Denitrifying and nitrous oxide reducing genotypes

Ecophysiology and niche differentiation

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Abstract

Denitrification is a biogeochemical process of major importance for nitrogen loss from ecosystems. This four-step pathway is modular, as organisms can have different subsets and variants of the genes involved in each step. The last step is the only known biological sink of nitrous oxide, a potent greenhouse gas and ozone depleting substance. The aim of this thesis was to assess whether specific environmental conditions favour certain denitrifying and nitrous oxide reducing genotypes. The effects of nitrogen, carbon and oxygen availabilities, as well as habitat type and diversity were examined in studies of denitrifying and nitrous oxide reducing microorganisms in pure cultures, enrichment cultures and natural communities from coastal sediments. By utilizing molecular techniques and directly targeting functional genes encoding for nitrite and nitrous oxide reductases, this work explores the link between genetic potential and functionality of denitrifying and nitrous oxide reducing microbial communities.

Microorganisms harbouring genes for complete denitrification dominated in coastal marine sediments, irrespective of oxygen regime and habitat type, which suggests they have an important role not only for nitrogen removal, but also nitrous oxide reduction in coastal ecosystems. However, oxygen affected the nitrous oxide reducing communities. The results indicate niche differentiation between nitrous oxide reducers in relation to oxic/anoxic conditions, as specific lineages within the nitrous oxide reductase gene phylogeny were favoured by certain oxygen regimes. In enrichment cultures, complete denitrifiers were competitive nitrous oxide reducers and could outcompete organisms only capable of nitrous oxide reduction when subjected to carbon and nitrous oxide limitation. For denitrifiers, functional difference between the genes *nirS* and *nirK* encoding two structurally different nitrite reductases within the same organism was observed, corroborating that closely related and almost identical genotypes differ in their denitrification activity. Furthermore, primers targeting nitrite reducing communities harbouring either *nirS* or *nirK* were re-evaluated and clade-specific primers are suggested. Finally, a framework for primer evaluation using metagenomes is suggested.

These results highlight the importance of accounting for the genetic potential of denitrifying and nitrous oxide reducing communities to better understand overall ecosystem constraints and how environmental factors might control this potential.

Keywords: Denitrifying microorganisms, nitrous oxide reducers, genetic potential, *nirK*, *nirS*, *nosZ*, marine sediment, gene expression, enrichment, diversity

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Denitrifierande och lustgasreducerande genotyper. Ekofysiologi och nischdifferentiering

Sammanfattning

Denitrifikation är en biogeokemisk process som har stor betydelse för kväveförlust från ekosystem. Processen består av fyra steg och är modulär; organismer kan ha genetisk potential för att utföra alla steg eller enbart vissa av stegen och i varje steg finns det varianter av generna. Det sista steget är den enda kända biologiska sänkan av dikväveoxid (lustgas), en potent växthusgas och ozonförstörande substans. Syftet med denna avhandling var att bedöma om specifika miljöförhållanden gynnar vissa denitrifierande och lustgasreducerande genotyper. Effekterna av kväve-, kol- och syretillgänglighet samt av habitattyp och diversitet undersöktes i studier av denitrifierande och lustgasreducerande mikroorganismer i renkulturer, anrikningskulturer och i naturliga samhällen från kustsediment. Detta arbete undersöker sambandet mellan genetisk potential och funktionalitet hos denitrifierande och lustgasreducerande mikrobiella samhällen genom att använda molekylära tekniker där funktionella gener som kodar för nitrit- och dikväveoxid-reduktaser utgör målet.

Mikroorganismer som har gener för fullständig denitrifikation dominerade i kustnära sediment, oberoende av syreförhållanden och habitattyp, vilket tyder på att de har en viktig roll inte bara för att ta bort kväve, utan också för att reducera lustgas i kustekosystem. Syre påverkade emellertid de lustgasreducerande samhällena. Resultaten indikerar att det förekommer nischdifferentiering mellan lustgasreducerande mikroorganismer i förhållande till oxiska/anoxiska betingelser, eftersom vissa syreregimer gynnade specifika fylogenetiska linjer av dikväveoxidreduktaser. I anrikningskulturer var kompletta denitrifierare konkurrenskraftiga och kunde konkurrera ut lustgasreducerande organismer när kol och lustgas var begränsande. För denitrifierare observerades funktionell skillnad mellan generna *nirS* och *nirK* som kodar för två strukturellt olika nitritreduktaser i samma organism, vilket bekräftar att även nära besläktade och nästan identiska genotyper skiljer sig åt i denitrifikationsaktivitet. Vidare utvärderades primrar för nitritreducerande samhällen, *nirS*- och *nirK*-primrar, och gruppspecifika primrar föreslås. Slutligen föreslås ett ramverk för primerutvärdering med hjälp av metagenom.

Resultaten i avhandlingen lyfter fram vikten av att räkna med den genetiska potentialen hos denitrifierande och lustgasreducerande mikrobiella samhällen för att bättre förstå övergripande ekosystembegränsningar och hur miljöfaktorer kan kontrollera denna potential.

For Ute

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This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Conthe M, Wittorf L, Kuenen JG, Kleerebezem R, van Loosdrecht MCM, Hallin S. Life on N_2O: deciphering the ecophysiology of N_2O respiring bacterial communities in a continuous culture. (submitted)

II Wittorf L, Bonilla-Rosso G, Jones CM, Bäckman O, Hulth S, Hallin S (2016). Habitat partitioning of marine benthic denitrifier communities in response to oxygen availability. *Environmental Microbiology Reports* 8 (4), 486-492.
Corrigendum: Wittorf L, Bonilla-Rosso G, Jones CM, Bäckman O, Hulth S, Hallin S (2016). Habitat partitioning of marine benthic denitrifier communities in response to oxygen availability. *Environmental Microbiology Reports* 8 (5), 936-936

III Wittorf L, Roger F, Alsterberg C, Gamfeldt L, Sundbäck K, Hulth S, Jones CM, Hallin S. Ecosystem complexity shapes nitrous oxide reducing and denitrifying communities in coastal sediments. (submitted)

IV Wittorf L, Jones CM, Bonilla-Rosso G, Hallin S. Expression of *nirK* and *nirS* genes encoding structurally distinct NO-forming nitrite reductases within the same strain of *Pseudomonas stutzeri*. (manuscript)

V Bonilla-Rosso G, Wittorf L, Jones CM, Hallin S (2016). Design and evaluation of primers targeting genes encoding NO-forming nitrite reductases: implications for ecological inference of denitrifying communities. *Scientific Reports* 6 (39208).

The contribution of Lea Wittorf to the papers included in this thesis was as follows:

I Contributed to the molecular work, data analysis, and writing.

II Performed the molecular work and data analysis and did a large part of the writing.

III Performed the molecular work and the data analysis as well as most of the writing.

IV Designed the experiment, performed all experimental work, the data analysis, and was main responsible for the writing.

V Contributed to the planning of the work, the analysis and interpretation and discussion of the results.

In addition to the listed papers included in this thesis, Lea Wittorf has contributed to the following papers within the time frame of the thesis work:

Roger F, Alsterberg C, Wittorf L, Sundbäck K, Hulth S, Hallin S, Gamfeldt L. Can we predict ecosystem functioning using tightly linked functional gene diversity? (manuscript)

Conthe M, Wittorf L, Parchen C, Kuenen JG, Kleerebezem R, Hallin S, van Loosdrecht MCM. Chemostat enrichment cultures of N_2O respiring bacteria – a sequel. (manuscript)

Abbreviations

anammox	anaerobic ammonium oxidation
DNRA	dissimilatory nitrate reduction to ammonium
N_2	dinitrogen/molecular nitrogen
N_2O	nitrous oxide
napA	gene encoding periplasmic nitrate reductase
narG	gene encoding cytoplasmic nitrate reductase
NH_4^+	ammonium
nirK	gene encoding copper nitrite reductase
nirS	gene encoding heme nitrite reductase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
norB	gene encoding nitric oxide reductase
nosZ	gene encoding nitrous oxide reductase

1 Introduction

Molecular nitrogen (N_2) is the major component of the atmosphere. It is inert and little involved in chemical reactions with other elements in the atmosphere. However, nitrogen is also one of the major elements in the biosphere and therefore of great importance for all forms of life. Its natural conversion to bioavailable ammonium (NH_4^+) is often viewed as the beginning of the nitrogen cycle, a set of five biogeochemical pathways involved in biological nitrogen transformations, mostly mediated by microorganisms (Fig. 1; Stein and Klotz, 2016). Since the beginning of the last century the bioavailable nitrogen pool generated by natural nitrogen fixation has been extended by the industrial production of ammonia through the Haber-Bosch process, which has led to a considerable increase in reactive nitrogen in the environment. This has led to serious environmental problems associated with eutrophication and emissions of nitrous oxide (N_2O; Howarth et al., 2011; Rockström et al., 2009; Thomson et al., 2012). This greenhouse gas and ozone-depleting substance N_2O has a 300 times higher warming potential compared to carbon dioxide over a time period of 100 years and is thereby very potent (IPCC, 2013; Ravishankara et al., 2009).

Denitrification is a process during which nitrate (NO_3^-) is reduced step-wise to N_2 and it thereby removes nitrogen from the environment. It is a facultative respiratory pathway mainly occurring under anaerobic conditions during which the involved microorganisms switch from oxygen respiration to respiration of nitrogen oxides for energy conservation (Zumft, 1997). As this process leads to the removal of nitrogen from the ecosystem, it can be an ecosystem service in systems with detrimental nitrogen loadings, such as coastal marine sediments or wastewater treatment plants. On the other hand, it can cause unwanted nitrogen losses from systems such as arable land, where nitrogen is added as fertilizer for crops. Similarly, the process of denitrification produces the greenhouse gas N_2O, which can be emitted to the atmosphere as an alternative end product of the pathway. However, denitrification is also the only known biological sink of N_2O as the last step in the pathway is the reduction of N_2O to N_2 (Hallin et al., 2017).

The ability to denitrify is a wide-spread trait harboured by a huge diversity of different bacterial, archaeal and fungal taxa present in most environments, both terrestrial as well as aquatic (Graf et al., 2014). Denitrifying microorganisms often do not account for more than ~5% of the total microbial community, even though they are of major importance for ecosystem functioning (Hallin et al., 2009; Henry et al., 2006; Jones et al., 2013). These circumstances make it difficult to study this functional group with methods such as traditional 16S rRNA sequencing, as the taxonomy of the organisms is not always linked to their ability to denitrify and even closely related organisms can differ in their ability to denitrify (Jones et al., 2008). Due to the low abundances of denitrifying microorganisms in the environment, it is also difficult to determine their abundance, diversity and community composition with methods such as metagenomics. Therefore, functional genes have been used as molecular markers to directly target the denitrifying communities. Most studies on the ecology of denitrifying communities are thus based on the genes for NO_3^- reduction (*narG*, *napA*), nitrite (NO_2^-) reduction (*nirS*, *nirK*), nitric oxide (NO) reduction (*norB*), or N_2O reduction (*nosZ*) (Braker et al., 1998; Braker and Tiedje, 2003; Flanagan et al., 1999; Hallin and Lindgren, 1999; Philippot, 2002; Philippot et al., 2007; Scala and Kerkhof, 1998; Throbäck et al., 2004). This trait-centred approach makes it possible to directly link the denitrifying community to the biogeochemical process denitrification, and thereby to community and ecosystem functioning (Philippot and Hallin, 2005).

