Process-understanding of marine nitrogen fixation under global change

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tr>
<td>CA</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>CCM</td>
<td>carbon concentrating mechanism</td>
</tr>
<tr>
<td>C_i</td>
<td>inorganic carbon</td>
</tr>
<tr>
<td>DIC</td>
<td>dissolved inorganic carbon</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>pCO₂</td>
<td>CO₂ partial pressure</td>
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<tr>
<td>POC</td>
<td>particulate organic carbon</td>
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<td>PON</td>
<td>particulate organic nitrogen</td>
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<td>PQ</td>
<td>plastoquinone</td>
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<td>PSI</td>
<td>photosystem I</td>
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<tr>
<td>PSII</td>
<td>photosystem II</td>
</tr>
<tr>
<td>TA</td>
<td>total alkalinity</td>
</tr>
</tbody>
</table>
CONTENTS

1.1 SUMMARY........................................................................................................................................... 1
1.2 ZUSAMMENFASSUNG........................................................................................................................... 3

2. INTRODUCTION

2.1 Marine cycles of C and N – the role of phytoplankton and future trends
   2.1.1 The marine carbon cycle........................................................................................................... 5
   2.1.2 The marine nitrogen cycle....................................................................................................... 7
   2.1.3 Global changes in C and N cycles............................................................................................ 9

2.2 N₂ fixing cyanobacteria
   2.2.1 Key players.............................................................................................................................. 12
   2.2.2 N₂ fixation.............................................................................................................................. 14
   2.2.3 Photosynthesis and respiration .............................................................................................. 16
   2.2.4 Carbon acquisition ................................................................................................................ 19

2.3 Aims and outline of the thesis........................................................................................................... 20

3. PUBLICATIONS

3.1 List of Publications & Declaration of own contribution.................................................................. 23
3.2 Publication I: Interactions of CCM and N₂ fixation in Trichodesmium............................................ 25
3.3 Publication II: Combined effects of N sources and pCO₂ levels on Trichodesmium.................... 39
3.4 Publication III: Cellular inorganic carbon fluxes in Trichodesmium.............................................. 57
3.5 Publication IV: Diversity of ocean acidification effects on marine N₂ fixers.............................. 83

4. SYNTHESIS

4.1 Physiological mechanisms behind ocean acidification responses -
   what we learned from Trichodesmium
   4.1.1 Ocean acidification and energy allocation................................................................................ 115
   4.1.2 Different N sources and energy allocation.............................................................................. 116
   4.1.3 Internal Cᵢ fluxes and energy availability................................................................................. 117

4.2 Response patterns in other N₂ fixers – can we generalize?......................................................... 119
4.3 Implications for future biogeochemical cycles of C and N.......................................................... 122
4.4 Perspectives for future research.................................................................................................... 125
4.5 Conclusion.......................................................................................................................................... 128

5. REFERENCES........................................................................................................................................... 129
1.1 SUMMARY

Diazotrophic cyanobacteria play an important role in the marine nitrogen cycle due to their ability to convert atmospheric N\textsubscript{2} to bioavailable N species. By supplying this fixed N to other phytoplankton, they fuel productivity of phytoplankton communities, especially in oligotrophic regions. With ongoing climate change, N\textsubscript{2} fixers are subject to an array of perturbations in their environment. Several previous experiments suggested the abundant N\textsubscript{2} fixer *Trichodesmium* to respond sensitively to ocean acidification as well as the concurrent changes in other environmental factors. The aim of this thesis was to improve understanding of the underlying mechanisms of these responses and determine whether they can be generalized to other diazotrophs.

In *Publication I*, previous experiments were reviewed, concordantly showing an increase in N\textsubscript{2} fixation rates as well as production of particulate organic carbon and nitrogen (POC and PON) with elevated pCO\textsubscript{2}. The magnitude of responses, however, differed strongly between studies. Growth responses were modulated by light intensity, explaining part of the variability between studies and furthermore suggesting a dependence of CO\textsubscript{2} effects on energy supply. Consequently, based on the observation of a down-regulation of the CCM under high pCO\textsubscript{2}, a reallocation of energy between the carbon concentrating mechanisms (CCM) and N\textsubscript{2} fixation was suggested to fuel the increase in production.

This reallocation of energy was further confirmed in *Publication II* by growing *Trichodesmium* on N sources with different energy demands (N\textsubscript{2} vs. NO\textsubscript{3}\textsuperscript{-}). Lower ATP requirements of NO\textsubscript{3}\textsuperscript{-} assimilation compared to N\textsubscript{2} fixation allowed for higher POC and PON production rates. Results from *Publication II*, however, also highlighted the limitations in energy reallocation imposed by differences in the stoichiometry of energy equivalents required (ATP:NADPH). pCO\textsubscript{2} effects on the CCM were less pronounced in NO\textsubscript{3}\textsuperscript{-} grown cells than in N\textsubscript{2} fixers. While the CCM as well as N\textsubscript{2} fixation require mainly ATP, readily allowing for reallocation of energy equivalents between the two processes under elevated pCO\textsubscript{2}, the high demand in reducing equivalents of NO\textsubscript{3}\textsuperscript{-} assimilation limits the potential for energy reallocation.

Estimating the potential consequences of a CCM down-regulation under ocean acidification for cellular energy budgets requires a detailed understanding of cellular inorganic carbon (C\textsubscript{i}) fluxes. In *Publication III*, the extra- and intracellular C\textsubscript{i} fluxes in
Trichodesmium were analyzed by a combined approach of direct flux measurements, isotope fractionation and modeling. Differing leakage estimates obtained by direct measurements and an isotope fractionation-based approach could be reconciled applying a model based on external as well as intracellular C\textsubscript{i} fluxes. Internal C\textsubscript{i} cycling was thereby shown to play an important role for C\textsubscript{i} acquisition as well as cellular energy budgets in Trichodesmium. The effects of internal C\textsubscript{i} cycling on \textsuperscript{13}C fractionation may have important implications also for interpretation of \textsuperscript{13}C signatures of other phytoplankton.

To estimate the applicability of the response mechanisms identified in Trichodesmium to other N\textsubscript{2} fixers, in Publication IV a species comparison was conducted. Comparison of \textit{pCO\textsubscript{2}} effects on functionally different N\textsubscript{2} fixers revealed high variability in responses between different groups and even species of diazotrophs. Some of the variability could be attributed to differences in nutrient housekeeping, cellular adaptations for protection of nitrogenase from O\textsubscript{2}, and CCM adaptations to different habitats. Although a broad range of N\textsubscript{2} fixers was investigated in this study, for making solid predictions of future N\textsubscript{2} fixation, further studies are necessary to confirm response patterns and include poorly characterized species such as the symbiotic N\textsubscript{2} fixers.
1.2 ZUSAMMENFASSUNG


Die in Publikation I zusammengefassten Publikationen zeigten übereinstimmend einen Anstieg in N\textsubscript{2}-Fixierung sowie Produktion von partikulärem organischem Kohlenstoff und Stickstoff (POC und PON) unter erhöhtem pCO\textsubscript{2}. Das Ausmaß der Effekte varierte dabei stark zwischen den Studien. Wachstumsreaktionen wurden durch die Lichtintensität im jeweiligen Experiment moduliert, was einen Teil der Variabilität zwischen Studien erklären kann, und darüber hinaus eine Abhängigkeit der Reaktionen von der vorherrschenden Energieverfügbarkeit nahelegt. Folglich wurde, basierend auf der Beobachtung einer Herunterregulierung der Kohlenstoffkonzentrierungs-Mechanismen (CCMs) unter erhöhtem pCO\textsubscript{2}, eine Reallokation von Energie zwischen CCM und N\textsubscript{2}-Fixierung als Grundlage für den Anstieg in POC- und PON-Produktion postuliert.

Diese Energierallokation wurde in Publikation II durch Anzucht von Trichodesmium auf Stickstoff-Quellen mit unterschiedlichem Energiebedarf (N\textsubscript{2} vs. NO\textsubscript{3}\textsuperscript{-}) bestätigt. Der niedrige ATP-Bedarf der Assimilation von NO\textsubscript{3}\textsuperscript{-} im Gegensatz zur N\textsubscript{2}-Fixierung ermöglichte höhere POC- und PON-Produktionsraten. Die Ergebnisse aus Publikation II zeigen jedoch auch Grenzen der Energierallokation auf, die durch Unterschiede in der Stöchiometrie der benötigten Energieäquivalente (ATP:NADPH) entstehen. CO\textsubscript{2}-Effekte auf den CCM waren weniger ausgeprägt in Zellen, die auf NO\textsubscript{3}\textsuperscript{-} angezogen wurden, als in N\textsubscript{2}-Fixierern. Während
sowohl CCM als auch N2-Fixierung hauptsächlich ATP benötigen, was eine Reallokation von Energie unter erhöhtem $pCO_2$ begünstigt, limitiert der hohe Bedarf an Reduktionsäquivalenten der NO3-Assimilation die Reallokation von Energie.

Um die möglichen Konsequenzen der Herunterregulierung des CCMs unter Ozeanversauerung für den zellulären Energiehaushalt abzuschätzen, bedarf es eines detaillierten Verständnisses der zellulären Kohlenstoff-Flüsse. In *Publikation III* wurden die extra- und intrazellulären Flüsse von inorganischem Kohlenstoff (Ci) in *Trichodesmium* mithilfe einer Kombination von direkten Flussmessungen, Isotopenfraktionierung und Modellierung untersucht. Unterschiedliche Ergebnisse für die *Leakage*, das Verhältnis von CO2-Efflux zur Gesamt-Aufnahme von Ci, aus direkten Messungen und fraktionierungs-basierten Methoden konnten mithilfe eines Modells zur Übereinstimmung gebracht werden. Hierfür wurden neben den externen Ci-Flüssen über die Plasmamembran auch die internen Ci-Flüsse berücksichtigt. Die Ergebnisse demonstrieren die Bedeutung der internen Flüsse für die Ci-Aufnahme sowie ihre Rolle im zellulären Energiehaushalt von *Trichodesmium*. Auswirkungen des internen Ci-Cyclings auf die $^{13}$C-Fraktionierung sollten auch bei der Interpretation der $^{13}$C-Signatur anderer Phytoplanktonarten beachtet werden.

2. INTRODUCTION

2.1 Marine cycles of C and N – the role of phytoplankton and future trends

2.1.1 The marine carbon cycle

Storing heat as well as carbon, the global oceans play a critical role for earth’s climate (Levitus et al., 2005; Sigman et al., 2010). Temperatures on earth are elevated by so-called greenhouse gases such as CO₂ that prevent the loss of heat from earth to the outer space, making it habitable for life as we know it (Mitchell, 1989). This greenhouse effect also implies that temperatures are sensitive to variations in atmospheric CO₂ concentrations such as those imposed by anthropogenic activity (Mitchell, 1989). In the current state of the system, 98% of carbon in the atmosphere-ocean system is stored in the oceans (Zeebe and Wolf-Gladrow, 2007), providing an enormous carbon pool to buffer changes in atmospheric pCO₂. In this system, equilibrium reactions with other carbon species as well as carbon fixation by phytoplankton play important roles in driving the uptake and storage of CO₂ in the oceans.

Most of the carbon in the oceans is not present in the form of gaseous CO₂, but is converted to an ionic form. When CO₂ dissolves in sea water, it reacts with water to form carbonic acid (H₂CO₃), which subsequently dissociates to form bicarbonate (HCO₃⁻), releasing a proton (H⁺) during the process (eq. 1). Bicarbonate further dissociates into a carbonate ion (CO₃²⁻) and another proton. Consequently, uptake of CO₂ and its subsequent dissolution ultimately decreases the pH of seawater. As a result of these equilibrium reactions, only about 1% of dissolved inorganic carbon (DIC) remains in the form of CO₂, while the bulk of carbon is in the form of HCO₃⁻ (~ 90%) at typical seawater pH (Zeebe and Wolf-Gladrow, 2007).

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{CO}_3^{2-} + 2\text{H}^+ \quad \text{(eq. 1)}
\]

As in reverse, HCO₃⁻ or CO₃²⁻ can also accept protons they are important components of total alkalinity (TA). This parameter can be described as the proton buffer capacity of seawater or, more specifically, the excess of proton acceptors over proton donors relative to a zero level of protons (Dickson, 1981, eq. 2).

\[
\text{TA} = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{B(OH)}_4^-] + [\text{OH}^-] + [\text{HPO}_4^{2-}] + 2[\text{PO}_4^{3-}] + [\text{H}_2\text{SiO}_4^-] + [\text{NH}_3] + [\text{HS}^-] - [\text{H}^+] - [\text{H}_2\text{SO}_4^-] - [\text{HF}] - [\text{H}_3\text{PO}_4] - [\text{H}_2\text{CO}_3] \quad \text{(eq. 2)}
\]
By determining how many of the protons released e.g. during CO2 dissolution (eq. 1) can be consumed, TA influences both the amount of CO2 that can dissolve in seawater and the change in pH for a given amount of CO2 dissolution. Apart from HCO3⁻ and CO3²⁻, a number of other ions, including nutrients such as phosphorus and ammonia, determine alkalinity of sea water (Wolf-Gladrow et al., 2007). Uptake of nutrients such as NO₃⁻ by phytoplankton can alter TA, as phytoplankton maintain cellular electroneutrality by pumping protons as a charge compensation (Wolf-Gladrow et al., 2007). For estimating biogeochemical effects of biological processes on seawater TA, the integrated effects on larger time and spatial scales need to be considered. For instance, while the process of N₂ fixation itself does not affect TA, N₂ fixation followed by remineralization of organic matter and nitrification decreases TA (Wolf-Gladrow et al., 2007).

The transport of carbon from the surface to the deep ocean is mediated by the physical and biological carbon pumps (Volk and Hoffert, 1985; Fig. 1). The physical pump denotes carbon transport driven by the global differences in sea water temperature and thus solubility for CO2. As CO2 is more soluble in cold water, sinking of cold and dense water masses at high latitudes transports CO2 to depth. At lower latitudes, warming of upwelling water masses can lead to subsequent release of CO2. The biological pumps, including the so-called soft-tissue pump and the carbonate counter pump, are driven by plankton in the surface ocean. The soft-tissue pump is based on the capacity of autotrophs to fix CO2 into organic matter during photosynthesis, which leads to a transient decrease in surface water CO2 concentrations. While a large fraction of this organic matter is remineralized in the surface ocean, releasing CO2, part of it is exported to the deep ocean in the form of sinking particles (export production). Subsequent re-equilibration of seawater with the atmosphere drives CO2 uptake. Some of these phototrophs are calcifiers and therefore also take part in the carbonate counter pump. Calcification leads to a decrease in DIC and TA in the surface ocean and shifts the carbonate system to higher CO2 concentrations. Consequently, the ratio of these two processes determines the net effect of the biological carbon pump, which under current conditions favors the uptake of CO2 from the atmosphere (Sarmiento et al., 2002). Aside from light driving photosynthesis, primary productivity is strongly dependent on the availability of different nutrients that phytoplankton need to build their enzymes, structural elements and nucleic acids allowing them to grow.
2.1.2 The marine nitrogen cycle

In many marine ecosystems, primary productivity is limited (or co-limited) by the availability of reactive nitrogen. Most phytoplankton is dependent on N in the form of NH$_4^+$ or NO$_3^-$, which can be supplied either by remineralization of organic matter in the euphotic zone (regenerated production) or from external sources (new production; Eppley and Peterson, 1979). As sources of ‘new N’ are scarce in most ocean regions, concentrations of reactive N are often depleted in the upper mixed layer (Gruber, 2005). The bulk of N is remineralized in the euphotic zone by zooplankton and small prokaryotes, releasing NH$_4^+$. Yet, as for carbon, also a fraction of the N sinks to the deep ocean as part of organic matter, entering a microbially driven chain of reactions that involves N compounds with a large range of different oxidation states (Fig. 1). The pathways taken and the chemical form of N that is finally resupplied to the surface ocean depend on how much O$_2$ the local environment contains (Canfield et al., 2010).

Fig. 1: Marine cycles of C (blue arrows) and N (green arrows). Starting from the left, the physical carbon pump, the carbonate counter pump, and the soft-tissue pump are shown. Please note that the carbonate counter pump and soft-tissue pump are in reality closely connected, as CaCO$_3$ can act as ballasting material in sinking POC particles. Suboxic processes of the N cycle are depicted in the shaded box in the lower right corner. Please note that the stoichiometry of C to N can be altered during transfer via the food web and the microbial loop as well as during sinking.
In oxic zones, nitrifiers convert PON to $\text{NO}_3^-$, which can be resupplied to the euphotic zone by upwelling. In $\text{O}_2$ depleted regions, $\text{NO}_3^-$ can be used as an alternative electron acceptor (‘$\text{NO}_3^-$ respiration’). During denitrification, $\text{NO}_3^-$ is reduced to $\text{N}_2\text{O}$ and further to $\text{N}_2$, both of which can be lost from the marine system by outgassing. More recently, another N loss process was discovered to play an important role the marine system, namely anaerobic ammonium oxidation (anammox), which includes the reaction of $\text{NH}_4^+$ with $\text{NO}_2^-$ yielding $\text{N}_2$. This previously unrecognized process was found to account for a considerable share of the loss of reactive N, both in shelf sediments (Thamdrup and Dalsgaard, 2002) and in oxygen minimum zones associated to major upwelling systems such as the Peruvian and Benguela upwelling systems (Hamersley et al., 2007; Kuypers et al., 2005).

In opposition to these N loss processes, $\text{N}_2$ fixers in the euphotic zone can access the virtually unlimited pool of atmospheric $\text{N}_2$ and convert it to forms available to other phytoplankton. In the tropical Atlantic and subtropical North Pacific Oceans, $\text{N}_2$ fixation has been estimated to support $\sim$ 25 to 50% of total primary production, with the remaining share being supported by $\text{NO}_3^-$ upwelling (Carpenter et al., 1999; Dore et al., 2002). The importance of marine $\text{N}_2$ fixation in the global N cycle has increasingly been recognized over the past few decades. Based on early rate measurements, marine $\text{N}_2$ fixation was thought to be relatively unimportant on a global scale (Capone and Carpenter, 1982; Lipschultz and Owens, 1996). This was later-on questioned by the observation that $\text{NO}_3^-$ supply by upwelling was far too low to support the N demand calculated from primary production measurements (reviewed by Arrigo, 2005). The conclusion that global $\text{N}_2$ fixation had been severely underestimated was initially based on the upward revision of $\text{N}_2$ fixation estimates for the abundant $\text{N}_2$ fixer *Trichodesmium* (Capone et al., 2005; Gruber, 2005). Additionally, with the development of new molecular tools in the past decade, previously unrecognized, single-celled $\text{N}_2$ fixers were discovered that are now believed to contribute a significant share of marine $\text{N}_2$ fixation (e.g. Moisander et al., 2010; Montoya et al., 2004). Furthermore, severe draw-backs in a commonly used protocol for determination of $\text{N}_2$ fixation rates were suggested to induce a significant underestimation of $\text{N}_2$ fixation estimates (Mohr et al., 2010b). With these new discoveries in both $\text{N}_2$ fixation and N loss processes, the picture of the marine N budget is rapidly changing, fueling an ongoing debate on the apparent imbalance in the marine N budget (e.g. Codispoti, 2007; Codispoti et al., 2001; Gruber and
Galloway, 2008; Voss et al., 2013). It is beyond question that $N_2$ fixers play a key role in determining this budget.

By supplying reactive N to N limited ecosystems, $N_2$ fixers not only play a crucial role for the N cycle but also provide a tight link to the C cycle (e.g. Arrigo, 2005). Through the release of reactive N either directly or subsequent to remineralization, $N_2$ fixation fuels productivity of the entire community. As several abundant diazotrophs, such as *Trichodesmium*, are buoyant and not heavily grazed and furthermore have been shown to excrete or release large amounts of N to their environment (e.g. Capone et al., 1997), the bulk of N fixed by these organisms is generally expected to be recycled in the euphotic zone rather than sinking to the deep ocean (La Roche and Breitbarth, 2005). This also implies that diazotrophs themselves are not an important part of the biological pump. By supplying ‘new N’ to other primary producers, however, diazotrophs can indirectly contribute to C export (Fig. 1). Some diazotrophs form symbioses with diatoms, which are effective export producers (Carpenter et al., 1999; Foster et al., 2007). The dense blooms formed by these diatom-diazotroph-associations have been suggested to contribute substantially to C export in the tropical North Atlantic (Subramaniam et al., 2008). Net export of organic matter from the euphotic zone is commonly related to new production based on upwelling of NO$_3^-$ or $N_2$ fixation (Eppley and Peterson, 1979). While NO$_3^-$ upwelling often involves upward flux of DIC close to the stoichiometric requirements of phytoplankton, however, $N_2$ fixation can introduce N to the marine system without any concurrent C inputs (Eppley and Peterson, 1979). When considering the potential of the biological carbon pump to lower atmospheric CO$_2$ concentrations, primary production based on $N_2$ fixation thus plays an important role in driving net CO$_2$ sequestration from the atmosphere.

### 2.1.3 Current changes in C and N cycles

Both the C cycle and the N cycle are subject to drastic changes due to anthropogenic activities. While atmospheric $p$CO$_2$ levels have oscillated between 180 and 280 µatm on glacial and interglacial time scales, anthropogenic CO$_2$ emissions have driven concentrations to a level unprecedented for the last 800,000 years due to the ongoing combustion of fossil fuels and land use changes (Lüthi et al., 2008; Petit et al., 1999; Siegenthaler et al., 2005). Since the industrial revolution, atmospheric $p$CO$_2$ has risen from 280 to 400 µatm (http://keelingcurve.ucsd.edu/). Yearly CO$_2$ emissions have been continuously increasing in
the past decades (Canadell et al., 2007), and until the end of this century, atmospheric $pCO_2$

is expected to reach 750 $\mu$atm (IPCC scenario IS92a; IPCC, 2007; Fig. 2) or even 1000 $\mu$atm

(Raupach et al., 2007). The oceans are the largest sink of this anthropogenic CO$_2$ and have
taken up about half of the CO$_2$ produced between 1800 and 1994 (Sabine et al., 2004). While
this buffers the effects of anthropogenic CO$_2$ emissions, it also has severe implications for
chemical conditions in the oceans. Due to the increasing uptake of CO$_2$, pH in the oceans is
decreasing, a phenomenon termed ocean acidification (Caldeira and Wickett, 2003; Fig. 2).

Sea water pH has decreased by 0.1 units since the industrial revolution and is expected to
drop by another 0.3 units until the end of this century (Feely et al., 2009; Fig. 2). This has
diverse impacts on marine organisms, including changes in the bioavailability of essential
nutrients, adverse effects on calcification, as well as the numerous effects of pH on cellular
metabolism (Riebesell and Tortell, 2011). At the same time, phytoplankton requiring CO$_2$ for
photosynthesis may benefit from the rising CO$_2$ availability (e.g. Beardall and Raven, 2004;
Rost et al., 2008). The balance of these potentially opposing effects differs between
organisms depending on their physiological adaptations, which results in highly diverse
response patterns between different groups and even species or strains of marine organisms
(Hendriks et al., 2010; Kroeker et al., 2010).

Aside from these direct impacts on marine carbonate chemistry, rising CO$_2$ in the
atmosphere also affects global temperatures by absorbing infrared radiation. About 80% of
the earth’s heat content originating from anthropogenic activity is absorbed by the oceans
(Levitus et al., 2005). Rising sea water temperatures affect the metabolism of marine
organisms in numerous ways and have already been shown to shift species distribution of
various taxa (e.g. Beaugrand and Reid, 2003; Hughes, 2000; Poertner, 2012; Toseland et al.,
2013). In addition to these direct effects, temperature indirectly affects phytoplankton by
altering their physical and chemical environment. As temperatures rise, stratification in the
oceans is expected to increase (Sarmiento et al., 2004; Fig. 2). This will, on the one hand,
increase the average light intensity phytoplankton is exposed to and, on the other hand,
decrease nutrient inputs from below the mixed layer (Sarmiento et al., 2004). At high
latitudes, the increase in light intensity due to stratification along with diminished sea ice
cover may stimulate primary production. In low latitudes, however, increasing nutrient
limitation is expected to decrease productivity (Arrigo et al., 2008; Behrenfeld et al., 2006;
Steinacher et al., 2010).
Introduction

Marine cycles of C and N

Fig. 2: Effects of rising atmospheric pCO₂ on the marine system. A) Direct effects of pCO₂ increase on marine carbonate chemistry (modified after Wolf-Gladrow et al., 1999). B) Effect of global warming on stratification and its consequences for irradiance and nutrient input to the upper mixed layer (modified after Rost and Riebesell, 2004). Predictions for year 2100 are based on the IPCC IS92a scenario (IPCC, 2007).

Owing to the potential effects on NO₃⁻ inputs to the euphotic zone, the expected changes in stratification also have significant implications for the N cycle. As NO₃⁻ supply by upwelling may decrease, it has been suggested that N₂ fixers may become more important in future oceans (Doney, 2006). Aside from these feedbacks of stratification, however, the N cycle is subject to significant direct perturbations by humankind, which have major implications for both terrestrial and marine ecosystems (e.g. Galloway et al., 2003). Especially in coastal areas, riverine and atmospheric N inputs are strongly increasing. On a global scale, anthropogenic production of fixed N has increased by a factor of 10 since the late 19ᵗʰ century, with the largest source being industrial N₂ fixation by the Haber-Bosch reaction (Erisman et al., 2008), followed by cultivation-induced biological N₂ fixation (e.g. cultivation of legumes) and fossil fuel combustion releasing the potent greenhouse gas N₂O (Galloway et al., 2003; Galloway et al., 2004; Manne and Richels, 2001). Recent studies showed that effects of anthropogenic N emissions are not limited to coastal areas but that a significant fraction is also deposited in the open oceans (Duce et al., 2008), antagonizing potential NO₃⁻ limitation induced by increasing stratification. In eutrophied marine ecosystems, where large amounts of organic matter are produced and subsequently remineralized, a substantial fraction of O₂ available in the water can be consumed by respiration (e.g. Arrigo, 2005). In the Baltic Sea, for instance, eutrophication is leading to formation of so-called ‘dead zones’, fuelling P release from anoxic sediments, which in turn fosters N₂ fixation (Vahtera et al.,...
In the open ocean, warming and increased stratification decrease O$_2$ concentrations and lead to the expansion of oxygen minimum zones (Helm et al., 2011). This favors N loss processes such as anammox and denitrification, not only reducing bioavailable N concentrations, but potentially also increasing radiative forcing due to increasing N$_2$O release to the atmosphere (reviewed by Gruber, 2011). On the other hand, ocean acidification has been suggested to decrease marine nitrification rates, reducing the production of N$_2$O but also the supply of NO$_3^-$ to phytoplankton (Beman et al., 2011). Predictions of the future N budget are difficult to make since the balance of these different trends remains poorly understood.

Yet, almost certainly, marine N$_2$ fixers will be affected by all of these changes in C and N cycles, in direct or more indirect ways: the competitive success of N$_2$ fixers depends on the changing supply of fixed N species; the geographical distribution of N$_2$ fixers may be extended to higher latitudes by warming (e.g. Breitbarth et al., 2007); and their physiological performance will be affected by ocean acidification (e.g. Hutchins et al., 2007; Kranz et al., 2009). As N$_2$ fixers play important roles in the N cycle as well as the C cycle, each of these effects, in turn, has the potential to feed back on these biogeochemical cycles. N$_2$ fixation is furthermore dependent on the availability of other elements such as phosphorus and iron, which, in turn, may be altered for instance by anthropogenically induced changes in dust deposition (Karl et al., 2002; Mills et al., 2004). To gain confidence in predicting the potential effects of this array of environmental changes, one needs to understand the physiological mechanisms by which N$_2$ fixers respond to their environment.

### 2.2 N$_2$ fixing cyanobacteria

#### 2.2.1 Key players

Long before the evolution of eukaryotes, even preceding the evolution of oxygenic photosynthesis, N$_2$ fixation evolved in anoxygenic photoautotrophs (Raymond et al., 2004). To date, it is confined to a range of archaea and bacteria, including cyanobacteria (Raymond et al., 2004). Cyanobacteria were the first to use light energy for fixing CO$_2$, producing O$_2$ as a by-product (oxygenic photosynthesis), and thereby initiated oxygenation of the oceans and the atmosphere (e.g. Kasting and Siefert, 2002). In the past millions of years, cyanobacteria have adapted to a wide range of habitats including most extreme conditions, such as hot
springs, hypersaline lakes and deserts, as well as symbioses with fungi (forming lichens; Whitton and Potts, 2002).

