Dinitrogen fixation in the unicellular diazotroph *Crocosphaera watsonii*

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Contents

Summary

Crocosphaera watsonii is an abundant organism in the tropical and subtropical ocean and is considered to be an important contributor to the marine nitrogen cycle due to its ability to fix dinitrogen (N_2). Light-dark cycles were used to determine the diel variation in N_2 fixation and photosynthesis in the unicellular diazotrophic cyanobacterium *C. watsonii* WH8501. A first set of experiments showed that N_2 fixation and photosynthesis were separated temporally with N_2 fixation during the dark and photosynthesis during the light periods. Due to the storage of carbon reserves during the day and subsequent respiration at night, *C. watsonii* links its cellular nitrogen and carbon metabolism. N_2 fixation and photosynthesis appear to be regulated and optimized by a circadian rhythm. Gene expression analysis demonstrated cyclic patterns of the major genes involved in nitrogen and carbon metabolism. However, the patterns of expression did not coincide with patterns of activity with the gene expression peaking several hours prior to the activity. This diel periodicity should be considered in the interpretation of environmental samples given the abundance of *C. watsonii* in the ocean. Protein analysis suggested that *C. watsonii* may retain functionality of the photosystems in the dark. This hypothesis was tested and confirmed in a second set of experiments using light-dark cycles and continuous-light. However, during the respective dark period, *i.e.* an artificial light period instead of a dark period, photosynthesis was also accompanied by N_2 fixation though the magnitude was decreased. Single-cell analysis using nanoSIMS revealed that the co-occurrence of N_2 fixation and photosynthesis at population level was probably due to each individual cell's capability of coping with the O_2-sensitivity of the nitrogenase enzyme. In addition, single-cell rates showed large variability with the highest rate about six times higher than the mean rate during the normal dark N_2 fixation. If all cells were fixing nitrogen at the maximum single-cell rate, the overall N_2 fixation would be much higher. Single-cell variability could have originated from stochasticity in gene expression or protein synthesis. If stochasticity at the single-cell level is controlling the overall population N_2 fixation, then this could imply an upper limit of N_2 fixation regardless of nutrient sufficiency.

The experimental work with a pure culture of a diazotroph provided the opportunity to compare the $^{15}N_2$ stable isotope method with acetylene reduction, two methods commonly used both in field and laboratory studies. Within the course of this comparison, discrepancies

between the acetylene reduction assay (ARA) and the $^{15}N_2$ tracer addition were found. A re-assessment of the $^{15}N_2$-tracer addition revealed that the currently applied direct injection of $^{15}N_2$ gas may have significantly underestimated N_2 fixation rates both in culture and field experiments because the equilibration of the injected $^{15}N_2$ gas is much slower than previously assumed. After an 8 h incubation, about 50% of the calculated enrichment are reached leading to an under-estimation of at least 50% of the real N_2 fixation rate dependent on the diel periodicity of N_2 fixation in the incubation. Shorter incubations tend to be further away from the real N_2 fixation rate whereas 24 h incubation will be closer to the real rate, still under-estimating though. Due to the time-dependent, slow equilibration the calculated isotopic enrichment assuming rapid equilibration of the bubble cannot be taken as a constant value. I designed a modified $^{15}N_2$ method based on the addition of $^{15}N_2$-enriched water. The application of the modified approach has the potential to diminish the gap between sources and sinks of fixed nitrogen in the ocean, *i.e.* the ‘missing N’.

Zusammenfassung

Crocosphaera watsonii ist ein in den tropischen und subtropischen Ozeangebieten häufig vorkommender Organismus. Er wird als wichtige Komponente im marinen Stickstoffkreislauf erachtet, da er die Fähigkeit besitzt, molekularen Stickstoff zu fixieren. Licht-Dunkel-Zyklen wurden genutzt, um die Änderung von Stickstoffixierung und Photosynthese im Tagesgang in *C. watsonii* zu ermitteln. In einer ersten Reihe von Experimenten wurde gezeigt, dass Stickstoffixierung und Photosynthese zeitlich getrennt waren, wobei Stickstoffixierung nachts und Photosynthese tagsüber erfolgten. Mithilfe der tagsüber gespeicherten Kohlenstoffreserven und ihrer darauffolgenden Respiration bei Nacht konnte *C. watsonii* seine zellulären Stickstoff- und Kohlenstoffkreisläufe miteinander koppeln. Stickstoffixierung und Photosynthese werden scheinbar über einen zirkadianen Rhythmus reguliert und optimiert. Die Analyse von Genexpression zeigte zyklische Muster der wesentlichen Gene für den Stickstoff- und Kohlenstoff-Metabolismus. Dennoch stimmten die zeitlichen Muster von Genexpression und Aktivität nicht überein. Die Genexpression war zeitlich nach vorne verschoben mit einem Höhepunkt von mehreren Stunden Unterschied im Vergleich zur Aktivität. Dieser tägliche Zyklus sollte bei der Interpretation von Felddaten berücksichtigt werden, besonders geachtet der Häufigkeit dieses Organismus im Ozean. Protein-Analysen zeigten, dass die Photosysteme von *C. watsonii* eventuell ihre Funktionalität im Dunkeln bewahren. Diese Hypothese wurde in einer zweiten Reihe von Experimenten mit der Nutzung von durchgängigen Lichtphasen getestet und bestätigt. Dennoch wurde während einer dieser Lichtphasen, diejenige, die normalerweise dunkel gewesen wäre, auch eine gleichzeitige, aber reduzierte Stickstoffixierung gemessen. Einzelzellanalysen mittels nanoSIMS deckten auf, dass das gleichzeitige Vorkommen von Stickstoffixierung und Photosynthese auf der Fähigkeit jeder individuellen Zelle mit der O_2-Sensitivität der Nitrogenase umzugehen beruhte. Zusätzlich zeigten die Einzelzellraten eine große Variabilität, wobei die höchste Rate während der ‚normalen' Dunkelphase sechs Mal höher war als die mittlere Rate an Stickstoffixierung. Dies bedeutet, dass, wenn die Zellen ihre Aktivität steigern könnten, die Gesamt-Stickstoffixierung steigen könnte. Wenn diese Variabilität aber durch Zufälligkeit in zellulären Prozessen oder anderen internen Faktoren zustande kommt, könnte die Höhe der Stickstoffixierung unabhängig von Nährstoffverfügbarkeit dadurch kontrolliert sein.

Die experimentelle Arbeit mit einer stickstoffixierenden Reinkultur ermöglichte einen Vergleich der in Feld- und Laborexperimenten üblich angewandten Messmethoden zur Stickstoffixierung. Im Rahmen dieses Vergleiches wurden Unstimmigkeiten zwischen der Acetylen-Reduktions-Methode und der stabilen-Isotopen-Methode ($^{15}N_2$) beobachtet. Die Neubewertung der $^{15}N_2$-Methode machte deutlich, dass die bisher angewandte, direkte Injektion von $^{15}N_2$-Gas Stickstoffixierungsraten signifikant unterschätzt hat, da sich das Isotopengleichgewicht zwischen der

injizierten $^{15}N_2$-Blase und dem umgebenden Wasser sehr langsam einstellt. Nach etwa 8 Stunden Inkubation sind nur etwa 50% der berechneten Anreicherung mit $^{15}N_2$ erreicht. Dies führt zu einer Unterschätzung der Fixierungsrate von mindestens 50% abhängig vom Tagesrhythmus der fixierenden Organismen während der Inkubation. Kürzere Inkubationen tendieren dazu, vom tatsächlichen Wert weiter entfernt zu sein, während 24 Stunden-Inkubationen näher an den realen Raten sind. Aufgrund dieser langsamen Einstellung des Isotopengleichgewichts kann der berechnete Wert unter der Annahme eines schnellen Ausgleiches nicht als konstant angenommen werden. Ich entwarf eine modifizierte Anwendung der $^{15}N_2$-Methode basierend auf der Zugabe von $^{15}N_2$-angereichertem Wasser. Die Anwendung dieser modifizierten Methode hat das Potential, die Lücke zwischen den Quellen und Senken von gebundenen Stickstoff im Ozean, der ‚vermisste Stickstoff', zu reduzieren.

I. INTRODUCTION

I. Introduction

Dinitrogen (N_2) fixation is the reduction of N_2 gas to ammonia (NH_3). Non-biological reduction of N_2 occurs naturally by lightning or industrially by the Haber-Bosch-process. The latter is performed at very high temperature (400-500 °C) and pressure (200 bar). Biological N_2 fixation (hereafter called only N_2 fixation) is an enzyme-catalyzed process carried out by organisms called diazotrophs. Diazotrophy is found exclusively in prokaryotes containing the enzyme complex nitrogenase. The nitrogenase complex catalyzes the reduction of N_2 to ammonia (NH_3) at ambient conditions. However, the process is very energy-demanding due to the strong triple bond between the two nitrogen (N) atoms in the N_2 molecule. For each N_2 reduced, 16 ATP are consumed:

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

Nevertheless, N_2 fixation can be observed in various habitats in terrestrial and aquatic ecosystems. Diazotrophs occupy a distinct ecological niche due to the capability of using the virtually un-exploitable dissolved N_2 pool as compared to non-diazotrophic organisms, which largely depend on the availability of combined (or fixed) nitrogen compounds. Nitrogen is an essential element for all living organisms and is incorporated into cellular components, mainly proteins and nucleic acids. Diazotrophic organisms and the magnitude of N_2 fixation in the terrestrial environment are much better known than for the ocean. Estimates of N_2 fixation in the ocean approach those of N_2 fixation by the land biosphere but are less well constrained. The research carried out during this Ph.D. focussed on marine diazotrophic organisms and marine N_2 fixation. Thus, only the marine environment will be considered in the following sections.

A. N_2 fixation in the marine environment

Dissolved N_2 gas constitutes the largest pool of nitrogen (N) in the ocean with about 1×10^7 Tg N (Gruber 2008). Other nitrogen species in the ocean are nitrate (NO_3^-), nitrite (NO_2^-), nitrous oxide (N_2O), ammonium (NH_4^+) and organic nitrogen, ranging in oxidation states from +V in NO_3^- to –III in NH_4^+ and organic nitrogen compounds (Figure I-1). With the exception of N_2, all other nitrogen species are considered 'fixed N' in the oceanic N budget and sum up to about 6.6×10^5 Tg N (Gruber 2008). N_2 fixation is the major source of fixed N in the ocean. Other sources of fixed N to the (open) ocean are atmospheric dry and wet deposition whereas in coastal areas riverine input may also contribute a substantial fraction.

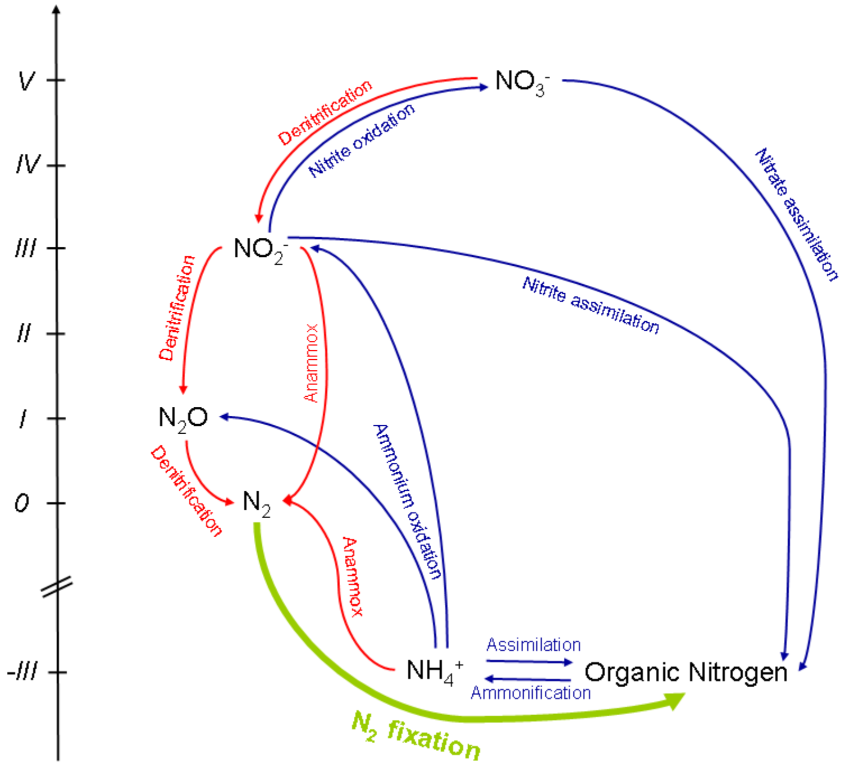

Fig. I-1: Overview of oxidation states of the different nitrogen species in the ocean and the processes of their production and consumption. Red arrows represent processes under anoxic conditions. The green arrow indicates the focus of this thesis, *i.e.* N_2 fixation. Figure redrawn from: Gruber (2008).

The main losses of fixed N from the ocean are through benthic and water column (pelagic) denitrification, here per definition, all processes that convert fixed nitrogen into N_2 gas. Minor sinks are the burial of organic matter in sediments as well as the loss of nitrous oxide (N_2O) to the atmosphere. Although the fixed N pool in the ocean is much smaller than the N_2 pool, it plays a pivotal role in the biogeochemical cycle of, for example, carbon, due to the coupling of carbon and nitrogen during primary production. Fixed nitrogen compounds are considered to be the (proximate) limiting nutrient for primary production in the euphotic zone of the open ocean (Tyrrell 1999) with the exception of high-nutrient-low-chlorophyll (HNLC) regions (*i.e.*, the subpolar North Pacific, the equatorial Pacific and the Southern

Ocean). There, dissolved iron (Fe) has been suggested to be the limiting (micro-) nutrient, and evidence for this has been obtained during iron-fertilisation experiments (Boyd *et al.* 2000).

Since both the major source and the major sink for fixed N in the ocean are coupled to biological processes, it will be mainly the balance or misbalance between these two processes that is likely to have influence on the extent of 'new' primary production and the air-sea exchange of atmospheric CO_2 (Karl *et al.* 2002). 'New' primary production is referred to as the primary production that is fuelled by nutrients that are physically transported into the euphotic zone as well as external sources, *i.e.* N_2 fixation and atmospheric deposition. Since the amount of new primary production by nutrients that are physically brought into the euphotic zone is balanced by the export of organic matter on large spatial and temporal scales, N_2 fixation and atmospheric deposition are the potential turning knobs for an increase or decrease in new primary production and hence CO_2 sequestration (Duce *et al.* 2008; Figure I-2). The increased atmospheric deposition of N compounds is likely to affect the surface ocean productivity since recent estimates of atmospheric deposition are around 67 Tg N a^{-1} and are expected to increase to about 77 Tg N a^{-1} in the next two decades (Duce *et al.* 2008).

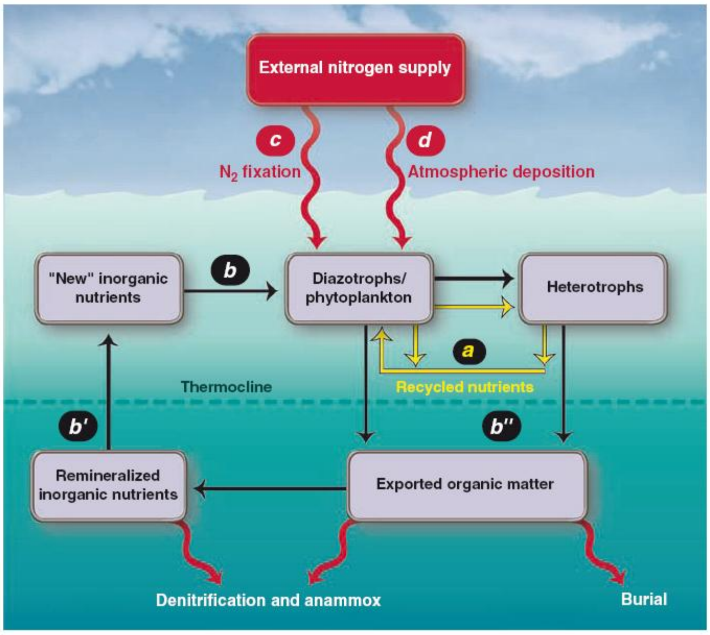

Figure I-2: Illustration of processes controlling surface ocean primary production and the major sinks and sources of fixed N (red arrows). The majority of total primary production is supported by remineralisation of nutrients within the euphotic zone, *i.e.* 'regenerated' production (*a*). 'New' production is maintained by physically transported nutrients into the surface ocean (*b*) and external sources of fixed N, *i.e.* N_2 fixation (*c*) and atmospheric deposition (*d*). At large spatial and temporal scale, the export production (*b''*) is balanced by the physical transport of remineralised nutrients from the deep ocean (*b'*). Source: Duce *et al.* (2008).

Current estimates of marine N_2 fixation are in the order of 100 – 200 Tg N a^{-1} (Karl *et al.* 2002, Gruber 2008) yet exceeding the atmospheric deposition. On a global scale, all sources of fixed N sum up to ~ 250 Tg N a^{-1} (Table I-1). Total losses of fixed N seem to exceed the sources with considerable debate about the magnitude, specifically denitrification (Codispoti *et al.* 2001; Galloway *et al.* 2004, Gruber 2004). The discrepancy between the total sources and sinks of fixed N in the ocean is also referred to as the 'missing N'.

Table I-1: Recent global estimates of nitrogen sinks and sources in the marine environment. Source: Gruber (2008).

Process	**Codispoti *et al.* (2001)**	**Galloway *et al.* (2004)**	**Gruber (2004)**
		Sources (Tg N a^{-1})	
Pelagic N_2 fixation	117	106	120
Benthic N_2 fixation	15	15	15
River input	76^a	48^a	80^a
Atmospheric deposition	86	33	50
Total sources	**294**	**202**	**265**
		Sinks (Tg N a^{-1})	
Organic N export	1		1
Benthic denitrification	300	206	180
Pelagic denitrification	150	116	65
Sediment burial	25	16	25
N_2O loss to atmosphere	6	4	4
Total sinks	**482**	**342**	**275**

[a] Sum of DON and PON

Although, it is generally accepted that the availability of nitrogen limits primary production, there is less agreement on the nutrient(s) that limits N_2 fixation, with experimental evidence for both the availability of phosphorus (P) (Sañudo-Wilhelmy *et al.* 2001) and iron (Fe) (Shi *et al.* 2007, Fu *et al.* 2008). The degree to which either one or both elements limits N_2 fixation may vary between ocean basins with the Atlantic Ocean being more phosphorus-limited and the Pacific Ocean being more iron-limited (Wu *et al.* 2000) although it has been shown that N_2 fixation in the Atlantic can be phosphorus and iron co-limited (Mills *et al.*

2004). The divergence between the Atlantic and Pacific oceans is attributed to the differing inputs of iron through mineral dust deposition with the Atlantic receiving higher deposition than the Pacific (Jickells *et al.* 2005; Figure I-3). However, theoretical arguments suggest that N_2 fixation by *Trichodesmium* could be limited by iron in 75% of the world's oceans (Berman-Frank *et al.* 2001*a*). Iron limitation is likely to result from the high Fe requirements of diazotrophs, especially when the prevailing diazotrophs are also photosynthetic organisms since both the nitrogenase as well as the photosynthetic apparatus requires iron.

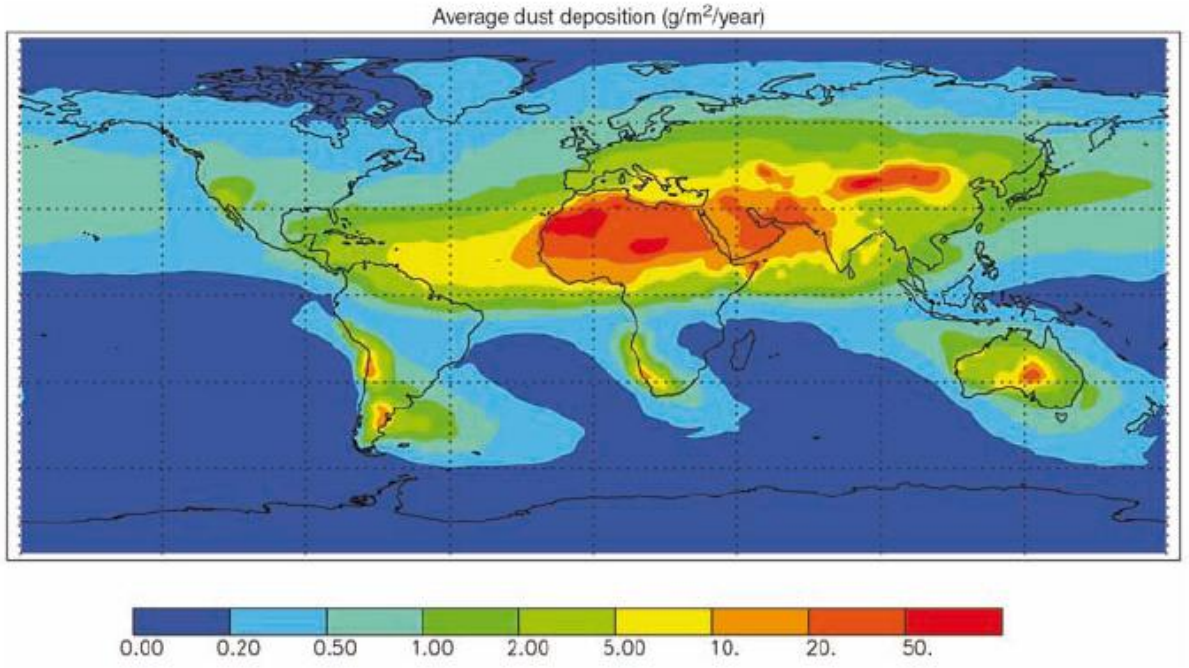

Figure I-3: Global dust deposition map. Source: Jickells *et al.* 2005.

Besides phosphorus and iron as limiting nutrients, the distribution of diazotrophs in the ocean appears to be a result of various other factors. Langlois *et al.* (2008) observed different temperature regimes for *Trichodesmium* and group A cyanobacteria (UCYN-A) in the Atlantic Ocean. The distribution and growth of the unicellular diazotroph *Crocosphaera watsonii* in the western South Pacific has also been attributed to temperature (Webb *et al.* 2009, Moisander *et al.* 2010). Diazotrophic cyanobacteria often comprise the majority of the diazotroph community (Church *et al.* 2005*a*, 2008; Langlois *et al.* 2008). For these diazotrophs, molecular oxygen (O_2) plays a special role since cyanobacteria are oxygenic phototrophs, *i.e.* they fix carbon dioxide (CO_2) and concomitantly evolve O_2. However, the N_2-fixing enzyme nitrogenase is inactivated upon contact with O_2 (Fay 1992, Gallon 1992) posing a serious dilemma for diazotrophic cyanobacteria. In addition to O_2 evolution by photosynthesis, most of the oceanic waters are well oxygenated with the exception of the oxygen minimum zones (*e.g.*, the upwelling areas off South America and Africa as well as the Arabian Sea). The O_2-production by (diazotrophic) cyanobacteria and the presence of dissolved O_2 in most of the ocean led to the evolution of several adaptations to avoid the contact of nitrogenase with O_2 since the nitrogenase existed well before the oxygenation of the atmosphere (Broda and Pescheck 1983). The different adaptations can generally be divided into two main strategies, spatial and temporal separation of photosynthesis and N_2 fixation. A representative example for the former is the development of cyanobacterial heterocysts, specialized cells that harbour the nitrogenase complex but lack the O_2-evolving photosystem II (PSII). In addition, these cells have a thick cell wall impeding diffusion of O_2 into the cell. Due to the lack of PSII, these cells are also incapable of fixing CO_2 and thus depend on the transfer of fixed carbon compounds from adjacent vegetative cells. The occurrence of heterocysts is hence limited to filamentous cyanobacteria. Heterocystous diazotrophs are usually constrained to fresh and brackish waters but have been observed in symbiosis with marine diatoms (Carpenter *et al.* 1999). Temporal separation of photosynthesis and N_2 fixation is largely carried out by unicellular, diazotrophic cyanobacteria. These diazotrophs photosynthesise during the light and fix N_2 during the dark (Sherman *et al.* 1998). While photosynthesising, the unicellular diazotrophs accumulate fixed carbon visible as glycogen granules (Schneegurt *et al.* 1997). The accumulated carbon is then respired at night to serve two purposes: to support the high energy requirements for N_2 fixation and the consumption of otherwise destructive O_2. This strategy allows unicellular diazotrophs to link the metabolism of carbon and nitrogen within one cell. A representative of the unicellular diazotrophic cyanobacteria, *Crocosphaera watsonii* WH8501, will be treated separately and in more detail in section B of this introduction. A combination of temporal and spatial separation is implemented in one of the most studied and well known diazotrophic cyanobacteria, *Trichodesmium* sp. *Trichodesmium* sp. is a non-heterocystous, filamentous organism that is very abundant in the tropical and subtropical regions of the ocean (Mulholland and Capone 1999; Tyrrell *et al.* 2003; Carpenter *et al.* 2004; Capone *et al.* 2005). It performs both photosynthesis and N_2 fixation during the day. The N_2 fixation peak occurs around midday and is accompanied by a decline in photosynthesis which consequently reduces the intracellular O_2 content to allow for N_2 fixation (Berman-Frank *et al.* 2001*b*). Additional processes such as respiration and the Mehler reaction are supposed to further decrease O_2 concentrations. *Trichodesmium* sp. does not fix N_2 at night suggesting a tight coupling of carbon and nitrogen metabolism. Indeed, it has been shown that CO_2 and N_2

fixation are tightly regulated by a so-called circadian rhythm (Chen *et al.* 1998). Circadian (daily) rhythms are internal programs that regulate and optimize processes to correspond to environmental cycles such as a light-dark cycles enhancing the fitness of the population (Mori and Johnson 2001). A set of proteins has been found to be responsible for the regulation, the KaiABC proteins which are encoded by the *kaiABC* genes. Mori and Johnson (2001) proposed the 'oscilloid' model with KaiC as a key component. In this model, the condensation/supercoiling of the bacterial chromosome oscillates and thereby globally regulates gene expression. Circadian regulation has not only been shown for *Trichodesmium* but also for unicellular diazotrophic cyanobacteria such as *Cyanothece* sp. ATCC 51142 (Colón-López *et al.* 1997, Toepel *et al.* 2008), *Synechococcus* sp. RF-1 (Huang *et al.* 1990) and *Gloeothece* sp. 68DGA (Taniuchi and Ohki 2007). The majority of the diazotrophic cyanobacteria including filamentous and unicellular types appears to optimize carbon and N_2 fixation under a circadian rhythm and thus links carbon and nitrogen metabolism.

