Ecology and Genomics of Microorganisms Reducing the Greenhouse Gas N_2O

Examples from the Rhizosphere

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Cover: Sunflower plants with root ball from the pot experiment in **paper III.** Inside the magnifying glass (free to use clip-art), a 16S rRNA maximum likelihood phylogeny of 652 microbial genomes containing either a *nir* or a *nosZ* gene (**paper II**) is shown. (photo and figure: Daniel Graf)

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Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas and the major ozone depleting substance in the stratosphere. One major source of N_2O is incomplete denitrification, whereas the only known tropospheric sink of N_2O is the microbial enzyme nitrous oxide reductase. Denitrification is defined as the stepwise reduction of nitrite to dinitrogen via nitric oxide and N_2O by facultative anaerobic microorganisms. This thesis aims to elucidate the phylogenetic diversity of the N_2O reductase encoding gene *nosZ* and its context in microbial genomes in relation to other genes in the denitrification pathway, as well as the relative influence of plants and soil on the activity, abundance and structure of N_2O-reducing communities in the rhizosphere.

Phylogenetic analysis of publicly available *nosZ* gene sequences revealed that its genetic diversity is divided into two distinct clades termed clade I and clade II, the latter having not been accounted for in previous studies. Newly developed molecular tools revealed that it is abundant in a wide range of environments. Analysis of microbial genomes showed that co-occurrence patterns of *nosZ* with other denitrification genes were neither randomly distributed among taxonomic units nor among habitats. Many genomes had truncated pathways as organisms possessing *nosZ*II often lacked other genes involved in denitrification, suggesting these organisms may act as N_2O-sinks in the environment. Pot experiments with sunflower and barley indicated a niche differentiation between the two *nosZ* gene variants, as *nosZ*I showed an affinity for plant roots while *nosZ*II was more abundant in the surrounding soil. However, denitrification and N_2O-production activity in soil were controlled by edaphic factors. Moreover, an intercropping experiment with cocksfoot and lucerne showed that intercropping had a negative influence on *nosZ*II abundances on cocksfoot roots which in conjunction with phylogenetic placement of sequencing reads indicated the presence of organisms with only *nosZ* lacking a denitrification pathway.

In conclusion, the development of new molecular tools combined with comparative genomic analysis sheds new light on the ecology of biological N_2O reduction in the rhizosphere.

Keywords: nitrous oxide, *nosZ*, *nirS*, *nirK*, microbial genomes, community assembly, rhizosphere, intercropping

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Dedication

To those who care to see behind the curtain.

I varje själ är tusen själar fångna, i varje värld är tusen världar dolda och dessa blinda, dessa undre världar är verkliga och levande fast ofullgångna, så sant som jag är verklig.

ur Färjesång - Gunnar Ekelöf

Contents

List of Publications

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

I Jones, C.M., Graf D.R.H., Bru, D., Philippot, L., Hallin, S. (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME Journal* 7, 417-426.

II Graf, D.R.H, Jones, C.M. and Hallin, S. (2014). Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N_2O emissions. *PLoS ONE*, 9(12):e114118.

III Graf, D.R.H, Jones, C.M., Zhao, M. and Hallin, S. Community assembly of N_2O-reducing denitrifiers associated with roots include selection and competition with community composition mainly depending on soil type. (manuscript)

IV Graf, D.R.H, Zhao, M., Carlsson, G. Jones, C.M., and Hallin, S. Composition and activity of N_2O-reducing communities associated with roots of *Medicago sativa* and *Dactylis glomerata* during intercropping. (manuscript)

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

I Performed a minor part of the laboratory work, the analysis and the writing.

II Participated in the planning of the study, performed half of the work and a major part of the analysis and the writing.

III Performed the majority of the planning of the experiment as well as of the work, the analysis and the writing.

IV Participated in the planning of the study and performed half of the laboratory work, the analysis and the writing

Abbreviations

ANAMMOX	anaerobic ammonia oxidation
DNRA	dissimilatory nitrate reduction to ammonium
N_2O	nitrous oxide
N_2OR	nitrous oxide reductase
NH_4^+	ammonium
NIR	nitrite reductase
nirK	gene encoding for copper-binding nitrite reductase
nirS	gene encoding for heme-binding nitrite reductase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOR	nitric oxide reductase
nosZ	gene encoding for nitrous oxide reductase
ppb	parts per billion

1 Introduction

Nitrous oxide (N_2O) is a colourless, odourless, non-toxic gas, commonly known as laughing gas. The Intergovernmental Panel on Climate Change (IPCC) report on climate change (2013) considers N_2O the third most important greenhouse gas. The atmospheric concentration levels of the gas have increased from 271 ppb in 1750, as determined by ice core analysis, to 324 ppb in 2011 of which 5 ppb have been added since 2005. Moreover the IPCC states that, considered over a 100 year period, the global warming potential for N_2O is 298 times higher than that of CO_2, thus making the gas responsible for around 7 % of the global radiative forcing despite occurring at far lower concentrations than CO_2. Historically, the concentration of N_2O currently observed in the atmosphere has not been as high since at least 800 000 years ago, and levels are rising with increasing speed. The stratosphere is considered the main sink for N_2O, where the gas undergoes photolysis to NO which in turn destroys ozone molecules. N_2O is now considered the most dominant ozone depleting substance (Ravishankara et al., 2009).

1.1 Nitrous oxide emissions from terrestrial ecosystems

Of global N_2O-emissions, 62% are attributed to terrestrial ecosystems where agricultural systems comprise the largest single source of N_2O (Skiba and Smith, 2000). The other third is produced in the oceans, whereas additional sources are the combustion of fossil fuels in power plants and vehicles as well as the production of nitric acid (IPCC, 2007). Smith et al. (2012) could effectively link the global rise of N_2O levels to overall inputs of reactive N into agricultural systems. This reactive N includes biologically fixed N and N fixed as synthetic fertilizer, as well as N mineralized from soil organic matter (SOM), when natural land is converted to agriculture, as well as NOx

deposition from combustion. Thus, they identified agricultural systems as the main source responsible for rising N_2O levels.

1.2 Biological processes regulating N_2O

N_2O is part of the N cycle, which begins with the fixation of atmospheric nitrogen into ammonium (NH_4^+) either by diazotrophic microorganisms or through the Haber-Bosch process in the industrial production of fertilizers. The NH_4^+ that is not incorporated into growing plant or microbial biomass can either be aerobically oxidized to NO_3^- via nitrification or converted to N_2 through anaerobic ammonia oxidation (ANAMMOX). The NO_3^- produced by nitrification can be reduced to N_2 via denitrification, which in addition to ANAMMOX is a major pathway by which fixed N is returned to the atmosphere. However, NO_3^- can also be reduced to NH_4^+ by the process dissimilatory nitrate reduction to ammonium (DNRA).

In soils, N_2O-emissions can be primarily attributed to nitrification and denitrification (Okereke, 1993; Zumft, 1997). The former is a two-step process where ammonia (NH_3) is first oxidized to nitrite (NO_2^-) via the intermediate hydroxylamine (NH_2OH) by ammonia oxidizing bacteria and archaea, and then to nitrate (NO_3^-) by nitrite oxidizing bacteria. While N_2O can be produced from oxidation of hydroxylamine, it has been pointed out that denitrification is the main cause of N_2O emissions also from ammonia oxidizers (Kool et al., 2011). Also DNRA can give rise to N_2O, here toxic NO can accumulate in the cell and is detoxified to N_2O (Spiro, 2012). However, this process is considered to contribute only marginally to overall N_2O budgets (Kool et al., 2011). Nevertheless, which process constitutes the dominant source of N_2O in a given system at a given point in space and time will depend on substrate availability and environmental conditions and can thus vary substantially (Baggs, 2011). In contrast, the only known biological sink for N_2O is the reduction to N_2 by nitrous oxide reductase, whose part in the denitrification pathway was in focus in this thesis.

1.3 The denitrification pathway

Denitrification is a facultative, anaerobic respiratory pathway in which NO_3^- or NO_2^- is reduced to N_2 with the intermediates NO and N_2O. N_2O can either be an intermediate or end product of denitrification, which is why the process is considered both a source and a sink for N_2O (Chapuis-Lardy et al. 2007; Jones

