On the role of dinitrogen (N_2) fixing cyanobacteria in marine environments with special focus on release and transfer of nitrogen (N)

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Summary

The aim of this PhD-thesis was to contribute to the existing knowledge on the role of diazotrophic cyanobacteria in marine environments. The basis of this study was the overarching hypothesis, that diazotrophy represents an important instantaneous source of “new” N in marine environments for bacteria and higher trophic level.

This thesis was part of the WGL (Wissenschaftsgemeinschaft Gottfried Wilhelm Leibniz) network TRACES (Ocean - Atmosphere-Land Impacts on Tropical Atlantic ecosystems) focusing on the exchange of matter between land-ocean and atmosphere and the influence of river inflow into the Tropical Atlantic Ocean.

In the first part of the thesis culture experiments on N_2 and C fixation, as well as N and C exudation were carried out to investigated metabolic processes in the course of a day. Moreover, light and phosphorous availability were tested as regulating factors of exudation. For this, experiments were carried out in the laboratory. N_2 and C fixations as well as the release of N and C compounds were tightly regulated over a diel cycle in *Nodularia spumigena* and *Trichodesmium erythreum*. The release of compounds was dominated by dissolved organic nitrogen (DON) and was presumably regulated by the amount of previously assimilated N. Integrated over a diel cycle 80% of N fixed was directly released. Changes in light intensity regulate and strongly enhance the exudation of N in both investigated species. We attribute this to a short-term excess supply of electron energy that is channelled out of the cell partly by using electrons to fix N_2 and subsequently release this excess N. Phosphorous availability showed no clear effect on the exudation of N compounds.

The second part aimed on the one hand, at the quantification of diazotrophic N that is transferred to mesozooplankton species and on the other hand on the distinction of direct grazing and microbial loop mediation to this transfer. For this, field experiments were carried out using stable isotope tracer addition (^{15}N) during

two simultanious cruises in the Baltic Sea with RV Heincke and RV Poseidon in July 2008 and during three cruises at the Cape Verde islands (Eastern Tropical Atlantic Ocean) with RV Islandia in July 2008. Samples were also provided by participants of the Mauritanian upwelling cruise with the RV Poseidon 348 in July 2007. These were used in this study to determine the impact of diazotrophy on N stable isotopes of PON and NO_3^-.

Up to 100% of diazotrophic fixed N was incorporated by mesozooplankton species in the experiments during the Baltic Sea studies. Because filamentous cyanobacteria, which were dominant during the investigation time, are grazed upon only by a limited number of zooplankter the profound transfer of newly fixed N in the Baltic Sea was mediated by microbial loop constituents (67%). Nevertheless, 33% of N resulted from direct grazing on filamentous cyanobacteria. The structure of the food web within the Baltic Sea differed from that in the Atlantic Ocean, with respect to the dominant diazotrophic species and the complexity of the mesozooplankton community structure. Unicellular cyanobacteria were present in the Atlantic Ocean and the food web was more complex. This lead to a change in the contribution of direct grazing to the overall gross transfer of diazotrophic N, because small celled primary producers are more readily grazed upon. Direct grazing accounted for 56% of gross N transfer and 44% were mediated by the microbial loop during the experiments in the Tropical Atlantic Ocean. Additionally, the analyses of N stable isotopes in PON and NO_3^- indicated that biological N_2 fixation appeared to be a plausible mechanism for introducing significant quantities of ^{15}N-depleted compounds into the Tropical Atlantic Ocean which in turn was available for further biological uptake.

Zusammenfassung

Das Ziel dieser Promotion war es, die Rolle N_2 fixierender (diazotrophe) Cyanobakterien in marinen Ökosystemen zu untersuchen. Die Basis hierfür bildete die Hypothese, dass N_2 fixierender Cyanobakterien eine wichtige sofort verfügbare Quelle "neuen" Stickstoffs für die bakterielle Produktion, sowie für höhere trophische Ebenen in marinen Habitaten bilden.
Die vorliegende Promotion ist Teil des WGL (Wissenschaftsgemeinschaft Gottfried Wilhelm Leibniz) Netzwerkes TRACES (Ocean - Atmosphere-Land Impacts on Tropical Atlantic ecosystems), welches sich mit der Interaktion von Land-Atmosphäe, Flüssen und dem Ökosystem Tropischer Atlantischer Ozean beschäftige.
Im ersten Teil der Arbeit wurden N_2 und C Fixierung genauer untersucht, sowie die anschließende Abgabe gelöster N und C Verbindungen im Laufe eines Tages. Darüber hinaus sollte festgestellt werden, ob die Licht und Phosphor Verfügbarkeit die Abgabe von N und C reguliert. Hierzu wurden Experimente im Labor durchgeführt.
Es stellte sich heraus, das N_2 und C Fixierung, sowie die Abgabe von N und C bei beiden untersuchten Arten eng miteinander verknüpft ist. Die Abgabe von N war von gelösten organischen N Verbindungen (DON) dominiert und eng an die Quantität des vorher fixierten N_2 gebunden.
Die Erhöhung der Lichtverfügbarkeit führte zudem zu einer Erhöhung der N Exudation bei beiden untersuchten Arten. Dies geht vermutlich auf ein kurzzeitiges Überangebot an Elektronenenergie zurück, welche jedoch nicht vollständig für metabolische Prozesse verwendet werden kann. Diese überschüssigen Elektronen werden daraufhin für die Fixierung von N_2 und die Synthese von ${NH_4}^+$ benutzt. Weitere Versuche zum Einfluß von P auf die Exudation von N Verbindungen zeigten jedoch keine Veränderung der Abgaberate.

Der zweite Teil hatte zum einen das Ziel, den Transfer von diazotrophen N in die Mesosooplankton Fraktion zu quantifizieren. Zum anderen sollte der dominante Weg des Transfers, direkter Fraß oder Transfer über das microbial loop, identifiziert werden. Hierzu wurden Freiland Experimente durchgeführt, bei denen die Zugabe von stabilen Isotopen (^{15}N) erfolgte. Die Freiland Experimente wurden zum einen während zweier gleichzeitig stattfindender Ausfahrten auf der Ostsee durchgeführt, it den FS Heincke und Poseidon im Juli 2007. Zum anderen wurden hierzu identische Versuche während dreier Ausfahrten mit dem FS Islandia auf den Kap Verdischen Inseln im Juli 2008 durchgeführt. Proben wurden zudem von den Forschern der FS Poseidon 348 im Juli 2007 zur Verfügung gestellt, welche genutzt werden sollten, um den Einfluß von Diazotrophie auf stabile Isotope im PON und NO_3^- des Atlantischen Ozeans zu ermitteln.
Bis zu 100% des diazotroph fixierten N wurde in den Ansätzen der Ostsee Versuche von der Mesozooplankton Gemeinschaft inkorporiert. Filamentöse Cyanobakterien werden jedoch nur selten direkt gefreßen, so dass der vorwiegende Anteil des diazotrophen N über das mikrobielle Nahrungsnetz in das Zooplankton gelangte (67%). Nichtsdestotrotz resultierten 33% der Aufnahme diazotrophen N aus direktem Fraß. Es stellte sich heraus, dass sich die Struktur des Nahrungsnetzes in den beiden untersuchten Gebieten voneinander unterschied. Zum einen, hinsichtlich der dominaten Cyanobakterien Art und zum anderen hinsichtlich der Komplexität der Mesozooplankton Gemeinschaft. Einzellige Cyanobakterien dominierten im Atlantischen Ozean, welche bevorzugt ingestiert werden. Filamentöse Cyanobakterien in der Ostsee, welche im generellen als Fraß resistent gelten. Des Weiteren war die Zooplankton Gemeinschaft im Atlantik im Vergleich zur Ostsee komplexer. Dies beiden Faktoren führten dazu, dass im Atlantischen Ozean der Anteil des direkten Frasses mit 56% höher war, als in der Ostsee. Zu 44% gelangte der diazotrophe N über das mikrobielle Nahrungsnetz in die Zooplankton Fraktion.
Die Analyse der stabilen Isotope im PON und NO_3^- wiesen darauf hin, dass N_2 Fixierung isotopisch leichte ^{15}N- Verbindungen in den Atlantischen Ozean einbringt, welche im Gegenzug für die biologische Produktion zur Verfügung steht.

Chapter 1

Introduction

1.1 General aspects

Nitrogen (N) is an essential nutrient for organisms in marine environments and can limit the production of organic matter (e.g. Zehr & Ward 2002). The input of reduced N compounds can take place via riverine inflow and atmospheric deposition. Furthermore, it can be mediated by biological dinitrogen (N_2) fixation, which is the reduction of atmospheric N_2 to ammonium (NH_4^+). N_2 fixation can only be performed by diazotrophic cyanobacteria, which comprise a diverse group of prokaryotes. The genetic lineage of cyanobacteria is evidently among the oldest on Earth (e.g. Brasier et al. 2002, Schopf 1993). Oxygenic photosynthesis originated in those organisms and created our present day oxygen-enriched atmosphere (e.g. Kasting & Siefert 2002, Williams 2006). Cyanobacteria absorb light energy for photosynthesis by synthesizing and utilizing the chlorophyll *a* molecule, phycobiliproteins, and accessory phycobilin pigments like phycoerythrin, allophycocyanin, and phycocyanin (e.g. Glazer 1977, Bryant 1982). High concentrations of the pigments phycocyanin and phycoerythrin often make these organisms appear greenish-blue, leading to their previous designation as “bluegreen algae” and current term of cyanobacteria (e.g. Glazer 1977, Bryant 1982). Nonetheless, cyanobacteria are metabolically a rather homogeneous group of organisms that are characterized by their general ability to perform oxygenic photosynthesis and to fix CO_2 through the Calvin reductive pentose phosphate path-way. On the other hand, some cyanobacteria are capable of

photo-heterotrophic or chemo-heterotrophic growth. With regard to the acquisition of N, most cyanobacteria assimilate NO_3^- and NH_4^+, and many strains are also able to assimilate urea and amino acids (e.g. Bronk 2007), besides fixing N_2. The assimilation of N compounds is subjected to tight regulation, such that NH_4^+ is assimilated with preference over other N sources when more than one N compound is available (Bronk et al. 1994).

Among the consortium of diazotrophs there are three groups distinguishable according to their different life strategies and environmental requirements (Tab. 1.1, Stal 1995). Group I comprise heterocystic cyanobacteria possessing specialized cells to protect the O_2 sensitive enzyme nitrogenase from the O_2 evolving process of photosynthesis. These protection mechanisms are discussed in subchapter 1.2.1. Group I cyanobacteria play a minor role in the pelagial of marine ecosystems, but are frequently found in brackish basins like the Baltic Sea and in freshwater ecosystems. In tropical and subtropical seas, the order Nostocales can be found, comprising another important heterocyst cyanobacterium species- *Richelia intracellularis*, which forms a tight symbiosis with diatoms like *Rhizosolenia* and *Hemiaulus* (e.g. Ferrario et al. 1995, diazotroph- diatom associations DDAs). *Nodularia spumigena*, a species that belongs to this group as well, will serve as a model organism to investigate N_2 fixation and N metabolism in chapter 2, exemplarily for Group I. Group II cyanobacteria are anaerobic non heterocystic species that will only fix N_2 under anoxic conditions. Such conditions mostly prevent oxygenic photosynthesis and therefore require an alternative electron source (e.g. H_2S) as it is found in microbial mats with steep gradients of oxygen and sulfide (Villbrandt & Stal 1996). This group of cyanobacteria will not be investigated in detail in this thesis, as they play a minor role the pelagic euphotic zone of marine habitats. Group III cyanobacteria are aerobic non heterocystic and filamentous (*Trichodesmium*) or unicellular (*Gloethece*), which fix N_2 under aerobic conditions. This is somewhat surprising, because they are not provided with a protective envelope like Group I cyanobacteria and therefore require a range of protective mechanisms (see subchapter 1.2.1 and 1.2.2). *Trichodesmium erythraeum* will serve as a model organism investigating N_2 fixation and N metabolism in chapter 2, exemplarily for this group.

Table 1.1: Species of N_2 fixing cyanobacteria and their importance in marine habitats (after Stal 1995).

Group	Characteristics
Group I heterocystic cyanobacteria	- filamentous - spatial separation of N_2 fixation (heterocycsts) oxygenic photosynthesis (vegetative cells) - e.g. *Anabaena*, *Nodularia*, *Aphanizomenon*, *Nostoc*, *Calothrix*, *Scytonema* - forming surface blooms, brackish seas, paddy fields, microbial mats symbiotic with numerous organisms - rare in marine ecosystems, occur in brackish basins (Baltic Sea)
Group II Non- heterocyctous anaerobic cyanobacteria	- filamentous, unicellular - induction of nitrogenase only under anoxia or low oxygen, sulphide may be necessary in order to inhibit oxygenic photosynthesis - e.g. *Plectonema boryanum*, *Oscillatoria limnetica*, *Synechococcus* sp. - ubiquist, especially in microbial mats
Group III Non- heterocyctous aerobic cyanobacteria	- filamentous, unicellular - temporal separation of N_2 fixation and oxygenic photosynthesis, different strategies of oxygenic protection mechanisms - diazotrophic growth possible under aerobic conditions - e.g. *Gloethece*, *Oscillatoria*, *Trichodesmium*, *Lyngbya* - pelagial of tropical oceans, paddy field, microbial mats

1.2 Diazotrophic N_2 fixation and N transfer on the cellular level

1.2.1 Nitrogenase – Structure and reaction mechanism

In many diazotrophs, nitrogenase comprises about 10% of total cellular proteins and consists of two distinct proteins, dinitrogenase and dinitrogenase reductase. Both proteins contain iron (Fe) and sulphur (S) and dinitrogenase also molybdenum (Mo). The molybdenum and iron in dinitrogenase are contributed by the cofactor known as iron-molybdenum centre (FeMo-Co , Postgate 1987). Nitrogenase is capable of catalysing the reduction of small molecules like dinitrogen (N_2) expressed by the following Equation 1.1:

$$N_2 + 8H^+ + 8e^- \longrightarrow 2NH_3 + H_2 \tag{1.1}$$

$$(\Delta G\text{= -8 kcal mol}^{-1})$$

The nitogenase reaction produces energy, indicated by the negative ΔG (change in free energy/ chemical potential= Gibbs free energy). Nevertheless, the first step of the reaction requires a lot of energy as a result of the triply bonded, extremely stable nature of the N_2 molecule (Postgate 1987). The ΔG of this reaction is +50 kcal mol^{-1}. In comparison to this, assimilatory $NO_3{}^-$ reduction to $NH_4{}^+$ is more favourable, yielding in -83 kcal mol^{-1}.

In particular, the Fe protein takes electrons from central metabolism electron carriers and transfers them to the Mo iron protein (MoFe) expending 12 to 24 ATP (Adenosine triphosphate, 4 to 5 ATPs are hydrolyzed for every 2e$^-$ transferred) per N_2 fixed. N_2 is reduced at the MoFe cofactor site with the intermediates N_2H_2 and N_2H_4 (hydrazine) being produced. Although N_2H_4 has been detected, N_2H_2 (diazene) is very unstable and tends to decompose back to N_2 + H_2. The final product from the operation of nitrogenase is ammonia (NH_3), which is subsequently converted to glutamate by the enzymes glutamine synthetase and glutamate synthase. Per N_2 reduced one H_2 is produced as part of its catalytic cycle, which represents a significant loss of energy. However, N_2 fixing cyanobacteria also possess the enzyme

hydrogenase that serves to recycle some of the electrons lost in H_2. ATP is recovered if those electrons are reintroduced into the electron transport chain. O_2 serves as a final electron acceptor in the so-called oxyhydrogen reaction (Saino & Hattori, 1982), leading to the recovery of ATP and the lowering of ambient O_2 levels. This process guarantees the function of nitrogenase, which would otherwise be damaged by O_2 radical species (singlet O_2, hydroxyl radicals, e.g. Gallon 1992). Nevertheless, aerobic diazotrophs need an adequate supply of O_2 for ATP production, but the supply must not exceed their demand for optimum nitrogenase activity. Thus a bell-shaped curve is obtained when nitrogenase activity is plotted against O_2 concentration (Gallon 1992 and references therein). At suboptimal O_2 the system is energy limited. As the O_2 supply increases and the optimum is passed nitrogenase activity declines. This inhibition of nitrogenase activity by excess O_2 is reversible in many diazotrophs and several protection mechanisms are found. They include temporal separation of N_2 fixation and oxygenic photosynthesis, a high rate of synthesis of nitrogenase to counteract losses of irreversibly inactivated enzyme and a switch off mechanism to respond to short-term exposures of O_2. Respiratory protection with activity of antioxidant enzymes and compounds such as superoxide dismutase and catalase (Mackey & Smith 1983, Stal & Krumbein 1985) is also found. Moreover, O_2 uptake via the Mehler reaction (generation of excess ATP via pseudocyclic photophosphorylation and simultaneous photoreduction of O_2 by PS 1) has been shown to be significant in cyanobacteria exposed to high light intensities (Hoch et al. 1963, Kana 1992). These high light intensities are accompanied by an increase in the cellular energy level, leading to an imbalance in the overall cellular energy status. It has been proposed, that electron consumption by N_2 fixation and production of N compounds is a mechanism to regulate and balance the cellular energy status (Lomas et al. 2000). In chapter 2 of this thesis this hypothesis is investigated experimentally for two diazotrophic species, *Nodularia spumigena* and *Trichodesmium erythraeum*. The need to fine tune N_2 fixation and O_2 evolving photosynthesis leads to characteristically patterns of fixation activity during the course of the day in the different cyanobacteria groups. This aspect is addressed in the following subchapter (1.2.2).

1.2.2 Physiology of heterocysts and diel cycles of N_2 and C fixation in different species

Not all diazotrophic cyanobacteria possess heterocysts. But wherever they are differentiated from vegetative cells, they remain the only compartments where N_2 is fixed. heterocystic cyanobacteria of Group I make up the majority of diazotrophs in the Baltic Sea, comprising the genus *Nodularia*, *Aphanizomenon* and *Anabaena*. In the summer time the Baltic Sea exhibits low NO_3^- concentrations while phosphate (PO_4^{3-}) is still present leading to favourable pre-conditions for the growth of diazotrophs. Overall, under conditions of N limitation vegetative cells differentiate to form heterocystic, non-growing, specialized cells in which N_2 is fixed into organic N like cyanophycin (e.g. Wolk 1996). These specialized cells differ from vegetative cells in many structural and functional features. At the molecular level, the different physiology of the heterocysts and vegetative cells is supported by different genes that are expressed, for example genes which are involved in the formation of the heterocysts envelope (e.g. Lynn et al. 1986, Buikema & Haselkorn 1993). Because the enzyme that catalyzes N_2 fixation, nitrogenase, is inhibited by O_2 (see subchapter 1.2.1, Stewart 1973, Smith et al. 1987), heterocysts must be physically isolated from nearby vegetative cells, which are sites of oxygenic photosynthesis and CO_2 fixation. This isolation is not complete, as vegetative and heterocyst cells must exchange energy, organic C and fixed N, as indicated in Figure 1.1. Connection to vegetative cells occurs through a pore, equipped with microplasmodesmata realising a rapid exchange of organic material between heterocysts and vegetative cells (Wolk et al. 1976, Meeks & Elhai 2002, Flores et al. 2005, Popa et al 2007). Heterocysts consume carbohydrates (mainly maltose and sucrose, e.g. Juttner 1983) and are on the other hand sources for N compounds (likely glutamine, e.g. Juttner 1983). Changes in the thylakoid structure of heterocysts are associated with synthesis of a glycolipid layer that is important in protection of nitrogenase from O_2 (e.g. Soriente et al. 1993). Hence, heterocysts maintain a relatively anoxic microenvironment in a filament that is predominantly oxic. The N demands of vegetative cells in the first hours of the day, is met by a very rapid export of organic N from heterocysts to vegetative cells. Therefore, reductants are almost exclusively channelled to the reduction of N to NH_3, which in turn reacts with glutamate derived from the im-

ported carbohydrates to form glutamine. Both O_2 and N_2 diffuse into the cells, but increased respiratory activity in membranes near to the polar ends of heterocysts depletes the O_2 concentration. The lack of N and C gradients in the vegetative cells suggests that N transport among vegetative cells is very rapid relative to the use of compounds.

It has been shown that *Nodularia* exhibits dark N_2 fixation, though the actual rates might be very low (e.g. Sanz- Alférerez & del Campo 1994). This reduction in activity of fixation reflects a high turn-over of proteins and enzymes involved in the N_2 fixation process and enzymes which have a catalytic effect or accessory functions (e.g. electron transfer, Ramos et al. 1985). Moreover, the shortening in availability of ATP in darkness decreases the protection of the Fe-protein component of nitrogenase against O_2 (Stewart 1980). After the onset of the light period energy is provided by photosynthesis and carbon supply is activated leading to a rapid increase in nitrogenase activity (Fig. 1.2). Nevertheless, next to the triggering by light, an endogenous rhythm has been implicated in the observed patterns, which is persistent under continuous light (e.g. Stal & Krumbein 1985a). The diel pattern in N_2 fixation has been reported to operate in several investigations of heterocystic cyanobacteria from the Baltic Sea (Evans et al. 2000, Gallon et al. 2002).
A representative of non-heterocystic filamentous cyanobacteria, *Trichodesmium*, is the dominant diazotroph in oligotrophic marine ecosystems like the Eastern Tropical Atlantic Ocean (e.g., Karl et al. 1997) being responsible for up to 50% of the new production in oligotrophic tropical and subtropical waters (Karl et al. 1997, Capone et al. 2005, Mahaffey et al. 2005). As they possess no differentiated heterocysts the separation of O_2 evolving photosynthesis and N_2 fixation is not spatial. Recently, Finzi et al. (2009) applied the novel method, the nanometer-scale secondary ion mass spectrometry (NanoSIMS) combined with transmission electron microscopy (TEM) imaging to elucidate the temporal uncoupling of N_2 and C fixation in single filaments during a diel cycle (Fig. 1.1). C fixation peaked early after the onset of the light period and was subsequently down regulation to ensure N_2 fixation, as earlier publications suggested (Fig. 1.2, e.g. Berman-Frank et al. 2001). On the other hand, they did not find any hints on a spatial separation of the two metabolic processes within a trichome (diazocyte), as Frederiksson & Bergmann (1996) and

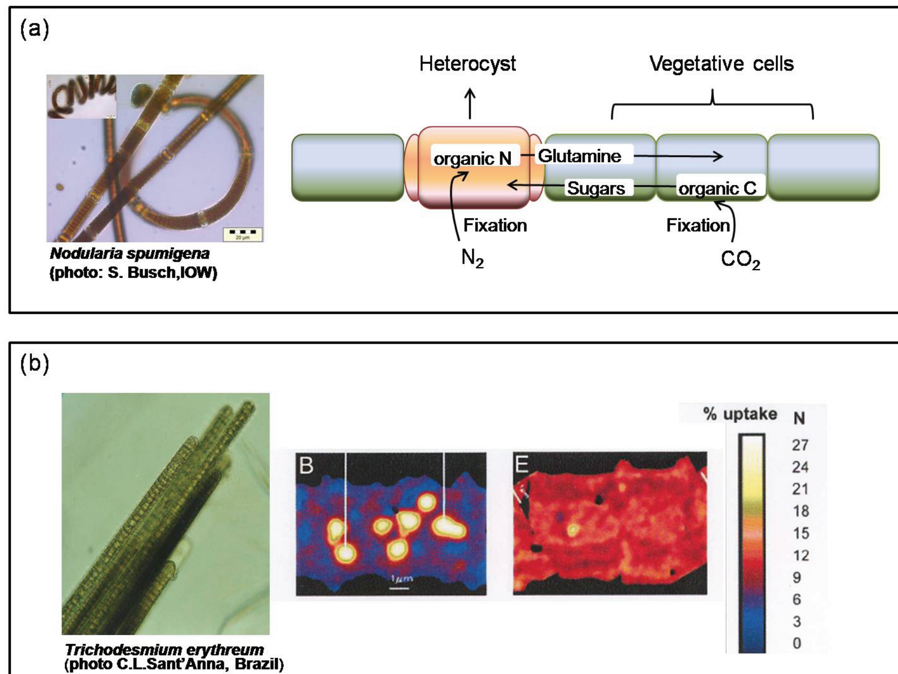

Figure 1.1: Illustration of N transfer on the cellular level of the heterocystic cyanobacterium *Nodularia spumigena* (a) and the non-heterocystic cyanobacterium *Trichodesmium erythraeum*(b). Figure adapted from Popa et al. 2007. (B) Light microscope and NanoSIMS (Fig. 3, Finzi-Hart et al. 2009) images of *Trichodesmium erythraeum.* NanoSIMS images demonstrate percentage of fixed ^{15}N after 8h (B) and 24h (E) (scale bar, 1 μm, Fig. 3 from Finzi-Hart et al. 2009).

Ohki (2008) earlier proposed. They concluded that a specialisation of single cells at the expense of C fixation is unlikely, unless this spatial separation is very transient and fixation metabolites are redistributed very rapidly. In *Trichodesmium* N_2 fixation is essentially light dependent (e.g. Mulholland & Capone 2000). At the onset of the light period (sunrise) nitrogenase is synthesized *de novo*, where the actual pattern is a function of growth rate and physiological status of the cell (e.g. Ohki et al .1992). During the day maximum N_2 fixation coincides with a down regulation of photosynthesis as e.g. Berman-Frank et al. (2001) reported. Moreover, a circadian clock is responsible for the coordination of expression of nifH genes and genes of the photosynthetic reaction centres (Chen et al. 1998, Kondo & Ishiura 2000). After the onset of the dark period (sunset) the nitrogenase activity rapidly ceases by protein hydrolysis (Chen et al. 1998).

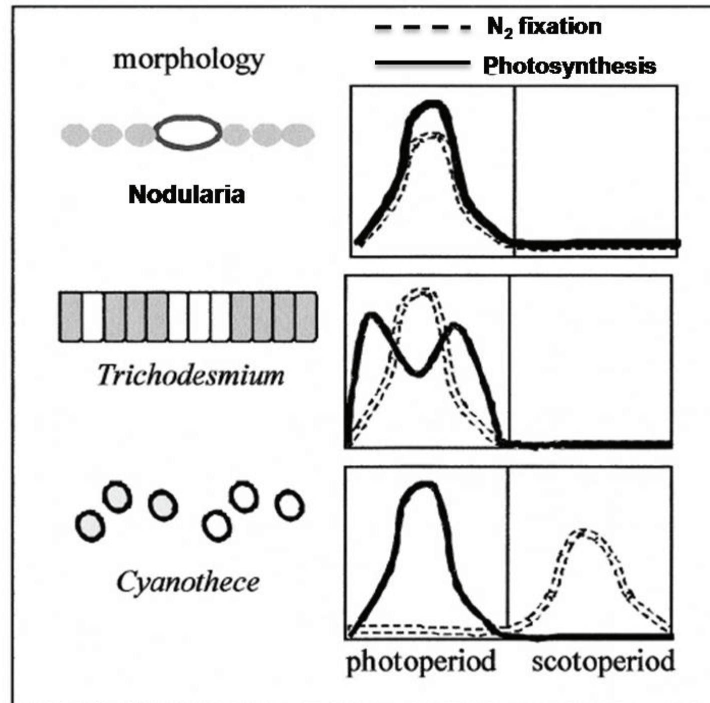

Figure 1.2: Schematic representation of the different diazotrophic morphologies, strategies of separation of N_2 fixation and C fixation). Grey shaded areas indicate the localization of nitrogenase within the cells. Dark gray indicates actively photosynthesizing vegetative cells. The graphs in the panels illustrate the relative timing of photosynthesis (solid black line) and N_2 fixation (dashed line) during the diel cycle. Figure adapted from Berman-Frank et al. (2003).

The diel cycles of N_2 and C fixation in Group I cyanobacteria and filamentous cyanobacteria of Group III are investigated exemplarily for *Nodularia* and *Trichodesmium* in chapter 2.

In contrast to Group I and filamentous cyanobacteria of Group III, unicellular diazotrophs of Group III (e.g., *Cyanothece*, *Croccosphaera*) fix N_2 only during the night when grown under a light: dark (L:D) cycle, or under the subjective dark-phase (scotophase) when grown under continuous light (Bergman et al. 1997). High nitrogenase activity coincides with high respiration rates; with a phase difference of up to 12 h from the peak of photosynthetic activity (Fig. 1.2). Energy and reductants are provided via respiration and use of photosynthetically fixed C (Tuit et al. 2004).

1.2.3 Acquisition of other N compounds

Inorganic (NO_3^-, NH_4^+, N_2) and organic (urea, amino acids) N sources are taken up trough permeases and ABC-type transporters. In Group I heterocystic cyanobacteria N compounds which derive from N_2 fixation have to be exported to vegetative cells. The majority of cyanobacteria preferentially assimilate NH_4^+ over other N sources, when more than one N source is available (Flore & Herrero 1994). This leads to a down regulation of the energy expensive N_2 fixation reaction, when inorganic N is available to save electron energy (e.g. Ohki et al. 1991, Mulholland et al. 2001, Fu et al. 2003). Intracellular NO_3^- is reduced to NO_2^- and NH_4^+, by nitrate-reductase and nitrite reductase. Organic sources of N, like urea are degraded to NH_4^+ and CO_2 by an urease (Valladares et al. 2002), whereas arginine is catabolised by an unusual pathway that combines the urea cycle and the arginase pathway rendering NH_4^+ and glutamate as final products (Quintero et al. 2000). Additionally, cyanobacteria bear broad specific amino acid transport systems and high-affinity permease for arginine and other basic amino acids (e.g. Montesinos et al. 1997) which are located in the cytoplasmatic membrane and are subsequently metabolized to NH_4^+, if needed (Fig. 1.3). Whatever the N source used for growth, intracellular NH_4^+ (in the form of glutamate) is incorporated into C skeletons through the glutamine synthetase-glutamate synthase pathway (GOGAT, reviewed in Flores & Herrero 1994). In cyanobacteria the main metabolic compound for N incorporation is 2-oxoglutarate, which originates from C fixation ($NADP^+$- isocitrate dehydrogenase, Muror-Pastor et al. 1992) and has to be translocated in heterocystic cyanobacteria from vegetative cells to heterocysts. Studies have suggested that 2-oxoglutarate plays a key role in perceiving the intracellular N status, where N deficiency is perceived by an increase in the intracellular 2-oxoglutarate pool and inversely, N excess is related to a decrease in this signalling molecule (Muro-Pastor et al. 2001). Finally, N is accumulated in multi-L-arginyl poly L- aspartic acid, a polymer of aspartate and arginine, also called cyanophycin which serves as a reserve material for C, N and energy in cyanobacteria.

The detailed discussion of N assimilation in cyanobacteria and its interrelationship to the C metabolism is the basis to understand the results and conclusion drawn in chapter 2, which will in part deal with the physiology of the N and C metabolism

and its regulation in *Nodularia spumigena* and *Trichodesmium erythraeum.*

1.2.4 Exudation of N compounds by cyanobacteria

To date there are different basic approaches to explain the release of N compounds by healthy growing cells, besides the theory of passive leakage of compounds through the cell wall. Firstly, it is believed that excess photosynthetats containing nitrogenous functional groups are released successive with photosynthesis, whenever the acquisition of exceeds the consumption of those compounds (e.g. Fogg 1983). Secondly,

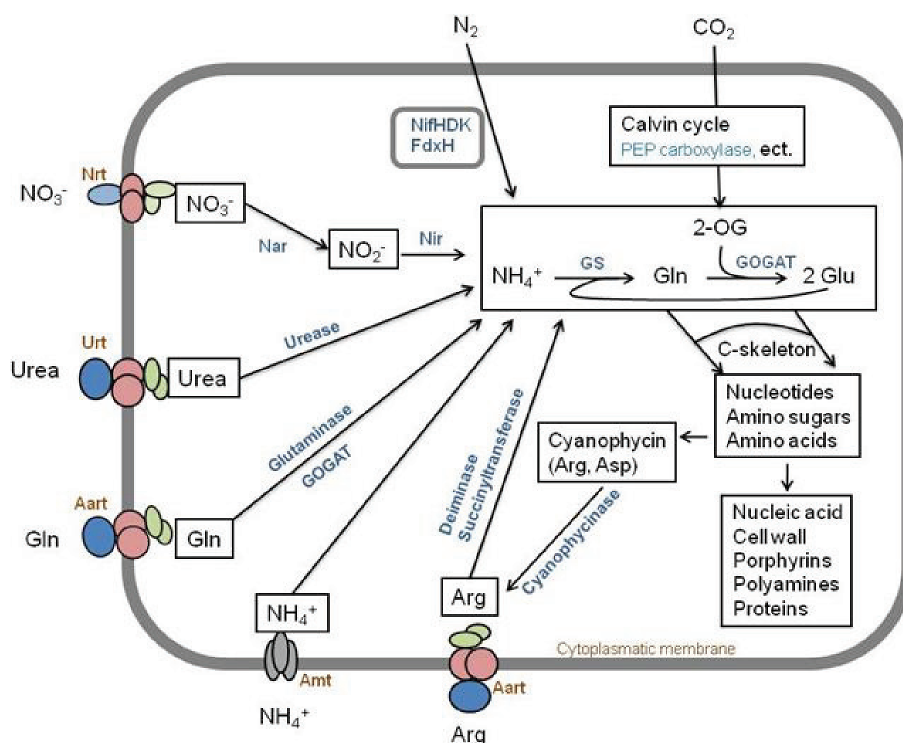

Figure 1.3: Main pathways of N assimilation in cyanobacteria (see text for further details). Abbreviations: 2-OG: 2- oxoglutarate; Arg: arginine; Asp: asparagine; Nrt: ABS-type nitrate/nitrite transporter; Urt: ABC- type urea transporter; Aart: ABC- type Amino acid transporter; Amt: ammonium permease; Nar: nitrate reductase; Nir: nitrite reductase; GS: glutamine synthase; NifHDK: nitrogenase complex; FdxH: heterocyst- specific ferredoxin; PEP carboxylase: phosphoenolpyruvate carboxylase. Nitrogenase and FdxH are boxed indicating that in some cyanobacteria N_2 fixation takes place in heterocycts. Figure adapted from Flores & Herrero (1994 and 2005).

it may be possible that diazotrophs, especially non heterocystic, supply other cells within the colony lacking nitrogenase with N compounds like amino acids (mainly Glu; e.g. Bermann & Bronk 2003 and references herein). Thirdly, Lomas et al. (2000) have shown for diatoms and dinoflagellates that active exudation of N compounds acts as a way to dissipate excess electron energy derived from a surplus in light energy. Fourthly, it has been hypothesised that diazotrophic populations are relieved from viral infection by exudation of N compounds. This is achieved, because the release of N compounds support associated bacterial populations who are more frequently infected with viruses (Murray 1995). The N compounds released are quantitatively dominated by dissolved organic N (DON, Capone et al. 1994, Glibert & Bronk 1994). DON is made up of low molecular weight compounds (LMW, < 10 kDa) and high molecular weight compounds (HMW, > 10 kDa). This classification is geared to the qualitative classification of dissolved organic carbon (DOC). LMW compounds are peptides, free dissolved amino acids, urea and nucleic acids. HMW compounds comprise proteins, enzymes, humic acids and cell wall compartments (Bermann & Bronk, 2003). To date, mechanisms and factors influencing the release of N, as well the chemical nature of theses exudates are poorly understood.

Questions that result from the literature review above and which are addressed in chapter 2 of this thesis are: Which N compounds are released at what time of the day? How much is quantitatively released by cyanobacteria? What factors influence and regulate this cellular N translocation. Is there a tight coupling between fixation and release in the course of the day? Are there differences between heterocystic and non-heterocystic cyanobacteria (*Nodularia* and *Trichodesmium*)? In the present subchapter (1.2.4) the cellular N metabolism has been examined and factors controlling N_2 fixation activity have been quoted.
In the next subchapter (1.3) these will be analysed in more detail.

