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1   MOLECULAR MARKERS DEFINE PROGRESSING STAGES OF PHOSPHORUS

2   LIMITATION IN THE NITROGEN-FIXING CYANOBACTERIUM, *CROCOSPHAERA*[1]

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17

18   Abstract

19   *Crocosphaera 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; *Crocosphaera*; 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 *Crocosphaera 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:

59   concentrations range from 0.2-5 nM in the North Atlantic (Wu et al. 2000, Cavender-Bares et al.

60   2001) and 10-100 nM in the North Pacific (Karl et al. 2001). Genes that are common markers for

61   detecting phosphorus stress are usually part of the Pho regulon (Vershinina and Znamenskaya

62   2002) and are under the control of a two-component regulatory system, where the response

63   regulator (PhoB) induces transcription under P-stress (Makino et al. 1989). Expression of the

64   gene encoding a high-affinity phosphate-binding protein, *pstS*, is a common response to

65   phosphorus deficiency because it increases the cellular efficiency of scavenging environmental

66   DIP (Suzuki et al. 2004, Dyhrman and Haley 2006). Cells utilize alkaline phosphatase (*phoA*) to

67   acquire phosphate from extracellular phosphomonoesters, another common mechanism for

68   adapting to phosphorus stress (Ray et al. 1991). Various cyanobacteria also have the capability to

69   hydrolyze phosphonates, facilitated by the *phn* gene cluster, as an extracellular dissolved organic

70   phosphate (DOP) source (Moore et al. 2005). Interestingly, *C.watsonii*, in contrast to

71   *Trichodesmium* (Dyhrman et al. 2006), is unable to use phosphonate as a phosphate source and

72   no homolog of the phosphonate transport gene cluster (*phnCDE*) has been found in its genome

73   (Dyhrman and Haley 2006). Polyphosphates often serve as an intracellular phosphorus reservoir;

74   thus, transcription of genes controlling polyphosphate synthesis (*ppK*) and hydrolysis (*ppX* and

75   *ppA*) can be turned on in response to phosphorus starvation (Gomez-Garcia et al. 2003, Schwarz

76   and Forchhammer 2005). Phytoplankton can also lower their cellular phosphorus requirement by

77   substituting sulfolipid for phospholipid to mitigate phosphorus limitation in the marine

78   environment (Van Mooy et al. 2009).

79   Genes that respond to phosphorus deficiency, even if they are not directly involved in alleviating

80   phosphorus stress, can also be used as molecular markers. For example, a gene coding for the

81   arsenite efflux pump, *arsB*, is upregulated under phosphorus stress as a protective mechanism for

82   the cell (Dyhrman and Haley 2011), since toxic arsenate (As) is a phosphate analog, and higher

83   As:P correlates with increasing phosphorus stress. Genes used as molecular diagnostic tools

84   should be stress-specific and their transcription should not be affected by other conditions (La

85   Roche et al. 1999). Since cellular processes in cyanobacteria, including *C.watsonii*, follow strict

86   circadian regulation and gene activity often exhibits a diel pattern (Shi et al. 2010), this creates

87   an additional complication when interpreting influences on transcriptional changes, especially in

88   natural populations. Thus, the transcription of these genes in response to increasing

89   environmental stress must be empirically validated.

90   While the expression of molecular markers are often used as indicators of phosphorus status of

91   cells, the response and regulation of nutrient utilization genes is complex. Examining the shifts in

92   the transcript abundance of relevant genes during different phases of phosphorus limitation will

93   help to better define the nutrient status of *C.watsonii* under variable conditions. Here we

94   analyzed the molecular responses to low availability of phosphorus in the unicellular

95   diazotrophic cyanobacterium *C. watsonii* strain WH8501 in order to better understand how to

96   apply and interpret well-characterized biomarkers for phosphorus stress in field applications.

97   *C.watsonii* has two main phenotypes: large-cells, which produce exopolysaccharides, and small-

98   cells. The decades-long cultured strain for *C. watsonii*, WH8501, is a small-cell type. It has a

99   larger genome (6.2 Mb) than the genomes of five other strains (4.5 Mb) and contains a higher

100   number of transposase genes and repeated genomic sequences (Bench et al. 2013). While *C.*

101   *watsonii* WH8501 is not the best representative of the natural populations, the genes shared

102   among strains have higher than 98% nucleotide identity (Bench et al. 2013). The high

103   conservation of genes among strains will allow the analyzed markers to be used for the *C.*

104   *watsonii* populations in environmental samples.

105   Materials and Methods

106   *Cell culturing*

107   *C. watsonii* WH8501 was grown at 27°C with 45 μmol quanta $\cdot$ m^{-2} $\cdot$ s^{-2} in a 12:12 light/dark

108   cycle. At this light level, *C. watsonii* WH8501 grew at a photosynthetic efficiency of Fv/Fm =

109   0.4, similar to other studies with small-celled *C. watsonii* (Fv/Fm = 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 μM K_2HPO_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 5x10^4 cells $\cdot$ 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 epifluoresence 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

126   polycarbonate, 0.22 µm pore filters (Poretics, Osmonics Inc., Minnetonka, MN, USA). Each

127   filter was mounted on a glass slide (Fischer Sci., Pittsburgh, PA, USA) and cells were counted

128   using 4,000× magnification under blue excitation light. For each slide, cell counts were obtained

129   by averaging counts from ten fields of view. Specific growth rates (k) were calculated per day,

130   using the formula: $k = \ln (N_2/N_1)/1$, where N_2 was the cell count 24 h after N_1. Growth curves

131   derived from cell counts were verified using relative fluorescence with a TD-700 fluorometer

132   (Turner Designs, Sunnyvale, CA, USA). Photosynthetic efficiency (F_v/F_m), which is the ratio of

133   the maximum change in variable fluorescence ($F_v = F_m-F_o$) to the maximum fluorescence yield

134   (F_m) was measured using a fast repetition rate fluorometer (FRRF) (Kolber et al. 1998). F_v/F_m is

135   a rapid and highly sensitive method for detecting stress, including P-limitation, in primary

136   producers (Beardall et al. 2001), and can be used as a proxy for cell health. Aliquots of 5 mL

137   culture samples for FRRF analysis were taken daily at the peak of the light cycle (L6) and

138   measured in triplicate.

139   *RNA extraction and cDNA synthesis*

140   RNA samples were collected by passing 10 mL of liquid culture through a 25 mm, 0.2 µm pore-

141   sized Suporfilter (Pall Corporation, Port Washington, NY, USA) using gentle vacuum filtration.

142   Filters were placed in bead beater tubes with β-mercaptoethanol and RLT buffer, then flash

143   frozen in liquid nitrogen immediately following filtration, after which they were stored at -80°C.

144   RNA extractions were carried out using a modified RNeasy Mini Kit protocol (Qiagen,

145   Germantown, MD, USA). Samples were thawed on ice and subjected to 2 min of bead-beating.

146   Filters were removed using sterile needles and the remaining buffer was placed on spin columns

147   for automated extraction in a QIAcube (Qiagen). The extraction protocol included a DNase step,

148   where each sample was treated with 10 µl RNase-Free DNase (Qiagen) to remove genomic

149   DNA. Aliquots of 8 µl extracted RNA were converted to single stranded complementary DNA

150   (cDNA) using the QuantiTect Reverse Transcription Kit (Qiagen) with random hexamer priming

151   according to manufacturer's guidelines. While generating cDNA, parallel reactions were run

152   with no reverse transcriptase to check for any residual DNA contamination. All cDNA was

153   subsequently stored at -20°C.