The denitrification pathway, as the nitrogen cycle in general, is highly modular. Thus, organisms can have different sub-sets of the denitrification genes and perform different reactions within the pathway (Graf et al., 2014; Zumft, 1997). In addition, some of the steps can be catalysed by two different enzymes. These enzymes have evolved over a long time frame and their phylogenies show distinct clades (Jones et al., 2008). Accordingly, the phylogeny of the N_2O reductase gene *nosZ*, was recently described to consist of two distinct clades with potential ecological differences (Hallin et al., 2017; Jones et al., 2013; Sanford et al., 2012). This results in i) a wide range of possible combinations of genes encoding for the different steps within the pathway, ii) the presence of multiple enzymes responsible for the same reactions, and iii) a great genetic diversity within each of the gene phylogenies. This raises questions about the differences between the specific denitrifying genotypes.

Patterns of specific denitrifying and N_2O reducing community composition have been reported, which suggests the possibility of niche differentiation between organisms with different denitrifying and N_2O reducing genotypes (e.g. Desnues et al., 2007; Enwall et al., 2010; Juhanson et al., 2017; Sun et al., 2017; Yuan et al., 2012). By determining which steps of the pathway, specific enzymes

and enzyme, variants are abundant within a community, the presence and abundance of different genotypes affects the genetic potential for denitrification and N_2O reduction. The genetic potential for denitrification is defined as the size, composition and diversity of the denitrifying and N_2O reducing community. Moreover, the presence of specific denitrifying and N_2O reducing genotypes has been shown to affect the enzymatic N_2O reduction potential from soils (Domeignoz-Horta et al., 2016; Philippot et al., 2011) and the genetic potential for N_2O reduction had a negative effect on actual fluxes measured in the field (Domeignoz-Horta et al., 2017). Therefore, understanding what factors favour specific denitrifying genotypes and thereby determine the genetic potential for denitrification and N_2O reduction in the environment is of major interest. To date, there is little known about the environmental factors affecting specific denitrifying and N_2O reducing genotypes. Thus, questions about their ecological roles remain, as well as how this affects the genetic potential for denitrification and N_2O reduction of the community, and thereby the nitrogen removal and N_2O reduction capacity of ecosystems.

1.1 Aims and objectives of the thesis

The main aim of this thesis was to address whether specific environmental factors favour or disfavour certain denitrifying and N_2O reducing genotypes and how this affects the genetic potential for denitrification and N_2O reduction of the communities. By assessing differences in the ecophysiology and niche differentiation between different genotypes, we were expecting to improve our understanding of the role of the substantial diversity of denitrifying and N_2O reducing genotypes and how that may affect functionality of denitrifying communities. Since the presence and abundance of specific genotypes in a community is linked to its genetic potential for denitrification and N_2O reduction, we aimed to further determine the effect of environmental factors controlling the nitrogen removal and N_2O reduction capacity of our model system, coastal sediments. To address which genotypes are favoured under which conditions we quantified denitrifier and N_2O reducer activity, abundance and diversity in response to environmental factors known to affect denitrifying communities, such as oxygen, nitrate and carbon availability as well as habitat type and diversity. This thesis focuses on denitrifying and N_2O reducing genotypes by directly targeting the *nirK* and *nirS* genes for NO_2^- reduction and the *nosZ* gene for N_2O reduction.

The aim of **paper I** was to compare the N_2O affinity and reduction efficiency between organisms harbouring *nosZ* genes from the two clades within the gene phylogeny. We investigated which N_2O reducing microorganisms were selected

from a complex community in sludge from a wastewater treatment plant in an enrichment culture exposed to either N_2O (electron-acceptor) or acetate (electron-donor) limiting conditions. This was expected to delineate differences in the ecophysiology of distinct N_2O reducing genotypes.

In **paper II** and **III** we expanded to natural sediment communities from two different coastal marine environments. **Paper II** aimed to specify which N_2O reducing genotypes were favoured under different oxygen regimes (oxic, anoxic, and fluctuating oxic and anoxic conditions), determine the overall effect of oxygen regimes on abundances of denitrifying and N_2O reducing genotypes, and identify potential niche differentiation between N_2O reducing genotypes in response to oxygen. **Paper III** investigated the importance of specific habitat types in an ecosystem for the abundance and diversity of distinct denitrifying and N_2O reducing genotypes. The aim was to determine whether habitat diversity had a positive effect on the genetic potential for nitrogen removal through denitrification in shallow-water sediments.

The following projects focused on the reduction of NO_2^-, a step that is catalysed by two structurally different, non-homologous enzymes encoded by the genes *nirS* and *nirK*. The aim of **paper IV** was to determine functional differences between the two NO_2^- reductases encoded by the genes *nirK* and *nirS* within the same organism. Specifically we examined if both *nir* genes are functional and therefore expressed in the same organism even though they catalyse the same reaction. In **paper V**, the aim was to improve the molecular tools to target NO_2^- reducers in the environment in order to get a more differentiated understanding of the role of different NO_2^- reducing genotypes in the future.

2 Denitrification and N_2O reduction

Denitrification is the microbial pathway reducing the soluble anions NO_3^- and NO_2^- to the gases NO, N_2O and N_2. The chemical reduction of NO_3^- and NO_2^- can also be catalysed by metal ions at low pH, but denitrification is mainly driven by microorganisms (Buchwald et al., 2016; van Cleemput, 1998). It is a facultative respiratory pathway and all steps are coupled to the electron transport chain and thereby involved, although indirectly, in generating a proton motive force across the cytoplasmic membrane (van Spanning et al., 2007; Zumft, 1997). Denitrification is usually an anaerobic process and most denitrifying microorganisms switch to oxygen respiration as soon as oxygen is available, as this leads to higher energy conservation and both processes use mostly the same respiratory machinery (Chen and Strous, 2013). In the environment, denitrification can be performed by a variety of different microbial taxa. Overall, denitrifying microorganisms across 18 different phyla are known (Graf et al., 2014). Bacterial taxa containing high numbers of denitrifying species are Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. In addition, some Fungi within the Ascomycota, and Archaea from different taxa have been described to denitrify (Graf et al., 2014; Philippot et al., 2007; Shoun and Tanimoto, 1991; Zumft, 1997). The modularity of the denitrification pathway allows for a wide variety of denitrifying genotypes with different sub-sets of the reduction steps, however, organisms capable of only a single reduction step might not always use this reaction for energy conservation. Therefore, the definition of denitrification used in this thesis is the reduction of the soluble anions NO_3^- or NO_2^- to the gases N_2O or N_2 for energy conservation. Organisms solely capable of N_2O reduction are considered its own functional group of non-denitrifier N_2O reducers. Primarily, because they contribute to the unique ecosystem service of acting as a N_2O sink by reducing N_2O.

2.1 Denitrification pathway

The denitrification pathway consists of four separate energy conserving reactions, some of which can be catalysed by several structurally different enzymes (Fig. 1). Thus, the first step, the reduction of NO_3^- to NO_2^-, is catalysed either by the cytoplasmic NO_3^- reductase encoded by the gene *narG* or the periplasmic NO_3^- reductase encoded by *napA* (Richardson et al., 2007). Organisms harbouring these genes are not necessarily denitrifiers and the NO_2^- generated in this reaction can be used in a variety of other nitrogen cycling pathways e.g. anaerobic oxidation of NH_4^+ (anammox) and dissimilatory NO_3^- reduction to NH_4^+ (DNRA; Stein and Klotz, 2016). Among the two different NO_3^- reductase systems the *napA* gene is more widespread among bacteria with different lifestyles such as fermentative, phototrophic or denitrifying, while *narG* is mostly connected to energy conservation and thus to denitrifying microorganisms (Chen and Strous, 2013; Simon and Klotz, 2013).

The second step is the reduction of NO_2^- to NO by two structurally different, non-homologous periplasmic enzymes containing either copper or heme as a cofactor. The copper-containing enzyme belongs to the multicopper oxidase enzymes and is encoded by the gene *nirK*, whereas the heme-containing enzyme is encoded by the *nirS* gene and contains a cytochrome cd_1 in its catalytic centre (Rinaldo and Cutruzzolà, 2007). This step is the first one within the denitrification pathway that produces a gas that can potentially be lost to the atmosphere. However, due to the cytotoxicity of NO as a free radical, most organisms that have the capacity to produce NO are also able to reduce it further to N_2O (Graf et al., 2014; Shapleigh, 2006; Zumft, 1997). This reaction constitutes the third step in the denitrification pathway, which is catalysed by an integral membrane protein with several variants (De Vries et al., 2007). The ability to reduce NO to N_2O is not restricted to denitrifying microorganisms and is often used for detoxification in non-denitrifying microorganisms (Philippot, 2005; Zumft, 1997). Within denitrifying microorganisms, variants of the *norB* gene encode for respiratory NO reductases (Zumft, 2005).

The final step of the denitrification pathway is the reduction of N_2O to N_2. This step is catalysed by a multicopper enzyme, the N_2O reductase, encoded by the gene *nosZ*. It is the only known enzyme capable of this reaction and therefore the only known biological sink of N_2O (Hallin et al., 2017; Zumft and Körner, 2007). This makes the enzyme and organisms with this function particularly interesting and indicates their importance as N_2O sink in ecosystems.

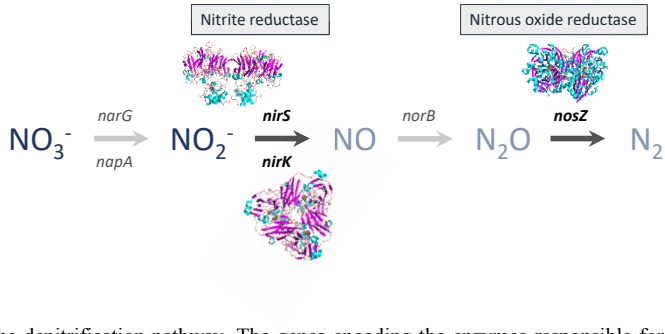

Figure 1. The denitrification pathway. The genes encoding the enzymes responsible for each step are indicated above and below the arrows. The two steps this thesis focuses on are highlighted, with the protein structures of the three enzymes catalysing these reactions. Soluble and gaseous products are indicated in dark and light blue, respectively.

