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Colimitation of the unicellular photosynthetic diazotroph *Crocosphaera watsonii* by phosphorus, light, and carbon dioxide

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Abstract

We describe interactive effects of total phosphorus (total P = 0.1–4.0 μmol L^{-1}; added as H_2NaPO_4), irradiance (40 and 150 μmol quanta m^{-2} s^{-1}), and the partial pressure of carbon dioxide (P_{CO_2}; 19 and 81 Pa, i.e., 190 and 800 ppm) on growth and CO_2- and dinitrogen (N_2)-fixation rates of the unicellular N_2-fixing cyanobacterium *Crocosphaera watsonii* (WH0003) isolated from the Pacific Ocean near Hawaii. In semicontinuous cultures of *C. watsonii*, elevated P_{CO_2} positively affected growth and CO_2- and N_2-fixation rates under high light. Under low light, elevated P_{CO_2} positively affected growth rates at all concentrations of P, but CO_2- and N_2-fixation rates were affected by elevated P_{CO_2} only when P was low. In both high-light and low-light cultures, the total P requirements for growth and CO_2- and N_2-fixation declined as P_{CO_2} increased. The minimum concentration (C_{min}) of total P and half-saturation constant ($K_{½}$) for growth and CO_2- and N_2-fixation rates with respect to total P were reduced by 0.05 μmol L^{-1} as a function of elevated P_{CO_2}. We speculate that low P requirements under high P_{CO_2} resulted from a lower energy demand associated with carbon-concentrating mechanisms in comparison with low-P_{CO_2} cultures. There was also a 0.10 μmol L^{-1} increase in C_{min} and $K_{½}$ for growth and N_2 fixation with respect to total P as a function of increasing light regardless of P_{CO_2} concentration. We speculate that cellular P concentrations are responsible for this shift through biodilution of cellular P and possibly cellular P uptake systems as a function of increasing light. Changing concentrations of P, CO_2, and light have both positive and negative interactive effects on growth and CO_2-, and N_2-fixation rates of unicellular oxygenic diazotrophs like *C. watsonii*.

Within the past 5 yr, new attention has focused on the effects of elevated partial pressures of carbon dioxide (P_{CO_2}) on marine dinitrogen (N_2) fixation. In particular, three studies initiated this interest by documenting increased N_2-fixation rates by *Trichodesmium erythraeum* in response to elevated P_{CO_2} (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007). It is, however, particularly important to answer questions about how multiple environmental factors might change and interact with rising P_{CO_2} to affect ocean biogeochemical cycles. Hutchins et al. (2007) examined the combined effects of P_{CO_2}, temperature, and P concentration on N_2-fixation rates by two strains of *T. erythraeum*. That study concluded that P_{CO_2} limitation of N_2-fixation rates by *T. erythraeum* was independent of P concentration because the relative magnitude of elevated P_{CO_2} effects on growth and N_2-fixation rates was nearly identical in P-limited and P-replete cultures. This finding suggests that decreases in P concentrations, which are likely to accompany future changes in surface ocean warming and stratification (Hutchins et al. 2009), would not influence the effect of elevated P_{CO_2} on oceanic N_2 fixation by *Trichodesmium*.

Recent studies indicate that the light environment can influence the effect of elevated P_{CO_2} on N_2-fixation rates by *T. erythraeum*; the stimulatory effect of elevated P_{CO_2} is reduced under high light relative to lower light intensities (Kranz et al. 2010; Garcia et al. 2011). While it is clear that interactive effects of multiple environmental factors must be considered for *Trichodesmium*, we also need to understand how unicellular N_2 fixers like *Crocosphaera watsonii* will respond to changing environmental conditions, as these organisms are now also recognized as major contributors to marine biological N_2 fixation (Zehr et al. 2001; Montoya et al. 2004; Moisander et al. 2010).

Two studies suggest that P_{CO_2} has a positive effect on N_2-fixation rates by *C. watsonii* and that light and iron interact with CO_2 in different ways to influence this effect (Fu et al. 2008; Garcia et al. 2013). The effect of elevated P_{CO_2} on N_2-fixation rates is thought to be less direct for *Crocosphaera* than it is for *Trichodesmium*, however, because of the different ways in which they fix nitrogen (i.e., night vs. day, respectively; Garcia et al. 2013). The temporal separation of CO_2 and N_2 fixation by *Crocosphaera* has different physiological and biogeochemical consequences in terms of how it responds to interacting environmental variables when compared to *Trichodesmium*, which fixes both CO_2 and N_2 simultaneously. For example, despite the large effects of elevated P_{CO_2} on growth and N_2 fixation by *Trichodesmium* (Hutchins et al. 2007; Kranz et al. 2010; Garcia et al. 2011), Garcia et al. (2013) did not find large differences in gross N_2-fixation rates by two Atlantic Ocean isolates of *C. watsonii* that were grown under present-day and elevated P_{CO_2} with high P concentrations (15–20 μmol L^{-1}). Here, we examine how the concentration of P, a key limiting nutrient for diazotrophs in the open ocean, interacts with CO_2 and light to control growth and CO_2- and N_2-fixation rates in a *C. watsonii* strain isolated from the subtropical Pacific Ocean.

Methods

We conducted a light experiment and a P-light-CO_2 experiment with *C. watsonii* (WH0003) that was isolated

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near Sta. ALOHA (A Long Term Oligotrophic Habitat Assessment) in the North Pacific Ocean near Hawaii (22°45′N, 158°00′W). Experimental cultures were grown with a semicontinuous culturing method at 28°C in autoclave-sterilized artificial seawater medium with nutrients added in concentrations equivalent to the recipe for the Aquil medium (except for NO_3^-), as in Garcia et al. (2011) and originally described by Morel et al. (1979). The total P concentration was altered in the P-light-CO_2 manipulation experiment by adding P as H_2NaPO_4 in aqueous solution.

In the light experiment, triplicate cultures were grown in 800 mL polystyrene flasks under five irradiances (18, 40, 100, 180, 300 μmol quanta m^{-2} s^{-1}) and diluted every 2–3 d to 10–20 $\times$ 10^3 cells mL^{-1}. In the P-light-CO_2 experiment, triplicate cultures were diluted every 2 d to 5 $\times$ 10^3 cells mL^{-1} with medium that contained treatment concentrations of PO_4^{3-} ranging from 0.1 to 4.0 μmol L^{--1}. Cells were counted microscopically in each replicate culture with a hemocytometer at the end of each dilution period, and steady state growth rates were calculated from an increase in culture cell number per unit volume between two and three dilution periods (4–6 d) after cultures were acclimated to treatment conditions for 7–10 generations. To calculate growth rates, we used the equation $N_T = N_0 e^{\mu T}$, where N_0 and N_T are the initial and final culture cell densities, respectively, and T is the amount of time in days between culture cell number estimates. With this method, the dilution rate is determined by the growth rate of the algae as determined by the experimental treatments rather than by controlling the growth rate through imposing a dilution rate as one does for continuous cultures.

We measured cell diameters of ~ 12 cells from treatment replicates with an ocular micrometer. In the light experiment, we measured cells in one replicate from each light treatment twice: once in the middle of the light period and once at the end of the light period on the same day. Although we did not measure cell diameters in every replicate culture, we used the Mann-Whitney test (described below) to test the effect of light on the means of cell diameters from replicate cultures in our P-light-CO_2 experiment.

A low cell biomass was necessary to control CO_2 concentrations in cultures, and a consistent dilution period reduced variations in growth rates between dilutions. In the P-light-CO_2 experiment, cultures were grown in 1 liter polycarbonate bottles at 40 or 150 μmol quanta m^{-2} s^{-1} and bubbled with 19 Pa or 81 Pa P_{CO_2} premixed air supplied and certified by Gilmore Liquid Air Company. Culture pH was measured with a pH meter using the National Bureau of Standards (NBS) scale for seawater pH measurements (model Orion 5 star, Thermo Scientific). For the P-light-CO_2 experiment, seawater was bubbled and pre-equilibrated with treatment concentrations of P_{CO_2} before measuring pH and adding nutrients. This was essential to maintain high pH values in the 19 Pa P_{CO_2} treatments. We excluded data from the high-light, 19-Pa P_{CO_2} treatment, where the pH was > 0.05 units lower than the expected pH range of 8.45–8.49 (specifically, the 0.4, 0.8, and 2.0 μmol total P L^{-1} treatments).

