Process-understanding of marine nitrogen fixation under global change

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ABBREVIATIONS

CA	carbonic anhydrase
CCM	carbon concentrating mechanism
C_i	inorganic carbon
DIC	dissolved inorganic carbon
ETC	electron transport chain
pCO_2	CO_2 partial pressure
POC	particulate organic carbon
PON	particulate organic nitrogen
PQ	plastoquinone
PSI	photosystem I
PSII	photosystem II
TA	total alkalinity

CONTENTS

1.1 SUMMARY

Diazotrophic cyanobacteria play an important role in the marine nitrogen cycle due to their ability to convert atmospheric N_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_2 fixers are subject to an array of perturbations in their environment. Several previous experiments suggested the abundant N_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_2 fixation rates as well as production of particulate organic carbon and nitrogen (POC and PON) with elevated pCO_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_2 effects on energy supply. Consequently, based on the observation of a down-regulation of the CCM under high pCO_2, a reallocation of energy between the carbon concentrating mechanisms (CCM) and N_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_2 vs. NO_3^-). Lower ATP requirements of NO_3^- assimilation compared to N_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_2 effects on the CCM were less pronounced in NO_3^- grown cells than in N_2 fixers. While the CCM as well as N_2 fixation require mainly ATP, readily allowing for reallocation of energy equivalents between the two processes under elevated pCO_2, the high demand in reducing equivalents of NO_3^- 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_i) fluxes. In *Publication III*, the extra- and intracellular C_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_i fluxes. Internal C_i cycling was thereby shown to play an important role for C_i acquisition as well as cellular energy budgets in *Trichodesmium.* The effects of internal C_i cycling on ^{13}C fractionation may have important implications also for interpretation of ^{13}C signatures of other phytoplankton.

To estimate the applicability of the response mechanisms identified in *Trichodesmium* to other N_2 fixers, in *Publication IV* a species comparison was conducted. Comparison of pCO_2 effects on functionally different N_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_2, and CCM adaptations to different habitats. Although a broad range of N_2 fixers was investigated in this study, for making solid predictions of future N_2 fixation, further studies are necessary to confirm response patterns and include poorly characterized species such as the symbiotic N_2 fixers.

1.2 ZUSAMMENFASSUNG

Diazotrophe Cyanobakterien spielen eine bedeutende Rolle im marinen Stickstoff-Kreislauf aufgrund ihrer Fähigkeit, atmosphärischen N_2 in biologisch verfügbare Stickstoff-Verbindungen zu konvertieren. Indem sie den fixierten Stickstoff anderen Phytoplankton-gruppen zugänglich machen, fördern sie die Produktivität von Phytoplankton-gemeinschaften insbesondere in oligotrophen Gebieten. Unter dem Einfluss des fortschreitenden Klimawandels sind N_2-Fixierer einer Reihe an Veränderungen in ihrer Umgebung ausgesetzt. In mehreren vorherigen Experimenten mit *Trichodesmium*, einem der häufigsten marinen N_2-Fixierer, wurde eine hohe Sensitivität gegenüber Ozeanversauerung und damit einhergehenden Veränderungen in anderen Umweltparametern beobachtet. Ziel dieser Arbeit war es, das Verständnis dieser Reaktionen durch Untersuchung der zugrundeliegenden Prozesse zu verbessern. Desweiteren wurde untersucht, ob die in *Trichodesmium* beobachteten Reaktionen auf andere Diazotrophe übertragen werden können.

Die in *Publikation I* zusammengefassten Publikationen zeigten übereinstimmend einen Anstieg in N_2-Fixierung sowie Produktion von partikulärem organischem Kohlenstoff und Stickstoff (POC und PON) unter erhöhtem pCO_2. Das Ausmaß der Effekte variierte 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_2, eine Reallokation von Energie zwischen CCM und N_2-Fixierung als Grundlage für den Anstieg in POC-und PON-Produktion postuliert.

Diese Energiereallokation wurde in *Publikation II* durch Anzucht von *Trichodesmium* auf Stickstoff-Quellen mit unterschiedlichem Energiebedarf (N_2 vs. NO_3^-) bestätigt. Der niedrige ATP-Bedarf der Assimilation von NO_3^- im Gegensatz zur N_2-Fixierung ermöglichte höhere POC- und PON-Produktionsraten. Die Ergebnisse aus *Publikation II* zeigen jedoch auch Grenzen der Energiereallokation auf, die durch Unterschiede in der Stöchiometrie der benötigten Energieäquivalente (ATP:NADPH) entstehen. CO_2-Effekte auf den CCM waren weniger ausgeprägt in Zellen, die auf NO_3^- angezogen wurden, als in N_2-Fixierern. Während

sowohl CCM als auch N_2-Fixierung hauptsächlich ATP benötigen, was eine Reallokation von Energie unter erhöhtem pCO_2 begünstigt, limitiert der hohe Bedarf an Reduktionsäquivalenten der NO_3^--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 (C_i) in *Trichodesmium* mithilfe einer Kombination von direkten Flussmessungen, Isotopenfraktionierung und Modellierung untersucht. Unterschiedliche Ergebnisse für die *Leakage*, das Verhältnis von CO_2-Efflux zur Gesamt-Aufnahme von C_i, aus direkten Messungen und fraktionierungs-basierten Methoden konnten mithilfe eines Modells zur Übereinstimmung gebracht werden. Hierfür wurden neben den externen C_i-Flüssen über die Plasmamembran auch die internen C_i-Flüsse berücksichtigt. Die Ergebnisse demonstrieren die Bedeutung der internen Flüsse für die C_i-Aufnahme sowie ihre Rolle im zellulären Energiehaushalt von *Trichodesmium*. Auswirkungen des internen C_i-*Cyclings* auf die ^{13}C-Fraktionierung sollten auch bei der Interpretation der ^{13}C-Signatur anderer Phytoplanktonarten beachtet werden.

Um die Übertragbarkeit der in *Trichodesmium* beobachteten Mechanismen auf andere N_2-Fixierer abzuschätzen, wurde in *Publikation IV* ein Artenvergleich durchgeführt. Die Untersuchung von pCO_2-Effekten auf funktionell unterschiedliche N_2-Fixierer zeigte große Unterschiede zwischen verschiedenen Gruppen sowie Arten von Diazotrophen. Ein Teil dieser Variabilität konnte auf Unterschiede im Nährstoffhaushalt und in den zellulären Adaptationen zum Schutz der Nitrogenase vor O_2, sowie auf Anpassungen des CCMs an unterschiedliche Habitate zurückgeführt werden. Obwohl in dieser Studie ein breites Spektrum an N_2-Fixierern untersucht wurde, sind weitere Studien notwendig, um Vorhersagen über zukünftige Veränderungen in der marinen N_2-Fixierung zu verbessern. Dazu müssen u.a. die beobachteten Reaktionsmuster bestätigt und bisher schlecht charakterisierte Arten, wie die symbiotischen N_2-Fixierer, mit eingeschlossen 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 CO_2 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 CO_2 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 pCO_2. 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 CO_2 in the oceans.

Most of the carbon in the oceans is not present in the form of gaseous CO_2, but is converted to an ionic form. When CO_2 dissolves in sea water, it reacts with water to form carbonic acid (H_2CO_3), which subsequently dissociates to form bicarbonate (HCO_3^-), releasing a proton (H^+) during the process (eq. 1). Bicarbonate further dissociates into a carbonate ion (CO_3^{2-}) and another proton. Consequently, uptake of CO_2 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 CO_2, while the bulk of carbon is in the form of HCO_3^- (~ 90%) at typical seawater pH (Zeebe and Wolf-Gladrow, 2007).

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad \text{(eq. 1)}$$

As in reverse, HCO_3^- or CO_3^{2-} 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).

$$TA = [HCO_3^-] + 2\,[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] + [HPO_4^{2-}] + 2\,[PO_4^{3-}] + [H_3SiO_4^-] + [NH_3] + [HS^-] - [H^+] - [HSO_4^-] - [HF] - [H_3PO_4] \qquad \text{(eq. 2)}$$

By determining how many of the protons released e.g. during CO_2 dissolution (eq. 1) can be consumed, TA influences both the amount of CO_2 that can dissolve in seawater and the change in pH for a given amount of CO_2 dissolution. Apart from HCO_3^- and CO_3^{2-}, 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 NO_3^- 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 N_2 fixation itself does not affect TA, N_2 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 CO_2. As CO_2 is more soluble in cold water, sinking of cold and dense water masses at high latitudes transports CO_2 to depth. At lower latitudes, warming of upwelling water masses can lead to subsequent release of CO_2. 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 CO_2 into organic matter during photosynthesis, which leads to a transient decrease in surface water CO_2 concentrations. While a large fraction of this organic matter is remineralized in the surface ocean, releasing CO_2, 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 CO_2 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 CO_2 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 CO_2 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).

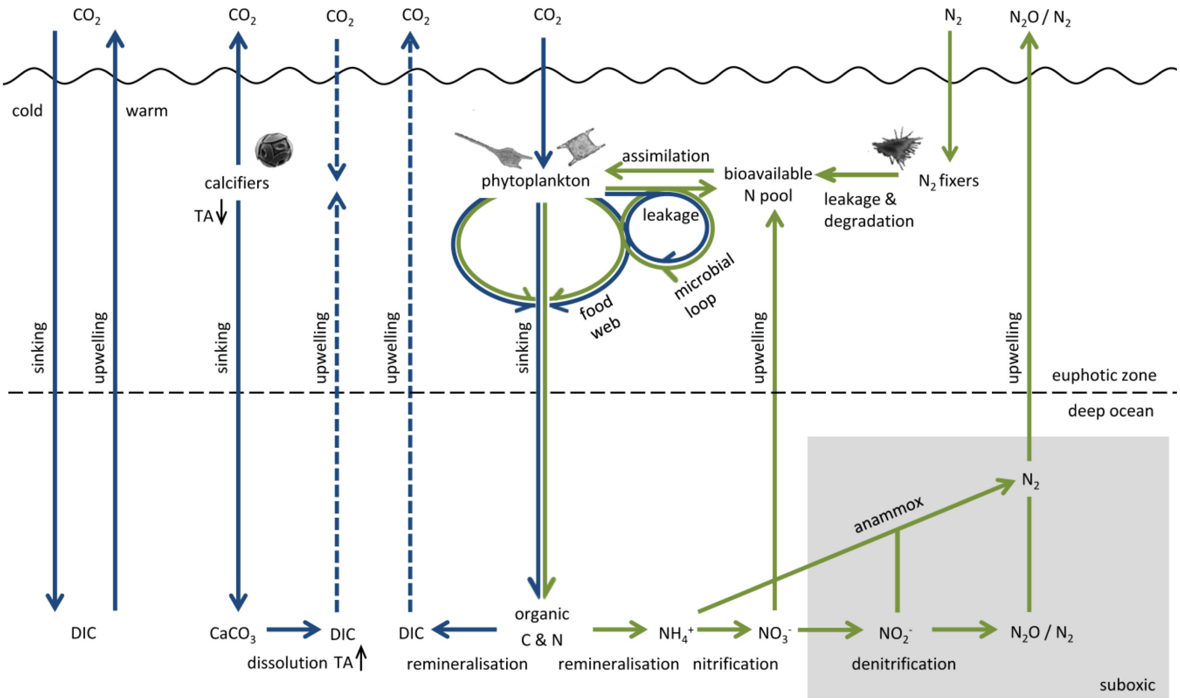

Fig. 1: Marine cycles of C (blue arrows) and N (green arrows). Starting from the left, the physical carbon pump, the carbonate counter pump, and the soft-tissue pump are shown. Please note that the carbonate counter pump and soft-tissue pump are in reality closely connected, as $CaCO_3$ can act as ballasting 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 NO_3^-, which can be resupplied to the euphotic zone by upwelling. In O_2 depleted regions, NO_3^- can be used as an alternative electron acceptor (‘NO_3^- respiration’). During denitrification, NO_3^- is reduced to N_2O and further to 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 NH_4^+ with NO_2^- yielding 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, N_2 fixers in the euphotic zone can access the virtually unlimited pool of atmospheric N_2 and convert it to forms available to other phytoplankton. In the tropical Atlantic and subtropical North Pacific Oceans, N_2 fixation has been estimated to support ~ 25 to 50% of total primary production, with the remaining share being supported by NO_3^- upwelling (Carpenter et al., 1999; Dore et al., 2002). The importance of marine N_2 fixation in the global N cycle has increasingly been recognized over the past few decades. Based on early rate measurements, marine 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 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 N_2 fixation had been severely underestimated was initially based on the upward revision of N_2 fixation estimates for the abundant 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 N_2 fixers were discovered that are now believed to contribute a significant share of marine 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 N_2 fixation rates were suggested to induce a significant underestimation of N_2 fixation estimates (Mohr et al., 2010b). With these new discoveries in both 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 $p\mathrm{CO_2}$ 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 $p\mathrm{CO_2}$ 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 μatm (IPCC scenario IS92a; IPCC, 2007; Fig. 2) or even 1000 μatm (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).

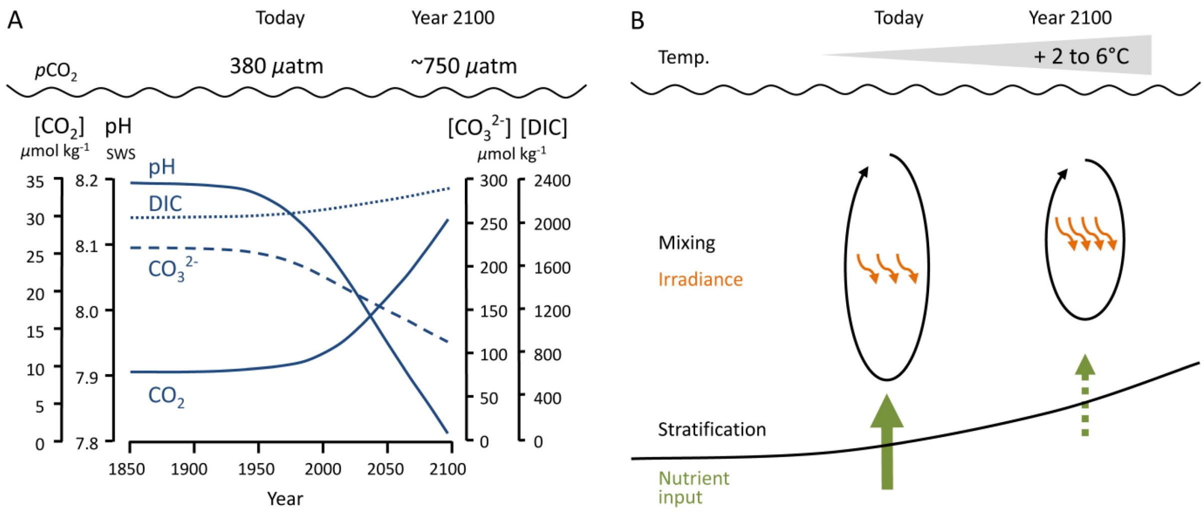

Fig. 2: Effects of rising atmospheric pCO_2 on the marine system. A) Direct effects of pCO_2 increase on marine carbonate chemistry (modified after Wolf-Gladrow et al., 1999). B) Effect of global warming on stratification and its consequences for irradiance and nutrient input to the upper mixed layer (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 NO_3^- inputs to the euphotic zone, the expected changes in stratification also have significant implications for the N cycle. As NO_3^- supply by upwelling may decrease, it has been suggested that N_2 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 N_2 fixation by the Haber-Bosch reaction (Erisman et al., 2008), followed by cultivation-induced biological N_2 fixation (e.g. cultivation of legumes) and fossil fuel combustion releasing the potent greenhouse gas N_2O (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 NO_3^- limitation induced by increasing stratification. In eutrophied marine ecosystems, where large amounts of organic matter are produced and subsequently remineralized, a substantial fraction of O_2 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 N_2 fixation (Vahtera et al., 2007). 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_2O 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_2O 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 N$_2$ fixation

Breaking the triple bond that connects two N atoms in a molecule of N_2 is an extremely energy demanding reaction. As much as 16 ATP molecules in addition to 8 electrons are needed to reduce N_2 to NH_4^+ (Postgate, 1998). The reaction involves formation of H_2, which, in fact, consumes a significant share of the energy required for N_2 fixation (Postgate, 1998). In addition to these direct costs of N_2 fixation, autotrophic diazotrophs have to invest a considerable amount of energy to cover secondary costs, which arise from the fact that their N_2 fixing enzyme nitrogenase, having evolved during times of low O_2 concentrations, is inhibited by O_2 (Fay, 1992; Robson and Postgate, 1980).

Different groups of cyanobacteria have evolved different mechanisms for protecting nitrogenase from photosynthetically produced O_2, separating the two processes in time and/or in space (Fig. 3). Single-celled cyanobacteria such as *Cyanothece* generally separate N_2 fixation from photosynthesis in time, fixing N_2 only during the night. This diurnal separation requires a circadian rhythm in N_2 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 N_2 fixing cells within their filaments. These cells termed heterocysts lack the O_2-evolving photosystem II (Wolk et al., 1994) and have a thick cell wall isolating them from O_2 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, O_2 fluxes and N_2 fixation are regulated in a concerted diurnal cycle, including a down-regulation of photosynthesis during the peak in N_2 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).

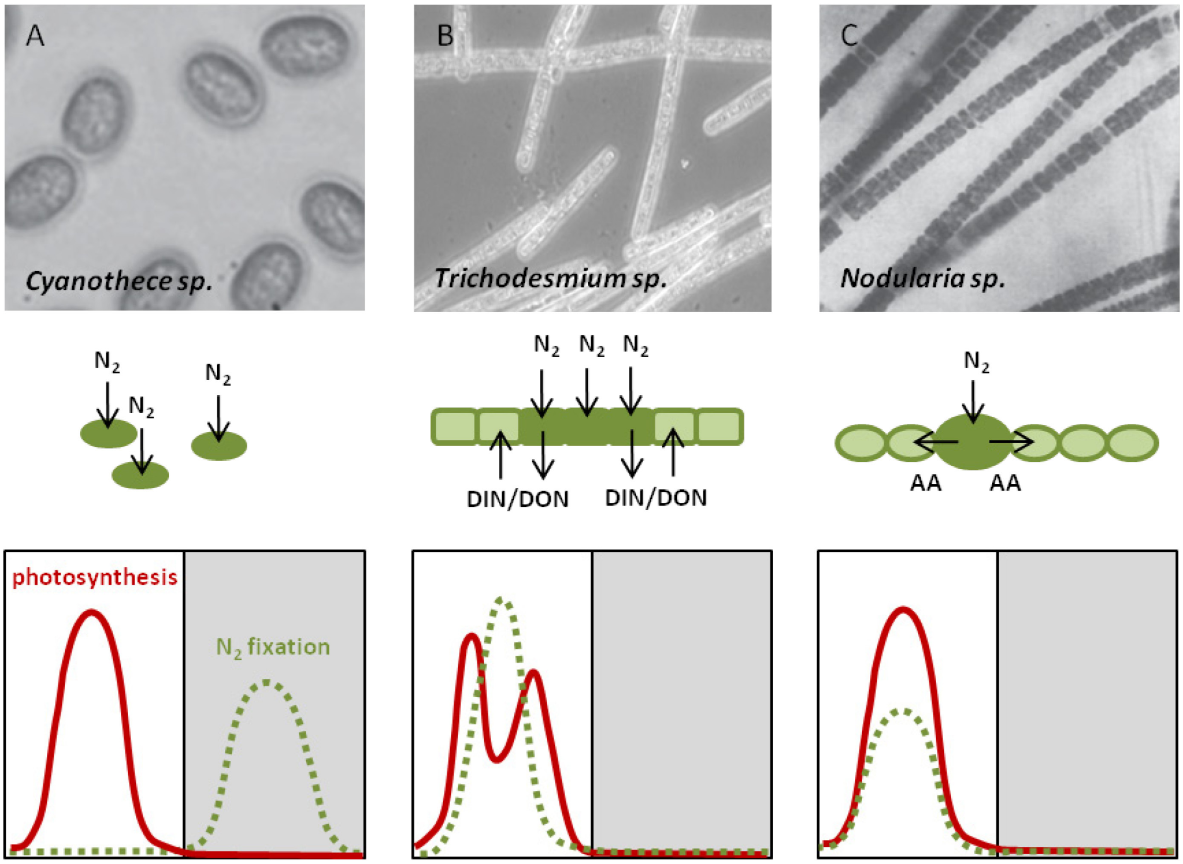

Fig. 3: Three mechanisms for separating N$_2$ fixation (green, dotted lines) from photosynthesis (red, solid lines) employed by different groups of N$_2$ fixing cyanobacteria. A) Single-celled cyanobacteria fix N$_2$ only during the night (photograph from Welkie et al., 2013). B) Diazocytes fix N$_2$ and subsequently excrete dissolved inorganic or organic N (DIN or DON) for use by the neighbouring cells in a filament. N$_2$ fixation is constrained to a period during midday, when photosynthesis is down-regulated (photograph by S. Kranz). C) In heterocystous species, N$_2$ fixed into amino acids (AA) is transferred directly from the heterocysts to the neighbouring cells in a filament (photograph from Hübel and Hübel, 1980). Figure modified after Berman-Frank et al. (2003).