et al. 2014; Philippot, Andert, et al. 2010). The first step in the denitrification pathway is the reduction of NO_3^- to NO_2^- which can either be catalyzed by a membrane associated nitrate reductase (NarGH) or its soluble periplasmic homologue (NapAB). This two electron step is considered to be the most energy conserving step in the denitrification pathway (Chen and Strous, 2013). The next step, the reduction of NO_2^- to NO, is regarded as the defining step of denitrification since nitrate reduction as a trait can exist decoupled from later steps in the denitrification pathway and be part of DNRA while NO as a free radical is cytotoxic and needs to be reduced further to N_2O (Shapleigh, 2006; Zumft, 1997). Dissimilatory nitrite reduction is performed by two functionally equivalent but evolutionarily distinct enzymes: the copper binding nitrite reductase, encoded by the gene *nirK*, and the heme binding reductase, encoded by the gene *nirS*. Both enzymes are located in the periplasm and until recently have been considered mutually exclusive in the genomes of denitrifying organisms. However, in **paper II** both genes were found in the genomes of a number of organisms, although whether both enzymes are functional in the same cell has not been shown so far. Reduction from NO to N_2O is carried out by nitric oxide reductase (NOR), which is a membrane bound protein with three structurally homologous variants. Since NO is a potent intercellular signalling compound and cytotoxin, its reduction to the relatively benign N_2O is not unique to denitrification and many microorganisms possess NOR to detoxify (Zumft, 2005). The last step of the pathway is nitrous oxide reductase (N_2OR) which is the only known sink of N_2O apart from stratospheric photolysis. N_2OR is a copper binding enzyme situated in the periplasm. Energetically, the complete denitrification pathway results in the transport of 30 protons across the cytoplasmic membrane to drive ATP synthesis when NADH is used as electron donor, six of which are contributed by N_2O-reduction (Richardson et al., 2009). Thus, N_2O reduction contributes about 20% to the overall energy gain of the denitrification pathway (Richardson et al., 2009), and many organisms have been shown to lack the *nosZ* encoding gene for N_2OR (**paper II**, Jones et al. 2008). On the other hand, bacteria such as *Wolinella succinogenes, Campylobacter fetus*, the thermophile *Geobacillus thermodenitrificans* and the soil bacterium *Anaeromyxobacter dehalogenans*, have been demonstrated to grow with N_2O as the sole electron acceptor (Liu et al. 2008; Payne et al. 1982; Sanford et al. 2002; Sanford et al. 2012) and many with the *nosZ* gene often lack other genes typically associated with the denitrification pathway (**paper II**, Jones et al. 2008). This has led to the conception that the denitrification pathway is a modular pathway where

organisms can have different combinations of denitrification genes (**paper II**; Zumft 1997).

1.4 Denitrifier community ecology

Denitrification as a facultative respiratory pathway is found amongst a wide variety of organisms (Philippot et al., 2007). In **paper II,** the genomes of 262 genera from 19 different phyla across bacterial, archaeal, and fungal domains were found to harbour either a *nirK*, *nirS* or *nosZ* gene. However, closely related microbial strains may or may not possess the ability to denitrify (**paper I**; **paper II**; Cavigelli & Robertson 2001; Jones et al. 2008). This effectively renders taxonomy-based approaches to assess the denitrifying microbial community useless. Instead, denitrifier communities are studied by using functional genes as opposed to 16S rRNA or other taxonomic marker genes, and PCR-based tools have been developed since the late 1990's (Braker et al., 1998; Hallin and Lindgren, 1999; Scala and Kerkhof, 1999). With the advent of bioinformatics and improved sequencing techniques these tools are being constantly refined in order to better capture the extant genetic diversity of denitrifying communities present in the environment (**paper I**; Dandie et al. 2007a; Throbäck et al. 2004; Verbaendert et al. 2014). Conceptually, this approach utilizes the idea of 'functional guilds' in which a group of organisms is defined by a shared ecosystem function rather than taxonomic affiliation (Simberloff and Dayan, 1991). It is thereby assumed that a functional guild can be targeted using one or several marker genes encoding the functional trait in question. The modularity of the denitrification pathway requires the employment of several functional gene markers in order to comprehensively assess communities that drive ecosystem functions such as N_2O-regulation. Another implication of the modular pathway concept is that a given denitrifier community might perform differently in situ depending on the community structure of organisms harbouring different set-ups of denitrification genes present (Philippot et al. 2011; Jones et al. 2014). Since denitrification is such a wide-spread function it is important to consider that other characteristics such as chemolithoautotrophy versus heterotrophy with different environmental cues may also play an important role in determining how denitrifier communities assemble in situ. While most studies point out habitat filtering as the driving force behind community assembly (e.g.Horner-Devine & Bohannan 2006; Jones & Hallin 2010), recent research shows that recruitment of microbial communities to a given niche from a surrounding matrix can also be based on stochastic processes (Burke et al., 2011a). Due to the modular nature of the

denitrification pathway, it is of importance whether a given N_2O-regulating community is the result of adaptation to different niches where denitrification might pose a competitive advantage or stochastic processes based on biogeography, or a combination of both.

1.5 Abiotic factors influencing denitrification activity

A basic driver of N_2O-regulation is soil moisture as it regulates the availability of oxygen in soil pores. While it has been postulated that N_2O-emissions have their optimum at 70-80% water-filled pore space (Davidson et al., 2000), a comprehensive comparison of 51 soils has shown that this is highly dependent on soil type (Butterbach-Bahl et al., 2013). Since denitrification is a facultative respiratory pathway in the absence of oxygen, low oxygen levels result in the upregulation of denitrification genes. Interestingly Bakken et al. (2012) found that N_2OR in contrast to NIR and NOR is upregulated before oxygen is completely depleted in *Paracoccous denitrificans*. Temperature is another important factor that can influence denitrification activity in soils. It has been found that the stimulation of denitrification by an increase in temperature by 10°C exceeded the observed stimulation of CO_2 emissions (Schaufler et al., 2010). This is attributed to the fact that the microbial C and N cycles are tightly interlinked and that increased soil respiration due to increased overall microbial activity result in lower oxygen levels in soils (Butterbach-Bahl et al., 2013). Another key factor for denitrification is NO_3^- availability. Here it is postulated that N_2O-emissions increase with N saturation in any given ecosystem where NO_3^- has the strongest stimulation effect on N_2O emissions of five investigated N compounds (Liu and Greaver, 2009). Soil pH also has a strong influence on denitrification activity (Bergaust et al., 2010; Clark et al., 2012; Simek and Cooper, 2002), especially the ratio of end products (Bakken et al., 2012; Šimek et al., 2002). It has been speculated that as N_2OR cannot assemble properly at low pH's (Bakken et al., 2012). Since many known denitrifiers depend on carbon compounds as electron donors, soluble carbon and thus soil organic matter content also affects denitrification rates (Bijay-Singh et al., 1988; Burford and Bremner, 1975). These general environmental drivers fluctuate largely both spatially and temporally, especially in a medium as heterogeneous as soil. Temporary water-logging, seasonal changes from drought to rewetting as well as transition zones between upland and wetland soils can result in 'hot spots' and 'hot moments' for denitrification (Groffman et al., 2009). Indeed, Parkin (1987) found that the bulk of denitrification activity in a soil core was localized in a minute fraction of the core constituting a decaying leaf.

1.6 Denitrifier community structure

Even though environmental factors play an important role determining denitrification activity and N_2O emissions, the actual outcome ultimately depends on the microbial community in situ. However, microbial communities in turn are shaped by environmental factors. The main factors shaping denitrifier communities in soils include carbon availability, soil moisture, temperature and pH (Wallenstein et al., 2006). In soil, these factors vary largely in conjunction with physical parameters such as particle size, soil organic matter content and porosity. This spatial heterogeneity could be shown to be reflected in the structure of denitrifier communities (Enwall et al. 2010; Philippot et al. 2009a). Apart from environmental cues, biogeographical distribution and site history are factors that cannot be neglected concerning microbial community composition in general (Green and Bohannan, 2006; Lindström and Langenheder, 2012; Martiny et al., 2006) and denitrifier community composition in particular (Jones and Hallin, 2010; Prasse et al., 2015; Zhu et al., 2015). An interesting feature in this context is that several steps of the denitrification pathway are encoded by different genes and gene variants, most prominently the *nirK* and *nirS* genes that encode two very different enzymes providing the same function but which have evolved independently (Jones et al. 2008). Since organisms in most cases only possess one of the two genes (**paper II**), it has been discussed whether these occupy different ecological niches and indeed indications for this have been found (Enwall et al., 2010; Hallin et al., 2009; Jones and Hallin, 2010). N_2OR has long thought to be encoded by one variant of the *nosZ* gene predominantly found in proteobacteria (Scala and Kerkhof, 1999) however a new gene variant from a greater variety of organisms has been detected more recently (**paper I**; Simon et al. 2004; Sanford et al. 2012). While the two clades of *nosZ* are phylogenetically related also here there is indication for niche differentiation between the two (**paper III** and **IV,** Jones et al. 2014).

1.7 Plants as factors regulating denitrification

The rhizosphere, defined as the soil influenced by plant roots, is a hotspot for denitrification due to higher inputs of organic carbon and nitrogen from plant roots via exudates, mucilage and shed root cells as well as fluctuating levels of oxygen (Henry et al., 2008; Klemedtsson et al., 1987; Prade and Trolldenier, 1988). However, this effect has been shown to vary across plant species (Crush, 1998; Wheatley et al., 1990). This may be of profound importance for N_2O-emissions, which are typically higher in soils planted with different agricultural crops when compared to that of bulk soils (Ding et al. 2007; Dong

et al. 2005; Hénault et al. 1998; Højberg et al. 1996; Klemedtsson et al. 1987; Ni et al. 2012; Sey et al. 2010; Verma et al. 2006). Moreover, plants have been found to influence denitrifier microbial community structure. Higher proportions of denitrifiers relative to other heterotrophic organisms have been reported in proximity to roots (Berg and Bothe, 1992; Clays-Josserand et al., 1995) and significant differences in denitrifier community composition have been observed between the rhizosphere and the surrounding bulk soil (Chèneby et al. 2004; Hamonts et al. 2013; Philippot et al. 2002; Ruiz-Rueda et al. 2009). Sharma et al. (2005) found that maize plants select denitrifiers carrying the *nirK* gene over those with *nirS* genes, further indicating a niche differentiation between organisms carrying these genes. Similarly, **paper III** indicates that organisms carrying the Clade I *nosZ* have an affinity to plant roots which is not shared by those with Clade II *nosZ*.

