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1 MOLECULAR MARKERS DEFINE PROGRESSING STAGES OF PHOSPHORUS
2 LIMITATION IN THE NITROGEN-FIXING CYANOBACTERIUM, *CROCOSPHERA*¹

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18 Abstract

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

37 Keywords

38 biomarkers; *Crocospaera*; cyanobacteria; nutrient stress; phosphorus

Abbreviations

DIP, dissolved inorganic phosphate

DOP, dissolved organic phosphate

MAGIC, magnesium induced co-precipitation

FRRF, fast repetition rate fluorometer

Introduction

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 *Crocospaera 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 in order to better understand how nutrients control the distributions and activities of nitrogen-fixing cyanobacteria (Webb et al. 2001, Dyhrman et al. 2002). *C. watsonii* must deal with varying dissolved inorganic phosphate (DIP) availability in its natural environment:

concentrations range from 0.2-5 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 genome (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 in order 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

C. watsonii WH8501 was grown at 27°C with $45 \mu\text{mol quanta} \cdot \text{m}^{-2} \cdot \text{s}^{-2}$ in a 12:12 light/dark cycle. At this light level, *C. watsonii* WH8501 grew at a photosynthetic efficiency of $F_v/F_m = 0.4$, similar to other studies with small-celled *C. watsonii* ($F_v/F_m = 0.35$) (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_2HPO_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 carryover. All cultures were inoculated at a starting density of $5 \times 10^4 \text{ cells} \cdot \text{mL}^{-1}$.

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 epifluorescence 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 $\times$ magnification under blue excitation light. For each slide, cell counts were obtained by averaging counts from ten 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 μ l RNase-Free DNase (Qiagen) to remove genomic DNA. Aliquots of 8 μ l 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 (qPCR) 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 hetero-dimers 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 ($\Delta\Delta C_t$) 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). Additionally, *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 μ l sterile water, 10 μ l SYBR Mastermix, 250 nM forward and reverse primers, and 1 μ l 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). 1 μ M 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 h and 36 h after T0. At these two timepoints, 2 L of seawater from each bottle was pre-filtered through 10 μ M filter and then a 2 μ M filter, which would later be extracted for RNA. Genes shown to have significant response to P-stress in previously conducted culture 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

212 We tested transcription of 11 genes affiliated with mediating the P-stress response, such as those
213 involved in gene regulation, cellular protection, P acquisition and substitution (Table 1). PCR
214 amplification efficiencies for each primer set were within $\pm 6\%$ of *rnpB* (Table 1), signifying that
215 every GOI amplified approximately equally with the endogenous control. The transcription of
216 each GOI in P-deficient cultures relative to P-replete cultures, or fold change, was analyzed.
217 Sampling from the middle of a P-starvation batch culture experiment showed 2 genes were
218 significantly up-regulated with a fold change > 2 , while all other genes responded with a fold
219 change < 2 (Fig. 2). The gene transcription of both *pstS* and *arsB* rose significantly in P-
220 deficient cultures, 5-fold and 11-fold, respectively. Other GOI's were not up-regulated with a
221 fold change > 2 at any other time point sampled during the P-stress experiments (data not
222 shown).

223 *Characterizing biomarkers of P status*

224 While *pstS* and *arsB* were shown to respond to P-stress, we wanted to further characterize this
225 response to evaluate their efficacy as biomarkers in *C. watsonii*. High-frequency sampling was
226 conducted in order to get a more accurate resolution of the sensitivity and variability of each
227 gene's response during batch culture P-starvation experiments. *pstS* responded quickly to initial
228 phosphate stress; a significant difference was detected as early as 1 h after cells were transferred
229 to low-P media (Fig. 3). In contrast, transcription of *arsB* was up-regulated only after 9 h and
230 transcription peaked 33 h after the initial transfer (Fig. 3). High resolution sampling revealed that
231 *pstS* exhibited strong diel regulation in both P-replete and P-deficient cultures. Despite this, it
232 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 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.

Discussion

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

260 Interestingly, transcription of the genes for DOP metabolism, both intracellularly (*ppK*, *ppX* and
261 *ppA*) and extracellularly (*phoA*) was not significantly upregulated during phosphorus limitation,
262 at least for the duration of this study. Intracellular cycling of polyphosphates is an adaptation
263 used by other marine cyanobacteria, such as *Synechococcus* (Gomez-Garcia et al. 2003) and
264 *Trichodesmium* (Orchard et al. 2010) under low-P conditions. The increasing activity of
265 phosphatases that enable cells to use alternative extracellular DOP sources in response to P-
266 stress, indicated by the transcription of *phoA* or its protein product, has been extensively used as
267 a biomarker (Moore et al. 2005, Orchard et al. 2009, Munoz-Martin et al. 2011). Transcription of
268 the *phoA* and *sphX* genes also did not increase under P-limitation in another study with *C.*
269 *watsonii* WH8501 (Dyhrman and Haley 2006). It is possible that the internal phosphorous
270 reserves prevented the cells from being under strong P-stress because the cells were grown with
271 replete DIP before being transferred into the medium with no added DIP.

