Combined effects of different CO₂ levels and N sources on the diazotrophic cyanobacterium *Trichodesmium*

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Received 11 November 2013; revised 17 January 2014

doi:10.1111/ppl.12172

To predict effects of climate change and possible feedbacks, it is crucial to understand the mechanisms behind CO₂ responses of biogeochemically relevant phytoplankton species. Previous experiments on the abundant N₂ fixers *Trichodesmium* demonstrated strong CO₂ responses, which were attributed to an energy reallocation between its carbon (C) and nitrogen (N) acquisition. Pursuing this hypothesis, we manipulated the cellular energy budget by growing *Trichodesmium erythraeum* IMS101 under different CO₂ partial pressure (pCO₂) levels (180, 380, 980 and 1400 μatm) and N sources (N₂ and NO₃⁻).

Subsequently, biomass production and the main energy-generating processes (photosynthesis and respiration) and energy-consuming processes (N₂ fixation and C acquisition) were measured. While oxygen fluxes and chlorophyll fluorescence indicated that energy generation and its diurnal cycle was neither affected by pCO₂ nor N source, cells differed in production rates and composition. Elevated pCO₂ increased N₂ fixation and organic C and N contents. The degree of stimulation was higher for nitrogenase activity than for cell contents, indicating a pCO₂ effect on the transfer efficiency from N₂ to biomass. pCO₂-dependent changes in the diurnal cycle of N₂ fixation correlated well with C affinities, confirming the interactions between N and C acquisition.

Regarding effects of the N source, production rates were enhanced in NO₃⁻ grown cells, which we attribute to the higher N retention and lower ATP demand compared with N₂ fixers. pCO₂ effects on C affinity were less pronounced in NO₃⁻ users than N₂ fixers. Our study illustrates the necessity to understand energy budgets and fluxes under different environmental conditions for explaining indirect effects of rising pCO₂.

**Introduction**

The release of anthropogenic carbon (C) has caused atmospheric CO₂ partial pressure (pCO₂) to increase from 280 to 390 μatm since pre-industrial times and pCO₂ levels are expected to rise further to 750 μatm or even beyond 1000 μatm by the end of this century (IPCC 2007, Raupach et al. 2007). As CO₂ is taken up by the ocean, seawater CO₂ concentrations increase and pH levels decrease, a phenomenon termed ocean acidification (Caldeira and Wickett 2003). These changes in carbonate chemistry are expected to have diverse effects

**Abbreviations** — ARA, acetylene reduction assay; CA, carbonic anhydrase; CCM, carbon concentrating mechanism; chl a, chlorophyll a; C₅, inorganic carbon; DIC, dissolved inorganic carbon; Fv/Fm, PSII photochemical quantum yield measured in dark-adapted state; Fv/Fm', PSII photochemical quantum yield measured in light-adapted state; HEPES, 4-(2-hydroxyethyl)-1-piperazin-ethanesulfonic acid; HSD, honest significant difference; K₁/₂, half-saturation concentration; pCO₂, CO₂ partial pressure; POC, particulate organic carbon; PON, particulate organic nitrogen; PQ, plastoquinone; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; TA, total alkalinity; Vₘₓ, maximum rate; σ, PSII functional absorption cross section; τ, QA re-oxidation time;
on marine phytoplankton (Rost et al. 2008, Riebesell and Tortell 2011). By fixing CO$_2$ into organic matter, phytoplankton acts as a C sink and plays a potential role as a negative feedback mechanism to atmospheric pCO$_2$ increase (Raven and Falkowski 1999, De La Rocha and Passow 2007).

In marine ecosystems, phytoplankton productivity is often limited by availability of nitrogen (N). Fixation of atmospheric N$_2$ by diazotrophic cyanobacteria thus plays a crucial role for primary productivity, particularly in oligotrophic regions of the world ocean. With global change, the marine N cycle is subject to an array of perturbations. On the one hand, increasing deposition of anthropogenic N leads to eutrophication in coastal regions (Duce et al. 2008). On the other hand, the expansion of oxygen minimum zones favors N loss processes such as denitrification and anammox (Lam and Kuyper 2011). Additionally, ocean acidification is expected to decrease marine nitrification rates (Beman et al. 2011), and global warming intensifies stratification and therewith lowers nutrient input into the upper mixed layer (Donef 2006). As the latter processes are likely to decrease the overall NO$_3^-$ availability in the surface ocean, marine N$_2$ fixation may become more important, helping to restore the global N budget.

The cyanobacterium *Trichodesmium* is considered one of the most important marine N$_2$ fixers with an estimated contribution of up to 50% to global marine N$_2$ fixation (Mahaffey et al. 2005). Previous studies found this diazotroph to be exceptionally sensitive to rising pCO$_2$. Laboratory experiments exposing cultures to pCO$_2$ levels projected for the end of this century showed significant increases in the production of particulate organic C and particulate organic nitrogen (POC and PON) as well as N$_2$ fixation rates (Barcelos é Ramos et al. 2007, Hutchins et al. 2007, 2013, Kranz et al. 2009, Levitan et al. 2010, Garcia et al. 2011); the magnitude of these effects yet differed strongly between investigations. In several follow-up studies, CO$_2$ effects on *Trichodesmium* were found to be strongly modulated by other environmental factors such as iron (Shi et al. 2012) and light (Kranz et al. 2010, Levitan et al. 2010, Garcia et al. 2011), the latter highlighting the importance of energy in the modulation of CO$_2$ effects.

Cyanobacteria have to invest a considerable share of energy into the accumulation of inorganic carbon (C$_i$) by carbon concentrating mechanisms (CCMs) owing to a competing reaction with O$_2$ and a particularly low CO$_2$ affinity of their ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Badger et al. 1998). The CCM of *Trichodesmium* involves a distinct assembly of RubisCO and carbonic anhydrase (CA) within carbonosomes, as well as two C$_i$ acquisition systems (Badger et al. 2006, Price et al. 2008). HCO$_3^-$ is taken up via a Na$^+$-dependent HCO$_3^-$ transporter (BicA) whereas diffusive uptake of CO$_2$ is facilitated by the so-called NDH-1$_4$ complex, converting CO$_2$ to HCO$_3^-$. Next to C$_i$ acquisition, another important energy sink in *Trichodesmium* is N$_2$ fixation (Kranz et al. 2011). As CCM activity was found to be downregulated at high pCO$_2$ levels, while N$_2$ fixation rates were simultaneously increased in this species, a reallocation of energy between C and N$_2$ fixing pathways has been suggested to fuel the increase in production at high pCO$_2$ (Kranz et al. 2010).

