete cultures (Fig. 1c). Within 24 h of inoculation, *pstS* transcript abundances increased over 2-fold in the P-deficient cultures. These elevated transcript levels were maintained from 24 h – 72 h as the P concentration continued to decrease, corresponding with [DIP] < 60 nmol L^{-1}. By 96 h, growth had ceased in P-deficient cultures and *pstS* was reduced to near basal levels, comparative with control cultures.

Crocosphaera WH8501 was also able to grow on phosphomonoesters as a sole source of added P. In a separate experiment, we tested growth on two alternative dissolved mono-phosphorus sources: GP and G6P. In the exponential phase, the growth rate of Pi-replete controls (0.39 ± 0.01 d^{-1}) were comparable to those in GP (0.37 ± 0.03 d^{-1}) and G6P (0.36 ± 0.03 d^{-1}) treatments (Fig. 1d).

3.2 Response to P re-supply

P re-supply experiments were conducted with both DIP and DOP additions in order to determine how quickly P additions influence *pstS* transcript levels, photosynthetic efficiency, and alkaline phosphatase activity (APA). Normalized *pstS* transcript levels in P-deficient cultures were initially 3-fold higher (0 h) than in P-replete cultures (Fig. 2a). Transcript levels of *pstS* in P-deficient *Crocosphaera*

cultures re-fed with DIP were significantly reduced ($p<0.2$) after 2 h compared to those in P-deficient cultures, and remained lower in all subsequent samples taken within 24 h. Photosynthetic efficiency in both P-replete and P-deficient cultures was ~0.5 at 24 h, but Fv/Fm in P-deficient treatments was significantly reduced compared to control cultures at 48 h and continued to steadily decrease until 120 h (Fig. 2c). We observed a higher Fv/Fm in re-fed treatments that was significantly different from that in P-deficient cultures 6 h after DIP addition. The photosynthetic efficiency in re-fed treatments was comparable to control cultures and remained elevated at Fv/Fm ~0.5 for the remainder of the experiment.

$\quad$ *Crocosphaera* cultures under P stress were also re-fed with DL-α-glycerol phosphate (GP) and glucose-6-phosphate (G6P) as DOP sources. There were no significant differences in responses of any of the parameters measured between cultures with the two DOP sources used for re-feeding. When P-deficient treatments were re-fed with DOP, *pstS* transcript levels were initially 2-fold higher than P-replete cultures (0 h) (Fig. 2b). Unlike DIP re-fed treatments, *pstS* transcript levels remained elevated at 2 h, 4 h, and 6 h in both DOP re-fed and P-deficient treatments. Transcription of *pstS* was significantly ($p<0.5$) down-regulated in both GP and G6P re-fed treatments at 10 h and remained reduced 24 h later. Similar to the DIP addition experiment, photosynthetic efficiency in P-replete and P-deficient cultures was ~0.5 at 24 h prior to the DOP addition (Fig. 2d). At 48 h, Fv/Fm was reduced in P-deficient cultures as well as DOP re-fed treatments compared to the control. Fv/Fm increased in re-fed treatments at 72 h, which was 30 h after the DOP addition. The

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photosynthetic efficiency continued to increase in re-fed treatments until the end of

the experiment, while steadily declining in P-deficient cultures. APA was also

measured as another metric of P stress during the DOP addition experiment. Initially

(T0), APA in P-deficient cultures was comparably low to that in control cultures.

After 24 h, APA had significantly increased in P-deficient treatments (Fig. 3). APA

incrementally decreased in DOP added treatments, and was significantly reduced

after 48 h. APA in re-fed treatments had returned to basal levels comparable to P-

replete cultures within 120 h.

3.3 Effect of changes in light intensity

To evaluate the effect of changing light intensity on photosynthetic efficiency,

pstS transcript abundances, and cell size in *Crocosphaera*, we analyzed P-replete and

P-deficient cultures at LL (55 μE m^{-2} s^{-1}), ML (180 μE m^{-2} s^{-1}), and HL (450 μE m^{-2} s^{-1}). Prior to the change in light levels at 72 h, P-replete treatments had a growth rate of

0.37 and P-deficient treatments had a similar growth rate of 0.38 (Fig. 4a). After the

transfer to higher light levels, the reduction of growth in P-deficient cultures,

indicated by biomass, was significantly lower in HL treatments compared to the LL

control at 120 h. At this time point, cells in P-deficient treatments at all irradiance

levels were in stationary phase. Similarly, both P-replete and P-deficient treatments

had similar photosynthetic efficiencies prior to the light increase (Fig. 4b). Six hours

after the light increase (72 h), Fv/Fm was significantly reduced in ML and HL

cultures compared to LL. Fv/Fm continued to decline in P-deficient treatments at all

irradiances until 144 h. The biomass and photosynthetic efficiency of P-replete treatments at all light intensities was comparable, with cells at higher light intensities exhibiting no increase in growth rate or Fv/Fm within the time-frame of the experiment.

Changes in transcript abundances of *pstS* were different depending on irradiance levels. Prior to the light increase at 72 h, *pstS* transcript abundance remained 2-fold higher in P-deficient cultures compared P-replete cultures (Fig. 4c). Four hours after the light increase (76 h), *pstS* transcript abundances were still significantly higher in LL and ML treatments in comparison to HL. By 84 h, there was a decrease in transcript levels in ML as well as HL, and at 96 h *pstS* transcript levels in P-deficient treatments at all irradiances had declined. Interestingly, we observed a 4-fold increase of *pstS* transcription in HL treatments compared to LL treatments in P-replete cultures (Fig. 4d). No further increase was observed 12 h after the change in light intensity.

Another metric used to assess the effect of light intensity on P-deficient cells was cell size. Cell diameter in P-replete cultures ranged from 3.1 ± 0.2 μm, and was generally higher than that of P-deficient cultures, which ranged from 2.7 ± 0.1 μm. After the light switch, cell diameters of P-replete cells at HL significantly increased ($p<0.02$) to 3.5 μm compared to LL cells at 120 h – 168 h (Fig. 5a). In P-deficient cultures, no significant difference was observed between irradiance treatments after the light increase from 72 h – 168 h (Fig. 5b).

4. Discussion

N$_2$-fixing cyanobacteria play an important role in open-ocean ecosystems, yet evaluating the factors that control their abundances and activities *in situ* remains a fundamental challenge (Fu and Bell 2003, Kustka et al. 2003, Garcia et al. 2015). Methods for determining species-specific nutrient control are needed to more accurately model the biogeography of N$_2$ fixation in the ocean and predict consequences of environmental changes (Lindell and Post 2001). In this study, we assessed the potential use and limitations of the P-regulated biomarker, *pstS*, to indicate P stress in the marine diazotroph *Crocosphaera watsonii*.

In order to evaluate *pstS* as an indicator of P stress, it is important to assess at what point transcript levels of this gene are sensitive to the depletion of Pi. In an experiment where cultures were exposed to an external [Pi] of 60 nmol L^{-1}, *pstS* transcription in P-deficient treatments was not initially upregulated (Fig. 1a). However, *pstS* transcript levels were elevated between 20 to 60 nmol L^{-1}, as external [Pi] was further depleted in P-deficient treatments. A threshold near 60 nmol L^{-1} for *Crocosphaera* is similar to the threshold [DIP] for *pstS* in a marine *Synechococcus* strain, and suggests that it would often experience P limitation in the natural environment (Scanlan et al. 1997). In the North Pacific, where surface DIP varies between 6-100 nmol L^{-1} (Bjorkman et al. 2000, Grabowski et al. 2008) and in the North Atlantic, DIP can be as low as 0.2 nmol L^{-1} (Wu et al. 2000). *Trichodesmium*, another diazotroph with similar global distribution to *Crocosphaera*, has a Pho regulon threshold of 7 nmol L^{-1} (Orchard, 2009). A lower Pho regulon threshold for

Trichodesmium indicates that it may outcompete *Crocosphaera* at extremely low [Pi]. A study in the North Pacific subtropical gyre found that *Crocosphaera* abundances increased with [Pi], while *Trichodesmium* was more abundant at low [Pi] (Robidart et al. 2014). In our P starvation *Crocosphaera* culture experiments, we found that *pstS* continued to be up-regulated at low Pi concentrations, until a concentration of ~15 − 20 nmol L^{-1} was reached, when cells entered into stationary phase. Below 15 nmol L^{-1}, *pstS* transcript levels are reduced, suggesting that this gene is not up-regulated under late stages of P-stress in *Crocosphaera*, as previously observed (Pereira et al., 2016). Similarly, the Pho regulon in *Prochlorococcus* is activated below 100 nmol L^{-1}, when most P-stress genes were found to be up-regulated (Martiny et al. 2009), but cells are growth-limited at a [Pi] of 30 nmol L^{-1} (Parpais et al. 1996). The variation in thresholds of *pstS* transcription among cyanobacteria may be attributed to differences in cellular P requirements, which can be affected by cell size or even genome size (Bertilsson et al., 2003).