154   *Quantitative PCR (qPCR) assays*

155   Primers were designed to target 11 genes known to be involved in phosphorus metabolism in

156   cyanobacteria (Table 1). Using BLAST, homologs of these genes were found in *C. watsonii*

157   WH8501 and primers were designed to specifically target these sequences. Primer3 (Untergasser

158   et al. 2012) was used to create primers with specifications for relative quantitative PCR (qPCR)

159   (primer3.sourceforge.net/). Candidate primers were each evaluated for their probability to form

160   hairpin loops, self-dimers and hetero-dimers using IDT Oligoanalyzer 3.1 web tool (Integrated

161   DNA technologies, Coralville, IO, USA). We did a BLAST search with the selected primers

162   against the National Center for Biotechnology Information (NCBI) nt database to verify that the

163   primers did not match anything other than their specific target gene sequence. Since the

164   expression of multiple genes was analyzed in this study, relative quantification was used to

165   compare gene expression profiles. The comparative (ΔΔCt) method was used to normalize the

166   expression of each gene. Transcription of each Gene of Interest (GOI) was normalized to *rnpB*,

167   which encodes the RNA component of RNase P, and whose transcript abundance does not

168   fluctuate with phosphorus stress (Gomez-Baena et al. 2009). This constitutively expressed

169   housekeeping gene is not cell-cycle dependent in the marine cyanobacterium *Prochlorococcus*

170 (Holtzendorff et al. 2001). Additionally, *rnpB* transcript abundance has been shown to be stable

171 under varying nutrient, light and temperature conditions in another diazotrophic marine

172 cyanobacterium, *Trichodesmium* (Chappell and Webb 2010). Finally, a diel transcriptome study

173 in *C.watsonii* WH8501 provides evidence that transcription of the *rnpA* gene, a protein subunit

174 of RNase P, does not exhibit diel variation (Shi et al. 2010). For each qPCR assay, the

175 amplification efficiency was first tested by normalizing each primer set to *rnpB*. Amplification

176 efficiencies of primer sets between 95% and 105% with an $r^2 \geq 0.98$ were determined to be

177 acceptable.

178 For each biological replicate, triplicate samples were run using SYBR Green Mastermix (Life

179 Technologies, Carlsbad, CA, USA) with reactions as follows: 8 µl sterile water, 10 µl SYBR

180 Mastermix, 250 nM forward and reverse primers, and 1 µl cDNA. The qPCR reactions were

181 prepared in 96-well plates (Applied Biosystems, Foster City, CA, USA) and run on an ABI 7500

182 Real-time PCR System (Applied Biosystems). Thermocycler conditions were as follows: 50°C

183 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

184 were run with every reaction to detect the occurrence of any nonspecific amplification. No

185 template controls were run in triplicate for each qPCR assay on each plate. A single T_0 time point

186 sample was used as a calibrator for calculating fold changes in each experiment. Fold changes

187 were calculated using Relative Quantification software (Applied Biosystems).

188 *Shipboard Incubations*

189 Whole water incubations were conducted at St. ALOHA (22° 45'N, 158° 00'W) in August 2010

190 during KM1016. Triplicate 4 L flasks were incubated with P, Fe and P+Fe treatments, alongside

191 controls (no addition). 1 µM of K_2HPO_4 and 2 nM of $FeCl_3$ were added to P and Fe treatments,

192   respectively. Along with an initial (T0) sample at the start of the incubation, RNA was taken at

193   two additional sampling points: 24 h and 36 h after T0. At these two timepoints, 2 L of seawater

194   from each bottle was pre-filtered through 10 μM filter and then a 2 μM filter, which would later

195   be extracted for RNA. Genes shown to have significant response to P-stress in previously

196   conducted culture starvation experiments were screened in each sample. The field products were

197   not sequenced in this study.

198

199   Results

200   *Growth of P-stressed cultures*

201   In batch culture experiments, *C. watsonii* WH8501 cells were exposed to P-replete and P-

202   deficient conditions. Twenty four h after transferring cells into low-P seawater medium, the

203   specific growth rates for day 1 in the two treatments were comparable: 0.34 ± 0.02 d^{-1} in P-replete

204   and 0.31 ± 0.03 d^{-1} in P-deficient cultures. Control cultures grew with an Fv/Fm~0.4, while cells

205   subjected to low-P had a 20% decline in Fv/Fm within 24 h (Fig. 1B). As the experiment

206   continued, growth rates of cells in P-replete cultures surpassed that of P-deficient cultures, which

207   entered stationary phase. The Fv/Fm in P-deficient cells steadily declined and was 16% of P-

208   replete cells by the end of the experiment (96 h). While both cultures started at a biomass of

209   5×10^4 cells $\cdot$ mL^{-1}, the final biomass in P-replete cultures was 2.4 times higher than in P-deficient

210   cultures (Fig. 1A).

211   *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 ± 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.

233   Although *pstS* was not significantly up-regulated in P-deficient versus P-replete cultures after 71

234   h, *arsB* continued to be up-regulated well after *pstS* transcription declined.

235   *Diel regulation of pstS*

236   *pstS* transcript abundance had a cyclic pattern of transcription in healthy P-replete cells, which

237   was enhanced in cells growing under P-deficiency. Since daily expression of *pstS* peaked at late-

238   light phase (L11) in both the control and treatment cultures (Fig. 4), the resulting fold change (–

239   P/+P) was lowest (~ 2) at that time. The highest fold change (~ 100) was displayed at late-dark

240   phase (D11), when *pstS* transcription was elevated in P-deficient cultures, but low in P-replete

241   cultures. Overall, we found that fold change was higher in the dark period (D3 – D7) than in the

242   light period (L5 – L11), but varied greatly depending on relative transcription in control cultures.

243   *Biomarkers in natural populations of Crocosphaera*

244   Using incubation experiments at St. ALOHA, natural populations of *C.watsonii* were screened

245   for *pstS* and *arsB* transcription. After 24 h, *pstS*/*arsB* > 1 in all treatments: Plus P, Plus Fe, Plus P

246   + Fe, and the no addition control (Fig. 5). At this time, transcription of *pstS* was elevated in all

247   bottles, while transcription of *arsB* was not detected. 39 h later, *arsB* transcript abundance was

248   detected at a significantly higher level than *pstS* in both the control and Plus Fe bottles,

249   presumably where P concentrations were reduced. However, *pstS*/*arsB* remained > 1 in Plus P

250   and Plus P + Fe treatments.

251

252   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-

275   limited cultures, however the ratio was not significantly different in *C.watsonii* (Van Mooy et al.

276   2009). In parallel to these results, we did not see a significant response in the gene regulating the

277   sulfolipid biosynthesis protein (*sqdB*) (Fig. 2). This inconsistent response between *C.watsonii*

278   and other cyanobacteria to P-stress suggests heterogeneity in the strategies of organisms that

279   compete for bioavailable phosphorus.

