

## Two subpopulations of *Crocospaera watsonii* have distinct distributions in the North and South Pacific

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Running title: Natural abundances of two *Crocospaera* sub-types

1 **Abstract**

2           *Crocospaera watsonii* is a unicellular nitrogen (N<sub>2</sub>)-fixing cyanobacterium with ecological  
3 importance in oligotrophic oceans. In cultivated strains there are two phenotypes of *C. watsonii* (large  
4 and small cell) with differences that could differentially impact biogeochemical processes. Recent  
5 work has shown the phenotypes diverged through loss or addition of type-specific genes in a fraction  
6 of their genomes, while the rest of the genomes were maintained at 99-100% DNA identity. Previous  
7 molecular assays for *C. watsonii* abundances targeted the conserved regions and therefore could not  
8 differentiate between phenotypes, so their relative distributions in natural communities were  
9 unknown. To determine phenotype distributions, this study developed and applied type-specific qPCR  
10 assays to samples from the North and South Pacific. Abundances of both *Crocospaera* types declined  
11 sharply with depth between 45 and 75 m in both sites. In surface water small cells were 10 to 100  
12 times more abundant than large cells in the N. Pacific; while in the S. Pacific the two phenotypes were  
13 nearly equal. Evidence for large cell aggregation was only found in N. Pacific samples. The differences  
14 in *C. watsonii* sub-populations in the North and South Pacific Ocean have direct implications for  
15 biogeochemistry and carbon export in oligotrophic gyres.

16

## 17 Introduction

18 *Crocospaera watsonii* is a species of unicellular nitrogen (N<sub>2</sub>)-fixing cyanobacteria (“UCYN”  
19 from here forward) that is important in marine primary production and biogeochemical cycling. This is  
20 especially true in oligotrophic waters where nitrogen is often a limiting nutrient, and *C. watsonii* is an  
21 important source of biologically available nitrogen (Zehr *et al.*, 2001; Falcon *et al.*, 2004; Montoya *et*  
22 *al.*, 2004; Kitajima *et al.*, 2009; Moisander *et al.*, 2010). UCYN are among the most abundant N<sub>2</sub>-fixers  
23 in oceanic systems and measurements of UCYN abundance by direct microscopy counts and qPCR of  
24 the *nifH* gene are important for determining their contributions to the N cycle. Direct counts of UCYN  
25 abundances have ranged from 10<sup>4</sup> to 10<sup>7</sup> cells per liter in the North Pacific (Zehr *et al.*, 2001; Church *et*  
26 *al.*, 2005), and near 10<sup>5</sup> cells per liter in the Atlantic (Falcon *et al.*, 2004), and qPCR studies have  
27 reported between 10<sup>3</sup> and 10<sup>6</sup> gene copies per liter in multiple ocean basins (Zehr *et al.*, 2001; Falcon  
28 *et al.*, 2004; Church *et al.*, 2005; Church *et al.*, 2008; Langlois *et al.*, 2008; Moisander *et al.*, 2008;  
29 Moisander *et al.*, 2010). However, microscopic and qPCR enumerations of natural populations have  
30 treated all *Crocospaera* cells as a single global population because the cells are morphologically  
31 similar by microscopy, and because there is a lack of genetic variation in the *nifH* gene.

32 The genetic conservation observed in *Crocospaera nifH* sequences was also observed in  
33 sequences encoding 16S rRNA and a number of other genes that were examined in natural populations  
34 and cultivated strains (Zehr *et al.*, 2007). Despite their gene sequence conservation, two distinct  
35 phenotypic categories have been described in *C. watsonii* isolates. The first (large cell) phenotype has  
36 a cell-diameter of 4-6 μm, produces abundant extracellular polysaccharide (EPS), has higher  
37 photosynthetic efficiencies ( $F_v/F_m$ ), and higher per-cell nitrogen fixation rates (Webb *et al.*, 2009; Sohm  
38 *et al.*, 2011). The other (small cell) type has a cell diameter less than 4 μm, and does not produce

39 noticeable amounts of EPS. There is also evidence that the small cell types grow in a narrower  
40 temperature range, and are missing some phosphorus scavenging genes that are found in the large cell  
41 types (Dyhrman and Haley, 2006; Webb *et al.*, 2009). More recently, genome comparisons showed  
42 that the large cell types contained a variety of genetic capabilities that were missing from the smaller  
43 genomes of the small cell types, such as EPS biosynthesis, iron stress response genes, and phosphorus  
44 metabolism genes (Bench *et al.*, 2011; Bench *et al.*, 2013).