Transcription of *pstS* in *Crocosphaera* was highly sensitive to the changes in DIP availability. In P-deficient cultures, *pstS* transcription was upregulated within 24 h, before changes in growth rate or photosynthetic efficiency were observed (Fig. 1 a-c). Previously, it was found that *pstS* transcripts return to basal levels within 24 h when cells are exposed to higher levels of exogenous Pi (Dyhrman and Haley 2006). Here we observed the decrease in *pstS* transcript abundances within 2h upon resupplying DIP (Fig. 2a). The sensitivity of *pstS* transcription to availability of DIP enables cells to conserve energy, as the high-affinity transport of Pi is an ABC-type

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transporter that requires ATP. Rapid transcriptional responses, within 2 h of nutrient resupply, have been identified for genes in response to N, P, and vitamin B12 resupply in other prokaryotes (Helliwell et al. 2011, Nakagawa and Stahl 2013, Wurch et al. 2014).

Similar to DIP, availability of DOP affected *pstS* transcription in *Crocosphaera*; however the response to DOP was slower than to DIP. The exponential growth rates were not significantly different between treatments growing on DIP versus those growing on DOP sources suggesting that *Crocosphaera* can grow competitively with phosphomonoester as a sole organic P source. This could help them persist in areas of the ocean with elevated DOP:DIP, such as the western North Atlantic, where the majority of the dissolved phosphorus pool is comprised of DOP (Wu et al. 2000). As expected, we found that bio-available DOP does repress the transcriptional activity of *pstS* in *Crocosphaera*. However, there was a lag time between the decline of *pstS* transcripts in response to both GP and G6P additions (10 h) versus DIP addition (2 h) (Fig. 2b). Since bio-available DOP must first be hydrolyzed before the associated Pi molecules can pass through the cell membrane, it is a less direct route for P-acquisition than DIP. However, AP hydrolysis occurred rapidly, and DIP concentrations in re-feed treatments was estimated to be approximately 38 µM after 24 h. The DOP conversion can be catalyzed by alkaline phosphatases localized in the periplasm or extracellular, suggesting that cells can still use *pstS* to scavenge DIP even when bioavailable DOP is present (Karl 2014). A lag time in the recovery of photosynthetic efficiency in treatments re-fed with DOP

versus DIP (Fig. 2c-d) also suggests the delayed availability of Pi from DOP re-feed treatments. This delay in molecular response and physiological recovery supports the suggestion that Pi is the preferential P source for phytoplankton (Falkowski and Raven 2007).

In addition to a slower downregulation of *pstS* transcription and recovery of photosynthetic efficiency, there was also a lag time in APA reduction after DOP additions (Fig. 3). The reduction of APA in DOP re-fed treatments likely resulted from feedback repression due to enzymatic hydrolysis of the added organic phosphorus sources (Huang and Hong 1999). APA detection 24 to 48 h after the addition of organic phosphates was similar to observations from previous studies with *Trichodesmium* (Stihl and Sommer 2001, Orchard et al. 2009). Such prolonged detection of APA suggests that the enzyme takes longer to degrade and that its detection may not always coincide precisely with P-stressed cells. It is also possible that heterotrophic alkaline phosphatases contribute to the measured APA in our experiments, although we tried to minimize these influences by measuring cell-associated activity on 2 μm filtrates instead of bulk APA. As in previous studies with *Crocosphaera* WH8501, the putative gene for alkaline phosphatase (*phoA*) was not differentially detected in P-deficient treatments (Dyhrman and Haley, 2006; Pereira et al., 2016). Since the observed transcription of *phoA* does not align with APA, it is possible that there are other unidentified genes that contribute to the APA. The discrepancy may also be attributed to a difference in regulation between *phoA* and *pstS* in this organism, since upregulation of *phoA* transcripts was not detected during

this experiment.

Interestingly, *pstS* transcripts were not always specifically elevated by low Pi, and also increased in response to high light intensity. A previous study detected *pstS* in *Synechocystis* after 3 h of exposure to a light intensity of 500 µE m^{-2} s^{-}, with transcripts appearing to increase until 8 h when the last measurement was taken (Bhaya et al. 2000). We found that *pstS* transcription could be induced in P-replete *Crocosphaera* with a light intensity of 450 µE m^{-2} s^{-1}, but not at 180 µE m^{-2} s^{-1}. Although we did not observe an increase in growth rate of cells in higher light treatments during our short-term experiment, it has previously been shown *Crocosphaera* cultures growing at higher light intensities have a shorter doubling time (Goebel et al. 2008). An increase in metabolic activity at the onset of high light, associated with increasing growth rates, may outstrip P-acquisition. In HL treatments, *pstS* was significantly up-regulated at 4 h compared to the LL control, but relative transcription decreased again at 12 h (Fig. 4c). It is possible that a few hours exposure to HL conditions results in the activation of *pstS* as cells transition to a higher P requirement. The switch to higher light intensity occurred at L0 in the 12:12 light: dark cycle. Thus, an increase in *pstS* transcripts corresponds with the growth and mitosis phases of the cell cycle, which occurs throughout the light phase in *Crocosphaera* (Dron et al. 2012). This upregulation of *pstS* may indicate an increase in P acquisition before cell division occurs. After 12 h, at the end of the light cycle, *pstS* transcripts are downregulated to basal levels again. The short interval between HL exposure and *pstS* upregulation suggests that *pstS* is responding to the immediate

impacts of increased light intensity, rather than the impending physiological changes that indirectly influence the cellular P requirements (i.e. increased cell size and growth rate). Because the external DIP concentration was 50 μM, *pstS* response under HL must be induced either by alternate regulation from the Pho regulon or due to a cytoplasmic Pi sensor that detects a decrease in internal Pi (Auesukaree et al. 2004).

In addition to affecting cells in P-replete conditions, we found that higher irradiance negatively impacted cells already under P stress. P-deficient treatments switched to higher light intensity exhibited significantly lower Fv/Fm compared to LL cultures (Fig. 4b). There was an incremental decrease in photosynthetic efficiency in cells at LL, ML, and HL. Additionally, the reduction in *pstS* transcripts in P-starved cells as cells were moving into stationary phase occurred more rapidly at HL and ML than at LL (Fig. 4c). We also observed that the average diameters of exponentially growing P-replete cells were 20% larger in HL than LL treatments, similar to a previous study with different *Crocosphaera* strains WH0401 and WH0402 (Garcia et al. 2013). This significant increase in cell size is detected 48 h after cells were transferred from LL to HL (Fig. 5a). The molecular response in HL treatments we observed when *pstS* transcription is induced may be due to an increased P requirement that cells have prior to an increase in size. Interestingly, this size increase due to light intensity is not observed when cells are experiencing P limitation (Fig. 5b). Larger cells have an increased P requirement, as they have more phospholipids in their cellular membrane. It is possible that cells already under P stress do not meet the increased P quotas needed to increase cell size. Thus,

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Crocosphaera may be able to more efficiently respond to low P conditions at lower light intensities due to the exacerbation of P stress effects at high light intensities.

5. Conclusions

Molecular proxies for nutrient stress are used to indicate the species-specific nutrient status of environmental populations *in situ* (Lindell and Post, 2001). Our findings suggest that *pstS* may not be a reliable predictor of P stress for *Crocosphaera* in the environment, except in field studies under controlled conditions. The viability of this marker as an indicator for P stress in natural populations of *Crocosphaera* depends on its specificity, sensitivity, and stability. While *pstS* transcription in *Crocosphaera* is induced at a low Pi threshold and responds rapidly to fluxes of DIP and DOP, similar to other cyanobacteria, its response is not consistently specific to low P conditions. Although an increased *pstS* signal can reflect instantaneous P deficiency, it can also be indicative of increased light intensity. This complexity suggests that the regulation of *pstS* in *Crocosphaera* is mediated by more than just sensing of Pi, and that its detection in the environment may not always reflect P limitation of natural assemblages. *pstS* is widely used as a proxy for P stress, however our work indicates that it may not be an ideal marker for every organism, especially since studies like this have not been done in most marine organisms. To fill this research gap, laboratory experiments focusing on taxon-specific responses should be used in order to interpret environmental applications of biomarkers. Further studies

that expand our understanding of the molecular mechanisms regulating nutrient stress

response will help to identify appropriate biomarkers for future use.