280   Of the genes that were screened, there were two that exhibited a significant response to DIP

281   depletion: *pstS* and *arsB* (Fig. 2). Similar to many marine cyanobacteria (Scanlan et al. 2009),

282   the genome of C.*watsonii* WH8501 has three copies of the *pstS* gene: CwatDRAFT_4928 (*pstS*),

283   CwatDRAFT_5160 (*sphX*), and CwatDRAFT_6534 (*pstS2*). The genes share little similarity

284   among them (21-24% amino acid identity): (Bench et al., 2013). Transcription of *sphX* did not

285   significantly change under P-stress for the duration of this study (Table 1). The *sphX* gene is

286   located 334 bp upstream of the *pstS* gene in the opposite orientation, and the lack of transcription

287   regulation by DIP availability indicates that regulatory regions in the *pstS* promoter are not used

288   for regulation of *sphX* transcription. Transcription of the *pstS2* gene was not analyzed in this

289   study. The *pstS* gene (CwatDRAFT_4928), with observed significantly up-regulated

290   transcription in response to P-deficiency, is part of the only complete *pstSCAB* operon (Dyhrman

291   and Haley 2006) which encodes the genes for the high-affinity phosphate transport system.

292   Notably, *Crocosphaera* genomes of the small cell-type (like WH8501) have 3-4 copies of the

293   *pstS* gene, while genomes of the large-cell types have 5-7 copies. It is possible that in the large

294   cell types, transcription of other *pstS* genes is also regulated based on the DIP availability.

295   Transcription of both *pstS* and *arsB* in *C.watsonii* was characterized with high-resolution

296   sampling during phosphorus starvation experiments (Fig. 3). High-affinity phosphate transport

297   may be an early response used by *C.watsonii*, since increased transcription of *pstS* was detected

298   within 1 h of cells exposed to low-P (Fig. 3). This highly sensitive response to changing

299   extracellular P is detected prior to physiological changes within the cell; photosynthetic

300   efficiency declined after 24 h and growth rates decreased after 48 h (Fig. 1). Natural populations

301   of *C.watsonii* may temporarily express *pstS* as a response to intermitant changes in

302   environmental [P], without experiencing changes in growth rate or photosynthetic efficiency.

303   The peak of *arsB* transcription under P-stress is slightly shifted from that of *pstS*, possibly in

304   response to the influx of arsenate due to a lower P:As ratio. While *C.watsonii arsB* can also be

305   directly upregulated by [As] > 30 nM (Dyhrman and Haley 2011), this condition does not occur

306   in the marine environment, where [As] ranges from 10-20 nM (Cutter et al. 2001, Cutter and

307   Cutter 2006); however, the internal cellular [As] is unknown. Neither marker singularly captures

308   the entire phosphorus stress response; at late P-limitation, the fold change of *pstS* declines, even

309   as stress increases, while transcription of *arsB* is up-regulated after a period of exposure to low-

310   P. Yet, used in tandem, these markers can effectively evaluate the P-status of *C.watsonii*. Both

311   *pstS* (CwatDRAFT_4928) and *arsB* genes are highly conserved (99-100 % nt identity) among

312   *C.watsonii* strains, at least among the six strains isolated from multiple oceanic regions and with

313   the genomes sequenced. Thus, the qPCR primers designed in this study can be used for

314   examining the cellular P status in natural *C.watsonii* populations.

315   We also found that regulation of *pstS* is different in *C.watsonii* compared to other cyanobacteria

316   (Scanlan et al. 1997), where increasing levels of *pstS* reflects increasing stress. Although *pstS* is

317   up-regulated immediately when external DIP falls below a threshold concentration, *pstS*

318   transcript abundance does not consistently correlate with increasing phosphorus stress, rather it

319   exhibits a strong diel pattern. The peak of *pstS* expression corresponds with the end of the light

320 cycle before S-phase, which occurs in the dark in *C.watsonii* (Dron et al. 2012) and may reflect

321 an increasing cellular requirement for phosphorus during replication. There are other instances

322 where P-stress response is uncoupled from exogenous DIP supply. In *Synechocystis*, phosphorus

323 starvation response can be triggered under high light, when growth rate surpasses the rate of

324 phosphorus assimilation (Bhaya et al. 2000). Basal *pstS* transcription raises the question of

325 alternative regulation of this gene outside of the P-stress response (Esteban et al. 2008). The

326 variable background levels of *pstS* in *C.watsonii* make it difficult to identify whether this

327 organism is responding to phosphorus stress from *pstS* expression alone; *pstS* transcripts may be

328 detected in cells that are not under phosphorus stress, depending on the time of day. Since the

329 detection of *pstS* is a common tool for predicting phosphorus stress in natural populations of

330 picocyanobacteria (Scanlan et al. 1997, Fuller et al. 2005, Hung et al. 2013), this finding has

331 implications for the use of *pstS* as an environmental biomarker for *Crocosphaera*. When working

332 with natural populations, late dark phase (D7 – D11) is the ideal time to sample for *pstS*, since

333 diel expression is lowest at that time (Fig. 4).

334 Shipboard incubation experiments were conducted in order to test transcription of *pstS* and *arsB*

335 genes in the environment. Since Fe and P are the major limiting nutrients for nitrogen-fixing

336 microorganisms (Mills et al. 2004), the experiments were conducted with both Fe and P

337 additions. We found that in natural populations of *C.watsonii*, using the combination of *pstS* and

338 *arsB* gene expression differentiates between early and late P-limitation. As expected, the ratio of

339 *pstS*/*arsB* transcript abundance > 1 after 24 h in all treatments, even in bottles treated with

340 phosphate (+P; +P+Fe), due to basal transcription. Fe was added to shipboard incubations in

341 order to facilitate the depletion of DIP in treatment bottles. In bottles without added P (control;

342 +Fe), basal transcription of *pstS* makes it difficult to differentiate any increased mRNA synthesis

343   that cells may produce due to early P-stress. However, after 39 h, the *pstS*/*arsB* < 1 in bottles

344   without added P, suggests a transition of cells to elevated levels of P-stress. This corresponds to

345   an increase of *pstS* transcription seen in cultures of *C.watsonii* initially exposed to low-P to a

346   shift of increasing *arsB* transcription at a later stage of P-stress (Fig. 3). This shift does not occur

347   in bottles treated with P (Fig 5). This illustrates consistency between the response of

348   environmental and cultured *C.watsonii* to P-limitation and provides evidence of how *pstS* and

349   *arsB* can be used to interrogate the P-status of natural populations.

350   Often, a single biomarker is used to identify stress in an organism. Here, we show the need for a

351   more comprehensive approach, with the use of multiple biomarkers that lead to a more accurate

352   analysis of P-status. The transcriptional response of *pstS* and *arsB* indicate different phases of

353   phosphorus stress. In the environment, detecting *arsB* transcripts would suggest that cells are

354   experiencing a higher level of phosphorus stress. Though it is widely used as a biomarker, cyclic

355   basal regulation of *pstS* in *C. watsonii* indicate possible alternative regulation and make it

356   unreliable as a sole indicator of P-stress in this open ocean diazotrophic organism. When using

357   these markers to interrogate field populations, we recommend sampling at night (6 h before

358   sunrise) if possible, to coincide with the lowest level of *pstS* basal transcription. During other

359   sampling times, *pstS* diel transcription will need to be considered. Future experiments that define

360   how rapidly *pstS* responds to added phosphorus could be used to infer P-status in environmental

361   populations of *C.watsonii* by assessing changes in transcript abundance. While our study

362   highlights potential markers, further experiments would be required to show that their

363   transcription is not also affected by conditions other than P-deficiency. In *Synechocystis* sp. PCC

364   6803, *pstS* had no significant change in response to Fe deficiency or reconstitution (Singh et al.

365   2003), however no comparative study has been done in *Crocosphaera*. Additionally, studies

366　using semi-continuous cultures would provide useful information about the response of these

367　genes under long-term P-stress. The results of this paper provide one step towards the full

368　evaluation of both genes as markers for use in the environment and emphasize the complexity of

369　gene transcription under stress.