B. *Crocosphaera watsonii*, a unicellular diazotrophic cyanobacterium

Crocosphaera watsonii is a unicellular, diazotrophic cyanobacterium and the reference strain WH8501 (Figure I-4) was isolated in 1984 in the (sub-) tropical South Atlantic Ocean (Rippka *et al.* 2001). It belongs to the group B of unicellular, diazotrophic cyanobacteria (Zehr *et al.* 2001). This strain is an obligate autotroph (*i.e.* it relies on CO_2 fixation as the sole carbon source), is between 2.5 – 4 µm in size and contains phycoerythrin in its light-harvesting complex (Rippka *et al.* 2001). Growth temperatures have a narrow range from 26 to 32 °C in *C. watsonii* WH8501 (Webb *et al.* 2009). Although this strain has been in culture for more than 25 years (Waterbury *et al.* 1988) and was known to be a diazotroph since isolation, only during the last decade studies started to investigate the ecophysiology and importance of this diazotrophic organism in more detail. The increased interest in *C. watsonii* results from the discovery of the importance of unicellular diazotrophs in the marine nitrogen cycle (Zehr *et al.* 2001, Montoya *et al.* 2004). As a unicellular diazotrophic cyanobacterium, *C. watsonii* fixes N_2 in the dark period of a light-dark cycle (Rippka *et al.* 2001, Tuit *et al.* 2004) to protect the nitrogenase complex from photosynthetically-evolved O_2 (Gallon 1992). Nevertheless it links carbon and nitrogen metabolism through the accumulation of carbon reserves during the day and subsequent respiration at night as described above. With respect to the above discussed nutrient limitations of N_2 fixation, *C. watsonii* seems to be quite well adapted and competitive in the oligotrophic open ocean environment. Under iron-stress, *C. watsonii* expresses an *idiA* gene homologue (Webb *et al.* 2001). The IdiA protein is thought to mainly protect photosystem II from oxidative damage (Exss-Sonne 2000). Photosystem I may also be protected against oxidative damage although by another protein, IsiA. Transcripts of the *isiA* gene have been found to be a dominant component in the metatranscriptome of a *C. watsonii* bloom in the South Pacific (Hewson *et al.* 2009). In this *C. watsonii* bloom, *isiA* gene transcripts also showed a diel variation with higher transcripts during the day which is consistent with recent transcriptomic data from pure culture experiments with *C. watsonii* WH8501 although cultures were grown Fe-replete (Shi *et al.* 2010). Thus, the expression of the *isiA* gene may have also resulted from oxidative stress (Shi *et al.* 2010) rather than iron-induced (oxidative) stress. Possibly, iron stress and oxidative stress may be interrelated. However, *C. watsonii* also appears to suffer from iron limitation as N_2 fixation and growth rates decline in Fe-deplete cultures (Fu *et al.* 2008).

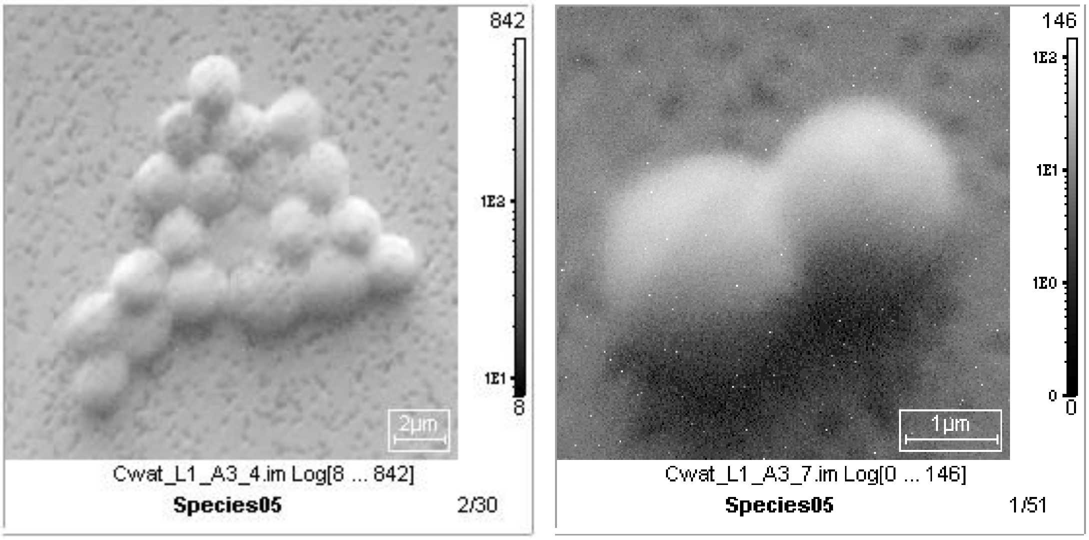

Figure I-4: Electron micrographs of *Crocosphaera watsonii* WH8501 obtained during nanometer-scale secondary ion mass spectrometry (nanoSIMS). The ‘clumping’ of cells in the left image was an artefact of preservation/filtration.

With regard to phosphorus, *C. watsonii* is distinct in comparison to other cyanobacteria. It is not capable of using phosphonates due to the lack of the *phn* gene cluster encoding for a C-P lyase (Dyhrman and Haley 2006). Phosphonates are organic phosphorus compounds with a carbon-phosphorus bond which can be utilized by *Trichodesmium* (Dyhrman *et al.* 2006) and possibly by *Cyanothece* sp., a unicellular cyanobacterium. On the other hand, *C. watsonii* may accommodate high- and low-affinity phosphate uptake systems whereas the latter has not been observed in other cyanobacteria (Dyhrman and Haley 2006). The presence of a low-affinity uptake system may render *C. watsonii* competitive in the environment (Dyhrman and Haley 2006). Some of the more recent studies on *C. watsonii* are based on genomic information. Sequencing of the *C. watsonii* WH8501 genome has been completed in 2004 and the publicly available data have been beneficial to the study of this diazotroph in both culture (Shi *et al.* 2010) and field (Hewson *et al.* 2009).

Since the initial work identified the importance of unicellular diazotrophs for marine N_2 fixation (Zehr *et al.* 2001, Montoya *et al.* 2004), a whole range of studies have quantified the abundance of these diazotrophs in the environment. *C. watsonii* (or *C. watsonii*-like cells or group B cells) have been detected in regions of the Atlantic Ocean (Falcón *et al.* 2004, Langlois *et al.* 2008, Foster *et al.* 2009) and Pacific Ocean (Falcón *et al.* 2004, Montoya *et al.* 2004, Church *et al.* 2005*a*, Zehr *et al.* 2007, Church *et al.* 2008) as well as in the Arabian Sea (Mazard *et al.* 2004). Abundances are in the range of about 1.7 x 10^3 cells L^{-1} (Church *et al.* 2005*a*) up to about 4 – 5 x 10^5 cells L^{-1} (Falcón *et al.* 2004, Zehr *et al.* 2007). However, abundances of about one order of magnitude higher (4 – 8 x 10^6 cells L^{-1}) have recently been observed in the South Pacific at around 40 m water depth (Hewson *et al.* 2009, Moisander *et al.* 2010). Attempts to quantify N_2 fixation by *C. watsonii* have until now been restricted to the isolation and culturing of environmental strains and the subsequent calculation of potential contribution to total N_2 fixation by either direct measurements of N_2 fixation (Tuit *et al.* 2004, Fu *et al.* 2008, Compaoré and Stal 2010, Webb *et al.* 2009) or modelling using the observed culture growth rates and cellular carbon to nitrogen (C:N) requirements (Goebel *et al.* 2007). Several isolated strains of *C. watsonii* (including strain WH8501) isolated from the North and South Atlantic as well as the North Pacific displayed N_2 fixation rates that varied 5-fold on a per-cell basis but were very similar when calculated to cellular volume (Webb *et al.* 2009). These strains also occupied two different size classes, one in the smaller range (2 – 4 µm diameter) and one in the larger range (5 – 6 µm diameter) with strains in the larger size class also excreting extracellular material sometimes resulting in an apparent buoyancy effect. In addition, the various strains exhibited different temperature ranges explaining the widespread distribution of *C. watsonii* in the ocean (Webb *et al.* 2009). This study shows that there is still considerable need for isolation and cultivation of new strains of marine diazotrophs and their subsequent physiological characterization. A very beneficial contribution to the assessment of N_2 fixation will be the application of nanometer-scale secondary ion mass spectrometry (nanoSIMS) to environmental samples which would yield a culture-independent direct measurement of N_2 fixation. This issue is the subject of manuscript C and will also be included in the discussion at the end of this thesis.

C. Measurements of N_2 fixation

Presently, the determination of N_2 fixation in the marine environment is achieved by a range of different approaches. They encompass the molecular assessment of diazotrophs, the indirect evaluation using geochemical estimates and direct measurements of N_2 fixation. As described above, nitrogenase is the enzyme responsible for the reduction of N_2 to bio-available NH_3. The iron subunit of the nitrogenase complex is encoded by the *nifH* gene which can be directly measured in filtered water samples using quantitative polymerase chain reaction (qPCR) after the extraction of total DNA from the filter. This measurement provides estimates of the abundance of different diazotrophic groups known today depending on the specific probes that are used. However, the abundance estimates rely on the assumption that the *nifH* gene is a single copy gene in each diazotroph species and that a single copy of the genome is present in each cell. For several marine diazotrophs, a single copy of the *nifH* gene per genome is true but there is uncertainty in the genome number present in a cell. Sequence analysis of published genomes suggests that *Trichodesmium*, *Cyanothece* and *Crocosphaera* contain only one *nifH* gene copy per genome whereas *Anabaena* and *Nostoc*, heterocystous cyanobacteria observed in fresh and brackish waters, appear to contain two or more *nifH* gene copies per genome. The estimates from qPCR are upper estimates, and therefore, the results are often presented as *nifH* gene copies per liter of seawater. However, the determination of *nifH* gene copies in a sample of seawater is a useful tool to describe the diazotrophic community and is a qualitative assessment of (potential) N_2 fixation. Molecular tools can also be used to yield a qualitative assessment of N_2 fixation activity through the determination of *nifH* gene transcripts with qPCR after the reverse transcription of RNA to cDNA. Gene transcripts can be detected in cells or a sample if a gene is actively transcribed indicating the usage of the encoded protein (Zehr and Montoya 2007). The determination of gene transcripts is thus a qualitative assessment of N_2 fixation activity because the presence of gene transcripts does not provide any quantitative information on N_2 fixation. Nevertheless, it is useful to determine which groups of diazotrophs are active in the entire community and supposedly contributing to N_2 fixation because the abundance of diazotrophs alone (*i.e.* abundance determined from DNA or microscopic counts) does not yield any information on the activity.

That abundance does not necessarily reflect activity was recently demonstrated by the measurement of $^{15}NH_4^+$ uptake using nanoSIMS combined with *in situ* hybridization where the least abundant organism (~0.3%) accounted for more than 40% of the total uptake (Musat *et al.* 2008).

The quantitative assessments of N_2 fixation can be sub-divided into indirect estimates and direct measurements. Indirect estimates of N_2 fixation have been based on geochemical estimates and modelling approaches. The latter involves abundances of different diazotrophic groups (assessed, for example, by molecular tools or direct microscopic counts), observed growth rates for these diazotrophs (usually representative cultured organisms) and their cellular C:N requirements. N_2 fixation rates (on a per cell basis) are then derived from the assumption of sustained cellular C:N ratios under certain growth rates. Regional or global estimates of N_2 fixation are then obtained by considering the cellular N_2 fixation rates and the abundances of the observed diazotrophs (Goebel *et al.* 2007). Cellular C:N ratios and elemental uptake ratios (C:N and N:P) are also of great importance for some geochemical estimates. Particulate material that is formed during primary production has an average molar C:N:P ratio of 106:16:1 (Redfield 1958, Klausmeier *et al.* 2004) and hence the uptake of the main nutrients, *i.e.* nitrate, the major constituent of fixed N, and phosphate must be close to 16:1. Surface ocean nitrate and phosphate concentrations are on average close to this ratio (Figure I-5, Gruber 2008). Nevertheless, deviations in either one of the two directions (*i.e.* lower or higher than 16:1) are attributed to the processes denitrification and nitrogen fixation. Both processes change the fixed N budget (see section A and Table I-2) by releasing or fixing N_2 but do not alter the overall phosphate quota. To quantify the importance of denitrification and N_2 fixation, the term N* was introduced with

$$N^* = [NO_3^-] - 16 \times [PO_4^{3-}] + 2.9\ \mu mol/kg \qquad \text{(Gruber 2008)}$$

where the constant 2.9 µmol/kg was added to obtain a global mean N* of zero (Gruber and Sarmiento 1997). Resulting positive and negative N* values denote areas of increased or reduced fixed N, respectively, relative to phosphate. Therefore, the balance between the main sink and the main source of fixed N, *i.e.* denitrification and N_2 fixation is reflected in the N* values. Global distributions of N* have suggested that the Atlantic Ocean serves as a net source of fixed N whereas the Pacific Ocean serves as a net sink of fixed N (Gruber 2008) despite the fact that higher rates of N_2 fixation have been proposed for the Pacific Ocean using a similar modelling approach of N:P stoichiometry (Deutsch *et al.* 2007).

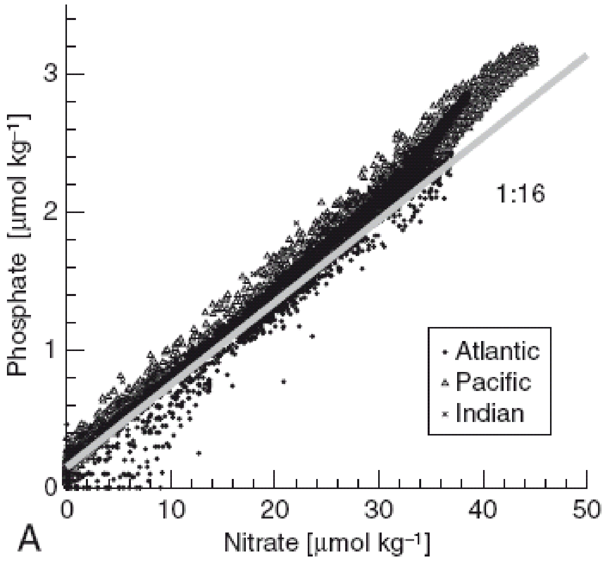

Figure I-5: Distributions of phosphate and nitrate concentrations from all depth and selected cruises of the World Ocean Circulation Experiment. The line indicates the canonical Redfield ratio. Source: Gruber (2008).

Table I-2: Oceanic budget for the main fixed nitrogen species. Source: Gruber (2008).

Nitrogen species	**Oceanic budget [Tg N]**
Nitrate (NO_3^-)	5.8 x 10^5
Nitrite (NO_2^-)	160
Ammonium (NH_4^+)	340
Dissolved organic N (DON)	7.7 x 10^4
Particulate organic N (PON)	400
Nitrous oxide (N_2O)	750
Total fixed N	**6.6 x 10^5**
Dissolved N_2 gas	**1 x 10^7**

Other geochemical estimates of N_2 fixation are based on the natural abundance of the stable nitrogen isotope ^{15}N. In the atmosphere, ^{15}N constitutes about 0.366% of all nitrogen atoms (*i.e.* atom% = ^{15}N / (^{15}N + ^{14}N)). For convenience, the isotope ratio of a sample is usually compared to the atmospheric isotope ratio using the δ notation with

$$\delta^{15}N\ [‰] = (IR_{SAMPLE} / IR_{ATM} - 1) \times 1000$$

where IR is the isotope ratio of either the sample or the atmosphere. Most biological processes discriminate against the heavier ^{15}N isotope resulting in an increase of the $\delta^{15}N$ of the substrate used. The discrimination for or against the ^{15}N isotope is generally referred to as fractionation. One of the main processes in the marine environment leading to increased $\delta^{15}N$ of the oceanic nitrate pool is (pelagic) canonical denitrification, *i.e.* nitrate serves as the terminal electron acceptor in respiration converting nitrate into N_2. In contrast, N_2 fixation has very little fractionation (Figure I-6). Since it is these two processes that are the main sink and source of oceanic fixed N and hence can alter the nitrogen mass balance in the ocean but have very different isotopic signatures, denitrification and N_2 fixation (as well as other processes) have been tracked using the isotopic signature of the oceanic fixed N (nitrate) pool.

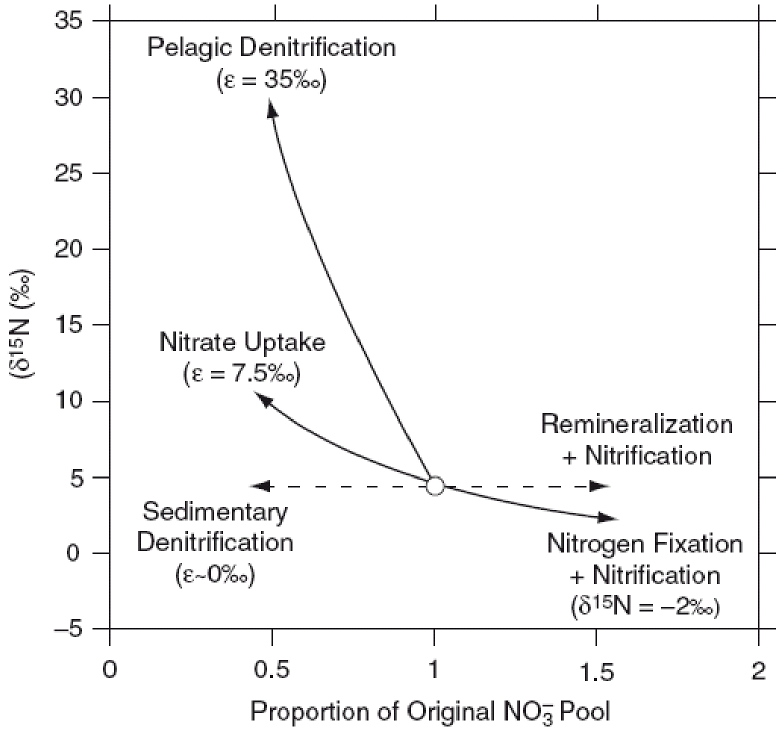

Figure I-6: Isotopic fractionation during major processes of the marine nitrogen cycle and their impact on the $\delta^{15}N$ of oceanic nitrate. Source: Montoya (2008).

The average $\delta^{15}N$ of nitrate is ~ 5‰ as determined in deep-ocean nitrate (Sigman *et al.* 2000). Although the assimilation of nitrate discriminates against ^{15}N leading to higher $\delta^{15}N$ in the residual nitrate and lower $\delta^{15}N$ in the particulate material formed, if all nitrate present is consumed, the $\delta^{15}N$ of the particulate material will reflect the $\delta^{15}N$ of the nitrogen source used. Particulate material with a $\delta^{15}N$ lower than average nitrate is assumed to have (at least partially) originated from diazotrophs since N_2 fixation has very little fractionation and leads to lower $\delta^{15}N$ values. The relationships between $\delta^{15}N$ values, fractionation and oceanic mass balances have been used to assess N_2 fixation (Montoya *et al.* 2002, Bourbonnais *et al.* 2009) and the oceanic fixed N budget (Brandes and Devol 2002).

Direct measurements of N_2 fixation are mostly carried out using the acetylene reduction assay (ARA; Capone 1993, Capone and Montoya 2001) and the $^{15}N_2$ tracer addition (Montoya *et al.* 1996, Capone and Montoya 2001). Mass balances are usually restricted to diazotroph pure cultures or on larger scales as isotope mass balances (Brandes and Devol 2002). The ARA has been developed in the 1960's and has since been widely used to measure N_2 fixation in laboratory and field studies. This method is based on the ability of nitrogenase to not only reduce N_2 but also other small triple-bonded molecules such as cyanide, azide, nitrous oxide and acetylene (C_2H_2). If added at saturating concentrations, C_2H_2 inhibits the reduction of N_2 but is itself reduced to ethylene (C_2H_4). Briefly, C_2H_2 is added to the headspace (>0.1 atm) of a closed vial containing seawater or culture (ratio of gas to liquid of 1:1 (Capone and Montoya 2001)) and is incubated for the desired time. The produced ethylene is afterwards measured in a gas chromatograph equipped with a flame ionization detector and the amount of C_2H_4 produced can be related to N_2 fixation with

$$N_2 \text{ fixed } [\text{mol } N_2] = C_2H_4 \text{ produced } / \text{ CF } [\text{mol } C_2H_4]$$

where CF = conversion factor (dimensionless). The reduction of N_2 to 2 NH_3 requires one pair of electrons for each bond in the N_2 molecule leading to a total of 6 electrons needed. The reduction of C_2H_2 to C_2H_4 requires 2 electrons which would lead to a conversion factor of 3, *i.e.* 3 mol of C_2H_2 are reduced instead of 1 mol N_2. But since N_2 fixation is accompanied by the formation of hydrogen (H_2) which requires another 2 electrons, a total of 8 electrons (and a conversion factor of 4) is needed for the reduction of N_2 to NH_3. There has been considerable debate (Capone 1993, Montoya *et al.* 1996, Zehr and Montoya 2007) about the use of 3 or 4 as a conversion factor and both are still applied (Czerny *et al.* 2009, Hamisi *et al.* 2009).

The application of $^{15}N_2$ gas to measure N_2 fixation was first applied in the 1940's (Burris and Miller 1941, Burris 1942). Measurements were mostly constrained to pure cultures or enriched samples due to the biomass needed for the mass spectrometric analysis of the particulate material. After the sensitivity of mass spectrometers had been improved, the $^{15}N_2$ tracer addition became a standard method for measuring N_2 fixation in (often) oligotrophic field samples (Montoya *et al.* 1996). Briefly, seawater incubations are started by the direct injection of $^{15}N_2$ gas to headspace-free filled bottles to yield an enrichment at around 2-5 atom% of the dissolved N_2 pool and samples are incubated for about 2-36 h (Zehr and Montoya 2007). At the end of the incubation, the particulate material is filtered onto a glass fibre filter (0.7 µm GF/F filter) and the isotopic composition of the particulate material is measured mostly using continuous flow isotope ratio mass spectrometry. The isotopic composition of the particulate material and the isotopic composition of the dissolved N_2 pool are later on used to calculate N_2 fixation rates (combined equations from Capone and Montoya 2001):

$$N_2 \text{ fixation [mol N } t^{-1}] = (AP\ PN_{final} - AP\ PN_{initial}) / (AP\ N_2 - AP\ PN_{initial}) \times PN_{final} \text{ [mol]} / \Delta t$$

where AP = atom% ^{15}N in the particulate organic nitrogen at the end (PN_{final}) or beginning ($PN_{initial}$) of the incubation or in the dissolved N_2 pool (AP N_2). Here, all parameters except the AP N_2 are measured components whereas the AP N_2 is a calculated value. Thus, the rapid equilibration between the added $^{15}N_2$ gas and the surrounding water and a uniform enrichment throughout the incubation period are of crucial importance for the N_2 fixation rates to be determined.

Simultaneous measurements of N_2 fixation rates using ARA and the $^{15}N_2$ tracer additions can be carried out to determine the conversion factor of C_2H_4 produced to N_2 fixed. Several studies found a good agreement between the two methods yielding ratios around the theoretical conversion factors (3 or 4) but also large discrepancies between methods have been observed (see summary in Mulholland 2007). These discrepancies have led to the operational definition of net and gross N_2 fixation (Gallon *et al.* 2002, Mulholland *et al.* 2004). The former describes the actual uptake of N_2 into biomass and the latter describes the total N_2 fixation, *i.e.* any N_2 that has been fixed and subsequently excreted as dissolved inorganic (DIN) or organic nitrogen (DON) in addition to the N_2 incorporated into biomass. While net N_2 fixation is obtained using the $^{15}N_2$ addition, gross N_2 fixation can be estimated by the ARA which accounts for the entire N_2 fixation occurring in the system. However, DIN and DON cannot always account for the discrepancies (Mulholland *et al.* 2004; Mulholland and Bernhardt 2005).

In addition to the discrepancies between N_2 fixation rates determined with ARA and $^{15}N_2$ tracer addition methods, mismatches between measured N_2 fixation rates and geochemical estimates are observed (Mahaffey *et al.* 2005) and the sinks and sources in the oceanic fixed N budget do not seem to be in balance (see section A). The use of a diazotrophic culture during this Ph.D. work facilitated an inter-comparison between the two methods in combination with a mass balance. In a diazotrophic culture grown in N-deplete medium, an increase in cellular nitrogen over time can be solely attributed to N_2 fixation and is a function of growth rate. Hence, direct measurements should balance the increase in cellular N over the same time. The overall result suggested that the $^{15}N_2$ tracer addition method was yielding incorrect N_2 fixation rates due to mismatches between the ARA and the $^{15}N_2$ method but also between the increase of particulate organic nitrogen and the $^{15}N_2$ method (mass balance). The results of these laboratory culture studies led to the re-assessment of the currently used $^{15}N_2$ tracer addition covered by manuscript A of this thesis. The results of the re-assessment have important practical implications.