45         Because of their genetic and metabolic differences, it is likely that the two phenotypes have  
46 different impacts on biogeochemical cycling. For example, EPS is a carbon-rich compound that can  
47 protect cells as well as cause aggregation which increases sinking rates (Passow *et al.*, 2001; Pereira *et al.*,  
48 2009; Sohm *et al.*, 2011). As such, EPS production could make the large cell types greater  
49 contributors to carbon export from surface water than the small cell types. The differences in iron and  
50 phosphorus related genes further suggest that the two *C. watsonii* types may be differently adapted to  
51 their chemical environment, and therefore may have different niches. However, because previous  
52 methods used to measure natural *Crocospaera* abundances were limited to viewing the species as a  
53 single population, very little is known about the distribution of the two types in the water column or  
54 the global oceans. In light of the ecologically relevant differences between types, it is important to  
55 determine their specific distributions in order to better understand the impact of the species on  
56 nutrient cycling and marine ecosystems. As a step towards that better understanding, this study was  
57 carried out with the following specific goals: 1) use recently identified phenotype-specific genes to  
58 develop specific and sensitive molecular assays to differentiate between *Crocospaera* phenotypes and  
59 2) apply those assays to water column samples from two regions in the Pacific Ocean in order to  
60 characterize differences in vertical and basin-wide distributions of the two *C. watsonii* types in natural  
61 populations.

## 62 Results

63           Recent genomic comparisons of multiple cultivated *Crocospaera watsonii* strains identified  
64 gene sequences that were strain-specific and phenotype-specific (Bench *et al.*, 2011; Bench *et al.*,  
65 2013). This enabled the design of the qPCR assays described in this study, which target four different  
66 genomic loci; two specific for large cell type strains, and two specific for small cell strains (Table 1).  
67 The previously used *Crocospaera nifH* (also referred to as “Group B”) primer probe sets (Moisander *et*  
68 *al.*, 2010) acted as the positive quantitative control during testing of each qPCR locus using DNA from  
69 six *C. watsonii* cultivated strains (see methods). All loci amplified quantitatively in reactions with DNA  
70 from strains with the targeted phenotype as expected, and did not amplify with DNA from non-target  
71 phenotype strains (Table S1). This was true for all loci, including reaction where DNA from a non-  
72 targeted phenotype strain (i.e. not expected to amplify) was ten-fold greater than DNA from the  
73 targeted strain.

74           After the qPCR assays were tested against individual strains, they were applied to samples from  
75 two research cruises (Fig. 1). There was good correlation across the N. Pacific samples between the  
76 two gene-specific assays within each phenotype (Fig. S1), and a paired t-test found no difference  
77 between the abundances reported by the assays ( $p = 0.69$  for small cell assays, and  $p = 0.71$  for large  
78 cell assays). The sum of the two phenotypes also correlated well with *nifH* abundances (Correlation  
79 coefficient = 0.9899, Fig. S2).

80           In the N. Pacific water column samples, total *nifH Crocospaera* abundance was often  $>10^6$   
81 gene copies per liter in samples from 50 m and shallower, and declined by 2 to 3 orders of magnitude  
82 between 50 and 100 meters, decreasing to abundances of  $\sim 10^3$  gene copies per liter at and below 150  
83 m (Fig. 2, upper panel). The small cell type accounted for the vast majority of total *Crocospaera* at

84 depths shallower than 75 m, while the large cell type was more abundant in samples deeper than 100  
85 m (Fig. 2). Small cell *Crocospaera* abundances ranged from over  $10^6$  gene copies per liter at the  
86 surface to less than  $10^3$  copies per liter at and below 125 m (Fig. 2, middle panel). The abundance of  
87 the large cell types in the upper water column was  $10^4$  to  $10^5$  gene copies per liter, decreasing to  $\sim 10^3$   
88 copies per liter below 75 m (Fig. 2, lower panel). The ratio of small cell to large cell *Crocospaera*  
89 (calculated by dividing the small cell gene copies by large cell gene copies for each sample) was  
90 typically between 10 and 1,000 in shallow samples, and between 0.1 and 1 in most samples below 75  
91 m (Fig. S3).

92 *Crocospaera* abundances were measured in sediment trap samples to assess possible  
93 differences in sinking processes between the two phenotypes. The ratio of the phenotypes in  
94 sediment traps was then compared with depth-integrated and spatially averaged values from water  
95 column samples collected during the same time (see methods). At 100 m, the average ratio of small  
96 cells to large cells was 51.4 in the water column and 42.9 in the sediment trap. At 150 m, the small:  
97 large ratio in the water column was 49.4 and the ratio in the sediment trap was 2.1.