Similarly to RubisCO, nitrogenase is characterized by a high sensitivity toward O$_2$ (Falkowski 1997). In consequence, while the fixation of N$_2$ is an extremely energy demanding reaction in itself (Eqn 1), diazotrophs face additional costs, which are related to the protection of nitrogenase from photosynthetically evolved O$_2$ (Groškopf and LaRoche 2012). To separate O$_2$ evolution from N$_2$ fixation, *Trichodesmium* has a tightly regulated diurnal cycle of N$_2$ fixation and photosynthesis (Berman-Frank et al. 2001), involving daily synthesis and degradation of nitrogenase (Capone et al. 1990, Sandh et al. 2009) and alternation of photosynthetic activity states (Küpper et al. 2004). Moreover, nitrogenase is expressed only in subsets of cells within filaments, the diazocytes (Lin et al. 1998, Berman-Frank et al. 2001). As no trans-cellular transport mechanisms for N compounds have been found in *Trichodesmium*, diazocytes have to release N for use by their neighboring cells (Mulholland and Capone 2000). Uptake mechanisms for N sources other than N$_2$ are thus indispensable for this species.

Laboratory studies have shown that *Trichodesmium* can use NO$_3^-$ and NH$_4^+$ as well as organic N compounds (glutamine, glutamate or urea; Mulholland et al. 1999), all of them requiring different amounts and types of energy equivalents. NO$_3^-$ is taken up in cyanobacteria by high-affinity ATP-dependent transporters and subsequently reduced to NH$_4^+$ in a two-step ferredoxin-dependent reaction catalyzed by nitrate reductase and nitrite reductase (Flores et al. 2005, Wang et al. 2000) (Eqn 2).

\[
\begin{align*}
N_2 + 8 H^+ + 8 e^- + 16 ATP & \rightarrow 2 NH_3 + H_2 \\
& + 16 (ADP + P_i) \quad (1)
\end{align*}
\]

\[
\begin{align*}
2 NO_3^- + 20 H^+ + 16 e^- + 2 ATP & \rightarrow 2 NH_4^+ + 6 H_2O \\
& + 2 (ADP + P_i) \quad (2)
\end{align*}
\]

In *Trichodesmium*, N$_2$ fixation was shown to be inhibited in cultures grown in NO$_3^-$-containing media (Ohki et al. 1991, Fu and Bell 2003, Holl and Montoya 2005,
Sandh et al. 2011). As the uptake and reduction of NO$_3^-$ requires little ATP (Eqn 2), it can be expected that NO$_3^-$ addition to culture media will alter the energy budget of the cells in comparison to N$_2$ fixing conditions.

In this study, *Trichodesmium erythraeum* IMS101 was acclimated in a matrix of four different pCO$_2$ levels (ranging from 180 to 1400 μatm) and two different N sources (N$_2$ and NO$_3^-$). In addition to acclimation effects on the level of growth and composition (C, N and pigments), physiological key processes (N$_2$ fixation, O$_2$ fluxes and electron transport) were analyzed to improve our understanding of the plasticity in energy and resource allocation under the different energetic requirements imposed by changing environmental conditions.

**Materials and methods**

**Culture conditions**

*Trichodesmium erythraeum* IMS101 was grown in semi-continuous batch cultures at 25°C and 150 μmol photons m$^{-2}$ s$^{-1}$ with a 12:12 h light:dark cycle. Light was provided by white fluorescent bulbs (BIOLEX, Osram, München, Germany). Cultures were grown in 0.2-μm-filtered artificial seawater (YBCII medium; Chen et al. 1996) and kept in exponential growth phase by regular dilution with culture medium. Cultures consisted of single trichomes and cell densities ranged between approximately 6000 and 180 000 cells ml$^{-1}$. Cells were acclimated in 1 l culture flasks, which were continuously bubbled with 0.2-μm-filtered air with pCO$_2$ levels of 180, 380, 980 and 1400 μatm. Gas mixtures were generated with a custom-made gas flow controller. Prior to experiments, cells were allowed to acclimate to the respective pCO$_2$ for at least 2 weeks. Cultures in which pH had drifted by >0.09 compared with cell-free reference media were excluded from further analysis. In treatments with NO$_3^-$ as the N source, 0.2-μm-filtered NaNO$_3$ was added to achieve mean concentrations of 97 ± 2 μM in the experiments, never falling below 65 μM. Concentrations were monitored photometrically according to Collos et al. (1999). Consumption of NO$_3^-$ by cellular uptake was compensated for by regular additions of NaNO$_3$. Cultures were acclimated to NO$_3^-$ for at least 1 week before measurements.

**Carbonate chemistry**

To compensate for an increase in total alkalinity (TA) due to NO$_3^-$ uptake (Wolf-Gladrow et al. 2007), appropriate quantities of HCl were added according to the daily changes in NO$_3^-$ concentration. TA was determined by potentiometric titration with a TitroLine alpha plus titrator (Schott Instruments, Mainz, Germany) and calculation from linear Gran plots (Gran 1952). Average precision was ±5 μmol kg$^{-1}$. Samples for dissolved inorganic carbon (DIC) analysis were filtered through 0.2 μm cellulose acetate filters and measured colorimetrically (TRAACS CS800 autoanalyzer, Seal, Norderstedt, Germany). Average precision was ±5 μmol kg$^{-1}$. Certified reference materials supplied by A. Dickson ( Scripps Institution of Oceanography) were used to correct for inaccuracies of TA and DIC measurements. pH values of the acclimation media were measured potentiometrically on the NBS scale [pH meter pH3110, Wissenschaftlich-Technische Werkstätten (WTW) GmbH, Weilheim, Germany]. Carbonate chemistry of the different pCO$_2$ and N treatments is shown in Table 1.