$N_2$ fixing cyanobacteria are common in freshwater as well as brackish systems such as the Baltic Sea, where they raise ongoing attention due to their dense, partly toxic, annual blooms causing significant nuisance to humans (e.g. Paerl and Huisman, 2009; Sivonen et al., 1989; Stal et al., 2003). Less conspicuous to humans, but all the more important for the global N cycle, are $N_2$ fixers in the open ocean. Observations of the bloom-forming cyanobacterium *Trichodesmium* date back to the voyages of Captain Cook (1770) and Charles Darwin (1839, Chancellor and Van Wyhe, 2009), yet their capacity for fixing $N_2$ was only discovered in the 20th century (Dugdale et al., 1961). Although the genus has been extensively studied since, many open questions as to its physiology remain, especially regarding the regulation of photosynthesis and $N_2$ fixation (Bergman et al., 2013; Capone et al., 1997). Vast surface blooms of *Trichodesmium* visible from space are common in oligotrophic open ocean regions of the tropics and subtropics, but also coastal blooms are known to occur frequently (La Roche and Breitbarth, 2005; Subramaniam and Carpenter, 1994). On a global scale, *Trichodesmium* has been estimated to contribute up to 50% to marine $N_2$ fixation (Mahaffey et al., 2005). Regarding diazotrophs other than *Trichodesmium*, unicellular $N_2$ fixers as well as diatom-diazotroph-associations are both believed to play important roles, yet their relative contribution to global marine $N_2$ fixation is still uncertain (e.g. Foster et al., 2007; Langlois et al., 2005; Villareal, 1994; Zehr et al., 1998). While unicellular marine diazotrophs have been overlooked until recently (Waterbury et al., 1979; Zehr et al., 1998), open ocean symbioses between heterocyst-forming, $N_2$ fixing cyanobacteria and the diatom *Rhizosolenia* were discovered already in the early 20th century (Lemmermann, 1905). Due to the difficulties in isolating and culturing these species, physiological traits of the key species of both groups are poorly characterized to date (Foster et al., 2007; Zehr et al., 2008).

In all $N_2$ fixing cyanobacteria, circumstances of their evolution during times of high atmospheric $CO_2$ and low $O_2$ concentrations are still reflected in the characteristics of two of their key enzymes, responsible for fixation of $CO_2$ and $N_2$. To efficiently use these enzymes under present-day conditions, a range of physiological adaptations is necessary, which impose considerable energy demands. The assimilation of C and N is thus tightly intertwined
not only due to common biochemical pathways but also since they compete for the energy produced in photosynthesis and respiration.

2.2.2 N₂ fixation

Breaking the triple bond that connects two N atoms in a molecule of N₂ is an extremely energy demanding reaction. As much as 16 ATP molecules in addition to 8 electrons are needed to reduce N₂ to NH₄⁺ (Postgate, 1998). The reaction involves formation of H₂, which, in fact, consumes a significant share of the energy required for N₂ fixation (Postgate, 1998). In addition to these direct costs of N₂ fixation, autotrophic diazotrophs have to invest a considerable amount of energy to cover secondary costs, which arise from the fact that their N₂ fixing enzyme nitrogenase, having evolved during times of low O₂ concentrations, is inhibited by O₂ (Fay, 1992; Robson and Postgate, 1980).

Different groups of cyanobacteria have evolved different mechanisms for protecting nitrogenase from photosynthetically produced O₂, separating the two processes in time and/or in space (Fig. 3). Single-celled cyanobacteria such as *Cyanothece* generally separate N₂ fixation from photosynthesis in time, fixing N₂ only during the night. This diurnal separation requires a circadian rhythm in N₂ fixation, respiration and photosynthesis as well as storage of C and N in the form of glycogen and cyanophycin (Mohr et al., 2010a; Schneegurt et al., 1994; Sherman et al., 1998). Most filamentous cyanobacteria, including many freshwater species as well as the Baltic *Nodularia* and diatom symbionts, have fully differentiated N₂ fixing cells within their filaments. These cells termed heterocysts lack the O₂-evolving photosystem II (Wolk et al., 1994) and have a thick cell wall isolating them from O₂ in their surroundings (Fay, 1992). Heterocysts and vegetative cells exchange C and N in the form of carbohydrates and amino acids (Popa et al., 2007). In *Trichodesmium*, separation in time and space are combined. Firstly, nitrogenase is only expressed in a subset of cells within a filament, the diazocytes (Lin et al., 1998). Additionally, O₂ fluxes and N₂ fixation are regulated in a concerted diurnal cycle, including a down-regulation of photosynthesis during the peak in N₂ fixation (Berman-Frank et al., 2001b). No transporters for direct N transfer between cells in a filament have been found, implying that non-diazotrophic cells have to rely on N released to the environment by the diazocytes (Mulholland and Capone, 2000). Accordingly, high N loss, reaching up to 80% of fixed N, has been measured in laboratory and
field studies, providing an important source of N also to associated organisms (reviewed in Mulholland, 2007).

Another important characteristic of nitrogenase is its exceptionally high requirement for iron (Berman-Frank et al., 2001a; Raven, 1988). Consequently, marine N$_2$ fixation is often limited by iron availability (Falkowski, 1997; Moore et al., 2009; Morel and Price, 2003; Paerl et al., 1994). Yet, regional differences have been observed, with iron limitation to N$_2$ fixers being especially prevalent in the Pacific, while in the Atlantic Ocean, phosphorus limitation is suggested to predominate (Sañudo-Wilhelmy et al., 2001; Sohm et al., 2008). Furthermore, N$_2$ fixation rates were demonstrated to be strongly correlated to light intensities in field and laboratory studies, which can be explained by its high energy demand (Breitbarth et al., 2008; Fu and Bell, 2003; Sañudo-Wilhelmy et al., 2001).
2.2.3 Photosynthesis and respiration

Cyanobacteria being the ancestors of eukaryotic chloroplasts (Margulis, 1971), they have no organelles comparable to chloroplasts or mitochondria. Consequently, photosynthesis and respiration are not physically separated in cyanobacterial cells. The two processes occur concurrently on the thylakoid membrane, even sharing some of the protein complexes of the electron transport chain (ETC, Fig. 4). The so-called light reactions involve production of O₂ and regeneration of energy equivalents (ATP and NADPH) in the ETC, which can subsequently be used for fixation of CO₂ into carbohydrates in the dark reactions. During respiration, these carbohydrates are broken down, ultimately leading to reduction of O₂ to H₂O as well as regeneration of ATP in the ETC (Falkowski and Raven, 2007).

The main components of the photosynthetic light reactions are the two photosystems (PSI and PSII). These contain chlorophyll a molecules in their reaction centers that can be excited by light energy, forcing them to emit an electron, which drives the transport of electrons along the series of protein complexes making up the ETC. To maximize light absorption, photoautotrophs have light harvesting complexes, which consist of pigments that capture light energy and transfer it to the reaction centers. In cyanobacteria, formerly named ‘blue-green algae’, these so-called phycobilisomes consist of the pigments phycoerythrin, phycocyanin and allophycocyanin that absorb efficiently between the red and blue region of the light spectrum (Sidler, 1994). Just like in other photoautotrophs, following excitation and charge separation in PSII, a water molecule is split to resupply the emitted electron. From PSII, electrons flow via several plastoquinones to the cytochrome b₆/f complex and further via a plastocyanin to PSI, substituting electrons emitted during charge separation in the reaction center. From PSI, electrons are transferred to ferredoxin, which can subsequently reduce NADP⁺ to NADPH+H⁺. For transporting electrons from PSII to the cytochrome b₆/f complex, the plastoquinones need to be reduced and subsequently reoxidized, which involves uptake of protons on the cytoplasmic side of the thylakoid membrane and subsequent release of protons into the thylakoid lumen. The resulting proton motive force fuels ATP synthase, which translocates protons via its membrane-spanning channel subunit to regenerate ATP from ADP and Pₐ. ATP and the reducing equivalent NADPH are subsequently distributed to the various biochemical pathways in the cell, including C fixation in the Calvin Cycle, C acquisition as well as N₂ fixation (Fig. 4).
Several alternative pathways to this so-called linear electron transport allow for adjusting energy generation to its consumption by downstream processes, depending on the environmental conditions. While in linear electron transport, ATP and NADPH are produced in a molar ratio of ~ 1.3 (Rochaix, 2011), the high ATP demands of C fixation and N₂ fixation require additional ATP production. This can be achieved by cyclic electron transport, where electrons are transferred from ferredoxin back to the PQ pool instead of reducing NADP⁺ (Fig. 4). Cyanobacteria have a higher ratio of PSI to PSII compared to eukaryotes, favoring processes such as cyclic electron transport around PSI that elevate the availability of ATP (Fujita et al., 1994). In addition to adjusting the stoichiometry of energy equivalents, cyanobacteria need to prevent the adverse effects of excess light energy (photodamage).
Introduction

Under circumstances when electrons cannot be drained fast enough from the ETC, they can react with O$_2$, forming radicals (e.g. Asada, 1999). Many cyanobacteria, such as *Trichodesmium*, have efficient mechanisms for adjusting to the very high light intensities encountered during surface blooms as well as lower light intensities encountered during mixing into deeper water layers (Andresen et al., 2009; Breitbarth et al., 2008). So-called state transitions allow for distribution of excitation energy between the photosystems to adjust to changes in the redox state of the ETC, presumably by detachment of the phycobilisomes from the photosystems (e.g. Andresen et al., 2009; Campbell et al., 1998). Several of the photo-protective mechanisms concomitantly alter the stoichiometry of energy equivalents as they divert electrons away from the usual pathways. For instance, the Mehler reaction involves transfer of electrons from ferredoxin onto O$_2$, which is subsequently reduced to water in a reaction presumably involving flavoproteins in cyanobacteria (Helman et al., 2003; Mehler, 1951). Thereby, the Mehler reaction contributes to ATP regeneration without concurrent production of NADPH and serves as an electron sink under high light intensities (Asada, 1999). Recently, a mechanism involving only PSII for increasing ATP production and dissipating energy (via plastoquinol oxidase) was suggested to play an important role in open ocean cyanobacteria, yet, the prevalence of this pathway is uncertain to date (Mackey et al., 2008; Zehr and Kudela, 2009).

In N$_2$ fixing cyanobacteria, regulation of photosynthetic and respiratory electron transport is also vital for controlling cellular O$_2$ concentrations. Especially in *Trichodesmium*, tight regulation of O$_2$ evolution and uptake processes is of great importance since N$_2$ fixation and photosynthesis are carried out concurrently, yet without heterocysts. Mehler reaction and respiration are suggested to protect nitrogenase in this genus (Berman-Frank et al., 2001b; Kana, 1993; Milligan et al., 2007), and are subject to substantial diurnal changes as demonstrated by measurements of O$_2$ fluxes as well as chlorophyll fluorescence (Berman-Frank et al., 2001b; Küpper et al., 2004). Also in single-celled as well as heterocystous cyanobacteria, respiration has been found to reduce O$_2$ concentrations and supply energy for N$_2$ fixation (e.g. Scherer et al., 1988). The interplay of N$_2$ fixation with O$_2$ evolution and uptake pathways is regulated by a combination of transcriptional and post-translational mechanisms that still bears many open questions. Both circadian rhythms (i.e., clock genes) and feedbacks induced by the redox state of the PQ pool are supposed to play important roles (e.g. Kumar et al., 2010; Küpper et al., 2004; Sherman et al., 1998; Toepel et al., 2008).
2.2.4 Carbon acquisition

The cyanobacterial C fixing enzyme RubisCO has a particularly low affinity to its substrate CO₂ compared to other autotrophs (Badger et al., 1998) and is furthermore susceptible to a competing reaction with O₂ (photorespiration), both of which can be traced back to the environmental conditions at the time of its evolution. Yet, by a range of structural and physiological adaptations, collectively termed carbon concentrating mechanisms (CCM), cyanobacteria are able to accumulate CO₂ in their cells by a factor of up to ~ 500 (Kaplan et al., 1980).

The CCM of cyanobacteria consists of several components, including mechanisms for the uptake of CO₂ and HCO₃⁻ as well as a protein microbody unique to cyanobacteria, the carboxysome (Fig. 4). While CO₂ can in principle diffuse into the cell and is therefore the least costly C source available, accumulation of Cᵢ against a concentration gradient requires active transport. Due to its low equilibrium concentration, slow interconversion of CO₂ and HCO₃⁻ and slow diffusion in seawater, CO₂ availability can quickly become limiting (Riebesell et al., 1993; Zeebe and Wolf-Gladrow, 2007). Moreover, as the plasmamembrane is permeable to CO₂, any accumulation of CO₂ in the cell entails leakage. To circumvent these draw-backs of CO₂ uptake, cyanobacteria additionally take up HCO₃⁻ by active transporters (BicA and SbtA; Price et al., 2002; Price et al., 2004). In addition to direct HCO₃⁻ uptake, the so-called NDH-1₃ and NDH-1₄ complexes convert CO₂ to HCO₃⁻ in the cytosol (Maeda et al., 2002). Prior to fixation into carbohydrates by RubisCO, HCO₃⁻ needs to be converted to CO₂. The conversion is accelerated by carbonic anhydrase (CA), which is located in the carboxysomes along with RubisCO. As carboxysomes are reported to have a much lower CO₂ permeability than the plasmamembrane (Dou et al., 2008), they allow for efficient accumulation of CO₂ in close vicinity of RubisCO.

While the CCM is generally hypothesized to consume a significant fraction of cellular energy reserves, the detailed requirements of the different components have not been quantified to date (e.g. Raven, 2010; Raven and Lucas, 1985). The HCO₃⁻ transporters are supposed to be fuelled directly or indirectly by ATP (Price et al., 2002). The NDH complex, in turn, is part of the photosynthetic/respiratory ETC, and, in fact, contributes to establishing the proton gradient required for ATP regeneration (Price et al., 2002). In addition to the costs imposed by active uptake processes, costs due to leakage as well as costs of building
Most seminal studies on the constituents and functioning of the CCM in cyanobacteria were conducted on the model organism *Synechococcus* (e.g. Badger and Price, 1989; Maeda et al., 2002; Price et al., 2004). With the sequencing of other cyanobacterial genomes, group- and species-specific differences in the suite of CCM components encoded by different cyanobacteria were revealed (Badger et al., 2006). Two major groups of cyanobacteria are distinguished with regard to their CCM, which are referred to as α- and β-cyanobacteria (Badger et al., 2002). This classification is based on the occurrence of one of the two different forms of RubisCO prevalent in cyanobacteria (forms 1A and 1B), which correlates with differences in the carboxysome protein structure as well as the suite of C\textsubscript{i} transporters (Badger et al., 2002). Sequence analysis of different cyanobacteria also showed that many strains encode for a range of C\textsubscript{i} transporters with different affinities, allowing them to adjust to changes in environmental conditions (Badger et al., 2006). In different groups of phytoplankton, CCM activity was shown to be modulated by light, temperature and availability of macro- and micronutrients (e.g. Beardall and Giordano, 2002). Yet, the effects seem to differ strongly between studies with different species and under different conditions, making it hard to deduce comprehensive response patterns (Giordano et al., 2005a). Relatively few studies have investigated the regulation of C\textsubscript{i} acquisition in ecologically important cyanobacteria, with the exception of *Trichodesmium* (e.g. Kranz et al., 2009; Kranz et al., 2010; Levitan et al., 2010a). Responses of the CCM to changes in CO\textsubscript{2} availability and potential feedbacks to other cellular processes as well as interactive effects with other environmental factors are of particular interest in the context of global change research.

### 2.3 Aims and outline of the thesis

With the ongoing anthropogenic perturbations of C and N cycles, marine N\textsubscript{2} fixing cyanobacteria are subject to manifold changes in their environment. Studies on climate change responses of N\textsubscript{2} fixers have so-far primarily focused on the abundant *Trichodesmium*, which showed an exceptionally high sensitivity to changing pCO\textsubscript{2} levels. Growth, production of POC and PON, as well as N\textsubscript{2} fixation rates were stimulated under high pCO\textsubscript{2}, N\textsubscript{2} fixation being increased by up to 140% at pCO\textsubscript{2} levels projected for the end of this century (Barcelos
The primary aim of this thesis is to improve understanding of the mechanisms behind the climate change responses of N$_2$ fixers. While the first three studies investigate several aspects of these physiological mechanisms in *Trichodesmium*, the fourth study addresses the question whether these responses can be generalized to other N$_2$ fixers.

Previous results on ocean acidification effects on *Trichodesmium* are characterized by a high variability between studies. Yet, pCO$_2$ responses were concordantly suggested to depend on interactions between C and N assimilation. Summarizing available literature data and pinpointing emerging patterns, *Publication I* reviews the current state of knowledge on the interactions between CCM and N acquisition in *Trichodesmium*.

A key aspect emerging from the first publication is the hypothesis that energy reallocation between CCM and N metabolism is an important driver behind CO$_2$ responses of *Trichodesmium*. In *Publication II*, this hypothesis is addressed by determining CO$_2$ effects under differing cellular energy states imposed by N sources with different energy demands. Analysis of growth, cellular composition as well as the underlying physiological processes permits new insights into the mechanisms of energy generation and allocation under different environmental conditions.

In view of the proposed down-regulation of the CCM as a possible source of energy under ocean acidification, cellular C$_i$ fluxes as well as their responses to environmental conditions are of key interest. In *Publication III*, C$_i$ fluxes in *Trichodesmium* are analyzed under different pCO$_2$ levels and N sources with a combination of methods, including isotope fractionation analyses, measurements of external C$_i$ fluxes by membrane inlet mass spectrometry as well as modeling of internal C$_i$ fluxes.

Finally, for being able to estimate implications of these findings on a global scale, it is essential to know whether the response patterns and mechanisms observed in *Trichodesmium* are valid also for other species. In *Publication IV*, ocean acidification responses of a range of functionally different N$_2$ fixers are investigated. Responses of three different species are determined in a pCO$_2$ manipulation experiment, compared to literature data and discussed in view of possible links of response patterns with ecological niches as well as physiological differences.
3. LIST OF PUBLICATIONS
& DECLARATION OF OWN CONTRIBUTION


*The review article was written in collaboration with the co-authors.*


*The experiment was designed together with the co-authors. I performed the experiments (with help by S.A. Kranz during N₂ fixation measurements) and analyzed the data. I drafted the manuscript and finalized it in collaboration with the co-authors.*


*The experiment was designed together with the co-authors. I performed the experiments, analyzed experimental and model results, and wrote the manuscript in collaboration with the co-authors.*


*The experiment was designed together with the co-authors. I performed the experiments and analyzed the data. I drafted the manuscript and finalized it in collaboration with the co-authors.*
Publication I

Interactions of CCM and N\textsubscript{2} fixation in *Trichodesmium*
Interactions between CCM and N₂ fixation in *Trichodesmium*

Sven A. Kranz · Meri Eichner · Björn Rost

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Abstract  In view of the current increase in atmospheric pCO₂ and concomitant changes in the marine environment, it is crucial to assess, understand, and predict future responses of ecologically relevant phytoplankton species. The diazotrophic cyanobacterium *Trichodesmium erythraeum* was found to respond strongly to elevated pCO₂ by increasing growth, production rates, and N₂ fixation. The magnitude of these CO₂ effects exceeds those previously seen in other phytoplankton, raising the question about the underlying mechanisms. Here, we review recent publications on metabolic pathways of *Trichodesmium* from a gene transcription level to the protein activities and energy fluxes. Diurnal patterns of nitrogenase activity change markedly with CO₂ availability, causing higher diel N₂ fixation rates under elevated pCO₂. The observed responses to elevated pCO₂ could not be attributed to enhanced energy generation via gross photosynthesis, although there are indications for CO₂-dependent changes in ATP/NADPH + H⁺ production. The CO₂ concentrating mechanism (CCM) in *Trichodesmium* is primarily based on HCO₃⁻ uptake. Although only little CO₂ uptake was detected, the NDH complex seems to play a crucial role in internal cycling of inorganic carbon, especially under elevated pCO₂. Affinities for inorganic carbon change over the day, closely following the pattern in N₂ fixation, and generally decrease with increasing pCO₂. This down-regulation of CCM activity and the simultaneously enhanced N₂ fixation point to a shift in energy allocation from carbon acquisition to N₂ fixation under elevated pCO₂ levels. A strong light modulation of CO₂ effects further corroborates the role of energy fluxes as a key to understand the responses of *Trichodesmium*.

Keywords  CO₂ concentrating mechanism · Diazotroph · Energy allocation · N acquisition · Ocean acidification · Photosynthesis

Introduction

Marine phytoplankton are responsible for almost half of all photosynthetic carbon fixation on Earth and play a vital role in altering the CO₂ exchange between ocean and atmosphere (Gruber 2004; Maier-Reimer et al. 1996). Some prokaryotic algae affect the primary productivity and thus CO₂ uptake capacity of the oceans by yet another process, the fixation of dinitrogen (N₂) into biomass. As nitrate is often limiting phytoplankton growth, so-called diazotrophs play a crucial role in many marine ecosystems by providing a new source of biologically available nitrogen.

One of these diazotrophs, *Trichodesmium*, is able to form massive blooms known as “sea-sawdust”, covering large areas of the surface ocean in the tropical and sub-tropical regions (Capone et al. 2005; Mahaffey et al. 2005). The first mention of *Trichodesmium* was made in 1770 by Captain Cook in the Coral Sea near Australia. In 1839, Charles Darwin described a bloom by this species during his cruise with the Beagle as “The whole surface of the water, as it appeared under a weak lens, seemed as if covered by chopped bits of hay, with their ends jagged.” In 1961, Dugdale and colleagues reported the “ability of *Trichodesmium* to fix atmospheric nitrogen.” Recent estimates on its contribution to overall marine N₂ fixation range up to 50% (Mahaffey et al. 2005) and in oligotrophic...
areas of the oceans, *Trichodesmium* is responsible for a major part of the primary production (Falkowski 1997; Gruber and Sarmiento 1997). Henceforward, *Trichodesmium* was acknowledged to exert a significant influence on the global nitrogen and carbon cycle.

As a key species in the marine ecosystem, *Trichodesmium* was used in several studies on the regulation of N\(_2\) fixation (e.g., Berman-Frank et al. 2001a, b, 2007; Capone et al. 2005; Kana 1993; Mulholland et al. 2004). Unlike other diazotrophs, this species has evolved special features allowing N\(_2\) fixation to occur during the photoperiod. To protect the oxygen-sensitive enzyme nitrogenase, which catalyzes the reduction of N\(_2\) to NH\(_3\), from photosynthetic O\(_2\) evolution, this species has developed distinct diurnal rhythms in photosynthesis and N\(_2\) fixation (Berman-Frank et al. 2001a; Breithbarth et al. 2008; Fu and Bell 2003). The potential influence of CO\(_2\)-induced changes in seawater chemistry or combined effects of different environmental factors have, however, been ignored for a long time.

Four recent studies tested the effect of different CO\(_2\) concentrations on growth, biomass production, and elemental composition of *Trichodesmium* (Barcelos é Ramos et al. 2007; Hutchins et al. 2007; Kranz et al. 2009; Levitan et al. 2007). They concordantly demonstrated higher growth and/or production rates under elevated pCO\(_2\), with a magnitude exceeding those CO\(_2\) effects previously seen in other marine phytoplankton. Nonetheless, the responses differed significantly in terms of absolute rates, cell quotas or C/N ratios (Table 1). For instance, the stimulation in N\(_2\) fixation and/or PON production between present-day pCO\(_2\) values (370–400 \(\mu\)atm) and those predicted for the year 2100 (750–1,000 \(\mu\)atm) ranged between 35 and 240%. Several of these laboratory studies also observed higher rates of growth or carbon fixation under elevated pCO\(_2\), yet the degree of stimulation differed. Responses in cell quotas or elemental stoichiometry varied in degree as well as direction with changes in pCO\(_2\).

These large differences in CO\(_2\) sensitivity obtained by the different studies raise questions about experimental conditions other than the applied pCO\(_2\) levels. It should be noted that all of the mentioned studies used the same *Trichodesmium* isolate (IMS101) from the Atlantic Ocean and even the same artificial seawater media (YBCII). They differed, however, in the light intensities applied during incubations, which may serve as an explanation for the differences in CO\(_2\) sensitivity. A recent companion study assessed the combined effect of pCO\(_2\) (150 vs. 900 \(\mu\)atm) and light (50 vs. 200 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) and could indeed show that light levels do modify the responses of *Trichodesmium* to pCO\(_2\) (Kranz et al. 2010a; Levitan et al. 2010b). Notably, the relative stimulation in growth, POC, or PON production rates was highest in the low-light treatment and diminished under high light (Fig. 1). These observations not only corroborate the exceptionally high CO\(_2\) sensitivity in the N\(_2\) fixing *Trichodesmium*, but also reconcile previous findings.

But how is the process of N\(_2\) fixation coupled to the availability of CO\(_2\)? Or why are CO\(_2\) effects on growth and

<table>
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<th>Conditions</th>
<th>Rates and elemental composition for <em>Trichodesmium</em> IMS 101 grown at different pCO(_2) levels and 25°C</th>
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<tr>
<td>Light ((\mu)mol photons m(^{-2}) s(^{-1}))</td>
<td>pCO(_2) ((\mu)atm)</td>
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<tr>
<td>80–120</td>
<td>400</td>
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<td></td>
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<td>(\sim) 100</td>
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The reported values relate to present day (370–400 \(\mu\)atm) and future scenarios for pCO\(_2\) (750–1,000 \(\mu\)atm) used in the different studies. Please note the differences in normalization and light levels.
biomass production modulated by light availability? These and other questions regarding the underlying reasons for the strong CO₂ effects in *Trichodesmium* can only be answered if the responses of physiological key processes are understood. In the following, the processes of N₂ fixation, photosynthesis, and carbon acquisition in *Trichodesmium* will be described and discussed, focusing on the influence of light and CO₂ as well as possible interactions.

**N₂ fixation**

Thriving in the oligotrophic regions of the ocean, *Trichodesmium* mainly fuels its N demand by fixation of N₂ (Mulholland et al. 2004). The reduction of N₂ is conducted by the enzyme nitrogenase, which consists of two proteins, the iron protein (dinitrogenase reductase) and the iron–molybdenum protein (dinitrogenase). This ancient enzyme evolved under O₂-free conditions in the Archean (Falkowski 1997; Falkowski and Raven 2007) and its iron protein is especially sensitive to O₂ (Bergman et al. 1997). Thus, photosynthetic energy generation and N₂ fixation within the same cell appear to be mutually exclusive processes. To circumvent this inhibitory effect, diazotrophic organisms evolved biochemical as well as morphological adaptations to separate photosynthetic O₂ evolution and N₂ fixation in time and space (Berman-Frank et al. 2007). *Trichodesmium* differs from other diazotrophs in this respect, as it lacks the clear spatial (i.e., heterocysts) and temporal separation (day vs. night activity) of the two processes (Berman-Frank et al. 2007).

In *Trichodesmium*, nitrogenase is localized in subsets of neighboring cells, so-called diazocytes, which comprise about 15–20% of cells within a trichome (Berman-Frank et al. 2003; Durner et al. 1996; Fredriksson and Bergman 1995). In contrast to heterocysts, diazocytes contain both, photosystem I (PSI) and photosystem II (PSII) (Bergman et al. 1997) and can, hence, conduct oxygenic photosynthesis (Fig. 2). To protect nitrogenase from photosynthetically produced O₂, *Trichodesmium* has developed a distinct diurnal rhythm in PSII activity and N₂ fixation (Berman-Frank et al. 2001b; Lin et al. 1999). In combination with O₂-reducing mechanisms like the Mehler reaction (Berman-Frank et al. 2001b; Küpper et al. 2004; Milligan et al. 2007), N₂ fixation in *Trichodesmium* reaches rates similar or even higher than those reported for heterocystous or other non-heterocystous cyanobacteria (Bergman et al. 1997). As only the diazocytes are capable of fixing N₂, the residual cells of a filament depend strongly on the supply of bioavailable N from the N₂ fixing cells. These N sources are either the direct outcome of the N₂ reduction, i.e., ammonia (NH₃) or ammonium (NH₄⁺), or the amino acid glutamine (Mulholland et al. 2004; Mulholland and Capone 2000; Wannicke et al. 2009), which is the product of the glutamine synthetase (GS) reaction. The inorganic or organic N sources released by the diazocytes...
can subsequently be taken up by the vegetative cells to fuel their N demand (Fig. 2, Mulholland et al. 2004).