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Figures
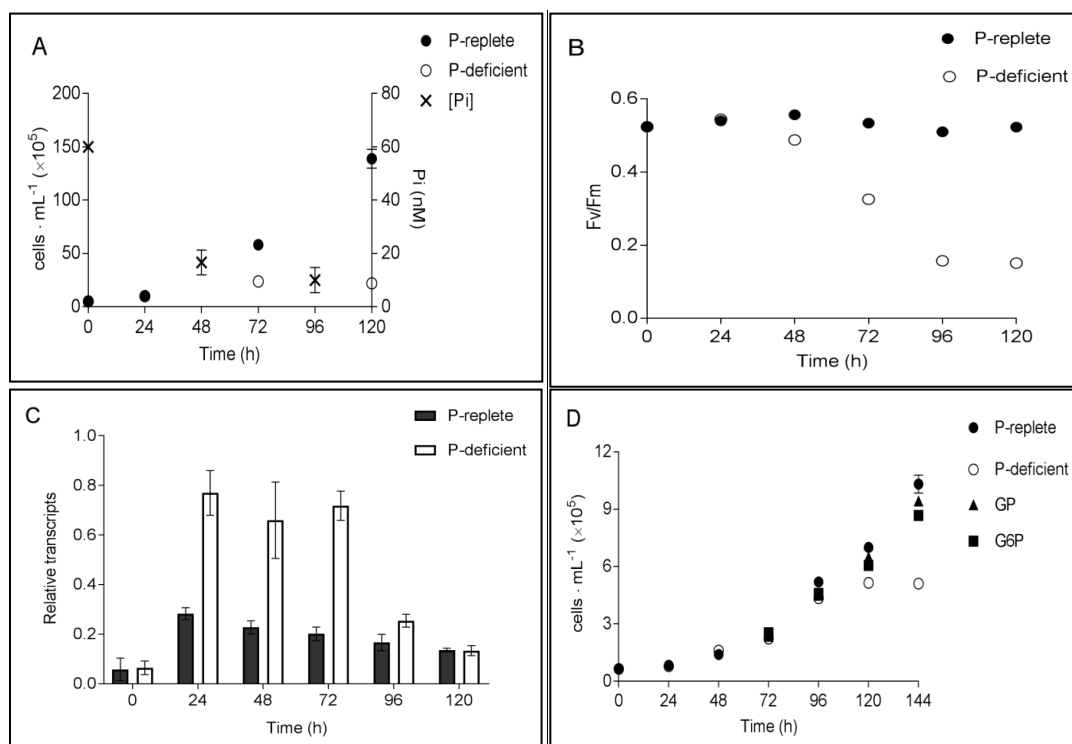
Figure 1. P stress response of *Crocosphaera watsonii* WH8501. Growth (A) and photosynthetic efficiency (B) shown in P-replete and P-deficient treatments. Pi concentrations in P-deficient treatments are also plotted at 0, 48 and 96 h. (C) *pstS* transcript levels in the same experiment in P-replete and P-deficient treatments. Transcripts of *pstS* were normalized to the housekeeping gene *rnpB*. Error bars represent the standard deviation of triplicate biological replicates for each treatment. (D) Growth of *C.watsonii* on two DOP sources, either GP (glyceraldehyde-3-phosphate) or G6P (glucose-6-phosphate) during a separate re-feed experiment.

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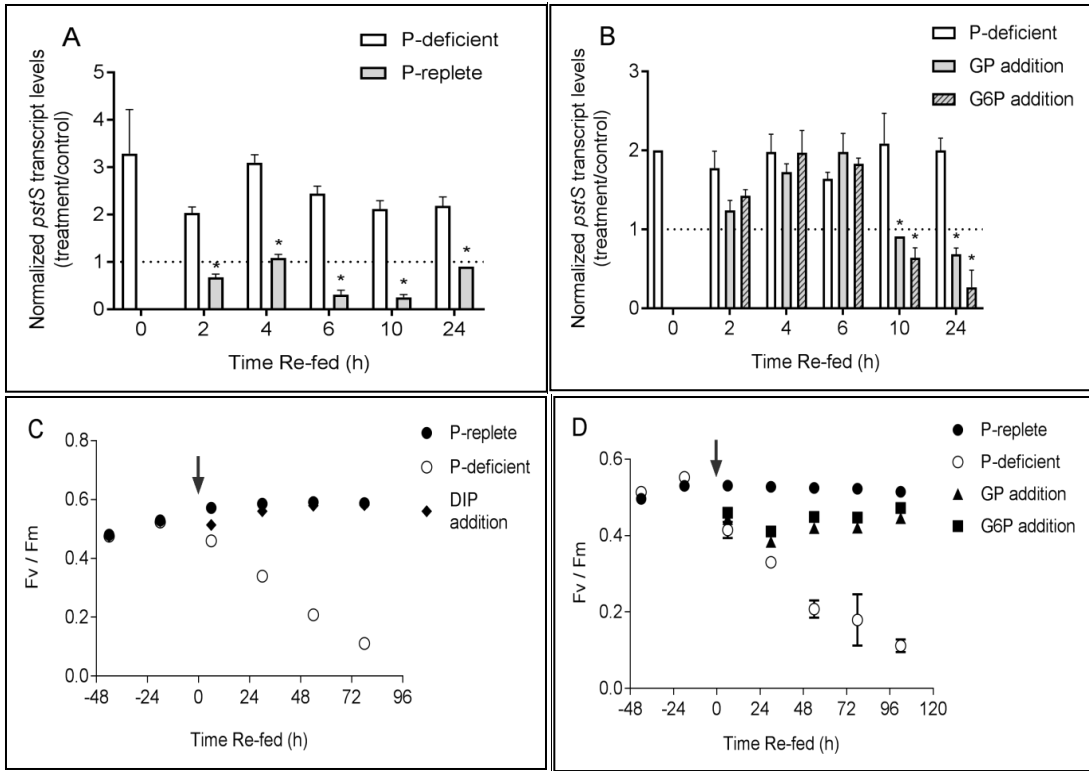
Figure 2. Transcript levels and photosynthetic efficiency in DIP (A and C) or DOP (B and D) re-feed experiments. *pstS* transcripts of either P-deficient or re-fed treatments shown as fold change, normalized to P-replete control cultures (A–B). Fold change of 1 (dotted line) represents transcript levels of P-replete control cultures. Arrows show where P-deficient treatments are re-fed, either with Pi (C) or DOP (D). An * indicates a *p*-value of less than 0.02 for the re-fed vs the P-deficient treatments at a single point in time for a paired *t*-test. Error bars indicate standard error of either duplicate or triplicate biological replicates.
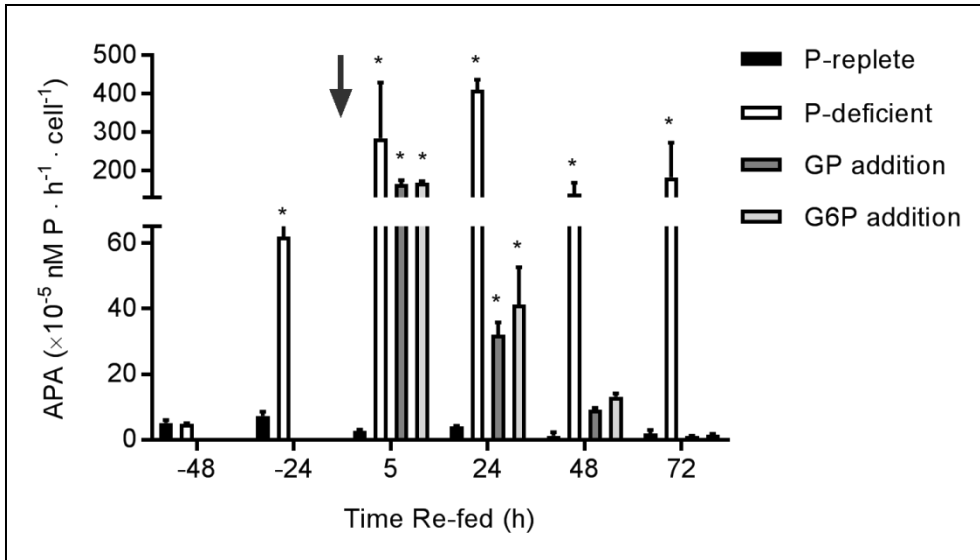
Figure 3. Effect of DOP additions on the APA activity. The arrow represents the point where P-deficient cultures were fed with 50 µM GP or G6P. An * indicates a $p<0.05$ for a paired t-test. Error bars indicate standard error of duplicate biological replicates.
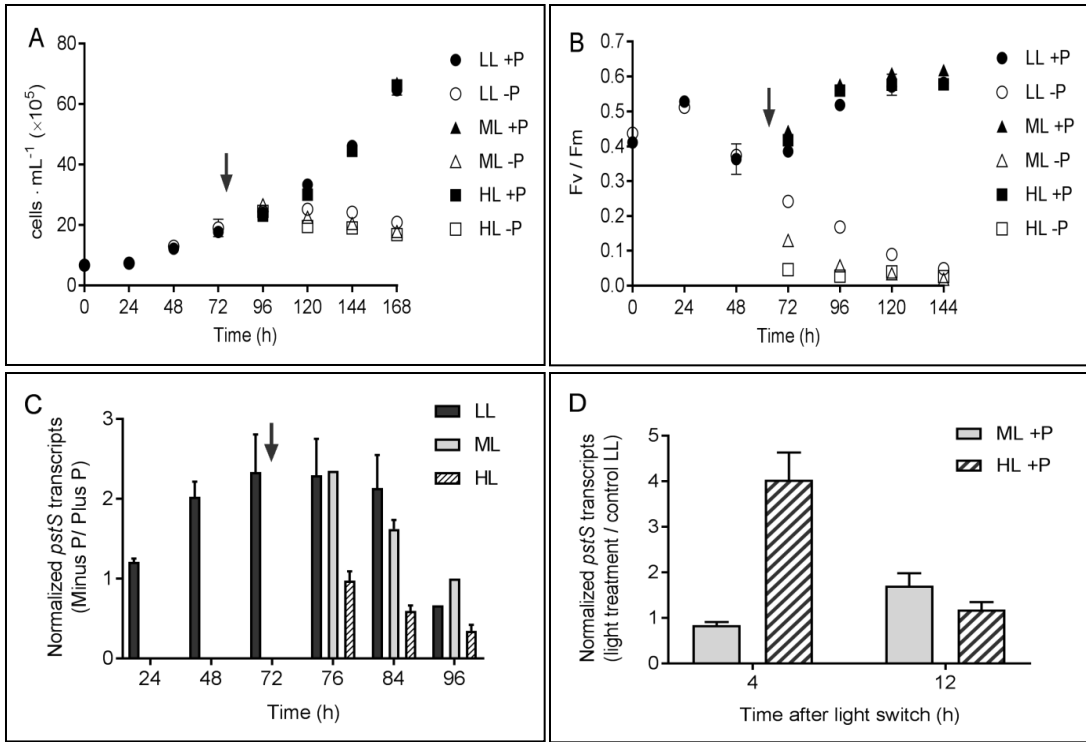
Figure 4. Effect of light intensity on responses to P stress. Biomass (A) photosynthetic efficiency (B), and normalized *pstS* transcripts (C–D). Arrows represent the time at which the light transfer from Low Light (LL) to Medium Light (ML) and High Light (HL) occurred, relative to when samples for either cell counts or Fv/Fm were taken. An * indicates a *p*-value of less than 0.05 for the HL vs the LL treatments at a single point in time for a paired *t*-test. Error bars indicate standard error of duplicate biological replicates.