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 O_2 and regeneration of energy equivalents (ATP and NADPH) in the ETC, which can subsequently be used for fixation of CO_2 into carbohydrates in the dark reactions. During respiration, these carbohydrates are broken down, ultimately leading to reduction of O_2 to H_2O 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 b_6/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 b_6/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 P_i. 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 N_2 fixation (Fig. 4).

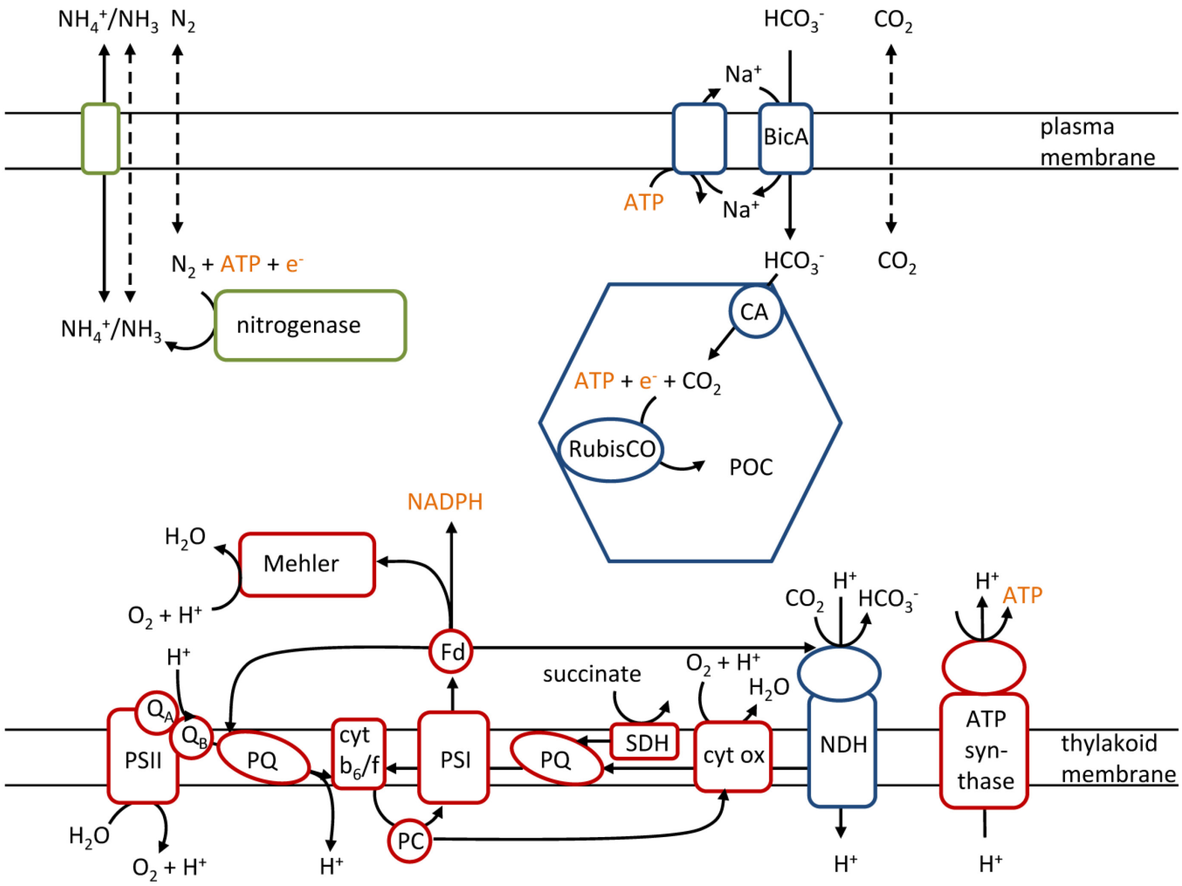

Fig. 4: Scheme of cellular fluxes involved in N assimilation (green), CCMs (blue) as well as photosynthesis and respiration (red) of N$_2$ fixing cyanobacteria, based on *Trichodesmium*. Please note that N$_2$ fixation and photosynthesis do not usually occur at the same time/in the same cell. CA, carbonic anhydrase; cyt b$_6$/f, cytochrome b$_6$/f complex; cyt ox, cytochrome oxidase; e$^-$, electron; Fd, ferredoxin; PC, plastocyanin; PQ, pool of plastoquinones; Q$_A$, primary quinone electron acceptor; Q$_B$, secondary quinone electron acceptor; SDH, succinate dehydrogenase. Dashed arrows denote diffusion.

Several alternative pathways to this so-called linear electron transport allow for adjusting energy generation to its consumption by down-stream 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 N$_2$ 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).

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 CO_2 compared to other autotrophs (Badger et al., 1998) and is furthermore susceptible to a competing reaction with O_2 (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 CO_2 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 CO_2 and HCO_3^- as well as a protein microbody unique to cyanobacteria, the carboxysome (Fig. 4). While CO_2 can in principle diffuse into the cell and is therefore the least costly C_i source available, accumulation of C_i against a concentration gradient requires active transport. Due to its low equilibrium concentration, slow interconversion of CO_2 and HCO_3^- and slow diffusion in seawater, CO_2 availability can quickly become limiting (Riebesell et al., 1993; Zeebe and Wolf-Gladrow, 2007). Moreover, as the plasmamembrane is permeable to CO_2, any accumulation of CO_2 in the cell entails leakage. To circumvent these draw-backs of CO_2 uptake, cyanobacteria additionally take up HCO_3^- by active transporters (BicA and SbtA; Price et al., 2002; Price et al., 2004). In addition to direct HCO_3^- uptake, the so-called NDH-1_3 and NDH-1_4 complexes convert CO_2 to HCO_3^- in the cytosol (Maeda et al., 2002). Prior to fixation into carbohydrates by RubisCO, HCO_3^- needs to be converted to CO_2. 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 CO_2 permeability than the plasmamembrane (Dou et al., 2008), they allow for efficient accumulation of CO_2 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 HCO_3^- 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 e.g. the carboxysomes and transporters need to be considered (e.g. Raven, 2010; Raven and Lucas, 1985).

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_i transporters (Badger et al., 2002). Sequence analysis of different cyanobacteria also showed that many strains encode for a range of C_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_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_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_2 fixing cyanobacteria are subject to manifold changes in their environment. Studies on climate change responses of N_2 fixers have so-far primarily focused on the abundant *Trichodesmium*, which showed an exceptionally high sensitivity to changing $p\mathrm{CO_2}$ levels. Growth, production of POC and PON, as well as N_2 fixation rates were stimulated under high $p\mathrm{CO_2}$, N_2 fixation being increased by up to 140% at $p\mathrm{CO_2}$ levels projected for the end of this century (Barcelos é Ramos et al., 2007; Hutchins et al., 2007; Kranz et al., 2009; Kranz et al., 2010; Levitan et al., 2007). 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

I. Kranz SA, Eichner M and Rost B (2011): Interactions between CCM and N_2 fixation in *Trichodesmium*. Photosynthesis Research 109(1-3): 73-84.

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

II. Eichner M, Kranz SA and Rost B (2014): Combined effects of different CO_2 levels and N sources on the diazotrophic cyanobacterium *Trichodesmium.* Physiologia Plantarum DOI: 10.1111/ppl.12172.

The experiment was designed together with the co-authors. I performed the experiments (with help by S.A. Kranz during N_2 fixation measurements) and analyzed the data. I drafted the manuscript and finalized it in collaboration with the co-authors.

III. Eichner M, Thoms S, Kranz SA, and Rost B: Cellular inorganic carbon fluxes in *Trichodesmium*: A combined approach of measurements and modeling. Submitted to Journal of Experimental Botany.

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.

IV. Eichner M, Rost B and Kranz SA (2014): Diversity of ocean acidification effects on marine N_2 fixers. Journal of Experimental Marine Biology and Ecology 457: 199-207.

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_2 fixation in *Trichodesmium*

Photosynth Res (2011) 109:73–84
DOI 10.1007/s11120-010-9611-3

REVIEW

Interactions between CCM and N_2 fixation in *Trichodesmium*

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Abstract In view of the current increase in atmospheric pCO_2 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 pCO_2 by increasing growth, production rates, and N_2 fixation. The magnitude of these CO_2 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 CO_2 availability, causing higher diel N_2 fixation rates under elevated pCO_2. The observed responses to elevated pCO_2 could not be attributed to enhanced energy generation via gross photosynthesis, although there are indications for CO_2-dependent changes in ATP/NADPH + H^+ production. The CO_2 concentrating mechanism (CCM) in *Trichodesmium* is primarily based on HCO_3^- uptake. Although only little CO_2 uptake was detected, the NDH complex seems to play a crucial role in internal cycling of inorganic carbon, especially under elevated pCO_2. Affinities for inorganic carbon change over the day, closely following the pattern in N_2 fixation, and generally decrease with increasing pCO_2. This down-regulation of CCM activity and the simultaneously enhanced N_2 fixation point to a shift in energy allocation from carbon acquisition to N_2 fixation under elevated pCO_2 levels. A strong light modulation of CO_2 effects further corroborates the role of energy fluxes as a key to understand the responses of *Trichodesmium*.

Keywords CO_2 concentrating mechanism · Diazotroph · Energy allocation · N acquisition · Ocean acidification · Photosynthesis

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Introduction

Marine phytoplankton are responsible for almost half of all photosynthetic carbon fixation on Earth and play a vital role in altering the CO_2 exchange between ocean and atmosphere (Gruber 2004; Maier-Reimer et al. 1996). Some prokaryotic algae affect the primary productivity and thus CO_2 uptake capacity of the oceans by yet another process, the fixation of dinitrogen (N_2) 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 subtropical 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 N_2 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. 2001b). By now, the genome of *Trichodesmium* IMS101 has been fully sequenced (US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/) and several aspects of its ecophysiology have been studied, e.g., effects of phosphorus, iron limitation, temperature, salinity, and irradiance (Berman-Frank et al. 2001a; Breitbarth 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 µatm) and those predicted for the year 2100 (750–1,000 µatm) 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 µatm) and light (50 vs. 200 µmol 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 1 Comparison of rates and elemental composition for *Trichodesmium* IMS 101 grown at different pCO_2 levels and 25°C

Conditions		Rates and elemental composition				References
Light (µmol photons m^{-2} s^{-1})	pCO_2 (µatm)	Growth μ (d^{-1})	C/N ratios (mol:mol)	N_2 fixation PON production	C fixation POC production	
80–120				nmol N (mg Chl a)$^{-1}$ h^{-1}	mol C (mol Chl a)$^{-1}$ s^{-1}	Levitan et al. (2007)
	400	0.17	6.5	1.3	0.126	
	900	0.26	7.0	3.1	0.155	
~100				µmol N (mg Chl a)$^{-1}$ h^{-1}	mg C (mg Chl a)$^{-1}$ h^{-1}	Hutchins et al. (2007)
	380	0.35	5.1	14.8	1.25	
	750	0.39	5.1	20.0	1.75	
~150				fmol N_2 (fmol P)$^{-1}$ h^{-1}	n.d.	Barcelos é Ramos et al. (2007)
	380	0.44	5.3	0.06		
	800	0.48	4.8	0.11		
~150				µmol N (mg Chl a)$^{-1}$ h^{-1}	µmol C (mg Chl a)$^{-1}$ h^{-1}	
	370	0.27	4.6	11.4	51.7	Kranz et al. (2009)
	1,000	0.34	4.5	14.9	67.6	

The reported values relate to present day (370–400 µatm) and future scenarios for pCO_2 (750–1,000 µatm) used in the different studies. Please note the differences in normalization and light levels

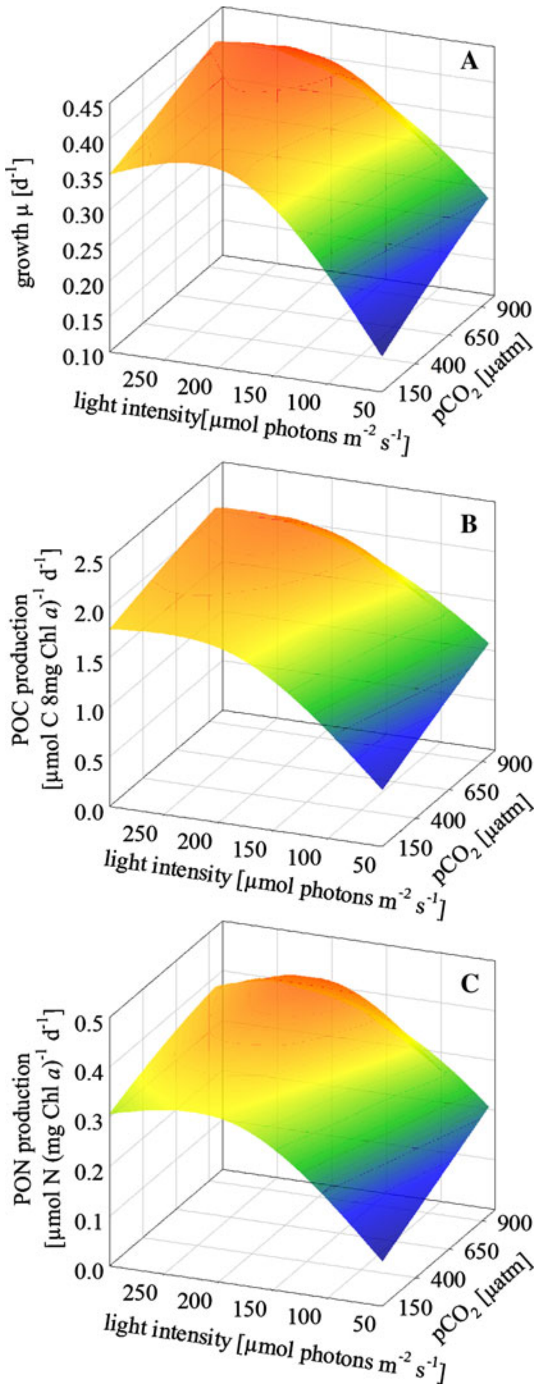

Fig. 1 Combined effects of pCO_2 and light. Plots are based on data taken from Kranz et al. (2010a) and Eichner et al. (unpublished; pCO_2 180 vs. 950 μatm at 300 μmol photons m^{-2} s^{-1}). **a** Growth rates, **b** production rates of particulate organic carbon (POC), and **c** production rates of particulate organic nitrogen (PON)

biomass production modulated by light availability? These and other questions regarding the underlying reasons for the strong CO_2 effects in *Trichodesmium* can only be answered if the responses of physiological key processes are understood. In the following, the processes of N_2 fixation, photosynthesis, and carbon acquisition in *Trichodesmium* will be described and discussed, focusing on the influence of light and CO_2 as well as possible interactions.

N_2 fixation

Thriving in the oligotrophic regions of the ocean, *Trichodesmium* mainly fuels its N demand by fixation of N_2 (Mulholland et al. 2004). The reduction of N_2 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 O_2-free conditions in the Archean (Falkowski 1997; Falkowski and Raven 2007) and its iron protein is especially sensitive to O_2 (Bergman et al. 1997). Thus, photosynthetic energy generation and N_2 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 O_2 evolution and N_2 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 O_2, *Trichodesmium* has developed a distinct diurnal rhythm in PSII activity and N_2 fixation (Berman-Frank et al. 2001b; Lin et al. 1999). In combination with O_2-reducing mechanisms like the Mehler reaction (Berman-Frank et al. 2001b; Küpper et al. 2004; Milligan et al. 2007), N_2 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 N_2, the residual cells of a filament depend strongly on the supply of bioavailable N from the N_2 fixing cells. These N sources are either the direct outcome of the N_2 reduction, i.e., ammonia (NH_3) or ammonium (NH_4^+), 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

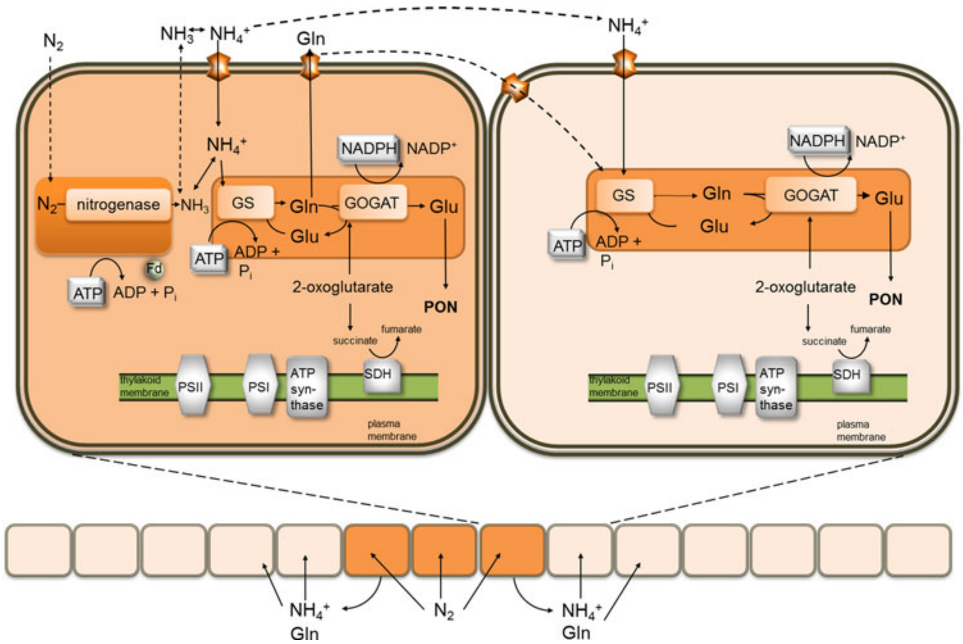

Fig. 2 A conceptual model of N_2 fixation and N cycling within for *Trichodesmium*. One filament is shown consisting of several single cells, with *dark orange* representing the N_2 fixing cells (diazocytes) while the *bright orange cells* represent the non-N_2-fixing vegetative cells. Processes of N acquisition in those types of cells are depicted. N_2 diffuses into the diazocytes, where it is reduced to NH_4^+. The latter is then converted into Glu by the GS/GOGAT reaction. The inorganic and organic N sources can be released/transported into the surrounding media and subsequently taken up by the non-N_2-fixing cells of *Trichodesmium*. Oxygenic photosynthesis as well as respiration is conducted in both types of cells, generating the energy and reductants as well as carbon skeletons required for N assimilation. *ADP* adenosine-5′-diphosphate, *ATP* adenosine-5′-triphosphate, *ATP synthase* adenosine-5′-triphosphate synthase, *Glu* glutamate, *GOGAT* glutamine oxoglutarate aminotransferase, *Gln* glutamine, *GS* glutamine synthetase, P_i inorganic phosphorus, *PON* particulate organic nitrogen, *PSI* photosystem 1, *PSII* photosystem 2, *SDH* succinate dehydrogenase

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, N_2 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 $MnCl_2$ 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 N_2 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 N_2 fixation, the splitting of the triple-bond of N_2 to form NH_3 requires at least 16 ATP as well as eight electrons:

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + 16ADP + 16Pi + H_2$$

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 + H^+ 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 N_2 fixation (Breitbarth et al. 2008; Kranz et al. 2010a) and as a cue for synthesis and/or demodification of nitrogenase (Zehr et al. 1993). N_2 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 N_2 fixation or PON production under elevated pCO_2 (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 N_2 fixation period (Kranz et al. 2010a). As these enhanced N_2 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 N_2 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 pCO_2, which could be expected from the stimulation in PON production, have, however, not been reported.