1.3 Factors controlling N_2 fixation

Factors controlling N_2 fixation can be grouped into physical, biological and biogeochemical factors. Physical factors are represented by turbulence, which has a negative influence on N_2 fixation rates by mixing cyanobacteria into areas of the

euphotic zone where light availability becomes limiting (e.g. Pearl 1985). Light is a very important controlling factor, because it provides energy for primary production as well as N_2 fixation. Two aspects are regulating, light quality and light quantity (intensity). Different cyanobacteria species are adapted to different light intensities. For example the Baltic Sea species *Aphanizomenon* requires lower intensities for growth, compared to *Anabaena* (e.g. De Nobel et al. 1998). This enables them to be mixed into deeper parts of the euphotic zone. Moreover, Cyanobacteria can acitvely control their position within the water column by positive buoyancy, to secure their light energy demand. In order to do so, they regulate gas vesicles and change their storage polymer content (e.g. Bormans et al. 1999). Not only light intensity itself, but also the ratio of light and dark phases during a diel cyle is important, especially in unicellular cyanobacteria. In this group N_2 and C fixation are separated temporarily (compare Fig. 1.2).
Biological (top down) effects on diazotrophs are associated with grazing. Direct grazing on filamentous cyanobacteria has been regarded as unimportant, so far (exception *Macrosetella* grazing actively on *Trichodesmium*, O'Neil et al. 1996), but recently studies gave hint to the significance of this controlling factor (Schaffner et al. 1994, Koski et al. 2002). Often this top down effect is insufficient to control blooms (Sellner 1997). Moreover, if filaments of cyanobacteria grow longer they better support heterocysts and are capable of more rapid growth (Howarth et al. 1999, Chan 2001). Contrasting to findings of Sellner et al. (1997), a recent publication proposed that zooplankton grazing can reduce cyanobacteria biomass provided they are present before cyanobacteria attain a size larger than the zooplankton species can handle (Marino 2006). In chapter 3 and 4 direct grazing on filamentous cyanobacteria by mesozooplankton species is addressed in more detail.
Lastly, there are biogeochemical factors that control N_2 fixation whereby the concentration, loading and bioavailability of micro- and macronutrients are of great importance (Fig. 1.4). Elements considered here are O_2, the macronutrients N and P, as well as the micronutrients Fe and Mo. Micronutrients have a more severe impact on diazotrophic growth, because the enzyme nitrogenase requires those elements for the function of its reaction centres (see subchapter 1.2.1).

O_2

N_2 fixation activity is negatively affected by O_2 (Fig. 1.4), as shown in subchapter

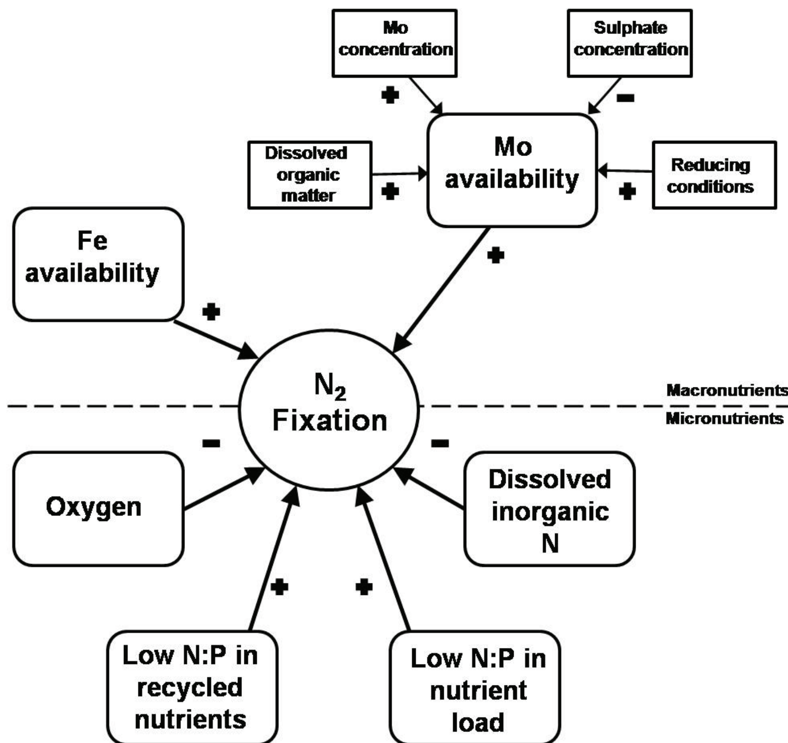

Figure 1.4: Biogeochemical control on N_2 fixation. Figure adapted from Howart et al. 1988.

1.2.1, because the enzyme nitrogenase is severely inhibited by O_2.

Macronutrients

Combined N compounds like NO_3^-, NO_2^- and especially NH_4^+ suppress N_2 fixation, whereby a concentration of 10 μmol l^{-1} DIN leads to complete decline of fixation activity within 2 hours (e.g. Mulholland et al. 2001). Nevertheless, N_2 fixation is sustained at high rates as long as the concentration of dissolved inorganic N (DIN) does not exceed 1 μmol l^{-1} (e.g. Fu & Bell 2003). This is valid especially for unicellular cyanobacteria, which show high fixation rates simultaneously with high NO_3^- concentrations in the plume of rivers (e.g. Grosse et al. in prep). Phosphorus is essential in the production within the cells to synthesize for example energy carrying phosphate compounds (ATP, NADPH), nucleic acids, several essential coenzymes and phospholipids (Marscher, 1995). The orthophosphate ion, PO_4^{3-}, is the principal form of P taken up by marine plants. Other sources include inorganic polyphosphates and dissolved organic phosphorus compounds (DOP). The

magnitude of P limitation is often rather a question of bioavailability than concentration, because DOP might be complexed to organic molecules and uptake requires complex extracellular cleavage and hydrolysis (e.g. Dyhrman et al. 2006). Processes such as luxury uptake of P by cyanobacteria might bias the cellular need for P compounds (e.g. Nausch et al. 2009). The ratio of C:P might even rise up to 400:1, indicating a smaller P demand of cyanobacteria than it would be expected from the Redfield ration (Larsson et al. 2001). Moreover, the ratio of N to P in the dissolved fraction in marine habitat governs the continuation of N_2 fixation. N_2 fixation occurs mainly when it provides a competitive advantage, because it is an energetically expensive process. If the N to P ratio is higher than the Redfield ratio (16:1), combined N will tend to be available after P is depleted. Under these circumstances N_2 fixation provides no competitive advantage, because diazotrophs exhibit a lower growth rate than other autotrophic phytoplankton. On the contrary, if the ratio of N:P in the habitat is lower than the Redfield ratio, N tends to be depleted before P is drawn down. Subsequently cyanobacteria are more competitive, because an otherwise unavailable N source is exploited (Tilman et al. 1982).

Micronutrients

Both micronutrients Fe and Mo, which are mentioned in the following subsection support N_2 fixation activity (Fig. 1.4). Cyanobacterial Fe requirements are typically higher than those of eukaryotic algae and its requirement is broad, although its use is restricted to catalytic processes, as no Fe is present in any structural components (Rueter & Unsworth 1991). Nevertheless, nitrogenase, which consists of two proteins, one large and one small (Postgate 1987), has been documented to contain up to 36 Fe atoms per complex (Rueter 1988). Fe is also present as a cofactor in various other enzymes (e.g. peroxidase, superoxide dismutases, aconitase, catalase and ribonucleotide diphosphate reductase). The uptake of Fe from the environment generally takes place through membrane transporters that directly access dissolved inorganic iron species (Wells et al. 1991). During periods of Fe deficiency, some cyanobacteria have the ability to produce siderophores, like *Anabaena* sp., *Nodularia* sp. The Baltic Sea species *Aphanizomenon* lacks this ability, which results in a competitive disadvantage over other species (e.g. Breitbarth et al. 2009). Siderophores are low molecular weight (400- 1200 Dalton) high affinity Fe(III) chelators, that are released into the external environment acting to solubilise

and chelate Fe adsorbed to particle surfaces, bound in minerals or existing complexes (Wilhelm 1995b). Those ferrisiderophore complexes can in turn be taken up by the cyanobacteria.
Mo is another essential component of nitrogenase. Neither protein can operate independently, highlighting the importance of this Fe-Mo complex. Mo is also essential in cyanobacteria that possess nitrate reductase. It is likely that Mo catalyses electron transfer in this oxidation-reduction process (Mengel & Kirkby 1987). It has been suggested that high concentrations of sulphate in seawater competes with Mo assimilation, making the acquisition of Mo more energetically expensive (Fig. 1.4, Howarth & Cole 1985).
The relative importance of some presented biogeochemical factors for marine habitats in the world ocean might be different and shall be analysed exemplarily for the brackish Baltic Sea and high saline Tropical Atlantic Ocean in the following subchapter (1.4).

1.4 Patterns of N_2 fixation in marine environments and biogeochemical control

The **Baltic Sea** is a shallow intra-continental shelf sea (415 023 km^2; mean depth 52 m) that is connected with the North Sea via the Skagerrak. The Kattegat Sound (Øresund) and Belt Sea (Great Belt, Little Belt, Kiel Bight and Mecklenburg Bight) represent the transitional area between North Sea and Baltic proper, and its shallow straits limit water exchange between the two. The hydrographic conditions especially in the deep basins (Bornholm Basin, Gdansk Deep and Gotland Basin) are dependent on the renewal of the bottom water through the inflow of high saline and oxygenated water masses from the North Sea. This event occurs infrequently (last big inflow 1993) and is governed by a combination of oceanographic and meteorological pre-conditions, low density in the bottom water of the Baltic (Matthäus & Franck 1992). During periods without an inflow, the deep water layers in the Baltic Sea tend to stagnate and a decrease in salinity, as well as O_2 concentration is detectable, while there is an increase in P. Vertically, a permanent halocline restricts the water exchange between the bottom water and the surface water. Salinity and

temperature in the upper water layer are influenced by freshwater run-off and air temperature (e.g. Malmberg & Svansson 1982), leading to a thermally stratified water body in the summer time. Blooms of diazotrophic cyanobacteria develop in summer in the Baltic Sea in areas where N:P ratios of dissolved compound are below the Redfield ratio of 16:1 (e.g. in the Baltic proper) normally from east of Bornholm up to the southern Bay of Bothnia (Niemi 1979). Typically, DIP here is drawn down from 800 nmol l^{-1} to near zero during the summer time (Nausch et al. 2004).
Growth, as well as N_2 fixation in the Baltic Sea are variable on the spatial and temporal scale showing great inter-annual fluctuations (e.g. Wasmund et al. 2001). Especially in the southern and western parts of the Baltic Proper high N_2 fixation rates are recorded (2-36 mol N m^{-2} yr^{-1}, Rahm et al. 2001). Annual estimates of N_2 fixation in the Baltic Sea are higher for open waters 60- 263 mmol N m^{-2}, Larsson et al. 2001, Wasmund et al. 2001) than for coastal habitats (21- 79 mmol N m^{-2}, Lindahl et al. 1978). This difference is attributed to the high nutrient loading in coastal waters originating from river run-off and other anthropogenic sources (Degerholm et al. 2008). Altogether, N_2 fixation can account for 3 to 13% of the total annual N to the Baltic Sea (e.g. Lindahl & Wallström 1985).
Fe concentrations in the Baltic Sea are one order of magnitude higher than in oligotrophic oceans (total Fe 9-13 nmol l^{-1}, dissolved Fe $\sim$ 3 nmol l^{-1}) and are not severely limiting N_2 fixation in this habitat. Up to 99% of this Fe is organically complexed, attributed to the high concentration of organic material with concentration of total organic carbon of $\sim$ 400 μmol l^{-1} in this environment (e.g. Breitbarth et al. 2009, Gelting et al. 2009). This richness in organic chelates leads to an accumulation of reduced and chelated Mo or Fe with concentrations of $\sim$ 10 nmol l^{-1} (Collier et al. 1985). heterocystic cyanobacteria dominate in the brackish, temperate Baltic Sea over non- heterocystic, because among other things the glycolipid envelop provides protection against O_2 and the relative high Fe availability supports N_2 fixation. Those potentially high rates of fixation facilitate growth, because more N compounds are made available for an increase in biomass, which compensates for the non-photosynthesizing heterocysts.

A different situation can be observed in the high saline and warm waters like the **Tropical Atlantic Ocean**, where non-heterocystic or unicellular cyanobacteria are dominating. Covering approximately 22% of Earth's surface, the Atlantic Ocean is

second to the Pacific Ocean in size. With its adjacent seas it occupies an area of about 106 400 000 km^2; without them, it has an area of 82 400 000 km^2. It exhibits an elongated, S-shaped basin extending longitudinally between the Americas to the west, and Eurasia and Africa to the east, and is divided into the North Atlantic and South Atlantic by equatorial counter currents, the South Atlantic Central Water (SACW) and the North Atlantic Central Water (NACW) at about 8° north latitude. The NACW can be distinguished from the SACW because it is saltier, warmer and contains less dissolved O_2. Water mass exchange in the Atlantic Ocean is accomplished by warm Tropical Surface Water (TSW), Central Water, Antarctic Intermediate Water (AAIW) and Upper Circumpolar Deep Water (UCDW), which are moving northward in the upper 1200 m. They are compensated by the cold North Atlantic Deep Water (NADW) moving southward at depths between 1200 and 4000 m. At the bottom, the northward directed Antarctic Bottom Water also carries a small amount of cold water into the northern hemisphere. Nevertheless, the surface layer of the Tropical Atlantic is occupied by the TSW, which exhibits a temperature of about 27°C. In the sharp thermocline underneath, the temperature drops from 25°C to 15°C. Imbedded in the TSW is the Salinity Maximum Water, characterized by a salinity maximum (34.8 - 35.8 psu) at about 100 m depth, while the overlying water is salinity poorer (34 psu) due to high precipitation in the tropics (e.g. Stramma & Schott 1999). The sea surface salinity (SSS) in the Tropical Atlantic shows a seasonal and cycle, affecting for example tropical cyclone formation (e.g. Giannini et al. 2003).
In the eastern Tropical Atlantic Ocean, 604 km off the western coast of Africa, opposite Mauritania and Senegal, the island country Republic of Cape Verde is situated (16°N 24°W). Studies for chapter 4 were carried out at the Cape Verde islands and a second set of samples were also taken off the Mauritanian coast. The size of Cape Verde is about 4000 km^2, composed of ten islands. These islands are divided into Barlavento (windward) islands (Sãnto Antão, São Vincente, Santa Luzia, São Nicolau, Sal, Boa Vista) and Sotavento (leeward) islands (Maio, Santiago, Fogo, Brava) with an estimated population of 500 000. The largest island is Santiago inhabiting the capital of Praia. Cape Verde is part of the Sahelian arid belt, lacking the rainfall level of other West African countries. This area is of special interest, because it is strongly influenced by trade winds and Saharan dust events which

lead to an input of nutrients into the oligotrophic habitat of the Tropical Atlantic Ocean (Fig. 1.5). This lead to the set-up of an oceanic time-series Observatory (TENATSO) site, north-east of São Vincente (17.4 °N, 24.5°W).

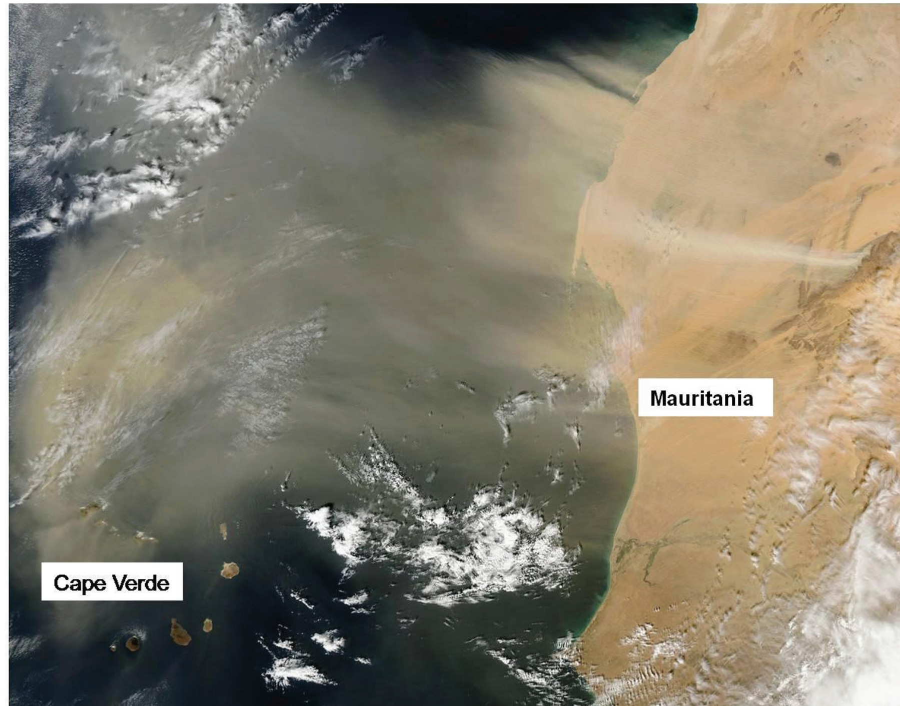

Figure 1.5: Saharan dust storm covering a large area of the Eastern Tropical Atlantic Ocean and the Cape Verde islands.

The upwelling region off the Mauritanian coast was the second area investigated in the Tropical Atlantic Ocean. It is influenced by equatorial and coastal upwelling and Saharan dust. At the Mauritanian coast for example persistent north-east trade winds drive upwelling of sub-surface waters which is rich in inorganic nutrients. Here highest euphotic layer-integrated chlorophyll a concentrations, primary production are found, resulting from fast growing phytoplankton (Marañón et al. 2000). On the other hand, open ocean regions of the Tropical Atlantic Ocean are oligotrophic, dominated by slow growing phytoplankton, with overall lower chlorophyll *a* concentrations (factor 3) and primary production (factor 10 - 20), compared to areas of upwelling (Marañón et al. 2000). Nevertheless, inputs of nutrients with Saharan dust temporarily enhance productivity, especially of diazotrophic cyanobacteria

(e.g. Mills et al. 2004). Open ocean pelagic cyanobacteria rely primarily on aeolian derived dust particles and deep upwelling of Fe rich water as their Fe source. Annual integrated rates of N_2 fixation for the Atlantic Ocean cover a range of 31-200 to 1010 mol N yr^{-1} (Gruber & Sarmiento 1997, Hansell et al. 2004) and seem to decrease towards the east (Montoya et al. 2007). N_2 fixation in the Tropical Atlantic Ocean is not limited by Mo availability (Collier et al. 1985). It is rather proposed to be Fe limited (inorganic dissolved Fe 10 pmol l^{-1}, organically complexed Fe $\sim$ 800 pmol l^{-1} Boyle et al. 2005), besides being controlled by P (Mills et al. 2004). Concentration alone is not a sufficient index for bioavailability, because Fe limitation temporarily resolved with Saharan dust storm (Boyle et al. 2004). The non-heterocystic *Trichodesmium* exhibits no siderophore mediated uptake system, creating a unique dependence for Fe-rich dust in this cyanobacterium. Rueter et al. (1992) hypothesised that *Trichodesmium* may be able to intercept, adsorb and solubilise the Fe from dust and thus allow a unique pathway for Fe acquisition. Dust particles have been shown to readily bind to *Trichodesmium* colonies and scanning electron microscopy has revealed dust particles adhered to *Trichodesmium* trichomes, covered in a sticky organic coating, confining them to the trichome's surface (Rueter et al., 1992). The influence of a low dust season and possible implications for the species composition and N_2 fixation rates in the Tropical Atlantic Ocean at the Cape Verde region is discussed in chapter 4.

The two different marine habitats, the Baltic Sea and the Tropical Atlantic Ocean, with different groups of cyanobacteria (heterocystic vs. non-heterocystic) being dominant were presented in this subchapter, showing different local controlling factors for N_2 fixation. To investigate *in situ* N_2 fixation patterns and the importance of diazotrophic N for the local food web, the Baltic Sea and the Tropical Atlantic Ocean were compared in chapter 3 and 4 of this thesis and subsequently Synthesis and future outlook) is presented in chapter 5.

1.5 The marine N cycle and transfer of diazotrophic N within the food web

N_2 fixed by diazotrophs can be readily exudated or released (see subchapter 1.2). These dissolved compounds are potentially available again for uptake by bacteria and primary producer (including diazotrophic cyanobacteria). New production based on N_2 fixation can also be transferred to higher trophic level of the food web. Diazotrophs and other primary producer remove NO_3^-, NO_2^- and NH_4^+ from the euphotic zone during the course of their metabolism and growth (Fig. 1.6). This biological uptake transforms dissolved inorganic nitrogen (DIN) to particulate organic nitrogen (PON), which in turn serves as food resource for higher trophic level. Dead particulate organic matter from all trophic levels is bacterially decomposed either in the euphotic zone or after sedimentation into the aphotic zone by ammonification and nitrification, leading to NO_3^-. This in turn can re-enter the euphotic zone by diffusion or advection. Direct predation on filamentous cyanobacteria by zooplankton has so far mostly been ignored, because cyanobacteria avoid predation by morphological adaptations (filamentous cells) and production of toxic substances (Fulton 1988, DeMott & Moxter 1991, Kirk & Gilbert 1992, Sellner et al. 1994, 1996). Chapter 3 and 4 will present results, which approves direct ingestion of filamentous cyanobacteria by mesozooplankton species. Yet, there are top down effects like "sloppy feeding" caused by zooplankton grazing, fecal pellet dissolution (Dagg 1974, Jumars 1989, O'Neil et al. 1996) and viral lysis (e.g. Fuhrman 1999) that lead to DN liberation. The process of autolysis, controlled by programmed cell death (PCD), leads to the release of DON particularly at the end of cyanobacteria blooms (Madeo et al. 2002, Segovia et al. 2003, Berman-Frank et al. 2004). It is assumed that PCD is triggered by nutrient stress (Brussaard et al. 1995, Berges & Falkowski 1998, Segovia et al. 2003).

When dead cyanobacteria are decomposed a lot of organisms like bacteria, fungi, diatoms, ciliates and juvenile decapods benefit from higher nutrient availabilities within the close environment of cyanobacterial colonies (Devassy 1979, Walsh & Steidinger 2001, Mulholland et al. 2006). These small organisms can then be ingested by mesozooplankton or other grazers channelling fixed N_2 to higher trophic levels. The importance of this so-called microbial loop is subject of the following

paragraph.

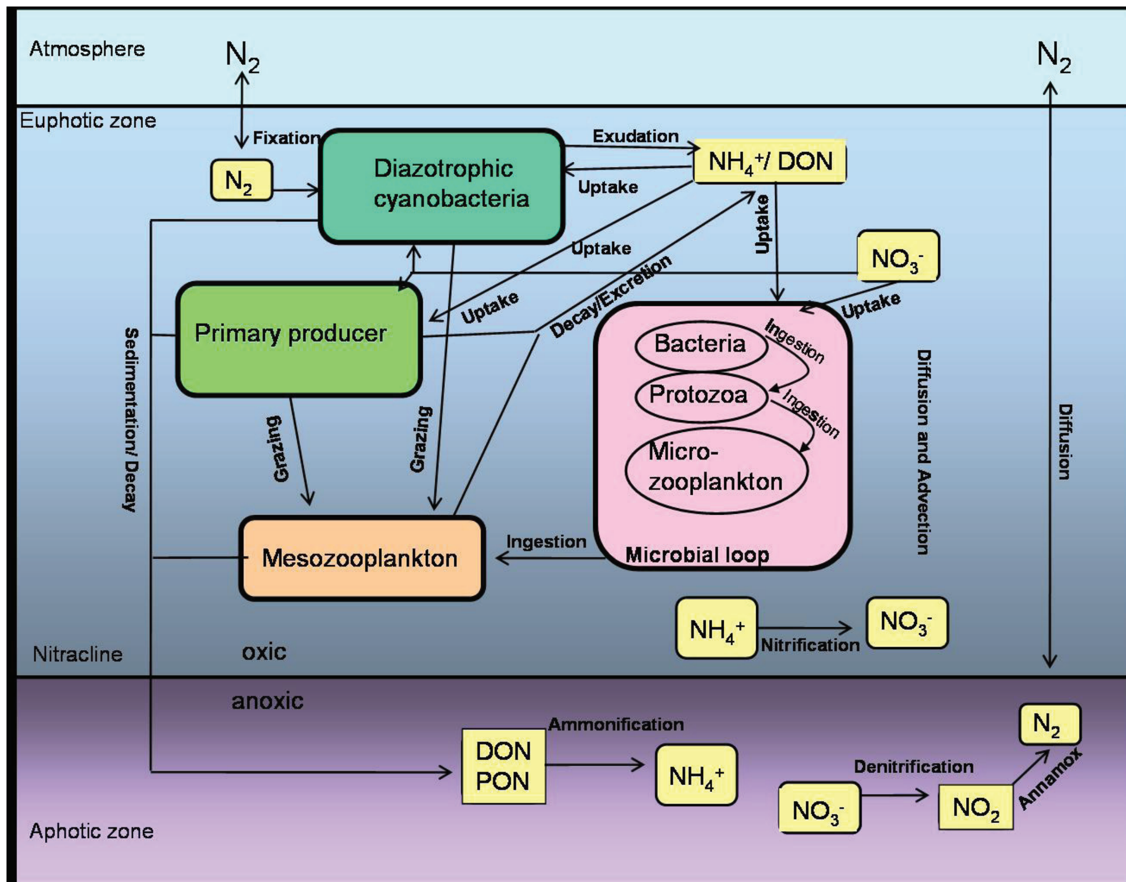

Figure 1.6: Schematic representation of the marine N cycle. (See text for further details.)

Specific aspects of the microbial loop

Azam et al. (1983) were the first who described in detail the concept of the microbial loop, where energy and nutrients are funnelled through a diverse collection of heterotrophs (viruses, bacteria, ciliates, heterotrophic nanoflagellates, HNF) before re-entering the classical food web. The microbial components are actually a series of additional trophic levels, which interact extensively with metazoans and thus link the microbial and classical grazing food web which would otherwise exist in parallel. An illustration of the interrelationships and the flux of dissolved nitrogen in the microbial loop are given in Figure 1.6. The sizes of the microbial loop species are 15 - 300 nm for viruses, < 1 μm for bacteria, 2 - 20 μm for HNF and 20 - 200 μm for the microzooplankton (microcrustacea, dinoflagellates, ciliates, rotifers).

Phytoplankton and mesozooplankton are assigned to the classical grazing food web. Bacteria, autotrophs and metazoans release DON and DIN directly in the course of metabolic activity and indirectly via lysis after viral infection and prey handling. In turn, bacteria, phytoplankton and viruses are consumed by heterotrophic protists and microzooplankton, which are subsequently grazed upon by mesozooplankton.
Contributors to the microbial food web- Abundance and productivity. Viruses are the most abundant biological entity in the water column of the world's ocean. There is evidence that they can exceed the abundance of bacteria by an order of magnitude and reach particle densities of up to 10^8 viruses ml^{-1} (Seymour et al. 2007, Suttle 2007). Almost all components of the microbial loop, as well as phytoplankton groups (including cyanobacteria) and crustacean zooplankton are infected by viruses (break up of cell compartments after infection, e.g. (Suttle 2007, Suttle et al. 1990, Brussaard 2004, Culley & Steward 2007). Thus, they are responsible for a substantial fraction of mortality in aquatic environments. Because accurate estimates of virus-mediated mortality remain elusive (Suttle 2005, Suttle 2007), a generally accepted estimate is that 20 to 40% of daily bacteria production is transformed into DOC by viruses (Suttle et al. 1990) which is of similar magnitude as the mortality through microzooplankton grazing (Fuhrman & Noble 1995). Viruses can also affect community composition, because infection is generally both host specific and density dependent (Fuhrman & Schwalbach 2003). A high level of viral lysis diverts the flow of nutrients, such as nitrogen into a semi enclosed cycle of bacterial uptake and release of organic matter. This process is known as the "viral shunt" (Fig. 1.7). Suttle (2005) concluded that a net effect of the "viral shunt" is to convert particulate organic matter (POM) into dissolved organic matter (DOM), resulting in more carbon being respired in oceanic surface waters, as viruses do not sink unless aggregated. Viral lysis thus shunts organic matter from bacteria (and phytoplankton) towards the dissolved organic pool, with a corresponding decrease in the transfer of carbon and nitrogen to metazoans. The burst size, that is the number of viruses produced per bacterial cell, ranges from 10 - 50 (Wommack & Colwell 2000) and accounts for an input of 4 - 40 nM N d^{-1} into the DOM pool. In addition, viral lysis underlies diel variability (Winter et al. 2004a, Winter et al. 2004b) with infection occurring mostly during night and lysis at noon. Viruses are altogether an important, but seldom studied group of marine organisms which play

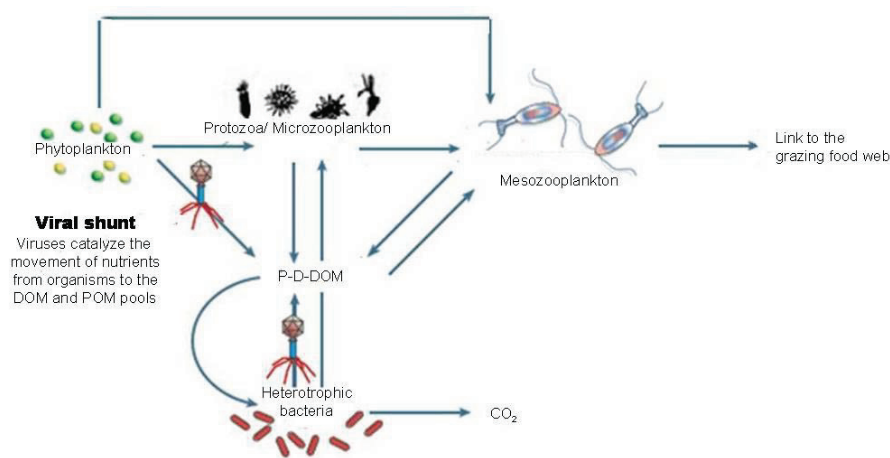

Figure 1.7: "Viral shunt" adapted from Suttle (2005). Viral lysis short-circuits the nitrogen flow from the pool of particulate organic matter to the dissolved pool, preventing nutrient and energy transfer to higher trophic level. Thereby, trophic transfer efficiency is decreased.

an important role for the recycling of nutrients.

Not only does the viral shunt cause shifts in the flow of nutrients and energy through the food web, but viral infection can also alter the gene pool of food web components. Horizontal gene transfer from hosts towards viruses and viruses towards new hosts leads to viable exchange of DNA and introduction of new genes (Chiura 1997). The range of **bacteria** abundance found in marine ecosystems is narrow. The numbers may be regulated by top down control attributed to the high grazing pressure (e.g. Troussellier et al. 2005), or bottom up by the substrate availability which controls bacterial abundance in coastal and estuarine habitats. It is generally accepted that bacterial populations decline in size from estuaries and inshore areas of greater organic and inorganic enrichment toward the more oligotrophic open sea (Sieburth 1979). Bacteria dominate the cycling of organic carbon in pelagic marine and freshwater environments, and account for a large fraction of heterotrophic community respiration (Sherr & Sherr 1996, Rivkin & Legendre 2002, Robinson & Williams 2005). Bacterial production is positively correlated to dissolved organic carbon (Carlson et al. 1996), inorganic nutrients (Kirchman & Rich 1996) and highly regulated by the temperature (Ducklow 2000). Bacterial thymidine incorporation rates can be considered as an index of the nutrient richness of an ecosystem (Billen

et al. 1990, Ducklow 1992), i.e. the higher the abundance and activity the richer the ecosystem. Noteworthy is, that bacterial production is maintained in a remarkably constant ratio to primary production, averaging about 0.15 - 0.2 across marine ecosystems (Ducklow 2000). Rates of bacterial production in coastal waters often reflect rates of phytoplankton production being the first link in the microbial loop in which DON of phytoplankton origin (exudates, cell contents released during sloppy feeding) is used. Exudation of DON by phytoplankton can for example sustain up to 90% of bacterial growth (Kristensen & Suraswadi 2002). Overall, bacteria are regarded as a sink for nutrients and are more nutrient consumers than producers (Goldman et al. 1985, Le Corre et al. 1996, Jürgens & Güde 1990).

Heterotrophic nanoflagellates (HNF) reach abundances of 10^3 - 10^4 ml^{-1} (Bano et al. 1997), but occasionally exceeding 10^5 ml^{-1} in extremely eutrophic waters (Sanders et al. 1992). They also reintroduce nutrients into the food web by remineralizing organic material to inorganic nutrients, because their prey (bacteria) act as nutrient sinks (Jürgens & Güde 1990). Their growth rate ranges from 0.043 to 0.27 h^{-1}, depending on abiotic and biotic factors (e.g. Bjørnsen et al. 1988, Laybourn-Parry & Walton 1998). HNFs are capable of active food selection and ingest preferential metabolic active food such as bacteria. They separate growing from dormant cells by sensing the presence or absence of signal molecules attached to the bacterial cell surface. This molecule is a polysaccharidic capsule which has to be continuously renewed and which is rapidly lost once the bacteria switch to dormancy (Heissenberger et al. 1996, Stoderegger & Herndl 1998). HNFs are not strictly bacteriovores, but omnivores stabilizing the structure and functioning of pelagic food webs (Strom et al. 2000).

Microzooplankton comprises a diverse assemblage of protists and metazoans of varying size, taxonomic groupings, trophic relationships and nutritional strategies (including mixotrophy). Microzooplankton also appears frequently as dominant grazer in coastal ecosystems with abundances in the range of 5 - 500 cells ml^{-1} (e.g. Buskey 1993, Gallegos et al. 1996, Lehrter et al. 1999). Recent studies indicate that microzooplankton is generally the primary herbivores in oceanic, coastal, and estuarine waters (Calbet & Landry 2004). They occupy a key position in the marine food web as major consumers of primary production (Calbet & Landry 2004), as intermediaries between primary producers and copepods (Calbet & Saiz 2005,

Gifford & Dagg 1991), and as key components of the microbial loop (Azam et al. 1983, Sherr & Sherr 2002). Growth rates of microzooplankton are in the range of 0.67 ± 0.05 d^{-1} (Landry & Calbet 2004). Secondary production rates of microzooplankton are typically in the range of 21- 38% of primary production (Landry & Calbet 2004, depending on the gross growth efficiency) but are depending substantially more (6 - 7 times) on production from phytoplankton than from heterotrophic bacteria. However, multiple trophic transfers within the microbial community can further enhance total microzooplankton production by an additional third to a half. Transfer efficiencies (TE, i.e. proportion of prey production that is converted to predator production) of microzooplankton production to mesozooplankton depend critically on the number of predatory interactions among microconsumers, and may be one way in which eutrophic and oligotrophic systems differ substantially.
Overall, the importance of N transfer from primary producers to heterotrophic organism is controversially discussed in the literature. In chapter 3 and 4 the following questions are discussed: To which extend is diazotrophic fixed N transferred instantaneously and is it possible to separate direct grazing from microbial loop mediation? The flow of N in the food web will be analysed with the application of stable isotopes.

1.6 Stable isotopes as a tool in biogeochemistry

The term isotopes refer to atoms of the same chemical elements, which have the same nuclear charge or atomic number (number of protons) but a different numbers of neutrons. Therefore they vary in their atomic mass (mass number). Isotopes are classified into two groups: stable isotopes which do not decay on geological timescales and non-stable (radioactive) isotopes which decay. Stable isotopes occur in different proportions in all elements with a "light" isotope (less neutron) being the predominating and a"heavier" isotope which is found only in traces (e.g. $^{12}C/^{13}C$ in CO_2, $^{14}N/^{15}N$ in N_2). For example the proportion of ^{14}N:^{15}N in atmospheric N_2 is 271:1 (Kendall 1998). Isotope-ratios are reported as delta (δ) values in permill (‰), relative to a standard of known composition, calculated using Equation 1.2:

$$^{15}N_{sample}(‰) = 1000 \cdot (R_{sample} - R_{standard}) \tag{1.2}$$

R represents the ratio of heavy to light isotope. The international standard for N isotopes is N_2 gas, for C it is Pee Dee Belemnite (PDE), where δ^{15}-N_2 and δ^{13}C-PDB are set to 0 ‰. Samples are measured along with reference gases (N_2, CO_2), which are calibrated relative to the international standards (IAEA-International Atomic Energy Agency). In the course of the measurement for N and C isotopes, the sample and internal standards are combusted to N_2 and CO_2 respectively, subsequently ionized in an Isotope Ratio Mass Spectrometer (IRMS), separated in a magnetic field according to their mass- to- charge ratio before being measured alternating with reference substances.
Mass variances of the single isotopes do not alter the behaviour of an element in chemical or biochemical reactions (Peterson & Fry 1987). Still in elements with low atomic numbers like N and C, the addition of a neutron leads to an isotope effect and changes in the proportion of the light and heavy isotopes in reaction products. The lighter isotope is usually accumulated in the product compared to the substrate, because it diffuses faster and requires lower activation energy (e.g. Owens 1987). This fractionation is reported as the isotope fractionation factor e (enrichment factor) and can be described by the “Rayleigh equations” (1.3):

$$R_t = R_0 f^{(1-\alpha)} \tag{1.3}$$

R_t and R_0 are the isotope ratios at t and at t=0, f is the fraction remaining at t, α is the equilibrium fractionation factor. Originally adapted for open equilibrium systems, it can also be used to approximate the evolution of isotope values during kinetic unidirectional reactions in closed systems, where the amount of substrate is finite (Mariotti et al. 1988) and depleted during a physicochemical reaction, while the product is removed from system. Uptake of N and C by organisms is a process which also leads to fractionation, because ^{14}N and ^{12}C are preferentially used (Wada & Hattori 1978), whereby the magnitude of fractionation is dependent on the nutrient source (Tab. 1.2, Pennock et al. 1996, Waser et al. 1998a). Moreover it is influenced by the plankton species (Montoya & McCarthy 1995, Needoba et al. 2003) and their growth conditions (Needoba et al. 2004).