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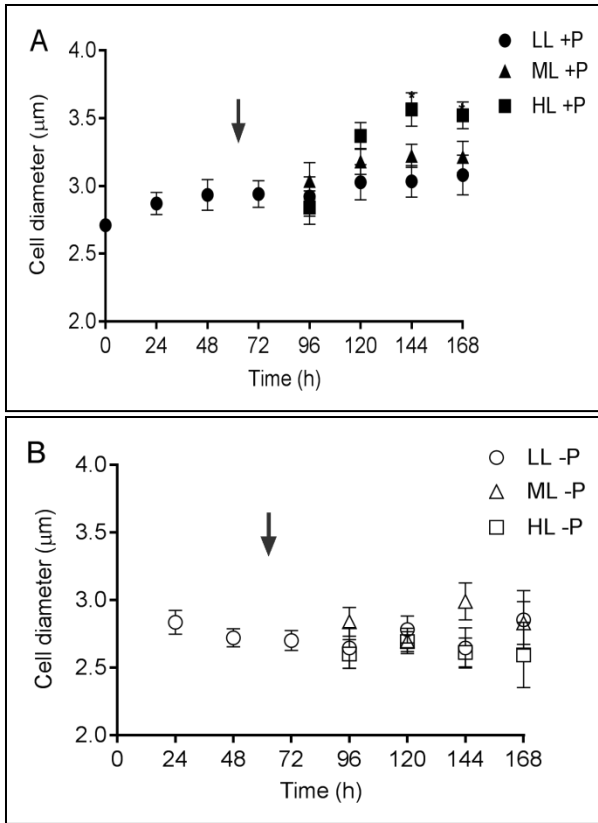
Figure 5. Cell diameter variation between three light treatments: Low Light (LL), Medium Light (ML), and High Light (HL), in (A) P-replete and (B) P-deficient cultures. Arrows represent the time at which the light transfer from LL to ML and HL occurred. An * indicates a *p*-value of less than 0.05 for the HL vs the LL treatments at a single point in time for a paired *t*-test. Error bars indicate standard error of duplicate biological replicates.

CHAPTER 3: USING BIOMARKERS TO DETERMINE THE P STATUS OF TWO N2-FIXING CYANOBACTERIA IN THE NORTH PACIFIC OCEAN

Abstract

Phosphorus (P) availability in the North Pacific Subtropical Gyre (NPSG) can limit nitrogen fixation by marine microorganisms, known as diazotrophs, which assimilate dinitrogen gas. However, the environmental controls that affect P stress in diazotroph assemblages are not well understood. Determining when natural populations experience P stress can help to identify environmental conditions that cause P limitation in microbial communities. Diagnostic biomarkers for P stress in the nitrogen-fixing cyanobacteria *Crocosphaera* and *Trichodesmium* were used to assess the P status of these organisms under a range of environmental conditions along a transect in the NPSG, and the relationship between environmental variables and P biomarker transcript levels was evaluated statistically. In natural populations of *Crocosphaera*, the biomarker for the arsenite efflux gene (*arsB)* was found to be a reliable indicator for low P concentrations. Elevated *arsB* transcripts strongly correlated with the principal component that reflected lower P and a decrease in *Crocosphaera* abundance, especially large-cell phenotypes. These results suggest that P concentrations can influence the proliferation and strain composition of *Crocosphaera* assemblages. In contrast, the *Trichodesmium* biomarker for the alkaline phosphatase gene (*phoX)* was up-regulated only at the lowest P concentration in this study (20 nmol L^{-1}), suggesting that populations were typically not stressed by P-availability, even at sites with high abundances and low P concentrations. These

findings emphasize the value of using molecular tools in concert with environmental data for predicting the competitive abilities of diazotrophs as a function of nutrient stress.

1. Introduction

The N_2-fixing cyanobacteria *Crocosphaera* and *Trichodesmium* are globally significant contributors to the marine nitrogen (N) cycle (Capone et al., 1997, Moisander et al., 2010). The contribution of fixed N by these diazotrophs is especially important in marine ecosystems where fixed inorganic N is present in limiting concentrations, such as in the North Pacific Subtropical Gyre (NPSG) (Carpenter and Capone, 2008, Church et al., 2008). Studies, including a recent one in the NPSG, provide evidence that phosphorus (P) availability controls N_2-fixation rates (Fu and Bell, 2003, Mills et al., 2004, Rees et al., 2006, Watkins-Brandt et al., 2011). Thus, the predicted climate-driven shift toward increasing P deficiency within the NPSG (Karl et al., 2001a) has implications for diazotroph populations and would confer an advantage to diazotrophs that are better competitors at low P.

Although *Crocosphaera* and *Trichodesmium* often co-occur in the marine environment, their genetic and physiological differences suggest their populations may respond differently to low P conditions. In order to mitigate a reduction in growth due to P deficiency, organisms induce a set of genes to increase the rates of dissolved inorganic phosphorus (DIP) uptake and dissolved organic phosphorus (DOP) hydrolysis. In *Trichodesmium*, the P stress response is activated at

approximately 9 nmol L^{-1} DIP (Moutin et al., 2008), while in *Crocosphaera*, P stress genes are up-regulated below a DIP concentration of 60 nmol L^{-1}, but may be expressed at higher DIP concentrations as well (Pereira et al., 2016). *Trichodesmium* has access to a greater portion of the DOP pool than *Crocosphaera*, due to a variety of alkaline phosphatases as well as genes for phosphonate metabolism that are not present in *Crocosphaera* (Dyhrman and Haley, 2006, Van Mooy et al., 2012).

There are two phenotypically distinct ecotypes of *Crocosphaera* that differ in size: small-cell (2-4 μm) and large-cells (5-6 μm) (Webb et al., 2009). The higher per-cell N_2-fixation rates and extracellular polysaccharide (EPS) production characteristic of the larger cells may contribute to higher P requirements (Sohm et al., 2011, Webb et al., 2009) compared to the small-cell types. The genomes of large-cell strains also possess more copies of P-acquisition genes and a greater variety of alkaline phosphatases than the small-cell strains (Bench et al., 2013). The variation in P stress genes between *Crocosphaera* phenotypes suggests that natural assemblages of these ecotypes could respond differently to P limitation.

Molecular markers provide a valuable means of detecting P stress of individual species in the environment (Fuller et al., 2005, La Roche et al., 1999). The high-affinity P-uptake gene, *pstS*, and the arsenite efflux gene, *arsB*, are two markers that have been used previously to evaluate the P status of *Crocosphaera* (Dyhrman and Haley, 2006, Dyhrman and Haley, 2011, Pereira et al., 2016). Since arsenate is an analog of phosphate, it is transported into cells along with P, especially when the high-affinity phosphate uptake system is activated under P stress. *pstS* gene

expression responds rapidly to P deficiency in *Crocosphaera*, but can also be transiently elevated in the presence of a replete exogenous P supply due to diel regulation and high light intensity (Dyhrman and Haley, 2006, Dyhrman and Haley, 2011, Pereira et al., 2016, Shi et al., 2010). *arsB* transcription is usually characteristic of later stages of P starvation, and is up-regulated as the phosphate to arsenate ratio declines (Dyhrman and Haley, 2011, Pereira et al., 2016). In *Trichodesmium*, the alkaline phosphatase gene, *phoX*, is an ideal marker for P stress because it exhibits a much stronger response to P deficiency than the frequently used alkaline phosphatase gene, *phoA*, and was shown to be elevated below 7 nmol L^{-1} DIP (Orchard et al., 2009, Orchard, 2010).

In this study, genus-specific P stress biomarkers for *Crocosphaera* (*pstS* and *arsB*) and *Trichodesmium* (*phoX*) were used to identify P-limiting conditions and evaluate environmental controls on cyanobacterial abundance. Samples from the Biosensing Lagrangian Instrumentation and Nitrogen Cycling Systems (BioLINCS) research cruise conducted near the long-term time series Station ALOHA in the NPSG were analyzed for expression of P stress biomarkers. As previously shown by Robidart et al. (2014), DIP concentrations correlated strongly with salinity during the cruise, making it an ideal location for this study (Fig. 1). This study demonstrates the contrasting effect of DIP availability to the P stress response in *Crocosphaera* and *Trichodesmium* populations, which will help predict how these ecologically significant diazotrophs may respond to P deficiency in future oceans.