As a direct effect of CO_2 on nitrogenase seems unlikely, a higher share of energy to nitrogen fixation was suggested to be the cause for the observed stimulation in N_2 fixation and PON production at high pCO_2 (Kranz et al. 2010a). This explanation is further substantiated by the observation that the CO_2 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 N_2 fixation and PON production under elevated pCO_2? To answer this question, one must first look at photosynthetic energy production and how it responds to changes in CO_2 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 b_6f (cyt b_6f) 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

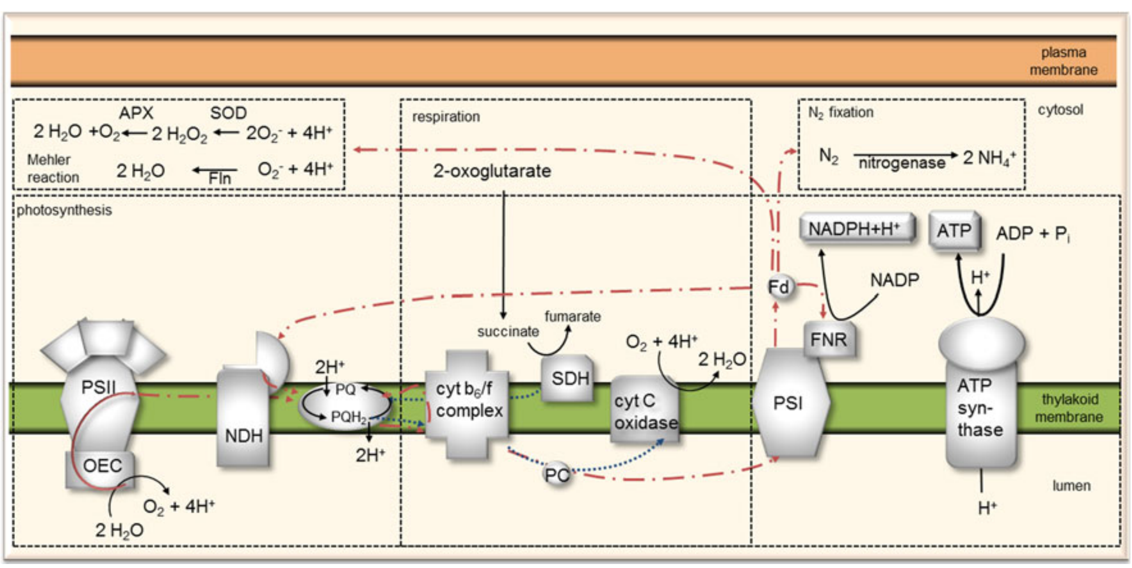

Fig. 3 Schematic representation of major cellular complexes involved in energy transport (photosynthesis, respiration, N_2 fixation, and Mehler reaction). *Red lines* represent photosynthetic electron flux, while *blue lines* represent respiratory electron flux. *ADP* adenosine-5′-diphosphate, *APX* ascorbate peroxidase, *ATP* adenosine-5′-triphosphate, *ATP synthase* adenosine-5′-triphosphate synthase, *Cyt b_6/f complex* cytochrome b_6/f 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_2O. 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_2O 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 NADPH + 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_6f 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_2O_2 production by superoxide dismutase (SOD) as superoxide can directly be reduced to H_2O by a flavoprotein in cyanobacteria (Helman et al. 2003). Nonetheless, H_2O_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 *Trichodesmium*

Process	Energy demand			References
	ATP	Electrons from $NADPH + H^+$ or Fd_{red}	$ATP:NADPH + H^+$	
C fixation	18	24	1.5	Allen (2002)
N acquisition	9	6	3	Flores and Herrero (1994)

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 N_2 to glutamate. For calculation of the $ATP:NADPH + H^+$ ratios, the quantity of electrons needed in N_2 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 N_2 can diffuse into the cell. The energetic demand of the different C acquisition systems is, however, still under debate

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 O_2 production and PSII chlorophyll fluorescence were not affected by the availability of CO_2, although changes were found in the composition of the ETC, with an increased ratio of PSI to PSII at elevated pCO_2 (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 N_2 fixation (Table 2), this may in turn contribute to the observed stimulation in nitrogenase activity under elevated pCO_2. Nonetheless, as the applied pCO_2 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 pCO_2. As inorganic carbon (C_i) uptake and its subsequent fixation represent the largest energy sink (Table 2), these processes certainly need to be considered to understand CO_2-dependent changes in the energy budget of *Trichodesmium*.

Inorganic carbon acquisition

As autotrophs, cyanobacteria use energy to produce organic carbon compounds from inorganic CO_2 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 CO_2 levels and low O_2 concentrations and is characterized by a very low-affinity for its substrate CO_2, a slow maximum turnover rate, as well as a susceptibility to a competing reaction with O_2 (Badger and Andrews 1987; Tortell 2000). Cyanobacterial RubisCO has one of the highest half-saturation concentrations for CO_2 of all autotrophic organisms (K_M of 105–185 µmol l^{-1} CO_2; Badger et al. 1998). Consequently, RubisCO represents a potential bottle neck 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 CO_2 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 *Synechocystis* PCC6803 (Eisenhut et al. 2008). If operative in *Trichodesmium*, this pathway would allow decreasing the O_2 concentration in the vicinity of nitrogenase.

In cyanobacteria, the operation of a CCM generally involves a suite of CO_2 and HCO_3^- 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 C_i uptake has thus far revealed five different systems, primarily based on studies with cyanobacterial strains such as *Synechococcus* spp. and *Synechocystis* spp. These five C_i acquisition systems comprise two inducible high-affinity HCO_3^- transporters (BCT1, Omata et al. 1999; SbtA, Shibata et al. 2002), a constitutive low-affinity HCO_3^- transporter (BicA, Price et al. 2004), a constitutive low-affinity system to convert CO_2 into HCO_3^- (CO_2 uptake facilitator) at the NDH-1 complex located at the thylakoid membrane (NDH-1_4, Maeda et al. 2002; Price et al. 2002; Shibata et al. 2001), as well as an inducible high-affinity CO_2 uptake facilitator based on a modified NDH-1 complex (NDH-1_3, Maeda et al. 2002; Shibata et al. 2001). All of these measures function to increase the HCO_3^- concentration in the cytosol of the cell. The accumulated HCO_3^- passes the protein shell of the carboxysome and, catalyzed by a CA, gets converted to CO_2 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_i concentrations. Differences in CCMs may, however, explain why certain species like *Trichodesmium* respond strongly to changes in C_i 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_i acquisition systems have been identified in *Trichodesmium*, the HCO_3^- transporter BicA and the CO_2 uptake facilitator NDH-1_4 (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_i acquisition systems (NDH-1_3, BCT1, SbtA; Price et al. 2008). In view of the strong CO_2 dependence in growth and biomass production (Barcelos é 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

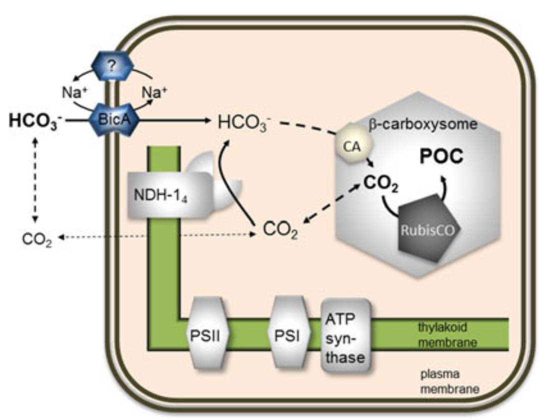

Fig. 4 A schematic model for the CO_2 concentrating mechanism in *Trichodesmium*, based on genetic homologies (Price et al. 2008). BicA represents the low-affinity, high-flux rate HCO_3^- transporter, which is driven by a Na^+ gradient. The buildup of the Na^+ gradient needed to drive the BicA transporter is not fully understood yet. NDH-1_4 represents a low-affinity CO_2 uptake facilitator located at the thylakoid membrane. The NDH-1_4 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

C_i 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 energize 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-1_4) 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-1_4 plays only a minor role in C_i uptake of *Trichodesmium*, this component of the CCM might be important to avoid efflux of CO_2 from the cytosol. NDH-1_4 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-1_4 increases with elevated pCO_2 as well as light intensities, which may consequently enhance the production of ATP and fuel the respective needs of processes such as N_2 fixation. Based on isotopic fractionation data as well as MIMS-based carbon fluxes, it was further demonstrated that the CO_2 efflux in *Trichodesmium* is moderate (20–50% of total C_i uptake) and internal C_i cycling via the NDH-1_4 is highly efficient, especially under high CO_2 concentrations (Kranz et al. 2010a). This C_i 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 affected by light intensities, competing processes like N_2 fixation, as well as by changes in CO_2 availability. This complex interplay of factors can be explained by the high energetic costs of the CCM. An effect of light levels on C_i 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 C_i uptake. Energy limitation on the CCM can, however, also be imposed by N_2 fixation, simply by competing for energy at times of high nitrogenase activities. This would explain the diurnal changes in C_i affinities following N_2 fixation (Fig. 5). Last but not least, similarly to many other species (Giordano et al. 2005), the availability of CO_2 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 CO_2-dependent stimulation in growth and biomass production. Considering energy and its allocation as a key to the observed phenomena also explains why the benefit from down-regulation of the CCM is highest under low light intensities (Figs. 1, 5).

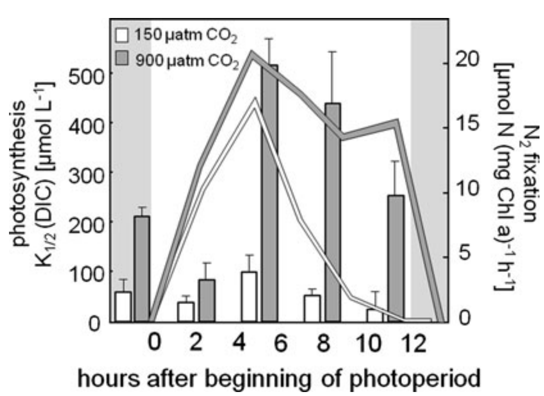

Fig. 5 Half-saturation concentrations ($K_{1/2}$ DIC) of photosynthesis (*bars*) and rates of N_2 fixation (*lines*) in *Trichodesmium* over a diurnal cycle. $K_{1/2}$ values were taken from Kranz et al. (2009), while rates of N_2 fixation were taken from Kranz et al. (2010a). Please note the differences 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 hundreds of 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 C_i 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 CO_2 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 N_2 fixers are not dependent on scarce nitrogen sources such as NO_3^- or NH_4^+ 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 NH_4^+ instead of NO_3^-, 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 NH_4^+ (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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Publication II

Combined effects of N sources and pCO$_2$ levels on *Trichodesmium*

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Combined effects of different CO_2 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_2 responses of biogeochemically relevant phytoplankton species. Previous experiments on the abundant N_2 fixers *Trichodesmium* demonstrated strong CO_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_2 partial pressure (pCO_2) levels (180, 380, 980 and 1400 µatm) and N sources (N_2 and NO_3^-). Subsequently, biomass production and the main energy-generating processes (photosynthesis and respiration) and energy-consuming processes (N_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_2 nor N source, cells differed in production rates and composition. Elevated pCO_2 increased N_2 fixation and organic C and N contents. The degree of stimulation was higher for nitrogenase activity than for cell contents, indicating a pCO_2 effect on the transfer efficiency from N_2 to biomass. pCO_2-dependent changes in the diurnal cycle of N_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_3^- grown cells, which we attribute to the higher N retention and lower ATP demand compared with N_2 fixation. pCO_2 effects on C affinity were less pronounced in NO_3^- users than N_2 fixers. Our study illustrates the necessity to understand energy budgets and fluxes under different environmental conditions for explaining indirect effects of rising pCO_2.

Introduction

The release of anthropogenic carbon (C) has caused atmospheric CO_2 partial pressure (pCO_2) to increase from 280 to 390 µatm since pre-industrial times and pCO_2 levels are expected to rise further to 750 µatm or even beyond 1000 µatm by the end of this century (IPCC 2007, Raupach et al. 2007). As CO_2 is taken up by the ocean, seawater CO_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

Abbreviations – ARA, acetylene reduction assay; CA, carbonic anhydrase; CCM, carbon concentrating mechanism; chl *a*, chlorophyll *a*; C_i, inorganic carbon; DIC, dissolved inorganic carbon; Fv/Fm, PSII photochemical quantum yield measured in dark-adapted state; Fv′/Fm′, PSII photochemical quantum yield measured in light-adapted state; HEPES, 4-(2-hydroxylethyl)-1-piperazine-ethanesulfonic acid; HSD, honest significant difference; $K_{1/2}$, half-saturation concentration; pCO_2, CO_2 partial pressure; POC, particulate organic carbon; PON, particulate organic nitrogen; PQ, plastoquinone; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; TA, total alkalinity; V_{max}, maximum rate; σ, PSII functional absorption cross section; τ, Q_A re-oxidation time;

on marine phytoplankton (Rost et al. 2008, Riebesell and Tortell 2011). By fixing CO_2 into organic matter, phytoplankton acts as a C sink and plays a potential role as a negative feedback mechanism to atmospheric pCO_2 increase (Raven and Falkowski 1999, De La Rocha and Passow 2007).

In marine ecosystems, phytoplankton productivity is often limited by availability of nitrogen (N). Fixation of atmospheric N_2 by diazotrophic cyanobacteria thus plays a crucial role for primary productivity, particularly in oligotrophic regions of the world ocean. With global change, the marine N cycle is subject to an array of perturbations. On the one hand, increasing deposition of anthropogenic N leads to eutrophication in coastal regions (Duce et al. 2008). On the other hand, the expansion of oxygen minimum zones favors N loss processes such as denitrification and anammox (Lam and 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 NO_3^- availability in the surface ocean, marine N_2 fixation may become more important, helping to restore the global N budget.

The cyanobacterium *Trichodesmium* is considered one of the most important marine N_2 fixers with an estimated contribution of up to 50% to global marine N_2 fixation (Mahaffey et al. 2005). Previous studies found this diazotroph to be exceptionally sensitive to rising pCO_2. Laboratory experiments exposing cultures to pCO_2 levels projected for the end of this century showed significant increases in the production of particulate organic C and particulate organic nitrogen (POC and PON) as well as N_2 fixation rates (Barcelos é Ramos et al. 2007, Hutchins et al. 2007, 2013, Kranz et al. 2009, Levitan et al. 2007); the magnitude of these effects yet differed strongly between investigations. In several follow-up studies, CO_2 effects on *Trichodesmium* were found to be strongly modulated by other environmental factors such as iron (Shi et al. 2012) and light (Kranz et al. 2010, Levitan et al. 2010, Garcia et al. 2011), the latter highlighting the importance of energy in the modulation of CO_2 effects.

Cyanobacteria have to invest a considerable share of energy into the accumulation of inorganic carbon (C_i) by carbon concentrating mechanisms (CCMs) owing to a competing reaction with O_2 and a particularly low CO_2 affinity of their ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Badger et al. 1998). The CCM of *Trichodesmium* involves a distinct assembly of RubisCO and carbonic anhydrase (CA) within carboxysomes, as well as two C_i acquisition systems (Badger et al. 2006, Price et al. 2008). HCO_3^- is taken up via a Na^+-dependent HCO_3^- transporter (BicA) whereas diffusive uptake of CO_2 is facilitated by the so-called NDH-1_4 complex, converting CO_2 to HCO_3^-. Next to C_i acquisition, another important energy sink in *Trichodesmium* is N_2 fixation (Kranz et al. 2011). As CCM activity was found to be downregulated at high pCO_2 levels, while N_2 fixation rates were simultaneously increased in this species, a reallocation of energy between C and N_2 fixing pathways has been suggested to fuel the increase in production at high pCO_2 (Kranz et al. 2010).

Similarly to RubisCO, nitrogenase is characterized by a high sensitivity toward O_2 (Falkowski 1997). In consequence, while the fixation of N_2 is an extremely energy demanding reaction in itself (Eqn 1), diazotrophs face additional costs, which are related to the protection of nitrogenase from photosynthetically evolved O_2 (Großkopf and LaRoche 2012). To separate O_2 evolution from N_2 fixation, *Trichodesmium* has a tightly regulated diurnal cycle of N_2 fixation and photosynthesis (Berman-Frank et al. 2001), involving daily synthesis and degradation of nitrogenase (Capone et al. 1990, Sandh et al. 2009) and alternation of photosynthetic activity states (Küpper et al. 2004). Moreover, nitrogenase is expressed only in subsets of cells within filaments, the diazocytes (Lin et al. 1998, Berman-Frank et al. 2001). As no trans-cellular transport mechanisms for N compounds have been found in *Trichodesmium*, diazocytes have to release N for use by their neighboring cells (Mulholland and Capone 2000). Uptake mechanisms for N sources other than N_2 are thus indispensable for this species.

Laboratory studies have shown that *Trichodesmium* can use NO_3^- and NH_4^+ as well as organic N compounds (glutamine, glutamate or urea; Mulholland et al. 1999), all of them requiring different amounts and types of energy equivalents. NO_3^- is taken up in cyanobacteria by high-affinity ATP-dependent transporters and subsequently reduced to NH_4^+ in a two-step ferredoxin-dependent reaction catalyzed by nitrate reductase and nitrite reductase (Flores et al. 2005, Wang et al. 2000) (Eqn 2).

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,\left(ADP + P_i\right) \quad (1)$$

$$2\,NO_3^- + 20\,H^+ + 16\,e^- + 2\,ATP \rightarrow 2\,NH_4^+ + 6\,H_2O + 2\,\left(ADP + P_i\right) \quad (2)$$

In *Trichodesmium*, N_2 fixation was shown to be inhibited in cultures grown in NO_3^--containing media (Ohki et al. 1991, Fu and Bell 2003, Holl and Montoya 2005, Sandh et al. 2011). As the uptake and reduction of NO_3^- requires little ATP (Eqn 2), it can be expected that NO_3^- addition to culture media will alter the energy budget of the cells in comparison to N_2 fixing conditions.

In this study, *Trichodesmium erythraeum* IMS101 was acclimated in a matrix of four different pCO_2 levels (ranging from 180 to 1400 µatm) and two different N sources (N_2 and NO_3^-). In addition to acclimation effects on the level of growth and composition (C, N and pigments), physiological key processes (N_2 fixation, O_2 fluxes and electron transport) were analyzed to improve our understanding of the plasticity in energy and resource allocation under the different energetic requirements imposed by changing environmental conditions.

Materials and methods

Culture conditions

Trichodesmium erythraeum IMS101 was grown in semi-continuous batch cultures at 25°C and 150 µmol photons $m^{-2} s^{-1}$ with a 12:12 h light:dark cycle. Light was provided by white fluorescent bulbs (BIOLUX, Osram, München, Germany). Cultures were grown in 0.2-µm-filtered artificial seawater (YBCII medium; Chen et al. 1996) and kept in exponential growth phase by regular dilution with culture medium. Cultures consisted of single trichomes and cell densities ranged between approximately 6000 and 180 000 cells ml^{-1}. Cells were acclimated in 1 l culture flasks, which were continuously bubbled with 0.2-µm-filtered air with pCO_2 levels of 180, 380, 980 and 1400 µatm. Gas mixtures were generated with a custom-made gas flow controller. Prior to experiments, cells were allowed to acclimate to the respective pCO_2 for at least 2 weeks. Cultures in which pH had drifted by >0.09 compared with cell-free reference media were excluded from further analysis. In treatments with NO_3^- as the N source, 0.2-µm-filtered $NaNO_3$ was added to achieve mean concentrations of 97 ± 2 µ*M* in the experiments, never falling below 65 µ*M*. 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 ($\delta^{15}N$) 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 pCO_2 and N treatment acquired in daily measurements during the experiment. Attained pCO_2 of the media was calculated from pH, TA, $[PO_4^{3-}]$, temperature and salinity using the CO2sys program (Pierrot et al. 2006) with equilibrium constants K_1 and K_2 given by Mehrbach et al. (1973), refit by Dickson and Millero (1987). Errors denote 1 SD ($n \geq 6$).

pCO_2 treatment (µatm)	NO_3^-	pH (NBS)	TA (µmol kg^{-1})	DIC (µmol kg^{-1})	pCO_2 attained (µatm)
180	−	8.48 ± 0.04	2415 ± 11	1847 ± 10	173 ± 12
	+	8.49 ± 0.04	2438 ± 37	1868 ± 22	168 ± 4
380	−	8.26 ± 0.03	2408 ± 15	2014 ± 6	329 ± 22
	+	8.26 ± 0.04	2389 ± 7	2003 ± 12	325 ± 10
980	−	7.88 ± 0.02	2377 ± 15	2142 ± 17	918 ± 12
	+	7.89 ± 0.03	2392 ± 19	2166 ± 26	912 ± 35
1400	−	7.74 ± 0.02	2399 ± 44	2231 ± 78	1354 ± 30
	+	7.76 ± 0.05	2413 ± 37	2237 ± 20	1298 ± 74

O_2 fluxes

Cellular O_2 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-hydroxylethyl)-1-piperazine -ethanesulfonic acid (HEPES, 50 m*M*, pH 8.0) at 25°C and acclimation light intensity unless otherwise specified. To account for the diurnal cycle of O_2 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. For normalization of the O_2 traces, duplicate samples for chl *a* analysis were taken after each measurement.

In the first set of measurements, net O_2 evolution was determined as a function of DIC using the disequilibrium method described by Badger et al. (1994). O_2 fluxes were monitored during consecutive dark and light phases typically lasting 4 min, starting with DIC concentrations close to zero (media bubbled with CO_2-free air), which were subsequently increased by step-wise addition of $NaHCO_3$ up to a maximum of approximately 4500 µ*M* DIC. DIC-saturated rates of photosynthesis [V_{max} (DIC)] and half-saturation concentrations [$K_{1/2}$ (DIC)] were obtained by fitting a Michaelis–Menten function to the data.

In a second approach, O_2 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 N_2 to remove $^{16}O_2$, then spiked with $^{18}O_2$ gas (Chemotrade, Düsseldorf, Germany) and allowed to equilibrate for >30 min, reaching O_2 concentrations of approximately 21%. DIC concentration was adjusted to approximately 2000 µ*M* by addition of 1 *M* $NaHCO_3$ solution prior to measurements. Fluxes of $^{16}O_2$ and $^{18}O_2$ 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 (I_k) 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 $^{18}O_2$ 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 Q_A 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 $P \leq 0.05$ was applied.