Table 1.2: Processes associated with ^{15}N and ^{13}C fractionation (ϵ) in microalgae. Values are taken from Sigman & Casciotti (2001) and Goericke et al. (1994). Question marks indicate uncertainties concerning the fractionation factor of the respective process.

Reaction	Substrate	ϵ
NO_3^- assimilation	NO_3^-	4 - 6
N_2 fixation	N_2	0 - 2
NH_4^+ assimilation	NH_4^+	6.5 - 8
Nitrification	NH_4^+	15
Denitrification	NO_3^-	20 - 30
CO_2 diffusion	CO_2	<0.7
Passive DIC uptake	HCO_3^-	<0.7
Active DIC uptake	CO_2	small?
Rubisco carboxylation	CO_2	20 - 29

The fractionation factor will be applied in chapter 4, trying to identify processes of N assimilation within the water column. Isotopes are not only fractionated during uptake of nutrients by organisms, but also during their transfer within the food web. On the other hand, mixture counteracts the effect of fractionation, because it recombinates heavy and light isotopes from different sources. Fractionation and mixing results in characteristic stable isotope signatures of food web components. This can be exploited in order to identify food web relationships and food sources. It appears that consumers are slightly enriched in $\delta^{13}C$ values relative to their food leading to a difference between the two compartments of 0.5 - 1 ‰per trophic position (e.g. Rau & Anderson 1981, Peterson & Fry 1987). This is attributed to preferential loss of $^{12}CO_2$ during digestion and metabolic fractionation during tissue synthesis (e.g. DeNiro & Epstein 1978). Caution has to be taken, because individuals of the same species feeding on the same diet might vary up to 2 ‰(DeNiro & Epstein 1978). $\delta^{13}C$ values are conserved within the food web, but vary at the base of the food web. Typical $\delta^{13}C$ values of phytoplankton are in the order of -15 to -19 ‰and -21 to -25 ‰(nano/picoplankton) (Fry & Sherr 1984, Rau et al. 1990). Within cells metabolic gradients show different $\delta^{13}C$ values, e.g. lipids are usually more depleted in $\delta^{13}C$ compared to carbohydrates (DeNiro & Epstein 1977). Zooplankton usually exhibits a $\delta^{13}C$ values in the range of -16 to -21 ‰(e.g. Fry & Sherr 1984), depending on their food source (e.g. benthic algae are more depleted in $\delta^{13}C$ than planktonic algae due to the acquisition of respired CO_2 which is lighter than assimilated C, DeNiro

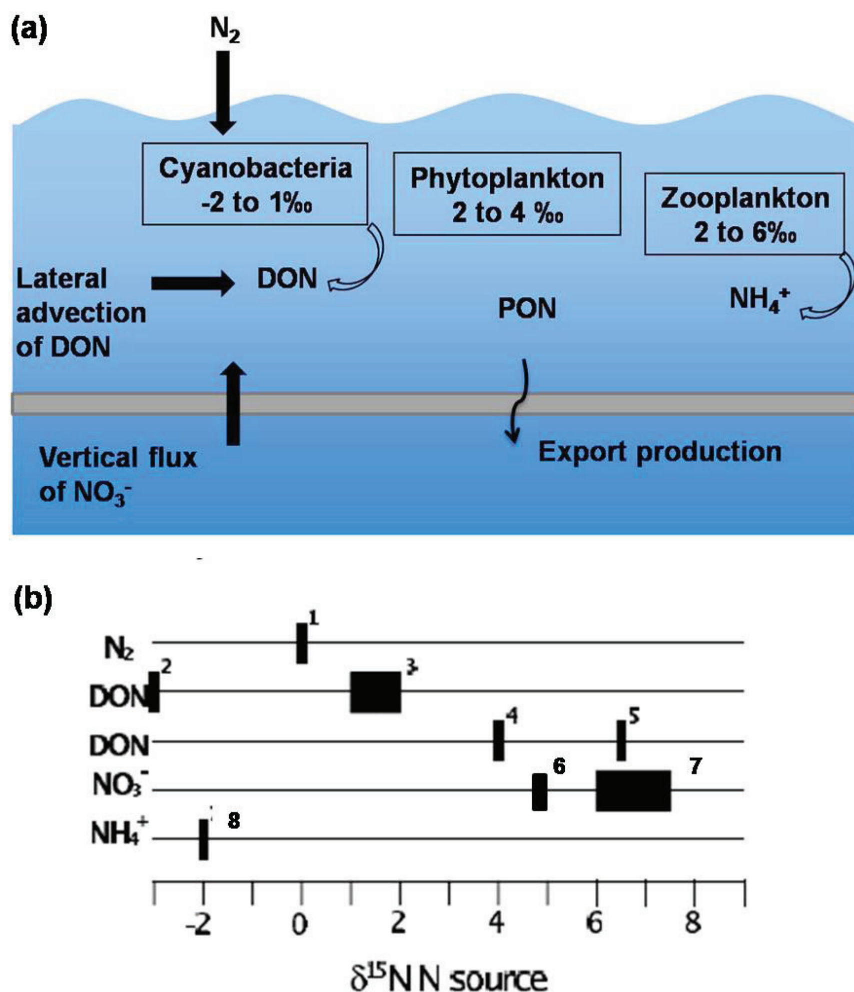

Figure 1.8: Schematic representation of the impact of diazotrophic N_2 fixation on N stable isotopes of PON in the surface ocean (a), where the actual δ^{15}N-PON signature is a mixture of N_2 fixation lateral advection of nitrate and dissolved organic nitrogen (DON), vertical transfer of nitrate (NO_3^-) from deep ocean and ammonium (NH_4^+) excreted by zooplankton. (b) Represents the stable N isotopic values associated with the different N source. 1: atmospheric N_2 (Minagawa & Wada, 1986). 2 and 3: DON from a N_2 fixing source (Liu et al. 1996, Abell et al. 2000). 4 and 5: DON from non-N_2 fixing source (Benner et al. 1997, Knapp & Sigman 2003). 6 and 7: NO_3^- from deep ocean (Liu & Kaplan 1989, Montoya et al. 2001). 8: NH_4^+ (Checkley & Miller 1989). Figure adapted from Mahaffey et al. (2004).

& Epstein 1978) and their mode of nutrition (omnivory, i.e. feeding on more than one trophic level, McCutchan et al. 2003). In contrast to C stable isotopes, δ^{15}N values show a clear increase as N moves through the food web. Other than that, the δ^{15}N value also reflects the inputs of N supporting production of primary producer. Deep- water NO_3^- has an average δ^{15}N of 4.5%, resulting in a δ^{15}N value of phytoplankton biomass of 3.5 to 10 ‰(Liu and Kaplan 1989, Sigman et al. 1997). Atmospheric N_2 exhibiting a value of 0 ‰lowers the δ^{15}N of diazotrophic cyanobacteria to -2 ‰(Fig. 1.8, e.g. Montoya et al. 2002). The δ^{15}N of autochthonous PON in phytoplankton is therefore determined by the δ^{15}N of the N substrate and the fractionation factor (Altabet & Francois 2001). Consumers are enriched by 3 to 4 ‰compared to their direct food source (e.g. Peterson & Fry 1987), presumably due to preferential excretion of ^{14}N (e.g. Minagawa & Wada 1984). The δ^{15}N of zooplankton might change from values of 5 to 12 ‰, when ingesting NO_3^- depending phytoplankton and deep water NO_3^-, to values of 2 to 6 ‰, when ingesting diazotrophs or phytoplankton (Fig. 1.8, Liu & Kaplan 1989, Montoya et al. 2002). The impact of diazotrophy on stable isotopes of NO_3^- and PON, which renders their δ^{15}N value, as seen in Figure 1.8, is discussed in chapter 4. Moreover, N stable isotopes and their measurement are the foundation of experiments of all chapters. The specific application of stable isotopes in the mode, in which they are used, is explained in detail in the “Material and Method” section of the single chapters.

1.7 Aims of this dissertation

The aim of this dissertation is to contribute to the existing knowledge on the role of diazotrophic cyanobacteria in the marine environments. The overarching hypothesis is that diazotrophy represents an important instantaneous source of new N for bacteria, but also higher trophic levels. This was studied in different marine environments, in the brackish eutrophic Baltic Sea, the oligotrophic Tropical Atlantic Ocean and the coastal upwelling region off Mauritania. This thesis is integrated and financed by the WGL (Wissenschaftsgemeinschaft Gottfried Wilhelm Leibniz) network TRACES (Ocean - Atmosphere-Land Impacts on Tropical Atlantic ecosystems) focusing on the exchange of matter between land-ocean and atmosphere and the influence of river inflow into the Tropical Atlantic Ocean. Chapter 2 will focus

on the determination of abiotic factors which regulate the exudation of N and C during a diel cycle, exemplary for two cyanobacterial species (*Nodularia spumigena* and *Trichodesmium erythraeum*). Species were selected according to their dominance in the marine environment sampled for chapter 3 and 4. Laboratory studies were carried out to estimate the quantity of N compounds, which are released and to clarify the reason for differences between the investigated species. Moreover, light intensity and P availability were tested for their potential to increase N exudation. This chapter provides fundamental findings for the field experiments discussed in chapter 3 and 4. It was co-authored by Boris Koch and Maren Voss and is accepted for publication in "Aquatic Microbial Ecology". Chapter 3 reports N_2 fixation rates and the transfer of diazotrophic fixed N within the food web of the Baltic Sea to higher trophic level. *Nodularia spumigena* was one of the dominant species during the investigation period. Moreover, the dominant pathway of N channelling is identified. It was co-authored by Frederike Korth and Maren Voss and is currently revised for "Marine Ecology Progress Series". Chapter 4 combines three approaches to elucidate the importance of diazotrophic N_2 fixation in the Eastern Tropical Atlantic Ocean for the nutrition of higher trophic level. Firstly, the natural abundance of δ^{15}N- NO_3^- within the water column at the Mauritanian coast is investigated. Samples were taken by Herman Bange and participants of the R/V Poseidon cruise 348.
A two source mixing model using natural abundance of δ^{15}N- PON was applied the Cape Verde Islands. Moreover, ^{15}N tracer addition experiments at the Cape Verde serve to identify diazotrophic N transfer within the food web. Hereto, samples were taken in cooperation with Julie LaRoche during the R/V Islandia cruises.
A detailed list of chapters and a statement on my contribution the manuscripts can be found on pages xix and xxi.

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Chapter 2

Factors influencing the release of fixed N_2 and C as dissolved compounds (TDN and DOC) by *Trichodesmium erythraeum* and *Nodularia spumigena*.

Abstract

Diel variations of N_2 and C fixation rates, as well as subsequent release of total dissolved nitrogen (TDN) and dissolved organic carbon (DOC) were determined for *Trichodesmium erythraeum* and *Nodularia spumigena*. A circadian rhythm of N_2 and C fixation, as well as a periodicity in the calculated release of dissolved compounds was observed. From the amount of nitrogen and inorganic carbon fixed by *T. erythraeum* during the light period of the experiment 71% and 50% were released as TDN and DOC, respectively. For the species *N. spumigena* we found a release of 89% and 53% during the light period. Additionally, two controlling factors (light and nutrient concentrations) for the release of TDN and DOC were studied. The data suggest that rapid shifts towards higher light intensity lead to a pronounced exudation of TDN and DOC. On a

short-term basis (first 30 minutes after exposure) the exudation of NH_4^+ and DON consumed up to 52% of electrons harvested by the cells in the same time interval. Thus, TDN release serves as a potential electron sink and protects cells from photodestruction. On the other hand, there was no clear effect of phosphorus concentration on the release of TDN and DOC. Our results indicate that uptake and subsequent exudation of TDN and DOC might be induced by abiotic parameters, besides being regulated endogenously by multiple feedback loops.

2.1 Introduction

In marine waters N availability generally controls primary production (e.g. Ryther & Dunstan 1971, Hecky & Kilham 1988, Falkowski 1997) and is an important potential growth limiting factor with ambient dissolved inorganic nitrogen concentrations of < 1 μM (Flores & Herrero 2005). In such environments cyanobacteria that can fix atmospheric dinitrogen (N_2) have a competitive advantage over most other photoautotrophic species. In the tropical and temperate oceans N_2 fixing cyanobacteria can be extremely abundant and account for a considerable input of combined N into the upper mixed layer (Montoya et al. 2002, Capone et al. 2005), with a strong impact on local community production (e.g. Tseng et al. 2005). Thereby they transiently dominate primary productivity and the N cycling (Bowman & Lancaster 1965, Capone et al. 1998, Karl et al. 1992). Estimates of global biological N_2 fixation are in the range of 80-110 Tg N yr $^{-1}$ (Gruber & Sarmiento 1997, Capone & Carpenter 1999). Local N_2 fixation rates for the North Atlantic Ocean are in the range of 259-864 mmol N m $^{-2}$ yr^{-1} (Capone 2005a) and are mainly attributed to the activity of *Trichodesmium*. For the Baltic Sea estimations of N_2 fixation are in the range of 55-840 mmol N m^2 yr^{-1} (Wasmund et al. 2005). A significant fraction of this recently fixed N can be directly released by cyanobacteria dispensing up to 80% of the N into the surrounding environment (Glibert & Bronk 1994, Bronk et al. 1994, Nagao 1999, Slawyk 2000, Ohlendieck 2000, Mulholland & Capone 2004). This total dissolved nitrogen (TDN) is composed of dissolved inorganic (DIN: NH_4^+, NO_2^-, NO_3^-) and organic compounds (DON: e.g. dissolved free amino acids, DFAA). The latter might even be quantitatively dominating (Capone et al. 1994, Glibert &

Bronk 1994, Vidal et al. 1999, Berman & Bronk 2003). Several explanations for the active exudation of dissolved organic matter (DOM) can be found in the literature such as the release of excess photosynthetats (Fogg 1983) or of DON to supply cells within the colony lacking the enzyme nitrogenase (Capone 1994, Mulholland et al. 2004a). Indirectly N, incorporated into diazotrophic biomass, can be liberated by processes like "sloppy feeding" and excretion from zooplankton (Dagg 1974, Jumars 1989, O'Neil et al. 1996), viral lyses (Fuhrman 1999) and programmed cell death (Madeo et al. 2002, Segovia et al. 2003, Berman-Frank et al. 2004). To date, the knowledge about the dynamics of DOM, especially DON production in marine ecosystems is still limited. It is not clear which factors regulate the exudation of DON in cyanobacteria. Lomas et al. (2000) postulated an internal factor being responsible for the increased release of nitrogenous compounds during periods of cellular imbalanced energy conditions in diatoms and flagellates. This effect occurred to accelerate the dissipation of excitation energy through processes other than the photosynthetic C metabolism. On the other hand, nutrient supply and physiological condition may stimulate the exudation of DON as shown in batch culture studies (Watt 1969, Fogg 1983, Chrost & Faust 1983, Sundh 1989). Consequently, algae that are replete in N and whose cellular N demand is fulfilled in excess tend to release more DON, especially in the exponential growing phase (Myklestad et al. 1989, Bronk 1999). Moreover, Nagao & Miyazaki (2002) showed that the release is dependent on the nitrogen source (NO_3^- vs. NH_4^+) and that release not necessarily derives from recently assimilated N. Additionally, N_2 fixation itself can be limited by iron and phosphorus (Karl et al. 1995, Sañudo-Wilhelmy et al. 2001, Mills et al. 2004, Mulholland & Bernhardt 2005). Thus, any enhancement in available limiting nutrients should increase the release of TDN, as soon as the cells are more N replete. The aim of this study was to examine possible factors that regulate the release of dissolved nitrogen (TDN) and dissolved organic carbon (DOC) in N_2 fixing cyanobacteria. Three questions are addressed: 1) Are DON and DOC released during the course of a diel cycle which is unaffected by any stress through high light or nutrient concentrations? 2) Which role does short-term cellular energy imbalanced conditions play? 3) Does the metabolic condition, like phosphorus availability, influence the release of TDN and DOC? The questions were studied for two marine species, the tropical, non-heterocystic *Trichodesmium erythraeum* and the temperate, hete-

rocystic *Nodularia spumigena* (Cyanobacteria).

2.2 Material and Methods

Culture condition and survey of a diel cycle — The heterocystic cyanobacterium *N. spumigena* was isolated from the Baltic Sea and maintained at the Leibniz Institute for Baltic Sea Research in batch cultures on F/2 medium free of any combined N compounds. The non-heterocystic *T. erythraeum*, strain IMS101 was originally isolated from the Atlantic Ocean and was obtained from the IFM-GEOMAR, Kiel. *T. erythraeum* was grown in batch cultures on medium YBCII (Chen et al. 1996) at 30°C under an alternating cycle of 12 h light (cool, white fluorescent lighting, = normal light NL 100 μmol photons m^{-2} s^{-1}) and 12 h darkness. *N. spumigena* was cultured at 15°C in a walk-in incubation chamber supplied with a light cycle of 16:8 (cool, white fluorescent lighting, = normal light NL 60 μmol photons m^{-2} s^{-1}). Cultures of both species were axenic when starting the experiments. Overall bacterial biomass during the course of the long-term experiments ($>$ 1 d) never exceeded 1% of cyanobacterial biomass. Cultures were routinely mixed to prevent adhesion of cyanobacteria to the sides of the culture vessels. To initiate the investigation of the diel cycle, duplicate 250 ml polycarbonate incubation bottles containing N-free medium were inoculated with equal volumes of an exponentially growing Trichodesmium or Nodularia parent culture and the stable isotope tracer ($^{15}N_2$, $NaH^{13}CO_3$) at the same starting point. Trichome number of both species was identical in all set ups. To follow the diel cycle of N_2 and C fixation, particulate organic nitrogen (PON) and carbon (POC), chlorophyll *a*, as well as the concentrations of dissolved inorganic nitrogen (DIN), total dissolved nitrogen (TDN), dissolved organic carbon (DOC) and dissolved free amino acids (DFAA) were measured in duplicates by gently vacuum filtrating over GF/F (Whatman) filter every 2 hours for a total of 24 to 26 hours. This set-up was carried out twice for both species. A summary of experimental conditions is given in Table 2.1).

Experimental design. Light shift and nutrient supply experiments — Light shift experiments were initiated by inoculating replicate culture vessels containing N-free medium with equal volumes of an exponentially growing parent culture. The first subset of triplicate vessels were exposed to the light intensity ap-

Table 2.1: Overview of experimental set up. Detailed information is given in the text. NL normal light, HL high light.

Treatment	*T. erythraeum*			*N. spumigena*		
(1)Diel cycle						
Growth media		YBCII			F/2	
Light: Dark		12:12			16:8	
Light intensity (μmol photons m^{-2} $^{-1}$)		100			60	
PO_4^{3-} (μM)		10			10	
Time of incubation (h)		24			26	
No. of experimental runs		2			2	
No. of replicate incubation bottles		2			2	
(2)Light	NL		HL	NL		HL
Growth media		YBCII			F/2	
Light: Dark		12:12			16:8	
Light intensity (μmol photons m^{-2} s^{-1})	100		200	60		120
PO_4^{3-} (μM)		10			10	
Time of incubation (h)		6			6	
No. of experimental runs		1			1	
No. of replicate incubation bottles		3			3	
(3)Phosphorus	Low P	Mid P	High P	Low P	Mid P	High P
Growth media		YBCII			F/2	
Light: Dark		12:12			16:8	
Light intensity (μmol photons m^{-2} s^{-1})		100			60	
PO_4^{3-} (μM)	1	10	20	1	10	20
Time of incubation (d)		5			5	
No. of experimental runs		1			1	
No. of replicate incubation bottles		2			2	

plied during the normal culturing process (NL: 100 μmol photons m^{-2} s^{-1} and 60 μmol photons m^{-2} s^{-1}, for *T. erythraeum* and *N. spumigena*, respectively), while the second subset of triplicates were simultaneously exposed to high light intensities with twice as much light intensity (HL: 200 μmol photons m^{-2} s^{-1} and 120 μmol photons m^{-2} s^{-1}, for *T. erythraeum* and *N. spumigena*, respectively). A summary of the experimental conditions is given in Table 2.1. Concentrations of DIN, DFAA, TDN, DOC and chlorophyll *a* were measured at: 30, 60, 120, 180 and 360 minutes after exposure to the respective light regime. Rate of N_2 and C fixation, as well as PON and POC concentrations were obtained by using particulate matter collected on pre-combusted glass fibre filter (GF/F, Whatman) for the measurements.
Additionally, a control set was run in *N. spumigena* cultures to test for photochemical driven elevation in dissolved compounds. Cyanobacteria were filtered through GF/F filters prior to light exposure. Light intensity was measured in the 400-700 nm range (photosynthetically available radiation, PAR) using a spherical quantum sensor (QSL-101, Biospherical Instruments, San Diego, CA).
The cultures which were used to investigate the influence of the phosphorus concentrations were pre-incubated three days in medium containing very low phosphorus concentrations ($<$0.1 μM) in order to empty all intracellular P-storages. Subsequently, three subsets of triplicates were used to study the effect of low (1 μM) medium (10 μM) and high (20 μM) phosphorus concentration in 2.5 l polycarbonate incubation bottles. Inoculation of all replicates started simultaneously by adding the same amount of cyanobacterial parent culture and stable isotope tracer. Sub samples were sacrificed on a daily basis for five consecutive days to measure rates of N_2 and C fixation, concentration of DIN, DFAA and TDN and DOC in the growth medium. Daily rate measurements were always made at 16 hours.
Nutrient and chlorophyll *a* analysis — Subsamples of the filtrates were taken for the analysis of dissolved nutrients (${NH_4}^+$, ${NO_2}^-$, ${NO_3}^-$ and ${PO_4}^{3-}$) and measured colorimetrically in a spectrophotometer U 2000 (Hitachi-Europe GmBH, Krefeld, Germany) according to Grasshoff et al. (1983) with a precision of 0.1μM. ${NO_3}^-$ and ${NO_2}^-$ concentrations remained undetectable in the course of all culture experiments. chlorophyll *a* filters were extracted in ethanol prior to fluorometrical determination of concentration.
Analysis of dissolved free amino acids (DFAA) — The dissolved free amino

acids were separated via HPLC (Elite LaChrom VWR) using a reversed phase column (5 h, LiChroCart 125-4, MERCK) at a temperature of 55°C. A multi-step gradient elution was used with a flow-rate of 1 ml min^{-1}. Solvent A contained 50 mM formic acid and 60 mM acetic acid (pH 2.9). Solvent B contained 50 mM formic acid and 60 mM acetic acid and 50% 2-propanol (pH 2.9). Prior to measurement, the amino acids were derivatised with dansyl chloride (Wiedmeyer et al. 1982). The quantification of the dansyl derivatives of alanine (Ala); arginine (Arg); asparagine (Asp), glutamine (Glu); glycine (Gly); leucine (Leu); lysin (Lys), proline (Prol); serine (Ser); taurine (Tau); valine (Val) were performed by fluorescence detection (excitation 320 nm; emission 490 nm).

Total dissolved N (TDN), DOC and DON — Total dissolved nitrogen (TDN) and dissolved organic carbon (DOC) concentrations were determined simultaneously in the filtrate by high temperature catalytic oxidation with a Shimadzu TOC-VCPN analyser. In the auto sampler 6 ml of sample volume (in pre-combusted vials) were acidified with 0.12 ml HCl (2 M) and sparged with oxygen (100 ml min^{-1} for 5 min to remove inorganic C. 50 μl sample volume was injected directly on the catalyst (heated to 680°C). Detection of the generated CO_2 was performed with an infrared detector. Final DOC concentrations were average values of triplicate measurements. If the standard variation or the coefficient of variation exceeded 0.1 μM or 1%, respectively, up to 2 additional analyses were performed and outliers were eliminated. Total N is quantified by a chemiluminescence detector (gas flow oxygen: 0.6 l min $^{-1}$. After every 5 samples one blank and one standard was measured for quality control. The concentration of DON was obtained indirectly by subtracting the measured values of TDN and DIN.

Isotopic analysis and rates measurements — Stable N and C isotope ratios (δ^{15}N-PON, δ^{13}C-POC) as well as PON and POC concentration were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020°C in a Thermo Finnigan Delta S mass-spectrometer. Filters containing particle samples were trimmed, sectioned and then loaded into tin capsules and pelletised for isotopic analysis. The stable N and C isotope ratios measured for each sample were corrected against the values obtained from standards with defined N and C element and isotopic compositions (International Atomic Energy Agency IAEA: IAEA-N1, IAEA-N_2, NBS 22 and IAEA-CH-6) by mass balance. Values are reported relative to atmospheric N_2

(δ^{15}N) and VPDB (δ^{13}C- Vienna Peedee belemnite). The analytical precision for both stable isotope ratios was 0.2 ‰. Calibration material for C and N analysis was acetanilide (Merck). N_2 fixation activity was measured using the ^{15}N-N_2 assay, C fixation using ^{13}C-$NaHCO_3$. Tracer incubations were terminated by gentle vacuum filtration through pre-combusted GF/F filters. These filters were dried at 60 °C and stored for isotopic analysis. Rates were calculated using the approach of Montoya et al. (1996). To compare these results to literature data and to relate them to biomass, rates were chlorophyll *a* normalized. Atom percentage excess enrichment in the DON and DIN pool was not measured. Since the cultures used were axenic during the start of the experiments, release and uptake of compounds were computed by determining the difference in concentration of NH_4^+, DON and DOC for each time point in comparison to the previous sampling:

$$\text{c TDN/DOC} = \text{c TDN/DOC}_{\,t} - \text{c TDN/DOC}_{\,t-1}.$$

Positive values reflect a surplus in concentrations and therefore release, whereas negative values reflect loss in concentration due to uptake. Release rates were chlorophyll *a* normalized.

Statistical analysis—Statistical analysis was done using SPSS (SPSS Inc). Student's t-test (Tukey method of multiple comparisons) was conducted to determine whether the results obtained from individual treatment incubations on the effects of light intensity were significantly different. Statistical comparisons on the effect of different phosphorus concentrations were made using either one-way analysis of variance (ANOVA) for normally distributed data or H-test for data sets which showed no normal distribution (Maxwell & Delaney 2003). The check for normal distribution was done using the Kolmogorov-Smirnov-test. For analysing the homogeneity of variances the Levene test was applied (Maxwell & Delaney 2003).

2.3 Results

Diurnal variation in N_2 and C fixation and release of nitrogenous compounds in the course of the day—The batch culture of *T. erythraeum* and *N. spumigena* exhibited a characteristic diurnal pattern of N_2 and C fixation (Fig. 2.1).

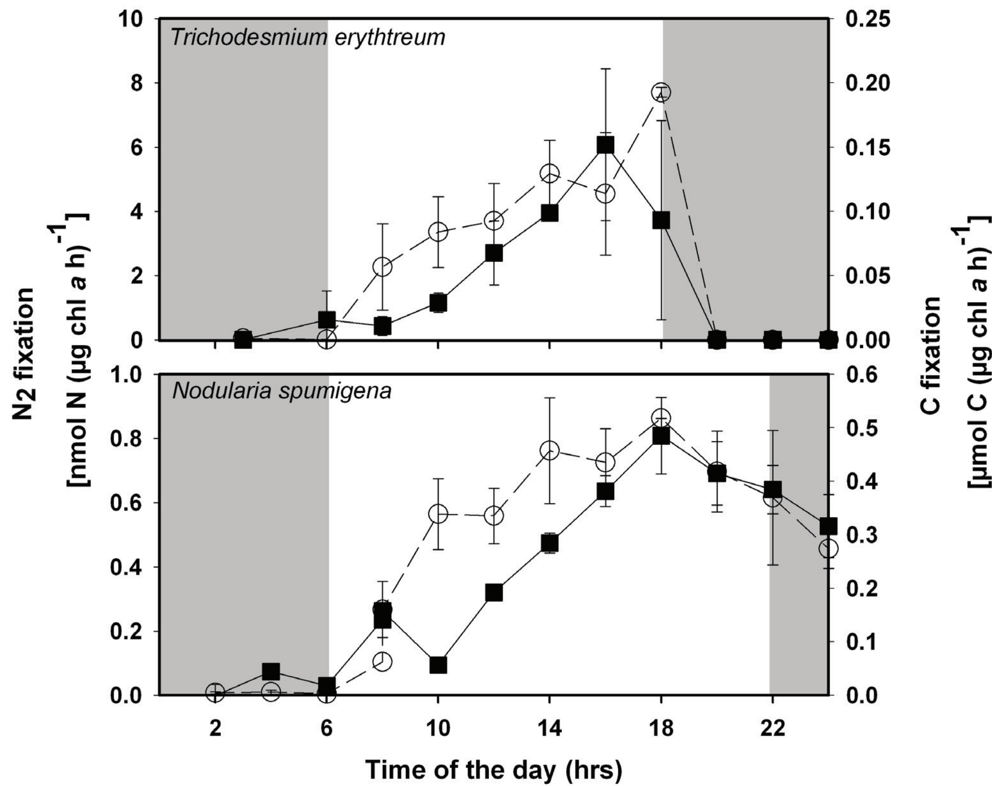

Figure 2.1: Diel variation of N_2 (solid line, filled circles) and C fixation (dashed line, open circles) of *Trichodesmium erythraeum* and *Nodularia spumigena.* Values are means and standard deviations of four replicates. Grey areas indicate dark periods.

N_2 fixation started with the onset of the photoperiod (6 hours). Maximum N_2 fixation rates were reached at 16 hours with 6.07 ± 2.36 nmol N (Chl *a* h)$^{-1}$ (n = 4) in *T. erythraeum* and at 18 hours in *N. spumigena* with a rate of 0.81 ± 0.12 nmol N (Chl *a* h)$^{-1}$ (n = 4). The highest rates of C fixation were found at 18 hours with maximum rates in *T. erythraeum* of 0.19 ± 0.003 μmol C (n = 4) and 0.52 ± 0 μmol (Chl *a* h)$^{-1}$ (n = 4) in *N. spumigena.* The specific growth rates for the time observed were equivalent to 0.324 d^{-1} for *T. erythraeum* and 0.319 d^{-1} for *N. spumigena.* The ratios of POC to PON and C to N fixed levelled at 7.29 ± 0.7 and 10.6 ± 3.2, respectively in *T. erythraeum.* During growth in *N. spumigena* the ratio of POC to PON was 6.5 ± 0.8. The ratio of C and N uptake was 6.15 ± 4.8. The concentration of NH_4^+ in the batch medium of *T. erythraeum* and *N. spumigena,* showed two maxima during the light period at 8 hours and 20 hours and one mini-

mum at 14 hours (Fig. 2.1 a,b). The absolute concentrations were 3.7 times higher for *N. spumigena* than for *T. erythraeum*. Normalised release rates of NH_4^+ in *T. erythraeum* showed a maximum of 0.06 ± 0.02 μmol N (Chl *a* h)$^{-1}$ (Fig.2.4 a). Maximum release rate in *N. spumigena* was 0.10 ± 0.067 μmol N (Chl *a* h)$^{-1}$ (Fig.2.4 b). DON concentrations in the batch media of both species seemed to fluctuate with a distinct minimum in concentration at 14 hours in *N. spumigena* and two minima at 8 hours and 18 hours in *T .erythraeum* (Fig. 2.2 c, d). Absolute values of ambient concentration were lower for *T. erythraeum* by a factor of 4.7 (8.4 μM vs. 38.5 ± 0.7 μM). Release rates of DON calculated from the differences of the maximum release rate in *T. erythraeum* was 0.35 ± 0.07 μmol N (Chl *a* h)$^{-1}$) and in *N. spumigena* 1.35 ± 0.18 μmol N (Chl *a* h)$^{-1}$ (Fig. 2.4 c, d). DFAA contribute to the DON pool with 7.9% in *T. erythraeum* and 1.3% in *N. spumigena* with maximum concentrations of 0.48 ± 0.06 μM and 0.66 ± 0.06 μM, respectively (Fig. 2.2 e, f). Glu and Gly accounted for 80% of bulk DFAA, but the average percentage of the dominant amino acid differed significantly between the two species ($p < 0.001$, $n = 12$). In *T. erythraeum* Glu was the most abundant amino acid (Glu 47.7%, Gly 32.3%), in *N. spumigena* Gly (Gly 44.6%, Glu 27.4%, Fig. 2.3). Other detectable DFAA were Ala, Val, Prol, Asp, Arg, Tau, and Lys. Maximum release rates of bulk DFAA in *N. spumigena* was 0.012 ± 0,001 μmol N (Chl *a* h)$^{-1}$ (Fig.2.4 f). Maximum release rate in *T. erythraeum* was 0.008 ± 0.002 μmol N (Chl *a* h)$^{-1}$ (Fig. 2.4 e). There was a pronounced maximum in the DOC concentration in *T. erythraeum* at 22 hours of 189.2 ± 26.2 μmol l^{-1} (Fig. 2.2 g) and maximum release rate of DOC occurred at 18 hours with a rate of 10.9 ± 0.8 μmol (Chl *a* h)$^{-1}$(Fig.4 g). In *N. spumigena* there was a distinct minimum and fluctuation in DOC concentration visible at 14 hours (Fig.2.2 h). The maximum release rate of 8.4 ± 1.1 μmol occurred at 16 hours (Fig. 2.4 h).

