Distribution and activity of nitrogen-fixing bacteria in marine and estuarine waters

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Distribution and activity of nitrogen-fixing bacteria in marine and estuarine waters

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Abstract

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In aquatic environments the availability of nitrogen (N) generally limits primary production. N_2-fixing prokaryotes (diazotrophs) can convert N_2 gas into ammonium and provide significant input of N into the oceans. Cyanobacteria are thought to be the main N_2-fixers but diazotrophs also include a wide range of heterotrophic bacteria. However, their activity and regulation in the water column is largely unknown.

In this thesis the distribution, diversity, abundance, and activity of marine and estuarine heterotrophic diazotrophs was investigated. With molecular methods targeting the *nifH* gene, encoding the nitrogenase enzyme for N_2 fixation, it was shown that diverse *nifH* genes affiliating with heterotrophic bacteria were ubiquitous in surface waters from ten marine locations world-wide and the estuarine Baltic Sea. Through enrichment cultures of Baltic Sea surface water in anaerobic N-free medium, heterotrophic N_2 fixation was induced showing that there was a functional N_2-fixing community present and isolates of heterotrophic diazotrophs were obtained. In Sargasso Sea surface waters, transcripts of *nifH* related to heterotrophic bacteria were detected indicating heterotrophic N_2-fixing activity.

Nitrogenase expression is thought to be highly regulated by the availability of inorganic N and the presence of oxygen. Low oxygen zones within the water column can be found in association with plankton. The presence of diazotrophs as symbionts of heterotrophic dinoflagellates was investigated and *nifH* genes related to heterotrophic diazotrophs rather than the cyanobacterial symbionts were found, suggesting that a symbiotic co-existence prevailed. Oxic-anoxic interfaces could also be potential sites for heterotrophic N_2 fixation. The Baltic Sea contains large areas of anoxic bottom water. At the chemocline and in anoxic deep water heterotrophic diazotrophs were diverse, abundant and active. These findings extend the currently known regime of N_2 fixation to also include ammonium-rich anaerobic waters.

The results of this thesis suggest that heterotrophic diazotrophs are diverse and widely distributed in marine and estuarine waters and that they can also be active. However, limits in the knowledge on their physiology and factors which regulate their N_2 fixation activity currently prevent an evaluation of their importance in the global marine N budget.

Keywords: 454-pyrosequencing, diazotrophs, heterotrophic bacteria, marine bacteria, marine microbial ecology, microbiology, *nifH,* nitrogenase, nitrogen fixation, PCR, qPCR.

This is the Ocean, silly,
we're not the only two in here

Dory in Finding Nemo

SAMMANFATTNING

Alla alger och bakterier behöver kväve i sin tillväxtmiljö. I akvatiska miljöer är tillgången till kväve låg och kvävetillgången begränsar därmed tillväxt och produktion. Detta gynnar en speciell grupp av mikroorganismer som har förmåga att omvandla gasformigt kväve som finns löst i vatten till ammonium genom en process som kallas kvävefixering. Fotosyntetiserande cyanobakterier (blågröna alger) är en känd grupp kvävefixerare som är vanligt förekommande i Östersjön och i andra marina miljöer, men även icke fotosyntetiserande heterotrofa bakterier kan fixera gasformigt kväve. Kunskapen om heterotrofa kvävefixerande bakterier är mycket begränsad och i nuläget anses deras bidrag till den marina kvävecykeln vara obetydligt.

Målet med denna avhandling är att öka kunskapen om heterotrofa kvävefixerande bakterier i havet genom att studera vilka de är, var de finns och var de är aktiva. För att utforska de genetiska förutsättningarna för kvävefixering studerades nitrogenasgenen, *nifH*, som kodar för en del av enzymet som möjliggör kvävefixering. I en världsomspännande studie undersöktes förekomsten och uttrycket av *nifH* i ytvatten. Mångfalden och förekomsten av heterotrofa kvävefixerare var stor och utgjorde en betydande del av den sammanslagna *nifH* genpoolen. Således visades att förekomsten av heterotrofa kvävefixerare var utbredd i världshaven och att de därmed kan ha en betydande roll för den marina kvävecykeln. Även en majoritet av *nifH* sekvenser från fritt levande bakterier i ytvatten från Östersjön var från heterotrofa bakterier. Närvaron av funktionella heterotrofa kvävefixerare i Östersjöns ytvatten påvisades genom att kvävefixering inducerades vid odling i anaerobt kvävefattigt medium. I denna studie erhölls även isolat av kvävefixerande heterotrofa bakterier som i framtiden kan användas för att utforska deras roll för kvävetillförsel i Östersjön.

Kvävefixering är en process som är mycket syrekänslig och därför krävs låga syrekoncentrationer för att möjliggöra kvävefixering. Dessa förutsättningar kan bland annat finnas i association med partiklar eller i syrefattiga områden i djupvatten. För att undersöka om symbionter av heterotrofa dinoflagellater var kvävefixerare analyserades de för

förekomsten av *nifH* gener. Studien visade på en samexistens av icke kvävefixerande cyanobakterier och kvävefixerande heterotrofa bakterier hos de studerade dinoflagellaterna.

I Östersjön finns utbredda områden av syrefattiga bottnar. Detta gör att det finns en syregradient mellan syrerikt och syrefattigt vatten som kan vara av betydelse för heterotrofa kvävefixerare. Eftersom djupvattnen är rika på tillgängligt kväve har kvävefixering ej ansetts vara av fördel för organismer i denna miljö. Dock påvisades att *nifH* gener från en stor mängd olika heterotrofa bakterier var vanligt förekommande och aktivt uttryckta vid gränsen mellan syrerikt och syrefattigt vatten i Östersjön. Kvävefixering uppmättes även i syrefattigt djupvatten vilket tyder på att kvävefixering sker i större utbredning än vad som tidigare var känt.

Sammanfattningsvis visar de molekylära studierna i denna avhandling på en stor mångfald och utbredning av heterotrofa kvävefixerare och att de är aktiva i marina miljöer. Denna kunskap är av stor vikt för att möjliggöra en framtida kartläggning av betydelsen av heterotrofa bakterier i den marina kvävecykeln och den globala kvävebudgeten.

LIST OF PAPERS

I. **Farnelid, H.**, Riemann, L. (2008). Heterotrophic N_2-fixing bacteria: overlooked in the marine nitrogen cycle? In: Couto G. N. (ed). Nitrogen Fixation Research Progress. Nova Science Publishers: New York, pp 409-423.

II. **Farnelid, H.**, Andersson, A. F., Bertilsson, S., Al-Soud, W., Hansen, L., Sørensen, S., Steward, G.F., Hagström, Å., Riemann, L. (2011). Nitrogenase gene amplicons from global marine surface waters are dominated by genes of non-cyanobacteria. PLoS ONE 6: e19223.