2. Methods

2.1 Cruise sample collection and environmental data

Samples for this study were acquired from the BioLINCS research cruise between 9/8/2011 – 9/18/2011 aboard the *R/V Kilo Moana*. Seawater was collected in Niskin bottles mounted to a SeaBird 911 Plus CTD at approximately 1200 hrs at each station, from depths of 5, 25, 45, and 75m (Table S1). Hydrographic data for salinity, temperature, and light was recorded by the CTD at each site. For RNA analysis, 2 L of seawater was pre-filtered through 10 µm, and then collected by filtration through a 0.22 µm Supor (Pall Life Sciences, Ann Arbor, MI, USA) filter using gentle peristaltic pumping. The filters were preserved in liquid nitrogen and stored at -80 °C until further processing. Nutrient analysis was conducted from CTD samples taken concurrently at each station (Robidart et al., 2014). Phosphorus samples were analyzed for DIP according to Karl and Tien (Karl and Tien, 1992) and nitrogen samples were analyzed for NH_4 according to Dore and Karl (Dore and Karl, 1996). Fluorometric chlorophyll *a* was measured via the CTD fluorescence sensor and calibrated against discrete extracts using fluorometric analysis (Strickland and Parsons, 1972). Abundances of *Crocosphaera* and *Trichodesmium* were previously determined by Bombar et al. (2015) using quantitative PCR (qPCR) to determine the *nifH* gene copies per liter of seawater. Similarly, abundances of small-cell and large-cell ecotypes of *Crocosphaera* were previously quantified by Bench et al. (2016) using qPCR primers that were designed to differentiate between the different phenotypic strains.

2.2 RNA extraction and quantitative PCR

Total RNA was extracted from the 0.22 µm filters using a modified RNeasy Mini Kit protocol (Qiagen, Germantown, MD, USA). RNA extractions and reverse transcription assays were preformed as previously described by Pereira *et al.* (2016). The first extraction steps were carried out manually, and the column-based extraction portions were completed using a Qiacube (Qiagen) automated system according to the manufacturer's instructions.

Relative quantitative PCR (qPCR) was used to assess the transcript levels of P stress induced genes in *Crocosphaera* and *Trichodesmium*. Genus-specific primers for *pstS* and *arsB* genes developed for qPCR assays targeted *Crocosphaera*, and *phoX* was used to target *Trichodesmium* (Orchard et al., 2009; Pereira et al., 2016). Relative gene expression was obtained by normalizing each sample to a reference (housekeeping) gene and to a control sample, using the comparative ($\Delta\Delta$Ct) method. Samples were normalized to the constitutively expressed housekeeping gene, *rotA,* which encodes for a peptidyl-prolyl *cis-trans* isomerase. Expression of *rotA* has been shown not to vary under P stress conditions in both *Crocosphaera* and *Trichodesmium*, making it an ideal reference gene for this study (Orchard et al., 2009; Dyhrman and Haley, 2011). Each target gene had an amplification efficiency between 97% and 100% of *rotA*.

Each sample was run in duplicate using SYBR Green Mastermix (Life Technologies, Carlsbad, CA, USA) with reactions as follows: 8 µl sterile water, 10 µl SYBR Mastermix, 250 nM forward and reverse primers, and 1 µl cDNA. The qPCR

reactions were prepared in 96-well plates (Applied Biosystems, Foster City, CA, USA) and run on an ABI 7500 Real-time PCR System (Applied Biosystems). Thermocycler conditions were as follows: 50°C for 2 min, 95°C for 10 min, and then 45 cycles of 95°C for 15 s and 60°C for 1 min. Melt curves were run with every reaction to detect the occurrence of any nonspecific amplification. No template controls were run in triplicate for each qPCR assay on each plate. A replicate of a single sample of DNA extracted from the BioLINCS cruise was run on each qPCR plate as a control sample to calibrate for any differences between plate runs.

2.3 Statistical analyses

The relationship between gene transcripts of *pstS*, *arsB*, and *phoX* to the auxiliary environmental data (temperature, light, salinity, chlorophyll *a*, ammonium, diazotroph abundance, and *Crocosphaera* ecotype, see Table 1), was evaluated using statistical methods. Linear regressions were initially used to assess correlations between gene transcript levels and the environmental variables. A principal component analysis (PCA) was run on the environmental variables to account for multicollinearity within the dataset (F-ratio > 4). Prior to the PCA, normal distribution of each environmental parameter was checked using the Shapiro-Wilk normality Test. Log transformations were used to normalize some variables in order to meet assumptions of normality. Using the PCA, the environmental variables were grouped into components, with eigenvalues < 1 discarded. Factor scores from the PCA were used in a multiple regression analysis (MRA) to further analyze the relationship between transcript abundance and environmental components. MRA's

were run using the PCA factor scores as dependent variables, and the gene as the

independent variable. From the results of each MRA, both negative and positive

correlations between environmental variables and gene transcripts were determined,

and statistical significance was obtained. The PCA, MRAs, and normality tests were

conducted using Systat 13.1 (Systat Software Inc., Chicago, Illinois, USA).

3. Results

3.1 Gene transcription

Of the six BioLINCS sites sampled in this study, stations 2, 3, and 5 had

relatively low salinity ($\leq$35.1) and high DIP concentrations ($\geq$81 nmol L^{-1}), and

stations 4, 10, and 12 had relatively high salinity ($\geq$35.2) and low DIP concentrations

($\leq$60 nmol L^{-1}) (Fig. 1). Transcript levels of P stress marker genes from

Crocosphaera and *Trichodesmium* varied in abundance at the different sites.

Transcription of a gene was considered up-regulated when transcript levels were

above the calculated average transcript abundance across all samples. In

Crocosphaera, *pstS* was up-regulated at sites with DIP concentrations between 21 and

127 nmol L^{-1} (Fig. 2a). Elevated transcripts were observed mainly at stations with low

DIP concentrations (4, 10, and 12, but also at station 2) where DIP concentrations

ranged from 115-127 nmol L^{-1} (Fig. S1). *Crocosphaera arsB* transcripts were up-

regulated only at sites with DIP concentrations $\leq$ 47 nmol L^{-1} (Fig. 2b) and transcript

levels were below average at all sites with DIP $\geq$ 60 nmol L^{-1} (Fig. S1). In

Trichodesmium, *phoX* transcripts were up-regulated at one site, which corresponded

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to the lowest DIP concentration of 20 nmol L^{-1} at station 4 (Fig. 2c).

3.2 Principal Component Analysis (PCA)

Data for 13 environmental variables (Table 1) from each site were analyzed by linear regression and PCA. Significant correlations were found only in regressions of the environmental variables with the *arsB* gene (Table S2). Multicollinearity within the environmental dataset resulted in weak correlations with gene transcript levels, and therefore PCA components were used in place of the environmental variables. There were three significant principal components that accounted for 77.2% of the total variance. The first principal component (PC1) explained the largest total variance (41.2%) and consisted of DIP, salinity, chlorophyll *a*, *Crocosphaera* abundance, and large-cell ecotypes. *Crocosphaera* abundances, large-cell ecotypes, and DIP loaded positively, and salinity and chlorophyll *a* loaded negatively (Fig. 3a). The second principal component (PC2) was comprised of *Trichodesmium*, and ammonium, explaining 18.4% of total variance, with both variables loading positively. *Trichodesmium* abundance loaded positively with ammonium (Fig. 3b). Principal component three (PC3) explained 17.6% of total variance and was comprised of small cell ecotypes of *Crocosphaera*, temperature, and light. All three of the variables in PC3 loaded positively (Fig. 3c).

3.3 Multiple Regression Analysis (MRA)

The factor scores of each principal component were used as dependent variables to assess relationships between environmental conditions and P stress gene transcript levels. *Crocosphaera pstS* gene transcripts did not significantly correlate

with any of the principal components (Table 2). Transcript levels of the *Crocosphaera* gene *arsB* exhibited a significant negative correlation with PC1 and PC3 (Table 2). Thus, *arsB* transcript levels were negatively associated with DIP, temperature, light, and *Crocosphaera* abundances, in particular the large-cell ecotypes. Conversely, salinity and chlorophyll *a* were positively correlated with *arsB* transcript levels. Since PC2 did not significantly correlate with *arsB* expression, it was assumed that *Trichodesmium* abundances and ammonium concentrations were not correlated with transcript levels. The *Trichodesmium phoX* gene did not have a significant correlation with any of the principal components. This was not surprising, since the dataset for this study only contained one site where *phoX* was up-regulated.

4. Discussion

Genus-specific P stress biomarkers were used to assess the environmental factors that contribute to P limitation in two marine diazotrophs: *Crocosphaera* and *Trichodesmium*. *Crocosphaera* and *Trichodesmium* populations grouped into separate components by PCA, suggesting that their abundances and distributions were regulated by different factors. Robidart et al. (2014) previously showed that the two diazotroph populations had inverse relationships with DIP concentrations along this cruise track. The distribution of *Crocosphaera* seemed to be explained mainly by PC1, where its abundance, especially large-cell ecotypes, increased with higher DIP at lower salinity and lower total biomass. *Trichodesmium* abundances were a component of PC2, and subsequently had a weaker negative association with DIP

concentration. Similar to findings from Robidart et al. (2014), the strong covariance between *Crocosphaera* abundances and DIP concentrations suggests that P-availability is a major control on the distribution of this diazotroph.