Fixation and release of dissolved compounds in response to shift in light intensity—Compared with the normal light (NL) conditions both species showed a significant rise in C fixation under high light (HL) influence ($p < 0.05$, Tab. 2.3). The mean values refer to measurements of three replicate incubation bottles after 180 and 360 minutes after the beginning of the experiment. In contrast, N_2 fixation in *T. erythraeum* cultures did not change significantly with doubling in light intensity (HL and NL: 1.19 ± 0.52 (μg Chl *a* h)$^{-1}$ and 0.86 ± 0.41 nmol (μg Chl

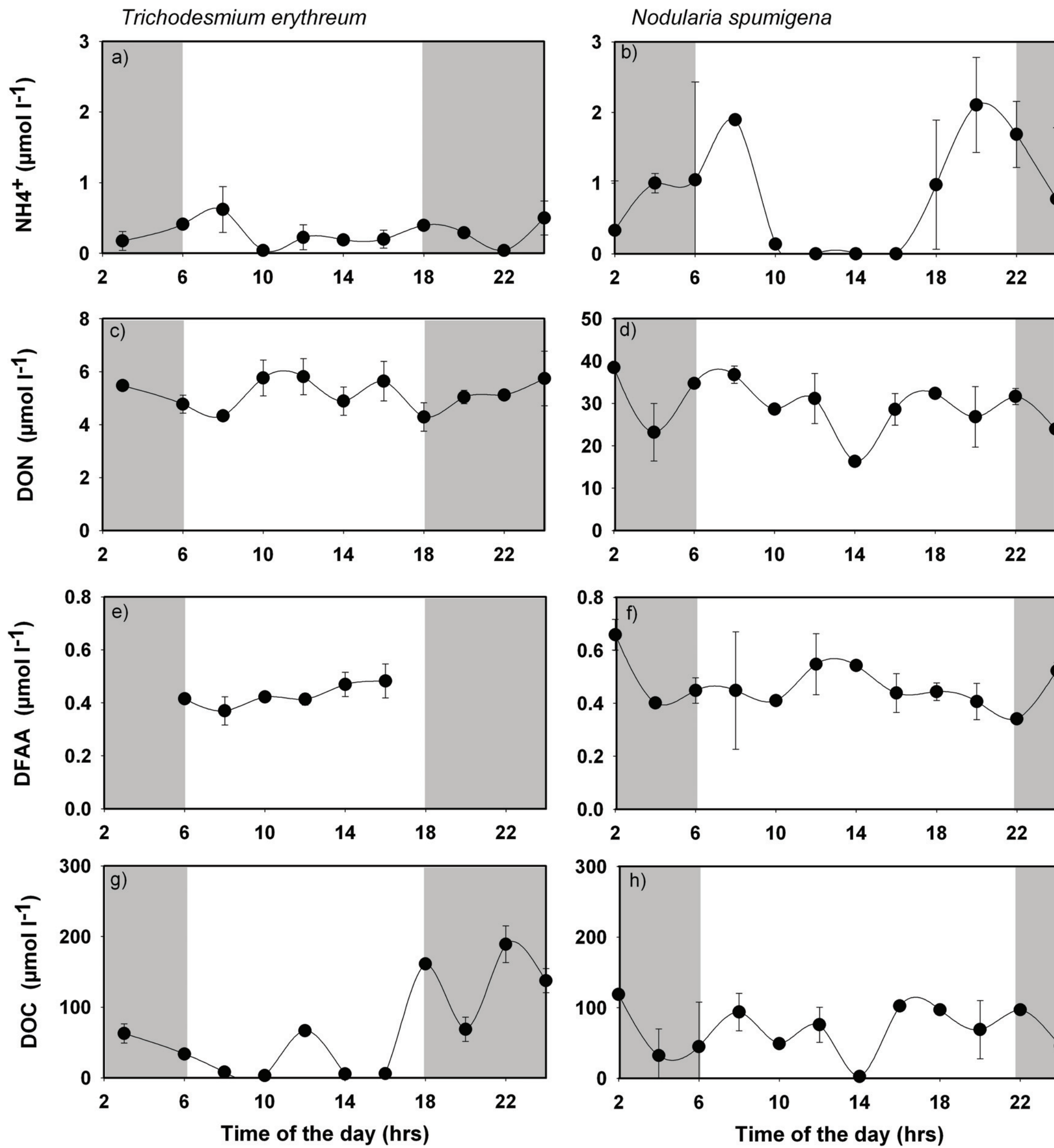

Figure 2.2: Diel variation in extracellular NH_4^+ (a,b), DON (c,d), DFAA (e,f) and DOC (g,h) concentrations in *Trichodesmium erythraeum* and *Nodularia spumigena* cultures. Symbols represent the mean value of four replicates and standard deviation. Grey areas indicate dark periods. Note the different scales for DON concentrations of the two species.

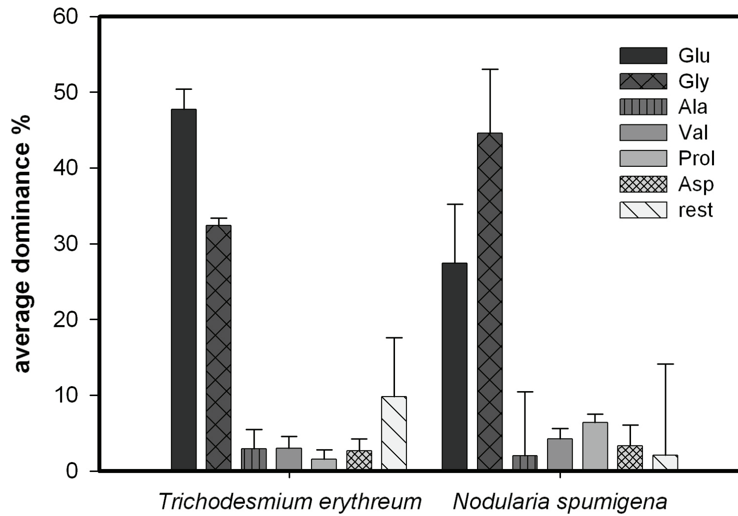

Figure 2.3: Average percentage dominance of extra-cellular dissolved free amino acids in *Trichodesmium erythraeum* and *Nodularia spumigena* during the course of a day. Amino acids Taurine, Leucine and Lysine are combined in rest.

a h)$^{-1}$, respectively, n=6), whereas N_2 fixation in *N. spumigena* was significantly higher in HL compared with NL treatment ($p < 0.05$, Tab. 2.2, n=6). Overall rates were within the range of values obtained during the survey of the diel cycle (Fig. 2.1) with the exception of C fixation in *T. erythraeum*, which was elevated in both light regimes by a factor of 20 and 13 compared to rate measurement during the day survey. In *T. erythraeum* the ratio of C: N fixed was 463 ± 80 in HL treatments and 375 ± 94. Ratios of C: N fixed in HL treatments were 113 ± 38 and in *N. spumigena* 65 ± 21. Changes in concentrations of $NH_4{}^+$, DON, DFAA and DOC in the batch medium after exposure to HL are presented in Figure 2.5 and Table 2.2. A significant increase was measurable in the ambient concentrations of all compounds in both species within the first 30 minutes upon exposure to HL in comparison to NL conditions, except for DFAA and DOC in *N. spumigena*. After 60 minutes the concentrations of compounds in the HL treatments resembled those of the NL treatment again. *T. erythraeum* exhibited a net release of DON, $NH_4{}^+$ and DOC, whereas *N. spumigena* showed only a net release of DON and $NH_4{}^+$ under the increased experimental irradiance within the first 30 minutes (Tab. 2.2).

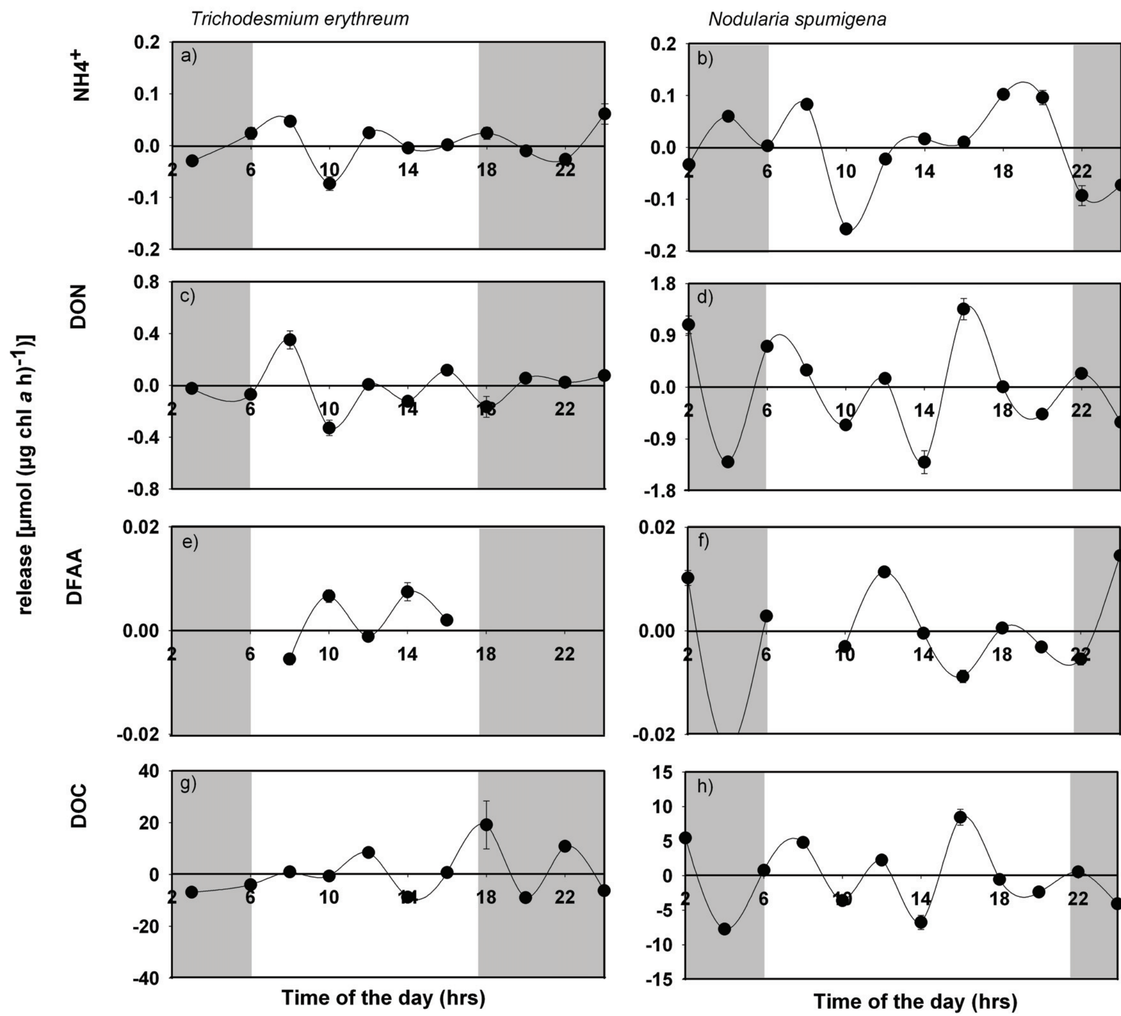

Figure 2.4: Diel variation in release or uptake of NH_4^+(a,b), DON (c,d), DFAA (e,f) and DOC (g,h) for *Trichodesmium erythraeum* and *Nodularia spumigena*. Symbols represent the mean value of four replicates and standard deviation. Positive values indicate release, negative values uptake of compounds Grey areas indicate dark periods.

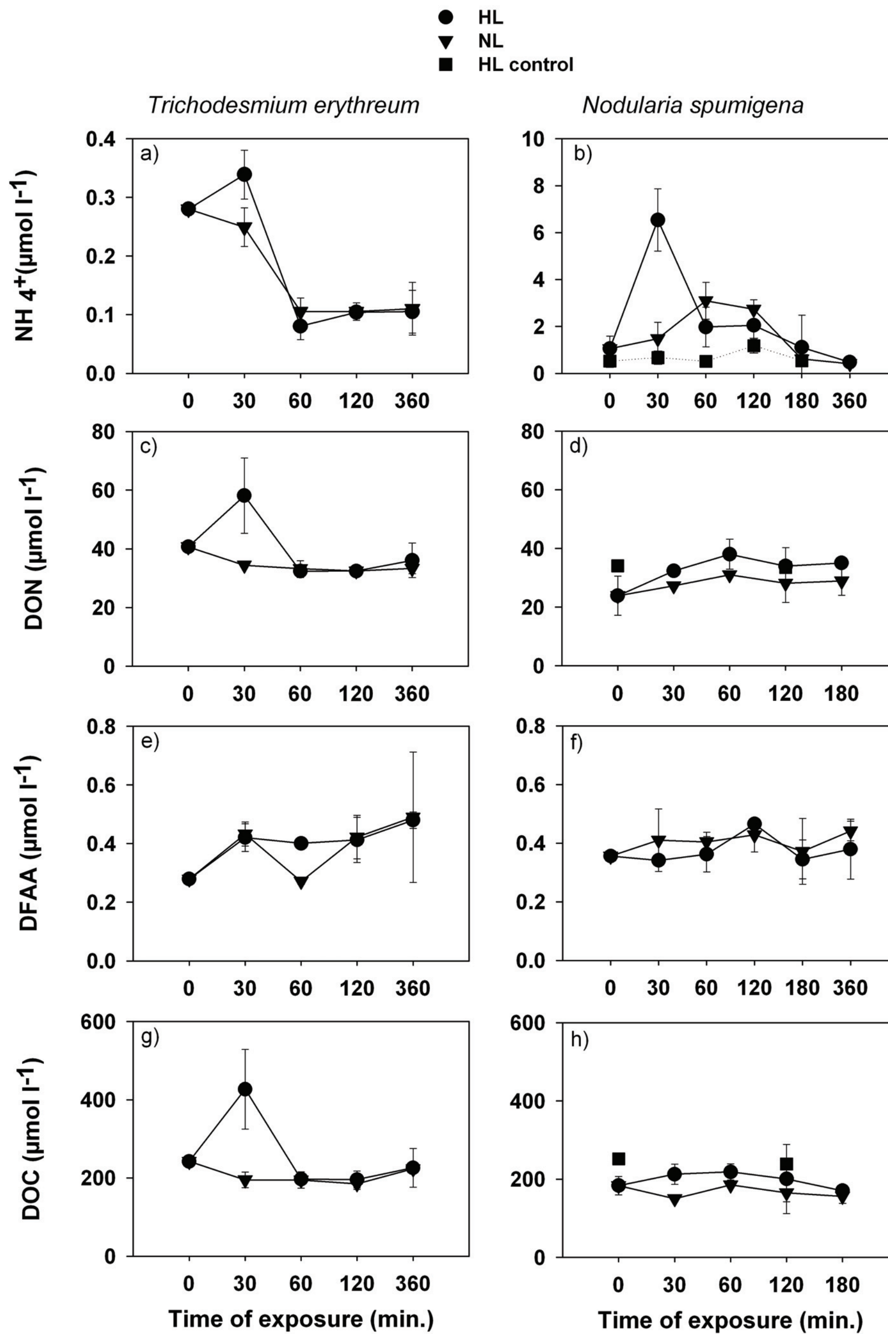

Figure 2.5: Time dependent variability of $NH_4{}^+$(a,b), DON (c,d), DFAA (e,f) and DOC (g,h) concentration for *Trichodesmium erythraeum* and *Nodularia spumigena* and the two light regimes, respectively: high light (HL, circles, 200 or 100 μmol photons m^{-2} s^{-1}) vs. normal light (NL, triangle, 100 or 60 μmol photons m^{-2} s^{-1}). HL control (square) treatments with no *Nodularia spumigena* present. Symbols represent the mean value of three replicates and standard deviation.

To check for photochemically derived increase of TDN and DOC concentrations, a control set-up was carried out using the same *N. spumigena* parent culture and the same light treatments. Prior to the start of the experiment algae were removed by filtering over GF/F filter (Whatman) to stop any biological activity. The control indicated no elevation in concentration of NH_4^+ ($p > 0.5$, $n = 3$), DON and DOC between HL and NL treatments (Fig. 2.5 b, d, h). The ratios of POC to PON in the treatments were not different in either cultures or between light treatments. In *T. erythraeum* POC to PON ratios were 8.8 ± 1.2 and 8.6 ± 1.6 in (HL and NL, respectively), in *N. spumigena* the ratios were 6.6 ± 0.1 and 6.6 ± 0.2 (HL and NL, respectively).

Fixation and release of dissolved compounds under different phosphorus concentrations— The concentrations of TDN in the treatments did not differ significantly from each other and were on average 11 μmol l^{-1} in *T. erythraeum* and 30 μmol l^{-1} in *N. spumigena* cultures. The resulting TDN to DIP ratios were 11,

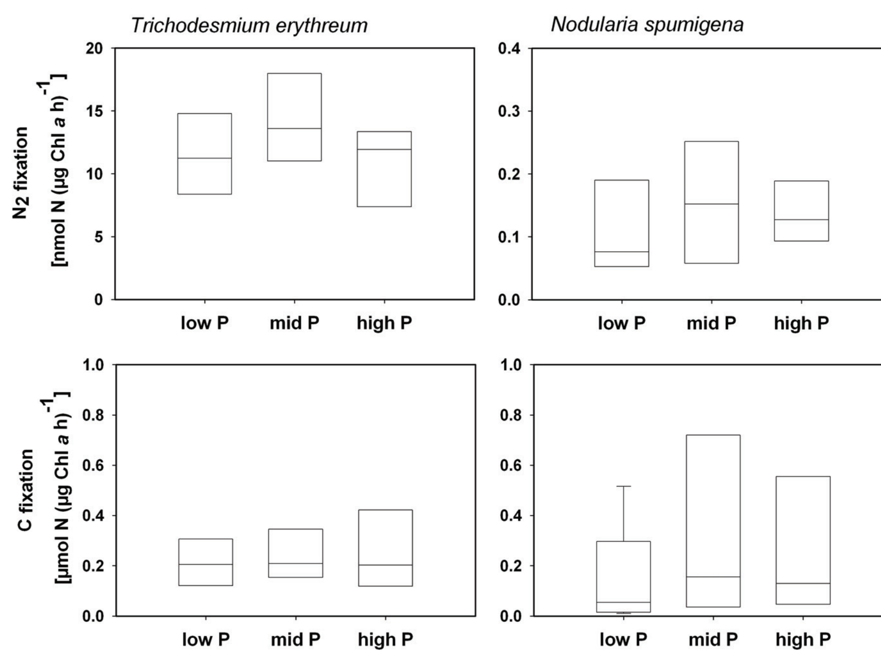

Figure 2.6: Box plot (n=10) of N_2 and C fixation for two cyanobacterial species *Trichodesmium erythraeum* and *Nodularia spumigena* and three different phosphorus concentrations: low P (1 μM), mid P (10 μM) and high P (20 μM).

1 and 0.6 (low P, mid P and high P, respectively) in *T. erythraeum* and 31, 3 and 2 (low P, mid P and high P, respectively) in *N. spumigena*. N_2 and C fixation rates did not show any significant trend in both species treated with the different phosphorus concentrations (Fig. 2.6, $p > 0.1$, $n = 10$). Moreover, no significant differences occurred in the concentration of nitrogenous compounds and DOC between the three applied phosphorus concentrations during the time of observation. The uptake rate of phosphorus (calculated from mass balance) for the different treatments were highly variable within the triplicates, leading to high standard deviations (low P vs. mid P vs. high P in *T. erythraeum* 0.01 $\pm$ 0.04 μmol (l d)$^{-1}$ vs. 0.01 $\pm$ 0.14 μmol (l d)$^{-1}$ vs. 0.04 $\pm$ 0.18 μmol (l d)$^{-1}$ and *N. spumigena* 0.02 $\pm$ 0.10 μmol (l d)$^{-1}$ vs. 0.27 $\pm$ 1.1 μmol (l d)$^{-1}$ vs. 0.68 $\pm$ 1.63 μmol (l d)$^{-1}$. The growth rates obtained from the *T. erythraeum* were not statistically different between the treatments low P, mid P and high P (0.28 d^{-1} , 0.28 d^{-1} and 0.35 d^{-1} ; $p > 0.5$, $n = 5$). Growth in *N. spumigena* was significantly higher in the mid P treatment than in high P (0.35 d^{-1} and 0.13 d^{-1} ; $p < 0.05$, n= 5), but not in low P (0.35 d^{-1} and 0.21 d^{-1} , $p > 0.1$, $n = 5$). Again there was no significant influence of ambient phosphorus concentration in the media and the ratio of POC to PON in both species (low P vs. mid P. vs. high P: in *T. erythraeum* 6.5 $\pm$ 0.8 vs. 6.4 $\pm$ 0.6 vs. 6.4 $\pm$ 0.5 and in *N. spumigena* 7.21 $\pm$ 0.4 vs. 7.58 $\pm$ 0.30 vs. 7.64 $\pm$ 0.40, $p > 0.1$, $n = 5$). C to N uptake ratio (C:N fixed) did not differ significantly within the applied phosphorus treatments (*T. erythraeum* 26 $\pm$ 8 vs. 28 $\pm$ 9 vs. 24 $\pm$ 7 and *N. spumigena* 28 $\pm$ 9 vs. 19 $\pm$ 13 vs. 18 $\pm$ 11, $p > 0.1$, $n = 5$).

2.4 Discussion

Endogenous control and diel pattern—This study provides fixation rate measurements and release of both N and C compounds by diazotrophic cyanobacteria over a diel cycle. The overall fixation rates are at the lower end of previous studies with maximum N_2 fixation rates below 10 nmol N (Chl *a* h)$^{-1}$ and maximum C fixation below 1 μmol C (μg Chl *a* h)$^{-1}$ (Berman-Frank et al. 2001, Mulholland et al. 2004a, Mulholland & Bernhardt 2005). The diel pattern of N_2 and C fixation with a constant rise to the late afternoon as shown here (Fig. 2.1) has been reported before

Table 2.2: Student's t-test statistic on the significance of difference in N_2 and C fixation for the observed time interval (360 minutes), NH_4^+, DON and DOC release for the first 30 minutes between the two experimental irradiance HL (200 or 120 μmol photons m^{-2} s^{-1}) and NL (120 or 60 μmol photons m^{-2} s^{-1}). Units of single parameter are: N_2 fixation [nmol N(μg Chl *a* h)$^{-1}$], C fixation [μmol C (μg Chl *a* h)$^{-1}$], release of NH_4^+, DON, DFAA and DOC [μmol C (μg Chl *a* h)$^{-1}$]. Values are mean value of n measurements ± standard deviation.

	T. erythraeum				*N. spumigena*			
	HL	NL	p	*n*	HL	NL	p	*n*
N_2 fixation	1.19 ± 0.52	0.86 ± 0.41	0.2	*6*	1.71 ± 0.54	0.75 ± 0.32	0.01*	*6*
C fixation	4.37 ± 1.14	2.47 ± 1.14	0.001**	*6*	1.50 ± 0.68	0.28 ± 0.07	0.002**	*6*
Release of								
NH_4^+	0.05 ± 0.03	-0.05 ± 0.05	0.02*	*3*	3.05 ± 0.92	0.13 ± 0.22	0.01*	*3*
NH_4^+control					0.085 ± 0.104	0.059 ± 0.029	>0.5	*3*
DON	14.2 ± 95	-9.3 ± 1.2	0.02*	*3*	4.9 ± 2.1	1.2 ± 0.2	0.04*	*3*
DFAA	0.12 ± 0.05	0.37 ± 0.06	>0.5	*3*	-0.007 ± 0.004	0.02 ± 0.04	0.5	*3*
DOC	196 ± 22	-69 ± 88	0.01*	*3*	19 ± 6	-27 ± 35	0.1	*3*

* and ** indicate statistical significant and highly significant level of differences. n, number of single measurements.

(Dugdale et al. 1961, Chen et al. 1998, Mulholland & Capone 2004, Berman-Frank et al. 2001). The fact that two independent runs using the same exponential growing parent culture resulted in a similar diel pattern confirmed its persistence under constant environmental condition. Initiation of C fixation occurred rapidly after initiation of the light period reaching 40% in *T. erythraeum* and 20% in *N. spumigena* of its maximum rate after 2 hours and slowing down in the later photoperiod (Fig. 2.1). N_2 fixation started slowly and increased significantly after 4 hours in the light (Fig. 2.1). This temporal pattern has been shown for example by Gallon et al. (2002) and recently by Popa et al. (2007) using the heterocystic cyanobacterium *Anabaena*. The molecular mechanism of the circadian oscillation of N_2 and C fixation are light dependent and based on transcription and translation processes, and on the phosphorylation of enzymes to reset the inner clock (Dunlap 1999, Nishiwaki et al. 2000). In addition, N_2 fixation is dependent on energy stored by photosynthesis (ATP, $NADPH_2$), which adds to the circadian rhythm. Data collected in our study showed an identical progression of N_2 and C fixation in *N. spumigena* and no sequential down regulation of either processes, as in heterocystic cyanobacteria both processes are strictly spatially separated in order to create anaerobic conditions for the nitrogenase enzyme. The timing of N_2 and C fixation in the non-heterocystic cyanobacterium *T. erythraeum* exhibited a slight down regulation of C fixation between 16 and 18 hours, while N_2 fixation is optimal. This pattern has been observed in other investigations (e.g. Berman-Frank et al. 2001) and is caused by the need to separate N_2 and C fixation. This is necessary, because N_2 fixation depends on stored energy (ATP, $NADPH_2$) and is negatively influenced by the accumulation of oxygen over the light period. Moreover, both fixation processes are harmonized in non-heterocystic cyanobacteria by allocating cells with nitrogenase activity within the trichomes and colonies. Only 12% of trichomes in Trichodesmium seem to actively express nitrogenase (Bergman & Carpenter 1991). This in turn causes a supply problem within colonies in those trichomes that lack nitrogenase activity. The surrounding environment is then suggested to act as an extracellular vacuole (Flynn & Gallon 1990). The oscillation of dissolved compounds detected in the experiments is therefore not surprising (Fig. 2.2 and 2.4). However, there is a lack of explanation for the observed oscillation in the concentrations of dissolved compounds was also visible in *N. spumigena*. The relatively high amount of extracellular NH_4^+ (1 μM,

(Fig. 2.2 a, b) and DON (10-40 μM, (Fig. 2.2 c, d) presumably originated directly from the reduction of newly fixed N_2. The percentage of DFAA in the DON pool is 1-8%. No further identification of the remaining DON was done, but possible compounds reported in the literature comprise dissolved combined amino acids (DCAA), urea and ribonucleic acid (Bronk 2002). The concentration of NO_3^- and NO_2^- remained below the detection limit throughout all the investigations. In spite of NH_4^+ and DON being present, N_2 fixation continued (Fig. 2.1 and 2.2), which has been shown by other studies as well (e.g. Ohki et al. 1991, Fu et al. 2003). Concentrations of dissolved compounds changed throughout the day in almost sine-like oscillations, although the observation period of two hours is too coarse to fit the data points with a sine model. The observed changes in both algae had different amplitudes and frequencies. In *T. erythraeum* amplitudes were smaller and the periods longer than in *N. spumigena*. Only the change in the amount of DFAA (mainly Glu) in *T. erythraeum* was directly positively correlated with the rise in N_2 fixation. Either this was caused by differences in the release or uptake rates, such that in *T. erythraeum* these processes are quicker than the investigation time and accumulation in the external media was therefore not as pronounced as it was for *N. spumigena* (except for DOC). A surplus in concentration measured in the course of the study indicated release and a decrease uptake of compounds (Fig. 2.4), on the premise that experiments were done using axenic cultures. On average 71% of fixed N_2 was exudated by *T. erythraeum* as NH_4^+ (41%) and DON (30%) and 89% in *N. spumigena* as NH_4^+ (39%) and DON (50 %) during the light period. The total rates (NH_4^++DON) are high compared to available literature data, because in the present study the release of NH_4^+ and DON was considered, whereas in other studies often only one of these components was investigated. Rates of DON exudation obtained here are well within the rates published (e.g. Capone et al. 1994, Hutchins et al. 2007). Field studies using stable isotopes as a tracer (^{15}N-DON and ^{15}N - NH_4^+) yielded in DON release rates up to 50% (Glibert & Bronk 1994, Mulholland et al. 2006). Investigations using pure cultures of *Trichodesmium* resulted in rates of up to 81% (Hutchins et al. 2007). The average release rates of C as DOC in our study were $\sim$50% in both species, which is within the range of literature data (Antia et al. 1963, Sellner et al. 1997). To date several studies revealed that the release of both TDN and DOC is a considerable fraction of the net N and C uptake (Bronk &

Glibert 1994, Bronk et al. 1994 and 1998, Slawyk et al. 1998, Bronk & Ward 1999, Varela et al 2003a and 2006). Overall, we suggest that the release of compounds in our study is presumably not regulated by *de novo* synthesis of permease involved in the transport of compounds, but rather by the amount of previously assimilated N and C and the regulation of permease activity, as presented by Vincent (1992) and Flores & Herrero (2005). While uptake of compounds against a concentration gradient involves a membrane potential-driven transport and ATP costly fixation using the nitrogenase enzyme, the reverse transfer out of the cell coincides with a concentration gradient. Cells perceiving a sufficient N status upon fixing N_2 do not increase the synthesis of new permease transporter to exudate this N in excess but rather modulate the activity of transporter by post-translational regulation (Flores & Herrero 2005). Thus, the observed oscillation of TDN release rates reported here within the course of the day (Fig. 2.4) may derive from the consumption and fixation of N and C. Any over consumption of N relative to C should lead to a release of nitrogenous compounds. The connection between the C and N metabolism in cyanobacteria is the glutamine synthetase-glutamate synthase cycle (GS-GOGAT, Flores & Herrero 1994; Herrero et al. 2001). Intracellularly produced NH_4^+ is incorporated into the C skeletons through the GS-GOGAT in the form of 2-oxoglutarate, which in turn is used for biosynthesis of Glu and Glu-derived compounds. N deficiency is perceived as an increase in the intracellular 2-oxoglutarate level, N excess as a decrease in the intracellular 2-oxoglutarate level. Therefore, 2-oxoglutarate acts as a signal by which cyanobacteria perceive the intracellular N status, leading to a feedback signal that drives N uptake in the form of N_2 or NH_4^+ or N release as NH_4^+ or DON. The phasing of chlorophyll *a*-specific release rates in *T. erythraeum* was shorter than the changes in concentrations, suggesting again that uptake and release activity was regulated quicker and might not be fully resolved by an investigation time of two hours. In *T. erythraeum* DOC accumulates during the day with its maximum in concentration during the night (22 hours). When looking at the various patterns of cycles during the day two forms can be distinguished. There are long period cycles of fixation of N and C. On the one hand, there short period cycling (presumably < 2 hours) of TDN compounds on the other hand, suggesting a much faster feedback control regulated by the amount of fixed N and the N status of the individual cell itself (Lillo 2001). Rapid feedback occurs within seconds, slower

feedback depends on the further metabolization (Kerby et al. 1987). It should be remembered that individual cells within a trichome in *Trichodesmium* sp. are not necessarily in the same stage of the cell cycle. Temporal separation of N_2 fixation and O_2 evolving during photosynthesis may occur, depending on the present stage of the cell cycle of individual cells within the same trichome or in trichomes in the colony (Popa et al. 2007, Ohki 2008).

Exogenous control- Fluctuation in cellular light energy supply—Our experiments simulating changing light intensities support the hypothesis that an increase in cell energy may not be used completely for biosynthesis (Lomas et al. 2000). Instead, the energy is dissipated by the release of dissolved nutrients (NH_4^+, DON and DOC). We observed a rise in C fixation rates in *T. erythraeum*, whereas N_2 fixation did not increase significantly in this species under HL incubation (Tab. 2.2). It is known that *Trichodesmium* is strongly light adapted and needs higher irradiances for growth than other phytoplankton (Kana 1993). A linear rise was determined in the latter study in photosynthesis up to 600 μmol photons m^{-2} s^{-1} and saturation was reached at 1600 μmol photons m^{-2} s^{-1} (the light intensity of a full bright sunny day is 1000 μmol photons m^{-2} s^{-1}. We assume that the HL intensity in our study (200 μmol photons m^{-2} s^{-1}) was not sufficient to significantly increase N_2 fixation in *Trichodesmium* (Tab. 2.2). The N_2 fixation rates of *N. spumigena* increased significantly when the cells are exposed to higher light intensities in this study (Tab. 2.2) and in others, up to 400 μmol photons m^{-2} s^{-1} (Fig. 7b in Evans et al. 2000). The ratios of fixed C to N in the light experiments were very high in both species *T. erythraeum* and *N. spumigena*: HL treatment 463 and 113, NL treatment 375 and 65, respectively), indicating that C and N incorporation were not balanced relative to somatic demand in light experiments (in HL and NL treatments). Studies have shown that the ratio of C:N fixed can be much higher than the Redfield ratio (Orcutt et al. 2001) and even reach up to 700:1 (Carpenter and Price 1977, McCarthy and Carpenter 1979). The observed periodical uncoupling of primary production and N_2 fixation might be explained by C ballasting, storage of glucose, lipids and polyhydoxybutyrates (Stal & Walsby 1998, Romans et al. 1994, Villareal & Carpenter 2003, Ohlendieck et al. 2007) and/or exudation of newly fixed N (Mulholland et al. 2004). Uptake of C in excess often occurs when phytoplankton proceeds photosynthesizing under high light conditions (e.g. Mague et al. 1980) and dispose the

surplus of fixed C as DOC. This might account for the high ratio of C:N fixed in the HL treatments, compared to the ratio observed in the diel cycle experiments 10.6 ± 3.2 and 6.2 ± 4.8 in *T. erythraeum* and *N. spumigena*, respectively). Nevertheless, it does not explain high ratios of C:N fixed in the NL treatments, where identical experimental conditions were applied as in the diel cycle experiments. It has to be noted, that parent cultures for both experiments were taken at different stages of exponential growth. On the other hand fixation in the light experiments was only surveyed for 6 hours (6 to 12 am) and N_2 fixation might have increased during a longer observation period. Much of the C that exceeds the demands for somatic growth may be put to various other fitness-promoting uses (Hessen & Anderson 2008) or released as DOC. Furthermore, there was a striking short term effect of increasing light intensity on the concentration of dissolved compounds detectable (NH_4^+, DON, DFAA, DOC, (Fig. 2.5). Within the first 30 minutes after the shift from NL to HL, concentrations rose in nearly all compounds in both species (taking the concentrations of the NL treatment as a reference), except for DFAA. This effect diminished after 60 minutes of exposure. Therefore, only release rates for the first 30 minutes after exposure were considered in the following discussion and argumentation. A control set-up using *N. spumigena* was applied to prove that the increase in NH_4^+, DON and DOC (control measurement for DFAA were not available) concentrations solely resulted from the metabolism and physiology of the cyanobacteria and not from photochemical reactions of dissolved compounds in the extracellular media. The quantity in the control set-up of dissolved compounds in the extracellular media did not change (Fig. 2.5 b, c, h). The release rates calculated for each compound were significantly higher in HL treatments than in NL treatments (Tab. 2.2), except for the release of DFAA. The release of DON therefore must comprise other compounds than DFAA. DON release might be attributed to passive leakage or disrupted cells. In the latter case the dominance of several amino acids measured in our study should have been identical to those found intracellular. Glu and Gly were the dominant extracellular amino acids in this study (Fig. 2.3) but known from the literature (Flynn & Gallon 1990) dominating intracellular amino acids are usually Glu, Ala and Arg. This discrepancy in composition contradicts a passive efflux or the breakage of cells in our study. Overall, exudation is still controversially discussed in the literature. Whether it is an overflow mechanism where

excess photosynthetic products are actively released when the fixation rates exceed the rate of macromolecular synthesis (Fogg 1983, Wood & Van Valen 1990) or a passive diffusion of small metabolites through the cell membrane (Bjørnsen 1988) is still unclear. If the overflow mechanism dominates, significant DON and DOC production would preferentially occur under conditions of high irradiance and low nutrient concentration (molar N: P of 3.2 in Alcoverro et al. 2000) as a mechanism for dissipating cellular energy (Wood & Van Valen 1990, Smith et al. 2000). If passive diffusion is the main mechanism, DOC and DON production can take place whenever a pool of small, recently fixed metabolites is available. The findings in our study support the opinion that exudation is an active and adaptive process reacting towards changes in the energy status of cells. To underline our hypothesis we also tested the potential of active exudation of NH_4^+ and DON as a sink for electrons by quantifying the percentage of electrons consumed by these processes (Tab. 2.3) as it has been carried out by Lomas et al. (2000) for diatoms and flagellates. In particular the numbers of electrons that were required to yield the observed extracellular accumulation of NH_4^+ and DON in the HL treatment were calculated, relative to the number of electrons harvested during the given time interval. The chlorophyll *a*-specific release was multiplied by a total of 8 electrons required for fixation of N_2, production and release of NH_4^+ and DON. The number of electrons harvested by the cells (per chlorophyll *a*) in the same time period was calculated using the formula given by Lomas et al. (2000):

$$\text{Electrons harvested} = E \times T \times a^* \times 0.5$$

with E being the incident irradiance (photons m^{-2} s^{-1}), T the time interval (180 seconds in this case), a* the chlorophyll *a*-specific absorption (m^2 mg Chl a^{-1}) and 0.5 a constant, assuming a 50% distribution of chlorophyll between the photosystems (Falkowski & Raven 1997). Species-specific values for a* used in this study are given in Table 2.3. The percentages of electrons consumed supporting the observed accumulation of dissolved N compounds (Tab. 2.2) under HL treatment were 52% in *T. erythraeum* and ~16% in *N. spumigena* average over the first 30 minutes of exposure (Tab. 2.3). The results from the concentration measurements (Fig. 2.5) as well as the calculated number of electrons needed for the observed accumulation of

TDN in the media further support the hypothesis that release of N compounds is an active way to dissipate excess energy consumed. The differences in percentage between the two species might result from the differences in the overall available light intensity and resulting number of electrons possibly harvested (200 μmol m^{-2} s^{-1} in *T. erythraeum* and 120 μmol m^{-2} s^{-1} in *N. spumigena*). Additionally, release might be more instantaneous and pronounced in the non-heterocystic *T. erythraeum*, because cells within the filament fixing N_2 are not coated with a three layer envelop like in heterocysts, which in turn is impermeable to ions (Haselkorn 1978). N_2 fixed in heterocysts of *N. spumigena* cultures must firstly be exported via permease into adjacent vegetative cells where it subsequently is released into the extracellular environment. Overall, when light energy is available in excess, the release of NH_4^+ and DON may serve as a short-term sink for electrons, in addition to other dissipation processes being activated within minutes (Mehler reaction, heat dissipation). Adaptations on the macromolecular basis (e.g. the adaptation of abundance of the messenger RNA encoding the light-harvesting chlorophyll proteins) even take longer, from two hours after the onset of light shifts to 12 hours (Falkowski & Raven 1997, Fujita et al. 1994).