III. **Farnelid, H.**, Öberg, T., Riemann, L. (2009). Identity and dynamics of putative N_2-fixing picoplankton in the Baltic Sea proper suggest complex patterns of regulation. Environmental Microbiology Reports. 1. 145-154. *

IV. **Farnelid, H.**, Tarangkoon, W., Hansen, G., Hansen, P.J., Riemann, L. (2010). Putative N_2-fixing heterotrophic bacteria associated with dinoflagellate-*Cyanobacteria* consortia in the low-nitrogen Indian Ocean. Aquatic Microbial Ecology. 61. 105-117.

V. **Farnelid, H.**, Bentzon-Tilia, M., Andersson, A. F, Bertilsson, S., Jost, G., Labrenz, M., Jürgens, K., Riemann, L. Active nitrogen fixing heterotrophic bacteria at and below the chemocline of the central Baltic Sea. *Submitted Manuscript*.

VI. **Farnelid, H.**, Harder, J., Bentzon-Tilia, M., Riemann, L. Isolation of heterotrophic diazotrophs from surface water in the Baltic Sea. *Manuscript*.

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Additional published work executed during the Ph.D. study but not included in this thesis:

- Riemann, L., **Farnelid, H.**, Steward, G. F. (2010). Nitrogenase genes in non-cyanobacterial plankton: prevalence, diversity and regulation in marine waters. Aquat Microb Ecol 61:235-247.

- Alonso-Sáez, L., Waller, A. S., Mende, D. R., Bakker, K., **Farnelid, H.**, Yager, P. L., Lovejoy, C., Tremblay, J-É., Potvin, M., Heinrich, F., Estrada, M., Riemann, L., Bork, P., Pedrós-Alió, C., Bertilsson, S. (2012). Role for urea in nitrification by polar marine Archaea. Proc Natl Acad Sci USA 109:17989-17994.

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INTRODUCTION

Marine N_2 fixation

Around 70% of the global surface consists of ocean. In the oceans, marine bacterioplankton flourish (10^6 cells ml^{-1}; Hobbie *et al.*, 1977; Porter and Feig, 1980), making them the most abundant group of organisms on earth. These microorganisms engage in chemical interactions with their environment in diverse metabolic processes and are thereby fundamental for biogeochemical cycling. Through primary production, marine phytoplankton remove carbon dioxide (CO_2) from the atmosphere at a level which equals that of terrestrial plants (Lalli and Parsons, 1997; Field *et al.*, 1998).

In the marine environment primary production is thought to be nitrogen (N) limited (Dugdale, 1967; Howarth, 1988; Capone, 2000) meaning that bioavailable N is often present in lower concentrations compared to other necessary elements. N can in most organisms be assimilated in combined forms such as ammonium (NH_4), nitrate (NO_3), nitrite (NO_2), and urea, but the most abundant form dinitrogen gas (N_2; 78% of our atmosphere), is generally unavailable to most organisms. Some prokaryotes, including diverse microorganisms of *Bacteria* and *Archaea*, carry the nitrogenase enzyme, by which they can reduce N_2 gas to ammonium (NH_4^+) in an ATP-expensive process called biological N_2 fixation (Postgate, 1998; Zehr and Paerl, 2008):

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

The input of N into the oceans through N_2 fixation is substantial (Paerl and Zehr, 2000; Capone *et al.*, 2005) and can support up to 50% of the primary production in some areas (Karl *et al.*, 1997). Over the last decades the balance of the marine N cycle has been debated. Global marine N budgets indicate that N losses (mainly denitrification and anammox) far exceed that of N input (N_2 fixation, riverine and atmospheric deposition; Codispoti *et al.*, 2001; Galloway *et al.*, 2004).

Since biogeochemical studies indicate that the N budget should be in balance, and even conservative estimates of oceanic N sinks are higher than present estimates of N input, N_2 fixation rates could be largely underestimated (Mahaffey *et al.*, 2005; Codispoti, 2007). Rates of N_2 fixation in previously unexplored marine regions, and the discovery of previously unknown N_2-fixers (diazotrophs), may help to increase the estimated N input.

Measuring and estimating rates of N_2 fixation

Estimating the input by N_2 fixation to the global N budget is a major challenge. N_2 fixation is variable both spatially and temporally. In addition, the available N_2 fixation rates are often sampled during times when N_2 fixation is expected to be high (e.g. during extensive cyanobacterial blooms) making the data largely biased. These factors, combined with the low frequency of measurements generally make global estimates uncertain. Biogeochemical estimates based on ratios of dissolved inorganic N to phosphate (P) relative to the canonical Redfield ratio (16N:1P; Michaels *et al.*, 1996; Gruber and Sarmiento, 1997) greatly increased most previous estimates of global oceanic N_2 fixation (Karl *et al.*, 2002; Galloway *et al.*, 2004). However, one of the disadvantages with this method is that areas where denitrification and N_2 fixation are concurrent may appear as neutral. In addition, verifications of N_2 fixation rates are required.

To measure rates of N_2 fixation in aquatic environments the acetylene reduction assay (ARA) has been widely used (Stewart *et al.*, 1967; Capone, 1993). The nitrogenase enzyme can (in addition to N_2) break the triple bond of a number of other substances such as acetylene or cyanide. Thus the production of ethylene from acetylene can be measured to assess nitrogenase activity. A direct method to measure N_2 fixation is the $^{15}N_2$ incorporation assay (Montoya *et al.*, 1996), which has been used in numerous studies (Luo *et al.*, 2012). Recently, Mohr *et al.* (2010) showed that injection of $^{15}N_2$ as a gas bubble, as used in the traditional protocol, might be slow to equilibrate in the sample and therefore underestimate N_2 fixation rates. As a result, with the improved method involving the addition of $^{15}N_2$ in a solution of filtered seawater, Großkopf *et al.* (2012) estimated an increase of N_2 fixation rates from 103 ± 8 TgN yr^{-1} to 177 ± 8 TgN yr^{-1}. The resulting estimates could reduce the current gap between N losses and N inputs in the global N budget (White, 2012).

The *nifH* gene as a phylogenetic marker

A new era in marine microbiology, revolutionizing the knowledge of marine bacterial diversity and dynamics, evolved with molecular methods based on the 16S rRNA gene as a phylogenetic marker (Giovannoni *et al*., 1990; Schmidt *et al*., 1991; Britschgi and Giovannoni, 1991). The 16S rRNA gene displays a high degree of functional consistency and occurs in all prokaryotes (Olsen *et al*., 1986; Woese, 1987). Bacteria with >97% 16S rRNA gene homology are regarded as the same species (Stackebrandt and Goebel, 1994). However, the 16S rRNA gene alone does not provide information about the physiology or metabolic features of the bacterium, and without representative isolates the ecological functions are largely unknown (Achtman and Wagner, 2008; Fraser *et al*., 2009). For this purpose, molecular methods targeting functional genes can provide useful links to functions within the bacterial community.