Results

Growth and composition

Cellular chl *a* contents stayed relatively constant over the range of pCO_2 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 pCO_2 in both N treatments when all data were included (Fig. 1, ANOVA, $P < 0.005$). However, when testing CO_2 levels individually, the differences between 980 and 380 µatm as well as 180 µatm depended on the N source: with N_2 there was a significant pCO_2 effect (TukeyHSD, $adj_P < 0.05$) while with NO_3^- there was no effect (TukeyHSD, $adj_P > 0.05$). In contrast, contents of POC and PON significantly increased by approximately 33% from 180 to 1400 µatm pCO_2 (ANOVA, $P < 0.0001$). As a result of these opposing trends, production rates of POC and PON did not change over the range of pCO_2 levels tested (Table 2, ANOVA, $P > 0.05$). There was no clear trend in the pCO_2 effect on the POC:PON ratio (Table 2). Regarding effects of the N source, cells grown on NO_3^- had slightly higher growth rates than N_2 fixing cells (ANOVA, $P < 0.05$). Consequently, also production rates of POC and PON were higher in NO_3^- grown cultures (ANOVA, $P < 0.0001$), even though contents of POC and PON were not significantly affected (ANOVA, $P > 0.05$). POC:PON ratios were significantly lower in NO_3^- users than in N_2 fixers (Table 2, ANOVA, $P < 0.0001$).

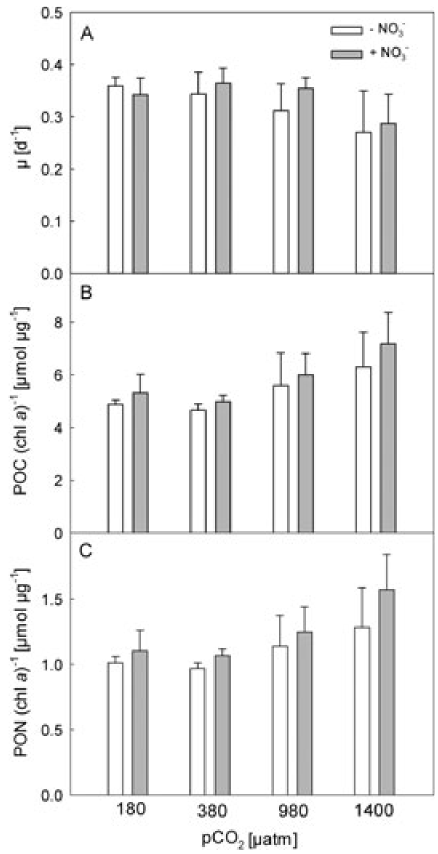

Fig. 1. Acclimation responses of *Trichodesmium* grown under different pCO_2 levels and N sources (N_2 and NO_3^-). (A) Growth rates ($n \geq 4$). (B) Ratios of POC to chl *a* ($n \geq 3$). (C) Ratios of PON to chl *a* ($n \geq 3$). Error bars denote 1 SD.

N_2 fixation

N_2 fixation was inhibited by the addition of NO_3^-, with N_2 fixation rates being close to detection limit (data not shown). Moreover, the $\delta^{15}N$ of PON clearly differed between treatments (ANOVA, $P < 0.0001$), with -1.3 ± 1.0‰ in N_2 fixing cells and $+4.8 \pm 1.3$‰ in NO_3^- grown cells. N_2 fixation rates displayed a typical diurnal cycle with high rates during midday (Fig. 2). At elevated pCO_2, there was a change in the diurnal pattern toward N_2 fixation rates remaining high until the end of the photoperiod. Consequently, integrated rates of N_2 fixation over the photoperiod increased by approximately 60% from 27 to 43 nmol N_2 (µg chl *a*)$^{-1}$ day^{-1} at 380 and 1400 µatm pCO_2, respectively. At 180 and 980 µatm pCO_2, integrated rates were 25 and 39 nmol N_2 (µg chl *a*)$^{-1}$ day^{-1}, respectively.

O_2 fluxes

To characterize the energy generating processes, O_2 evolution was firstly assessed as a function of DIC concentrations. In all treatments, maximal net O_2 evolution [V_{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 pCO_2 (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 pCO_2 on $K_{1/2}$ (DIC) were modulated by the N source and the time of day. While under NO_3^- grown conditions, there was no significant pCO_2 effect (TukeyHSD, $adj_P > 0.5$), $K_{1/2}$ (DIC) was significantly lower at 380 µatm than at 1400 µatm pCO_2 under N_2 fixing conditions (TukeyHSD, $adj_P < 0.005$). The difference between pCO_2 treatments in N_2 fixers was especially pronounced toward the evening (TukeyHSD, $adj_P < 0.0001$), with $K_{1/2}$ (DIC) decreasing in cells grown at 380 µatm but remaining high in cells grown at 1400 µatm pCO_2.

In a second approach, evolution and uptake of O_2 were assessed over a range of light intensities. Net O_2 evolution typically reached light compensation between 10 and 60 µmol photons m^{-2} s^{-1} and started to enter saturation (I_k) at approximately 280 µmol photons m^{-2} s^{-1} (Fig. 4). Light-dependent O_2 uptake, i.e. an excess of O_2 uptake in the light over O_2 uptake in the dark,

Table 2. POC and PON production and ratios of *Trichodesmium* grown under different pCO_2 levels and N sources (N_2 and NO_3^-). Errors denote 1 SD ($n \geq 3$).

pCO_2 treatment (μatm)	NO_3^-	POC production (μmol (μg chl *a*)$^{-1}$ day^{-1})	PON production (μmol (μg chl *a*)$^{-1}$ day^{-1})	POC:PON (mol:mol)
180	−	1.75 ± 0.10	0.36 ± 0.02	4.83 ± 0.10
	+	1.89 ± 0.24	0.39 ± 0.05	4.82 ± 0.06
380	−	1.60 ± 0.22	0.33 ± 0.04	4.81 ± 0.08
	+	1.82 ± 0.14	0.39 ± 0.03	4.68 ± 0.07
980	−	1.26 ± 0.42	0.26 ± 0.09	4.89 ± 0.09
	+	2.15 ± 0.19	0.45 ± 0.05	4.82 ± 0.12
1400	−	1.62 ± 0.20	0.33 ± 0.03	4.93 ± 0.21
	+	2.02 ± 0.15	0.44 ± 0.03	4.56 ± 0.07

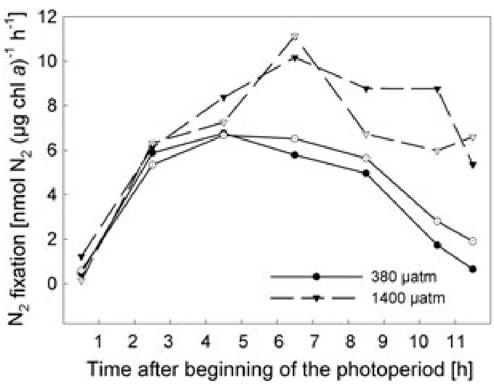

Fig. 2. Diurnal cycle of N_2 fixation in *Trichodesmium* grown at 380 μatm (circles) and 1400 μatm (triangles) pCO_2 in a 12:12 h light:dark cycle. Open and closed symbols represent biological duplicates.

was detected at irradiances >60 μmol photons m^{-2} s^{-1} (Fig. 4). Gross O_2 evolution typically saturated at higher light intensities than net photosynthesis, which is consistent with the increase in light-dependent O_2 uptake with increasing irradiance. At acclimation light intensity, diel mean values for dark respiration and light-dependent O_2 uptake amounted to 20 and 13% of gross O_2 evolution, respectively. With irradiances increasing beyond acclimation levels, light-dependent O_2 uptake increased further and equaled about 19% of gross O_2 evolution at 1300 μmol photons m^{-2} s^{-1}. Regarding effects of pCO_2 and N source on gross O_2 evolution and light-dependent O_2 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 pCO_2 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

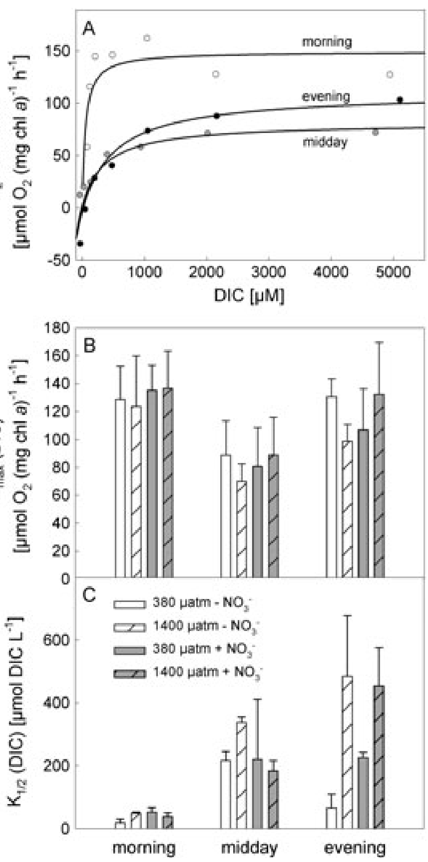

Fig. 3. Typical diurnal cycle of O_2 evolution as a function of DIC [(A) measured in *Trichodesmium* grown at 1400 μatm $-NO_3^-$]; Diurnal cycle of maximal net O_2 evolution (B) and half saturation DIC concentrations (C) in *Trichodesmium* grown under different pCO_2 levels and N sources (N_2 and NO_3^-). Error bars denote 1 SD ($n \geq 3$; except for 1400 μatm $-NO_3^-$ morning and midday and 380 μatm $+NO_3^-$ morning with $n = 2$).

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

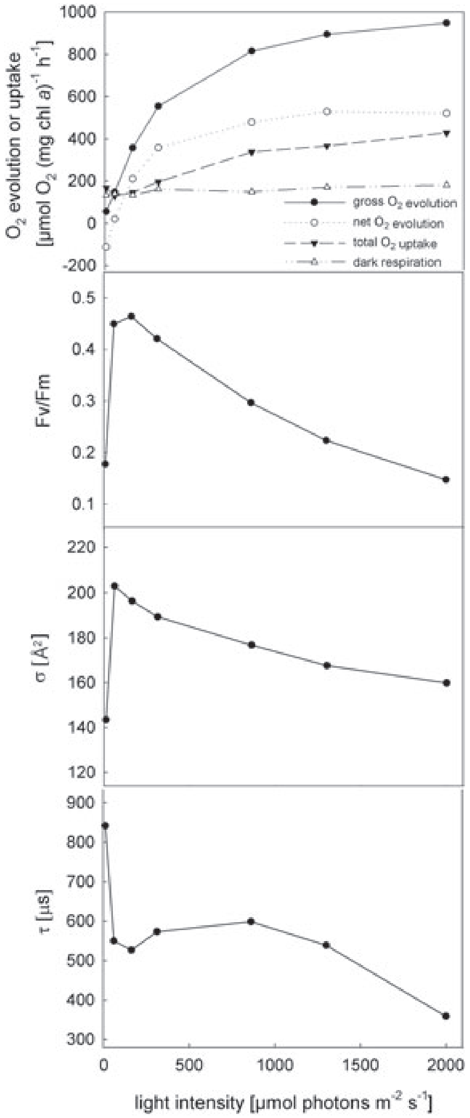

Fig. 4. Typical example of light dependence of O_2 fluxes and chlorophyll fluorescence parameters (measured in *Trichodesmium* grown at 380 µatm pCO_2 with NO_3^-). Total O_2 uptake consists of dark respiration and light-dependent O_2 uptake. Fv/Fm, PSII photochemical quantum yield; σ, PSII functional absorption cross section; τ, Q_A 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}). Q_A re-oxidation time (τ) was longest in the dark and decreased by approximately 40% from approximately 60 to 2000 µmol photons m^{-2} s^{-1}.

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

Concerning responses to pCO_2 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 pCO_2, however, only by approximately 10% (ANOVA, $P < 0.0001$). Neither τ_{dark} nor τ_{light} were significantly affected by pCO_2 or N source (ANOVA, $P > 0.5$).

Discussion

To investigate CO_2 effects on *Trichodesmium* under altered energy requirements, cultures were grown over a range of different pCO_2 levels under N_2 fixing conditions as well as with NO_3^-, the latter providing a N source with a significantly lower demand in ATP but higher electron requirements (Eqns 1 and 2). We also tested NH_4^+ 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 N_2 fixation. However, measurements revealed NH_4^+ to be toxic to *Trichodesmium* in concentrations as low as 10 µ*M* (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 NH_3 (data not shown), rendering it impossible to perform pCO_2 manipulations without simultaneously affecting the N availability. The addition of NO_3^-, on the other hand, had no negative effects on *Trichodesmium* and was not influenced by pH. As a consequence, we chose NO_3^- to impose a change in the energy status of cells. The change in N usage upon NO_3^- addition was demonstrated by direct measurements of nitrogenase activity as well as by the change in ^{15}N composition of PON. NO_3^- assimilation resulted in corresponding changes in TA, which were compensated by additions of HCl in equimolar amounts to keep the carbonate system comparable between N_2 fixing and NO_3^- using cultures. Growth rates and Fv/Fm indicate that cells were not stressed in any of the treatments.

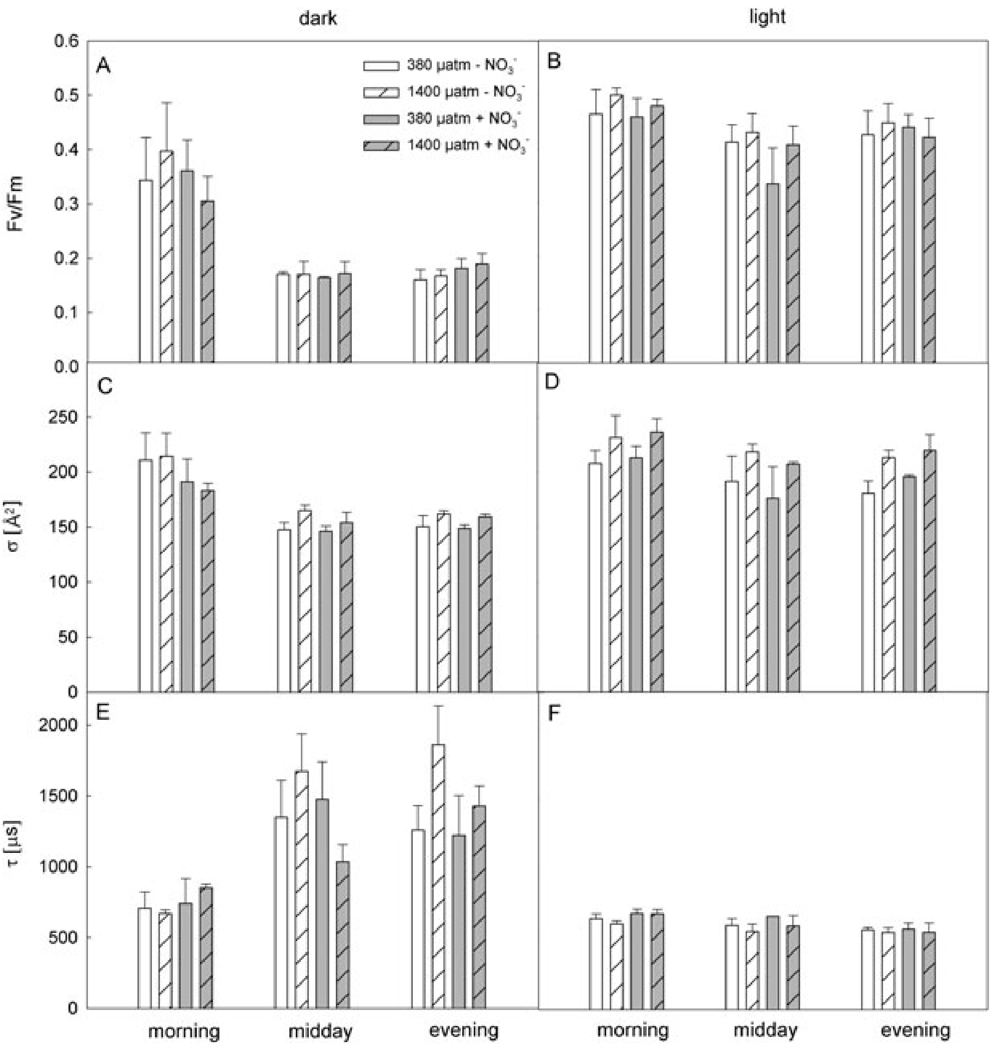

Fig. 5. Diurnal cycle of chlorophyll fluorescence of *Trichodesmium* cultures grown under different pCO_2 levels and N sources (N_2 and NO_3^-), measured in the dark (A, C and E) and at acclimation light intensity (B, D and F). Error bars denote 1 SD (n ≥ 3, except for τ of 380 µatm $+NO_3^-$ midday with n = 1). Fv/Fm, PSII photochemical quantum yield; σ, PSII functional absorption cross section; τ, Q_A re-oxidation time.

Please note that the light level applied in the acclimations (150 µmol photons m^{-2} s^{-1}) was below saturation (Fig. 4), imposing a general energy constraint in the cell.

Acclimation effects of different pCO_2 levels and N sources

The increase of POC as well as PON with pCO_2 (Fig. 1) is in accordance with previous results (Kranz et al. 2009, 2010). Respective production rates, however, stayed relatively constant due to the concomitant decrease in growth (Table 2, Fig. 1). In other words, cells contained less biomass and divided more quickly at low and medium pCO_2, while at high pCO_2, cell quotas were higher and cells divided more slowly. Among the previous studies on *T. erythraeum* IMS101 testing the effect of pCO_2 levels up to 750 or 1000 µatm, some showed an increase in growth rate with pCO_2 (Barcelos é Ramos et al. 2007, Levitan et al. 2007, Kranz et al. 2010, Garcia et al. 2011), while others did not find significant differences (Hutchins et al. 2007, Kranz et al. 2009). Only one study has tested CO_2 levels comparable to our highest CO_2 treatment, finding that positive effects on growth leveled off between 760 and 1500 µatm in *T. erythraeum* GBRTRL101 (Hutchins et al. 2007). In a recent study investigating pCO_2 effects under low iron availability representative for oligotrophic oceans, growth rates of *Trichodesmium* were shown to decrease with pCO_2 (380 vs 750 µatm; Shi et al. 2012).

The stimulation in PON production in NO_3^- grown cells may be directly attributed to the lower energy requirement for N assimilation (Table 2, Eqns 1 and 2) as well as the fact that filaments are not subject to N loss during transfer from diazocytes to non-diazotrophic cells when grown on NO_3^-. The effect on POC production, however, cannot be directly linked to N assimilation and suggests a more global effect of NO_3^- on the cells' metabolism such as reallocation of energy from N to C assimilation. The cost reduction associated with the switch from N_2 to NO_3^- assimilation is also reflected in the lower POC:PON ratios under these conditions (Table 2). Changes in POC:PON ratios have been found in response to nutrient limitation in different phytoplankton (Sterner and Elser 2002). Even though none of our treatments was N limited, the observed changes in POC:PON ratios may simply reflect the higher N assimilation costs in N_2 fixers.

O_2 fluxes and electron transport

To better understand how the observed effects of pCO_2 and N source on POC and PON were fuelled, we investigated photosynthesis as a measure of energy generation. Concerning treatment effects, neither pCO_2 nor the N source had a significant effect on net O_2 evolution (Fig. 3). However, net O_2 evolution can be uncoupled from energy generation by high rates of O_2 uptake or cyclic electron transport (Heber 2002). Thus, gross and net O_2 fluxes as well as chlorophyll fluorescence need to be considered to obtain a more complete picture of energy generating processes. In all cultures, irrespective of pCO_2 or N source, about one third of the gross O_2 evolved was consumed by dark respiration and light-dependent O_2 uptake (Fig. 4), the latter being indicative for either the classical Mehler reaction (Mehler 1951) or the equivalent reduction of O_2 by flavoproteins (Helman et al. 2003). High rates of O_2 uptake by dark respiration and Mehler reaction have been suggested to protect nitrogenase from O_2 inhibition in *Trichodesmium* (Kana 1993, Carpenter and Roenneberg 1995, Berman-Frank et al. 2001, Milligan et al. 2007). Rates of Mehler reaction equaled only about 10% of gross O_2 evolution at acclimation light intensity, yet rates increased when light intensities exceeded acclimation levels (Fig. 4). This light dependency could either indicate a role for Mehler reaction in photoprotection and/or reflect the enhanced need for nitrogenase protection at high gross O_2 evolution rates. Moreover, the fact that light-dependent O_2 uptake was not significantly affected by the N source seems surprising, considering the proposed role of Mehler reaction in nitrogenase protection in *Trichodesmium* (Milligan et al. 2007).

Chlorophyll fluorescence showed a light response typical for cyanobacteria, with dark-adapted fluorescence being controlled by respiratory electron flow that introduces electrons into the plastoquinone (PQ) pool (reviewed by Campbell et al. 1998). At low light intensities, electron flux through PSI is induced, oxidizing the PQ pool and thereby increasing Fv′/Fm′ and decreasing Q_A re-oxidation time (τ) (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 (σ) 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 Q_A re-oxidation time (τ) 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 Q_A (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 (σ, 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 τ_{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 (σ), 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^-). Theoretical demands of C fixation, CCM and POC production were normalized to mol N using the average POC:PON ratio measured in the experiment. CCM costs are based on 80% HCO_3^- use and a transport cost of 0.5 mol ATP per mol HCO_3^-, assuming 50% CO_2 leakage. Costs of NO_3^- assimilation include 1 mol ATP for uptake. Loss of fixed nitrogen (e.g. NH_4^+) is not accounted for. Please note that numbers given do not include costs for synthesis of enzymes and transporters, which would significantly increase the estimates for fixation of carbon as well as nitrogen (Brown et al. 2008). POM (particulate organic matter) is the sum of POC and PON.