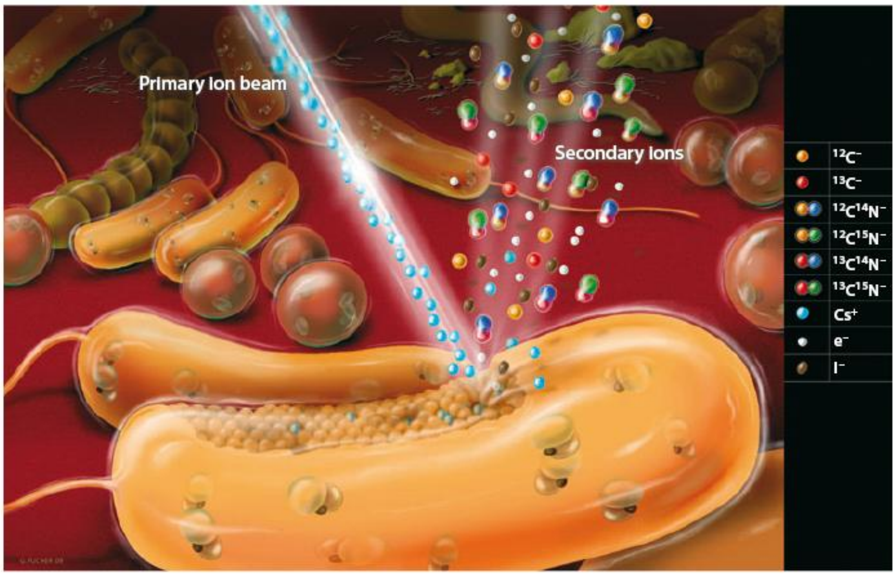

Figure I-7: Schematic illustration of a dynamic secondary ion mass spectrometry (SIMS) analysis of a bacterial community (not to scale). The bacterial cell sputtered with the primary ion beam is labelled with ^{15}N and ^{13}C. Only selected secondary ions are displayed. Note that nitrogen is detected as cyanide ion and that isobaric ions are formed (*e.g.*, $^{12}C^{15}N^-$ and $^{13}C^{14}N^-$), whose differentiation requires high mass resolution as offered, for example, by modern NanoSIMS instruments. Source: Wagner (2009).

In the study of N_2 fixation in the marine environment, the different approaches described above can be combined to reveal, for example, the magnitude of N_2 fixation using the direct measurements and the contribution by the different diazotrophic groups as determined by molecular methods. Nevertheless, until a few years ago, the diazotrophs actually fixing N_2 could not be identified at the cellular level even when gene transcripts were detected and N_2 fixation was measurable due to the lack of a link between the two. The recent improvements in methodologies and protocols using nanometer-scale secondary ion mass spectrometry (nanoSIMS) after incubation with stable isotopes enable scientists to link the identity of an organism to its' function/activity by coupling nanoSIMS to *in situ* hybridization (Behrens *et al.* 2008, Li *et al.* 2008, Musat *et al.* 2008). NanoSIMS can be used to reveal sub-cellular isotopic composition of (filtered) particulate material due to a resolution of about 50 nm. Coupled to stable isotope incubations such as the $^{15}N_2$ tracer addition, single-cell N_2 fixation rates can be assessed. During secondary ion mass spectrometry, a sample is exposed to a primary ion beam (*e.g.* Cs^+ ions) and atoms or molecules are sputtered of the sample surface (Figure I-7). Atoms and molecules of one polarity (negative or positive) can then be separated in an electric field, and the secondary ion beam can be analysed by mass spectrometry. Since several masses can be analysed simultaneously, this gives the opportunity to obtain isotope ratios of the sample under investigation. The application of nanoSIMS to determine N_2 fixation rates is the subject of manuscript C in this thesis.

D. Aim of thesis

Dinitrogen (N_2) fixation is the major source of fixed nitrogen (N) in the ocean. It is hence a crucial component in the marine N cycle since the availability of nitrogen controls the magnitude of primary production in the surface ocean. The diazotrophic community is often dominated by cyanobacteria including the well-known filamentous, non-heterocystous *Trichodesmium* and a range of unicellular cyanobacteria. Most unicellular cyanobacteria separate N_2 fixation and photosynthesis temporally to protect the nitrogenase from photosynthetically-evolved O_2. The activity of the different diazotrophs is commonly assessed using molecular methods based on the expression of the *nifH* gene. The *nifH* gene expression varies over diel cycles and this has been shown for several cultured species, for example, *Cyanothece* sp. ATCC 51142. At the beginning of this Ph.D. work, diel patterns of gene expression were not known for *Crocosphaera watsonii*, an abundant unicellular cyanobacterial diazotroph in the tropical and subtropical ocean. Experiments were carried out to confirm the temporal segregation of N_2 fixation and photosynthesis. The physiological characterization was complemented by gene expression and protein analysis to yield detailed information on temporal shifts between gene expression, protein synthesis and enzyme activity. This information will be helpful in the study of N_2 fixation in the environment.

Results from the first study indicated that *C. watsonii* may retain functionality of the photosystems which could be activated upon illumination. To test this hypothesis, further experiments were carried out using a dark-light cycle followed by continuous light. In addition, single-cell analyses were carried out to reveal population dynamics in the processes of N_2 fixation and photosynthesis. Population heterogeneity has been shown for several organisms at gene expression and protein level or in growth rates. Heterogeneity in metabolic processes, however, has been rarely shown due to methodological limitations. The application of nanoSIMS and stable isotope incubations (here $^{15}N_2$ and $DI^{13}C$) provided an excellent tool for the analysis of N_2 fixation and photosynthesis at the cellular level.

The simultaneous application of stable isotope incubations in a diazotroph pure culture and the comparison to the acetylene reduction assay (ARA) to measure N_2 fixation activity in the previous experiments was used to carry out an inter-comparison between direct measurements of N_2 fixation, *i.e.* the ARA and the $^{15}N_2$ tracer addition method. Preliminary results indicated that the currently used $^{15}N_2$ tracer addition method, *i.e.* the direct injection of $^{15}N_2$ gas to incubations may have severely underestimated N_2 fixation rates. Upon this finding, a series of experiments was set up to re-assess the currently used $^{15}N_2$ tracer addition method and to provide a modified approach to obtain real rates of N_2 fixation.

II. RESULTS

II. Results

This thesis is based on the following manuscripts:

A. **Mohr, W.**, Grosskopf, T., Wallace, D.W.R, LaRoche, J. (2010) Methodological underestimation of oceanic nitrogen fixation rates. Submitted to *PLos ONE*. (http://www.plosone.org/home.action)

Contribution: W.M. designed the research, carried out the experiments and measurements, analysed the data and wrote the manuscript. T.G., D.W.R.W. and J.L.R. assisted with the design and analysis of the data and commented on the prepared manuscript.

B. **Mohr, W.**, Intermaggio, M.P., LaRoche, J. (2010) Diel rhythm of nitrogen and carbon metabolism in the unicellular, diazotrophic cyanobacterium *Crocosphaera watsonii* WH8501. *Environmental Microbiology* **12**: 412-421. (http://www3.interscience.wiley.com/)

Contribution: W.M., M.P.I. and J.L.R. designed the research, W.M. and M.P.I. carried out the experiments and measurements and analysed the data, W.M. wrote the manuscript. J.L.R. assisted in preparation of manuscript and M.P.I. commented on the prepared manuscript.

C. **Mohr, W.**, Vagner, T., Kuypers, M.M.M., LaRoche, J. (2010) Tracing the high-performance cells: Heterogeneity in a diazotrophic population. Prepared for submission to *Proceedings of the National Academy of Sciences USA*. (http://www.pnas.org/)

Contribution: W.M. M.M.M.K. and J.L.R. designed the research. W.M. carried out the experiments. W.M. and T.V. carried out measurements. W.M. analysed the data and wrote the manuscript. M.M.M.K and J.L.R. assisted in data analyses and commented on the prepared manuscript.

A. Methodological underestimation of oceanic nitrogen fixation rates

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Abstract

The two commonly applied methods to assess dinitrogen (N_2) fixation rates are the $^{15}N_2$-tracer addition and the acetylene reduction assay (ARA). Discrepancies between the two methods as well as inconsistencies between N_2 fixation rates and biomass/growth rates in culture experiments have been attributed to variable excretion of recently fixed N_2. Here we demonstrate that the $^{15}N_2$-tracer addition method underestimates N_2 fixation rates significantly when the $^{15}N_2$ tracer is introduced as a gas bubble. The injected $^{15}N_2$ gas bubble does not attain equilibrium with the surrounding water leading to a $^{15}N_2$ concentration lower than assumed by the method used to calculate $^{15}N_2$-fixation rates. The resulting magnitude of underestimation varies with the incubation time, to a lesser extent on the amount of injected gas and is sensitive to the timing of the bubble injection relative to diel N_2 fixation patterns. Here, we propose and test a modified $^{15}N_2$ tracer method based on the addition of $^{15}N_2$-enriched seawater that provides an instantaneous, constant enrichment and allows more accurate calculation of N_2 fixation rates for both field and laboratory studies. We hypothesise that application of N_2 fixation measurements using this modified method will significantly reduce the apparent imbalances in the oceanic fixed-nitrogen budget.

Introduction

Biological dinitrogen (N_2) fixation is the major source of fixed nitrogen (N) in the oceanic N budget [1]. Current estimates of global oceanic N_2 fixation are ~ 100 - 200 Tg N a^{-1} [2]. N_2 fixation rates can be assessed through geochemical estimates, modelling of diazotroph abundances and growth rates [3] and direct measurements of N_2 fixation. Geochemical estimates rely on the measurement of, *e.g.*, nutrient stoichiometry and estimates or models of ocean circulation [4, 5] or the distribution of stable isotope abundances [*e.g.* 6]. Direct measurements of N_2 fixation are obtained either using the $^{15}N_2$-tracer addition method [7, 8] or the acetylene reduction assay (ARA) [9, 8]. However, direct measurements of N_2 fixation rates account for ≤50% of the geochemically-derived estimates [10]. Furthermore, the sink terms in the oceanic fixed N budget significantly exceed the current estimates of N_2 fixation and other source terms for fixed N [11, 12]. This gap between sources and sinks of fixed N implies an oceanic nitrogen imbalance, which may reflect non-stationarity of the oceanic fixed-N inventory, or result from over-estimation of loss processes and/or under-estimation of fixed nitrogen inputs [13, 10]. However isotopic signatures in sediments suggest that the fixed N budget is in a steady-state [14].

The comparison of N_2 fixation rates measured simultaneously using the $^{15}N_2$-tracer addition and the ARA shows that the $^{15}N_2$-tracer addition generally yields lower rates. In addition, mass balance analyses of $^{15}N_2$-based N_2 fixation rates measured in experiments with cultured diazotrophs, indicate that the $^{15}N_2$-tracer addition method yields rates that are too low for sustaining the observed growth rates and biomass [15, 16]. The discrepancies between the two methods and the lack of mass balance in culture experiments have often been attributed to the excretion of recently fixed nitrogen as ammonium (NH_4^+) or dissolved organic nitrogen (DON). The discrepancies have led to the operational definition of gross and net N_2 fixation [17, 15] as measured by the ARA and the $^{15}N_2$-tracer addition approaches, respectively. However, the measured release of NH_4^+ or DON is rarely sufficient to balance the observed growth in culture, and even invoking recycling of the dissolved fixed N rarely accounts for the observed discrepancies between N_2 fixation rate and growth rate/biomass [15].

The apparent oceanic N imbalance, differences between geochemical estimates and measured rates of N_2 fixation, and the difficulties in reconciling discrepancies between ARA and $^{15}N_2$-based estimates of N_2 fixation in the field and in culture experiments, led us to re-assess the $^{15}N_2$-tracer addition method. This method is based on the direct injection of a $^{15}N_2$ gas bubble into a seawater sample [7] sufficient to yield a final enrichment of 2-5 atom percent (atom%) and incubation for 2-36 hours [18]. N_2 fixation rates are then retrieved from the incorporation of $^{15}N_2$ into the particulate organic nitrogen (PON). The method assumes implicitly that the injected gas fully and rapidly equilibrates with the surrounding water, and this assumption is the basis for calculation of the initial ^{15}N enrichment of the dissolved N_2 pool. Knowledge of this enrichment is pivotal to the calculation of N_2 fixation rates with this method as seen in equation 1 (equations combined from [8]):

$$N_2\,fixation\ rate = \frac{(A_{PN}^{final} - A_{PN}^{t=0})}{(A_{N_2} - A_{PN}^{t=0})} \, x \, \frac{[PN]}{\Delta t}$$

where A = atom% ^{15}N in the particulate organic nitrogen (*PN*) at the end (*final*) or beginning ($t=0$) of the incubation or in the dissolved N_2 pool (N_2).

In applications of the method, all parameters of the equation are measured except for the atom% ^{15}N in the dissolved N_2 pool (A_{N2}). Equation 1 shows that calculation of N_2 fixation rates depends strongly on this value which is calculated from the predicted equilibrium dissolved N_2 concentration [19, 20], its natural ^{15}N abundance, and the amount of $^{15}N_2$ tracer added with the bubble. The calculation assumes that there is complete isotopic equilibration between the injected bubble of $^{15}N_2$ and the surrounding water at the start of the incubation.

Here we report results of experiments that were designed to assess the rate of equilibration of an introduced $^{15}N_2$ gas bubble with the surrounding water. Based on results of these experiments, we developed a modified approach involving addition of $^{15}N_2$-enriched seawater which assured a well-defined and constant ^{15}N enrichment of the dissolved N_2 gas at the beginning of the incubations. We propose the application of the modified approach for future assessments of N_2 fixation rates in natural microbial communities and in laboratory cultures.

Results

Time-resolved equilibration of a bubble of $^{15}N_2$ in seawater

A first set of experiments (isotopic equilibration experiments) was carried out to assess the time required to attain isotopic equilibrium in the dissolved pool of N_2 gas after injection of a known amount of $^{15}N_2$ gas as a bubble into sterile filtered seawater. A gas bubble of pure $^{15}N_2$ was injected directly into incubation bottles which were manually inverted fifty-times (~ 3 min agitation) and left standing for up to 24 h. Concentration of dissolved $^{15}N_2$ was followed over the 24 h period to assess the degree of equilibration of the $^{15}N_2$ gas bubble with the surrounding water as a function of time. Dissolved $^{15}N_2$ concentrations in the seawater increased steadily with the incubation time (Fig. 1A). After eight hours, dissolved $^{15}N_2$ concentrations reached about 50 % of the concentration calculated assuming complete isotopic equilibration of the injected bubble with the ambient dissolved N_2 gas in the seawater sample. At the end of the 24 h incubation, the dissolved $^{15}N_2$ concentration had increased to about 75% of the calculated concentration.

N_2 fixation rate underestimation due to incomplete $^{15}N_2$ gas bubble equilibration

Similar results were obtained in the incubation experiments with pure culture of *Crocosphaera watsonii* (culture experiments), which confirmed the incomplete and time-dependent equilibration of the injected bubble of $^{15}N_2$ gas with the surrounding water (Fig. 1B). These experiments also demonstrated the associated underestimation of N_2 fixation rates. Culture experiments were conducted after $^{15}N_2$ addition as a gas bubble and also after $^{15}N_2$ addition in the form of $^{15}N_2$-enriched seawater (our modified method, see Methods section). The incubation of *C. watsonii* after injection of a bubble of $^{15}N_2$ gas and without prior incubation of this bubble in algal-free media, gave a N_2 fixation rate which was only 40% of the maximum rate measured in the incubations to which $^{15}N_2$-enriched seawater had been added (Fig. 1B, black symbol). In other words, for the 12-h incubation period under the described experimental conditions, the N_2 fixation rate was underestimated by 60% when the $^{15}N_2$ was introduced as a gas bubble. In contrast, in both the isotopic equilibration and the culture experiments, the concentration of dissolved $^{15}N_2$ remained stable at the predicted value throughout the 24 h in incubations to which $^{15}N_2$-enriched water was added.

Factors influencing $^{15}N_2$ gas dissolution in N_2-saturated seawater

Continuous, vigorous shaking (50 rpm) greatly increased the concentration of $^{15}N_2$ in the media (Fig. 2) reaching ~ 67% of the calculated concentration after 30 minutes whereas the initial, manual agitation, *i.e.* inverting bottles 50 times (~3 min), resulted in only ~ 13% of the calculated concentration. Information on agitation is generally not provided in the published literature, but this is clearly a variable factor in incubations, especially if performed at sea. Continuous, vigorous shaking, as tested here (50 rpm; Fig. 2), is difficult to achieve in field experiments and may, in addition, be detrimental to some diazotrophs (*e.g. Trichodesmium* colonies).

Increasing the size of the incubation bottles, increasing the amount of gas injected per liter of seawater and the addition of dissolved organic matter (DOM) all led to slower equilibration of the $^{15}N_2$ gas bubble with the surrounding water (Fig. 3 and 4A), even when bottles were shaken continuously for one hour (Fig. 4B). Only with the injection of 8 ml of $^{15}N_2$ gas per liter of water and one hour of continuous, vigorous shaking, was near-complete equilibration achieved (97% of calculated concentration).

Discussion

Both the isotopic equilibration and the culture experiments demonstrated clearly that the equilibration of $^{15}N_2$ gas injected as a bubble into N_2-saturated seawater is time-dependent and incomplete, even after 24 hours. The lack of complete equilibration causes the resulting calculated N_2 fixation rates to be variably and significantly underestimated (see Equation 1). The equilibration, *i.e.* the isotopic exchange between the $^{15}N_2$ gas in the bubble and the surrounding water is controlled primarily by diffusive processes. The major variables that influence the rate of isotopic exchange include the surface area to volume ratio of the bubble, the characteristics of the organic coating on the bubble surface [21], temperature and the rate of renewal of the water-bubble interface [22]. The renewal of the water-bubble interface appears to have the greatest effect on the isotopic exchange, as continuous vigorous shaking of the incubation bottles generated the highest enrichment of $^{15}N_2$ in the water phase. However, the calculated (equilibrium) enrichment in $^{15}N_2$ was not attained fully even after one-hour of continuous shaking at 50 rpm on a rotary shaker. Incubations carried out on board a research vessel will provide some agitation of the bubble but this will not approach the high and constant agitation tested in our experiments. The implication is that variable sea-state conditions encountered during sea-going incubations, and the details of individual experiments, will lead to variable $^{15}N_2$ enrichments and hence variable underestimation of N_2 fixation rates. Further, N_2 fixation studies in the oligotrophic regions of the ocean usually require the use of large incubation volumes (*e.g.*, 2-4 L), so that continuous shaking for one hour or more is not practical, and in addition would likely be detrimental to the natural microbial communities.

The experiments with variable bottle sizes and DOM additions (Fig. 3 and 4) demonstrated that there are factors in addition to the bubble incubation time that affect the equilibration. On the other hand, the addition of $^{15}N_2$-enriched seawater to the incubations led to a stable enrichment over the 24 h incubation time which was instantaneous and independent of the agitation of the bottles.

This study was motivated partly by the mismatches between the ARA and $^{15}N_2$-based measurements of N_2 fixation as well as imbalances between $^{15}N_2$-fixation rates and biomass-specific rates (~ growth rate) or C:N fixation ratios (Table 1). Such mismatches have been observed in environmental studies and in culture studies, mainly with *Trichodesmium*. Although it has been shown that *Trichodesmium* can excrete recently fixed N_2 as NH_4^+ or DON [15, 26], the excretion of $^{15}NH_4^+$ or $DO^{15}N$ rarely accounts for the observed discrepancies [15, 16]. The operational definition of gross and net N_2 fixation as obtained through ARA and $^{15}N_2$ incubations, respectively, has been mainly based on the mismatch between the rates measured by the two methods. Our results demonstrate that N_2 fixation rates, as measured with the $^{15}N_2$ method [7] are underestimated. Therefore, the magnitude of the exudation of recently fixed nitrogen and the conditions promoting this process should be re-evaluated, taking into account the results presented here.

We reviewed published studies that have used the direct injection of a $^{15}N_2$ gas bubble to assess N_2 fixation rates in order to evaluate the magnitude of under-estimation. However, first attempts to assess the degree of underestimation of field and culture N_2 fixation rates were obscured by a wide range of experimental conditions among the studies. Bottle sizes ranged from 14 ml to 10 L, the amount of $^{15}N_2$ injected varied from 0.2 to 40.8 ml $^{15}N_2$ per L seawater and incubation times ranged from 0.25 to 48 hours, with the majority of the field studies using 2-4 L bottles and 24 h incubations. In addition, information on agitation was, in general, not available. There were no obvious trends of reported N_2 fixation rates with either bottle size, incubation time or the amount of injected $^{15}N_2$ gas probably because of the large variability of geographic locations and environmental conditions prevailing in the individual studies, which would have a dominant effect on the local diazotrophic communities and their N_2 fixation rates. An evaluation of the degree of possible underestimation of $^{15}N_2$ fixation rates in environmental studies is further confounded by diel periodicity of N_2 fixation [27-29]. The lack of knowledge on the exact timing and magnitude of the individual N_2 fixation activity of the different diazotrophs relative to the timing of $^{15}N_2$ gas injection hinders back-calculation of published N_2 fixation data. This can be illustrated, for example, with a hypothetical diazotroph community that is dominated by unicellular cyanobacteria which fix nitrogen during the night period only (Fig. 5A). In this microbial community, measurements of N_2 fixation using the direct injection of a $^{15}N_2$ gas bubble during a 24 hour incubation will lead to a variable underestimation of the true N_2 fixation rate, depending on the timing of the incubation start relative to the peak in the nitrogenase activity (Fig. 5C, solid lines). The underestimation will be more pronounced if the start of the incubation is coincident with the onset of the active N_2 fixation period. In contrast, incubations with enriched $^{15}N_2$ seawater, will not lead to an underestimate, regardless of the incubation start relative to the diel cycle (Fig. 5C, dashed lines).

The discrepancies and mismatches/imbalances observed in field and laboratory studies could, in part, be explained by the variable underestimation of the true N_2 fixation rate due to the methodological uncertainty reported here. We propose the addition of $^{15}N_2$-enriched seawater to incubations to assess N_2 fixation rates in laboratory and field studies. We suggest that measurements using this approach are likely to increase measurements and estimates of N_2 fixation at species, regional and global level and lead to a reduction in the apparent oceanic nitrogen imbalance.

Experimental procedures

Culture and growth conditions

The diazotrophic cyanobacterium *Crocosphaera watsonii* WH8501 was grown in batch cultures in N-free YBCII media [30] at 28°C in a temperature-controlled growth chamber. *C. watsonii* was subjected to 12:12 h dark:light cycles.

Direct injection of a $^{15}N_2$ gas bubble in water

We first examined the rate of equilibration between an injected bubble of $^{15}N_2$ gas and seawater. Two series of incubations were started by injecting 140 µl of $^{15}N_2$ into 133 ml of an artificial seawater media (YBCII) contained in headspace-free, septum-capped glass bottles. In the first series (isotopic equilibration experiments), all bottles were inverted fifty times (~ 3 min) after injection of the $^{15}N_2$ gas bubble and left at room temperature in the laboratory. One bottle was sampled immediately after the agitation in order to determine how much $^{15}N_2$ gas had dissolved initially. The other bottles were opened and sampled after standing for periods from 1 to 24 h. Upon opening of the bottles, samples to measure the dissolved $^{15}N_2$ were taken and stored in gas-tight glass vials (Exetainer®) until analysis.

In the second series (culture experiments), the YBCII media was pre-heated to 28°C in a temperature-controlled chamber before being used to fill septum-capped glass bottles. As with the first series, samples were agitated and left standing for varying periods of time after the injection of a $^{15}N_2$ gas bubble. Instead of taking subsamples for $^{15}N_2$ analysis, 13 ml of media were replaced by *C. watsonii* WH8501 culture upon opening of the bottles. This series of experiments was timed so that the introduction of culture into the media took place at the start of a dark phase of the 12:12h dark:light-adapted *C. watsonii* culture. The samples with the culture were then incubated for 12 h at culture growth conditions (28°C, dark phase, *i.e.* N_2-fixing) and filtered onto pre-combusted GF/F (Whatman; 450°C for 4 h) filters at the end of the incubation. Filters were dried immediately after (50°C, 6 h) and stored at room temperature until analysis. To obtain a measure of underestimation using the direct injection of a $^{15}N_2$ gas bubble, one bottle containing 13 ml of *C. watsonii* culture was incubated for 12 h after the injection of 140 µl $^{15}N_2$ gas at the start of the dark phase and without release of the bubble, essentially resembling a laboratory or field incubation.

Direct addition of $^{15}N_2$ tracer-enriched seawater

An alternative, modified $^{15}N_2$ tracer addition method was developed, which involved addition of an aliquot of $^{15}N_2$-enriched water to incubations. This alternative method was based on earlier approaches used to study oxygen cycling using $^{18}O_2$ [31] and the release of DON using $^{15}N_2$ [26]. The preparation of the $^{15}N_2$-enriched water was started by degassing 0.2 µm-filtered artificial seawater (YBCII media). Degassing was carried out by applying vacuum (≤200 mbar absolute pressure) to continuously stirred (stir bar) media for about 30 min. The degassed water was transferred rapidly but gently into septum-capped glass bottles until overflow, and 1 ml of $^{15}N_2$ gas (98 at%; Campro Scientific) was injected per 100 ml of media. The bottles were shaken vigorously until the bubble disappeared. Aliquots of this $^{15}N_2$-enriched water were then added to the incubation bottles, with the enriched water constituting no more than 10% of the total sample volume. This alternative enrichment method was applied to the two series of experiments described above.

Assessment of additional factors contributing to variation in $^{15}N_2$ enrichment

We assessed possible effects of varying bottle size, amounts of injected gas and different amounts of agitation on their contribution to the equilibration between a bubble of $^{15}N_2$ gas and the surrounding seawater. For the bottle size comparison, incubations were performed in 0.13 L bottles and in 1.15 L bottles. The amount of injected gas varied between 1 ml $^{15}N_2$ per 1 L seawater up to 8 ml $^{15}N_2$ per 1 L seawater. The incubations were agitated either by inverting fifty times manually (~ 3 min) or by continuous agitation on a rotating bench-top shaker (Biometra WT 17) at 50 rpm (rotations per minute). We also added marine broth (Difco 2216; 0.2 µm filter-sterilized) to some bottles to examine the effect of dissolved organic matter (DOM).

$^{15}N_2$ analysis in the artificial seawater and ^{15}N analysis in the particulate organic nitrogen (PON)

Subsamples taken during the equilibration experiments were analysed for $^{15}N_2$ concentration with a membrane-inlet mass spectrometer (MIMS; GAM200, IPI) within one week of subsampling. Dried GF/F filters were pelletized in tin cups and PON as well as isotope ratios were measured by means of flash combustion in an elemental analyser (Carlo Erba EA 1108) coupled to a mass spectrometer (Thermo Finnigan Delta S).

