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Interactions between changing pCO₂, N₂ fixation, and Fe limitation in the marine unicellular cyanobacterium *Crocospaera*

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Abstract

We examined the physiological responses of steady-state iron (Fe)-replete and Fe-limited cultures of the biogeochemically critical marine unicellular diazotrophic cyanobacterium *Crocospaera* at glacial (19 Pa; 190 ppm), current (39 Pa; 380 ppm), and projected year 2100 (76 Pa; 750 ppm) CO₂ levels. Rates of N₂ and CO₂ fixation and growth increased in step with increasing partial pressure of CO₂ (pCO₂), but only under Fe-replete conditions. N₂ and carbon fixation rates at 75 Pa CO₂ were 1.4–1.8-fold and 1.2–2.0-fold higher, respectively, relative to those at present day and glacial pCO₂ levels. In Fe-replete cultures, cellular Fe and molybdenum quotas varied threefold and were linearly related to N₂ fixation rates and to external pCO₂. However, N₂ fixation and trace metal quotas were decoupled from pCO₂ in Fe-limited *Crocospaera*. Higher CO₂ and Fe concentrations both resulted in increased cellular pigment contents and affected photosynthesis vs. irradiance parameters. If these results also apply to natural *Crocospaera* populations, anthropogenic CO₂ enrichment could substantially increase global oceanic N₂ and CO₂ fixation, but this effect may be tempered by Fe availability. Possible biogeochemical consequences may include elevated inputs of new nitrogen to the ocean and increased potential for Fe and/or phosphorus limitation in the future high-CO₂ ocean, and feedbacks to atmospheric pCO₂ in both the near future and over glacial to interglacial timescales.

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By the end of this century, CO₂ levels in the atmosphere and in surface seawater will roughly double, to 70–75 Pa (IPCC 2007). In the context of this anticipated doubling of the partial pressure of CO₂ (pCO₂), some culture and field studies have indicated that growth and carbon fixation by marine phytoplankton may increase (Kim et al. 2006; Fu et al. 2007; Hutchins et al. 2007). Anthropogenically driven changes in CO₂ availability may thus exert a strong control on algal physiology, nutrient cycling, and ecological interactions.

Experiments with natural phytoplankton communities indicate that elevated CO₂ could result in increased phytoplankton primary production in the open ocean (Hein and Sand-Jensen 1997) or in changes in competitive interactions among major marine phytoplankton groups (Tortell et al. 2002; Hare et al. 2007). Laboratory studies have shown that changes in CO₂ concentrations can result in species-specific modifications in cellular carbon acquisition pathways (Raven 1997; Tortell et al. 2000; Burkhardt et al. 2001) and elemental ratios (Tortell et al. 2000; Fu et al. 2007; Hutchins et al. 2007).

Many cyanobacteria possess active carbon concentrating mechanisms (CCMs) that facilitate HCO₃⁻ or CO₂ transport and interconversion in order to increase CO₂ concentration near ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO). These CCMs can support rapid growth even at low external dissolved inorganic carbon (DIC) concentrations (Badger et al. 2006). These active DIC transport processes, however, involve substantial energetic and metabolic costs. Consequently, it has been speculated that increased CO₂ availability could potentially reduce the allocation of energy or nutrients needed for carbon acquisition (Hutchins et al. 2007).

Recently several studies using cultures of the diazotrophic cyanobacterium *Trichodesmium* demonstrated that CO₂ availability controls nitrogen (N) and carbon (C) fixation rates and elemental ratios in this biogeochemically critical genus (Hutchins et al. 2007; Levitan et al. 2007; Ramos et al. 2007). These results indicate that *Trichodesmium*, which is thought to provide up to 50% of new nitrogen in the oligotrophic gyres (Karl et al. 2002), is carbon limited at present-day pCO₂. By the end of this century CO₂ enrichment could substantially increase N₂ and CO₂ fixation by *Trichodesmium*, fundamentally altering the current marine N and C cycles and providing a possible negative feedback on atmospheric pCO₂. Down-regulation of *Trichodesmium* N₂ fixation at low glacial pCO₂ levels could also play a pivotal role in global climatic shifts over longer periods, by reducing the inputs of new N that drive atmospheric CO₂ sequestration via the oceanic biological pump (Hutchins et al. 2007).

Although *Trichodesmium* has long been recognized as a dominant contributor to oceanic diazotrophy (Capone et al. 1997), recent evidence shows that total global marine N₂ fixation by unicellular N₂-fixing cyanobacteria probably equals or exceeds that of *Trichodesmium* (Montoya et al. 2004). Unicellular diazotrophs such as *Crocospaera watsonii* are a widespread diazotrophic group that can fix N₂ at high rates, and, hence, they can provide a substantial fraction of new N where they occur (Montoya et al. 2004; Falcon et al. 2005; Mulholland 2007).

In view of the new evidence for CO₂ control of carbon and N₂ fixation by *Trichodesmium*, the question arises as to whether other diazotrophic cyanobacteria exhibit similar responses to CO₂. In the non-nitrogen fixing unicellular cyanobacteria *Synechococcus* and *Prochlorococcus*, the responses of two common strains are quite different; there is a growth response of the former to changing pCO₂, but not of the latter (Fu et al. 2007). Thus, it is entirely possible that unicellular N₂-fixers might exhibit a unique set of

physiological changes in response to changing pCO₂. Despite the growing body of research investigating global change effects on marine phytoplankton, including diatoms (Burkhardt and Riebesell 1997; Tortell et al. 2002), cyanobacteria (Fu et al. 2007; Hutchins et al. 2007), coccolithophores (Feng et al. 2008), and harmful bloom flagellates (Fu et al. 2008), the effects of changing seawater pCO₂ on unicellular N₂-fixing cyanobacteria have not been examined.

Much recent research has focused on limiting factors for diazotrophic growth in the current ocean, particularly on the influences of potentially limiting nutrients such as iron (Fe) and phosphorus (P) (Sañudo-Wilhelmy et al. 2001; Mills et al. 2004; Fu et al. 2005a). Other studies have examined the effects of physical factors such as temperature and light availability (Bell and Fu 2005; Mulholland and Bernhardt 2005; Hutchins et al. 2007). Anthropogenic global change is of course by no means limited to pCO₂ increases, and far-reaching, comprehensive changes in the global environment are virtually certain to affect all of these other potential N₂ fixation-controlling variables as well. Rising sea surface temperature, increased stratification, shallower mixed layers, decreased vertical nutrient fluxes, and changing aeolian Fe inputs will all be likely consequences of near-future global change effects on the ocean (Sarmiento et al. 2004; Bopp et al. 2001; Boyd and Doney 2002). For instance, some projections indicate that future Fe supplies could increase to parts of the open ocean as a result of either accelerating human-induced desertification (Takeda and Tsuda 2005) or to deposition of anthropogenic fossil fuel aerosols (Sedwick et al. 2007). Although Fe limitation is now widely recognized as a major constraint on marine N₂ fixation (Kustka et al. 2003), how global change-driven shifts in Fe supply will interact with rising pCO₂ to influence the future N cycle is currently unknown.

A large body of existing literature on N₂ fixation has focused on understanding how Fe availability could control growth and N₂ fixation by *Trichodesmium* (Rueter et al. 1990; Berman-Frank et al. 2001; Fu and Bell 2003). Previous culture studies on *Trichodesmium* have demonstrated that photosynthesis, growth, and N₂ fixation were stimulated by the addition of Fe (Fu and Bell 2003), and modeling work by Berman-Frank et al. (2001) indicates that Fe availability restricts N₂ fixation by *Trichodesmium* in 75% of the global ocean. Fe requirements and cellular Fe quotas from both cultures and natural populations of *Trichodesmium* have been estimated (Berman-Frank et al. 2001; Kustka et al. 2003).

Past studies of the genus *Crocospaera* have included expression assays of genes responsible for N₂ fixation and P scavenging (Dyhrman and Haley 2006) and P uptake kinetics experiments (Falcon et al. 2005). However, to date, relatively little work has been done to determine the Fe requirements of unicellular diazotrophic cyanobacteria (Tuit et al. 2004; Berman-Frank et al. 2007). Based on the results of natural community incubation experiments, Mills et al. (2004) suggested that Fe limitation (or co-limitation, along with P) restricts the N₂ fixation of unicellular diazotrophs in the tropical North Atlantic.

Currently there is little published information on the Fe quotas and physiology of *Crocospaera* (Tuit et al. 2004) and none at all that concerns how Fe availability could interact with changing pCO₂ and potential consequent changes in N₂ fixation rates.