Process	Unit	ATP	e^-	ATP:NADPH + H^+	Reference
C fixation	mol (mol N)$^{-1}$	14	19	1.5	Allen 2002
CCM	mol (mol N)$^{-1}$	4	0		Hopkinson et al. 2011
POC production	mol (mol N)$^{-1}$	18	19	1.9	
N_2 assimilation to NH_4^+	mol (mol N)$^{-1}$	8	4	4.0	Flores and Herrero 1994
NO_3^- assimilation to NH_4^+	mol (mol N)$^{-1}$	1	8	0.3	Flores et al. 2005
NH_4^+ assimilation to glutamate	mol (mol N)$^{-1}$	1	2	1.0	Flores et al. 2005
PON production N_2 fixer	mol (mol N)$^{-1}$	9	6	3.0	
PON production NO_3^- user	mol (mol N)$^{-1}$	2	10	0.4	
POC production measured in N_2 fixer	µmol (µg chl a)$^{-1}$ day^{-1}	5.9	6.2	1.9	
POC production measured in NO_3^- user	µmol (µg chl a)$^{-1}$ day^{-1}	7.5	7.9	1.9	
POC production difference NO_3^- vs N_2	µmol (µg chl a)$^{-1}$ day^{-1}	1.6	1.7		
PON production measured in N_2 fixer	µmol (µg chl a)$^{-1}$ day^{-1}	2.9	1.9	3.0	
PON production measured in NO_3^- user	µmol (µg chl a)$^{-1}$ day^{-1}	0.8	4.2	0.4	
PON production difference NO_3^- vs N_2	µmol (µg chl a)$^{-1}$ day^{-1}	−2.0	2.3		
Total POM production in N_2 fixer	µmol (µg chl a)$^{-1}$ day^{-1}	8.8	8.2	2.2	
Total POM production in NO_3^- user	µmol (µg chl a)$^{-1}$ day^{-1}	8.3	12.1	1.4	
POM production difference NO_3^- vs N_2	µmol (µg chl a)$^{-1}$ day^{-1}	−0.5	3.9		

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.

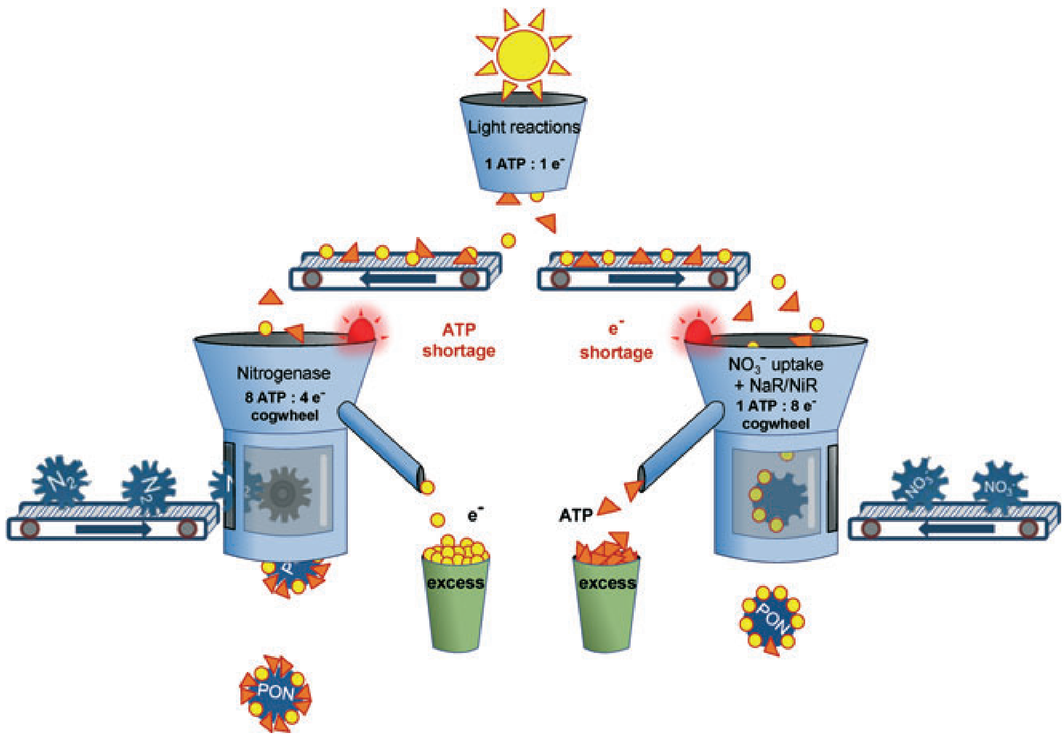

Fig. 6. Schematic diagram of the distribution of energy equivalents for PON production under different N sources (N_2 and NO_3^-). Due to the different requirements of N_2 and NO_3^- assimilation with respect to ATP and electron (e^-) stoichiometry, N_2 fixation is prone to limitation by ATP while NO_3^- 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 CO_2 sensitivity, previous studies found ARA-based estimates of N_2 fixation to increase more strongly with pCO_2 than estimates of PON production based on cell quotas or ^{15}N 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 pCO_2 according to our data. The high assimilation costs and unavoidable N loss in N_2 fixers impose higher energy requirements compared with NO_3^- users, especially under elevated pCO_2. As all treatments, however, showed the same energy generation, we expect changes in other energy sinks.

Inorganic C acquisition

Acquisition of inorganic C constitutes a major energy sink in *Trichodesmium* due to the high CCM activities required to compensate for the poor CO_2 affinity of its RubisCO (Kranz et al. 2009). Similarly to O_2 and electron fluxes as well as N_2 fixation, also the affinity for inorganic C was subject to a strong diurnal cycle (Fig. 3), which was previously described by Kranz et al. (2009). The high affinity for inorganic C in the mornings observed in all treatments is in line with the high rates of photosynthesis discussed above. The overall lower affinities at high pCO_2, especially during the second half of the day, suggest significantly lower operational costs for the CCM which, in turn, allow for the enhanced N_2 fixation observed (Figs 2 and 3). These CO_2-dependent changes in affinities and the anti-correlation with N_2 fixation are in agreement with previous results (Kranz et al. 2010). The fact that pCO_2 effects are larger in N_2 fixers than in NO_3^- using cells can be attributed to the higher overall energy requirements of N_2 fixation as well as differences in the stoichiometry of ATP and electron demand (Fig. 6): Provided that the downregulation of CCM activity mainly saves ATP, this surplus energy can be readily used in N_2 fixers to cover the high ATP demand of nitrogenase. In contrast, NO_3^- 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_i) fluxes, the cyanobacterium *Trichodesmium erythraeum* IMS101 was grown with different N sources (N_2 vs NO_3^-) and pCO_2 levels (380 vs. 1400 µatm). Cellular C_i fluxes were assessed by combining membrane inlet mass spectrometry (MIMS), ^{13}C fractionation measurements and modeling. Aside from a significant decrease in C_i affinity at elevated pCO_2 and changes in CO_2 efflux with nitrogen source, extracellular C_i fluxes estimated by MIMS were largely unaffected by the treatments. ^{13}C fractionation during biomass production increased with pCO_2, irrespective of the N source. Strong discrepancies were observed in CO_2 leakage estimates obtained by MIMS and a ^{13}C-based approach, which further increased under elevated pCO_2. These offsets could be explained by applying a model that comprises extracellular CO_2 and HCO_3^- fluxes as well as internal C_i cycling around the carboxysome via the CO_2 uptake facilitator NDH-1_4. Regarding the latter, assuming unidirectional, kinetic fractionation between CO_2 and HCO_3^- in the cytosol or enzymatic fractionation by the CO_2 uptake facilitator NDH-1_4, both significantly improved the comparability of leakage estimates. Our results highlight the importance of internal C_i cycling for ^{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_2, cyanobacteria, leakage, NDH, ocean acidification

Abbreviations

a_{carb}, fractional contribution of HCO_3^- to total C_i uptake into the carboxysome; a_{cyt}, fractional contribution of HCO_3^- to total C_i uptake into the cytosol; CA, carbonic anhydrase; CCM, carbon concentrating mechanism; chl *a*, chlorophyll *a*; C_i, inorganic carbon; DIC, dissolved inorganic carbon; $K_{1/2}$, half-saturation concentration; L_{13C}, leakage calculated from ^{13}C fractionation; L_{carb}, modeled leakage from the carboxysome; L_{cyt}, modeled leakage over the plasmamembrane; L_{MIMS}, leakage estimated by MIMS; MIMS, membrane inlet mass spectrometry; POC, particulate organic carbon; V_{max}, maximal rate; ε_{cyt}, ^{13}C fractionation in the cytosol; ε_{db}, ^{13}C equilibrium fractionation in the external medium, ε_p, total ^{13}C fractionation during POC formation; ε_{Rub}, ^{13}C fractionation by RubisCO

Introduction

Cyanobacteria are ancient organisms responsible for oxygenation of the atmosphere during times when CO_2 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 CO_2 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 CO_2 concentration in the vicinity of RubisCO. Currently, due to the ongoing anthropogenic CO_2 combustion, the availability and speciation of inorganic carbon (C_i) 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 CO_2 responses of different groups of phytoplankton (e.g. Rost et al. 2008). Among these studies, the abundant N_2 fixing cyanobacterium *Trichodesmium* stood out by showing an exceptionally high stimulation of biomass production and N_2 fixation in response to elevated pCO_2 (e.g. Hutchins et al. 2007, Kranz et al. 2009, Levitan et al. 2007). Further studies on the underlying reasons for these CO_2 effects showed a decrease in C_i affinity at high pCO_2 (Kranz et al. 2009, Kranz et al. 2010). Given the high energy demand of the CCM in cyanobacteria, a re-allocation of energy between C_i acquisition and N_2 fixation was suggested to stimulate production at high pCO_2 (Kranz et al. 2010).

Cellular C_i affinities of *Trichodesmium* are determined by the interplay of several transporters and structural adaptations composing the CCM. For understanding pCO_2 responses of the CCM as well as potential changes in energy demand it is necessary to distinguish between these different components. While CO_2 can diffuse through the cell membrane without energy investments, the low equilibrium concentrations, the slow interconversion with HCO_3^- (Zeebe and Wolf-Gladrow 2007) as well as its tendency to leak out of the cell compromise the use of CO_2 as the predominant C_i source. Therefore, cyanobacteria have evolved energy dependent transporters for taking up HCO_3^-, 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 HCO_3^- (Kranz et al. 2009, Kranz et al. 2010). Uptake of HCO_3^- in this species is catalyzed by the Na^+-dependent transporter BicA, which is fuelled by Na^+/HCO_3^- 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 HCO_3^- but pose a diffusion barrier for CO_2 (Dou et al. 2008, Espie and Kimber 2011), allowing for significant accumulation of CO_2 in the vicinity of RubisCO. Inside the carboxysomes, transformation of HCO_3^- to CO_2 is accelerated by carbonic anhydrase (CA; reviewed by Espie and Kimber 2011). In addition to direct HCO_3^- uptake and CO_2 diffusion, CO_2 uptake in *Trichodesmium* is facilitated by the NDH-1_4 complex, which converts CO_2 to HCO_3^- 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 CO_2, 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 CO_2 to HCO_3^- by the NDH complex has been proposed to drive an internal C_i recycling to minimize the loss via CO_2 efflux (Maeda et al. 2002, Price et al. 2002). Due to the strong CO_2 accumulation required in cyanobacteria, CO_2 efflux is a major challenge in these organisms. Despite the interplay of the carboxysome and proposed recapture of CO_2 by the NDH-1_4 complex, efflux of CO_2 has been shown to equal ~ 50 to 90% of gross C_i uptake in *Trichodesmium* (Kranz et al. 2009, Kranz et al. 2010). Next to the carbon source (CO_2 vs. HCO_3^-), leakage (i.e. CO_2 efflux : gross C_i uptake) can strongly affect isotopic composition of organic carbon produced during photosynthesis (Burkhardt et al. 1999, Sharkey and Berry 1985), and thus measurements of ^{13}C 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 C_i 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 N_2 fixation is a highly energy demanding process with a high demand in ATP, NO_3^- 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 C_i fluxes is as yet poorly characterized. To shed light on the extra- and intracellular C_i fluxes under the different energetic conditions, *Trichodesmium* was grown under different pCO_2 levels and N sources (N_2 vs. NO_3^-) and a combination of different methods, including MIMS and ^{13}C fractionation measurements as well as modeling was employed. While MIMS provides the perfect tool to investigate C_i fluxes over the cell membrane, internal fluxes cannot be directly measured and were therefore modeled. Model calculations of internal C_i fluxes made use of the measured extracellular C_i fluxes and the isotopic composition of particulate organic carbon ($\delta^{13}C_{POC}$), which reflects the integrated effects of extra- and intracellular C_i fluxes. Hereby, a common model of ^{13}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 µmol photons m^{-2} s^{-1} with a 12:12 h light:dark cycle. Cultures were grown in 0.2-µm-filtered artificial seawater (YBCII medium; Chen et al. 1996) and kept in exponential growth phase by regular dilution with culture medium. Culture bottles were continuously bubbled with 0.2-µm-filtered air with pCO_2 levels of 380 and 1400 µatm. Prior to experiments, cells were allowed to acclimate to the respective 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 NO_3^- as the N source, 0.2-µm-filtered $NaNO_3$ was added to achieve mean concentrations of 97 ± 2 µmol L^{-1} in the experiments, never falling below 65 µmol L^{-1}. Cultures were acclimated to NO_3^- for at least one week before measurements. Samples for the analysis of dissolved inorganic carbon (DIC) were filtered through 0.2 µm filters and measured colorimetrically (QuAAtro autoanalyzer, Seal, Norderstedt, Germany). Average precision was ± 5 µmol 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 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

C_i 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. CO_2 and O_2 fluxes were measured as a function of DIC, starting with concentrations close to zero (media bubbled with CO_2-free air), which were subsequently increased by step-wise addition of $NaHCO_3$ 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 HCO_3^- : CO_2 stays constant over the investigated DIC range. DIC-saturated rates of photosynthesis (V_{max}) and half-saturation concentrations ($K_{1/2}$ (DIC)) were obtained by fitting a Michaelis-Menten function to the data. Net O_2 evolution was converted to C fixation (F_{fix}) assuming a photosynthetic quotient of 1.34 (Williams and Robertson 1991). Net CO_2 uptake ($F_{cyt,\ netCO2}$) was calculated from the steady-state rate of CO_2 depletion at the end of the light period and corrected for the CO_2/HCO_3^- inter-conversion in the medium ($F_{ext,\ db}$). Using C fixation and net CO_2 uptake, HCO_3^- uptake rates ($F_{cyt,\ HCO3-}$) could be derived by a mass balance equation. For normalization of the CO_2 and O_2 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).

<u>Leakage estimation</u>

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 CO_2 efflux ($F_{cyt,\ out}$) to gross C_i uptake (i.e. the sum of HCO_3^- ($F_{cyt,\ HCO3-}$) and gross CO_2 uptake ($F_{cyt,\ CO2}$)):

$$L_{MIMS} = \frac{F_{cyt,out}}{F_{cyt,HCO_3^-} + F_{cyt,CO_2}} \qquad \text{(Eqn 1)}$$

$F_{cyt,\ out}$ was estimated from the initial increase in CO_2 concentration after switching off the light (Badger et al. 1994). These estimates are based on the assumption that the rate of diffusive CO_2 efflux during the light phase is well represented by the rate of CO_2 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 ($\delta^{13}C_{POC}$) and CO_2 ($\delta^{13}C_{CO2}$) in the medium according to Freeman and Hayes (1992). Duplicate samples for analysis of $\delta^{13}C_{POC}$ were filtered onto pre-combusted GF/F filters and acidified with 200 µl HCL (0.2 M) to remove all C_i prior to analysis. $\delta^{13}C_{POC}$ was measured with an EA mass spectrometer (ANCA SL 2020, SerCon Ltd., Crewe, UK). For analysis of the isotopic composition of DIC ($\delta^{13}C_{DIC}$), 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 $\delta^{13}C_{DIC}$, 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):

$$L_{13C} = \frac{\varepsilon_p - (a_{cyt}\varepsilon_{db})}{\varepsilon_{Rub}} \qquad \text{(Eqn 2)}$$

where ε_{Rub} is the intrinsic discrimination of ^{13}C by RubisCO (assumed to be +25‰; Guy et al. 1993, Roeske and O'Leary 1984) and ε_{db} represents the equilibrium fractionation between CO_2 and HCO_3^- (-9‰; Mook et al. 1974). The fractional contribution of HCO_3^- to gross C_i uptake (a_{cyt}), 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

<u>C_i fluxes under different $p$$CO_2$ levels and N sources</u>

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 ~ 20 and 500 µmol DIC L^{-1} (supplementary material, Fig. S1), which is equivalent to ~ 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 ~ 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 (~ 2100 µmol CO_2 L^{-1}), leakage measured by MIMS ranged between 0.3 and 0.7 (i.e.

CO_2 efflux equaled 30 to 70% of gross C_i uptake, Table 1). As a consequence of the high HCO_3^- contribution and the high CO_2 efflux, the net fluxes of CO_2 were generally directed out of the cell (cf. negative values for net CO_2 uptake, Table 1, Fig. 2).

Diurnal changes - The diurnal cycle was characterized by low $K_{1/2}$ 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 HCO_3^- to CO_2 uptake (Table 1, Fig. S1C), which were characterized by low CO_2 fluxes in the mornings (ANOVA, $p < 0.05$, Table 1), while HCO_3^- 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 HCO_3^- uptake, which is less prone to diffuse out of the cell, was thus correlated with lower leakage.

Treatment effects - The affinity for C_i was down-regulated at elevated pCO_2, as indicated by high $K_{1/2}$ values under these conditions (Fig. S1B). Under acclimation DIC, however, C_i fluxes (C fixation, C_i uptake, CO_2 uptake and efflux) were not significantly affected by pCO_2 (ANOVA, $p > 0.05$, Table 1), reflecting the cells' capacity to achieve similar C fixation over a range of pCO_2 levels by regulating their CCM. Regarding the N source, C fixation rates and CO_2 uptake and efflux under acclimation DIC were equally not affected (ANOVA, $p > 0.05$, Table 1). Although cells mainly used HCO_3^- as a C_i source in all treatments, HCO_3^- uptake at acclimation DIC decreased slightly with pCO_2 (~ 10%; ANOVA, $p < 0.05$, Table 1, Fig. S1C), yet was not affected by N source (ANOVA, $p > 0.05$, Table 1). Interestingly, CO_2 efflux was affected by the N source (ANOVA, $p < 0.01$, Table 1), with ~ 20% lower efflux in NO_3^- users compared to N_2 fixers, possibly due to differences in internal pH caused by the uptake/accumulation of NO_3^- vs. NH_4^+ in the cell. One could also speculate that growing cells on NO_3^- reduces the general membrane permeability, since NH_4^+ transfer between cells is only necessary under N_2 fixing conditions, which could also impact the permeability for CO_2. Leakage at acclimation DIC estimated by MIMS was, however, not significantly affected by pCO_2 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_i accumulation necessary for C fixation in cyanobacteria due to the poor CO_2 affinity of their RubisCO. Yet, leakage estimates obtained from $\delta^{13}C$ values (L_{13C}, Eqn 2) even exceeded MIMS-based estimates. Overall fractionation during formation of POC (ε_p) was not significantly affected by N treatment (ANOVA, $p > 0.05$) but increased with pCO_2 (ANOVA, $p < 0.0001$), ranging from 14.4 ± 1.0‰ at 380 µatm to 19.9 ± 0.9‰ at 1400 µatm pCO_2. Consequently, leakage estimates based on ε_p (Eqn 2) also increased with pCO_2, while estimates from MIMS measurements at acclimation DIC were constant over the range of pCO_2 levels. L_{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_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_2 efflux and gross C_i uptake. As CO_2 efflux cannot readily be determined during the light due to the concurrent C_i uptake, the rise in the CO_2 signal directly after switching off the light is taken as an estimate for CO_2 efflux during the light phase, assuming that the accumulated C_i pool and therewith gross CO_2 efflux are initially at the pre-darkness level (Badger et al. 1994). In case that active C_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 ^{13}C-based approach makes use of the effect of leakage on ε_p (Eqn 2; Sharkey and Berry 1985). Briefly, while the intrinsic fractionation by RubisCO (ε_{Rub}) is generally causing organic material to be depleted in ^{13}C (Fig. 1A), variation in ε_p can be induced by changes in the C_i source and/or leakage. Consequently, any errors in estimates of ε_{Rub} or a_{cyt} but also any unaccounted process affecting ε_p would cause ^{13}C-based leakage estimates to be biased.