Exogenous control- Fluctuation of the nutritional status—A co-limitation of N_2 fixation by iron and phosphorus has been documented by several studies (Sañudo-Wilhelmy et al. 2001, Mills et al. 2004, Mulholland & Bernhardt 2005, Degerholm et al. 2006). Our results are insufficient to clearly verify the hypothesis that increases in the supply of limiting nutrients like phosphorus fuel N_2 fixation and thus the release of nitrogenous compounds. There were no significant differences in fixation of both compounds when comparing the applied phosphorus concentrations in the batch media (Fig. 2.6). Overall, the experimental set-up testing the influence of PO_4-P addition on N_2 and C fixation and exudation of TDN and DOC was using phosphorus concentrations which turned out to be too high to identify any significant trend (low P: 1 μM, mid: P 10 μM and high P: 20 μM). The resulting N: P ratios of dissolved compounds were 10, 1 and 0.5, considering the ambient NH_4^+ and DON concentrations in the batch media (10 μM, data not shown). Besides the low N:P ratio, cyanobacteria were not N limited, because they actively fixed N_2. The ratio of C:N fixed ranged between 19 and 28 and was not significantly different between phosphorus treatments. The deviation of this ratio from the Redfield ratio

Table 2.3: Estimates of average percentage of electron consumption supporting the observed rates of NH_4^+ and DON release under the experimental irradiance HL (200 or 120 μmol photons m^{-2} s^{-1}) between 0 and 30 minutes. a* (m^{-2} mg Chl a^{-1}) represents the chlorophyll-specific absorption coefficient.

Species	a*	Compound released	%Electron consumption	
	(m^2 mg Chl a^{-1})		max	min
Trichodesmium erythraeum	0.0187	NH_4^+	0.18	0.03
	Subramaniam et al. 1999b	DON	52.4	10.5
Nodularia spumigena	0.024	NH_4^+	8.8	4.7
	Metsamaa et al. 2006	DON	15.5	4.6

(~ 6) is still in the range of literature data (Orcutt et al. 2001). N_2 fixation rates are found to be saturated at a P concentration of 1.2 μM in *Trichodesmium* (Fu et al. 2003). Still, in experiments using the same species significantly higher N_2 fixation rates were observed with extracellular P concentrations of 5 μM compared to 1 μM (Mulholland & Bernhardt 2005).

Conclusion

Cyanobacteria in natural environments are exposed to continuous changes in light intensity during passive or active movement within the upper water column. These constant shifts towards cellular energy imbalanced condition seem to lead to peaks of TDN and DOC exudation. The diel rhythm is probably controlled endogenously and exogenously creating temporarily patchy nutrient rich local habitats. Although excretion represents a physiological loss term, algae may gain in symbiotic like advantages within a planktonic community (Williams 1990). Furthermore, DOM is needed in colonies of non- heterocystic cyanobacteria to supply trichomes and cells that lack nitrogenase activity with nitrogenous compounds. Moreover, exudation might support the nutrient flow within the food web (Vidal et al. 1999, Tseng et al. 2005). Organisms like bacteria, fungi, diatoms, ciliates and juvenile decapods are found spatially and temporarily associated to cyanobacteria, benefit from higher nutrient availabilities in close connection with cyanobacterial colonies (e.g. Devassy 1979), pointing to a key position of cyanobacteria in marine food webs.

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Chapter 3

Incorporation of diazotrophic fixed N_2 by mesozooplankton species – Case studies in the southern Baltic Sea using ^{15}N - stable isotope tracer addition.

Abstract

During two simultaneous cruises in the Central Baltic Sea in July 2007 we applied a ^{15}N tracer addition approach to constrain the impact of N_2 fixation on mesozooplankton (>200 μm) production in the Central Baltic Sea. We determined rates of diazotroph $^{15}N_2$ fixation as well as uptake of diazotrophic derived ^{15}N by mesozooplankton species. Diazotrophic $^{15}N_2$ fixation rates were low representing pre-bloom situations. First order estimates using a two source mixing model of natural $\delta^{15}N$-PON abundance revealed that diazotrophic nitrogen contributed to 27 ± 8% to mesozooplankton biomass. Additionally, the application of the tracer showed that fixed ^{15}N was detectable in the mesozooplankton fraction within one hour after the onset of the incubation. On a daily basis 100% of newly fixed ^{15}N and 14% of cyanobacteria standing

stock were incorporated by mesozooplankton species in our experimental set ups. By applying size fractionation experiments, we determined that the majority of ^{15}N transfer (67%) was mediated by released nitrogenous compounds and their channelling trough the microbial loop towards the mesozooplankton community. Moreover, it was also possible to show that direct grazing on filamentous cyanobacteria accounted for 33% of gross ^{15}N incorporation. Thus, N_2 fixing cyanobacteria are ecologically more important as instantaneous sources of nitrogen for higher trophic level of the Baltic Sea food web than previously assumed.

3.1 Introduction

Over the recent years diazotrophic N_2 fixation has become recognized as a noteworthy component of the nitrogen cycle in marine ecosystems being one of the most important process that adds biologically active nitrogen to oligotrophic marine ecosystems. Open ocean habitats missing up- welling of nitrate often show mass occurrences of cyanobacteria, which have a distinct competitive advantage by fixing N_2 (Capone et al. 1997). In the Baltic Sea cyanobacteria can account for the majority of phytoplankton biomass in the Baltic Sea (700 mg C m^{-3}, Wasmund et al. 2006) during summer situations forming extensive surface blooms. *Nodularia spumigena* and *Aphanizomenon flos-aquae* are the dominant cyanobacteria in the Baltic Sea, fixing N_2 at a rate of up to 138 mmol N m^{-2} yr^{-1} (Wasmund et al. 2005). Nevertheless, it is still controversy discussed to which extent the diazotrophic fixed N is transferred to higher trophic level and whether direct grazing is important. In general direct grazing on cyanobacteria has been regarded to play a minor part, due to morphological adaptation and production of cyanotoxins (e.g. Fulton 1988; DeMott & Moxter 1991, Kirk & Gilbert 1992, Sellner et al. 1994, 1996). Moreover cyanobacteria provide low food quality for zooplankton species due to the lack of long chained poly unsaturated fatty acids (Ahlgren et al. 1992), which are essential for zooplankton growth and reproduction (Müller-Navarra et al. 2000). Several studies support this argumentation. For instance Meyer-Harms & von Bodungen (1997) stated that ingestion of cyanobacteria is avoided when a natural phytoplankton community is available. Evidence for reduced fertility of zooplankton upon

ingestion of cyanobacteria was given by Lampert (1987), Sellner et al. (1996) and Koski et al. (1999). Nevertheless, more recent publications present opposing findings on the relevance of grazing, such that copepods and cladocera exhibit high uptake rates of cyanobacteria as well as high reproduction rates in the presence of filamentous cyanobacteria (e.g. Burns & Xu 1990, Meyer-Harms et al. 1999b, Engström et al. 2000, Koski et al. 2002, Kozlowsky-Suzuki et al. 2003). Although grazing on cyanobacteria proves to be of more relevance than previously accepted, the majority of diazotrophic nitrogen is expected to remain in the particulate fraction and lost by sedimentation or to be released in the form of organic and inorganic N (DON, ${NH_4}^+$, ${NO_2}^-$, ${NO_3}^-$). It is well known that DON is actively released by growing cyanobacteria with a rate of up to 80% of recently fixed nitrogen (Bronk et al. 1994, Glibert & Bronk 1994, Bronk & Ward 1999, Nagao & Miyazaki 1999, Slawyk 2000, Diaz & Raimbault 2000, Ohlendieck et al. 2000). Even more, there are top down effects which lead to DON liberation like "sloppy feeding" caused by zooplankton grazing, faecal pellet dissolution (Dagg 1974, Jumars 1989, O'Neil et al. 1996) and viral lysis (Bratback 1998, Fuhrman 1999). The process of autolysis, controlled by programmed cell death (PCD), also adds to the release of DON particularly at the end of cyanobacteria blooms (Madeo et al. 2002, Segovia et al. 2003, Berman-Frank et. al. 2004). These dissolved nitrogenous compounds in turn can be taken up by bacteria and processed in the microbial loop before being reintroduced into the classic grazing food web and reach the mesozooplankton community (Ohlendieck et al. 2000). Sommer et al. (2006) argued based on mass balance of natural δ^{15}N abundance of cyanobacteria and mesozooplankton of the Baltic Sea, that 23-45% of diazotroph fixed N_2 is transferred to the mesozooplankton fraction via the microbial loop. These findings result from mesocosm experiments where a bloom of diazotrophs was induced. To give more precise conclusions on the importance of diazotrophic N_2 fixation to the production of mesozooplankton species (≥ 200 μm), and furthermore the proportion of direct grazing and microbial loop mediation under natural conditions, we carried out experiments during two simultaneous cruises conducted in the Central Baltic Sea in July 2007. Two methods were used. Firstly we estimate the percentage contribution based on natural abundance of ^{15}N values using a two source mixing model according to Montoya et al. (2002). Secondly we used ^{15}N labelling experiments to: (a) Determine diazotrophic $\delta^{15}N_2$ fixation rates.

(b) Determine ^{15}N uptake by mesozooplankton species using size exclusion filtration.
(c) Identify the significance of direct grazing and microbial loop mediation to the gross flux of ^{15}N.

3.2 Material and Methods

Experiments were conducted during cruises on board the RV “Heincke (HE 273)” and “Poseidon (POS 353)” from 10.07.2007 till 21.07.2007 in the Central Baltic Sea (Fig. 3.1). During the POS cruise the ship sampled a grid of stations in the Northern Gotland Basin. During the HE cruise the ship drifted in the Southern Gotland Basin.

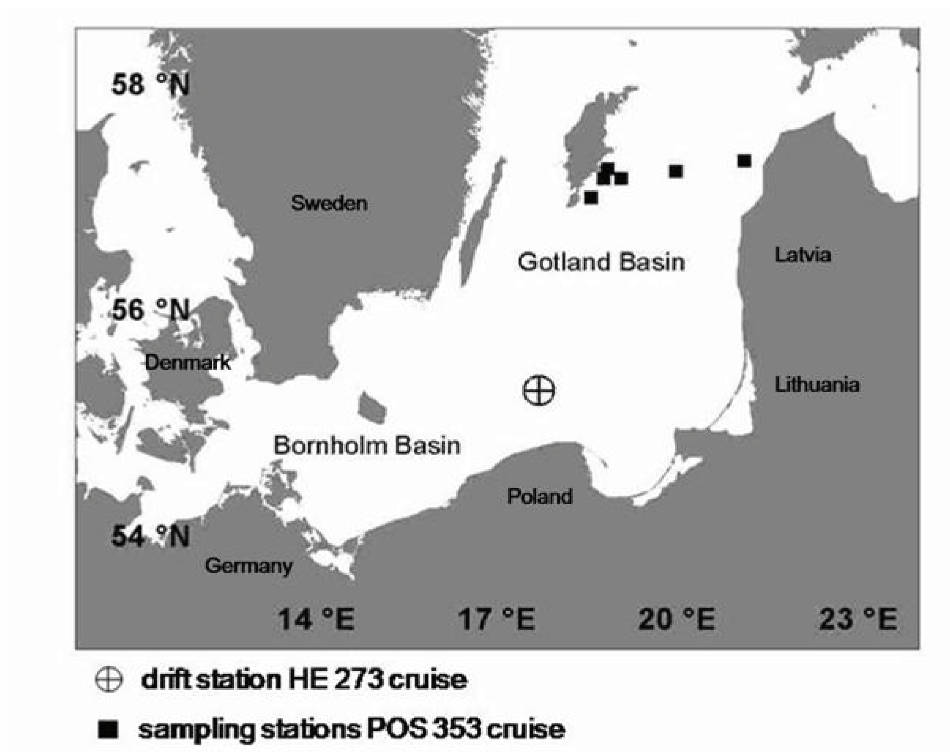

Figure 3.1: Map of the study area showing drift area for the Heincke 273 (HE) and sampling stations for the Poseidon 353 (POS) and in the southern Baltic Sea July 2007.

Environmental parameter and plankton composition.— A conductivity temperature- depth (CTD)-rosette system was used to collect water samples for the on board experiments, as well as for plankton and nutrient analysis. Water for on board experiments during the HE cruise was sampled using a bailer. Concentrations of NH_4^+, NO_2^-, NO_3^- and PO_4^- were determined on board according to the methods described by Grasshoff et al. (1983) with a precision of 0.1 μmol l^{-1}. During the POS cruise subsamples of the treatment bottle were filtrated over GF/F filter for determination of total dissolved nitrogen (TDN) concentrations by applying high temperature catalytic oxidation with a Shimadzu TOC-VCPN analyser to subsequently calculate DON concentrations (DON = TDN-DIN). chlorophyll *a* was analysed after Jeffrey and Humphrey (1975). Sub samples for phytoplankton analysis were preserved with 1 ml Lugol's solution per 250 ml of sample and stored at room temperature in the dark. Phytoplankton species were analysed quantitatively and qualitatively according to the Uthermöl method under an inverted microscope (Leica). Carbon biomass of phytoplankton was determined using biovolume- carbon conversion factors. To quantify mesozooplankton composition and abundance of the unbiased seawater, zooplankton was collected with a 200 μm mesh-sized WP-2 net by vertically towing from 20 and 50 m depth to the surface. Zooplankton was fixed with Formalin and identified and counted in the lab under a binocular microscope (Leica DMIRP, 100x, 200x, 400x). Prior to the start of the experiments, mesozooplankton samples from the net tow were incubated in a "light trap", in which positive phototactic and healthy zooplankton actively moves from a shaded into an illuminated container, to sort out detritus and filamentous cyanobacteria. After 30 minutes subsamples from the light trap were added to the treatment bottles. Analysis of abundance and composition of mesozooplankton in the experiments was done differently during the two cruises. During the POS cruise, determination was done directly by counting and identifying zooplankton using sub-samples of each single experiment. During the HE cruise determination was done indirectly by relating the carbon biomass (POC μg C l^{-1} of each experiment to the specific carbon content of those taxa present in the unbiased seawater samples and their percentage dominance of abundance. The specific carbon content of each taxon was calculated using the Equation 3.1,

$$\text{carbon content } (\mu\text{g C ind.}^{-1}) = a \cdot L^b \tag{3.1}$$

where L is the mean length (μm), determined microscopically, a and b are taxon specific factors gained from the literature (Kankaala & Johansson 1986, Postel et al. 2007).

Isotopic analysis.— Stable N isotope ratios (δ^{15}N-PON) as well as PON and POC concentration were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020 C in a Thermo Finnigan Delta S mass-spectrometer. Filters containing particle samples were trimmed, sectioned, then loaded into tin capsules and palletised for isotopic analysis. The stable N isotope ratios measured for each sample were corrected against the values obtained from standards with defined nitrogen and carbon isotopic compositions (IAEA-N1, IAEA-N_2, NBS 22, and IAEA-CH-6) by mass balance. Values are reported relative to atmospheric N_2 (δ^{15}N). The analytical precision for both stable isotope ratios was ± 0.2 ‰. Calibration material for N analysis was acetanilide (Merck). On board zooplankton samples for natural abundance of isotopes were thoroughly rinsed with surface seawater to remove cyanobacteria caught in the sample, separated into discrete size fractions by passage through a series of Nitex sieves (10, 200 μm) and filtered onto glass fibre filters (Whatman GF/F). The size fractionated samples were frozen for later isotopic analysis ashore. In the laboratory samples were dried at 60 C and packed into tin capsules for elemental and isotopic analysis.

First-order estimates of diazotrophic contribution to mesozooplankton biomass.— We used the mass-balance approach of Montoya et al. (2002) to give estimates on the contribution of diazotroph N to the biomass of mesozooplankton species (Eqn. 3.2).

$$\%\ \text{Diazotroph N} = 100 \cdot \left(\frac{\delta^{15}\text{N}_{\text{Zpl}} - \delta^{15}\text{N}_{\text{RefZpl}}}{\delta^{15}\text{N}_{\text{Diazotroph}} - \delta^{15}\text{N}_{\text{RefZpl}}} \right) \tag{3.2}$$

For zooplankton > 200 μm we used the highest N found during both cruises (8 ‰) as a reference value $\delta^{15}N_{RefZpl}$, representing zooplankton with minimal inputs of nitrogen from diazotrophs. The δ^{15}N value for diazotrophs used for the calculations was 0.2 ‰, which is the mean value for δ^{15}N of hand picked filaments.

Experimental set-up.— Two experimental set ups were conducted to investigate nitrogen transfer from cyanobacteria to the mesozooplankton community (Fig. 3.2). In the first type of experiment (set-up I, Fig. 3.2) unbiased surface water

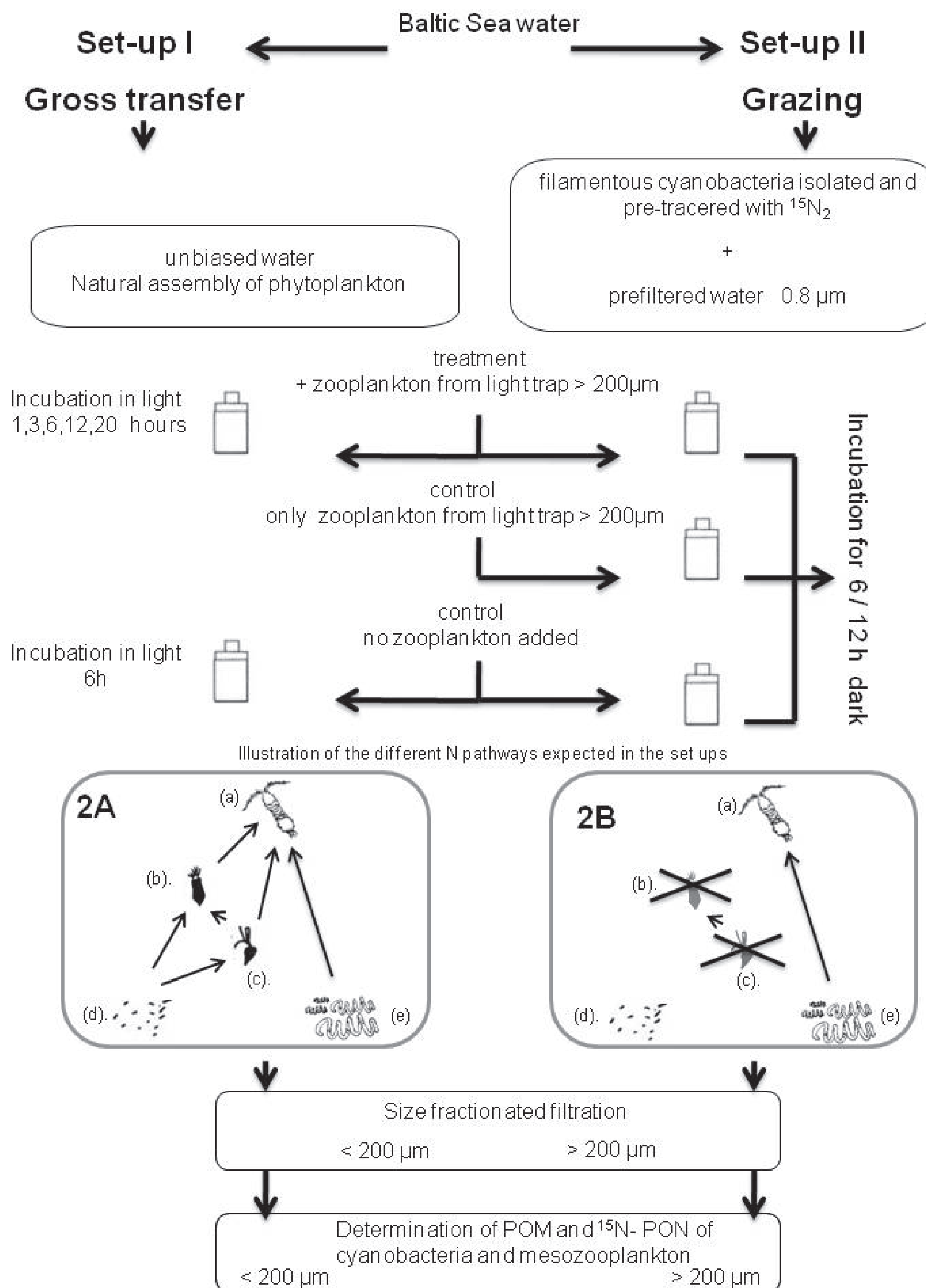

Figure 3.2: Experimental design illustrating the two different set ups. 2A represents set-up I- whole community incubation with all components of the food web present (2B) represents set-up II- direct grazing, components of the microbial loop and are excluded. (a) mesozooplankton (b) protists (c) heterotrophe flagellates (d) bacteria (e) cyanobacteria

with natural phytoplankton assemblage was filled into 2.5 l polycarbonate bottles. Zooplankton from net tows was kept in light traps for 30 minutes prior to the run and subsequently transferred into the incubation bottles with an abundance ranging from minimum of 51 to maximal 632 individuals per liter (Tab. 3.3). Bottles were filled, sealed gas tight and spiked with 2.5 ml $^{15}N_2$ (99% $^{15}N_2$, Campro Scientific). The flasks were placed in flow trough incubation tanks on board for 1, 3, 6, 12 or 20 h under 75% ambient irradiance and an incubation temperature of 16°C. At the end of the incubation period the samples were screened through 200 μm gauze to isolate the zooplankton from the phytoplankton. Each fraction >200 and <200 μm was collected on precombusted Whatman GF/F filters and stored frozen until analysis with a continuous-flow isotope ratio mass spectrometer. Either zooplankton was hand picked on board with a pipette prior to filtration onto Whatman GF/F (direct determination of ^{15}N uptake) or treatments were set off against control treatments with only phytoplankton being present.
In the second type of experiment (set-up II, Fig. 3.2) uptake of $^{15}N_2$ labelled cyanobacteria by mesozooplankton were determined. Unlike in the set-up I, here only direct grazing was possible, as microbial loop contributors were excluded by filtering the seawater used for the incubation over GF/F filter (0.8 mum), prior to adding the zooplankton. The pre-labelling started by enriching cyanobacteria from surface water using 10 μm mesh plankton net and adding them to 5 L polycarbonate bottles filled with filtered seawater. Bottles were sealed gas tight and spiked with 5 ml $^{15}N_2$ (99% $^{15}N_2$, Cambro Scientific). The flasks were placed in incubation tanks for 6 h under 75% ambient irradiance. At the end of the incubation cyanobacteria were isolated by sieving the sample trough 10 μm gauze. The $^{15}N_2$ labelled cyanobacteria were added to 2.5 l polycarbonate bottles filled with 0.8 μm filtered sea water. Additionally, mesozooplankton from the light traps were added to the same bottle. Samples were incubated in tanks for 12 or 20 h in the dark. At the termination of the experiment zooplankton and cyanobacteria colonies were apportioned by screening the sample through 200 μm gauze. Handling of filters and isotopic analysis was carried out as described above. We use the term gross incorporation or gross uptake of N for results of set-up I to distinguish ^{15}N incorporation by direct grazing plus uptake of ^{15}N labelled microbial loop components from results of set-up II, the net incorporation that results only from direct grazing.

$^{15}N_2$ fixation rates measurements and $^{15}N_2$ incorporation by mesozooplankton $^{15}N_2$ fixation and accumulation of ^{15}N in mesozoplankton species were measured using a $^{15}N_2$ assay. Tracer incubations were terminated by gentle vacuum filtration (100 cm Hg) through precombusted GF/F filters. These filters were dried at 60 °C and stored for isotopic analysis. Diazotroph N_2 fixation was determined using the approach of Montoya et al. (1996). A modified versions of Montoya et al. (1996) equations (6) and (7) were applied to calculate ^{15}N uptake by mesozooplankton,

$$V(T^{-1}) = \frac{1}{\Delta t} \cdot \left(\frac{A[PN]_{\mathrm{Zplf}} - A[PN]_{\mathrm{Zpl0}}}{A[PN]_{\mathrm{diazf}} - A[PN]_{\mathrm{Zpl0}}} \right) \tag{3.3}$$

$$\rho \left(M \cdot L^{-3} \cdot T^{-1} \right) \approx V[PN]_{\mathrm{Zplf}} \tag{3.4}$$

where equation 3.3 represents the specific rate of uptake of nitrogen (N) by mesozooplankton in the experimental bottle (V) per time (T) with $A[PN]_{Zplf}$ and $A[PN]_{Zplf0}$ being final and initial atomic enrichment of zooplankton and $A[PN]_{diazf}$ being the atomic enrichment of the diazotrophic food. Equation 3.4 represents the volumetric rate of nitrogen (N) uptake per time (T) and volume (L), where $[PN]_{Zplf}$ is the concentrations of zooplankton PN in the experimental bottle, which changed little during the short-term experiments. This approach is based on the general principle of tracer methodology (Sheppard 1962). ^{15}N uptake rates of mesozooplankton were related to their abundance within individual treatments.

Determination of functional response curve

To determine, whether there is a correlation of direct grazing (consumption) by zooplankton and the biomass of filamentous cyanobacteria according to the functional response model, consumption rates (Tab.3.1, 4th column) and biomass of filamentous cyanobacteria (Tab.3.3, 7th column) were plotted against each other. Subsquently, a fit using different functional response types: type I linear, type II , type III sigmoid (Holling 1965) was carried out. The goodness of fit for the functional response models in Figure 3.6 were determined using Sigma Plot, with the best fit being type III (Hill equation).

Statistical analysis. Statistical analysis was done using SPSS (SPSS Inc). Student's t-test (Tukey method of multiple comparisons) was conducted in order to determine whether the results obtained for the individual cruises HE and POS were

significantly different.

3.3 Results

Physical and chemical hydrography.— The temperature of the mixed layer during the HE cruise varied between 12.5 to 16.7 °C with a thermocline located between 10 and 17 m. The salinity of the mixed layer water body was on average 7.4. During the POS 353 cruise the mixed layer depth reached down to 20 m and showed a mean temperature range of 14 to 16 °C, as well as a salinity of 7.2 psu. Dissolved inorganic nitrogen (DIN) concentrations in the mixed layer during both cruises were below the detection limit. Phosphate concentrations decreased from 0.27 to 0.17 μmol l^{-1} during the HE cruise while phosphate concentrations during the POS cruise reached 0.2 ± 0.1 μmol l^{-1}. Concentrations of DON within the treatment bottles during POS cruise ranged from 18 to 25 μmol l^{-1}. The average wind speed during the cruises was 6.5 m s^{-1} (HE) and 9.5 m s^{-1} (POS). Maximum wind speed occurred on the 16.07.2007 in both investigation areas with 15 m s^{-1}.

Phytoplankton composition and N_2 fixation.— The phytoplankton community was dominated in both cruises by dinoflagellates (Tab. 3.1). Filamentous cyanobacteria made up 4 -19% of the total phytoplankton biomass, with *Anabaena* sp., *Aphanizomenon*sp., *Nodularia* sp. and *Pseudoanabaena* sp. being present. *Aphanizomenon* sp. was the most abundant species throughout the HE cruise, while the *Nodularia* sp. dominated cyanobacterial numbers during The POS cruise (Tab. 3.1). Standing stocks of filamentous cyanobacteria as well as the whole phytoplankton were lower during the POS cruise compared to the HE cruise showing only a weak significant trend (p = 0.3 and p = 0.2). The total chlorophyll *a* values for the HE cruise were on average 2.3 ± 0.2 μg l^{-1} and 3.18 ± 0.88 μg l^{-1} during the POS cruise. The natural abundance of ^{15}N-PON of the phytoplankton fraction was 3.3 ± 2‰. during both cruises. For the HE cruise the ratio of POC: PON of phytoplankton equalled on average 9, for the POS cruise this ratio was 8.

The accumulation of the $^{15}N_2$ tracer in the cyanobacteria fraction in the HE experiments showed a maximum at 12 hours incubation time (Fig. 3.3 A), while it rose nearly linear during the POS experiments (Fig. 3.3 C). Hourly $^{15}N_2$ fixation rates measured on the HE cruise had a mean value of 2.0 ± 4.5 nmol N l^{-1} h^{-1} and ranged

from 0.1 nmol N l^{-1} h^{-1} to 8.9 nmol N l^{-1} h^{-1} (Tab. 3.3). Rates determined during the POS cruise were significantly lower ($p = 0.05$) with a mean value of 0.8 ± 0.5 nmol N l^{-1} h^{-1} and variation from 0.2 nmol N l^{-1} h^{-1} to 1.7 nmol N l^{-1} h^{-1}.

Zooplankton composition and gross ^{15}N incorporation. The total abundance of mesozooplankton in the field (0- 20 m) during the HE cruise ranged from 8.8 ind. l^{-1} to 27.4 ind. l^{-1} (Fig. 3.4), during the POS cruise the abundance of zooplankton was significantly smaller ($p < 0.05$) and ranged from 8 ind. l^{-1} to 13.7 ind. l^{-1} (Fig. 3.4). Copepods identified in the natural assemblage were *Acartia* sp. *Eurytemora affinis*, *Temora longicornis*, *Pseudocalanus* sp. and *Centropages* sp. Moreover, the cladocera *Bosmina coregoni maritima*, *Evadne nordmanni* und *Podon leuckarti* were found. Zooplankton abundance and composition in the actual exper-

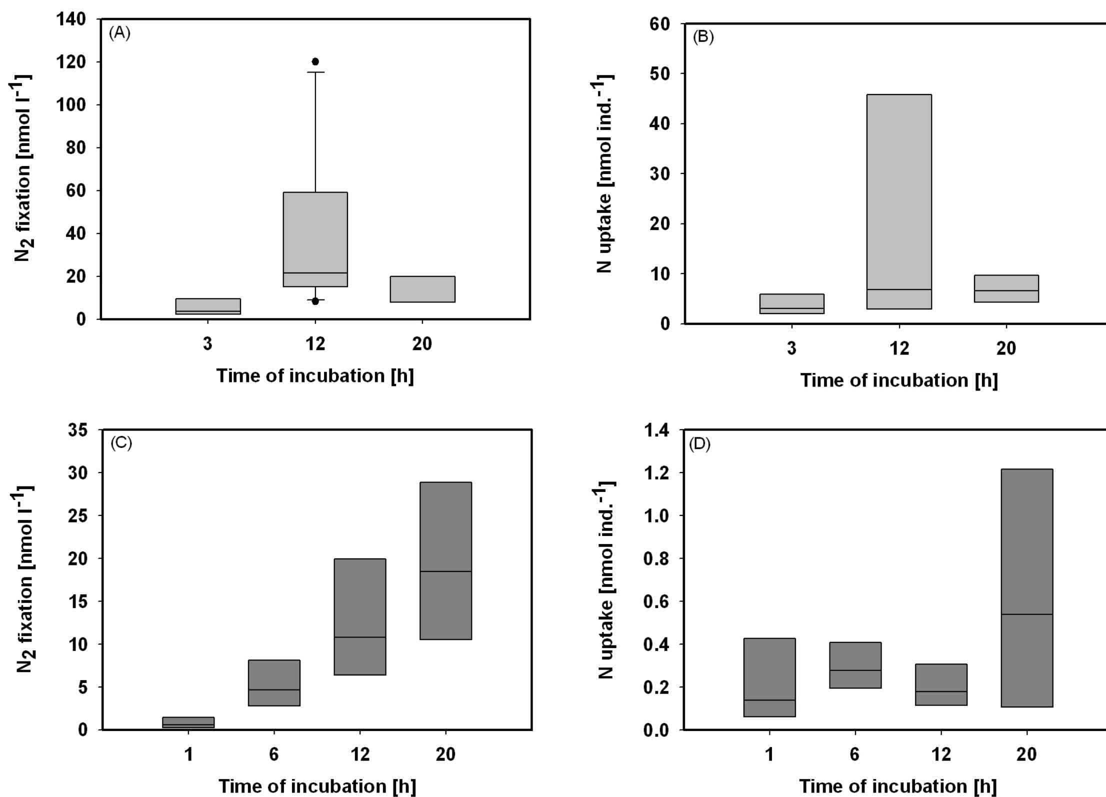

Figure 3.3: Incorporation of ^{15}N tracer by cyanobacteria (diazotroph $^{15}N_2$ fixation) for HE 273 cruise (A) and POS 353 cruise (C) and mesozooplankton >200 μm ^{15}15N incorporation for HE 273 cruise (B) and POS 353 cruise (D) with increasing incubation time.

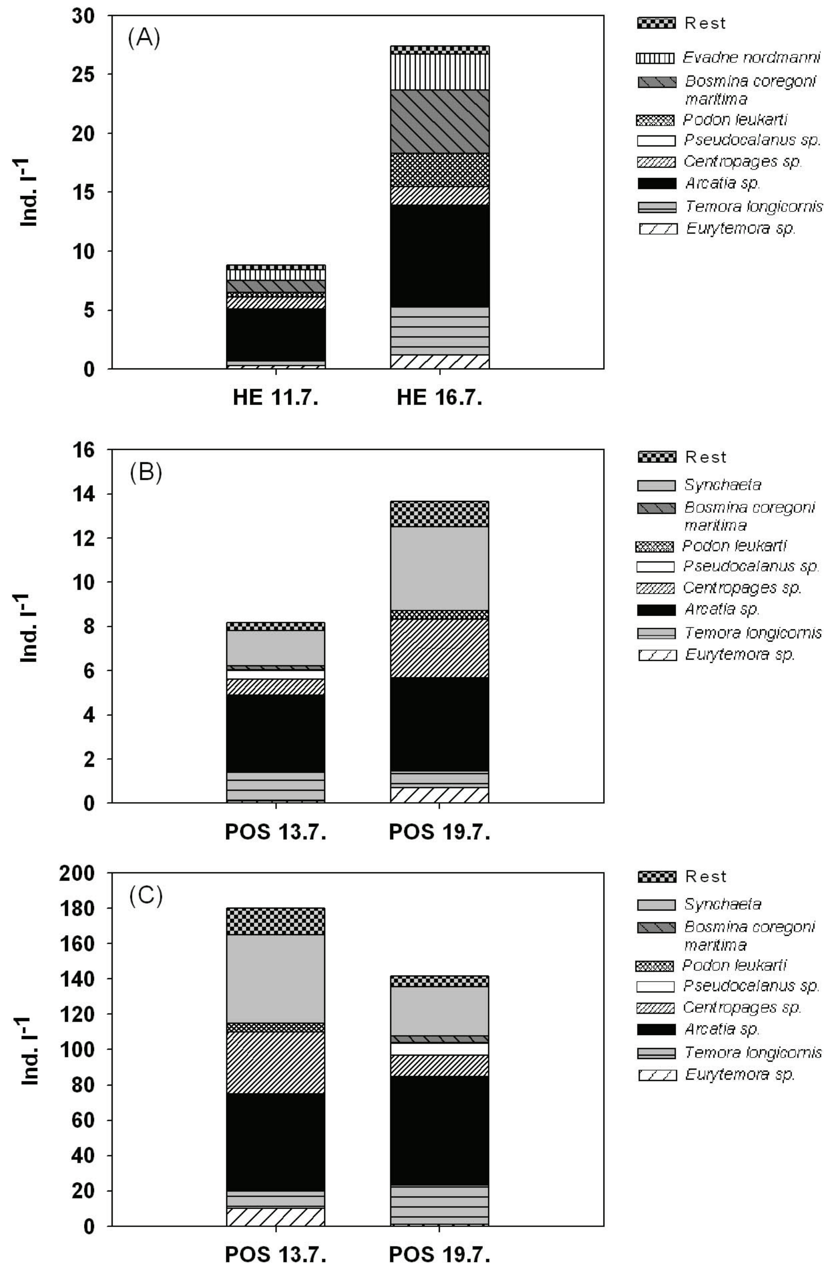

Figure 3.4: Abundance of zooplankton species in the field for 0-20 m of (A) Heincke HE 273 and (B) Poseidon POS 353 cruise and abundance within the treatment bottle during POS 353 cruise (C).

