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CO2 Control of Trichodesmium N-2 Fixation, Photosynthesis, Growth rates, and Elemental Ratios: Implications for Past, Present, and Future Ocean Biogeochemistry

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CO₂ control of *Trichodesmium* N₂ fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry

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Abstract

Diazotrophic marine cyanobacteria in the genus *Trichodesmium* contribute a large fraction of the new nitrogen entering the oligotrophic oceans, but little is known about how they respond to shifts in global change variables such as carbon dioxide (CO₂) and temperature. We compared *Trichodesmium* dinitrogen (N₂) and CO₂ fixation rates during steady-state growth under past, current, and future CO₂ scenarios, and at two relevant temperatures. At projected CO₂ levels of year 2100 (76 Pa, 750 ppm), N₂ fixation rates of Pacific and Atlantic isolates increased 35–100%, and CO₂ fixation rates increased 15–128% relative to present day CO₂ conditions (39 Pa, 380 ppm). CO₂-mediated rate increases were of similar relative magnitude in both phosphorus (P)-replete and P-limited cultures, suggesting that this effect may be independent of resource limitation. Neither isolate could grow at 15 Pa (150 ppm) CO₂, but N₂ and CO₂ fixation rates, growth rates, and nitrogen : phosphorus (N : P) ratios all increased significantly between 39 Pa and 152 Pa (1500 ppm). In contrast, these parameters were affected only minimally or not at all by a 4°C temperature change. Photosynthesis versus irradiance parameters, however, responded to both CO₂ and temperature but in different ways for each isolate. These results suggest that by the end of this century, elevated CO₂ could substantially increase global *Trichodesmium* N₂ and CO₂ fixation, fundamentally altering the current marine N and C cycles and potentially driving some oceanic regimes towards P limitation. CO₂ limitation of *Trichodesmium* diazotrophy during past glacial periods could also have contributed to setting minimum atmospheric CO₂ levels through downregulation of the biological pump. The relationship between marine N₂ fixation and atmospheric CO₂ concentration appears to be more complex than previously realized and needs to be considered in the context of the rapidly changing oligotrophic oceans.

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2005). Consequently, the potential for limitation of *Trichodesmium* dinitrogen (N$_2$) and carbon dioxide (CO$_2$) fixation by nutrients such as iron (Fe) and phosphorus (P) has been investigated (Sañudo-Wilhelmy et al. 2001; Mills et al. 2004). A further complication in our understanding of the interactions between oceanic N$_2$ fixation and the carbon (C) cycle is the unknown effect of global-scale anthropogenic perturbation of carbon biogeochemistry, following a century of unrestrained fossil fuel burning.

The atmospheric partial pressure of CO$_2$ (pCO$_2$) has already increased by roughly one-third, to the highest levels in at least half a million years (Houghton et al. 2001). Continuing emissions will nearly double current seawater pCO$_2$ to ~76 Pa by 2100, lowering ocean pH by about 0.35 units (Wolf-Gladrow et al. 1999). Marine phytoplankton respond to anthropogenic CO$_2$ increases and sea-surface warming by altering their physiology (Riebesell et al. 2000), relative abundance (Tortell et al. 2002), and biogeography (Boyd and Doney 2002). In particular, the detrimental effects of ocean acidification on marine calcifying organisms have received a great deal of recent attention (Riebesell et al. 2000; Orr et al. 2005; Feng et al. unpubl. data). Unicellular marine cyanobacteria such as *Synechococcus* and *Prochlorococcus* can also show taxon-specific responses to increasing pCO$_2$ and temperature (Fu et al. in press), but little information is currently available about global change effects on N$_2$-fixing cyanobacteria such as *Trichodesmium*.

Marine N$_2$ fixation may be coupled to long-term natural climate cycles through indirect feedbacks to atmospheric CO$_2$. For example, it has been hypothesized that increases in N$_2$ fixation from dust-driven iron fertilization of the ocean during glacial periods may shift the balance between global nitrogen fixation (N inputs) and denitrification (N removal). The resulting accumulation of fixed nitrogen in the ocean alleviates phytoplankton nitrogen limitation, although limitation by other nutrients such as P could potentially modify this scenario. Subsequently, increased carbon export then sequesters atmospheric CO$_2$, reducing greenhouse forcing and cooling the Earth. Thus, oceanic N$_2$ fixation may be an important driver of pCO$_2$ and climate regulation, even over geological timescales (Falkowsky 1997; Michaels et al. 2001).

Here, we present the results of experiments examining the relationship between major global change variables (pCO$_2$ and temperature) and diazotrophic growth of two *Trichodesmium* ecotypes. We grew cultures of both Pacific and Atlantic Ocean isolates across a range of CO$_2$ concentrations, at two different temperatures, and at sufficient and limiting P concentrations. Sea-surface temperature is increasing globally along with pCO$_2$ (Boyd and Doney 2002), and *Trichodesmium* distributions are thought to be limited geographically by temperature. Furthermore, diazotrophic growth across most of the ocean is believed to be limited by P or Fe (Sañudo-Wilhelmy et al. 2001; Mills et al. 2004; Fu et al. 2005a), so determining how variables like temperature and P availability interact with changing pCO$_2$ is critical. Cultured populations were used for these studies out of necessity, as acclimated growth is critical to evaluating steady-state physiological responses to elevated CO$_2$ and natural *Trichodesmium* samples cannot be maintained for extended periods in enclosed experiments at sea (Mulholland et al. 2001). Our results suggest that N$_2$ and carbon fixation, growth rates, and elemental stoichiometry in *Trichodesmium* are strongly affected by CO$_2$ levels during steady-state growth, a finding that could have large implications for nutrient cycling and food web dynamics in the past, present, and future ocean.