370

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545

546   Table

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

548

549   Figure legends

550

551   Fig. 1. Growth and photosynthetic efficiency of *Crocosphaera watsonii* WH8501. Cell cultures

552   grown in phosphate replete (60 μM; closed symbols) and phosphate depleted (60 nM; open

553   symbols) media. Cell counts (A) taken for cultures starting at T0 and photosynthetic efficiency

554   (Fv/Fm) (B) measured during the same experiment. All error bars indicate standard deviation of

555   triplicate biological replicates.

556   Fig. 2. Transcription of 6 genes relative to reference gene *rnpB* and secondarily normalized to P-

557   replete cultures. Genes with a fold change between P-deplete/P-replete > 2 were considered

558   significantly upregulated. Error bars indicate standard error of triplicate biological replicates.

559   Fig. 3. Transcription profiles of *pstS* and *arsB* during a batch culture experiment where

560   phosphorus is limited. *Crocosphaera watsonii* WH8501 cultures grown over 85 h and sampled

561   approximately every 4 h during the light and dark (shaded area) cycles. Relative Quantification

562   (RQ) of each gene is normalized to a reference gene, *rnpB*, and time zero time point (T0). Error

563   bars indicate the standard deviation of triplicate biological replicates.

564   Fig. 4. Daily gene expression of *pstS* in a batch culture experiment. *Crocosphaera 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 μM K_2HPO_4), Plus Fe (2 nM $FeCl_3$), and Plus P+Fe. All error bars represent

573   standard deviation of triplicate biological replicates.

574

575

1   MOLECULAR MARKERS DEFINE PROGRESSING STAGES OF PHOSPHORUS

2   LIMITATION IN THE NITROGEN-FIXING CYANOBACTERIUM, *CROCOSPHAERA*[1]

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17

18   Abstract

19   *Crocosphaera 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; *Crocosphaera*; 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 *Crocosphaera 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:

59 concentrations range from 0.2-5 nM in the North Atlantic (Wu et al. 2000, Cavender-Bares et al.

60 2001) and 10-100 nM in the North Pacific (Karl et al. 2001). Genes that are common markers for

61 detecting phosphorus stress are usually part of the Pho regulon (Vershinina and Znamenskaya

62 2002) and are under the control of a two-component regulatory system, where the response

63 regulator (PhoB) induces transcription under P-stress (Makino et al. 1989). Expression of the

64 gene encoding a high-affinity phosphate-binding protein, *pstS*, is a common response to

65 phosphorus deficiency because it increases the cellular efficiency of scavenging environmental

66 DIP (Suzuki et al. 2004, Dyhrman and Haley 2006). Cells utilize alkaline phosphatase (*phoA*) to

67 acquire phosphate from extracellular phosphomonoesters, another common mechanism for

68 adapting to phosphorus stress (Ray et al. 1991). Various cyanobacteria also have the capability to

69 hydrolyze phosphonates, facilitated by the *phn* gene cluster, as an extracellular dissolved organic

70 phosphate (DOP) source (Moore et al. 2005). Interestingly, *C.watsonii*, in contrast to

71 *Trichodesmium* (Dyhrman et al. 2006), is unable to use phosphonate as a phosphate source and

72 no homolog of the phosphonate transport gene cluster (*phnCDE*) has been found in its genome

73 (Dyhrman and Haley 2006). Polyphosphates often serve as an intracellular phosphorus reservoir;

74 thus, transcription of genes controlling polyphosphate synthesis (*ppK*) and hydrolysis (*ppX* and

75 *ppA*) can be turned on in response to phosphorus starvation (Gomez-Garcia et al. 2003, Schwarz

76 and Forchhammer 2005). Phytoplankton can also lower their cellular phosphorus requirement by

77 substituting sulfolipid for phospholipid to mitigate phosphorus limitation in the marine

78 environment (Van Mooy et al. 2009).

79 Genes that respond to phosphorus deficiency, even if they are not directly involved in alleviating

80 phosphorus stress, can also be used as molecular markers. For example, a gene coding for the

81 arsenite efflux pump, *arsB*, is upregulated under phosphorus stress as a protective mechanism for

82   the cell (Dyhrman and Haley 2011), since toxic arsenate (As) is a phosphate analog, and higher

83   As:P correlates with increasing phosphorus stress. Genes used as molecular diagnostic tools

84   should be stress-specific and their transcription should not be affected by other conditions (La

85   Roche et al. 1999). Since cellular processes in cyanobacteria, including *C.watsonii*, follow strict

86   circadian regulation and gene activity often exhibits a diel pattern (Shi et al. 2010), this creates

87   an additional complication when interpreting influences on transcriptional changes, especially in

88   natural populations. Thus, the transcription of these genes in response to increasing

89   environmental stress must be empirically validated.

90   While the expression of molecular markers are often used as indicators of phosphorus status of

91   cells, the response and regulation of nutrient utilization genes is complex. Examining the shifts in

92   the transcript abundance of relevant genes during different phases of phosphorus limitation will

93   help to better define the nutrient status of *C.watsonii* under variable conditions. Here we

94   analyzed the molecular responses to low availability of phosphorus in the unicellular

95   diazotrophic cyanobacterium *C. watsonii* strain WH8501 in order to better understand how to

96   apply and interpret well-characterized biomarkers for phosphorus stress in field applications.

97   *C.watsonii* has two main phenotypes: large-cells, which produce exopolysaccharides, and small-

98   cells. The decades-long cultured strain for *C. watsonii*, WH8501, is a small-cell type. It has a

99   larger genome (6.2 Mb) than the genomes of five other strains (4.5 Mb) and contains a higher

100   number of transposase genes and repeated genomic sequences (Bench et al. 2013). While *C.*

101   *watsonii* WH8501 is not the best representative of the natural populations, the genes shared

102   among strains have higher than 98% nucleotide identity (Bench et al. 2013). The high

103   conservation of genes among strains will allow the analyzed markers to be used for the *C.*

104   *watsonii* populations in environmental samples.

105   Materials and Methods

106   *Cell culturing*

107   *C. watsonii* WH8501 was grown at 27°C with 45 µmol quanta $\cdot$ m^{-2} $\cdot$ s^{-2} in a 12:12 light/dark

108   cycle. At this light level, *C. watsonii* WH8501 grew at a photosynthetic efficiency of Fv/Fm =

109   0.4, similar to other studies with small-celled *C. watsonii* (Fv/Fm = 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 µM K_2HPO_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 5×10^4 cells $\cdot$ 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 epifluoresence 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

126   polycarbonate, 0.22 μm pore filters (Poretics, Osmonics Inc., Minnetonka, MN, USA). Each

127   filter was mounted on a glass slide (Fischer Sci., Pittsburgh, PA, USA) and cells were counted

128   using 4,000× magnification under blue excitation light. For each slide, cell counts were obtained

129   by averaging counts from ten fields of view. Specific growth rates (k) were calculated per day,

130   using the formula: $k = \ln(N_2/N_1)/1$, where N_2 was the cell count 24 h after N_1. Growth curves

131   derived from cell counts were verified using relative fluorescence with a TD-700 fluorometer

132   (Turner Designs, Sunnyvale, CA, USA). Photosynthetic efficiency (F_v/F_m), which is the ratio of

133   the maximum change in variable fluorescence ($F_v = F_m - F_o$) to the maximum fluorescence yield

134   (F_m) was measured using a fast repetition rate fluorometer (FRRF) (Kolber et al. 1998). F_v/F_m is

135   a rapid and highly sensitive method for detecting stress, including P-limitation, in primary

136   producers (Beardall et al. 2001), and can be used as a proxy for cell health. Aliquots of 5 mL

137   culture samples for FRRF analysis were taken daily at the peak of the light cycle (L6) and

138   measured in triplicate.