During the last decades the *nifH* gene has been widely used to study the presence and diversity of N_2-fixers. The *nifH* gene contains relatively conserved regions and can be amplified using degenerate primers (Zehr and McReynolds, 1989; Kirshtein *et al*., 1991). The *nifH* gene encodes the iron (Fe)-protein of the nitrogenase enzyme known as dinitrogenase reductase. It transfers electrons to the second part of the enzyme, the molybdenum (Mo)Fe-protein, which further reduces N_2 to NH_4^+ (Howard and Rees, 1996). The MoFe-protein, or dinitrogenase, is encoded by *nifD* and *nifK*, and together the three genes encoding the nitrogenase enzyme are organized into one operon, *nifHDK*. N_2 fixation occurs through the transcription (Fig. 1) and translation of the *nifHDK* genes. Some diazotrophs have alternative non-Mo containing dinitrogenase reductases, which contain vanadium (*vnfH*) or Fe (*anfH*; Bishop *et al*., 1986; Fallik *et al*., 1991). However, the ecological significance of alternative nitrogenases is not yet known.

The high sequence similarity of the *nifH* gene among diverse microorganisms suggests an early origin or lateral gene transfer among prokaryotic lineages (Zehr *et al*., 2003b; Raymond *et al*., 2004). Phylogenetically, the *nifH* gene has been divided into four Clusters (Chien and Zinder, 1996; Fig. 2). Cluster I includes cyanobacteria and *Alpha*-, *Beta*- and *Gammaproteobacteria*. Within Cluster I, *nifH* phylogeny largely resembles that of the 16S rRNA gene, making cross system comparisons possible (Zehr *et al*., 2003b). However there are several examples of possible gene transfers (Cantera *et al*., 2004; Kechris *et al*., 2006; Bolhuis *et al*., 2010). Cluster II includes alternative Fe-only nitrogenases (*anfH*) and Cluster III represents a divergent assemblage of phylotypes related to anaerobic bacteria and *Archaea* such as *Desulfovibrio*, *Clostridium* and *Methanosarcina*. Cluster IV contains a

divergent group of non-functional *nifH*-like sequences from methanogens and some anoxygenic photosynthetic bacteria.

Today, molecular methods targeting the *nifH* gene are commonly used to identify diazotrophs and to quantify their abundance (e.g., quantitative polymerase chain reaction, qPCR; Short *et al.*, 2004; Short and Zehr, 2005). These methods have significantly increased the knowledge on diversity and distribution of putative diazotrophs. In addition, *nifH* gene expression, in the form of mRNA can be considered an indicator of active N_2 fixation (Chien and Zinder, 1996; Sicking *et al.*, 2005). With the use of reverse transcriptase-PCR (RT-PCR), the presence of *nifH* mRNA can be determined and quantified (RT-qPCR; Fig. 1), providing information about the activity of the diazotrophic community and specific phylotypes therein.

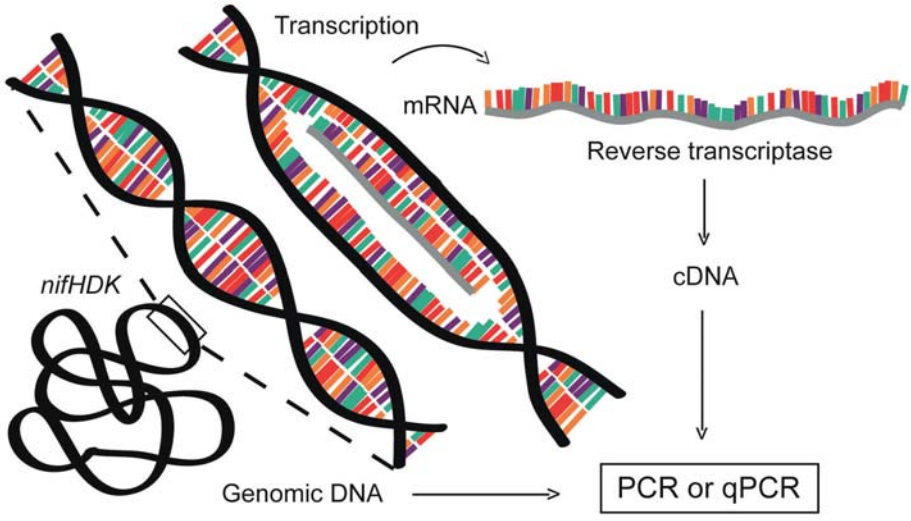

Figure 1. Illustration of the nitrogenase structural genes (nifHDK) of genomic DNA and transcription of mRNA.

Regulation of nitrogenase expression

It has been argued that because of the ability of N_2-fixers to supply N to N-deficient regions, N should not be the ultimate nutrient limiting primary productivity (Redfield, 1958). However, although theoretically not limited by N, diazotrophs face environmental constraints such as oxygen (O_2) tension, turbulence, temperature, availability of nutrients, trace metals, and energy to sustain marine N_2 fixation (Paerl, 1985; Howarth *et al*., 1988; Paerl, 1990; Karl *et al*., 2002).

It is not well known what controls the distribution and activity of diazotrophs in aquatic environments. Nitrogenase synthesis is thought to be highly regulated by the availability of N (particularly NH_4^+) and the presence of O_2 (Dixon and Kahn, 2004). Protection of the nitrogenase enzyme from inactivation or inhibition by O_2 is essential for N_2 fixation and many diazotrophs have evolved strategies to avoid O_2 exposure (Gallon, 1981; Gallon, 1992). For example, some cyanobacteria have specialized non-photosynthesizing cells, called heterocysts, which spatially separate N_2 fixation from O_2 producing photosynthesis (Fay, 1992; Gallon, 1992), while other bacteria may require microaerophilic conditions for N_2 fixation (Marchal and Vanderleyden, 2000). As NH_4^+ uptake is energetically favorable compared to N_2 fixation (Zehr and Ward, 2002), it has been assumed that the presence of more accessible N sources precludes N_2 fixation. Moreover, in cultivation studies, nitrogenase activity has been shown to decrease upon NH_4^+ addition (Klugkist and Haaker, 1984; Fritzsche and Niemann, 1990). However, evidence of substantial N_2 fixation in N-replete waters (Knapp, 2012) highlights that factors which regulate diazotrophic activity are not yet understood.

Among marine microbes it is often assumed that genomic traits provide a selective advantage (e.g. genomic divergence between *Prochlorococcus* ecotypes; Rocap *et al*., 2003). Consequently, although the presence of *nifH* genes is not necessarily an indicator of active N_2 fixation, these genes would be expected to acquire mutations and eventually be excluded from the genome if they were not used (Berg and Kurland, 2002). Thus, if there is a genetic potential for N_2 fixation within the microbial community there should be occasions when these diazotrophs are active in N_2 fixation.

