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Physiological Response of *Crocosphaera watsonii* to Enhanced and Fluctuating Carbon Dioxide Conditions

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Abstract

We investigated the effects of elevated pCO_2 on cultures of the unicellular N_2-fixing cyanobacterium *Crocosphaera watsonii* WH8501. Using CO_2-enriched air, cultures grown in batch mode under high light intensity were exposed to initial conditions approximating current atmospheric CO_2 concentrations (~400 ppm) as well as CO_2 levels corresponding to low- and high-end predictions for the year 2100 (~750 and 1000 ppm). Following acclimation to CO_2 levels, the concentrations of particulate carbon (PC), particulate nitrogen (PN), and cells were measured over the diurnal cycle for a six-day period spanning exponential and early stationary growth phases. High rates of photosynthesis and respiration resulted in biologically induced pCO_2 fluctuations in all treatments. Despite this observed pCO_2 variability, and consistent with previous experiments conducted under stable pCO_2 conditions, we observed that elevated mean pCO_2 enhanced rates of PC production, PN production, and growth. During exponential growth phase, rates of PC and PN production increased by ~1.2- and ~1.5-fold in the mid- and high-CO_2 treatments, respectively, when compared to the low-CO_2 treatment. Elevated pCO_2 also enhanced PC and PN production rates during early stationary growth phase. In all treatments, PC and PN cellular content displayed a strong diurnal rhythm, with particulate C:N molar ratios reaching a high of 22:1 in the light and a low of 5.5:1 in the dark. The pCO_2 enhancement of metabolic rates persisted despite pCO_2 variability, suggesting a consistent positive response of *Crocosphaera* to elevated and fluctuating pCO_2 conditions.

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Introduction

Anthropogenic emissions and land use change are increasing the concentration of carbon dioxide (CO_2) in the atmosphere and surface ocean waters [1]. The predicted effects of elevated CO_2 partial pressure (pCO_2) and consequent ocean acidification (OA) on marine ecosystems include a potential upregulation of metabolic processes by CO_2-limited phytoplankton species [2]. Because the carboxylating enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) is typically not saturated under ambient surface seawater pCO_2, many phytoplankton groups invest energy in carbon concentrating mechanisms (CCMs) to increase CO_2 concentrations at the catalytic site [3]. Hence, under future OA conditions, phytoplankton with low-affinity RuBisCO could potentially down-regulate CCMs and reallocate energy and elemental resources to allow for increased carbon (C) fixation and growth rates [4]. Indeed, elevated pCO_2 has been shown to stimulate C fixation by select monocultures of phytoplankton and natural assemblages (reviewed in [5]).

Elevated pCO_2 appears to have a particularly strong metabolic enhancement in dinitrogen (N_2)-fixing (diazotrophic) cyanobacteria. Initial laboratory experiments using strain IMS101 of *Trichodesmium*, a group of filamentous, non-heterocystous cyanobacteria, showed that doubling pCO_2 increases N_2 fixation rates by 35–138% and C fixation rates by 23–40% [4,6–8]. Likewise, the unicellular cyanobacterium *Crocosphaera* strain WH8501 displays increased rates of C and N_2 fixation under elevated pCO_2 conditions [9]. *Trichodesmium* and *Crocosphaera* are dominant diazotrophic taxa in oligotrophic open-ocean environments [10], where the bioavailable nitrogen (N) from N_2 fixation can fuel up to half of production exported from the euphotic zone [11]. In theory, a global pCO_2 enhancement of marine N_2 fixation could increase oceanic C uptake and export, producing a negative feedback to climate change [6].

In contrast to laboratory findings, recent field experiments using natural diazotrophic assemblages do not reliably show that raising pCO_2 enhances N- or C-based productivity. Elevating pCO_2 in bottle incubations has been shown to increase N_2 fixation rates by *Trichodesmium* colonies isolated from the Subtropical Atlantic and the Gulf of Mexico [12,13] but not by *Trichodesmium* colonies isolated from the North Pacific Subtropical Gyre [14]. Furthermore, experiments using whole water diazotrophic assemblages from the North and South Pacific gyres have found no relationship between pCO_2 and N_2 fixation rates [15,16]. The inconsistent results from field incubations highlight the importance of assessing how the effect of pCO_2 on diazotrophs is influenced by other factors, including community composition [14,17], physiology, and environmental conditions [18].