In both experiments, light was supplied on a 12 : 12 light : dark cycle with cool-white fluorescent bulbs. We terminally sampled each replicate culture 24 h after the last dilution for N_2-fixation rates and CO_2-fixation rates, and at this point we also sampled for P-uptake rate measurements and cellular P content from each replicate in the P-light-CO_2 experiment. To acclimate cultures to low-P conditions in the P-light-CO_2 experiment, we consecutively reduced the concentration of P by transferring cultures acclimated to neighboring P concentrations in the experimental matrix. Steady-state growth was not achievable in treatments with the lowest P concentrations because growth rates continuously declined when the concentration of P was reduced to those concentrations. In these cases, we sampled cultures before growth rates became negative, except for the low-light, low-P, low-P_{CO_2} treatment, which did have a negative growth rate.

Nitrogen-fixation rates were determined with the acetylene reduction method as described in Garcia et al. (2013). Briefly, duplicate 50 mL culture samples were collected from experimental replicates, and 4 mL of acetylene were injected into 30 mL headspace at the beginning of the dark period of the light cycle. Samples were gently agitated to equilibrate gas concentrations between the headspace and culture samples after injecting acetylene and before measuring ethylene concentrations. We used a Bunsen coefficient for ethylene of 0.082 (Breitbarth et al. 2004) and an ethylene production : N_2 fixation rate ratio of 3 : 1, and we calculated N_2-fixation rates over 14 h (this included the 12 h dark cycle and the first 2 h of the light cycle).

Samples for measurements of total CO_2 (T_{CO_2}) were preserved with 0.05% mercuric chloride (final concentration) in glass bottles without headspace and determined using a carbon coulomb meter (model CM 140, UIC Inc.). For these analyses, we acidified 5 mL with a 10% phosphoric acid solution (1–2% final concentration), quantified the CO_2 trapped in an acid sparging column, and calculated T_{CO_2} with reference material provided by Andrew Dickson's laboratory (batch 95). We calculated P_{CO_2} with the CO_2 System Calculations program using K_1 and K_2 constants from Mehrbach et al. (1973), refit by Dickson and Millero (1987) and the NBS pH scale (Lewis and Wallace 1998; *see* Table 1 for T_{CO_2} measurements and P_{CO_2} calculations in the P-light-CO_2 experiment).

We determined CO_2-fixation rates using a Multi-Purpose Scintillation Counter (model LS-6500, Beckman Coulter) similar to the method described by Garcia et al. (2011). Briefly, we inoculated 40 mL samples from each treatment replicate with 0.925 KBq mL^{-1} $H^{14}CO_3^-$. The concentration of $H^{14}CO_3^-$ added to the sample was negligible in comparison with the T_{CO_2} concentration of the sample. Samples were incubated for 12 h under treatment-specific conditions of irradiance and temperature and then filtered onto Whatman GF/F filters and rinsed three times with ~ 5 mL filtered seawater to remove extracellular $H^{14}CO_3^-$. The incubation was initiated at the beginning of the light period and terminated at the end of the 12 h light period. Total CO_2 concentrations were multiplied by the ratio of radioactivity of cellular incorporation of ^{14}C to the total radioactivity of $H^{14}CO_3^-$. For CO_2-fixation rate calculations in our P-light-CO_2 experiment, we pooled ~ 25 mL from each of three treatment replicates into one sample for

Table 1. Total CO_2 (T_{CO_2}, μmol L^{-1}) and pH measurements were used to calculate the partial pressure of CO_2 (P_{CO_2}, Pa) for treatments with total phosphorus (P, μmol L^{-1}) and irradiance (μmol quanta m^{-2} s^{-1}). Means and standard errors (SE) of three experimental replicates are reported.

[Total P] (μmol L^{-1})	pH	SE	TCO_2 (μmol L^{-1})	P_{CO_2}
81 Pa treatment				
150 μmol quanta m^{-2} s^{-1}				
0.20	8.00	0.00	2350	78.8
0.25	7.99	0.01	2341	79.3
0.40	8.01	0.00	2291	74.1
0.80	8.00	0.00	2319	76.5
2.0	8.00	0.01	2289	76.6
4.0	8.01	0.01	2206	71.5
40 μmol quanta m^{-2} s^{-1}				
0.10	8.01	0.00	2325	75.9
0.15	8.00	0.00	2371	78.7
0.20	8.01	0.00	2240	72.0
0.25	8.01	0.00	2300	73.9
4.0	8.02	0.01	2261	71.8
19 Pa treatment				
150 μmol quanta m^{-2} s^{-1}				
0.25	8.47	0.00	1923	19.3
0.30	8.46	0.01	1909	19.6
1.4	8.47	0.00	1911	18.8
4.0	8.49	0.00	1881	17.9
40 μmol quanta m^{-2} s^{-1}				
0.15	8.48	0.00	1983	19.4
0.20	8.46	0.03	1954	20.0
0.3	8.43	0.02	1761	19.4
0.4	8.45	0.01	1746	18.5
4.0	8.45	0.00	1948	20.6

T_{CO_2} measurements. To calculate CO_2-fixation rates in the light experiment, we used a mean concentration of 2061 μmol L^{-1} T_{CO_2} that was measured in identical nonbubbled experiments with other isolates of *C. watsonii* (Garcia et al. 2013). Nonphotosynthetically driven ^{14}C incorporation was determined by incubating replicate culture samples (40 mL) for 12 h during the same time period in opaque bottles at 28°C with the same concentration of $H^{14}CO_3^-$; these values were subtracted from measured total ^{14}C incorporation to estimate photosynthetic incorporation. The total radioactivity of $H^{14}CO_3^-$ was determined by stabilizing 50 μL of the 37 MBq $H^{14}CO_3^-$ with 100 mL of a basic solution of phenylethylamine (99%) before adding 4 mL of Ultima Gold® XR (PerkinElmer).

Phosphorus-uptake rates were determined with radioactive $^{33}PO_4^{3-}$ over 24 h similarly to the method used for $H^{14}CO_3^-$ uptake described here. We inoculated 200 mL culture samples from each treatment replicate with 0.46 KBq $^{33}PO_4^{3-}$ mL^{-1}, yielding a final added concentration of 0.33 pmol $^{33}PO_4^{3-}$ mL^{-1}. We accounted for $^{33}PO_4^{3-}$ that was not incorporated into the cell by inoculating parallel 200 mL culture samples (pooled from three experimental replicates) with the same final activity and concentration of $^{33}PO_4^{3-}$ just before filtering at the end of the 24 h incubation period.

Table 2. Best-fit Monod function kinetics (r^2) as a function of irradiance (μmol quanta m^{-2} s^{-1}) for *Crocosphaera watsonii* (WH0003). Maximum rates (μ_{max}, d^{-1}; or V_{max}, fmol C or N $cell^{-1}$ h^{-1}), half-saturation constants ($K_{1/2}$, μmol quanta m^{-2} s^{-1}), and light compensation points (E_c, μmol quanta m^{-2} s^{-1}) for cellular growth and CO_2- and N_2-fixation rates with respect to light.

	μ_{max} or V_{max}	$K_{1/2}$	E_c	r^2
Specific growth	0.86	76	16	0.99
CO_2 fixation	193	169	15	0.99
N_2 fixation	22	95	16	0.99

Near the end of the light period (9th–11th hour), we filtered samples for cellular P content (50 mL) from each replicate onto combusted (450°C, 4 h) Whatman GF/F filters and measured them as in Fu et al. (2005). Filtered samples were rinsed three times with 2 mL 0.17 mol L^{-1} Na_2SO_4 and placed in 20 mL glass scintillation vials with 2 mL 0.017 $MgSO_4$, which was evaporated at ~ 80°C over a few days. Filters were combusted at 450°C for 2 h to release P from organic compounds. After cooling, filters were reheated to 80°C along with 5 mL 0.2 mol L^{-1} HCl for 30 min, and phosphate concentrations were estimated spectrophotometrically with the colorimetric assay described by Lebo and Sharp (1992).