98 For all water column samples, phenotype-specific assays were performed separately on the  
99 10 $\mu$ m and the 0.2  $\mu$ m filters that were collected in-line (see methods) and the relative contribution of  
100 the 0.2  $\mu$ m filter to the total for each sample was calculated. For nearly all samples, close to 100% of  
101 small cells were found on the 0.2  $\mu$ m filter (Fig. 3, upper panel and Fig. S4). Only two samples (out of  
102 60) showed less than 60% of small cells on the 0.2  $\mu$ m filter, and in the other 58 samples an average of  
103 94% of small cell abundance was found on the 0.2  $\mu$ m filter. In contrast, for many samples, especially  
104 in the first half of the cruise, less than 40% of the total large cell copies were found on the 0.2  $\mu$ m filter  
105 (Fig. 3, lower panel and Fig. S5). In other samples (e.g. samples collected from below 75 m depth and

106 surface samples from the last two stations), nearly all large cells were found on the 0.2  $\mu\text{m}$  filter,  
107 similar to the small cells.

108 A principal component analysis of environmental variables was used to investigate potential  
109 correlations between *Crocospaera* abundances in BioLINCS samples and water column parameters.  
110 The first principal component (PC1) explained 48% of the observed variation, and was mainly related to  
111 depth, density, temperature, light, ammonia and nitrite. The second principal component (PC2)  
112 explained 30% of the variation and was mainly composed of oxygen, salinity, chlorophyll, phosphate  
113 and silicate. The two dimensional projection shows clustering of the water samples into three depth-  
114 related groups (Fig. 4): surface (orange symbols), chlorophyll maximum and adjacent depths (hereafter  
115 referred to as chl max, green symbols), and deeper water (blue symbols). In contrast to the chl max  
116 samples, the surface and deep samples showed little variation along PC1, but are well spread along  
117 PC2. Abundances of both *Crocospaera* cell types (as well as total *Crocospaera*) showed significant  
118 negative correlation with PC1.

119 The three clusters (surface, chl max, and deep) were also tested separately to identify  
120 correlations with environmental factors that were independent from depth and abundance (Table S2).  
121 In surface water samples, large cell abundances were positively correlated with phosphate and silicate  
122 and negatively correlated with salinity. In the chl max samples, both cell types were significantly  
123 positively correlated with temperature and PAR, and negatively correlated with salinity, density,  
124 chlorophyll, nitrate, and silicate. In addition, large cells had a negative correlation with ammonia. In  
125 the deep samples, no significant relationships were found between environmental variables and either  
126 cell type.

127 Abundances of the two *Crocospaera* phenotypes were also measured in samples collected at  
128 three stations during a research cruise in the South Pacific Ocean in the austral fall of 2007. An in-

129 depth characterization of oceanographic conditions and total diazotroph population distributions are  
130 described in Moisander *et al.* (2010). In these samples, abundances of both *Crocospaera* types were  
131 approximately  $10^5$  to  $10^6$  gene copies per liter near the surface, dropping to between  $10^2$  to  $10^4$  in  
132 deeper water (Fig. 5, C). The ratio of large cells to small cells was relatively constant throughout the  
133 water column, typically between 0.4 and 4 with an average of 1.5 for all samples (Fig. 5, D). Over 80%  
134 of the total abundance of small cells was found on the 0.2  $\mu\text{m}$  filter for all S. Pacific samples, with many  
135 close to 100% and an overall average of nearly 93% (Table S3). Over 80% of the large cell types were  
136 also captured on the 0.2  $\mu\text{m}$  filter in all but three samples, with an average over 89% for all samples.

137

## 138 **Discussion**

139 Despite known phenotypic differences in cultivated strains, *Crocospaera watsonii* has only  
140 been assayed as a single population in the environment because of a lack of known genetic variation  
141 and an assumption that it behaves ecologically as a single population. The qPCR assays described in  
142 this study are necessary to examine natural populations for differences in the phenotypically distinct  
143 groups of *C. watsonii*. Testing of the novel qPCR primers and probes demonstrated that all four qPCR  
144 assays were robust with no evidence of cross-reactivity or inhibition from un-targeted *C. watsonii*  
145 phenotypes. Strong agreement between the total *Crocospaera nifH* abundances and the sum of the  
146 two sub-types (Fig. S2) illustrated that these two types make up the entire natural *Crocospaera*  
147 community, at least for the samples in this study. As such, this study presents important details of *C.*  
148 *watsonii* distributions in the Pacific Ocean, as well as new and robust tools that can be used to further  
149 examine *C. watsonii* populations in other ocean basins and during other seasons and years.