Calculations

The expected concentration of $^{15}N_2$ following bubble injections was calculated assuming rapid and complete isotopic equilibration between bubble and surrounding seawater and considering atmospheric equilibrium concentrations of dissolved N_2 [20]. When $^{15}N_2$-enriched aliquots were added, the amount of $^{15}N_2$ originally dissolved in the degassed seawater and the volume of the aliquot added were taken into account. The calculations of N_2 fixation rates in the culture incubations were made according to Equation 1 and are presented as a percentage of the highest rate measured. For the comparison between methods, the measured $^{15}N_2$ concentrations are presented as a percentage of the expected concentration calculated as follows

$$V_{15N2} \text{ x } MV^{-1} \text{ x } V_{TOTAL}^{-1} = [\text{mol } ^{15}N_2 \text{ } L^{-1}] \text{ (\textit{i.e.} 100\%)} \qquad \text{(Equation 2)}$$

for the direct injection of a $^{15}N_2$ gas bubble where V_{15N2} is the volume of the $^{15}N_2$ gas bubble, MV is the molar volume and V_{TOTAL} is the total (water) volume of the incubation. The expected concentration was corrected for the amount of $^{15}N_2$ gas which remains in the bubble at isotopic equilibrium with the surrounding water. For the addition of $^{15}N_2$-enriched water the expected concentration is

$$V_{15N2} \text{ x } MV^{-1} \text{ x } V_{DG}^{-1} \text{ x } V_{EW} \text{ x } V_{TOTAL}^{-1} = [\text{mol } ^{15}N_2 \text{ L}^{-1}] \ (\textit{i.e.}\ 100\%) \qquad \text{(Equation 3)}$$

where V_{DG} is the volume of degassed water, V_{EW} is the volume of $^{15}N_2$-enriched water added to the incubation and V_{TOTAL} is the total (water) volume of the incubation.

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Figure legends

Figure 1. **Dependence of the degree of equilibration of a $^{15}N_2$ gas bubble and the surrounding water.** Results are presented as a function of the time after bubble injection (white symbols). For comparison, the addition of $^{15}N_2$-enriched water to samples yielded a constant $^{15}N_2$ enrichment over 24 h (grey symbols). The immediate incubation of culture after the injection of a $^{15}N_2$ gas bubble without pre-incubation (black symbol) was intended to resemble laboratory and environmental studies. (A) Measured dissolved $^{15}N_2$ concentrations as percentage of calculated concentration assuming rapid and complete isotopic equilibrium. (B) N_2 fixation rates by *C. watsonii* as percentage of the maximum rate measured during the experiments.

Figure 2. Agitation-dependent increase in dissolved $^{15}N_2$ using bubble incubations. Values are presented as a percentage of the calculated concentration. The manually-shaken (3 min) sample was added to the plot for comparison (grey symbol).

Figure 3. Dissolved $^{15}N_2$ concentration as a function of bottle size and amount of injected $^{15}N_2$ gas. Values are presented as a percentage of the calculated concentration. Bottles were incubated for 1 hour. Black bars, 0.13 L bottle and white bars, 1.15 L bottle.

Figure 4. Dissolved $^{15}N_2$ concentration as a function of the amount of injected gas and agitation. Values are presented as a percentage of the calculated concentration (A) after 1 hour incubation in manually (3 min shaking and 1 h subsequent incubation), and (B) in continuously (1 h) shaken samples.

Figure 5. Influence of diel N_2 fixation patterns on the magnitude of N_2 fixation rates. Schematic diagram illustrating the influence of diel N_2 fixation patterns on N_2 fixation rates

when determined with the direct injection of a $^{15}N_2$ gas bubble. A hypothetical diel N_2 fixation pattern is shown (A) with a duration of the N_2-fixing period of 12 h. Three possible time periods for 24 h incubations are indicated by the solid bars. The corresponding ^{15}N enrichment in the dissolved N_2 pool (B) is shown for the three incubation periods using the direct injection of a $^{15}N_2$ gas bubble (solid lines) and the addition of $^{15}N_2$-enriched seawater (dashed line). The resulting cumulative N_2 fixation in each of the incubations (C) demonstrates that the timing of the incubation relative to diel N_2 fixation patterns introduces a variable underestimation in the total N_2 fixation rate measured during the incubation after a $^{15}N_2$ gas bubble is injected (solid lines) as compared to the N_2 fixation measured with the addition of $^{15}N_2$-enriched seawater (dashed lines). The diagram is based on the observations made in the experiments described in this study.

Table 1: Discrepancies observed between $^{15}N_2$ fixation, ARA and carbon fixation or biomass-specific rates [a].

Organism / area	C_2H_2 : $^{15}N_2$	C:N fixation ratio	biomass-specific rate [d^{-1}]	Reference
Trichodesmium / Pacific, Atlantic, north of Australia		808[a]		[23]
cyanobacterial bloom / Baltic	3 – 20			[17]
Trichodesmium IMS 101	3 – 22	75 – 133		[16]
Trichodesmium IMS 101	1.5 – 6.9		0.002 – 0.011[c]	[15]
Trichodesmium / Gulf of Mexico		10-107[b]		[24]
Trichodesmium / Bermuda Atlantic Time Series station (BATS)		13 – 437	0.006-0.03[d]	[25]

[a] C:N fixation ratio is based on $^{15}N_2$-fixation measurements

[b] Ratio calculated from $DI^{13}C$ and $^{15}N_2$ fixation rates.

[c] Calculated from $^{15}N_2$ fixation rate divided by PON.

[d] Calculated from doubling time with biomass-specific rate = ln (2) / doubling time.

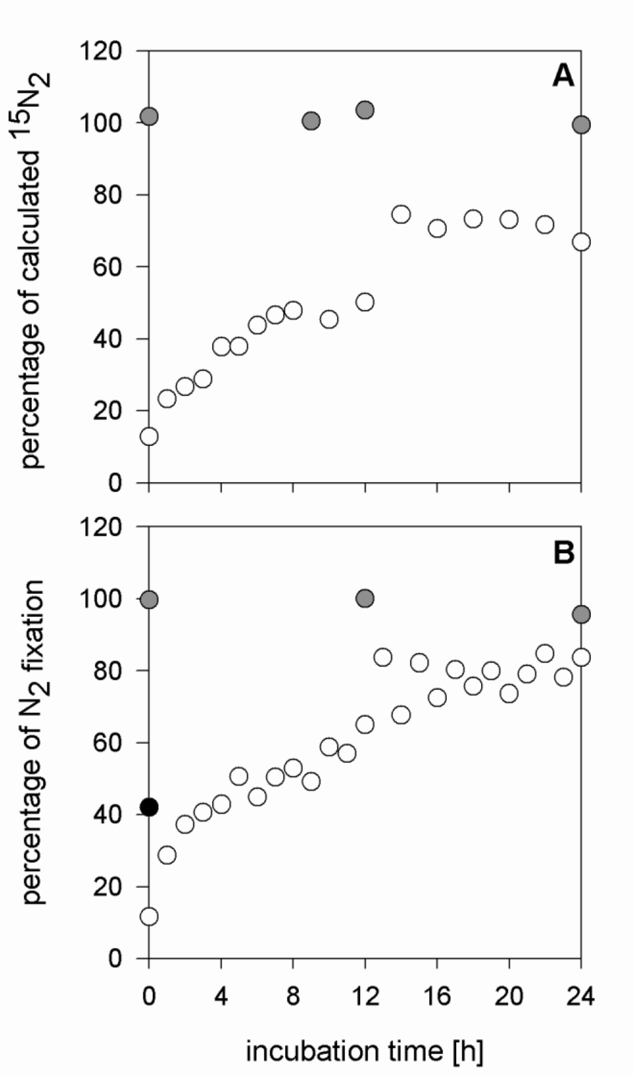

Figure 1

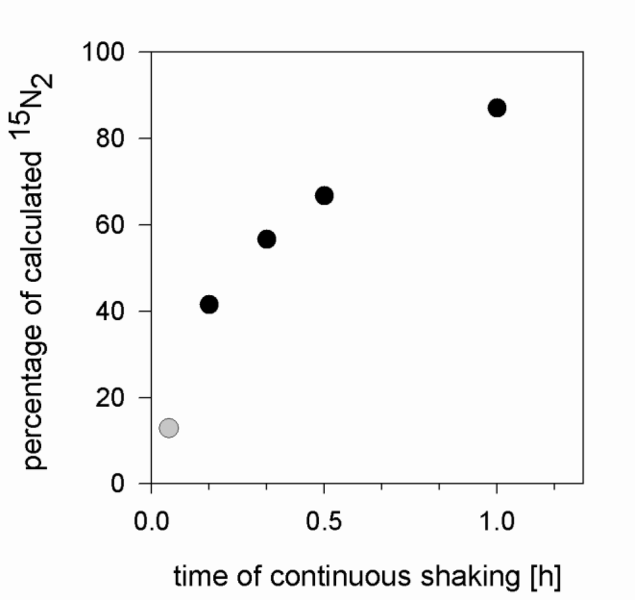

Figure 2

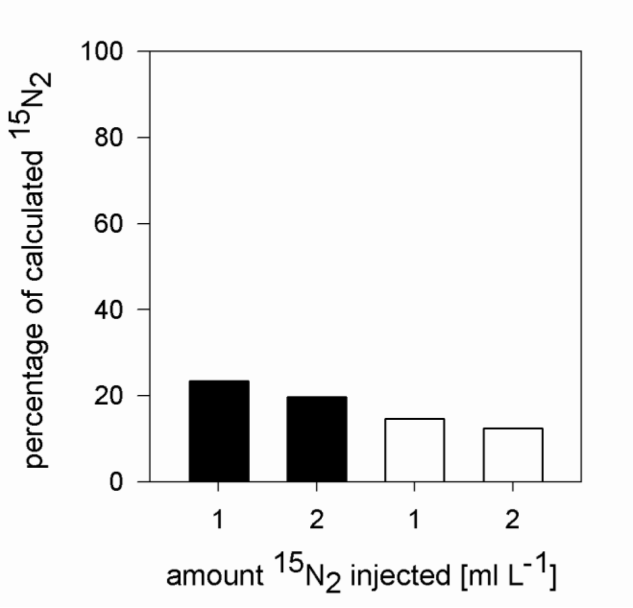

Figure 3

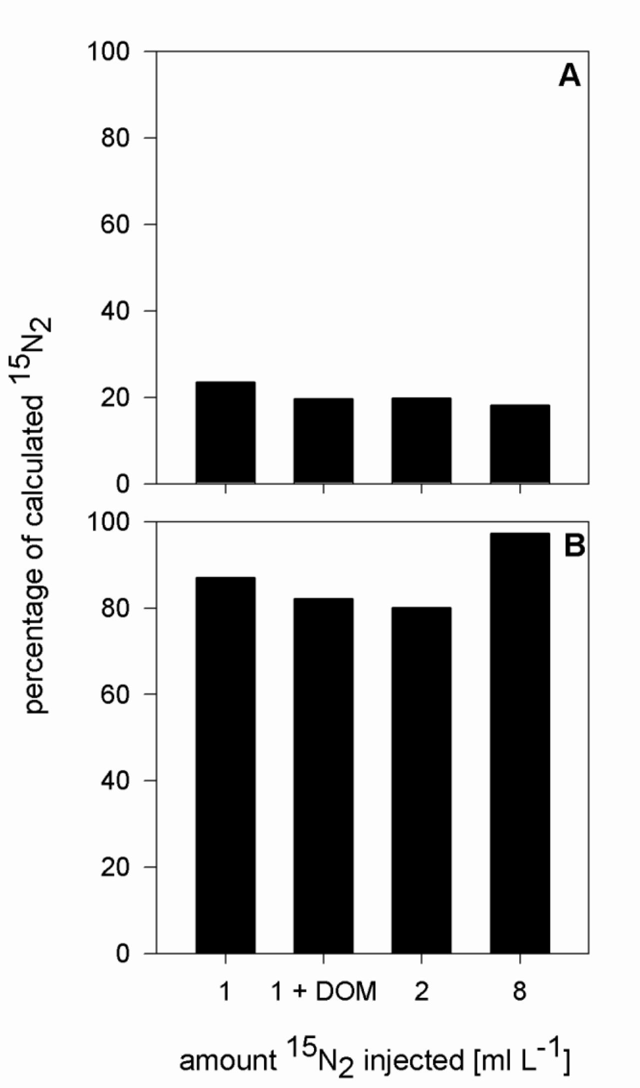

Figure 4

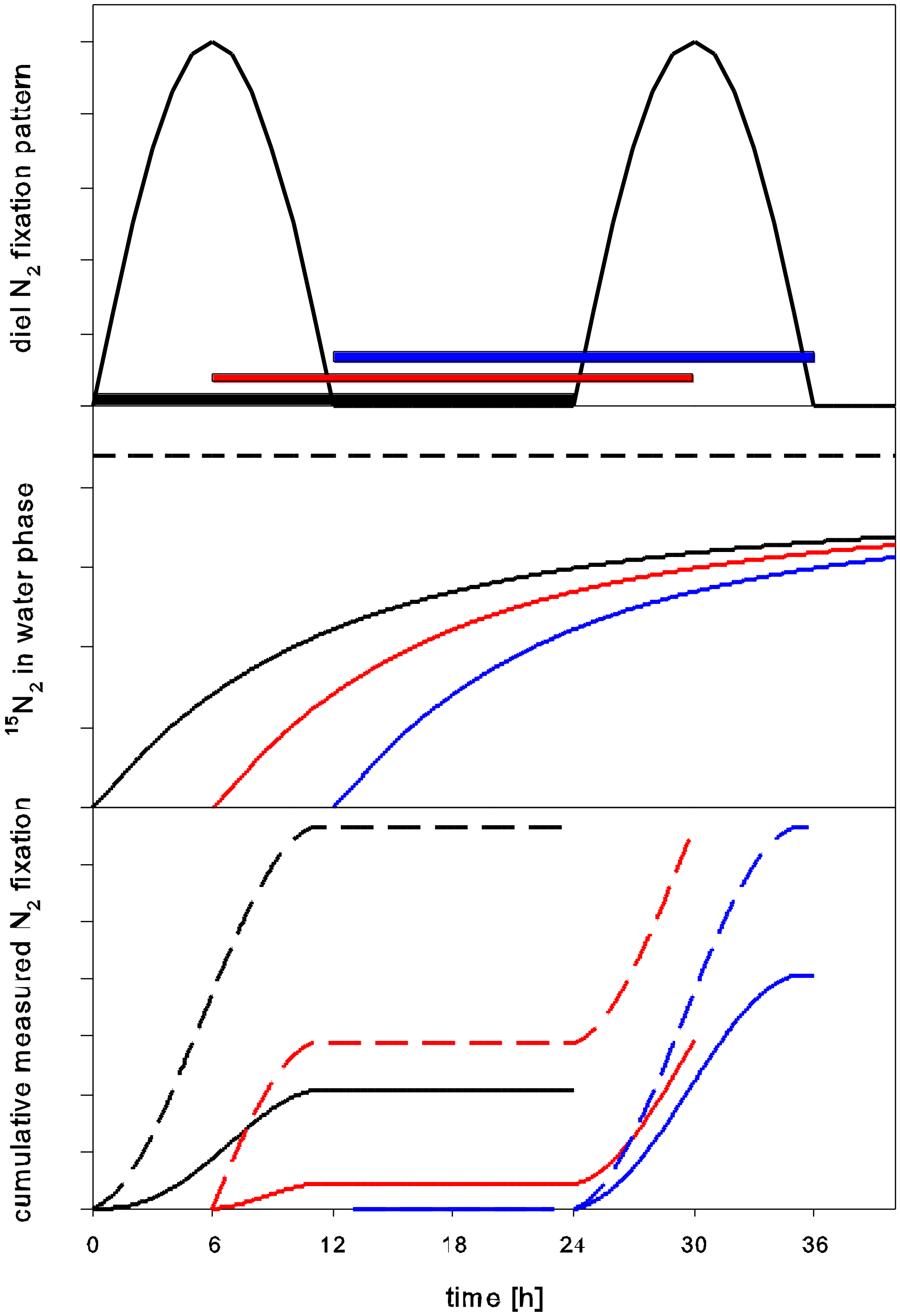
diel N_2 fixation pattern
$^{15}N_2$ in water phase
cumulative measured N_2 fixation
0
6
12
18
24
30
36
time [h]

Figure 5

B. Diel rhythm of nitrogen and carbon metabolism in the unicellular, diazotrophic cyanobacterium *Crocosphaera watsonii* WH8501

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Diel rhythm of nitrogen and carbon metabolism in the unicellular, diazotrophic cyanobacterium *Crocosphaera watsonii* WH8501

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Summary

We examined the diel variation in nitrogen and carbon metabolism in *Crocosphaera watsonii* WH8501 at the physiological and gene expression level in order to determine the temporal constraints for N_2 fixation and photosynthesis. N_2 fixation and photosynthesis were restricted to the dark and light periods, respectively, during a 24 h light–dark cycle. All genes studied here except one (*psbA2*) showed diel variations in their expression levels. The highest variation was seen in *nifH* and *nifX* relative transcript abundance with a factor of $3–5 \times 10^3$ between light and dark periods. Photosynthesis genes showed less variation with a maximum factor of about 500 and always had high relative transcript abundances relative to other genes. At the protein level, the photosystems appeared more stable than the nitrogenase complex over a 24 h light–dark cycle, suggesting that *C. watsonii* retains the ability to photosynthesize during the dark period of the diel cycle. In contrast, nitrogenase is synthesized daily and exhibits peak abundance during the dark period. Our results have implications for field studies with respect to the interpretation of environmental gene expression data.

Introduction

Diazotrophic cyanobacteria have to cope with the predicament of exhibiting oxygenic photosynthesis, i.e. oxygen (O_2)-evolving, while preserving the integrity of the O_2-sensitive nitrogenase enzyme complex in order to carry out dinitrogen (N_2) fixation. Several mechanisms have evolved to resolve this metabolic dilemma. Some filamentous cyanobacteria carry out N_2 fixation in specialized cells called heterocysts that lack the O_2-evolving photosystem II. This allows N_2 fixation to take place concurrently with photosynthesis during the light period (Popa *et al.*, 2007). Although N_2 fixation and photosynthesis are tightly regulated by a circadian rhythm in the filamentous, non-heterocystous *Trichodesmium erythraeum,* both processes take place concurrently during the light period (Chen *et al.*, 1996; 1998; 1999), through the spatial and temporal separation of the two processes (Berman-Frank *et al.*, 2001). The peak in N_2 fixation, which occurs at midday, is accompanied by a decrease in the photosynthesis rate.

Most unicellular diazotrophic cyanobacteria separate photosynthesis and N_2 fixation temporally with photosynthesis occurring in the light and N_2 fixation in the dark when grown under light–dark conditions. Extensive experiments on *Cyanothece* sp. have shown a strong diel regulation of these two processes (Reddy *et al.*, 1993; Schneegurt *et al.*, 1994; Meunier *et al.*, 1997; Colón-López *et al.*, 1997; Colón-López and Sherman, 1998; Sherman *et al.*, 1998). *Cyanothece* sp. accumulates fixed carbon from photosynthetic activity in intracellular granules (Reddy *et al.*, 1993; Schneegurt *et al.*, 1994). This stored carbon is thought to serve as a depot for the energy-demanding N_2 fixation process and is degraded during the night as indicated by high respiration rates (Colón-López *et al.*, 1997; Taniuchi *et al.*, 2008). Other metabolic pathways associated with either N_2 fixation or photosynthesis such as the synthesis of cyanophycin, a cyanobacterial nitrogen storage molecule, might also be under circadian control (Li *et al.*, 2001).

Crocosphaera watsonii WH8501 was isolated in the mid-eighties in the western tropical South Atlantic Ocean (Waterbury and Rippka, 1989; Rippka *et al.*, 2001). This unicellular cyanobacterium is capable of N_2 fixation and belongs to the group B of unicellular diazotrophs (Zehr *et al.*, 2001). It grows at water temperatures of 26–34°C (Waterbury and Rippka, 1989; Webb *et al.*, 2009). The contribution of unicellular diazotrophic cyanobacteria to oceanic N_2 fixation is still under debate (Goebel *et al.*, 2008). However, the numerical abundance of the unicellular diazotrophs in certain areas such as the North Pacific

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subtropical gyre (Church *et al.*, 2008) shows that they can at times dominate the diazotroph community. *Crocosphaera watsonii* cell abundance as high as 4.7×10^6 l^{-1} were observed at a depth of approximately 40 m in the South Pacific (Hewson *et al.*, 2009). Abundances of the order of 10^5 *nifH* copies l^{-1} of group B phylotype were detected in the North Pacific subtropical gyre (Church *et al.*, 2008).

Although *C. watsonii* and *Cyanothece* sp. are both unicellular cyanobacteria, differences at the genomic level and in their physical habitat preference (Langlois *et al.*, 2008) make it necessary to characterize *C. watsonii* given its dominance under certain environmental conditions (Church *et al.*, 2008; Hewson *et al.*, 2009). The occurrence of N_2 fixation at night in *C. watsonii* has been previously demonstrated (Tuit *et al.*, 2004), but the timing of other physiological processes and the peak in expression of the corresponding genes are lacking so far. The availability of a complete genome sequence for *C. watsonii* makes it an attractive organism to follow in field population (Hewson *et al.*, 2009). In order to study *C. watsonii* and other unicellular diazotrophic cyanobacteria in environmental samples, it is important to first establish the temporal expression patterns for N_2 fixation and photosynthesis in culture in order to interpret information on gene expression obtained from natural microbial populations. Unlike the identification of *nifH* phylotypes and the estimation of their abundance from environmental DNA samples, measurements of *nifH* transcript levels are expected to vary widely over a diel cycle. Assessing the diel pattern of *nifH* expression is therefore important for designing field sampling strategies. Here we present a detailed study that demonstrates the clear separation of photosynthesis and N_2 fixation during a diel cycle in laboratory cultures.

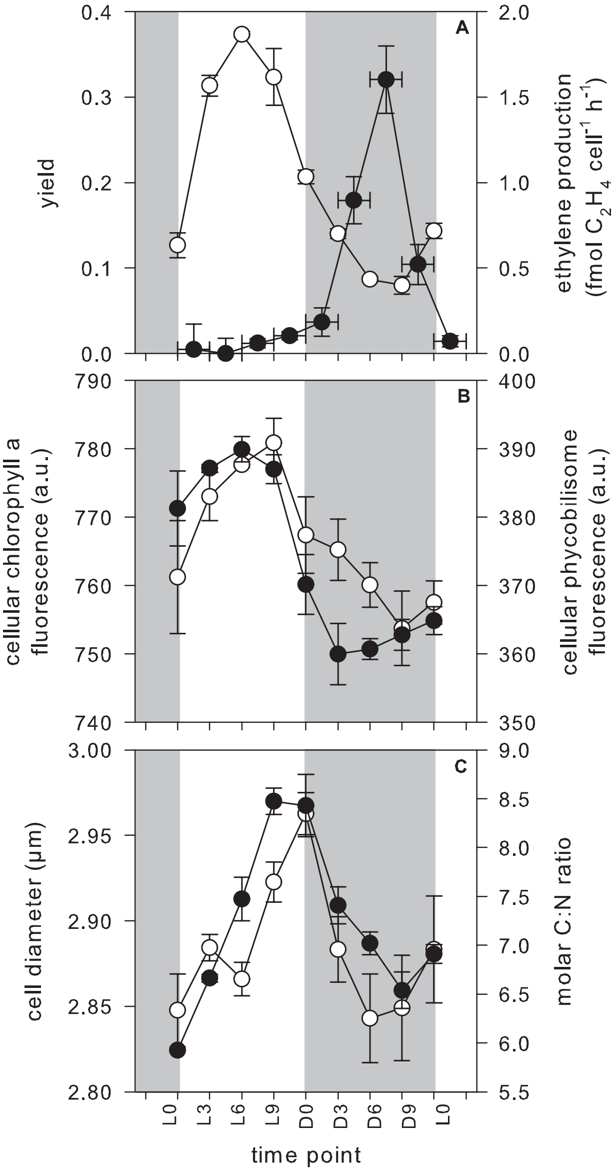

Fig. 1. Physiological properties in *C. watsonii* WH8501 in light–dark conditions. Shaded areas correspond to the dark period. Cultures for light and dark periods were kept in opposite light–dark regimes to facilitate sampling. Symbols represent mean ± standard error of triplicate cultures, except for D0, which is the mean of two sets of triplicate cultures kept in opposite light–dark regimes. All measurements were done at the beginning of the 3 h sampling intervals except the N_2 fixation (see below).

A. Photosynthetic quantum yield (open circle) and N_2 fixation [filled circle; presented as ethylene (C_2H_4) production rates] as measured by PAM fluorescence and acetylene reduction respectively. The *x*-axis error bars represent the 3 h incubation time for the N_2 fixation measurement.

B. Cellular chlorophyll *a* (open circle) and phycobilisome (filled circle) fluorescence as measured through flow cytometry.

C. Cell size (open circle) and molar C : N ratio (filled circle) as measured with a coulter counter and an elemental analyser respectively.