In fact, despite the increasing amount of attention that has been devoted to the responses of *Trichodesmium* to growth-limiting nutrients and to changes in pCO₂, nothing is known about their combined effects on N₂ fixation and growth of any other marine diazotrophs. The purpose of this study was to fill this gap and gather basic information on the growth, photosynthetic physiology, elemental ratios, and Fe quotas of a Sargasso Sea isolate of the unicellular N₂-fixer *Crocospaera* (Dyhrman and Haley 2006) under conditions of glacial era, present day, and projected year 2100 pCO₂. We examined all of these pCO₂ treatments under both Fe-limited (present day scenario) or Fe-replete (glacial and projected scenarios) growth conditions. The effect of variable pCO₂ on molybdenum (Mo) quotas, another metal required for N fixation, was also investigated.

Methods

Cultures and growth conditions—Experiments used *Crocospaera watsonii* WH8501, which is genetically nearly identical (>99% identity) to sequences found in North Pacific metagenomic collections (Zehr et al. 2007). Stock and experimental cultures were grown at 28°C in 0.2 μm-filtered, microwave-sterilized surface Sargasso seawater, which lacked added fixed N but was enriched with AQUIL levels of phosphate and trace nutrients (Morel et al. 1979). Fe stock was chelated with ethylenediaminetetraacetic acid (EDTA) before being added to the medium at final concentrations of 5.0 μmol L⁻¹ EDTA and 0.45 μmol L⁻¹ Fe, respectively. Light was provided on a 12:12 dark:light cycle using cool white fluorescent bulbs at 80 μmol photons m⁻² s⁻¹.

Experimental set-up—Semicontinuous culturing methods were used in order to measure Fe and pCO₂ effects during acclimated, steady-state growth (Fu et al. 2007; Hutchins et al. 2007). For all experiments, final sampling occurred once steady-state growth was obtained for each growth condition. Cultures were deemed to be at steady state when there were no significant differences in growth rates ($p > 0.05$) for at least seven generations. Experiments were conducted in triplicate 1-liter acid-washed and microwaved polycarbonate bottles containing 0.9 liters of medium.

Semicontinuous cultures were diluted every 2 d with medium that was previously adjusted to the appropriate temperature and pCO₂. Each bottle was diluted individually based on the cell count-derived growth rate calculated for that bottle, allowing each culture to independently reach its steady-state growth rate under a given experimental treatment. Samples from each culture bottle were always taken at the same time in the diel cycle (Tuit et al. 2004), between 09:00 h and 10:00 h in the morning, to measure cell density and, thus, to determine changes in growth rate.

The water for the experimental culture medium was the same Sargasso Sea seawater used for stock cultures, but it was collected using a shipboard trace metal-clean Teflon diaphragm pump system coupled to an acid-washed in-line 0.2-μm cartridge filter. The water was stored in the dark at 4°C in acid-washed polycarbonate carboys until use. All medium handling, culturing, and manipulation used careful trace metal-clean methods under class 100 conditions (Hutchins et al. 1998; Fu and Bell 2003; Hare et al. 2007).

Added Fe concentrations were 450 and 10 nmol L⁻¹ for the Fe-replete and Fe-limited cultures, respectively. Within each Fe condition, triplicate bottles were equilibrated at three different CO₂ concentrations: 19 Pa, 39 Pa, and 76 Pa. These concentrations were chosen to simulate a range of pCO₂ spanning glacial atmospheric levels (Petit et al. 1999), current concentrations, and the values predicted to occur by the end of this century (IPCC 2007). Seawater media was gently bubbled with commercially prepared air and CO₂ mixtures to achieve targeted CO₂ concentrations (Scott Gas). In-line high-efficiency particulate air filters were used to avoid Fe contamination from particles in the gas tanks or lines. CO₂ equilibration was monitored daily by measuring pH and DIC. The pH under steady-state growth conditions was ~8.5 at low pCO₂, ~8.1 at ambient pCO₂, and ~7.9 at elevated pCO₂. In general, total DIC concentrations were ~6% and ~14% higher in the 76-Pa pCO₂ treatments than in the 39-Pa pCO₂ and 19-Pa pCO₂ treatments, respectively (data not shown).

Analytical methods—N₂ fixation rates were estimated during the dark period using both acetylene reduction and ¹⁵N₂ incorporation methods, as described previously in Hutchins et al. (2007) and Mulholland and Bernhardt (2005). Samples for the analysis of particulate organic carbon (POC), particulate organic nitrogen (PON), and particulate organic phosphorus (POP) were collected onto precombusted (450°C for 5 h) 25-mm GF/F glass-fiber filters under low vacuum. POC and PON were analyzed on a 440 Elemental Analyzer following the protocol of Fu et al. (2007). The POP measurement is described in detail in Fu et al. (2005b). Cells were counted in a hemacytometer using epifluorescence microscopy.

Short-term production vs. irradiance (P-E) curves and 24-h primary production measurements under experimental conditions were conducted as described in Fu et al. (2008). Photosynthetic rates for the P-E curves were measured by incubating 5-mL culture samples in 7-mL vials with 0.1 μCi mL⁻¹ ¹⁴C sodium bicarbonate at 28°C for 30 min. The incubations were terminated by rapid filtration directly after the cells were killed with glutaraldehyde. Irradiance was provided by a photosynthetron (Composite High Pressure Technologies) and ranged from 10 to 900 μmol photons m⁻² s⁻¹. Duplicate vials for each treatment were wrapped in aluminum foil to estimate any dark uptake. All ¹⁴C uptake rates were corrected for dark uptake. Cell-normalized data were fitted to the equation of Platt et al. (1980) using the curve-fitting program in SigmaPlot to obtain the photosynthetic parameters. These included photosynthetic efficiency, α [ng C (cell)⁻¹ h⁻¹ (μmol quanta m⁻² s⁻¹)⁻¹], and the maximum cell-specific C

fixation rate, P_{Bmax} [ng C (cell)⁻¹ h⁻¹]. E_k , the light saturation point and index of light adaptation, was calculated as P_{Bmax}/α . Primary production was measured in triplicate using 24-h incubations with H¹⁴CO₃ under the appropriate experimental growth conditions for each treatment (Fu et al. 2007). All C-fixation rates were calculated using initial experimental DIC concentrations and cell numbers for each treatment. For the analysis of total dissolved inorganic C, 25-mL samples from each bottle were preserved with 200 μ L 5% HgCl₂ L⁻¹ and stored at 4°C until analysis in triplicate. Details of the DIC analyses are given in Fu et al. (2007).

The cyanobacterial accessory pigments phycoerythrin (PE) and phycocyanin (PC) were determined from 50–70-mL samples filtered through GF/F glass-fiber filters and then immediately placed in the freezer at –80°C until analysis. Frozen sample filters were cut into strips and placed into a 10-mL all-glass tissue grinder with 3 mL of 50 mmol L⁻¹ phosphate buffer (pH 7.0) containing 2 mg mL⁻¹ lysozyme. Each filter was thoroughly homogenized and then the homogenates were immersed in an ice bath and sonicated with a micro-tip sonication probe for 1 min to rupture the cell wall and increase the extraction efficiency. After sonication, the samples were kept in the dark at 4°C for 1 h and then filtered through a 0.45- μ m membrane filter. The filtrate was then withdrawn, and concentrations of PE and PC were determined using a spectrophotometer. Quantitative estimations of PE per cell were determined using the molar extinction coefficient from Wyman (1992). PC concentrations were determined spectrophotometrically as in Fu et al. (2007). GF/F glass-fiber filter samples for chlorophyll *a* (Chl *a*) analysis were stored frozen at –20°C. Chlorophyll was extracted in the dark for 24 h in 6 mL of a mixture of ice-cold 90% acetone and 10% water. The extracted Chl *a* was measured fluorometrically in duplicate on a Turner Designs 10AU fluorometer.

Trace metal quota measurements—Samples for analysis of cellular Fe and Mo quotas were filtered onto acid-washed 0.2- μ m polycarbonate filters under class 100 conditions using trace metal-clean handling and filtering techniques (Frew et al. 2006). The samples were rinsed with oxalate reagent to remove surface-adsorbed Fe, according to the method of Tovar-Sanchez et al. (2003); this was followed by three rinses with trace metal-clean 0.2 μ m-filtered seawater. Inductively coupled plasma mass spectrometry (ICPMS) was used for the determination of trace metals and P. The procedures for the filter digestions and trace metal analyses were those described in Sañudo-Wilhelmy et al. (2001), with minor modifications. Briefly, the filter samples were digested with 1.5 mL concentrated Q-HCL, 0.5 mL of concentrated Q-HNO₃, and 25 μ L Q-HF at room temperature for 2 weeks initially, and then for an additional 4 months. The second 4-month digestion was carried out to assess whether additional cellular metals were solubilized by the long-term acid digestion. Trace element concentrations of the digested samples were determined twice, the first time immediately after the 2-week acid digestion and a second time after the 4-month digestion. Procedural blank filters for each treatment were

carried through the filtration, rinsing, digestion, incubation, and analysis processes, and these blank values were subtracted from measured values.