150 In the upper water column N. Pacific samples, small cell *Crocospaera* abundances and  
151 distributions were similar to total *nifH* abundances, and the large cell type *Crocospaera* were much  
152 less abundant. However, in deeper water, large cell abundances were often much higher than the  
153 small cell abundances in the same samples. Intriguingly, while total *Crocospaera* abundances were  
154 similar in surface water of the two locations, small cell types did not dominate the *Crocospaera*  
155 populations in the S. Pacific. In fact, large cell abundances were on par with small cell abundances  
156 indicating that, at the time of sampling, conditions in the surface mixed layer of the S. Pacific were  
157 more favorable for the large cell type than conditions in the N. Pacific during the BioLINCS cruise (Fig.  
158 5). However, at both locations, there was a slight dominance of large cell *Crocospaera* below 75 m.  
159 In addition, the dominance of total *Crocospaera* populations by the small cell population (i.e. the high  
160 ratio of small: large cells when integrating over the water column) was reduced in sediment trap  
161 samples relative to water column values collected in the N. Pacific, particularly at 150 m depth. The  
162 dominance of large cells in deeper water and the overrepresentation of large cell *Crocospaera* in the  
163 sediment traps relative to the water column could be explained by any or all of the following; 1) faster  
164 sinking of the large cell type, 2) slower degradation of large cells during sinking, or 3) preferential  
165 grazing of the small cell type. All three mechanisms would be enhanced by EPS production in the large  
166 cells (Passow et al., 2001; Pereira et al., 2009; Sohm et al., 2011), emphasizing the importance of  
167 distinguishing the two types of *Crocospaera* in the environment, since the two populations appear  
168 capable of playing different ecological roles. A previous study carried out microscopic cell counts to  
169 quantify relative abundance of two cell size classes of *Crocospaera* in the western South Pacific (Webb  
170 et al., 2009). In that study, the smaller cells were slightly more abundant than the larger cells, but  
171 populations were only examined within a narrow depth range near the surface (6-14 m), so deeper  
172 water patterns cannot be compared to the qPCR assays results of this study. Evidence of cell

173 aggregation was seen in the large cells (Webb *et al.*, 2009), which supports the possibility of differential  
174 export dynamics of the two phenotypes, and is different from the results presented here which did not  
175 show evidence of aggregation in the S. Pacific. Future experiments with additional environmental  
176 samples will be needed to fully understand the export processes and the conditions that favor the two  
177 phenotypes.

178         The principal component analyses carried out on the N. Pacific samples offer some clues about  
179 which environmental factors may affect *Crocospaera* abundances. The largest component (PC1) was  
180 driven by factors that vary strongly with depth, and resulted in the samples clustering into three  
181 groups along the PC1 axis (Fig. 4). The statistically significant negative correlations with PC1 for both  
182 *Crocospaera* phenotypes probably illustrate their observed depth-related decline as well as their lack  
183 of dependence on bioavailable nitrogen. In addition, the larger spread in PC1 for chl max samples was  
184 expected for samples that span the thermocline and contain the associated variability in depth-related  
185 factors (Robidart *et al.*, 2014). The second principal component (PC2) was driven by factors that  
186 varied more from station to station, (as opposed to depth) and resulted in a spreading of the surface  
187 and deep samples along this axis (Fig. 4).

188         The negative correlation with ammonia may be an indication of the type of N<sub>2</sub>-fixation  
189 inhibition by ammonia that was observed in *Crocospaera* culture experiments (Dekaezemacker, 2011;  
190 Garcia, 2014b) . Those studies found the effect of N<sub>2</sub>-fixation inhibition was stronger under low light  
191 growth conditions (Garcia, 2014b), and also stronger in large cell strains compared to small cell strains  
192 (Dekaezemacker, 2011). Given those results, it is notable that there was a dominance of large cells in  
193 deeper water where ammonia concentrations are higher and light levels are lower. A factor that  
194 would favor the dominance of actively growing larger cells in deeper water is the observation that

195 certain small-cell strains will not grow under the same low light conditions where large cells are able  
196 to grow (Garcia, 2013b).