Results

Physiological properties

Crocosphaera watsonii cultures grown at 50 µE m^{-2} s^{-1} and 28°C showed strong diel variation in photosynthesis and N_2 fixation. Photosynthetic quantum yield (F_v/F_m) increased throughout the light period with a maximum (0.37) at 6 h into the light period (L6) and decreased afterwards (Fig. 1A). The minimum yield (0.08) was reached 6–9 h into the dark period (D6–D9). Relative cellular phycobilisome and chlorophyll *a* fluorescence, measured by flow cytometry, also increased during the light period, reaching its maximum in the middle of the light period (Fig. 1B). Lowest phycobilisome and chlorophyll *a* fluorescence was at 3 (D3) and 9 h (D9) into the dark period respectively. N_2 fixation rates were low to undetectable during the light period, rapidly increasing to a maximum by 6 h in the dark (D6) and rapidly declining

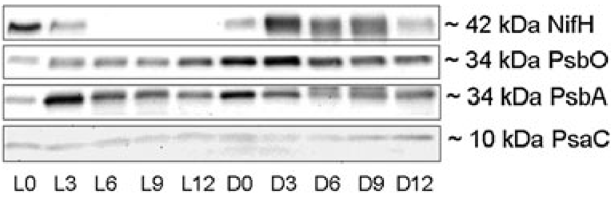

Fig. 2. Western blots from NifH, PsbO, PsbA and PsaC protein analysis. This analysis was done on samples from a replicated experiment (which had identical patterns for physiology and gene expression) because protein samples from this experiment were lost during analysis. Equal amounts of protein (total of 9 μg) were loaded into each well on the gel and were separated in SDS-PAGE. The corresponding protein and the estimated size are indicated on the right side. Note that L12 and D0 as well as D12 and L0 are the same time.

towards the end of the dark period (Fig. 1A). These results demonstrate that N_2 fixation is largely confined to the dark period in a 12–12 h light/dark cycle. Consistent with this pattern, the C : N molar ratio increased to a maximum of 8.5 at 9 h into the light period (L9), indicating a net accumulation of fixed organic carbon during the day (Fig. 1C). Conversely, the C : N ratio decreased until it reached its minimum of 6.5 at 9 h into the dark period (D9), probably resulting from active N_2 fixation, combined with the breakdown of stored carbon compounds. Cell size of *C. watsonii* increased during the light until the beginning of the dark period and declined subsequently (Fig. 1C). The maximum cell diameter of 2.96 μm at the beginning of the dark period (D0) was significantly different ($P < 0.05$, paired *t*-test) from the minimum cell diameter of 2.84 μm at 6 h (D6) into the dark period.

Relative protein abundance

Western blot analysis showed diel variation of NifH and PsbO proteins with the maximum relative protein abundance in the early dark period (Figs 2 and 3A). Minimum abundances of PsbO were observed during the light period. The NifH virtually disappeared during the light period with abundances of about 0.5% of its maximum. The PsaC and PsbA proteins were present throughout the entire 24 h cycle with lowest abundances of 34% and 32% of their respective maximum and generally slightly higher abundances during the light period than during the dark period (Figs 2 and 3B). The PsaC had its lowest protein abundance at the end of the light and the beginning of the dark period, and abundances were close to 100% at the beginning of the light period. The PsbA was maximal at the beginning of the light period (L3) and decreased until 6 h into the dark period (D6).

Gene expression patterns

Expression patterns of selected genes encoding for components of the photosynthetic apparatus, N_2 fixation machineries, for the synthesis of the associated storage forms of carbon and nitrogen and for the regulation of nitrogen assimilation were assessed using reverse transcription followed by quantitative PCR (RT-qPCR). Relative quantitation is represented as enrichment factor with respect to the mean relative expression of the RNA polymerase *rpbl* throughout the diel cycle. Transcript levels of two genes belonging to the *nif* operon, *nifH* and *nifX*, coding for the nitrogenase iron protein and a protein involved in the nitrogenase FeMo-cofactor biosynthesis, respectively, reached a minimum at 3 h into the light period (L3) and thereafter started to increase (Fig. 4A). Their maximum was at the start of the dark period (D0), slowly declining until 9 h into the dark period (D9) and then rapidly declining to the initial level when the light period starts (L0). *nifH* and *nifX* had almost identical expression patterns although the relative amount of *nifH*

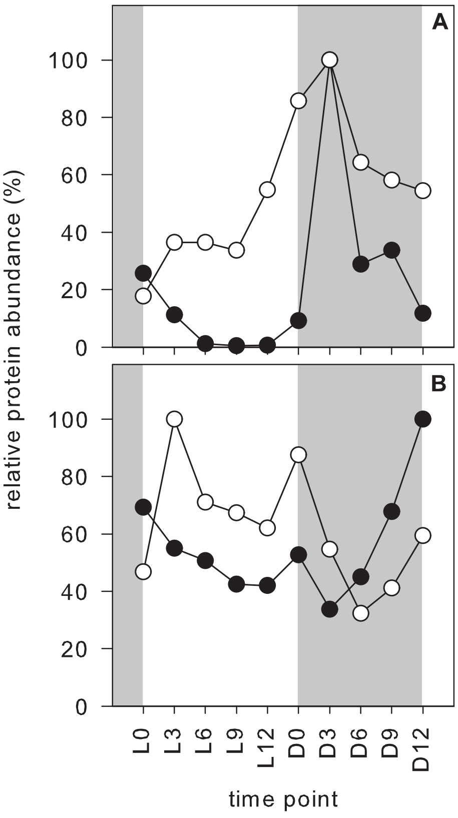

Fig. 3. Densitometric analysis of Western blots showing the abundance of protein as a percentage of the strongest band that was manually set to 100%; the other bands were calculated accordingly. Note that L12 and D0 as well as D12 and L0 are the same time.
A. Densitometry for the NifH (filled circle) and PsbO (open circle) bands.
B. Densitometry for PsbA (open circle) and PsaC (filled circle) bands. The mean abundance of proteins from L3 to L9 and D3 to D9 were significantly different for NifH ($P = 0.005$) and for PsbA ($P = 0.05$). Mean abundance was not significantly different for PsaC ($P > 0.9$) and for PsbO ($P > 0.09$).

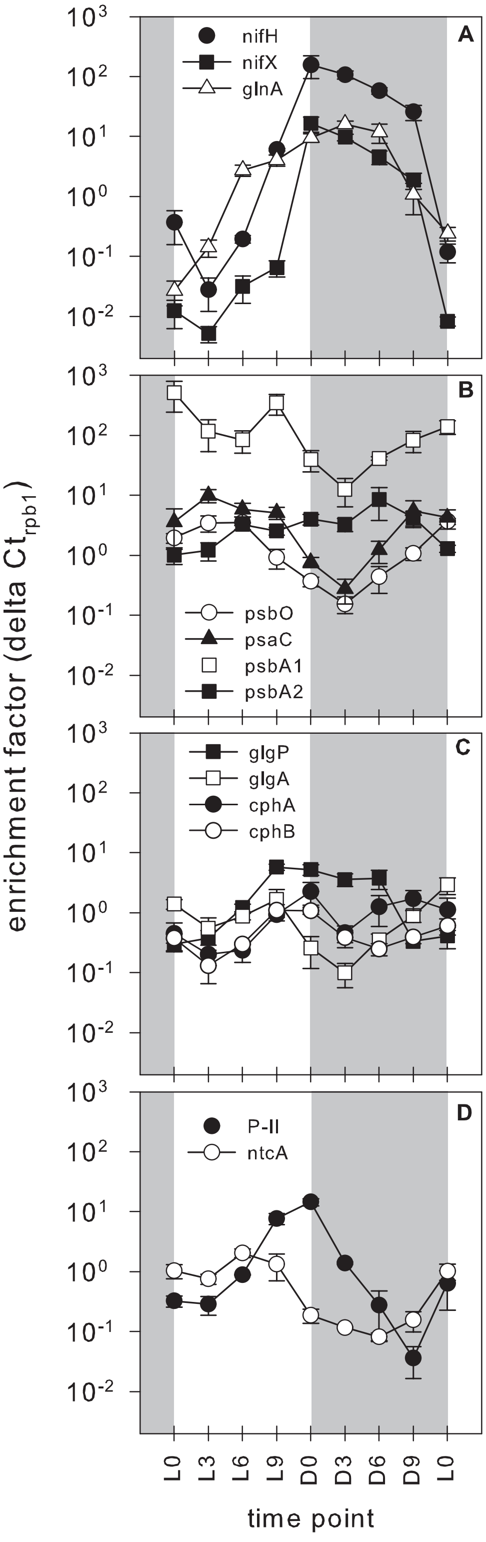

Fig. 4. Relative gene transcript abundance during the time-course experiment shown as enrichment factor relative to the RNA polymerase *rpbl* (see *Experimental procedures*). Symbols represent mean ± standard error of triplicate cultures except for time point L6, which is only from duplicate samples, and D0, which is from two times triplicate cultures kept in opposite light–dark regimes. The relative position of the curves to each other reflect the relative amount of transcripts between genes, i.e. the *psbA1* gene was always higher expressed than all other photosynthesis genes studied here.

A. Relative enrichment in *nifH* (filled circle), *nifX* (filled square) and *glnA* (open triangle) transcripts.

B. Relative enrichment in *psbA1* (open square), *psbA2* (filled square), *psbO* (open circle) and *psaC* (filled triangle) transcripts.

C. Relative enrichment in *glgA* (open square), *glgP* (filled square), *cphA* (filled circle) and *cphB* (open circle) transcripts.

D. Relative enrichment in *ntcA* (open circle) and P-II (filled circle) transcripts.

transcripts was about 10 times higher than for *nifX*. The expression of *glnA*, the gene coding for glutamine synthetase, also showed diel variation with maximum abundances during the early dark period and a minimum at the beginning of the light period. This was expected since glutamine synthetase is essential for the incorporation of newly fixed N_2 by catalysing the condensation of glutamate and ammonia to form glutamine. Three genes coding for, respectively, the photosynthetic proteins photosystem I subunit VII (*psaC*), the photosystem II D1 protein (*psbA1*) and the photosystem II O_2-evolving complex protein (*psbO*) followed similar expression patterns with higher transcript levels during the day and lower transcript levels at night (Fig. 4B). Transcript levels remained constant during the day until 9 h into the light period (L9) when they started to decline. Minimum transcript levels were at 3 h into the dark period (D3) and increased afterwards reaching a steady level at the point when the light turns on. The second *psbA* gene (*psbA2*) increased steadily from the beginning of the diel cycle (L0) to 6 h into the dark period (D6) and declined afterwards to the initial level when the light turns on (L0) (Fig. 4B). The glycogen synthase encoded by the *glgA* gene is taking part in the formation of glycogen by building α-1,4-glucan chains from ADP-glucose. The *glgP* gene encodes a glycogen phosphorylase that produces glucose-1-phosphate through the addition of orthophosphate to the non-reducing end in, e.g. glycogen. Both *glgA* and *glgP* genes showed variable gene transcription during the diel cycle (Fig. 4C). Minimum transcript level was at the start of the light period (L0) for *glgP* whereas *glgA* transcripts had their maximum at L0. *glgP* transcripts increased during the light period with a maximum at 9 h into the light period (L9), slowly decreased until 6 h into the dark period (D6) and then returned to the initial level at the start of the light period (L0). As expected, transcript levels of *glgA* showed the opposite trend. The pattern in transcript levels for *glgA* and *glgP* genes mirrored each other, suggesting that carbon synthesis and degradation are taking place during light and dark periods respectively. Transcript levels for the genes encoding the cyanophycin synthetase (*cphA*) and cyanophycinase (*cphB*) were also assessed. While *cphA* transcripts showed a variable expression pattern, *cphB* transcripts increased from 3 to 9 h into the light period (L3 to L9) and stayed constant through a large part of the dark period (D0–D6), declining again after 9 h in the dark (Fig. 4C). Transcript levels of the two nitrogen regulatory genes *ntcA* and *glnB*-like (nitrogen-regulatory protein P-II) were also monitored throughout the diel cycle (Fig. 4D). P-II transcript abundance had its minimum at 9 h into the dark period (D9) and its maximum at the start of the dark period (D0) with a relatively steep increase and decrease. *ntcA* transcripts peaked earlier with a smaller amplitude in the variation of transcript abundance throughout the 24 h cycle.

Discussion

Diazotrophic cyanobacteria evolve O_2 through oxygenic photosynthesis even though nitrogenase, the enzyme complex responsible for N_2 fixation, is O_2-sensitive, emphasizing the need to regulate these two processes. When grown under light–dark conditions in nitrogen-depleted medium, *C. watsonii* showed strong diel variations in nitrogen and carbon assimilation as well as in the expression of the genes and accumulation of the proteins involved in these biochemical processes.

One of the most striking results in our experiments was the cycling of the physiological and biochemical parameters related to N_2 fixation. N_2 fixation was low to undetectable during the light period and peaked at the middle of the dark period. Our results show that the nitrogenase protein NifH is rapidly degraded during the light period and synthesized *de novo* in the dark period. However, in contrast to *Cyanothece* sp. where NifH was absent for the entire light period (Colón-López *et al.*, 1997), some NifH is remaining in *C. watsonii* at the beginning of the light period. The nitrogen fixation results, however, suggest that the residual nitrogenase may have been inactivated upon contact with O_2 generated at the onset of photosynthesis (Fay, 1992). Similarly, an inactive form of NifH protein was observed in *Oscillatoria limosa* throughout a 16:8 h light : dark cycle whereas the active NifH form was present only during N_2 fixation (Villbrandt *et al.*, 1992). Diel patterns in the expression of *nif* genes reflect the diel cycle in N_2 fixation; the peak in transcript abundance, however, preceded the maximum N_2 fixation rate. The strongest variation in gene expression was seen in *nifH* and *nifX* transcripts between light and dark period with a difference of about $3–5 \times 10^3$-fold in relative transcript abundance. These were by far the strongest changes in transcript abundance for all the genes studied here. In *Cyanothece* sp. and *Synechococcus* sp. RF-1, *nifH* transcripts were not detectable during the light period in studies using Northern blot analysis (Huang and Chow, 1990; Colón-López *et al.*, 1997). However, low transcript abundance was detected during the light period in a recent study of the whole *Cyanothece* sp. ATCC51142 transcriptome (Stöckel *et al.*, 2008), showing nearly identical expression patterns for *nifH* and *nifX* when compared with the results in *C. watsonii* from this study. The higher *nifH* transcript abundance relative to *nifX* abundances is also consistent with other transcriptional studies of the *nif* operon. Toepel *et al.* (2008) consistently found higher transcript abundances of *nifH* compared with other *nif* gene transcripts in *Cyanothece* sp. ATCC51142 during the dark period. A possible explanation might be that *nifH* and *nifX* are arranged in two operons with the *nifENX* operon downstream of the *nifHDK* operon. The *nifX* gene might even be transcribed separately within the *nifENX* operon (Huang *et al.*, 1999). Alternatively, it is also likely that both mRNAs have different turnover rates (for review see Deana and Belasco, 2005). The large difference in relative expression of genes involved in N_2 fixation between light and dark phases and the degradation and *de novo* synthesis of NifH during the diel cycle emphasizes the relative importance of N_2 fixation in the activity of unicellular, photosynthetic diazotrophs during a diel cycle (Toepel *et al.*, 2008).

Physiological and biochemical measurements of photosynthetic activity in *C. watsonii* also showed diel variations but not as strong as N_2 fixation. The photosystem (PS) I protein PsaC is responsible for the electron transfer from the core reaction centres to the stromal ferredoxin (for review see Fromme *et al.*, 2001) and is fundamental for photochemical activity as well as the stable assembly of PSI (Takahashi *et al.*, 1991). The PsaC abundance varied during the day with higher values during the late dark and early light period that might be due to a structural change of PSI in order to favour cyclic electron flow during the dark period (Colón-López and Sherman, 1998). The PsbA (D1 protein) is a core reaction centre protein in PSII. Western blot analysis showed a significantly ($P = 0.05$) higher abundance of the D1 protein (PsbA) during the light period but also its persistence during the dark period. The latter is in accordance with findings for *Cyanothece* sp. where the authors found a difference between the abundance of the two different forms of PsbA with D1 form 2 having a higher abundance during the light and D1 form 1 having a steady abundance throughout a 24 h light–dark cycle (Colón-López and Sherman, 1998). The relative abundance of the two known *psbA* genes in *C. watsonii* showed a maximum of about 500-fold difference with *psbA1* always having the higher transcript level. The diel variation was different for *psbA1* and *psbA2* with 40-fold and eightfold differences respectively. Also, the *psbA1* transcripts showed diel variation similar to *psaC* whereas *psbA2* transcripts showed a slightly increased abundance during the dark period. Detailed experiments, beyond the scope of this study, would be needed to understand the subtle differences in the timing of the peak in expression of a gene and the maximum abundance in its corresponding protein. Nevertheless, both the presence of essential photosystem proteins and continued gene expression throughout the dark period indicate that *C. watsonii* may retain functionality of the photosynthetic apparatus in the dark that could be activated by illumination.

The accumulation of carbon during the light period and its degradation accompanied by N_2 fixation during the dark period was suggested by the increasing and decreasing cell size and the molar C : N ratio (Colón-López *et al.*, 1997; Li *et al.*, 2001; Tuit *et al.*, 2004). Two key proteins in the regulation of cellular nitrogen, NtcA and P-II signalling protein (for review see Luque and Forchhammer, 2008), are thought to be sensors for the cellular C : N balance by perceiving the intracellular level of 2-oxoglutarate (Forchhammer, 2004), which is a precursor of ammonia assimilation (for review see Leigh and Dodsworth, 2007). Both gene transcripts showed diel variation with maxima during the light and minima during the dark period, suggesting the involvement of both proteins in the assimilation or regulation of nitrogen in *C. watsonii*. Further evidence for the assimilation and storage of carbon during the light and its degradation in the dark is provided by the reciprocal expression patterns of *glgA* and *glgP* that encode a glycogen synthase and glycogen phosphorylase respectively. Cyanophycin, a cyanobacterial nitrogen storage molecule, has been shown to be synthesized and degraded during dark and light phase, respectively, in *Cyanothece* sp. (Li *et al.*, 2001). The expression patterns for the genes encoding cyanophycin synthetase (*cphA*) and cyanophycinase (*cphB*) are similar with respect to their maximum at the change from light to dark periods. Although these two enzymes catalyse opposing reactions, i.e. the synthesis and degradation of cyanophycin, the transcript patterns indicate cotranscription of *cphA* and *cphB*. The approximately 10-fold difference in relative expression between minimum and maximum of the analysed *glg* and *cph* genes suggests that the storage molecules glycogen and cyanophycin, and hence their synthesis and degradation, are involved in the carbon and nitrogen metabolism but may not play a major role in the diel cycle of *C. watsonii* compared with N_2 fixation and photosynthesis.

Recent whole-genome microarray data for *Cyanothece* sp. cultures showed that about 30% of the analysed genes exhibit diurnal fluctuations (Stöckel *et al.*, 2008; Toepel *et al.*, 2008). Metatranscriptomic analysis of natural marine microbial assemblages enriched in *Crocosphaera*-like cells and RT-qPCR analysis of nucleic acid samples collected from depth profiles ranging from surface to about 100 m depth, nevertheless, showed no pronounced difference in diel expression of nitrogenase cluster and *nifH* transcript abundance respectively (Hewson *et al.*, 2009). Besides methodological biases mentioned by the authors, the sampling time is one of the most crucial steps in gene expression analysis. The sampling for day and night expression was about 8 and 6 h after sunrise and sunset respectively, and thus may not have coincided with the minimum and maximum expression of *nifH* for *C. watsonii*. Recently, Webb and colleagues (2009) showed that *C. watsonii* strains displayed a fivefold difference in N_2 fixation rate per cell, a fact that demonstrates the need for isolation of unicellular diazotrophs into pure cultures as well as further physiological characterization of isolates in order to obtain more precise N_2 fixation rates combined with environmental abundance data for modelling studies (Goebel *et al.*, 2007). *Crocosphaera watsonii* can be abundant in environmental samples and its presence can easily be assessed from their *nifH* sequence in DNA samples (Falcón *et al.*, 2004; Montoya *et al.*, 2004; Church *et al.*, 2005a; 2005b; 2008; Langlois *et al.*, 2005; Fong *et al.*, 2008; Hewson *et al.*, 2009; Webb *et al.*, 2009). However, the design of sampling strategies for transcriptomic studies and the interpretation of field data related to the detection of *C. watsonii nifH* transcripts are more complex given the strong diel cycle in N_2 fixation observed in this species. Our study provides basic information on the diel cycle of *nifH* transcript levels in relation to the peak in N_2 fixation and nitrogenase abundance, which should be considered in the interpretation of field studies.

Experimental procedures

Culture conditions and experimental set-up

Batch cultures of *C. watsonii* WH8501 were grown at 28°C in a 12:12 h light : dark cycle with a light intensity of approximately 50 $\mu E\ m^{-2}\ s^{-1}$. Cultures were kept in YBCII medium (Chen *et al.*, 1996) without combined nitrogen in 1 l polycarbonate bottles. Only axenic cultures were used to perform the experiments. This was ensured by inoculating an aliquot of the culture into liquid marine broth (Difco 2216), as well as inoculating positive and negative controls, consisting of tapwater and sterile distilled water respectively. Bacterial growth was monitored visually for a minimum of 1 week. Positive controls and a (discarded) contaminated culture showed visible growth within 1 day, and axenic cultures and negative controls had no visible growth after 1 week (Bertilsson *et al.*, 2003). Microscopic verification with acridine orange stained aliquots of controls and axenic cultures confirmed the results of the visual test. Experiments were conducted during the exponential growth phase, which was monitored through cell counts using a coulter counter (Beckman). To facilitate sampling both light and dark periods at the same time, two sets of triplicate cultures were grown in incubators set with opposite light/dark conditions during the period of 08:00–20:00. Subsamples were collected every 3 h from 08:00 to 20:00. Results are reported as means with standard error of triplicate cultures except for the Western blots and the associated densitometric analysis that are from a single sample of a replicated experiment.

Cell size and abundance, pigment analysis, elemental stoichiometry and photosystem response

Subsamples for analytical flow cytometry were preserved with 2.0% (w/v) glutaraldehyde (final concentration) for 24 h at 4°C and stored at −80°C until further analysis. Frozen samples were thawed and analysed for cell abundance, and chlorophyll *a* and phycobilisome relative fluorescence in a FACScalibur (Becton Dickinson) flow cytometer equipped with a 488 nm laser. Cell size was assessed with a Beckman coulter counter. Cellular carbon : nitrogen (C : N) molar ratios were determined by filtration of 50 ml subsample onto precombusted (450°C, overnight) GF/F filters (Whatman) and storage at −20°C until analysis. Thawed filters were oven-dried for 6 h at 60°C and acid-fumed (HCl) for 24 h at room temperature prior to measurements of particulate organic carbon and particulate nitrogen in an Euro-EA (Hekatech) elemental analyser. Photosynthetic quantum yield was assessed using a PAM-fluorometer (PhytoPAM, WALZ) after a dark adaptation of 20–30 min.

N_2 fixation measurements

Rates of N_2 fixation were measured using the acetylene reduction assay (Capone, 1993). Ethylene produced was calculated using the Bunsen gas solubility coefficients from Breitbarth and colleagues (2004). N_2 fixation is presented as ethylene production rates. For the assay, 19 ml of culture was placed in 20.9 ml glass vials and capped with PTFE-coated septum caps. Gas-tight syringes were used to inject 300 μl of acetylene into the headspace. In order to reduce the contamination of background ethylene, the acetylene was cryogenically batch-purified and stored in a SilcoCan canister. Samples were incubated for 3 h at the previously described growth conditions. A 300 μl headspace sample was then analysed in a Shimadzu GC14B gas chromatograph equipped with a flame ionization detector and a Rt-Alumina PLOT column. Headspace ethylene concentration was calibrated using a dilution series of pure ethylene ranging from 1 to 1000 p.p.m.

Protein extractions and Western blotting

Subsamples of 10^7 total cells were collected by centrifugation (10 min, 5000 r.p.m., 4°C) and cell pellets were flash-frozen in liquid nitrogen until extraction. Whole-cell extracts were prepared by addition of 50 μl lysis buffer (LaRoche *et al.*, 1993), with final concentrations of 4% sodium dodecyl sulphate (SDS), 68 mM Na_2CO_3, 0.4 nM phenylmethylsulfonyl fluoride and subsequent sonication (2 × 10 s). Samples were centrifuged for 4 min at maximum speed and the cell debris discarded. Protein concentrations were assessed with the BCA Protein Assay Kit (Pierce), and remaining sample was diluted with sample buffer with final concentrations of 50 mM dithiothreitol, 7.5% glycerol, 1% SDS, 17 mM Na_2CO_3, 0.25% bromophenol blue. The sample was then heated for 2 min at 98°C, flash-frozen in liquid nitrogen and stored at −80°C until Western blotting. Equivalent amounts (9 μg) of extracted proteins were separated in a SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12% Tris-HCl and 10–20% Tris-HCl precast Ready Gels from BIO-RAD for NifH/PsbA and PsaC/PsbO respectively) for 60 min at 130 V. Separated proteins were transferred onto Immun-Blot PVDF (polyvinylidene difluoride) membranes (0.2 μm, BIO-RAD) for 60 min at 100 V in a Mini-PROTEAN II cell. Blots were blocked in 4% (w/v; non-fat dry milk) milk solution for 2 h prior to each immunoblotting. Primary antibodies were Anti-PsaC, Anti-PsbA, Anti-PsbO and Anti-NifH [Anti-NifH kindly provided by Paul Ludden, all others from Agrisera AB; diluted in 1% (w/v) milk solution prior to immunoblotting]. Anti-PsaC (1:2500 dil.) and Anti-PsbO (1:1000 dil.) were done on the same blot as were Anti-NifH (1:5000 dil.) and Anti-PsbA (1:1000 dil.). Secondary antibody was rabbit Anti-IgG (1:15000 dil.; Pierce). Blots were developed with the chemiluminescence system SuperSignal West Pico (Pierce) and protein size was estimated using the Spectra Multicolor Broad Range Protein Ladder (Fermentas).

RNA extraction, cDNA synthesis and RT-qPCR

RNA samples were collected by centrifugation (10 min, 5000 r.p.m., 4°C) of 10^7 total cells. The cell pellet was immediately flash-frozen in liquid nitrogen and stored at −80°C until extraction. RNA was extracted using the RiboPure-Bacteria Kit (Ambion) following the manufacturer's manual, starting with addition of RNAwiz solution to the frozen cell pellet. Extracted RNA was eluted in two times 25 μl (total 50 μl) of the supplied elution solution. Eluted RNA was then treated with TURBO DNA-free (Ambion) and reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) and random hexamers. RNA concentrations were measured with the RiboGreen Kit (Molecular Probes). For RT-qPCR, 15 *C. watsonii* specific primers (Table 1) were designed using the software Primer Express v2.0 (Applied Biosystems) and based on the draft genome sequence of *C. watsonii* (permanent draft assembly 30 January 2004, last update 17 October 2007) available from the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/. Each RT-qPCR reaction (25 μl) consisted of 12.5 μl Platinum SYBRGreen qPCR SuperMix-UDG with ROX (2×), 6.5 μl PCR-H_2O, 0.5 μl of each forward and reverse primer (10 pmol μl^{-1}) and 5 μl template cDNA. Samples were run in duplicates and no-template controls were included for each primer pair. At the beginning of the analysis, RNA samples were used as template in RT-qPCR to check for genomic DNA contamination. In most samples no genomic DNA contamination was present and where present it contributed equal or less than 0.01% of the measured amount of total DNA (DNA and cDNA). The RT-qPCR reactions were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the following thermal cycling conditions: 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s and 60°C for 30 s, followed by a dissociation stage of 95°C for 15 s, 60°C for 20 s and 95°C for 15 s. The resulting Ct values were used to calculate the relative enrichment of transcript copies by applying the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) according to the following equations:

Table 1. Primer sequences used in RT-qPCR.