Most denitrifying microorganisms have highly regulated pathways to keep NO_2^- and especially NO below cytotoxic concentration and there are shared as well as specific regulators for the different steps. Different denitrifying organisms might have distinct regulatory systems, but oxygen and the involved nitrogen compounds are key components affecting gene expression, and thereby denitrification activity (Ka et al., 1997; Rodionov et al., 2005; van Spanning, 2011).

2.2 Modularity of the denitrification pathway

The modularity of the denitrification pathway partly derives from the fact that organisms do not always harbour the full set of genes for complete denitrification. This is further complicated by the existence of different reductase systems for some of the steps and the two clades in the *nosZ* phylogeny (Jones et al., 2013; Sanford et al., 2012). There are several possible combinations, but genome comparisons have illustrated a prevalence of specific combinations of genes in the genomes of denitrifying microorganisms (Graf et al., 2014). About 80% of the genomes that harbour a *nirS* gene also harbour the gene for *nosZ* and are thus capable of complete denitrification from NO_2^- to N_2. By contrast, 70% of genomes that harbour a *nirK* gene do not have a *nosZ* gene, which makes these organisms potential sources of the greenhouse gas N_2O. There is also a clear trend among genomes containing *nosZ* genes belonging to either of the two

clades. While 83% of genomes with *nosZ* clade I also harbour a *nir* gene and thus are able to reduce NO_2^- to N_2, 52% of organisms with *nosZ* clade II are non-denitrifier N_2O reducers (Fig. 2; Graf et al., 2014). This makes organisms with *nosZ* clade II particularly interesting as potential N_2O sinks.

The above-mentioned gene co-occurrences are not randomly distributed among denitrifying organisms from different taxa. Most Proteobacteria, especially Beta- and Gammaproteobacteria, are unlikely to have a partial pathway and most often possess *nir* and *nosZ* genes. Within the Proteobacteria, *nirK* occurs most often within the Rhizobiales, whereas *nirS* is overrepresented in denitrifying Pseudomonadales. Moreover, non-denitrifying N_2O reducers are common among Bacteroidetes, Deltaproteobacteria, Firmicutes and Euryarchaeota (Graf et al., 2014). This is in congruence with research showing the presence of *nirS* and *norB* associated to Beta- and Gammaproteobacteria and *nosZ* associated to Gemmatimonadetes and Myxococcales in metagenomes from estuary sediments (Baker et al., 2015). This suggests that the denitrification pathway can be split between these organisms and that N_2O is not produced and reduced within the same organism.

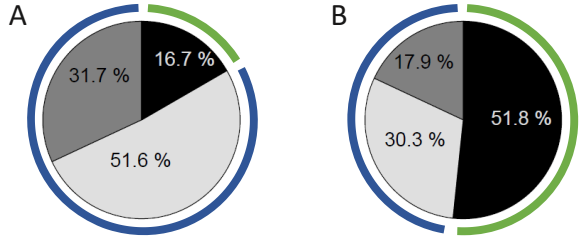

Figure 2. Gene co-occurrences of nitrous oxide reductase genes A) *nosZ* clade I and B) clade II with nitrite reductase genes *nirS* and *nirK* in genomes of denitrifying microorganisms. Numbers within the pie chart indicate percentage of genomes with the respective combination of *nirS* and *nosZ* (dark grey), *nirK* and *nosZ* (light grey) and only *nosZ* (black). Colours on the outside of the pie charts highlight the relative amount of genomes harbouring *nir* and *nosZ* genes (blue) and genomes with only *nosZ* (green). Based on data from Graf et al., 2014.

2.3 Denitrifying and N_2O reducing communities in natural environments

Denitrifying and N_2O reducing communities are present in most environments and, on a global scale, denitrification activity is particularly high in continental shelf sediments and soils (Seitzinger et al., 2006). Other environments with high denitrification rates are oceanic oxygen minimum zones, estuaries, wetlands and engineered systems, such as wastewater treatment plants. Denitrification rates and the end-product ratio between N_2O and N_2 can be influenced by a variety of abiotic factors, such as oxygen concentrations, temperature, nitrate levels, pH, and availability of organic carbon (Cuhel et al., 2010; Davidson and Swank, 1986; Seitzinger et al., 2006; Šimek and Cooper, 2002; Wallenstein et al., 2006). It has also been discussed that denitrification peaks under particularly favourable conditions in both space and time (Groffman et al., 2009). However, denitrification and N_2O reduction is ultimately determined by the abundance, structure and composition of the denitrifying and N_2O reducing microbial community (Wallenstein et al., 2006). Models predicting process rates of carbon and nitrogen cycling improved by incorporation of microbial community data (Graham et al., 2016; Powell et al., 2015). Accordingly, community composition and the presence and abundance of specific denitrifying and N_2O reducing genotypes has an influence on the denitrification end-product ratio (N_2O:N_2) and denitrifying community functioning (Cavigelli and Robertson, 2001; Domeignoz-Horta et al., 2016; Jones et al., 2014; Morales et al., 2010; Philippot et al., 2011, 2009).

Due to the considerable diversity of denitrifying and N_2O reducing genotypes, their communities can be influenced by a variety of different environmental factors, for example by pH and salinity (Enwall et al., 2005; Jones and Hallin, 2010; Lee and Francis, 2017; Santoro et al., 2006), two variables that have been shown to have major effects on microbial community composition in general (Fierer and Jackson, 2006; Lozupone and Knight, 2007). Although the importance of specific environmental factors can vary between habitats, other important factors seem to be oxygen concentrations, carbon and nitrogen availabilities, soil or sediment texture and ratios between carbon and nitrogen (e.g. Bowen et al., 2013; Dini-Andreote et al., 2016; Graf et al., 2016; Graves et al., 2016; Oakley et al., 2007; Santoro et al., 2006; **paper II**). Furthermore, biotic and spatial factors have been discussed in different environments for communities of NO_2^- reducers as well as N_2O reducers (Enwall et al., 2010; Juhanson et al., 2017; Lee and Francis, 2017; Scala and Kerkhof, 1999; Tsiknia et al., 2015). Denitrifying organisms harbouring either *nirS* or *nirK* have been shown to respond differently to environmental factors, which suggests niche differentiation between these organisms (Desnues et al., 2007; Enwall et al.,

2010; Jones and Hallin, 2010; Yuan et al., 2012). Similarly, contrasting patterns of abundance, diversity and community structure between organisms harbouring *nosZ* clade I compared to clade II genes have also been observed in different environments (Hallin et al., 2017; Juhanson et al., 2017; **paper II**, **paper III**). Considering the co-occurrence patterns of these genes with other denitrification genes, this might have direct effects on the genetic potential for denitrification. This highlights the importance of community composition and structure for denitrification and ecosystem functioning.

3 Ecophysiology and niche differentiation of N_2O reducers

3.1 N_2O reduction

The *nosZ* gene for the reduction of N_2O is present in a wide variety of bacterial and archaeal phyla and its phylogeny is divided into two distinct clades, called clade I and clade II (Hallin et al., 2017; Jones et al., 2013; Sanford et al., 2012). The distribution of taxonomic groups within each of the clades mostly follows the taxonomy of the organisms. While clade I mainly consists of genes belonging to the Alpha-, Beta-, and Gammaproetobacteria, clade II harbours genes from a wider variety of bacterial and archaeal groups, such as hyperthermophilic archaea, Delta- and Epsilonproteobacteria, Gemmatimonadetes and Bacteroidetes (Jones et al., 2013). The separation of the *nosZ* phylogeny into two clades opens up for questions about differences in the function and ecological roles of the two clades.

The genomic context of *nosZ* clade I and clade II genes differs. The *nosZ* gene is part of an operon consisting of several genes involved in protein maturation and electron transport to the N_2O reductase (Zumft and Kroneck, 2007). A major difference between the gene clusters of *nosZ* clade I and II is the presence of *nosR* in organisms possessing *nosZ* clade I, and the predicted protein *nosB* in organisms harbouring *nosZ* clade II, which both are most likely involved in electron transport (Hallin et al., 2017; Sanford et al., 2012; van Spanning, 2011). The only exception to this pattern are the halophilic archaea, which cluster within *nosZ* clade I but have a different genomic context compared to other organisms within clade I (Hallin et al., 2017). In addition to the differences in the genomic context, the *nosZ* clade II genes of the Epsilonproteobacteria and some other Proteobacteria possess a C-terminal extension, which could be involved in electron transfer (Jones et al., 2013; Simon et al., 2004). Moreover,

the translocation mechanisms used to transport the proteins from the cytoplasm to the periplasm differ between the clades and it is not resolved why organisms might benefit from harbouring the different secretory pathways (Jones et al., 2013; Lee et al., 2006). Altogether, these results suggest differences in the ecology and functioning of N_2O reductases encoded by *nosZ* clade I or clade II.

It has been proposed that N_2O reduction could be involved in detoxification, as high levels of N_2O could have cytotoxic effects (Sullivan et al., 2013). Moreover, the use of N_2O reductases as electron sinks during short periods of low oxygen availabilities has been proposed (Park et al., 2017). However, this is unrelated to the functional difference between *nosZ* clade I and II as it is well known that N_2O reduction is used for energy conservation by organisms harbouring both *nosZ* clade I and clade II (Payne et al., 1982; Sanford et al., 2012).

3.2 Ecophysiology of *nosZ* clade I and II organisms

N_2O reduction has been extensively studied in pure cultures of Proteobacteria with a complete denitrification pathway, such as *Paracoccus denitrificans* and *Pseudomonas stutzeri*, and there are a few early studies focusing on non-denitrifying N_2O reducing bacteria like *Wollinella succinogenes* and *Campylobacter fetus* (Payne et al., 1982; Simon et al., 2004). Due to the increased knowledge about the diversity of N_2O reducers during recent years, several studies have since been focusing on non-denitrifying N_2O reducers harbouring *nosZ* clade II, e.g. *Anaeromyxobacter dehalogenans*, *W. succinogenes*, *Bacillus vireti* and *Dyadobacter fermentas*, trying to understand the efficiency and regulation of N_2O reduction in these organisms as well as their ecological role as N_2O sinks in the environment (Domeignoz-Horta et al., 2016; Kern and Simon, 2016; Mania et al., 2016; Sanford et al., 2012). The first results about substrate affinity and N_2O consumption rates indicate that *nosZ* clade II organisms have a higher affinity to N_2O (lower whole-cell saturation constant K_s) and lower N_2O consumption rates (maximum N_2O reduction rate V_{max}), though higher biomass yields when growing on N_2O compared to organisms of clade I (Yoon et al., 2016). This indicates higher metabolic efficiency of organisms harbouring *nosZ* clade II when growing on N_2O, and Yoon et al. (2016) therefore suggested that substrate affinity is more important than consumption rate for the competition between organisms with clade I and II *nosZ* genes. If that is the case, *nosZ* clade II organisms would have a competitive advantage under substrate limitation. Since these studies only tested a few strains in pure cultures, it is not possible to generalize this to all organisms harbouring *nosZ* clade II.