Statistical analysis—Effects of the two independent variables pCO₂ and Fe on each dependent variable (including growth rates, N₂ fixation rates, photosynthetic parameters, cell quotas, and elemental ratios) in the experiments were assessed using two-way ANOVA to detect two-way interactions (Systat software). This test is based on the method of Zar (1999). Differences between treatment groups were determined using a Tukey multiple comparisons test. Differences were termed significant when $p < 0.05$. Three replicate bottles for every treatment were each sampled for each analysis, so for all data presented, $n = 3$. There are six (two \times three) combinations of the two factors; therefore, there are a total of $N = 18$ data in this experiment.

Results

Growth rates—In Fe-replete cultures, growth rates increased with increasing CO₂ concentrations (Fig. 1A). Growth rates in the 19-Pa treatment were significantly lower than in the other two treatments ($F_{1,12} = 74.36$, $p = 0.00005$; $F_{1,12} = 39.2$, $p = 0.0005$). They were also slightly higher in the 76-Pa CO₂ treatment than in the 39-Pa CO₂ treatment, although these differences were not significant ($F_{1,12} = 2.3$, $p = 0.3$).

As expected, the growth rates of Fe-limited cultures (Fig. 1B) were significantly lower (~ 50 – 60% , $F_{1,12} = 248$, $p = 0.0005$) than those of Fe-replete cultures (Fig. 1A) grown under the same CO₂ conditions. As in the Fe-replete cultures, there was no significant difference between the 76-Pa CO₂ treatment and the 39-Pa CO₂ treatment for the Fe-limited treatments ($F_{1,12} = 2.3$, $p = 0.3$). Growth rates in the Fe-limited 19-Pa treatment were significantly lower than those in the 76-Pa treatment ($F_{1,12} = 33$, $p = 0.003$), but they were not significantly different from those in the 39-Pa treatment ($F_{1,12} = 1.0$, $p = 0.5$).

N₂ fixation—Elevated CO₂ stimulated the rates of N₂ fixation in Fe-replete cultures, with a progressive increase in fixation rates across the three pCO₂ treatments (Fig. 2A). C-normalized N₂ fixation rates in the 76-Pa treatment were 1.4-fold and 1.8-fold higher than in the 39-Pa and 19-Pa treatments, respectively ($F_{1,12} = 30.2$, $p = 0.03$; $F_{1,12} = 513$, $p = 0.002$). The cultures grown at 39-Pa CO₂ also exhibited significantly higher N₂ fixation rates (~ 1.3 fold, $F_{1,12} = 18.6$, $p = 0.05$) compared with the 19-Pa treatment. In contrast, the rates of N₂ fixation by Fe-limited cultures were much lower and relatively constant with increasing CO₂ concentrations ($F_{1,12} = 3.2$, $p = 0.15$; Fig. 2B). For both Fe-replete and Fe-limited cultures, N₂ fixation rates calculated from acetylene reduction measurements showed the same trends as those obtained using ¹⁵N₂ incorporation (data not shown).

It is not surprising to find that in general, N₂ fixation rates were higher in Fe-replete cultures than in Fe-limited cultures. With regard to the interaction between Fe and

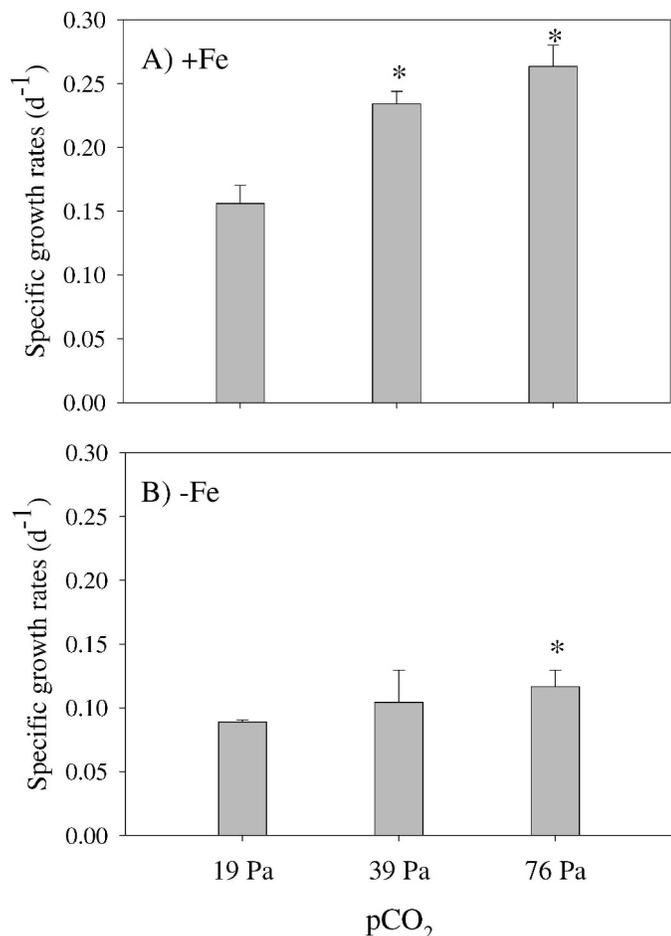


Fig. 1. Cell-specific growth rates of *Crocosphaera* grown at three different pCO₂ levels (19 Pa, 39 Pa, and 76 Pa) under (A) Fe-replete or (B) Fe-limited growth conditions. Values are the means and error bars are the standard deviations of triplicate cultures for each treatment ($n = 3$). Asterisks above histogram bars indicate a treatment average that was significantly different from that of the 19-Pa pCO₂ treatment.

CO₂ condition, it is noteworthy that under elevated CO₂ conditions (76 Pa), the N₂ fixation rates of the Fe-limited cultures (Fig. 2B) were only slightly lower than those of the Fe-replete cultures grown at 19-Pa CO₂ (Fig. 2A, $F_{1,12} = 18.3$, $p = 0.05$).

C, Fe, and Mo cell quotas and Fe and Mo use efficiencies—Cellular C quota measurements (Q_c) in the experiments indicated that there were differences in cell sizes between treatments (Table 1). Under Fe-replete conditions there were no differences in Q_c between the 39-Pa and 76-Pa treatments, but the cellular C quotas in the 19-Pa treatment were only 68–73% of those in the two higher pCO₂ levels ($F_{1,12} = 31.6$, $p = 0.0001$; $F_{1,12} = 21$, $p = 0.0006$). Q_c values in Fe-limited treatments were only 39–73% of those in the Fe-replete cultures at the corresponding pCO₂ values ($F_{1,12} = 174$, $p < 0.0001$), but there were no differences ($p > 0.05$) in C quotas between the three Fe-limited pCO₂ treatments (Table 1). Similar effects of Fe limitation on cell size have

been noted in other species of phytoplankton in the past (Sunda and Huntsman 1995; Hutchins et al. 1998).

Our ICPMS results were used to test for a possible relationship between pCO₂, N₂ fixation rates, and cellular quotas of Fe and Mo, the two trace metal cofactors required for the *Crocosphaera* nitrogenase enzyme (Tuit et al. 2004). The C-normalized Fe and Mo quotas (Fe:C and Mo:C ratios) were significantly higher in Fe-replete cultures (Fig. 2C,E) than in Fe-limited ones (Fig. 2D,F), regardless of CO₂ condition ($F_{1,12} = 849$, $p = 0.0005$; $F_{1,12} = 49$, $p = 0.0005$). In Fe-replete cultures, it is also apparent that the C-normalized Fe (Fig. 2C) and Mo (Fig. 2E) quotas increased significantly with increasing CO₂ concentrations ($F_{1,12} = 73$, $p = 0.005$), but this was not the case in Fe-limited cultures (Fig. 2D,F). In general, the overall responses of Fe:C ratios (Fig. 2C,D) and Mo:C ratios (Fig. 2E,F) to changing pCO₂ and Fe availability were nearly identical to those of N₂ fixation rates (Fig. 2A,B).