One methodological challenge in pCO_2 manipulation studies is producing realistic timescales for pCO_2 perturbations. In nature, seawater pCO_2 varies spatially and temporally: phytoplankton experience pCO_2 fluctuations on diurnal, seasonal, episodic, and

long-term (e.g. OA-driven) timescales [19]. However, most laboratory OA studies grow cultures under stable pCO_2 treatments, allowing cultures to acclimate to a steady pCO_2 for multiple generations. These stable pCO_2 conditions may affect phytoplankton differently than the dynamic pCO_2 experienced in marine ecosystems; for instance, energetic costs associated with resource allocation may be minimized under stable pCO_2 regimes [20].

Predicting the future response of diazotrophic assemblages to OA will require an assessment of how pCO_2 affects marine diazotrophs under variable environmental conditions and physiological states. Here, we present data from experiments tracking the growth, PC and PN production rates of *Crocosphaera watsonii* WH8501 cultures bubbled with air at three CO_2 levels (~400, 750,1000 ppm), while allowing for biologically induced pCO_2 variability in the culture medium resulting from photosynthesis and respiration. This approach contributes to the existing literature on potential responses of marine diazotrophs to future OA, but expands from previous studies by testing the effect of elevated pCO_2 under a dynamic pCO_2 environment. Our results suggest a consistent response of this organism to elevated pCO_2 under variable pCO_2 conditions.

Methods

Culture conditions

Unialgal stock cultures of *Crocosphaera watsonii* strain WH8501 were grown in 0.2 μm-filtered, nitrogen-free YBCII medium [21] using 40 μmol L^{-1} K_2HPO_4. Cultures were not axenic, but heterotrophic bacterial counts were kept at low levels (1.4–2.1×10^5 cells mL^{-1}). Light was provided using cool white fluorescent bulbs set on a 12:12 light/dark cycle. Stock cultures were grown at 24°C and 250 μmol quanta m^{-2} s^{-1}. For the experiment, cultures were grown at 30°C, a temperature which promotes optimal growth of *Crocosphaera* in the laboratory [22] and at which high abundances of *Crocosphaera* cells have been observed at sea [23]. Incoming irradiance for the experiment was 1000 μmol quanta m^{-2} s^{-1} as measured by a Biospherical light meter, a level which is saturating but not inhibitory for *Crocosphaera* WH8501 [24]. Cultures were stirred at least once a day with magnetic stir bars to minimize cells sticking to the glass. The pCO_2 was manipulated by gently bubbling cultures with commercially prepared air/CO_2 mixtures of ~400 ppm ('low-CO_2'), ~750 ppm ('mid-CO_2'), and ~1000 ppm ('high-CO_2'). Parent cultures were grown under these CO_2, light, and temperature conditions for seven days (~3–4 generations) before productivity rates were measured.

Experimental Design

Crocosphaera cultures were grown under three CO_2 treatments and monitored over a six-day period (day 0–day 5). Triplicate bottle replicates were used for each CO_2 treatment. Preceding the experiment, 2 L glass bottles were filled with 0.2 μm-filtered media that was pre-equilibrated to target pCO_2 levels. Initial CO_2 equilibration of the media was verified by measuring a stable pCO_2 in outflowing gas (>24 h equilibration) using a LI-840 LI-COR gas analyzer (Biosciences). The pH of each replicate was measured and converted through CO2calc (see below) to produce initial pCO_2 values of 404±23, 724±51, and 916±34 μatm for low-, mid-, and high-CO_2 treatments, respectively. To initiate the experiment, parent cultures in exponential growth phase were diluted into the pre-equilibrated media, producing initial biomass concentrations of 3.9–4.6 μg chlorophyll *a* (Chl *a*) L^{-1} (Table 1). Because parent cultures were shifting media pCO_2 through biological processes, the addition of parent cultures to pre-equilibrated media altered the initial pCO_2 values to 355±14, 600±21, and 788±11 μatm (Table S1). Bottles were gently bubbled with air/CO_2 mixtures at 50 mL min^{-1} throughout the experiment. Each replicate was sampled once daily after the sixth hour of light (L6) from day 0–day 2, then four times daily after the sixth and twelfth hours of light and darkness (L6, L12, D6, and D12) from day 3–day 5. The pH in each replicate was measured at the time of sampling and samples were preserved for particulate carbon (PC), particulate nitrogen (PN), Chl *a*, and flow cytometric cell counts (FCM).