Target gene	Gene ID	Forward/reverse	Primer sequence (5′-3′)
16S	CwatDraft_R0029	F	CAT CAA ACC CAG CCT CAG TTC
		R	TTC ATG CTC TCG AGT TGC AGA
cphA	CwatDraft_0476	F	ACA AAT GGC CCA GGT AAA AGG
		R	GCC ATA GCT GCT ACC AAA GGA
cphB	CwatDraft_0475	F	TCG GCC AAT AAG CCA CAC AGT
		R	ATC GCG ACC AAG GAA ACG A
glgA	CwatDraft_6377	F	GGA ACT GTT CCC ATT GTG CGT
		R	ACG GTG CAT GGC TGA TTC TAA G
glgP	CwatDraft_4758	F	TAA CGG CCC ACG AAG TTT ACG
		R	CCG CTA GAA ATG CGA TCG AT
glnA	CwatDraft_5271	F	GCC TTT TTT GGA CCA GAA GCA
		R	TTC TCG GCC AGT ATT CCA ACG
nifH	CwatDraft_3818	F	TGC TGA AAT GGG TTC TGT TGA A
		R	TCA GGA CCA CCA GAT TCT ACA CAC T
nifX	CwatDraft_3824	F	TGT CCA TGT CAA TGC CCA CTT
		R	TCG CCA CCG AAC TCT AAG GTA
ntcA	CwatDraft_0834	F	TGG TTC AAC CCG TGT GAC AGT
		R	TCT TGG CGT AAA TCT CCG AGA
P-II	CwatDraft_5924	F	TCG CGG TTC GGA ATA TAC G
		R	AGC CCC TCT CAG CTT GTC AAT
psaC	CwatDraft_5974	F	TTG CTT CCT CCC CTC GTA CA
		R	TTT CGC ATC GCT TAC AGC C
*psbA*1	CwatDraft_1423	F	CTT CCT TCA ACA ACA GCC GTG
		R	CAG GCC ATG CAC CTA AGA AGA
*psbA*2	CwatDraft_4668	F	AGG ATT CCA AGC TGA CCA CAA
		R	GAA ACC GCC AAC ATA TGC AA
psbO	CwatDraft_4858	F	AAC ACC GGA ATT GCC AAC A
		R	TTG CAA GCA CAG ATC GTC AAC
rpb1	CwatDraft_3959	F	ACC GAA GCG GCT ATT GAA GGT
		R	TCC GGC AGG AAT CAA ACG A

The gene ID of the permanent draft genome assembly (30 January 2004, last update 17 October 2007) is as reported on the Integrated Microbial Genomes website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) provided by the Joint Genome Institute, U.S. Department of Energy.

$$\Delta \mathrm{Ct}_{\mathrm{GOI}} = \mathrm{Ct}_{\mathrm{GOI}} - \mathrm{Ct}_{16\mathrm{S}} \qquad \text{(Eqn 1)}$$

and

$$\Delta\Delta \mathrm{Ct}_{\mathrm{GOI}} = \Delta \mathrm{Ct}_{\mathrm{GOI}} - \mathrm{mean}(\Delta \mathrm{Ct}_{rpbI}) \qquad \text{(Eqn 2)}$$

In Eqn 1, the Ct values of the genes of interest (GOI) are normalized to the Ct values of an endogenous reference gene, here taken as the 16S rRNA gene, at each time point. In Eqn 2, the $\Delta\mathrm{Ct}_{\mathrm{GOI}}$ is normalized to a calibrator that was chosen here as the mean of $\Delta\mathrm{Ct}_{rpbI}$ for the entire diel cycle. The *rpbI* gene was chosen as the calibrator because it did not vary more than twofold relative to the 16S rRNA endogenous control within the 24 h cycle. Calculating ΔΔCt from time-course experiments using a specific time point (such as time point zero) as the calibrator (Livak and Schmittgen, 2001) results in a loss of information regarding the abundance of transcripts of different genes relative to each other. In order to retain this information, here we calculate ΔΔCt with respect to the *rpbI*, which enables one to see the relative expression not just within one gene throughout the experiment, but also the relative transcript abundance among the different genes. In cases where Ct values were undetermined, samples were assigned a Ct value of 40 (corresponding to the last cycle number) to enable the calculation of ΔCt. Nevertheless, in only two instances transcripts were not detectable in any of the replicates, i.e. at D9 for P-II and at D12 for *nifX*, which might lead to an overestimation of transcript abundance or *vice versa* to an underestimation of variation.

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C. Tracing the high-performance cells: Heterogeneity in a diazotrophic population

Classification: Biological Sciences, Environmental Sciences

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Abstract

The unicellular diazotrophic cyanobacterium *Crocosphaera watsonii* is abundant in the subtropical and tropical areas of the Atlantic and Pacific Oceans. Due to its capability of fixing atmospheric dinitrogen (N_2) and providing thus new nitrogen to the upper ocean it is an important contributor to the oceanic nitrogen cycle. Here, using nanometer-scale secondary ion mass spectrometry (nanoSIMS) technology, we show that a population of *C. watsonii* can be quantitatively analysed at the single-cell level demonstrating population heterogeneity in N_2 fixation and photosynthetic rates. During a 12:12 h dark:light cycle, single-cell rates varied from 0 – 2.3 fmol N cell^{-1} h^{-1} and from 0 – 11.2 fmol C cell^{-1} h^{-1} for N_2 and inorganic carbon fixation, respectively. During a subsequent 24 h continuous light period, we show that the *C. watsonii* population was capable of both inorganic carbon as well as N_2 fixation within the respective dark period by circumventing the dilemma of an O_2-sensitive nitrogenase and photosynthetically-derived O_2 at the cellular rather than population level. Gene expression analysis suggested that N_2-fixing activity during that period was regulated at the protein level. Highest rates of N_2 fixation and inorganic carbon fixation were respectively 6 and 2.4 times higher than the bulk mean rate of the population indicating that *C. watsonii* has a potential for increased N_2 fixation and photosynthesis which in turn may be controlled by yet-unknown factors including stochasticity. In the case of an internal control, this could limit the input of new nitrogen via N_2 fixation into the surface ocean.

Introduction

Single-cell measurements have intrigued scientists for more than a few decades. Numerous observations in population heterogeneity have been made using mostly flow cytometry or microscopic methods (*e.g.*, Schuster *et al.*, 2000; Elfwing *et al.*, 2004; Ingham *et al.*, 2008; Strovas and Lidstrom, 2009). Differences have been recognized in, *e.g.*, behaviour (Spudich and Koshland, 1976), DNA content (Lebaron and Joux, 1994), gas vesicle volume (Brookes *et al.*, 2000), growth rate (Kelly and Rahn, 1932; Strovas *et al.*, 2007) and (m)RNA (Strovas *et al.*, 2007) which encompass variation in behavioural, genetic and physiological properties. The scope of population heterogeneity in metabolic processes, however, is much smaller (Brookes *et al.*, 2000; Lechene *et al.*, 2007; Popa *et al.*, 2007). The more recent single-cell studies used either multiple-isotope imaging mass spectrometry (MIMS) or nanometer-scale secondary ion mass spectrometry (nanoSIMS) to reveal sub-cellular isotopic composition coupled to stable-isotope incubations (Lechene *et al.*, 2007; Popa *et al.*, 2007; Finzi-Hart *et al.*, 2009). In environmental studies, nanoSIMS is also combined with *in situ* hybridization in order to link the identity of an organism to its activity (Behrens *et al.*, 2008; Li *et al.*, 2008; Musat *et al.*, 2008). The approach to measure single-cell dinitrogen (N_2) fixation rates, though, has only been done very few times using cultures of, for example, *Anabaena oscillarioides* (Popa *et al.*, 2007), *Teredinibacter turnerae* (Lechene *et al.*, 2007) or *Trichodesmium* sp. (Finzi-Hart *et al.*, 2009). However, N_2 fixation rates were only presented as relative rates, increase in atom percent or rates relative to biomass. Variability in N_2 fixation between individual cells was shown for *A. oscillarioides* (Popa *et al.*, 2007) and *T. turnerae* (Lechene *et al.*, 2007) which are a heterocystous cyanobacterium and a symbiotic proteobacterium, respectively. In trichomes of *Trichodesmium* sp., a non-heterocystous, filamentous cyanobacterium, single-cell rates did not show much variation except for decreased rates at the centre of trichomes which may have derived from trichome splitting (Finzi-Hart *et al.*, 2009).

The unicellular, diazotrophic cyanobacterium *Crocosphaera watsonii* separates N_2 fixation and photosynthesis temporally with N_2 fixation occurring in the dark and photosynthesis in the light (Tuit *et al.*, 2004; Mohr *et al.*, 2010; Shi *et al.* 2010; this study). The two processes are often regulated under a circadian rhythm (*e.g.*, Schneegurt *et al.*, 1994; Chow and Tabita, 1994; Chen *et al.*, 1998; Colón-López and Sherman, 1998), and diel expression of genes related to N_2 fixation and photosynthesis is known for *C. watsonii* (Mohr *et al.*, 2010; this study). *C. watsonii* is abundant in the tropical and subtropical areas of the Atlantic and Pacific oceans (Fong *et al.*, 2008; Church *et al.*, 2008; Langlois *et al.*, 2008; Church *et al.*, 2009; Hewson *et al.*, 2009). It is believed to markedly contribute to total N_2 fixation, *i.e.* 'new nitrogen', into the surface ocean (Zehr *et al.*, 2001; Montoya *et al.*, 2004; Goebel *et al.*, 2007). Nevertheless, the magnitude of its contribution still remains ambiguous as indicated by the recent discovery of different phenotypes of *C. watsonii* which varied in N_2 fixation rates by a factor of five (Webb *et al.*, 2009). Using stable isotope incubations and nanoSIMS technology, we show here that *C. watsonii* strain WH8501 displayed heterogeneity in single-cell N_2 fixation and photosynthetic rates within a population. The single-cell rates presented here are, to our knowledge, the first quantitative single-cell N_2 fixation rates for a unicellular diazotroph. The average nitrogen and carbon fixation rates calculated from more than 40 individual cells as well as the highest single-cell rates are compared to bulk measurements of N_2 fixation and photosynthesis during a 12:12 h dark:light cycle followed by a 24 h continuous light cycle.

Results and Discussion

Bulk analysis

N_2 fixation rates in *C. watsonii* determined via acetylene reduction assay (ARA) followed the diel pattern known for this organism (Tuit *et al.*, 2004; Mohr *et al.*, 2010) during the first 24 h of the experimental period encompassing a 12:12 h dark:light cycle with N_2

fixation occurring during the dark period (Figure 1A). N_2 fixation rates measured via the incorporation of $^{15}N_2$ into biomass (Figure 1A) closely matched the observed reduction of acetylene with a ratio of 4.5:1 of ethylene produced to N_2 fixed which compared well with the conversion factor of 4:1 (Capone, 1993). Inorganic carbon fixation as a measure of photosynthetic activity was constrained to the light period of the dark:light cycle (Figure 1B). It is very well known, that unicellular, diazotrophic cyanobacteria separate N_2 fixation and photosynthesis temporally (Colón-López *et al.*, 1997; Colón-López and Sherman, 1998; Taniuchi and Ohki, 2007; Taniuchi *et al.*, 2008) to counteract the inactivation of the nitrogenase enzyme by molecular oxygen (O_2) (Fay, 1992; Gallon, 1992). We exposed *C. watsonii* to a 24 h continuous light cycle just after a 12:12 h dark:light cycle to determine how *C. watsonii* regulates both N_2 fixation and photosynthesis under the stress that continuous light poses on the population. Surprisingly, both inorganic carbon and N_2 fixation rates were measurable concurrently at the population level (Figure 1) during the respective dark period in which the cells were kept in continuous light rather than returned to the expected dark phase. However, N_2 fixation was about 86% lower than the maximum observed during the regular dark period. Photosynthetic rates were maintained during the respective dark period at about 71% of its regular light phase activity providing evidence for, at least partially, functional photosystems during the dark that could be activated upon illumination. Both processes were integrated in the molar C:N (carbon:nitrogen) ratio (Figure 1B), as indicated by the increase in the C:N ratio compared to the previous dark and light periods, which reflected the accumulation of both carbon and nitrogen in the biomass during the respective dark period. The accumulation of glycogen has been observed in *Cyanothece* sp. during a respective dark period as well as concurrent, but decreased N_2 fixation which was attributed to decreased respiration leading to a shortage in energy supply (Toepel *et al.*, 2008). Taking into account the rates of N_2 fixation and photosynthesis measured here as well as the molar C:N ratio, the flattened slope in the C:N ratio (as compared to the regular light period) could be attributed to the maintained but lower rate of photosynthesis and N_2 fixation which together only account for 67% of the lowered slope. Another 33% could be contributed by additional respiration which supports nitrogenase activity in the light (Maryan *et al.*, 1986).

Expression of genes related to N_2 fixation and photosynthesis was analysed to identify possible deviations between the regular dark and light phases and the artificial continuous light phase. Special emphasis was on the respective dark phase due to the co-occurrence of N_2 fixation and photosynthetic activity. However, gene expression levels for *nifH* abundance did not show any differences in peak abundance between the regular and the respective dark period (Figure 2A). Given the reduced N_2 fixation in the respective dark period, the regulation of N_2-fixing activity must be at the protein level (Toepel *et al.*, 2008), resulting from the irreversible inactivation of the nitrogenase complex by the copious amount of O_2 produced as a result of photosynthetic activity during the respective dark period (Fay, 1992). We cannot distinguish whether the slight deviations that were observed resulted from the artificial light period or were within the natural variation in culture experiments (Stöckel *et al.*, 2008). However, all genes analysed here showed remarkable cycling as in the regular dark period (Figure 2) suggesting circadian regulation of N_2 fixation and photosynthesis in *C. watsonii* (Pennebaker *et al.*, 2010). We also analysed two genes coding for proteins of the cyanobacterial circadian clock. The *kaiC* gene codes for the essential circadian clock protein KaiC (Ishiura *et al.*, 1998) which is involved in the setting of the circadian clock via the ratio of phosphorylated to unphosphorylated KaiC (Xu *et al.*, 2003). KaiA, another essential circadian clock protein (Ishiura *et al.*, 1998), promotes the phosphorylation of KaiC and thus the acceleration of the circadian clock (Xu *et al.*, 2003). Both genes showed cyclic expression patterns throughout the entire experimental period giving further evidence for circadian regulation. The circadian clock proteins appear to globally regulate gene expression through the condensation status of the DNA (Mori and Johnson, 2001a) and are assumed to play a role in the gene expression patterns in *C. watsonii* (Pennebaker *et al.* 2010). Detailed expression studies supplemented with protein analysis would give further insight into the possible deviations in expression patterns observed here and regulatory processes but are beyond the scope of this manuscript.

Eventually, all of these measurements including the gene expression analysis only represented the population average.

Single-cell analysis

The co-occurrence of N_2 fixation and photosynthesis during the respective dark period indicates that the population was heterogeneous with assemblages of cells that either fixed N_2 or photosynthesized due to the incompatibility of both processes. The development of nanoSIMS protocols to measure single-cell isotopic composition after stable isotope incubations provided the means to determine single-cell N_2 fixation as well as photosynthetic rates and to yield further evidence for population heterogeneity in metabolic processes. N_2 fixation rates within the regular dark period varied from 0 to 2.3 fmol N cell^{-1} h^{-1} (Figures 3 and 4) with a mean of 0.4 fmol N cell^{-1} h^{-1}. Regarding total N_2 fixation of the population, 37% of the cells with higher than average rates contributed about 88% of the total N_2 fixation. Single-cell photosynthetic rates in the regular light period also showed variability ranging from 0 to 11.2 fmol C cell^{-1} h^{-1} with a mean of 4.6 fmol C cell^{-1} h^{-1} (Figures 3 and 4B) but not as pronounced as for N_2 fixation. These results demonstrated high cell-to-cell variability within the population in both N_2 fixation and photosynthesis. Average values of the single-cell rates compared well to the bulk measurements (Figure S1) and likewise N_2 fixation was restricted to the regular and respective dark periods, and photosynthesis was measurable throughout all periods receiving light. In the regular and the last light period, only cells carrying out inorganic carbon fixation were found. In the regular and respective dark periods, some cells showed a labelling with ^{13}C and ^{15}N (Figure 4A and C) suggesting uptake of inorganic carbon and N_2 fixation within the same cell. For the regular dark period, we believe this represents a true intracellular uptake of $H^{13}CO_3^-$ but that the carbon was not further incorporated in complete darkness. In the respective dark period, the dual-labelling could also result from the incubation such that N_2 fixation was carried out at the beginning of the incubation and inorganic carbon uptake towards the end of the incubation because nitrogenase may have been inactivated by O_2 produced within the population. Anyhow, the simultaneous measurement of both N_2 fixation and inorganic carbon uptake in individual cells revealed different assemblages of cells. In the regular dark period (Figure 4A) there were mainly three clusters of cells: 1) cells that carried out N_2 fixation and did not take up carbon, 2) cells that did not fix N_2 but had a varying uptake of carbon and 3) cells that showed labelling with both isotopes (which was already discussed above). When compared to the respective dark period, the first assemblage (almost) disappeared suggesting that the N_2-fixing assemblage of the population had to suffer from the photosynthetically-evolved O_2. In agreement with this is the fact that cells with the highest inorganic carbon fixation did not fix N_2 and *vice versa* (Figure 4C). Taken together, these results show that *C. watsonii* circumvented the dilemma of the O_2-sensitivity of the nitrogenase at the cellular rather than population level. The definite mechanisms behind this stay elusive, though. The assemblage of cells that did not fix N_2 during the regular dark period accounted for about 36% of the total population. If we assume, for example, that these 36% of the population were cells undergoing division, then this would reveal a growth rate between 0.2 and 0.3 d^{-1} depending on whether it is assumed that cells are about to divide or have just divided. In the latter case, the growth rate would be 0.2 d^{-1} which is close to the observed growth rate in the culture (0.17-0.2 d^{-1}). Hence, the non-N_2-fxing assemblage could have consisted of cells that had just divided especially because *C. watsonii* does not seem to divide equally within the day. Shi *et al.* (2010) though suggested that *C. watsonii* would most likely divide during the early light period due to up-regulated genes that are involved in cellular division. Lechene and colleagues (2007) suggested that differences in the physiological state could explain individual N_2 fixation rates. These differences in physiological state are often attributed to stochastic processes (for review see Kærn *et al.*, 2005; Davidson and Surette, 2008) including gene expression (Chabot *et al.*, 2007; McAdams and Arkin, 1997) and protein synthesis (Cai *et al.*, 2006). Stochastic processes influencing gene expression and protein synthesis are also likely to have occurred in *C. watsonii* since stochasticity preserves the population's capability to react to environmental perturbations (Booth, 2002; Kærn *et al.*, 2005). Also, since global gene expression regulation by circadian control appears to be independent of cellular division (Mori and Johnson, 2001b), it is even more likely that stochastic processes contributed to the cell-to-cell variability. We could here show that the decrease in N_2 fixation and the concurrent photosynthesis in the population is the result of each individual cells' capacity to cope with the incompatibility of N_2 fixation and photosynthesis as a response to a short-term change in the environment (during the respective dark period). The comparison of single-cell rates to bulk measurements of N_2 and inorganic carbon fixation shows that nanoSIMS measurements can be a useful tool in quantifying absolute contributions of diazotrophic groups to total N_2 fixation due to the consistency of both measurements.

Environmental impact

During the regular dark period, the highest single-cell N_2 fixation rate was almost six times higher than the mean rate of the population illustrating that some cells are capable of comparatively high N_2 fixation. This was even more pronounced in the respective dark period where the maximum N_2 fixation rate was nine times higher than the population mean of the regular dark period. The ratio between highest rate and mean rate was only 2.4 for photosynthetic rates but still showed that *C. watsonii* has a potential for increased photosynthesis. These results suggest that: 1) if every cell could be as active as the most active one, total N_2 fixation and photosynthesis would increase. There may hence be a potential for an increase in these processes in the environment leading to an increased input of "new nitrogen" into the surface ocean as well as increased carbon sequestration, and 2) if it is an internal (population-inherent) factor controlling the N_2 fixation and photosynthetic rates of a population, then there could be a limit on how much these populations contribute to "new nitrogen" and CO_2 sequestration into the surface ocean independent of iron- and phosphorus sufficiency. Iron- and phosphorus fertilization (Mills *et al.*, 2004) as well as increased carbon dioxide concentration (Fu *et al.*, 2008) may increase N_2 fixation but in case of an internal control in diazotrophic populations, there may be an upper limit of N_2 fixation and carbon sequestration by unicellular, diazotrophic cyanobacteria.

Materials and Methods

Experimental Setup

Axenic batch cultures of *Crocosphaera watsonii* WH8501 were kept at 28°C in YBCII medium (Chen *et al.*, 1996) without combined nitrogen in a 12:12 h dark:light cycle for several generations followed by a 24 h continuous light cycle leading to an experimental period of 48 h. Cultures were kept in temperature- and light-controlled incubators with opposite dark:light regimes to facilitate sampling. Subsamples for stable isotope incubations and acetylene reduction assay to assess N_2 fixation rates were taken every 4 and 3 h, respectively. Samples for gene expression analysis were taken every four hours during the experimental period. Cell abundance for calculations of cell-based rates was assessed at the beginning of each experimental dark or light phase using analytical flow cytometry. Subsamples for nanoSIMS analysis were taken from the stable isotope incubations at the end of the following incubation periods: the middle of the regular dark period, the middle of the regular light period, the middle of the respective dark period and the end of the continuous light period.

Acetylene reduction assay (ARA)

N_2 fixation rates were assessed by incubating 3 ml of culture in 8.65 ml septum-capped vials containing 650 µl of acetylene in the headspace. Incubations lasted for ~ 3 h and ethylene (C_2H_4) concentrations were then measured in a 250 µl headspace sample using a GC-14B gas chromatograph equipped with a flame ionization detector (FID) and a RT Alumina Plot column. C_2H_4 concentrations were calibrated with a dilution series ranging from 1 to 1000 ppm C_2H_4. C_2H_4 production was converted to N_2 fixation with a conversion factor of 4:1 (C_2H_4 produced:N_2 fixed) (Capone, 1993).

Gene expression analysis

RNA extraction, cDNA synthesis and real-time quantitative PCR were carried out as described previously (Mohr *et al.* 2010). Transcripts levels of genes related to N_2 fixation, photosynthesis and the cyanobacterial circadian clock were detected using *C. watsonii*-specific primers (Table S1). Transcript levels were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) and are presented as enrichment factor relative to the expression of *rpbI* (RNA polymerase) which was chosen as the calibrator.

Stable isotope incubations

N_2 and inorganic carbon fixation rates were determined by simultaneous incubation of 133 ml of culture with 280 µl $^{15}N_2$ and $NaH^{13}CO_3$ (1% of ambient bicarbonate) every 4 h during the 48 h experimental period. Aliquots for elemental stoichiometry and bulk stable isotope analysis as well as for nanoSIMS (nanometer-scale secondary ion mass spectrometry) analysis were taken at the end of each ~ 3-h incubation. Samples for bulk stable isotope analysis were filtered onto pre-combusted (450°C, 4h) GF/F filters (Whatman), oven-dried (60°C for 6h) and stored until analysis. Filters were pelletized in tin cups and analysed using isotope ratio monitoring mass spectrometry. Samples for nanoSIMS were preserved with formaldehyde (1% (v/v) final) for up to 24 h at 4°C and subsequently filtered onto Au/Pd-sputtered GTTP filters (Isopore, 0.22 µm pore size, 25 mm). Filters were rinsed with sterile-filtered (0.2 µm) phosphate-buffered saline solution (PBS buffer), air-dried for 20 min and stored at -20°C until analysis.

NanoSIMS analysis

NanoSIMS samples from the stable isotope incubations were analysed using a NanoSIMS 50L (CAMECA). Analysis was done according to Musat *et al.* (2008) except that we did not record the $^{19}F^-$ ion and the image fields varied in size. Thirty to fifty consecutive images were recorded for each image field, and several image fields were analysed per sample. A single sample from each dark or light period was selected for analysis.

Calculation of N_2 fixation and inorganic carbon uptake rates

Biomass-specific N_2 fixation and inorganic carbon uptake rates were calculated based on the atom percent of ^{15}N and ^{13}C within either the bulk or the single-cell measurements as well as the enrichment achieved by the addition of $^{15}N_2$ and $NaH^{13}CO_3$ within the ~ 3-h incubation. Cellular rates were based on the bulk nitrogen and carbon content of the population.

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Figure legends

Fig. 1: N_2 fixation and photosynthesis during the dark:light and continuous light periods. A. N_2 fixation measured via acetylene reduction assay (ARA; filled circle) and $^{15}N_2$ incubation (open circle). B. Photosynthesis measured via $H^{13}CO_3^-$ incubation (filled circle) and molar C:N ratio (open circle). The grey bars indicate the regular dark period and the striped grey bars indicate the respective dark period. Symbols and error bars represent mean ± SE of triplicate cultures.

Fig. 2: Gene expression analysis shown as enrichment factor of relative transcript abundance. The genes are indicated in the top right corner of each panel. Filled circles represent the experimental data during the 48 h period. The open circles are data superimposed onto the respective dark and continuous light period and are the same data from the first 24 h to facilitate the display of deviations in gene expression versus the regular dark:light cycle. The grey bars indicate the regular dark period and the striped grey bars indicate the respective dark period. Symbols and error bars represent mean ± SE of triplicate cultures.