197 In chl max samples (Fig. 4, green symbols), both cell types were significantly correlated with  
198 salinity. As salinity is a conservative property of seawater, it appears that, within the chlorophyll max,  
199 *Crocospaera* distributions may be driven by mixing of disparate water types, rather than biological  
200 interactions or nutrient variation. In surface samples, there was a correlation with phosphate and  
201 silicate in large cells, and not small cells, indicating the small cell type may be less dependent on  
202 phosphate. This is in contrast to genomic evidence (i.e. fewer copies of phosphorus related genes) that  
203 suggests the small cell type is less adapted to low phosphorus conditions than the large cell strains  
204 (Bench *et al.*, 2013). Of course, the small cell phenotype has a higher surface to volume ratio, so it may  
205 also have a reduced need for phosphorus scavenging capabilities. There is also recent evidence of a  
206 dramatic reduction in cell size in both phenotypes of cultured *Crocospaera* under P and Fe co-  
207 limitation (Jacq, 2014; Garcia 2014a). Those results underscore the importance of measuring both Fe  
208 and P concentrations in the water where the two types are quantified as well as making microscopic  
209 measurements of cell-size in natural populations. Future measurements of abundances of the two cell  
210 types in other marine samples, particularly in the Atlantic where Fe is higher and P is lower than in the  
211 Pacific, will help strengthen (or refute) the correlations observed in this study, and refine how  
212 researchers understand *Crocospaera* ecology.

213 Because all *C. watsonii* isolates have cell diameters that range from 3.5  $\mu\text{m}$  to  $\sim 6 \mu\text{m}$  (Webb *et*  
214 *al.*, 2009; Sohm *et al.*, 2011), it is expected that the vast majority of naturally occurring *C. watsonii* cells  
215 should pass through the 10  $\mu\text{m}$  filter and be captured on the 0.2  $\mu\text{m}$  filter (which were arranged in-line  
216 as described in the methods). However, as discussed above, the large cell phenotype is also known to  
217 produce copious amount of EPS, and to form multi-cell aggregates as a result (Webb *et al.*, 2009; Sohm

218 *et al.*, 2011). Such aggregates could contribute to retention of the large cell type on the larger pore  
219 size filter, as was observed in microscopic examination of the *Crocospaera* community in the western  
220 South Pacific (Webb *et al.*, 2009). In this study, the small cell type was found almost exclusively on the  
221 0.2µm filter in all samples in both ocean basins. However, in many of the N. Pacific samples, the  
222 majority of large cells were found on 10µm filters, supporting previous evidence of aggregation in the  
223 large cell type.

224 The smaller fraction of large cells captured on 10µm filters in the deeper N. Pacific samples  
225 could be explained by aggregates breaking up as they sink, or by grazing. The release of cells by these  
226 processes would result in a larger proportion passing through the 10 µm filter. This explanation would  
227 mean that the presence of *C. watsonii* below 75 m is not indicative of those cells being active and  
228 dividing (i.e. adapted to that depth), but rather a result of sinking and mixing. On the other hand, the  
229 apparent lack of aggregation observed in large cell *Crocospaera* in all S. Pacific samples (rather than  
230 just deeper samples) suggests those cells were producing less EPS. There is some possibility that EPS  
231 production within a genotype may be influenced by environmental factors. For example, a study of  
232 *Crocospaera* growth responses to inorganic nitrogen observed a plasticity in the C:N ratio of the large-  
233 cell strain WH0003 (DeKaezemacker, 2011). While those authors did not observe changes in EPS  
234 production, they suggested that modifying EPS production could be a mechanism that large cell  
235 *Crocospaera* could use to modulate their cellular C:N ratios. Further evidence from cultures and  
236 genomic data strongly suggests that EPS production is an inherent, rather than inducible, trait in  
237 *Crocospaera* (Sohm *et al.*, 2011; Bench *et al.*, 2013). As such, the much lower incidence of capture on  
238 the 10 µm in the S. Pacific surface samples suggests that basin may have had a different large cell sub-  
239 type than the N. Pacific, where aggregation was more often observed. If that is the case, it will be  
240 important to distinguish between the two large cell sub-types because a non-aggregating sub-type

241 would likely have export properties more similar to the small cell types. Distinct responses between  
242 two small cell strains to changing CO<sub>2</sub> concentrations (Hutchins et al. 2013) provides evidence that  
243 there are sub-types with different ecological adaptations within the larger two phenotypic categories  
244 examined in this study. Additional genetic markers will be needed to design and carry out experiments  
245 that could differentiate between the potential sub-types, and identify the physical and/or chemical  
246 conditions that are more favorable to each type.