In terms of the diurnal regulation, N2 fixation was shown to be controlled on different levels. Diazocytes develop primarily during the dark period at the same time when cell division takes place in *Trichodesmium* (Sandh et al. 2009). In these diazocytes, the nitrogenase is synthesized de novo each morning and for the most part degraded during the afternoon and night (Capone et al. 1990; Levitan et al. 2010a; Sandh et al. 2009). On the post-translational level, part of the iron protein pool is modified to an inactive form in the afternoon, which can persist throughout the night, being activated again in the morning (Zehr et al. 1993). The switch between active and inactive state of the iron protein is regulated by ADP-ribosylation via the activating enzyme dinitrogenase reductase-activating glycohydrolase (DRAG; Halbleib and Ludden 2000), which requires ATP and MnCl2 in a specific ratio (Saari et al. 1986). Both, the transcription of nitrogenase and the post-translational modification of its iron protein were shown to be controlled by an endogenous rhythm (Chen et al. 1998). Rates of N2 fixation followed the diurnal pattern in nitrogenase abundance and its post-translational modification, with highest activities during midday (Berman-Frank et al. 2001b; Levitan et al. 2010a, 2007; Milligan et al. 2007). The timing of both, demodification of the iron protein and nitrogenase activity was shown to be affected by light intensities (Kranz et al. 2010a; Zehr et al. 1996). The described diurnal pattern in cell division, protein build-up and degradation, as well as physiological activity may represent a mean for *Trichodesmium* to optimize its energy budget.

Regarding energy requirements for N2 fixation, the splitting of the triple-bond of N2 to form NH3 requires at least 16 ATP as well as eight electrons:

\[
\text{N}_2 + 16\text{ATP} + 8e^- + 8\text{H}^+ \rightarrow 2\text{NH}_3 + 16\text{ADP} + 16\text{Pi} + \text{H}_2
\]

The subsequent production of glutamine and glutamate within the glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) reaction further demands energy in form of 1 ATP and 1 NADPH per glutamate produced. Thus, N acquisition in *Trichodesmium* is strongly dependent on availability of energy. Although respiratory processes may contribute some of the energy, most of the ATP and reductants are provided through photosynthesis (Bergman et al. 1997; Breitbarth et al. 2008). Light appears to play a crucial role both as the...
source of energy for N₂ fixation (Breitbarth et al. 2008; Kranz et al. 2010a) and as a cue for synthesis and/or demodification of nitrogenase (Zehr et al. 1993). N₂ fixation rates, obtained from acetylene reduction assays, have been shown to increase up to light intensities of about 300 μmol photons m⁻² s⁻¹ (Bell and Fu 2005; Breitbarth et al. 2008). As cell densities of Trichodesmium are often highest at depths of 20–40 m (Capone et al. 1997), prevailing light intensities may commonly limit the energy supply to nitrogenase in natural populations (Sanudo-Wilhelmy et al. 2001).

The increase in N₂ fixation or PON production under elevated pCO₂ (Barcelos é Ramos et al. 2007; Hutchins et al. 2007; Kranz et al. 2009; Levitan et al. 2007) was found to be caused by a prolongation of the N₂ fixation period (Kranz et al. 2010a). As these enhanced N₂ fixation rates were not accompanied by larger protein pools of nitrogenase (Levitan et al. 2010b), they may have been achieved by post-translational modification and/or higher energy availability for nitrogenase activity. Higher rates of N₂ fixation also requires more C skeletons in the form of 2-oxoglutarate, which are provided via the tri-carboxylic acid pathway, for instance, during high rates of respiration in the early hours of the photoperiod (Berman-Frank et al. 2001b; Kranz et al. 2009). Enhanced respiration rates at elevated pCO₂, which could be expected from the stimulation in PON production, have, however, not been reported.

As a direct effect of CO₂ on nitrogenase seems unlikely, a higher share of energy to nitrogen fixation was suggested to be the cause for the observed stimulation in N₂ fixation and PON production at high pCO₂ (Kranz et al. 2010a). This explanation is further substantiated by the observation that the CO₂ effects in Trichodesmium were strongly modulated by light (Fig. 1). Yet, what is the source of the additional energy and resources supporting the observed stimulation in N₂ fixation and PON production under elevated pCO₂? To answer this question, one must first look at photosynthetic energy production and how it responds to changes in CO₂ availability.

### Photosynthesis and electron transport

The energy for metabolic processes is provided by the phosphorylation of ADP as well as the reduction of NADP⁺ due to photosynthetic and respiratory electron transport. The electron transport chain (ETC) in the thylakoid membrane of cyanobacteria differs in several important aspects from that of eukaryotes. As cyanobacteria lack chloroplasts, both photosynthesis and respiration are conducted on the thylakoid membrane, sharing specific protein complexes (Schmetterer 1994), including the plastoquinone (PQ) pool, the cytochrome b₆f (cyt b₆f) complex, and plastocyanin (Fig. 3). A concerted regulation of photosynthetic and respiratory electron flow is, hence, required to avoid detrimental feedbacks and/or photodamage.

During photosynthesis, electrons are introduced into the ETC via PSII, while respiration feeds electrons into the PQ complex via the cytochrome b₆f protein complex, Cyt C oxidase cytochrome C oxidase, Fd ferredoxin, Fln flavoprotein, FNR ferredoxin NADP reductase, H⁺ proton, NADP nicotinamide-adenine-dinucleotide-phosphate, NDH NADPH dehydrogenase, OEC oxygen evolving complex, PC plastocyanin, PQ plastoquinone, PSI photosystem 1, PSII photosystem 2, SDH succinate dehydrogenase, SOD superoxide dismutase.
pool via a succinate dehydrogenase (SDH) (Schmetterer 1994). Thus, photosynthetic as well as respiratory electron transport generate the proton motive force needed for ATP production (Mitchell 1961). Electrons from plastocyanin can be allocated to cytochrome C oxidase (cyt C oxidase), the terminal electron acceptor of the respiratory ETC, leading to the reduction of O$_2$ to H$_2$O. During the dark, all electrons from respiration are donated to the cyt C oxidase, while in the light, cyt C oxidase competes with PSI for electrons (Milligan et al. 2007). The flux of electrons to O$_2$, producing H$_2$O at the cyt C oxidase, is likely to be influenced by the ratio of PSI:PSII and the location of the phycobilisomes (Küpper et al. 2004), but may also be affected by kinetic constraints of the terminal oxidase and PSI (Milligan et al. 2007). In contrast to eukaryotes, the ratio of PSI:PSII in cyanobacteria is usually above one and highly variable (Fujita et al. 1994). For *Trichodesmium*, reported values cover a large range from 1.3 (Berman-Frank et al. 2001b, 2007) to values between 2 and 4 (Brown et al. 2008; Levitan et al. 2010b, 2007) and up to values as high as 24 (Subramaniam et al. 1999). Thus, the high ratio of PSI:PSII allows for efficient electron flow through PSI, preventing an overly high-redox state of the components of the ETC.

From PSI, electrons can be transferred to ferredoxin and allocated to a ferredoxin NADP reductase (FNR), producing the reductant NADP + H$^+$ in the so-called linear electron transport (Vermaas 2001). In addition to linear electron transport, several alternative electron transport routes have been found to operate. In the so-called cyclic electron transport around PSI, electrons transferred from PSI to ferredoxin can be fed back to the cyt b$_{6}f$ complex, thereby increasing proton translocation and thus ATP synthesis (Stroebel et al. 2003). Electrons needed in N$_2$ fixation are allocated from the reduced ferredoxin onto nitrogenase (Flores and Herrero 1994). Therefore, N$_2$ fixation represents a strong quenching mechanism in *Trichodesmium*, yielding an oxidized PQ pool and improved electron transport. Alternatively, electrons can be transferred from ferredoxin onto O$_2$ in the pseudo-cyclic electron transport called the Mehler reaction. Such an electron transfer does not necessarily lead to H$_2$O$_2$ production by superoxide dismutase (SOD) as superoxide can directly be reduced to H$_2$O by a flavoprotein in cyanobacteria (Helman et al. 2003). Nonetheless, H$_2$O$_2$ was shown to be present in *Trichodesmium* cells during the time of N$_2$ fixation, which could only be explained by SOD activity (Berman-Frank et al. 2001b). High rates of Mehler reaction have been found in *Trichodesmium* both in laboratory experiments (Kranz et al. 2010a; Levitan et al. 2007; Milligan et al. 2007) and in the field (Berman-Frank et al. 2001b; Kana 1993). The Mehler reaction has been suggested to serve as a nitrogenase protection mechanism as it can strongly decrease the O$_2$ concentrations in the vicinity of nitrogenase (Berman-Frank et al. 2001b; Kana 1993; Milligan et al. 2007). However, light-dependent $^{18}$O$_2$ uptake was also observed at times of low nitrogenase activity (Kranz et al. 2010a) or under conditions when N$_2$ fixation was repressed (Milligan et al. 2007; Eichner et al. unpublished).

In addition, measurements of $^{18}$O$_2$ uptake showed that Mehler reaction can function as a photoprotection mechanism in *Trichodesmium* at high light intensities (Kranz et al. 2010a). In any case, the transfer of electrons to either N$_2$ fixation or Mehler reaction needs to be balanced in order to prevent inhibition of nitrogenase by O$_2$ and electron shortage for nitrogenase.

The energy produced in photosynthesis and respiration is subsequently allocated according to the needs of the different metabolic pathways. The largest share of ATP and reductants is used for the fixation of CO$_2$ in the Calvin Cycle (Table 2). Other major energy sinks in *Trichodesmium* are the fixation of N$_2$ and the active uptake of inorganic carbon. All these pathways compete for energy but differ in their proportional demands for ATP and reductants (Table 2). CO$_2$ fixation needs a ratio of ATP:NADPH + H$^+$ of 3:2, while N$_2$ fixation requires ATP and electrons in a ratio of 2:1. Inorganic carbon uptake also requires energy in the form of ATP and reductants, however, not much is known about respective requirements for this process in *Trichodesmium*. To cover the energetic demand of these processes, which vary strongly over the photoperiod, a concerted regulation in the production of ATP and reductants is necessary. Linear electron transport yields ATP and NADPH + H$^+$ in a ratio close to one, while non-linear electron flow raises this ratio significantly. In *Trichodesmium*, the high PSI:PSII ratios and alternative electron flow indicate generally higher generation of ATP than of NADPH + H$^+$, which may serve to satisfy the need for a high ATP:NADPH + H$^+$ ratio imposed by N$_2$ fixation.

As energy supply to the different metabolic pathways is first and foremost driven by light availability, efficient photosynthesis is crucial for an organism like *Trichodesmium* with its high energy demands. In its natural habitat, *Trichodesmium* is subject to a highly variable light regime and so it is not surprising that *Trichodesmium* shows high plasticity in light acclimation (Andresen et al. 2010). These acclimation mechanisms include modifications in pigment composition, enhanced protein turnover (Andresen et al. 2010) and state transition, i.e., reversible uncoupling of phycobilisomes or individual pycobiliproteins from PSI and PSII (Küpper et al. 2004). This high flexibility in light capture efficiency enables effective energy generation by *Trichodesmium*, thereby avoiding drawbacks like D1 protein degradation or superoxide production at PSII.

The effects of different pCO$_2$ levels on photosynthetic electron generation and its subsequent utilization in
Table 2  Relative energy demand for carbon and nitrogen fixation for \textit{Trichodesmium}

<table>
<thead>
<tr>
<th>Process</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>C fixation</td>
<td>Allen (2002)</td>
</tr>
<tr>
<td>N acquisition</td>
<td>Flores and Herrero (1994)</td>
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<table>
<thead>
<tr>
<th>Process</th>
<th>Energy demand</th>
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<tr>
<td></td>
<td>ATP</td>
<td>Electrons from</td>
</tr>
<tr>
<td>C fixation</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>N acquisition</td>
<td>9</td>
<td>6</td>
</tr>
</tbody>
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Estimated values relate to the proportional demand relative to units of N, assuming a C:N ratio of 6. N acquisition includes assimilation steps from N₂ to glutamate. For calculation of the ATP:NADPH + H⁺ ratios, the quantity of electrons needed in N₂ fixation was converted to a corresponding amount of NADPH + H⁺ produced in linear electron transport. Please note that the active C uptake would add to the energetic cost for C assimilation, while N₂ can diffuse into the cell. The energetic demand of the different C acquisition systems is, however, still under debate.

\textit{Trichodesmium} have been examined using MIMS measurements (Kranz et al. 2009, 2010a; Levitan et al. 2007), chlorophyll fluorescence measurements (Levitan et al. 2007, 2010a), and protein analysis (Levitan et al. 2010b). These studies showed that gross O₂ production and PSII chlorophyll fluorescence were not affected by the availability of CO₂, although changes were found in the composition of the ETC, with an increased ratio of PSI to PSII at elevated pCO₂ (Levitan et al. 2010b). This finding indicates a higher capacity for cyclic electron transport around PSI, allowing for an increase in ATP production at the expense of NADP reduction. Considering the high ratio of ATP-reductant required in N₂ fixation (Table 2), this may in turn contribute to the observed stimulation in nitrogenase activity under elevated pCO₂. Nonetheless, as the applied pCO₂ levels did not alter the overall energy generation it cannot explain the observed stimulation in growth and biomass production. In consequence, one must search for other causes of the apparent surplus of energy under elevated pCO₂. As inorganic carbon (Cᵢ) uptake and its subsequent fixation represent the largest energy sink (Table 2), these processes certainly need to be considered to understand CO₂-dependent changes in the energy budget of \textit{Trichodesmium}.

\textbf{Inorganic carbon acquisition}

As autotrophs, cyanobacteria use energy to produce organic compounds from inorganic CO₂ in the Calvin Cycle, the first step being catalyzed by the enzyme Ribulose bisphosphate carboxylase/oxygenase (RubisCO). This ancient enzyme evolved, similarly to the nitrogenase, during times of elevated CO₂ levels and low O₂ concentrations and is characterized by a very low-affinity for its substrate CO₂, a slow maximum turnover rate, as well as a susceptibility to a competing reaction with O₂ (Badger and Andrews 1987; Tortell 2000). Cyanobacterial RubisCO has one of the highest half-saturation concentrations for CO₂ of all autotrophic organisms (\(K_M\) of 105–185 \(\mu\)mol l\(^{-1}\) CO₂; Badger et al. 1998). Consequently, RubisCO represents a potential bottleneck for primary production by cyanobacteria in today’s oceans. All cyanobacterial species investigated to date, however, possess mechanisms to enhance the carboxylation efficiency of their RubisCO, reducing the risk of carbon limitation. These processes are commonly referred to as CO₂ concentrating mechanisms (CCMs; Badger et al. 2006; Giordano et al. 2005; Kaplan and Reinhold 1999). Although CCM functioning should significantly repress the oxygenase function of RubisCO (Colman 1989), photorespiration has recently been found to occur under certain conditions in \textit{Synechocystis} PCC6803 (Eisenhut et al. 2008). If operative in \textit{Trichodesmium}, this pathway would allow decreasing the O₂ concentration in the vicinity of nitrogenase.

In cyanobacteria, the operation of a CCM generally involves a suite of CO₂ and HCO₃⁻ acquisition systems, as well as a distinct assembly of RubisCO and carbonic anhydrase (CA) within so-called carboxysomes (Badger and Price 2003; Cannon et al. 2001; Price et al. 2008; Raven 2003). Characterization of cyanobacterial Cᵢ uptake has thus far revealed five different systems, primarily based on studies with cyanobacterial strains such as \textit{Synechococcus} spp. and \textit{Synechocystis} spp. These five Cᵢ acquisition systems comprise two inducible high-affinity HCO₃⁻ transporters (BCT1, Omata et al. 1999; SbtA, Shibata et al. 2002), a constitutive low-affinity HCO₃⁻ transporter (BicA, Price et al. 2004), a constitutive low-affinity system to convert CO₂ into HCO₃⁻ (CO₂ uptake facilitator) at the NDH-1 complex located at the thylakoid membrane (NDH-1, Maeda et al. 2002; Price et al. 2002; Shibata et al. 2001), as well as an inducible high-affinity CO₂ uptake facilitator based on a modified NDH-1 complex (NDH-1, Maeda et al. 2002; Shibata et al. 2001). All of these measures function to increase the HCO₃⁻ concentration in the cytosol of the cell. The accumulated HCO₃⁻ passes the protein shell of the carboxysome and, catalyzed by a CA, gets converted to CO₂ for subsequent fixation by RubisCO. The differences in the composition of the carboxysomes and the details of their genetic assembly allow
differentiating the genera of cyanobacteria into two classes, the α- and β-cyanobacteria. Further information on the role of carboxysomes in the cyanobacterial CCM can be found in several reviews (Badger et al. 2006; Cannon et al. 2001; Kaplan and Reinhold 1999; Price et al. 2002). All in all, a large diversity in CCMs has evolved that allows cyanobacteria to grow over a wide range of $C_4$ concentrations. Differences in CCMs may, however, explain why certain species like *Trichodesmium* respond strongly to changes in $C_4$ availability.

Genomic analysis for the CCM in *Trichodesmium* revealed that it belongs to the β-cyanobacteria and its CCM comprises only few of the known CCM components (Badger et al. 2006; Price et al. 2008). Two $C_4$ acquisition systems have been identified in *Trichodesmium*, the HCO$_3^-$ transporter BicA and the CO$_2$ uptake facilitator NDH-14 (Fig. 4). In contrast to other marine β-cyanobacteria (e.g., *Anabaena*, *Crocosphaera*, *Lyngbya*, *Nodularia*, *Synechococcus PCC7002*), *Trichodesmium* lacks CO$_2$-responsive genes (*CcmR/CmpR*) as well as genes associated with high-affinity $C_4$ acquisition systems (NDH-13, BCT1, SbtA; Price et al. 2008). In view of the strong CO$_2$ dependence in growth and biomass production (Barcelos e´ Ramos et al. 2007; Hutchins et al. 2007; Kranz et al. 2009, 2010a; Levitan et al. 2007), one could assume that the CCM of *Trichodesmium* functions only insufficiently to saturate the carboxylation reaction of RubisCO (Raven et al. 2005). Physiological studies have, however, revealed $C_4$ affinities high enough to saturate rates of photosynthesis under CO$_2$ concentrations typical for the present-day ocean (Kranz et al. 2009).

In *Trichodesmium*, inorganic carbon was found to be supplied mainly by the uptake of HCO$_3^-$ via the BicA transporter (Kranz et al. 2009, 2010a). This transporter was first described in the marine cyanobacterium *Synechococcus PCC7002* (Price et al. 2004) as being a low-affinity, high-flux transporter for HCO$_3^-$; further characterization revealed that BicA is dependent on a sodium (Na$^+$) gradient across the plasma membrane, which energizes a Na$^+$/HCO$_3^-$ symporter (Espie and Kandasamy 1994). The Na$^+$ extrusion is thought to be operated via a plasma membrane located, so-called Mnh complex (Woodger et al. 2007). Alternatively, the Na$^+$ gradient might be generated by a H$^+$/Na$^+$ antiport via PxcA (Price et al. 2008). Despite uncertainties about the actual functioning, genes encoding both Mnh and PxcA are present in *Trichodesmium*. Provided that these complexes exchange H$^+$ and Na$^+$, the overall process might mitigate the cytosolic alkalinization resulting from HCO$_3^-$ uptake.

The gene encoding BicA appears to be constitutively expressed (Price et al. 2004), at least in the freshwater cyanobacteria *Synechocystis PCC6803*. Only recently, also in *Trichodesmium* the regulation of CCM genes was investigated over a range of CO$_2$ concentrations, different temperatures and over the photoperiod (Levitan et al. 2010c), showing constitutive gene expression for bicA1, ndhF4 (encoding subunits of BicA and NDH-14) as well as carboxysomal-related genes. Despite constitutive gene expression, a high plasticity in response to different pCO$_2$ (150–1,000 μatm) was found in the affinities of the HCO$_3^-$ transport (Kranz et al. 2009). The apparent discrepancy between CCM gene expression and CCM activities points to a post-translational modification of BicA or alterations in the Na$^+$ gradient driving this transporter. Next to the influence of pCO$_2$ on HCO$_3^-$ uptake affinities, the CCM activity of *Trichodesmium* was greatly affected by the diurnal rhythm in N$_2$ fixation and photosynthesis (Kranz et al. 2009).

Even though CO$_2$ uptake via NDH-14 plays only a minor role in $C_4$ uptake of *Trichodesmium*, this component of the CCM might be important to avoid efflux of CO$_2$ from the cytosol. NDH-14 is a protein complex located at the thylakoid membrane and its subunit, the chpX, converts CO$_2$ into HCO$_3^-$, by using electrons from reduced ferredoxin or NADPH + H$^+$ (Fig. 4). Due to its ability to use electrons from the ETC and to drive H$^+$ translocation through the thylakoid membrane, this protein may be involved in cyclic electron flow around PSI and thus contribute to ATP production (Price et al. 2002). In Kranz et al. (2010a), it was shown that CO$_2$ utilization by NDH-14 increases with elevated pCO$_2$ as well as light intensities, which may

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**Fig. 4** A schematic model for the CO$_2$ concentrating mechanism in *Trichodesmium*, based on genetic homologies (Price et al. 2008). BicA represents the low-affinity, high-flux rate HCO$_3^-$ transporter, which is driven by a Na$^+$ gradient. The buildup of the Na$^+$ gradient needed to drive the BicA transporter is not fully understood yet. NDH-14 represents a low-affinity CO$_2$ uptake facilitator located at the thylakoid membrane. The NDH-14 is thought to be involved in cyclic electron transport of photosynthesis. *ATP synthase* adenosine-5′-triphosphate synthase, CA carbonic anhydrase, *POC* particulate organic carbon, *PSI* photosystem 1, *PSII* photosystem 2, *RubisCO* ribulose bisphosphate carboxylase/oxygenase.
consequently enhance the production of ATP and fuel the respective needs of processes such as N₂ fixation. Based on isotopic fractionation data as well as MIMS-based carbon fluxes, it was further demonstrated that the CO₂ efflux in *Trichodesmium* is moderate (20–50% of total Cᵢ uptake) and internal Cᵢ cycling via the NDH-1₄ is highly efficient, especially under high CO₂ concentrations (Kranz et al. 2010a). This Cᵢ cycling might yield an energetic surplus, both by preventing the efflux of previously taken up carbon as well as by the buildup of the H⁺ gradient over the thylakoid membrane.

In conclusion, the CCM of *Trichodesmium* appears to be strongly influenced by light intensities, competing processes such as N₂ fixation, as well as by changes in CO₂ availability. This complex interplay of factors can be explained by the high energetic costs of the CCM. An effect of light levels on Cᵢ affinities has been observed in several species from different taxa (Beardall and Giordano 2002), which was ascribed to changes in the degree of energy limitation on active Cᵢ uptake. Energy limitation on the CCM can, however, also be imposed by N₂ fixation, simply by competing for energy at times of high nitrogenase activities. This would explain the diurnal changes in Cᵢ affinities following N₂ fixation (Fig. 5). Last but not least, similarly to many other species (Giordano et al. 2005), the availability of CO₂ was shown to strongly alter the CCM in *Trichodesmium* (Kranz et al. 2009, 2010a). Such high plasticity in CCM regulation may serve as a reason for the observed CO₂-dependent stimulation in growth and bio-plasticity in CCM regulation may serve as a reason for the differences in light intensities between studies (150 vs. 200 µmol photons m⁻² s⁻¹)

**Ecological implications**

Owing to the poor catalytic properties of RubisCO (Badger et al. 1998), the energetic costs of the CCM are high but its operation is nonetheless crucial for *Trichodesmium*. This is especially true during bloom conditions, when pH levels rise and dissolved inorganic carbon (DIC) concentrations can be significantly lowered in the ambient seawater (Kranz et al. 2010b). In addition, the aggregation of several hundred filaments to so-called puffs or tufts, a typical phenomenon for *Trichodesmium* under bloom conditions, can lead to a distinct boundary layer effect with lowered DIC concentration in the close vicinity of the cells (Kranz et al. 2010b). The reduced Cᵢ availability during the progression of a bloom coincides with decreasing levels of other nutrients, which may in turn also influence the CCM.

Regulation of the CCM in response to changes in N availability has been reported for different phytoplankton species (Beardall and Giordano 2002). In *Chlamydomonas reinhardtii*, for instance, the CCM was down-regulated under N depletion (Giordano et al. 2003), which may reflect lower carbon demand and serves to maintain the C:N ratio relatively constant. Controlling elemental stoichiometry is of fundamental importance for ensuring optimal functioning of the enzymatic machinery (Beardall and Giordano 2002). In *Dunaliella tertiolecta* and *Chlorella emersonii*, however, affinities for CO₂ were enhanced under N depletion (Beardall et al. 1991; Young and Beardall 2005). This type of CCM regulation may serve to improve the N use efficiency by reducing the N requirement for synthesis of RubisCO (Beardall et al. 1991; Raven 1991). By suppressing photorespiration, up-regulation of CCMs under N-limitation may also prevent the exudation of downstream products such as glycolate and thus the loss of N (Giordano et al. 2005).

For diazotrophs like *Trichodesmium*, a regulation of the CCM would intuitively seem to be uncoupled from N availability as N₂ fixers are not dependent on scarce nitrogen sources such as NO₃⁻ or NH₄⁺ or dissolved organic nitrogen. The acquisition of C and N are, however, coupled via their demand for energy (cf. Fig. 5). In *Dunaliella salina*, an increase in CCM activity was measured in cells grown on NH₄⁺ instead of NO₃⁻, supposedly due to increased energy availability to the CCM in the case of the more reduced nitrogen source (Giordano 1997; Giordano and Bowes 1997). Similarly, changes in the availability of dissolved organic nitrogen compounds or NH₄⁺ (released by the diazocytes or provided by other sources) might influence CCM activities in *Trichodesmium* by altering the energy demand of N assimilation. As C and N assimilation compete for energy, the interaction will be strongly influenced by the overall energy supply.
For *Trichodesmium*, effects of CO$_2$ on growth and production were shown to be light dependent (Fig. 1). In its natural environment, *Trichodesmium* is subject to a wide range of light intensities, mostly because of the vertical motion of the cells in the euphotic zone (Villareal and Carpenter 1990). The CO$_2$ effect on *Trichodesmium* will therefore differ depending on its vertical distribution in the water column. As CO$_2$ effects on *Trichodesmium* proved to be diminished at higher light intensities, which prevail close to the ocean’s surface, the current rise in pCO$_2$ levels will mainly affect cells residing in deeper water layers. The predicted shoaling of the upper mixed layer will yield an increase of average light intensities, while nutrient availability will diminish (Doney 2006). As NO$_3^-$ depleted areas will expand, the importance of diazotrophs like *Trichodesmium* may increase. Data on CO$_2$ dependency of N$_2$ fixation rates from recent publications suggest that N$_2$ fixation by *Trichodesmium* spp. might increase by more than 20 Tg N a$^{-1}$ to about 100 Tg N a$^{-1}$ until the end of this century (Hutchins et al. 2009). These predictions, however, do not consider the light modulation of CO$_2$ effects nor the possible changes in the distribution pattern of *Trichodesmium*. Future predictions on the overall N$_2$ fixation should thus include interactive effects on physiological and ecological aspects, i.e., changes in the rates of processes and shifts in the dominance of diazotrophs.