Table 3.1: Carbon biomass (mg C m^{-3}) of phytoplankton groups of Heincke 273 and Poseidon 353 cruises (0- 10 m, mixed samples). T Phyto: total phytoplankton, Cyano: cyanobacteria (*Aphanizomenon* sp., *Nodularia* sp., *Pseudoanabaena* sp),Rest: (Dinophyceae; Cryptophyceae, Diaptomophyceae, Prasinohyceae, Chlorophyceaea).

Cruise	Date	Total Phyto.	Cyano.	Dominant Cyano.	Rest
HE 273	11.07.2007	1226	162	*Aphanizomenon*	1064
	19.07.2007	1484	335	*Aphanizomenon*	1149
	21.07.2007	1220	216	*Aphanizomenon*	1004
POS 353	13.07.2007	973	105	*Pseudoanabaena*	868
				Aphanizomenon	
	16.07.2007	877	163	*Nodularia*	714
	19.07.2007	1638	188	*Nodularia*	1450

imental treatment of the POS cruise is shown in Figure 3.4 C. Species composition in the treatments did not deviate from *in situ* conditions, which is assumed for the HE cruise as well. Nevertheless, there was a difference in cladoceran composition between the two cruises with *Evadne nordmanni* being present only in the HE cruise. Overall abundance of zooplankton species for the single parallel treatments of both cruises is given in Table 3.3. The ^{15}N-PON of the mesozooplankton >200 mum was significantly lower during the HE cruise 5.6 ± 0.2 ‰(p = 0.05) than values from the POS cruise 6.3 ± 0.5 ‰.
The first order estimates of diazotroph contribution to mesozooplankton biomass, based on mass balance of natural abundance of ^{15}N-PON were on average 30 ± 9% during HE cruise and 24 ± 14% during POS cruise (Tab. 3.2). The accumulation of ^{15}N in the mesozooplankton fraction of the tracer addition experiments are shown in Figure 3.3, revealing that the accumulation rose to a maximum after 12 hours of incubation during HE cruise experiments (Fig. 3.3 B). During POS cruise experiments the accumulation increased linear till 20 hours of incubation (Fig. 3.3 D). The hourly gross ^{15}N incorporation rates of mesozooplankton (set-up I) are presented in Table 3.3 showing high variances between the individual replicates. Gross ^{15}N incorporation rates ranged from 0 to 4043 mol N ind.$^{-1}$ h^{-1} during the HE cruise experiments with an average of 883 pmol N $^{-1}$ h^{-1}. Gross incorporation rates gained through POS cruise experiments were significantly lower (p = 0.01) compared to HE rates ranging from 12 to 196 pmol N ind.$^{-1}$ h^{-1}, with an average of

Table 3.2: Estimated contribution of N_2 fixation to the biomass of mesozooplankton during Heincke 273 and Poseidon 353 cruises in July 2007. The diazotroph contribution is calculated using mass balance approach (Eqn. 3.2. Standard deviations from replicates are in parentheses. The reference ^{15}N values used in these calculations are given in the text.

Cruise	Date	Long	$^{15}N_{Zpl}$ ((‰)	Diazotr.contribution (%)
HE 273	14.07.2007	17.92	5.4 (0.7)	34 (9)
	15.07.2007	18.02	5.5 (0.2)	32 (2)
	17.07.2007	17.89	5.8	28
	18.07.2007	17.90	5.6	30
	20.07.2007	17.82	5.9	34 (9)
	21.07.2007	17.74	5.5	32
POS 353	13.07.2007	18.55	5.9 (0.6)	26 (8)
	14.07.2007	18.76	6.3 (0.9)	28 (11)
	16.07.2007	18.83	6.1	24
	17.07.2007	19.05	5.8 (0.4)	12 (5)
	19.07.2007	19.96	6.8	22
	20.07.2007	21.10	7.1 (1.7)	30 (0.3)

67 pmol N ind.$^{-1}$ h^{-1}. Within the individual treatments 100% of new diazotrophic fixed $^{15}N_2$ was transferred to the mesozooplankton community during both cruises.

Distinction between direct grazing and microbial loop mediated transfer of ^{15}N.— To be able to distinguish between direct grazing on filamentous cyanobacteria and transfer of labelled nitrogenous compounds via the microbial loop, we applied an experimental set-up, allowing only active grazing on pre-labelled diazotrophs (3.2, set-up II). Values for hourly ^{15}N uptake by direct grazing are given in Table 3.3 (set-up II). Direct grazing contributed from 11 to 53% during the HE cruise and 5 to 77% during the POS cruise to the gross uptake of ^{15}N by mesozooplankton species (Tab. 3.3). Those values were not significantly different between cruises. The mediation of ^{15}N uptake of the zooplankton community by microbial loop contributors accounted for 47% to 89% (HE cruise) and 23% to 95% (POS cruise).

Table 3.3: Diazotrophic $^{15}N_2$ fixation rates for Heincke 273 and Poseidon 353 cruises in July 2007 and the gross incorporation of recently fixed nitrogen into the mesozooplankton fraction (set-up I) determined using $^{15}N_2$ tracer addition. St-up II (microbial loop excluded = direct grazing). Percentage contribution of direct grazing (set-up II) to the gross uptake (set-up I) is given in row 8. Units are: N_2 fixation (nmol l^{-1} h^{-1}), mesozooplankton biomass (μg C l^{-1}), mesozooplankton abundance (ind. l^{-1}), $^{15}N_2$-incorporation rate (pmol ind.$^{-1}$ h^{-1}), proportion of set-up II (%). Standard deviations from replicates and error of zooplankton counting are in parentheses.zpl: zooplankton

Cruise	Date	$^{15}N_2$ fixation	Mesozooplankton		^{15}N-incorporation zpl		Proportion of
			Biomass	Abundance	Set-up I	Set-up II	Set-up II
HE 273	11.07.2007	0.4	197 (7)	124 (4)	345 (200)		
	12.07.2007	0.8 (0.5)	413 (109)	261 (70)	318 (160)	-	-
	13.07.2007	1	63	51	330	183 (11)	31 (2)
	15.07.2007	1.2 (0.4)	216 (32)	165 (85)	764 (100)	358	47 (6)
	16.07.2007	2.1 (0.2)	420 (270)	385 (247)	472 (221)	-	-
	20.07.2007	5.1 (3.8)	343 (38)	314 (35)	3068 (975)	423 (32)	15 (4)
	21.07.2007	2.4 (2.4)	333 (133)	305 (122)	1690 (2046)	-	-
POS 353	13.07.2007	1.3 (0.4)	198 (39)	141 (81)	57 (24)	12 (1)	40 (28)
	16.07.2007	0.5 (0.3)	361 (122)	340 (48)	41 (22)	3 (1)	13 (8)
	19.07.2007	0.7 (0.3)	189 (52)	180 (77)	104 (92)	37 (10)	54 (23)

3.4 Discussion

Quantification of gross incorporation.— The results of the present study imply that a significant proportion of new diazotrophic fixed N is transferred to the mesozooplankton fraction. First order estimates using the approach of Montoya et al. (2002) and a mass balance calculation with natural abundance ^{15}N values for cyanobacteria and mesozooplankton revealed that in our study N_2 fixation contributed to the mesozooplankton biomass production with an average percent of 26 ± 8% (Tab. 3.2). This is a conservative estimate of the role of diazotrophs, where especially the application of the zooplankton reference value (Eqn 4.3) is potentially blended with unknown proportion of newly fixed and isotopic lighter N. Montoya et al. (2002) published contribution values of N_2 fixation to zooplankton production in the Atlantic Ocean of 6 to 65%. Loick et al. (2007) used the same approach to show that 13% of zooplankton production was supported by diazotroph N_2 fixation in South China Sea. In addition Sommer et al. (2006) applied the isotopic mixing model in experiments using Baltic Sea plankton communities in mesocosm experiments. Their results were similar to ours and other studies (Hawser et al. 1992, Capone et al. 1997, Letelier & Karl 1996). The advantage of taking natural abundance and a mixing model to evaluate the contribution of diazotrophic N to the production of higher trophic level is that long term trends of the biochemical fluxes are mirrored. Nevertheless, the instantaneous role of N_2 fixation for nutrition is missed. Apart from using the two source mixing model we also measured the efficiency of N transfer by adding a ^{15}N stable isotope tracer to the community and examining the instantaneous transfer to the mesozooplankton community. This presents a snap-shot of N transfer which might be higher than results gained when using the two source mixing model of natural ^{15}N-PON. The daily integral of uptake of diazotrophic derived ^{15}N by mesozooplankton in our study exceeds diazotrophic N_2 fixation with a strong variance between single measurements. Moreover, it has to be considered that N_2 fixation itself was highly variable (Tab. 3.3). On average rates of fixation and biomass of diazotrophs were in the range for pre-bloom situations during both cruises. The average cyanobacterial biomass of 174 mg C m^{-3} during the HE cruise and 152 mg C m^{-3} during the POS cruise were much lower than values reported for the Central Baltic Sea at similar times of the year, e.g.

700 mg C m^{-3} (Wasmund et al. 2006). Even maximal N_2 fixation rates were by a factor of 1.5 (HE cruise) and 8 (POS cruise) lower than rates published previously during bloom events in the same area of investigation (e.g. Ohlendieck et al. 2007). Additionally, N_2 fixation activity was significantly lower by a factor of 5 during POS cruise experiments compared to values gained on HE cruise. This difference might be attributed to the spatial variation of N_2 fixation activity and differences in the community composition of cyanobacteria. Above all strong wind prevented the onset of cyanobacteria bloom development during both cruises due to physiological effect of mixing and turbulent shear on the cells (Paerl 1985, Sellner 1997, Moisander & Pearl 2000) and due to mixing of cells into deeper water layers where light becomes limited (Levine & Lewis 1987, Howarth et al. 1993). Overall lower N_2 fixation activity and abundance of mesozooplankton species during the POS cruise resulted in proportional lower ^{15}N uptake rates of mesozooplankton compared to the HE cruise in the Northern Baltic Sea. On a daily basis 14% of the diazotroph PON and on average 100% of the fixed N were consumed in our set-ups by the mesozooplankton community during both cruises (Fig. 3.5 A and B, values in parentheses). We propose that in this case the ^{15}N tracer approach was more sensible to the concurrent zooplankton composition, the actual N_2 fixation activity and to additional interactions with other food web components or among mesozooplankton species. Moreover, N_2 fixation activity might have been underestimated when labelled ^{15}N, originally fixed by diazotrophs, is lost to the DON pool (50% of fixation might be exudated according to e.g. Bronk et al. 2007) and subsequently transferred. Additionally, abundance of zooplankton within the experimental set-up was enriched by a factor of 10 and 20 (HE and POS cruise respectively) compared to in situ conditions, which results in an overall increase of N incorporation by the whole mesozooplankton community. The individual incorporation rate should depend on the prey abundance (functional response). Moreover, incorporation is also influenced by the predator density and prey-predator ratio (numerical response), because of density dependent physical and social interaction. Experimentally, predator dependence is rarely confirmed (e.g. Fussmann et al 2005). Applying the incorporation rates obtained in this study (Tab. 3.3) and using the natural abundance of zooplankton and *in situ* N_2 fixation rate, an idealized daily N budged can be calculated for the two cruises, which is represented in Figure 3.5. Standing stocks of cyanobacteria are 2.78 μmol N l^{-1}

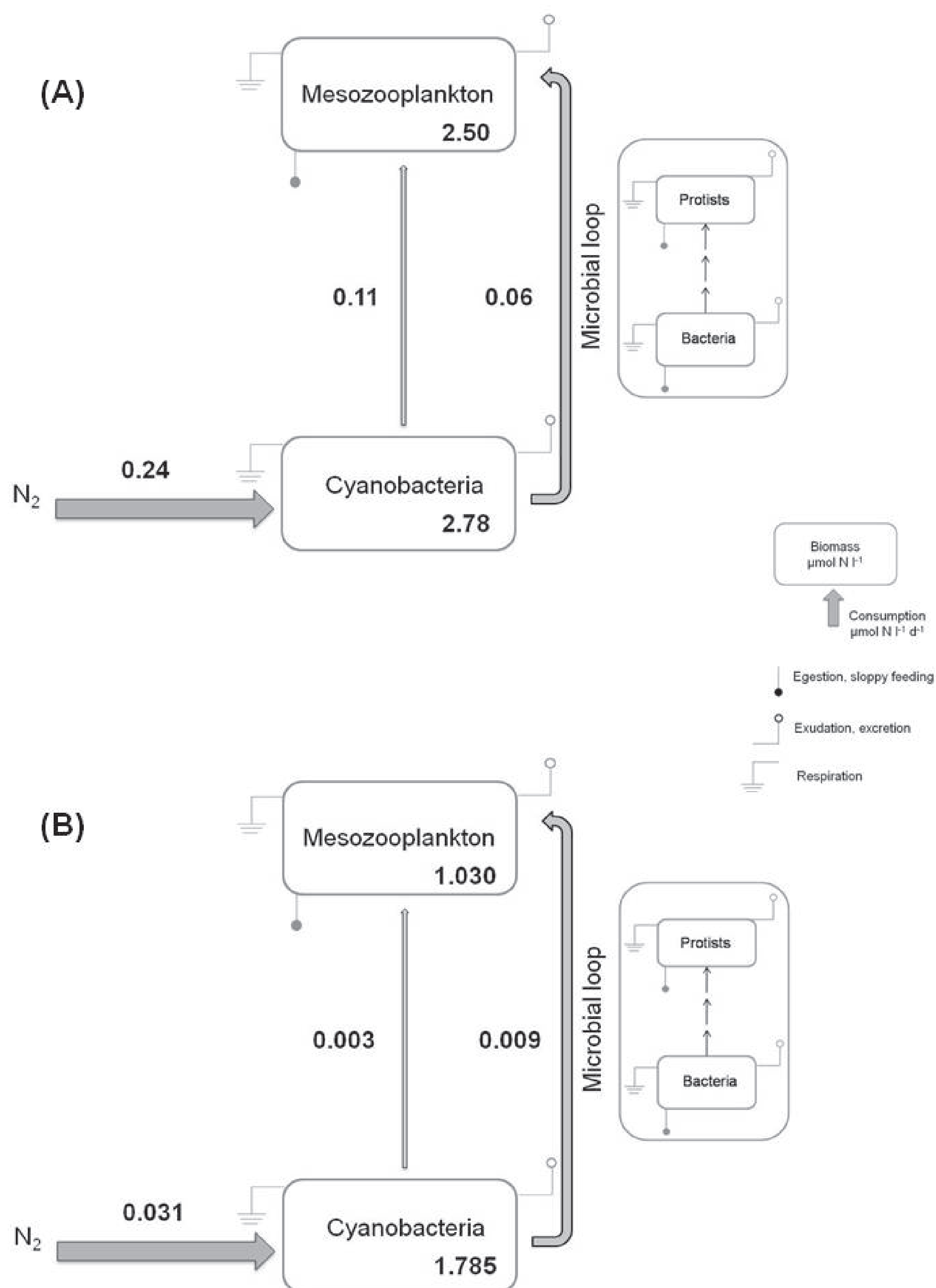

Figure 3.5: Idealized N budget using minimal and maximal (numbers in parenthesis) *in situ* abundance of mesozooplankton and the determined average N incorporation rates of HE 272 (6A) and POS 353 (6B) cruise. The numbers in the box are the mean standing stock (μmol N l^{-1}). Numbers next to the arrow are fluxes of N (μmol N $l^{-1}d^{-1}$). Thicknesses of arrows are roughly proportional to the N flux. Egestion, sloppy feeding, exudation, excretion and respiration were not quantified and are only used to illustrate loss terms for the single trophic positions.

and 1.78 μmol N l^{-1} for the HE and POS cruise, respectively and of mesozooplankton 2.50 μmol N l^{-1} and 1.03 μmol N l^{-1}. The average daily N_2 fixation rate is 1.29 μmol N l^{-1} d^{-1}. Mesozooplankton would have incorporated on average 36% during HE cruise and 25% during the POS cruise of this daily N_2 fixation. This equals to 6% and 1.2% of mesozooplankton body N during HE and POS cruise respectively, that is incorporated per on a daily basis. If we assume a C:N ratio of 7:1 for prey ingested and 6:1 for zooplankton biomass, we can convert N incorporation to C incorporation. On the whole, 1.190 μmol C l^{-1} d^{-1} and 0.084 μmol C l^{-1} d^{-1} would be ingested accounting for 7.9% and 1.35% of mesozooplankton body C deriving from cyanobacteria. Calbet (2001) published data indicating that in intermediate productive marine ecosystems 19.9 $\pm$ 0.04% of mesozooplankton body C is ingested based on the whole phytoplankton community. Compared to this, the value gained in this study for the HE cruise is lower by a factor 2.5. This indicates that potentially, cyanobacteria are an important source of nutrition in mesozooplankton. Values gained from the POS cruise are lower by one order of magnitude (factor 14). It has to be kept in mind, that C ingestion rates were not directly measured and derived from conservative estimates using an average of C:N of cyanobacteria. C ingestion rates might be higher than expected, when food species temporarily gain C:N ratios up to 28, due to carbon ballasting (Ohlendieck et al. 2007).

The zooplankton community during HE cruise may have starved without ingesting diazotrophic derived nitrogen (through direct ingestion of filamentous cyanobacteria and ingestion of microbial loop components). Compared hereto, less diazotrophic biomass was taken up during the POS by zooplankton species, attributed possibly to the dominance of *Nodularia* during the cruise in the Northern Gotland Basin, while *Aphanizomenon* was the most abundant filamentous cyanobacterium during the HE cruise in the Southern Gotland Basin. It is known that, although both species are potentially toxic, *Nodularia* is responsible for the hepatotoxicity of the cyanobacterial blooms in the Baltic Sea (Sivonen et al. 1989a), while *Aphanizomenon* is more common in the non-toxic blooms (Sivonen et al. 1990). Nevertheless, as our results indicate, filamentous cyanobacteria present as a mixture of *Aphanizomenon*, *Nodularia*, and *Pseudoanabaena* are grazed upon directly (direct grazing is discussed in subsection below). To our knowledge, only O'Neil et al. (1996) have used the same ^{15}N tracer addition approach investigating grazing on filamentous cyanobac-

teria *Trichodesmium* sp. grazed by *Macrosetella gracilis.* Results from their study indicated that up to 100% of fixed N and 33-54% of diazotroph colony N was ingested by *M. gracilis* on a daily basis. These values are very similar to our results most surprisingly remembering that *M. gracilis* has been known for actively feeding on filamentous diazotrophs (e.g. O'Neil & Roman 1994), while ingestion by Baltic Sea mesozooplankton is negotiated to a large degree (e.g. Sellner et al. 1996, Engström et al. 2000). In order to compare the ^{15}N incorporation rates gained in this study with other published ingestion rates of mesozooplankton species using the ^{14}C method, we converted N uptake to C uptake rates using POC to PON ratio of the cyanobacteria food resource. On average the calculated C uptake rates of mesozooplankton in this study range from 18 to maximal 509 ng C ind.$^{-1}$ h^{-1} during the HE cruise, assuming a POC: PON ration of phytoplankton food of 9. During the POS cruise carbon uptake rates derived from ^{15}N incorporation rates ranged from 2 to maximal 25 ng C ind.$^{-1}$ h^{-1}. Considering periodical uncoupling of primary production and N_2 fixation of recently fixed compounds (C:N_{rate} of maximal 28, Ohlendieck et al. 2007) would raise the values of the POS cruise to 5 and 77 ng C ind.$^{-1}$ h^{-1}. These calculated carbon uptake rates are at the lower end for the POS cruise and even higher for the HE cruise compared to results published earlier for monocultures of cyanobacteria and zooplankton species of Baltic Sea, ranging from 30 to 114 ng C ind.$^{-1}$ h^{-1} (Engström et al. 2000, Koski et al. 2002, Kozlowsky-Suzuki et al. 2003). Nevertheless, it has to been considered that determination of food ingestion using monospecific diet (as done in the aloft mentioned studies) of normally omnivorous zooplankton species alters the overall incorporation rate to the lower end.

Proportion of direct grazing to the gross incorporation.— Although ^{15}N incorporation rates differed between the two individual cruises according to the predominant abundance of plankton and N_2 fixation activity, the proportion of grazing to the gross uptake did not differ significantly between cruises ($p = 0.5$, Tab. 3.3). Nevertheless, the results show a large variance between parallels within the treatments of each cruise. This may be partly explained by the differing contribution of mesozooplankton known to be active grazers on filamentous cyanobacteria. Active grazers like *Arcatia* sp., *Eurytemora* sp. and *Bosmina* made up 50% of total mesozooplankton carbon biomass in this study. The daily average of grazing proportion

to the gross N transfer was on average 31% during the HE cruise and 26% during the POS cruise. Grazing rates of zooplankton determined in this study were strongly correlated to diazotrophic carbon biomass during both cruises, showing a best fit for a functional response type III relation (Fig. 3.6). The functional response model type III applies to a predator that will not begin feeding until there have been several encounters with their prey. Nevertheless, it has to be kept in mind that a consortium of mezozooplankton grazer (copepods as well as cladocera) accounts for the ingestion of diazotroph N, representing different types of food uptake strategies. The model also implies that uptake rates decrease with decreasing food concentration. Literature data supporting functional response type III are scare although it is most consistent model resulting in a stable strategy of co-existence between zooplankton and phytoplankton, unlike the model of functional response type I and II (Steele 1974). Functional response model type III has nevertheless been applied for daphniids (Chow-Fraser & Sprules 1992) and marine copepods (Gismervik & Andersen 1997, Evjemo et al. 1999). Up to now, grazing on filamentous cyanobacteria is controversially discussed in the literature. In studies consumer control of heterocystic cyanobacteria blooms is effective in some instances, but not others. Thus, there are contradicting findings on avoidance of filamentous cyanobacteria by *A. bifilosa* and *E. affinis* (Sellner et al. 1996, Engström et al. 2000) and active grazing by the latter on the other hand (Kozlowsky-Suzuki et al. 2003). Several studies give proof that *A. bifilosa*, *A. tonsa* and *E. affinis* graze actively on *Nodularia spumigena* even when other food items are present (Meyer Harms et al. 1999, Kozlowsky-Suzuki et al. 2003, Koski 2002, Chan 2001 Chan et al. 2006) and moreover survived and sustained egg production. Holm et al. (1983) reported that *Daphnia pulex* is capable of grazing single filaments (200 mm long, broken from colonies) and small colonies (1.5 mm long) of *Aphanizomenon flos-aquae*, which suggests that *A. flos-aquae* is readily assimilated once it is ingested. The results published by Wilson et al. (2006) are in the line of our argumentation. They presented statistically analysed literature data on grazing of zooplankton on size fractions of cyanobacteria showing that filamentous cyanobacteria and algae were a better food source than single celled cyanobacteria, compared with control food (Lynch 1980, Porter & McDonough 1984, Gliwicz 1990). Moreover, they postulated that there was no strong evidence in the literature to support the generalization that the presence or

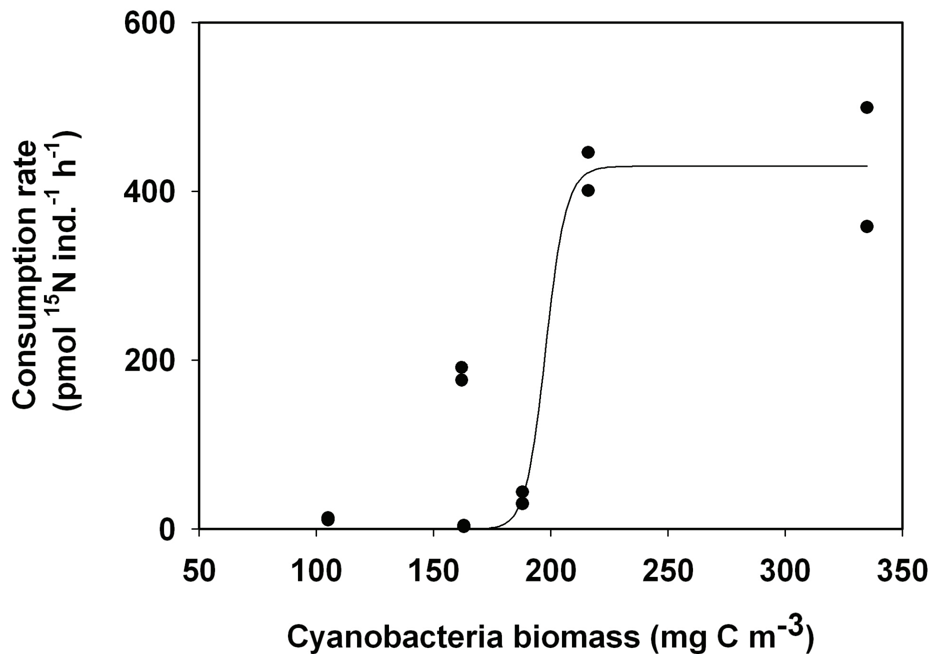

Figure 3.6: Consumption rate of mesozooplankton as a function of cyanobacterial biomass. Solid line represents fitted curve for Holling type III functional response using a Hill function ($y=429*x^{45}/ 197^{45} + x^{45}$, $R^2= 0.899$, $n=12$, $p = 0.05$)

absence of described cyanotoxins is an important factor driving the poor quality of cyanobacteria as food for zooplankton in general. More likely it has been attributed to the absence of long-chain polyunsaturated fatty acids (PUFAs, Gugger et al. 2002) and sterols (Volkman 2003, Summons et al. 2006). PUFAs and sterols are essential dietary compounds in arthropods that cannot be synthesized de novo (Harrison 1990; Grieneisen 1994) and serve as precursors of many bioactive molecules. Martin-Creuzburg et al. (2008) published results, stating that the absence of PUFAs in cyanobacteria appears to be of minor importance for somatic growth of Daphnia but potentially affects egg production, while the absence of sterols has to be considered the major food-quality constrain. On the contrary, besides the documented low food quality of cyanobacteria concerning PUFAs and sterols, they might provide a source of essential growth factors especially in N-limited habitats which are important in maturation and reproduction of crustaceans, e.g. amino acids for syntheses of peptide hormones and egg yolk proteins (Harrison 1990). Cyanobacteria have been

found to have a very high content of amino acids (Ahlgren et al. 1992). Moreover, they exhibit high N:P stoichiometry due to the accumulation of proportionally more light harvesting machinery components, needed to fuel the energy demand of the N_2 fixation process (Klausmeier et al. 2004). Overall, the majority of recent publications and the results of our study do not support the general view of detrimental effects of cyanobacteria on copepod feeding. They rather stress the importance of studies that include the entire plankton community rather than monocultures or mixtures of a few species. Zooplankton grazing on cyanobacteria can occur, but it is often insufficient to control the gross growth rate of the heterocystic cyanobacteria and so the potential for a bloom to develop (Carpenter et al. 1995, Sellner 1997). On the other hand, Marino et al. (2002) showed that in more saline ecosystems grazing might act as a large constraint on the production of cyanobacterial biomass when growth rates are slow, but it is far less constraining in freshwaters and brackish waters where heterocystic cyanobacteria can achieve high cell growth rates.

Proportion of microbial loop mediation to the gross incorporation.— On the whole, 67% of incorporated ^{15}N by mesozooplankton derived from mediation of microbial loop constituents. So far, several studies gave evidence that the primary route of N transfer from diazotrophs to the mesozooplankton community is through the microbial food web (e.g. O'Neil et al. 1996, Capone et al. 1997, Rolff 2000, Ohlendieck et al. 2000, Sommer et al. 2006). Diazotrophic fixed N_2 is either retained in POM itself, or lost to the pool of dissolved N (Bronk et al. 1994). It is commonly known that cyanobacteria release up to 80% of fixed N_2 as DON (Bronk et al. 2007 and references within). This DON is available for uptake by bacteria which are subsequently ingested by ciliates and protozoa and finally reach mesozooplankton species. Furthermore, NH_4^+ plays a crucial role in the regeneration of diazotrophic N. For example O'Neil et al. (1996) assumed that NH_4^+ regeneration in a *Trichodesmium* consortium through breakage of cells and release as well as excretion by microheterotrophs is responsible for the majority of N transfer determined in their study. In addition to these findings, several other investigations have shown that zooplankton produces considerable amounts of DON and NH_4^+ through sloppy feeding, leakage from faecal pellets, and excretion (Gardner & Paffenhöfer 1982, Hasegawa et al. 2001, Møller et al. 2003, Vincent et al. 2007), providing again bioavailable substrate for bacterial uptake. Sloppy feeding has been suggested to

depend on the shape and size of the prey (Møller 2005). Large prey, like filamentous cyanobacteria, seems to produce large amounts of DOC and probably DON (Roy et al. 1989, Hasegawa et al. 2001, Møller et al. 2003), while no DOC is produced when the prey is small relative to the copepod (Strom et al. 1997, Møller & Nielsen 2001). The number of trophic steps took on channelling energy and N trough the microbial loop before entering the mesozooplankton fraction, and thus the trophic position of the mesozooplankton results in substantial consequences for the energy transfer within the food web. Pathways that involve multiple trophic links provide a greater opportunity for respiratory and other losses than in two-step pathways that occur between algae and zooplankton (Sanders & Wickham 1993). Ultimately, the trophic position of zooplankton differs when C and N are incorporated directly by grazing or indirectly via the microbial loop. The trophic transfer efficiency decreases when trophic links are added (90% of energy is lost per trophic position). These increased energy losses caused by an additional trophic link might be to some extent counterbalanced by 'trophic upgrading', i.e. by protozoa being better copepod food than algae. However, an increase of ecological efficiency from ca. 10% to ca. 30% would be needed to compensate for an additional tropic level. Nevertheless, results from field studies by Koshikawa et al. (1996) and Havens et al. (2000) revealed that the transfer efficiencies were nearly the same for both pathways and that the microbial loop is possibly a link of magnitude similar to that of the photosynthetic food chain.

Ecological implications— The results of our study indicate that: (1) N derived from diazotroph fixation reaches the mesozooplankton community within one hour. (2) The profound transfer of newly fixed nitrogen is mediated by microbial loop constituents (67% of gross transfer). (3) Zooplankton species of the Baltic Sea are able to use cyanobacteria as a direct food source (33% of gross N transfer). The transfer of diazotrophic fixed N in our study, via direct consumption of cyanobacteria and indirect consumption via microbial loop indicates that N_2 fixing cyanobacteria are ecologically more important as instantaneous sources of nitrogen for higher trophic level of the Baltic Sea food web than previously assumed. This holds true even for grazers like mysids as shown recently by Gorokhova (2009). A simple calculation may clarify this by applying our data set to a full bloom situation with maximal N_2 fixation rates of 2.2 μg N l^{-1} d^{-1}, (Ohlendieck et al. 2007) and in situ values of plankton biomass. Diazotrophic N_2 fixation would in this case provide 50% of N for

mesozooplankton production on a daily basis.

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Chapter 4

Impact of diazotrophy on N stable isotope signatures of NO_3^- and PON and transfer of diazotrophic fixed N to mesozooplankton species – Case studies in North - Eastern Tropical Atlantic Ocean.

4.1 Introduction

Diazotrophs play a critical role in supporting oceanic new production in the Atlantic Ocean, by fixing atmospheric N_2 and subsequently introducing reduced N compounds into a open ocean ecosystems. Two major groups were regarded to be responsible for the majority of N_2 fixation: cyanobacteria from the genus *Trichodesmium* and diazotroph- diatom associations (DDAs) with *Richelia intracellulans* or *Calothrix* being the diazotrophic endosymbiont (Carpenter et al. 1999, Karl et al. 2002, Capone et al. 2005, Foster et al. 2007). Moreover, recent studies have strengthened the idea, that a significant amount of fixation is carried out by unicellular anaerobic non-heterocystic picocyanobacteria < 10 μm in diameter (Zehr

et al. 2001, Montoya et al. 2004 and 2006). Unlike *Trichodesmium* and DDAs, which reside in the shallower portions o the upper euphotic zone (Carpenter et al. 2004), unicellular diazotrophs seem more uniformly distributed through the water column (Montoya et al. 2004, Langlois et al. 2005). N_2 fixation in the Tropical Atlantic Ocean is influenced by different local physical and chemical forcing factors making it spatially variable. Additionally, diazotrophic production is limited by P and micro/macro nutrients like Fe (see also Introduction, subchapter 1.4, Mills et al. 2004), which may be delivered by high aeolian inputs of dust from the African continent to the Atlantic Ocean (Jickells et al. 2005). This process is thought to relieve N_2 fixation from limitation by this micronutrient (Falkowski 1997, Mills et al. 2004, Voss et al. 2004) driving this oceanic ecosystem toward P limitation (Wu et al. 2000).

There is a strong seasonality in this dust transport. During summer time long- range transport at high latitudes (1.5 km above sea level) within the so called Saharan Air layer (S.A.L.) leads to low dust precipitation over the Eastern tropical Atlantic Ocean in the vicinity of the Cape Verde Islands. In winter time this region receives a higher amount of dust, because the islands are localized across the main path of African dust transport (Chiapello et al. 1995). This seasonality in the transport mechanisms affects the production in the Atlantic Ocean. Blooms of diazotrophs are detected after such dust events (e.g. Langlois et al. 2008), with *Trichodesmium* being the dominant species. Not only the presence or absence, but also the type of dominating diazotrophic group has an impact on the food web (see also Tab. 1.1). For example the flux of new N entering the food web is altered when *Trichodesmium* is the dominant diazotroph, because it is only grazed upon by a limited number of herbivores (e.g. *Macrosetella*, Roman 1978, Hawser et al. 1991, O'Neil et al. 1996). Consequently, the majority of N bound in its biomass enters the food web through the microbial loop and recycling processes (Capone et al. 1994, 1997, Letelier & Karl 1996, Capone 2001), unicellular diazotrophs may be grazed upon directly.

Regardless from which different diazotrophic group new nitrogen emerged, a signal in the composition of stable isotopes δ^{15}N: the proportion of heavier to lighter ^{14}N ^{15}N) of the particulate fractions of food web should be measurable. As the δ^{15}N-PON values of phytoplankton are influenced by variations in the isotopic composition of inorganic N in surface waters (Waser et al. 2000, Mino et al. 2002, Montoya et

al. 2002), the isotopic composition of bacteria and other components of the food web should likewise be affected by the $\delta^{15}N$ variability of their N source (Montoya et al. 2002). N_2 fixation produces biomass with a low $\delta^{15}N$ between -1 ‰ and -2 ‰, ultimately lowering the $\delta^{15}N$ of all organic pools in the food web (Montoya et al. 2002), as well as dissolved inorganic nitrogen (DIN, e.g. NO_3^-) compounds produced in the course of remineralisation. Field studies from Montoya et al. (2002) and Sommer et al. (2006) using natural plankton communities have already proofed this correlation in the Atlantic Ocean and Baltic Sea, respectively. Moreover, it has been shown that a significant proportion of diazotrophic N ultimately reaches higher trophic level via direct grazing upon filamentous cyanobacteria, like mesozooplankton (O'Neil et al. 1996) and mysids (Gorokhova 2009), which contrasted the predominant opinion on cyanobacteria being of poor food quality, toxic and not actively ingested.
In the absence of significant N_2 fixation, particulate material will be produced, reflecting the stable isotope composition of deep water NO_3^-. Thus, an estimate of the importance of diazotrophy can be made when analyzing the natural abundance of stable isotopes in various dissolved and particulate compounds using a two source mixing model (Montoya et al. 2002). The aim of this chapter is to give a quantitative estimate of the impact of N_2 fixation on the natural abundance of N stable isotopes in NO_3^- and PON of phytoplankton and zooplankton. Furthermore, the aim is to quantitatively determine the instantaneous transfer of diazotrophic fixed N to mesozooplankton species in the Eastern Tropical Atlantic Ocean and to identify the dominating pathway of N transfer, i.e. direct grazing or through microbial loop mediation. Three approaches were applied to achieve the aims: 1) The natural abundances of $\delta^{15}N$-NO_3^-, 2) $\delta^{15}N$-PON of cyanobacteria and bulk phytoplankton and the apllication of a 2- source mixing model and 3) pulse chase experiments using stable isotope tracer addition. The study was carried out in the Mauritanian upwelling region and close to São Vincente, Cape Verde.