At an Fe-limited growth rate of 0.1 d⁻¹ under a present day pCO₂ of 39 Pa, *Crocosphaera* had a Fe:C ratio of ~27 μmol mol⁻¹, and Fe:C ratios were very similar in the other two Fe-limited pCO₂ treatments (Fig. 2D). Increased Fe availability at high concentrations (i.e., 450 nmol L⁻¹) resulted in an accumulation of intracellular Fe in substantial excess of that required to maintain algal growth rates at 0.1 d⁻¹. For instance, Fe-replete *Crocosphaera* accumulated 290 μmol Fe mol⁻¹ C at a growth rate of ~0.27 d⁻¹ in the 76-Pa CO₂ treatment (Fig. 2C), an 11-fold higher value than the values required to support the Fe-limited growth rates of about 0.1 d⁻¹ observed in all three Fe-limited pCO₂ treatments (Fig. 1B).

We estimated the cellular net use efficiencies for Fe and Mo (IUE and MoUE, respectively) as indices of how efficiently our cultures were able to grow and fix C as a function of cellular Fe or Mo content under our experimental treatments. Net IUE and MoUE values were calculated as the specific growth rates divided by the Fe:C or Mo:C ratios. In Fe-limited cultures, the net Fe use efficiencies ranged from 3214 to 3703 mol C mol Fe⁻¹ d⁻¹, with no apparent trend with pCO₂ (Table 1). For Fe-replete cultures, net IUE values were substantially lower, ranging from 897 to 1300 mol C mol Fe⁻¹ d⁻¹, and showed a modest but significant inverse trend with pCO₂ (Table 1). These Fe-replete net IUE values represent a lower limit estimate, since the cells undoubtedly contained storage Fe under these growth conditions. For Fe-limited cultures, the net Mo use efficiency ranged from 225,000 to 342,857 mol C mol Mo⁻¹ d⁻¹ (Table 1). Contrary to net IUE, effects of pCO₂ on net MoUE were not apparent under either Fe-limited or Fe-replete growth.

C fixation—As with the other rate measurements, most short-term C fixation parameters from P-E curves were significantly lower in Fe-limited cultures than in Fe-replete cultures, regardless of CO₂ conditions (Table 2; $F_{1,12} = 1053$, $p = 0.0005$; Table 2). CO₂-driven changes in photosynthetic parameters were also evident ($F_{2,12} = 186$, $p = 0.0005$), regardless of growth limitation by Fe. Under both Fe conditions, the two highest CO₂ concentration

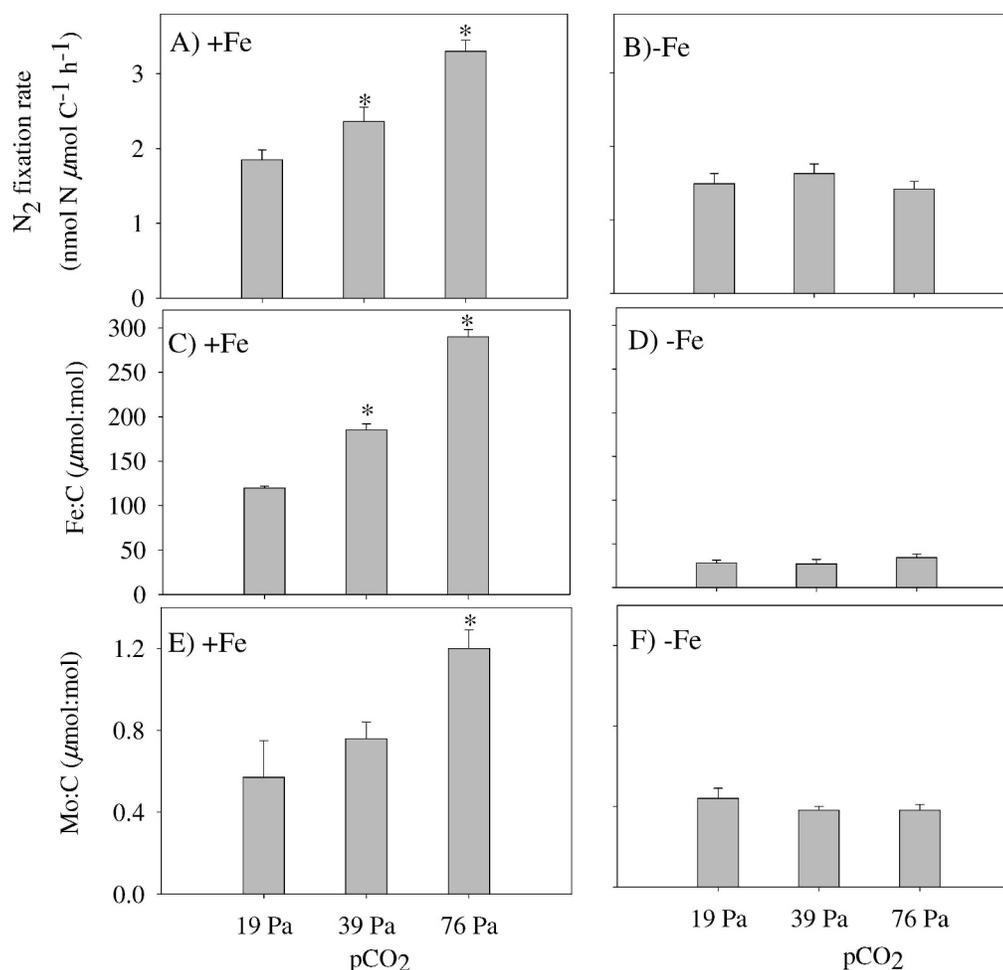


Fig. 2. *Crocospaera* cultures were grown at three different pCO₂ levels (19 Pa, 39 Pa, and 76 Pa) under Fe-replete and Fe-limited growth conditions. Shown are carbon-specific N₂ fixation rates of (A) Fe-replete and (B) Fe-limited cultures; Fe:C ratios of (C) Fe-replete and (D) Fe-limited cultures; and Mo:C quotas of (E) Fe-replete and (F) Fe-limited cultures. Values are the means and error bars are the standard deviations of triplicate cultures for each treatment, and symbols are as in Fig. 1.

treatments of 76 Pa and 39 Pa had P_{Bmax} values that were significantly higher than the 19-Pa values in the corresponding Fe treatment ($F_{1,12} = 309.4$, $p < 0.0001$; $F_{1,12} = 69.7$, $p = 0.005$; $F_{1,12} = 91.7$, $p = 0.001$; $F_{1,12} = 8.4$, $p = 0.01$). These pCO₂-mediated increases were 64% (76 Pa) and 30% (39 Pa) in Fe-replete cultures and 76% and 24% in Fe-limited cultures, respectively.

Treatment-related trends in the slope of the light-limited portion of the P-E curve, α , were in general similar to those for P_{Bmax} (Table 2). In Fe-replete cultures, the values of α increased with increasing CO₂ concentrations. In comparison, the values of α varied slightly and showed no significant differences between 19 Pa and 39 Pa CO₂ in Fe-limited cultures ($F_{1,12} = 1.5$, $p = 0.40$), but they had

Table 1. Net iron (Fe) use efficiency (IUE, mol carbon [C] mol Fe⁻¹ d⁻¹); net molybdenum (Mo) use efficiency (MoUE, mol C mol Mo⁻¹ d⁻¹); C:nitrogen (N), N:phosphorus (P), and C:P ratios (mol: mol); chlorophyll *a* (Chl *a*) to C ratios (μmol: mol); and cellular C quota or content (Qc, fmol C cell⁻¹) of *Crocospaera* during steady-state Fe-replete and Fe-limited growth in three different partial pressure of CO₂ (pCO₂) treatments. Values given are the means and values in parentheses are the standard deviations of measurements on triplicate cultures.