139   *RNA extraction and cDNA synthesis*

140   RNA samples were collected by passing 10 mL of liquid culture through a 25 mm, 0.2 μm pore-

141   sized Suporfilter (Pall Corporation, Port Washington, NY, USA) using gentle vacuum filtration.

142   Filters were placed in bead beater tubes with β-mercaptoethanol and RLT buffer, then flash

143   frozen in liquid nitrogen immediately following filtration, after which they were stored at -80°C.

144   RNA extractions were carried out using a modified RNeasy Mini Kit protocol (Qiagen,

145   Germantown, MD, USA). Samples were thawed on ice and subjected to 2 min of bead-beating.

146   Filters were removed using sterile needles and the remaining buffer was placed on spin columns

147   for automated extraction in a QIAcube (Qiagen). The extraction protocol included a DNase step,

148   where each sample was treated with 10 µl RNase-Free DNase (Qiagen) to remove genomic

149   DNA. Aliquots of 8 µl extracted RNA were converted to single stranded complementary DNA

150   (cDNA) using the QuantiTect Reverse Transcription Kit (Qiagen) with random hexamer priming

151   according to manufacturer's guidelines. While generating cDNA, parallel reactions were run

152   with no reverse transcriptase to check for any residual DNA contamination. All cDNA was

153   subsequently stored at -20°C.

154   *Quantitative PCR (qPCR) assays*

155   Primers were designed to target 11 genes known to be involved in phosphorus metabolism in

156   cyanobacteria (Table 1). Using BLAST, homologs of these genes were found in *C. watsonii*

157   WH8501 and primers were designed to specifically target these sequences. Primer3 (Untergasser

158   et al. 2012) was used to create primers with specifications for relative quantitative PCR (qPCR)

159   (primer3.sourceforge.net/). Candidate primers were each evaluated for their probability to form

160   hairpin loops, self-dimers and hetero-dimers using IDT Oligoanalyzer 3.1 web tool (Integrated

161   DNA technologies, Coralville, IO, USA). We did a BLAST search with the selected primers

162   against the National Center for Biotechnology Information (NCBI) nt database to verify that the

163   primers did not match anything other than their specific target gene sequence. Since the

164   expression of multiple genes was analyzed in this study, relative quantification was used to

165   compare gene expression profiles. The comparative ($\Delta\Delta$Ct) method was used to normalize the

166   expression of each gene. Transcription of each Gene of Interest (GOI) was normalized to *rnpB*,

167   which encodes the RNA component of RNase P, and whose transcript abundance does not

168   fluctuate with phosphorus stress (Gomez-Baena et al. 2009). This constitutively expressed

169   housekeeping gene is not cell-cycle dependent in the marine cyanobacterium *Prochlorococcus*

170   (Holtzendorff et al. 2001). Additionally, *rnpB* transcript abundance has been shown to be stable

171   under varying nutrient, light and temperature conditions in another diazotrophic marine

172   cyanobacterium, *Trichodesmium* (Chappell and Webb 2010). Finally, a diel transcriptome study

173   in *C.watsonii* WH8501 provides evidence that transcription of the *rnpA* gene, a protein subunit

174   of RNase P, does not exhibit diel variation (Shi et al. 2010). For each qPCR assay, the

175   amplification efficiency was first tested by normalizing each primer set to *rnpB*. Amplification

176   efficiencies of primer sets between 95% and 105% with an $r^2 \geq 0.98$ were determined to be

177   acceptable.

178   For each biological replicate, triplicate samples were run using SYBR Green Mastermix (Life

179   Technologies, Carlsbad, CA, USA) with reactions as follows: 8 µl sterile water, 10 µl SYBR

180   Mastermix, 250 nM forward and reverse primers, and 1 µl cDNA. The qPCR reactions were

181   prepared in 96-well plates (Applied Biosystems, Foster City, CA, USA) and run on an ABI 7500

182   Real-time PCR System (Applied Biosystems). Thermocycler conditions were as follows: 50°C

183   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

184   were run with every reaction to detect the occurrence of any nonspecific amplification. No

185   template controls were run in triplicate for each qPCR assay on each plate. A single T_0 time point

186   sample was used as a calibrator for calculating fold changes in each experiment. Fold changes

187   were calculated using Relative Quantification software (Applied Biosystems).

188   *Shipboard Incubations*

189   Whole water incubations were conducted at St. ALOHA (22° 45'N, 158° 00'W) in August 2010

190   during KM1016. Triplicate 4 L flasks were incubated with P, Fe and P+Fe treatments, alongside

191   controls (no addition). 1 µM of K_2HPO_4 and 2 nM of $FeCl_3$ were added to P and Fe treatments,

192   respectively. Along with an initial (T0) sample at the start of the incubation, RNA was taken at

193   two additional sampling points: 24 h and 36 h after T0. At these two timepoints, 2 L of seawater

194   from each bottle was pre-filtered through 10 µM filter and then a 2 µM filter, which would later

195   be extracted for RNA. Genes shown to have significant response to P-stress in previously

196   conducted culture starvation experiments were screened in each sample. The field products were

197   not sequenced in this study.

198

199   Results

200   *Growth of P-stressed cultures*

201   In batch culture experiments, *C. watsonii* WH8501 cells were exposed to P-replete and P-

202   deficient conditions. Twenty four h after transferring cells into low-P seawater medium, the

203   specific growth rates for day 1 in the two treatments were comparable: 0.34 ±0.02 d^{-1} in P-replete

204   and 0.31±0.03 d^{-1} in P-deficient cultures. Control cultures grew with an Fv/Fm~0.4, while cells

205   subjected to low-P had a 20% decline in Fv/Fm within 24 h (Fig. 1B). As the experiment

206   continued, growth rates of cells in P-replete cultures surpassed that of P-deficient cultures, which

207   entered stationary phase. The Fv/Fm in P-deficient cells steadily declined and was 16% of P-

208   replete cells by the end of the experiment (96 h). While both cultures started at a biomass of

209   5×10^4 cells · mL^{-1}, the final biomass in P-replete cultures was 2.4 times higher than in P-deficient

210   cultures (Fig. 1A).

211   *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 ± 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.

233   Although *pstS* was not significantly up-regulated in P-deficient versus P-replete cultures after 71

234   h, *arsB* continued to be up-regulated well after *pstS* transcription declined.

235   *Diel regulation of pstS*

236   *pstS* transcript abundance had a cyclic pattern of transcription in healthy P-replete cells, which

237   was enhanced in cells growing under P-deficiency. Since daily expression of *pstS* peaked at late-

238   light phase (L11) in both the control and treatment cultures (Fig. 4), the resulting fold change (–

239   P/+P) was lowest (~ 2) at that time. The highest fold change (~ 100) was displayed at late-dark

240   phase (D11), when *pstS* transcription was elevated in P-deficient cultures, but low in P-replete

241   cultures. Overall, we found that fold change was higher in the dark period (D3 – D7) than in the

242   light period (L5 – L11), but varied greatly depending on relative transcription in control cultures.