Marine and estuarine diazotrophs

Cyanobacteria

Cyanobacteria are considered the main contributors to marine N_2 fixation (Zehr, 2011; Luo *et al*., 2012). The filamentous cyanobacterium *Trichodesmium*, which often forms colonies or aggregates, is widespread in tropical and subtropical oceans and is assumed to be the dominant diazotroph (Capone *et al*., 1997; 2005) and is also the most well studied (Dugdale *et al*., 1961; Carpenter and Romans, 1991; LaRoche and Breitbarth, 2005). Heterocystous cyanobacteria, forming symbionts with unicellular eukaryotic algae (e.g., *Richelia intracellularis* and *Calothrix rhizosoleniae*), are also among the few diazotrophs that could be identified with conventional techniques (Villareal, 1991; Gómez *et al*., 2005), and can in some regions be quantitatively significant (Carpenter *et al*., 1999; Subramaniam *et al*., 2008).

The use of molecular studies amplifying *nifH* genes in seawater revealed an unexpected large phylogenetic diversity of previously unrecognized marine N_2-fixing microorganisms (Zehr *et al*., 1998; 2000). This led to the discovery that several groups of diazotrophic unicellular cyanobacteria were widely distributed in subtropical and tropical oceans (Zehr *et al*., 2001; Langlois *et al*., 2005; Church *et al*., 2005a), sometimes at equal or greater abundance compared to other cyanobacterial diazotrophs (Foster *et al*., 2007; 2008; Kong *et al*., 2011). The uncultivated unicellular cyanobacterial Group A (UCYN-A) are found also in cooler waters compared to *Trichodesmium* and thus occupy a larger geographic area (Needoba *et al*., 2007; Langlois *et al*., 2008; Moisander *et al*., 2010; Mulholland *et al*., 2012). Consequently, although they appear to be less important than *Trichodesmium*, N_2 fixation by unicellular cyanobacteria can be significant in certain areas (Montoya *et al*., 2004; Goebel *et al*., 2007; 2010).

In the estuarine Baltic Sea the heterocystous cyanobacteria *Nodularia, Anabaena* and *Aphanizomenon* are considered the main N_2-fixers (Wasmund *et al*., 2005; Ohlendieck *et al*., 2007). During summer, induced by low N:P ratios (Granéli *et al*., 1990), extensive cyanobacterial blooms occur (Finni *et al*., 2001; Stal *et al*., 2003) resulting in significant N input through N_2 fixation (Rahm *et al*., 2000; Wasmund *et al*., 2005; Degerholm *et al*., 2008). Some studies indicate that there may also be N_2 fixation in the picoplankton size fraction (Larsson *et al*., 2001; Wasmund *et al*., 2001) but currently there is no evidence for N_2 fixation by unicellular cyanobacteria in the Baltic Sea.

Heterotrophic bacteria

Among the previously unrecognized diazotrophs, *nifH* genes and transcripts from non-cyanobacterial heterotrophic diazotrophs were also detected (e.g., Zehr *et al.*, 1998; Falcón *et al.*, 2004; Langlois *et al.*, 2005; Moisander *et al.*, 2008). Heterotrophic diazotrophs are diverse and include a broad range of prokaryotes with *nifH* genes distributed over the defined *nifH* Clusters. In **Paper I**, reports of heterotrophic diazotrophs and the factors that may regulate their activity and distribution are discussed. It is not known how heterotrophic diazotrophs protect their nitrogenase from inactivation by O_2 or how they obtain energy to support the expensive process of N_2 fixation in the water column. At present, due to the general difficulty of cultivating marine microbes (Staley and Konopka, 1985; Connon and Giovannoni, 2002), and particularly diazotrophs, the few isolates of marine heterotrophic diazotrophs (e.g., Maruyama *et al.*, 1970; Werner *et al.*, 1974; Wynn-Williams and Rhodes, 1974; Guerinot and Colwell, 1985; Tibbles and Rawlings, 1994; Boström *et al.*, 2007) limit the understanding of how they function and what factors regulate their N_2 fixation activity.

As they have not received a lot of attention, information on the distribution, diversity and activity of heterotrophic diazotrophs in marine and estuarine waters is limited (reviewed in Riemann *et al.*, 2010). The studies which have investigated heterotrophic diazotrophs using qPCR have generally found them in low abundances (e.g., Church *et al.*, 2005a; Zehr *et al.*, 2007; Hewson *et al.*, 2007a; Langlois *et al.*, 2008; Church *et al.*, 2008) and their dilute nature make them difficult to study. For instance although diazotrophs are present in the population, *nifH* genes can be undetected in metagenome libraries (Johnston *et al.*, 2005). Their low abundance may also prevent detection of transcripts or measurements of N_2 fixation rates due to methodological limitations or technical difficulties such as contamination. How, where, and when heterotrophic diazotrophs are active is therefore largely unknown.

AIMS

Heterotrophic N_2-fixing bacteria are widespread in marine and estuarine environments. However there is a lack of knowledge on their metabolic functions and factors which regulate their N_2-fixing activity. Consequently, their contribution to N input in local and regional scales cannot be evaluated and they are currently thought to be insignificant to the global marine N budget.

Using molecular and cultivation techniques the following questions were addressed in this thesis:

- What is the spatial distribution and diversity of *nifH* genes in global marine surface waters?
- Which factors control the composition of diazotrophic populations?
- Is there a N_2 fixation potential in the picoplankton fraction of the central Baltic Sea?
- Are symbionts of heterotrophic dinoflagellates diazotrophic?
- Does the chemocline of the central Baltic Sea provide suitable conditions for heterotrophic N_2 fixation?
- Are marine heterotrophic bacteria fixing N_2?

RESULTS AND DISCUSSION

Distribution, diversity and abundance of heterotrophic diazotrophs

To investigate the diversity and distribution of *nifH* phylotypes in marine locations worldwide, *nifH* genes were amplified and sequenced using 454-pyrosequencing (**Paper II**). A great divergence in sequence composition with distinct geographic distributions was observed between sites. *nifH* genes from cyanobacteria were most frequent among amplicons from the warmest waters, but overall the diversity and relative number of sequences was dominated by *nifH* genes from non-cyanobacteria. Similarly, although cyanobacteria have been the focus of most studies on N_2 fixation, non-cyanobacterial *nifH* phylotypes compose a large part of published clone libraries (average 82%; **Paper I**).

In a compilation of *nifH* sequences of marine and estuarine origin from a database on available *nifH* sequences (http://pmc.ucsc.edu/~wwwzehr/research/database/), 66% of sequences were related to non-cyanobacteria (Fig. 2; Riemann *et al.*, 2010). The occurrence of heterotrophic diazotrophs is frequent in coastal and estuarine areas (Affourtit *et al.*, 2001; Zehr *et al.*, 2003b; Jenkins *et al.*, 2004; Man-Aharonovich *et al.*, 2007; **Paper III**), and generally there seem to be a greater genetic potential for N_2 fixation in these waters compared to oceanic waters. In the database compilation, 36% of the sequences from the open ocean were from non-cyanobacteria compared to 80% of those from coastal or estuarine plankton samples (Fig. 2; Riemann *et al.*, 2010). Notably, these studies are often limited to surface waters, but the relative abundance of non-cyanobacteria may increase with depth (e.g., Hewson *et al.*, 2007a; **Paper I**). Therefore, the prevalence of non-cyanobacterial phylotypes is likely underestimated. However, **Papers I, II** and **III** show that heterotrophic diazotrophs are diverse and widely distributed also in surface waters.