1.8 Aim and outline of the thesis

The aim of this thesis was to investigate the ecology of the N_2O-reducing microbial community both in general and in the rhizosphere as a model system. Since N_2O-reduction is taxonomically wide spread the microbial community providing this function was assessed using the *nosZ* gene as a proxy.

In **paper I** the phylogenetic diversity of N_2O-reducing microorganisms and to develop tools to comprehensively target them in a large variety of environments. Moreover, co-occurrence patterns of denitrification genes relevant for N_2O-regulation in microbial genomes were put into context regarding taxonomy and habitat preferences of respective organisms, generating hypotheses about their ecology (**paper II**). The tools and hypotheses developed in **paper I** and **II** were applied in the rhizosphere as a relevant model system for N_2O-regulation. Here, the relative influence of soil type and plant species (**paper III**) and intercropping (**paper IV**) on denitrification activity and the community assembly of N_2O-reducing microorganisms in the rhizosphere were investigated**.**

2 Diversity of nitrous oxide reductase

2.1 Nitrous oxide reductase

N_2OR is a soluble enzyme located in the periplasm. Structurally, the enzyme is a homodimer with each monomer binding six copper ions in the form of two copper clusters, a di-nuclear electron transfer and storage site, CuA and a tetra-nuclear cluster, CuZ (Dell'Acqua et al., 2011). Enzyme synthesis is upregulated by transcription factors sensitive to oxygen (FNR), nitric oxide and nitrite (NNR), and nitrate (NarR) levels (Richardson et al., 2009). The CuZ center can be damaged by oxygen and is inactivated by transient oxygen (Frunzke and Zumft, 1986) which likely can be the cause for elevated N_2O emissions in environments with highly fluctuating O levels (Richardson et al., 2009). Since the two copper centres together require 12 copper ions (Haltia et al. 2003; Paraskevopoulos et al. 2006) the enzyme is highly copper dependent and it has been shown that N_2O emissions rise in copper deficient media (Granger and Ward, 2003; Matsubara et al., 1982). Compared to other denitrification enzymes, the activity of N_2OR has also been shown to be more affected by low temperatures (Bakken et al., 2002) which putatively could explain observed peaks in N_2O emissions during winter time (Dörsch et al., 2004; Sehy et al., 2003). However these peaks could also be attributed to nutrient release during freeze-thaw cycles (Christensen and Christensen, 1991). Nevertheless, the abiotic factor affecting N_2OR that is most widely discussed is pH (Simek and Cooper, 2002). Indeed the enzyme has been shown to be sensitive to pH and to have its activity optimum at pH > 7 in vitro, which seems, however, to be dependent on the electron donor (Berks et al., 1993; Dell'Acqua et al., 2008). Accordingly, a number of studies report higher N_2O-emissions from denitrification at low pH (Baggs et al., 2010; Čuhel and Šimek,

2011; Kesik et al., 2006). However, others report adaptation of denitrifier communities to low pH levels in situ (Parkin et al., 1985; Šimek et al., 2002; Yamulki et al., 1997) and Palmer et al. (2010) could demonstrate consumption of N_2O at low pH.

2.2 *nosZ* as a molecular marker

While abiotic factors certainly are of importance for the formation and functioning of N_2OR, the ultimate factor regulating whether N_2O will be reduced to N_2 in a given environment is whether there are organisms present that harbour the gene encoding the enzyme - *nosZ*. Denitrification as a trait is widespread among many bacterial and archaeal phyla, and even fungi. Moreover, since the trait is facultative, denitrification genes are likely to be horizontally transferred (Alvarez et al., 2013; Jones et al., 2008; Philippot, 2002). Since the energy gain from N_2O-reduction is comparatively low, it has been discussed whether *nosZ* is more prone to gene loss, as suggested by the observation that a large fraction of denitrifiers do not possess the gene (**paper II**; Jones et al. 2008). Although it has been shown that *nosZ* phylogenies are most congruent with 16S rRNA phylogenies from the same organisms compared to other denitrification genes (Dandie et al. 2007a; Jones et al. 2008), closely related genes can be found in distantly related organisms and vice versa and closely related organisms may or may not possess a *nosZ* gene (Philippot, 2002). Of this follows that comprehensive studies of the N_2O-reducing microbial community cannot rely on 16 rRNA as a molecular marker but need to address the functional gene itself.

The PCR-based assays used in microbial community ecology rely on primer sequences specific to the target gene. Primers for functional genes typically target regions within the gene that encode for conserved structural features of the enzyme such as catalytic domains, which in the case of *nosZ* include regions encoding for the CuA and the CuZ active sites (**paper I**). The comprehensiveness and specificity of a given primer pair depends on the variation present in the sequences used in their design. In case of *nosZ*, the primers used during the last decade were based on sequences obtained from cultured proteobacterial species (Henry et al., 2006; Scala and Kerkhof, 1999; Throbäck et al., 2004) that were relevant for human health and agriculture. However, the increasing number of sequenced genomes from a more diverse range of organisms available in the databases, combined with indications for a wider range of N_2OR diversity (Jones et al., 2011; Sanford et al., 2002; Simon et al., 2004), prompted a re-evaluation of the *nosZ* sequence diversity present

in the databases, and new primers were designed to target a hitherto unaccounted clade of the gene **(paper I**).

2.3 *nosZ* sequence diversity and abundance

The diversity of *nosZ* genes described in previous studies consisted primarily of sequences from α-, β- and γ- proteobacteria due to the limitations of sequence databases (Jones et al. 2008; Palmer et al. 2009). However, the rapid increase of available genome sequences since the advent of affordable next generation sequencing methods has resulted in a dramatic expansion of the known *nosZ* diversity. For comparison, while Jones et al. (2008) found 43 distinct sequences of *nosZ* in public databases, 142 sequences were obtained in **paper I** and 314 in **paper II**. The maximum likelihood phylogeny of the 142 full length *nosZ* sequences in **paper I** revealed an entire new clade of potential N_2O reducers belonging to a wide range of bacterial and archaeal phyla, designated clade II (Figure 1). Organisms possessing a *nosZ* from this new clade included hyperthermophilic archaea, δ- and ε-proteobacteria, Gemmatimonadetes and Bacteroidetes. The latter three groups have been shown to be highly abundant in soils and fresh water lakes (Lauber et al., 2009; Newton et al., 2011), thus hinting at the relative ecological importance of *nosZ* Clade II. This was corroborated by quantitative PCR showing *nosZ* Clade II to be equally abundant or in even higher abundance than the traditional *nosZ* Clade (subsequently designated *nosZ* Clade I) in a variety of environments, spanning from arable soils and rice paddys to lake sediment and waste water treatment plants (**paper I**). Earlier studies have found that, compared to the abundance of the nitrite reductase genes *nirS* and *nirK* potentially producing N_2O, the *nosZ* gene abundance was 2-10 times lower (Bru et al., 2011; Hallin et al., 2009; Henry et al., 2006). The discovery that *nosZ* Clade II is similar abundance in many environments as *nosZ* clade I has diminished this gap, however *nir* abundances are still substantially higher. Interestingly, Sanford et al. (2012) demonstrated that a strain of the *nosZ* Clade II harbouring bacterium *Anaeromyxobacter dehalogenans* could grow more efficiently with N_2O as electron acceptor compared to a *nosZ* Clade I harbouring *Pseudomonas stutzeri* strain, indicating a higher efficiency of the clade II N_2OR. In addition, while several denitrifying species with *nosZ* Clade I have been shown to exhibit inhibition of N_2O respiration in the presence of nitrate (Blackmer and Bremner, 1978; Richardson et al., 2009), this was not observed for *Anaeromyxobacter*. This could indicate that i) Clade II N_2OR might be affected differently by environmental factors, which is supported by the fact that it has a different secretion pathway, and ii) that organisms possessing *nosZ* Clade II might be

adapted to different ecological niches than canonical denitrifiers with *nosZ* Clade I (cf. **paper III**). This is underscored by the fact that most *nosZ* Clade II organisms as shown earlier belong to different bacterial and archaeal phyla. In **paper I**, many species with *nosZ* Clade II were among the Bacteroidetes. Interestingly Philippot et al. (2009a) found that Bacteroidetes (*nosZ* Clade II) and α-proteobacteria (*nosZ* Clade I) showed contrasting spatial patterns across a pasture thus indicating adaptation to different environmental conditions in situ which, one might speculate, inter alia could be temperature and O, NO_3^- and pH levels affecting denitrification.