243   *Biomarkers in natural populations of Crocosphaera*

244   Using incubation experiments at St. ALOHA, natural populations of *C.watsonii* were screened

245   for *pstS* and *arsB* transcription. After 24 h, *pstS*/*arsB* > 1 in all treatments: Plus P, Plus Fe, Plus P

246   + Fe, and the no addition control (Fig. 5). At this time, transcription of *pstS* was elevated in all

247   bottles, while transcription of *arsB* was not detected. 39 h later, *arsB* transcript abundance was

248   detected at a significantly higher level than *pstS* in both the control and Plus Fe bottles,

249   presumably where P concentrations were reduced. However, *pstS*/*arsB* remained > 1 in Plus P

250   and Plus P + Fe treatments.

251

252   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-

13

275   limited cultures, however the ratio was not significantly different in *C.watsonii* (Van Mooy et al.

276   2009). In parallel to these results, we did not see a significant response in the gene regulating the

277   sulfolipid biosynthesis protein (*sqdB*) (Fig. 2). This inconsistent response between *C.watsonii*

278   and other cyanobacteria to P-stress suggests heterogeneity in the strategies of organisms that

279   compete for bioavailable phosphorus.

280   Of the genes that were screened, there were two that exhibited a significant response to DIP

281   depletion: *pstS* and *arsB* (Fig. 2). Similar to many marine cyanobacteria (Scanlan et al. 2009),

282   the genome of C.*watsonii* WH8501 has three copies of the *pstS* gene: CwatDRAFT_4928 (*pstS*),

283   CwatDRAFT_5160 (*sphX*), and CwatDRAFT_6534 (*pstS2*). The genes share little similarity

284   among them (21-24% amino acid identity): (Bench et al., 2013). Transcription of *sphX* did not

285   significantly change under P-stress for the duration of this study (Table 1). The *sphX* gene is

286   located 334 bp upstream of the *pstS* gene in the opposite orientation, and the lack of transcription

287   regulation by DIP availability indicates that regulatory regions in the *pstS* promoter are not used

288   for regulation of *sphX* transcription. Transcription of the *pstS2* gene was not analyzed in this

289   study. The *pstS* gene (CwatDRAFT_4928), with observed significantly up-regulated

290   transcription in response to P-deficiency, is part of the only complete *pstSCAB* operon (Dyhrman

291   and Haley 2006) which encodes the genes for the high-affinity phosphate transport system.

292   Notably, *Crocosphaera* genomes of the small cell-type (like WH8501) have 3-4 copies of the

293   *pstS* gene, while genomes of the large-cell types have 5-7 copies. It is possible that in the large

294   cell types, transcription of other *pstS* genes is also regulated based on the DIP availability.

295   Transcription of both *pstS* and *arsB* in *C.watsonii* was characterized with high-resolution

296   sampling during phosphorus starvation experiments (Fig. 3). High-affinity phosphate transport

297   may be an early response used by *C.watsonii*, since increased transcription of *pstS* was detected

298   within 1 h of cells exposed to low-P (Fig. 3). This highly sensitive response to changing

299   extracellular P is detected prior to physiological changes within the cell; photosynthetic

300   efficiency declined after 24 h and growth rates decreased after 48 h (Fig. 1). Natural populations

301   of *C.watsonii* may temporarily express *pstS* as a response to intermitant changes in

302   environmental [P], without experiencing changes in growth rate or photosynthetic efficiency.

303   The peak of *arsB* transcription under P-stress is slightly shifted from that of *pstS*, possibly in

304   response to the influx of arsenate due to a lower P:As ratio. While *C.watsonii arsB* can also be

305   directly upregulated by [As] > 30 nM (Dyhrman and Haley 2011), this condition does not occur

306   in the marine environment, where [As] ranges from 10-20 nM (Cutter et al. 2001, Cutter and

307   Cutter 2006); however, the internal cellular [As] is unknown. Neither marker singularly captures

308   the entire phosphorus stress response; at late P-limitation, the fold change of *pstS* declines, even

309   as stress increases, while transcription of *arsB* is up-regulated after a period of exposure to low-

310   P. Yet, used in tandem, these markers can effectively evaluate the P-status of *C.watsonii*. Both

311   *pstS* (CwatDRAFT_4928) and *arsB* genes are highly conserved (99-100 % nt identity) among

312   *C.watsonii* strains, at least among the six strains isolated from multiple oceanic regions and with

313   the genomes sequenced. Thus, the qPCR primers designed in this study can be used for

314   examining the cellular P status in natural *C.watsonii* populations.

315   We also found that regulation of *pstS* is different in *C.watsonii* compared to other cyanobacteria

316   (Scanlan et al. 1997), where increasing levels of *pstS* reflects increasing stress. Although *pstS* is

317   up-regulated immediately when external DIP falls below a threshold concentration, *pstS*

318   transcript abundance does not consistently correlate with increasing phosphorus stress, rather it

319   exhibits a strong diel pattern. The peak of *pstS* expression corresponds with the end of the light

320   cycle before S-phase, which occurs in the dark in *C.watsonii* (Dron et al. 2012) and may reflect

321   an increasing cellular requirement for phosphorus during replication. There are other instances

322   where P-stress response is uncoupled from exogenous DIP supply. In *Synechocystis*, phosphorus

323   starvation response can be triggered under high light, when growth rate surpasses the rate of

324   phosphorus assimilation (Bhaya et al. 2000). Basal *pstS* transcription raises the question of

325   alternative regulation of this gene outside of the P-stress response (Esteban et al. 2008). The

326   variable background levels of *pstS* in *C.watsonii* make it difficult to identify whether this

327   organism is responding to phosphorus stress from *pstS* expression alone; *pstS* transcripts may be

328   detected in cells that are not under phosphorus stress, depending on the time of day. Since the

329   detection of *pstS* is a common tool for predicting phosphorus stress in natural populations of

330   picocyanobacteria (Scanlan et al. 1997, Fuller et al. 2005, Hung et al. 2013), this finding has

331   implications for the use of *pstS* as an environmental biomarker for *Crocosphaera*. When working

332   with natural populations, late dark phase (D7 – D11) is the ideal time to sample for *pstS*, since

333   diel expression is lowest at that time (Fig. 4).

334   Shipboard incubation experiments were conducted in order to test transcription of *pstS* and *arsB*

335   genes in the environment. Since Fe and P are the major limiting nutrients for nitrogen-fixing

336   microorganisms (Mills et al. 2004), the experiments were conducted with both Fe and P

337   additions. We found that in natural populations of *C.watsonii*, using the combination of *pstS* and

338   *arsB* gene expression differentiates between early and late P-limitation. As expected, the ratio of

339   *pstS*/*arsB* transcript abundance > 1 after 24 h in all treatments, even in bottles treated with

340   phosphate (+P; +P+Fe), due to basal transcription. Fe was added to shipboard incubations in

341   order to facilitate the depletion of DIP in treatment bottles. In bottles without added P (control;

342   +Fe), basal transcription of *pstS* makes it difficult to differentiate any increased mRNA synthesis

343   that cells may produce due to early P-stress. However, after 39 h, the *pstS/arsB* < 1 in bottles

344   without added P, suggests a transition of cells to elevated levels of P-stress. This corresponds to

345   an increase of *pstS* transcription seen in cultures of *C.watsonii* initially exposed to low-P to a

346   shift of increasing *arsB* transcription at a later stage of P-stress (Fig. 3). This shift does not occur

347   in bottles treated with P (Fig 5). This illustrates consistency between the response of

348   environmental and cultured *C.watsonii* to P-limitation and provides evidence of how *pstS* and

349   *arsB* can be used to interrogate the P-status of natural populations.