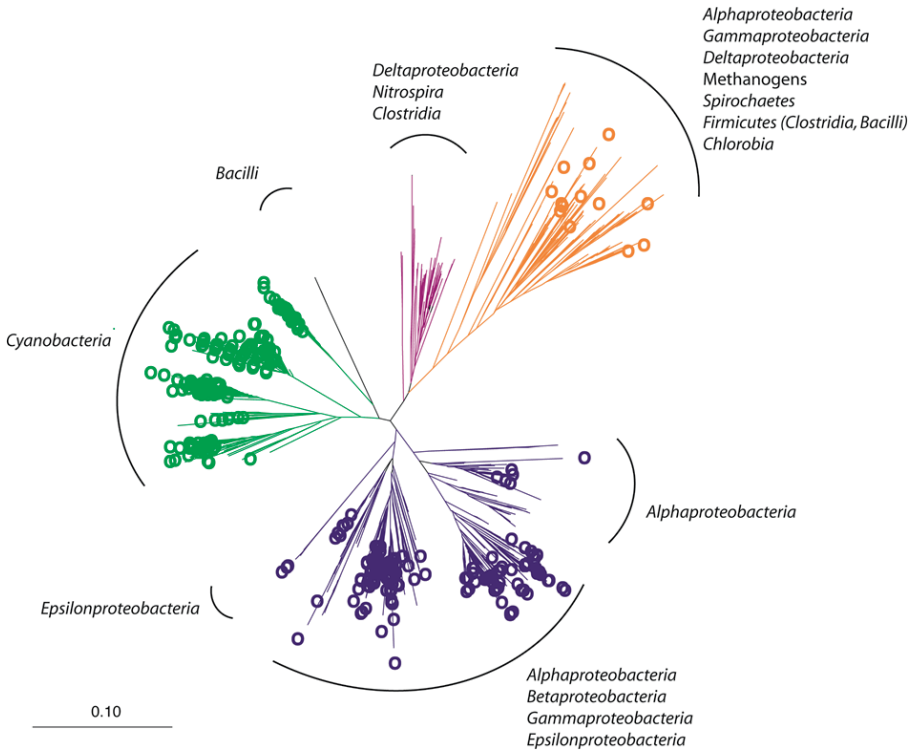

Figure 2. Phylogenetic tree illustrating the diversity among 2570 nifH genes amplified from microorganisms in plankton samples from marine and estuarine environments (Riemann et al., 2010, with permission from Inter Research). Green and blue branches show Cyanobacteria and Proteobacteria within Cluster I, orange branches are affiliated with Clusters II and III. Purple branches are not assigned to the traditionally defined clusters. Branches derived from sequences from open ocean samples are marked with a "o" (n = 809). Open ocean samples include those from the Atlantic and Pacific Oceans and the Arabian Sea, excluding near shore environments (gulfs, bays, harbors, fjords) and inland seas (Baltic, Mediterranean). Clusters are labeled to indicate the phylogenetic affiliations of cultivated microorganisms whose nifH sequences most closely match those from the uncultivated microorganisms shown in the tree.

In **Papers II** and **III**, sequences within *nifH* Cluster I (Fig. 2) related to *Alpha-*, *Beta-*, and *Gammaproteobacteria*, were most common. Cluster III *nifH* phylotypes (diverse anaerobic diazotrophs; Fig. 2), appear to be uncommon in open ocean samples, but have been frequently detected in coastal and estuarine areas (Fig. 2; Affourtit *et al.*, 2001; Moisander *et al.*, 2007; Foster *et al.*, 2009; Mulholland *et al.*, 2012). In the Baltic Sea, 21% of the *nifH* clones from surface waters (**Paper III**) and diverse clusters at and below the chemocline affiliated with Cluster III (**Paper V**). Recent studies report *nifH* Cluster III phylotypes from oxygen minimum zones (OMZs; Fernandez *et al.*, 2011; Hamersley *et al.*, 2011;

Jayakumar *et al*., 2012) suggesting that they may thrive in these low O_2 habitats. Interestingly, Cluster III *nifH* phylotypes were also identified as symbionts of dinoflagellates from the Indian Ocean (**Paper IV**), indicating that the association may provide low O_2 conditions. In marine surface samples, *nifH* sequences within Cluster III were largely absent but appeared to be prominent in cold waters, especially the Arctic sample where cyanobacterial *nifH* sequences were completely absent (**Paper II**). Notably, Díez *et al*. (2012) recently reported cyanobacterial *nifH* genes in Arctic seawater highlighting that the geographical distribution of specific *nifH* phylotypes is largely unknown.

A PCR bias towards amplification of a gammaproteobacterial phylotype was recently shown (Turk *et al*., 2011). In addition, *nifH* sequences clustering with *Proteobacteria* have been detected in PCR reagents (Zehr *et al*., 2003a; Goto *et al*., 2005) and in ultra-pure water (Kulakov *et al*., 2002). Consequently some of the reported *nifH* sequences may have originated from contaminants or have been overestimated compared to cyanobacteria. One way to detect *nifH* phylotypes originating from PCR reagents is to excise a gel piece of the expected PCR product size from no template control samples, purify, clone and sequence (**Papers II, III, IV, V** and **VI**). In **Paper III** it was demonstrated that *nifH* sequences derived from control samples clustered separately from the samples. However, distinguishing between contaminants and true environmental sequences can be difficult. In **Paper V** the occurrence of a *nifH* phylotype closely affiliated with contaminants was investigated. Using qPCR, generally low concentrations of the contaminant-like *nifH* phylotype was detected, but the abundance and distribution did not reflect the relative abundance in sequence libraries (**Paper V**). Hence, the occurrence of *nifH* sequences in clone libraries should be interpreted with caution, as they may not always reflect abundances of specific *nifH* phylotypes *in situ*.