Treatments	pCO ₂ (Pa)	IUE	MoUE	C:N	N:P	C:P	Chl <i>a</i> :C	Qc
+Fe	76	897	216,667	7.3 (0.2)	19.2 (0.4)	140 (5)	73 (2)	0.26 (0.03)
	39	1243	302,632	6.9 (0.2)	18.8 (0.4)	130 (2)	83 (12)	0.28 (0.02)
	19	1333	280,702	7.4 (0.2)	15.6 (0.8)	116 (4)	67 (10)	0.19 (0.02)
-Fe	76	3529	342,857	6.5 (0.1)	16.2 (0.9)	104 (6)	51 (10)	0.12 (0.005)
	39	3703	285,714	6.3 (0.0)	16.1 (0.2)	101 (1)	44 (2)	0.11 (0.004)
	19	3214	225,000	6.4 (0.2)	15.8 (0.5)	101 (6)	44 (13)	0.14 (0.01)

Table 2. Functional photosynthetic performance parameters for *Crocospaera* during steady-state iron (Fe)-replete and Fe-limited growth in three different partial pressure of CO₂ (pCO₂) treatments. Shown are maximum cell-specific carbon (C) fixation rate (P_{Bmax} , ng C cell⁻¹ h⁻¹), α [ng C h⁻¹ (cell)⁻¹ (μ mol photon m⁻² s⁻¹)⁻¹], and the light saturation point and index of light adaptation (E_k , μ mol photon m⁻² s⁻¹). Values given are the means and values in parentheses are the standard deviations of measurements on triplicate cultures.

Treatments	pCO ₂ (Pa)	P_{Bmax}	α	E_k
+Fe	76	133 (5)	0.70 (0.015)	190 (8)
	39	106 (3)	0.56 (0.04)	190 (10)
	19	81 (5)	0.27 (0.03)	300 (20)
-Fe	76	67 (3)	0.55 (0.08)	122 (27)
	39	47 (02)	0.32 (0.04)	147 (19)
	19	38 (1)	0.23 (0.02)	165 (28)

significant increases from 39 Pa to 76 Pa CO₂. α values in Fe-replete cultures were significantly greater relative to Fe-limited cultures at all CO₂ concentrations ($F_{1,12} = 25$, $p = 0.0008$), except for the lowest pCO₂ at 19 Pa, which had very similar α values under both Fe conditions ($F_{1,12} = 1.7$, $p = 0.37$).

The light saturation constant E_k was evaluated from the ratios of P_{Bmax} to α (Table 2). As a result of greater increases in P_{Bmax} than in α for Fe-replete cultures, the ratios of P_{Bmax} to α increased modestly relative to values in the Fe-limited cultures ($F_{1,12} = 63$, $p = 0.0005$). In Fe-limited cultures the values of both P_{Bmax} and α were enhanced with increasing CO₂ concentrations, and, hence, resulted in no evident CO₂ effects on E_k ($F_{2,6} = 2.7$, $p = 0.5$). However, in Fe-replete cultures, the greater relative decreases in α than in P_{Bmax} under the 19-Pa treatment resulted in significant increases in E_k relative to the invariant values in the other two CO₂ concentrations ($F_{2,6} = 52.4$, $p = 0.0005$; $F = 22$, $p = 0.003$).

The pCO₂-mediated trends for daily primary production in *Crocospaera* were in general similar to those observed for N₂ fixation (Fig. 3). Like the trends in P_{Bmax} obtained from short-term P-E curves (Table 2), increases in CO₂ in Fe-replete cultures stimulated 24-h primary production rates under the experimental culture light conditions (Fig. 3A). Primary production rates in the Fe-replete 76-Pa treatment were twofold higher than in the Fe-replete 19-Pa treatment. For Fe-limited cultures grown at a pCO₂ of 76 Pa, primary productivity was elevated by 150–230% compared with the other two pCO₂ treatments (Fig. 3B). Paired comparisons of Fe-replete and Fe-limited cultures at the same pCO₂ concentration showed significantly elevated primary production at each pCO₂ with increasing Fe availability. To some extent the effects of increasing CO₂ and decreasing Fe offset each other; for instance, there was no significant difference in primary production rates between the Fe-replete 19-Pa (Fig. 3A) and the Fe-limited 76-Pa treatments (Fig. 3B; $F_{1,12} = 3.0$, $p = 0.17$).

Photosynthetic pigments—In general, the C normalized content of photosynthetic pigments was affected much more by Fe than by CO₂. Fe-replete *Crocospaera* cells

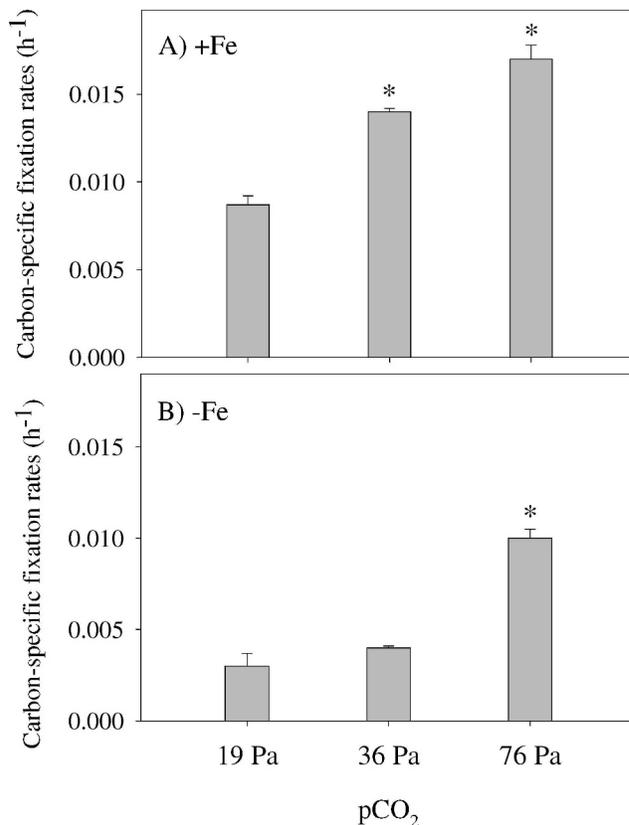


Fig. 3. Carbon-specific CO₂ fixation rates of *Crocospaera* grown at three different pCO₂ levels (19 Pa, 39 Pa, and 76 Pa) under (A) Fe-replete or (B) Fe-limited growth conditions. Values are the means and error bars are the standard deviations of triplicate cultures for each treatment, and symbols are as in Fig. 1.

grown under two elevated-pCO₂ conditions show somewhat higher PE content than do cells grown at a pCO₂ of 19 Pa ($F_{1,12} = 8.3$, $p = 0.02$), but there was no significant difference between these two elevated-pCO₂ conditions (Fig. 4A; $F_{1,12} = 0.1$, $p = 0.92$). For PC, Fe-replete cells grown at a pCO₂ of 76 Pa had a significantly higher pigment content than those grown at 19 Pa ($F_{1,12} = 6.4$, $p = 0.03$), but PC content was not significantly different between 19 Pa and 39 Pa or between 39 Pa and 76 Pa ($F_{1,12} = 3.4$, $p = 0.1$; $F_{1,12} = 1.6$, $p = 0.2$). Elevated CO₂ did not change the much lower and invariant cellular phycobilin pigment content of Fe-limited cultures (Fig. 4C). A comparison of C normalized Chl *a* cells in Fe-replete cultures (Fig. 4B) with the Fe-limited cultures (Fig. 4D) showed a decrease in the cell-normalized amount of this pigment. In contrast, in both Fe-replete and Fe-limited cultures, Chl *a* contents were relatively unchanged between pCO₂ treatments (Fig. 4B,D).

Elemental ratios—Fe availability had a minor effect on the ratios of C to N, regardless of pCO₂ concentrations (Table 1). In general, the molar ratios of C to N for the cells grown under Fe-replete conditions were slightly but significantly higher than the values from Fe-limited cultures, varying from 6.4 to 7.4. However, for both Fe-