Kranz et al. (2010) suggested that internal C_i cycling within the cell may affect ε_p in general. The CO_2-dependence of the offset between MIMS- and ^{13}C-based leakage estimates was furthermore suggested to reflect a CO_2 effect on the NDH complex driving this internal C_i cycling (Kranz et al. 2010), in line with early observations of the C_i dependence of CO_2 uptake by the NDH complex (Price and Badger 1989a, c). In Fig. 1, the effects of internal C_i cycling on the isotopic composition are illustrated. While Fig. 1A assumes an equilibrium situation and does not include any internal C_i cycling, Fig. 1B illustrates non-equilibrium situations caused by internal C_i cycling. The degree of ^{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_i fluxes on carbon fractionation

Model setup - To test our concept (Fig. 1) and quantitatively describe the possible effect of internal cycling on $\delta^{13}C$, intracellular C_i fluxes and their effects on isotopic ratios in different cellular C_i pools were modeled. For parameterizations, HCO_3^- and gross CO_2 fluxes measured by MIMS as well as measured fractionation values ε_p were used. The model is based on flux balance equations for the individual isotope species. The flux balance of total carbon (^{12}C + ^{13}C) in the cytosol and in the carboxysome, respectively, is given by the following equations:

$$F_{cyt,CO_2} + F_{cyt,HCO_3^-} + F_{carb,out} - F_{cyt,out} - F_{carb,CO_2} - F_{carb,HCO_3^-} = 0 \qquad \text{(Eqn 3)}$$

$$F_{carb,HCO_3^-} + F_{carb,CO_2} - F_{carb,out} - F_{fix} = 0 \qquad \text{(Eqn 4)}$$

As about 99% of carbon is ^{12}C, i.e. $F = {}^{12}F + {}^{13}F \equiv {}^{12}F$, the flux balance equations for ^{13}C can be derived by multiplying the fluxes F with the isotopic ratio R = $^{13}C/^{12}C$. The isotopic fractionation factor α_{db} is defined by the isotopic ratio of CO_2 divided by the isotopic ratio of HCO_3^-, i.e. $\alpha_{db} = R_{CO_2} / R_{HCO_3^-}$. Using the equilibrium fractionation (ε_{db}), the fractionation factor between CO_2 and HCO_3^- can be calculated for the external medium as well as for the cytosol according to:

$$\alpha_{db,ext} = 1 + \varepsilon_{db}/1000 \qquad \text{(Eqn 5)}$$

$$\alpha_{db,cyt} = 1 + \varepsilon_{cyt}/1000 \qquad \text{(Eqn 6)}$$

While the equilibrium value ε_{db} is -9‰ (i.e. CO_2 is isotopically lighter than HCO_3^-; Mook et al. 1974), ε_{cyt} can significantly deviate from this value due to kinetic effects. The uncatalyzed conversion of HCO_3^- to CO_2 shows a kinetic fractionation of -22‰ whereas the formation of

HCO_3^- from CO_2 is associated with a kinetic fractionation of +13‰. Hence, the actual value of ε_{cyt} is determined by the disequilibrium between CO_2 and HCO_3^- in the cytosol, which depends on all fluxes in and out of the cytosol and on the internal CO_2 and HCO_3^- concentrations, which cannot be calculated in the framework of a flux-based model. Assuming a unidirectional conversion of CO_2 to HCO_3^- 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-1_4 complex will be taken into account. The case that the conversion of CO_2 to HCO_3^- 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 ^{13}C by RubisCO described by the factor $\alpha_{Rub} = R_{carb} / R_{POC}$, where R_{carb} is the isotopic ratio of CO_2 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):

$$\alpha_{Rub} = 1 + \varepsilon_{Rub}/1000 \qquad \text{(Eqn 7)}$$

Given the isotopic ratios R of CO_2 and the isotopic fractionation factors between HCO_3^- and CO_2 expressed as $\alpha_{bd} = 1 / \alpha_{db}$, the flux balance equations for ^{13}C can be derived from equations 3 & 4 for the cytosol and the carboxysome, respectively:

$$\begin{aligned} &R_{ext}F_{cyt,CO_2} + \alpha_{bd,ext}R_{ext}F_{cyt,HCO_3^-} + R_{carb}F_{carb,out} - R_{cyt}F_{cyt,out} - R_{cyt}F_{carb,CO_2} \\ &- \alpha_{bd,cyt}R_{cyt}F_{carb,HCO_3^-} = 0 \end{aligned} \qquad \text{(Eqn 8)}$$

$$\alpha_{bd,cyt}R_{cyt}F_{carb,HCO_3^-} + R_{cyt}F_{carb,CO_2} - R_{carb}F_{carb,out} - R_{carb}F_{fix}/\alpha_{Rub} = 0 \qquad \text{(Eqn 9)}$$

R_{cyt} is the isotopic ratio of CO_2 in the cytosol. The overall isotopic fractionation by the cell is defined with respect to the isotopic composition of CO_2 in the external medium (R_{ext}):

$$\varepsilon_p = \left(\frac{R_{ext}}{R_{POC}} - 1\right)\cdot 1000 = \left(\alpha_{Rub}\frac{R_{ext}}{R_{carb}} - 1\right)\cdot 1000 \qquad \text{(Eqn 10)}$$

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:

$$\frac{R_{ext}}{R_{carb}} = \frac{\left(F_{fix}/\alpha_{Rub} + F_{carb,out}\right)\left(F_{carb,CO_2} + \alpha_{bd,cyt}F_{carb,HCO_3^-} + F_{carb,out}\right)}{\left(F_{cyt,CO_2} + \alpha_{bd,ext}F_{cyt,HCO_3^-}\right)\left(F_{carb,CO_2} + \alpha_{bd,cyt}F_{carb,HCO_3^-}\right)} - \frac{F_{carb,out}}{\left(F_{cyt,CO_2} + \alpha_{bd,ext}F_{cyt,HCO_3^-}\right)}$$

$$= \frac{F_{cyt,out}}{\alpha_{Rub}\left(F_{cyt,CO_2} + \alpha_{bd,ext}F_{cyt,HCO_3^-}\right)}\left(\frac{F_{fix}}{F_{cyt,out}} + \frac{F_{fix} + \alpha_{Rub}F_{carb,\ out}}{F_{carb,CO_2} + \alpha_{bd,cyt}F_{carb,HCO_3^-}}\right)$$

(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:

$$F_{fix} = F_{carb,CO_2} + F_{carb,HCO_3^-} - F_{carb,out} = F_{cyt,CO_2} + F_{cyt,HCO_3^-} - F_{cyt,out} \qquad \text{(Eqn 12)}$$

Given the fractional contribution of HCO_3^- to total C_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:

$$\varepsilon_p = \frac{a_{cyt}\varepsilon_{db}}{1 - a_{cyt}\varepsilon_{db}/10^3} + L_{cyt}\frac{\left(a_{carb}\varepsilon_{cyt} + L_{carb}\varepsilon_{Rub}\right)}{\left(1 - a_{cyt}\varepsilon_{db}/10^3\right)\left(1 - a_{carb}\varepsilon_{cyt}/10^3\right)}$$

(Eqn 13)

$$\approx a_{cyt}\varepsilon_{db} + L_{cyt}\left(a_{carb}\varepsilon_{cyt} + L_{carb}\varepsilon_{Rub}\right).$$

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

$$L_{cyt} = \frac{\varepsilon_p - a_{cyt}\ \varepsilon_{db}}{a_{carb}\ \varepsilon_{cyt} + L_{carb}\varepsilon_{Rub}} \qquad \text{(Eqn 14)}$$

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 ε_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}C fractionation. For membrane permeability exceeding 10^{-4} cm s^{-1}, as proposed for a diatom (~ 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 ε_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 ε_p by not more than 0.7‰. Thus, a_{cyt} can be excluded as a main driver behind the variability in ε_p (or leakage estimates), even if variability in a_{cyt} is severely underestimated. Applying the model to our measured fluxes and ε_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 (ε_{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 ε_p in both of our pCO_2 treatments, the high ε_p measured in cells grown at 1400 µatm constrains the range of possible values, while ε_p of cells grown at 380 µatm 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 ε_{cyt} to explain our measured ε_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:

$$\frac{F_{carb,\,CO_2} + F_{carb,\,HCO_3^-}}{F_{cyt,\,CO_2} + F_{cyt,\,HCO_3^-}} = \frac{1 - L_{cyt}}{1 - L_{carb}} \qquad \text{(Eqn 15)}$$

For the chosen value for L_{carb} of 0.9 and the measured L_{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 CO_2 to HCO_3^- is +13‰ (O'Leary et al. 1992). With this kinetic fractionation, ε_p values measured for the two $p CO_2$ levels can be explained by leakage from the cytosol (L_{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_{13C} = 1.1 and 0.8, respectively). The remaining difference to leakage estimates obtained by MIMS (L_{MIMS} = 0.5) could be explained by an underestimation of leakage by the MIMS approach, as discussed above. Assuming that the conversion between CO_2 and HCO_3^- in the cytosol was not completely unidirectional, ε_{cyt} could range between +13‰ and -9‰ (equilibrium fractionation; O'Leary et al. 1992). To simulate this intermediate scenario, an ε_{cyt} of +8‰ is assumed (Scenario 4), yielding an L_{cyt} of 0.9 for the high 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 HCO_3^- to CO_2. Mutants of *Synechococcus* expressing human CA in the cytosol were unable to accumulate C_i (Price and Badger 1989b), suggesting that HCO_3^- is accumulated in the cytosol, and that a chemical disequilibrium in the cytosol favors the reaction from HCO_3^- to CO_2 rather than the opposite direction. This strongly argues for a fractionating enzyme instead of a purely chemical disequilibrium driving unidirectional CO_2 to HCO_3^- conversion in the cytosol. Assuming that NDH not only prevents the back-reaction but also discriminates against ^{13}C during the reaction, leakage estimates by our model can be further reconciled with MIMS-based estimates. With an ε_{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 $p CO_2$ treatment ($L_{MIMS} = L_{cyt}$ = 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 CO_2 to 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-1_4 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 pCO_2 and the slight changes in a_{cyt} could not explain the observed variation in ε_p, a_{carb} and/or ε_{cyt} would need to vary with pCO_2 for explaining the observed CO_2-dependence of ε_p. Any pCO_2-dependent changes in cytosolic pH and/or a_{cyt} would be expected to decrease a_{carb} at elevated pCO_2, and can thus not explain the increase in ε_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_{carb} as well as ε_{cyt} and therewith ε_p (Fig. 5, lower branch). With an increase in a_{carb} from 0.7 to 1 and an increase in ε_{cyt} from 20 to 30‰, the measured increase in ε_p between 380 and 1400 µatm can be explained, almost reproducing MIMS-measured leakage in both scenarios (Scenarios 5 & 6, Table 2). A higher activity of NDH at high pCO_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 pCO_2 would not provide any energetic benefit to the cell. A positive correlation with pCO_2 might be coupled to the higher CO_2 availability and resulting small change in a_{cyt} at high pCO_2 levels in the acclimations (Table 1, Fig. S1C, Fig. 5). Please note that the change in a_{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 pCO_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 pCO_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 pCO_2 treatment with light intensities similar to our experiment (200 µmol photons m^{-2} s^{-1}, Scenario 3, Table 2). In high pCO_2 and low light treatments, the difference between L_{MIMS} and L_{cyt} was larger and could consequently only be reconciled with ε_{cyt} values much larger than +30‰. Short-term exposure to high light intensities (300 µmol photons m^{-2} s^{-1}) in our experiment affected CO_2 efflux in cells acclimated to high pCO_2 (ANOVA, $p < 0.05$, data not shown). Such light-sensitivity generally suggests CO_2 efflux to be closely associated to photosynthetic electron transport. As this light effect was only observed under high pCO_2, i.e. conditions associated with higher NDH activity according to our model results, the interrelation of CO_2 efflux and the NDH complex is further corroborated. The observed light effects on CO_2 efflux (this study) and ε_p (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 pCO_2 as well as light on internal C_i fluxes and potential feedbacks on cellular energy budgets of this key N_2 fixer.

Conclusion & Outlook

In conclusion, this study demonstrates that internal C_i fluxes via the NDH-1_4 complex need to be considered not only in terms of cellular C_i acquisition, but also with regard to carbon isotopic fractionation and cellular energy status. The comparison of direct measurements of C_i fluxes with estimates based on isotopic composition revealed that ^{13}C fractionation in *Trichodesmium* cannot be described adequately by considering only the external C_i fluxes. Compatibility with direct leakage measurements was improved by applying a model accounting for internal C_i 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 C_i fluxes. The agreement of model results with measured values could further be improved by accounting for possible ^{13}C enrichment of CO_2 leaking out of the cell as well as a disequilibrium situation in the surroundings of the cell (altering the isotopic signature of HCO_3^- taken up). The model applied here could be used to improve estimates of leakage based on ^{13}C signatures also for other species in which several compartments and/or internal C_i 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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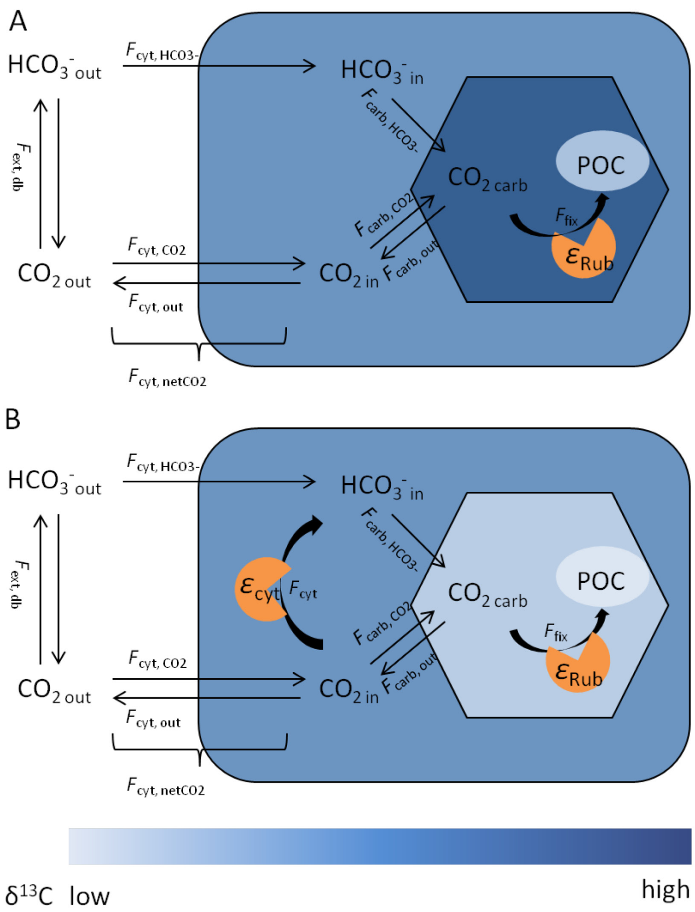
Table 1: Diurnal cycle of C_i fluxes measured by MIMS under acclimation DIC levels (~ 2100 µmol L^{-1}) in *Trichodesmium* acclimated to two different $p$$CO_2$ levels (380 vs 1400 µatm) and N sources (N_2 vs NO_3^-). All C_i fluxes are given in µmol C (mg chl a)$^{-1}$ h^{-1}. Errors are standard deviations for biological replicates (1 SD; n = 3 except 1400 +NO_3^- morning with n = 1).

	380 µatm -NO_3^-			380 µatm +NO_3^-			1400 µatm -NO_3^-			1400 µatm +NO_3^-		
	morning	midday	evening	morning	midday	evening	morning	midday	evening	morning	midday	evening
net C fixation	91±15	57±14	87±3	95±20	56±22	70±18	80±13	39±14	61±4	91	61±19	63±16
gross C_i uptake	157±18	144±24	167±17	135±23	128±16	143±25	144±7	124±9	142±6	144	127±22	131±17
HCO_3^- uptake	134±18	117±22	133±12	118±21	108±14	113±14	126±11	95±9	112±2	120	97±17	102±12
gross CO_2 uptake	23±4	27±4	34±6	17±3	20±2	30±13	18±4	28±5	30±6	24	30±9	29±6
net CO_2 uptake	-45±10	-60±12	-46±9	-23±2	-52±8	-42±11	-46±5	-56±8	-51±4	-28	-36±8	-39±9
HCO_3^- : C_i uptake	0.85±0.03	0.81± 0.03	0.80 ± 0.02	0.87±0.01	0.84±0.01	0.80±0.06	0.88±0.03	0.77±0.04	0.79±0.03	0.83	0.76±0.05	0.78±0.01
CO_2 efflux	69±12	87±13	79±13	40±3	72±6	72±8	64±7	85±14	81±3	53	66±4	67±10
leakage	0.44 ± 0.05	0.61±0.05	0.47±0.03	0.30±0.03	0.57±0.11	0.51±0.04	0.45±0.07	0.69±0.10	0.57±0.01	0.37	0.53±0.07	0.52±0.07

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

Scenario	pCO_2	ε_p	a_{cyt}	L_{MIMS}	L_{13C}	L_{cyt}	L_{carb}	a_{carb}	ε_{cyt}
		measured					modeled		
1	1400	20	0.8	0.5	1.1	0.8	0.9	1	13
2	380	14	0.8	0.5	0.8	0.6	0.9	1	13
3	180	7	0.8	0.4	0.6	0.4	0.9	1	13
4	1400	20	0.8	0.5	1.1	0.9	0.9	1	8
5	1400	20	0.8	0.5	1.1	0.5	0.9	1	30
6	380	14	0.8	0.5	1.1	0.6	0.9	0.7	20



$HCO_3^-{}_{out}$	HCO_3^- concentration in the medium
$CO_{2\,out}$	CO_2 concentration in the medium
$HCO_3^-{}_{in}$	HCO_3^- concentration in the cytoplasm
$CO_{2\,in}$	CO_2 concentration in the cytoplasm
$CO_{2\,carb}$	CO_2 concentration in the carboxysome
POC	Cellular pool of organic carbon
$F_{ext, db}$	Rate of net equilibrium reaction from CO_2 to HCO_3^- in the medium
$F_{cyt, HCO3-}$	Rate of cellular HCO_3^- uptake
$F_{cyt, CO2}$	Rate of cellular gross CO_2 uptake
$F_{cyt, out}$	Rate of cellular CO_2 efflux
$F_{cyt, netCO2}$	Rate of cellular net CO_2 uptake
$F_{carb, HCO3-}$	Rate of HCO_3^- uptake into the carboxysome
$F_{carb, CO2}$	Rate of gross CO_2 influx into the carboxysome
$F_{carb, out}$	Rate of CO_2 efflux from the carboxysome
F_{fix}	Rate of carbon fixation by RubisCO
F_{cyt}	Rate of CO_2 to HCO_3^- conversion in the cytosol

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_{fix}) were measured by MIMS, while fluxes in and out of the carboxysome were modeled. Color code denotes $\delta^{13}C$ 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. ε_{Rub} fractionation by RubisCO; ε_{cyt}, fractionation in the cytosol.

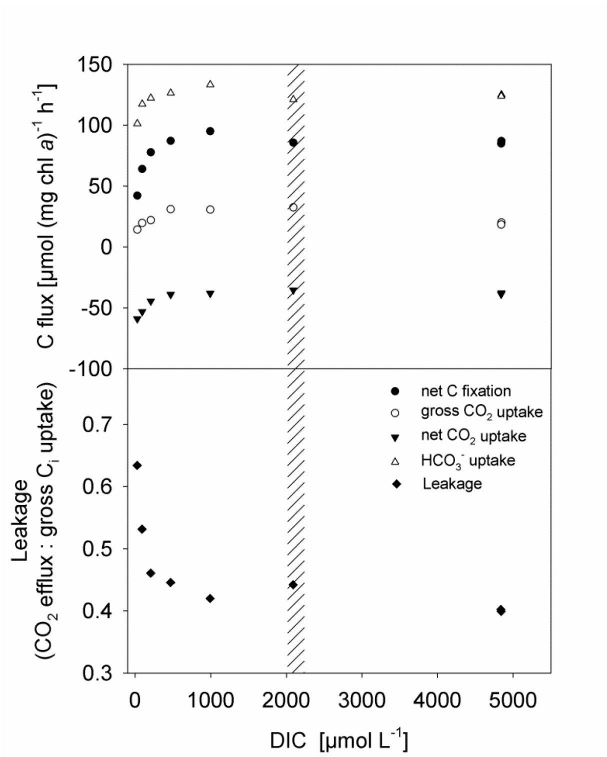


Fig. 2: Example showing the dependence of C_i fluxes measured by MIMS in *Trichodesmium* on the DIC concentration in the assay. Data shown were measured in the evening in a culture grown at 380 µatm pCO_2 without NO_3^-. The shaded area denotes the range of acclimation DIC levels.

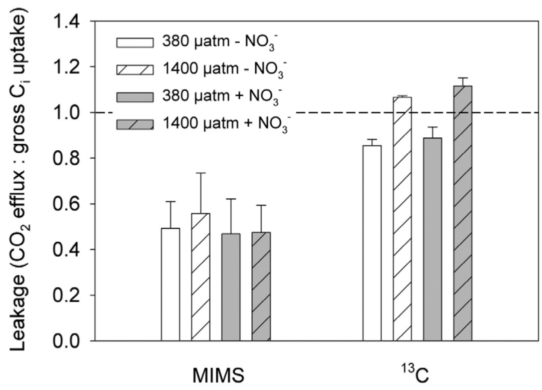


Fig. 3: Leakage estimates by MIMS (mean values of measurements conducted at three time points over the day; Badger et al. 1994) and ^{13}C fractionation (Sharkey and Berry 1985) determined in *Trichodesmium* grown under two pCO_2 levels and N sources ($n \geq 3$).