One way to avoid biases associated with end-point PCR and clone libraries is to use qPCR to quantify specific phylotypes. An increasing number of studies report abundance and transcript abundance data of heterotrophic diazotrophs (Table 1). In some studies the *nifH* copies L^{-1} is comparable to those reported for cyanobacteria (e.g., Foster *et al*., 2008; Fong *et al*., 2008; Mulholland *et al*., 2012). Among the most recognized phylotypes is an uncultivated gammaproteobacterial group, which has been found to be widely distributed (e.g., Langlois *et al*., 2005; Moisander *et al*., 2008; Langlois *et al*., 2008; Turk *et al*., 2011) and expressed (Bird *et al*., 2005; Church *et al*., 2005b). In **Paper II,** a cluster related to this gammaproteobacterial group (UMB) was found in high relative abundance in the Sargasso Sea and was also transcribed indicating that this group is an active part of the N_2-fixing community in surface waters.

nifH copies L^{-1}	Phylotype (Reference/Accession)	Location	Depth (m)	Reference
19000[b]	Alphaproteobacterial isolate (BAL398)	Baltic Sea	0-20	**Paper VI** (this thesis)
143000[a]	*Alpha/Betaproteobacteria* (CB912H4, AY224022)	Chesapeake Bay	1 to 20	Short *et al.* (2004)
60000[a]	*Alphaproteobacterium* (24809A06, EU052488)	South China Sea	Epipelagic	Moisander *et al.* (2008)
700[a]			Mesopelagic	
700000[a]	*Alphaproteobacterium* (HQ586648)	South China Sea	Epipelagic	Zhang *et al*. (2011)
500-4000			Mesopelagic	
110 (<10 µm) 560 (>10 µm)	*Alphaproteobacterium* (Moisander *et al*. 2008)	Pacific Ocean	350	Hamersley *et al.* (2011)
34000[a]	Gammaproteobacterial isolate (BAL281, AY972874)	Baltic Sea	Surface	Boström *et al*. (2007)
13000[b]	Gammaproteobacterial isolate (BAL354)	Baltic Sea	0-20	**Paper VI** (this thesis)
0-7736000, 0-148000[c]	*Gammaproteobacteria* (HM210377, HM210643, HM210397, HM210363)	South Pacific Ocean	0-220	Halm *et al*. (2012)
5000[a]	*Gammaproteobacterium* (24774A11, EU052413)	South China Sea	Mesopelagic	Moisander *et al*. (2008)
750-3300, 5400-88000[c]	*Gammaproteobacterium* (Moisander *et al*. 2010)	South China Sea	Surface	Bombar *et al*. (2011)
8700[a, c]	*Gammaproteobacterium* (Moisander *et al*. 2010)	North Atlantic Ocean	Surface	Turk *et al*. (2011)
0-92000 ± 27000	*Gammaproteobacterium* (AO15)	North Pacific Ocean	25	Zehr *et al*. (2007)
2	*Gammaproteobacterium* (BT19215A01)	North Atlantic Ocean	5948	Hewson *et al*. (2007a)
2	*Gammaproteobacterium* (EP19212A01)	Pacific Ocean	1389	
500-10000	*Gammaproteobacterium* (HQ586273)	South China Sea	Epipelagic	Zhang *et al*. (2011)
400[b, c]	*Gammaproteobacterium* (AY706889 and AY706890)	North Pacific Ocean	0-175	Church *et al*. (2005a)
10000-100000	*Gammaproteobacterium* (Church *et al*. 2005a)	North Pacific Ocean	0-100	Fong *et al*. (2008)
570[a]	*Gammaproteobacterium* (Church *et al*. 2005a)	Red Sea	0-80	Foster *et al*. (2009)
700	*Gammaproteobacterium* (Church *et al*. 2005a)	North Pacific Ocean	10	Church *et al.* (2008)
1000-10000	*Gammaproteobacterium* (Gamma A, AY896371)	North Atlantic Ocean	5-120	Langlois *et al*. (2008)
0-1000	*Gammaproteobacterium* (Gamma P, AY896428)		5-120	
324700[c]	*Gammaproteobacterium* (Langlois *et al*. 2008)	South Pacific Ocean	Surface	Halm *et al*. (2012)
10000[a]	*Gammaproteobacterium* (Langlois *et al*. 2008)	North Atlantic Ocean	Surface	Rijkenberg *et al.* (2011)
0-10000	*Gammaproteobacterium* (Langlois *et al*. 2008)	Atlantic Ocean	0-80	Großkopf *et al*. (2012)
520000[a]	*Gammaproteobacterium* (EQF91)	Baltic Sea	100-200	**Paper V** (this thesis)
3000000[a]	*Gammaproteobacterium* (ALHOU)		48-200	

Table 1A. Summary of reported abundances of heterotrophic nifH phylotypes (Cluster I) and transcripts. [a]maximum abundance, [b]average abundance, [c]nifH transcripts L^{-1}.

nifH copies L^{-1}	Phylotype (Reference/Accession)	Location	Depth (m)	Reference
43	Cluster III (BT5667A01)	North Atlantic Ocean	1000	Hewson *et al*. (2007a)
0-300, 0-2400[c]	Cluster III (Langlois *et al*. 2008)	South Pacific Ocean	0-200	Halm *et al*. (2012)
340000[a]	Cluster III (CB907H22, AY223963)	Chesapeake Bay	1 to 20	Short *et al*. (2004)
2300000[a], 32000[a, c]	Cluster III (DOCY3)	Baltic Sea	38-200	**Paper V** (this thesis)
22000000[a]	Cluster III (ECl27)		41-200	

Table 1B. Summary of reported abundances of heterotrophic nifH phylotypes (Cluster III) and transcripts. [a]maximum abundance, [b]average abundance, [c]nifH transcripts L^{-1}.

In the central Baltic Sea the abundances of two gammaproteobacterial phylotypes were investigated in depth profiles with high resolution around the chemocline. Although the phylotypes could not be detected in surface waters one of the phylotypes reached abundances of up to 3 x 10^6 *nifH* copies L^{-1} at the chemocline (**Paper V**). Abundances in the same order of magnitude were recently reported for gammaproteobacterial phylotypes from the South Pacific Gyre, where heterotrophic diazotrophs were found to dominate N_2 fixation (Halm *et al*., 2012). In **Paper VI**, the *nifH* abundances of a *Gammaproteobacteria* and an *Alphaproteobacteria* isolated from surface water from the central Baltic Sea was investigated. The results showed that these phylotypes were almost consistently present throughout the season, indicating that they were a stable part of the bacterial community. The abundances of the alphaproteobacterial isolate (average 1.9 x 10^4 copies L^{-1}) were similar to what has previously been reported for alphaproteobacterial phylotypes in the South China Sea (Moisander *et al*., 2008; Zhang *et al*., 2011; Table 1A).

Few studies have targeted Cluster III phylotypes using qPCR and generally low abundances have been detected (e.g., Church *et al*., 2005a; Langlois *et al*., 2008; Table 1B). In **Paper V**, abundances of two Cluster III phylotypes were up to 3.3 x 10^6 and 2.2 x 10^7 copies L^{-1} respectively at and below the chemocline of the Baltic Sea proper, being among the highest abundances of *nifH* phylotypes ever reported (but see Halm *et al*., 2012; Mulholland *et al*., 2012). In summary, the increasing number of studies which have quantified heterotrophic diazotrophs in marine and estuarine environments indicate that they are present and in some areas as abundant as cyanobacterial phylotypes (Church *et al*., 2005a; Fong *et al*., 2008), suggesting that they could also be significant to global marine N input.