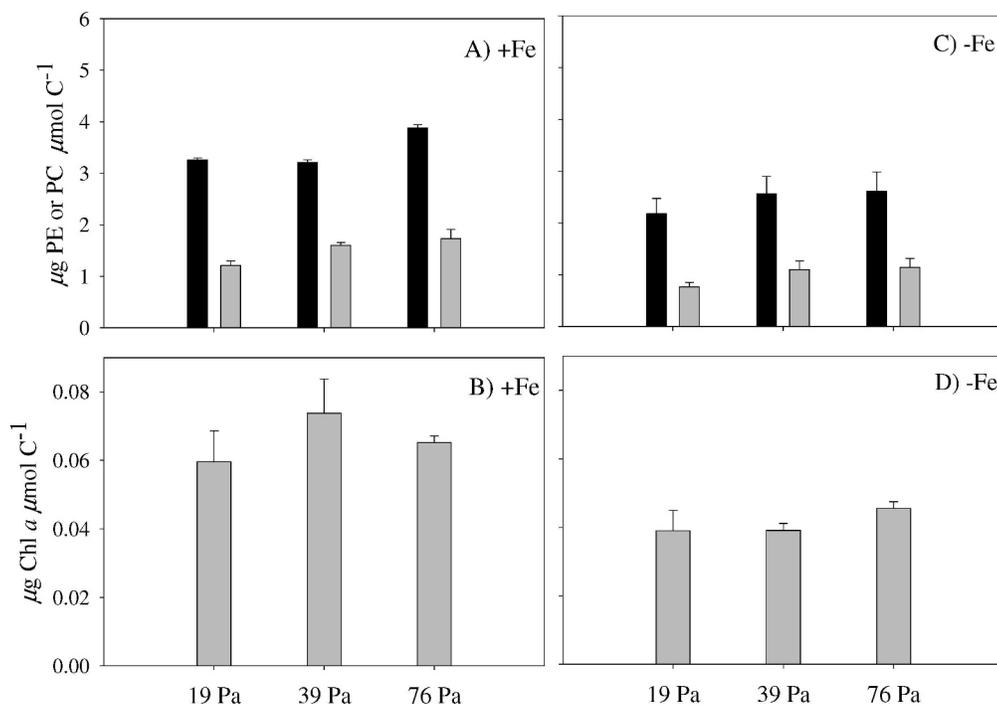


Fig. 4. Photosynthetic pigments ($\mu\text{g C}^{-1}$) of *Crocospaera* grown at three different pCO₂ levels (19 Pa, 39 Pa, and 76 Pa) under Fe-replete or Fe-limited growth conditions. Phycoerythrin (gray bars) and phycocyanin (black bars) in (A) Fe-replete and (C) Fe-limited cultures and Chl *a* in (B) Fe-replete and (D) Fe-limited cultures. Values given are the means and error bars are the standard deviations of measurements on triplicate cultures.

replete and Fe-limited cultures, the cellular C:N ratios were relatively invariant among the three pCO₂ treatments.

Molar N:P ratios of *Crocospaera* cells ranged from 14 to 17 for all the treatments, and there was no Fe effect (Table 1). Only in Fe-replete cultures did N:P ratios increase by 21% at 39 Pa and 76 Pa pCO₂, as compared to 19 Pa, but there was no significant difference between the two higher pCO₂ treatments (Table 1; $F_{1,12} = 0.52$, $p = 0.48$). The N:P ratio remained unchanged in Fe-limited cultures across the CO₂ gradient.

In Fe-replete cultures the molar C:P ratios increased with increasing pCO₂ concentrations; however, in Fe-limited cultures, the cellular C:P ratios were not significantly different from each other in the three CO₂ concentrations (Table 1; $F_{2,12} = 0.35$, $p = 0.72$). The cellular C:P ratios for both the 76-Pa and 39-Pa treatments were significantly higher in Fe-replete grown cultures relative to Fe-limited ones (Table 1; $F_{1,12} = 186$, $p = 0.0005$). The differences in C:P ratios between Fe treatments were, however, relatively minor under the 19-Pa CO₂ conditions ($F_{1,12} = 16.6$, $p = 0.02$; Table 1).

Discussion

Biogeochemical implications—Recently, several studies examining the marine diazotrophic cyanobacterium *Trichodesmium* have shown significant increases in N₂ fixation and photosynthesis in response to elevated CO₂ concentration (Hutchins et al. 2007; Levitan et al. 2007; Ramos et al. 2007). Our data extend these findings to encompass the marine unicellular N₂-fixing cyanobacterium *Crocospaera*.

This group is now recognized as being perhaps equally as important as *Trichodesmium* to the ocean N cycle (Montoya et al. 2004).

As with *Trichodesmium*, there is a strong positive correlation between pCO₂ levels and N₂ fixation rates in *Crocospaera* grown under Fe-replete conditions. In contrast, N₂ fixation in Fe-limited *Crocospaera* cultures was not responsive to changing pCO₂ levels. Thus, the relationship between pCO₂, N₂ fixation, and Fe limitation in *Crocospaera* differs in a fundamental way from the interactions between pCO₂ and N₂ fixation and P limitation in *Trichodesmium*. Hutchins et al. (2007) showed that P-limited *Trichodesmium* at present day pCO₂ rates should really be considered to be co-limited by both P and C, since growth and N and C fixation rates will increase in response to either phosphate or CO₂ additions. Fe limitation in *Crocospaera*, however, negates the effects of changing pCO₂ on N₂ fixation, indicating that future CO₂-mediated increases in unicellular diazotrophy may ultimately be governed by Fe availability.

In fact, the results of our study demonstrate that the tight coupling between pCO₂ and N₂ fixation rates in *Crocospaera* also implies a similar, albeit indirect, coupling between pCO₂ and cellular Fe requirements. This interaction can be illustrated by re-plotting the data shown in Fig. 2. Over the three CO₂ concentrations we examined, the cellular Fe quota of Fe-replete *Crocospaera* is linearly related to N₂ fixation rates (Fig. 5A). Because N₂ fixation is in turn a linear function of pCO₂ (Fig. 5B), Fe quotas are therefore also a linear function of CO₂ concentration. A similar relationship applies to cell quotas of the other

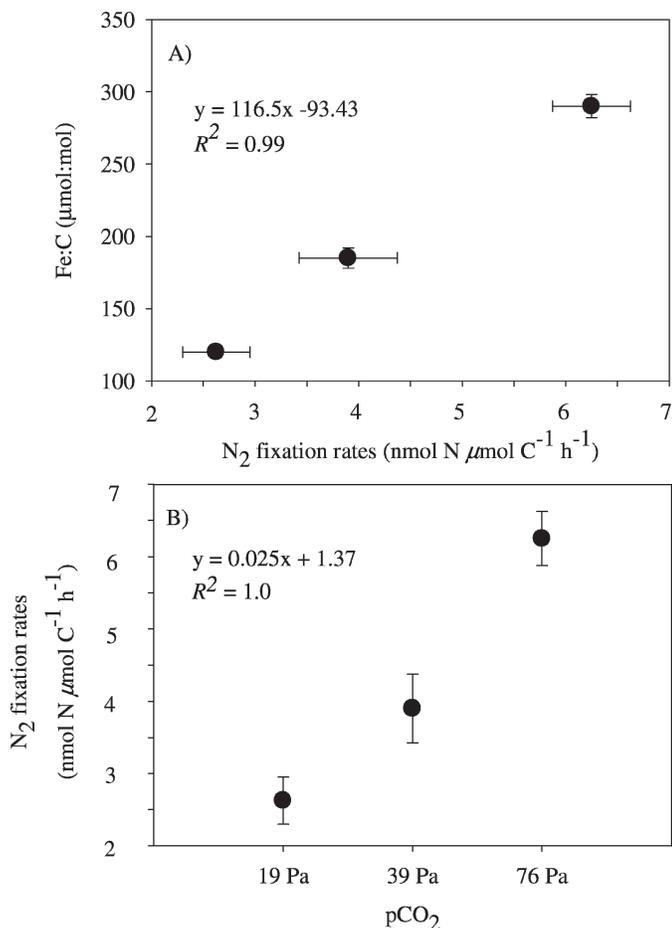


Fig. 5. (A) Fe:C ratios vs. N₂ fixation rates and (B) N₂ fixation rates vs. pCO₂ for Fe-replete *Crocosphaera* grown at three different pCO₂ levels (19 Pa, 39 Pa, and 76 Pa). Values given are the means and error bars are the standard deviations of measurements on triplicate cultures.

element involved as a co-factor in N₂ fixation, Mo (data not shown).

Thus, it appears likely that future rising pCO₂ will have a major effect on not only the biogeochemical cycle of N (by stimulating N₂ fixation) but also secondarily on the biogeochemistry of Fe (and Mo). If N₂ fixation by *Crocosphaera* increases as a result of “CO₂ fertilization” of the ocean over the coming century, the consequent elevated cellular Fe requirements necessary to support these higher N₂ fixation rates may also simultaneously drive this cyanobacterium toward Fe limitation.

Paleo-climate regulation implications—Hutchins et al. (2007) found that as a result of severe C limitation, *Trichodesmium* are unable to survive in long-term acclimated growth at a low pCO₂ of 15 Pa. They suggested that under glacial era low-pCO₂ atmospheric conditions, this constraint could result in down-regulation of new N inputs to the ocean from N₂ fixation and, thus, could potentially lead to lower oceanic inventories of fixed N. Ultimately this down-regulation could result in a weakened biological pump, reduced oceanic CO₂ uptake, and stabilization of or

even an increase in atmospheric pCO₂. Our results demonstrating C limitation of N₂ fixation by *Crocosphaera* grown at glacial pCO₂ levels of 19 Pa support and strengthen this negative feedback scenario. In fact, the observed decrease in N₂ fixation and growth rates that we observed from 39 Pa to 19 Pa was much larger than the decrease we observed from 76 Pa to 39 Pa, indicating that the magnitude of glacial era to present day differences could be much larger than the magnitude of present day to year 2100 differences. It may not be coincidental that the limiting pCO₂ levels that virtually shut down N₂ fixation by both *Trichodesmium* and *Crocosphaera* are quite close to the minimum atmospheric values at the last glacial maximum (~19 Pa; Petit et al. 1999). Future investigations will be needed to examine this potential atmosphere and ocean biology feedback loop in more detail and to determine the possible consequences for long-term climate regulation processes.