4.2 Material and Methods

Cruise tracks.—Samples for isotopic analysis in nitrate ($\delta^{15}N$-NO_3^-) were taken using a CTD at three stations (244: 19°30.0'N, 17°0.0 W; 245: 19°30.0'N, 17°30.0'W

and 246: 20°0.0'N, 18°0.0'W) on board the RV Poseidon 348 during an upwelling situation along the Mauritanian coast in July 2007 (Fig. 4.1). The Cape Verde

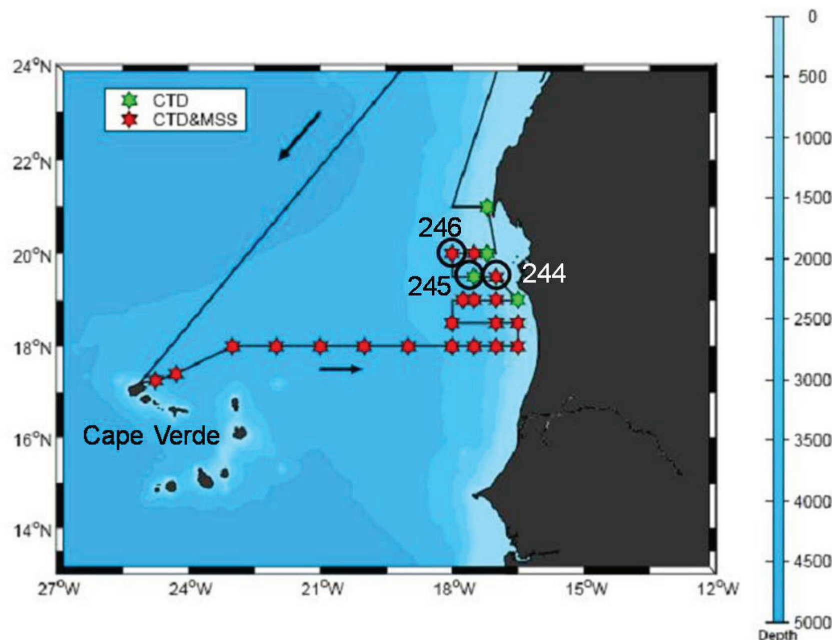

Figure 4.1: Sampling grid of the POS 348 cruise in July 2007. Note that samples for $\delta^{15}N\text{-}NO_3^-$ were taken only at three stations (244, 245, 246) during the cruise, highlighted by the black circle.

studies were carried out in July 2008 sampling three different stations in the course of three different cruises with the RV/Islandia (CV1 on the 16.7.2008: southwest of São Vincente 16°45.897'N, 25°07.367'W; CV2 on the 20.7.2008: northeast of São Vincente 17°03.980'N, 24°51.489'W; CV3 on the 24.07.2008: northeast of São Vincente 17°04.081'N, 24°49.471'W) (Fig. 4.2). Water sampling was done using a continuous flow sampling device (FISH).

Environmental parameter and plankton composition of POS 348 and Islandia cruises.—Concentrations of NO_3^- were determined on board according to the methods described by Grasshoff et al. (1983) with a precision of 0.1 μmol l^{-1}. During the POS 348 cruise subsamples for pigment analysis were filtered onto GF/F filters (Whatman), placed into Cryovials and frozen for later analysis at -80 °C. Analysis was done at the IFM-GEOMAR, Kiel by Ilka Peeken. During the Cape Verde

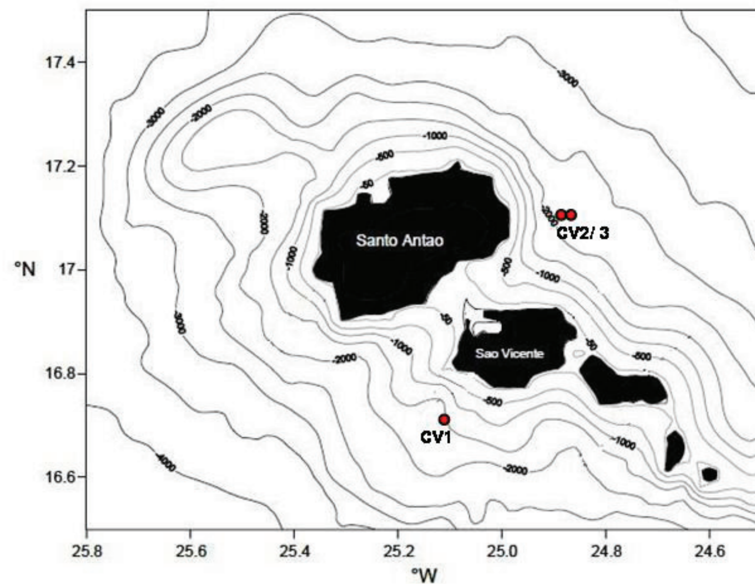

Figure 4.2: Sampling stations of the Cape Verde cruises in July 2008

cruises chlorophyll *a* filters were extracted in ethanol prior to fluorometrical determination of concentration. Abundance of *Trichodesmium* was determined from NifH gene copy number (results provided by LaRoche and Mohr) assuming one copy per cell and 100 cells per trichome for filamentous cyanobacteria.

To quantify mesozooplankton composition and abundance during the RV/Islania cruises, zooplankton was collected with a 200 μm mesh-sized Bongo net by vertically towing from 20 and 50 m depth to the surface. To quantify zooplankton abundance in the treatment bottles subsamples were preserved, with Formalin and identified as well as counted in the lab under a binocular microscope (Leica DMIRP, 100x,200x, 400x).

Isotopic analysis

Natural abundance of δ^{15}N-NO_3^- during the POS 348 cruise.—Samples were acidified to a pH below 3, stored, and transported to the Leibniz-Institute for Baltic Sea Research, Warnemuende (IOW) for further treatment. Stable isotope abundance of NO_3^- was analyzed by the so-called diffusion method where NO_3^- is reduced to NH_4^+, and subsequently trapped on an acidified GF/F filter according

to Sigman et al. (1997). Samples were analysed in parallels and a mean value was calculated.

Calculation of δ^{15}N-NO_3^- isotope fractionation factor ϵ— The determination of the isotopic fractionation factor ϵ (‰) was performed graphically assuming a closed system, where the δ^{15}N-NO_3^- (ordniate) was plotted against the ln transformed NO_3^- concentration (abscissa). The slope of the linear regression lines yielded an estimate of ϵ. This relation can be approximated by the Rayleigh fractionation equation 4.1, where $\delta^{15}NO_3^-$ and [NO_3^-] are known values.

$$\delta^{15}NO_3^- = -\epsilon \cdot ln[NO_3^-] \qquad (4.1)$$

ϵ corresponds to (R_{15}/R_{14} -1) · 1000, where R_{15}/R_{14} is the ratio of the specific reaction rates for ^{14}N and ^{15}N, respectively. In the literature R_{15}/R_{14} is also referred to as the fractionation factor α.

Natural abundance of δ^{15}N-PON during the RV/Islandia cruises.— Bongo net hauls were taken ashore for further treatment in the laboratory of the INDP (Instituto Nacional de Desenvolvimento das Pescas, Mindelo). The zooplankton and phytoplankton fraction were separated using a “light trap”, in which positive phototactic and healthy zooplankton actively moves from a shaded into an illuminated container, to sort out detritus. Subsamples for the two fractions for analysis of natural abundance of isotopes were separated into discrete size fractions by passage through a series of Nitex sieves (10, 200μm) and filtered onto glass fibre filters (Whatman GF/F). The size fractionated samples were frozen for later isotopic analysis at the IO-Warnemünde. For this, the filters were dried at 60 °C and packed into tin capsules and pelletised for elemental and isotopic analysis.

The stable N isotope ratios (δ^{15}N-PON) as well as PON concentration were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020 °C in a Thermo Finnigan Delta S mass-spectrometer. Filters containing particle samples were trimmed, sectioned, then loaded into tin capsules and palletised for isotopic analysis. The stable N isotope ratios measured for each sample were corrected against peptone which was calibrated using standards with defined nitrogen and carbon isotopic compositions (IAEA-N1, IAEA-N2, NBS 22, and IAEA-CH-6) by mass balance. Values are reported relative to atmospheric N_2 (δ^{15}N). The analytical

precision for both stable isotope ratios was ± 0.2 ‰. Calibration material for N analysis was acetanilide (Merck).

First-order estimates of diazotroph contributions to the abundances of δ^{15}N- NO_3^- during the POS 348 cruise.— We used the mass-balance approach of Montoya et al. (2002) to give estimates on the contribution of diazotroph N to the δ^{15}N- NO_3^- (Eqn. 4.2).

$$\%\text{Diazotroph N} = 100 \cdot \left(\frac{\delta^{15}\text{N}_{\text{NO}_3^-} - \delta^{15}\text{N}_{\text{RefNO}_3^-}}{\delta^{15}\text{N}_{\text{Diazotroph}} - \delta^{15}\text{N}_{\text{RefNO}_3^-}} \right) \tag{4.2}$$

As a reference value for δ^{15}N-NO_3^- we used the highest δ^{15}N value of NO_3^- found during the three cruises (^{15}N $\text{Ref}_{\text{NO3}-}$= 6.65 ‰), representing minimal inputs of N from diazotrophs. The δ^{15}N value for diazotrophs used for the calculations was 0.5 ‰, which is the mean value for δ^{15}N of hand picked filaments.

First-order estimates of diazotroph contributions to PON of phytoplankton and zooplankton during RV/Islania cruises.— We used the same mass-balance approach of Montoya et al. (2002) to give estimates on the contribution of diazotroph N to the biomass of phytoplankton species (Eqn. 4.3).

$$\% \text{ Diazotroph N} = 100 \cdot \left(\frac{\delta^{15}\text{N}_{\text{PON}} - \delta^{15}\text{N}_{\text{RefPON}}}{\delta^{15}\text{N}_{\text{Diazotroph}} - \delta^{15}\text{N}_{\text{RefPON}}} \right) \tag{4.3}$$

As a reference value for PON of phytoplankton we used the highest δ^{15}N found during the three cruises (^{15}N Ref_{PON}=9.9 ‰), representing PON with minimal inputs of N from diazotrophs. The reference value for zooplankton was 13 ‰, representing PON with minimal inputs of N from diazotrophs. The δ^{15}N value for diazotrophs used for the calculations was 0.5 ‰, which is the mean value for δ^{15}N of hand picked filaments.

Pulse chase experiment using ^{15}N tracer addition during the RV/Islandia cruises.—Two experimental set-ups, identical to the set-up used in chapter 3, were conducted to investigate N transfer from cyanobacteria to the mesozooplankton community (Fig. 3.2).

In the first type of experiment, which was identical to the set-up in chapter 3 (set-up I, Fig. 3.2 A) unbiased surface water with natural phytoplankton assemblage was

filled into 2.5 l polycarbonate bottles. Zooplankton from net tows was transferred into the incubation bottles. Bottles were filled, sealed gas tight and spiked with 2 ml $^{15}N_2$ (99% ^{15}N, Campro Scientific). The flasks were placed in flow trough incubation tanks on board for 1, 3, 6, 12 or 20 h under 75% ambient irradiance. At the end of the incubation period the samples were screened through 200 μm gauze to isolate the zooplankton from the phytoplankton. Each fraction >200 and <200 μm was collected on precombusted Whatman GF/F filters and stored frozen until analysis with a continuous-flow isotope ratio mass spectrometer. Either zooplankton was hand picked on board with a pipette prior to filtration onto Whatman GF/F (direct determination of ^{15}N uptake) or treatments were set off against control treatments with only phytoplankton being present.
In the second type of experiment (set-up II, Fig. 3.2 B) uptake of pre-labelled cyanobacteria by mesozooplankton were determined. Unlike in the set-up I, only direct grazing was possible, as microbial loop contributors were excluded by filtering the seawater used for the incubation over GF/F filter (0.8 μm), prior to adding the zooplankton. The pre-labelling started by enriching cyanobacteria from surface water using a GF/F filter allowing the filter not to run dry during enrichment and adding them to 2.5 l polycarbonate bottles filled with filtered seawater. Bottles were sealed gas tight and spiked with 1 ml l^{-1} $^{15}N_2$ (99% ^{15}N, Cambro Scientific). The flasks were placed in incubation tanks for 6 h under 75% ambient irradiance. To start the actual experiment, the pre-labelled cyanobacteria and zooplankton were added to 2.5 l polycarbonate bottles filled with 0.8 μm filtered sea water. Samples were incubated in tanks for 12 or 20 h in the dark. At the termination of the experiment zooplankton and cyanobacteria colonies were separated by screening the sample through 200 μm gauze. Organisms smaller than that were sampled on GF/F filters. Handling of filters and isotopic analysis was carried out as described above.
We use the term gross incorporation or gross uptake of N for results of set-up I to distinguish ^{15}N incorporation by direct grazing plus uptake of ^{15}N labelled microbial loop components from results of set-up II, the net incorporation that results only from direct grazing.
Rate calculation for the pulse chase experiments.— $^{15}N_2$ fixation and accumulation of ^{15}N in mesozoplankton species were measured using a $^{15}N_2$ assay. Tracer incubations were terminated by gentle vacuum filtration (100 cm Hg) through pre-

Table 4.1: Pigment distribution (ng l^{-1}) in the upper 10 m of the water column for the Eastern tropical North Atlantic station determined during POS 348 in July 2007.

Station	Lat/ Long	Chl *a*	Fucoxanthin	Zeaxanthin
244	19°30/17°00	4000	1700	10
245	19°30/17°30	2000-3000	900-1200	10
246	20°00/18°00	2000	600	30

combusted GF/F filters. These filters were dried at 60°C and stored for isotopic analysis. Diazotroph N_2 fixation was determined using the approach of Montoya et al. (1996). A modified version of Montoya et al. (1996) equations (6) and (7) were applied to calculate ^{15}N uptake by mesozooplankton (identical to chapter 3, Eqn. 3.3 and 3.4).
Statistical analysis— Statistical analysis was done using SPSS (SPSS Inc). Student's t-test (Tukey method of multiple comparisons) was conducted to determine whether the results obtained from individual cruises were significantly different, while the significance of correlation between individual parameter was tested using Pearson's correlation.

4.3 Results

Evidence from a Mauritanian upwelling study 2007 during the POS 348.— The concentration of NO_3^- in the water column rose at station 244 from an average concentration of 13.7 ± 1.0 μmol l^{-1} in the surface water to maximal 22.7 ± 2.0 μmol l^{-1} below the nitracline (between 50 and 100 m; Fig. 4.3), a clear sign of high upwelling intensity. NO_3^- concentrations at stations 245 and 246 were below (31 ± 4 μmol l^{-1} and 29 ± 4 μmol l^{-1} and above the nitracline (4.2 ± 0.1 μmol l^{-1} 6.2 μmol l^{-1} were not statistically different from each other, but significantly lower in the upper water column ($<$ 100 m) compared to 244 (p= 0.001).
Depth profiles of $\delta^{15}N$ of NO_3^- showed enrichment between 300 and 800 m (Fig. 4.3). Furthermore there is a decrease in the $\delta^{15}N$- NO_3^- from 50 to 300 m. At the surface ($<$ 50 m) these values rose again to $\sim$ 6. Altogether, depth profile values of $\delta^{15}N$-NO_3^- gained for station 245 were significantly lighter compared station 246 (p= 0.05). At station 244 the $\delta^{15}N$-NO_3^- above the nitracline was significantly lower

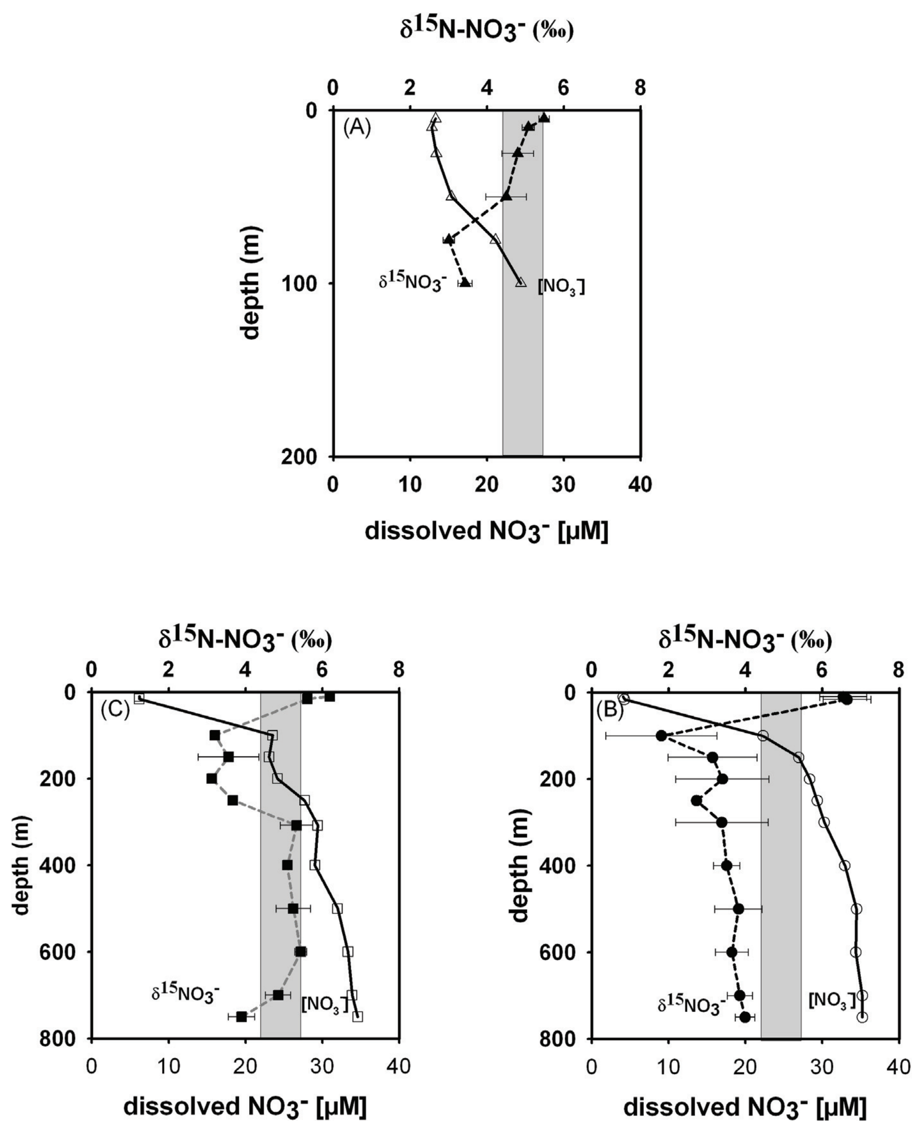

Figure 4.3: Depth profile of NO_3^- concentrations and δ^{15}N- NO_3^- values for the Eastern tropical North Atlantic stations 244 (solid triangles), 245 (circles) and 246 (gray squares) of the POS 348 cruise. Symbols represent mean value and standard deviation of replicate analyses on individual δ^{15}N- NO_3^- samples (except stat. 246: 400, 16 and 10 m). Grey box in δ^{15}N- NO_3^- plot represents the range of literature values of δ^{15}N deep NO_3^- (Knapp et al. 2005, Liu & Kaplan 1989, Sigman et al. 1997).

(5.0 ± 0.4 ‰) than at station 245 (6.6 ± 0.1 ‰) and 246 (5.9 ± 0.4 ‰) (p= 0.005). Moreover, the δ^{15}N-NO_3^- values were always significantly lower below the nitracline than above (p= 0.001).
Evaluating the horizontal distribution of phytoplankton pigment marker (Tab. 4.1) there is a shift visible from fucoxanthin (diatom specific) to zeaxanthin (cyanobacteria specific) from the more coastal station (244) to the more offshore stations (245 and 246).

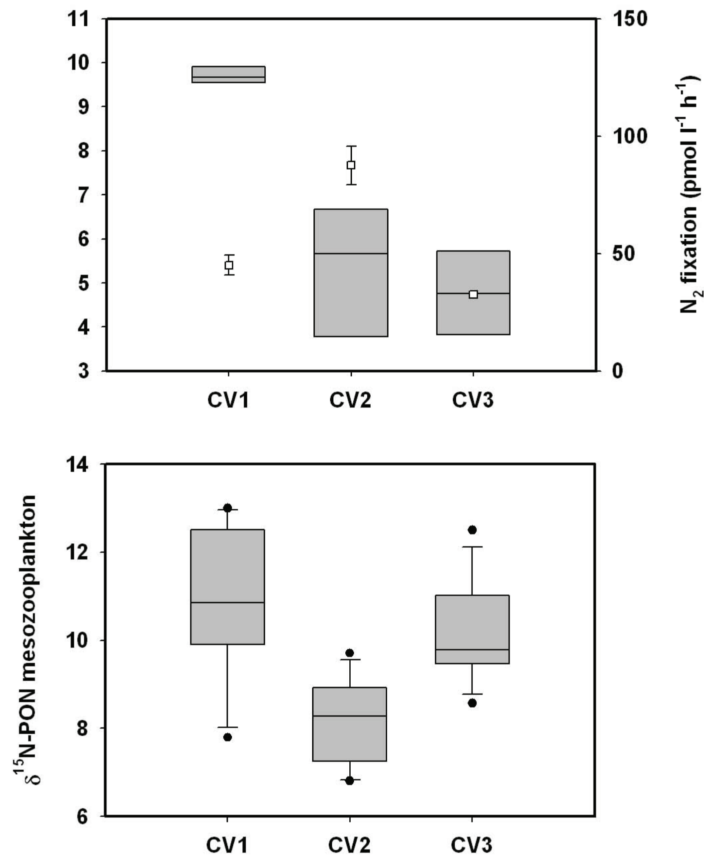

Figure 4.4: N_2 fixation rates from the three RV/Islandia cruises (white squares) and natural abundance of δ^{15}N-PON of phytoplankton (grey box plot) and mesozooplankton grey (box plot).

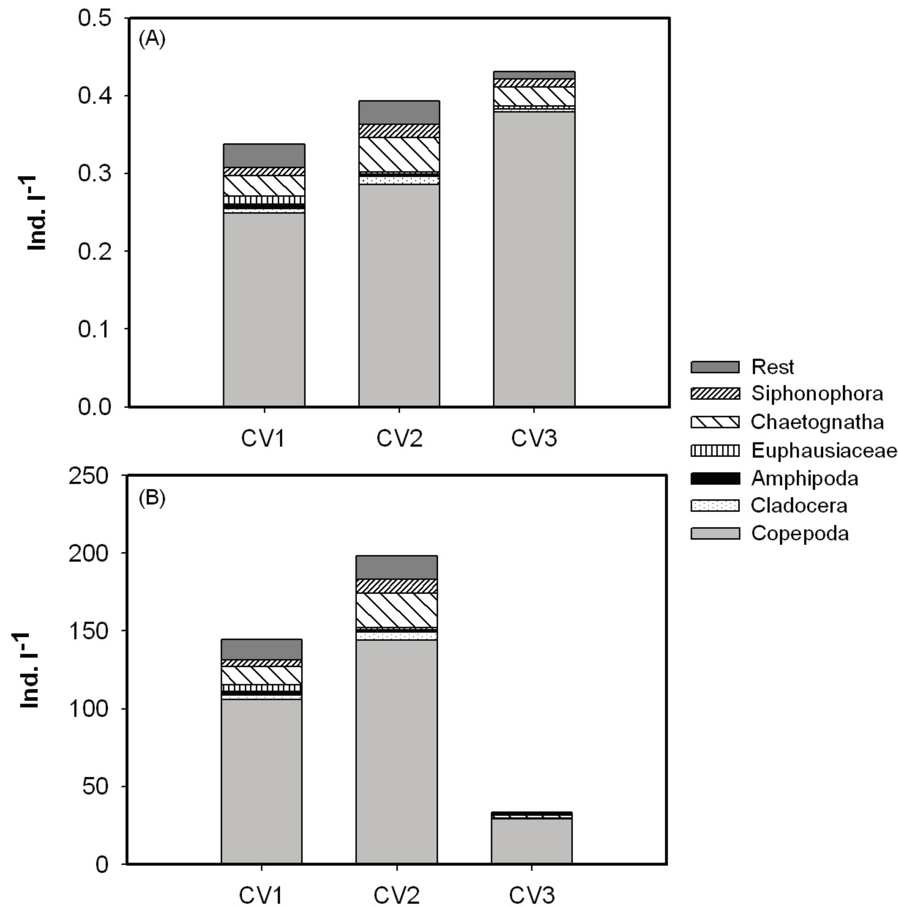

Figure 4.5: Abundance of zooplankton species in the field for 0- 30 m of water column (A) and abundance within the treatment bottles during the ^{15}N uptake experiments (B) during the RV/Islandia cruises in summer 2008. Note the different scales of y-axis in A and B.

Isotopic fractionation factor ϵ.

The isotopic fractionation factor ϵ of $NO_3{}^-$ for the three stations 244, 245 and 246 and two distinct depth intervals (0-100m and 100 to 700/750m) is given in Table 4.1. The lowest value for ϵ was found at station 246 in the watercolumn above 100 m, while the highest is found at the same station at the depth interval below 100 m. For station 245 ϵ did not statistically differ between the intervals. For station 244 there are only measurements in the interval of 0-100 m, which are in the same range as in the other stations.

Table 4.2: Isotopic fractionation factor ϵ for regression of δ^{15}N-NO_3^- and NO_3^- concentration for the three stations sampled during the POS 348 July 2008 cruise (244, 245, 246). Standard deviation is given in parentheses

Station	Depth (m)	ϵ (‰)	R^2	n	p
244	0-100	3.3 (0.6)	0.925	6	0.008
245	0-100	2.9 (0.1)	0.999	3	0.02
245	101-750	3.1 (0.8)	0.812	8	0.008
246	0-100	2.0 (0.4)	0.982	3	0.1
246	101-700	5.5 (1.4)	0.842	8	0.008

Evidence from the Cape Verde study 2008.—
Physical and chemical hydrography.
The temperature of the mixed layer during the Cape Verde cruises varied between 23 to 25 °C with a thermocline located between 40 and 50 m. The salinity of the mixed layer was on average 35. The average wind speed during the CV1, CV2 and CV3 cruises were 6.5±1.4 m s^{-1}, 4.0 ± 0.9 m s^{-1} and 5.2 ± 2.1 m s^{-1}, respectively.
Abundance of *Trichodesmium* sp. and N_2 fixation
The diazotrophic community was dominated by picocyanobacteria, while filamentous cyanobacteria, represented by *Trichodesmium*, were less abundant (= 100 trichomes l^{-1}, personnel comment Julie LaRoche). The total chlorophyll *a* values for the three RV/Islandia cruise were on average 0.075 μg l^{-1} (Tab. 4.1). The natural abundance of ^{15}N-PON of the phytoplankton fraction was significantly higher during CV1 cruise (9.6 ± 0.1 ‰) compared to CV2 (5.4 ± 1.5 ‰, p= 0.001) and CV3 cruise (4.8 ± 0.9 ‰, p= 0.05) (Fig. 4.4). Daily $^{15}N_2$ fixation rates were significantly higher during CV 2 cruise (2.1±0.2 nmol N l^{-1} d^{-1}) in comparison to CV1 (1.1±0.1 nmol N l^{-1} d^{-1}, p= 0.05) and CV3 (0.8 nmol N l^{-1} d^{-1}, p= 0.001, Fig. 4.4).
Zooplankton composition.
The total *in situ* abundance of mesozooplankton in the first 20 m of the water column during the individual cruises did not differ significantly (Fig. 4.5). The majority of mesozooplankton species identified belonged to the class of Copepoda with the major contributors of the genus *Eucheata*, *Paraeucheata* and *Nanocalanus*. Moreover, Cladocera, Amphipoda, Euphyausiaceae, Chaetognatha and Siphonophora were found. Species that were not sufficiently identified were pooled in the group

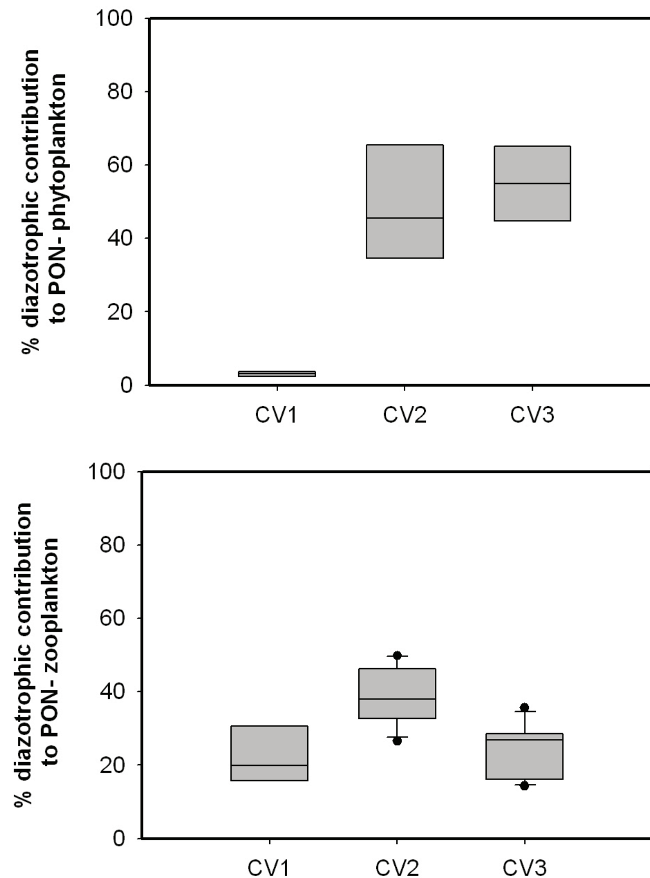

Figure 4.6: Percentage contribution of diazotrophic N to PON during the three RV/Islandia cruises in summer 2008.

"Rest". Zooplankton abundance and composition in the actual experimental treatment of the individual cruise is shown in Figure 4.5. Species composition in the treatments did not deviate from *in situ* conditions, but total abundance was enriched by a factor of 60 to 433. Moreover, abundance was significantly lower in the treatments of cruise CV3 compared to CV1 and CV2 ($p= 0.001$). The ^{15}N-PON of the mesozooplankton >200 μm was significantly lower during the CV2 (8.2 ± 1.9 ‰) cruise compared to CV1 (10.9 ± 1.6 ‰, $p= 0.001$) and CV3 (10.2 ± 1.1 ‰, $p= 0.001$, Fig. 4.5).

Two source mixing model.

The first order estimates of diazotroph contribution to PON of phytoplankton, based

on mass balance of natural abundance of ^{15}N-PON was significantly higher during the CV2 and CV3 cruise (48.5 $\pm$ 15.7% and 54.9 $\pm$ 14.4%), compared to the CV1 cruise (3.1 $\pm$ 0.6%, p= 0.001), but differed not significantly between CV2 and CV3 (Fig. 4.6). Diazotrophic contribution to mesozooplankton biomass during the CV1 crusise was 16.8 $\pm$ 12.8%. The contribution during the CV2 (38.4 $\pm$ 15.2) cruise was higher than during CV3 cruise (21.6 $\pm$ 8.8), nevertheless this tendency was not statistically significant.

Gross ^{15}N incorporation.

The accumulation of ^{15}N in the mesozooplankton fraction of the tracer addition experiments are shown in Figure 4.7, revealing that the accumulation rose to a maximum after 3 hours of incubation and decreased again after 12 hours. The hourly gross ^{15}N incorporation rates of mesozooplankton (set-up I) are presented in Table 4.3. Individual gross incorporation rates gained during all cruises ranged from 1.6 to 20.6 pmol ind.$^{-1}$ h^{-1}. Average incorporation rates were not statistically different during the individual cruises.

To distinguish between direct grazing on filamentous cyanobacteria and transfer of labelled nitrogenous compounds via the microbial loop, we applied an experimental set-up, allowing only active grazing on pre-labelled diazotrophs. The average value for ^{15}N hourly uptake by direct grazing was 3.2 $\pm$ 2.8 pmol ind^{-1} h^{-1}. Direct grazing contributed on average 56 $\pm$ 33% to the gross uptake of ^{15}N by mesozooplankton species. The mediation of ^{15}N uptake of the zooplankton community by microbial loop contributors accounted for 46 $\pm$ 33% and considering the standard deviation not different from the proportion of direct grazing.

4.4 Discussion

Impact of N_2 fixation on natural abundance of δ^{15}N-NO_3^- during the Mauritanian study

Cyanobacteria make use of N_2 fixation an alternative N source in habitats exhibiting low input of NO_3^-. In reverse conclusion, if N_2 fixation is of importance one should be able to detect changes in the δ^{15}N values of NO_3^-, due to the fact that isotopically light N (with δ^{15}N of -2.1 $\pm$ 1‰) is introduces to the ocean (e.g. Minagawa & Wada 1986, see also Introduction). On the other hand, ^{15}N values for deep water

NO_3^- are in the range of 4-5 ‰(Knapp et al. 2005, Liu & Kaplan 1989, Sigman et al. 1997). Values in between this range may originate from mixing of N_2 fixation and NO_3^- assimilation. There is a consistent pattern in the isotopic composition of NO_3^- at all stations sampled during this study, revealing three zones with distinct δ^{15}N- NO_3^- values. Firstly, in surface water above 50 m the δ^{15}N- NO_3^- is on average 5.6 ± 0.8 ‰, while NO3 concentrations are low. Secondly, in subsurface waters (50 to 100m) the δ^{15}N- NO_3^- is significantly lower (2.96 ± 0.7 ‰). Thirdly, there is an increase in the δ^{15}N- NO_3^- from 100 to the maximal observed depth of 750 m (4.0 ± 0.8 ‰). These isotopic shifts can be explained by different predominant metabolic processes. Before I want to discuss the possible influencing factors and the contribution of diazotrophy on the δ^{15}N- NO_3^- it is necessary to make two asumptions: Unlike NO_3^- consumption, the production of NO_3^- should have little effect on the N stable isotope composition in oxic waters (e.g. Sigman et al. 2005). The δ^{15}N of NO_3^- produced is primarily controlled by the δ^{15}N of the organic matter that is remineralized (Sigman et al. 2005). Thus, the fractionation factor ϵ of NO_3^- production mirrors the dominant source of N acquisition of organisms in the water column, i.e. production based on deep water NO_3^- or diazotrophy and N_2 (compare subchapter 1.5 and Fig. 1.8).

The isotopic fractionation factor ϵ of NO_3^- assimilation is usually in the range of 4-6 ‰(e.g. Altabet et al. 2001). In this study the ϵ was lower than this literature value ($p = 0.001$). The higher fractionation factor below the thermocline might as well result from reminerlisation or lateral input of other water masses. In addition to this, ϵ at the investigated stations is statistically lower ($p = 0.05$) above the thermocline (>100m) than below (Tab. 4.2).

The overall prevailing lowest δ^{15}N- NO_3^- from 50 to 100 m might result from substantial N_2 fixation activity and introduction of isotopically light N compounds, which are processed by other components of the food web and reintroduced after remineralisation as NO_3^-. The contribution of N_2 fixation to the isotopic composition of NO_3^- can be calculated assuming a mixture of two sources, upwelling NO_3^- with δ^{15}N of 4.7 ‰ and regenerated from diazotrophs with δ^{15}N of 0.56 ‰. In this water mass the contribution of N_2 fixation to the dissolved NO_3^- pool would account for 47 ± 20%. This value is quite close to that published by Lui et al. (1996) for the water mass below the euphotic zone (40 ± 15%). Although N_2 fixa-

tion is of importance in surface waters as well, the higher δ^{15}N- NO_3^- might result from the discrimination of bulk phytoplankton against ^{15}N- NO_3^- in favour of ^{14}N-NO_3^- (Altabet & McCarthy 1985) during uptake and assimilation. On the other hand studies have shown a significant decrease of δ^{15}N- NO_3^- in the euphotic zone (<80 m) attributed to N_2 fixation and thus introduction of isotopically light N compounds (Montoya et al. 2002, Brandes et al. 1998). This effect was not visible in our data, presumably because N_2 fixation was not as pronounced as in other studies, regardless that actual N_2 fixation measurements for the Mauritanian upwelling campaign are lacking. The contribution of N_2 fixation to the δ^{15}N- NO_3^- in the euphotic zone 50 to 100 m adds up to 40 ± 10%. In deep water masses from 100 to 750 m depth N_2 fixation contributes with 16 ± 20% to the δ^{15}N- NO_3^-. The large standard deviation results from differences between station 245 and 246. Considering these stations separately, the contribution on station 244 would be 33 ± 15%. This is consistent with findings from Montoya & Voss (2006), who postulated that a significant proportion of diazotrophic derived N in the Arabian Sea is remineralized in depths greater than 300 m, reflecting either preferential routes of transport or resistance to microbial breakdown. For station 246 it would result in a contribution of 9 ± 22% fixed N. This deviation between the stations 245 and 246 might reflect a shift in phytoplankton community assemblage (the proportion of species specific pigment marker, Tab. 4.1), from diatom to cyanobacteria from the more productive coastal upwelling station 245 to the oligotrophic open ocean station 246 (0.73, p= 0.05). Nonetheless, there is no information about the proportion of diazotroph-diatom associations (DDAs) to the bulk diatom biomass, which can add a substantial fraction to the community N_2 fixation (Carpenter et al. 1999, Karl et al 2002, Capone 2005, Foster et al. 2007), wherever enough silicate is imported into the water column (e.g. by riverine inflow, upwelling of deep water masses).