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References


Publication II

Combined effects of N sources and $p\text{CO}_2$ levels on *Trichodesmium*
Combined effects of different CO2 levels and N sources on the diazotrophic cyanobacterium Trichodesmium

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To predict effects of climate change and possible feedbacks, it is crucial to understand the mechanisms behind CO\textsubscript{2} responses of biogeochemically relevant phytoplankton species. Previous experiments on the abundant N\textsubscript{2} fixers Trichodesmium demonstrated strong CO\textsubscript{2} 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 CO\textsubscript{2} partial pressure (pCO\textsubscript{2}) levels (180, 380, 980 and 1400 \textmu atm) and N sources (N\textsubscript{2} and NO\textsubscript{3}−). Subsequently, biomass production and the main energy-generating processes (photosynthesis and respiration) and energy-consuming processes (N\textsubscript{2} fixation and C acquisition) were measured. While oxygen fluxes and chlorophyll fluorescence indicated that energy generation and its diurnal cycle was neither affected by pCO\textsubscript{2} nor N source, cells differed in production rates and composition. Elevated pCO\textsubscript{2} increased N\textsubscript{2} fixation and organic C and N contents. The degree of stimulation was higher for nitrogenase activity than for cell contents, indicating a pCO\textsubscript{2} effect on the transfer efficiency from N\textsubscript{2} to biomass. pCO\textsubscript{2}-dependent changes in the diurnal cycle of N\textsubscript{2} 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 NO\textsubscript{3}− grown cells, which we attribute to the higher N retention and lower ATP demand compared with N\textsubscript{2} fixers. pCO\textsubscript{2} effects on C affinity were less pronounced in NO\textsubscript{3}− users than N\textsubscript{2} fixers. Our study illustrates the necessity to understand energy budgets and fluxes under different environmental conditions for explaining indirect effects of rising pCO\textsubscript{2}.

Introduction

The release of anthropogenic carbon (C) has caused atmospheric CO\textsubscript{2} partial pressure (pCO\textsubscript{2}) to increase from 280 to 390 \textmu atm since pre-industrial times and pCO\textsubscript{2} levels are expected to rise further to 750 \textmu atm or even beyond 1000 \textmu atm by the end of this century (IPCC 2007, Raupach et al. 2007). As CO\textsubscript{2} is taken up by the ocean, seawater CO\textsubscript{2} 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 even beyond 1000 \textmu atm by the end of this century (IPCC 2007, Raupach et al. 2007). As CO\textsubscript{2} is taken up by the ocean, seawater CO\textsubscript{2} 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...
on marine phytoplankton (Rost et al. 2008, Riebesell and Tortell 2011). By fixing CO₂ into organic matter, phytoplankton acts as a C sink and plays a potential role as a negative feedback mechanism to atmospheric pCO₂ 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 N₂ 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 Kuypers 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 (Doney 2006). As the latter processes are likely to decrease the overall NO₃⁻ availability in the surface ocean, marine N₂ fixation may become more important, helping to restore the global N balance.

The cyanobacterium *Trichodesmium* is considered one of the most important marine N₂ fixers with an estimated contribution of up to 50% to global marine N₂ fixation (Mahaffey et al. 2005). Previous studies found this diazotroph to be exceptionally sensitive to rising pCO₂. Laboratory experiments exposing cultures to pCO₂ 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 N₂ fixation rates (Barcelos é Ramos et al. 2007, Hutchins et al. 2007, 2013, Kranz et al. 2009, Levitan et al. 2007); the magnitude of these effects yet differed strongly between investigations. In several follow-up studies, CO₂ 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 CO₂ effects.

Cyanobacteria have to invest a considerable share of energy into the accumulation of inorganic carbon (Cᵢ) by carbon concentrating mechanisms (CCMs) owing to a competing reaction with O₂ and a particularly low CO₂ 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 carbonysomes, as well as two Cᵢ acquisition systems (Badger et al. 2006, Price et al. 2008). HCO₃⁻ is taken up via a Na⁺-dependent HCO₃⁻ transporter (BicA) whereas diffusive uptake of CO₂ is facilitated by the so-called NDH-1 complex, converting CO₂ to HCO₃⁻. Next to Cᵢ acquisition, another important energy sink in *Trichodesmium* is N₂ fixation (Kranz et al. 2011). As CCM activity was found to be downregulated at high pCO₂ levels, while N₂ fixation rates were simultaneously increased in this species, a reallocation of energy between C and N₂ fixing pathways has been suggested to fuel the increase in production at high pCO₂ (Kranz et al. 2010).

Similarly to RubisCO, nitrogenase is characterized by a high sensitivity toward O₂ (Falkowski 1997). In consequence, while the fixation of N₂ 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 O₂ (Großkopf and LaRoche 2012). To separate O₂ evolution from N₂ fixation, *Trichodesmium* has a tightly regulated diurnal cycle of N₂ 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 (Muholland and Capone 2000). Uptake mechanisms for N sources other than N₂ are thus indispensable for this species.

Laboratory studies have shown that *Trichodesmium* can use NO₃⁻ and NH₄⁺ as well as organic N compounds (glutamine, glutamate or urea; Muholland et al. 1999), all of them requiring different amounts and types of energy equivalents. NO₃⁻ is taken up in cyanobacteria by high-affinity ATP-dependent transporters and subsequently reduced to NH₄⁺ in a two-step ferredoxin-dependent reaction catalyzed by nitrate reductase and nitrite reductase (Flores et al. 2005, Wang et al. 2000) (Eqn 2).

\[
\text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- + 16 \text{ATP} \rightarrow 2 \text{NH}_3 + \text{H}_2 + 16 (\text{ADP} + \text{Pi}) \quad (1)
\]

\[
2 \text{NO}_3^- + 20 \text{H}^+ + 16 \text{e}^- + 2 \text{ATP} \rightarrow 2 \text{NH}_4^+ + 6 \text{H}_2\text{O} + 2 (\text{ADP} + \text{Pi}) \quad (2)
\]

In *Trichodesmium*, N₂ fixation was shown to be inhibited in cultures grown in NO₃⁻-containing media (Ohki et al. 1991, Fu and Bell 2003, Holl and Montoya 2005, Physiol. Plant. 2014).
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 (BIOLUX, Osram, München, Germany). Cultures were grown in 2.8-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 m$^{-1}$. Cells were acclimated in 11 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, [PO₄], 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).

<table>
<thead>
<tr>
<th>pCO₂ treatment</th>
<th>NO₃⁻</th>
<th>pH (NBS)</th>
<th>TA (μmol kg⁻¹)</th>
<th>DIC (μmol kg⁻¹)</th>
<th>pCO₂ attained (μatm)</th>
</tr>
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<tbody>
<tr>
<td>180</td>
<td>–</td>
<td>8.48 ± 0.04</td>
<td>2415 ± 11</td>
<td>1847 ± 10</td>
<td>173 ± 12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.49 ± 0.04</td>
<td>2438 ± 37</td>
<td>1868 ± 22</td>
<td>168 ± 4</td>
</tr>
<tr>
<td>380</td>
<td>–</td>
<td>8.26 ± 0.03</td>
<td>2408 ± 15</td>
<td>2014 ± 6</td>
<td>329 ± 22</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.26 ± 0.04</td>
<td>2389 ± 7</td>
<td>2003 ± 12</td>
<td>325 ± 10</td>
</tr>
<tr>
<td>980</td>
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<td>7.88 ± 0.02</td>
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<td>2142 ± 17</td>
<td>918 ± 12</td>
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<tr>
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<td>2392 ± 19</td>
<td>2166 ± 26</td>
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</tr>
<tr>
<td>1400</td>
<td>–</td>
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<td>2231 ± 78</td>
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<td>7.76 ± 0.05</td>
<td>2413 ± 37</td>
<td>2237 ± 20</td>
<td>1298 ± 74</td>
</tr>
</tbody>
</table>

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-piperazine-ethanesulfonic acid (HEPES, 50 mM, pH 8.0) at 25°C and acclimation light intensity unless otherwise specified. To account for the diurnal cycle of O₂ fluxes in Trichodesmium, measurements were performed three times over the day, during time intervals from 0 to 1.5, 5.5 to 9, and 9 to 10.5 h after beginning of the photoperiod. For normalization of the O₂ traces, duplicate samples for chl a analysis were taken after each measurement.

In the first set of measurements, net O₂ evolution was determined as a function of DIC using the disequilibrium method described by Badger et al. (1994). O₂ fluxes were monitored during consecutive dark and light phases typically lasting 4 min, starting with DIC concentrations close to zero (media bubbled with CO₂-free air), which were subsequently increased by step-wise addition of NaHCO₃ up to a maximum of approximately 4500 μM DIC. DIC-saturated rates of photosynthesis (Vₘₐₓ (DIC)) and half-saturation concentrations [K₁/₂ (DIC)] were obtained by fitting a Michaelis–Menten function to the data.

In a second approach, O₂ evolution and uptake were assessed as a function of light intensity according to Fock and Sültemeyer (1989). Prior to measurements, HEPES-buffered YBCII media were bubbled with N₂ to remove ¹⁶O₂, then spiked with ¹⁸O₂ gas (Chemotrade, Düsseldorf, Germany) and allowed to equilibrate for >30 min, reaching O₂ concentrations of approximately 21%. DIC concentration was adjusted to approximately 2000 μM by addition of 1 M NaHCO₃ solution prior to measurements. Fluxes of ¹⁶O₂ and ¹⁸O₂ were monitored during consecutive 4 min dark and light intervals, applying a range of light intensities from 8 to 2000 μmol photons m⁻² s⁻¹. The light intensity at which photosynthesis starts to enter saturation (Iₜ) 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 (Fv/Fm: measured in dark-adapted state; Fv'/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 ± 0.28 pg chl a cell⁻¹, and were therefore used for normalization. Growth decreased slightly with increasing pCO₂ in both N treatments when all data were included (Fig. 1, ANOVA, P < 0.005). However, when testing CO₂ levels individually, the differences between 980 and 380 μatm as well as 180 μatm depended on the N source: with N₂ there was a significant pCO₂ effect (TukeyHSD, adj P < 0.05) while with NO₃⁻ 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 μatm pCO₂ (ANOVA, P < 0.0001). As a result of these opposing trends, production rates of POC and PON did not change over the range of pCO₂ levels tested (Table 2, ANOVA, P > 0.05). There was no clear trend in the pCO₂ effect on the POC:PON ratio (Table 2). Regarding effects of the N source, cells grown on NO₃⁻ had slightly higher growth rates than N₂ fixing cells (ANOVA, P < 0.05). Consequently, also production rates of POC and PON were higher in NO₃⁻ 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 NO₃⁻ users than in N₂ fixers (Table 2, ANOVA, P < 0.0001).

**N₂ fixation**

N₂ fixation was inhibited by the addition of NO₃⁻, with N₂ fixation rates being close to detection limit (data not shown). Moreover, the δ¹⁵N of PON clearly differed between treatments (ANOVA, P < 0.0001), with −1.3 ± 1.0‰ in N₂ fixing cells and +4.8 ± 1.3‰ in NO₃⁻ grown cells. N₂ fixation rates displayed a typical diurnal cycle with high rates during midday (Fig. 2). At elevated pCO₂, there was a change in the diurnal pattern toward N₂ fixation rates remaining high until the end of the photoperiod. Consequently, integrated rates of N₂ fixation over the photoperiod increased by approximately 60% from 27 to 43 nmol N₂ (μg chl a)⁻¹ day⁻¹ at 380 and 1400 μatm pCO₂, respectively. At 180 and 980 μatm pCO₂, integrated rates were 25 and 39 nmol N₂ (μg chl a)⁻¹ day⁻¹, respectively.

**O₂ fluxes**

To characterize the energy generating processes, O₂ evolution was firstly assessed as a function of DIC concentrations. In all treatments, maximal net O₂ evolution [V_{max} (DIC)] followed a typical diurnal cycle with lowest rates during midday (Fig. 3, ANOVA, P < 0.0001). Values of V_{max} (DIC) were not affected by the N source or pCO₂ (ANOVA, P > 0.5). Half saturation concentration (K_{1/2} (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 pCO₂ on K_{1/2} (DIC) were modulated by the N source and the time of day. While under NO₃⁻ grown conditions, there was no significant pCO₂ effect (TukeyHSD, adj P > 0.5), K_{1/2} (DIC) was significantly lower at 380 μatm than at 1400 μatm pCO₂ under N₂ fixing conditions (TukeyHSD, adj P < 0.005). The difference between pCO₂ treatments in N₂ fixers was especially pronounced toward the evening (TukeyHSD, adj P < 0.0001), with K_{1/2} (DIC) decreasing in cells grown at 380 μatm but remaining high in cells grown at 1400 μatm pCO₂.

In a second approach, evolution and uptake of O₂ were assessed over a range of light intensities. Net O₂ evolution typically reached light compensation between 10 and 60 μmol photons m⁻² s⁻¹ and started to enter saturation (Iₗ) at approximately 280 μmol photons m⁻² s⁻¹ (Fig. 4). Light-dependent O₂ uptake, i.e. an excess of O₂ uptake in the light over O₂ 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).

<table>
<thead>
<tr>
<th>pCO₂ treatment (µatm)</th>
<th>NO₃⁻</th>
<th>POC production (µmol (µg chl a)⁻¹ day⁻¹)</th>
<th>PON production (µmol (µg chl a)⁻¹ day⁻¹)</th>
<th>POC:PON (mol:mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>180</td>
<td>–</td>
<td>1.75 ± 0.10</td>
<td>0.36 ± 0.02</td>
<td>4.83 ± 0.10</td>
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<td></td>
<td>+</td>
<td>1.89 ± 0.24</td>
<td>0.39 ± 0.05</td>
<td>4.82 ± 0.06</td>
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<tr>
<td>380</td>
<td>–</td>
<td>1.60 ± 0.22</td>
<td>0.33 ± 0.04</td>
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<td>1.82 ± 0.14</td>
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<td>980</td>
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<td>1.26 ± 0.42</td>
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<tr>
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<td>2.15 ± 0.19</td>
<td>0.45 ± 0.05</td>
<td>4.82 ± 0.12</td>
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<tr>
<td>1400</td>
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<td>4.93 ± 0.21</td>
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<td>2.02 ± 0.15</td>
<td>0.44 ± 0.03</td>
<td>4.56 ± 0.07</td>
</tr>
</tbody>
</table>

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...
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, QA re-oxidation time.

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). QA 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. QA re-oxidation time, which was measured in the dark (τdark), was significantly lower in the morning than during the rest of the day (ANOVA, P < 0.0001), while τlight 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 τdark nor τlight 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.
Diurnal cycle of chlorophyll fluorescence of *Trichodesmium* cultures grown under different pCO$_2$ levels and N sources (N$_2$ and NO$_3^-$), measured in the dark (A, C and E) and at acclimation light intensity (B, D and F). Error bars denote 1SD (n $\geq$ 3, except for $\tau$ of 380 $\mu$atm +NO$_3^-$ midday with n = 1). Fv/Fm, PSII photochemical quantum yield; $\sigma$, PSII functional absorption cross section; $\tau$, QA re-oxidation time.

Please note that the light level applied in the acclimations (150 $\mu$mol 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 $\mu$atm, 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 $\mu$atm 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 $\mu$atm; 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 cell’s 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 flow 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\(^{-}\)) costs of cellular processes and costs calculated for the observed POC and PON production rates under two different N sources (N\(_2\) and NO\(_3\))

<table>
<thead>
<tr>
<th>Process</th>
<th>Unit</th>
<th>ATP</th>
<th>e(^{-})</th>
<th>ATP:NADPH + H(^{+})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C fixation</td>
<td>mol (mol N(^{-1}))</td>
<td>14</td>
<td>19</td>
<td>1.5</td>
<td>Allen 2002</td>
</tr>
<tr>
<td>CCM</td>
<td>mol (mol N(^{-1}))</td>
<td>4</td>
<td>0</td>
<td>1.9</td>
<td>Hopkinson et al. 2011</td>
</tr>
<tr>
<td>POC production</td>
<td>mol (mol N(^{-1}))</td>
<td>18</td>
<td>19</td>
<td>1.9</td>
<td>Flores et al. 1994</td>
</tr>
<tr>
<td>N(_2) assimilation to NH(_4)(^{+})</td>
<td>mol (mol N(^{-1}))</td>
<td>8</td>
<td>4</td>
<td>4.0</td>
<td>Flores et al. 2005</td>
</tr>
<tr>
<td>NO(_3)(^{-}) assimilation to NH(_4)(^{+})</td>
<td>mol (mol N(^{-1}))</td>
<td>1</td>
<td>8</td>
<td>0.3</td>
<td>Flores et al. 2005</td>
</tr>
<tr>
<td>NH(_4)(^{+}) assimilation to glutamate</td>
<td>mol (mol N(^{-1}))</td>
<td>1</td>
<td>2</td>
<td>1.0</td>
<td>Flores et al. 2005</td>
</tr>
<tr>
<td>PON production N(_2) fixer</td>
<td>mol (mol N(^{-1}))</td>
<td>9</td>
<td>6</td>
<td>3.0</td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>PON production NO(_3)(^{-}) user</td>
<td>mol (mol N(^{-1}))</td>
<td>2</td>
<td>10</td>
<td>0.4</td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>POC production measured in N(_2) fixer</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>5.9</td>
<td>6.2</td>
<td>1.9</td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>POC production measured in NO(_3)(^{-}) user</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>7.5</td>
<td>7.9</td>
<td>1.9</td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>POC production difference NO(_3)(^{-}) vs N(_2)</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>1.6</td>
<td>1.7</td>
<td></td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>PON production measured in N(_2) fixer</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>2.9</td>
<td>1.9</td>
<td>3.0</td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>PON production measured in NO(_3)(^{-}) user</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>0.8</td>
<td>4.2</td>
<td>0.4</td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>PON production difference NO(_3)(^{-}) vs N(_2)</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>−2.0</td>
<td>2.3</td>
<td></td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>Total POC production in N(_2) fixer</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>8.8</td>
<td>8.2</td>
<td>2.2</td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>Total POC production in NO(_3)(^{-}) user</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>8.3</td>
<td>12.1</td>
<td>1.4</td>
<td>(Sandh et al. 2011)</td>
</tr>
<tr>
<td>POC production difference NO(_3)(^{-}) vs N(_2)</td>
<td>μmol (μg chl a(^{-1})) day(^{-1})</td>
<td>−0.5</td>
<td>3.9</td>
<td></td>
<td>(Sandh et al. 2011)</td>
</tr>
</tbody>
</table>

evolution as well as Mehler reaction, while dark respiration increased. Interestingly, the diurnal cycle of O\(_2\) evolution and uptake as well as electron transport was maintained also in NO\(_3\)\(^{-}\) 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\(_3\))\(^{-}\) (Ohki et al. 1991). These findings suggest that although nitrogenase was not active, NO\(_3\))\(^{-}\) 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\(_2\) also in NO\(_3\))\(^{-}\) grown cells (i.e. O\(_2\) 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\(_2\) fluxes observed in our study. There is, however, also data suggesting significantly lower expression levels of nitrogenase subunits NifK and NifH in NO\(_3\))\(^{-}\) grown cells (Sandh et al. 2011).

In summary, the lack of a clear pCO\(_2\) 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\(_2\) fixation and C acquisition.

**N\(_2\) fixation**

In agreement with previous results (Kranz et al. 2010), a characteristic change in the diurnal pattern of N\(_2\) fixation was observed at elevated pCO\(_2\), with the phase of high nitrogenase activity being prolonged toward the evening (Fig. 2). Although integrated daily N\(_2\) fixation rates increased by as much as 60% between 380 and 1400 μatm pCO\(_2\), PON production was not significantly affected by the different pCO\(_2\) levels. The ARA used for estimating N\(_2\) fixation rates gives a measure of the maximal nitrogenase enzyme activity under the respective assay conditions (approximating gross N\(_2\) fixation) while PON production rates reflect how much N is ultimately incorporated into the cells (approximating net N\(_2\) fixation). 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\(_2\) 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 (N₂ and NO₃⁻). Due to the different requirements of N₂ and NO₃⁻ assimilation with respect to ATP and electron (e⁻) stoichiometry, N₂ fixation is prone to limitation by ATP while NO₃⁻ assimilation tends to be limited by e⁻ 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⁻ available, which is, in turn, controlled by the ratio of (pseudo-) cyclic to linear e⁻ 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 CO₂ sensitivity, previous studies found ARA-based estimates of N₂ fixation to increase more strongly with pCO₂ than estimates of PON production based on cell quotas or ¹⁵N fixation (Kranz et al. 2010, Garcia et al. 2011). In the natural environment, N release by *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 pCO₂ according to our data. The high assimilation costs and unavoidable N loss in N₂ fixers impose higher energy requirements compared with NO₃⁻ users, especially under elevated pCO₂. 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 *Trichodesmium* due to the high CCM activities required to compensate for the poor CO₂ affinity of its Rubisco (Kranz et al. 2009). Similarly to O₂ and electron fluxes as well as N₂ 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 pCO₂, especially during the second half of the day, suggest significantly lower operational costs for the CCM which, in turn, allow for the enhanced N₂ fixation observed (Figs 2 and 3). These CO₂-dependent changes in affinities and the anti-correlation with N₂ fixation are in agreement with previous results (Kranz et al. 2010). The fact that pCO₂ effects are larger in N₂ fixers than in NO₃⁻ using cells can be attributed to the higher overall energy requirements of N₂ 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 N₂ fixers to cover the high ATP demand of nitrogenase. In contrast, NO₃⁻ 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.

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Publication III

Cellular inorganic carbon fluxes in *Trichodesmium*
Cellular inorganic carbon fluxes in *Trichodesmium*: A combined approach of measurements and modeling

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Abstract

To predict effects of climate change on phytoplankton, it is crucial to understand how their mechanisms for carbon acquisition respond to environmental conditions. Aiming to shed light on the responses of extra- and intracellular inorganic carbon (C\textsubscript{i}) fluxes, the cyanobacterium *Trichodesmium erythraeum* IMS101 was grown with different N sources (N\textsubscript{2} vs NO\textsubscript{3}\textsuperscript{-}) and pCO\textsubscript{2} levels (380 vs. 1400 µatm). Cellular C\textsubscript{i} fluxes were assessed by combining membrane inlet mass spectrometry (MIMS), \textsuperscript{13}C fractionation measurements and modeling. Aside from a significant decrease in C\textsubscript{i} affinity at elevated pCO\textsubscript{2} and changes in CO\textsubscript{2} efflux with nitrogen source, extracellular C\textsubscript{i} fluxes estimated by MIMS were largely unaffected by the treatments. \textsuperscript{13}C fractionation during biomass production increased with pCO\textsubscript{2}, irrespective of the N source. Strong discrepancies were observed in CO\textsubscript{2} leakage estimates obtained by MIMS and a \textsuperscript{13}C-based approach, which further increased under elevated pCO\textsubscript{2}. These offsets could be explained by applying a model that comprises extracellular CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} fluxes as well as internal C\textsubscript{i} cycling around the carboxysome via the CO\textsubscript{2} uptake facilitator NDH-1\textsubscript{4}. Regarding the latter, assuming unidirectional, kinetic fractionation between CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} in the cytosol or enzymatic fractionation by the CO\textsubscript{2} uptake facilitator NDH-1\textsubscript{4}, both significantly improved the comparability of leakage estimates. Our results highlight the importance of internal C\textsubscript{i} cycling for \textsuperscript{13}C composition as well as cellular energy budgets of *Trichodesmium*, which ought to be considered in process-studies on climate change effects.

Keywords

Carbon acquisition, CCM, CO\textsubscript{2}, cyanobacteria, leakage, NDH, ocean acidification

Abbreviations

\( a\text{\textsubscript{carb}} \), fractional contribution of HCO\textsubscript{3}\textsuperscript{-} to total C\textsubscript{i} uptake into the carboxysome; \( a\text{\textsubscript{cys}} \), fractional contribution of HCO\textsubscript{3}\textsuperscript{-} to total C\textsubscript{i} uptake into the cytosol; CA, carbonic anhydrase; CCM, carbon concentrating mechanism; chl \( a \), chlorophyll \( a \); C\textsubscript{i}, inorganic carbon; DIC, dissolved inorganic carbon; K\textsubscript{1/2}, half-saturation concentration; \( L_{13C} \), leakage calculated from \textsuperscript{13}C fractionation; \( L\text{\textsubscript{carb}} \), modeled leakage from the carboxysome; \( L\text{\textsubscript{cys}} \), modeled leakage over the plasmamembrane; \( L\text{\textsubscript{MIMS}} \), leakage estimated by MIMS; MIMS, membrane inlet mass spectrometry; POC, particulate organic carbon; V\textsubscript{max}, maximal rate; \( \varepsilon\text{\textsubscript{cys}} \), \textsuperscript{13}C fractionation in the cytosol; \( \varepsilon\text{\textsubscript{db}} \), \textsuperscript{13}C equilibrium fractionation in the external medium, \( \varepsilon\text{\textsubscript{ps}} \), total \textsuperscript{13}C fractionation during POC formation; \( \varepsilon\text{\textsubscript{Rub}} \), \textsuperscript{13}C fractionation by RubisCO
Introduction

Cyanobacteria are ancient organisms responsible for oxygenation of the atmosphere during times when CO₂ concentrations were about 2 orders of magnitude higher than today (cf. Buick 1992, Kasting and Siefert 2002). Possibly due to their origin at that time, the CO₂ fixing enzyme RubisCO of cyanobacteria has one of the lowest affinities among all autotrophic organisms (Badger et al. 1998, Tortell 2000). Consequently, cyanobacteria are dependent on high activities of carbon concentrating mechanisms (CCM) for increasing the CO₂ concentration in the vicinity of RubisCO. Currently, due to the ongoing anthropogenic CO₂ combustion, the availability and speciation of inorganic carbon (Cᵢ) in seawater is changing at a rapid pace (IPCC 2007). In view of this ocean acidification (Caldeira and Wickett 2003), a number of studies in recent years have focused on the mechanisms of carbon acquisition and CO₂ responses of different groups of phytoplankton (e.g. Rost et al. 2008). Among these studies, the abundant N₂ fixing cyanobacterium *Trichodesmium* stood out by showing an exceptionally high stimulation of biomass production and N₂ fixation in response to elevated pCO₂ (e.g. Hutchins et al. 2007, Kranz et al. 2009, Levitan et al. 2007). Further studies on the underlying reasons for these CO₂ effects showed a decrease in Cᵢ affinity at high pCO₂ (Kranz et al. 2009, Kranz et al. 2010). Given the high energy demand of the CCM in cyanobacteria, a re-allocation of energy between Cᵢ acquisition and N₂ fixation was suggested to stimulate production at high pCO₂ (Kranz et al. 2010).

Cellular Cᵢ affinities of *Trichodesmium* are determined by the interplay of several transporters and structural adaptations composing the CCM. For understanding pCO₂ responses of the CCM as well as potential changes in energy demand it is necessary to distinguish between these different components. While CO₂ can diffuse through the cell membrane without energy investments, the low equilibrium concentrations, the slow interconversion with HCO₃⁻ (Zeebe and Wolf-Gladrow 2007) as well as its tendency to leak out of the cell compromise the use of CO₂ as the predominant Cᵢ source. Therefore, cyanobacteria have evolved energy dependent transporters for taking up HCO₃⁻, which can be accumulated in the cell more efficiently (Badger et al. 2006). *Trichodesmium* has been found to cover ~ 90% of its carbon demand by HCO₃⁻ (Kranz et al. 2009, Kranz et al. 2010). Uptake of HCO₃⁻ in this species is catalyzed by the Na⁺-dependent transporter BicA, which is fuelled by Na⁺/HCO₃⁻ symport or via a H⁺/Na⁺ antiport mechanism (Price et al. 2008). Cyanobacterial RubisCO is localized in distinct compartments within the cell, the so-called carboxysomes. The protein shells of these microbodies are permeable for HCO₃⁻ but pose a diffusion barrier for CO₂ (Dou et al. 2008, Espie and Kimber 2011), allowing for significant
accumulation of CO₂ in the vicinity of Rubisco. Inside the carboxysomes, transformation of HCO₃⁻ to CO₂ is accelerated by carbonic anhydrase (CA; reviewed by Espie and Kimber 2011). In addition to direct HCO₃⁻ uptake and CO₂ diffusion, CO₂ uptake in *Trichodesmium* is facilitated by the NDH-1 complex, which converts CO₂ to HCO₃⁻ in the cytoplasm, presumably in a CA-like reaction (Price et al. 2002). The protein complex is supposed to be located on the thylakoid membrane and form part of the photosynthetic/ respiratory electron transport chain, being fuelled by electrons donated from NADPH or ferredoxin, which are subsequently transferred to the plastoquinone pool (Price et al. 2002). After the hydration of CO₂, a proton is supposed to be released into the thylakoid lumen, contributing to the pH gradient necessary for ATP synthesis and making the reaction irreversible in the light (Price et al. 2002).