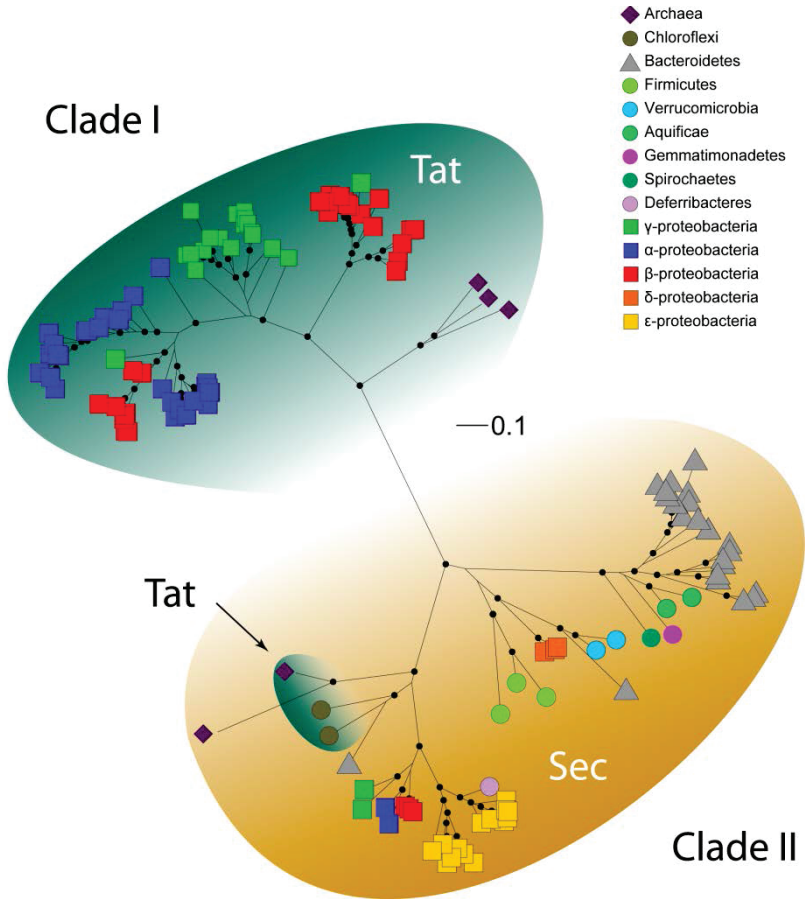

Figure 1. Unrooted maximum likelihood phylogeny of full-length *nosZ* amino-acid sequences obtained from genomes. The distribution of signal peptide motif detected for each clade is indicated. Symbols on tree tips specify major taxonomic groups, and scale bar indicates corrected substitutions per site. Nodes with > 70% bootstrap support (n=500) are denoted by dots.

2.4 Secretion pathways of N_2OR

An interesting observation in **paper I** was that the two different *nosZ* clades had signal peptides coding for different protein translocation pathways. With the exemption of the archaeal genus *Ferroglobus* and some Chloroflexi, all members of *nosZ* Clade II had the signal peptide coding for the widely used secretory pathway (sec) where proteins are transported unfolded across the cytoplasmic membrane. By contrast, all sequences coding for *nosZ* Clade I had the signal peptide coding for the twin arginine translocation (tat) pathway, in which proteins are transported across the membrane already folded (Pohlschröder et al., 2005). This pathway is used by many organisms to

transport redox proteins (Lee et al., 2006). Rose et al. (2002) could see that the tat pathway was much more extensively used among halophilic archaeal strains compared to mesophilic or thermophilic archaeal strains and thus drew the conclusion that the pathway might be an adaptation to high salt concentrations outside the cytoplasm which interfere with protein folding. However, the halophilic bacterium *Salinibacter ruber* did not show the same extensive use of the Tat-pathway in general (Dilks and Giménez, 2005) and for N_2OR in particular **(paper I)**. Energetically the Tat-pathway requires the equivalent of 10 000 ATP to transport a folded protein while the Sec-pathway requires 1 ATP per 20 amino acids (Lee et al., 2006). This large difference raises the question why organisms use a secretion pathway as comparatively costly as the Tat-pathway? One rationale is that complex co-factors can be inserted in the cytoplasm which sidesteps the requirement for additional mechanisms to export the cofactor and to catalyse its insertion into the protein in the periplasm (Palmer and Berks, 2012). However even though the CuZ center of N_2OR is a complex co-factor it is inserted in the periplasm (Zumft, 2006). Although this puzzle is not yet solved it is intriguing to think that using the less costly Sec-pathway might contribute to more efficient usage of N_2O as electron acceptor as observed for *Anaeromyxobacter dehalogenans* by Sanford et al. (2012). Additionally, operons encoding for Clade II type N_2OR include accessory genes different from those encoding Clade I N_2OR (Sanford et al. 2012; Simon et al. 2004) indicating different mechanisms of protein folding, translocation and assembly.

2.5 Does N_2O reductase diversity matter?

An ongoing debate in ecosystem ecology is whether biological diversity matters for ecosystem functioning. In ecosystem models, microbial diversity and community structure are usually not addressed and instead put into a black box of kinetic constants and response functions (Schimel, 2001). This is based on the assumption that microbial communities are resistant, resilient and functionally redundant as well as so taxonomically diverse that incorporation into models is deemed neither necessary nor feasible (Allison and Martiny, 2008). Here it is assumed that turnover rates do not change with environmental conditions and that microbial processes are never limited by the abundance of any microorganism (Schimel, 2001). Microbial communities are believed to be resistant in the sense that disturbances do not result in changes of community composition, and resilient, meaning that if they change they quickly return to their original stage. However in accordance with Hooper et al. (2005), who

state that higher levels of biodiversity correspond to increased ecosystem functioning, an extensive review has found that a majority of microbial communities are neither resistant nor resilient, especially when longer time periods and repeated disturbances are considered (Allison and Martiny, 2008).

In case of denitrifying bacteria, Philippot et al. (2013) could show that removal of 75% of bacterial OTU's (operational taxonomic units) led to a decrease in potential denitrification activity of about 48 to 88 %, and that the diluted communities stabilized at this lower level showing that these communities were neither resistant nor resilient. Functional redundancy in turn means that loss of diversity does not alter the functional performance of a given biological system and thus figures as a null hypothesis of biodiversity (Loreau, 2004; Nannipieri et al., 2003). The common definition for functional redundancy is in terms of niche similarity, where species with identical niches may still differ in their ability to exploit resources. This definition is however flawed since organisms in a changing environment such as soil that differ in their ability to exploit resources, are not redundant. While a community might temporarily perform equally after diversity loss, differences in performance would become evident over time with changing conditions. This can be exemplified with denitrifiers that perform alike under certain conditions, but have different niches when it comes to e.g. low temperature. Here, loss of those organisms adapted to low temperatures would lead to lower overall community performance in winter time even though a measurement at warmer temperatures would not show this. Consequently, one needs to ask whether organisms can be perfectly redundant, meaning that losing any species from the community would not alter the function within the range of environmental conditions prevalent in situ (Johnson, 2000).
Cavigelli & Robertson (2001) observed that different denitrifier communities from geomorphically similar soils had different N_2O-emission rates and that each community was regulated differently by environmental factors. Moreover inocculation with *Agrobacterium tumefaciens* lacking the *nosZ* gene led to an increase in N_2O-emissions in three different soils (Philippot et al., 2010b).

In **paper I,** a previously unknown diversity of N_2O-reducing organisms from very different microbial phyla is described. These *nosZ* clade II bearing organisms have been shown to have different N_2O-sink capacities and to be regulated differently by environmental factors compared to *nosZ* clade I (Jones et al., 2014). According to Loreau (2004) perfect redundancy requires stable coexistence while stable coexistence in turn requires differences between organisms which lead to functional complementarity and not redundancy

which is why he argues that perfect functional redundancy cannot exist. However, he further argues that spatial and temporal environmental variability makes functional redundancy possible at small spatial and temporal scales. This was confirmed by studies on plants showing that while plant communities appeared to be redundant in terms of biomass in short term, diversity increased biomass production in the long run (Reich et al., 2012). In conclusion it seems thus unlikely that a given N_2O-reducing community is redundant over long periods of time and changing environmental conditions.

2.6 Conclusions and perspectives

Given that *nosZ* Clade II was in equal or higher abundance than *nosZ* Clade I in most environments investigated in **paper I,** its ecological significance in terms of N_2O reduction should not be underestimated, as demonstrated in Jones et al. (2014). Future studies quantifying the genetic potential for microbial N_2O reduction need to address both *nosZ* Clades in order to assess the N_2O-reducing community comprehensively. Since most studies concerning the influence of abiotic factors on denitrification and N_2O reduction have been carried out using canonical denitrifiers carrying *nosZ* clade I, effort should be put into investigating whether *nosZ* Clade II carriers are affected differently by these factors and thus maybe occupy different ecological niches. The latter is corroborated by the observations in **paper III** where the two clades differ in their affinity to plant roots and should be investigated further. The environments where *nosZ* gene abundances were assessed in **paper I,** were limited in number and no marine samples were investigated. Future studies should investigate more environments and include marine samples in order to assess the significance of *nosZ* Clade II for N_2O reduction on a global level. Furthermore, studies regarding differences in cellular regulatory mechanisms between *nosZ* clade I and *nosZ* Clade II N_2OR should be performed in order to possibly relate different ecological niches to physiological constrains.

3 Co-occurences of denitrification genes

3.1 Modularity of the denitrification pathway

That the denitrification pathway is modular, meaning that organisms do not always have the complete set of denitrification genes, has been previously suggested (Zumft, 1997). However, the scope of this modularity and how it relates to taxonomy and habitat preferences has not been addressed. This is perhaps unsurprising since studying the genetic potential of microorganisms requires fully sequenced microbial genomes, which until recent years have not been available in sufficient numbers. **Paper I** was focused on the N_2OR gene *nosZ*, and the next logical step appeared to be to investigate how N_2O-reduction is connected to N_2O-production in microbial genomes. Since NO needs to be detoxified to N_2O, nitrite reductase is usually considered to be the N_2O-producing step in the denitrification pathway. Two evolutionary different and most often mutual exclusive (**paper II**) nitrite reductases exist encoded by the genes *nirS* and *nirK* and it has been indicated that these occupy different ecological niches (Enwall et al., 2010; Hallin et al., 2009; Jones and Hallin, 2010). One of the aims of **paper II** was to investigate the frequence of co-occurrence of *nosZ* with either *nirS* or *nirK* since if the *nosZ* gene would be lacking more frequently in genomes with one or the other nitrite reductase gene this would theoretically increase the potential for N_2O-emissions in a given environment dominated by this gene.