**Methods**

*Culturing techniques*—Stock and experimental cultures of *Trichodesmium erythraeum* strains GBRTRL1101 from the Pacific Ocean (GBR) and IMS101 from the Atlantic Ocean (IMS) were grown without combined nitrogen in modified YBCII seawater medium (Chen et al. 1996; Fu et al. 2005a,b) supplemented with growth-replete levels of Fe and other trace metals (Fu et al. 2005a). Unialgal stock cultures were grown at 25°C with a light–dark cycle of 12 : 12 light : dark (LD) and an incident photon flux density of 100μmol photons m$^{-2}$ s$^{-1}$.

*Experimental design*— Cultures were grown semicontinuously by diluting with fresh microwave-sterilized, pCO$_2$-equilibrated medium every 2 days. Cell numbers were monitored microscopically to calculate growth rates (Fu et al. 2006, in press). Cultures were kept optically thin (< ~150 filaments mL$^{-1}$) to avoid self-shading and dissolved inorganic carbon (DIC) or nutrient depletion. Bottles were equilibrated at the target pCO$_2$ by gentle bubbling at uniform rates in all treatments with commercially prepared air : CO$_2$ mixtures (Scott Gas) for all treatments except the ambient pCO$_2$ bottles, which were bubbled at the same rate with fresh filtered ambient air using an aquarium pump. CO$_2$ equilibration was verified in all experiments by daily pH measurements and by total DIC analyses (see Analytical methods). Steady-state growth was considered attained when measured cell-specific growth rates were constant for 7–10 consecutive generations, at which time the cultures were sacrificed for measurements of N$_2$ fixation rates, CO$_2$ fixation rates, photosynthesis versus irradiance curves, and elemental ratios (see Analytical methods). Significant differences ($p < 0.05$) between means of triplicate bottles for each treatment were determined using a Student’s $t$-test.

The first set of experiments used GBR grown under P- and Fe-replete conditions at 25°C, with triplicate bottles equilibrated at 15, 39, 76, 127, and 152 Pa CO$_2$ (150, 380, 750, 1250, and 1500 ppm). For the second set of experiments, cultures of both GBR and IMS were acclimated in triplicate 1-liter polycarbonate bottles under the stock culture growth conditions at either 25°C or 29°C. For each temperature treatment, triplicate bottles were incubated at present-day pCO$_2$ (~39 Pa) or at projected year 2100 pCO$_2$ (76 Pa) levels as described above. The four treatments used in this part of the study were 25°C and 39 Pa CO$_2$ (control); 29°C and 39 Pa CO$_2$ (high temperature); 25°C and 76 Pa CO$_2$ (high CO$_2$); and 29°C and 76 Pa CO$_2$ (greenhouse). Both isolates were maintained in steady-state growth, in
triplicate, under all four treatment conditions in both P-replete (20 μmol L⁻¹ phosphate) and P-limited (0.2 μmol L⁻¹ phosphate) media (Fu et al. 2005a,b).

Analytical methods—Rates of N₂ fixation in the experiments were estimated by the acetylene reduction method using a ratio of 3:1 to convert ethylene production to N₂ fixation. N₂ fixation rates were also estimated by ¹⁵N₂ incorporation in some cases (Mulholland and Bernhardt 2005). Trends in ¹⁵N₂ uptake rates were very similar to those obtained from acetylene reduction (data not shown), but are not presented as they were not performed for every experiment. Particulate organic C and N and isotope ratios were determined using a Europa 20:20 isotope ratio mass spectrometer equipped with an automated nitrogen and carbon analysis for gas, solids, and liquids (ANCA-GSL) preparation unit (Mulholland and Bernhardt 2005); intracellular particulate organic P and chlorophyll a (Chl a) were measured as in Fu et al. (2005b).

CO₂ fixation rates under experimental conditions were measured for the first GBR experiment using H¹³CO₂⁻ (Mulholland and Bernhardt 2005), and for the IMS experiment and the second GBR experiment using H¹⁴CO₂⁻ (Fu et al. in press). All CO₂ fixation rates were calculated using experimental DIC concentrations for each pCO₂ treatment. Total DIC was determined by injecting 1.25-mL samples into an acid sparging column, and the CO₂ resulting from acid conversion of the DIC pool was quantified with a Licor infrared analyzer with high precision flow control (Fu et al. in press). This instrument has replicate precision of ±0.06% for seawater samples. Triplicate subsamples for DIC analysis were preserved with HgCl₂ (0.44 μmol L⁻¹) and stored at 4°C in the dark until analysis.

Photosynthesis versus irradiance (P–E) curves measured ¹⁴C fixation rates across a range of light intensities using a CHPT photosynthesetron with a fiber optics–based light source (Fu et al. in press). Triplicate 5-mL culture samples from each treatment were placed in 7-mL glass vials, and 3,700 Bq mL⁻¹ ¹⁴C sodium bicarbonate was added. Samples were then incubated at appropriate experimental growth temperatures under light intensities ranging from 10 μmol photons m⁻² s⁻¹ to 900 μmol photons m⁻² s⁻¹. To correct for dark uptake, parallel incubations were carried out in duplicate foil-covered vials. Duplicate killed control blanks were also performed for each treatment and subtracted from reported live cell activities. After 30-min incubations, ¹⁴C uptake was stopped by fixation with gluteraldehyde, and the samples were filtered (0.4 μm polycarbonate), thoroughly rinsed with 0.2-μm-filtered seawater, and counted using a Wallac System 1400 liquid scintillation counter. CO₂ fixation rate calculations used measured DIC values in the seawater for each experimental treatment, as described above. P–E parameters were calculated as in Platt et al. (1980), including corrections for potential photoinhibition at elevated irradiance (β). These included the initial slope of the P–E curve x [μg C (mg Chl a)⁻¹ h⁻¹ (μmol quanta m⁻² s⁻¹)⁻¹], the maximum Chl a-specific carbon fixation rate Pₘₐₓ [μg C (mg Chl a)⁻¹ h⁻¹], and the light saturation point Eₘ (Pₘₐₓ : 2).