247         The three processes proposed above to explain the abundance patterns of the two size  
248 fractions (aggregate break-down, grazing, and separate sub-types) have different predictions for the  
249 metabolic state of the deeper population of cells. If the cells at those depths have simply sunk from  
250 shallower water, they would not be expected to contribute fixed N to the deeper water where they  
251 were observed. However, if they are a separate large-cell population that is adapted to those depths,  
252 their contribution of new N to deeper water will need to be considered. Future experiments could  
253 assess the biogeochemical contributions of the two sub-types using a variety of methods, including  
254 measuring sinking rates, measuring N<sub>2</sub>-fixation rates in deep vs. shallow water, and by assessing the  
255 viability of the deeper population through physiological fluorescence measurements and/or gene  
256 expression levels of the two groups.

257

## 258 **Conclusions**

259         The qPCR assays developed for this study provide a novel method for quantifying two  
260 phenotypes of *Crocospaera* that were previously treated as a single, globally distributed population.  
261 Resulting water column distribution patterns demonstrated, for the first time, that each phenotype has  
262 a distinct biogeography, consistent with known phenotypic differences and suspected ecological

263 distinctions. Small cells were 10 to 1,000 times more abundant than large cells in the N. Pacific upper  
264 water column, but the two types were nearly equally represented throughout the water column in the  
265 S. Pacific. Furthermore, the evidence of large cell aggregation observed in the N. Pacific samples was  
266 not seen in the S. Pacific large cell populations. These patterns indicate that further sub-types of large  
267 cell *Crocospaera* exist, with unique distributions the North and South Pacific, or alternatively,  
268 differences in physiology and/or grazing rates between the phenotypes result in differing degrees of  
269 aggregation in each basin. The observed patterns indicate that distinct controls determine the  
270 distributions of the two *Crocospaera* phenotypes, many of which could vary over time as well as  
271 space. Because of the ecologically important differences between the two phenotypes demonstrated  
272 here, quantifying *C. watsonii* as two distinct groups is necessary for determining the global contribution  
273 of this keystone species to the carbon pump and marine biogeochemical cycles.

274

## 275 Experimental Procedures

### 276 Design and testing of qPCR assays

277 Previous comparisons of six *Crocospaera watsonii* genomes identified genes unique to each  
278 phenotype (Bench *et al.*, 2011; Bench *et al.*, 2013). Two genes were chosen for each phenotype that  
279 were found in all genomes of one type, and were absent from all genomes of the other type. A primer-  
280 probe set was designed for each gene using Primer 3 (Rozen and Skaletsky, 1999) with a goal of  
281 obtaining a  $T_m$  of 64°C for primers, and 74°C for probes. All four genes and corresponding primer and  
282 probe sequences were used in nucleotide BLAST searches against the CAMERA (Sun *et al.*, 2011) and  
283 GenBank NT and WGS (Benson *et al.*, 2003) databases to verify that they did not have significant  
284 sequence similarity to other known organisms. The genes used for primer and probe design and the  
285 resulting sequences for all loci are listed in Table 1, with the design and testing of the *nifH* locus  
286 previously described (Moisander *et al.*, 2010). Dual-label probes were synthesized with FAM  
287 fluorescent tags and TAMRA quenchers. Reactions were set up in sterile PCR hoods using UV sterilized  
288 optical tubes or plates and contained 1.5 - 2  $\mu$ l of template DNA plus 1  $\mu$ l of each primer (10  $\mu$ M), 0.5  
289  $\mu$ l of probe (10  $\mu$ M), 12.5  $\mu$ l TaqMan Gene Expression 2X Master Mix (Life Technologies, Grand Island,  
290 NY, USA), and water to a final volume of 25  $\mu$ l. Amplification and detection was carried out on an ABI  
291 7500 instrument using the following 2-step reaction: initial steps of 50°C for 2 minutes, then 95°C for  
292 10 minutes, then 45 cycles of 90°C for 15 seconds, then 60°C for 60 seconds. Each run included 3 or 4  
293 no template controls (NTCs) and a set of standards, in triplicate, with known gene copies from  $10^0$  to  
294  $10^7$  per reaction. Following each run, the threshold cycle ( $C_t$ ) values for each standard were plotted  
295 versus the log of its gene copy number to create a standard curve. The equation for that standard  
296 curve was used to calculate the gene copies in each of the sample reactions from the same run.