Transcript levels of *Crocosphaera* P stress biomarkers were elevated in multiple samples from the BioLINCS cruise track. As expected, transcript levels of both *pstS* and *arsB* genes increased at low DIP sites (< 50 nmol L^{-1} DIP) (Figure 2a,b). The up-regulation of *pstS* at station 2 was unexpected, since the DIP concentration was relatively high for the mixed layer of the NPSG (> 100 nmol L^{-1}) (Karl et al., 2001b, Wilson et al., 2015). Although *Crocosphaera pstS* gene transcription has diel regulation, the samples were taken at the same time each day (~5 h after sunrise), which corresponds to the time of lowest basal expression (Shi et al., 2010 ; Pereira et al, 2016). Higher light intensity can also result in elevated *pstS* transcript levels in *Crocosphaera* (Pereira unpublished), however light levels at station 2 were lower than at another high DIP sites. Past exposure to low P concentrations can influence the P stress response by affecting the DIP threshold that signals gene transcription and resulting in persistent gene expression after the signal is reduced (Igoshin et al., 2008, Lambert and Kussell, 2014, Wagner et al., 1995). However, since these samples provide only a snapshot of the environment, we cannot ascertain the history of *Crocosphaera* populations at station 2.

Although *pstS* is often used as a field marker for P stress, its detection in the natural populations does not always reflect a low P environment (Bhaya et al., 2000, Fischer et al., 2006, Pereira et al., 2016). This study observed similar patterns, and

although it is an integral part of the P stress response in *Crocosphaera, pstS* transcript

levels did not correlate with environmental variables in this study, including light and

DIP (Table S2). Since *pstS* transcript levels were elevated over a range of DIP, it was

not surprising that there was not a significant correlation with PC1. Unlike *pstS*, a

more consistent relationship was observed between *Crocosphaera arsB* transcripts

and DIP concentrations. arsB gene transcript levels differed between low DIP and

high DIP stations (Fig. S1); there was a significant negative correlation ($p<0.05$)

between *arsB* and DIP (Table S2); and the highest transcript levels of *arsB* were

observed at the lowest DIP sites (Fig. 2). While *Crocosphaera pstS* transcript levels

increase rapidly in response to low P, *arsB* is expression is more indicative of

sustained P deficiency (Pereira et al., 2016). The prevalence of *arsB* at low DIP sites

implies that *Crocosphaera* populations in those sites had experienced a longer period

of P stress.

 As the more reliable biomarker for P stress, *arsB* transcript levels were used to

determine the environmental factors that correlated with the P status of

Crocosphaera. The MRA identified PC1 as the most influential determinant of P

stress (Table 2). Unsurprisingly, this gene was not correlated with PC2, indicating

that *Trichodesmium* abundance has no significant influence on P limitation in

Crocosphaera. The positive relationship between *arsB* and PC1 shows that transcripts

are elevated in water masses characterized by high salinity and low DIP, and that

Crocosphaera populations comprise less of the total biomass in these regions. This

suggests that *Crocosphaera* does not compete well against other microorganisms in

low P environments, possibly due to a high cellular P demand (Garcia et al., 2013).

While total *Crocosphaera* biomass is affected by DIP availability, there is also a distinction between the abundances of the large and small cell ecotypes at low DIP. The stronger correlation of large-cell ecotypes with DIP concentrations indicates that fluctuations in P have a greater influence on the abundance of the large-cell type *Crocosphaera* compared to the small-cell types (Bench et al., 2013). Genomic evidence (more copies of P acquisition genes in large-cell ecotypes) might indicate a higher P requirement or an increased competitive ability of this phenotype at low P. The increase in *arsB* transcript levels that correlates with a decrease in abundances of large-cell ecotypes as a factor of PC1 implies the former, suggesting that P limitation is a driver of the decrease in abundance observed in the large-cell phenotype. With a lower surface to volume ratio, large cells likely have an increased need for P scavenging capabilities, as well as a higher cellular P requirement.

In contrast to *Crocosphaera*, populations of *Trichodesmium* did not show evidence of P limitation in this study area. While *Crocosphaera* P stress biomarkers were elevated at a variety of locations, *Trichodesmium phoX* was only elevated at one site, suggesting that the high abundance of *Trichodesmium* cells present at lower DIP are not experiencing physiological P limitation. Significantly higher transcript levels (13-fold above average) of *phoX* were observed at station 4 at 45 m, which corresponded with the lowest DIP in the dataset at 20 nmol L^{-1}. However, *phoX* was not elevated at other station 4 sites, where DIP concentrations were similar at 21 nmol L^{-1}. It was surprising that *phoX* was significantly higher at a DIP concentration of 20

nmol L^{-1}, since P becomes limiting for *Trichodesmium* at an estimated DIP threshold concentration of 9 nmol L^{-1} (Moutin et al., 2005). However, DIP thresholds calculated in lab-grown cultures can vary from those in natural populations (Karl, 2014).

Since *phoX* transcript abundances were largely constant in the samples, it was not surprising that the transcript levels of this gene were not correlated with any environmental factors. The PCA showed a positive relationship between *Trichodesmium* and ammonium (Fig. 3b). Since nitrogen depletion confers a selective advantage to diazotrophs (Karl et al., 2002, Knapp et al., 2012), it is improbable that *Trichodesmium* abundances increase with higher ammonium concentrations, and more likely that the increased ammonium concentrations are the result of cellular leakage (Gardner and Boyle, 2017, Glibert and Bronk, 1994).

5. Conclusions

As climate change is expected to result in increasing stratification and further depletion of nutrients in the NPSG, those environmental conditions will select for the growth of diazotrophs. The resulting elevated N$_2$-fixation is hypothesized to shift the NPSG toward an increasingly P-limited environment (Karl et al., 2001a). Thus, it is important to understand how natural populations of diazotrophs adapt to P deficiency. The use of genus-specific P stress biomarkers in this study revealed a contrast between *Trichodesmium* and *Crocosphaera* in abundance, distribution, and P status. The findings suggest that *Trichodesmium* has a competitive advantage over other diazotrophs in low P environments, because it is not under physiological P stress until

DIP is extremely depleted. In contrast, *Crocosphaera*, especially large-cell ecotypes, relies on higher DIP availability to meet cellular P requirements. Future work using molecular markers that differentiate between *Crocosphaera* ecotypes can be used to further evaluate the response to P limitation in these two phenotypes.

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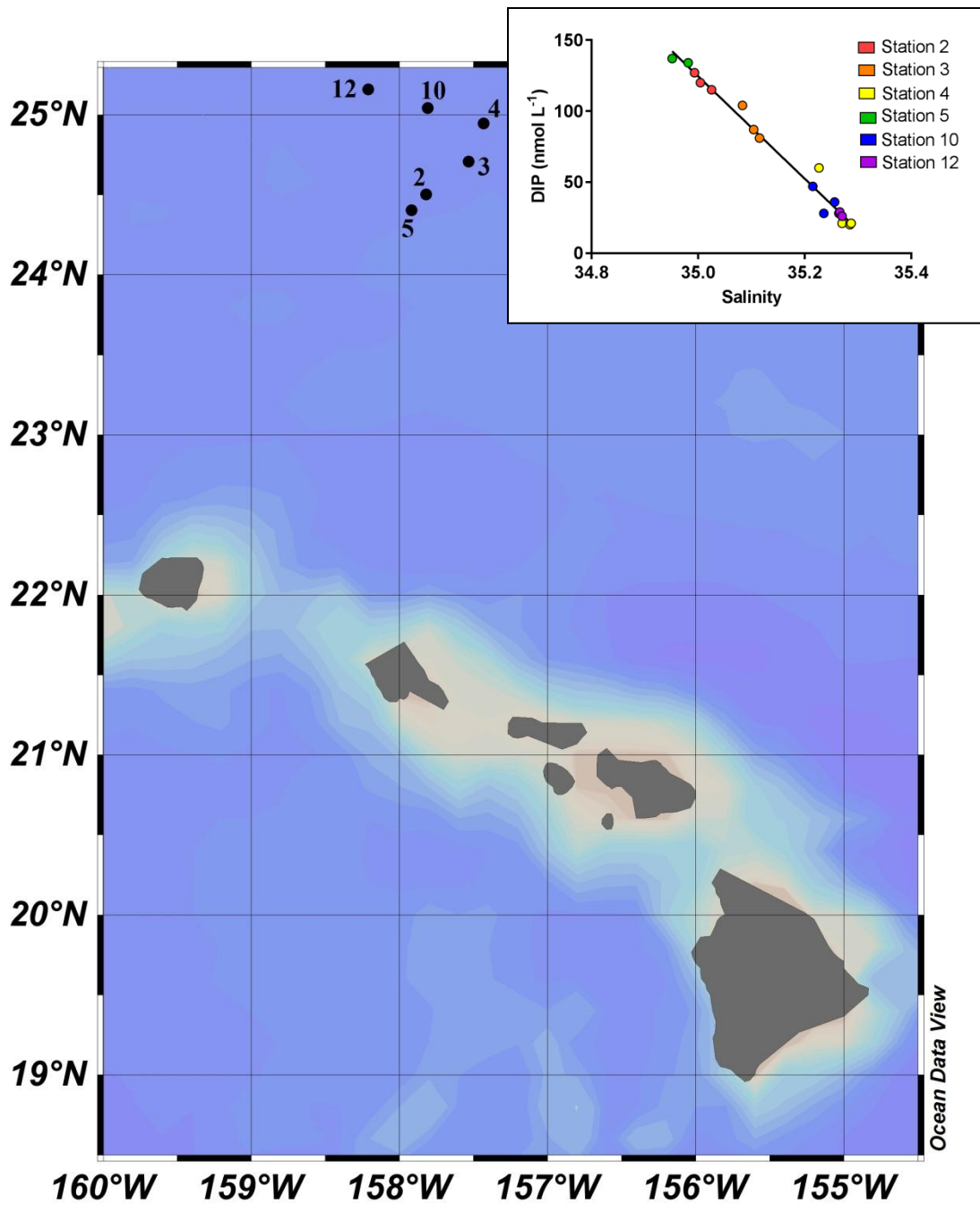
Figures

Figure 1. Sample locations and station numbers in the NPSG. Linear regression shows the relationship between DIP and salinity at these stations (inset).