Environmental controls on heterotrophic diazotrophs

In **Paper I**, the current knowledge on the regulation of heterotrophic diazotrophs in terms of carbon availability, presence of inorganic N and O_2 tension is discussed. The factors which regulate *nifH* diversity *in situ* are largely unknown. For example a large genetic potential for N_2 fixation has been observed in N-replete waters (e.g., Chesapeake Bay; Jenkins *et al.*, 2004; Moisander *et al.*, 2007). In **Paper III,** covariance between *nifH* composition and several environmental factors was shown but no strong links could be established. This suggests a variable and complex regulation of diazotrophic groups within Baltic Sea picoplankton. Similarly, in a recent study in the North Atlantic Ocean, no single factor controlling the distribution patterns of the *nifH* gene abundance of a gammaproteobacterial group and N_2 fixation rates could be found (Rijkenberg *et al.*, 2011). In the South Pacific Ocean, Moisander *et al.* (2012) observed increased abundances of the gammaproteobacterial group γ-24774A11 with iron (Fe) and P-Fe additions in some parts of the study area, suggesting that the growth of these diazotrophs was Fe limited. However, the environmental factors, which control the abundance, distribution, and diversity of heterotrophic diazotrophs, are not yet well understood.

Sites within the water column for heterotrophic N_2 fixation

N_2 fixation requires reduction of intracellular O_2 or low ambient O_2 concentrations. In the oxygenated water column, this can for example be achieved by colonizing particles with interior low O_2 micro-zones (Guerinot and Colwell, 1985; Paerl and Carlton, 1988), or in association with plankton assemblages (e.g. Proctor, 1997; Braun *et al.*, 1999). In **Paper IV**, the association of diazotrophs as symbionts of heterotrophic dinoflagellates was studied. Using light microscopy and transmission electron microscopy cyanobacteria, heterotrophic bacteria, and eukaryotic algae were recognized as symbionts of heterotrophic dinoflagellates. Analysis of *nifH* sequences amplified from individual dinoflagellates revealed that the majority of sequences were from heterotrophic diazotrophs suggesting a symbiotic co-existence of non-diazotrophic cyanobacteria and N_2-fixing heterotrophic bacteria in heterotrophic dinoflagellates (**Paper IV**).

Low O_2 biomes, such as transition zones between oxic and anoxic layers in the water column and OMZs have also been suggested as possible sites for heterotrophic N_2 fixation (Zehr *et al.*, 2006; Riemann *et al.*, 2010). OMZs occupy large areas (Helly and Levin, 2004;

Paulmier and Ruiz-Pino, 2009; Fig. 3) and are expanding (Stramma *et al.*, 2008). At low O_2 concentrations, anaerobic metabolism is induced making these regions important for the global N cycle. Within OMZs 30-50% of the oceanic N loss (Gruber and Sarmiento, 1997) is estimated to occur mainly through the processes of denitrification and anaerobic ammonium oxidation (Ward *et al.*, 2009; Lam and Kuypers, 2011; Ulloa *et al.*, 2012). The characteristics of OMZs, with low O_2 and relatively low N:P ratios, particularly near the oxic-anoxic interface (Carpenter and Capone, 2008), could provide suitable conditions for heterotrophic N_2 fixation.

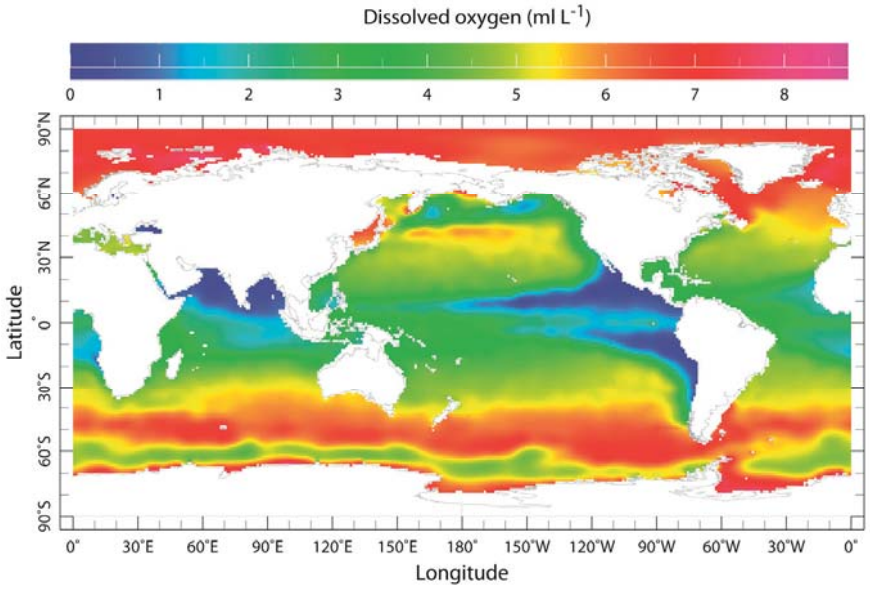

Figure 3. Global map of major oceanic oxygen minimum zones (Riemann et al., 2010, with permission from Inter Research). Annual mean dissolved oxygen levels at 200 m below the surface are illustrated as a color contour plot. Areas in dark blue indicate regions with particularly pronounced subsurface O_2 minima. Data from the IRI/LDEO Climate Data Library, Columbia University (http://iridl.ldeo.columbia.edu/). Original raw data from World Ocean Atlas 2005 (Garcia et al., 2006).

In a geochemical model, Deutsch *et al.* (2007) examined the decrease in excess P in upwelling waters from OMZs and predicted high N_2 fixation rates suggesting coexistence of N_2 fixation and denitrification. Recently N_2 fixation rates and diverse putative heterotrophic diazotrophs were reported from hypoxic waters in the eastern tropical South Pacific (Fernandez *et al.*, 2011) and the Southern Californian Bight (Hamersley *et al.*, 2011). Similarly, *nifH* genes and transcripts related to heterotrophs were found in the Arabian Sea OMZ (Jayakumar *et al.*, 2012). Together these observations provide the first evidence for N_2 fixation within oceanic OMZs and a co-occurrence with water column denitrification.

Because of their high concentrations of NH_4^+, anoxic basins have received limited attention as potential sites for N_2 fixation (Zehr *et al.*, 2006). In **Paper V**, the diversity, abundance, and transcription of *nifH* genes in two Baltic Sea basins characterized by permanent anoxic bottom water was examined. Rates of N_2 fixation were measured at the surface and below the chemocline. The suboxic and anoxic waters of the central Baltic Sea were found to harbor diverse and active heterotrophic N_2-fixing communities (**Paper V**). These findings highlight the lack of understanding of regulatory mechanisms among heterotrophic marine N_2-fixers in response to availability of inorganic N and extend the distribution of active diazotrophs to NH_4^+ rich sulfidic-anoxic waters.