Conversion of CO₂ to HCO₃⁻ by the NDH complex has been proposed to drive an internal Cᵢ recycling to minimize the loss via CO₂ efflux (Maeda et al. 2002, Price et al. 2002). Due to the strong CO₂ accumulation required in cyanobacteria, CO₂ efflux is a major challenge in these organisms. Despite the interplay of the carboxysome and proposed recapture of CO₂ by the NDH-1 complex, efflux of CO₂ has been shown to equal ~ 50 to 90% of gross Cᵢ uptake in *Trichodesmium* (Kranz et al. 2009, Kranz et al. 2010). Next to the carbon source (CO₂ vs. HCO₃⁻), leakage (i.e. CO₂ efflux : gross Cᵢ uptake) can strongly affect isotopic composition of organic carbon produced during photosynthesis (Burkhardt et al. 1999, Sharkey and Berry 1985), and thus measurements of ¹³C fractionation can provide complementary information on this aspect of CCM regulation (e.g. Keller and Morel 1999, Laws et al. 1997, Rost et al. 2006, Tchernov and Lipschultz 2008). In fact, differences in leakage estimates based on membrane inlet mass spectrometry (MIMS; Badger et al. 1994) and carbon isotope fractionation (Sharkey and Berry 1985) have been attributed to internal Cᵢ cycling driven by NDH (Kranz et al. 2010).

In a previous study (Eichner et al. 2014), the energy allocation to different physiological processes in *Trichodesmium* under varying energetic states was addressed by altering the cellular energy budget through addition of different nitrogen sources: while N₂ fixation is a highly energy demanding process with a high demand in ATP, NO₃⁻ requires very little ATP (only for uptake) but instead has a high electron demand. The study highlighted the dependence of energy reallocation on the stoichiometry in energy demands (ATP vs. NADPH) of the respective pathways involved. The energy demand of the CCM in *Trichodesmium* remains uncertain, however, especially because the regulation of internal Cᵢ fluxes is as yet poorly characterized. To shed light on the extra- and intracellular Cᵢ fluxes
under the different energetic conditions, *Trichodesmium* was grown under different \( p\text{CO}_2 \) levels and N sources (\( \text{N}_2 \) vs. \( \text{NO}_3^- \)) and a combination of different methods, including MIMS and \(^{13}\text{C}\) fractionation measurements as well as modeling was employed. While MIMS provides the perfect tool to investigate \( \text{C}_i \) fluxes over the cell membrane, internal fluxes cannot be directly measured and were therefore modeled. Model calculations of internal \( \text{C}_i \) fluxes made use of the measured extracellular \( \text{C}_i \) fluxes and the isotopic composition of particulate organic carbon (\( \delta^{13}\text{C}_{\text{POC}} \)), which reflects the integrated effects of extra- and intracellular \( \text{C}_i \) fluxes. Hereby, a common model of \(^{13}\text{C}\) fractionation (Sharkey and Berry 1985) was extended by including internal fluxes around the carboxysome.

**Materials and Methods**

**Culture conditions**

*Trichodesmium erythraeum* IMS101 was grown in semi-continuous batch cultures at 25°C and 150 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) with a 12:12 h light:dark cycle. Cultures were grown in 0.2-\( \mu \text{m} \)-filtered artificial seawater (YBCII medium; Chen et al. 1996) and kept in exponential growth phase by regular dilution with culture medium. Culture bottles were continuously bubbled with 0.2-\( \mu \text{m} \)-filtered air with \( \text{pCO}_2 \) levels of 380 and 1400 \( \mu \text{atm} \). Prior to experiments, cells were allowed to acclimate to the respective \( \text{pCO}_2 \) for at least two weeks. Cultures in which pH had drifted by > 0.09 units compared to cell-free reference media were excluded from further analysis. In treatments with \( \text{NO}_3^- \) as the N source, 0.2-\( \mu \text{m} \)-filtered \( \text{NaNO}_3 \) was added to achieve mean concentrations of 97 ± 2 \( \mu \text{mol L}^{-1} \) in the experiments, never falling below 65 \( \mu \text{mol L}^{-1} \). Cultures were acclimated to \( \text{NO}_3^- \) for at least one week before measurements. Samples for the analysis of dissolved inorganic carbon (DIC) were filtered through 0.2 \( \mu \text{m} \) filters and measured colorimetrically (QuAAtro autoanalyzer, Seal, Norderstedt, Germany). Average precision was ± 5 \( \mu \text{mol kg}^{-1} \). pH values of the acclimation media were measured potentiometrically (pH meter pH3110, WTW, Weilheim, Germany). For further details on culture conditions as well as carbonate chemistry parameters, please refer to Eichner et al. (2014).

**MIMS measurements**

Cellular \( \text{C}_i \) fluxes (Fig. 1) were obtained using a custom-made MIMS system (Rost et al. 2007), applying a disequilibrium approach described by Badger et al. (1994). Assays were performed in YBCII medium buffered with HEPES (50 mM, pH 8.0) at acclimation temperature and light intensity, unless otherwise specified. To account for the diurnal cycle of
C₁ fluxes in *Trichodesmium*, measurements were performed three times over the day, during time intervals from 0 to 1.5, 5.5 to 7 and 9 to 10.5 h after beginning of the photoperiod. CO₂ and O₂ fluxes were measured as a function of DIC, starting with concentrations close to zero (media bubbled with CO₂-free air), which were subsequently increased by step-wise addition of NaHCO₃ up to concentrations of ~5000 μM. Please note that as the assay medium is buffered, unlike the conditions during acclimation of the cells, the ratio of HCO₃⁻ : CO₂ stays constant over the investigated DIC range. DIC-saturated rates of photosynthesis (V_max) and half-saturation concentrations (K₁/₂ (DIC)) were obtained by fitting a Michaelis-Menten function to the data. Net O₂ evolution was converted to C fixation (Ffix) assuming a photosynthetic quotient of 1.34 (Williams and Robertson 1991). Net CO₂ uptake (Fcyt, netCO₂) was calculated from the steady-state rate of CO₂ depletion at the end of the light period and corrected for the CO₂/HCO₃⁻ inter-conversion in the medium (Fext, db). Using C fixation and net CO₂ uptake, HCO₃⁻ uptake rates (Fcyt, HCO₃⁻) could be derived by a mass balance equation. For normalization of the CO₂ and O₂ traces, duplicate samples for chlorophyll a (chl a) analysis were taken after each measurement. Chl a was extracted in acetone for > 12 h and determined fluorometrically (TD-700 fluorometer, Turner Designs, Sunnyvale, CA; Holm-Hansen and Rieman 1978).

**Leakage estimation**

Cellular leakage was estimated by two different methods. Firstly, leakage was determined by MIMS measurements using the disequilibrium approach (Badger et al. 1994). Cellular leakage (L_MIMS) is defined as the ratio of CO₂ efflux (Fcyt, out) to gross C₁ uptake (i.e. the sum of HCO₃⁻ (Fcyt, HCO₃⁻) and gross CO₂ uptake (Fcyt, CO₂)):

\[
L_{MIMS} = \frac{F_{cyt, out}}{F_{cyt, HCO_3} + F_{cyt, CO_2}}
\]

(Eqn 1)

Fcyt, out was estimated from the initial increase in CO₂ concentration after switching off the light (Badger et al. 1994). These estimates are based on the assumption that the rate of diffusive CO₂ efflux during the light phase is well represented by the rate of CO₂ efflux during the first seconds of the subsequent dark phase.

In the second approach, leakage was estimated from the isotopic fractionation during POC formation (ε_p), which was calculated from the difference in isotopic composition between POC (δ¹³C_POC) and CO₂ (δ¹³C_CO₂) in the medium according to Freeman and Hayes (1992). Duplicate samples for analysis of δ¹³C_POC were filtered onto pre-combusted GF/F filters and acidified with 200 μl HCL (0.2 M) to remove all C₁ prior to analysis. δ¹³C_POC was measured
with an EA mass spectrometer (ANCA SL 2020, SerCon Ltd., Crewe, UK). For analysis of the isotopic composition of DIC ($\delta^{13}$C$_{\text{DIC}}$), filtered samples were fixed with HgCl$_2$ (final concentration 110 mg L$^{-1}$). Subsequent to acidification of the samples, isotopic composition of CO$_2$ in the headspace was analyzed with an isotope ratio mass spectrometer (GasBench-II coupled to Delta-V advantage, Thermo, Bremen, Germany). The isotopic composition of CO$_2$ was calculated from $\delta^{13}$C$_{\text{DIC}}$, following a mass balance equation (Zeebe and Wolf-Gladrow 2007). Leakage ($L_{13C}$) was subsequently derived using an extended equation from Sharkey and Berry (1985):

$$L_{13C} = \frac{\varepsilon_{e} - (a_{cyc}\varepsilon_{db})}{\varepsilon_{Rub}}$$

(Eqn 2)

where $\varepsilon_{Rub}$ is the intrinsic discrimination of $^{13}$C by RubisCO (assumed to be $+25\%o$; Guy et al. 1993, Roeske and O’Leary 1984) and $\varepsilon_{db}$ represents the equilibrium fractionation between CO$_2$ and HCO$_3^-$ ($-9\%o$; Mook et al. 1974). The fractional contribution of HCO$_3^-$ to gross C$_i$ uptake ($a_{cyc}$), being introduced by Burkhardt et al. (1999), has been determined by MIMS measurements for the respective treatments. These calculations assume an equilibrium situation and further consider the cell as a single compartment.

**Results & Discussion**

$C_i$ fluxes under different $p$CO$_2$ levels and N sources

**General CCM characteristics** - MIMS measurements showed a highly efficient CCM with a high capacity for regulation of the C$_i$ affinity over the diurnal cycle as well as with different $p$CO$_2$ levels, in agreement with previous studies on *Trichodesmium* (e.g. Kranz et al. 2009, Kranz et al. 2010). Half-saturation DIC concentrations for C fixation ($K_{1/2}$) ranged between $\sim$ 20 and 500 µmol DIC L$^{-1}$ (supplementary material, Fig. S1), which is equivalent to $\sim$ 0.2 and 4 µmol CO$_2$ L$^{-1}$ and thus substantially lower than the $K_M$ of cyanobacterial RubisCO (105 - 185 µmol CO$_2$ L$^{-1}$; Badger et al. 1998). Taking the ratio of $K_M$ to $K_{1/2}$ as a measure of CO$_2$ accumulation in the vicinity of RubisCO (assuming a $K_M$ of 150 µmol CO$_2$ L$^{-1}$), our data suggest accumulation factors between $\sim$ 35 and 900 and indicate that the degree of RubisCO saturation is always larger than 80%. Accordingly, under the applied external CO$_2$ concentrations, concentrations in the carboxysome typically exceed 600 µmol CO$_2$ L$^{-1}$. The CCM was primarily based on active HCO$_3^-$ uptake, accounting for 82 ± 4% of gross C$_i$ uptake (Table 1). As gross C$_i$ uptake was approximately twice as high as net C fixation at acclimation DIC ($\sim$ 2100 µmol CO$_2$ L$^{-1}$), leakage measured by MIMS ranged between 0.3 and 0.7 (i.e.
CO₂ efflux equaled 30 to 70% of gross Cᵢ uptake, Table 1). As a consequence of the high HCO₃⁻ contribution and the high CO₂ efflux, the net fluxes of CO₂ were generally directed out of the cell (cf. negative values for net CO₂ uptake, Table 1, Fig. 2).

**Diurnal changes** - The diurnal cycle was characterized by low K₁/₂ values in the morning and a down-regulation in C fixation rates during midday (ANOVA, p < 0.001, Figure S1A&B). Leakage estimated by MIMS at acclimation DIC was lowest in the morning, increased towards midday and decreased again towards the evening (ANOVA, p < 0.05, Table 1). Leakage estimates for DIC levels approaching zero (obtained by curve fits of leakage plotted over DIC concentration, Fig. 2) varied even more over the course of the day, yielding values around 0.3 in the mornings, while at midday and in the evening, ratios approached 1.0 (data not shown). These diurnal changes in leakage could be explained by the concurrent changes in the ratio of HCO₃⁻ to CO₂ uptake (Table 1, Fig. S1C), which were characterized by low CO₂ fluxes in the mornings (ANOVA, p < 0.05, Table 1), while HCO₃⁻ uptake was higher in the morning than during midday and increased again towards the evening (ANOVA, p < 0.05, Table 1). Over the day, a higher share of HCO₃⁻ uptake, which is less prone to diffuse out of the cell, was thus correlated with lower leakage.

**Treatment effects** - The affinity for Cᵢ was down-regulated at elevated pCO₂, as indicated by high K₁/₂ values under these conditions (Fig. S1B). Under acclimation DIC, however, Cᵢ fluxes (C fixation, Cᵢ uptake, CO₂ uptake and efflux) were not significantly affected by pCO₂ (ANOVA, p > 0.05, Table 1), reflecting the cells' capacity to achieve similar C fixation over a range of pCO₂ levels by regulating their CCM. Regarding the N source, C fixation rates and CO₂ uptake and efflux under acclimation DIC were equally not affected (ANOVA, p > 0.05, Table 1). Although cells mainly used HCO₃⁻ as a Cᵢ source in all treatments, HCO₃⁻ uptake at acclimation DIC decreased slightly with pCO₂ (~ 10%; ANOVA, p < 0.05, Table 1, Fig. S1C), yet was not affected by N source (ANOVA, p > 0.05, Table 1). Interestingly, CO₂ efflux was affected by the N source (ANOVA, p < 0.01, Table 1), with ~ 20% lower efflux in NO₃⁻ users compared to N₂ fixers, possibly due to differences in internal pH caused by the uptake/accumulation of NO₃⁻ vs. NH₄⁺ in the cell. One could also speculate that growing cells on NO₃⁻ reduces the general membrane permeability, since NH₄⁺ transfer between cells is only necessary under N₂ fixing conditions, which could also impact the permeability for CO₂. Leakage at acclimation DIC estimated by MIMS was, however, not significantly affected by pCO₂ or N source at any time of the day (ANOVA, p > 0.05, Table 1).
Offsets in leakage estimates

High leakage values obtained in MIMS measurements reflect the strong C\textsubscript{i} accumulation necessary for C fixation in cyanobacteria due to the poor CO\textsubscript{2} affinity of their RubisCO. Yet, leakage estimates obtained from δ\textsubscript{13}C values (L\textsubscript{13C}, Eqn 2) even exceeded MIMS-based estimates. Overall fractionation during formation of POC (ε\textsubscript{p}) was not significantly affected by N treatment (ANOVA, p > 0.05) but increased with pCO\textsubscript{2} (ANOVA, p < 0.0001), ranging from 14.4 ± 1.0‰ at 380 µatm to 19.9 ± 0.9‰ at 1400 µatm pCO\textsubscript{2}. Consequently, leakage estimates based on ε\textsubscript{p} (Eqn 2) also increased with pCO\textsubscript{2}, while estimates from MIMS measurements at acclimation DIC were constant over the range of pCO\textsubscript{2} levels. L\textsubscript{13C} was calculated to range between 0.82 and 1.14, exceeding MIMS-based measurements by ~30 to 60% (Fig. 3) and even reaching theoretically impossible values (> 1). A similar discrepancy between these two approaches, that was equally dependent on pCO\textsubscript{2} acclimation, has been observed previously (Kranz et al. 2010). In the following paragraph, possible reasons for the deviations between estimates are outlined.

Following the approach by Badger et al. (1994), leakage is directly calculated from the measured CO\textsubscript{2} efflux and gross C\textsubscript{i} uptake. As CO\textsubscript{2} efflux cannot readily be determined during the light due to the concurrent C\textsubscript{i} uptake, the rise in the CO\textsubscript{2} signal directly after switching off the light is taken as an estimate for CO\textsubscript{2} efflux during the light phase, assuming that the accumulated C\textsubscript{i} pool and therewith gross CO\textsubscript{2} efflux are initially at the pre-darkness level (Badger et al. 1994). In case that active C\textsubscript{i} uptake as well as C fixation by RubisCO do not cease immediately upon darkening, leakage estimates could be biased and likely underestimated. Despite these potential uncertainties, this is a more direct approach than the alternative method, which infers leakage from the isotopic composition of cells. The 13C-based approach makes use of the effect of leakage on ε\textsubscript{p} (Eqn 2; Sharkey and Berry 1985). Briefly, while the intrinsic fractionation by RubisCO (ε\textsubscript{Rub}) is generally causing organic material to be depleted in 13C (Fig. 1A), variation in ε\textsubscript{p} can be induced by changes in the C\textsubscript{i} source and/or leakage. Consequently, any errors in estimates of ε\textsubscript{Rub} or α\textsubscript{crt} but also any unaccounted process affecting ε\textsubscript{p} would cause 13C-based leakage estimates to be biased.

Kranz et al. (2010) suggested that internal C\textsubscript{i} cycling within the cell may affect ε\textsubscript{p} in general. The CO\textsubscript{2}-dependence of the offset between MIMS- and 13C-based leakage estimates was furthermore suggested to reflect a CO\textsubscript{2} effect on the NDH complex driving this internal C\textsubscript{i} cycling (Kranz et al. 2010), in line with early observations of the C\textsubscript{i} dependence of CO\textsubscript{2} uptake by the NDH complex (Price and Badger 1989a, c). In Fig. 1, the effects of internal C\textsubscript{i} cycling on the isotopic composition are illustrated. While Fig. 1A assumes an equilibrium
situation and does not include any internal C\textsubscript{i} cycling, Fig. 1B illustrates non-equilibrium situations caused by internal C\textsubscript{i} cycling. The degree of \textsuperscript{13}C enrichment in the cytosol and within the carboxysome, according to this concept, would be dependent on the type of kinetic fractionation in the cytosol. This could include complete or incomplete unidirectional fractionation as well as enzymatic fractionation by the NDH complex. Accounting for these processes requires the introduction of a second compartment. The approach taken here can be considered as an extension of the model by Sharkey and Berry (1985), which considers the cell as one compartment. In order to avoid the errors being introduced by large uncertainties, e.g. in permeability of the plasma membrane and carboxysome in *Trichodesmium*, a flux-based model that is independent of these assumptions is employed, rather than a full kinetic model. Our approach is similar to the model by Schulz et al. (2007), yet also disequilibrium situations are considered.

**Effects of internal C\textsubscript{i} fluxes on carbon fractionation**

*Model setup* - To test our concept (Fig. 1) and quantitatively describe the possible effect of internal cycling on δ\textsuperscript{13}C, intracellular C\textsubscript{i} fluxes and their effects on isotopic ratios in different cellular C\textsubscript{i} pools were modeled. For parameterizations, HCO\textsubscript{3}\textsuperscript{-} and gross CO\textsubscript{2} fluxes measured by MIMS as well as measured fractionation values \(\varepsilon_p\) were used. The model is based on flux balance equations for the individual isotope species. The flux balance of total carbon (\textsuperscript{12}C + \textsuperscript{13}C) in the cytosol and in the carboxysome, respectively, is given by the following equations:

\[
F_{\text{cyt,CO}_2} + F_{\text{cyt,HCO}_3} + F_{\text{carb,out}} - F_{\text{cyt,out}} - F_{\text{carb,CO}_2} - F_{\text{carb,HCO}_3} = 0 \quad \text{(Eqn 3)}
\]

\[
F_{\text{carb,HCO}_3} + F_{\text{carb,CO}_2} - F_{\text{carb,out}} - F_{\text{fix}} = 0 \quad \text{(Eqn 4)}
\]

As about 99% of carbon is \textsuperscript{12}C, i.e. \(F = ^{12}F + ^{13}F = ^{12}F\), the flux balance equations for \textsuperscript{13}C can be derived by multiplying the fluxes \(F\) with the isotopic ratio \(R = \text{^{13}C/^{12}C}\). The isotopic fractionation factor \(\alpha_{\text{db}}\) is defined by the isotopic ratio of CO\textsubscript{2} divided by the isotopic ratio of HCO\textsubscript{3}\textsuperscript{-}, i.e. \(\alpha_{\text{db}} = R_{\text{CO}_2} / R_{\text{HCO}_3}\). Using the equilibrium fractionation (\(\varepsilon_{\text{db}}\)), the fractionation factor between CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{-} can be calculated for the external medium as well as for the cytosol according to:

\[
\alpha_{\text{db,ext}} = 1 + \varepsilon_{\text{db}} / 1000 \quad \text{(Eqn 5)}
\]

\[
\alpha_{\text{db,cyt}} = 1 + \varepsilon_{\text{cyt}} / 1000 \quad \text{(Eqn 6)}
\]

While the equilibrium value \(\varepsilon_{\text{db}}\) is -9‰ (i.e. CO\textsubscript{2} is isotopically lighter than HCO\textsubscript{3}\textsuperscript{-}; Mook et al. 1974), \(\varepsilon_{\text{cyt}}\) can significantly deviate from this value due to kinetic effects. The uncatalyzed conversion of HCO\textsubscript{3}\textsuperscript{-} to CO\textsubscript{2} shows a kinetic fractionation of -22‰ whereas the formation of
HCO₃⁻ from CO₂ is associated with a kinetic fractionation of +13‰. Hence, the actual value of ε_{cyt} is determined by the disequilibrium between CO₂ and HCO₃⁻ in the cytosol, which depends on all fluxes in and out of the cytosol and on the internal CO₂ and HCO₃⁻ concentrations, which cannot be calculated in the framework of a flux-based model. Assuming a unidirectional conversion of CO₂ to HCO₃⁻ in the cytosol, a value of +13‰ for ε_{cyt} will be adopted. By setting ε_{cyt} to +30‰, a potential fractionation by the NDH-1₄ complex will be taken into account. The case that the conversion of CO₂ to HCO₃⁻ in the cytosol is not completely unidirectional will be considered by setting ε_{cyt} to +8‰.

The R associated with F_{fix} can be written in terms of the isotopic fractionation against ¹³C by RubisCO described by the factor α_{Rub} = R_{carb} / R_{POC}, where R_{carb} is the isotopic ratio of CO₂ in the carboxysome and R_{POC} is the isotopic ratio of POC. The value of α_{Rub} is calculated from the intrinsic RubisCO fractionation ε_{Rub} (assuming an intermediate value of +25‰; Guy et al. 1993, Roeske and O’Leary 1984):

\[ α_{Rub} = 1 + \frac{ε_{Rub}}{1000} \]  
(Eqn 7)

Given the isotopic ratios R of CO₂ and the isotopic fractionation factors between HCO₃⁻ and CO₂ expressed as α_{bd} = 1 / α_{db}, the flux balance equations for ¹³C can be derived from equations 3 & 4 for the cytosol and the carboxysome, respectively:

\[ R_{ext} F_{cyt,CO₂} + \alpha_{bd,ext} R_{ext} F_{cyt,HCO₃⁻} + R_{carb} F_{carb,out} - R_{cyt} F_{cyt,out} - R_{cyt} F_{carb,CO₂} - \alpha_{bd,cyt} R_{cyt} F_{carb,HCO₃⁻} = 0 \]  
(Eqn 8)

\[ \alpha_{bd,cyt} R_{cyt} F_{carb,HCO₃⁻} + R_{cyt} F_{carb,CO₂} - R_{carb} F_{carb,out} - R_{carb} F_{fix} / \alpha_{Rub} = 0 \]  
(Eqn 9)

R_{cyt} is the isotopic ratio of CO₂ in the cytosol. The overall isotopic fractionation by the cell is defined with respect to the isotopic composition of CO₂ in the external medium (R_{ext}):

\[ ε_p = \left( \frac{R_{ext}}{R_{POC}} - 1 \right) \cdot 1000 = \left( \frac{α_{Rub} R_{ext}}{R_{carb}} - 1 \right) \cdot 1000 \]  
(Eqn 10)

The ratio R_{ext} / R_{carb} reflects the impact of the inner compartment on the isotopic fractionation and can be calculated from flux balance equations 8 & 9. Eqn 9 can be solved for R_{cyt}, which in turn is substituted into Eqn 8, yielding the ratio:
\[ \frac{R_{ext}}{R_{carb}} = \left( \frac{F_{fix} \alpha_{Rab} + F_{carb, out} \left( F_{carb, CO_2} + \alpha_{bd, cyt} F_{carb, HCO_3} + F_{carb, out} \right)}{F_{cyt, CO_2} + \alpha_{bd, ext} F_{cyt, HCO_3} \left( F_{carb, CO_2} + \alpha_{bd, cyt} F_{carb, HCO_3} \right)} \right) - \frac{F_{carb, out}}{F_{cyt, CO_2} + \alpha_{bd, ext} F_{cyt, HCO_3}} \]

(Eqn 11)

Please note that this solution is valid for arbitrary combinations of fluxes as long as the constraints imposed by flux balance equations 3 & 4 are obeyed:

\[ F_{fix} = F_{carb, CO_2} + F_{carb, HCO_3} - F_{carb, out} = F_{cyt, CO_2} + F_{cyt, HCO_3} - F_{cyt, out} \quad \text{(Eqn 12)} \]

Given the fractional contribution of HCO_3^- to total C\textsubscript{i} uptake into the cytosol (\(a_{cyt}\)) and the carboxysome (\(a_{carb}\)) as well as the leakage out of the cytosol (\(L_{cyt}\)) and the carboxysome (\(L_{carb}\)), Eqn 5 to 7 and Eqn 10 to 12 can be used to derive the overall isotopic fractionation:

\[ \varepsilon_p = \frac{a_{cyt} \varepsilon_{db}}{1-a_{cyt} \varepsilon_{db} / 10^3} + \frac{L_{cyt} \left( a_{carb} \varepsilon_{c} + L_{carb} \varepsilon_{Rab} \right)}{1-a_{carb} \varepsilon_{cyt} / 10^3} \]

(Eqn 13)

Solving the approximated solution for \(L_{cyt}\) yields the following:

\[ L_{cyt} = \frac{\varepsilon_p - a_{cyt} \varepsilon_{db}}{a_{carb} \varepsilon_{cyt} + L_{carb} \varepsilon_{Rab}} \quad \text{(Eqn 14)} \]

The approximated solution can be considered as a generalization of the original function given by Sharkey and Berry (1985), accounting for two compartments. The authors assumed that the cell takes up HCO_3^- into a single compartment and subsequently convert it to CO_2, hence, there is no HCO_3^- inside the cell. The compatibility of our model with the original function can be confirmed by comparing \(\varepsilon_p\) for \(L_{carb} = 1\) (i.e. no second compartment) and \(a_{carb} = 0\) (i.e. only CO_2 uptake into the carboxysome). As pointed out by Schulz et al. (2007), diffusive CO_2 fluxes generally need to be added to cellular fluxes measured by MIMS (Badger et al. 1994) when relating them to \(^{13}\text{C}\) fractionation. For membrane permeability exceeding \(10^{-4}\) cm s\(^{-1}\), as proposed for a diatom (\(\sim 10^{-2}\) cm s\(^{-1}\); Hopkinson et al. 2011), diffusive CO_2 fluxes are high and internal CO_2 concentrations approach those of the cell’s exterior (Fig. S2). In this case, gross CO_2 efflux estimated by MIMS would be underestimated, which could explain part of the discrepancy between MIMS-based leakage and estimates based on Eqn 3 (Sharkey and Berry 1985). Assuming that the membrane
permeability of cyanobacteria is significantly lower, approaching $10^{-5}$ cm s$^{-1}$ (Badger et al. 1985, Marcus et al. 1986), diffusive CO$_2$ fluxes are however low enough to allow for considerable CO$_2$ accumulation in the cell (Fig. S2). Using this permeability, the effect of CO$_2$ diffusion on leakage obtained by our model was estimated, yielding maximum changes in the order of a few percent, which were thus neglected.

**Model application** - To test the sensitivity of our model, the potential effect of changes in $a_{cyt}$ on $\varepsilon_p$ was quantified, using the maximum variability observed in our study (0.84 vs. 0.76) while leaving all other parameters constant. This variability can explain a change in $\varepsilon_p$ by not more than 0.7‰. Thus, $a_{cyt}$ can be excluded as a main driver behind the variability in $\varepsilon_p$ (or leakage estimates), even if variability in $a_{cyt}$ is severely underestimated. Applying the model to our measured fluxes and $\varepsilon_p$ values, a range of different possible scenarios for intracellular fluxes and fractionation in the cytosol is obtained (Fig. 4).