Table 1. Environmental data from six stations over the BioLINCS cruise track

Variable	Units	Range	Mean	SD
DIP	nmol L^{-1}	20-137	68	45
Salinity	PSS	34.95-35.29	35.16	0.12
Chlorophyll *a*	µg L^{-1}	0.05-0.21	0.09	0.04
Crocosphaera	*nifH* copies liter^{-1}	1.3×10^3-3×10^6	1.1×10^6	7.5×10^5
Large-cell ecotypes	*gene* copies liter^{-1}	6.1×10^2-9.7×10^4	2.8×10^4	2.9×10^4
Small-cell ecotypes	*gene* copies liter^{-1}	2.1×10^3-3.2×10^6	1.5×10^6	9.1×10^5
Trichodesmium	*nifH* copies liter^{-1}	1.6×10^3-7.8×10^5	1.9×10^5	2.3×10^5
NH$_4$	nmol L^{-1}	3-91	31	21
Temperature	°C	25.5-26.4	26	0.3
Light	PAR (V)	24-1014	342	340

Range, mean and standard deviation of ten environmental variables from the six stations in this study.
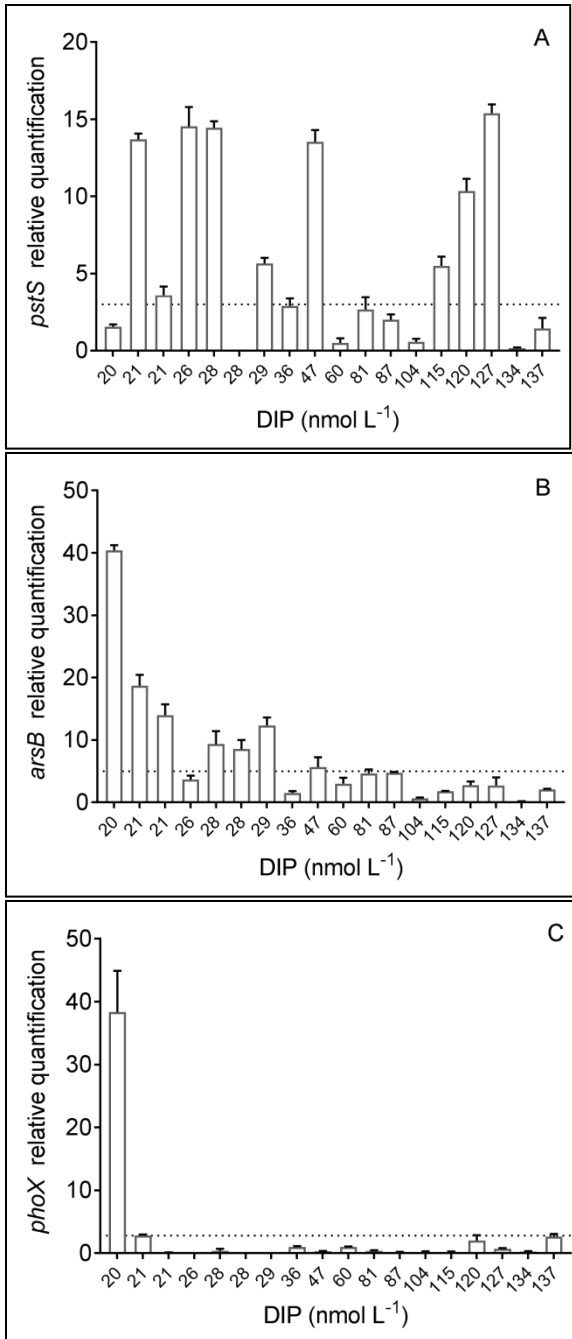
Figure 2. Relative quantification of three P stress biomarkers over a range of DIP concentrations. *Crocosphaera pstS* (A) and *arsB* (B), and *Trichodesmium phoX* (C) transcripts relative to the housekeeping gene *rotA*. Dotted lines represent the average transcription for each gene during this study. Error bars represent the standard deviation between statistical replicates.
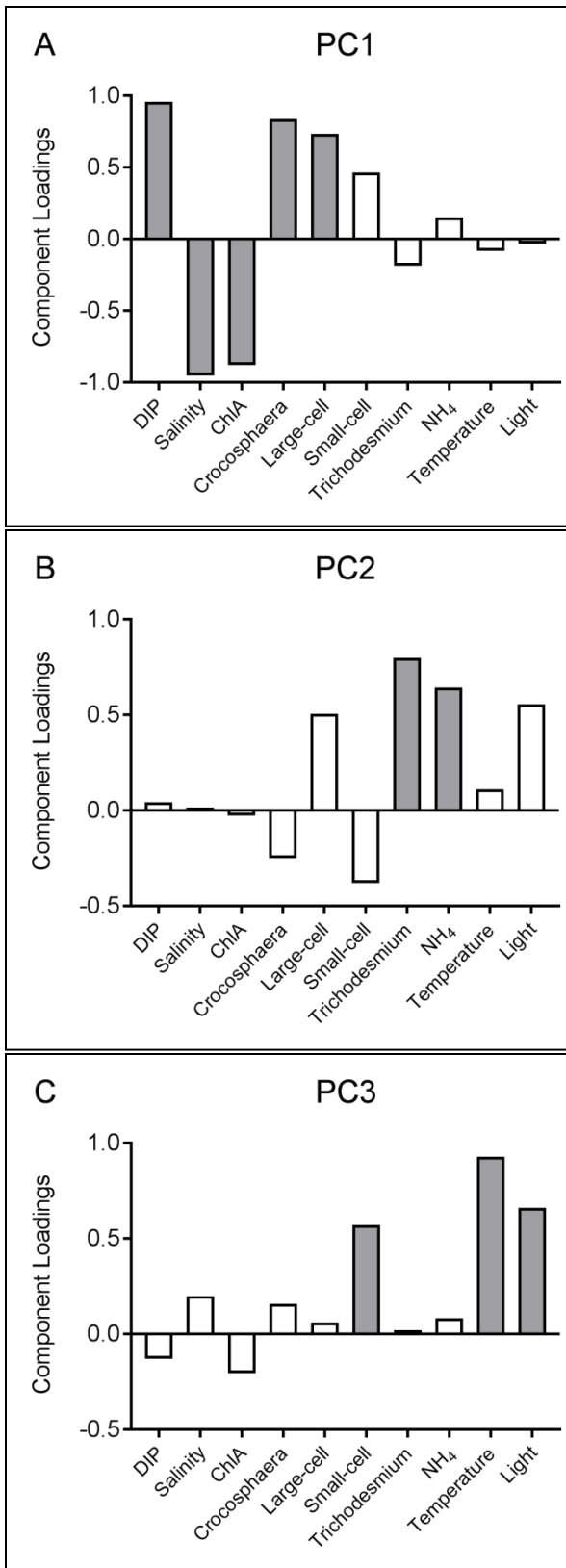
Figure 3. Principal component analysis of ten environmental variables. The variables were grouped into three principal components: PC1 – PC3, and each panel displays the component loadings for that principal component. PC1 grouped DIP, salinity, Chl *a*, *Crocosphaera*, and large-cell ecotypes (A). PC2 grouped *Trichodesmium*, ammonium (NH_4) (B). PC3 grouped temperature, light, and small-cell ecotypes of *Crocosphaera* (C).

Table 2. Multiple regression analysis evaluating principal components versus *Crocosphaera* biomarkers

Principal Component	*pstS* Regression Coefficient	*p*-value	*arsB* Regression Coefficient	*p*-value
1	0.01	0.962	-0.424	0*
2	0.036	0.866	0.091	0.247
3	-0.072	0.378	-0.239	0.007*

Regression coefficients identifying the relationship between *pstS* and *arsB* to the three principal components. * indicates a significant relationship between the gene and principal component.

Table S1. Locations and sampling dates for the six BioLINCS cruise stations assessed in this study.