Growth, N₂ fixation, and C fixation under variable pCO₂ and Fe—N₂ fixation by cyanobacteria is dependent on supplies of reductant, adenosine triphosphate (ATP), and C-derived metabolites. Hence, it is not surprising that under Fe-replete and Fe-elevated pCO₂ conditions, increases in both light-limited and light-saturated photosynthetic rates produced a larger pool of C and energy to support N₂ fixation and growth. This is consistent with the increased slope of the light-limited photosynthetic rate α with elevated pCO₂ and Fe repletion, indicating that the cells grown under these conditions would possess a higher capacity to assimilate C. By contrast, in Fe-limited cultures N₂ fixation was unchanged across a range of pCO₂, despite similar increases in parameters such as P_{Bmax} and α , which is inconsistent with this model of N₂ fixation stimulation driven mainly by enhanced C fixation rates. The cultures grown under Fe-limited conditions showed much smaller increases in growth rates with increasing pCO₂ concentrations compared to cultures grown under Fe-replete conditions, indicating that the cells were largely unable to compensate for the low availability of Fe despite greater external CO₂ availability.

Crocosphaera segregates N₂ fixation from photosynthesis by fixing N₂ only at night, a fundamentally different strategy from that of *Trichodesmium*, which fixes N₂ and CO₂ simultaneously during the day. Thus, in *Crocosphaera* energy and reductant to fuel energetically expensive N₂ fixation comes from respiratory pathways using stored photosynthate. Competition between N₂ and CO₂ fixation pathways for photosynthetically derived energy and reductant has been suggested as a possible reason for enhancement of both processes under high-CO₂ conditions in *Trichodesmium* (Hutchins et al. 2007). This type of competition may also occur in *Crocosphaera*, despite the temporal decoupling of C fixation and N₂ fixation in this genus.

As long as ample Fe supplies are available for both processes, increased C fixation due to elevated CO₂ should also result in the simultaneous stimulation of N₂ fixation in order to maintain balanced cellular C:N ratios. Fe limitation in particular affects the photosynthetic appara-

tus and electron transport (Raven et al. 1999), resulting in decreased photosynthesis for Fe-limited N₂-fixers (Fu and Bell 2003). The major electron carriers requiring Fe, such as cytochromes and Fe-S proteins, are all involved in both photosynthesis and respiration. Thus, Fe limitation impinges upon a wide range of relevant cellular processes, from synthesis of nitrogenase to supplies of photosynthetically generated ATP and/or reducing equivalents to support C fixation, N₂ fixation, and DIC transport.

Pigments under variable pCO₂ and Fe—Reductions in cellular pigments are commonly observed in Fe-limited phytoplankton cells. Reductions in cellular Chl *a* and accessory pigments PC and PE also indicated that Fe availability affected the production of photosynthetic pigments by *Crocospaera* in our study. However, our study showed that Chl *a*-normalized light-saturated photosynthesis was also affected by Fe availability, contrary to the results of a study of Trick et al. (1995) using the cyanobacterium *Oscillatoria*. This indicates that light-saturated photosynthesis is not limited by Chl *a* content in *Crocospaera* and confirms the results of the study of Sandmann (1985), in which the Chl *a* of Fe-limited *Aphanocapsa* was in excess, was associated nonfunctionally, or was less efficiently utilized.

Regardless of Fe condition, cellular Chl *a* content remained unchanged under different pCO₂ concentrations. This result is contrary to those of a study of the non-diazotrophic unicellular marine cyanobacterium *Synechococcus*, in which cellular Chl *a* content increased with increasing pCO₂ levels (Fu et al. 2007). However, invariant cellular Chl *a* in response to changing availability of DIC or pCO₂ has been reported in other cyanobacteria, such as *Anacytis nidulans*, as well as in some eukaryotic algae, such as *Scenedesmus obtusius*, *Dunaliella viridis*, and the raphidophyte *Heterosigma* (Gordillo et al. 2003; Müller et al. 1993; Fu et al. 2008). Gordillo et al. (2003) suggested that this could be because qualitative rather than quantitative changes in the light harvesting machinery are involved in reaching higher photosynthetic rates under CO₂ enrichment.

Crocospaera cellular PC and PE pigments in the 75-Pa treatment were slightly higher than in the 19-Pa treatment under Fe-replete conditions. No effect of CO₂ was observed on phycobilin content in *Crocospaera* under Fe-limited conditions. PC is the main component of phycobiliproteins in *Crocospaera*, and the ratio of PC to Chl *a* was significantly higher in Fe-replete cultures compared to Fe-limited ones. This is in agreement with the study of Trick et al. (1995), since in our experiment the ratio significantly increased under elevated pCO₂ in Fe-replete cultures but not in Fe-limited ones. Hence, Fe availability by itself has an effect on the ratio. In general, it appears that the photosynthetic apparatus of *Crocospaera* is more affected by changes in Fe availability than by changes in CO₂ availability.

Implications for interactions between the C, N, P, and Fe cycles—There is relatively little information on how the availability of Fe affects the elemental ratios of marine

diazotrophs, especially marine unicellular N₂-fixers such as *Crocospaera*. Studies using *Trichodesmium* IMS101 and the unicellular N₂-fixer *Cyanothece* (Berman-Frank et al. 2001, 2007) showed that Fe availability has little effect on the C:N:P elemental ratios. Our *Crocospaera* results showed that increased Fe availability increased C:N and C:P ratios, but N:P ratios remained stable. Also, it is noteworthy that in general, the C:P and N:P ratios of *Crocospaera* are higher than the ratios of *Trichodesmium* from the study of Berman-Frank et al. (2001), particularly in Fe-limited cultures. Assuming that differences between the growth conditions of the two studies were not significant, our data indicate that *Crocospaera* require less P and N relative to C to maintain growth under Fe-limited conditions.

Levitan et al. (2007) reported changes in *Trichodesmium* C:N ratios from enhanced pCO₂, but these changes were not observed by Hutchins et al. (2007). Regardless of Fe conditions, the C:N ratios of *Crocospaera* were not affected by changing CO₂ concentration. As suggested by Hutchins et al. (2007) for *Trichodesmium*, it appears that simultaneous stimulation of both N₂ fixation and C fixation by rising CO₂ allows *Crocospaera* to maintain a balanced C:N ratio. Since both diazotrophs ultimately transfer much of their fixed C and N to the rest of the plankton community (Mulholland 2007), this indicates that overall biological C:N stoichiometry could remain relatively stable despite changes in past and future atmospheric pCO₂.

CO₂ enrichment did not affect the ratios of C to P or of N to P for *Crocospaera* cultures grown under Fe-limited conditions. *Crocospaera* grown under Fe-replete conditions did, however, show similar trends to *Trichodesmium* (Hutchins et al. 2007), in that C:P and N:P ratios increased with increasing pCO₂. In general, though, pCO₂-driven changes in *Crocospaera* C:P and N:P ratios were smaller than those observed for *Trichodesmium*.

These higher C:P and N:P ratios at enhanced CO₂ levels in both *Crocospaera* and *Trichodesmium* are consistent with the suggestion of Hutchins et al. (2007) that unlike N₂ and CO₂ fixation, phosphate uptake by marine N₂-fixers is not regulated by changing pCO₂. The possible biogeochemical consequences of enhanced N and C incorporation without a comparable increase in P incorporation could include P limitation of both the diazotrophs themselves as well as an increased likelihood of P limitation in the rest of the phytoplankton community, since supplies of new fixed N will be enhanced relative to P supply. Higher C:P ratios with rising CO₂ are also biogeochemically significant, since they could increase C storage by the ocean's biological pump relative to P utilization by the biota.