According to these interrelations, while at $L_{cyt}$ according to our MIMS measurements (0.5), only a very high fractionation in the cytosol ($\varepsilon_{cyt}$) can explain our results, at $L_{cyt} \geq 0.7$, there is a large range of possible combinations of parameters (see shaded areas in Fig. 4). As we aim to find parameters that can explain $\varepsilon_p$ in both of our $p$CO$_2$ treatments, the high $\varepsilon_p$ measured in cells grown at 1400 $\mu$atm constrains the range of possible values, while $\varepsilon_p$ of cells grown at 380 $\mu$atm could be explained by a larger range of values for $a_{carb}$ and $L_{carb}$ (Fig. 4). High values for $a_{carb}$ and $L_{carb}$ (both approaching 1) allow for a larger range of possible values of $\varepsilon_{cyt}$ to explain our measured $\varepsilon_p$ (Fig. 4). Due to the high contribution of HCO$_3^-$ to C$_i$ uptake and the additional conversion of CO$_2$ to HCO$_3^-$ by the NDH complex, $a_{carb}$ is likely to be close to 1, most probably exceeding $a_{cyt}$ measured in our experiment (0.82). Moreover, high diffusive CO$_2$ influx into the carboxysome seems unlikely in view of the supposed function of the carboxysome as a diffusion barrier to CO$_2$ (e.g. Reinhold et al. 1989). While comparison experiments with CA knock-out mutants with intact and broken carboxysomes confirmed that the carboxysome shell impedes diffusion of CO$_2$ (Dou et al. 2008), the pores in the hexamer protein subunits of the shell are supposed to be permeable to small, negatively charged molecules such as HCO$_3^-$ (Espie and Kimber 2011, Klein et al. 2009, Tsai et al. 2007). Despite the low CO$_2$ permeability, high rates of CO$_2$ efflux, and thus high $L_{carb}$, are likely due to the very high accumulation factor (two to three orders of magnitude, this study and Kaplan et al. 1980). A value for $L_{carb}$ of 0.9 is therefore used in the model scenarios described in the following (Table 2). Using Eqn 12, the following expression for the ratio of internal to external C$_i$ fluxes can be derived:
\[
\frac{F_{\text{carb, CO}_2} + F_{\text{carb, HCO}_3^-}}{F_{\text{cyt, CO}_2} + F_{\text{cyt, HCO}_3^-}} = \frac{1 - L_{\text{cyt}}}{1 - L_{\text{carb}}} \tag{Eqn 15}
\]

For the chosen value for \(L_{\text{carb}}\) of 0.9 and the measured \(L_{\text{cyt}}\) of 0.5, Eqn 5 yields a ratio of internal vs. external \(C_i\) cycling of 5.

Compared to estimates based on Sharkey and Berry (1985), our model significantly improved the compatibility of leakage estimates with those obtained by MIMS measurements (Table 2). The maximum fractionation that could be achieved in an uncatalyzed reaction from \(\text{CO}_2\) to \(\text{HCO}_3^-\) is +13‰ (O’Leary et al. 1992). With this kinetic fractionation, \(\varepsilon_p\) values measured for the two \(p\text{CO}_2\) levels can be explained by leakage from the cytosol \(L_{\text{cyt}}\) of 0.8 and 0.6, respectively (Scenarios 1 & 2, Table 2), which is significantly lower than the estimate based on the function by Sharkey and Berry \((L_{13\text{C}} = 1.1\) and 0.8, respectively). The remaining difference to leakage estimates obtained by MIMS \((L_{\text{MIMS}} = 0.5)\) could be explained by an underestimation of leakage by the MIMS approach, as discussed above. Assuming that the conversion between \(\text{CO}_2\) and \(\text{HCO}_3^-\) in the cytosol was not completely unidirectional, \(\varepsilon_{\text{cyt}}\) could range between +13‰ and -9‰ (equilibrium fractionation; O’Leary et al. 1992). To simulate this intermediate scenario, an \(\varepsilon_{\text{cyt}}\) of +8‰ is assumed (Scenario 4), yielding an \(L_{\text{cyt}}\) of 0.9 for the high \(p\text{CO}_2\) treatment.

Kinetic fractionation could be achieved by the NDH complex or by creation of a strong disequilibrium in the cytosol, preventing any back-reaction from \(\text{HCO}_3^-\) to \(\text{CO}_2\). Mutants of \(\text{Synechococcus}\) expressing human CA in the cytosol were unable to accumulate \(C_i\) (Price and Badger 1989b), suggesting that \(\text{HCO}_3^-\) is accumulated in the cytosol, and that a chemical disequilibrium in the cytosol favors the reaction from \(\text{HCO}_3^-\) to \(\text{CO}_2\) rather than the opposite direction. This strongly argues for a fractionating enzyme instead of a purely chemical disequilibrium driving unidirectional \(\text{CO}_2\) to \(\text{HCO}_3^-\) conversion in the cytosol. Assuming that NDH not only prevents the back-reaction but also discriminates against \(^{13}\text{C}\) during the reaction, leakage estimates by our model can be further reconciled with MIMS-based estimates. With an \(\varepsilon_{\text{cyt}}\) of +30‰ (Scenario 5, Table 2), which is within the range of fractionation measured in other enzymes such as RubisCO, our MIMS-measured data can be reproduced even for the high \(p\text{CO}_2\) treatment \((L_{\text{MIMS}} = L_{\text{cyt}} = 0.5,\) Table 2). Although CA and NDH have been proposed to have similar reaction mechanisms (Price et al. 2002), our model results suggest that the fractionation by the NDH complex is different from that of CA (1‰ for conversion of \(\text{CO}_2\) to \(\text{HCO}_3^-\); Paneth and O’Leary 1985). This might be due to the fact that the subunit carrying out the hydration reaction in the NDH-14 complex (chpX) is embedded in
a larger functional unit including the transmembrane proton channel and is associated to the electron transport chain.

Since our MIMS measurements showed that leakage was unaffected by $p$CO$_2$ and the slight changes in $a_{\text{cyt}}$ could not explain the observed variation in $\varepsilon_p$, $a_{\text{carb}}$ and/or $\varepsilon_{\text{cyt}}$ would need to vary with $p$CO$_2$ for explaining the observed CO$_2$-dependence of $\varepsilon_p$. Any $p$CO$_2$-dependent changes in cytosolic pH and/or $a_{\text{cyt}}$ would be expected to decrease $a_{\text{carb}}$ at elevated $p$CO$_2$, and can thus not explain the increase in $\varepsilon_p$ observed in our measurements (Fig. 5, upper branch). An increase in the activity of the NDH complex, however, could yield an increase in $a_{\text{carb}}$ as well as $\varepsilon_{\text{cyt}}$ and therewith $\varepsilon_p$ (Fig. 5, lower branch). With an increase in $a_{\text{carb}}$ from 0.7 to 1 and an increase in $\varepsilon_{\text{cyt}}$ from 20 to 30‰, the measured increase in $\varepsilon_p$ between 380 and 1400 $\mu$atm can be explained, almost reproducing MIMS-measured leakage in both scenarios (Scenarios 5 & 6, Table 2). A higher activity of NDH at high $p$CO$_2$ may seem unexpected in view of its supposed role as part of the CCM. Yet, in contrast to other components of the CCM such as HCO$_3^-$ transporters, the reaction catalyzed by the NDH complex contributes to ATP regeneration rather than consuming energy, and thus a down-regulation of NDH at high $p$CO$_2$ would not provide any energetic benefit to the cell. A positive correlation with $p$CO$_2$ might be coupled to the higher CO$_2$ availability and resulting small change in $a_{\text{cyt}}$ at high $p$CO$_2$ levels in the acclimations (Table 1, Fig. S1C, Fig. 5). Please note that the change in $a_{\text{cyt}}$ may have been underestimated in our study due to the constant pH in MIMS measurements. Elevated cytosolic CO$_2$ availability could increase the activity of the NDH complex due to an increased availability of its substrate CO$_2$ (Fig. 5, lower branch). Due to its function as a proton pump in the thylakoid membrane, activity of the NDH complex can increase the ratio of ATP to NADPH available in the cell. The production of ATP by high NDH activity at high $p$CO$_2$ could, in turn, contribute to the increased ATP requirement to fuel N$_2$ fixation in *Trichodesmium* under ocean acidification (e.g. Eichner et al. 2014, Kranz et al. 2010).

Kranz et al. (2010) compared leakage estimates based on MIMS and $^{13}$C for *Trichodesmium* grown under different $p$CO$_2$ levels as well as light intensities. Applying our model to this dataset, MIMS-based leakage estimates could be reproduced with merely unidirectional fractionation for a low $p$CO$_2$ treatment with light intensities similar to our experiment (200 $\mu$mol photons m$^{-2}$ s$^{-1}$, Scenario 3, Table 2). In high $p$CO$_2$ and low light treatments, the difference between $L_{\text{MIMS}}$ and $L_{\text{cyt}}$ was larger and could consequently only be reconciled with $\varepsilon_{\text{cyt}}$ values much larger than +30‰. Short-term exposure to high light intensities (300 $\mu$mol photons m$^{-2}$ s$^{-1}$) in our experiment affected CO$_2$ efflux in cells acclimated to high $p$CO$_2$ (ANOVA, $p < 0.05$, data not shown). Such light-sensitivity generally
suggests CO₂ efflux to be closely associated to photosynthetic electron transport. As this light effect was only observed under high pCO₂, i.e. conditions associated with higher NDH activity according to our model results, the interrelation of CO₂ efflux and the NDH complex is further corroborated. The observed light effects on CO₂ efflux (this study) and εp (Kranz et al. 2010) impose interesting questions with regard to a potential regulation of the NDH complex by the electron transport chain (redox state and/or proton gradient), which should be investigated in future studies. A better understanding of the regulation of the NDH complex is essential to improve confidence in explaining the effects of pCO₂ as well as light on internal Cᵢ fluxes and potential feedbacks on cellular energy budgets of this key N₂ fixer.

Conclusion & Outlook

In conclusion, this study demonstrates that internal Cᵢ fluxes via the NDH-1₄ complex need to be considered not only in terms of cellular Cᵢ acquisition, but also with regard to carbon isotopic fractionation and cellular energy status. The comparison of direct measurements of Cᵢ fluxes with estimates based on isotopic composition revealed that ¹³C fractionation in *Trichodesmium* cannot be described adequately by considering only the external Cᵢ fluxes. Compatibility with direct leakage measurements was improved by applying a model accounting for internal Cᵢ fluxes around the carboxysome, e.g. via the NDH complex. These model calculations provide a generalization of the model by Sharkey and Berry (1985) and are thus independent of potentially uncertain assumptions of e.g. permeability of the cell membrane and carboxysome and pH values for the different compartments. Once future studies improve confidence in these parameters, a kinetic model can be used to predict internal concentrations and individual Cᵢ fluxes. The agreement of model results with measured values could further be improved by accounting for possible ¹³C enrichment of CO₂ leaking out of the cell as well as a disequilibrium situation in the surroundings of the cell (altering the isotopic signature of HCO₃⁻ taken up). The model applied here could be used to improve estimates of leakage based on ¹³C signatures also for other species in which several compartments and/or internal Cᵢ fluxes play an important role. For phytoplankton groups that are relevant in terms of paleo-proxies, this could have important implications for the interpretation of carbon isotope signals.
Supplementary Material

Fig. S1 shows DIC-saturated rates of C fixation ($V_{max}$), half saturation DIC concentrations ($K_{1/2}$) and HCO$_3^-$ : $C_i$ uptake ratios measured at three time points during the day in *Trichodesmium* grown under two $p$CO$_2$ levels and N sources (N$_2$ and NO$_3^-$). Fig. S2 shows the dependence of intracellular CO$_2$ concentrations on membrane permeability.

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transporters, diversity, genetic regulation and prospects for engineering into plants. Journal of Experimental Botany 59(7), 1441-1461.


Table 1: Diurnal cycle of $C_i$ fluxes measured by MIMS under acclimation DIC levels (~ 2100 µmol L$^{-1}$) in *Trichodesmium* acclimated to two different $p$CO$_2$ levels (380 vs 1400 µatm) and N sources (N$_2$ vs NO$_3^-$). All $C_i$ fluxes are given in µmol C (mg chl a)$^{-1}$ h$^{-1}$. Errors are standard deviations for biological replicates (1 SD; n = 3 except 1400 +NO$_3^-$ morning with n = 1).

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Table 2: Different scenarios of external and internal $C_i$ fluxes that can reconcile measurements of $C_i$ fluxes by MIMS and $\varepsilon_p$ values obtained in this study (Scenarios 1 to 5) and by Kranz et al. (2010, Scenario 3). $\varepsilon_p$, $a_{cyt}$ and $L_{MIMS}$ were measured, $L_{13C}$ was calculated from $\varepsilon_p$ according to Sharkey and Berry (1985), the remaining values are model input parameters and model results ($L_{cyt}$, $L_{carb}$, $a_{carb}$ and $\varepsilon_{cyt}$).

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<td>0.9</td>
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Fig. 1: Scheme of the cellular C_i pools and fluxes characterized by measurements and modeling. Fluxes and concentrations in the external medium and fluxes over the cell membrane as well as C fixation ($F_{\text{fix}}$) were measured by MIMS, while fluxes in and out of the carboxysome were modeled. Color code denotes δ^{13}C values of different cellular C_i pools. A: Fractionation during C fixation by RubisCO leads to depletion of POC in ^{13}C and enrichment of ^{13}C in the carboxysomal C_i pool. B: Fractionation during internal C_i cycling, e.g. via NDH, leads to ^{13}C depletion of the carboxysomal C_i pool. Consequently, the POC formed is isotopically lighter than in scenario A. $\varepsilon_{\text{Rub}}$, fractionation by RubisCO; $\varepsilon_{\text{cyt}}$, fractionation in the cytosol.
Fig. 2: Example showing the dependence of $C_i$ fluxes measured by MIMS in *Trichodesmium* on the DIC concentration in the assay. Data shown were measured in the evening in a culture grown at 380 µatm $pCO_2$ without $NO_3^-$. The shaded area denotes the range of acclimation DIC levels.
Fig. 3: Leakage estimates by MIMS (mean values of measurements conducted at three time points over the day; Badger et al. 1994) and $^{13}$C fractionation (Sharkey and Berry 1985) determined in *Trichodesmium* grown under two $p$CO$_2$ levels and N sources ($n \geq 3$).
Fig. 4: Interrelation of $L_{\text{carb}}$, $\varepsilon_{\text{cyt}}$ and $a_{\text{carb}}$ in the model, depicted for different values of $L_{\text{cyt}}$ and $\varepsilon_p$. The shaded areas mark the range of possible values for $L_{\text{carb}}$ and $\varepsilon_{\text{cyt}}$ that could reconcile our measurements of isotopic composition with measured external C$_i$ fluxes. Black and grey lines are based on $\varepsilon_p$ measured in cells acclimated to 380 and 1400 µatm $p$CO$_2$, respectively.
Fig. 5: Hypothetical effect of elevated $p$CO$_2$ on $\varepsilon_p$ with and without involvement of the NDH-1$_4$ complex. Without the NDH complex, elevated $p$CO$_2$ might decrease $\varepsilon_p$ due to the lower share of HCO$_3^-$ uptake ($a_{cyt}$ and $a_{carb}$; upper branch). Induction of the NDH-1$_4$ complex by a higher availability of its substrate CO$_2$ in the cytosol might, however, override these effects, elevating $a_{carb}$ and $\varepsilon_{cyt}$, both of which would increase $\varepsilon_p$ (lower branch).
Publication IV

Diversity of ocean acidification effects on marine $N_2$ fixers
Diversity of ocean acidification effects on marine N\textsubscript{2} fixers

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A B S T R A C T

Considering the important role of N\textsubscript{2} fixation for primary productivity and CO\textsubscript{2} sequestration, it is crucial to assess the response of diazotrophs to ocean acidification. Previous studies on the genus Trichodesmium suggested a strong sensitivity towards ocean acidification. In view of the large functional diversity in N\textsubscript{2} fixers, the objective of this study was to improve our knowledge of the CO\textsubscript{2} responses of other diazotrophs. To this end, the single-celled Cyanothecae sp. and two heterocystous species, Nodularia spumigena and the symbiotic Calothrix rhizopenaeae, were acclimated to two pCO\textsubscript{2} levels (380 vs. 980 μatm). Growth rates, cellular composition (carbon, nitrogen and chlorophyll a) as well as carbon and N\textsubscript{2} fixation rates (14C incorporation, acetylene reduction) were measured and compared to literature data on different N\textsubscript{2} fixers. The three species investigated in this study responded differently to elevated pCO\textsubscript{2}, showing enhanced, decreased or unaltered growth and production rates. For instance, Cyanothecae increased production rates with pCO\textsubscript{2}, which is in line with the general view that N\textsubscript{2} fixers benefit from ocean acidification. Due to lowered growth and production of Nodularia, nitrogen input to the Baltic Sea might decrease in the future. In Calothrix, no significant changes in growth or production could be observed, even though N\textsubscript{2} fixation was stimulated under elevated pCO\textsubscript{2}. Reviewing literature data confirmed a large variability in CO\textsubscript{2} sensitivity across diazotrophs. The contrasting response patterns in our and previous studies were discussed with regard to the carbonate chemistry in the respective natural habitats, the mode of N\textsubscript{2} fixation as well as differences in cellular energy limitation between the species. The group-specific CO\textsubscript{2} sensitivities will impact differently on future biogeochemical cycles of open-ocean environments and systems like the Baltic Sea and should therefore be considered in models estimating climate feedback effects.

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1. Introduction

Owing to their ability to fix atmospheric N\textsubscript{2}, diazotrophic cyanobacteria play a crucial role in the biogeochemical cycles of nitrogen as well as carbon. N being the limiting nutrient in vast regions of the ocean (e.g. Moore et al., 2013), N\textsubscript{2} fixers fuel primary productivity by providing a source of new N to the phytoplankton community. While primary production based on remineralization in the upper mixed layer does not lead to C export, at least on longer time and spatial scales, the so-called new production based on N\textsubscript{2} fixation can significantly foster net CO\textsubscript{2} sequestration (Eppliy and Peterson, 1979). Since nutrient concentrations in the low latitude surface ocean are expected to decrease with the predicted increase in stratification, N\textsubscript{2} fixation may become more important as global warming progresses (Doney, 2006). Aside from global warming, rising atmospheric CO\textsubscript{2} levels cause ocean acidification, which can affect phytoplankton in numerous ways (e.g. Rost et al., 2008). Considering the important role of N\textsubscript{2} fixation for primary productivity and CO\textsubscript{2} sequestration, it is crucial to assess the response of diazotrophs to ocean acidification. Laboratory experiments on the abundant diazotroph Trichodesmium sp. suggested a strong CO\textsubscript{2} sensitivity for this species, with an increase in N\textsubscript{2} fixation or particulate organic nitrogen (PON) production ranging between 35 and 140% for pCO\textsubscript{2} levels expected by the end of this century (e.g. Hutchins et al., 2007; Kranz et al., 2011; Levitan et al., 2007). While Trichodesmium is undoubtedly the most prominent diazotroph in the current ocean, contributing up to 50% of marine N\textsubscript{2} fixation (Mahaffey et al., 2005), the development of new methods has shed light on the importance of previously unrecognized N\textsubscript{2} fixers (e.g. Moisander et al., 2010; Thompson and Zehr, 2013; Zehr, 2011).

Diazotrophs differ with regard to cellular structure and physiological key mechanisms, which are linked to the different strategies they evolved to protect their N\textsubscript{2} fixing enzyme, nitrogenase, from O\textsubscript{2}. Single-celled species generally separate photosynthesis and N\textsubscript{2} fixation in time, fixing N\textsubscript{2} only during the night, whereas photosynthesis is carried out during the day. This day-night-cycle requires a concerted

Abbreviations: CCM, carbon concentrating mechanism; chl a, chlorophyll a; DIC, dissolved inorganic carbon; POC, particulate organic carbon; PON, particulate organic nitrogen; TA, total alkalinity.

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regulation of nitrogenase synthesis and degradation as well as respiration and photosynthesis, which is driven largely by a circadian rhythm (Mohr et al., 2010; Sherman et al., 1998; Toepel et al., 2009). Photosynthetic products are stored within glycogen granules that are broken down by respiration during the night, providing ATP for the highly energy-demanding process of $N_2$ fixation (Saito et al., 2011; Schneggurt et al., 1994). In most filamentous species, photosynthesis and $N_2$ fixation are separated in space, with only certain cells within a filament containing nitrogenase. These heterocysts are freely differentiated cells that lack photosystem II and are surrounded by a thick cell wall, which allows for $N_2$ fixation during the day by posing a diffusion barrier to $O_2$. N fixed in these cells is transported along the filament in the form of amino acids, while heterocysts are supplied with carbohydrates by the vegetative cells (reviewed in Böhme, 1998; Kumar et al., 2010). In *Trichodesmium*, photosynthesis and $N_2$ fixation are separated in space as well as time, with so-called diazocytes fixing $N_2$ during midday when photosynthesis is typically down-regulated (Berman-Frank et al., 2001; Fredriksson and Bergman, 1997).

The role of unicellular diazotrophs may have been severely underestimated, in terms of their ecology as well as biogeochemistry. Field studies using molecular tools to target nitrogenase genes have revealed high abundances of small, unicellular diazotrophic cyanobacteria (UCYN, which may contribute a significant share of marine $N_2$ fixation (Langlois et al., 2005; Moisander et al., 2010; Montoya et al., 2004; Zehr et al., 1998). Cyanobacteria belonging to group UCYN-A, which lack the genes for photosystem II and RubisCO and are therefore assumed to be symbiotic (Zehr et al., 2008), appear to be highly abundant, their global $nifH$ gene abundance exceeding that of *Trichodesmium* (Luo et al., 2012).

While most of these newly discovered species are uncultivated and thus underestimated, in terms of their ecology as well as biogeochemistry. To assess the diversity in $CO_2$ responses of $N_2$ fixers other than *Trichodesmium* have indeed yielded different $CO_2$ response patterns (e.g. Czerny et al., 2009; Fu et al., 2008; Garcia et al., 2013b; Wannicke et al., 2012), but very few investigations have compared multiple diazotrophic species or strains under the same experimental conditions (Garcia et al., 2013b; Hutchins et al., 2013). To assess the diversity in $CO_2$ responses of $N_2$ fixers with very different physiology, we determined $CO_2$ effects on the single-celled *Cyanotheca* sp. and two heterocystous species, *Nodularia spumigena* and the symbiotic *Calothrix rhusoselainea* by growing them under present-day (380 μatm) and future pCO$_2$ levels (980 μatm). These results are then compared to literature data to relate the response patterns to specific physiological traits and structural characteristics of the different types of $N_2$ fixers.

2. Material and methods

2.1. Culture conditions

Cultures of *Calothrix rhusoselainea* SC01, *Cyanotheca* sp. ATCC51142 and *Nodularia spumigena* IOW-2000/1 were grown in semi-continuous dilute batch cultures at 25 °C and 150 μmol photons m$^{-2}$ s$^{-1}$ with a 12:12 h light:dark cycle, using 0.2-μm-filtered artificial seawater (YBCII medium; Chen et al., 1996). Salinity of the media was set to 33 for *Cyanotheca* and *Calothrix* and 9 for *Nodularia* (measured with Autosol 8400B, Guildline). Cells were kept in exponential growth phase by regular dilution with culture medium. Cultures were grown in 1 L (Cyanotheca and Nodularia) or 2 L (*Calothrix*) culture flasks, which were continuously bubbled with 0.2-μm-filtered air with pCO$_2$ levels of 380 and 980 μatm. As *Calothrix* cultures tended to form small, sinking aggregates, they were additionally placed on a shaker (ShakerX, Kuhner). Gas mixtures were generated with a gas flow controller (CGM 2000, MCZ Umwelttechnik), mixing pure CO$_2$ (Air Liquide Deutschland) and CO$_2$-free air (CO2RP280, Dominick Hunter). Culture media were equilibrated with the respective pCO$_2$ for at least 24 h before usage. Prior to experiments, cells were allowed to acclimate to the respective pCO$_2$ for two weeks (~5 divisions). Cultures with a pH drift of ≥0.09 compared to cell-free reference media were excluded from further analysis.

2.2. Carbonate chemistry

Total alkalinity (TA) was determined by duplicate potentiometric titration (TitroLine alpha plus, Schott Instruments) and calculation from linear Gran plots (Gran, 1952). Reproducibility was ±5 μmol kg$^{-1}$. Samples for dissolved inorganic carbon (DIC) analysis were filtered through 0.2 μm cellulose acetate filters and measured colorimetrically (QuAAtro autoanalyzer, Seal, reproducibility ±5 μmol kg$^{-1}$). Certified Reference Materials supplied by A. Dickson ( Scripps Institution of Oceanography, USA) were used to correct for inaccuracies of TA and DIC measurements. Daily pH values of the media were measured photometrically on the NBS scale (pH meter pH3110, WTW, uncertainty 0.02 units), pCO$_2$ of the media was calculated from pH and DIC using CO2sys (Pierrot et al., 2006) with equilibrium constants K1 and K2 given by Mehrbach et al.

<table>
<thead>
<tr>
<th>Target pCO$_2$</th>
<th>pH (NBS)</th>
<th>TA [μmol kg$^{-1}$]</th>
<th>DIC [μmol kg$^{-1}$]</th>
<th>pCO$_2$ attained [μatm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>380</td>
<td>8.20 ± 0.01</td>
<td>2356 ± 2</td>
<td>1969 ± 1</td>
<td>380 ± 9</td>
</tr>
<tr>
<td>980</td>
<td>7.86 ± 0.02</td>
<td>2368 ± 13</td>
<td>2154 ± 10</td>
<td>974 ± 20</td>
</tr>
<tr>
<td>380</td>
<td>8.18 ± 0.02</td>
<td>1524 ± 28</td>
<td>1357 ± 12</td>
<td>391 ± 7</td>
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<tr>
<td>980</td>
<td>7.80 ± 0.02</td>
<td>1515 ± 17</td>
<td>1399 ± 25</td>
<td>974 ± 39</td>
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<tr>
<td>380</td>
<td>8.22 ± 0.02</td>
<td>2348 ± 16</td>
<td>1950 ± 13</td>
<td>340 ± 30</td>
</tr>
<tr>
<td>980</td>
<td>7.93 ± 0.03</td>
<td>2339 ± 28</td>
<td>2102 ± 18</td>
<td>832 ± 70</td>
</tr>
</tbody>
</table>
(1973), refit by Dickson and Millero (1987). Carbonate chemistry parameters for the different treatments are shown in Table 1.

2.3. Growth and elemental composition

Samples for determination of growth and elemental composition were generally taken between 1.5 and 3 h after the beginning of the photoperiod to account for changes due to the diurnal rhythm in cell metabolism. Cell densities and cell size of *Cyanothec*ae were determined using a Coulter counter (MultiSizer III, Beckman Coulter). Cell densities of *Nodularia* cultures were determined using a Sedgewick Rafter Cell (Graticules Ltd.) and light microscope (Axiovert 200 M, Zeiss). Duplicate samples for chlorophyll *a* (chl *a*) determination were filtered onto cellulose-nitrate filters (Whatman) and immediately transferred to −80°C. Chl *a* was extracted in acetone (90%) for ~24 h and treated by ultrasound (~10 sec, Sonifer 250, Branson Ultrasonics). Chl *a* concentrations were determined fluorometrically (TD-700 Fluorimeter, Turner Designs) and corrected for fluorescence of phycobilipigments (Holm-Hansen and Riemsma, 1978). Calibration was performed measuring absorbance of a chl *a* standard (*Anacystis nidulans*, Sigma) spectrophotometrically (Spectronic Genesys 5, Milton Roy) according to Jeffrey and Humphrey (1975). Specific growth rates (μ) were calculated from daily increments in cell density (*Cyanothec*ae and *Nodularia*) or chl *a* concentrations (*Calothrix*) monitored over 6–7 days:

\[
\mu(\text{d}^{-1}) = \frac{\text{ln}(c_{1}) - \text{ln}(c_{0})}{\text{d}t}
\]  

where \(c_{0}\) and \(c_{1}\) are the initial and final cell density or chl *a* concentration and \(\text{d}t\) is the time interval between samplings. Duplicate samples for analysis of POC and PON were filtered onto pre-combusted (500 °C, 10 h) GF/F filters. Prior to analysis, samples were acidified with 200 µl ultrapure HCl (0.2 M) to remove all inorganic C. Particulate organic carbon and nitrogen (POC and PON) contents were measured with an elemental analyzer (EuroEA, Euro Vector). Daily production rates of POC and PON were obtained by multiplication of the respective elemental quotas and growth rates.