Analytical Measurements

For PC/PN and Chl *a* measurements, three subsamples of 5–50 mL (depending on cell density) were withdrawn from each replicate and filtered onto glass fiber filters (GF/F, Whatman), using pre-combusted GF/F filters for PC/PN. Samples were immediately frozen at −80°C (PC/PN) or −20°C (Chl *a*). PC/PN samples were dried at 60°C overnight, packaged into silver and tin capsules, and analyzed using a Carlo Erba elemental analyzer. Acetanilide (71.09% C and 10.36% N by weight) served as a standard, and filter blanks were <10% of total C and N content. Chl *a* was extracted in 90% acetone at −20°C for 48 hours and analyzed with a Turner Model 10-AU fluorometer using the acidification method of Strickland and Parsons [25]. On day 0 and day 5 L6 time points, 25 mL samples were withdrawn from GF/F filtrate and immediately frozen for soluble reactive phosphorus (assumed to be equivalent to PO_4) and NH_4 analyses. NH_4 concentrations were measured with a Technicon AutoAnalyzer II, using a modified indophenol blue method [26] and PO_4 via the standard ascorbic acid-molybdate method [25].

Crocosphaera and heterotrophic bacterial cell densities were measured using FCM. Two 3-mL subsamples were withdrawn from each replicate, pipetted into 4 mL cryovials, and fixed with paraformaldehyde at a final concentration of 1% (volume $volume^{-1}$). Samples were inverted and allowed to sit in the dark for ~10 minutes before being frozen at −80°C. For analysis of *Crocosphaera* cell densities, samples were thawed on ice in the dark then spiked with a known number of 3 μm Polysciences Fluoresbrite yellow-green beads and run on a Becton-Dickinson FASCaliber flow cytometer with a 488 nm laser. *Crocosphaera* cells and beads were distinguished from other particulate matter by their side light scatter and fluorescence in orange wavelengths. The bead count determined the volume of sample run, and thus the concentration of *Crocosphaera* cells. A similar method was used to enumerate the background heterotrophic bacteria in these cultures. The samples were spiked with Fluoresbrite 1 μm beads, stained with SYBR Green I according to the method of Marie et al. [27], and differentiated by their side light scatter and green fluorescence.

The pH of each replicate was measured directly using a VWR sympHony electrode calibrated with VWR buffers (NBS scale). pH values were converted to pCO_2 by assuming a constant total alkalinity (TA) for the YBCII medium (2500 μM). This TA value was determined by analyzing DIC and pCO_2 of a separate batch of YBCII medium according to the methods of Bandstra et al. [28], then the program CO2calc [29] was used to convert DIC and pCO_2 to TA (with CO_2 constants from Merbach et al. [30] refit by Dickson and Millero [31], and a correction to the NBS scale by the CO2calc program). Finally, CO2calc was used to calculate pCO_2 from our measured pH data and the constant TA.

Our assumption of constant TA of the YBCII medium throughout the experiment is based on the observation that bubbling with air/CO_2 mixtures perturbs DIC but not TA [32];

Table 1. Time series biomass measurements for cultures of *Crocosphaera watsonii* WH8501 grown under three CO_2 treatments.

	Low-CO_2 treatment				Mid-CO_2 treatment				High-CO_2 treatment			
Time point	**PC**	**PN**	**Cells**	**Chl *a***	**PC**	**PN**	**Cells**	**Chl *a***	**PC**	**PN**	**Cells**	**Chl *a***
Day 0 L6	124 (12)	8.8 (1)	-	4.3 (0.6)	120 (4)	8.5 (0.7)	-	4.6 (0.3)	118 (2)	7.6 (0.1)	-	3.9 (0.4)
Day 1 L6	166 (24)	14.2 (2)	5.6E+05 (7.4E+04)	7.3 (1.6)	192 (1)	16.2 (0.2)	8.0E+05 (6.3E+04)	9 (0.4)	172 (21)	13.2 (2)	7.5E+05 (1.9E+04)	7.9 (0.4)
Day 2 L6	374 (145)	22 (4)	7.4E+05 (1.2E+05)	11.6 (1.5)	424 (73)	28.2 (3)	1.2E+06 (1.1E+05)	16.3 (3)	547 (63)	28.3 (2)	1.2E+06 (9.7E+03)	15.1 (1)
Day 3 L6	542 (65)	42.5 (6)	1.4E+06 (2.5 E+05)	24.8 (3)	706 (88)	55.4 (6)	2.4E+06 (1.9E+05)	39.6 (6)	740 (44)	55.9 (4)	2.6E+06 (1.3E+05)	35.3 (6)
Day 4 L6	833 (121)	66 (7)	2.3E+06 (3.1E+05)	31.6 (2.6)	1144 (174)	91.7 (10)	3.6E+06 (1.5E+06)	50.6 (9)	1308 (121)	97.4 (7)	4.8E+06 (2.1E+05)	49.1 (9)
Day 5 L6	1037 (58)	84.2 (3)	3.3E+06 (2.9E+05)	55.4 (2.1)	1589 (186)	130.2 (12)	6.7E+06 (4.4E+05)	82.5 (8)	1852 (65)	155.1 (2)	8.1E+06 (3.8E+05)	101 (2)

Concentrations of particulate carbon (μmol L^{-1}; PC), particulate nitrogen (μmol L^{-1}; PN), cells (# mL^{-1}), and chlorophyll *a* (Chl *a*; μg L^{-1}) are provided for L6 time points. Data are mean values from three replicate bottles; standard deviations are presented in parentheses.