272 Another commonly used adaptation to phosphorus stress in cyanobacteria is the substitution of
273 sulfolipids for phospholipids. Multiple species of *Synechococcus*, *Prochlorococcus* and
274 *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, *Crocospaera* 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 10-20 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 of *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* (CwatDRAFT_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 *Crocospaera*. 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 in order 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 in order 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 of *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. Though 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 *Crocospaera*. Additionally, 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 towards 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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Table

Table 1. *Crocospaera watsonii* WH8501 specific primers for phosphate metabolism genes.

Figure legends

Fig. 1. Growth and photosynthetic efficiency of *Crocospaera watsonii* WH8501. Cell cultures grown in phosphate replete (60 μ M; 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.

Fig. 2. Transcription of 6 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. *Crocospaera 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.

Fig. 4. Daily gene expression of *pstS* in a batch culture experiment. *Crocospaera watsonii* WH8501 cultures grown in P-replete and P-depleted media. Relative transcripton 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 μ M K₂HPO₄), Plus Fe (2 nM FeCl₃), and Plus P+Fe. All error bars represent standard deviation of triplicate biological replicates.

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

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Abstract

Crocospaera 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, in order 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. Additionally, 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.

Keywords

biomarkers; *Crocospaera*; cyanobacteria; nutrient stress; phosphorus

39 Abbreviations

40 DIP, dissolved inorganic phosphate

41 DOP, dissolved organic phosphate

42 MAGIC, magnesium induced co-precipitation

43 FRRF, fast repetition rate fluorometer

44

45 Introduction

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

55 Molecular diagnostic tools that target cell-specific responses to nutrient limitation have been
56 developed in order to better understand how nutrients control the distributions and activities of
57 nitrogen-fixing cyanobacteria (Webb et al. 2001, Dyhrman et al. 2002). *C. watsonii* must deal
58 with varying dissolved inorganic phosphate (DIP) availability in its natural environment:

concentrations range from 0.2-5 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 genome (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 in order 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.

105 Materials and Methods

106 *Cell culturing*

107 *C. watsonii* WH8501 was grown at 27°C with 45 $\mu\text{mol quanta} \cdot \text{m}^{-2} \cdot \text{s}^{-2}$ in a 12:12 light/dark
108 cycle. At this light level, *C. watsonii* WH8501 grew at a photosynthetic efficiency of $F_v/F_m =$
109 0.4, similar to other studies with small-celled *C. watsonii* ($F_v/F_m = 0.35$) (Sohm et al. 2011). We
110 found that growth at higher light intensities led to photoinhibition in this lab strain. For all
111 experiments, triplicate cultures were grown in seawater based media (SO; pH 8.0, salinity 28),
112 prepared without added nitrogen (Waterbury et al. 1986). Phosphate-replete (+P) cultures were
113 grown in SO medium, prepared with a 0.2 μm -filtered North Pacific (Station ALOHA, HI)
114 seawater base and 60 $\mu\text{M K}_2\text{HPO}_4$. Phosphate-deplete (-P) cultures were grown in SO medium
115 without added phosphate. Residual phosphate in the seawater base used for SO medium was
116 measured at 60 nM using (MAGIC) (Karl and Tien, 1992). Cells used as the inocula for all
117 treatments were grown with replete phosphate and harvested with a 0.2 μm filter at mid-log
118 phase from a single mother culture, then washed three times and re-suspended in medium
119 without added phosphate to restrict carryover. All cultures were inoculated at a starting density
120 of $5 \times 10^4 \text{ cells} \cdot \text{mL}^{-1}$.

121 *Microscopy and photosynthetic efficiency*

122 In each culture experiment, samples were taken every 24 h to monitor cellular physiology. Cell
123 growth and abundance of all cultures was monitored using epifluorescence microscopy with an
124 Axioplan 2 Zeiss microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA). Aliquots of 1
125 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 $\times$ magnification under blue excitation light. For each slide, cell counts were obtained by averaging counts from ten 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 μ l RNase-Free DNase (Qiagen) to remove genomic DNA. Aliquots of 8 μ l 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 (qPCR) 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 hetero-dimers 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 ($\Delta\Delta C_t$) 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). Additionally, *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 μ l sterile water, 10 μ l SYBR Mastermix, 250 nM forward and reverse primers, and 1 μ l 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). 1 μ M 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 h and 36 h after T0. At these two timepoints, 2 L of seawater from each bottle was pre-filtered through 10 μ M filter and then a 2 μ M filter, which would later be extracted for RNA. Genes shown to have significant response to P-stress in previously conducted culture 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: $0.34 \pm 0.02 \text{ d}^{-1}$ in P-replete and $0.31 \pm 0.03 \text{ d}^{-1}$ in P-deficient cultures. Control cultures grew with an $F_v/F_m \sim 0.4$, while cells subjected to low-P had a 20% decline in F_v/F_m 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 F_v/F_m 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 $5 \times 10^4 \text{ cells} \cdot \text{mL}^{-1}$, 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 $\pm 6\%$ 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 in order to get a more accurate resolution of the sensitivity and variability of 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 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.