Fig. 3: Enrichment in ^{15}N ($^{15}N/^{14}N$ images, colour scale: absolute ratio of ^{15}N / (^{15}N + ^{14}N)) due to N_2 fixation by individual *C. watsonii* cells. The aggregation of cells was an artefact of filtration. A. Regular dark period. B. Regular light period. C. Respective dark period. (Scale bars: 5µm). The continuous light period (last 12 h of the experiment) displayed the same results as shown in panel B for the regular light period.

Fig. 4: $^{15}N_2$ fixation and $H^{13}CO_3^-$ uptake rates as calculated from the isotopic enrichment of individual cells (each symbol represents one individual cell). The corresponding dark or light period is indicated in the upper right corner of each panel.

Fig. S1: Comparison of bulk mass spectrometric analysis (black bars) and nanoSIMS analysis (white bars) of inorganic carbon fixation (upper panel) and N_2 fixation (lower panel). D1: regular dark period, L1: regular light period, D2: respective dark period, L2: continuous light period.

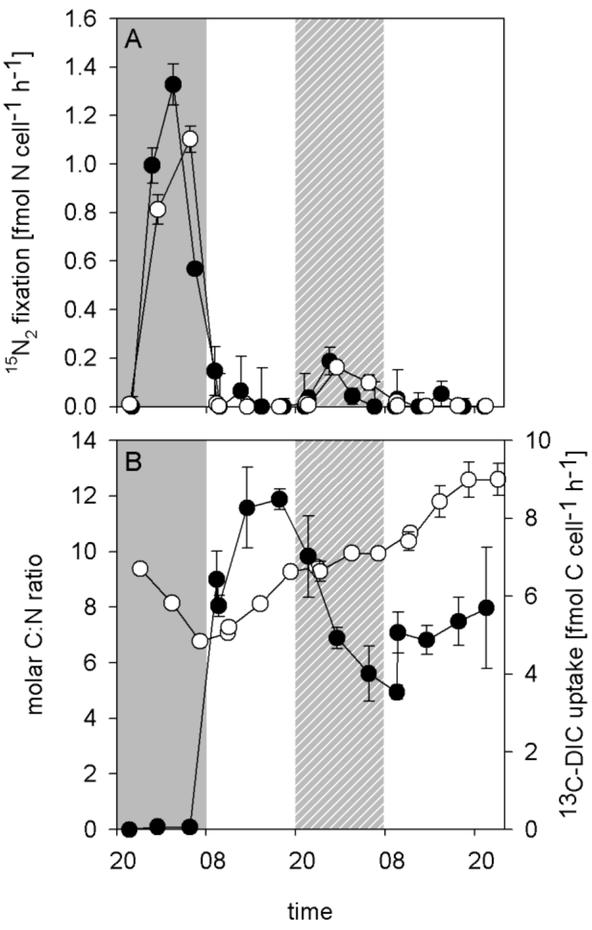
A
B
$^{15}N_2$ fixation [fmol N cell^{-1} h^{-1}]
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
molar C:N ratio
0
2
4
6
8
10
12
14
13C-DIC uptake [fmol C cell^{-1} h^{-1}]
0
2
4
6
8
10
20
08
20
08
20
time

Fig. 1

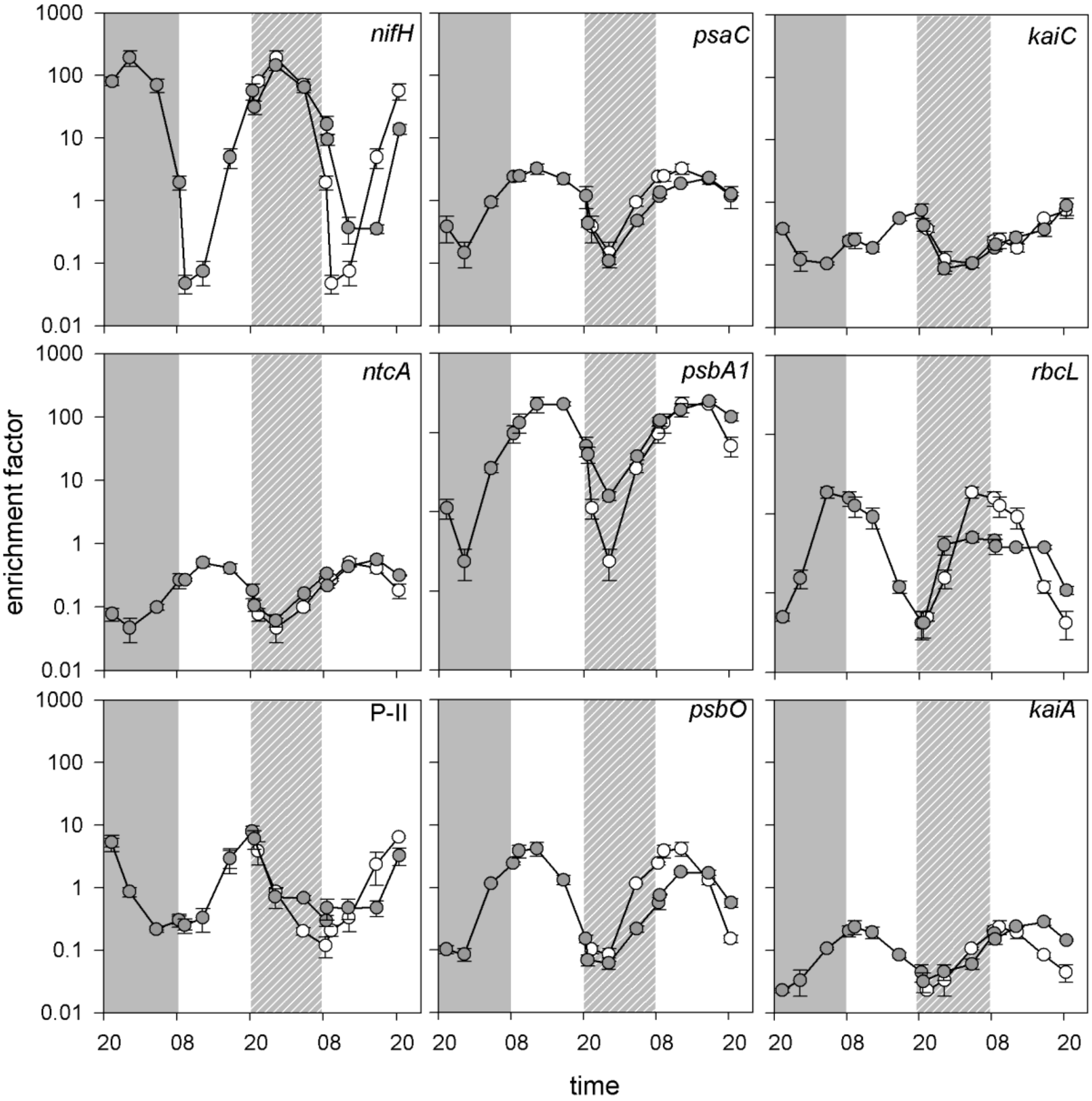
nifH
psaC
kaiC
ntcA
psbA1
rbcL
P-II
psbO
kaiA
enrichment factor
1000
100
10
1
0.1
0.01
20
08
time

Fig. 2

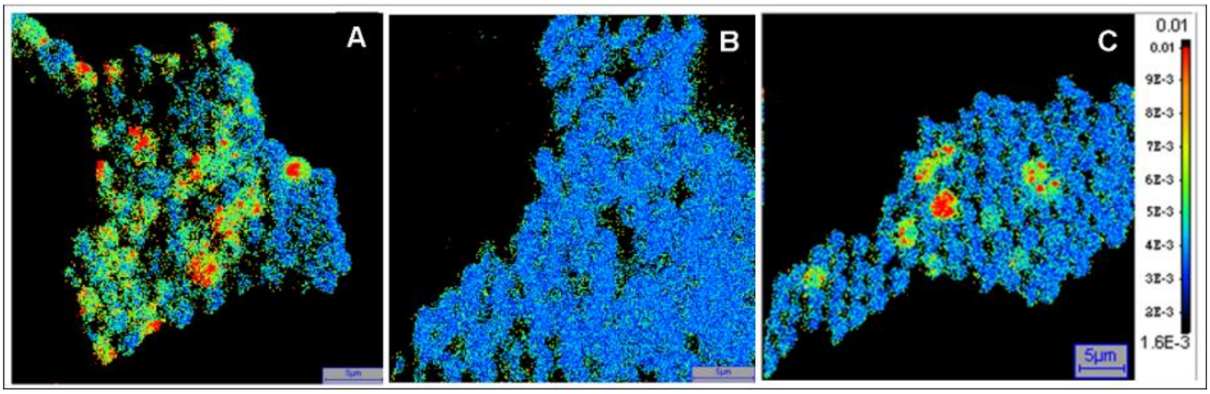

Fig. 3

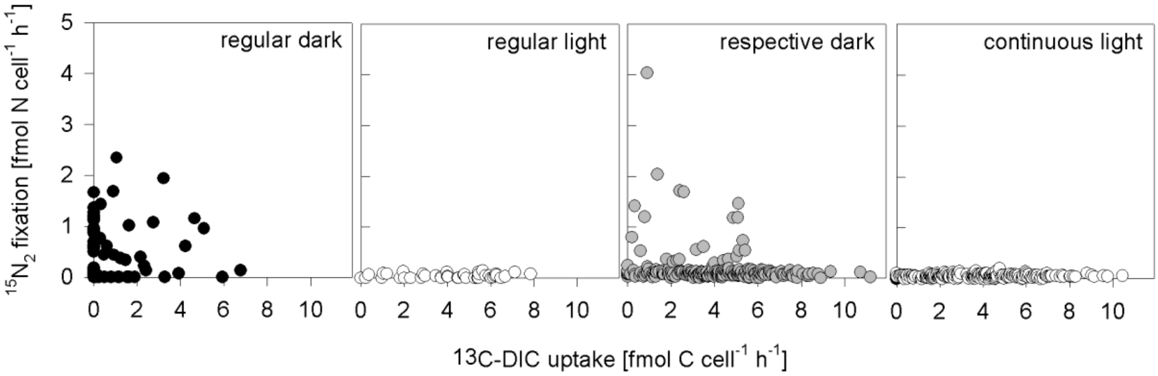
regular dark
regular light
respective dark
continuous light
$^{15}N_2$ fixation [fmol N cell^{-1} h^{-1}]
0
1
2
3
4
5
0
2
4
6
8
10
13C-DIC uptake [fmol C cell^{-1} h^{-1}]

Fig. 4

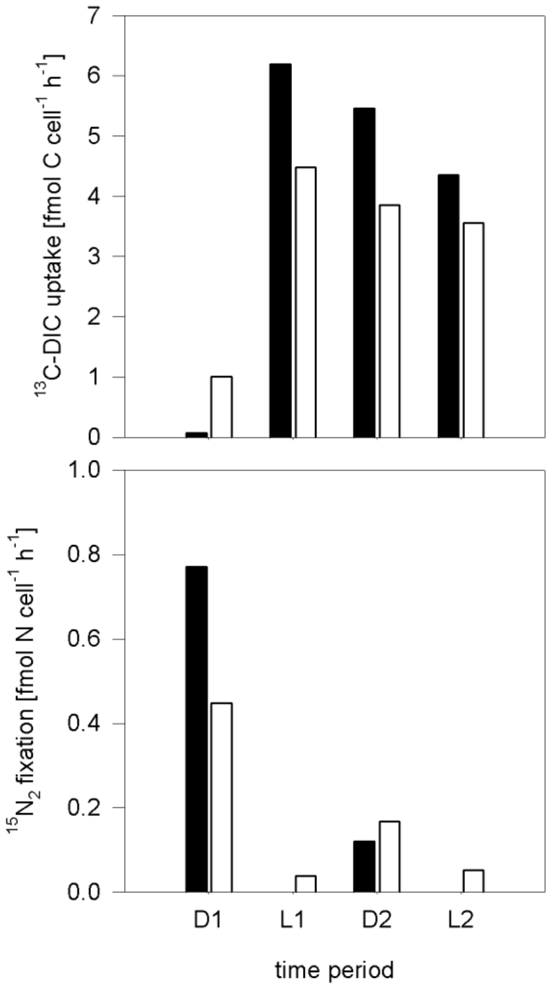
^{13}C-DIC uptake [fmol C cell^{-1} h^{-1}]
0
1
2
3
4
5
6
7
^{15}N$_2$ fixation [fmol N cell^{-1} h^{-1}]
0.0
0.2
0.4
0.6
0.8
1.0
D1
L1
D2
L2
time period

Fig. S1

Table legends

Table S1: Primer sequences used in real-time quantitative PCR (RT-qPCR). The CwatDraft # of the permanent draft genome assembly (30 Jan 04, last update 17 Oct 2007) is as reported on the Integrated Microbial Genomes website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) provided by the Joint Genome Institute, U.S. Department of Energy.

Table S1

Target gene	CwatDraft #	Forward Reverse	Primer sequence (5' – 3')
16S	R0029	F R	CAT CAA ACC CAG CCT CAG TTC TTC ATG CTC TCG AGT TGC AGA
kaiA	4942	F R	TGG CGA AGA TGC CGA CAT TA CGT CCA TCA GTT CCA TGT GCA
kaiC	4944	F R	TCC ATC GAT TCG GTT ACT GCA GCG AAA AAT CTC CCG TCT CAC
nifH	3818	F R	TGC TGA AAT GGG TTC TGT TGA A TCA GGA CCA CCA GAT TCT ACA CAC T
ntcA	0834	F R	TGG TTC AAC CCG TGT GAC AGT TCT TGG CGT AAA TCT CCG AGA
P-II	5924	F R	TCG CGG TTC GGA ATA TAC G AGC CCC TCT CAG CTT GTC AAT
psaC	5974	F R	TTG CTT CCT CCC CTC GTA CA TTT CGC ATC GCT TAC AGC C
*psbA*1	1423	F R	CTT CCT TCA ACA ACA GCC GTG CAG GCC ATG CAC CTA AGA AGA
psbO	4858	F R	AAC ACC GGA ATT GCC AAC A TTG CAA GCA CAG ATC GTC AAC
rbcL	2714	F R	CTT CCG CAT GAC TCC CCA TGC TGC ACC GGC TTC TTC
*rpb*1	3959	F R	ACC GAA GCG GCT ATT GAA GGT TCC GGC AGG AAT CAA ACG A

III. DISCUSSION

III. Discussion

N_2 fixation in unicellular diazotrophs and emerging issues

Unicellular diazotrophs have been recognized as important contributors to the marine nitrogen cycle (Zehr *et al.* 2001, Montoya *et al.* 2004). They are abundant in oceanic regions, however, only a few organisms have been isolated and are in culture (*e.g.*, *Synechococcus* sp. RF-1, *Cyanothece* sp. ATCC 51142, *Crocosphaera watsonii* WH8501). An abundant unicellular diazotroph in the open ocean is *Crocosphaera watsonii*. *C. watsonii* is a diazotrophic cyanobacterium, *i.e.* it carries out inorganic carbon fixation under O_2-evolution and N_2 fixation. Since the nitrogenase enzyme is very sensitive to oxygen, *C. watsonii* separates CO_2 and N_2 fixation temporally with CO_2 fixation during the light and N_2 fixation during the dark in light:dark cycles. Dark N_2 fixation in *C. watsonii* has been known since the strain WH8501 was isolated in 1984 and circadian regulation has been suggested (Pennebaker *et al.* 2010). In manuscript B, I could show that photosynthesis and N_2 fixation were indeed temporally separated and that the nitrogenase enzyme was synthesized *de novo* each dark period as hypothesized earlier (Tuit *et al.* 2004).

The abundance of *C. watsonii* in field samples is often determined using molecular methods (Church *et al.* 2005*a*, 2008; Langlois *et al.* 2008). But also the relative activity of *C. watsonii* and other diazotrophs in field samples is assessed using molecular methods based on the expression of the *nifH* gene (Church *et al.* 2005*b*). Manuscript B demonstrated that gene expression for the major nitrogen and carbon metabolic processes (*i.e.* N_2 fixation and photosynthesis) exhibits diel cycling with the peak abundance of the *nifH* gene at the beginning of the dark period. However, gene transcripts are already measurable during the day whereas declining transcript abundances can be observed at the end of the dark period. This diel periodicity is important for the assessment of N_2-fixing activity in field samples. Due to ship schedules, it may not always be possible to sample at the minimum and maximum of diel gene expression in the field but knowledge of the diel variation may hence be taken into account when determining *C. watsonii nifH* gene transcripts. The diel variation in gene transcripts also raises further evidence for a circadian regulation of CO_2 and N_2 fixation and thus optimization of the cellular metabolism in *C. watsonii*. Circadian rhythms are characterized by three criteria: 1) oscillating patterns of ~ 24 h duration, 2) patterns are temperature-compensated, *i.e.* the oscillating behaviour is sustained at different growth temperatures and 3) rhythms can be entrained by environmental cycles such as light-dark cycles. The entrainment by environmental cycles is probably the most important characteristic for the optimization of cellular metabolism. However, *C. watsonii* may retain the ability to photosynthesize during the night (upon illumination) because essential proteins are not entirely degraded as is, for example, the nitrogenase for N_2 fixation (manuscript B). This hypothesis was tested and confirmed by exposing *C. watsonii* to a continuous light period following a dark:light cycle (manuscript C). N_2 fixation and photosynthesis rates using stable isotope incubations as well as gene expression were followed throughout the experimental period. The gene expression analysis confirmed the diel cycling of genes involved in nitrogen and carbon metabolism even throughout the continuous light period yielding further evidence for a circadian regulation of N_2 fixation and photosynthesis. Surprisingly, concurrent, though decreased, N_2 fixation and photosynthesis were measurable in that part of the continuous light period that would have otherwise been a dark period (respective dark). Generally, these two processes should not occur at the same time (or at least not in the same cell) due to the incompatibility of the nitrogenase with O_2 (Gallon 1992). The use of stable isotope incubations gave the opportunity to measure N_2 fixation (N_2 incorporated into biomass) and inorganic carbon fixation at the single-cell level by employing nanoSIMS technology. The results suggested that cells either fixed N_2 or CO_2 but in general not both at the same time. The discrimination between N_2 and CO_2 fixation was likely a result of the O_2-dilemma of the nitrogenase and the photosynthetically-evolved O_2 during the respective dark period. Combining gene expression analysis and single-cell measurements showed that N_2 and CO_2 fixation appear to be regulated by a circadian rhythm for optimization of the cellular metabolism in general but can be influenced by deviations from the ‘normal’ light-dark cycles, *i.e.* environmental perturbations (Pennebaker *et al.* 2010). A striking feature of the single-cell measurements by nanoSIMS was the high variability of the single-cell N_2 fixation and photosynthetic rates. The highest single cell rate was up to 9-fold higher when compared to the mean of the single-cell rates. This high variability has two important implications for N_2 fixation measurements in the field: 1) If every cell could be as active as the most active one, then N_2 fixation could be several-fold higher and 2) if, for example, stochastic processes are the cause for the high variability they may hence constrain the magnitude of N_2 fixation in the environment independent of sufficient nutrient availability such as iron and phosphorus. The results of culture experiments like the ones carried out during this Ph.D. work are helpful tools in the study of environmental N_2 fixation and will contribute to the determination of limiting factors which may otherwise not be discovered if not studied in culture. The results (manuscript B and C) also emphasize the necessity of isolating and culturing environmental diazotrophic strains for their physiological characterization.

However, isolation and culturing are not straightforward. Abundant diazotrophs in the ocean designated the group A cyanobacteria (UCYN-A) have not yet been successfully isolated although this group seems to be widespread in the ocean (Church *et al.* 2005*a*; Zehr *et al.* 2008; Moisander *et al.* 2010). Until recently, the UCYN-A cyanobacteria had only been described by their *nifH* gene sequence. Using analytical flow cytometry, Zehr and colleagues (2008) sorted small-sized cells with a dim autofluorescence and shot-gun sequencing of the sorted cells yielded complete genomic information on the UCYN-A. The metagenome analysis of the UCYN-A indicates that although this group can carry out N_2 fixation and thus should contribute to fixed N, they cannot directly contribute to CO_2 sequestration because they lack genes for oxygenic photosynthesis and carbon fixation (Tripp *et al.* 2010). Due to the high abundance of UCYN-A and their potential contribution to total N_2 fixation but the lack of contribution to CO_2 sequestration, this phenomenon should be incorporated into biogeochemical models of oceanic carbon and nitrogen cycles.

The discovery of the UCYN-A diazotrophs (which are non-O_2-evolving), the presence of heterotrophic diazotrophs in the ocean and the O_2-sensitivity of the nitrogenase draw the attention to distinct regions, the oxygen minimum zones (OMZs). There, the low oxygen content, the low concentrations of fixed N or low N:P ratios due to denitrification could provide potential hot spots for N_2 fixation. To my knowledge, there are no direct measurements of N_2 fixation within the OMZs but geochemical estimates suggest high N_2 fixation rates, for example, for the Eastern South Pacific, *i.e.* the OMZ and upwelling region off South America (Deutsch *et al.* 2007). An expansion of the oceanic OMZs is predicted in the course of global change (Stramma *et al.* 2008). If increased N_2 fixation would be a general phenomenon in OMZs, global inputs of fixed N could increase due to the expansion. However, OMZs are also regions of high denitrification rates and the balance between denitrification and N_2 fixation suggests that the Pacific is a net sink for fixed N (Gruber and Sarmiento 1997). Therefore, an expansion of the OMZs and a resulting increase in denitrification may counterbalance an increase in N_2 fixation. However, the expansion of the OMZs is not the only proposed change associated with global climate change. Transitions in the Saharan mineral dust deposition over the Atlantic could change the availability of iron to the surface ocean (Rijkenberg *et al.* 2008) and result in an increase or decrease in N_2 fixation.

But mineral dust also carries nitrogen and phosphorus compounds (Baker *et al.* 2007) which could alter the impact of dust on N_2 fixation and overall new primary production. An increase or decrease in N_2 fixation would in return influence the oceanic fixed N budget and result in changes of new primary production and CO_2 sequestration. Although increasing CO_2 itself may promote N_2 fixation (Hutchins *et al.* 2007; Fu *et al.* 2008), the availability of iron may diminish this effect (Shi *et al.* 2007, Fu *et al.* 2008).

The ‘missing N’ challenge

Current estimates of sources and sinks of fixed N in the ocean deviate by up to ~ 200 Tg N a^{-1}. However, isotopic analysis of sediments suggests that the oceanic N cycle is in balance (Altabet 2007). Thus the difference between sinks and sources of fixed N in the ocean is also termed the ‘missing N’. The divergence could result from over-estimation of the sinks, mainly denitrification, as well as under-estimation of the sources, mainly N_2 fixation. Estimates of N_2 fixation range between 100 – 200 Tg N a^{-1} (Karl *et al.* 2002, Gruber 2008). However, direct measurements of N_2 fixation do not match N_2 fixation determined through biogeochemical estimates (Mahaffey *et al.* 2005). Part of this discrepancy may have been introduced by the lack of temporal and spatial coverage of direct measurements but even at regional scale where N_2 fixation has been measured, the rates do not correspond to biogeochemical estimates (Orcutt *et al.* 2001). Furthermore, N_2 fixation rates in laboratory and field studies do not meet N requirements as calculated by growth rate or C:N ratio (see manuscript A). There are often large discrepancies between the two direct methods to determine N_2 fixation rates, *i.e.* the acetylene reduction assay (ARA) and the $^{15}N_2$ tracer addition (Gallon *et al.* 2002). In laboratory studies carried out during this Ph.D. work I observed discrepancies between the ARA and the $^{15}N_2$ tracer approach. Since the experiments were carried out with a diazotrophic pure culture, mass balance calculations were applied resulting in a mismatch between N_2 fixation rates determined with the $^{15}N_2$ tracer addition and the increase in cellular N during the experiment. In natural environments, mass balance approaches for discrete samples is often not possible because an increase in cellular N could result not only from N_2 fixation but also from other sources of fixed N, *e.g.* nitrate, ammonium or organic N compounds. However, comparisons of direct measurements of N_2 fixation in the natural environment with geochemical estimates suggest an under-estimation of N_2 fixation (Mahaffey *et al.* 2005). In addition, imbalances between sources and sinks of fixed N, *i.e.* the 'missing N' are observed and together with the other discrepancies point to the need to evaluate the direct methods further. Taken together, the discrepancies in the published literature as well as the discrepancies between my own $^{15}N_2$ tracer addition and mass balance calculations from culture experiments led to the re-assessment of the $^{15}N_2$ tracer addition method. The existing method relies on the rapid equilibration of the injected $^{15}N_2$ gas bubble with the surrounding water to yield a uniform $^{15}N_2$ enrichment of the dissolved N_2 pool (see Introduction section C). Laboratory experiments showed that the equilibration of the injected $^{15}N_2$ is strongly time-dependent spanning incubation times generally applied in laboratory and field studies of N_2 fixation (manuscript A). The direct injection of $^{15}N_2$ gas into a diazotrophic culture and subsequent incubation for 12 h (here the N_2-fixing dark period with a peak activity) yielded only 40% of the actual rate, *i.e.* the N_2 fixation rate was underestimated by 60%. The magnitude of the underestimation, though, varies with the incubation time. Due to the slow equilibration of the $^{15}N_2$ gas bubble, shorter incubations tend to be further away from the real N_2 fixation rate. To determine real N_2 fixation rates, an improved $^{15}N_2$ tracer addition was carried out. This approach relies on the addition of sterile-filtered, $^{15}N_2$-enriched water to the incubations. The $^{15}N_2$-enriched water had previously been degassed, and $^{15}N_2$ gas was dissolved in it. I could show in direct measurements of dissolved $^{15}N_2$ as well as N_2 fixation rates by *C. watsonii* that the addition of $^{15}N_2$-enriched water yielded a uniform enrichment of dissolved $^{15}N_2$ throughout 24 h incubations. Also, the highest rates were achieved using this improved tracer addition. Therefore, I suggest the addition of $^{15}N_2$-enriched water to determine N_2 fixation rates in laboratory and field studies. The results of this study imply that the application of the conventional $^{15}N_2$ tracer approach, *i.e.* the direct injection of a $^{15}N_2$ gas bubble, may have severely underestimated N_2 fixation rates both in culture and field experiments. Attempts to correct for the underestimation in published N_2 fixation data were not successful for several reasons. The enrichment of $^{15}N_2$ in the dissolved N_2 pool is strongly dependent on the incubation time but also on the amount of agitation, the amount of $^{15}N_2$ injected, dissolved organic matter and the bottle size used for the incubation. However, even if several parameters would be known, the amount of agitation is mostly an uncontrolled parameter especially when incubations are carried out on board a ship. In addition, and maybe most importantly, rates of N_2 fixation are substantially influenced by diel periodicity (Colón-López *et al.* 1997; Chen *et al.* 1998; manuscript B). These factors will prevent an accurate determination of the magnitude of underestimation. The application of the method described here may contribute significantly to increase the global estimates of N_2

fixation and thereby diminish the ‘missing N’ especially when combined with a larger spatial and temporal coverage of direct measurements of N_2 fixation.