**Growth and elemental composition**

Samples for determination of growth and elemental composition of cells were generally taken between 1 and 2.5 h after beginning of the photoperiod to account for changes due to the diurnal rhythm of *Trichodesmium*. Duplicate samples for chlorophyll a (chl a) determination were extracted in acetone for >12 h and determined fluorometrically (TD-700 Fluorometer, Turner Designs, Sunnyvale, CA; Holm-Hansen and Riemann 1978). Specific growth rates (μ) were estimated by exponential regression through chl a concentrations measured daily over at least 4 days. Duplicate samples for analysis of POC and PON were filtered onto pre-combusted GF/F filters and stored at −20°C. Prior to analysis, filters were acidified with 200 μl HCl (0.2 M) to remove all inorganic C. POC and PON contents as well as PON isotopic composition (δ$^{15}$N) were measured with an EA mass spectrometer (ANCA SL 20-20, Sercon Ltd, Crewe, UK). Daily production rates of POC and PON were obtained by multiplication of the respective elemental contents and growth rates.

**N$_2$ fixation**

N$_2$ fixation rates were determined using the acetylene reduction assay (ARA) (Capone 1993). Samples were spiked with acetylene (20% of head space volume) in crimp vials followed by incubation for 1 h at acclimation light and temperature with continuous agitation to avoid aggregation of cells. The amount of acetylene reduced to ethylene was then measured by gas chromatography (Trace GC, Thermo Finnigan, Bremen, Germany). Solubility of acetylene in the aqueous phase was taken into account by applying the Bunsen coefficient (0.088 at 25°C and salinity 32; Breitbarth et al. 2004). A conversion factor of 4:1 (Capone and Montoya 2001) was used to convert acetylene reduction rates to N$_2$ fixation rates.
Table 1. Carbonate chemistry for each pCO2 and N treatment acquired in daily measurements during the experiment. Attained pCO2 of the media was calculated from pH, TA, [PO43−], temperature and salinity using the CO2sys program (Pierrot et al. 2006) with equilibrium constants K1 and K2 given by Mehrbach et al. (1973), refit by Dickson and Millero (1987). Errors denote 1 SD (n ≥ 6).

| pCO2 treatment (μatm) | NO3− | pH (NBS) | TA (μmol kg⁻¹) | DIC (μmol kg⁻¹) | pCO2 attained (μatm) |
|-----------------------|-------|----------|----------------|-----------------|----------------------|
| 180                   | −     | 8.48 ± 0.04 | 2415 ± 11   | 1847 ± 10     | 173 ± 12             |
|                       | +     | 8.49 ± 0.04 | 2438 ± 37   | 1868 ± 22     | 168 ± 4              |
| 380                   | −     | 8.26 ± 0.03 | 2408 ± 15   | 2014 ± 6      | 329 ± 22             |
|                       | +     | 8.26 ± 0.04 | 2389 ± 7    | 2003 ± 12     | 325 ± 10             |
| 980                   | −     | 7.88 ± 0.02 | 2377 ± 15   | 2142 ± 17     | 918 ± 12             |
|                       | +     | 7.89 ± 0.03 | 2392 ± 19   | 2166 ± 26     | 912 ± 35             |
| 1400                  | −     | 7.74 ± 0.02 | 2399 ± 44   | 2231 ± 78     | 1354 ± 30            |
|                       | +     | 7.76 ± 0.05 | 2413 ± 37   | 2237 ± 20     | 1298 ± 74            |

O2 fluxes

Cellular O2 fluxes were measured by means of membrane inlet mass spectrometry (MIMS) as described by Rost et al. (2007). Assays were performed in YBCII medium buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 50 mM, pH 8.0) at 25°C and normalization of O2 traces. For O2 fluxes were monitored during consecutive 4 min dark and light intervals, applying range of light intensities from 8 to 2000 μmol photons m⁻² s⁻¹. The light intensity at which photosynthesis starts to enter saturation (Iq) was obtained by a curve fit as specified by Rokitta and Rost (2012).

Fluorescence measurements

Chl a fluorescence was measured using the Fluorescence Induction and Relaxation (FIRE) method with a FiRe Fluorometer System (Satlantic, Halifax, Canada) and the associated actinic light source. Measurements were performed in parallel to the 18O2 assays, following the same protocol of dark and light intervals as well as light intensities. PSII photochemical quantum yield (F/Fm: measured in dark-adapted state; F'/Fm': measured in light-adapted state) and functional absorption cross section of PSII (σ) as well as QA re-oxidation time (τ) were assessed by analysis of the single turnover flash response using the Fireworx matlab code (http://sourceforge.net/projects/fireworx, written by Audrey Barnett).

Statistical analysis

Data were analyzed using R for significance of differences by two-way analysis of variance (ANOVA) tests, followed by Tukey’s test for Honest Significant Differences (TukeyHSD) for specification of differences between means where appropriate. A significance level of P ≤ 0.05 was applied.

Results

Growth and composition

Cellular chl a contents stayed relatively constant over the range of pCO2 levels and N sources tested, with
a mean of $1.04 \pm 0.28 \text{pg chl cell}^{-1}$, and were therefore used for normalization. Growth decreased slightly with increasing pCO$_2$ in both N treatments when all data were included (Fig. 1, ANOVA, $P < 0.005$). However, when testing CO$_2$ levels individually, the differences between 980 and 380 $\mu$atm as well as 180 $\mu$atm depended on the N source: with N$_2$ there was a significant pCO$_2$ effect (TukeyHSD, adj$p < 0.05$) while with NO$_3^-$ there was no effect (TukeyHSD, adj$p > 0.05$). In contrast, contents of POC and PON significantly increased by approximately 33% from 180 to 1400 $\mu$atm pCO$_2$ (ANOVA, $P < 0.0001$). As a result of these opposing trends, production rates of POC and PON did not change over the range of pCO$_2$ levels tested (Table 2, ANOVA, $P > 0.05$). There was no clear trend in the pCO$_2$ effect on the POC:PON ratio (Table 2). Regarding effects of the N source, cells grown on NO$_3^-$ had slightly higher growth rates than N$_2$ fixing cells (ANOVA, $P < 0.05$). Consequently, also production rates of POC and PON were higher in NO$_3^-$ grown cultures (ANOVA, $P < 0.0001$), even though contents of POC and PON were not significantly affected (ANOVA, $P > 0.05$). POC:PON ratios were significantly lower in NO$_3^-$ users than in N$_2$ fixers (Table 2, ANOVA, $P < 0.0001$).