297 Standards were made from amplified genomic DNA from *C. watsonii* strains of the appropriate  
298 phenotype. To avoid amplifying DNA from contaminants in non-axenic cultures, cells were sorted  
299 using a flow cytometer prior to whole genome amplification (WGA) with Repli-g (Qiagen, Germantown,  
300 MD, USA). The sorting and WGA were carried out as described in the methods used for genome  
301 sequencing of *C. watsonii* strains (Bench *et al.*, 2011; Bench *et al.*, 2013). Amplified genomic DNA was  
302 quantified using Pico Green (Life Technologies, Grand Island, NY, USA), and genome copies/ $\mu$ l were  
303 calculated based on the DNA concentration and the genome sizes (Bench *et al.*, 2013). Appropriate  
304 dilutions were made to generate a set of standards that contained  $10^0$  to  $10^7$  genome copies in 2  $\mu$ l  
305 (the volume used in each reaction). Multiple sets of the prepared genomic standards were compared  
306 in triplicate to *nifH* linearized plasmid standards to verify the DNA quantification, and relative reaction  
307 efficiency, and no significant differences were observed between the plasmid and any of the genomic  
308 standards.

309 Tests for cross reactivity and inhibition were carried out for all loci using multiple mixtures of  
310 DNA from different *C. watsonii* strains. Names and phenotypes of *C. watsonii* strains used are listed in  
311 Table S1. Four test mixtures contained genomic DNA from WH8501 and WH0003 strains in the  
312 following ratios: 1:3, 3:1, 1:10, and 10:1. Eight additional test mixtures contained WH8501 or WH0003  
313 DNA mixed with 3-fold more DNA (final ratio of 1:3) from one of four additional strains (WH8502,  
314 WH0401, WH0401, and WH0005). The primer-probe set for each locus was tested for amplification  
315 and inhibition in triplicate qPCR reactions with the 12 different mixtures, which ranged over an order in  
316 magnitude in target DNA and non-target DNA concentrations, and included samples that contained  
317 only target DNA as well as only non-target DNA. Copy numbers from qPCR reactions were consistent  
318 with DNA concentrations used in each reaction, and there was no amplification in any of the samples  
319 that did not contain target DNA (Table S1). Because there was no observed cross-reactivity or



320 inhibition from non-target strains, all four primer-probe sets were determined to be appropriate for  
321 use in environmental samples.

322

### 323 Sample collection, DNA extraction and qPCR of cruise samples

324 South Pacific samples were collected during the R/V Kilo Moana cruise KM0703 in March and  
325 April of 2007. Cruise station locations as well as methods for water sample collection and processing  
326 and DNA extraction were described previously (Moisander *et al.*, 2010). North Pacific samples were  
327 collected at 8 stations during the BioLINCS cruise in September of 2011 just north of Station Aloha (Fig.  
328 1). At each station, water samples were collected from multiple discrete depths (5, 25, 45, 75, 100,  
329 125, 150, 175 m) with Niskin bottles mounted on a CTD rosette. Two to three liters of collected water  
330 was filtered through two in-line Durapore filters (10 µm pore size, followed by 0.2 µm pore size).  
331 Filters were placed in bead beater tubes with sterile glass beads, immediately flash-frozen in liquid  
332 nitrogen and subsequently stored at -80°C until DNA was extracted. Sediment trap samples were  
333 collected from seven depths (the three used in this study are 100, 150 and 500m) using a drifting  
334 sediment trap that was deployed near Station 5 (Fig. 1). The trap drifted northeastward during the  
335 BioLINCS cruise, on a track that was approximately 30° northeastward of the ship transit. In order to  
336 relate qPCR abundances of ecotypes sampled from the ship's CTD Niskin bottles to samples collected in  
337 the drifting sediment traps, samples exclusively from the ship's northeast trajectory were used. Details  
338 of the sediment trap deployment, drift track and sample collection are described in Wilson *et al.*  
339 (2014). Because the volume of sea water that contributed to the sediment trap samples is not known,  
340 *Crocospaera* abundances "per sample" are reported, and ratios of the two phenotypes (rather than  
341 actual abundances) are used for comparisons between sediment trap samples and water column data.  
342 In addition, water column abundances of each *Crocospaera* type were integrated over equivalent

343 depths to the sediment traps (100 m, or 150 m), and the depth-integrated totals were used to calculate  
344 the ratio of small to large cells at each station. The ratios were then averaged over the six stations that  
345 approximated the multi-day drift track of the sediment trap in order to approximate the average water  
346 column populations above the sediment trap during its deployment.