We calculated the light compensation point (E_c, where net rates are zero) and the minimum concentration (C_{min}) of total P for growth, CO_2- and N_2-fixation rates using the hyperbolic function ($y = [a \cdot x]/[b + x]$) with the software program Sigma Plot 10. All three replicates in the 0.15 μmol total P L^{-1} low-light, low-P_{CO_2} treatment had slightly negative growth rates, so we assumed net growth rates of zero in those replicates, as was done in a prior study of phytoplankton growth kinetics (Hutchins et al. 2007). Next, we calculated the values of "a" (the maximum rate) and "b" (the half-saturation concentration, $K_{½}$) after aligning the data set as a whole along the x-axis, with respect to the origin, to yield the highest r^2 value. We then realigned the data to their original values along with the best-fit hyperbolic functions. The C_{min} and E_c are equivalent to the origin before the realignment. This method yields realistic Monod hyperbolic maximum rates, $K_{½}$, and C_{min} or Ec values (Tables 2, 3; Figs. 1–3). We also calculated 95% confidence bands on the hyperbolic functions using Sigma Plot 10 (Figs. 1–3). For the light experiment, the hyperbolic function of CO_2- and N_2-fixation rates were fitted to irradiance without including the rates measured at 100 μmol quanta m^{-2} s^{-1} due to problems with an altered light level for this treatment just prior to sampling for CO_2- and N_2-fixation rates (Fig. 1b,c).

In our P-light-CO_2 experiment, we tested the effects of light and CO_2 on growth and N_2- and CO_2-fixation rates in P-replete treatments with the Mann–Whitney nonparametric statistical test presented in Zar (1999) and report the *U*-statistic, which is calculated with the number of samples in each treatment group (n) and by ranking values used in the

Table 3. Best-fit Monod kinetics (r^2) as a function of total phosphorus (P) (μmol L^{-1}) in a factoral matrix of two P_{CO_2} levels (19 and 81 Pa) and two light levels (40 and 150 μmol quanta m^{-2} s^{-1}) for *Crocosphaera watsonii* (WH0003) with maxima (μ_{max}, d^{-1}; or V_{max}, fmol C or N $cell^{-1}$ h^{-1}), half-saturation constants ($K_{1/2}$, μmol total P L^{-1}), and minimum concentrations of total P (C_{min}, μmol total P L^{-1}) for cellular growth and CO_2-, and N_2-fixation rates with respect to total P.

	Specific growth (d^{-1})				CO_2 fixation (fmol C $cell^{-1}$ h^{-1})				N_2 fixation (fmol N $cell^{-1}$ h^{-1})			
	μ_{max}	$K_{1/2}$	C_{min}	r^2	V_{max}	$K_{1/2}$	C_{min}	r^2	V_{max}	$K_{1/2}$	C_{min}	r^2
40 μE m^{-2} s^{-1}												
19 Pa	0.10	0.16	0.15	0.98		No data	No data		3.9	0.16	0.14	0.99
81 Pa	0.25	0.10	0.10	0.93		No data	No data		4.4	0.15	0.09	0.96
150 μE m^{-2} s^{-1}												
19 Pa	0.44	0.25	0.25	0.99	55	0.26	0.24	0.97	10	0.25	0.25	0.99
81 Pa	0.70	0.20	0.20	0.99	77	0.21	0.20	0.96	16	0.21	0.20	0.98

test. We define P-replete treatments as those that have mean rate responses > 75% of hyperbolic maximum rates that we calculated from Monod functions in Table 3. Thus, our set P-replete conditions yielded different sample sizes between treatments of light and P_{CO_2} that were determined from Table 3 and Figs. 2, 3. This test is robust in detecting significant differences between two populations of data that are not normally distributed and that have unequal variances and unequal sample sizes. We used a one-tail test where we expected a priori that values would be lower in 19 Pa P_{CO_2} and 40 μmol quanta m^{-2} s^{-1} treatments in comparison with 81 Pa P_{CO_2} and 150 μmol quanta m^{-2} s^{-1} treatments, respectively, and a two-tailed test elsewhere. Each datum plotted in Figs. 1–5 is from an individual culture and is treated as an independent sample in statistical comparisons.

Results

Functional response curves with respect to light suggest that growth and N_2-fixation rates at 150 μmol quanta m^{-2} s^{-1} represented ~ 67% of maximum rates, indicating that growth and N_2-fixation rates were not light limited in our P-light-CO_2 experiment (Table 2; Fig. 1). Half-saturation constants ($K_{1/2}$) for growth and N_2-fixation rates of *C. watsonii* (WH0003) with respect to light were relatively close to each other (76 and 94 μmol quanta m^{-2} s^{-1}; Table 2; Fig. 1a,b). Although the E_c for growth and N_2- and CO_2-fixation rates were similar, the $K_{1/2}$ for CO_2 fixation was roughly twice as high as those for N_2-fixation and growth rates (Table 2; Fig. 1a–c).

In general, growth and N_2- and CO_2-fixation rates as a function of light were similar between our light experiment and our P-light-CO_2 experiment (Figs. 1–3). Culture pH and T_{CO_2} concentrations in treatments of P_{CO_2} were close to ranges documented in previous culture studies with *C. watsonii* grown at 19 and 76 Pa P_{CO_2} (Table 1; Garcia et al. 2013). The effect of light on CO_2-fixation rates was strongly positive at 81 Pa P_{CO_2} (U_{1tail} = 211, $n_{lowlight}$ = 15, $n_{highlight}$ = 15, $p < 0.001$) but was not significant (ns) at 19 Pa P_{CO_2} ($U_{1tai\ 1}$ =63, $n_{lowlight}$ = 15, $n_{highlight}$ = 6, p = ns; Fig. 3c,d). Despite these differential effects of light on CO_2-fixation rates, light had a significant positive effect on N_2-fixation rates (for 19 Pa P_{CO_2}, U_{1tail} = 108, $n_{lowlight}$ = 12, $n_{highlight}$ = 9, $p < 0.001$; for 81 Pa P_{CO_2}, U_{1tail} = 120, $n_{lowlight}$ = 8, $n_{highlight}$ = 15, $p < 0.001$; Fig. 3a,b) and growth rates (for 19 Pa P_{CO_2}, U_{1tail} = 108, $n_{lowlight}$ = 12, $n_{highlight}$ = 9, $p <$ 0.001; for 81 Pa P_{CO_2}, U_{1tail} = 180, $n_{lowlight}$ = 12, $n_{highlight}$ = 15, $p < 0.001$; Fig. 2a,b) under both low- and high-P_{CO_2} conditions.

Under 150 μmol quanta m^{-2} s^{-1}, 19 Pa P_{CO_2} conditions, cell growth and N_2- and CO_2-fixation rates at 0.25 μmol total P L^{-1} were below the $K_{1/2}$ with respect to total P, while these rates were near hyperbolic maximum rates in the 81 Pa P_{CO_2} treatment at 0.25 μmol total P L^{-1} (Table 3; Figs. 2a, 3a,c). Thus, mean rates increased by 240%, 540%, and 250%, respectively, as a function of elevated P_{CO_2} in the 0.25 μmol total P L^{-1} treatment (Fig. 2a,b). In P-replete cultures, however, hyperbolic maximum growth and N_2- and CO_2-fixation rates increased by only 60%, 59%, and 40%, respectively, in response to elevated P_{CO_2} (Table 3; Figs. 2a, 3a,c). The Mann–Whitney test indicated that the effect of elevated P_{CO_2} on P-replete growth rates (U_{1tail} = 134, n_{19Pa} = 9, n_{81Pa} = 15, $p < 0.001$), N_2-fixation rates (U_{1tail} = 131, n_{19Pa} = 9, n_{81Pa} = 15, $p < 0.001$), and CO_2-fixation rates (U_{1tail} = 88, n_{19Pa} = 6, n_{81Pa} = 15, $p <$ 0.001) was significantly positive in the 150 μmol quanta m^{-2} s^{-1} treatment (Figs. 2a, 3a,c). Thus, under high light, the effect of elevated P_{CO_2} on growth and N_2- and CO_2-fixation rates was magnified by 4, 8.9, and 6.3 times, respectively, under low total P in comparison with P-replete conditions.