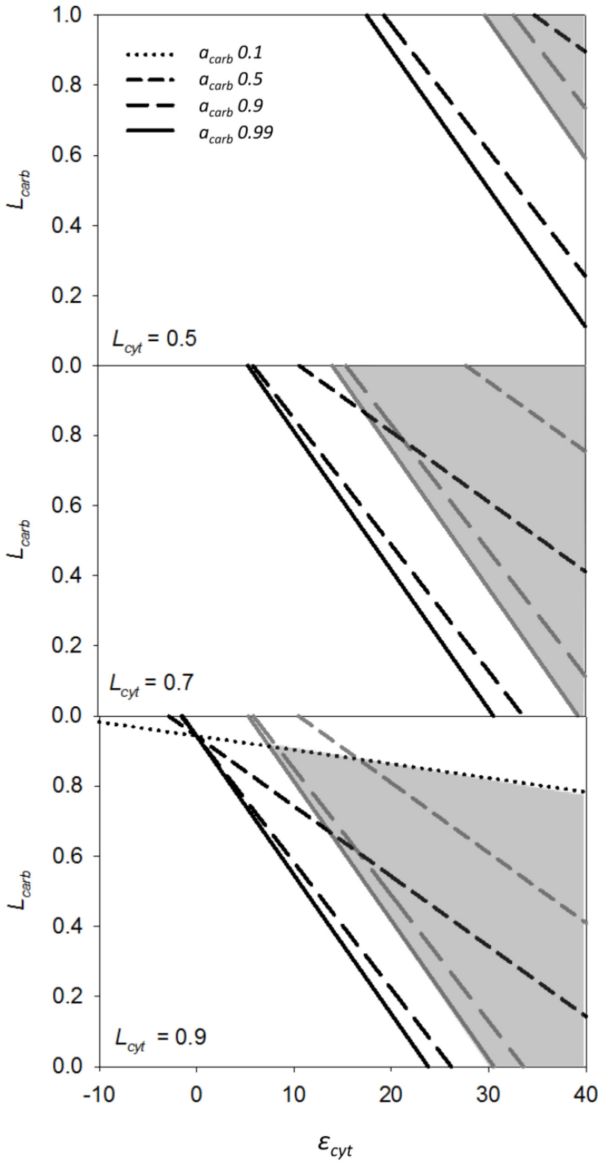


Fig. 4: Interrelation of L_{carb}, ε_{cyt} and a_{carb} in the model, depicted for different values of L_{cyt} and ε_p. The shaded areas mark the range of possible values for L_{carb} and ε_{cyt} that could reconcile our measurements of isotopic composition with measured external C_i fluxes. Black and grey lines are based on ε_p measured in cells acclimated to 380 and 1400 µatm pCO_2, respectively.

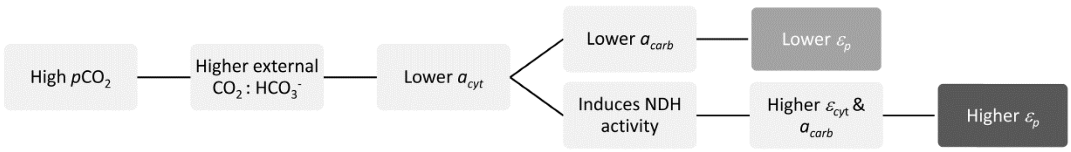


Fig. 5: Hypothetical effect of elevated pCO_2 on ε_p with and without involvement of the NDH-1_4 complex. Without the NDH complex, elevated pCO_2 might decrease ε_p due to the lower share of HCO_3^- uptake (a_{cyt} and a_{carb}; upper branch). Induction of the NDH-1_4 complex by a higher availability of its substrate CO_2 in the cytosol might, however, override these effects, elevating a_{carb} and ε_{cyt}, both of which would increase ε_p (lower branch).

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Diversity of ocean acidification effects on marine N_2 fixers

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ABSTRACT

Considering the important role of N_2 fixation for primary productivity and CO_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_2 fixers, the objective of this study was to improve our knowledge of the CO_2 responses of other diazotrophs. To this end, the single-celled *Cyanothece sp.* and two heterocystous species, *Nodularia spumigena* and the symbiotic *Calothrix rhizosoleniae*, were acclimated to two pCO_2 levels (380 vs. 980 μatm). Growth rates, cellular composition (carbon, nitrogen and chlorophyll *a*) as well as carbon and N_2 fixation rates (^{14}C incorporation, acetylene reduction) were measured and compared to literature data on different N_2 fixers. The three species investigated in this study responded differently to elevated pCO_2, showing enhanced, decreased as well as unaltered growth and production rates. For instance, *Cyanothece* increased production rates with pCO_2, which is in line with the general view that N_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_2 fixation was stimulated under elevated pCO_2. Reviewing literature data confirmed a large variability in CO_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_2 fixation as well as differences in cellular energy limitation between the species. The group-specific CO_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.

1. Introduction

Owing to their ability to fix atmospheric N_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_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_2 fixation can significantly foster net CO_2 sequestration (Eppley and Peterson, 1979). Since nutrient concentrations in the low latitude surface ocean are expected to decrease with the predicted increase in stratification, N_2 fixation may become more important as global warming progresses (Doney, 2006). Aside from global warming, rising atmospheric CO_2 levels cause ocean acidification, which can affect phytoplankton in numerous ways (e.g. Rost et al., 2008). Considering the important role of N_2 fixation for primary productivity and CO_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_2 sensitivity for this species, with an increase in N_2 fixation or particulate organic nitrogen (PON) production ranging between 35 and 140% for pCO_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_2 fixation (Mahaffey et al., 2005), the development of new methods has shed light on the importance of previously unrecognized N_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_2 fixing enzyme, nitrogenase, from O_2. Single-celled species generally separate photosynthesis and N_2 fixation in time, fixing N_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*; DDA, diatom-diazotroph-association; 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; Schneegurt 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 fully 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 poorly characterized, the physiology of *Crocosphaera* (UCYN-B) and *Cyanothece* (closely related to group UCYN-C) has been investigated in laboratory experiments. Heterocystous diazotrophs play an important ecological role in many fresh water as well as brackish systems, not only due to their ability to fix N_2 but also due to their tendency to form extensive, partly toxic blooms. In the Baltic Sea, for instance, blooms formed by *Nodularia spumigena, Aphanizomenon sp.* and *Anabaena sp.* have attracted attention for many decades primarily due to their broad impact on the ecosystem and nuisance to humans (Sivonen et al., 1989, 1990; Stal et al., 2003). The dense surface scums building up during blooms form highly productive microenvironments (Ploug, 2008) and provide a significant source of N to the ecosystem (Larsson et al., 2001). In open ocean environments, heterocystous species have been observed to form symbioses with diatoms. These so-called diatom-diazotroph-associations (DDAs) reach especially high abundances in the tropical Atlantic Ocean, where they benefit from nutrient inputs by the Amazon River plume (Carpenter et al., 1999; Foster et al., 2007; Luo et al., 2012; Subramaniam et al., 2008; Villareal, 1994). Global N_2 fixation rates by DDAs have been tentatively estimated to equal those of *Trichodesmium* (Foster et al., 2011). These symbioses may furthermore be especially important in the context of the biological C pump, as the heavy silica shells of the diatoms act as ballast material (Karl et al., 2012; Subramaniam et al., 2008; Yeung et al., 2012). Symbiotic heterocystous cyanobacteria identified to date include *Richelia intracellularis* found in symbioses with diatoms of the genera *Rhizosolenia* and *Hemiaulus*, and *Calothrix rhizosoleniae,* a symbiont of *Chaetoceros*, which has been isolated and successfully maintained in lab cultures (Foster et al., 2010). While relatively little is known about the physiological interactions between symbiont and host, cyanobacteria in symbioses have been shown to fix N_2 in excess of their needs and transport large amounts of fixed N to the host diatoms (Foster et al., 2011).

While a number of studies addressed the CO_2 sensitivity of *Trichodesmium* (e.g. Barcelos é Ramos et al., 2007; Hutchins et al., 2007; Kranz et al., 2009; Levitan et al., 2007), the response patterns can most likely not be extrapolated to other diazotrophs due to the fundamental differences described above. Several recent studies on 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 diazotroph 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 *Cyanothece sp.* and two heterocystous species, *Nodularia spumigena* and the symbiotic *Calothrix rhizosoleniae* 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 rhizosoleniae* SC01, *Cyanothece 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 *Cyanothece* and *Calothrix* and 9 for *Nodularia* (measured with Autosal 8400B, Guildline). Cells were kept in exponential growth phase by regular dilution with culture medium. Cultures were grown in 1 L (*Cyanothece* 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 potentiometrically 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 1
Parameters of the carbonate system for each pCO_2 treatment acquired in daily measurements (pH) or at the start and end of growth curves (DIC and TA). Attained pCO_2 of the media was calculated from pH, DIC, phosphate concentration, temperature and salinity using CO2sys (Pierrot et al., 2006). Errors denote 1 SD (n ≥ 6).

	target pCO_2 [μatm]	pH (NBS)	TA [μmol kg^{-1}]	DIC [μmol kg^{-1}]	pCO_2 attained [μatm]
Cyanothece	380	8.20 ± 0.01	2356 ± 2	1969 ± 1	380 ± 9
	980	7.86 ± 0.02	2368 ± 13	2154 ± 10	974 ± 20
Nodularia	380	8.18 ± 0.02	1524 ± 28	1357 ± 12	391 ± 7
	980	7.80 ± 0.02	1515 ± 17	1399 ± 25	974 ± 39
Calothrix	380	8.25 ± 0.02	2348 ± 16	1950 ± 13	340 ± 30
	980	7.93 ± 0.03	2339 ± 28	2102 ± 18	832 ± 70

(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 *Cyanothece* were determined using a Coulter counter (Multisizer III, Beckman Coulter). Cell densities of *Nodularia* cultures were determined using a Sedgwick 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, Sonifier 250, Branson Ultrasonics). Chl *a* concentrations were determined fluorometrically (TD-700 Fluorometer, Turner Designs) and corrected for fluorescence of phycobilipigments (Holm-Hansen and Riemann, 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 (*Cyanothece* and *Nodularia*) or chl *a* concentrations (*Calothrix*) monitored over 6–7 days:

$$\mu[d^{-1}] = (\ln(c_1) - (\ln(c_0))/dt \tag{1}$$

where c_0 and c_1 are the initial and final cell density or chl *a* concentration and dt 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. N_2 fixation

N_2 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 avoid aggregation 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 (Breitbarth et al., 2004).

2.5. Carbon fixation

C fixation rates were determined using ^{14}C fixation assays (Steemann-Nielsen, 1952). Samples were transferred to 25 ml vials with < 4% headspace and spiked with 20 μCi $H^{14}CO_3^-$ (1.576 GBq/mmol $NaH^{14}CO_3$ 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 (C_{fixed}) was calculated using the following equation:

$$C_{fixed} = (dpm_{sample} x[DIC]x1.05)/dpm_{total} \tag{2}$$

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 $H^{14}CO_3^-$ 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 (*Cyanothece* and *Nodularia*).

3. Results and discussion

The three species investigated in this study showed very different responses to elevated pCO_2. 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 *Cyanothece*, growth rates were not affected by pCO_2 (*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 pCO_2 (*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 pCO_2 (*t*-test, $p > 0.05$, Table 2). Also N_2 fixation was not significantly affected by pCO_2 (*t*-test, $p > 0.05$, Fig. 2). C fixation as determined in ^{14}C-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 ^{14}C fixation being lower than POC production. As ^{14}C 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 ^{14}C-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 *Cyanothece*, which has a strong diurnal cycle in cellular C and N quotas due to the temporal separation of N_2 fixation and photosynthesis. In the heterocystous *Nodularia*, less diurnal variability can be expected and accordingly, ^{14}C 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 N_2 fixation, including any N that might be excreted from the cell subsequent to fixation. Absolute values of N_2 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 N_2 fixation have not been verified for the here tested species. Despite these uncertainties in absolute numbers, trends with pCO_2 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 *Cyanothece*, 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

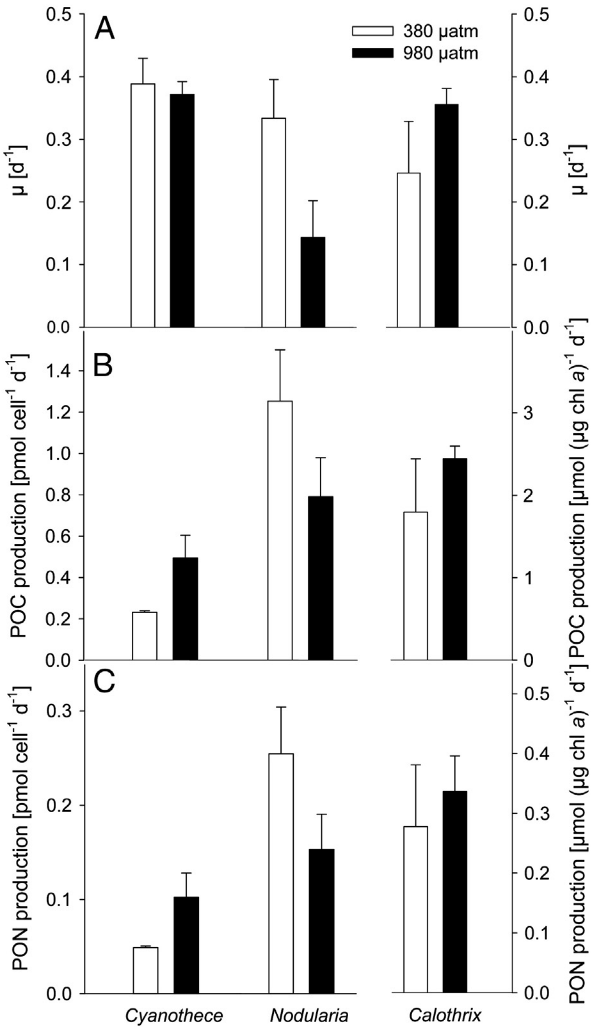

Fig. 1. Growth rates (A) and production rates of POC and PON (B & C) of *Cyanothece*, *Nodularia* and *Calothrix* grown at two different pCO_2 levels. Growth rates of *Cyanothece* and *Nodularia* were based on cell densities, growth rates of *Calothrix* were based on chl *a* concentrations. Errors denote 1 SD (n ≥ 3, except for POC & PON production in *Cyanothece* at 980 µatm with n = 2).

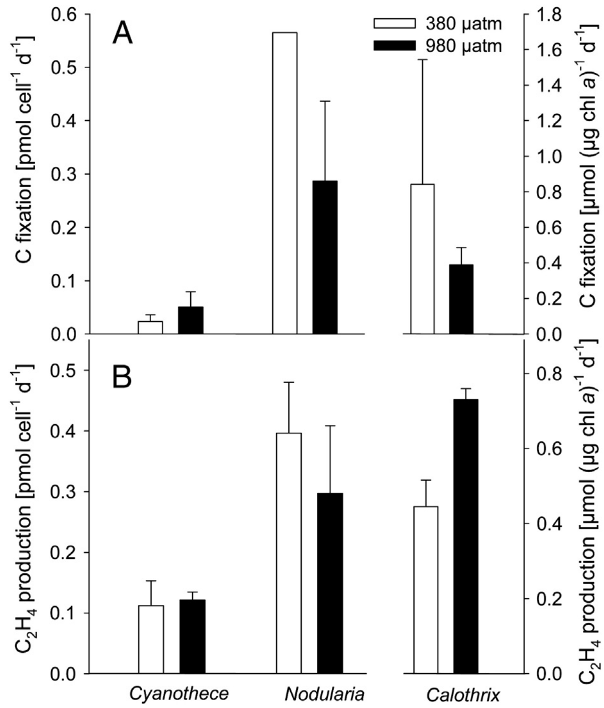

Fig. 2. C fixation (A) and ethylene (C_2H_4) production rates (B) of *Cyanothece*, *Nodularia* and *Calothrix* grown at two different pCO_2 levels. Errors denote 1 SD (n ≥ 3, except for C fixation in *Nodularia* at 380 µatm with n = 1).

mechanisms for C and N that are required due to the diurnal cycle of photosynthesis and N_2 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 pCO_2 is consistent with the increase in glycogen contents found in a previous study testing effects of very high pCO_2 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 pCO_2 (*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 pCO_2 (*t*-test, $p < 0.05$, Table 2). POC and PON production generally showed a decreasing trend in response to elevated pCO_2, which was, however, only significant for PON production (*t*-test, $p < 0.05$, Fig. 1). The decrease in growth and production with increasing pCO_2 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 pCO_2. In the latter study, however, cultures were limited by phosphate and were not continuously mixed. The negative effect of high pCO_2 on growth and production rates of *Nodularia* has been attributed to a detrimental effect of low pH on N transfer between cells along the filament (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 pCO_2 (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 ^{14}C 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 pCO_2 levels. Errors denote 1 SD (n ≥ 3, except for POC & PON in *Cyanothece* at 980 µatm with n = 2). n.d.: not determined.

	Target pCO_2 [µatm]	Chl *a* content [pg cell^{-1}]	POC content [pmol cell^{-1}]	PON content [pmol cell^{-1}]	POC : chl *a* [µmol µg^{-1}]	PON : chl *a* [µmol µg^{-1}]	POC:PON [mol:mol]
Cyanothece	380	0.036 ± 0.007	0.60 ± 0.06	0.13 ± 0.01	17.0 ± 4.6	3.6 ± 0.8	4.75 ± 0.23
	980	0.034 ± 0.007	1.27 ± 0.17	0.26 ± 0.04	42.5 ± 0.1	8.8 ± 0.3	4.85 ± 0.13
Nodularia	380	0.65 ± 0.12	4.08 ± 1.50	0.83 ± 0.29	6.4 ± 2.2	1.3 ± 0.5	4.92 ± 0.14
	980	0.53 ± 0.05	4.17 ± 0.36	0.81 ± 0.07	8.0 ± 1.2	1.5 ± 0.2	5.16 ± 0.04
Calothrix	380	n.d.	n.d.	n.d.	7.3 ± 0.2	1.1 ± 0.1	6.47 ± 0.13
	980	n.d.	n.d.	n.d.	6.9 ± 0.7	0.9 ± 0.1	6.64 ± 0.15

suggesting 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 pCO_2 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 pCO_2 is consistent with the decrease in POC production (Figs. 1 and 2). Likewise, N_2 fixation was not significantly affected by pCO_2 (*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 pCO_2 on excretion of C or N for *Nodularia*. This is in agreement with previous results showing no effect of pCO_2 on cell-specific production of mucinous substances and dissolved organic carbon (Endres et al., 2012).

In *Calothrix*, mean values of growth rates increased with pCO_2, 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, N_2 fixation showed a significant increase with pCO_2 (*t*-test, $p < 0.05$, Fig. 2). C fixation was not significantly different between pCO_2 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 CO_2 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 N_2 fixation rates (Foster et al., 2011). While N_2 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 N_2 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 μmol ethylene (mg chl $a)^{-1}$ h^{-1}; Foster et al., 2010). In fact, N_2 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 $cell^{-1}$ 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 N_2 fixers are highly variable

As outlined above, the three investigated species responded very differently to elevated pCO_2. A review of literature data showed that this large diversity in response patterns holds also for other species of N_2 fixing cyanobacteria (Table 3). To identify characteristics that might explain these differences, we grouped N_2 fixers according to their mechanisms for N_2 fixation (Table 3). This comparison revealed a considerably large variability of pCO_2 responses within groups and even species (Table 3). Part of this can be attributed to species- or strain-specific differences in CO_2 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 pCO_2 (e.g. Garcia 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 CO_2 sensitivity.

Especially for heterocystous species, there is little data with often contrasting results, indicating stimulation in N_2 fixation for *Calothrix* and negative as well as positive responses towards elevated pCO_2 for *Nodularia* (Table 3). The diazocystous *Trichodesmium*, however, has been extensively studied and the data are more coherent, showing a positive response of N_2 fixation in most cases (Table 3). Also single-celled species responded positively to elevated pCO_2 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 N_2 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 pCO_2, it thus seems insufficient to consider only the functional diversity in modes of N_2 fixation. Looking at the present-day growth conditions of a species may also provide hints about its sensitivity towards ocean acidification.

3.3. CO_2 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 CO_2 supply on the three species investigated here, adaptation of their carbon concentrating mechanisms (CCM) to the respective habitats might thus cause different CO_2 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 pCO_2 levels exceeding 380 μatm by assuming that their mode of CCM and pH homeostasis has evolved to function optimally under low pCO_2/high pH. In fact, Czerny et al. (2009) hypothesized maximal growth rates even below a pCO_2 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 CO_2 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 pCO_2 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 pCO_2 responses.

3.4. Magnitudes of CO_2 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 pCO_2 responses of different N_2 fixing cyanobacteria. Arrows indicate direction of trend with increasing pCO_2; numbers in brackets indicate percent change relative to low pCO_2 level calculated from original data; significance was taken from the respective publications. For comparison reasons, only data for pCO_2 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}.