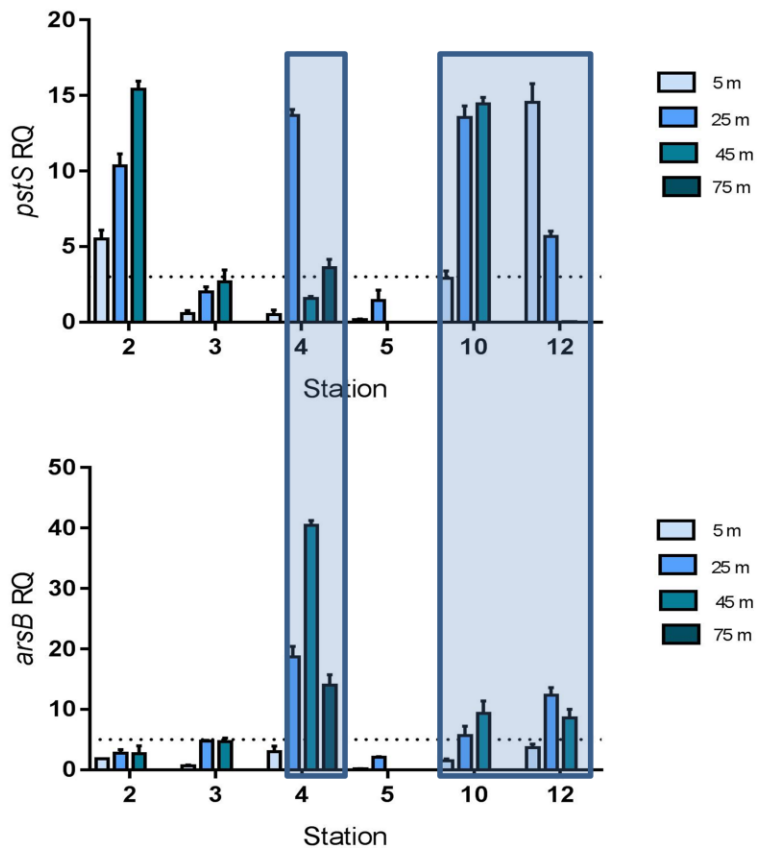| Station	Date	Latitude	Longitude
2	9-08-2011	24.505	157.75
3	9-09-2011	24.724	157.554
4	9-10-2011	24.902	157.427
5	9-11-2011	24.405	157.875
10	9-16-2011	25.037	157.725
12	9-18-2011	25.128	158.199

Figure S1. Relative quantification of *Crocosphaera* P stress biomarkers at six stations and over four depths: 5m, 25m, 45m, and 75m. *pstS* (top panel) and *arsB* (bottom panel) transcripts relative to the housekeeping gene *rotA*. Dotted lines represent the average transcription for each gene during this study. Error bars represent the standard deviation between statistical replicates. Shaded areas represent low P sites (<60 nmol L^{-1}).

Table S2. Pearson correlation values between environmental variables and P stress genes.

Environmental Variables	*pstS*	*arsB*	*phoX*
DIP	-0.097	-0.732*	0.38
Salinity	0.044	0.684*	-0.379
Chl *a*	0.011	0.692*	-0.13
NH$_4$	-0.275	-0.111	-0.192
Crocosphaera	0.043	-0.667*	0.207
Trichodesmium	0.333	0.32	0.299
Small ecotypes	0.223	-0.605	0.01
Large ecotypes	0.152	-0.451	0.398
Temperature	-0.346	-0.394	-0.269
Light	0.048	-0.142	-0.282

Significant correlations ($p<0.05$) are indicated by *.

CONCLUSION

In oligotrophic regions of the ocean, the abundance and distribution of the marine cyanobacterium *Crocosphaera* is controlled by nutrient availability. Although N is often the key limiting nutrient in oligotrophic ocean gyres, *Crocosphaera* has access to an abundant source of dinitrogen gas, so its growth is limited by other nutrients such as P. Determining when environmental populations of *Crocosphaera* are experiencing P stress can aid in understanding the ecological drivers of such assemblages. Genetic biomarkers are commonly used to evaluate whether natural populations are under P stress. They confer taxon specificity, have high sensitivity, due to the rapid induction and degradation of mRNA, and transcript levels are quantifiable through qPCR. Yet, because the Pho regulon is not well characterized in most marine cyanobacterial species, it is difficult to predict which biomarkers will be ideal for identifying P stress. Furthermore, the transcript levels of potential biomarkers must be characterized in controlled culture experiments before their environmental detection is interpretable. This body of work culminates in identifying key diagnostic markers for P stress in *Crocosphaera*, characterizing their transcription under various cultured conditions, testing their efficacy in the environment, and utilizing them to assess the P status of natural assemblages in comparison to another important marine diazotroph, *Trichodesmium*.

Out of the 11 biomarkers that were screened in *Crocosphaera*, two had a significant increase in transcript levels during P deprivation. These were genes coding for the high-affinity P binding protein (PstS) and the arsenite efflux protein (ArsB).

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Interestingly, the transcript levels of each gene were elevated at different stages of P stress. *pstS* was up-regulated within 1 h of exposure to low concentrations of orthophosphate (Pi), while *arsB* was elevated at later stages of P starvation. Since As is an analog for phosphate, it is unsurprising that as external P becomes scarce, cells need to contend with an increase in As influx. The segregated response in up-regulation suggests that while one marker would not be ideal to represent the entire P stress response in *Crocosphaera*, the two could be used in tandem. Transcript levels of these markers were compared in shipboard bottle experiments in the North Pacific subtropical gyre where natural assemblages were forced into P stress by the addition of Fe. While *pstS* was initially detected at higher levels, *arsB* transcripts became elevated in treatments without added P. Using high-resolution sampling to characterize biomarker transcript levels, it was determined that *pstS* exhibited a diel pattern of transcription in P replete conditions, and that this pattern was elevated under P stress. Identifying that *pstS* transcript levels were highest at the late phase of the light cycle allows for a more accurate interpretation of its detection in field populations.

As basal transcription of *pstS* was detected in *Crocosphaera*, additional culture experiments were conducted to assess its suitability as an environmental marker for P stress. Transcript levels of *pstS* were elevated when Pi concentrations dropped below 60 nmol L^{-1}, indicating that *Crocosphaera* would be experiencing P stress at concentrations typical in oligotrophic regimes. Since natural populations often contend with constant fluctuations in P supply, it was important for *pstS* to

rapidly respond to changes in bioavailable P. While *pstS* transcripts were repressed by all P sources tested, transcripts were downregulated faster in response to inorganic P than organic P sources. Induction of *pstS* at low Pi and the rapid and specific repression by both inorganic and organic P sources indicated that it was a suitable marker for P. However, since prior work in the marine cyanobacterium *Synecocystis* showed that *pstS* transcripts could increase in response to light, *pstS* was additionally evaluated under high light conditions. It was then discovered that *Crocosphaera pstS* is also elevated transiently under high light (450 μE m^{-2} s^{-1}). *pstS* constitutes an adaptive cellular response that mitigates P deficiency to avoid cells becoming P limited. Therefore, it is conceivable that cells may use a high-affinity P binding protein under conditions where rapid P acquisition is required. This could be in response to high light conditions that may result in an increase in growth rate or prior to the start of S phase in the diel cycle. The non-specific induction of *pstS* suggests that although this gene is a key component of the P stress response, it would not be an ideal standalone biomarker in *Crocosphaera*.

Because the projected goal of this work was the application of biomarkers to interrogate the P status of environmental populations, biomarkers evaluated in previous chapters were used to screen natural assemblages of diazotrophs in the North Pacific subtropical gyre. *Crocosphaera* biomarkers *pstS* and *arsB* were used along with a *Trichodesmium* biomarker for P stress, *phoX*, which codes for alkaline phosphatase. *arsB* negatively correlated with dissolved inorganic phosphorus (DIP) and was elevated below 50 nmol L-1 DIP. While *pstS* was also mainly elevated at low

P locations, elevated transcripts were detected at some higher P sites. This was unsurprising, given the findings from previous chapters, however diel transcription and elevated light levels did not account for higher transcripts at these sites. To account for co-linearity between environmental factors in this study, they were grouped into principal components (PC) that were regressed against P stress biomarkers. When *Crocosphaera* was under P stress, indicated by high *arsB* transcript levels, both DIP concentrations and the abundance of this diazotroph were low. Interestingly, the abundance of large cell ecotypes of *Crocosphaera* was more negatively affected under P stress than small cell ecotypes. This suggests that although large cell ecotypes contain more P stress genes, they may be less competitive in low P environments, possibly due to a higher P requirement or higher surface: volume ratio than smaller cell ecotypes. While P concentrations seemed to be an important driver of *Crocosphaera* populations, it was not determined to be an influencing factor of *Trichodesmium*. Rather, *Trichodesmium* populations were increasingly abundant at low DIP and did not exhibit the P stress response, except at one site. The difference between the P status of these diazotrophs within the same study area indicates that *Trichodesmium* may be a better competitor at low P than *Crocosphaera*.

Taken together, these data advances our understanding of the P stress response in *Crocosphaera* and underscores the importance of evaluating biomarkers in culture to allow for a more accurate interpretation of their detection in the environment. While taxon-specific markers are valuable tools for evaluating nutrient stress in field

populations, this work highlights the difficulty in finding ideal biomarkers due to the variability and complexity of the P stress response within cyanobacteria. Research clarifying the regulatory controls on cyanobacterial P stress genes will be key in identifying and evaluating future genetic markers. Specifically, determining whether *Crocosphaera pstS* is under regulatory control separate from the Pho regulon would help to better inform the conditions under which immediate P acquisition is necessary. Chapter 3 touches upon the potential disparity in P stress response between *Crocosphaera* ecotypes, and phenotype-specific markers would be useful to assess how P concentrations influence their distinct distributions in future.