To increase the understanding of the ecophysiology, the physiological response and adaptation of N_2O reducing organisms to environmental conditions, we worked with a continuous culture of N_2O reducing microorganisms enriched from activated sludge (**paper I**). Continuous culture is preferred over batch culture in this case, as it allows to apply truly limiting conditions in the culture, so that the organisms compete for growth based on their substrate affinity. Besides, continuous cultivation is more reliable to obtain growth yields than batch cultivation as the conditions are controlled and constant over longer time periods (Kuenen and Johnson, 2009). It also allowed us to study organisms with different growth rates since the dilution rate can be controlled. The use of enrichment cultures rather than pure cultures of N_2O reducers limits the bias of using well characterized model organisms, and allowed for monitoring the selection of the best adapted organisms from a community of naturally occurring N_2O reducers under a defined set of conditions. This approach has previously been beneficial when studying the ecophysiology of organisms involved in nitrogen cycling pathways (Kraft et al., 2014; van den Berg et al., 2015).

We were able to cultivate an enrichment culture growing on N_2O as the sole electron acceptor and acetate as the electron donor for an extended amount of time with growth yields similar to that of full denitrification (**paper I**), which confirms predictions about N_2O reduction and previous results (Chen and Strous, 2013; Koike and Hattori, 1975; van Spanning et al., 2007). In contrast to the hypothesis proposed by Yoon et al. (2016) that *nosZ* clade II organisms would have a competitive advantage under substrate limiting conditions, we selected for a community of denitrifying bacteria harbouring *nosZ* clade I in the enrichment (**paper I**). This was consistent over all conditions tested, irrespective whether N_2O or acetate was growth limiting. Thus, availability of N_2O was not a selective driver for neither N_2O reducers harbouring *nosZ* clade II nor non-denitrifier N_2O reducers. We selected for communities dominated by *P. stutzeri* and *Azoarcus*, depending on the dilution rate. Both harbour *nir* and *nosZ* genes and can be considered complete denitrifiers (Graf et al., 2014). Even the most abundant organism harbouring *nosZ* of clade II in the enrichment was closely related to *Dechlormonas*, which has also been shown to be involved in complete denitrification (Graf et al., 2014). The most pronounced change in community composition in the enrichment was observed after the change in dilution rate and not when switching between N_2O and acetate limiting conditions. On account of this and because *P. stutzeri* is known to have high maximum growth rates (Lalucat et al., 2006), we assume that growth rate was the main driver for the competitive advantage of organisms harbouring *nosZ* clade I in this system. Our results do not directly contradict to the results from Yoon et al. (2016) because

we did not measure a higher affinity for N_2O in organisms with *nosZ* clade I compared to those with clade II, although it is likely that substrate affinities were relatively high in the enrichment as conditions were limiting. However, our results emphasize the importance of growth rate even under limiting conditions.

Other factors that might have been responsible for the selection of organisms in the enrichment could be their ability to cope with high levels of N_2O. High levels of N_2O can limit growth as N_2O inactivates vitamin B_{12} and organisms have to adjust their metabolism using vitamin B_{12} independent pathways instead (Sullivan et al., 2013). This might explain the lower growth yields of the community that was detected under acetate limiting conditions when N_2O was highly abundant (**paper I**).

3.3 Genetic potential for N_2O reduction

The selection of a community of complete denitrifiers in the continuous culture of **paper I** indicates that complete denitrifiers are highly competitive N_2O reducers and emphasizes their importance also in natural communities. Presumably, we selected for this community because of its fast growth rate, which might not be equally important in natural communities as those tend to grow slow in the environment. Nevertheless, our results highlight that it might be advantageous to have a relatively high affinity for N_2O even for microorganisms with the complete denitrification pathway. This might increase the flexibility of the organism when conditions are changing or certain nitrogen compounds become limiting. This was proposed to be the case for *P. denitrificans*, since the organism produced N_2O under NO_3^- sufficient conditions, but optimized its use of nitrogen under limiting conditions (Felgate et al., 2012). The advantage of increased flexibility might also be the reason why we found high genetic potential for complete denitrification and high nitrogen removal capacities in the natural communities in marine sediment (**paper II** and **III**). In communities that were exposed to frequent fluctuations of oxygen availability, which most likely results in repeated switches between oxygen respiration and denitrification, the genetic potential for complete denitrification was increased (**paper II**). This directly increases the genetic potential for nitrogen removal from these coastal ecosystems.

Links between the genetic potential for N_2O reduction of a community and the end product ratio of N_2O:N_2 have been investigated in experiments altering soil communities by addition of denitrifying bacteria without the ability to reduce N_2O or non-denitrifier N_2O reducer, which either decreased or increased the N_2O sink capacity of the soil (Domeignoz-Horta et al., 2016; Philippot et al., 2011). Furthermore, N_2O reducers with *nosZ* clade II and non-denitrifier N_2O

reducers were associated with high N_2O sink capacities and low N_2O fluxes from soils (Domeignoz-Horta et al., 2017; Jones et al., 2014). In the enrichment, we selected for a community based on its capacity to use N_2O, however, this community was also capable to rapidly grow on NO_3^- when the electron acceptor regime was changed as it consisted of complete denitrifiers harbouring the genes *nirS* and *nosZ* clade I (**paper I**). Altogether, these results suggest direct links between the ecophysiology of N_2O reducers, the genetic potential for denitrification and N_2O reduction of microbial communities, and the capacity of an ecosystem to remove excess nitrogen and reduce N_2O.

3.4 Niche differentiation among N_2O reducing microorganisms

Most studies targeting *nosZ* genes of clade I and II consistently found contrasting patterns in the abundance and diversity for both clades. While detailed information is available about specific factors positively affecting *nosZ* clade I communities, there is still little information about the effects of environmental factors favouring *nosZ* clade II communities, and the mechanisms, and drivers of this probable niche differentiation are still little understood (Hallin et al. 2017). In soils, edaphic factors, such as nutrient and mineral content, soil texture, agricultural practices, or biochar amendment affected the abundance of the two clades differently (Domeignoz-Horta et al., 2017, 2015; Harter et al., 2016; Juhanson et al., 2017). Within the soil environment it has also been shown that N_2O reducers with *nosZ* clade I are associated with roots and the rhizosphere, whereas clade II is more abundant in the bulk soil (Graf et al., 2016; Truu et al., 2017). In aquatic systems, the abundance of both clades varies according to nutrient loading, oxygen availabilities and physical gradients, such as depth and sediment structure (Highton et al., 2016; Saarenheimo et al., 2015; Sun et al., 2017). Consistently, we found differences in the response towards oxygen regimes between *nosZ* clade I and II communities (**paper II**). While both communities were favoured under oscillating oxygen conditions, this effect was more pronounced for the clade I community. Moreover, community assembly processes were described to be different for *nosZ* clade I and clade II (Juhanson et al., 2017). Clade I community assembly was more often shaped by deterministic processes, such as habitat filtering and competition, which might be explained by the higher diversity found within organisms harbouring *nosZ* clade II compared to clade I. Similarly, we could observe a higher level of betadispersal in the comparably heterogeneous cyanobacterial mat sediments for *nosZ* clade I but not for *nosZ* clade II communities (**paper III**). Overall, these

results suggest niche differentiation between organisms harbouring the two clades.

In **paper II** we detected specific lineages within the *nosZ* phylogeny that were affected differentially by the imposed oxygen regimes. While community membership in terms of presence/absence did not vary substantially between the oxygen regimes, changes in relative abundance of specific lineages could be detected according to oxygen regime. We used the phylogeny-based method pplacer for this analysis, which places all obtained *nosZ* reads into a reference phylogeny containing full-length *nosZ* sequences from the genomes of known N_2O reducers (Matsen et al., 2010). Subsequent analysis of the phylogenetic placement by edge principal component analysis revealed a separation of the samples by oxygen regime (Fig. 3). The method detects which edges in the overall phylogeny contributed most to the differences between the samples. The relative contribution of the individual edges to each principal component axis is then mapped into the reference phylogeny (Matsen and Evans, 2011).

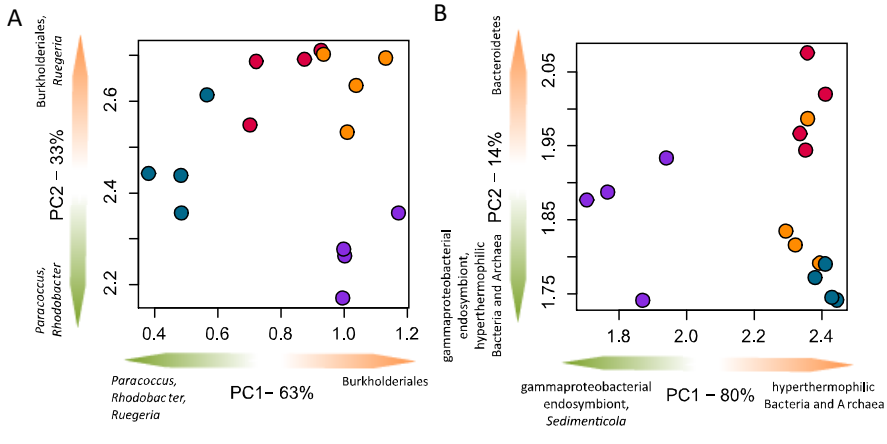

Figure 3. Edge principal component analysis of A) *nosZ* clade I and B) *nosZ* clade II communities showing differences in community structure in response to oxygen regimes. The lineages in the reference phylogeny that contribute the most to the separation in principal components (PC) 1 and 2 are indicated for each axis. The variance explained is indicated for each axis. Colours correspond to the oxygen regimes constantly anoxic (purple), constantly oxic (blue), oscillating conditions, starting oxic (orange), and oscillating conditions starting anoxic (red). From **paper II**.

Under constantly oxic conditions we could detect an increase of reads closely related to *Paracoccus* spp. and *Rhodobacter* spp. and organisms within these two taxa are usually capable of complete denitrification (Fig. 3). *Paracoccus denitrificans* has been described to denitrify aerobically linking heterotrophic nitrification and denitrification, which might be particularly useful under nitrate limitation (Arts et al., 1995; Robertson and Kuenen, 1990). The oscillating conditions, supported communities with higher relative abundance of reads closely related to Burkholderiales and *Ruegeria*, both of which are capable of complete denitrification (Graf et al., 2014). Oscillating oxygen conditions could be favourable for organisms capable of denitrification, as it is a facultative anaerobic respiratory pathway. Additionally, many *nosZ* clade II reads within these samples were closely related to Bacteroidetes, a high proportion of which are described to solely harbour the genes for N_2O reduction.