Under low light, P also influenced the effect of P_{CO_2} on growth. Elevated P_{CO_2} affected growth rates at all P concentrations, but the effect was greatest when P was low. In P-replete cultures, P_{CO_2} had a strong positive effect on growth rates (U_{1tail} = 144, n_{19Pa} = 12, n_{81Pa} = 12, $p <$ 0.001), yielding a large percent difference in hyperbolic maximum growth rates between the 19 and 81 Pa P_{CO_2} treatments (150%), in comparison with high-light treatments (60%; Table 3; Fig. 2a,b). Growth rates at 0.15 μmol total P L^{-1}, 19 Pa P_{CO_2}, were so drastically reduced, however, that they were in fact negative (a growth rate of zero was assumed for the curve-fitting function in Fig. 2b), while cultures bubbled with 81 Pa $P_{CO_2\,2}$ at 0.15 μmol total P L^{-1} grew close to the hyperbolic maximum rate (Table 3; Fig. 2b).

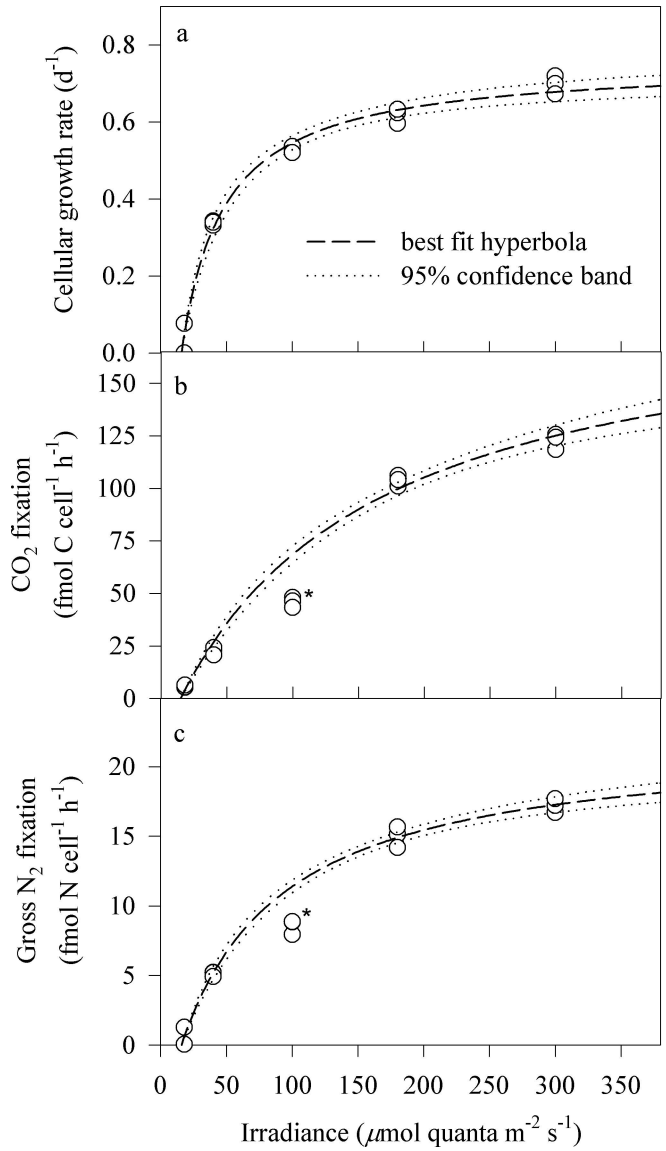

Fig. 1. (a) Cellular growth rates (d^{-1}), (b) CO_2-fixation rates (fmol C cell^{-1} h^{-1}), and (c) N_2-fixation rates (fmol N cell^{-1} h^{-1}) by *Crocosphaera watsonii* (WH0003) as a function of irradiance (18–300 μmol quanta m^{-2} s^{-1}). *Data were removed from calculations of Monod hyperbolic parameters. Hyperbolic response curves (dashed lines) were best fit to all the data, which are from individual cultures. Dotted lines represent the 95% confidence band on hyperbolas.

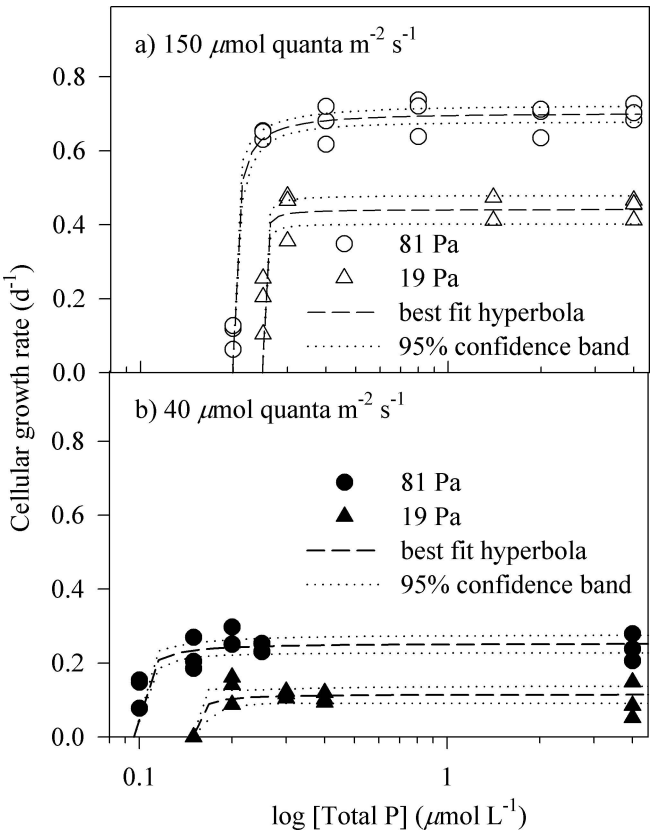

Fig. 2. Cellular growth rates (d^{-1}) of *Crocosphaera watsonii* (WH0003) as a function of phosphate concentration (0.1–4 μmol total P L^{-1}) in 19 Pa (triangles) and 81 Pa P_{CO_2} (circles) treatments under (a) high (150 μmol quanta m^{-2} s^{-1}, open symbols) and (b) low (40 μmol quanta m^{-2} s^{-1}, closed symbols) light. Hyperbolic response curves (dashed lines) were best fit to all the data, which are from individual cultures. Dotted lines represent the 95% confidence band on hyperbolas.

Despite this large effect on growth rates in low-light cultures, effects of elevated P_{CO_2} on both CO_2- and N_2-fixation rates were very small. Elevated P_{CO_2} under low light did not significantly affect CO_2-fixation rates (U_{1tail} = 81, n_{19Pa} = 15, n_{81Pa} = 15, p = ns), and we could not determine meaningful kinetic parameters for CO_2 fixation in low-light treatments because they did not follow a clear trend as a function of P and P_{CO_2} (Fig. 3d). The effect of elevated P_{CO_2} on N_2-fixation rates in P-replete cultures was not statistically significant either (U_{1tail} = 47, n_{19Pa} = 12, n_{81Pa} = 7, p = ns; Fig. 3b). Thus, under low light, P_{CO_2} had a major effect on growth rates but no significant affect on N_2-fixation or CO_2-fixation rates.

Overall, $K_{½}$ values for growth with respect to total P were close to previously reported values for large unicellular diazotrophs (Falcon et al. 2005) and varied as a function of light and P_{CO_2} (Table 3; Figs. 2, 3). Both the $K_{½}$ and the C_{min} of total P for growth and N_2-fixation rates decreased by approximately 0.1 μmol total P L^{-1} as a function of decreasing irradiance and by approximately 0.05 μmol total P L^{-1} as a function of increasing P_{CO_2}. We documented this shift as a function of P_{CO_2} under both 40 and 150 μmol quanta m^{-2} s^{-1} (Figs. 2a,b, 3a,b), and nonoverlapping 95% confidence intervals indicated that this shift in C_{min} of total P for growth and N_2 and CO_2 fixation was significant ($n_{highlight81Pa}$ = 18, $n_{highlight19Pa}$ = 12, $n_{lowlight81Pa}$ = 15, $n_{lowlight19Pa}$ = 15, $p < 0.05$; Figs. 2a,b, 3a–c).