350   Often, a single biomarker is used to identify stress in an organism. Here, we show the need for a

351   more comprehensive approach, with the use of multiple biomarkers that lead to a more accurate

352   analysis of P-status. The transcriptional response of *pstS* and *arsB* indicate different phases of

353   phosphorus stress. In the environment, detecting *arsB* transcripts would suggest that cells are

354   experiencing a higher level of phosphorus stress. Though it is widely used as a biomarker, cyclic

355   basal regulation of *pstS* in *C. watsonii* indicate possible alternative regulation and make it

356   unreliable as a sole indicator of P-stress in this open ocean diazotrophic organism. When using

357   these markers to interrogate field populations, we recommend sampling at night (6 h before

358   sunrise) if possible, to coincide with the lowest level of *pstS* basal transcription. During other

359   sampling times, *pstS* diel transcription will need to be considered. Future experiments that define

360   how rapidly *pstS* responds to added phosphorus could be used to infer P-status in environmental

361   populations of *C.watsonii* by assessing changes in transcript abundance. While our study

362   highlights potential markers, further experiments would be required to show that their

363   transcription is not also affected by conditions other than P-deficiency. In *Synechocystis* sp. PCC

364   6803, *pstS* had no significant change in response to Fe deficiency or reconstitution (Singh et al.

365   2003), however no comparative study has been done in *Crocosphaera*. Additionally, studies

366   using semi-continuous cultures would provide useful information about the response of these

367   genes under long-term P-stress. The results of this paper provide one step towards the full

368   evaluation of both genes as markers for use in the environment and emphasize the complexity of

369   gene transcription under stress.

370

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546 Table

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

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549 Figure legends

550

551 Fig. 1. Growth and photosynthetic efficiency of *Crocosphaera watsonii* WH8501. Cell cultures

552 grown in phosphate replete (60 µM; closed symbols) and phosphate depleted (60 nM; open

553 symbols) media. Cell counts (A) taken for cultures starting at T0 and photosynthetic efficiency

554 (Fv/Fm) (B) measured during the same experiment. All error bars indicate standard deviation of

555 triplicate biological replicates.

556 Fig. 2. Transcription of 6 genes relative to reference gene *rnpB* and secondarily normalized to P-

557 replete cultures. Genes with a fold change between P-deplete/P-replete > 2 were considered

558 significantly upregulated. Error bars indicate standard error of triplicate biological replicates.

559 Fig. 3. Transcription profiles of *pstS* and *arsB* during a batch culture experiment where

560 phosphorus is limited. *Crocosphaera watsonii* WH8501 cultures grown over 85 h and sampled

561 approximately every 4 h during the light and dark (shaded area) cycles. Relative Quantification

562 (RQ) of each gene is normalized to a reference gene, *rnpB*, and time zero time point (T0). Error

563 bars indicate the standard deviation of triplicate biological replicates.

564   Fig. 4. Daily gene expression of *pstS* in a batch culture experiment. *Crocosphaera 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 μM K_2HPO_4), Plus Fe (2 nM $FeCl_3$), and Plus P+Fe. All error bars represent

573   standard deviation of triplicate biological replicates.

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

Gene	Annotation	CwatDRAFT	NCBI GI	Forward primer 5'→3'	Reverse primer 5'→3'	Amplification Efficiency[b]
pstS	Phosphate substrate binding protein	4928	67921475	TTGTGCAACTCAACACAGCA	TTGGGATCATTCCAGTTGGT	105%
sphX	Phosphate substrate binding protein	5160	67921474	CAGCAACACCAAGATAAACTCAAAG	GAAACAAAGGACGGGATAAAGG	103%
arsB	Arsenate resistance protein	5214	67921397	GTGTGGCCGAGACATTAGACG	TTGCTGCTTGCACTGCTTG	101%
sqdB	Sulfolipid biosynthesis protein	5287	67920767	CGAGCCACTGATCTAAACCAAGG	CGCCGTACCAAAGACACCATC	102%
phoH	Unknown	4455	67922063	TCCCAAACCCTGCAATTACC	GACCAAGTTAGCTCCCG	106%

					TATGAC	
phoU	Negative regulator	5911	67920826	TGGATGATGCTTACGAGGAAC	GCGTGATCAGCCATTCTTTC	100%
phoA	Alkaline phosphatase	1549	67924612	CACCGCTGATGCTAACTTG	ATTCGTACCGCTTCTGTTCC	105%
phoB	Transcriptional regulator	2775	67923615	AACCGTCGATGTTCATATTCG	TCCAAACCGATAACCAAAGC	101%
ppK	Polyphosphate kinase	6491	67920515	GTGGTGGTTCGTGATCCTG	AGCTGTTTGCTCGTCTTCTTG	101%
ppX	Exopolyphosphate	1948	67924239	CGTTCCCTAGCTTCTTTACGAC	GTGATGCCAACTTCTGCTTG	106%

| *ppA* | Inorganic pyrophosphatase | 2235 | 67924135 | CGGGTTGTGTCATTGCAG | AGCGTGGATCTTCATCA GG | 95% |
| *rnpB*[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 ± 6%.
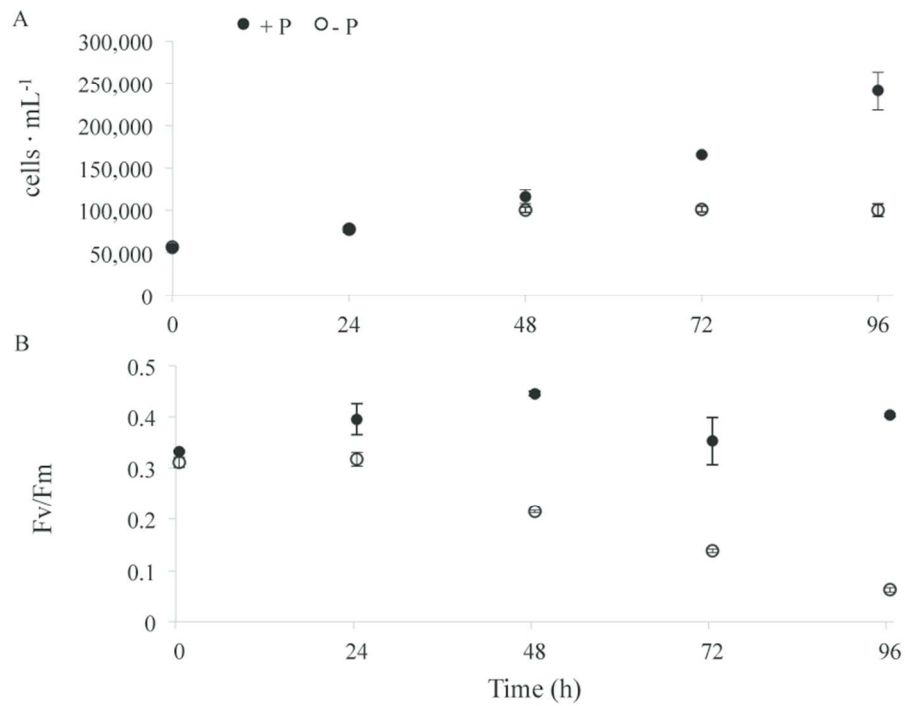
Fig. 1. Growth and photosynthetic efficiency of *Crocosphaera watsonii* WH8501. Cell cultures grown in phosphate replete (60 µM; closed symbols) and phosphate depleted (60 nM; open symbols) media. Cell counts (A) taken for cultures starting at T0 and photosynthetic efficiency (Fv/Fm) (B) measured during the same experiment. All error bars indicate standard deviation of triplicate biological replicates.
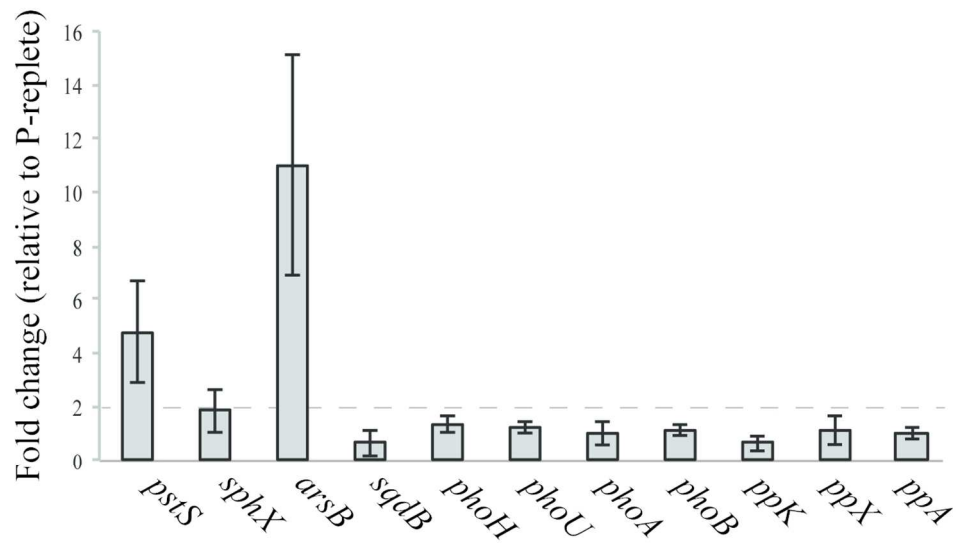81x60mm (300 x 300 DPI)