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thus, any change in TA through the experiment was due to biological activity. While the process of N_2 fixation does not affect TA, photosynthesis can have a small effect due to hydrogen ion uptake to balance anionic nutrient (N, P, and S) acquisition [33]. Assuming no inorganic N uptake (as cultures were grown in N-free media) and a 2.4:1 S:P uptake ratio (as in [33]), we estimate that the average PO_4 drawdown of $\sim$9 μM observed in our experiment (see *Results and Discussion*) increased TA by an average of $\sim$52 μM by day 5, generating a maximum pCO_2 error of $\sim$2%. In addition, we assume that calcium carbonate ($CaCO_3$) minerals were not precipitated in our experiment, as the presence of PO_4 has been shown to inhibit $CaCO_3$ precipitation, even at high $CaCO_3$ saturation states [34,35].

Rate calculations

Specific growth rates were calculated for each of the biomass parameters measured: cell density, PC, PN, and Chl *a* (Table 2). Growth rates (μ) were determined using Eq. 1,

$$\mu(\mathrm{d}^{-1}) = \frac{\ln(N_T/N_0)}{\Delta T} \quad (1)$$

where N_T is the biomass at day 3, N_0 is the biomass at day 1, and ΔT is the time interval in days. The day 1–day 3 time interval was chosen for growth rate calculations because this was the phase of exponential growth (Fig. 1A).

Carbon-normalized PC and PN production rates ($\sim$net C and N_2 fixation rates) were calculated using Eq. 2,

$$\text{Production rate} = \frac{(N_T - N_0)/PC_0}{\Delta T} \quad (2)$$

where N_T is the biomass (PC or PN) at the final time point, N_0 is the biomass at the initial time point, PC_0 is the initial PC concentration, and ΔT is the time interval in days. Production rates were calculated for both exponential (day 1–day 3) and early stationary (day 3–day 5) growth phases.

Growth rates, PC and PN production rates were all calculated using data from L6 time points. Day 0 was excluded from these analyses due to missing FCM samples on this day.

Statistics

The effects of pCO_2 on growth rates, PC production, PN production, and molar C:N ratios were assessed using the one-way ANOVA. Differences between CO_2 treatments were determined using the Tukey Honest Significance Difference (HSD) test of multiple comparisons. All data reported in this study are averages from triplicate bottles. Statistical tests were run using the program R (http://www.r-project.org/).

Results and Discussion

Our study tested how enhanced pCO_2 affects the growth, PC and PN production rates of high-density *Crocosphaera* cultures. In agreement with a previous study [9], we found that PC production, PN production, and growth rates were all positively correlated with pCO_2 (Table 2, Fig. 2). This pCO_2 enhancement was observed for *Crocosphaera* cultures during both exponential and early stationary growth phases (Fig. 2). The high growth rates and cell densities observed in our study produced a strong diurnal rhythm of C and N metabolism in *Crocosphaera* cultures as well as daily pCO_2 variability (Fig. 1).

Table 2. Biomass-specific growth rates of *Crocosphaera watsonii* WH8501 cultures grown under three CO_2 treatments.

	Specific Growth rate μ (d^{-1})			Tukey HSD *p*-value	
	Low-CO_2	Mid-CO_2	High-CO_2	Low-CO_2 vs. high-CO_2	Low-CO_2 vs. mid-CO_2
Cell density	0.45 (0.02)	0.54 (0.02)	0.60 (0.02)	<0.001	<0.01
Particulate nitrogen	0.54 (0.02)	0.60 (0.05)	0.71 (0.04)	<0.01	0.2
Particulate carbon	0.58 (0.02)	0.63 (0.06)	0.71 (0.06)	<0.05	0.44
Chlorophyll *a*	0.60 (0.06)	0.72 (0.05)	0.72 (0.07)	0.12	0.14