It is important to note that denitrifying and N_2O reducing organisms might not necessarily respond to biotic and abiotic factors due to their ability to denitrify, but possibly due to other traits they possess. However, their relative abundance directly affects the genetic potential for denitrification of the community. For example, Juhanson et al. (2017) revealed a predominance of *nosZ* clade I organisms belonging to *Bradyrhizobium* and *Mesorhizobium* in agricultural fields that were not fertilized with inorganic nitrogen fertilizer. Both bacterial taxa are capable of nitrogen fixation, which could explain their increased abundance in these samples.

In conclusion, there is indication of complex niche differentiation among N_2O reducers both between and within the two clades and oxygen gradients have the potential to drive community structure of N_2O reducing communities in the environment.

4 Denitrifying and N_2O reducing communities in coastal marine sediments

4.1 Nitrogen cycling in marine sediments

About 230 Tg nitrogen is processed in the marine environment every year, an amount that is similar to that of terrestrial systems. A high proportion of this nitrogen is introduced into the system via biological nitrogen fixation. However, about 80-100 Tg nitrogen per year is also brought in from terrestrial systems into the marine system, a large proportion of which is directed through freshwaters (Billen et al., 2013; Fowler et al., 2013). Nitrogen concentrations in the oceans are subject to large temporal and spatial variation with particularly high levels in coastal zones and colder climates. Furthermore, the transport of nitrogen compounds in the oceans is slow, which reduces turnover rates. Therefore, oceans retain nitrogen for long time periods. This highlights the importance of nitrogen removal processes in coastal regions to prevent nitrogen from terrestrial systems to enter the open ocean (Fowler et al., 2013).

Coastal sediments are hot spots of nitrogen turnover. They are crucial for benthic nitrogen removal (Codispoti, 2007; Gruber, 2008) and denitrification is the major nitrogen removal pathway (Fig. 4; Deutsch et al., 2010; Hulth et al., 2005; Marchant et al., 2014; Middelburg et al., 1996; Seitzinger et al., 2006). This is consistent with denitrification gene abundance data from fjord sediments in **paper II**, where the denitrification genes had significantly higher abundances than the genes for anammox or DNRA. In addition to denitrification, nitrogen can be removed from marine systems by anammox, which occurs under anaerobic conditions and can contribute considerably to the nitrogen removal from the marine environment globally (Fig. 4; Dalsgaard et al., 2005). Beyond that, DNRA is an important NO_3^- reduction pathway in coastal sediments, but it

leads to nitrogen retention rather than removal from the ecosystem (Dong et al., 2011; Gardner et al., 2006; Giblin et al., 2013). Denitrification and DNRA compete for NO_3^- and, even though denitrification is the major process in many marine sediments, DNRA can outcompete denitrification when carbon to nitrogen ratios are high in an environment (Kraft et al., 2014; Marchant et al., 2014; van den Berg et al., 2015). This competition has been suggested to favour anammox, which then can act as the major nitrogen removal pathway in sediments where DNRA is dominating (Kartal et al., 2007; Lam et al., 2009; Salk et al., 2017).

The prevalence and relative importance of each of the nitrogen cycling pathways in marine sediments is influenced by multiple factors, such as nitrogen availabilities, organic carbon levels, and oxygen concentrations. In **paper II** we tested the effect of oxygen on the nitrogen cycling communities. Even though denitrification was always the dominant pathway according to gene abundances, we could observe relatively higher abundances of the genes for DNRA in constantly anoxic sediments. By contrast, genes for nitrification became more abundant in constantly oxic sediments. As expected, denitrification genes were relatively more abundant in sediments with oscillating oxygen regimes. However, we also detected a high abundance of these genes under oxic conditions, suggesting the presence of aerobic denitrification. This has been reported in permeable sediments as an adaptation to fluctuating redox conditions (Gao et al., 2010; Marchant et al., 2017; Rao et al., 2007).

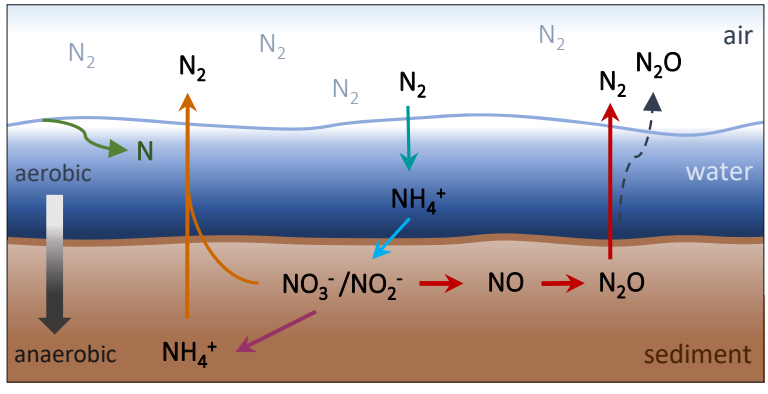

Figure 4. Schematic of the inorganic nitrogen cycle in marine systems. Arrows with different colours indicate the different pathways; nitrogen fixation (turquoise), nitrification (blue), denitrification (red), anammox (orange) and DNRA (purple). Nitrogen entering from terrestrial systems is indicated in green. Nitrous oxide losses to the atmosphere are included as dotted arrow.

In addition to the nitrogen removal or retention capacity of marine sediments, the prevalence of any of the three NO_2^- reducing pathways can influence N_2O emissions from coastal ecosystems. High levels of denitrification in marine sediments can lead to emissions of N_2O, whereas anammox does not produce N_2O, and sediments where DNRA dominates seem to emit relatively low levels of N_2O (Kartal et al., 2012; Salk et al., 2017). Our data suggests that all sediments investigated within this thesis harboured mostly denitrifying microorganisms capable of complete denitrification (**paper II** and **III**). Within the illuminated shallow-water sediments we found, additionally, high abundances of organisms harbouring *nosZ* clade II genes (**paper III**). Altogether, these results indicate a high genetic potential for N_2O reduction especially in the shallow-water sediments. This is in agreement with ratios of N_2O:N_2 fluxes of less than 2% in estuarine environments (Murray et al., 2015).

4.2 Abundance of denitrifying and N_2O reducing genotypes in marine sediments

In marine ecosystems, denitrifying communities are often dominated by organisms harbouring *nirS* rather than *nirK* nitrite reductase genes, whereas both *nir* genes show similar abundances in soil environments (Abell et al., 2010; Enwall et al., 2010; Hallin et al., 2009; Jones and Hallin, 2010; Mosier and Francis, 2010). This was consistent with our results from **paper II** and **III**, as *nirS* harbouring organisms were dominating denitrifying communities in both deep fjord and illuminated shallow-water sediments. This pattern was even more pronounced in the deeper fjord sediments, where *nirS* genes accounted for the majority of total *nir* gene abundance (**paper II**). In the shallow-water sediments, *nirK*/*nirS* ratios were higher, which might be due to the influence of the adjacent terrestrial system and its communities (**paper III**). Comparably, *nosZ* clade I is most often equally or more abundant than clade II in aquatic systems (Hallin et al., 2017), and the deeper fjord sediments were dominated by N_2O reducers with *nosZ* clade I (**paper II**). This was in contrast to abundances in shallow-water sediments where *nosZ* clade II was slightly more abundant than clade I (**paper III**).

In general, salinity, nutrient loading – especially nitrogen availability – oxygen concentration, and sediment structure have been shown to affect denitrifying genotypes harbouring *nirS* or *nirK* and *nosZ* clade I or II differently (Desnues et al., 2007; Graves et al., 2016; Lee and Francis, 2017; Santoro et al., 2006). The results from the microcosm experiment in **paper II** indicate that oscillating oxygen availabilities positively affect the denitrifier community and can lead to increased abundances of all denitrification genes, but especially *nirS*

and *nosZ* clade I were affected, both of which are associated with complete denitrification (Graf et al., 2014). As denitrification is a facultative pathway it gives the organisms a competitive advantage under fluctuating conditions that other organisms might lack. Additionally, a complete denitrification pathway might be energetically favourable under NO_3^- limited conditions (Felgate et al., 2012). This was the case in these microcosms as they were not supplemented with nitrogen during the incubation and all NO_3^- was either originally present in the sediment or produced from NH_4^+ by nitrifying organisms under oxic conditions.

We observed a clear difference in denitrification gene abundance between the four different habitats from the shallow-water ecosystem (**paper III**). All three genes, *nirS*, *nirK* and *nosZ* clade I and II, were significantly more abundant in silty-mud and sandy sediments compared to *Ruppia maritima* meadow and cyanobacterial mat sediments. Overall, these results emphasize the importance of these habitat types and the positive effect of fluctuating oxygen regimes on the genetic potential for denitrification, and thereby on the nitrogen removal capacity of the ecosystem.

4.3 Diversity and composition of N_2O reducing communities

The structure of the N_2O reducing communities was significantly affected by habitat type (**paper III**). This highlights the importance of potentially complex environmental factors shaping denitrifying and N_2O reducing communities in coastal sediment ecosystems, as these habitats have been described as quite variable regarding their environmental descriptors (Alsterberg et al., 2017). The relative abundance and community composition of N_2O reducing microorganisms with clade I and II has been shown to be affected by oxygen, nutrient availability and salinity in marine systems (Graves et al., 2016; Sun et al., 2017; **paper II**). We therefore expected that community composition would differ between habitat types. Our results demonstrate that the diversity of the *nosZ* clade I community was positively affected by dissolved organic nitrogen in the pore water of the sediments (**paper III**). While this was consistent with previous findings that could detect responses of the denitrifying and N_2O reducing communities to nitrogen availabilities (Graves et al., 2016; Hannig et al., 2006; Liu et al., 2003), this was not apparent for the *nosZ* clade II community. This might be due to the fact that many organisms carrying *nosZ* clade II are not denitrifying and might respond differently to nitrogen availabilities (Graf et al., 2014). Accordingly, co-occurrences of *nosZ* clade II and the genes for DNRA have been detected in some genomes and might

indicate that these N_2O reducers are involved in this pathway (Giblin et al., 2013; Sanford et al., 2012). Furthermore, some microorganisms with *nosZ* clade II have been found to be obligate anaerobic in contrast to mostly facultative anaerobic denitrifiers (Payne et al., 1982; Simon et al., 2004).