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.
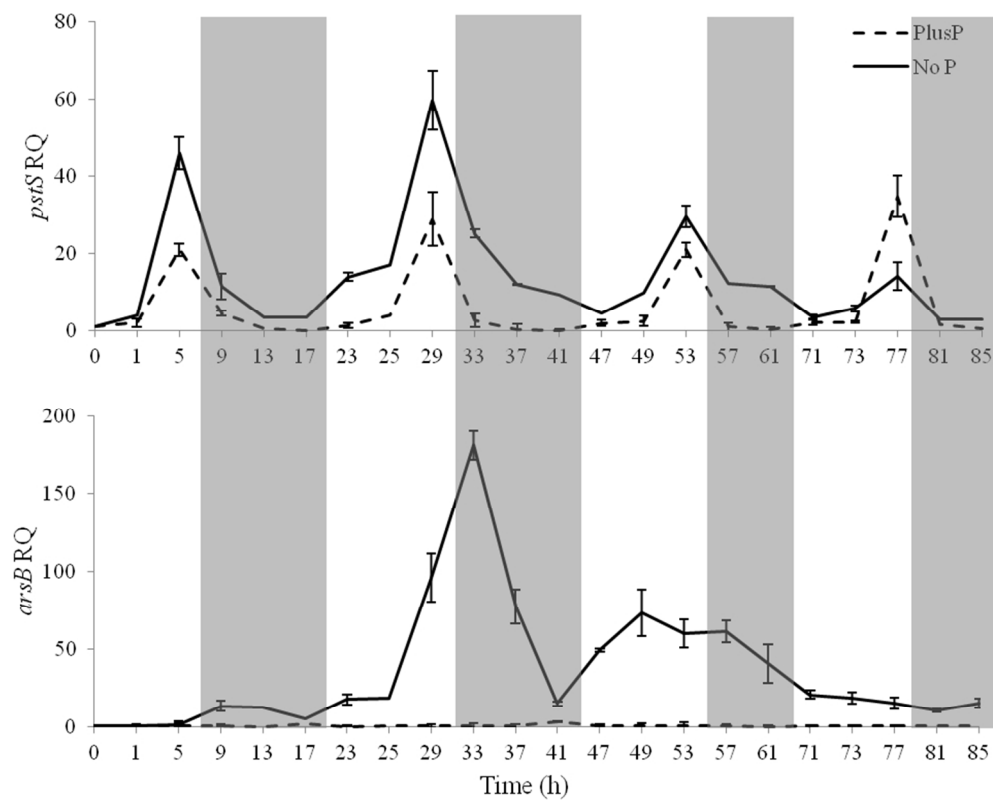122x74mm (300 x 300 DPI)

Fig. 3. Transcription profiles of *pstS* and *arsB* during a batch culture experiment where phosphorus is limited. *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.
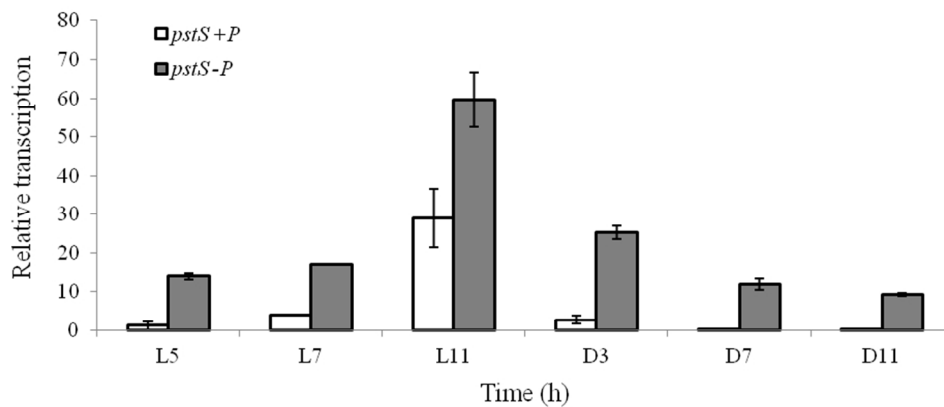82x65mm (300 x 300 DPI)

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 *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.
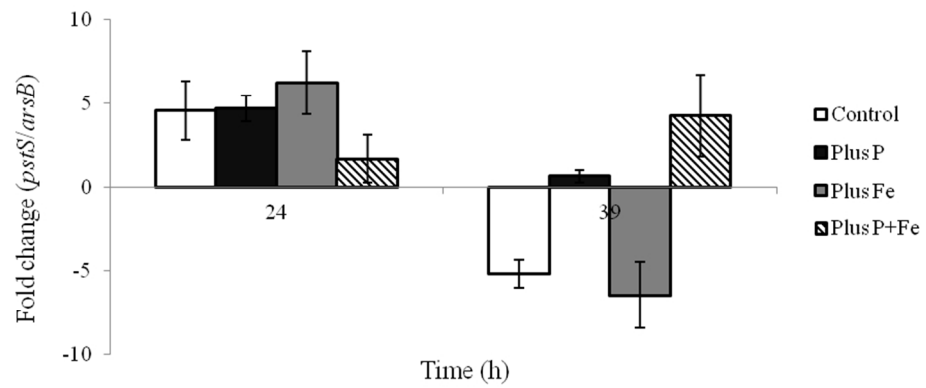82x35mm (300 x 300 DPI)

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 µM 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)