Rates were calculated from L6 time points between day 1 and day 3 (exponential growth phase). Tukey HSD *p*-values are provided for comparisons among CO_2 treatments. Standard deviations of growth rates from three replicate bottles are presented in parentheses.
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Diurnal rhythm in growth and pCO_2

Diazotrophic cyanobacteria employ various mechanisms to separate the oxygen (O_2) evolved through photosynthesis from the enzyme nitrogenase, which catalyzes biological N_2 fixation and is irreversibly inactivated by O_2 [36]. *Crocosphaera* circumvents this problem by restricting N_2 fixation to the nighttime, when O_2 is not being produced. The energy needed to fix N_2 is generated photosynthetically in the light and stored primarily as carbohydrate granules [37]; respiration of these organic C reserves fuels N_2 fixation in the dark. The temporal separation and energetic linkage of photosynthesis and N_2 fixation in *Crocosphaera* produces a daily pattern in the timing and magnitude of PC and PN production and loss. In our study, cultures were grown under high light conditions, producing high growth rates and an especially pronounced diurnal rhythm.

We observed a strong daily pattern in PC production, PN production, and cell division by *Crocosphaera* in all CO_2 treatments (Fig. 1). The PC concentration of *Crocosphaera* cultures fluctuated widely between the light and dark periods: PC increased 48–216% in the light (~C fixation) and decreased 17–79% in the dark (~respiration) (Fig. 1B). This substantial dark PC loss is consistent with previous studies of *Crocosphaera* and reflects the respiration of carbohydrate reserves to fuel N_2 fixation [37–39]. A fraction of PC may have also been exuded from cells, as it has been shown that *Crocosphaera* WH8501 can release ~10% of total C content daily as extracellular polymeric substances [38]; however, dissolved organic C was not measured in our study. The sharp increase in cell concentration following the D12 measurement indicates that cells divided in the first half of the light period (Fig. 1C).

PN production was restricted to the dark period, when PN concentrations increased between 48 and 93% (Fig. 1A). Rates of PN increase approximate net N_2 fixation rates, though we cannot rule out NO_3 or NH_4 utilization as driving some small fraction of PN production. While cultures were grown in N-free media [21], the initial dilution of the parent cultures into fresh media resulted in NH_4 concentrations of 0.3 ± 0.1 μM on day 0. By day 5, NH_4 had increased to 1.1 ± 0.3 μM, supporting the interpretation that the rate of accumulation of inorganic plus particulate N in our cultures was fueled by N_2 fixation. The accumulated NH_4 had presumably been fixed by *Crocosphaera* and released from cells; *Crocosphaera* have been previously observed to release 23–67% of recently fixed N [38]. PN decreased slightly (1–8%) during the light period of the experiment (Fig 1A), which may indicate daily NH_4 release. The total NH_4 accumulated through the experiment (0.8 μM) represents 0.5–1% of total PN production (day 5–day 0, Table 1).

Together, the high rates of PN production, PC production in the day (~C fixation) and PC loss in the night (~C respiration) led to large fluctuations in the particulate C:N ratio over the daily cycle: molar C:N ratios in our study ranged from 5.5–22.1 (Fig. 1D). The C:N ratios at D12 time points had a relatively consistent range (~5.5–8) encompassing the 6.6 ratio predicted from Redfield stoichiometry [40]. Elevated C:N ratios at L6, L12, and D6 time points were driven by the accumulation of organic C reserves to fuel dark N_2 fixation and other cellular processes. These daily C:N deviations were independent of pCO_2 treatment. Previous studies have reported less dramatic stoichiometric fluctuations in *Crocosphaera*, with daily C:N content ranging from 6.5–8.5 [39] and 5.0–8.8 [38]. The strong daily C:N deviations observed in our study are consistent with metabolic rates at high growth rates (~0.5 d^{-1}) of cultures grown at optimum temperature (30°C, [20]) and saturating incoming irradiance (1000 μmol quanta m^{-2} s^{-1}, [24]). Our experiment spanned both exponential and early stationary growth phases. Cultures grew exponentially from day 0 to day 3, at which point growth rates began to decline (Fig. 1A). The shift in growth phase is evident from non-linearity of natural log-normalized PN growth curves at L6 time points (Fig. 1A) and from decreased PC and PN production rates from day 3–day 5 (Fig. 2). Declines in the magnitude of daily PC fluctuations indicate decreased rates of photosynthesis (~positive derivative, in light) and respiration (~negative derivative, in dark) after day 3 (Fig. 1B). *Crocosphaera* cultures were grown in an artificial medium initially containing an ample supply of macro and micronutrients [16], and PO_4 remained replete throughout the experiment (PO_4 decreased from 36 ± 1.8 μM on day 0 to 27 ± 1.8 μM on day 5, data not shown). Thus, although we cannot exclude the possibility of limitation by a micronutrient, we hypothesize that the shift to early stationary growth phase resulted from self-shading or a decreased RuBisCO carboxylation efficiency during the second half of the photoperiod, either through direct CO_2 limitation or through competitive inhibition of carboxylation from photorespiration at high O_2:CO_2 ratios [41].