2.4. N2 fixation

N2 fixation rates were determined using the acetylene reduction assay (Capone, 1993). Samples were transferred to crimp vials and spiked with acetylene (20% of head space volume) followed by incubation for 24 h at acclimation light and temperature with continuous shaking to minimize aggregation and sinking of cells. DIC consumption during the course of the assay was found to be insignificant (~4%). The amount of acetylene reduced to ethylene was then measured with a gas chromatograph (5890 Series II Plus, Hewlett Packard), which was calibrated on a daily basis with an ethylene standard (Sigma-Aldrich). Solubility of acetylene in the aqueous phase was taken into account by applying the Bunsen coefficient for the respective temperature and salinities (Breitharth et al., 2004).  

2.5. Carbon fixation

C fixation rates were determined using 14C fixation assays (Steemann-Nielsen, 1952). Samples were transferred to 25 ml vials with < 4% headspace and spiked with 20 µCi H14CO3 (1.576 GBq/mmol NaH14CO3 solution, Perkin Elmer), followed by incubation for 24 h at acclimation light and temperature with continuous shaking to minimize aggregation and sinking of cells. At the end of the incubation time period, samples were acidified with 6 N HCl (1 mL) to a final pH < 1, followed by degassing for ~3 days until all liquid had evaporated. Radioactivity of the samples was measured in a Packard Tri-Carb Liquid scintillation counter (GMI) between 24 and 48 h after addition of scintillation cocktail (UltimaGold AB, Packard). Counts were corrected for background radioactivity using samples that were acidified directly after addition of spike solution. The amount of C fixed during the assay (Cfixed) was calculated using the following equation:

\[
C_{\text{fixed}} = \frac{\text{dpm}_{\text{sample}} \times [\text{DIC}] \times 1.05}{\text{dpm}_{\text{total}}}
\]

where dpm_{sample} is the blank-corrected radioactivity of the acidified sample in dpm, [DIC] the respective concentration for each treatment, 1.05 the fractionation factor (Vogel et al., 1970) and dpm_{total} the total radioactivity of H14CO3 added to the sample. C fixation rates were normalized using chl *a* samples that were taken on the same day (*Calothrix*) and mean chl *a* cell quotas measured during the experiment (*Cyanothec*ae and *Nodularia*).

3. Results and discussion

The three species investigated in this study showed very different responses to elevated pCO2. In the following paragraphs, we first discuss the response patterns for each species in view of its respective ecophysiology. Subsequently, literature data on other species are synthesized and response patterns are related to ecological and physiological characteristics of the different diazotrophs.

3.1. Species-specific response patterns reflect cellular mechanisms of nutrient housekeeping

In *Cyanothec*ae, growth rates were not affected by pCO2 (t-test, p > 0.05, Fig. 1). In contrast, cell quotas of POC and PON increased to values more than twice as high at 980 compared to 380 µatm (t-test, p < 0.05, Table 2). Consequently, also production rates of POC and PON increased with pCO2 (t-test, p < 0.05, Fig. 1). Since cell size was not significantly affected (t-test, p > 0.05, data not shown), cells must have strongly increased their C and N content per volume. Neither cellular chl *a* content nor the ratio of POC:PON were affected by pCO2 (t-test, p > 0.05, Table 2). Also N2 fixation was not significantly affected by pCO2 (t-test, p > 0.05, Fig. 2). C fixation as determined in 14C-fixation assays showed no significant difference between treatments due to high variability (t-test, p > 0.05, Fig. 2), yet indicated the same trend as POC production. Both methods, however, yielded different rates in absolute terms, with 14C fixation being lower than POC production. As 14C fixation samples were not filtered but acidified to degas all inorganic C, these values potentially also include dissolved organic C produced by the cells. Thus, it is even more puzzling why 14C-based estimates were in fact lower than POC production rates. Part of this discrepancy could be explained by an overestimation of POC production due to diurnal variability in C quotas. The largest offset between methods was observed in *Cyanothec*ae, which has a strong diurnal cycle in cellular C and N quotas due to the temporal separation of N2 fixation and photosynthesis. In the heterocystous *Nodularia*, less diurnal variability can be expected and accordingly, 14C fixation and POC production were more consistent. This aspect can, however, not fully account for the observed offsets between methods. The acetylene reduction assay gives a measure of gross N2 fixation, including any N that might be excreted from the cell subsequent to fixation. Absolute values of N2 fixation and PON production are not directly comparable due to methodological issues, as pointed out previously by Mulholland and Capone (2001), and since the conversion factors from acetylene reduction to N2 fixation have not been verified for the here tested species. Despite these uncertainties in absolute numbers, trends with pCO2 can be interpreted for C as well as N. Furthermore, differences in trends between methods can give valuable indications of changes in N or C excretion. For *Cyanothec*ae, release of organic C and N measured in laboratory cultures was low (~2 and ~1% of fixed C and N, respectively; Benavides et al., 2013), suggesting a high nutrient retention potential. This is in line with the high variability in cellular contents (Table 2), which reflects effective storage...
mechanisms for C and N that are required due to the diurnal cycle of photosynthesis and N₂ fixation in this species. In each of the cells, fixed C is stored in glycogen granules and N can be directly used for synthesis of amino acids or cyanophycin, thus neither C nor N is subject to loss during intercellular transfer. The increase in POC with pCO₂ is consistent with the increase in glycogen contents found in a previous study testing effects of very high pCO₂ levels (10,000 and 80,000 μatm) on the same strain of Cyanothece (Stöckel et al., 2013).

In Nodularia, growth rates decreased significantly with increasing pCO₂ (t-test, p < 0.05, Fig. 1), while neither cellular chl a content nor POC and PON contents were significantly affected (t-test, p > 0.05, Table 2). POC:PON increased slightly with pCO₂ (t-test, p > 0.05, Table 2). POC and PON production generally showed a decreasing trend in response to elevated pCO₂, which was, however, only significant for PON production (t-test, p < 0.05, Fig. 1). The decrease in growth and production with increasing pCO₂ is in agreement with previous results by Czerny et al. (2009). In contrast, Karlberg and Wulff (2013) showed no effect, while Wannicke et al. (2012) found an increase in growth with pCO₂. In the latter study, however, cultures were limited by phosphate and were not continuously mixed. The negative effect of high pCO₂ on growth and production rates of Nodularia has been attributed to a detrimental effect of low pH on N transfer between cells along the filaments (Czerny et al., 2009). This explanation has been debated, however, since transfer likely occurs via a continuous periplasm, preventing any direct contact of fixed N compounds with the outer medium (Flores et al., 2006; Wannicke et al., 2012). In our study, C:N ratios were increased only slightly with pCO₂ (Table 2), which also argues against any considerable obstruction of trans-cellular N transfer unless C transfer is equally inhibited. Aside from the uncertainty in absolute values derived by the two methods, the ratio of ¹⁴C fixation to POC production was higher in Nodularia than in Cyanothece (Figs. 1 and 2).

Table 2
Cellular composition of Cyanothece, Nodularia and Calothrix grown at two different pCO₂ levels. Errors denote 1 SD (n ≥ 3, except for POC & PON in Cyanothece at 980 μatm with n = 2).

<table>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanothece</td>
<td>380</td>
<td>0.036 ± 0.007</td>
<td>0.60 ± 0.06</td>
<td>0.13 ± 0.01</td>
<td>17.0 ± 4.6</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>980</td>
<td>0.034 ± 0.007</td>
<td>1.27 ± 0.17</td>
<td>0.26 ± 0.04</td>
<td>42.5 ± 0.1</td>
<td>8.8 ± 0.3</td>
</tr>
<tr>
<td>Nodularia</td>
<td>380</td>
<td>0.65 ± 0.12</td>
<td>4.08 ± 1.50</td>
<td>0.83 ± 0.29</td>
<td>6.4 ± 2.2</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>980</td>
<td>0.53 ± 0.05</td>
<td>4.17 ± 0.36</td>
<td>0.81 ± 0.07</td>
<td>8.0 ± 1.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Calothrix</td>
<td>380</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>7.3 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>980</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>6.9 ± 0.7</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>
suggested higher excretion of dissolved organic carbon in Nodularia. This is in line with previous studies showing significant excretion of fixed C and N by this species (Ploug et al., 2011; Wannicke et al., 2009). Regarding pCO₂ effects, while our C fixation data do not allow statistical evaluation due to the lack of replication for one of the treatments (Fig. 2), the decreasing trend in C fixation with elevated pCO₂ is consistent with the decrease in POC production (Figs. 1 and 2). Likewise, N₂ fixation was not significantly affected by pCO₂ (t-test, p > 0.05, Fig. 2), but mean values showed a decreasing trend, in line with PON production. The similarity in these trends suggests no appreciable effect of pCO₂ on excretion of C or N for Nodularia. This is in agreement with previous results showing no effect of pCO₂ on cell-specific production of mucinous substances and dissolved organic carbon (Endres et al., 2012).

In Calothrix, mean values of growth rates increased with pCO₂, yet the trend was not significant due to high standard deviations (t-test, p > 0.05, Fig. 1). POC and PON contents (relative to chl a) and production rates as well as the POC:PON ratio were not significantly affected (t-test, p > 0.05, Fig. 1, Table 2). In contrast, N₂ fixation showed a significant increase with pCO₂ (t-test, p < 0.05, Fig. 2). C fixation was not significantly different between pCO₂ treatments and was subject to high variability between replicates (t-test, p > 0.05, Fig. 2). Aggregation of filaments caused patchiness in our cultures, which precluded cell counts and resulted in deviations between triplicates for cellular composition, despite using large sample volumes. Results from bioassays are even more variable due to restrictions in sample volumes in the assays. Direct measurements of growth and cellular composition of Calothrix have to our knowledge not been performed previously and little is known about the physiology of Calothrix and other symbiotic cyanobacteria. While our study gives first indications of their CO₂ responses, it should be noted that the applicability to the natural environment is compromised by the fact that cells were cultured without the host. Cells in our cultures formed small aggregates, which may have caused microenvironments to slightly differ from the bulk medium. A similar situation in fact occurs in natural symbioses, where epibionts such as Calothrix are located in the boundary layer of the diatom cell, i.e. in a microenvironment shaped by the diatom’s metabolism (Flynn et al., 2012). Moreover, the host cell seems to directly influence the cyanobacterial metabolism in symbioses, considerably increasing the N₂ fixation rates (Foster et al., 2011). While N₂ fixation rates measured in our study cannot be directly compared with previous data since cellular chl a quotas have not been determined, assuming a chl a quota similar to Trichodesmium the N₂ fixation rates obtained in our experiment (Fig. 2) are higher than those of free-living filaments measured in the field (0.17 fmol N per cell h⁻¹; Foster et al., 2011) and in a previous laboratory study (-1 to 4 moln ethylene (mg chl a)⁻¹ h⁻¹; Foster et al., 2010). In fact, N₂ fixation rates are more similar to those of cells in symbiosis measured in field studies (70 fmol N per cell h⁻¹; Foster et al., 2011; 50 fmol N per cell h⁻¹ for Richelia, Carpenter et al., 1999), suggesting that the physiological status in our cultures may indeed be comparable to symbiotic Calothrix in their natural environment.

3.2. Ocean acidification responses across different N₂ fixers are highly variable

As outlined above, the three investigated species responded very differently to elevated pCO₂. A review of literature data showed that this large diversity in response patterns holds also for other species of N₂ fixing cyanobacteria (Table 3). To identify characteristics that might explain these differences, we grouped N₂ fixers according to their mechanisms for N₂ fixation (Table 3). This comparison revealed a considerably large variability of pCO₂ responses within groups and even species (Table 3). Part of this can be attributed to species- or strain-specific differences in CO₂ sensitivities (e.g. Hutchins et al., 2013; Langer et al., 2006; Schaum et al., 2013). It also has to be noted that growth conditions can significantly modulate the responses to elevated pCO₂ (e.g. García et al., 2011; Kranz et al., 2010; Shi et al., 2012), which complicates linking response patterns to specific traits. Lastly, the low number of studies on certain groups does not permit deducing clear statements on their CO₂ sensitivity.

Especially for heterocystous species, there is little data with often contrasting results, indicating stimulation in N₂ fixation for Calothrix and negative as well as positive responses towards elevated pCO₂ for Nodularia (Table 3). The diazocystous Trichodesmium, however, has been extensively studied and the data are more coherent, showing a positive response of N₂ fixation in most cases (Table 3). Also single-celled species responded positively to elevated pCO₂ in many of the studies, yet the magnitude of effects was generally smaller than in Trichodesmium. For instance, growth was significantly increased in about half of the observations on Trichodesmium but was not altered in most observations on single-celled species (Table 3). Also, in cases where an increase in N₂ fixation was observed, the magnitude of effects ranged between ~35 and ~140% in Trichodesmium, while for single-celled species it ranged only between ~20 and 45% (Table 3). Regarding cellular composition and production of POC and PON, data are scarce for single-celled species (Table 3). While we observed a strong increase in C and N quotas for Cyanothece, no such changes were observed in the only other study investigating C quota of Crocosphaera (Fu et al., 2008; Table 3). For understanding the variable responses to rising pCO₂, it thus seems insufficient to consider only the functional diversity in modes of N₂ fixation. Looking at the present-day growth conditions of a species may also provide hints about its sensitivity towards ocean acidification.

3.3. CO₂ responses are dependent on ecological niches

The species investigated in our study grow in different habitats, which strongly diverge with respect to carbonate chemistry. Compared to the relatively stable conditions experienced in open-ocean environments by diazotrophs such as Cyanothece, species in the Baltic Sea such as Nodularia are subject to highly variable carbonate chemistry over time. This is due to the low alkalinity as well as high biological activity of dense cyanobacterial blooms inducing strong seasonal variability (Thomas and Schneider, 1999). Regarding effects of increased CO₂ supply on the three species investigated here, adaptation of their carbon concentrating mechanisms (CCM) to the respective habitats might thus cause different CO₂ sensitivities. In surface scums formed by summer blooms of Nodularia and Aphanizomenon, pH can reach values as high as 9 during illumination (Ploug, 2008). Thus, one could explain the negative response of Nodularia to pCO₂ levels exceeding 380 µatm by assuming that their mode of CCM and pH homeostasis has evolved to function optimally under low pCO₂/high pH. In fact, Czerny et al. (2009) hypothesized maximal growth rates even below a pCO₂ of 150 µatm. In the case of symbiotic Calothrix, carbonate chemistry in the immediate surroundings of the cell is modulated by the diatom host, which can significantly alter CO₂ concentrations and pH by respiration and photosynthesis. Since diurnal variability within the microenvironment of photoautotrophs is directly dependent on their size (Flynn et al., 2012), variability can be expected to be larger for a symbiont on a diatom chain than for a single cyanobacterial cell or filament, though by far not as large as in surface scums of Nodularia in the Baltic. In our experiment, Calothrix did not show negative responses as observed in Nodularia, but tended to respond positively to elevated pCO₂ like the single-celled Cyanothece (Table 3). Thus, while Nodularia and Calothrix are both heterocystous, adaptation of their CCM and/or their mode of pH homeostasis to different environmental conditions might explain the differences in pCO₂ responses.

3.4. Magnitudes of CO₂ responses can be attributed to cellular energy demand

Due to the strong interdependence of different physiological pathways in the cell, several other factors apart from carbonate chemistry
Table 3
Overview of literature data on pCO2 responses of different N2 fixing cyanobacteria. Arrows indicate direction of trend with increasing pCO2; numbers in brackets indicate percent change relative to low pCO2 level calculated from original data; significance was taken from the respective publications. For comparison reasons, only data for pCO2 levels closest to our treatments and normalized to cell or chl a (where available) are shown. For studies with varying nutrient conditions, data from replete conditions are shown, unless stated otherwise. Light intensity is given in μmol photons m⁻² s⁻¹.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species/strain</th>
<th>pCO2 [μatm]</th>
<th>Light intensity</th>
<th>Growth</th>
<th>POC content</th>
<th>PON content</th>
<th>POC:PON</th>
<th>N2 fixation</th>
<th>O2 evol. or C fix.</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Single cells</td>
<td>Crocosphaera watsonii WH8501</td>
<td>380 vs 750 80</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↑(+40%)</td>
<td>↑(+20%)²</td>
<td>(Fu et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>watsonii WH003</td>
<td>280 vs 750 120</td>
<td>↑(+60%)</td>
<td>↑(+20%)</td>
<td>↑(+45%)</td>
<td>↑(+60%)²</td>
<td>↑(+70%)</td>
<td>(Hutchins et al., 2013)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>watsonii WH0402</td>
<td>380 vs 750 100</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↑(+35%)</td>
<td>↑(+17%)²</td>
<td>(Hutchins et al., 2013)</td>
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<tr>
<td></td>
<td>watsonii WH0401</td>
<td>380 vs 750 100</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↑(+35%)</td>
<td>↑(+20%)²</td>
<td>(Hutchins et al., 2013)</td>
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<td></td>
<td>Cyanothece ATCC51142</td>
<td>380 vs 980 150</td>
<td>↑(+110%)</td>
<td>↑(+100%)</td>
<td>↑(+140%)²</td>
<td>↑(+70%)</td>
<td>↑(+60%)²</td>
<td>(Barcelos é Ramos et al., 2007)</td>
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<td></td>
<td>Trichodesmium erythraeum IMS101</td>
<td>380 vs 850 150</td>
<td>↑(−60%)</td>
<td>↑(−60%)</td>
<td>↑(−60%)</td>
<td>↑(+110%)</td>
<td>↑(+50%)</td>
<td>(Hutchins et al., 2007)</td>
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<td>Heterocysts</td>
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<td>Calothrix rhizosoleniae SC01</td>
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¹ O2 evolution.
² C fixation.
³ Low iron.
⁴ Field incubations.
⁵ Low phosphate.
can affect C acquisition and thus alter the overall CO₂ responses of phytoplankton. Studies on the underlying mechanisms of CO₂ responses suggested a reallocation of energy between the CCM and N₂ fixation in *Trichodesmium* (Kranz et al., 2009, 2010). This was confirmed by a strong modulation of CO₂ effects by light intensity (Kranz et al., 2010), suggesting that the degree of energy limitation plays an important role in controlling CO₂ responses in this species. A dependence of CO₂ effects on energy limitation has also been suggested for *Crococphera* in a study on combined effects of pCO₂ and phosphorus limitation, which linked the magnitude of pCO₂ effects to ATP pool size (Garcia et al., 2013a). Daily synthesis of nitrogenase and the photosynthetic and respiratory protein complexes is supposed to consume a significant fraction of cellular resource and energy costs in *Trichodesmium* (Brown et al., 2008). For single-celled species such as *Crococphera* and *Cyanothece*, it can be assumed that the day-night separation of photosynthesis and N₂ fixation similarly imposes high energy demands for the turnover of enzymes and storage products. Thus, while single-celled species may respond positively to elevated pCO₂ due to the lowered energy demand of the CCM, just as proposed for *Trichodesmium*, the difference in magnitudes of responses might be associated to differences in energy limitation under the experimental conditions. While *Crococphera* occurs with maximum abundance at −35 – 60 m depth (Hewson et al., 2009; Montoya et al., 2004), *Trichodesmium* is known to form surface blooms (Capone et al., 1997). One could therefore speculate that *Trichodesmium* experienced relatively stronger energy limitation under the light conditions typically applied in the experiments (Table 3). Not only the overall magnitude of CO₂ effects but also the relative sensitivity of C and N₂ fixation differed between *Trichodesmium* and single-celled species: In *Trichodesmium*, N₂ fixation was strongly increased in many of the studies (up to 140%; Table 3), while C fixation/O₂ evolution were increased only in part of the studies and to a lesser extent (up to 60%; Table 3). In contrast, single-celled species showed an increase in N₂ fixation only in half of the observations (reaching up to 45%), while C fixation was significantly increased (up to 60%) in all except our study (Table 3). This pattern suggests that the reallocation of energy from C acquisition to N₂ fixation in the single-celled species was not as pronounced as in *Trichodesmium*. The physiology of C acquisition in *Crococphera* or *Cyanothece* has not been characterized to date. However, based on the differences in cell/filament size and thus diffusive boundary layers, it could be expected that small, single-celled species can cover more of their C demand by CO₂ diffusion than the large filaments/aggregates of *Trichodesmium* (Edwards et al., 2011; Finkel et al., 2010; Wolf-Gladrow and Riebesell, 1997). If cells rely to a large extent on diffusive CO₂ supply rather than active uptake of HCO₃⁻, elevated CO₂ concentrations will directly increase C influx with supposedly little effect on the cellular energy budget, and consequently also little feedback on N₂ fixation.

In heterocystous species such as *Nodularia*, overall energy demands connected to N assimilation might be lower than in single-celled species or *Trichodesmium*, since daily synthesis and degradation of nitrogenase as well as high turn-over of storage products are not necessary. The N transfer between cells also seems to be more efficient in *Nodularia* (excretion up to 33%; Ploug et al., 2011) than in *Trichodesmium* (excretion up to 80%; Mulholland, 2007), which is in line with transfer being conducted directly between adjoining cells rather than extracellularly (Flores et al., 2006). If the magnitude of CO₂ responses is directly dependent on the level of energy limitation, as proposed for *Trichodesmium* (Kranz et al., 2010), the lower energy demands for N assimilation may explain why *Nodularia* did not respond positively to high pCO₂ in our study. In line with this, the positive CO₂ responses observed in *Nodularia* by Wannicke et al. (2012) might reflect energy constraints induced by phosphate limitation in that experiment. However, neither the costs nor the regulation of C acquisition in *Nodularia* have been investigated to date. Knowledge on the physiology of *Calothrix* is even more limited and energy demands are most likely highly variable depending on whether cells occur in free-living filaments or in symbioses.

3.5. Benefits from CO₂ concentration outbalance negative pH effects in most, but not all N₂ fixers

For most of the N₂ fixers tested to date, a benefit from either the direct effect of CO₂ supply or the energy saved from down-regulation of CCM activity seems to allow positive responses to ocean acidification. While differences in the magnitude of these CO₂ effects could be attributed to cellular energy limitation, negative responses, as observed in *Nodularia*, cannot be explained by the costs of C acquisition. The concurrent decrease in seawater pH under ocean acidification affects many cellular processes, such as enzyme functioning, transport processes and ion balance, as well as speciation of nutrients (e.g. Hinga, 2002). In *Trichodesmium* grown under low iron concentrations, low pH has also been shown to negatively affect nitrogenase efficiency (Shi et al., 2012). As nitrogenase is a highly conserved protein complex (Zehr et al., 2003), this pH effect on the enzyme seems likely to be relevant also for other diazotrophs. In previous DIC manipulation experiments with *Trichodesmium* under nutrient replete conditions, this negative effect was apparently outbalanced by the strong positive effect of high CO₂ concentrations. In *Nodularia*, on the other hand, positive effects of high pCO₂ seemed to be attenuated, possibly due to adaptation to low pCO₂ levels and a lower energy demand of N assimilation, which allowed negative effects of the pH to dominate. For a better understanding of these opposing effects, both factors should be tested independently in a setup with uncoupled carbonate chemistry (e.g. Bach et al., 2013).

3.6. Biogeochemical implications differ between ecosystems and depend on ecological interactions

Concerning implications on the ecosystem level, the wide variety in pCO₂ responses of N₂ fixers is likely to affect community composition in future oceans (Hutchins et al., 2013). Since N₂ fixing species differ with regard to important ecological and physiological characteristics, such as susceptibility to grazing, sinking and nutrient release, these species shifts will further impact on biogeochemical cycles of C and N (Soehn et al., 2011). For instance, the increase in POC and PON production rates at high pCO₂ observed in *Cyanothece* would increase C and N input into the photic zone. The possible increase in N₂ fixation rates by single-celled species as well as *Calothrix* adds on to the increase in N₂ fixation projected for *Trichodesmium*. The fate of fixed N within the ecosystem, however, is likely to differ between diazotroph species (Thompson and Zehr, 2013). While *Trichodesmium* is hardly grazed and releases large amounts of fixed N that is directly available to other phytoplankton (e.g. Mulholland, 2007), in *Cyanothece* fixed N seems to be efficiently stored and may thus be transferred more directly to higher trophic levels. In *Calothrix*, the bulk of fixed N is transferred to the diatom host cells (Foster et al., 2011). While diazotrophs are often neutrally buoyant, diatoms sink relatively fast due to their dense silica shells. A possible increase in DDA blooms due to the stimulation in N₂ fixation would thus have profound impacts on the global C cycle, fueling the biological pump and therewith CO₂ sequestration (Karl et al., 2012; Subramaniam et al., 2008).

In contrast to the stimulating effect of high pCO₂ on open-ocean symbiotic and single-celled species, our study as well as Czerny et al. (2009) showed decreasing growth and production rates for the Baltic *Nodularia*. Since results from different studies on this species are conflictive (cf. Karlberg and Wulff, 2013; Wannicke et al., 2012), further investigations are necessary before making conclusive predictions of ecosystem responses, which should also include other important Baltic diazotrophs, such as *Aphanizomenon*. *Nodularia* and *Aphanizomenon* frequently co-occur in cyanobacterial blooms and their relative abundance has been shown to be strongly dependent on environmental perturbations (Stal et al., 2003). Species interactions and possible differences in CO₂ sensitivity could change community composition and therewith toxicity of future blooms, as *Nodularia* produces the hepatotoxin...
nodiularin (Karlberg and Wulf, 2013; Sivonen et al., 1989). Aggregates and surface scums, such as those formed by Nodularia, are usually heavily colonized by heterotrophic bacteria and play an important role in promoting regenerated production due to the excretion of inorganic C and N (Ploug et al., 2011; Simon et al., 2002). A decrease in Nodularia productivity may therefore have profound impacts on Baltic nutrient fluxes, depending on the response of cyanobacteria with similar ecological function such as Aphanizomenon.

4. Conclusions

In summary, the three species of functionally different N2 fixers investigated in this study responded differently to elevated pCO2, showing enhanced, decreased as well as unaltered growth and production rates. This confirms the large diversity of pCO2 responses found among previous studies and suggests that the deviations between studies were not merely due to discrepancies in experimental conditions. Our review showed that while CO2 effects on Trichodesmium have been extensively studied, we cannot extrapolate these responses to other diazotrophs. Based on results from this and previous studies, we were able to draw connections between response patterns and regional differences in carbonate chemistry as well as species-specific differences in energy requirements. These interrelations yet need to be confirmed by physiological studies to enable more robust predictions of response patterns.

The observed differences in CO2 sensitivities will impact on species distribution and biogeochemical cycles and should therefore be considered in ecosystem and global models estimating climate feedback effects. In areas of strong competition between N2 fixers, contrasting responses to rising pCO2 can lead to considerable regional changes, which should be considered in small-scale, regional models. In addition to Trichodesmium, also single-celled and symbiotic open-ocean species were found to respond positively to high pCO2. Despite species-specific differences observed in the magnitude of responses, our study thus confirms the predictions of an increase in Nfixation with ocean acidification on a global scale. In contrast, N input to the Baltic Sea might decrease due to potentially lowered productivity of Nodularia.

Large uncertainty remains with regard to the poorly characterized UCYN-A cyanobacteria, which are increasingly recognized to have a high biogeochemical importance. Their pCO2 responses and underlying mechanisms are most probably very different from the diazotrophs studied so far, as they do not fix C and are thus unlikely to react to between nitrogen and phosphorus competitive abilities and cell size in phytoplankton. Ecology 92 (11), 2085–2097.