	Genus	Species/strain	pCO_2 [µatm]	Light intensity	Growth	POC content	PON content	POC:PON	N_2 fixation	O_2 evol. or C fix.	Reference
Single cells	*Crocosphaera*	*watsonii* WH8501	380 vs 750	80	↔	↔		↔	↑ (+40%)	↑ (+20%)[2]	(Fu et al., 2008)
			280 vs 750	120					↑ (+20%)		(Hutchins et al., 2013)
		watsonii WH0003	280 vs 750	120					↔		(Hutchins et al., 2013)
			190 vs 810	150	↑ (+60%)				↑ (+45%)	↑ (+60%)[2]	(Garcia et al., 2013a)
		watsonii WH0402	380 vs 750	100	↔				↔	↑ (+35%)[2]	(Garcia et al., 2013b)
		watsonii WH0401	380 vs 750	100	↔				↔	↑ (+17%)[2]	(Garcia et al., 2013b)
			280 vs 750	120					↑ (+35%)		(Hutchins et al., 2013)
	Cyanothece	*sp.* ATCC51142	380 vs 980	150	↔	↑ (+110%)	↑ (+100%)	↔	↔	↔[2]	(This study)
Diazocytes	*Trichodesmium*	*erythraeum* IMS101	380 vs 850	150	↔	↓ (−60%)	↓ (−60%)	↔	↔		(Barcelos é Ramos et al., 2007)
			380 vs 750	100				↔	↑ (+35%)	↑ (+40%)[2]	(Hutchins et al., 2007)
			400 vs 900	80–120	↑ (+50%)			↑ (+8%)	↑ (+140%)	↔[1]	(Levitan et al., 2007)
			370 vs 1000	150	↔	↑ (+30%)	↑ (+30%)	↔			(Kranz et al., 2009)
			150 vs 900	200	↑ (+11%)	↑ (+9%)	↑ (+20%)	↓ (−8%)	↑ (+110%)	↔[1]	(Kranz et al., 2010)
			380 vs 750	100	↑ (+30%)	↔	↔		↑ (+70%)	↑ (+60%)[2]	(Garcia et al., 2011)
			380 vs 750	90	↓ (−20%)			↔	↓ (−50%)	↔[2]	(Shi et al., 2012)[3]
		erythraeum GBRTRLI101	380 vs 750	100				↔	↑ (+45%)	↑ (+30%)[2]	(Hutchins et al., 2007)
			380 vs 750	220	↔				↔	↑ (+17%)[2]	(Garcia et al., 2011)
			370 vs 750	120					↑ (+50%)		(Hutchins et al., 2013)
		erythraeum KO4-20	280 vs 750	120					↑ (+35%)		(Hutchins et al., 2013)
		contortum 2174	280 vs 750	120					↑ (+30%)		(Hutchins et al., 2013)
		thiebautii H9-4	280 vs 750	120					↔		(Hutchins et al., 2013)
		thiebautii	380 vs 800	?				↔	↑ (+55%)	↔[2]	(Lomas et al., 2012)[4]
Heterocysts	*Nodularia*	*spumigena* IOW 2000/1	380 vs 800	85	↓ (−30%)	↑ (+20%)	↔	↑ (+17%)	↓ (−15%)		(Czerny et al., 2009)
			380 vs 980	150	↓ (−60%)	↔	↔	↑ (+5%)	↔		(This study)
		spumigena	~400 vs ~500	200	↑ (+40%)	↓	↓	↔	↑ (+35%)	↑ (+20%)[2]	(Wannicke et al., 2012)[5]
	Calothrix	*rhizosoleniae* SC01	380 vs 980	150	↔	↔	↔	↔	↑ (+60%)	↔[2]	(This study)

[1] O_2 evolution.
[2] C fixation.
[3] Low iron.
[4] Field incubations.
[5] Low phosphate.

can affect C acquisition and thus alter the overall CO_2 responses of phytoplankton. Studies on the underlying mechanisms of CO_2 responses suggested a reallocation of energy between the CCM and N_2 fixation in *Trichodesmium* (Kranz et al., 2009, 2010). This was confirmed by a strong modulation of CO_2 effects by light intensity (Kranz et al., 2010), suggesting that the degree of energy limitation plays an important role in controlling CO_2 responses in this species. A dependence of CO_2 effects on energy limitation has also been suggested for *Crocosphaera* in a study on combined effects of pCO_2 and phosphorus limitation, which linked the magnitude of pCO_2 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 *Crocosphaera* and *Cyanothece*, it can be assumed that the day-night separation of photosynthesis and N_2 fixation similarly imposes high energy demands for the turnover of enzymes and storage products. Thus, while single-celled species may respond positively to elevated pCO_2 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 *Crocosphaera* 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 CO_2 effects but also the relative sensitivity of C and N_2 fixation differed between *Trichodesmium* and single-celled species: In *Trichodesmium*, N_2 fixation was strongly increased in many of the studies (up to 140%; Table 3), while C fixation/O_2 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 N_2 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 N_2 fixation in the single-celled species was not as pronounced as in *Trichodesmium*. The physiology of C acquisition in *Crocosphaera* 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 CO_2 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 CO_2 supply rather than active uptake of HCO_3^-, elevated CO_2 concentrations will directly increase C influx with supposedly little effect on the cellular energy budget, and consequently also little feedback on N_2 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 CO_2 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 pCO_2 in our study. In line with this, the positive CO_2 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 CO_2 concentration outbalance negative pH effects in most, but not all N_2 fixers

For most of the N_2 fixers tested to date, a benefit from either the direct effect of CO_2 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 CO_2 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 CO_2 concentrations. In *Nodularia*, on the other hand, positive effects of high pCO_2 seemed to be attenuated, possibly due to adaptation to low pCO_2 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 pCO_2 responses of N_2 fixers is likely to affect community composition in future oceans (Hutchins et al., 2013). Since N_2 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 (Sohm et al., 2011). For instance, the increase in POC and PON production rates at high pCO_2 observed in *Cyanothece* would increase C and N input into the photic zone. The possible increase in N_2 fixation rates by single-celled species as well as *Calothrix* adds on to the increase in N_2 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 N_2 fixation would thus have profound impacts on the global C cycle, fuelling the biological pump and therewith CO_2 sequestration (Karl et al., 2012; Subramaniam et al., 2008).

In contrast to the stimulating effect of high pCO_2 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 CO_2 sensitivity could change community composition and therewith toxicity of future blooms, as *Nodularia* produces the hepatotoxin nodularin (Karlberg and Wulff, 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 N_2 fixers investigated in this study responded differently to elevated pCO_2, showing enhanced, decreased as well as unaltered growth and production rates. This confirms the large diversity of pCO_2 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 CO_2 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 CO_2 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 N_2 fixers, contrasting responses to rising pCO_2 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 pCO_2. Despite species-specific differences observed in the magnitude of responses, our study thus confirms the predictions of an increase in N_2 fixation 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 pCO_2 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 CO_2 concentrations directly, but may instead be more influenced by pH and/or their possible host species. The physiology and abundance of this group of cyanobacteria as well as the response of symbionts within the microenvironment of their host clearly need further investigation before CO_2-dependent alterations in the N cycle can be predicted.

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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_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_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_2 fixation constituting major energy sinks in N_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_2 and chlorophyll *a* fluorescence measurements indicated that photosynthetic and respiratory pathways were not altered under different pCO_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_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_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_2, down-regulation of the CCM allows for energy reallocation to N_2 fixation (*Publications I & II*, Fig. 5A).

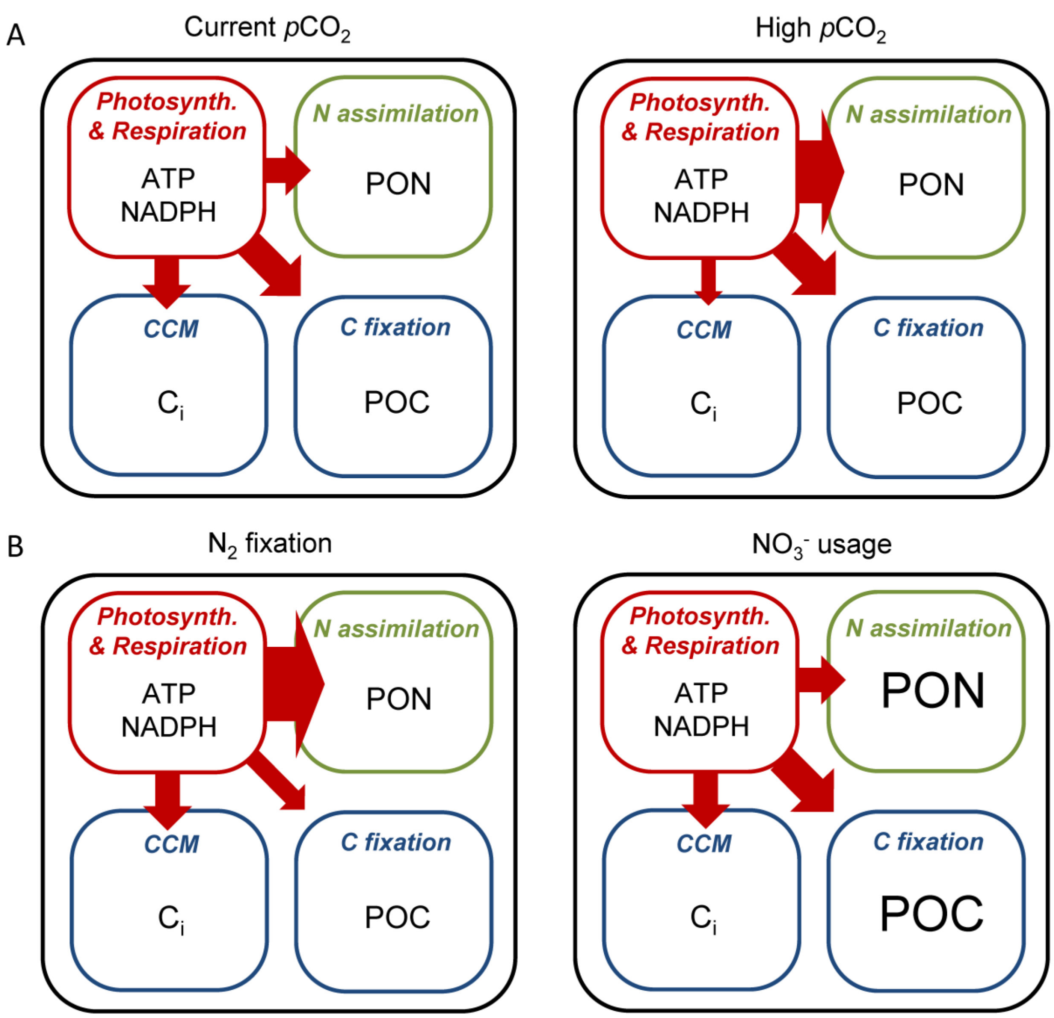

Fig. 5: Scheme of energy reallocation between the major cellular energy sinks under different *p*CO_2 levels and N sources according to results from *Publication II*. In all treatments, total energy supply by photosynthesis and respiration was unaltered. A) Under high *p*CO_2, the CCM is down-regulated, allowing for energy reallocation to N assimilation. B) Under N_2 fixation, a significant share of energy is needed for N assimilation, while in NO_3^- grown cells, lower operational costs of N assimilation and a subsequent reallocation of energy to POC assimilation allow for higher production rates of PON as well as POC.

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 CO_2 effects on the CCM were less pronounced in NO_3^- users than N_2 fixers (*Publication II*), it can be hypothesized that cells grown with NO_3^- could not benefit from a down-regulation in the CCM to the same extent as those grown on N_2 due to differences in energy requirements of the two N sources. CCM and N_2 fixation both have high ATP requirements, favoring energy reallocation between the two processes due to a compatible stoichiometry, while NO_3^- 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 pCO_2 with different N sources. Owing to the different stoichiometry in energy equivalents required by N_2 fixation and NO_3^- assimilation, energy can be readily reallocated from the CCM to N_2 fixation, while in NO_3^- 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 reconstellation 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 C_i 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 C_i source, since HCO_3^- uptake is mediated by ATP-dependent transporters, but also on the amount of C_i 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 ^{13}C–based approach relating fractionation to external C_i fluxes. These offsets could be explained by applying a model accounting for the effects of internal C_i fluxes on fractionation. According to this model, fractionation in the cytosol during C_i cycling, e.g. by unidirectional kinetic fractionation or by enzymatic fractionation by the NDH-1_4 complex, leads to a depletion of organic matter in ^{13}C (Fig. 7).

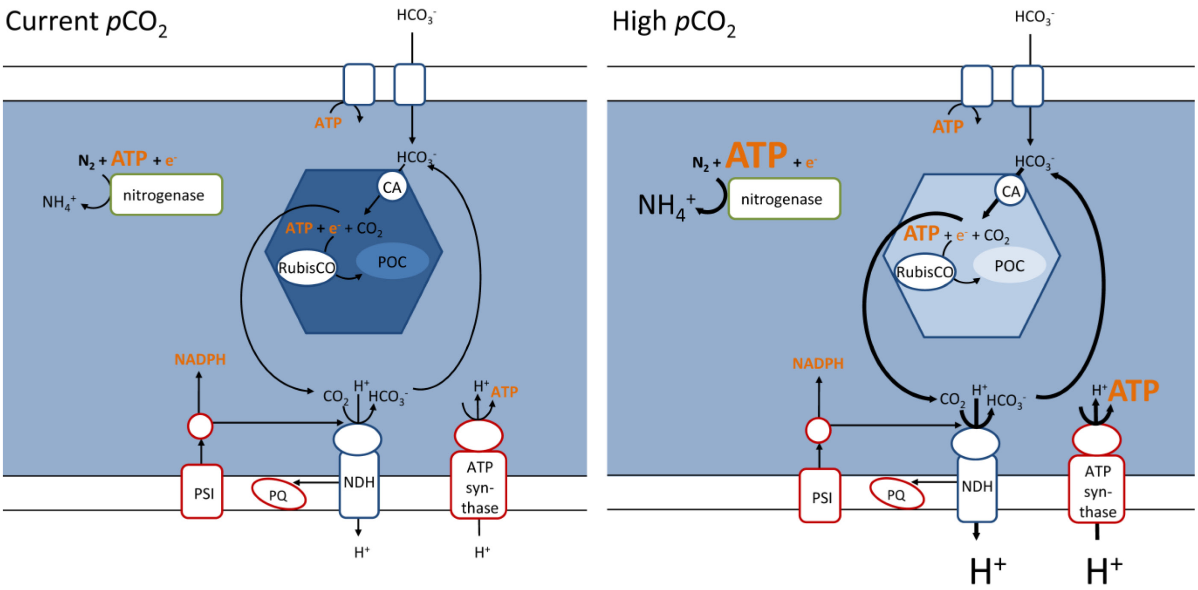

Fig. 7: Hypothetical effect of elevated pCO_2 on internal C_i fluxes, isotopic composition of C_i pools and energy availability. Different shades of blue denote isotopic composition of C pools, with darker colour indicating higher $\delta^{13}C$. At high pCO_2, internal cycling of C_i via the NDH complex 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_2 fixation rates at high pCO_2.

Results from *Publication III* highlight the importance of internal C_i cycling via the NDH complex in *Trichodesmium* with regard to several aspects: In addition to its role as a CO_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_4 complex to be affected by light intensities and pCO_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_2 conditions (Fig. 7). Accordingly, it has been suggested that higher NDH-1_4 activity may fuel the increased N_2 fixation rates observed at high pCO_2 (Kranz et al., 2010; Fig. 7). Although the molecular regulation mechanisms remain unclear, considering that NDH-1_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-1_4, 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-1_4) and furthermore lacks the CO_2 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 N_2 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 N_2 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 O_2 and CO_2 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 N_2 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 N_2 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.

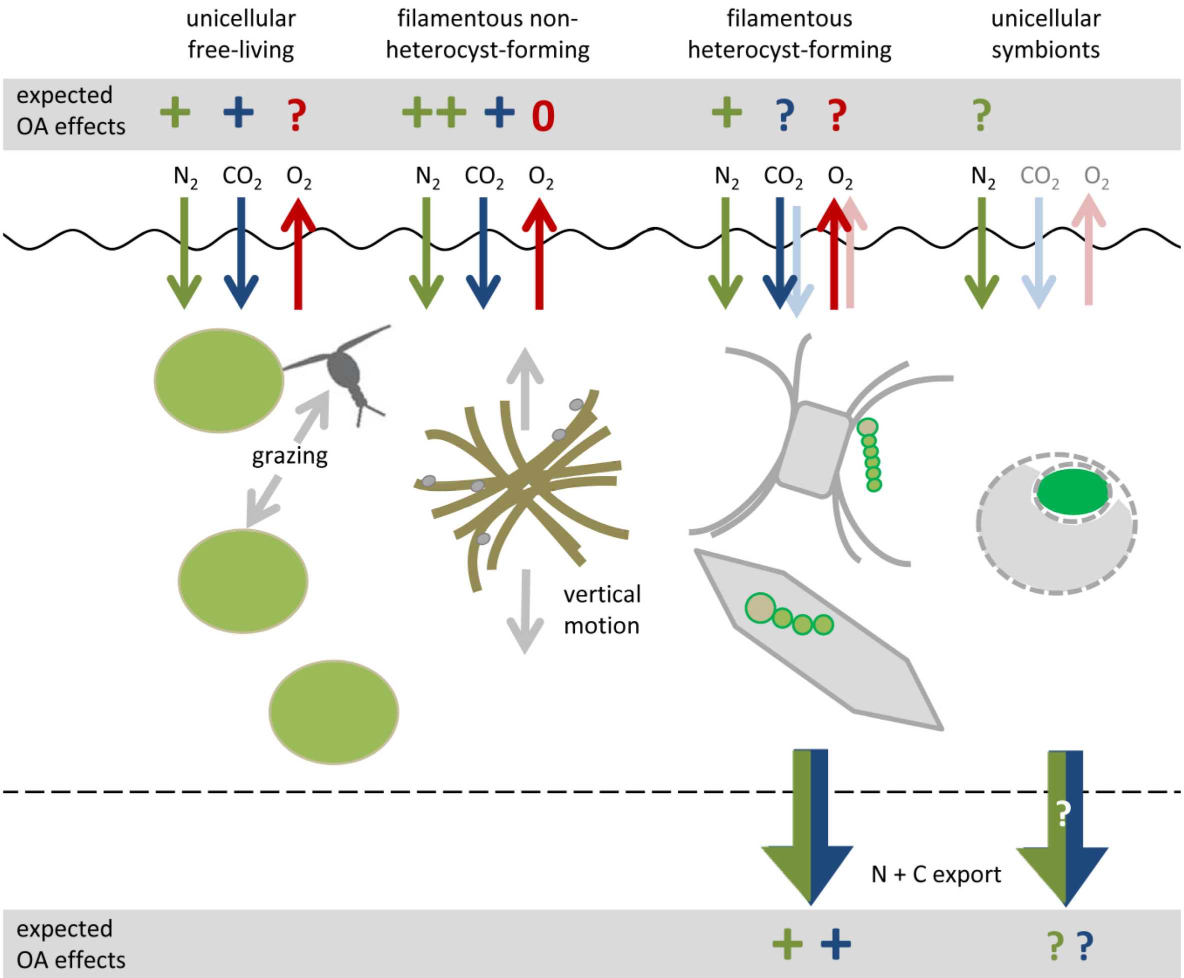

Fig. 8: Differential biogeochemical impacts of major groups of open ocean N_2 fixers as well as potential trends with ocean acidification (OA) in fluxes of N, C and O_2. Light-colored arrows for CO_2 and O_2 denote fluxes due to metabolic activity of the hosts. Please note that predictions of OA effects 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 conflictive 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 N_2 fixer productivity in the Baltic Sea may decrease. Trends in both regions are, however, subject to uncertainties due to possible modulation of CO_2 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 N_2 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 N_2 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 N_2 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 N_2 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 N_2 fixers have continued to change the picture of the marine N cycle. These include indications for N_2 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 N_2 fixation rates by the commonly used ^{15}N-based method (Mohr et al., 2010a), the authors recently reported on field data obtained with a revised method suggesting almost double N_2 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 N_2 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, O_2 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 N_2 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 N_2 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 C_i 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 pCO_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 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_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 *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_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 *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_2 and O_2 (Ploug et al., 2011) can be expected to have implications for multiple physiological processes in N_2 fixers. For instance, pH influences iron bioavailability (e.g. Shi et al., 2010), O_2 inhibits nitrogenase (e.g. Fay, 1992) and C_i availability and speciation determine the requirements for a CCM (e.g. Raven, 1997; Reinfelder, 2011). CO_2 draw-down in the diffusive boundary layer would have implications also for ^{13}C signatures and internal C_i 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 N_2 fixers.

4.5 Conclusion

This thesis focused on the underlying mechanisms of the responses of N_2 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 CO_2 in the vicinity of RubisCO (Fig. 4), N_2 fixation is subject to high loss rates and requires complex regulation mechanisms (Fig. 3). Accordingly, energy allocation between the CCM and N_2 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 C_i (*Publication III*) are major findings of this thesis, which improve our process-understanding for the abundant N_2 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 CO_2 concentrations, the rapid rate of current changes and the multiple interactions with their environment and 'younger' cohabitants make for challenging questions.

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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.