N$_2$ fixation

N$_2$ fixation was inhibited by the addition of NO$_3^-$, with N$_2$ fixation rates being close to detection limit (data not shown). Moreover, the $\delta^{15}$N of PON clearly differed between treatments (ANOVA, $P < 0.0001$), with $-1.3 \pm 1.0\%$ in N$_2$ fixing cells and $+4.8 \pm 1.3\%$ in NO$_3^-$ grown cells. N$_2$ fixation rates displayed a typical diurnal pattern with high rates during midday (Fig. 2). At elevated pCO$_2$, there was a change in the diurnal pattern toward N$_2$ fixation rates remaining high until the end of the photoperiod. Consequently, integrated rates of N$_2$ fixation over the photoperiod increased by approximately 60% from 27 to 43 nmol N$_2$ (µg chl a)$^{-1}$ day$^{-1}$ at 380 and 1400 $\mu$atm pCO$_2$, respectively. At 180 and 980 $\mu$atm pCO$_2$, integrated rates were 25 and 39 nmol N$_2$ (µg chl a)$^{-1}$ day$^{-1}$, respectively.

O$_2$ fluxes

To characterize the energy generating processes, O$_2$ evolution was firstly assessed as a function of DIC concentrations. In all treatments, maximal net O$_2$ evolution ($V_{\text{max}} \text{DIC}$) followed a distinct diurnal pattern with significantly lower values in the morning than for the rest of the day (Fig. 3, ANOVA, $P < 0.0001$). Effects of pCO$_2$ on $K_{1/2}$ (DIC) were modulated by the N source and the time of day. While under NO$_3^-$ grown conditions, there was no significant pCO$_2$ effect (TukeyHSD, adj$p > 0.05$), $K_{1/2}$ (DIC) was significantly lower at 380 $\mu$atm than at 1400 $\mu$atm pCO$_2$ under N$_2$ fixing conditions (TukeyHSD, adj$p < 0.005$). The difference between pCO$_2$ treatments in N$_2$ fixers was especially pronounced toward the evening (TukeyHSD, adj$p < 0.0001$), with $K_{1/2}$ (DIC) decreasing in cells grown at 380 $\mu$atm but remaining high in cells grown at 1400 $\mu$atm pCO$_2$.

In a second approach, evolution and uptake of O$_2$ were assessed over a range of light intensities. Net O$_2$ evolution typically reached light compensation between 10 and 60 µmol photons m$^{-2}$ s$^{-1}$ and started to enter saturation ($I_s$) at approximately 280 $\mu$mol photons m$^{-2}$ s$^{-1}$ (Fig. 4). Light-dependent O$_2$ uptake, i.e. an excess of O$_2$ uptake in the light over O$_2$ uptake in the dark,
**Table 2.** POC and PON production and ratios of *Trichodesmium* grown under different pCO2 levels and N sources (N2 and NO3\(^-\)). Errors denote 1 SD (n ≥ 3).

| pCO2 treatment (μatm) | NO3\(^-\) | POC production (μmol (μg chl a\(^-1\)) day\(^{-1}\)) | PON production (μmol (μg chl a\(^-1\)) day\(^{-1}\)) | POC:PON (mol:mol) |
|------------------------|---------|--------------------------------|--------------------------------|-----------------|
| 180 – 1.75 ± 0.10      | 0.36 ± 0.02 | 4.83 ± 0.10                      |
| 380 – 1.60 ± 0.22      | 0.33 ± 0.04 | 4.81 ± 0.08                      |
| 980 – 1.26 ± 0.42      | 0.26 ± 0.09 | 4.68 ± 0.07                      |
| 1400 – 1.62 ± 0.20     | 0.33 ± 0.03 | 4.93 ± 0.21                      |

**Fig. 2.** Diurnal cycle of N2 fixation in *Trichodesmium* grown at 380 μatm (circles) and 1400 μatm (triangles) pCO2 in a 12:12 h light:dark cycle. Open and closed symbols represent biological duplicates.

was detected at irradiances >60 μmol photons m\(^{-2}\) s\(^{-1}\) (Fig. 4). Gross O2 evolution typically saturated at higher light intensities than net photosynthesis, which is consistent with the increase in light-dependent O2 uptake with increasing irradiance. At acclimation light intensity, diel mean values for dark respiration and light-dependent O2 uptake amounted to 20 and 13% of gross O2 evolution, respectively. With irradiances increasing beyond acclimation levels, light-dependent O2 uptake increased further and equaled about 19% of gross O2 evolution at 1300 μmol photons m\(^{-2}\) s\(^{-1}\). Regarding effects of pCO2 and N source on gross O2 evolution and light-dependent O2 uptake at acclimation light, no clear trend was found and rates followed a diurnal pattern with highest values in the morning (data not shown). Also dark respiration was not affected by pCO2 or N source, yet rates were lowest in the morning (data not shown).

**Chlorophyll fluorescence**

PSII photochemical quantum yield (Fv/Fm and Fv′/Fm′) was higher at acclimation light than in the dark and decreased with increasing light intensities beyond acclimation level, covering a range between approximately 0.5 and 0.1 (Fig. 4). At low irradiances, the functional absorption cross section of PSII (σ) increased with light (by approximately 30% from approximately
8 to 60 μmol photons m⁻² s⁻¹) while at higher light intensities, it slightly decreased (by approximately 10% from approximately 60 to 2000 μmol photons m⁻² s⁻¹). Q_A re-oxidation time (τ) was longest in the dark and decreased by approximately 40% from approximately 60 to 2000 μmol photons m⁻² s⁻¹.