In **paper II,** all microbial genomes available at the time (November 2012) containing either the nitrite reductase genes *nirK* or *nirS* or the nitrous oxide reductase gene *nosZ* were downloaded from the National Center for Biotechnology Information database (NCBI, USA). At that point, the NCBI contained around 5000 microbial genomes including fungi, of which many were drafts. As a side note, today (August 2015) there are 47 777 fully

sequenced prokaryotic genomes alone. Nevertheless, after curating the data there was a dataset of 652 genomes containing either a *nir* or a *nos* gene (Figure 2) in no less than seven different combinations. Distinct co-occurrence patterns of the two genes with *nosZ* were detected where *nosZ* co-occurred much more often with *nirS* than with *nirK* (Figure 3). This could be interpreted such that the *nirS* gene can serve as an indicator for canonical denitrifiers who reduce nitrite all the way to dinitrogen which was emphasized by the fact that one of the nitric oxide reductase genes *qnorB* and *cnorB* almost always co-occurred with *nirS* while it was often lacking with *nirK*. In contrast, the lack of a *nosZ* gene in the majority of all cases in organisms with *nirK* could be interpreted such that *nirK* might be an indicator for nitrite reducers putatively giving rise to N_2O-emissions. That many *nirK*-type organisms lacked a *nor* gene is surprising given the fact that nitric oxide is a toxic free radical. However these organisms might be detoxifying using a third nitric oxide reductase named qCu_ANor that has been described in *Bacillus azotoformans* (Suharti et al., 2004) which was not included in the study.

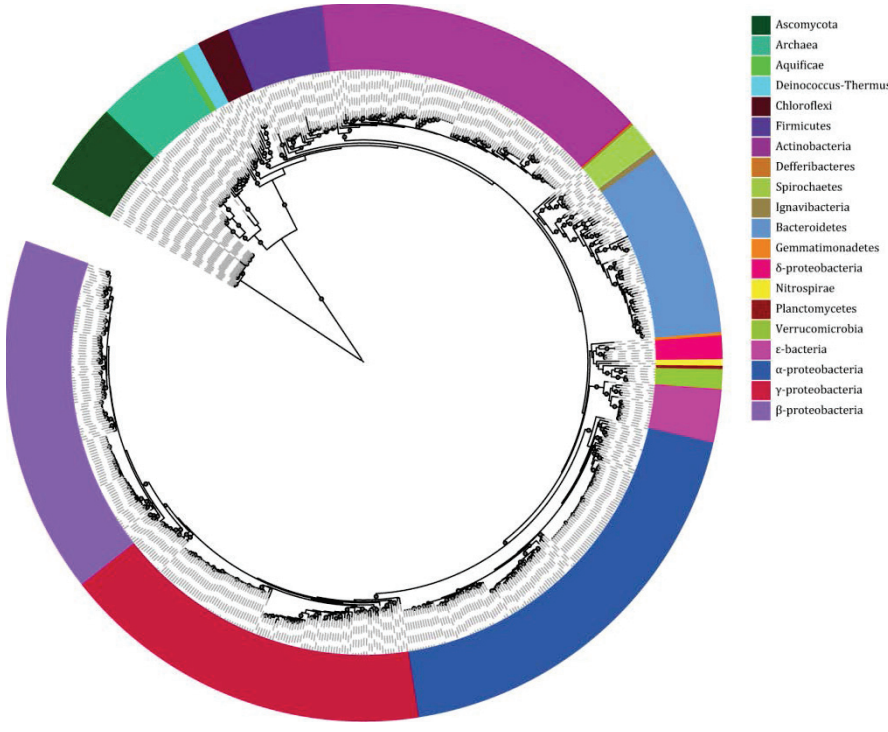

Figure 2. Maximum likelihood phylogeny of full-length 16S/18S rRNA sequences from 652 organisms with denitrification genes. The coloured ring represents taxonomic affiliation as indicated by the legend. Bootstrap values > 70% are indicated by black circles. Classification is based on the SILVA database with denomination according to NCBI taxonomy.

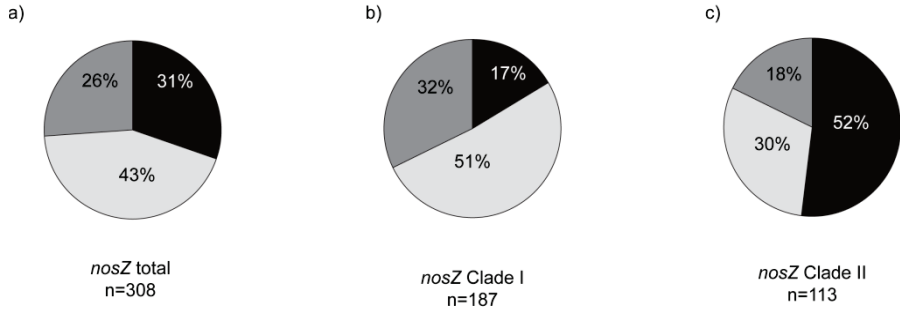

Figure 3. Pie charts depicting genomic co-occurrence of the *nosZ* gene with the nirK and nirS genes in percentage of a) the total number of organisms harbouring *nosZ*, b) organisms from *nosZ* Clade I and c) from *nosZ* Clade II. Genomes with only *nosZ* (black), *nosZ* and *nirK* (light grey) and *nosZ* and *nirS* (dark grey) are indicated. Six genomes harbouring both *nirS* and *nirK* in addition to *nosZ* are excluded, as well as eight halophilic Archaea that group outside Clade I and II in b) and c).

3.2 nirK and *nirS* niche partitioning

Niche partitioning between *nirK* and *nirS* communities has been suggested in several studies (Desnues et al. 2007; Jones & Hallin 2010; Junier et al. 2008; Knapp et al. 2009; Smith & Ogram 2008). Especially soil moisture has been pointed out as a partitioning factor since *nirS* has been found to be dominating in waterlogged and wet soils (Ligi et al. 2013; Kim et al. 2008; Petersen et al. 2012; Philippot et al. 2009c) as well as sediments (Abell et al., 2010; Nogales et al., 2002) whereas *nirK* communities dominated in comparatively dry soils (Babić et al., 2008; Bremer et al., 2009; Dandie et al., 2008). Moreover, it has been suggested that organisms harbouring *nirS* are better adapted to stable anoxic environments, while *nirK* communities tend to dominate under more recurrently changing conditions (Graham et al., 2010; Knapp et al., 2009; Petersen et al., 2012).

In an evolutionary context, the existence of more than one allele in a population is prohibited by genetic drift and allele fixation. With the caveat that evolutionary theory was originally developed with monophyletic animal populations in mind and providing that horizontal gene transfer occurs in denitrifier communities (Jones et al. 2008; Alvarez et al. 2013) one can extrapolate that: Two functionally equivalent genes can co-exist only if i) communities are geographically or temporally separated so that exchange of genetic material cannot occur, ii) communities harbour only one of the genes due to genetic barriers prohibiting gene transfer (Kurland et al., 2003), or iii) the genes are not truly functionally equivalent meaning that possessing the one or the other provides a selective advantage under different environmental conditions. In case of the two functionally equivalent genes *nirS* and *nirK* we

know that this is unlikely to be the case since the two genes have been found in the same environment in numerous studies (e.g. Bañeras et al. 2012; Enwall & Hallin 2009; Smith & Ogram 2008). Considering ii, in **paper II** we can see that Ascomycota and Actinobacteria exclusively have a *nirK* gene while in contrast ε-bacteria only have *nirS*, thus indicating putative genetic barriers in these phyla. The study however, also shows that both genes occur in closely related strains of α-, β-, and γ-proteobacteria indicating that no genetic constraints concerning transfer of the *nir* genes exist in these classes. It has been shown that a *nirK* gene from a *Pseudomonas aureofaciens* strain could replace a knocked out *nirS* gene in a *Pseudomonas stutzeri* strain and encode a functional nitrite reductase (Glockner et al., 1993). Thus there might be genetic constraints concerning the acquisition of one of the *nir* genes in some microbial phyla but not in others. Concerning the latter the most plausible explanation why there are still two functionally equivalent genes left is iii; adaptation to different environmental conditions.