The effects of CO₂ on elemental ratios in phytoplankton are species-specific. C:N:P ratios in the cyanobacterium *Prochlorococcus* grown under nutrient-replete conditions do not vary with pCO₂ changes; however, the ratios of C to P and N to P of *Synechococcus* are considerably higher at increased pCO₂ (Fu et al. 2007). It has been suggested that groups like *Synechococcus* that are able to take up CO₂ actively are likely to show much larger cellular stoichiometry

etry changes with varying $p\text{CO}_2$ than are those (like *Prochlorococcus*) that are thought to rely largely on HCO_3^- uptake (Burkhardt et al. 1999; Fu et al. 2007). This line of reasoning is based on the fact that the concentration of HCO_3^- changes only marginally with doubled $p\text{CO}_2$. However, many cyanobacteria are known to possess a wide variety of CCMs (Badger et al. 2006), so predictions based on changing external DIC species alone could be misleading. To date there is no published information on potential CCMs in *Crocospaera*.

C, Fe, and Mo quotas and use efficiencies—Recently Fe requirements of both cultures and natural populations of *Trichodesmium* and lab cultures of the unicellular N_2 -fixers *Crocospaera* and *Cyanothece* have been investigated (Kustka et al. 2003; Tuit et al. 2004; Berman-Frank et al. 2007). In general, these studies showed that Fe:C ratios are correlated positively to Fe availability and negatively to cell size. The Fe:C ratios for *Crocospaera* in our study ranged from 27 to 290 $\mu\text{mol mol}^{-1}$ and varied with both Fe availability and (in Fe-replete cultures only) with $p\text{CO}_2$. The minimum values we obtained for *Crocospaera* in Fe-limited cultures were lower ($\sim 27 \mu\text{mol mol}^{-1}$) than the values (38–48 $\mu\text{mol mol}^{-1}$) from Kustka et al. (2003) and Berman-Frank et al. (2001) for *Trichodesmium* growing at the same rate of $\sim 0.1 \text{ d}^{-1}$. Likewise, our estimation of net Fe use efficiency by Fe-limited *Crocospaera* was 40–80% higher than that of Fe-limited *Trichodesmium* at the same growth rate (Kustka et al. 2003). These results imply that this unicellular N_2 -fixer probably has a lower Fe requirement than does *Trichodesmium*, and it may therefore be less likely to be Fe limited in the open ocean.

Smaller cells with higher surface:volume ratios are in general less vulnerable to Fe limitation (Sunda and Huntsman 1997). The cell diameter of *Crocospaera* is $\sim 3.0 \mu\text{m}$, about threefold smaller than the cells of *Trichodesmium*. This would allow for higher growth rates of *Crocospaera* in Fe-limited waters, as its higher surface:volume ratio would allow for a higher cell volume-normalized Fe uptake rate if Fe uptake per unit cell surface is constant at a given Fe concentration, as has been found previously (Sunda and Huntsman 1997). Thus, *Crocospaera* should be able to out-compete *Trichodesmium* in Fe-limited waters in two ways, via higher uptake rates per unit of cell volume or biomass and lower Fe:C ratios needed to support growth. In addition, the fact that the former is unicellular and that the latter often forms large colonies likely affects their relative Fe uptake abilities and requirements as well.

The higher Fe quota of *Trichodesmium* may also be related to its differing N_2 fixation strategy. As noted above, *Trichodesmium* fixes N_2 only during the day, while *Crocospaera* does so only in the dark (Tuit et al. 2004; Berman-Frank et al. 2007). To protect its nitrogenase from inactivation by oxygen during photosynthesis, *Trichodesmium* is thought to depend upon elevated respiration rates, cytochrome oxidase activity, and/or antioxidant enzymes, all of which require Fe (Gallon 1992). The higher PSI:PSII ratios of *Trichodesmium* compared to unicellular diazotrophs (Berman-Frank et al. 2007; Milligan et al. 2007) may

also tend to increase the Fe requirements of the former, since PSI has a sixfold higher Fe content than PSII (Strzepek and Harrison 2004).

Crocospaera Fe:C and Mo:C ratios determined by Tuit et al. (2004) varied on a diel basis and ranged from 7 to 97 and 0.45 to 0.90, respectively, during the N_2 -fixing (dark) period. In general, the observed values from our culture study are considerably higher than their reports. Our lowest values of Fe:C ($\sim 27 \mu\text{mol:mol}$) and Mo:C ($\sim 0.38 \mu\text{mol:mol}$) were obtained from the Fe-limited cultures; all of our sampling was carried out in the daytime.

Although we cannot provide a conclusive explanation for the difference in metal quotas obtained in our study and that of Tuit et al. (2004), the close correlations between our Fe:C ratios and Fe availability, N_2 fixation rates, and $p\text{CO}_2$ (Figs. 2A, 5) indicate that our higher values are unlikely to be due to accidental Fe contamination of cultures or samples. Such contamination would inevitably result in a great deal of random variability between samples, obscuring the clear relationship between Fe quotas and the experimental variables. It is notable, though, that we have found that *Crocospaera* cells are surprisingly resistant to lysis and acid digestion, and therefore cellular trace metals appear to be only incompletely extracted from filter samples by a typical short-term digestion protocol, as in Tuit et al. (2004). Our trace metal acid digest samples from this experiment were measured twice: once soon after digestion and a second time after allowing the samples to extract at room temperature for another 4 months. ICPMS-derived Fe and Mo values from the second analysis were two- or threefold higher than values from the first one; a similar comparison of digestion time effects on trace metal concentrations of eukaryotic phytoplankton samples shows an enhancement of only 20–30% (data not shown). These results indicate that for *Crocospaera* samples, extended acid digestion times may be required to avoid serious underestimation of trace metal quotas. Issues with incomplete acid digestion may also explain why our Fe:C ratios for *Crocospaera* are ~ 36 -fold higher than those reported by Berman-Frank et al. (2007) for another related unicellular diazotrophic cyanobacterium, *Cyanothece*.

Our study confirms and extends a growing body of evidence indicating that currently rising atmospheric CO_2 levels will tend to significantly increase N_2 fixation in tropical and subtropical marine ecosystems by the end of this century (Hutchins et al. 2007; Levitan et al. 2007; Ramos et al. 2007). Our culture studies predict that the relative magnitude of this increase may be somewhat greater for *Trichodesmium* (35–66% increase; Hutchins et al. 2007) than for *Crocospaera* ($\sim 24\%$; this study), but the general trend appears to be similar for both of these biogeochemically critical groups of globally distributed diazotrophs. This CO_2 enhancement effect needs to be evaluated on several different timescales, including those that are relevant for plankton ecology (seasonal cycles of mixed-layer $p\text{CO}_2$), anthropogenic global change (decade- and century-scale increases in atmospheric $p\text{CO}_2$), and natural climatic cycles (glacial and interglacial $p\text{CO}_2$ changes). The results of our experiments indicate that

CO₂ enrichment could alter new N inputs to the ocean by these two ecologically dominant groups on all of these timescales and that major feedbacks to not only N biogeochemistry but also to the C, P, and Fe cycles are to be expected. This study highlights some of these potential interactive feedbacks between pCO₂, N₂ fixation, and Fe availability.

Unlike the predictable trend of rising pCO₂, the possible future trends in Fe supplies to the oceans are relatively unknown. Some studies have predicted climate-driven reductions in aeolian Fe inputs (Mahowald and Luo 2003), while others suggest that Fe supplies could increase to parts of the open ocean as a result of either accelerating human-induced desertification or of deposition of anthropogenic fossil fuel aerosols (Sedwick et al. 2007). Predicted increased stratification and mixed-layer shoaling may also lead to reduced supplies of Fe from vertical advection and mixing (Boyd and Doney 2002). Our results and those of Hutchins et al. (2007) indicate that increasing pCO₂ and consequent stimulation of N₂ fixation may increase the potential for Fe and P limitation of diazotrophs in particular, and of the whole biological community in general, throughout the oligotrophic ocean. Fe limitation in particular seems to have the potential to constrain future CO₂-mediated increases in oceanic diazotrophy, unless changing climate regimes are also accompanied by enhanced Fe supplies. Under such hypothetical future conditions, warmer, dust-fertilized and CO₂ enriched, N₂-fixing cyanobacteria appear likely to thrive and could ultimately drive major regime shifts in the gyre ecosystems.

It is becoming obvious that other environmental factors, such as temperature, light, and Fe and nutrient availability, can interactively modify growth and N₂ fixation responses to changing pCO₂. These interactive effects need to be carefully considered in designing and interpreting experiments and models aimed at determining rising pCO₂ effects on natural populations of phytoplankton. Future work focused on these combined effects will help us to build a much more complete picture of the intricate web of feedback interactions among elevated pCO₂, other global change processes, and the biogeochemistry and biology of the ocean.

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