Table 1. Measured steady-state pH and total dissolved inorganic carbon (DIC) values in semicontinuous cultures of Trichodesmium GBR grown across a pCO₂ range. Shown are the known pCO₂ of the air: CO₂ mixture with which the cultures were bubbled and the calculated pCO₂ in the seawater medium based on measured pH and total DIC concentrations.

<table>
<thead>
<tr>
<th>Known air pCO₂ (Pa)</th>
<th>pH</th>
<th>DIC (μmol kg⁻¹)</th>
<th>Calculated seawater pCO₂ (Pa)</th>
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</thead>
<tbody>
<tr>
<td>15</td>
<td>8.52</td>
<td>1844</td>
<td>15</td>
</tr>
<tr>
<td>39</td>
<td>8.20</td>
<td>2056</td>
<td>40</td>
</tr>
<tr>
<td>76</td>
<td>7.96</td>
<td>2169</td>
<td>76</td>
</tr>
<tr>
<td>127</td>
<td>7.76</td>
<td>2283</td>
<td>130</td>
</tr>
<tr>
<td>152</td>
<td>7.69</td>
<td>2339</td>
<td>157</td>
</tr>
</tbody>
</table>

Results

Seawater medium pCO₂ values calculated from pH and DIC measurements were all within <3% of the known concentrations in the commercial air: CO₂ mixtures used to bubble the cultures (Table 1). Total DIC concentrations in the present-day control (2056 μmol kg⁻¹) fall within the natural annual range observed in areas where Trichodesmium blooms, like the Sargasso Sea (~2010–2070 μmol kg⁻¹, Bates 2001). Likewise, changes in pH and DIC values in the elevated pCO₂ treatments (Table 1) were closely comparable to those expected in future surface seawater (Houghton et al. 2001). Thus, the carbonate buffer system parameters in our artificial seawater culture medium were virtually identical to those found in natural seawater across the relevant range of pCO₂ values.

Chl a–normalized N₂ fixation rates of GBR increased significantly by 63% between present day CO₂ (39 Pa) and projected year 2100 pCO₂ (76 Pa) (p < 0.05, Student’s t-test), and then remained relatively steady between 76 Pa and 152 Pa (p > 0.05, Fig. 1a). CO₂ fixation rates also increased incrementally by 54% (p < 0.05) between 39 Pa CO₂ and 152 Pa CO₂ (Fig. 1b), and cellular growth rates increased by 37% (p < 0.05) for the same pCO₂ range (Fig. 1c). Cellular C:N ratios were relatively invariant (5.2–5.9, p > 0.05) between treatments (data not shown). However, cellular N:P ratios (and by extension C:P ratios, data not shown) increased significantly (p < 0.05) from near-Redfield values of 17 at 39 Pa CO₂ to 21–23 at 76–152 Pa (Fig. 1d). N:P ratios were not significantly different from each other in the three highest CO₂ treatments (p > 0.05).

Three independent trials using triplicate cultures demonstrated that neither the Pacific GBR strain nor the Atlantic Trichodesmium strain IMS101 were capable of surviving for extended periods at 15 Pa CO₂ (data not shown). Therefore, N₂ and CO₂ fixation and growth rates were zero at this pCO₂ (Fig. 1a–c), and elemental ratios could not be determined (Fig. 1d). After transfer to this low pCO₂ treatment, all cultures ceased growing within 2 days, individual filaments of cells fragmented, and both strains ultimately died within a week or so.

In the second experiment, the effects of 39 Pa CO₂ and 76 Pa CO₂ were examined at two temperatures during P-
replete and P-limited steady-state growth for both GBR and IMS in a complete eight-treatment matrix for each isolate. In P-replete cultures of both strains, neither Chl \(a\)-normalized (Fig 2a,b) nor cell-normalized (Fig 2c,d) \(N_2\) fixation rates were affected by a 4°C temperature increase for either isolate \((p > 0.05)\). As in the first experiment, however, P-replete \(N_2\) fixation rates were significantly higher in every case \((p < 0.05)\) at elevated pCO\(_2\) (Fig. 2). \(N_2\) fixation rates in P-replete cultures grown at 76 Pa CO\(_2\) were 42–66% (GBR) and 35% (IMS) higher than in cultures grown at the same temperatures at 39 Pa CO\(_2\) (Table 2).

As expected, severely P-limited cultures of both the Pacific and Atlantic isolates fixed much less \(N_2\) than P-replete cultures (Table 2). Despite this, relative increases in \(N_2\) fixation rates under high CO\(_2\) were similar to, or even greater than, in the P-replete cultures (Fig. 3). CO\(_2\)-mediated increases in \(N_2\) fixation rates in the P-limited samples were 66–100% (GBR) and 35–39% (IMS) in the 76 Pa cultures relative to the 39 Pa cultures (Table 2). Again, similar increases were observed whether \(N_2\) fixation rates were normalized to Chl \(a\) (Fig. 3a,b) or cell number (Fig. 3c,d). Similar results were also recorded regardless of whether \(N_2\) fixation rates were measured using the acetylene reduction method (Fig. 3), or by \(^{15}\)N\(_2\) incorporation (data not shown).