Regarding changes in the diurnal cycle, light-adapted Fv/Fm' values were highest in the morning (0.48 ± 0.04 at acclimation light) and lowest at midday (0.40 ± 0.05 at acclimation light, ANOVA, P < 0.0001, Fig. 5). Variability over the course of the day was even more pronounced regarding dark-adapted Fv/Fm (ANOVA, P < 0.0001), with values ranging from 0.35 ± 0.07 (morning) to 0.17 ± 0.01 (midday). Likewise, functional absorption cross section of PSII (σ) was always highest in the morning (ANOVA, P < 0.0001), irrespective of light conditions. Q_A re-oxidation time, which was measured in the dark (τ_{dark}), was significantly lower in the morning than during the rest of the day (ANOVA, P < 0.0001), while τ_{light} decreased slightly by approximately 10% over the course of the day (ANOVA, P < 0.05).

Concerning responses to pCO₂ and the N source, Fv/Fm and Fv'/Fm' were not significantly affected by either of the two parameters (Fig. 5, ANOVA, P > 0.05). Irrespective of light conditions, functional absorption cross section of PSII (σ) was not affected by the N source (ANOVA, P > 0.05), while it was slightly higher at 1400 μatm than 380 μatm pCO₂, however, only by approximately 10% (ANOVA, P < 0.0001). Neither τ_{dark} nor τ_{light} were significantly affected by pCO₂ or N source (ANOVA, P > 0.5).

**Discussion**

To investigate CO₂ effects on *Trichodesmium* under altered energy requirements, cultures were grown over a range of different pCO₂ levels under N₂ fixing conditions as well as with NO₃⁻, the latter providing a N source with a significantly lower demand in ATP but higher electron requirements (Eqns 1 and 2). We also tested NH₄⁺ as an alternative N source, which would have altered the energy requirements most strongly, lowering the ATP as well as the electron demand compared with N₂ fixation. However, measurements revealed NH₄⁺ to be toxic to *Trichodesmium* in concentrations as low as 10 μM (data not shown), which equaled the average daily N consumption in our cultures, and therefore argued against the applicability in dilute batch incubations. Additionally, concentrations could not be kept stable because of pH-dependent out-gassing of NH₃ (data not shown), rendering it impossible to perform pCO₂ manipulations without simultaneously affecting the N availability. The addition of NO₃⁻, on the other hand, had no negative effects on *Trichodesmium* and was not influenced by pH. As a consequence, we chose NO₃⁻ to impose a change in the energy status of cells. The change in N usage upon NO₃⁻ addition was demonstrated by direct measurements of nitrogenase activity as well as by the change in ¹⁵N composition of PON. NO₃⁻ assimilation resulted in corresponding changes in TA, which were compensated by additions of HCl in equimolar amounts to keep the carbonate system comparable between N₂ fixing and NO₃⁻ using cultures. Growth rates and Fv/Fm indicate that cells were not stressed in any of the treatments.

![Diagram](image_url)

**Fig. 4.** Typical example of light dependence of O₂ fluxes and chlorophyll fluorescence parameters (measured in *Trichodesmium* grown at 380 μatm pCO₂ with NO₃⁻). Total O₂ uptake consists of dark respiration and light-dependent O₂ uptake. Fv/Fm, PSII photochemical quantum yield; σ, PSII functional absorption cross section; τ, Q_A re-oxidation time.
Please note that the light level applied in the acclimations (150 μmol photons m\(^{-2}\) s\(^{-1}\)) was below saturation (Fig. 4), imposing a general energy constraint in the cell.

**Acclimation effects of different pCO\(_2\) levels and N sources**

The increase of POC as well as PON with pCO\(_2\) (Fig. 1) is in accordance with previous results (Kranz et al. 2009, 2010). Respective production rates, however, stayed relatively constant due to the concomitant decrease in growth (Table 2, Fig. 1). In other words, cells contained less biomass and divided more quickly at low and medium pCO\(_2\), while at high pCO\(_2\), cell quotas were higher and cells divided more slowly. Among the previous studies on *T. erythraeum* IMS101 testing the effect of pCO\(_2\) levels up to 750 or 1000 μatm, some showed an increase in growth rate with pCO\(_2\) (Barcelos é Ramos et al. 2007, Levitan et al. 2007, Kranz et al. 2010, Garcia et al. 2011), while others did not find significant differences (Hutchins et al. 2007, Kranz et al. 2009). Only one study has tested CO\(_2\) levels comparable to our highest CO\(_2\) treatment, finding that positive effects on growth leveled off between 760 and 1500 μatm in *T. erythraeum* GBRTRL101 (Hutchins et al. 2007). In a recent study investigating pCO\(_2\) effects under low iron availability representative for oligotrophic oceans, growth rates of *Trichodesmium* were shown to decrease with pCO\(_2\) (380 vs 750 μatm; Shi et al. 2012).
The stimulation in PON production in NO$_3^-$ grown cells may be directly attributed to the lower energy requirement for N assimilation (Table 2, Eqns 1 and 2) as well as the fact that filaments are not subject to N loss during transfer from diazocytes to non-diazotrophic cells when grown on NO$_3^-$. The effect on POC production, however, cannot be directly linked to N assimilation and suggests a more global effect of NO$_3^-$ on the cells’ metabolism such as reallocation of energy from N to C assimilation. The cost reduction associated with the switch from N$_2$ to NO$_3^-$ assimilation is also reflected in the lower POC:PON ratios under these conditions (Table 2). Changes in POC:PON ratios have been found in response to nutrient limitation in different phytoplankton (Sterner and Elser 2002). Even though none of our treatments was N limited, the observed changes in POC:PON ratios may simply reflect the higher N assimilation costs in N$_2$ fixers.