The high *Crocosphaera* growth rates and cell densities affected the stability of C chemistry within CO_2 treatments: photosynthesis and respiration produced pCO_2 variability despite continuously bubbling cultures with air/CO_2 mixtures (Fig. 1E). The magnitude of pCO_2 variability increased throughout the experiment, and by day 5, the high-CO_2 treatment had fluctuated between 1216 and 96 μatm (Fig. 1E). In addition, by day 4, all cultures, independent of pCO_2 treatment, reached consistent minimum pCO_2 values close to 100 μatm in measurements taken at the end of the light cycle (L12); hence, ~100 μatm appears to represent a physiological limit for the uptake of inorganic C by this organism. Despite the extreme pCO_2 fluctuations within treatments, mid- and high-CO_2 treatments always had higher pCO_2 values than the low-CO_2 treatment (which fluctuated between 376 and 74 μatm), with the exception of the final time point. To separate the effect of

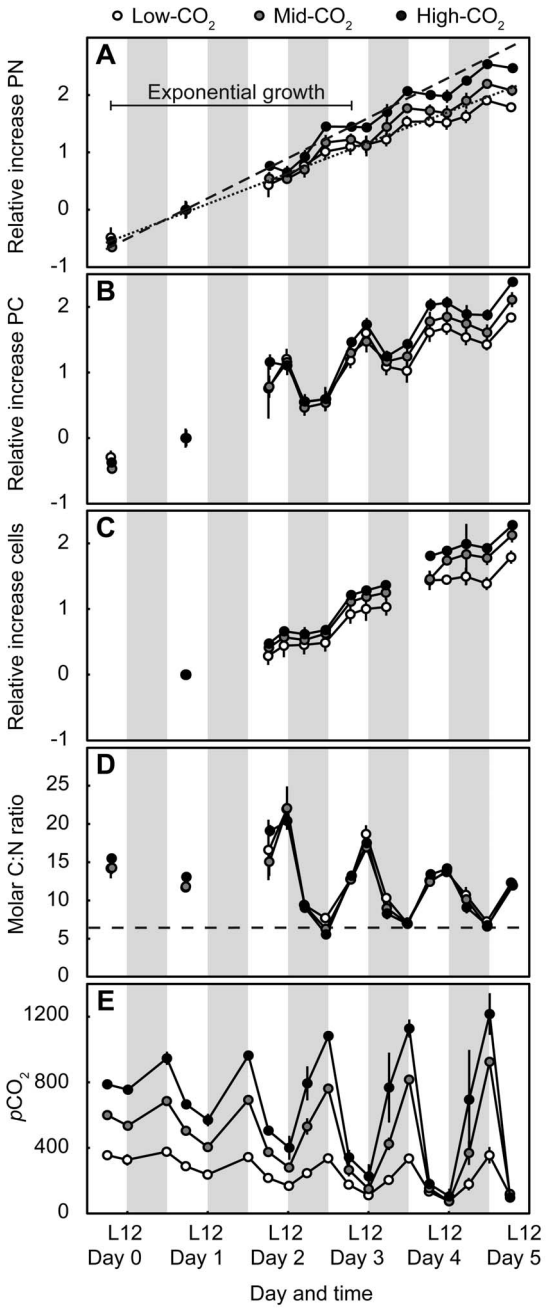

Figure 1. Growth of *C. watsonii* WH8501 batch cultures over a 6-day period under three CO_2 treatments. Shown are concentrations of PN (a), PC (b), and cells (c), molar C:N ratios (d), and pCO_2 in μatm (e) within each treatment. For (a–c), the concentrations for each time point (Table S1) were first normalized to the concentration at the day 1 L6 time point, then ln-transformed. The derived slopes between day 1 L6 and day 3 L6 time points correspond to the exponential growth rates (μ) as shown in Table 2. The lines in (a) represent linear regressions through the day 1, day 2, and day 3 L6 time points for high-CO_2 (dashed line) and low-CO_2 (dotted line) treatments. The regression lines have been extended to the full time period (day 0–5) for visualization of exponential growth (day 0–3 L6 time points) transitioning to early stationary growth (L6 time points after day 3). The dotted line in (d) represents the 6.6 C:N ratio expected from Redfield stoichiometry. Shaded areas represent the dark periods. Error bars represent standard deviations from three replicates.
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inflowing pCO_2 from the possible confounding effect of cell densities, we present growth rates from day 1 to day 3 when pCO_2 fluctuations were less extreme and treatments did not overlap in pCO_2 range (Table 2); the mean pCO_2 levels measured in this time interval were 252, 477, and 665 μatm for the low-, mid-, and high-CO_2 treatments, respectively. The response of *Crocosphaera* to elevated pCO_2 combined with daily, biologically induced pCO_2 fluctuations may have ecological implications for bloom scenarios (discussed below).