When comparing the 652 genomes, 10 were found to harbour both *nirS* and *nirK*, which earlier had been thought to be mutually exclusive in the genomes of denitrifiers. The question whether both genes translate into functional enzymes remains however unanswered. It is intriguing to speculate whether possessing both gene variants is a result of adaptation to certain environmental conditions, which is indicated by the fact that these organisms were significantly overrepresented in wastewater treatment plants and among extremophilic organisms (**paper II**). Wastewater treatment plants built to optimize nitrogen removal use a two-step process where aerobic ammonia oxidation to nitrite and further to nitrate is facilitated by aeration and denitrification occurs under anaerobic conditions so that oxygen levels fluctuate rather rapidly and substantially. Four of the organisms harbouring both nitrite reductases that were isolated from wastewater were *Pseudomonas stutzeri* strains. *Pseudomonas stutzeri* occurs naturally in soils and sediments and can be an opportunistic pathogen (Lalucat et al., 2006). Strains of this species have been shown to denitrify under high oxygen levels (Ji et al., 2015; Su et al., 2001), a characteristic which is thought to be an adaptation to highly fluctuating oxygen levels such as in tidal sand flats (Gao et al., 2010). Under such rhythmically changing conditions it might be of advantage to employ both enzymes in order to gain maximum efficiency. Given that other environmental factors are kept relatively constant in a wastewater treatment plant, such fine-tuned adaptation to one factor is not unlikely to happen. However research is required to elucidate if, how and under which conditions the two enzymes are active in the same cell.

3.3 Taxonomy as a proxy for co-occurrence types

Although 16S rRNA based taxonomy at species level cannot serve as a proxy for N_2O-regulation as a trait, the question of whether genomic co-occurrence patterns of denitrification genes are independent of taxonomy remains unanswered. The ecological coherence of high taxonomic ranks, defined such that members of a taxon share general life strategies or traits that distinguish them from other taxa, has been proposed previously (Philippot, Andersson, et al. 2010). The rationale is that phylogenetic relationships and ecotypes coincide to a certain extent in monophyletic lineages with the underlying assumption that the particular lineage has evolved to occupy a specific ecological niche. A prominent example here is the phylum Cyanobacteria, in which all representatives are photoautotrophs. However, such a straight-forward pattern is only the case for a few microbial phyla, whereas the majority has members at lower taxonomic ranks that are adapted to many different ecological niches, thus obscuring possible coherent ecological characteristics (Philippot, Andersson, et al. 2010). Nonetheless members of theses phyla or other higher taxonomic ranks can still have an overall preference for a particular habitat or environmental parameter setting which is not strong enough to be detectable among its subtaxa, but becomes apparent when higher taxonomic units are considered (Koeppel and Wu, 2012). A number of studies have associated shifts in higher order community structure with health conditions such as diabetes (Giongo et al., 2011) obesity (Ley et al., 2005) and cancer (Turnbaugh et al., 2007), while other studies could associate higher taxonomic ranks with particular environments such as marine or fresh water (Glockner et al., 1999; Zwart et al., 2002). However, a pit-fall of such association studies is the inability to determine whether the higher order associations truly reflect habitat associations of the whole taxonomic unit or only of a few highly abundant lower level taxa. Re-evaluation of two studies of the human skin and gut microbiome show this to be the case for the phylum Firmicutes in human guts that had been associated with obesity, where only the *Clostridiales* within the Firmicutes phylum were associated with obesity (Koeppel and Wu, 2012).

In case of co-occurrence types of *nir* and *nosZ* genes, seven different types were found to not be randomly distributed among the different taxonomic divisions of organisms in the dataset (**paper II**). Instead, significant patterns could be discerned at the phylum, class and order levels. The Ascomycota and Actinobacteria had the same pattern (*nirK*-only) throughout all taxonomic ranks, whereas *nosZ*-only types were overrepresented among members of the Bacteroidetes, even though other patterns could also be observed in this

phylum. However, a third of these organisms also did not have a *nor* gene, which could be attributed to the order *Flavobacteriales* making this pattern more specific to the *Flavobacteriales* than the Bacteroidetes in general. In contrast, patterns of partial pathways were significantly underrepresented among Proteobacteria, especially β- and γ-proteobacteria. The picture that is thus emerging when N_2O-regulation is considered is one of three different groups of organisms: one that potentially produces N_2O but does not reduce it (Actinobacteria and Ascomycota), one that consists of canonical denitrifiers capable of producing and reducing N_2O (Proteobacteria), and one that potentially reduces but does not produce N_2O (Bacteroidetes or Flavobacteriales). This coincides with the results of Philippot et al (2009a, 2009b) who investigated the abundance of different taxa at high taxonomic ranks, as well as the abundance of different denitrifier genes and potential N_2O production and denitrification rates on a field that was subjected to different cattle grazing regimes. Interestingly, the percentage of N_2O to total denitrification activity ($N_2 + N_2O$) was lowest in the region of the field with the highest relative abundance of Bacteroidetes and lowest abundance of Actinobacteria. Furthermore, regions of the field in which *nirS* and *nosZ* Clade I were most abundant also had the highest relative abundance of Betaproteobacteria, corresponding to our finding that the co-occurrence of *nirS* and *nosZ* is the predominant pattern of denitrification genes within this class. Thus, taxonomic information in conjunction with the co-occurrence patterns described in **paper II** have the potential power to predict N_2O-emission potential in situ. However, a distinction has to be made between taxonomic units that always show the same pattern such as the *Actinobacteria* and the *nirK* only pattern, and those where there is a significant association only such as the Betaproteobacteria and the *nirS* and *nosZ* pattern, where by no means do all Betaproteobacteria have this pattern. Even though there are significant trends among the Proteobacteria, we observe all co-occurrence patterns within this phylum indicating that denitrification as a facultative trait is evolutionarily labile among taxa at species and strain level, with frequent transitions between genetic set-ups. Martiny et al. (2009) state that for *Prochlorococcus*, the transition between high-light and low-light habitats occurs at greater phylogenetic depth than the transition between different temperatures, which in turn occurs at greater depth than transitions between habitats with different nitrate concentrations, indicating that some transitions are more difficult to make than others. Analogously, the acquisition of denitrification genes may be more difficult for Ascomycota and Actinobacteria, resulting in nitrite reduction being conserved at greater phylogenetic depth, whereas it might be

comparatively easy for Proteobacteria resulting in gene transfer occuring at species and strain level.

3.4 Conclusions and perspectives

Paper II confirmed that the denitrification pathway is highly modular with a range of different possible co-occurrence patterns between the *nir* and *nos* genes. However, the co-occurrence patterns were neither randomly distributed among taxa nor among habitats. Even though there are exceptions, we identified a significant pattern of *nirS* genes being representative of canonical denitrifiers and *nirK* as being more likely associated with nitrite reducers with truncated denitrification pathways. This corroborates earlier suggestions that organisms with either gene residing in their respective genome occupy different ecological niches. The two different clades of *nosZ* also show non-random co-occurrence patterns where organisms with *nosZ* Clade II in more than one third of all cases do not have a *nir* gene while this is much more rare in organisms with *nosZ* Clade I. Thus *nosZ* Clade II organisms are more prone to act as N_2O sinks which has been demonstrated recently (Jones et al., 2014). **Paper II** was a hypothesis generating study and indeed many questions arise from it. The basis of the study were fully sequenced microbial genomes from public databases which at the time of download were still heavily biased towards culturable strains important for human health and agriculture. It would thus be interesting to see whether the observed patterns can be confirmed in genome studies focusing on environmental samples. In addition, since the number of available genome sequences in the databases rises exponentially over time, it would be of interest to do a follow up study in some years and see if our observations are corroborated with a larger dataset. The hypothesis of whether organisms with *nirS* or *nirK* occupy different niches regarding stable versus fluctuating anoxic conditions could be tested in microcosm studies that are subjected to different oxygen regimes with subsequent gene quantification. These could be combined with metagenomic analyses and denitrification activity measurements in order to test the hypothesis that *nirS* organisms represent canonical denitrifiers, which if dominant may lead to lower N_2O-emissions. Establishing a link between the *nirK*/*nirS* ratio, fluctuating conditions, and high N_2O-emissions could then also answer why we see hotspots and hot moments of N_2O-emissions in environments with fluctuating oxygen levels. *Pseudomonas stutzeri* strains are relatively easy to cultivate so that investigating if, when and how the four strains which have both *nirS* and *nirK* residing in their genomes use the enzymes should probably not prove too difficult. Maybe this could lead to the

discovery of a highly efficient waste water treatment strain? Another interesting question that arises from **paper II** is whether taxonomy can serve to predict denitrification gene co-occurrence patterns and thus N_2O-emission potential. Here it would be of special interest to explore the N_2O-reducing Bacteroidetes and see if a putative N_2O sink function can be narrowed down to *Flavobacteriales* or whether this observation merely is a case of database bias or if it is reflected in nature and if so, what are the underlying ecological and evolutionary mechanisms?

4 Community assembly in the rhizosphere

The rhizosphere of agricultural crops is an import model system to study denitrification and N_2O-emission dynamics since denitrification is stimulated by a living root system (Woldendorp, 1962) and because the major part of anthropogenic N_2O-emissions originates from agricultural soils (IPCC, 2013; Smith et al., 2012). The relative influence of plant species versus edaphic factors on denitrification rates and N_2O-emissions and how this relates to the microbial community structure has long be discussed (Philippot et al., 2009c) and was a focus in the experiment in **paper III.** Here, a pot experiment was set up in growth chambers growing barley (*Hordeum vulgare*) and sunflower (*Helianthus anuus*) in two agricultural soils, one with a high clay content and one with a high portion of sand (Figure 4).

Monoculture systems that are prevailing in today's agricultural systems have been shown to give rise to higher N_2O-emissions compared to those with mixed plant species (Niklaus et al., 2006; Sun et al., 2013) which is why **paper IV** focused on the effect of intercropping on denitrification rates and the community structure of N_2O-reducing microorganisms. In this experiment lucerne (*Medicago sativa*) and cocksfoot (*Dactylis glomerata*) were grown in rhizoboxes either as single crops or intercropped in an agricultural soil from a site near Alnarp, Sweden (Figure 5).