**O$_2$ fluxes and electron transport**

To better understand how the observed effects of pCO$_2$ and N source on POC and PON were fuelled, we investigated photosynthesis as a measure of energy generation. Concerning treatment effects, neither pCO$_2$ nor the N source had a significant effect on net O$_2$ evolution (Fig. 3). However, net O$_2$ evolution can be uncoupled from energy generation by high rates of O$_2$ uptake or cyclic electron transport (Heber 2002). Thus, gross and net O$_2$ fluxes as well as chlorophyll fluorescence need to be considered to obtain a more complete picture of energy generating processes. In all cultures, irrespective of pCO$_2$ or N source, about one third of the gross O$_2$ evolved was consumed by dark respiration and light-dependent O$_2$ uptake (Fig. 4), the latter being indicative for either the classical Mehler reaction (Mehler 1951) or the equivalent reduction of O$_2$ by flavoproteins (Helman et al. 2003). High rates of O$_2$ uptake by dark respiration and Mehler reaction have been suggested to protect nitrogenase from O$_2$ inhibition in *Trichodesmium* (Kana 1993, Carpenter and Roenneberg 1995, Berman-Frank et al. 2001, Milligan et al. 2007). Rates of Mehler reaction equaled only about 10% of gross O$_2$ evolution at acclimation light intensity, yet rates increased when light intensities exceeded acclimation levels (Fig. 4). This light dependency could either indicate a role for Mehler reaction in photoprotection and/or reflect the enhanced need for nitrogenase protection at high gross O$_2$ evolution rates. Moreover, the fact that light-dependent O$_2$ uptake was not significantly affected by the N source seems surprising, considering the proposed role of Mehler reaction in nitrogenase protection in *Trichodesmium* (Milligan et al. 2007).

Chlorophyll fluorescence showed a light response typical for cyanobacteria, with dark-adapted fluorescence being controlled by respiratory electron flow that introduces electrons into the plastoquinone (PQ) pool (reviewed by Campbell et al. 1998). At low light intensities, electron flux through PSI is induced, oxidizing the PQ pool and thereby increasing Fv'/Fm' and decreasing QA re-oxidation time ($\tau$) (Fig. 4). When light intensities increase beyond acclimation light, input of excitation energy can become higher than the cells’ capacity of ferredoxin re-oxidation, making cells vulnerable to photodamage. However, being adapted to high and variable light regimes, *Trichodesmium* employs effective photoprotective mechanisms (Breitbarth et al. 2008, Andresen et al. 2009). First of all, state transitions lead to a re-arrangement of phycobilisomes toward PSI, decreasing the PSII functional absorption cross section ($\sigma$) and therewith Fv'/Fm' (Fig. 4). Second, the enhanced rates of Mehler reaction dissipate electrons at high light (Fig. 4). The effectiveness of these photoprotective mechanisms is reflected in a decreasing QA re-oxidation time ($\tau$) whilst gross O$_2$ evolution increases with light (Fig. 4).

To cover the high ATP demand of N$_2$ fixation (Table 3; Eqn 1), *Trichodesmium* depends on high rates of cyclic electron transport and Mehler reaction, increasing the ATP:NADPH ratio beyond that provided by linear photosynthetic electron transport. High rates of cyclic electron transport have been proposed to result in chemical reduction of the PQ pool, increasing re-oxidation time of QA (Berman-Frank et al. 2001). Assuming that cells adjust their energy generation closely to their needs, we expected the treatment-dependent differences in energy demand to be reflected in chlorophyll fluorescence. Contrary to our assumption, none of the fluorescence parameters measured was affected by pCO$_2$ or N source with the exception of a small pCO$_2$ effect on functional absorption cross section of PSII ($\sigma$, Fig. 5).

Regarding the diurnal cycle, there was a characteristic downregulation of maximal net photosynthesis as well as Fv/Fm during midday, which has been shown previously in *Trichodesmium* as part of the cells’ mechanisms to reduce O$_2$ concentrations during the phase of highest N$_2$ fixation (Berman-Frank et al. 2001). In the morning, highly efficient electron transport was indicated by high Fv/Fm and a large PSII functional absorption cross section (Fig. 5), which is in line with the high gross O$_2$ evolution (data not shown). Dark respiration, as indicated by $\tau_{\text{dark}}$ and O$_2$ flux measurements, was lowest in the morning, while rates of Mehler reaction were at their maximum. Later during the day, rates of photosynthetic electron transport decreased, reflected by lower Fv/Fm, functional absorption cross section of PSII ($\sigma$), O$_2$
Table 3. Theoretical ATP and electron (e\textsuperscript{-}) costs of cellular processes and costs calculated for the observed POC and PON production rates under two different N sources (N\textsubscript{2} and NO\textsubscript{3}\textsuperscript{-}). Theoretical demands of C fixation, CCM and POC production were normalized to mol N using the average POC:PON ratio measured in the experiment. CCM costs are based on 80% HCO\textsubscript{3}\textsuperscript{-} use and a transport cost of 0.5 mol ATP per mol HCO\textsubscript{3}\textsuperscript{-}, assuming 50% CO\textsubscript{2} leakage. Costs of NO\textsubscript{3}\textsuperscript{-} assimilation include 1 mol ATP for uptake. Losses of fixed nitrogen (e.g. NH\textsubscript{4}\textsuperscript{+}) is not accounted for. Please note that numbers given do not include costs for synthesis of enzymes and transporters, which would significantly increase the estimates for fixation of carbon as well as nitrogen (Brown et al. 2008). POM (particulate organic matter) is the sum of POC and PON.