pCO_2 enhancement of growth, PC and PN production

Consistent with previous studies of *Crocosphaera* WH8501 [7] and *Trichodesmium* IMS101 [4,6–8], we found that elevating pCO_2 increased *Crocosphaera* growth, PC and PN production rates (Table 2, Fig. 2). Growth in the high-CO_2 treatment was significantly higher than in the low-CO_2 treatment for growth rates specific to PC, PN, and cell density (Table 2). Cell-specific growth rates were also significantly higher in the mid-CO_2 treatment than the low-CO_2 treatment (Table 2). Chl *a* growth rates were not significantly different between CO_2 treatments, possibly because of the large coefficient of variation between replicates (7–10%) (Table 2). Biomass-normalized PC and PN

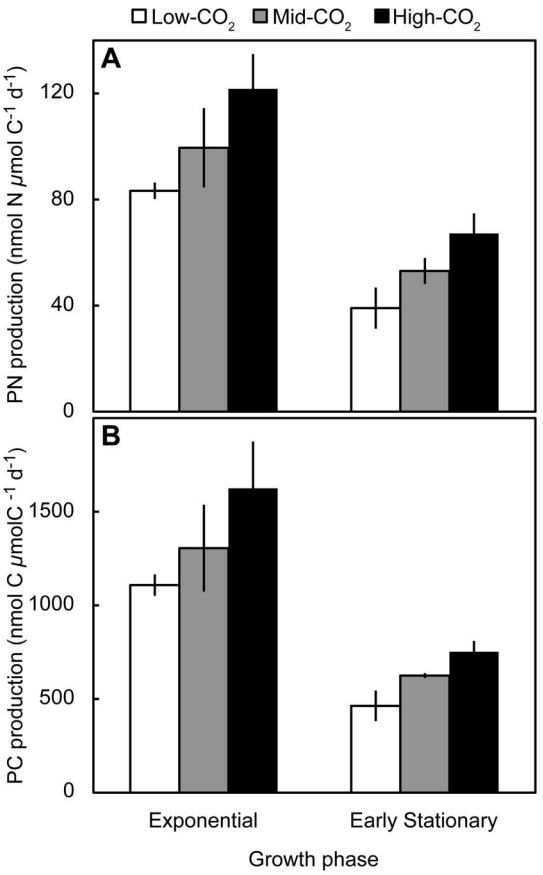

Figure 2. Carbon-normalized PN (a) and PC production rates (b) of *Crocosphaera* WH8501 cultures grown under three CO_2 treatments during periods of exponential (day 1–day 3) and early stationary (day 3–day 5) growth phases. Production rates are calculated as increases in PC and PN concentrations (data provided in Table 1) per time normalized to initial PC concentrations within the time interval. Error bars represent standard deviations from three replicates.
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production rates were significantly enhanced in mid-CO_2 and high-CO_2 treatments compared to the low-CO_2 treatment (Fig. 2). The pCO_2 enhancement of PC and PN production rates was observed both during exponential (day 1–day 3) and early stationary (day 3–day 5) growth phases (Fig. 2).

The only previous CO_2 manipulation study using *Crocosphaera* strain WH8501 tested a pCO_2 range of 190–50 μatm and observed that raising ambient pCO_2 (380 μatm) to 750 μatm produced 1.2- and 1.4-fold higher rates of C and N_2 fixation, respectively [9]. In our study, PC and PN production rates during exponential growth were both ~1.2-fold higher in the mid-CO_2 treatment than the low-CO_2 (Fig. 2). The magnitude of pCO_2 enhancement we observed is strikingly similar to those reported by Fu et al. [9], especially considering that the environmental conditions utilized in our study differed from the low light (80 μmol quanta m^{-2} s^{-1}), steady pCO_2 conditions of Fu et al. [9]. In our study, including a higher pCO_2 treatment displayed even larger enhancements: both PC and PN production rates were ~1.5-fold higher in the high-CO_2 treatment than the low-CO_2 treatment. Higher pCO_2 treatments would need to be included to determine the threshold pCO_2 condition that saturates C and N_2 fixation rates of *Crocosphaera* WH8501.