Overall, alpha diversity of the N_2O reducing community was significantly increased in silty-mud and sandy sediments, which suggests an important role of these two habitat types for sustaining high nitrogen removal capacities in coastal ecosystems. Lower diversity of denitrifying communities was previously shown to be associated to lower potential denitrification activity in agricultural soil (Philippot et al., 2013). Another interesting aspect of our results was the increase in gamma diversity with increasing habitat diversity that was detected for both clades of *nosZ* (**paper III**). This indicates that loss of habitat diversity on the ecosystem scale would lead to lower gamma diversity. Thus, even though the effect of habitat type was more pronounced, habitat diversity would be important for the overall ecosystem.

It has been shown that high diversity in the denitrifying communities results in broader operating ranges for denitrification in soil (Hallin et al., 2012), as it could increase the chance of maintaining high denitrification activities, and thereby high levels of nitrogen removal, under fluctuating or changing conditions (Yachi and Loreau, 1999). This could explain the discrepancy between the denitrifier abundances and diversity observed in **paper III,** and the denitrification rates measured on the same samples by Alsterberg et al. (2017). The lack of congruence between the genetic potential for denitrification, regarding both abundance and diversity, and denitrification rates is in contrast to other studies on denitrifying communities in sediment environments (Lisa et al., 2015; Repert et al., 2014; Smith et al., 2007).

5 The role of *nirS* and *nirK* in denitrification

5.1 NO_2^- reduction

The existence of two different enzymes catalysing the reduction of NO_2^- to NO in the denitrification pathway is particularly interesting because both enzymes have been described as functionally equivalent, even though they are structurally different and consequently use different catalytic mechanisms (Rinaldo and Cutruzzolà, 2007). In addition, there are structural variants of the *nirK*-type NO_2^- reductase. While most *nirK*-type NO_2^- reductases consist of three subunits, the NO_2^- reductase found in *Hyphomicrobium* spp. is structurally distinct and has six identical subunits (Nojiri et al., 2007). Genes for both enzymes are found in a wide variety of microbial taxa and habitats, and the type of *nir* gene harboured by an organism is not entirely linked to its taxonomy (Coyne et al., 1989; Graf et al., 2014; Jones et al., 2008; Jones and Hallin, 2010). For instance, *Pseudomonas* spp. have been shown to harbour either *nirS* or *nirK* genes and the NO_2^- reductase genes from this genus fall into several clades within the gene phylogenies (Graf et al., 2014; Jones et al., 2008). Both genes *nirS* and *nirK* have evolved separately and were shaped by different evolutionary driving forces (Jones et al., 2008). An apparent question is whether there is some degree of functional and ecological difference which could explain the maintenance of two *nir* systems during evolution.

Alternative functions have been described for both *nirS*- and *nirK*-type NO_2^- reductases, such as the reduction of oxygen and selenite, respectively (Basaglia et al., 2007; Rinaldo and Cutruzzolà, 2007). It has also been shown that *nirS* and *nirK* are involved in the reduction of NO_2^- to NO within the anammox pathway (Hira et al., 2012; Kraft et al., 2011; Strous et al., 2006). However, the functional equivalence of both NO_2^- reductases was shown within a strain of *P. stutzeri*, in which the *nirS*-type NO_2^- reductase was complemented successfully with a *nirK*-

type reductase that still maintained the original denitrifying activity (Glockner et al., 1993).

The two NO_2^- reductase systems are vastly different regarding the genomic context of their genes. The *nirS* gene is located within an operon with many accessory proteins that are mostly involved in enzyme maturation of the NO_2^- reductase, whereas *nirK* often occurs alone or in close proximity to only one accessory gene (Philippot, 2002). In some species, such as *P. stutzeri*, the *nirS* operon is interlinked with the genes for NO reduction forming a joint gene cluster. In many genomes, NO_2^- reductase genes occur in close proximity to other denitrification genes, which is especially pronounced in the genomes of *P. stutzeri* that possess extensive denitrification cluster with the operons of *nosZ*, *nirS* and *norB* in direct neighbourhood (Lalucat et al., 2006; Philippot, 2002).

Consistent with the assumption that both NO_2^- reductase systems are functionally redundant, it was long assumed that the two genes encoding the enzymes were mutually exclusive in the genomes of denitrifying microorganisms. However, genome comparisons have identified a few bacteria that harbour both genes, which reinforces discussions about their functional redundancy in general and raises questions about their respective function particularly within the same organism (Graf et al., 2014).

5.2 Physiology of NO_2^- reduction in the denitrification pathway

Four of the denitrifying genomes that harboured both NO_2^- reductase genes belong to the species *P. stutzeri*, as a comparison of more than 650 denitrifying genomes indicated (Graf et al., 2014). *P. stutzeri* is a well characterized species and many strains have been used as model organisms to study denitrification for a long time (Lalucat et al., 2006). Denitrifying *P. stutzeri* usually harbour the genes for the complete denitrification pathway and it was long assumed that the *nirS*-type NO_2^- reductase system was the only NO-forming NO_2^- reductase present in this species. Consistently, the *P. stutzeri* strains harbouring both NO_2^- reductase genes possess *nirS* genes with relatively similar phylogenetic signal, all clustering within the proteobacterial clade of the *nirS* phylogeny, whereas the *nirK* genes have different phylogenetic signals (Fig. 5 and 6; Graf et al., 2014). The strains *P. stutzeri* SDM-LAC and JM300 have closely related *nirK* genes, which fall within the proteobacterial clade of the *nirK* phylogeny. By contrast, the genes of T13 and AN10 are closely related to *nirK* in alphaproteobacterial *Hyphomicrobia* spp., which have been described to harbour a *nirK*-type NO_2^- reductase with a structural arrangement of six instead of three subunits (Fig. 5; Nojiri et al., 2007).

Our results indicate that both *nir* genes can be expressed within the same organism, as we detected parallel expression of *nirS* and *nirK* in *P. stutzeri* JM300 (**paper IV**). However, the strains *P. stutzeri* JM300 and AN10, showed a differential expression pattern in response to growth conditions, which suggests differences in denitrification activity between these closely related strains and similar denitrifying genotypes. Accordingly, both strains displayed different NO_3^- consumption patterns which further supports this. In addition, while *nirS* was expressed by both strains when supplemented with NO_3^-, *nirK* was only overexpressed in aerobic JM300 cultures with NO_3^- but none of the AN10 cultures. Consequently, we assume that NO_3^- is important for the regulation of *nirS* but not *nirK*. The different expression patterns of *nirS* and *nirK* genes within the same strain corroborates that the NO_2^- reductases are not fully functionally equivalent in these organisms even though they catalyse the same reaction.

Aerobic denitrification has been described earlier for different strains of *P. stutzeri* (Lloyd et al., 1987; Robertson and Kuenen, 1984; Takaya et al., 2003), even though it seems bioenergetically unfavourable and should only occur under rapidly fluctuating conditions or when the direct surroundings of the organism have low levels of oxygen (Chen and Strous, 2013). Our results, however, indicate high expression levels of *nirS*, and in the case of JM300 considerable levels of *nirK* expression, even under aerobic conditions provided that NO_3^- is present in the culture (**paper IV**). Previous work on denitrifying *Thauera* strains harbouring two distinct copies of *nirS* has indicated *nirS* gene expression under aerobic growth conditions, irrespective of the presence of NO_3^- in the medium (Etchebehere and Tiedje, 2005). For the strains we studied, the presence of two distinct NO_2^- reductase systems as well as the *nirS* gene expression under NO_3^- rich conditions might give them a competitive advantage. Accordingly, aerobic denitrification has been reported for natural communities (Gao et al., 2010; Marchant et al., 2017; Rao et al., 2007) and, although we did not measure activity, we could detect comparable abundances of denitrification genes in both constantly anoxic and oxic sediment communities (**paper II**).

Consistent with the different phylogenetic signal of the *nirS* and *nirK* genes from JM300 and AN10, the genomic context of all four genes differed substantially (**paper IV**). The *nirS* genes of JM300 and AN10 cluster in different subclades within the proteobacterial clade (Graf et al., 2014) and had a different arrangement and number of accessory genes. The *nirK* gene of JM300 was associated with the accessory gene *nirV*, whereas the *nirK* gene in the AN10 genome was directly upstream of a *norB* gene cluster. This cluster had an arrangement atypical for *P. stutzeri*, but similar to those described for Alphaproteobacteria and *Hyphomicrobium* spp. (Lalucat et al., 2006; Martineau

et al., 2015), which is consistent with the phylogenetic signal of the *nirK* gene. The *nirS* genes of both strains had binding sites for well-known transcription factors of denitrification in their genomes directly upstream of the *nirS* gene sequence. This was also the case for *nirK* of JM300 but not for AN10, which suggests that this gene is regulated in a different way and might be expressed under a set of conditions that we did not test in this experiment. The difference observed in expression of *nirK* genes in both strains might be explained by their differences in phylogenetic signal and genomic context, stressing the importance of the denitrifying genotypes and their potential effect on the functionality of the denitrifying communities they belong to.

5.3 Ecological differences between NO_2^- reducers carrying *nirS* or *nirK*

Differences in the response of *nirS* and *nirK* communities to biotic and abiotic factors have been described in various studies and indicate different ecological roles for organisms harbouring either NO_2^- reductase system. Processes underlying NO_2^- reducing community assembly seem to differ between *nirS* and *nirK* communities, but niche-based processes, such as environmental filtering, play an important role in structuring both (Jones and Hallin, 2010). Studies have shown that soil moisture and structure as well as copper content have a strong effect on *nirK* communities (Enwall et al., 2010; Smith and Ogram, 2008). On the other hand nitrogen and oxygen availabilities more directly affects *nirS* communities (Cole et al., 2004; Enwall et al., 2010; Oakley et al., 2007; Philippot et al., 2009). Similarly, in **paper II** we saw a strong effect of oxygen availability on *nirS* with oscillating oxygen regimes promoting *nirS* abundances. This is in contrast with previous results that indicate the predominance of *nirS* in more stable and *nirK* under fluctuating conditions (Desnues et al., 2007; Knapp et al., 2009). However, considering that *nirS* genes are more often associated with complete denitrification, direct effects of nitrogen and oxygen concentrations on *nirS* abundance seems expectable as both of these factors directly influence denitrification. Compared to the detected difference between *nirS* and *nirK* gene abundance under different oxygen regimes, we found no difference in their respective response towards the habitat types in **paper III**. Although *nirS* was dominating the denitrifying community, the *nirK*/*nirS* ratios were similar in all four sediments.

Lee et al. (2017) could detect OTUs, and thereby indicator species and specific lineages within the *nirS* phylogeny, associated with high or low salinity along a salinity gradient within estuary sediments. This study was comparable to what was done for the *nosZ* communities in **paper II** in the sense that it allows

for a better understanding of the factors affecting specific denitrifier genotypes and lineages within the gene phylogenies. These type of studies contribute to increase our understanding of how environmental factors shape the genetic potential for denitrification.