347         The DNA extraction protocol used for N. Pacific samples is a slight adaptation of the modified  
348 DNeasy Plant MiniKit (Qiagen) protocol used to extract the S. Pacific samples (Moisander *et al.*, 2008;  
349 Moisander *et al.*, 2010). Filters were thawed and 400  $\mu$ l of AP1 buffer (provided in kit) was added to  
350 each tube. Samples were subjected to three freeze-thaw cycles of rapid freezing in liquid N<sub>2</sub>, followed  
351 by rapid thawing in a 65°C heat block. The samples were then bead-beat in Mini-Beadbeater-96  
352 (Biospec Inc.) for 2 minutes. Tubes were centrifuged briefly prior to addition of 45  $\mu$ l (20 mg/ml) of  
353 Proteinase K (Qiagen), vortexed briefly and incubated (with rocking) at 55°C for 1 hour. An RNaseA  
354 digestion was then carried out by adding 4  $\mu$ l of RNaseA to each sample, vortexing and incubating at  
355 65°C for 10 minutes. The filters were removed from the tubes, and 130  $\mu$ l of AP2 buffer (provided in  
356 kit) was added to each tube followed by a brief vortex and a 10 minute incubation on ice. Tubes were  
357 spun for 5 minutes at 14,000 RPM to pellet beads large precipitates, and the supernatant for each  
358 sample was transferred to sterile 2 ml locking Sample tubes RB (Qiagen). DNA was extracted from the  
359 transferred supernatant using the standard reagents and protocols for “Plant Cell & Tissues” with the  
360 “DNeasy Plant Mini” kit in the QIAcube instrument (Qiagen). The final elution volume for each sample  
361 was 100  $\mu$ l.

362         The qPCR assays of environmental samples used the same reaction contents (except template  
363 DNA), genomic DNA standards, and cycling conditions were as described for primer-probe testing  
364 above. DNA extracts were diluted 1:5 (N. Pacific samples) or 1:1 (S. Pacific samples) and 1.5  $\mu$ l of the  
365 dilution was used in triplicate reactions. For the N. Pacific samples, *nifH* reactions contained 2  $\mu$ l of

366 undiluted DNA extract in duplicate reactions. For the S. Pacific samples, previously determined *nifH*  
367 abundance values (Moisander *et al.*, 2010) were used.

368

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375

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469 **Table and Figure legends**

470

471 Table 1. Primer and probe sequences and gene source for qPCR assays. (All sequences area shown in 5'  
472 to 3' direction).

473

474 Figure 1. Sample locations and station numbers in the Pacific Ocean. South Pacific samples were  
475 collected in 2007, and North Pacific samples (details in inset) were collected in 2011.

476

477 Figure 2. Abundances for total (top panel) and two phenotypic sub-groups (middle and bottom) of  
478 *Crocospaera watsonii* in samples collected during the BioLINCS cruise (station locations shown in Fig.  
479 1). Gene copy numbers for all three assays are the sum of both size fractions for each sample, see  
480 supplemental material for plots of abundances of each size fraction.

481

482 Figure 3. Fraction of total abundance (i.e. the sum of both size fractions) found on 0.2µm filter for the  
483 small-cell phenotype of *Crocospaera watsonii* (upper panel) and for the large-cell phenotype (lower  
484 panel) in samples collected during the BioLINCS cruise (station locations shown in Fig. 5, and  
485 abundances on each filter are shown in supplemental material (figures S4 and S5).

486 Figure 4. Principal component analysis of BioLINCS (N. Pacific) water samples. Symbols indicate depth  
487 where water sample was collected and are clustered into three depth-related groups, with surface  
488 samples in orange symbols, chl max in green, and deep samples in blue. Projections of environmental  
489 variables (red arrows) and *Crocospaera watsonii* abundances (sum of both filters, purple arrows) are  
490 shown in the PC space multiplied by 10 and 5 respectively. PCA analysis did not include the depth as a  
491 variable. Total variance covered by the two components is 78% (48% by PC1 and 30% by PC2).

492 Figure 5. Abundances of two *Crocospaera watsonii* phenotypes in North Pacific (A) and South Pacific  
493 (C) plotted according to the depth where each water sample was collected. The ratio of the two  
494 phenotypes (small:large) is also shown for each of the N. Pacific (B) and the S. Pacific (D) samples.

495