The pCO\(_2\)-mediated trends for CO\(_2\) fixation rates in both Pacific and Atlantic Trichodesmium strains were generally similar to those observed for \(N_2\) fixation (Fig. 4). As with \(N_2\) fixation, the relative degree of rate enhancement at high pCO\(_2\) was similar or even greater in P-limited cultures as compared to P-replete ones. For P-replete GBR (Fig. 4a) and P-replete or P-limited IMS (Fig. 4b, d) grown at a pCO\(_2\) of 76 Pa, CO\(_2\) fixation rates were significantly higher than in cultures grown at the same temperatures at 39 Pa CO\(_2\) \((p < 0.05, \text{Table 2})\). P-limited GBR cultures showed a slightly different response, with the highest CO\(_2\) fixation rates in the greenhouse and high-temperature treatments (Fig. 4c). At the higher pCO\(_2\), CO\(_2\) fixation rates were elevated by 27–38% and 15–24% for P-replete and P-limited GBR, respectively, and by 39–51% and 97–128% for P-replete and P-limited IMS, respectively, relative to the cultures grown at the same temperatures at present day pCO\(_2\) (Table 2).

Molar C:N ratios of the GBR and IMS cells in the second experiment ranged from 4.7 to 5.9, and as in the first experiment there was no systematic influence of the two pCO\(_2\) treatments (Table 2). In contrast, paired comparisons of P-replete GBR and IMS at the same temperatures show that N:P ratios increased by 16–21% \((p < 0.05)\) at 76 Pa CO\(_2\) as compared to 39 Pa CO\(_2\). The CO\(_2\)-

Fig. 1. \(N_2\) fixation rates, CO\(_2\) fixation rates, cell-specific growth rates, and elemental ratios of the Pacific Trichodesmium isolate GBR across a pCO\(_2\) range from 15 Pa to 152 Pa. (a) Chl \(a\)-normalized \(N_2\) fixation rates, (b) Chl \(a\)-normalized CO\(_2\) fixation rates, (c) cell-specific growth rates, and (d) cellular molar N:P ratios. In panels a, b, and c arrows indicate approximate last glacial maximum pCO\(_2\) (~19 Pa, dotted arrow), present day pCO\(_2\) (~39 Pa, solid arrow), and projected year 2100 pCO\(_2\) (~76 Pa, dashed arrow). \(N_2\) fixation, CO\(_2\) fixation, and growth rates at 15 Pa were zero, as Trichodesmium was unable to survive at this pCO\(_2\). Consequently, no elemental ratio measurements were possible for the 15 Pa culture, and these data and the arrow indicating 19 Pa were omitted in panel d. Values are the means and error bars are the standard deviations of triplicate cultures for each treatment.
mediated N : P increase in P-limited cultures was 10–27% (p < 0.05, Table 2).

As with the other rate measurements, short-term CO₂ fixation rates from the P–E curves were invariably much lower (≈25%) in P-limited cultures than in P-replete cultures. However, CO₂- and temperature-driven changes in photosynthetic parameters were evident regardless of growth limitation by P. Maximum light–saturated Chl a-normalized photosynthetic rates (Pₐmax) in both P-replete and P-limited GBR cultures increased 27–55% in the high CO₂ and high-temperature treatments relative to the respective controls (Fig. 5a,c). In contrast, under both conditions of P availability, increases in both CO₂ and temperature in the greenhouse treatment increased Pₐmax significantly more (p < 0.05), by 88% and 64% over the control values in the P-replete and P-limited IMS cultures, respectively (Table 2).

The response of IMS Pₐmax to temperature and CO₂ increases differed somewhat from that of GBR, but was again consistent between P-replete and P-limited cultures (Fig. 5b,d). Unlike the GBR isolate, the IMS Pₐmax was not significantly increased under high CO₂ alone as compared to the control values (p > 0.05). Increased temperature alone, however, did increase Pₐmax significantly relative to the respective control levels (100% in the P-replete and 46% in the P-limited IMS treatments, Table 2). However, as with the GBR results, the greenhouse treatments had much higher Pₐmax under both nutrient conditions than in any of the other treatments, 70–178% higher than in the controls (Fig. 5b,d).

Treatment-related trends in the slope of the light-limited portion of the P–E curve, α, were generally similar to those for Pₐmax for the GBR experiment (Table 2). In P-limited cultures of this Pacific isolate, α was marginally enhanced relative to the control by either elevated temperature or CO₂, but values in the combined greenhouse treatment were significantly greater relative to the other treatments (p < 0.05). P-replete GBR and IMS cultures had a slightly different trend, with significant increases in α from high temperature alone but not from high CO₂ alone, but again the greenhouse treatment had the highest α values (38% to 307% higher than in the control, p < 0.05). In contrast to P-replete conditions, α increased only modestly in the P-limited IMS greenhouse treatment. This slight increase was not significant, and all four treatments had similar α values (p > 0.05, Table 2).

Eₖ (Pₐmax : α) represents the light saturation point, where light absorption is in balance with the capacity of the photosynthetic system to process this incoming energy. Eₖ was elevated in all three high-temperature and/or CO₂ treatments in P-replete GBR relative to the control, mainly because of greater increases in Pₐmax than in α under all of these growth conditions. These three treatments did not, however, differ significantly from each other (p > 0.05, Table 2). In contrast, in the P-limited GBR cultures, Eₖ decreased significantly in the greenhouse treatment relative to the fairly invariant values in the other three treatments, in this case because Pₐmax showed a smaller relative increase than α under the combined high CO₂ and high-temperature conditions. IMS Eₖ showed this same trend but only in the P-replete cultures; in P-limited cultures, both high-temperature treatments had higher α values than both low-temperature treatments, with no evident CO₂ effect (Table 2).
Table 2. Cell-specific growth rates $\mu$ (d$^{-1}$), Chl $a$-normalized $N_2$ fixation rates [µmol N (mg Chl $a$)$^{-1}$ h$^{-1}$], $CO_2$ fixation rates [µg C (mg Chl $a$)$^{-1}$ h$^{-1}$], C : N and N : P ratios (mol : mol), and the P - E parameters $P_{\text{max}}$, [µg C (mg Chl $a$)$^{-1}$ h$^{-1}$], $z$ (µg C (mg Chl $a$)$^{-1}$ h$^{-1}$ (µmol photons$^{-2}$ s$^{-1}$)$^{-1}$, and $E_k$ (µmol photons$^{-2}$ s$^{-1}$) of Pacific (GBR) and Atlantic (IMS) *Trichodesmium* strains during steady-state P-replete and P-limited growth in the four $CO_2$ and temperature treatments. Values are the means and numbers in parentheses are the standard deviations of triplicate bottles for each treatment.