5.4 Targeting NO_2^- reducers in the environment

To study the ecology of denitrifying communities, the genes *nirS* and *nirK* have since a long time been used to directly target denitrifying microorganisms in the environment and primers have been recurrently updated (Braker et al., 1998; Hallin and Lindgren, 1999; Throbäck et al., 2004; Wei et al., 2015a). Even though PCR-based methods rely heavily on the coverage and specificity of the primers used, they are still preferred over methods such as metagenomics because of the low abundance of denitrifying communities in the environment. Metagenomics mostly show low coverage of *nirS* and *nirK* genes, which impedes estimations about their abundance and diversity and make it difficult to draw conclusions about their ecological roles (**paper V**). It has been known that existing primer sets mainly cover proteobacterial *nirS* and *nirK* genes and that there are many understudied clades within the *nirS* and *nirK* phylogenies that are not targeted (Ellis et al., 2007; Jones et al., 2008). Primer bias of the earlier primer sets for *nirS* and *nirK* has been discussed and efforts have been made to overcome this bias by designing primers specific for the organisms of interest in different studies (Green et al., 2012; Maeda et al., 2015; Verbaendert et al., 2014; Wei et al., 2015b). Recent attempts to design new primers for both genes followed a similar approach to what was done for *nosZ* and designed primers separately for different clades within the *nirS* and *nirK* gene phylogeny (Wei et al., 2015a).

In **paper V** we designed a framework to better estimate primer coverage and specificity by testing primer pairs first against phylogenies of full-length *nirS* and *nirK* genes from sequenced genomes (Fig. 5 and 6) and, in a next step, using phylogenies of *nirS* and *nirK* genes from metagenomes from different environments. We found no published primer pair that achieved complete coverage across all sequences for neither *nirS* nor *nirK*. As expected, our results indicate that many of the earlier published and widely used primers are highly specific to the proteobacterial clades of *nirS* and *nirK*. However, their good coverage of both genome and metagenome sequences in these clades makes them suitable candidates for clade-specific proteobacterial primers. Furthermore we found that even though the clade-specific primers designed by Wei et al. (2015a) were highly specific, their coverage was low.

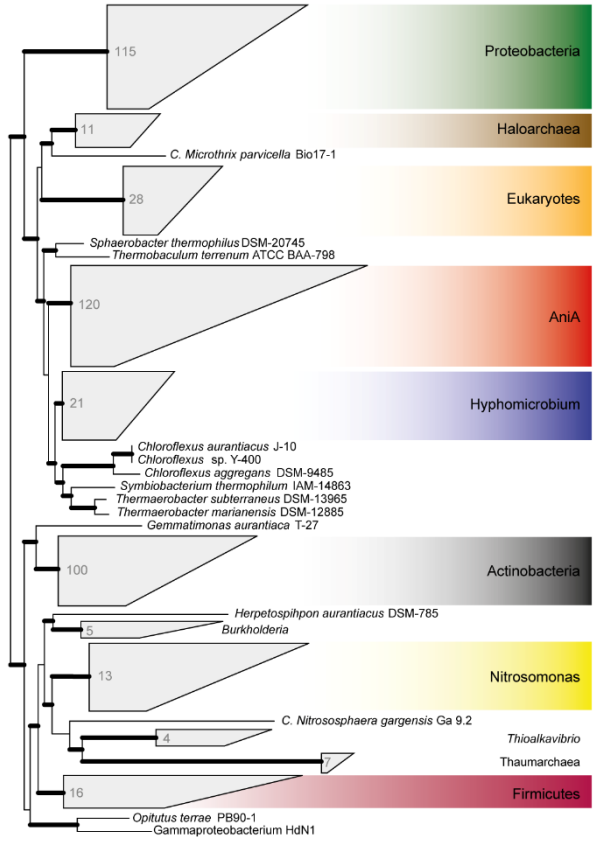

Figure 5. Reference phylogeny of full-length *nirK* sequences based on figure 3 in **paper V**. The number of sequences within collapsed clades and branches with >65% bootstrap support are indicated on the tree.

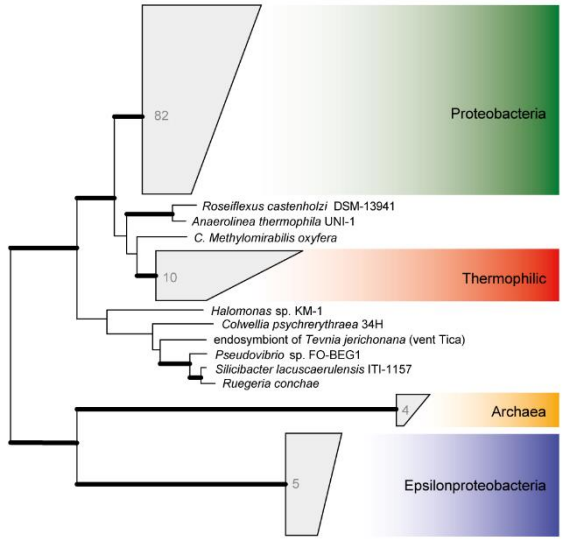

Figure 6. Reference phylogeny of full-length *nirS* sequences based on figure 3 in **paper V**. The number of sequences within collapsed clades and branches with >65% bootstrap support are indicated on the tree.

Our attempt of designing primers covering the complete phylogeny was only successful for *nirK* and only when allowing a high level of degeneracy, which increases the risk of unpredictable biases. We therefore investigated the option of using primer pairs for each of the evolutionary conserved clades within the *nirS* and *nirK* gene phylogenies (Fig. 5 and 6) and could suggest either already published or newly designed primers for most groups. The approach of using clade-specific primers prevents the characterization of the total community structure, however, the existence of conserved clades in the *nirS* and *nirK* phylogeny points towards the existence of ecologically distinct groups, and targeting them separately might increase our understanding of NO_2^- reducers more than looking at them as one single functional group. This was also the case in **paper IV** as the *nirK* genes from the two strains have a different phylogenetic signal and are not expressed under the same conditions.

Problems associated with the use of several clade-specific primer pairs to target *nirS* and *nirK* genes depends on the application. High specificity is important for quantitative studies so that denitrifier gene abundance is not overestimated, but even when primers are highly specific it is not possible to sum up gene abundances detected with different primer pairs as this can heavily

inflate estimations of the size of overall denitrifying communities. On the other hand, non-specific primers are a possible option for sequence based applications as reads clustering in non-target clades can be excluded during the analysis. Furthermore, we detected differences between primer coverage and specificity in the metagenomes of different habitats investigated in **paper V**. Altogether, our results indicate that primer options should be carefully evaluated and selected depending on the application, the environment, and the ecological question of the study.

6 Conclusions and future perspectives

Denitrification dominates as nitrogen removal pathway in many ecosystems and is directly involved in nitrogen regulation in the environment. The process can mitigate high nitrogen loadings from anthropogenic sources and can thereby have a direct effect on ecosystem functioning. The modularity of the pathway has been recognized together with the impact this could have on nitrogen mitigation and N_2O emissions. Thus, new insights into the genetic diversity within denitrifying and N_2O reducing genotypes have raised questions about their ecophysiology, niche differentiation and general importance in the environment.

In this thesis, denitrifying microorganisms with a complete pathway were important in the systems we investigated, including activated sludge enrichment cultures and deep as well as shallow coastal marine sediments (**paper I-III**). This emphasizes the relevance of microorganisms with the genetic potential for complete denitrification, and suggests that they have a major role for nitrogen removal as well as N_2O reduction in coastal areas. Niche differentiation among organisms harbouring *nosZ* clade I or II could be detected in response to oxygen regimes. Oscillating oxygen regimes as well as two specific coastal habitats, sandy and silty-mud sediments, were shown to positively affect the genetic potential for nitrogen removal through denitrification. We could further conclude that complete denitrifiers were competitive N_2O reducers – both under electron donor and N_2O limiting conditions.

Regarding denitrification, our results suggest a functional difference between the genes *nirS* and *nirK* encoding two structurally different NO_2^- reductases within the same organism, and corroborate that even closely related and almost identical denitrifying genotypes differ in their denitrification activity (**paper IV**). Finally, we could propose improved molecular tools to better target denitrifying (i.e. NO_2^- reducing) communities. The primers we are suggesting within this thesis work in **paper V** will likely enhance the possibilities to target NO_2^- reducers in different environments and will aid in advancing our

understanding about factors driving NO_2^- reducer abundance and diversity in the environment as well as effects of environmental factors on distinct NO_2^- reducer genotypes. This will eventually give deeper insight into the importance of genotypes from the different clades within the gene phylogenies, which will complement the knowledge we have to date about the clades within the *nirS* and *nirK* gene phylogenies.

This thesis emphasizes the importance of assessing what drives the genetic potential for denitrification and N_2O reduction in order to understand and predict the nitrogen removal capacity of ecosystems. To address this further, gene expression data can be an option, especially in cultures and enrichments, as it gives an overview of the active metabolism within the community. This kind of data is difficult to obtain for soil and sediment samples due to the instability of RNA. In that case, it could be an option to assess the total microbial community in addition to the denitrifying and N_2O reducing community members similar to what was done for the enrichment culture in **paper I**. This can give insights on broad changes in the overall community, either taxonomic or functional, that may be associated with the ability to denitrify or respire N_2O.

Increasing our knowledge of the extant variation in gene content and gene sequence diversity among denitrifying genotypes also opens up questions about how environmental heterogeneity influences diversification of the denitrification pathway. In addition, whether the modularity of the denitrification pathway can lead to a division of the pathway between organisms where different steps are performed by separate organisms has been suggested but not yet confirmed for denitrification. This has been discussed extensively for nitrifying communities and shortening of pathways might be advantageous as it could increase the growth rate of an organism (Costa et al., 2006).

Further research in these directions will hopefully increase our knowledge about factors shaping denitrifying and N_2O reducing communities and contribute to our ability to predict and mitigate N_2O emissions and the effects of growing nitrogen loadings in ecosystems worldwide.

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Popular science summary

Nitrogen is a major component in our atmosphere where most of it is deposited in the form of molecular nitrogen (N_2). However, nitrogen is also of major importance for all life. Therefore, N_2 has to be converted to bioavailable ammonium in a reaction called nitrogen fixation, which, in nature, is carried out entirely by microorganisms. Since the beginning of the industrial revolution and the invention of the chemical production of ammonia through the Haber-Bosch process, nitrogen loadings in the environment have only increased. This is mainly due to the importance of nitrogen for e.g. fertilizer in agriculture, explosives and the chemical industry. High levels of nitrogen cause great problems as they can lead to water and air pollution with negative consequences for natural ecosystems, the climate, as well as human health. Within water systems increased nitrogen concentrations often lead to deterioration of water quality, unusually low oxygen levels and subsequent degeneration of habitats and ecosystems.