In the light experiment, mean cell diameter increased with increasing light intensity (Fig. 4a). In our P-light-CO_2 experiment, the means of cell diameters in replicates were higher in the high-light, high-P_{CO_2} treatment in comparison with both the 81 Pa and the 19 Pa P_{CO_2} treatments at low light (for both comparisons, U_{1tail} = 132, $n_{lowlight}$ = 15,

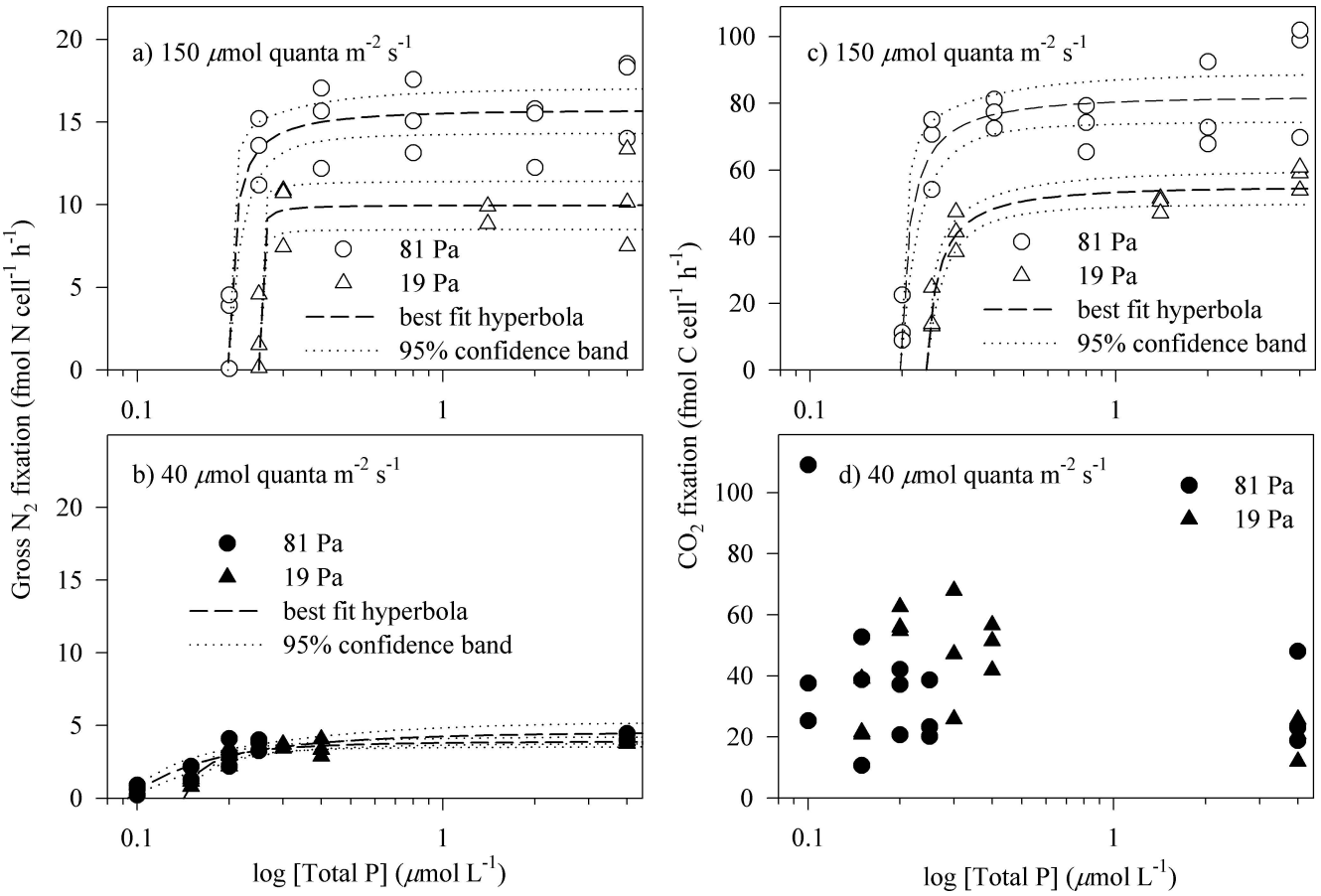

Fig. 3. Gross N_2-fixation rates (fmol N cell^{-1} h^{-1}) by *Crocosphaera watsonii* (WH0003) as a function of phosphate concentration (0.1–4 μmol total P L^{-1}) in 19 Pa (triangles) and 81 Pa P_{CO_2} (circles) treatments under (a) high (150 μmol quanta m^{-2} s^{-1}, open symbols) and (b) low (40 μmol quanta m^{-2} s^{-1}, closed symbols) light and CO_2-fixation rates (fmol C cell^{-1} h^{-1}) in the same phosphate and CO_2 treatments under (c) high and (d) low light. Hyperbolic response curves (dashed lines) were best fit to all the data, which are from individual cultures. Dotted lines represent the 95% confidence band on hyperbolas.

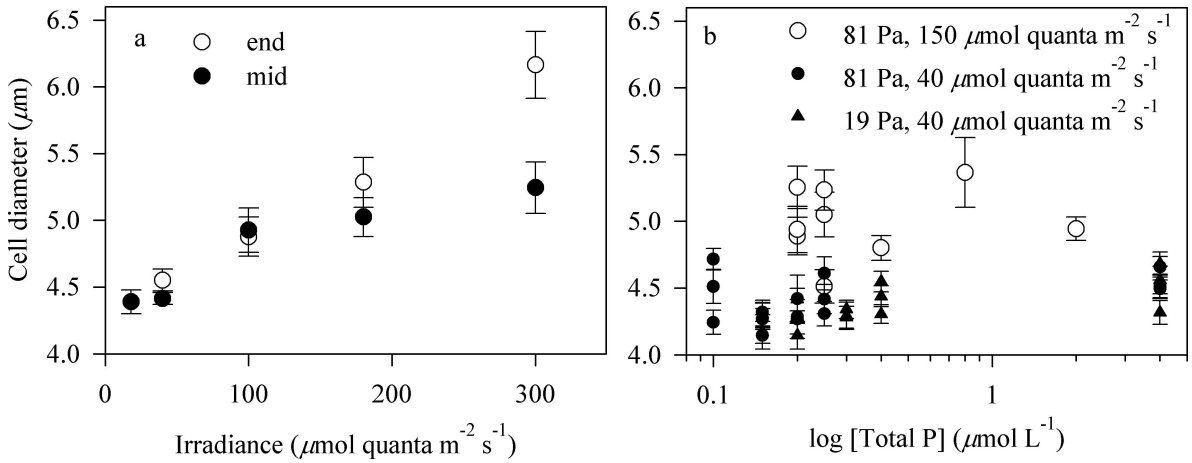

Fig. 4. Cell diameter of *Crocosphaera watsonii* (WH0003) in response to irradiance in (a) the light experiment at the mid and end points of the light period and (b) as a function of total P concentration (0.1–4 μmol total P L^{-1}) in high-light (150 μmol quanta m^{-2} s^{-1}) and low-light (40 μmol quanta m^{-2} s^{-1}) treatments grown under high P_{CO_2} (81 Pa) and low P_{CO_2} (19 Pa) in the P-light-CO_2 experiment. (b) No data were collected for the high-light, low-P_{CO_2} treatment. All data are mean cell diameters with standard errors from a single culture, calculated as described in *Methods*.