<table>
<thead>
<tr>
<th></th>
<th>GBR</th>
<th></th>
<th></th>
<th></th>
<th>IMS</th>
<th></th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
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<td>P-limited</td>
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<td>High CO$_2$</td>
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<td>High CO$_2$</td>
<td>Greenhouse</td>
</tr>
<tr>
<td>Growth rate $\mu$</td>
<td>0.29 (0.04)</td>
<td>0.36</td>
<td>0.36</td>
<td>0.40</td>
<td>0.08 (0.01)</td>
<td>0.08</td>
<td>0.12</td>
<td>0.10</td>
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<tr>
<td>$N_2$ fixation rate</td>
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<td>16.4</td>
<td>26.1</td>
<td>27.2</td>
<td>5.9 (0.2)</td>
<td>5.3</td>
<td>11.9</td>
<td>8.9</td>
</tr>
<tr>
<td>$CO_2$ fixation rate</td>
<td>2.37 (0.1)</td>
<td>2.57</td>
<td>3.01</td>
<td>3.54</td>
<td>0.56 (0.1)</td>
<td>0.87</td>
<td>0.69</td>
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<tr>
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<td>5.0</td>
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<td>5.5 (0.1)</td>
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<td>N : P ratio</td>
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<td>19.4</td>
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<td>36.2 (7.8)</td>
<td>39.0</td>
<td>45.3</td>
<td>49.4</td>
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<td>$P_{\text{max}}$</td>
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<td>6.1</td>
<td>7.9</td>
<td>1.1 (0.2)</td>
<td>1.4</td>
<td>1.5</td>
<td>1.8</td>
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<tr>
<td>$z$</td>
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<td>0.062</td>
<td>0.053</td>
<td>0.072</td>
<td>0.018 (0)</td>
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<tr>
<td>$E_k$</td>
<td>81 (4)</td>
<td>107</td>
<td>115</td>
<td>110</td>
<td>59 (9)</td>
<td>63</td>
<td>52</td>
<td>49</td>
</tr>
</tbody>
</table>
Fig. 3. $N_2$ fixation rates in P-limited Pacific (GBR, open bars) and Atlantic (IMS, closed bars) *Trichodesmium* cultures during steady-state growth under four experimental combinations of $pCO_2$ and temperature. (a) GBR, chl $a$-normalized $N_2$ fixation rates, (b) IMS, chl $a$-normalized $N_2$ fixation rates, (c) GBR, cell-normalized $N_2$ fixation rates, and (d) IMS, cell-normalized $N_2$ fixation rates. Values are the means and error bars are the standard deviations of triplicate cultures for each treatment.

Fig. 4. Chl $a$-normalized CO$_2$ fixation rates of P-replete and P-limited Pacific (GBR) and Atlantic (IMS) *Trichodesmium* cultures during steady-state growth under four experimental combinations of $pCO_2$ and temperature. (a) P-replete GBR, (b) P-replete IMS, (c) P-limited GBR, and (d) P-limited IMS. Note differences in y-axis scales for P-replete cultures (a and b) as compared to P-limited cultures (c and d). Values are the means, and error bars are the standard deviations of triplicate cultures for each treatment.
Discussion

Our results demonstrate that *Trichodesmium* N\textsubscript{2} and CO\textsubscript{2} fixation rates, growth rate, and N:P ratios are all strongly dependent on the CO\textsubscript{2} concentrations experienced during growth. We found remarkably consistent responses to pCO\textsubscript{2} changes in two physiologically different *Trichodesmium* isolates from distinct biogeochemical regimes in two major ocean basins (Fu et al. 2005\textsuperscript{a}), under varying temperature and nutrient regimes. This suggests that these results may be broadly applicable to natural *Trichodesmium* populations in the oligotrophic oceans. Enhanced N\textsubscript{2} fixation in response to increasing CO\textsubscript{2} has been reported in some terrestrial ecosystems such as agricultural soils (Cheng et al. 2001), but these results provide evidence that marine nitrogen fixation may also be sensitive to ongoing rapid changes in atmospheric CO\textsubscript{2} levels.

In contrast, nitrogen fixation in our cultures was not responsive to a 4°C temperature increase, consistent with results previously reported for the IMS strain (Mulholland and Bernhardt 2005; Breitbarth et al. 2006). It has, however, been suggested that the spatial extent and thus the magnitude of *Trichodesmium* N\textsubscript{2} fixation could increase in the future because of sea-surface warming and consequent expansion of the subtropical biome into higher latitudes (Boyd and Doney 2002). It is also suggested that *Trichodesmium* N\textsubscript{2} fixation could decrease, if warming exceeds optimal temperatures for growth (Breitbarth et al. 2006). Increased stratification and mixed layer shoaling are other global change processes not investigated here that may either enhance or inhibit future *Trichodesmium* diazotrophy by leading to higher mean light exposures or reduced vertical supplies of nutrients like P or Fe.