Impact on natural abundance of δ^{15}N- PON during the Cap Verde study

Just as δ^{15}N-PON phytoplankton is influenced by variations in the isotopic composition of DIN in surface waters (Karl et al. 1997, Waser et al. 2000, Mino et al. 2002, Montoya et al. 2002, Capone et al. 2005), the isotopic composition of mezozooplankton should likewise be affected by the δ^{15}N-variability of their N source. We tested the influence of N_2 fixation on δ^{15}N-PON in phyto- and mesozooplankton by correlating the data obtained in this study. N_2 fixation is negatively correlated with

both δ^{15}N-PON of phytoplankton and zooplankton (-0.623, p= 0.01), indicating a significant influence of diazotrophy on suspended particulate organic matter in the surveyed area. Altabet (1988) and Mahaffey et al. (2003) have earlier drawn this conclusion based on variations of bulk δ^{15}N-PON, while McClelland et al. (2003) analysed the δ^{15}N of amino acids of zooplankton and their food source. Moreover, it has been shown that variations in the sources of N supporting production generate gradients in δ^{15}N of surface PON both with latitude (Mino et al. 2002) and longitude (Waser et al. 2000, Montoya et al. 2002) in mesotrophic and oligotrophic regions of the North Atlantic Ocean. The isotopically light δ^{15}N value for summer PN export was attributed to relatively higher N inputs via N_2 fixation during this season, particularly due to blooms of *Trichodesmium* spp. (Karl et al. 1997) in the Pacific Ocean. In general, there is a strong dominance of N_2 fixation by *Trichodesmium* in the western part of the tropical North Atlantic to a rising contribution of picocyanobacteria in the eastern part of the basin (Montoya et al. 2004, Langlois et al. 2008). This trend is also reflected by the cyanobacterial composition during the Cap Verde studies, with very low abundances of *Trichodesmium* spp as shown by Tyrell et al. (2003) for the same region (1-10 filaments per 50 ml). In turn, the pattern of diazotrophic assemblage has implications for the predominant path of channelling diazotroph N into higher trophic level (see sub-section below). To give further evidence to the impact of diazotrophy we used a mass balance calculation with a two source mixing model of natural abundance ^{15}N values for cyanobacteria and mesozooplankton. The results reveal that in our study the contribution of N_2 fixation to the mesozooplankton biomass production was highest at station 245 with an average percent of 38.6 ± 7.8%. This diazotrophic contribution to zooplankton biomass was positively correlated with the N_2 fixation rate measured (0.668, p= 0.01). This is a conservative estimate of the role of diazotrophs, ranging in the same order as Montoya et al. (2002) with 13 to 40% and Capone et al. (2005) who found 36% contribution to the bulk PN in the tropical Atlantic Ocean.

Instantaneous transfer of diazotrophic N during the Cap Verde study (pulse chase experiments).

N_2 fixation, especially by filamentous cyanobacteria is strongly influenced by mixing due to increased wind speed, turbulent shear on the cells (Paerl 1985, Sellner 1997, Moisander & Pearl 2000) and mixing of cells into deeper water layers where

Table 4.3: Chlorophyll *a*, N_2 fixation and incorporation of diazotrophic fixed ^{15}N by mesozooplankton species of RV/Islandia cruises in July 2008. ^{15}N uptake rate are given for set-up I (whole planktonic community = gross uptake) and set-up II (direct grazing). Standard deviations from replicates are in parentheses.zpl: zooplankton.

Cruise	Date	Lat/ Long	Chl *a*	$^{15}N_2$ fixation	Set-up I	Set-up II
					^{15}N-incorporation zpl	^{15}N-incorporation zpl
			(μg l^{-1})	(pmol l^{-1} h^{-1})	(pmol ind.$^{-1}$ h^{-1})	(pmol ind.$^{-1}$ h^{-1})
CV1	16/07/08	55.26/17.65	0.08	44(3)	9.4(7.8)	-
CV2	20/07/08	55.29/17.74	0.06	88(5)	4.2(0.6)	5.22
CV3	24/07/08	55.29/17.81	0.06	32	11.4(9.2)	1.28

light becomes limited (Levine & Lewis 1987, Howarth et al. 1993). Wind speed was lowest during CV2 cruise which might have supported N_2 fixation activity, resulting in significantly higher N_2 fixation rate compared to the cruises CV1 and 3 (p=0.05 and 0.001, respectively). We hypothesize that N_2 fixation resulted equally from *Trichodesmium* and unicellular picocyanobacteria, as abundance of *Trichodesmium* can be very low, also shown by e.g. Tyrell et al. (2003). Our N_2 fixation rates are at the lower end of rates published for this region and time of the year (e.g. Voss et al. 2004, Capone et al. 2005). Especially subsequent to dust events N_2 fixation can increase, as suggested by bioassay experiments from Mills et al. (2004) and the occurrence of blooms of *Trichodesmium* in areas of higher aeolian dust import (e.g. Bermann-Frank et al. 2001, Voss et al. 2004). Diazotrophic derived N was detectable in the mesozooplankton after 1 hour of investigation time (Fig. 4.7) and rate calculation revealed that 100% of recently fixed $N_2$was incorporated by the whole zooplankton community in the treatment bottles. This is consistent to findings of O'Neil et al. (1996).

Applying the incorporation rates obtained in this study (Tab. 4.3) and using the natural abundance of zooplankton and *in situ* N_2 fixation rate, an idealized daily

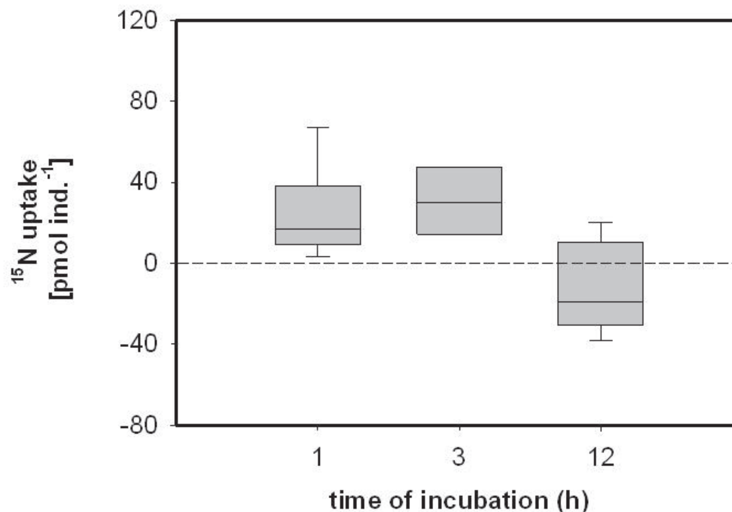

Figure 4.7: Incorporation of ^{15}N tracer by mesozooplankton (>200μm) with increasing incubation time.

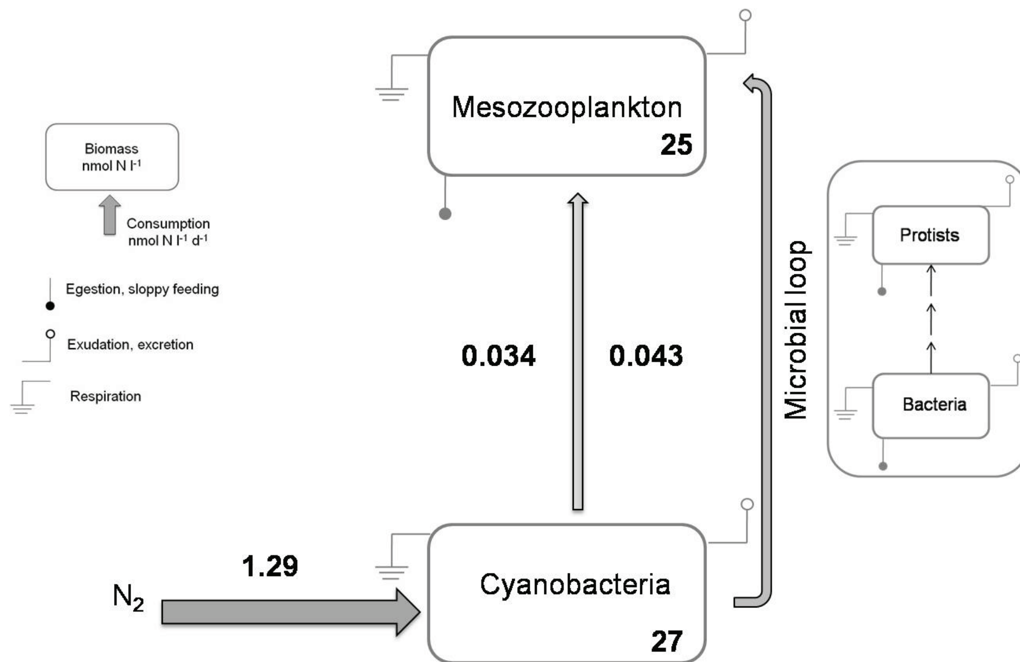

Figure 4.8: Idealized N budget using *in situ* abundance of mesozooplankton and the determined average N incorporation rates from Table 4.3. The numbers in the box are the mean standing stock (nmol N l^{-1}). Numbers next to the arrow are fluxes of N (nmol N $l^{-1}d^{-1}$). Thicknesses of arrows are roughly proportional to the N flux. Egestion, sloppy feeding, exudation, excretion and respiration were not quantified and are only used to illustrate loss terms for the single trophic positions.

N budged can be calculated, which is represented in Figure 4.8. Standing stocks of cyanobacteria are 27 nmol N l^{-1} and of mesozooplankton 25 nmol N l^{-1}. The average daily N_2 fixation rate is 1.29 nmol N l^{-1} d^{-1}. Mesozooplankton incorporated on average 6% of this daily N_2 fixation. This equals to 0.3% of mesozooplankton body N that is incorporated on a daily basis. If we assume a C:N ratio of 7:1 for prey ingested and 6:1 for zooplankton biomass, we can convert N incorporation to C incorporation. On the whole, 0.539 nmol C l^{-1} d^{-1} would be ingested accounting for 0.36% of mesozooplankton body C deriving from cyanobacteria. Calbet (2001) published data indicating that in unproductive marine ecosystems 5.90 $\pm$ 0.01% of mesozooplankton body C is ingested based on the whole phytoplankton community. Compared to this, the value gained in this study is lower by one order of magnitude (factor 16). This indicates that other phytoplankton species than cyanobacteria make up the majority of food ingested by zooplankton. It has to be

kept in mind, that C ingestion rates were not directly measured and derived from conservative estimates using an average of C:N of cyanobacteria. C ingestion rates might be higher than expected, when food species temporarily gain C:N ratios up to 28, due to carbon ballasting (Ohlendieck et al. 2007). The incorporation of diazotropic N by zooplankton resulted from both direct grazing (56%) on the dominant picocyanobacteria and via channelling trough the microbial loop (44%). In this study, *Trichodesmium* exhibited only minor abundance, therefore grazing activity might have resulted from incorporating small cells diazotrophs, leading to a higher contribution of grazing to the gross incorporation rate as results from the Baltic Sea revealed (33%), where filamentous cyanobacteria dominated (compare Chapter 4). In general, grazing on filamentous cyanobacteria like *Trichodesmium* in the Atlantic Ocean is confined to a specialized zooplankter like *Macrosetella* (Roman 1978, Hawser et al. 1991, O'Neil et al. 1996). Our findings from this study differ from suggestions made in earlier publications were the microbial loop is expected to be the dominant pathway of introducing diazotrophic N to higher trophic level (e.g. Sommer et al. 2006).
However, it is well known that cyanobacteria release a substantial fraction of fixed N as DON (up to 50%, e.g. Chapter 3 in this study, Bronk et al. 2007). This DON is rapidly recycled (e.g. Chapter 2 in this study, Knapp et al 2005) and it is not questionable that heterotrophic bacteria play an important role in the redistribution and trafficking of this new ^{15}N-deplete N into the food web (e.g. Meador 2007).

Conclusion and outlook

The results presented reveal that biological N_2 fixation appears to be a plausible mechanism for introducing significant quantities of ^{15}N-depleted compounds into the Northern Tropical Atlantic Ocean which in turn is available for further biological uptake. Moreover, tracer addition experiments add evidence to the importance of the instantaneous transfer of diazotrophic derived N to other trophic levels in the food web.
N_2 fixation in this study was compatible with both the pattern and the magnitude of the isotopic depletion of dissolved NO_3^-, as well as PON in zooplankton and phytoplankton.
Conclusions drawn in this chapter are based on two different approaches. Firstly, on the investigating of long term influence of diazotrophy on natural abundance of

δ^{15}N- NO_3^- and PON. Secondly, on snapshot experiments which determined the instantaneous transfer of diazotrophic N to higher trophic level. Both approaches highlighted the importance of diazotrophic N input to the marine environment of the North-Eastern Tropical Atlantic Ocean. To complete the picture several missing variables should be analysed. There is missing data from the Cape Verde study on the δ^{15}N- NO_3^-, as well as a complete identification of the diazotrophic community and abundance of individual groups. Additionally, there is no data available on actual N_2 fixation rate measurements during the Mauritanian campaign, but future cruises into this region should produce data addressing this question.

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Chapter 5

Synthesis and future outlook

Diazotrophy represents a large incoming flux of N to the global Ocean, potentially fuelling production of other trophic level. Yet, present day estimates of N_2 fixation rates and subsequently release of N vary widely within and between regions (e.g. Galloway et al. 2004). Moreover the importance of diazotrophic N, especially for higher trophic level is only beginning to emerge in recent publications (e.g. Gorokhova 2009). The discrepancy of published data associated with this input and its significance in part reflects our incomplete knowledge of the factors regulating diazotrophic N_2 fixation activity and subsequently release and insufficient datasets using natural compositions of phytoplankton instead of monoculture cyanobacteria. The chapters of this thesis touch upon specific aspects of the physiological and physical controls of the N_2 fixation and release of N compounds in marine habitats. Moreover, the studies using natural communities gave evidence to the quantity and quality N transfer within the food web (chapter 3 and 4). Interessting conclusion that can be drawn from results of this thesis will be presented in the following subchapter (5.1 and 5.2).

5.1 Nitrogen uptake and release on the cellular level

N_2 and C fixation, as well as the release of N compounds were investigated for two different diazotrophic species, the heterocystic *Nodularia* and the non-heterocystic *Trichodesmium*. Firstly, the results from chapter 2 revealed a tight regulation of

N_2 and C fixation and the release of N and C compounds over the diel cycle in both investigated species. The underlying regulation was based upon a circadian clock that regulates fixation activity and synchronises the two opposing processes N_2 fixation and the oxygen evolving photosynthesis. The release of compounds, with DON being the major part, was more likely regulated by the amount of previously assimilated N. Additionally, it turned out that integrated over a diel cycle a major fraction of fixed N is directly released (average from both species investigated 80%). It has to be remembered that this value derived from optimal growing monospecies laboratory cultures and that under natural conditions it might alter to an unknown extent. It was not possible to fully clarify the chemical composition of the DON fraction released, as dissolved free amino acids (DFAA) made up maximal 8%. Further studies should address this issue with regard to possible contributors like urea and combined amino acids.

Secondly, in chapter 2 one possible abiotic regulating mechanism that drives N release by cyanobacteria was identified. Changes in light intensity strongly enhanced the exudation of N in both investigated species. We attribute this to a short-term excess supply of electron energy that is channelled out of the cell partly by using electrons to fix N_2 and subsequently release this excess N. Additional research should investigate other possible regulating abiotic factors such as the influence of shear and temperature. Overall, chapter 2 points out that when investigating the release of N one has to carefully consider the physiological status of the cell.

Exudation of N might create microenvironments within the water column enriched in N compounds. A simple calculation should clarify this. If we take published areal daily N_2 fixation rate into consideration and apply the exudation rates from this thesis (80%) we get an upper maximal estimate how much new N derived from diazotrophic production potentially can be exudated and is subsequently available for the food web. For the Baltic Sea Wasmund et al. (2005) published an areal daily of 1841 μmol N $m^{-2}d^{-1}$, while for the Northern Tropical Atlantic Ocean Capone (2005) presented 1893 μmol N $m^{-2}d^{-1}$. Set into relation we end up with a maximum of 1472 μmol N $m^{-2}d^{-1}$ new N entering the Baltic Sea and 1514 μmol N $m^{-2}d^{-1}$ is theoretically released in the Northern Tropical Atlantic Ocea. This N, which is directly released is readily available for e.g. bacterial uptake. Overall, his "new" N is important for local food webs of the Baltic Sea and Atlantic Ocean and can be

tranferred to higher trophic level. Chapter 3 and 4 dealt with this question. The results from both chapters are comparatively discussed in the following subchapter (5.2)

5.2 Transfer of diazotrophic N within the food web

Two marine environments were comparatively sampled in the course of this stuy. In the brackish, temperate Baltic Sea, as well the high saline Eastern Tropical Atlanitc

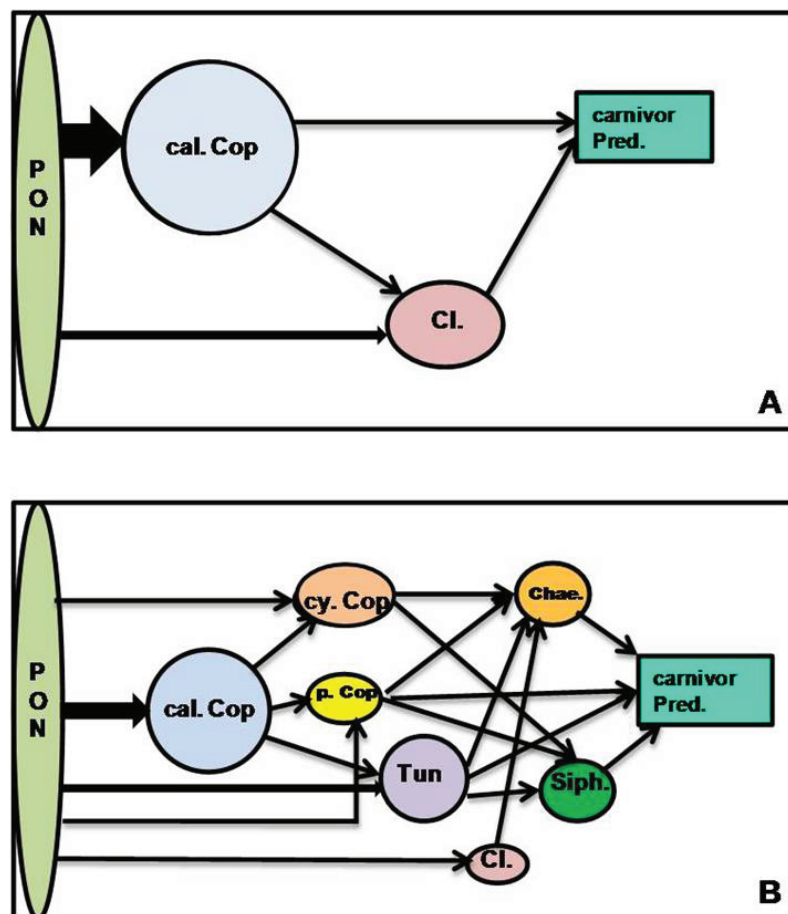

Figure 5.1: Illustration of the mesozooplankton community structure from Chapter 4 (mesotrophic, Baltic Sea) and 5 (oligotrophic, Northern Atlantic Ocean). Abreviations are: PON (particulate organic nitrogen), cal. Cop (calanoid copepod), Cl. (Cladocera), carnivore Pred. (carnivore predator), cy.Cop (cyclopoid copepod), p.Cop (poicilost copepod), Tun (Tunicata), Siph. (Siphonophora), Chae. (Cheatognatha).

Ocean, identical experiments were carried out, which investigated the transfer of diazotrophic N within the food web towards higher trophic level. Both habitats differ in physical and biogeochemical forcing factors (see subchapter 1.4) and predominant plankton species composition. The dominant diazotrophic species during the investigation time in the Baltic Sea were *Nodularia* and *Aphanizomenon*. Both are heterocystic, being protected from increased influx of O_2 inlfux by a glycolipid envelop (Staal et al. 2003), which is toxic to the N_2 fixing enzyme nitrogenase. Because N_2 fixation in the Baltic Sea is not limited by Fe and Mo and P limitation can be overcome by the exploitation of dissolved organic phosphorous (DOP), as it has been shown in the study of Vahtera et al. (2007b). Fixation rates usually are higher in the Baltic Sea, compared to rates measured in the oligotrophic Atlantic Ocean. This was also the case in this study.

N_2 fixation in the Atlantic Ocean is mainly limited by P and Fe acquisition. Gener-

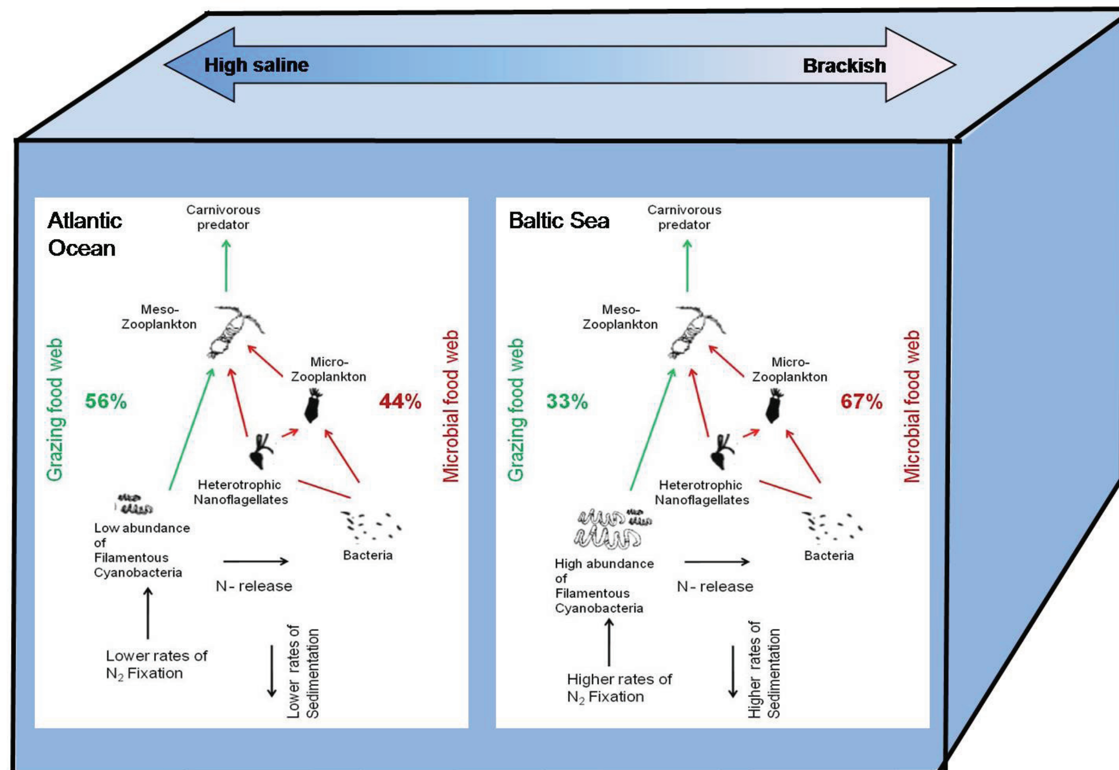

Figure 5.2: Transfer of diazotrophic N in the Atlantic Ocean and the Baltic Sea.

ally, *Trichodesmium* gains high abundance in the Tropical Atlantic Ocean, especially in the western part and successively following Saharan dust storm events, where it exploits temporal pulses of nutrient input (Lenes et al. 2001). Other diazotrophic species found are unicellular and diatom- associated diazotophs (DDAs). In these regions fixation rates can be in the same range as rates gained in the Baltic Sea. Nevertheless, during low dust seasons and in the more eastern part of the ocean *Trichodesmium* is found at lower abundance (Tyrell et al. 2003). Here unicellular diazotrophs are more abundant. Because of their more favourable surface-to-area ratio and higher growth rate compared to the filamentous *Trichodesmium* they are more competetive in low nutrient regions. As seen in chapter 4, unicellular cyanobacteria were dominant in the low dust season near the Cape Verde islands. Rates of N_2 fixation were by the order of 10 lower than in the Baltic Sea.

Not only the diazotrophic composition differed between the investigation areas, but also the zooplankton composition. Figure 5.1 is a simplified illustration of the mesozooplankton community present at the time of investigation in the two habitats. In the Baltic Sea calanoid copepods dominated the mesozooplankton community, with a few cladoceran species being present (Fig. 5.1 A). There are fewer trophic level, compared to the Atlantic Ocean. Thus, losses of energy, that occur at each trophic level due to respiration and excretion, are smaller (transfer efficency, TE, of energy is higher). Energy in this system is often lost to sedimentation. In the Atlantic Ocean there are several mesozooplankton species and interrelationships (Fig. 5.1 B). There are more trophic levels than in the Baltic Sea. Siphonophora are usually preying on other mesozooplankton species. The transfer efficiency in this system is lower. This food web is based on the regeneration of nutrients within the water column and upwelling of nutrients from below the thermocline occurs rarely (apart from coastal upwelling regions, like the coast of Mauritania, where constant winds drive the input of deep water nutrients). Because primary producers in this habitat are small, herbivorous zooplankton species are predominantly fine filterer (Tunicata, Copepoda), or carnivorous. Energy in this system is predominantly conserved in biomass.
Overall, the structure of the food web influences the transfer of diazotrophic N within the food web. Still, both studies proved that N_2 fixing cyanobacteria are

ecologically more important as instantaneous sources of N for higher trophic level of the food web than previously assumed.
The above mentioned differences in the structure of the food webs between the two investigated ecosystems strongly influenced the pathway of N flow through the food web. Because filamentous cyanobacteria are grazed upon only by a limited number of zooplankton species, the prevailing transfer of diazotrophic N in the Baltic Sea was mediated by microbial loop constituents (67%, Fig. 5.2). Nevertheless, 33% of incorporated N resulted from direct grazing on filamentous cyanobacteria. This means that 0.5-4.5% of mesozooplankton body N derived from diazotrophic production (Fig. 3.5. Although filamentous cyanobacteria being regarded as poor food, they might add otherwise missing growth factors to the diet of zooplankter. Future studies should take emphasis on these unknown growth factors. For example, Loick-Wilde et al. (2007 and unpubl. data) investigate the transfer of essential amino acids (Guillaume 1997) from cyanobacteria to mesozooplankton of the Baltic Sea using a compound specific isotope analysis approach. Their results show, that zooplankton gain especially essential amino acids from filamentous cyanobacteria, when they are offered as food in a mixture together with other phytoplankton species (diatoms, Chlorophyta).
In the Tropical Atlantic Ocean the majority of N transfer seemed to result from direct grazing upon unicellular cyanobacteria, as indicated from results in chapter 4 (56%, Fig. 5.2), while microbial loop mediation accounted for 44% of gross transfer to mesozooplankton. Although unicellular diazotrophs are more easily ingestible a smaller amount of diazotrophic N fuelled mesozooplankton body N on a daily basis (0.3% of mesozooplankton body N derived from diazotroph production, Fig. 4.8) compared to the Baltic Sea study.

This thesis highlighted the important role of N_2 fixing cyanobacteria in marine environments, because of their potential to exudate large quantities of N compounds and their contribution to the nutrition of mesozooplankton species. How the importance might chance in the future ocean is discussed in the following subchapter 5.3.

5.3 N_2 fixation in the future ocean

Recent research has strengthened the impending climate change and its consequences for the world oceans. Ocean surface waters are warming and become increasingly acidic (e.g. WBGU, Berlin 2006). This will have a severe impact on the marine ecosystem at all levels.
To date there are no sufficient data sets available on the performance of Baltic Sea species. But studies using *Trichodesmium* have shown that elevated concentration of dissolved inorganic carbon (DIC, i.e. CO_2) from 400 to 900 ppm stimulates growth and N_2 fixation (Levitan et al. 2006, Hutchins et al. 2007, Barcelos e Ramos et al. 2007). They predicted a doubling in N_2 fixation by the year 2100. This effect occurs, because the enzyme for C acquisition (ribulose-1.5-bisphosphate carboxylase oxygenase, RuBisCO) is an unspecific enzyme which usually invests energy to concentrate C (i.e. Carbon Concentration Mechanism CCM, Tortell 2000). At higher DIC concentrations in the water this CCM is down-regulated and energy can be allocated to other processes (Giordano et al. 2005). But it also turned out, that in *Trichodesmium* cellular C, N and P content is reduced, because cell size decreases and cell division rate accelerates (Barcelos e Ramos et al. 2007). Thus, cellular N:P ratio rises by up to 50%. The question remains to which extend excess N compounds are exudated? Moreover, what effect will the possible alteration of the nutritional content of cyanobacteria have, e.g. are they more likely to be ingested. Will the reduced cell size provoke a higher grazing pressure on filamentous cyanobacteria?
The importance of N_2 fixing cyanobacteria in the future ocean is still unknown, so is the future ocean itself, but both have to be investigated gradually.

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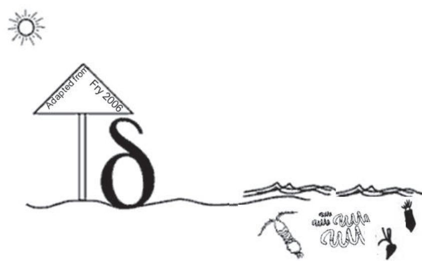
Danksagung



In completing one discovery we never fail to get an imperfect knowledge of others of which we could have no idea before, so that we cannot solve one doubt without creating several new ones.— Joseph Priestley: Experimente and Observationen on Different Kinds of Air (1786)

Diese Dissertation repräsentiert einen dreijährigen Forschungsweg, der sich mit der Biologie und Ökologie N_2 fixierender Cyanobakterien und der Ernährung kleiner, manchmal farbenfroh leuchtender, Copepoden beschäftigte. Diese Arbeit entwickelte sich nicht nur auf Grund meiner Anstrengungen, sondern auch durch die Anregungen meiner Doktor-Mutter Maren Voss. Maren inspirierte mich, als Wissenschaftlerin zu wachsen, lehrte mich, Sachen in Frage zu stellen und ermutigte mich immer, meinen Interessen zu folgen. Nicht zu vergessen, dass sie meine Freude am wissenschaftlichen Schreiben weckte. Zumindest schien es nach jeden weiteren hundert Manuskriptseiten leichter von der Hand zu gehen. Ich möchte mich ebenso dafür bedanken, die Möglichkeit zur Teilnahme an mehreren Ausfahrten gehabt zu haben. Danke an den Fahrtleiter des FS Poseidon 353, Ulli Lass. Danke auch an Julie LaRoche, die mich auf die holprigen Fahrten mit dem FS Islandia mitgenommen hat. Danke in diesem Zusammenhang auch an Wiebke und Maija für die wunderbare Zeit im heißen und staubigen Mindelo. Die Forschungsfahrten

haben den Ozean wortwörtlich begreifbar gemacht. Ich möchte mich auch bei meinen Ko-Autoren der Veröffentlichungen, die aus dieser Dissertation enstanden sind, bedanken: Maren Voss, Frederike Korth, Boris Koch. Ich habe es auch sehr genossen, in einer Arbeitsgruppe zu arbeiten, die eine so angenehme Atmosphäre verbreitet. Danke an die "AG Stabilen Isotope" (Maren, Iris, Rike, Natalie, Barbara, Claudia, Deniz, Julia), auch wenn wir jetzt "AG Mariner Stickstoffkreislauf" heißen und nicht "AG Pfütze". Besonders möchte ich mich bei Natalie Liock-Wilde und Barbara Deutsch bedanken, für die zahlreichen Diskussionen, Anregungen und die Hilfe bei den verzwickten Berechnungen und Formulierungen. Danke auch an Frederike Korth für das Aufmuntern und das solidarische betrübt sein. Und Entschuldigung für die Niesanfälle in unserem Büro und die daraus entstandene (eingebildete) Allergie gegen mich. Auf keinen Fall möchte ich vergessen, die Arbeit von Iris Liskow zu würdigen- unsere gute Seele im Labor. Ohne dich wären wir nichts. Auch für die technische Unterstützung von Anke Gerber und Birgit Sadkowiak möchte ich mich bedanken.
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Am Schluss möchte ich mich bei meiner Schwester Diana für die 21 Jahre bedanken, die wir zusammen hatten. Diese Arbeit ist für dich, auch wenn du sie nicht mehr lesen kannst.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe. Ich habe keine ausser die von mir angegebenen Hilfsmitteln und Quellen hierzu verwendet und die den benutzen Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht.

Rostock, den

Curriculum vitae

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List of chapters

This doctoral thesis includes the following publications/manuscripts:

1. Subchapter 1.5. is part of a book chapter "Internal cycling of nitrogen and nitrogen transformations" within a new edition of "Treatise on estuarine and coastal science" which is edited by Remi Laane and Jack Middelburg. The book chapter was written in cooperation with Maren Voss, Deborah Bronk, Barbara Deutsch, R. Purvaja, R. Ramesh Tim Rixen, and Rachel Sipler and will be published in 2010.

2. Factors influencing the release of fixed N_2 and C as dissolved compounds (TDN and DOC) by Trichodesmium erythreum and Nodularia spumigena. Wannicke N., Koch B and Voss M (2009) Aquatic Microbial Ecology (in press.)

3. Incorporation of diazotrophic fixed N_2 by mesozooplankton species - Case studies in the southern Baltic Sea using ^{15}N - stable isotope tracer addition. Wannicke N., Korth F. and Voss M. (2009) Marine Ecology Progress Series (under revision)

4. Impact of diazotrophy on N stable isotope signature of NO_3^- and PON and transfer of diazotrophic fixed N to mesozooplankton species – Case studies in North - Eastern Tropical Atlantic Ocean.

5. Synthesis and future outlook

Statement on my contribution to the publications/manuscripts

Publication 1
Subchapter 1.5 of the introduction reviews aspects of the N cycle within coastal and estuarial marine habitats. It is part of a book chapter "Internal cycling of nitrogen and nitrogen transformations" within a new edition of "Treatise on estuarine and coastal science" which is edited by Remi Laane and Jack Middelburg. The section I wrote independently based on a literature review summarizes the current knowledge of the role of the microbial loop. In specific, components are presented with abundances and activity parameter, their interplay and their significance for the food web.
Publication 2
Laboratory experiments were planned, conducted and analysed by me. Measurements of TN were done by Boris Koch and working group. The concept for this paper was drafted by me. It was written by me, with scientific advice and editing by Maren Voss and Boris Koch.
Manuscript 3
Laboratory experiments were planned, conducted and analyzed by me and Frederike Korth. The manuscript was written by me. Maren Voss and Nathalie Liock-Wilde edited the manuscript and provided scientific advice.
Manuscript 4
The experiments were planned and conducted by me. Herman Bange and cruise participants of the POS 348 cruise contributed to the data collection. Julie LaRoche and participants of the RV/Islandia cruises provided data on NifH gene copy number. The manuscript was written by me. Maren Voss and Nathalie Liock-Wilde edited the manuscript and provided scientific advice.