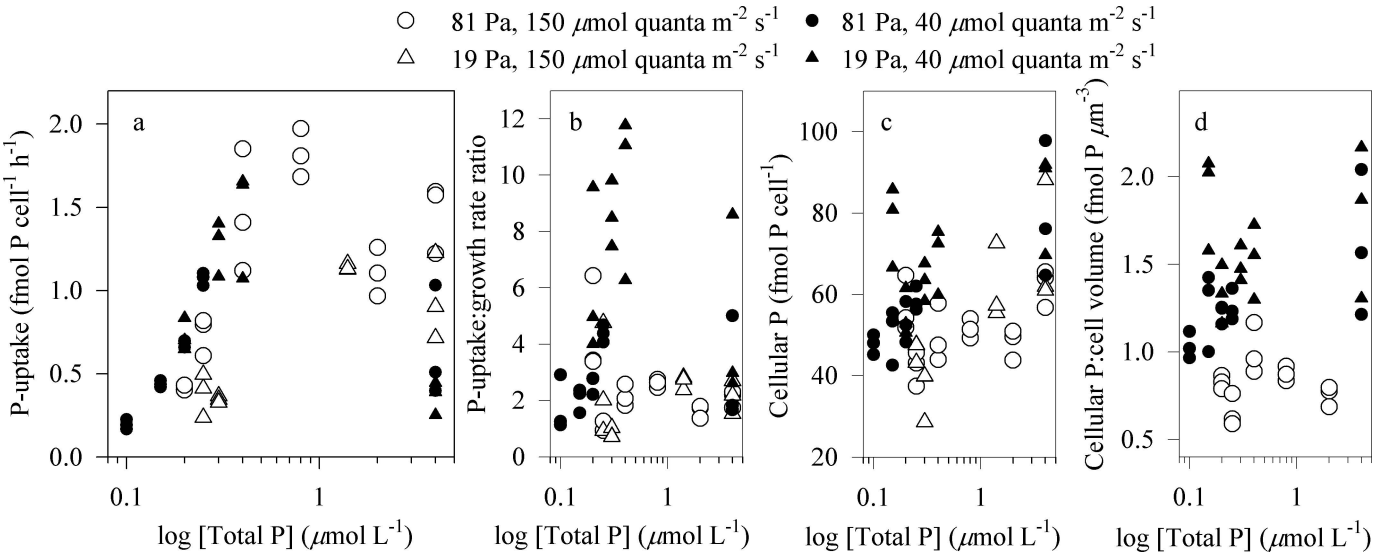

Fig. 5. (a) Phosphate-uptake rates as a function of total P concentration (0.1–4 μmol total P L^{-1}), in high-light (150 μmol quanta m^{-2} s^{-1}) and low-light (40 μmol quanta m^{-2} s^{-1}) treatments grown under high P_{CO_2} (81 Pa) and low P_{CO_2} (19 Pa). (b) Phosphate uptake rates (fmol P $cell^{-1}$ h^{-1}) relative to cellular growth rates (d^{-1}), (c) cellular P content, and (d) cellular P per unit cell volume as a function of total P concentration (0.1–4 μmol total P L^{-1}) in response to the same treatment matrix of light and P_{CO_2}. All data are from individual cultures.

$n_{highlight} = 9$, $p < 0.001$; Fig. 4b). Although we do not have data describing cell diameter at 19 Pa P_{CO_2} under high light, cell diameter was not different between the 19 Pa and 81 Pa P_{CO_2} treatments under low light ($U_{2tail} = 97$, $n_{19Pa} = 15$, $n_{81Pa} = 15$, p = ns; Fig. 4b).

In 4.0 μmol total P L^{-1} treatments, P-uptake rates were high in high-light cultures relative to those in low-light cultures, a trend that is similar to trends in growth rates. In low-P cultures (< 0.3 μmol total P L^{-1}; Fig. 5a), however, P-uptake rates were higher in low-light cultures than in high-light cultures. The P-uptake : growth rate ratio did not vary widely in high-light cultures but was elevated in low-light, low-P_{CO_2} cultures relative to other treatments (Fig. 5b), and as a result, cellular P content (fmol P $cell^{-1}$) was highest in low-light, low-P_{CO_2} cultures in comparison with other treatments of light and P_{CO_2} ($n_{lowlight19Pa} = 15$; compared to low light, 81 Pa P_{CO_2}, $U_{2tail} = 177$, $n_{81Pa} = 15$, $p < 0.01$; compared to high light, 19 Pa P_{CO_2}, $U_{2tail} = 144$, $n_{highlight19Pa} = 12$, $p < 0.01$; compared to high light, 81 Pa P_{CO_2}, $U_{2tail} = 241$, $n_{highlight81Pa} = 18$, $p < 0.001$; Fig. 5c). When normalized to cell volume, cellular P content (fmol P μm^{-3}) was lower in the high-light, 81 Pa P_{CO_2} treatment relative to the two low-light treatments ($n_{highlight81Pa} = 15$; for low light, 81 Pa P_{CO_2}, $U_{2tail} = 220$, $n_{1owlight81Pa} = 15$, $p < 0.001$; for low light, 19 Pa P_{CO_2}, $U_{2tail} = 224$, $n_{lowlight19Pa} = 15$, $p < 0.001$; Fig. 5d).

The cellular P-use efficiency for N_2 fixation (mol N h^{-1} : mol P) was significantly higher in high-light cultures relative to low-light cultures at both P_{CO_2} concentrations (for 19 Pa P_{CO_2}, $U_{1tail} = 108$, $n_{lowlight} = 12$, $n_{highlight} = 9$, $p < 0.001$; for 81 Pa P_{CO_2}, $U_{1tail} = 120$, $n_{lowlight} = 8$, $n_{highlight} = 15$, $p < 0.001$; Fig. 6a), but for CO_2 fixation, light had a significant effect only at 81 Pa P_{CO_2} ($U_{1tail} = 210$, $n_{lowlight} = 14$, $n_{highlight} = 15$, $p < 0.001$; Fig. 6b). Elevated P_{CO_2} had a significant effect on the P-use efficiency for N_2 and CO_2 fixation under high light (for N_2 fixation, $U_{1tail} = 117$, $n_{19Pa} = 9$, $n_{81Pa} = 15$, $p < 0.003$; for CO_2 fixation, $U_{1tail} = 90$, $n_{19Pa} = 6$, $n_{81Pa} = 15$, $p < 0.001$) but not under low light (for N_2

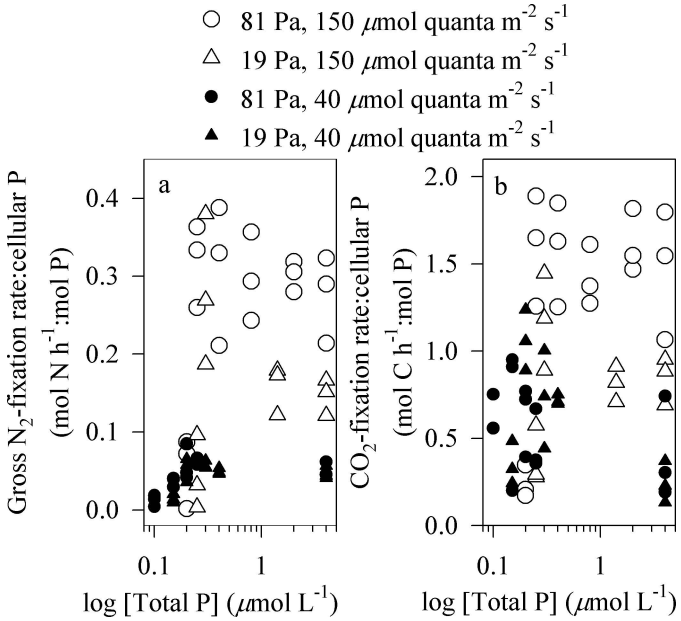

Fig. 6. Efficiency of cellular P for (a) gross N_2-fixation rates and (b) CO_2-fixation rates (mol N_2 or CO_2 fixed $cell^{-1}$ h^{-1} per mol cellular P) by *Crocosphaera watsonii* WH0003 in P-replete cultures (4.0 μmol total P L^{-1}) as a function of high light (open symbols) and low light (filled symbols), high P_{CO_2} (81 Pa), and low P_{CO_2} (19 Pa). All data are from individual cultures.

fixation, $U_{1tail} = 63$, $n_{19Pa} = 12$, $n_{81Pa} = 8$, p = ns; for CO_2 fixation, $U_{1tail} = 66$, $n_{19Pa} = 12$, $n_{81Pa} = 14$, p = ns; Fig. 6a,b).