Discussion

When cells progress towards 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 (F_v/F_m) 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, *Crocospaera* 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 10-20 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 of *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* (CwatDRAFT_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 *Crocospaera*. 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 in order 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 in order 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 of *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. Though 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 *Crocospaera*. Additionally, 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 towards 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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Table

Table 1. *Crocospaera watsonii* WH8501 specific primers for phosphate metabolism genes.

Figure legends

Fig. 1. Growth and photosynthetic efficiency of *Crocospaera watsonii* WH8501. Cell cultures grown in phosphate replete (60 μ M; 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.

Fig. 2. Transcription of 6 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. *Crocospaera 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.

564 Fig. 4. Daily gene expression of *pstS* in a batch culture experiment. *Crocospaera watsonii*
565 WH8501 cultures grown in P-replete and P-depleted media. Relative transcripton of each gene
566 indicates the fold change between the sample expression and the zero time point, normalized to
567 housekeeping gene *rnpB*. Samples were taken at intervals during light (L; 5, 7 11 h) and dark (D;
568 3, 7, 11 h) phases within a 12:12-h light-dark cycle. All error bars represent standard deviation of
569 triplicate biological replicates.

570 Fig. 5. Natural samples of *C.watsonii* during an incubation experiment at St. ALOHA. Gene
571 expression showing the fold change between *pstS* and *arsB* in control (no addition) and treatment
572 bottles: Plus P (1 μ M K₂HPO₄), Plus Fe (2 nM FeCl₃), and Plus P+Fe. All error bars represent
573 standard deviation of triplicate biological replicates.

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Table 1. *Crocospaera* WH8501 specific primers for phosphate metabolism genes.

Gene	Annotation	CwatDRAFT	NCBI	Forward primer	Reverse primer	Amplification
			GI	5'→3'	5'→3'	Efficiency ^b
<i>pstS</i>	Phosphate substrate binding protein	4928	67921475	TTGTGCAACTCAACACAGCA	TTGGGATCATTCCAGTTG	105%
					GT	
<i>sphX</i>	Phosphate substrate binding protein	5160	67921474	CAGCAACACCAAGATAAAC	GAAACAAAGGACGGGAT	103%
				TCAAAG	AAAGG	
<i>arsB</i>	Arsenate resistance protein	5214	67921397	GTGTGGCCGAGACATTAGAC	TTGCTGCTTGCACTGCTT	101%
				G	G	
<i>sqdB</i>	Sulfolipid biosynthesis protein	5287	67920767	CGAGCCACTGATCTAAACCA	CGCCGTACCAAAGACAC	102%
				AGG	CATC	
<i>phoH</i>	Unknown	4455	67922063	TCCCAAACCCTGCAATTACC	GACCAAGTTAGCTCCCG	106%

					TATGAC	
<i>phoU</i>	Negative	5911	67920826	TGGATGATGCTTACGAGGAA	GCGTGATCAGCCATTCTT	100%
	regulator			C	TC	
<i>phoA</i>	Alkaline	1549	67924612	CACCGCTGATGCTAACTTG	ATTCGTACCGCTTCTGTT	105%
	phosphatase				CC	
<i>phoB</i>	Transcriptional	2775	67923615	AACCGTCGATGTTTCATATTC	TCCAAACCGATAACCAA	101%
	regulator			G	AGC	
<i>ppK</i>	Polyphosphate	6491	67920515	GTGGTGGTTCGTGATCCTG	AGCTGTTTGCTCGTCTTC	101%
	kinase				TTG	
<i>ppX</i>	Exopolyphosphate	1948	67924239	CGTTCCTAGCTTCTTTACG	GTGATGCCAACTTCTGCT	106%
				AC	TG	

<i>ppA</i>	Inorganic pyrophosphatase	2235	67924135	CGGGTTGTGTCATTGCAG	AGCGTGGATCTTCATCA GG	95%
<i>rnpB</i> ^a	RNase	4794	67856398	GACTCCCGAAAGATCAGACT TG	GTTTACCGAGCCAGTAC CTCAC	100%

^a *rnpB* is used as a housekeeping gene.

^b All primers had an amplification efficiency of $\pm 6\%$.

Fig. 1. Growth and photosynthetic efficiency of *Crocosphaera watsonii* WH8501. Cell cultures grown in phosphate replete (60 μ M; 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.

81x60mm (300 x 300 DPI)

Fig. 2. Transcription of 6 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.
122x74mm (300 x 300 DPI)

Fig. 3. Transcription profiles of *pstS* and *arsB* during a batch culture experiment where phosphorus is limited. *Crocospheara 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.

82x65mm (300 x 300 DPI)

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 transcripton of each gene indicates the fold change between the sample expression and the zero time point, normalized to housekeeping gene *mpB*. 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.

82x35mm (300 x 300 DPI)

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 μ M K_2HPO_4), Plus Fe (2 nM $FeCl_3$), and Plus P+Fe. All error bars represent standard deviation of triplicate biological replicates.

82x34mm (300 x 300 DPI)