| Process | Unit | ATP | e\textsuperscript{-} | ATP:NADPH+H\textsuperscript{+} | Reference |
|---------|------|-----|---------------------|-------------------------------|-----------|
| C fixation | mol (mol N\textsuperscript{-1}) | 14 | 19 | 1.5 | Allen 2002 |
| CCM | mol (mol N\textsuperscript{-1}) | 4 | 0 | 0.3 | Hopkinson et al. 2011 |
| POC production | mol (mol N\textsuperscript{-1}) | 18 | 19 | 1.9 | Flores and Herrero 1994 |
| N\textsubscript{2} assimilation to NH\textsubscript{4}\textsuperscript{+} | mol (mol N\textsuperscript{-1}) | 8 | 4 | 4.0 | Flores et al. 2005 |
| NO\textsubscript{3}\textsuperscript{-} assimilation to NH\textsubscript{4}\textsuperscript{+} | mol (mol N\textsuperscript{-1}) | 1 | 8 | 0.3 | Flores et al. 2005 |
| NH\textsubscript{4}\textsuperscript{+} assimilation to glutamate | mol (mol N\textsuperscript{-1}) | 1 | 2 | 1.0 | Flores et al. 2005 |
| PON production N\textsubscript{2} fixed | mol (mol N\textsuperscript{-1}) | 9 | 6 | 3.0 | |
| PON production NO\textsubscript{3}\textsuperscript{-} user | mol (mol N\textsuperscript{-1}) | 2 | 10 | 0.4 | |
| POC production measured in N\textsubscript{2} fixed | μmol (μg chl a\textsuperscript{-1}) day\textsuperscript{-1} | 5.9 | 6.2 | 1.9 | |
| POC production measured in NO\textsubscript{3}\textsuperscript{-} user | μmol (μg chl a\textsuperscript{-1}) day\textsuperscript{-1} | 7.5 | 7.9 | 1.9 | |
| POC production difference NO\textsubscript{3}\textsuperscript{-} vs N\textsubscript{2} | μmol (μg chl a\textsuperscript{-1}) day\textsuperscript{-1} | 1.6 | 1.7 | |
| PON production measured in N\textsubscript{2} fixed | μmol (μg chl a\textsuperscript{-1}) day\textsuperscript{-1} | 2.9 | 1.9 | 3.0 | |
| PON production difference NO\textsubscript{3}\textsuperscript{-} vs N\textsubscript{2} | μmol (μg chl a\textsuperscript{-1}) day\textsuperscript{-1} | 0.8 | 4.2 | 0.4 | |
| Total POM production in N\textsubscript{2} fixed | μmol (μg chl a\textsuperscript{-1}) day\textsuperscript{-1} | 8.8 | 8.2 | 2.2 | |
| Total POM production in NO\textsubscript{3}\textsuperscript{-} user | μmol (μg chl a\textsuperscript{-1}) day\textsuperscript{-1} | 8.3 | 12.1 | 1.4 | |
| POM production difference NO\textsubscript{3}\textsuperscript{-} vs N\textsubscript{2} | μmol (μg chl a\textsuperscript{-1}) day\textsuperscript{-1} | −0.5 | 3.9 | |

Evolution as well as Mehler reaction, while dark respiration increased. Interestingly, the diurnal cycle of O\textsubscript{2} evolution and uptake as well as electron transport was maintained also in NO\textsubscript{3}\textsuperscript{-} grown cultures. Studies on the diurnal cycle of nitrogenase protein abundance in *Trichodesmium* showed that nitrogenase is synthesized de novo every day (Zehr et al. 1996), resulting in a significant energy demand for protein synthesis (Brown et al. 2008). Nitrogenase was found to be synthesized, yet not activated by post-translational modification, in cells grown even at high levels of NO\textsubscript{3}\textsuperscript{-} (Ohki et al. 1991). These findings suggest that although nitrogenase was not active, NO\textsubscript{3}\textsuperscript{-} grown cells in our study may still have invested a considerable amount of energy for synthesis of nitrogenase. This would cause similar energy requirements as well as protection of nitrogenase from O\textsubscript{2} also in NO\textsubscript{3}\textsuperscript{-} grown cells (i.e. O\textsubscript{2} consumption by dark respiration and Mehler reaction as well as downregulation of photosynthesis during midday), explaining the lack of N effects on chlorophyll fluorescence and O\textsubscript{2} fluxes observed in our study. There is, however, also data suggesting significantly lower expression levels of nitrogenase subunits NifK and NifH in NO\textsubscript{3}\textsuperscript{-} grown cells (Sandh et al. 2011).

In summary, the lack of a clear pCO\textsubscript{2} or N effect on photosynthesis, dark respiration or Mehler reaction confirms that there was no difference in energy generation (ATP and reducing equivalents). The observed treatment effects on contents and production of POC and PON can thus not be explained by differences in the overall energy availability, indicating potential changes down-stream of the electron transport chain. To identify alterations in the energy consuming processes we therefore measured rates of N\textsubscript{2} fixation and C acquisition.

**N\textsubscript{2} fixation**

In agreement with previous results (Kranz et al. 2010), a characteristic change in the diurnal pattern of N\textsubscript{2} fixation was observed at elevated pCO\textsubscript{2}, with the phase of high nitrogenase activity being prolonged toward the evening (Fig. 2). Although integrated daily N\textsubscript{2} fixation rates increased by as much as 60% between 380 and 1400 μatm pCO\textsubscript{2}, PON production was not significantly affected by the different pCO\textsubscript{2} levels. The ARA used for estimating N\textsubscript{2} fixation rates gives a measure of the maximal nitrogenase enzyme activity under the respective assay conditions (approximating gross N\textsubscript{2} fixation) while PON production rates reflect how much N is ultimately incorporated into PON. While there are indications that a considerable share of fixed N is lost before incorporation into PON (Mulholland and Capone 2000, Mulholland 2007), significant uncertainties remain with respect to the absolute values due to methodological issues (Mulholland and Capone 2001 and references therein). It also has to be noted that, in contrast to acetylene reduction during ARA, actual N\textsubscript{2} fixation is dependent on ammonium consumption by downstream metabolism (e.g. Herrero et al.)
Fig. 6. Schematic diagram of the distribution of energy equivalents for PON production under different N sources (N\textsubscript{2} and NO\textsubscript{3}\textsuperscript{−}). Due to the different requirements of N\textsubscript{2} and NO\textsubscript{3}\textsuperscript{−} assimilation with respect to ATP and electron (e\textsuperscript{−}) stoichiometry, N\textsubscript{2} fixation is prone to limitation by ATP while NO\textsubscript{3}\textsuperscript{−} assimilation tends to be limited by e\textsuperscript{−} supply. Please note that the ultimate outcome in terms of PON production in the different N treatments is strongly dependent on the ratio of ATP per e\textsuperscript{−} available, which is, in turn, controlled by the ratio of (pseudo-) cyclic to linear e\textsuperscript{−} transport and the use of energy equivalents by other cellular processes. NaR, nitrate reductase; NiR, nitrite reductase.