Activity of heterotrophic diazotrophs

Although *nifH* genes from heterotrophic diazotrophs were present and diverse in surface waters of ten marine locations world-wide (**Paper II**) and the Baltic Sea (**Paper III**), it cannot be inferred from fragmented sequence data alone if these sequences were derived from functioning diazotrophs. Considering the diversity among diazotrophs, absence of N_2 fixation is unlikely caused by the lack of genetic potential but rather by physical and chemical constraints in the environment. In **Paper VI**, a cultivation effort was made to isolate functional N_2-fixers from cold N-replete well oxygenated waters (>10 ml L^{-1}) from the Baltic Sea. Indeed, when provided with low O_2 conditions in N-free medium, N_2 fixation was shown in enrichment cultures (**Paper VI**) indicating that with favorable conditions heterotrophic diazotrophs can be active.

Since the review in **Paper I**, documenting an average of 44% of non-cyanobacterial *nifH* transcripts from clone library studies, reports of transcripts of heterotrophic diazotrophs are increasing (e.g., Bombar *et al.*, 2011; Jayakumar *et al.*, 2012; Halm *et al.*, 2012). In **Paper II**, 42% of the identified non-cyanobacterial *nifH* clusters from the corresponding DNA samples were also detected in cDNA, showing that parts of the heterotrophic community are also expressing *nifH*. However,

comparisons of present and transcribed diazotrophs are often deviating (e.g., Man-Aharonovich *et al*., 2007; Hewson *et al*., 2007b; Zehr and Paerl, 2008; Rees *et al*., 2009), suggesting that only a small part of the heterotrophic community is active at a given time. This is likely reflected by environmental factors making *nifH* expression spatially and temporally variable. However, the occurrence of transcripts could also have been biased due to changes of environmental conditions in association with sample processing which could affect expression patterns (Feike *et al*., 2012).

Using qPCR, the *nifH* transcript abundance of heterotrophic diazotrophs has been investigated (Table 1). In some areas, transcripts of heterotrophic diazotrophs have been reported in high abundances (e.g., Bombar *et al*., 2011; Halm *et al*., 2012) suggesting that they are active in N_2 fixation and could also be significant. In a recent study in the South Pacific Gyre, N_2 fixation was largely from the contribution of *Gammaproteobacteria* and was not light dependent (Halm *et al*., 2012). In **Paper V**, N_2 fixation was measured in dark hypoxic waters of the Baltic Sea with co-occuring *nifH* transcripts of a Cluster III phylotype. N_2 fixation was also reported from hypoxic waters in the eastern tropical South Pacific and the southern California Bight (Fernandez *et al*., 2011; Hamersley *et al*., 2011). These studies indicate that heterotrophic N_2 fixation is distributed in regions that have not previously been recognized as sites of N_2 fixation.

Interestingly, in a recent study, Großkopf *et al*. (2012) found that the conventional method for $^{15}N_2$ incorporation was biased towards the composition of the diazotrophic community, largely underestimating rates when unicellular, symbiotic cyanobacteria and *Gammaproteobacteria* dominated the diazotrophic community. Consequently, measurements of N_2 fixation in communities dominated by *Gammaproteobacteria* may have been underestimated and heterotrophic N_2 fixation could have been overlooked.

Taken together, the relative expression and abundance of *nifH* transcripts in marine and estuarine environments indicate that heterotrophic diazotrophs are active N_2-fixers (Table 1; **Paper V** and **VI**). Observations of heterotrophic N_2 fixation also extend regions of N_2 fixation to dark, colder and coastal waters (e.g., Rees *et al*., 2009; Mulholland *et al*., 2012) which were previously thought to be insignificant areas of N_2 fixation. As a result, extrapolation of rate estimates, although low at local scales, over large areas could make heterotrophic N_2 fixation significant to the overall N budget.

CONCLUSIONS AND FUTURE PERSPECTIVES

The marine N cycle is essential in the function of the oceanic ecosystem and plays a central role in the response to global environmental change. N_2 fixation by diazotrophic bacterioplankton is a significant source of fixed N into the open ocean and thereby controls primary production and carbon flux. Geochemical analyses indicate that marine N_2 fixation may be underestimated. It is therefore essential to identify the distribution, abundance and activity of N_2-fixers in order to understand their influence on the N cycle. Currently heterotrophic diazotrophs are not considered significant in marine N_2 fixation, however the knowledge of their ecological role in the water column is limited and the factors which control their diversity and distribution in space and time are not understood.

The results of this thesis show that heterotrophic diazotrophs are diverse and widespread in marine and estuarine environments and that they are also active. In marine surface waters collected world-wide, *nifH* sequences of non-cyanobacteria dominated the sequence libraries and some of these were also expressed. In the Baltic Sea, diverse *nifH* sequences affiliating with heterotrophic bacteria were found in surface water and with cultivation techniques it was shown that bacteria within Baltic Sea surface water could fix N_2. It was also shown that the chemocline of the Baltic Sea harbors a diverse assemblage of heterotrophic diazotrophs, and that *nifH* phylotypes related to anaerobic bacteria were abundant and transcribed at and below the chemocline.

Measurements of N_2 fixation rates and *nifH* transcripts in N-replete areas underlines that the regulation of N_2 fixation activity is complex. An increased research focus on the activity of diazotrophs *in situ* with rate measurements and molecular methods targeting *nifH* mRNA can provide information on the activity of specific *nifH* phylotypes. With the advancements of sampling techniques, extraction efficiencies and the use of cDNA standards, the abundance of transcripts of these dilute

bacteria can also be more accurately determined. However, to gain insights into the physiology and metabolic functions of diazotrophs, and to ultimately predict their N_2 fixation activity in the water column, isolates and culture manipulation experiments will be necessary. Once more genomes become available, these can also provide information about the characteristics and lifestyles of marine diazotrophs and provide a platform for further molecular studies.

In this thesis, symbionts of heterotrophic dinoflagellates were investigated for their ability to fix N_2. *nifH* genes related to heterotrophic bacteria were found in samples of individual dinoflagellates, suggesting a symbiotic co-existence of non-diazotrophic cyanobacteria and N_2-fixing heterotrophic bacteria in heterotrophic dinoflagellates. In the future, single-cell techniques may be used to study and quantify the metabolic activities of these consortia to further understand the possible symbiotic relationship.

In the light of recent findings of underestimation of the $^{15}N_2$ incorporation technique (the most commonly used method to determine N_2 fixation rates), the gap between N loss and gain may not be as large as previously thought. However, the large distribution of heterotrophic diazotrophs found in this thesis, also in areas void of cyanobacteria, suggests that measurements of heterotrophic N_2 fixation and sampling in waters, which have previously not been investigated for N_2 fixation, could increase the current estimates of N input.

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