Discussion

Our main finding is that P_{CO_2} strongly affected the $K_{½}$ with respect to total P and the C_{min} of total P for growth and CO_2- and N_2-fixation rates by *C. watsonii* (WH0003). We documented a 0.05 μmol total P L^{-1} shift in the $K_{½}$ and C_{min} of total P for growth and N_2-fixation rates between cultures growing under the last glacial maximum level of atmospheric CO_2 and the year-2100 predicted concentration (Figs. 2, 3; Caldiera and Wickett 2001). As a result of this shift, we documented an increase in the effect of elevated P_{CO_2} on growth and N_2-fixation rates in low-P-acclimated cultures in comparison with P-replete cultures under both high light (150 μmol quanta m^{-2} s^{-1}) and low light (40 μmol quanta m^{-2} s^{-1}; Figs. 2, 3).

The value of the shift in $K_{½}$ and C_{min} of total P that we documented is constrained by our experimental design in that we used 0.05 μmol total P L^{-1} increments in our P treatments near the growth-limiting range of P. Had we used smaller increments, the value of this shift may have been different. Furthermore, we assume that our method for determining the best-fit hyperbolic function is valid for determining the C_{min} of total P. In support of our method, growth-limited P treatments nearest the C_{min} of total P for growth progressively declined between dilutions and were sampled before growth rates became negative (Fig. 2).

These results indicate that the effect of elevated P_{CO_2} on growth and CO_2 and N_2 fixation by *C. watsonii* is dependent on the concentration of P and are in contrast to the independent relationship between P_{CO_2} and P documented for *Trichodesmium* (Hutchins et al. 2007). The reason for this difference between *Trichodesmium* and *Crocosphaera* in the interdependency of P_{CO_2} and P is not presently known but may be caused by the difference in N_2-fixation strategies used by these two very different oceanic diazotrophs (i.e., temporal vs. spatial separation of N_2 fixation from CO_2 fixation; Berman-Frank et al. 2007).

We speculate that the decrease in C_{min} of total P for growth associated with high P_{CO_2} is caused by a decreased energy demand by carbon concentrating mechanisms (CCMs). In high-P_{CO_2}-acclimated cultures, the energy used by CCMs to import bicarbonate (HCO_3^-) and interchange CO_2 and HCO_3^- intracellularly is thought to be low relative to that in low-P_{CO_2} cultures (Price et al. 2008; Kranz et al. 2011), thereby leaving more energy for other processes, such as cell division and CO_2 and N_2 fixation. High CO_2-fixation rates probably lead to high glycogen production during the light period, and in *Crocosphaera*, glycogen seems to be a major energy source for N_2 fixation during the dark period as it gets respired (Saito et al. 2011). Alternatively, high CO_2-fixation rates might result in high cellular 2-oxoglutarate concentrations that are known to induce N uptake and mechanisms related to N_2 fixation in other cyanobacteria (Laurent et al. 2005; Muro-Pastor et al. 2005).

In high-light, low-P_{CO_2} treatments, this type of energetic deficit might also contribute to the low P-uptake rates in comparison with high-light, high-P_{CO_2} treatments. Dyhrman and Haley (2006) identified genes for an inducible high-affinity P transport system (PstS) in *C. watsonii* that is likely an energetically demanding mechanism (Scanlan et al. 1993). Thus, we propose that a reduced adenosine triphosphate (ATP) pool available for high-energy–demanding processes directly causes a high C_{min} of total P for growth under low P_{CO_2}.

This interpretation implies N and C also limit *C. watsonii* growth when P is limiting. As P approaches the C_{min} for cell division, the capacity to maintain high cellular ATP concentrations may become compromised, thereby limiting N_2-fixation rates. This colimitation relationship between N and P is further exacerbated because N_2 fixation by *C. watsonii* is also dependent on stored cellular glycogen, which is also heavily dependent on ATP through membrane transport of HCO_3^- and CO_2 fixation. Thus, we propose that low P induces a negative feedback on *C. watsonii* growth through colimitation with N and C. In support of this, the rectilinear nature of the hyperbola functions in Figs. 2, 3 is indicative of a negative feedback type of relationship. In this respect, one might expect the C_{min} of P for growth to change as function of reactive N availability (e.g., urea, NO_3^-, NH_4^+).

The apparent effects of a low-P_{CO_2}–induced energy limitation are different under low light. At low light, CO_2-fixation rates were not different between P_{CO_2} treatments and are probably responsible for the minor effect of elevated P_{CO_2} on N_2-fixation rates in comparison with that at higher irradiance because N_2 fixation is dependent on stored cellular C. In support of these findings, the positive effect of elevated P_{CO_2} on CO_2-fixation rates increased with increasing irradiance in *T. erythraeum* (Garcia et al. 2011) and an isolate of *C. watsonii* isolated from the Atlantic Ocean (Garcia et al. 2013).

At low light, CO_2- and N_2-fixation rates were not different between the two P-replete P_{CO_2} treatments (Figs. 3b,d), but growth rates in P-replete cultures were 150% higher in high-P_{CO_2} than in low-P_{CO_2} treatments (Fig. 2b). These data imply that N was fixed in excess of that needed to support the observed low growth rates in the low-light, 19 Pa P_{CO_2} treatments. In support of these data, gross : net N_2-fixation rate ratios of *C. watsonii* (by acetylene reduction and $^{15}N_2$-isotope uptake methods, respectively) reported by Garcia et al. (2013) suggested that cellular retention of fixed N increased as a function of increasing light and P_{CO_2}. Garcia et al. (2011) also reported similar findings for *T. erythraeum*. Collectively, these data indicate that CO_2- and N_2-fixation rates were decoupled from growth rates under low light and low P_{CO_2} and that CO_2 and N_2 fixation take precedence over cell division in terms of using cellular energy.

In P-limited cultures, P-uptake rates were several times higher relative to growth rates in the low-light, low-P_{CO_2} treatment than they were other treatments (Fig. 5b), enhanced, perhaps, by the high cell surface area : volume ratio that accompanies small cells (Fig. 4b). As proposed by Sunda and Huntsman (2005) for zinc transport, cell membrane P transporters may become concentrated as cell growth is becomes limited. Thus, under high light,

biodilution of P transporters embedded in the cell membrane resulting from high growth rates might cause a high C_{min} of total P and low P-uptake rates. In addition, transcription rates and ratios of various P-acquisition mechanisms may vary as function of light and P_{CO_2}, which could also affect C_{min}.

Alternatively, cells might have a high cellular P demand for N_2 and CO_2 fixation due to a slow ATP turnover rate when cellular energy is low. For instance, cellular P was highest in low-light, low-P_{CO_2} cultures, while N_2- and CO_2-fixation rates were low (Figs. 3, 5c). A reduced cell size (Fig. 4b) and slow growth rates and high P-uptake rates in low-light cultures (Figs. 2b, 5a,b) yielded an increase in cellular P per unit cell volume, further saturating the cell with P. Conversely, in high-light cultures, N_2- and CO_2-fixation rates were high, while cellular P per unit cell volume was relatively low and cells were large (Figs. 3, 5c, 4b). Thus, a bioconcentration of cellular P under low light might be responsible for the lack of effect of elevated P_{CO_2} on N_2- and CO_2-fixation rates under low light (Figs. 3b,d), while a biodilution of cellular P might be responsible for the increasing effect of elevated pCO_2 under high light (Fig. 3a,c). This high P use efficiency for N_2 and CO_2 fixation in high-light, high-P_{CO_2} cultures of *C. watsonii* (Fig. 6a,b) may be related to a high turnover rate of ATP, relative to that under low light and probably differences in relative ratios of cellular P pools such as ribosomal ribonucleic acid, messenger RNA, phospholipids, and polyphosphates.