2001). However, interpretation of trends within results of each of the methods should be valid. In accordance with our findings on CO\textsubscript{2} sensitivity, previous studies found ARA-based estimates of N\textsubscript{2} fixation to increase more strongly with pCO\textsubscript{2} than estimates of PON production based on cell quotas or \textsuperscript{15}N fixation (Kranz et al. 2010, Garcia et al. 2011). In the natural environment, N release by \textit{Trichodesmium} has been suggested to provide an important N source for a range of associated organisms (Mulholland and Capone 2000, Mulholland 2007), which may be enhanced under elevated pCO\textsubscript{2} according to our data. The high assimilation costs and unavoidable N loss in N\textsubscript{2} fixers impose higher energy requirements compared with NO\textsubscript{3}\textsuperscript{−} users, especially under elevated pCO\textsubscript{2}. As all treatments, however, showed the same energy generation, we expect changes in other energy sinks.

Inorganic C acquisition

Acquisition of inorganic C constitutes a major energy sink in \textit{Trichodesmium} due to the high CCM activities required to compensate for the poor CO\textsubscript{2} affinity of its RubisCO (Kranz et al. 2009). Similarly to O\textsubscript{2} and electron fluxes as well as N\textsubscript{2} fixation, also the affinity for inorganic C was subject to a strong diurnal cycle (Fig. 3), which was previously described by Kranz et al. (2009). The high affinity for inorganic C in the mornings observed in all treatments is in line with the high rates of photosynthesis discussed above. The overall lower affinities at high pCO\textsubscript{2}, especially during the second half of the day, suggest significantly lower operational costs for the CCM which, in turn, allow for the enhanced N\textsubscript{2} fixation observed (Figs 2 and 3). These CO\textsubscript{2}-dependent changes in affinities and the anti-correlation with N\textsubscript{2} fixation are in agreement with previous results (Kranz et al. 2010). The fact that pCO\textsubscript{2} effects are larger in N\textsubscript{2} fixers than in NO\textsubscript{3}\textsuperscript{−} users indicates that higher overall energy requirements of N\textsubscript{2} fixation as well as differences in the stoichiometry of ATP and electron demand (Fig. 6): Provided that the downregulation of CCM activity mainly saves ATP, this surplus energy can be readily used in N\textsubscript{2} fixers to cover the high ATP demand of nitrogenase. In contrast, NO\textsubscript{3}\textsuperscript{−} usage requires only little ATP (for uptake) and is, instead, likely to be limited by the supply of reducing equivalents. Consequently, a downregulation of the
CCM in NO$_3^-$ users would not have the same stimulatory effect on PON production as in N$_2$ fixers.

Energy requirements of the CCM are generally dependent on the C sources and uptake mechanisms. CCM operation in *Trichodesmium* is considered to predominantly consume ATP, as the main C source for this species is HCO$_3^-$ (approximately 80%; Kranz et al. 2009, 2010), which is taken up via a transporter fuelled indirectly by ATP (BicA; Price et al. 2008). Such HCO$_3^-$ transporters are dependent on a Na$^+$ gradient across the plasma membrane and presumably consume 0.5 mol ATP per mol HCO$_3^-$ (Espie and Kandasamy 1994, Hopkinson et al. 2011). Furthermore, the so-called NDH-1$_4$ complex converts CO$_2$ to HCO$_3^-$, thereby driving uptake of CO$_2$ as well as an internal recycling to prevent CO$_2$ leakage (Price et al. 2002, 2008). The reaction is involved in the electron transport chain, receiving electrons from NADPH or ferredoxin that are subsequently transferred to PQ. Intriguingly, NDH-1$_4$ activity leads to a release of protons into the thylakoid lumen, which in turn increases the pH gradient used for ATP synthesis. The observation that this complex seems to be especially active at high pCO$_2$ (Kranz et al. 2010) is in line with the increased ATP demand by enhanced N$_2$ fixation under these conditions (Fig. 2). It has to be noted that the operational costs for BicA and NDH-1$_4$ are still under debate. Provided that the two CCM components have opposing effects on cellular ATP levels, it is crucial to investigate their differential regulation in response to different environmental conditions.

**Conclusions**

Despite the change in energy demand imposed by the different pCO$_2$ levels and N sources, *Trichodesmium* showed no alteration in energy producing pathways. Yet, elevated pCO$_2$ increased cellular POC and PON contents in both N treatments. In N$_2$ fixers, also nitrogenase activity was strongly enhanced with pCO$_2$. Concurrently, CCM activity was downregulated, reducing the use of ATP in active HCO$_3^-$ uptake and allowing its allocation to N$_2$ fixation. The increase in N$_2$ fixation was, however, not reflected in PON production, possibly due to an increase in N loss with increasing pCO$_2$. In NO$_3^-$ users, the lower N-normalized ATP demand for PON production (Table 3) and the better N retention allowed for higher production rates of POC as well as PON compared with N$_2$ fixers. A calculation of the theoretical energy demands of the measured POC and PON production rates (Table 3) revealed that most of the ATP saved from the switch to NO$_3^-$ use (approximately 80%) was invested into increasing the production rates of POC and PON, resulting in almost unaltered ATP demand in our cultures (0.5 ATP residue, Table 3). The concomitant increase in the demand of reducing equivalents may have prevented a full implementation of ATP savings into the production of particulate organic matter (POM). The effects of pCO$_2$ on CCM activity were smaller in NO$_3^-$ users than in N$_2$ fixers, highlighting the dependence of energy reallocation on the stoichiometric demands in energy equivalents: As NO$_3^-$ assimilation requires only little ATP and is limited by electrons (Fig. 6), any spare ATP arising from downregulation of the CCM would not have the same stimulatory effect as in N$_2$ fixers. Interestingly, the diurnal pattern in O$_2$ fluxes usually attributed to protection of nitrogenase was maintained also in NO$_3^-$ grown cells. Further studies are necessary to unravel the effects of different environmental conditions on cellular energy budgets, focusing on energization of the CCM as well as the intricate effects of the NDH-1$_4$ complex on C use efficiency and energy balance.

**Author contributions**

M. E., S. A. K. and B. R. conceived and designed the experiment. M. E. and S. A. K. performed the experiments. M. E. analyzed the data; M. E., S. A. K. and B. R. wrote the paper.

**Acknowledgements** We thank Ulrike Richter, Jana Hölscher and Klaus-Uwe Richter for laboratory assistance and technical support. Grant support was provided by the European Research Council under the European Community’s Seventh Framework Programme (FP7/2007-2013)/ERC grant agreement (205150).

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