Figure 4. Pot experiment with barley and sunflower as well as unplanted soil randomized on a tray in a growth chamber kept at 20°C during day time and 15° during night with 18h day length. (Photo: Daniel Graf)

Figure 5. Sketch of a rhizobox, indicating the height, width and depth (left), and photo of a rhizobox with intercropped cocksfoot (*Medicago sativa*) and lucerne (*Dactylis glomerata*) taken at the soil and root sampling occasion (Photo: Georg Carlsson).

4.1 Denitrification and N_2O-emissions in the rhizosphere

Increased rates of denitrification in rhizosphere compared to bulk soil have been measured in a number of studies (Bakken, 1988; Højberg et al., 1996; Klemedtsson et al., 1987). The activity was positively correlated to NO_3^-, where at low NO_3^- concentrations, denitrification rates could even be lower in the rhizosphere compared to bulk soil (Qian et al., 1997; Smith and Tiedje, 1979). The positive rhizosphere effect on denitrification activity has been shown to decrease with moisture tension (Bakken, 1988) and was confined to an air-filled pore space below 10-12% (v/v) (Prade and Trolldenier, 1988). However, studies on rice paddy fields have found that N_2O-emissions occur mainly during periods when the fields are not flooded (Lindau et al., 1990; Xing, 1998). The primary driver behind the rhizosphere effect is postulated to be low molecular organic compounds from roots and denitrification rates have been positively correlated to soluble organic C (Baggs and Blum, 2004) and the addition of root exudates or mucilage (Henry et al., 2008; Mounier et al., 2004). However, the latter studies could not observe any differences in the denitrifier community composition depending on rhizodeposits. Differences between plant species with regard to their effect on denitrification activity have been observed for legumes and cereals (Kilian and Werner, 1996; Scaglia et al., 1985; Svensson et al., 1991), with legumes generally stimulating higher activity, but also for different grass species (Patra et al., 2006) and wetland species (e.g. Ruiz-Rueda et al. 2009). Some studies also report plant species to significantly influence the denitrifier community composition (Bremer et al., 2007; Chèneby et al., 2004; Hamonts et al., 2013; Patra et al., 2006; Philippot et al., 2002; Ruiz-Rueda et al., 2009) while others could not find such a connection (Deiglmayr et al., 2004).

Emissions of N_2O have been observed to be greater in the presence of growing plants, particularly legumes, than from bare soil (Klemedtsson et al. 1987; Højberg et al. 1996; Ni et al. 2012; Dong et al. 2005; Ding et al. 2007; Sey et al. 2010; Hénault et al. 1998; Verma et al. 2006). Emission factors vary from 0.1% to 7% of nitrogen applied in different agricultural systems (Skiba and Smith 2000), reflecting differences in vegetation type, crop management, inherent soil properties and climate. In **papers III** and **IV** we could not observe any differences between planted and unplanted soil concerning denitrification activity in any of the soils used in the experiments. This might be attributed to the fact that we did not add any fertilizer during the experiment, putatively resulting in comparatively low NO_3^- levels. However no N limitation of the plants could be observed in the three soils. In addition no plant species effect could be observed for either *nosZ* Clade I or Clade II communities in the soil.

However, a significant plant effect could be observed for the *nosZ* Clade I community in **paper IV,** which mainly could be attributed to compositional differences between the unplanted soil and the soil planted with lucerne (Figure 6) which indicates that a putative plant effect on the N_2O-reducing community both depends on the plant and the clade of *nosZ* involved. **Paper III** also showed that soil effects overrode plant effects even in association with roots concerning both the community structure of *nosZ* Clade I and II and the gene abundances of 16S rRNA, *nirK*, *nirS* and *nosZ* II. This is in accordance with a number of recent studies that have shown that soil effect overrides plant effect considering microbial community structure in the rhizosphere (Bulgarelli et al., 2012; Edwards et al., 2015; Lundberg et al., 2012; Prasse et al., 2015).

Interestingly, denitrification and N_2O production could only be measured from barley and cocksfoot roots, but not from sunflower and lucerne even though gene abundance data showed that bacteria in general, as assessed by 16S rRNA genes and N_2O-reducing bacteria were present in similar abundances on the roots of all species (**papers III** and **IV**). However, Li et al. (2007) reported significantly higher concentrations of malate and citrate in the rhizosphere of faba bean compared to the rhizosphere of maize, which is in accordance with previous observations that dicots, particularly legumes, produced and excreted more organic acids to the rhizosphere than monocots (Raghothama 1999). The denitrification activity assay protocol used in **papers III** and **IV** was based on glucose, acetate and succinate, and denitrifying and N_2O-reducing microorganisms specialized on the usage of specific exudates that putatively dominate the root surface of lucerne and sunflower might not have been activated and thus, no activity could be measured.

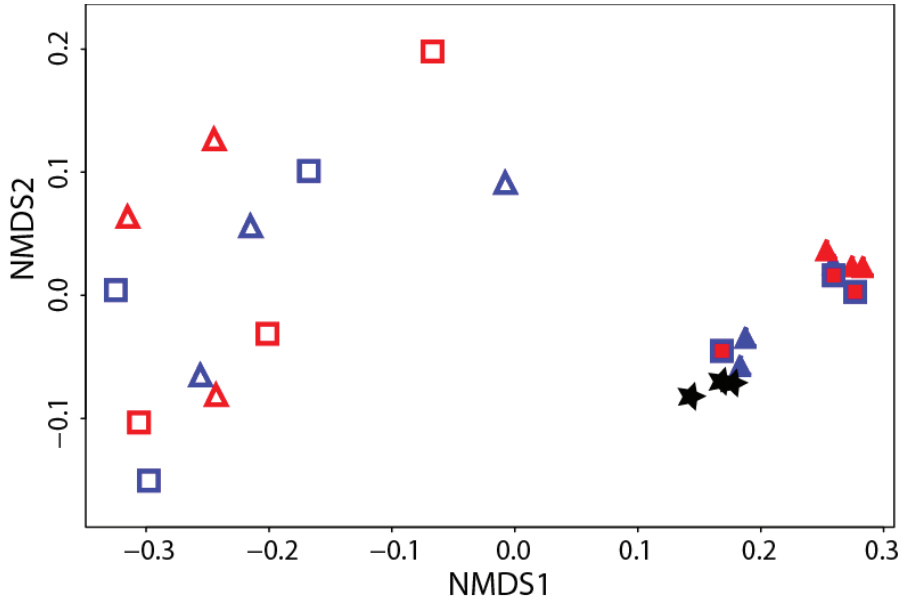

Figure 6. Non-metric multidimensional scaling of generalized UniFrac distances) of *nosZ* Clade I (stress: 0.06) where open symbols depict communities on root samples and closed symbol denote soil samples where blue=cocksfoot and red=lucerne. Triangles symbolize samples from rhizoboxes with monocultures and squares from those with intercropping. Black stars depict unplanted soil.

4.2 Community assembly processes in the rhizosphere

The principles underlying assembly and structure of microbial communities, especially in relationship to ecosystem functioning, are of general concern in microbial ecology. Traditionally, community assembly has been regarded as the result of a continuum of abiotic and biotic factors driving community structure (Weiher and Keddy, 1995), where the driving forces are habitat filtering, dispersal, diversification and drift (cf. Vellend 2010; Nemergut et al. 2013). Neutral theory as proposed by Hubbell (2001) is based on the assumption that in case of ecological redundancy of organisms the composition of communities at a local scale is influenced only by random immigration, birth and death events (Woodcock et al., 2007). While neutral theory applies the concept of ecological redundancy broadly, the competitive lottery model (Sale, 1978) applies it within the frame of a given niche such as the root or soil. Burke et al. (2011) could explain the large variation in the community structure of microbial communities based on taxa between individual specimens of the coastal macro algae *Ulva australis* with the competitive lottery model. Nevertheless, they identified a core of functional genes present in all taxa

associated with the surface of the algae suggesting that assembly is not random in terms of function. Thus,a given microbial community in situ is most likely the result of a combination of both niche driven and neutral processes. In fact, Ferrenberg et al. (2013) could demonstrate that while neutral processes dominate in shaping forest soil communities shortly after a wildfire, the community is later predominantly shaped by habitat filtering, thus indicating that different processes take effect under different circumstances.

From an ecological point of view, conventional agricultural fields constitute a habitat subjected to recurring perturbations, thus keeping it at a pre-successional stage, and unlike natural systems with permanent vegetation, living roots are a temporary phenomenon in this system. Hence, every year a new microbial community is recruited from the soil to constitute the root microbiome of the annual crop and the question arises whether community assembly is based on ecological niche partitioning, neutral processes or a combination of both. One aim of **paper III** was to study whether *nosZ* Clade I and II denitrifiers are recruited to roots stochastically or whether they occupy particular niches in the rhizosphere. Both edge principal component analysis and non-parametric multidimensional scaling in **paper III** and **IV** showed that the structure of root sample N_2O reducing communities was more variable than in the soil communities. The net relatedness index further showed that the *nosZ* communities in the root samples tended to be relatively more phylogenetically dispersed across the phylogeny than soil samples irrespective of the soil type. Taken together this indicated that community assembly on roots is not based on niche partitioning since that would imply selection of close relatives based on advantageous traits for that particular habitat, but rather an important role for competition between close relatives where traits are conserved (Webb et al., 2002). Thus, in accordance with Burke *et al.* (2011a, 2011b) it seems likely that the community assembly of *nosZ* Clade I and Clade II communities on roots in the rhizosphere might be based on the lottery hypothesis which states that within a pool of organisms capable of utilizing resources in a given habitat priority effects take place. However this data only describes the community assembly within each clade of *nosZ* and whether members of the two clades assemble to a common community based on neutral processes or if they separate due to habitat filtering remains unanswered.