Based on data by Montoya et al. (2004), blooms of unicellular diazotrophs that might resemble *C. watsonii* had pigment maxima between 50 and 60 m in the Arafura Sea. We expect light intensity to range between $\sim$ 150 and 200 μmol quanta m^{-2} s^{-1} at this depth, based on the light correlation with depth presented by Breitbarth et al. (2008). Certainly, however, *C. watsonii* is known to occupy deeper waters as well (Hewson et al. 2009), where concentrations of dissolved inorganic P, reactive N (e.g., NO_3^-), and P_{CO_2} would increase with depth, potentially affecting N_2-fixation rates. Thus, warmer surface waters and shallower mixed layers in future oceans would have a complex effect on water column N_2 fixation by *C. watsonii*–like diazotrophs.

In summary, our main findings indicate that the effects of elevated P_{CO_2} on growth and N_2 and CO_2 fixation varied as a function of light and P and illustrate that P requirements for growth of *C. watsonii* shifted as a function of P_{CO_2} and light. We surmise that the strong percent differences in the effects of elevated P_{CO_2} on growth and N_2- and CO_2-fixation rates under high and low P may be related to changes in the cellular energy budget. Thus, in regions where P is low, we expect that a doubling of the present-day concentration of P_{CO_2} would yield larger effects on *C. watsonii* than those described by Garcia et al. (2013), which indicated that N_2-fixation rates were not different between present-day and 100-yr predicted P_{CO_2} concentrations under high P concentrations (15–20 μmol L^{-1}). Changes in the cellular energy budget imply that the interactive effects of P, light, and P_{CO_2} on growth and N_2 fixation in our experiment would be multiplicative. In general, however, Leibig-like colimitation of growth by nutrients, which is based on biomass concentrations, likely occurs simultaneously with multiplicative effects (*see* discussion of cell biomass concentrations in relation to total P below). In an effort to minimize effects of Leibig-like colimitation in our experiments, all cultures were diluted to 0.5×10^7 cells L^{-1} during dilutions. Thus, where treatment effects limited growth rates, we assume that effects of Leibig-like limitation of growth were also minimized. For example, in high-light cultures at 0.25 μmol total P L^{-1}, cell growth was limited in the 19 Pa P_{CO_2} treatment. Because growth was not limited in the 81 Pa P_{CO_2} treatment at 0.25 μmol total P L^{-1}, we assume that effects of Leibig-like limitation was minimized in the 19 Pa P_{CO_2} treatment (Fig. 2a,b).

In conclusion, these apparent effects of elevated P_{CO_2} on N_2 fixation by *C. watsonii* most likely do not apply to all oceanic unicellular N_2 fixers. For instance, nonoxygenic diazotrophs (e.g., Candidatus *Atelocyanobacterium thalassa*; Thompson et al. 2012) do not fix CO_2, and the primary benefit of high P_{CO_2} seems to be associated CO_2 fixation, whereas N_2 fixation likely receives secondary benefits (Law et al. 2012). Thus, assuming that oceanic P_{CO_2} will increase within the next 100 yr, N_2 fixation by unicellular oxygenic diazotrophs will likely be affected by future concentrations of P_{CO_2}, mostly in areas where they reach high densities, which are known to include tropical regions of the Atlantic Ocean (Langlois et al. 2008) and parts of the Pacific Ocean near northern Australia and Hawaii (Montoya et al. 2004; Church et al. 2005; Goebel et al. 2007).

In consideration of effects of global change on unicellular oxygenic diazotrophs, the predicted decrease in P concentrations in stratified mixed layers of the ocean will likely have a negative effect on oceanic N_2 fixation (Hutchins et al. 2009). This potential negative effect will likely be offset however, by the predicted concomitant increase in oceanic P_{CO_2}. In addition, the overall effect of increasing light on N_2 fixation by *C. watsonii* seems to be positive by increasing the P-use efficiency but may also act to increase P requirements for growth. Thus, we propose an integrated effect of these three changing variables on growth and CO_2 and N_2 fixation by marine unicellular oxygenic diazotrophs. This integrated effect assumes that these environmental variables will change in the future in the directions that have been predicted. When considering a typical water column and approaching the ocean surface from depth, the combined effect of increasing light and decreasing P concentrations might constrain upper water column depth limits on growth and CO_2 and N_2 fixation, while lower limits on growth within the column may be controlled by light and retention of fixed N. We then expect future increases in mean mixed layer irradiance and prospective decreases in P concentrations to reduce this upper limit into deeper water, but the contrary effect of elevated P_{CO_2} might permit an extension of this upper limit back into shallower water. Such an extension of the present-day upper limit into shallower water could result in a stronger effect on CO_2 fixation relative to N_2 fixation because of the high light saturation intensity for CO_2 fixation relative to that for N_2 fixation (Fig. 1b,c) and

thereby potentially influence the efficiency of the biological carbon pump. In this respect, more data describing the fate of extracellular polysaccharides that are produced by *C. watsonii* as a function of light are needed, as some isolates of *C. watsonii* are known to exude large amounts of extracellular polysaccharides (Webb et al. 2009; Sohm et al. 2011).

Although recent experiments identified areas of P limitation of N_2 fixation in the North Pacific Subtropical Gyre (Watkins-Brandt et al. 2011), P concentrations are generally thought to be lower in the Atlantic than in the North Pacific Ocean (Wu et al. 2000). Mean total phosphate concentrations in the Sargasso Sea are $\sim$ 0.075 μmol L^{-1}, while those in the North Pacific have been measured at much higher concentrations between 0.2 and 0.3 μmol L^{-1} (Wu et al. 2000; Watkins-Brandt et al. 2011). Considering that the cell biomass in our experiments ($0.5–2 \times 10^7$ L^{-1}) was 25–100 times higher than that which one might find in blooms of *C. watsonii* (2×10^5 cells L^{-1}; Montoya et al. 2004), 0.2–0.3 μmol total P L^{-1} would most likely represent a P-replete environment, while P concentrations below 0.075 μmol total P L^{-1} would likely represent a P-limited environment for *C. watsonii*.

White et al. (2010) documented no difference in the ability of *Trichodesmium* to grow on inorganic or organic P. Although this finding needs to be confirmed for *C. watsonii*, we expect the net effect of global change on N_2 fixation by unicellular oxygenic diazotrophs to vary by ocean region based, in part, on differences in mean total P concentrations in different regions. For example, if *Crocosphaera* is confined to shallow depths in a warmer, more stratified water column, higher P concentrations in the Pacific might allow higher rates of N_2 fixation in shallow regions of the water column. Alternatively, we expect the effect of elevated P_{CO_2} on N_2 fixation to be higher when P is low, as it is in the Atlantic Ocean.

We must also consider other variables, however, as Mills et al. (2004) have documented simultaneous P and Fe limitation of N_2 fixation in the western tropical Atlantic Ocean. In a *C. watsonii* culture study, Fu et al. (2008) documented interacting effects of P_{CO_2} and Fe on N_2 fixation, where Fe limitation canceled out the stimulatory effects of high CO_2. This is important because Wu et al. (2000) reported low Fe concentrations in the Pacific that probably limit N_2 fixation in vast regions of the Pacific Ocean. Overall, future trends in Fe supply and availability are difficult to predict because the effects of global change on atmospheric Fe inputs to the oceans are not well understood (Mahowald et al. 2009). To add to the complexity of this picture, Dutkiewicz et al. (2012) modeled interactions of diazotrophs with non–N_2-fixing phytoplankton in a complex spatial framework of fixed N and Fe in the Pacific Ocean.

Assuming that P, P_{CO_2}, and light will change in the next 100 yr in the directions that have been predicted within the water column, we anticipate both positive and negative effects of global change on growth and N_2 fixation by *C. watsonii*. The net effects of these positive and negative effects are difficult to quantify, and we must also consider interactive effects of other key variables, such as temperature, iron, and reactive N availability on oceanic N_2-fixation rates. It will be a challenging task to fully explore the interdependent relationships of these many other global change variables with phosphorus, P_{CO_2}, and light availability as they affect this key component of the marine N cycle.

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