IV. CONCLUSIONS AND OUTLOOK

IV. Conclusions and outlook

The laboratory experiments carried out during this Ph.D. work using *Crocosphaera watsonii* demonstrated evidence for a tight (circadian) regulation of nitrogen and carbon metabolism in unicellular, diazotrophic cyanobacteria. The timing of metabolic processes does not coincide with the corresponding gene expression which has practical implications for the interpretation of gene expression data obtained from field samples. The magnitude of N_2 fixation in *C. watsonii* may be (partially) controlled by population-inherent factors such as stochastic processes in gene expression and protein synthesis. If an internal factor, such as stochasticity, (partially) controls the magnitude of N_2 fixation within the population then this may imply an upper limit of N_2 fixation in the environment independent of nutrient sufficiency. Stochastic processes lead to unequal distributions of, for example, transcript abundances per cell within a population. Thus, not each cell will be able to produce the same amount of enzyme needed and will hence have differing enzyme activities. These activities may be independent of available nutrients because they will be limited by gene expression or protein synthesis. Whether stochasticity in gene expression or protein synthesis actually applies in C. watsonii, still needs to be tested. Respective experiments have already been carried out in conjunction with the Max Planck Institute for Marine Microbiology in Bremen (MPI Bremen) and are awaiting analysis. Furthermore, variability in single-cell N_2 fixation rates may have been regulated or influenced by the capability of each individual cell to cope with the incompatibility of nitrogenase and O_2. The single-cell measurements with a diazotroph culture demonstrated that stable isotope incubations linked to nanoSIMS can be used to quantitatively analyse single-cell N_2 fixation rates and hence could be very useful in determining the contribution of different phylotypes to total N_2 fixation in field experiments.

The realization that the most commonly used method (N_2fixation rates determined by the direct injection of $^{15}N_2$ gas) to assess N_2 fixation in the field under-estimates the true rate by a variable amount led to the design of a more reproducible method. The subsequent improvement of the $^{15}N_2$ tracer addition method has the potential to diminish the gap between sources and sinks of fixed N in the ocean, *i.e.* the ‘missing N’. If our proposal proves to be true then this study will have a significant influence on current global estimates of N_2 fixation and subsequent biogeochemical modelling of the marine nitrogen cycle. A protocol for the application of the $^{15}N_2$-enriched water addition to determine rates of N_2 fixation will be provided at the end of this section.

Field experiments at the Cape Verde Islands

Besides the culture experiments presented above, I participated in two large field campaigns at the Cape Verde Islands to study the 'Ecosystem effects of dust deposition (iron and nitrogen) on phytoplankton productivity and nitrogen fluxes' as part of the German SOPRAN project (Surface Ocean PRocesses in the ANthropocene). The Cape Verde Islands are located in the eastern tropical North Atlantic Ocean off the coast of Mauretania and Senegal, western Africa. The area around Cape Verde is characterized by a sub-surface maximum of chlorophyll *a*, typical for tropical open ocean regions (Figure IV-1).

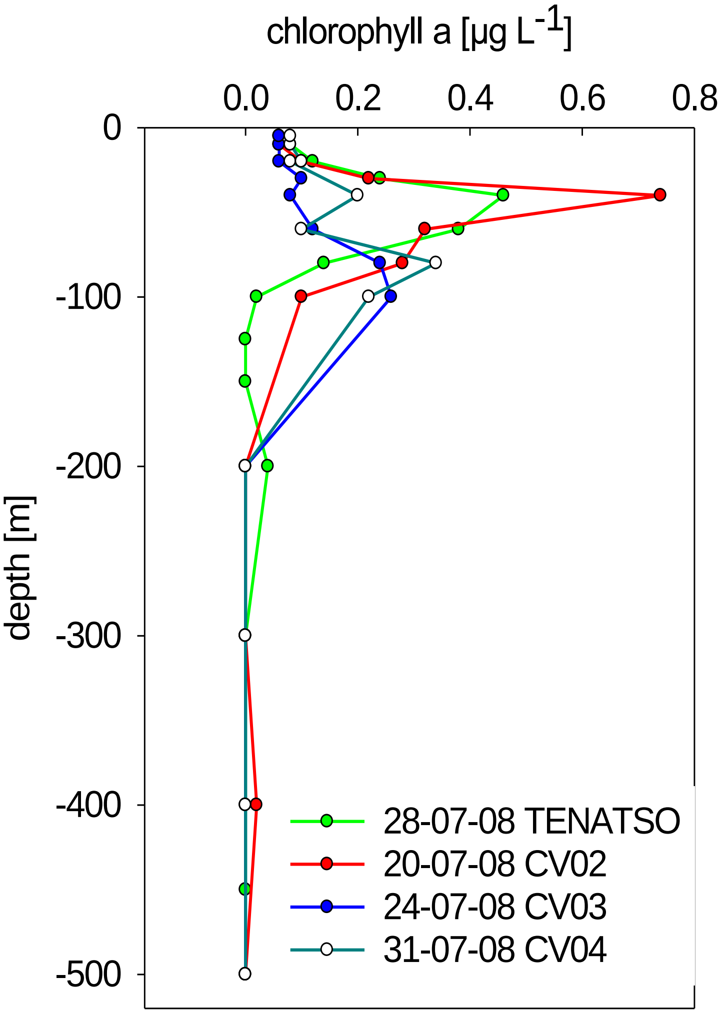

Figure IV-1: Vertical profiles of chlorophyll *a* at various stations CV02-04 in July 2008.

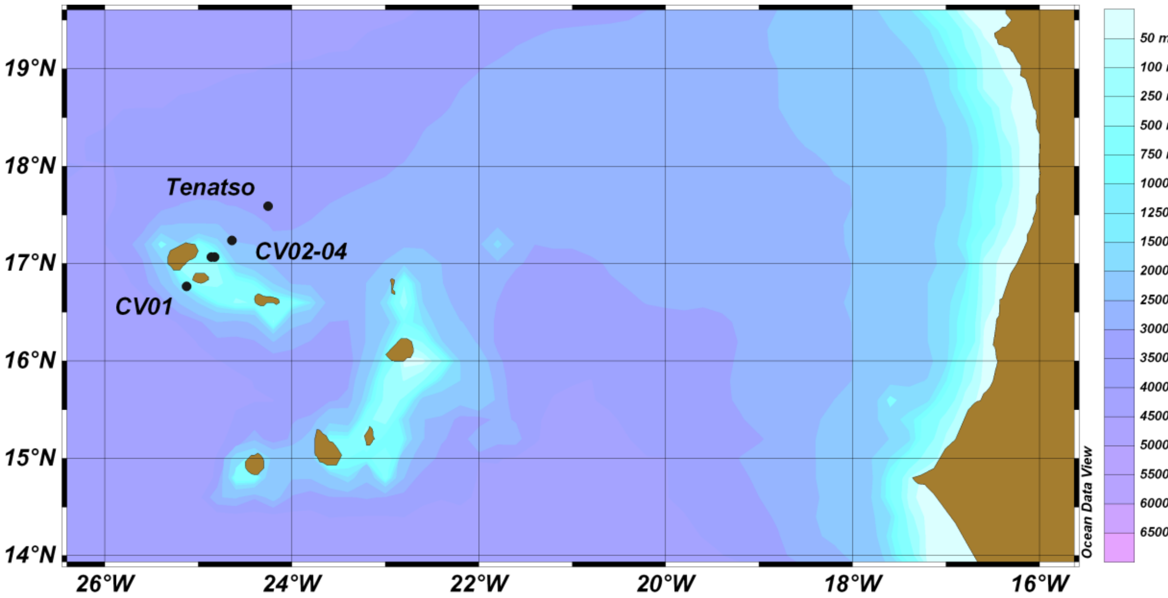

Figure IV-2: Map of the Cape Verde Islands and the surrounding bathymetry. Red symbols indicate the stations which were sampled in July/August 2008. *Tenatso* indicates the ocean site of the Tropical Eastern North Atlantic Time-Series Observatory.

The first field campaign was carried out in July/August 2008, including four bioassay experiments (Figure IV-2; CV01-CV04). Seawater was collected trace metal-clean on board R/V Islandia and distributed into 4.5 L polycarbonate bottles. Samples were transported back to the onshore laboratory in Mindelo, Sao Vicente. Upon arrival, seawater samples were prepared and incubated as described in Mills *et al.* (2004) to determine rates of primary production and N_2 fixation under the influence of nutrient and dust additions. Preliminary results showed that primary production and N_2 fixation were nitrogen and phosphorus-iron co-limited, respectively (Figure IV-3). Additions of Saharan dust stimulated N_2 fixation but not primary production indicating that the added dust did not release significant amounts of nitrogen to promote phytoplankton growth. The dust was most likely a source of iron or possibly iron and phosphorus although the magnitude of N_2 fixation was more similar to the N_2 fixation in the Fe-only treatment. The addition of phosphorus and iron resulted in the highest N_2 fixation rate among treatments but also in the highest abundance of diazotrophs as measured by *nifH* gene copies (Figure IV-3, lower panel). The enormous increase in diazotroph abundance was due to a ‘bloom’ of UCYN-A (data not shown). Furthermore, UCYN-A were the dominant phylotype in all four experiments carried out in 2008 (Figure IV-4) suggesting that these unicellular cyanobacteria are an important component of the nitrogen cycle in the eastern tropical North Atlantic.

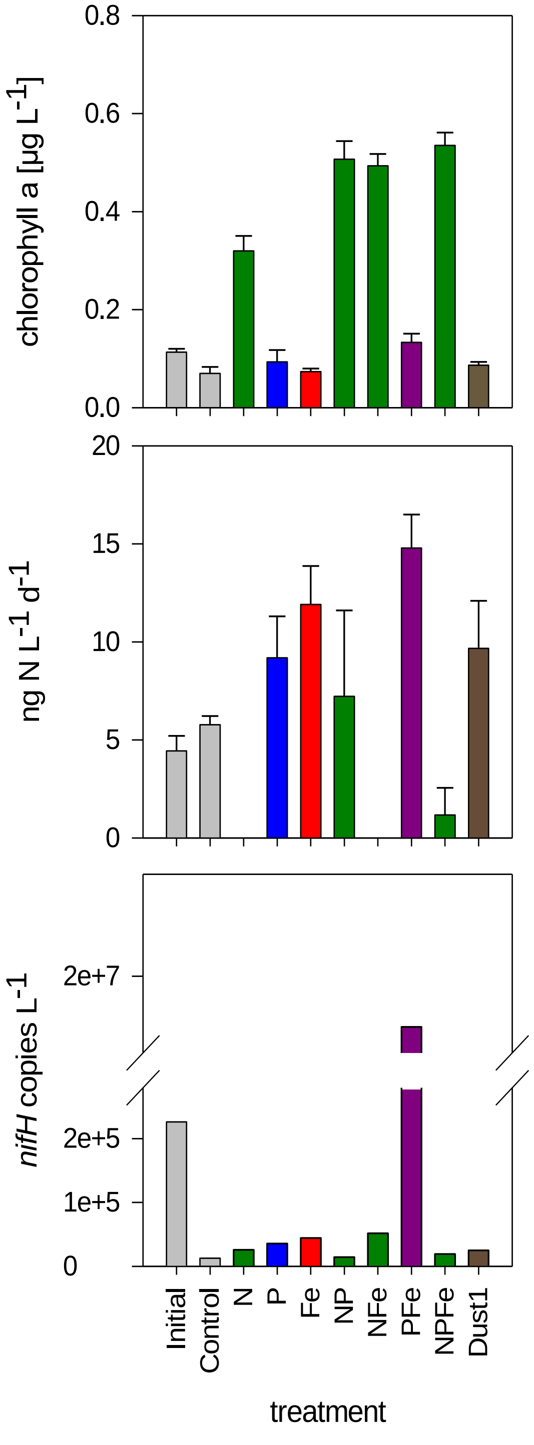

Figure IV-3: Primary production (given as chlorophyll *a* concentration), N_2 fixation and diazotroph abundance (as *nifH* gene copies L^{-1}) from bioassay experiment CV01. Treatment colour codes are the same in each panel. N amendments were + 1 µmol L^{-1} NH_4NO_3, P + 0.2 µmol L^{-1} NaH_2PO_4, Fe + 2 nmol L^{-1} $FeCl_3$, Dust1 + 2 mg L^{-1} Saharan dust.

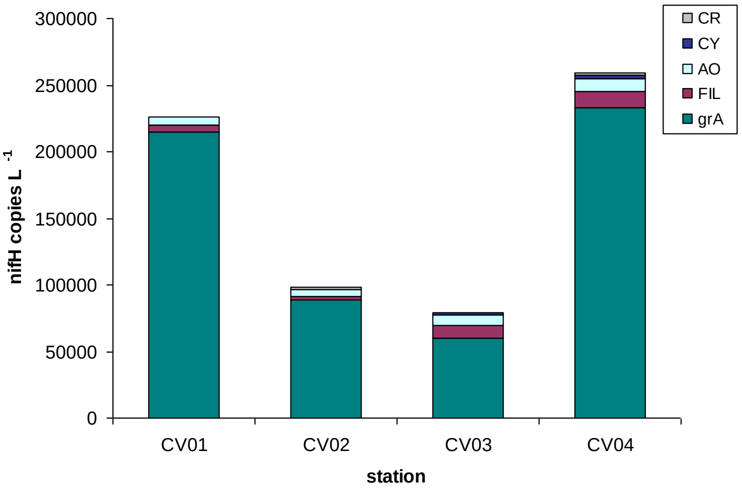

Figure IV-4: Initial diazotroph community composition in the four bioassay experiments carried out in July/August 2008. CR = *Crocosphaera*; CY = *Cyanothece*; AO = γ-proteobacterium AO; FIL = filamentous cyanobacteria; grA = UCYN-A.

Samples taken during the 2009 campaign will be processed this year but preliminary chlorophyll *a* data suggested that nitrogen was limiting primary productivity as in the previous experiments 2008. In 2009, additional experiments were carried out to determine the magnitude of light- and dark-dependent N_2 fixation. In April/May of this year, I will participate in the ANTXXVI-4 cruise of R/V Polarstern from Punta Arenas, Chile to Bremerhaven, Germany. On this 6-week transect, I will take regular surface water samples for molecular analysis of the *nifH* gene at abundance and gene expression level. In addition, regular seawater incubations using stable isotopes ($^{15}N_2$ and $NaH^{13}CO_3$) will be performed to assess N_2 fixation and primary production. Data from this cruise will provide information on the spatial and temporal variation of N_2 fixation in the North Atlantic Ocean especially since previous cruises followed the same transect but have been conducted during different seasons. The combination of these data with future cruises on the same transect will give insights into the interannual variability of both N_2 fixation and diazotrophs abundances throughout the Atlantic Ocean. Data from the Cape Verde Islands will hopefully provide information on “who is out there” and especially on “who does how much”. The question on “who does how much” is very intriguing but has been largely un-answered due to the lack of methods that couple the identity of an organism to its activity. Method developments, *e.g.*, the combination of microautoradiography with *in situ* hybridization (Micro-FISH), have provided insights into this question but require the use of radioisotopes (Micro-FISH). I could show in manuscript C that single-cell N_2 fixation rates can be quantitatively determined and related to bulk N_2

fixation rates which will constrain the magnitude of N_2 fixation by the different phylotypes if combined with in situ hybridization (HISH-SIMS). A recent HISH-SIMS study on NH_4^+-uptake showed that the most abundant organism does not have to be responsible for the bulk activity (Musat *et al.* 2008). And even though the question on "who is out there" has been addressed by molecular studies, both questions still remain widely open especially with respect to the spatial and temporal variation and the response to Saharan dust deposition in the North Atlantic Ocean.

Protocol for the addition of $^{15}N_2$-enriched water to determine N_2 fixation rates

The principal of this protocol is based on previous methods to study the cycling of oxygen using $^{18}O_2$ (Kana 1990) and the exudation of DON using $^{15}N_2$ (Glibert and Bronk 1994). The use of specific equipment and the application of this protocol were designed by me.

The protocol is divided into several stages (for a schematic diagram see Figure IV-5): (1) preparation of seawater, (2) degassing of seawater, (3) the addition and dissolution of $^{15}N_2$ gas and (4) the addition of the $^{15}N_2$-enriched water to incubations.

(1) Preparation of seawater: On-site collected seawater is 0.2 µm filtered and pumped through a ≤10 µm pre-filter (*a*). This pre-filter is incorporated into the system to protect the subsequent membrane. The use of a peristaltic pump or membrane pump is suggested but care has to be taken that no air bubbles are introduced into the line (tubing). After pre-filtration, the seawater line is leading into a 'degassing membrane' (*b*). Instead of 0.2 µm filtering the seawater and applying a 10 µm pre-filter, an in-line 0.2 µm filter could also be used prior to the degassing. The preparation of this water should at best be done with the same water that is used for the incubations. If this is not possible, seawater with the same nutrient concentrations should be used in order to not contaminate the incubations.

(2) Degassing of seawater: The 'degassing membrane' is a membrane contactor made of Celgard® microporous polypropylene hollow fibers housed in a polycarbonate shell. Here, the seawater flows on the lumen side of the fibers and vacuum is applied on the shell side of the membrane. The use of a vacuum of ≤70 mbar (absolute) pressure is recommended which can be achieved by the use of a diaphragm vacuum pump or, alternatively, a water jet pump if sufficient water flow is available. The outflow of the membrane, *i.e.* the degassed seawater, is transferred into Tedlar® gas sampling bags (fitted with a septum port and an inlet/outlet port or a dual port system; evacuated prior to filling with seawater) using gas-tight tubing (*e.g.* Tygon® SE 200; clear tubing to assure that any contaminating air bubbles in the line are visible) (*c*).

(3) No air bubbles should enter the Tedlar® bag while filling with seawater. When the Tedlar® bag is filled, $^{15}N_2$ gas is injected through the septum port at a ratio of 1 ml $^{15}N_2$ gas per 100 ml seawater (*d*). This amount corresponds roughly to the maximum amount of N_2 gas which can be dissolved taking into account that the degassing

efficiency will probably never reach 100%. Dissolve the $^{15}N_2$ gas by breaking up the large bubble into smaller bubbles (*e.g.* clapping with a ruler) but great care should be taken not to damage the Tedlar® bag (*e*).

(4) Once the $^{15}N_2$ gas has dissolved, aliquots of the water can be taken out of the Tedlar® bag through the inlet/outlet port. Add an aliquot of the $^{15}N_2$-enriched water to the incubation bottle which has been filled with on-site collected seawater (*f*) leaving a small headspace for mixing (*g*). Invert the incubation bottle with the added $^{15}N_2$-enriched water several times. Try to keep the time as short as possible but also gently handle the seawater. After mixing, fill the incubation bottles to the rim and cap leaving no headspace in the bottle (*h*). Samples can now be incubated for the desired incubation time and processed as routinely done for mass spectrometric analysis.

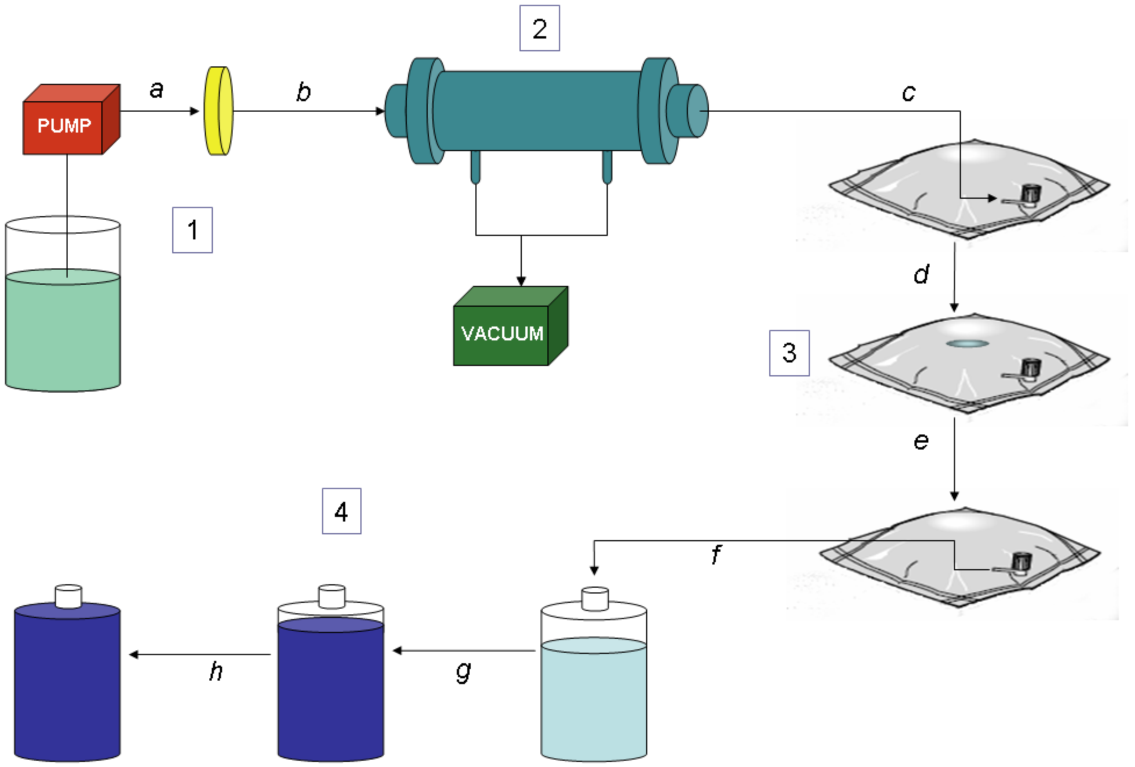

Figure IV-5: Schematic diagram (not to scale) for the preparation and addition of $^{15}N_2$-enriched water (for details see protocol). Source of Tedlar® bag icon: Operation manual http://www.skcinc.com/instructions/3781.pdf

The addition of 50 ml $^{15}N_2$-enriched water to 1 L seawater will lead to an enrichment of roughly 5 atom% $^{15}N_2$ depending on salinity and temperature of the ambient water. The rough calculation is based on the addition of 1 ml $^{15}N_2$ gas to 100 ml of degassed seawater during the preparation. Thus, as a rule of thumb, a replacement of 1% of the original seawater in the incubation with $^{15}N_2$-enriched seawater will lead to an increase in $^{15}N_2$ enrichment of ~ 1 atom% independent of the bottle size used. If 5% of the seawater are replaced by $^{15}N_2$-enriched water, the final enrichment in the dissolved N_2 pool will hence be ~ 5 atom% which is sufficient for N_2 fixation measurements in the oligotrophic regions of the ocean (Zehr and Montoya 2007). The most accurate determination of $^{15}N_2$ that has been added to the incubations by $^{15}N_2$-enriched water, though, would be the direct measurement of $^{15}N_2$ in the enriched water or the incubation by, *e.g.*, membrane-inlet mass spectrometry (MIMS). However, the subsequent calculations of N_2 fixation rates remain the same as for the direct injection of $^{15}N_2$ gas (Capone and Montoya 2001).

The application of this protocol using the membrane contactor and the Tedlar® bags is still under investigation in field experiments. If I can demonstrate that this protocol is satisfactory for the use of incubations with $^{15}N_2$ for N_2 fixation measurements, we will publish a user-friendly protocol for public access. For information on the membrane and its technology, please visit www.liqui-cel.com or www.membrana.com

The membrane contactor that we are currently testing is a 1.7 x 5.5 MiniModule®. This membrane has a maximum flow rate of $\leq$2500 ml/min. We determined that a flow rate of 500-600 ml/min is sufficient for the proposed application. An increase in the flow rate of the membrane results in a decreased degassing efficiency. Thus, a compromise between flow rate and degassing efficiency has to be taken. The production of ~ 500 ml degassed water per minute should be adequate for ship-based large scale experiments encompassing about fifty 4.5 L bottles. To reach an enrichment of about 5 atom%, a total of 10 L (degassed) and $^{15}N_2$-enriched water would be needed for such an experiment. This could be achieved within ~ 20 min at the above mentioned flow rates. The dissolution of the $^{15}N_2$ gas will take about 5-10 minutes (T. Grosskopf, pers. comm.). The application of this protocol does not disturb the simultaneous incubation with other stable isotopes such as $NaH^{13}CO_3$ for determination of photosynthetic rates in the same bottle. The isotope can be added at the same time as the $^{15}N_2$-enriched seawater. Larger membranes are available if the experiment requires a higher amount of $^{15}N_2$-enriched water. Therefore, I consider this protocol practicable for field assays of N_2 fixation.

V. References

VI. Acknowledgements

VII. Eidesstattliche Erklärung

V. References

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VII. Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass die von mir vorgelegte Dissertation, abgesehen von der Beratung durch meine Betreuerin, nach Inhalt und Form meine eigene Arbeit ist und unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft (DFG) angefertigt wurde. Genutzte Quellen, Hilfsmittel und Zusammenarbeit mit anderen Wissenschaftlern wurden kenntlich gemacht. Desweiteren versichere ich, dass die von mir vorgelegte Dissertation weder im Ganzen noch zum Teil einer anderen Fakultät oder anderen Hochschule im Rahmen eines Prüfungsverfahrens vorgelegen hat. Veröffentlichte oder zur Veröffentlichung eingereichte Manuskripte wurden kenntlich gemacht.

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