Similar to natural nitrogen fixation the natural removal of excess nitrogen from an ecosystem is accomplished by microorganisms, which are capable to recycle nitrogen back to the atmosphere. Denitrification is often the major nitrogen removal pathway in many ecosystems and leads to the stepwise reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to N_2. On the other hand, the pathway can also lead to emissions of nitrous oxide (N_2O), a potent greenhouse gas with a warming potential 300 times higher than carbon dioxide (CO_2), which also has the ability to destroy the ozone layer. However, the last step within this pathway is the reduction of N_2O and this is the only known biological sink for this greenhouse gas. Therefore, microorganisms capable of denitrification can be divided into three groups, those that can produce N_2O but are incapable of its reduction, those that have the complete denitrification pathway and therefore the capacity to reduce the N_2O produced by themselves, and finally those that are solely capable of N_2O reduction. This last group can be considered as a true sink of N_2O in the environment.

This thesis aims to understand how specific environmental factors affect these groups of microorganisms. The questions that we try to answer with this research are whether the activity, presence and abundance of organisms with different abilities for denitrification is influenced by environmental factors. Moreover, we are interested in whether that has an effect on the genetic potential for denitrification of the community or the ecosystem. The genetic potential for denitrification is estimated by assessing the presence and abundance of organisms within the three groups of denitrifying microorganisms. As this gives an estimate about the nitrogen removal capacity of an ecosystem and its potential to contribute to N_2O emission, this information is valuable in natural and engineered systems with high nitrogen loadings, such as coastal areas and wastewater treatment plants. Within the thesis work we approached these questions from different angles by working on single organisms, simple communities and complex natural communities from coastal marine sediments.

The work on cultures of one single denitrifying bacterium at a time indicated that even organisms from the same group of denitrifying microorganisms can show differences in denitrification activity when exposed to the exact same conditions. This highlights the importance of detailed knowledge about the microorganisms present in a community. Our experiments with simple communities revealed that denitrifying microorganisms with the complete pathway are successful N_2O reducers. They were dominating the microbial communities in all experiments and were more competitive than those organisms solely capable of N_2O reduction. This emphasizes that complete denitrifiers might be of major importance for N_2O reduction even in natural environments.

In a next step, we investigated the effects of oxygen and habitat type on natural microbial communities from coastal marine sediments. A set of sediment samples from Gullmars Fjord at the west coast of Sweden was subjected to different oxygen regimes to assess the effect of oxygen on the different groups capable of denitrification. We could see that, even though those organisms capable of the complete pathway were dominating the denitrifying communities in all samples, they were favoured and increased in abundance under oxygen regimes that switched from oxic to anoxic and back every five days. This was expected as these organisms are especially suited to live under fluctuating oxygen conditions. The second set of sediment samples originated from a shallow-water bay close to Gothenburg, Sweden, where several different habitats are present in close proximity. Quantification of the size and diversity of the denitrifying community indicated that microorganisms with the complete pathway were highly abundant as well, but in addition we also found a high proportion of the community to be capable of only N_2O reduction. This suggests that these sediments could have low net emissions of N_2O. Comparisons of the

denitrifying communities in the different habitats highlighted the importance of sandy and silty mud sediments for the nitrogen removal capacity of the environment. As coastal areas are heavily affected by human activities this kind of information is important to be able to predict possible negative effects of habitat destruction and homogenization.

Finally, we proposed new molecular tools to better target denitrifying microorganisms, which might improve our knowledge about denitrifying microorganisms with the complete pathway or those involved in N_2O production in the future.

Within this thesis work we could highlight the importance of microorganisms capable of complete denitrification in the environment and as successful reducers of the greenhouse gas N_2O. Moreover, our results indicate that oscillating oxygen regimes and the presence of sandy and silty mud sediments can positively affect denitrifying communities and improve the nitrogen removal capacity of coastal ecosystems. This thesis contributes to ongoing research about how denitrifying microorganisms react to environmental conditions and whether that influences the nitrogen removal capacity of ecosystems. Many questions remain unanswered but the new molecular tools developed in this study might further our understanding about these interesting microorganisms and their contribution to the functioning of ecosystems.

Populärvetenskaplig sammanfattning

Kväve är en betydande komponent i vår atmosfär, där den största delen utgörs av molekylär kvävgas (N_2). Kväve har dock stor betydelse för allt liv. Därför behöver N_2 omvandlas till biologiskt tillgänglig ammonium i en reaktion som kallas kvävefixering, vilken i naturen utförs av mikroorganismer. Sedan början av den industriella revolutionen och uppfinnandet av den kemiska produktionen av ammonium genom Haber-Boschprocessen har kväveutsläppen i miljön bara ökat. Detta beror framför allt på betydelsen av kväve i t.ex. gödsel i jordbruk, explosiva ämnen och den kemiska industrin. Höga nivåer av kväve kan orsaka problem och leda till vatten- och luftföroreningar med negativa konsekvenser för naturliga ekosystem, klimatet, så väl som människors hälsa. Inom vattensystem kan ökade kvävekoncentrationer leda till försämrad vattenkvalitet, ovanligt låga syrehalter och påföljande försämringar av habitat och ekosystem.

I likhet med naturlig kvävefixering så utförs det naturliga avlägsnandet av överskottskväve från ekosystem av mikroorganismer, vilka kan återföra kväve tillbaka till atmosfären. Denitrifikation är ofta den huvudsakliga reaktionsvägen för avlägsnande av kväve i många ekosystem och leder till en stegvis reduktion av nitrat (NO_3^-) och nitrit (NO_2^-) till N_2. Å andra sidan kan reaktionsvägen också leda till utsläpp av kväveoxid (N_2O), en potent växthusgas med 300 gånger högre uppvärmningspotential än koldioxid (CO_2), vilken också har förmågan att förstöra ozonlagret. Det sista steget i den här reaktionsvägen är dock reduktion av N_2O och detta är den enda kända biologiska sänkan för denna växthusgas. Därför kan mikroorganismer som har förmåga till denitrifikation delas upp i tre grupper, de som kan producera N_2O men inte reducera den, de som har den kompletta reaktionsvägen av denitrifikation och därför kapaciteten att reducera den N_2O som de själva producerat, och slutligen de som bara kan reducera N_2O. Denna sista grupp kan betraktas som en sann sänka av N_2O i miljön.

Den här avhandlingen syftar till att förstå hur specifika miljöfaktorer påverkar dessa grupper av mikroorganismer. Frågorna som vi försöker besvara med denna forskning är om aktiviteten, förekomsten och abundansen av

organismer med olika förmåga till denitrifikation påverkas av miljöfaktorer. Dessutom är vi intresserade av om det påverkar den genetiska potentialen för denitrifikation av samhället eller ekosystemet. Den genetiska potentialen för denitrifikation mäts genom att bedöma förekomst och abundans av organismer inom de tre grupperna av denitrifierande mikroorganismer. Eftersom detta ger ett mått på förmågan till borttagande av kväve från ekosystem och dess förmåga att bidra till utsläpp av N_2O så kan denna information vara värdefull för naturliga och skapade system med höga kvävehalter, så som kustområden och vattenreningsverk. Inom ramen för arbetet med den här avhandlingen närmade vi oss dessa frågor från olika vinklar genom att arbeta med enskilda organismer, enkla samhällen och komplexa, naturliga samhällen från havskustsediment.

Arbetet med kulturer av en enskild denitrifierande bakterie vid ett tillfälle indikerade att även organismer från samma grupp av kvävefixerande mikroorganismer kan visa skillnader i denitrifikationsaktivietet när de exponeras för exakt samma förhållanden. Detta belyser vikten av detaljerad kunskap om de mikroorganismer som finns i ett samhälle. Vårt experiment med enkla samhällen visade att denitrifierande mikroorganismer med den kompletta reaktionsvägen är framgångsrika N_2O-reducerare. De dominerade de mikrobiella samhällena i alla experimenten och var mer konkurrenskraftiga än de organismer som bara hade förmåga till N_2O-reduktion. Detta understryker att kompletta denitrifierare kan vara av störst betydelse för N_2O-reduktion även i naturliga miljöer.

I ett senare steg undersökte vi effekterna av syre och typ av habitat på naturliga, mikrobiella samhällen från havskustsediment. En uppsättning av sedimentprover från Gullmarsfjorden på den svenska västkusten utsattes för olika syreförhållanden för att bedöma effekten av syre på olika grupper med denitrifikationsförmåga. Vi kunde se att, även om de organismerna som hade förmåga till den kompletta reaktionsvägen dominerade denitrifierande samhällen i alla proverna, så gynnades de och ökande i abundans under syretillgång som skiftade från oxiska till anoxiska och tillbaka var femte dag. Detta var förväntat eftersom dessa organismer är speciellt anpassade för att leva under fluktuerande syretillgång. Den andra uppsättningen av sedimentprover kom från en långgrund bukt när Göteborg i Sverige, där flera olika habitat finns nära varandra. Kvantifiering av storleken och diversiteten hos det denitrifierande samhället tydde på att mikroorganismerna med den kompletta reaktionsvägen var abundanta i hög grad, men dessutom fann vi att en hög andel av samhället hade förmåga till enbart N_2O-reduktion. Detta tyder på att sedimenten skulle kunna ha låga nettoutsläpp av N_2O. Jämförelser av de denitrifierande samhällena i de olika habitaten belyser vikten av sandiga och slammiga lersediment för avlägsnande av kväve från miljön. Eftersom kustområden är kraftigt påverkade

av mänsklig aktivitet är den här typen av information viktig för att kunna förutsäga möjliga effekter av förstörelse och homogenisering av habitat.

Slutligen föreslog vi nya molekylära verktyg för att undersöka denitrifierande mikroorganismer med bättre precision, vilka kan förbättra vår kunskap om denitrifierande mikroorganismer med den kompletta reaktionsvägen eller de som deltar i N_2O-produktion i framtiden.

Inom ramen för arbetet med den här avhandlingen kunde vi belysa betydelsen av mikroorganismer med förmågan till total denitrifikation i miljön och som framgångsrika reducerare av växthusgasen N_2O. Dessutom tyder våra resultat på att oscillerande syretillgång och förekomsten av sandiga och slammiga lersediment kan påverka denitrifierande samhällen positivt och förbättra kustekosystemens kapacitet till kväveavlägsnande. Denna avhandling bidrar till den pågående forskningen om hur denitrifierande mikroorganismer reagerar på miljöförhållanden och om det påverkar ekosystemens kapacitet till kväveavlägsnande. Många frågor är fortfarande obesvarade men nya molekylära metoder som utvecklats i den här studien kan öka vår förståelse av dessa intressanta mikroorganismer och deras bidrag till ekosystemens funktionalitet.

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DANKE!