Conclusions and ecological implications

We observed that elevated pCO_2 conditions significantly enhanced PC production, PN production, and growth rates of *Crocosphaera* strain WH8501. This pCO_2 enhancement persisted despite biologically induced pCO_2 variability in all treatments. By allowing photosynthesis and respiration to drive pCO_2 deviations from target values, our methods contrast with those of many previous OA studies, which often keep cultures optically dilute and/or do not report the measured pCO_2 time course for each replicate. Though pCO_2 in our study varied within treatments, the mid- and high-CO_2 treatments had higher pCO_2 values than the low-CO_2 treatment for nearly all time points (Fig. 1E). Thus, the higher rates of growth, PC and PN production observed in mid- and high-CO_2 treatments can be attributed to the elevated pCO_2. Furthermore, the differences between treatments observed in the early stationary growth phase reflect low-end estimates of potential pCO_2 enhancements: cell densities were highest in the elevated CO_2 treatments, possibly leading to more severe growth limitations and dampening evidence for CO_2 enhancement.

Our study allows for new insights into the response of *Crocosphaera* to enhanced pCO_2 under a variable pCO_2 environment. Elevated mean pCO_2 enhanced the growth of *Crocosphaera* cultures despite large pCO_2 fluctuations on timescales of less than a generation time, showing that this organism does not need to be acclimated to a stable pCO_2 regime to benefit from elevated pCO_2. Testing the response of microbes to CO_2 perturbations on multiple timescales is ecologically relevant, because net community metabolism, temperature and salinity effects on CO_2 solubility, and advective processes cause pCO_2 fluctuations on episodic, diurnal, and seasonal timescales. Phytoplankton will experience the long-term OA pCO_2 signal superimposed onto this existing pCO_2 variability. Furthermore, future OA will increase the DIC:TA ratio of surface waters, leading to a reduced capacity to buffer processes like photosynthesis and respiration, ultimately increasing the magnitude of pCO_2 fluctuations [42].

The pCO_2 fluctuations observed in our study are probably more extreme than the natural variability experienced by *Crocosphaera* populations in open-ocean habitats. In the North Pacific Subtropical Gyre, surface pCO_2 varies by ~20–50 μatm seasonally [43]; mesoscale features in this region may cause biologically induced pCO_2 swings of ~150 μatm [44]. However, aggregated cells may experience larger pCO_2 swings; for example, *Crocosphaera nifH* genes have been observed associated with *Trichodesmium* colonies [14] and could thus experience more extreme pCO_2 fluctuations during blooms and subsequent crashes in these concentrated-biomass microhabitats [45]. Regardless of the large magnitude of pCO_2 fluctuations employed in our study, our results suggest that elevated mean pCO_2 impacts the growth response of *Crocosphaera* despite short-term variability.

Overall, our study contributes to the growing literature on the response of marine diazotrophs to elevated pCO_2. We observed that growth, PC and PN production rates of *Crocosphaera* WH8501 were enhanced under elevated and variable pCO_2 in both exponential and early stationary growth phases. It should be noted that a recent study by Garcia et al. [46] found that *Crocosphaera* strains WH0401 and WH0402 appear to be fully saturated under present day pCO_2 conditions (~400 μatm); thus, elevated pCO_2 seems to have strain-specific effects within *Crocosphaera*. Further research investigating how community composition and environmental conditions regulate the response of marine diazotrophs to elevated pCO_2 will be key to predicting whether global rates of N_2 fixation will increase under future OA scenarios.

Supporting Information

Table S1 Time series measurements for cultures of *Crocosphaera watsonii* WH8501 grown under three pCO_2 treatments. Measured pH, calculated pCO_2 (μatm, see Methods) and concentrations of particulate carbon (μmol L^{-1}; PC), particulate nitrogen (μmol L^{-1}; PN), cells (# mL^{-1}), and chlorophyll *a* (Chl *a*; μg L^{-1}) are provided for every time point available. Data are mean values from three replicate bottles; standard deviations are presented in parentheses. Dashes indicate no data available.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MRG AEW RML. Performed the experiments: MRG. Analyzed the data: MRG AEW RML. Contributed reagents/materials/analysis tools: AEW RML. Wrote the paper: MRG.

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