Current global estimates of N\textsubscript{2} fixation by *Trichodesmium* are about 60 \times 10^9 kg N yr\textsuperscript{-1} (Mahaffey et al. 2005), and diazotrophic growth represents upward of 50% of the new production in some oligotrophic tropical and subtropical oceans (Capone et al. 2005; Mahaffey et al. 2005). If our experimental results can be directly extrapolated to the ocean (which remains to be tested), by 2100 this amount could increase to between 81 \times 10^9 kg N yr\textsuperscript{-1} (35% increase) and 120 \times 10^9 kg N yr\textsuperscript{-1} (100% increase) because of anticipated CO\textsubscript{2} increases alone. This calculation assumes that the relative influences of other limiting factors such as P, Fe, and light availability do not change.

If these estimates are coupled with modeling predictions of a 27% warming-induced expansion of suitable habitat (Boyd and Doney 2002), calculations suggest that global N\textsubscript{2} fixation by *Trichodesmium* alone could range from 103 \times 10^9 kg N yr\textsuperscript{-1} to 152 \times 10^9 kg N yr\textsuperscript{-1} by the end of this century. Because *Trichodesmium* carries out 40–59% of the geochemically inferred N\textsubscript{2} fixation in the North Atlantic and Pacific (Mahaffey et al. 2005), these values would substantially increase recent estimates for total pelagic N\textsubscript{2} fixation of \sim 100–200 \times 10^9 kg N yr\textsuperscript{-1} (Galloway et al. 2004). Our results also suggest, however, that N\textsubscript{2} fixation rates may saturate at about 76 Pa CO\textsubscript{2} (Fig. 1a) and that further enhancement due to subsequent CO\textsubscript{2} increases may be unlikely.

![Fig. 5. P–E curves for P-replete and P-limited Pacific (GBR) and Atlantic (IMS) *Trichodesmium* cultures during steady-state growth in control (circle), high temperature (triangle), high CO\textsubscript{2} (square), and greenhouse (diamond) treatments. (a) P-replete GBR, (b) P-replete IMS, (c) P-limited GBR, and (d) P-limited IMS. Note differences in y-axis scales for P-replete cultures (a and b) as compared to P-limited cultures (c and d). Values are the means and error bars are the standard deviations of triplicate cultures for each treatment.](image-url)
Free-living unicellular cyanobacteria are now believed to fix at least as much nitrogen as *Trichodesmium* in the ocean (Montoya et al. 2004). In addition, endosymbiotic cyanobacteria contribute substantially to N\textsubscript{2} fixation, at least regionally (Carpenter et al. 1999). If N\textsubscript{2} fixation rates in these groups show commensurate increases with rising CO\textsubscript{2}, the cumulative effect on the global nitrogen cycle could be considerably larger (e.g., a doubling). Thus, determining the influence of pCO\textsubscript{2} on all important ocean N\textsubscript{2}-fixing groups, including free-living and symbiotic cyanobacteria, heterotrophic bacteria, and other common *Trichodesmium* species such as *Trichodesmium thiebautii*, should be a priority for future research. Of course, the net effect of increased N\textsubscript{2} fixation on the future fixed nitrogen inventory of the ocean will also depend on how global change variables such as temperature and stratification affect the availability of nutrients such as P or Fe or major N removal processes such as denitrification.

Our results predict that, like N\textsubscript{2} fixation, CO\textsubscript{2} fixation by *Trichodesmium* should also increase dramatically in the future because of CO\textsubscript{2} enrichment. Unlike N\textsubscript{2} fixation, CO\textsubscript{2} fixation did respond somewhat to increases in temperature. For example, CO\textsubscript{2} fixation rates in P-limited GBR (Fig. 4c) were dependent on temperature as well as pCO\textsubscript{2}. Likewise, maximum photosynthetic rates from the P-E curves (P\textsubscript{Bmax}) increased with either temperature alone or pCO\textsubscript{2} alone (GRB) or with temperature alone but not pCO\textsubscript{2} alone (IMS). Although this observation suggests interesting differences in the short-term photosynthetic physiological responses of the two isolates, the highest P\textsubscript{Bmax} values in both IMS and GBR were always found in the combined high-temperature and high CO\textsubscript{2} greenhouse treatment. This trend was also observed in the longer-term productivity estimates.

The temperature effect on maximum photosynthetic rates is not surprising, because carbon fixation is enzymatically controlled under saturating light conditions (Falkowski and Raven 1997). Temperature effects on light-related photosynthetic parameters, however, did not translate into temperature-mediated effects on growth or N\textsubscript{2} fixation rates (Table 2). Carbon fixation and growth rates have often been observed to be decoupled in other phytoplankton (Raven et al. 1993; Beardall et al. 1998), which has been attributed to processes such as dissolved organic carbon exudation (Fu et al. in press; Mulholland 2007). Differences in time scales between short-term CO\textsubscript{2} fixation measurements and longer-term growth rate measurements could also account in part for this difference.

The slope of the light-limited portion of the P-E curve (α) increased under greenhouse conditions for both strains, suggesting that *Trichodesmium* light-harvesting efficiency for photosynthesis may be more efficient under future sea-surface warming and high CO\textsubscript{2} regimes. Likewise, the light saturation constant E\textsubscript{s} also changed under our experimental treatments in both *Trichodesmium* strains. In nutrient replete cultures, shifts in E\textsubscript{s} under greenhouse conditions in the GBR isolate were largely driven by greater changes in P\textsubscript{Bmax} relative to changes in α, whereas the opposite effect was noted in the IMS isolate. Interestingly, these corresponding changes were the direct opposite for each strain when maintained under P-limited conditions (e.g., P\textsubscript{Bmax} changed less in the GBR isolate, whereas the IMS isolate had a greater change in P\textsubscript{Bmax} relative to α). Although beyond the scope of this present study, analyses of pigment quotas and absorption efficiencies should further insight into how these variables may change in light of altered nitrogen assimilation (discussed below) and help determine their effect on photosynthetic capacity in different isolates of *Trichodesmium*.