References
4. SYNTHESIS

4.1 Physiological mechanisms behind ocean acidification responses - what we learned from *Trichodesmium*

4.2.1 Ocean acidification and energy allocation

In the first part of this thesis, the physiological mechanisms behind ocean acidification effects on the N\textsubscript{2} fixer *Trichodesmium* were investigated. *Trichodesmium* was shown to respond sensitively to ocean acidification scenarios not only in cellular pathways directly linked to carbon acquisition (CCM and POC production) but also by strongly increasing N\textsubscript{2} fixation and PON production (*Publications I & II; Hutchins et al., 2007; Kranz et al., 2009; Kranz et al., 2010; Levitan et al., 2007*). These effects were attributed to the tight links between C and N metabolism by shared biochemical pathways as well as competition for energy equivalents, CCM and N\textsubscript{2} fixation constituting major energy sinks in N\textsubscript{2} fixing cyanobacteria (*Publication I*). The dependence of the ocean acidification responses on energy availability was emphasized by a strong modulation of effects by light intensity (*Publication I*).

Fluxes of O\textsubscript{2} and chlorophyll *a* fluorescence measurements indicated that photosynthetic and respiratory pathways were not altered under different pCO\textsubscript{2} levels or N sources despite the differences in energy requirements imposed by the treatments (*Publication II*). This is in agreement with several other studies showing that rates of photosynthesis and respiration were largely unaffected by pCO\textsubscript{2} (*Publication I; Kranz et al., 2009; Kranz et al., 2010; Levitan et al., 2010c*). In view of the efficient mechanisms in *Trichodesmium* for adjusting electron transport to different light intensities as well as controlling O\textsubscript{2} fluxes over the day (e.g. Andresen et al., 2009; Berman-Frank et al., 2001b), it remains puzzling that cellular energy regeneration was not altered in the experiments. Yet, the observed changes in energy consuming processes suggest that *Trichodesmium* can efficiently redistribute energy among the sinks according to the requirements imposed by different environmental conditions (Fig. 5). More specifically, it was hypothesized that under high pCO\textsubscript{2}, down-regulation of the CCM allows for energy reallocation to N\textsubscript{2} fixation (*Publications I & II, Fig. 5A*).
4.2.2 Different N sources and energy allocation

Efficient mechanisms for energy reallocation were confirmed by growing *Trichodesmium* with different N sources, which induced changes in assimilation of N as well as C (Publication II, Fig. 5B). Higher POC and PON production in cells grown on NO$_3^-$ rather than N$_2$ was attributed to the lower energy costs associated with NO$_3^-$ assimilation in addition to better N retention. The comparison of $p$CO$_2$ effects between cells grown on NO$_3^-$ vs. N$_2$ furthermore revealed the constraints of reallocation, showing that the capacity for energy reallocation is strongly dependent on the stoichiometry of energy requirements (ATP:NADPH) of sinks and
sources (Publication II, Fig. 6). As CO₂ effects on the CCM were less pronounced in NO₃⁻ users than N₂ fixers (Publication II), it can be hypothesized that cells grown with NO₃⁻ could not benefit from a down-regulation in the CCM to the same extent as those grown on N₂ due to differences in energy requirements of the two N sources. CCM and N₂ fixation both have high ATP requirements, favoring energy reallocation between the two processes due to a compatible stoichiometry, while NO₃⁻ assimilation requires mainly reducing equivalents, limiting the potential for energy reallocation from the CCM (Fig. 6).

Fig. 6: Potential for energy reallocation from the CCM to N assimilation at elevated pCO₂ with different N sources. Owing to the different stoichiometry in energy equivalents required by N₂ fixation and NO₃⁻ assimilation, energy can be readily reallocated from the CCM to N₂ fixation, while in NO₃⁻ users there is only limited potential for reallocation due to the low ATP per NADPH demand.

The importance of the availability of energy equivalents in regulation of ocean acidification responses has been emphasized also in studies on other phytoplankton such as coccolithophores, where a reconstitution of metabolic fluxes was suggested to be induced by changes in the redox equilibria of NAD⁺ and NADP⁺ (Rokitta et al., 2012). Publication II illustrates that it is not only the redox state and overall availability of energy equivalents but also the relative proportion of ATP to reduction equivalents that determines ocean acidification responses. This is in close analogy to the concept of ecological stoichiometry highlighting the importance of the ratio of elements in nutrient limitation (e.g. Sterner and Elser, 2002).

4.2.3 Internal Cᵢ fluxes and energy availability

Considering the importance of energy reallocation for explaining ocean acidification effects, it is of key interest to quantify energy demands and understand the regulation of key physiological pathways such as the CCM. Energy demands of the CCM generally depend on the Cᵢ source, since HCO₃⁻ uptake is mediated by ATP-dependent transporters, but also on the amount of Cᵢ lost by leakage. In Publication III, offsets in leakage estimates obtained by two different methods were demonstrated, comparing results of direct flux measurements with a common ¹³C–based approach relating fractionation to external Cᵢ fluxes. These offsets
could be explained by applying a model accounting for the effects of internal C\textsubscript{i} fluxes on fractionation. According to this model, fractionation in the cytosol during C\textsubscript{i} cycling, e.g. by unidirectional kinetic fractionation or by enzymatic fractionation by the NDH-1\textsubscript{4} complex, leads to a depletion of organic matter in $^{13}$C (Fig. 7).

![Diagram of Current pCO\textsubscript{2} and High pCO\textsubscript{2}]

**Fig. 7:** Hypothetical effect of elevated pCO\textsubscript{2} on internal C\textsubscript{i} fluxes, isotopic composition of C\textsubscript{i} pools and energy availability. Different shades of blue denote isotopic composition of C\textsubscript{i} pools, with darker colour indicating higher $\delta^{13}$C. At high pCO\textsubscript{2}, internal cycling of C\textsubscript{i} via the NDH complex is increased, leading to formation of isotopically lighter POC. Due to proton pumping through the thylakoid membrane by the NDH complex, an increasing amount of ATP is generated, which can be used for to fuel elevated N\textsubscript{2} fixation rates at high pCO\textsubscript{2}.

Results from *Publication III* highlight the importance of internal C\textsubscript{i} cycling via the NDH complex in *Trichodesmium* with regard to several aspects: In addition to its role as a CO\textsubscript{2} uptake mechanism and its effects on $^{13}$C composition of POC, NDH affects the cellular energy status by contributing to ATP regeneration. Model calculations suggested internal cycling via the NDH-1\textsubscript{4} complex to be affected by light intensities and pCO\textsubscript{2}, with higher activity in ocean acidification scenarios (*Publication III*). Consequently, changes in NDH activity can be assumed to alter cellular ATP:NADPH ratios under different pCO\textsubscript{2} conditions (Fig. 7). Accordingly, it has been suggested that higher NDH-1\textsubscript{4} activity may fuel the increased N\textsubscript{2} fixation rates observed at high pCO\textsubscript{2} (Kranz et al., 2010; Fig. 7). Although the molecular regulation mechanisms remain unclear, considering that NDH-1\textsubscript{4} is directly involved in the ETC it seems likely to be affected by the redox state and proton gradient. Both have been suggested as regulators for numerous cellular processes, including the Calvin Cycle and N metabolism (Durnford and Falkowski, 1997; Enser and Heber, 1980; Giordano et al., 2005b).
While the transcriptional regulation of the inducible CCM components has been proposed to be independent of the redox state of the ETC, these mechanisms may not be valid for NDH-14, which is constitutively expressed (Woodger et al., 2003).

Especially in view of the results from Publication II highlighting the importance of the stoichiometry of energy equivalents, the capacity of the NDH complex for fueling ATP regeneration may play an important role in *Trichodesmium*. Quantitative estimates of energy savings by CCM down-regulation can only be made once the regulation of the NDH complex in dependence of environmental conditions is understood. Yet, results from Publications I & II also demonstrate that competition for energy between physiological key processes induces indirect effects of rising $pCO_2$, which can only be understood if the cellular metabolism is investigated in a holistic approach rather than looking at single pathways in isolation.

### 4.2 Response patterns in other N$_2$ fixers – can we generalize?

The comparison of ocean acidification responses of different N$_2$ fixers revealed a high variability (Publication IV), implying that response patterns observed in *Trichodesmium* cannot be generalized to other marine N$_2$ fixers. The basic mechanisms for reallocation of energy under ocean acidification (e.g. energy reallocation from CCMs to other processes) are most likely a universal pattern, and similar effects have been suggested also for other phytoplankton (e.g. Rokitta and Rost, 2012). Yet, results from Publication IV demonstrate that the degree to which these mechanisms are expressed seems to be dependent on many external and intrinsic, group-specific factors. N$_2$ fixers have a diverse physiology, part of which can be attributed to the different adaptations for separating N$_2$ fixation and photosynthesis (Fig. 3), such as cell differentiation into heterocysts, circadian rhythms as well as the metabolism required for storage of C and N. Yet, also within groups with the same N$_2$ fixation mechanisms, there is considerable variability in ocean acidification responses (Publication IV). Several characteristics that could be related to the observed response patterns were identified in Publication IV, including differences in cellular energy limitation, different strategies of nutrient ‘housekeeping’ as well as adaptations to different habitats with regard to carbonate chemistry.

Energy requirements of cellular metabolism could differ between species with the energy costs for turnover of enzymes and storage compounds, regulation of O$_2$ fluxes, CCM
demands due to size differences, as well as the efficiency of nutrient transfer between cells (Publication IV). Reallocation of energy between different pathways may play a more important role in species with generally higher energy demands relative to the energy supply. For instance, *Trichodesmium* could be speculated to have higher energy demands compared to the heterocystous *Nodularia*, as it synthesizes a new pool of nitrogenase every day (Capone et al., 1990) and is furthermore subject to a substantial loss of N during extracellular transfer of N (Mulholland, 2007). While both species frequently form surface blooms and are thus subject to very high light intensities, under limiting light conditions such as those imposed in a laboratory experiment, *Trichodesmium* may be more dependent on efficient energy reallocation mechanisms. It has to be noted, however, that laboratory experiments with light intensities differing from natural conditions, which are typically dynamic and often higher than those applied in laboratory studies, can only give limited information about species-specific differences in energy limitation.

N$_2$ fixers have different mechanisms for adjusting their metabolism in response to changes in energy availability. Several patterns of ‘nutrient housekeeping’ could be deduced from comparisons of net vs. gross C and N uptake as well as the plasticity in cellular C and N quotas in different species (Publication IV). In the unicellular *Cyanothece*, efficient mechanisms for storage of C and N, which are fundamental to support the diurnal cycle in N$_2$ and C fixation, are also the basis for strong variations in cellular POC and PON quotas in response to elevated pCO$_2$ (Publication IV). In the filamentous *Trichodesmium* and *Nodularia*, excretion rates of C and N are generally high (e.g. Mulholland, 2007; Ploug et al., 2011), which is, at least in the case of *Trichodesmium*, due to the requirement for transfer of N and C between cells. Variability in cell quotas was lower in these species than in *Cyanothece*, and, instead, increasing nutrient release may provide a mechanism to shunt excess energy under high light or ocean acidification (Publications II & IV; Wannicke et al., 2009).

The ecophysiology of the CCM has been investigated in several studies on *Trichodesmium* (Publication III; Kranz et al., 2009; Kranz et al., 2010; Levitan et al., 2010b), though not in any of the other N$_2$ fixers examined in this thesis. However, genetic evidence suggests that the CCM of *Trichodesmium* might respond differently to environmental changes than that of other important marine N$_2$ fixers. Many cyanobacteria (e.g. *Nodularia, Crocosphaera* and *Anabaena*) encode for a combination of constitutive, low-affinity HCO$_3^-$ transporters and CO$_2$
uptake systems as well as inducible, high-affinity transporters (Price et al., 2008). In contrast, *Trichodesmium* has only constitutive low-affinity transporters (BicA and NHD-14) and furthermore lacks the CO₂ responsive genes *CcmR* and *CmpR* (Price et al., 2008). Such differences in the suite of CCM components and regulation mechanisms could induce differences in the energy costs of the CCM and may explain some of the species-specific responses of N₂ fixers towards ocean acidification.

Finally, part of the differences in response patterns may be explained by adaptations to different habitats. For instance, the Baltic Sea differs strongly from open ocean regions with respect to carbonate chemistry, with considerably lower alkalinity and DIC as well as high seasonal variability (Thomas and Schneider, 1999). Assuming that their CCM is adapted to the characteristic conditions in their natural environment, these differences could explain the distinct response of *Nodularia* compared to other N₂ fixers (*Publication IV*; Czerny et al., 2009). Moreover, also environmental differences on a small scale such as those imposed by microenvironments of symbionts may modulate responses to global change. Epibionts such as *Calothrix* are likely adapted to a strong variability in O₂ and CO₂ concentrations as well as pH induced by the metabolic activity of their hosts (Flynn et al., 2012). The direct environment of other symbionts such as UCYN-A and *Richelia* is poorly characterized to date, with uncertainties remaining with regard to their location on/within the host cell (Thompson and Zehr, 2013).

Classification of phytoplankton according to ‘functional traits’ is commonly used for instance in models that predict their biogeography (e.g. Barton et al., 2013; Follows et al., 2007). Similarly, attributing response patterns to certain characteristics of N₂ fixers could help to predict effects of ocean acidification. Yet, the literature review in *Publication IV* showed that further studies are necessary before solid predictions can be made. Firstly, more comparison studies are required that assess responses of multiple species under similar experimental conditions (similar to *Publication IV* or Hutchins et al., 2013) in order to identify the characteristics that cause similar response patterns. Furthermore, in-depth physiological studies are necessary to generate an understanding of the underlying response mechanisms in species other than *Trichodesmium* that allows for extrapolating to other N₂ fixers and different conditions.
4.3 Implications for future biogeochemical cycles of C and N

The diversity in ocean acidification responses of N$_2$ fixers also implies diverse impacts on future biogeochemical cycles. Species- or group-specific trends in productivity (Publication IV, Fig. 8) have different biogeochemical implications depending on the interactions of N$_2$ fixers with their environment as well as with other cells (Thompson and Zehr, 2013; Fig. 8). Results from Publications I, II & IV highlight the need to consider cellular processes other than growth when addressing climate change effects. While growth rates were often not altered, concurrent changes in production rates of POC and PON can be much larger and will have direct biogeochemical impacts.

Fig. 8: Differential biogeochemical impacts of major groups of open ocean N$_2$ fixers as well as potential trends with ocean acidification (OA) in fluxes of N, C and O$_2$. Light-colored arrows for CO$_2$ and O$_2$ denote fluxes due to metabolic activity of the hosts. Please note that predictions of OA effects are highly tentative since also within the groups summarized here, there is high variability in responses. N and C export strongly depend on OA responses of the host. The potential for export by symbioses of unicellular diazotrophs with calcifiers is still uncertain. Figure modified after Thompson and Zehr (2013).
Laboratory experiments with *Trichodesmium* generally showed a strong stimulation in productivity and N$_2$ fixation with increasing pCO$_2$ (*Publications I & II*, Fig. 8). Furthermore, the efficiency in N transfer between cells of *Trichodesmium* seemed to be decreased under high pCO$_2$, which might in turn stimulate productivity of associated microorganisms (*Publication II*). Results on *Trichodesmium* were characterized by a large diversity between studies that can partly be explained by a modulation of CO$_2$ effects by differences in experimental conditions (*Publications I & IV*). Consequently, the future potential for N$_2$ fixation by this genus depends strongly on interactive effects with environmental factors that are changing along with ocean acidification (Fig. 2B). For instance, increasing light exposure with stratification may decrease the magnitude of CO$_2$ effects in *Trichodesmium* (Kranz et al., 2010). Iron limitation has also been shown to diminish ocean acidification effects in this species (Shi et al., 2012), yet, future iron availability is uncertain due to the potentially opposing effects of increasing dust deposition (Karl et al., 2002), increasing stratification (Behrenfeld et al., 2006) and pH effects on the bioavailability of iron (Shi et al., 2010).

The productivity of the single-celled *Cyanothece* and heterocystous *Calothrix* also increased in ocean acidification scenarios (*Publication IV*, Fig. 8). While *Cyanothece* responded very strongly to elevated pCO$_2$, almost doubling production rates under 980 compared to 380 µatm pCO$_2$ (*Publication IV*), responses of other unicellular cyanobacteria may be very different, especially when it comes to the presumably symbiotic UCYN-A cyanobacteria, which have the genes for PSI but lack PSII as well as a subunit of RubisCO (Zehr et al., 2008; Fig. 8). Also for *Calothrix*, host-symbiont interactions can have strong impacts on cellular metabolism. In a recent study on different heterocystous symbionts, growth rates of the N$_2$ fixers in symbioses were very similar to those of their hosts, but diverged strongly in free-living N$_2$ fixers of the same species (Foster et al., 2011). Regarding the Baltic Sea, there are indications for a decrease in productivity and N$_2$ fixation of *Nodularia* with ocean acidification, yet different studies have yielded conflicting results (*Publication IV* and references therein). Species interactions between *Nodularia* and other N$_2$ fixers such as *Aphanizomenon* may furthermore modulate ocean acidification effects on Baltic diazotroph populations (Karlberg and Wulff, 2013).

In conclusion, the productivity of N$_2$ fixers in open ocean regions can generally be expected to increase, antagonizing possible decreases in N input induced by stratification
(Behrenfeld et al., 2006), while N₂ fixer productivity in the Baltic Sea may decrease. Trends in both regions are, however, subject to uncertainties due to possible modulation of CO₂ effects by interactions with other species as well as other environmental factors. Increasing confidence in predictions of future environmental conditions as well as further matrix experiments investigating the interactive effects of these parameters on diazotrophs will improve estimates of productivity trends. An increase in productivity can have different biogeochemical impacts depending on the diazotroph species, since the fate of fixed N differs between the major groups of N₂ fixers (Fig. 8), N being directly excreted or remineralized in the euphotic zone, transferred to grazers or the host species in case of symbionts, and/or sinking to deeper water layers.

The export of organic material from the euphotic zone depends on the size and density of the sinking particles (De La Rocha and Passow, 2007). Ballasting material such as silica and calcite shells of diatoms and coccolithophores as well as grazing and sinking of fecal pellets can enhance the efficiency of the biological carbon pump (De La Rocha and Passow, 2007). Growing evidence has been gathered in recent years that symbioses of heterocystous N₂ fixers with diatoms promote carbon export (Karl et al., 2012; Kemp and Villareal, 2013; Yeung et al., 2012; Fig. 8). Moreover, indications for a symbiosis between unicellular N₂ fixers and calcifiers were found, which may also contribute to export production (Thompson et al., 2012; Fig. 8). While under physiologically active conditions, *Trichodesmium* is buoyant due to gas vesicles that allow them to vertically migrate in the water column (Villareal and Carpenter, 1990; Fig. 8), the demise of *Trichodesmium* blooms by programmed cell death has been reported to involve the loss of these gas vesicles (Berman-Frank et al., 2004). Consequently, a large fraction of the senescent bloom sinks out of the euphotic zone, while a smaller fraction remains suspended in the water column (Berman-Frank et al., 2004; Berman-Frank et al., 2007). While *Trichodesmium* is supposedly not heavily grazed, some species being toxic, direct excretion of large quantities of fixed N by *Trichodesmium* is supposed to supply N to a range of organisms, including bacteria directly associated to the colonies (Fig. 8) as well as free-living phytoplankton in the water column (e.g. Capone et al., 1997). Thus, under non-bloom conditions, *Trichodesmium* supposedly contributes little to export production but fuels the microbial loop as well as productivity of other phytoplankton via N release (La Roche and Breitbarth, 2005). Less is known about the ecology and biogeochemical impacts of single-celled N₂ fixers. The share of N directly excreted by
unicellular cyanobacteria is uncertain as estimates diverge strongly between studies (Benavides et al. 2013, Dron et al. 2012). Due to their small size, single-celled cyanobacteria are generally not prone to sinking, yet symbioses as well as grazing may increase their contribution to export production (De La Rocha and Passow, 2007; Thompson and Zehr, 2013; Fig. 8).

Even in the few years since the beginning of this PhD project, new results on N₂ fixers have continued to change the picture of the marine N cycle. These include indications for N₂ fixation in the Arctic Ocean (Blais et al., 2012; Diez et al., 2012) as well as new information on the abundance and symbiotic interactions of UCYN-A cyanobacteria (Krupke et al., 2013; Thompson et al., 2012). Furthermore, following the discovery of significant underestimations of N₂ fixation rates by the commonly used ¹⁵N-based method (Mohr et al., 2010a), the authors recently reported on field data obtained with a revised method suggesting almost double N₂ fixation rates compared to previous estimates (Groszkopf et al., 2012). While these revised estimates seem to close the gap in the current marine N budget, the future budget will depend on the balance in climate change responses of N₂ fixation and N loss processes. On the one hand, global change responses of the newly discovered diazotrophs need to be included in predictions. On the other hand, also other prokaryotes driving the N cycle may well be affected by changing pH, O₂ concentrations and warming. While denitrification rates have been found to decrease under ocean acidification scenarios (Beman et al., 2011), the pH sensitivity of other processes, such as N remineralization, is poorly characterized to date (Clark et al., 2014; Voss et al., 2013). The integrated effect of the multitude of responses thus remains to be clarified.

4.4 Perspectives for future research

As this thesis aimed to shed light on the ocean acidification responses of N₂ fixers, many new questions emerged, some of which address more in-depth aspects of the physiological processes investigated in this thesis, while others regard knowledge gaps on environmental interactions as well as poorly studied N₂ fixers that currently hamper large scale predictions of global change effects. In the following paragraphs, some of these open questions as well as possible methodological approaches will be discussed, focusing on cellular mechanisms of energy allocation and C₄ fluxes as well as the potential use of field studies and investigations of microenvironments.
Energy allocation between different cellular processes proved to be an important mechanism behind climate change responses. For being able to quantify the energy demands and allocation to different cellular processes, physiological rate measurements should be combined with a more detailed analysis of cellular composition under different conditions. Macromolecular C pools such as lipids, carbohydrates and proteins, for instance, have different reduction states and therefore their build-up requires different amounts of reducing equivalents per unit of C. In an experiment with a coccolithophore, the lipid composition was shown to vary with $p\text{CO}_2$ (Riebesell et al., 2000). While in this thesis, only total POC quotas were quantified, variations in its composition could alter energy demands (Publications I & II) as well as isotopic composition (Publication III) and might thus help explain some of the response patterns observed in *Trichodesmium* and other species. Direct methods for assessing cellular pools of energy equivalents and their redox state could furthermore help to unravel energy fluxes, in particular if cellular accumulation of energy equivalents could be linked with the activity of certain physiological processes.

Covering relatively poorly characterized processes, our investigations of intracellular $C_i$ fluxes also opened up a range of new questions. Overall energy demands of cyanobacterial CCMs are still uncertain (Raven et al., 2014), partly due to an incomplete understanding of the reaction mechanisms of $\text{HCO}_3^-$ transporters and the NDH complex. The findings of this thesis emphasize that a more detailed analysis of the regulation mechanisms of cellular $C_i$ fluxes is necessary for understanding the mechanisms of energy reallocation under ocean acidification. In particular the NDH complex and its potential regulation by photosynthetic and respiratory electron transport should be of key interest. Since intracellular $C_i$ fluxes cannot be directly measured, indirect tools need to be exploited to improve understanding of these processes. *Publication III* demonstrated that the combination of physiological measurements with modeling is a very useful approach, as models can for instance be used to test hypotheses generated from physiological data. A more sophisticated model of cellular $C_i$ fluxes could help to estimate e.g. intracellular concentrations of different $C_i$ species. A lot of new knowledge on the functioning of the CCM has been generated in recent years using molecular tools, revealing e.g. the transcriptional regulation mechanisms of major CCM genes (Daley et al., 2012). Transcriptomic and/or proteomic methods could also be exploited to characterize the potential for intracellular $C_i$ fluxes via the NDH complex under different environmental conditions, allowing insights into the regulation mechanisms.
While these methods can only indicate the capacity for fluxes via certain pathways, they can still give valuable information in cases where actual rate measurements are not feasible. Combining these with measurements of cellular pools of energy equivalents could advance understanding of the effects of the NDH complex on cellular energy stoichiometry.

For improving predictions of ocean acidification effects on an ecosystem or global level, further laboratory studies on other species of N\textsubscript{2} fixers should be conducted to confirm response patterns such as those deduced in Publication IV and to identify new patterns. Field studies should furthermore be used to test applicability of the concepts derived in laboratory studies. If properly designed, field incubation experiments can account for possible modulation of effects by environmental factors such as light and iron limitation, which are often not representative in laboratory studies, as well as interactions with other microorganisms. In the case of \textit{Trichodesmium}, for example, interactions with the diverse assemblage of heterotrophic bacteria associated to colonies in the field (Hmelo et al., 2012) determine the transfer of responses to the ecosystem. The manifold interactions of symbionts with their hosts (Foster et al., 2011) as well as the lack of photosynthesis in UCYN-A cyanobacteria (Zehr et al., 2008) may cause response patterns that differ substantially from those in other diazotrophs. As neither UCYN-A cyanobacteria nor symbionts in association with their hosts could as yet be cultured, these responses have to be investigated in field incubation experiments with natural communities.

Another important aspect when it comes to choosing representative study systems is the fact that many species of N\textsubscript{2} fixers form filaments and/or aggregates, symbioses, colonies or dense surface blooms in their natural environments, all of which lead to the formation of distinct microenvironments. Based on a recent modeling study investigating the size-dependent variability in carbonate chemistry in the boundary layer of phytoplankton cells (Flynn et al., 2012), a considerable increase in variability with climate change can be expected in the large aggregates formed by some cyanobacteria. Using microsensors to directly determine chemical conditions within \textit{Nodularia} aggregates, microenvironments have been shown to differ significantly from the bulk seawater (Ploug, 2008; Ploug et al., 2011). The observed offsets in absolute values as well as variability over time in pH and concentrations of CO\textsubscript{2} and O\textsubscript{2} (Ploug et al., 2011) can be expected to have implications for multiple physiological processes in N\textsubscript{2} fixers. For instance, pH influences iron bioavailability (e.g. Shi et al., 2010), O\textsubscript{2} inhibits nitrogenase (e.g. Fay, 1992) and C\textsubscript{i} availability and
speciation determine the requirements for a CCM (e.g. Raven, 1997; Reinfelder, 2011). CO₂ draw-down in the diffusive boundary layer would have implications also for ¹³C signatures and internal C₅ fluxes as modeled in *Publication III*. According to *Publications I & II*, these changes in physiological key processes could be expected to feedback to cellular energy budgets and consequently modulate the ocean acidification responses of N₂ fixers.

### 4.5 Conclusion

This thesis focused on the underlying mechanisms of the responses of N₂ fixing cyanobacteria to the current anthropogenic climate changes. Many of these, in fact, go back to the long evolutionary history of cyanobacteria, which requires energy-demanding mechanisms to run the ‘out-dated’ C and N fixing enzymes under present-day conditions: while C fixation requires accumulation of CO₂ in the vicinity of RubisCO (Fig. 4), N₂ fixation is subject to high loss rates and requires complex regulation mechanisms (Fig. 3). Accordingly, energy allocation between the CCM and N₂ fixation was shown to play an important role in driving climate change responses (*Publication I*). The conditions for this energy reallocation (i.e. compatible energy stoichiometry, *Publication II*) and the important role and multiple implications of intracellular fluxes used to concentrate C₅ (*Publication III*) are major findings of this thesis, which improve our process-understanding for the abundant N₂ fixer *Trichodesmium*. Yet, while nitrogenase and RubisCO are highly conserved enzymes, during their long evolution, cyanobacteria have developed different nitrogenase protection strategies and adaptations of the CCM, and therefore have distinct physiological characteristics that make them respond differently to the current anthropogenic changes (*Publication IV*). Future studies will have to account for the high diversity in life styles between cyanobacteria (Fig. 8) when aiming to assess their role in the quickly changing C and N cycles. Although during their evolution, cyanobacteria may have ‘seen it all’ when it comes to CO₂ concentrations, the rapid rate of current changes and the multiple interactions with their environment and ‘younger’ cohabitants make for challenging questions.
5. REFERENCES


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Erklärung

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel

*Process-understanding of marine nitrogen fixation under global change*

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Bei dieser Version handelt es sich um eine für die Veröffentlichung in Einzelheiten überarbeitete, inhaltlich jedoch im Wesentlichen unveränderte Version der Doktorarbeit.