Whereas the effect of N\textsubscript{2} fixation on C budgets has not been widely assessed, the available paired estimates of N\textsubscript{2} and CO\textsubscript{2} fixation suggest that C:N fixation ratios often exceed the cellular C:N ratio (Mulholland et al. 2006). Assuming conservatively that C:N fixation proceeds at rates similar to the cellular C:N ratio of about 5.0 (Table 2), the projected increases in global *Trichodesmium* N\textsubscript{2} fixation rates calculated above would support CO\textsubscript{2} fixation rates of 515–760 × 10\textsuperscript{9} kg C yr\textsuperscript{-1} by this genus under end-of-the-century conditions. Although the ultimate biogeochemical fate of *Trichodesmium*-fixed elements is not fully understood (Mahaffey et al. 2005; Mulholland et al. 2006; Mulholland 2007), any fraction of this enhanced primary productivity that is exported to underlying waters will contribute to sequestration of atmospheric carbon.

Increases in algal carbon fixation rates at elevated CO\textsubscript{2} concentration are well documented (Riebesell 2004), but our work demonstrates that increased pCO\textsubscript{2} can also stimulate N\textsubscript{2} fixation. Similar enhancement of N\textsubscript{2} fixation at elevated pCO\textsubscript{2} was also reported for the IMS *Trichodesmium* strain by Levitan et al. (2007). Genomic evidence suggests that this strain possesses homologues for genes encoding a low-affinity HCO\textsubscript{3}transport system (BicA) and a low affinity form of the NDH-1 complex (a dehydrogenase possibly involved in active CO\textsubscript{2} uptake), although it apparently lacks the high-affinity DIC transport mechanisms present in some other marine cyanobacteria (Badger et al. 2006). Regardless of genetic evidence for putative *Trichodesmium* inorganic carbon transport systems, our physiological data emphatically demonstrate that this species is vulnerable to DIC limitation at present day (or lower) CO\textsubscript{2} levels.

We suggest that the enhancement of N\textsubscript{2} fixation at higher pCO\textsubscript{2} may be an indirect effect resulting from alleviation of C-limitation of CO\textsubscript{2} fixation, rather than being a consequence of direct CO\textsubscript{2} regulation of N\textsubscript{2} fixation pathways such as nifH gene transcription. Carbon and nitrogen fixation are both energy-intensive processes that compete directly for the products of the light reactions of photosynthesis (Berman-Frank et al. 2001). Growth at elevated CO\textsubscript{2} may thus provide a photosynthetic subsidy that enables diversion of increased amounts of energy to support diazotrophy. Our results with both Atlantic and Pacific *Trichodesmium* strains in multiple experiments robustly confirm the phenomenon, but further elucidation of the cellular genetic and metabolic pathways involved is still needed. In ecological and biogeochemical terms, though, it is immaterial whether CO\textsubscript{2} control of diazotrophy is mediated through direct genetic regulation or indirectly through other physiological processes. The salient fact is that it has the potential to profoundly affect global ocean nutrient and carbon cycling.
Carbon limitation of growth and N₂ fixation is also strongly suggested by the inability of either *Trichodesmium* strain to grow at 15 Pa CO₂. This indicates that under the last glacial maximum atmospheric CO₂ levels of ~19 Pa (Petit et al. 1999), growth and N₂ fixation by *Trichodesmium* were likely to have been marginal (Fig. 1). It is conceivable that the interaction between CO₂ limitation and N₂ fixation could contribute to determining a set point for minimum CO₂ levels during glacial periods. When atmospheric CO₂ falls to some critical level (above 15 Pa but much lower than 39 Pa), our experiments have insufficient resolution to determine the exact value. *Trichodesmium* becomes severely carbon-limited. Whether this is also the case for other marine diazotroph groups still remains to be tested. At this point, new N inputs by oceanic N₂ fixation might be significantly reduced. This in turn would tend to downregulate oceanic CO₂ sequestration by the biological pump, thus stabilizing atmospheric CO₂ against further decreases. Reduced glacial atmospheric CO₂ levels could thus act as a governor on oceanic N₂ fixation, regardless of other external drivers like Fe or P supply. Thus, CO₂ control of marine diazotrophy offers a possible corollary to the “glacial N₂ fixation hypothesis” (Falkowski 1997; Michaels et al. 2001) that provides a crucial missing element: e.g., an “off switch” mechanism for increased glacial productivity.

One of our striking results was that increased pCO₂ enhanced N₂ and CO₂ fixation and growth rates even under severely P-limited steady-state growth conditions (Fig. 2; Table 2). This finding has major implications, because limitation of *Trichodesmium* growth and N₂ fixation by P, Fe, or light appears to be widespread (Fu et al. 2005a; Moutin et al. 2005; Mulholland and Bernhardt 2005). Although P-limited cultures fix much less N₂ and CO₂ and have lower growth rates than P-replete cultures, higher pCO₂ leads to rate increases of similar relative magnitude in both. Thus, P-limited cells can also be considered to be simultaneously limited by CO₂. This surprising observation is difficult to reconcile with the concept of a single Liebig-type limiting nutrient and lends support to the emerging paradigm and abundant evidence for various types of nutrient co-limitation (Arrigo 2005; Hutchins and Fu in press). Our results with both *Trichodesmium* isolates conclusively demonstrate that CO₂ availability exerts an overarching control on N and C acquisition, superimposed upon the growth-regulating effects of P availability.

Future CO₂ increases may have the potential to at least partially offset any intensified P-limitation of *Trichodesmium* from expected stratification-driven decreases in P supply. For instance, our P-limited cultures grown at present-day pCO₂ fixed only 34–39% as much N₂ as P-replete cultures grown at the same temperatures (Table 2). When pCO₂ was raised to 2100 levels, however, P-limited cultures were able to fix N₂ at rates that were 61% (IMS) and 48–73% (GBR) of those measured in the P-replete present-day pCO₂ cultures. Higher pCO₂ thus compensates to a large extent for P-limited growth, offering yet another potential feedback mechanism between atmospheric CO₂ and ocean nutrient biogeochemistry. Further work will be required to determine whether pCO₂ increases also promote N₂ and CO₂ fixation regardless of limitation by other resources such as Fe or light.

Based on our results with P-limited cultures, it seems likely that elevated atmospheric CO₂ levels will boost new nitrogen inputs by *Trichodesmium* despite widespread P limitation in regimes like the Sargasso Sea or the North Pacific Central Gyre (Sañudo-Wilhelmy et al. 2001; Karl et al. 2002). This may increase biological N:P ratios in these areas and tend to drive the biota further toward P limitation. Indeed, just such a progressive shift toward P limitation has been observed in the North Pacific since the early 1990s, driven by apparent increases in N₂ fixation. The reasons for this shift may include changing climate regimes and aeolian iron fluxes (Karl et al. 2001), but the fact that atmospheric pCO₂ has increased from ~35 Pa to ~39 Pa in the intervening years (Houghton et al. 2001) could also be contributing to this trend.

C:N ratios did not change with pCO₂ in our experiments, likely because both N₂ and CO₂ fixation increase simultaneously with rising CO₂. Both isolates did exhibit markedly increased N:P ratios at higher pCO₂ under all growth conditions tested. Although we did not measure phosphate uptake rates in our experiments, this observation suggests that unlike N₂ and CO₂ fixation, P incorporation is not responsive to varying CO₂ concentrations. Changing pCO₂ does not affect phosphate uptake in incubated natural populations of the coccolithophorid *Emiliania huxleyi* either (Engel et al. 2005).

The observed increases in N:P ratios and proportional trends in C:P ratios (not shown) match closely with observed paired increases of 11–25% in cell-specific growth rates between 39 Pa CO₂ and 76 Pa CO₂ (Table 2, discounting a single outlier increase of 50% at 25°C in P-limited GBR). This is consistent with the suggestion that elemental ratio changes are driven by increases in cellular N and C incorporation, with P quotas remaining relatively constant under elevated pCO₂. The relative degree of cellular N:P ratio and growth rate enhancement by increased pCO₂ is, however, somewhat smaller than the observed increases in N₂ and CO₂ fixation rates, suggesting that an increased fraction of fixed N and C must be released from the cells at elevated pCO₂.

*Trichodesmium* often exudes a significant and highly variable amount of recently fixed N as dissolved organic nitrogen and NH₄⁺ even under present-day pCO₂ conditions, and this flux is a major route by which new N enters the marine food web (Mulholland and Bernhardt 2005; Mulholland 2007). The “missing” fixed N in our experiments suggests that N exudation may be further increased at elevated pCO₂. Fixed N release can be indirectly estimated by the difference between paired N₂ fixation rates measured using acetylene reduction (gross N₂ fixation) and ¹⁵N₂ incorporation (net N₂ fixation, Mulholland 2007). This calculation suggested that in some of our experiments, N release increased from negligible to as much as 50% of total fixed N between 39 Pa CO₂ and 76 Pa CO₂ (data not shown).

The magnitude of the changes in N and C release in our first GBR experiment can also be estimated by comparing the relative increases in N₂ and CO₂ fixation rates with the increases in cellular growth rate (Fig. 1), assuming that...
any C and N not appearing in cell biomass was lost to exudation. These calculations suggest that N and C exudation accounted for 41% and 31% of the increase in N₂ and CO₂ fixation rates, respectively, as pCO₂ increased from 39 Pa to 152 Pa. Similar increases in dissolved organic carbon release have been observed in eukaryotic phytoplankton communities incubated at elevated pCO₂ (Engels et al. 2005). For *Trichodesmium*, direct measurements of fixed N and C release are needed in future work to confirm our preliminary indirect estimates and to determine exactly how newly fixed N and C is partitioned under varying CO₂ availability.

Together, our observations suggest that globally increasing pCO₂ could have large consequences both for biological elemental stoichiometry and for supplies of *Trichodesmium*-derived dissolved organic N and C available to other phytoplankton and to the marine microbial food web (Mulholland et al. 2006; Mulholland 2007). Such C and N flux changes could alter the taxonomic and trophic structures of present day marine biological communities and affect the strength of the biological pump relative to the remineralizing microbial loop. Remineralization of fixed carbon by an enhanced microbial food web could also at least partially offset any elevated carbon sequestration from increased new production by N₂ fixation under future CO₂ regimes.

CO₂ control of N₂ fixation by *Trichodesmium* could therefore have implications for understanding the future responses of the marine biosphere and global ecosystem to ongoing anthropogenic change. Likewise, carbon limitation of diazotrophy may be linked with the mechanisms controlling climate and CO₂ levels across glacial and interglacial timescales. Many of our current concepts describing the interactions between oceanic nitrogen fixation, atmospheric CO₂, nutrient biogeochemistry, and global climate may need re-evaluation to take into account these previously unrecognized feedback mechanisms between atmospheric composition and ocean biology.

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