As described in **papers I and II**, the two clades of *nosZ* have a distinct taxonomic affiliation and show distinct co-occurrence patterns with other denitrification genes. Hence, it seems logical to ask whether organisms representing either clade occupy different ecological niches in different

environments. This idea is in accord with Jones *et al.* (2014) who found that phylogenetic diversity and abundance of *nosZ* Clade I and II were differently affected by various edaphic factors in bulk soil, and the observation that *nosZ* Clade I, but not Clade II could be readily amplified in roots from various macrophyte species (Hallin et al., 2015). In **paper III,** gene abundance data indicated an affinity to plant roots of *nosZ* Clade I organisms, whereas the abundance and structure of *nosZ* Clade II organisms was mainly governed by a soil effect. While the pattern was the same for *nosZ* Clade II in **paper IV,** this could not entirely be corroborated for *nosZ* Clade I, where no significant difference in abundance between root and soil communities could be observed. Still, the idea that *nosZ* Clade I has an affinity to plant roots is asserted by earlier studies showing that the gene was in greater abundance in the proximity to roots compared to bulk soil (Hamonts et al., 2013; Ruiz-Rueda et al., 2009). Overall, data indicates that *nosZ* Clade II has a preference for soil, which putatively implies that these organisms predominantly are subject to different environmental cues than *nosZ* Clade I organisms which should be investigated further.

4.3 Effects of intercropping

Intercropping is the agricultural practice of growing two or more crops simultaneously in the same location, and is considered to be a more sustainable method for increasing crop yields compared to high-impact practices associated with monoculture-based farming systems (Brooker et al., 2015). The increase in plant biomass resulting from intercropping relies on efficient use of resources via niche complementarity (Hooper and Vitousek, 1997). In particular, enhanced N use efficiency is a key feature of intercropping (Brooker et al., 2015) and several studies have shown that soil N pools increase with decreasing plant diversity (Mueller et al., 2013; Niklaus et al., 2006; Zak et al., 2003). Interestingly, higher N_2O-emissions have been observed in monoculture systems compared to intercropped systems (Niklaus et al., 2006; Sun et al., 2013), indicating an effect of intercropping on microbial communities either producing or reducing N_2O. In **paper IV** we observed that the abundance of *nosZ* Clade II was significantly lower in association with cocksfoot roots from rhizoboxes that were intercropped with cocksfoot and lucerne compared to those planted with cocksfoot alone. This coincided with a higher ratio of potential N_2O-production to total denitrification activity on intercropped cocksfoot roots compared to single cropped ones. Moreover, amplicon sequencing data showed that most *nosZ* Clade II reads associated with roots were most similar to sequences associated with Ignavibacteria,

Gemmatimonades and Opitutaceae. The two known Ignavibacteria genomes harbouring *nosZ* genes are *Ignavibacterium album* and *Melioribacter roseus* and both possess the *nosZ* Clade II gene, but no *nir* or *nor* genes (Graf et al., 2014). These organisms as well as a number of species within the Opitutaceae do, however, possess *nrfA* gene (R. Sanford et al., 2012; Song et al., 2014). Sanford et al. (2012) found *nosZ* Clade II in 15 genomes of organisms that also possessed the *nrfA*, encoding the enzyme catalysing the reduction of nitrite to ammonium within the dissimilatory nitrate reduction to ammonium (DNRA) pathway. The process has been shown to be positively correlated to high C:N ratios (Schmidt et al., 2011; Song et al., 2014), which is found in direct association with roots of non-legume plants such as cocksfoot where the plant competes for NO_3^- and provides low molecular weight C (Danso et al., 1987; Ehrmann and Ritz, 2013). In cereal-legume intercropping systems, competition for soil N by the cereal results in depletion of N in the rhizosphere of the legume, which in turn stimulates N-fixation activity (Danso et al., 1987; Ehrmann and Ritz, 2013; Hauggaard-Nielsen et al., 2001). This in turn probably decreases the C:N ratio making it plausible that heterotrophic microorganisms associated with lucerne roots became C limited due to growth stimulation by excess N and started to compete for C produced by the cocksfoot roots with the DNRA organisms, resulting in lower numbers of the latter. Thus, one possible scenario is that organisms harbouring *nosZ* Clade II in association with cocksfoot roots perform DNRA and reduce N_2O, while intercropping with lucerne results in a decrease in the C:N ratio, and subsequently organisms performing DNRA decrease in number which leads to an increase of the N_2O-production/denitrification ratio on intercropped cocksfoot roots. Alternatively it could be that when N is not limiting due to the presence of lucerne, denitrifiers become more dominant. Among those many could lack *nosZ* resulting in a higher N_2O-production/denitrification ratio.

4.4 Conclusions and perspectives

The rhizosphere of agricultural crops is an important system to study N_2O-reducing microorganisms due to the often high denitrification activity, which increases the risk for N_2O-emissions. **Paper III** and **IV** indicated a niche differentiation between *nosZ* Clade I and II organisms. Here, one needs to keep in mind that the majority of studies on N_2O-reducing organisms have been conducted targeting *nosZ* Clade I only. The present papers emphasize that conclusions drawn from these studies cannot simply be expanded on N_2O reducing organisms in general. This is also affirmed by the observation that the dominant group of *nosZ* Clade II organisms associated with roots in paper IV probably were organisms performing DNRA. Thus, future research needs to

address the environmental factors controlling organisms harbouring *nosZ* Clade II in further detail as well as their abilities to perform DNRA. In addition, the affinity of *nosZ* Clade I to plant roots should be studied further in order to understand the mechanisms behind this. One particular difficulty in these studies was to develop assays to assess potential denitrification and N_2O-production rates in association with roots. The harvested root biomass was comparatively low so that the assay had to be scaled down substantially. While the results showed a distinct difference in root-associated denitrifier activities between the different plant species, development of more sensitive assays is required.

5 General conclusions and perspectives

Due to accelerating climate change, the mitigation of anthropogenic N_2O-emissions is an urgent matter. During the recent decades significant progress has been made studying microorganisms as driving forces of the N-cycle even though many open questions remain. Although the activity, abundance and community structure of denitrifying microorganisms is dependent on abiotic factors, whether N_2O is emitted or reduced to N_2 in a given environment ultimately depends on whether there are microorganisms present that are capable of doing so. Thus, understanding the effects of environmental drivers on microbial biodiversity, and in turn the effect of changes in biodiversity on N_2O-regulation, is crucial. The advent of molecular methods in general and high-throughput sequencing in particular has greatly facilitated this endeavour. This thesis aimed to further elucidate the ecology of N_2O-reducing microorganisms by exploring the genetic diversity, the genomic context, and the relation to plants as habitat shaping factors in the rhizosphere, the results of which are summarized in the following conclusions.

- A new gene variant of the N_2OR encoding gene *nosZ* (Clade II) was described, which largely belongs to organisms taxonomically distinct from the previously known *nosZ* (Clade I) harbouring organisms, and is in equal abundance in many environments (**paper I**). Future research needs to consider *nosZ* Clade II whenever the N_2O-reducing microbial community is assessed.

- Investigation of microbial genomes revealed that co-occurrences patterns of N_2O-reducing *nosZ* with *nir* and *nor* genes were non-randomly distributed among taxonomic groups and habitats (**paper II**). Seven different co-occurrences patterns were observed elucidating the modularity of the denitrification pathway. Thus, denitrifiers with a complete pathway constitute only one group of organisms providing the ecosystem function of

denitrification and the existence of various consortia of NO_2^-- and N_2O-reducers in a given environment needs to be taken into consideration when denitrification and N_2O-reduction are assessed as functions.

- Indications for niche differentiation between organisms harbouring either *nirS* or *nirK* (**paper II**) and *nosZ* Clade I or II (**paper III and IV**) have been found. However, whether these organisms differentiate due to different substrate affinities, adaptation to certain environmental factors or ecological strategies needs to be further investigated.

- In soil, denitrification and N_2O-production as well as the abundance and structure of denitrifying and N_2O-reducing communities was mostly affected by edaphic factors compared to plant factors, even though *nosZ* Clade I abundance could not be explained by either (**paper III**). Thus, future studies ought to address the circumstances in terms of soil type, plant species and environmental conditions under which a rhizosphere effect on N_2O-emissions occurs.

- N_2O-reducing communities on roots differed both in terms of abundance and composition from those in the corresponding soil (**paper III and IV**). While abundance and structure of *nosZ* Clade II communities could predominantly be explained by soil even in association with roots the *nosZ* Clade I community structure was explained by soil and a comparatively large plant factor which together with higher abundances of this clade in association with roots (**paper III**) might indicate niche differentiation between the two clades in the rhizosphere.

- Intercropping with *Medicago sativa* negatively affected the abundance of *nosZ* Clade II in association with roots of *Dactylis glomerata* and increased the N_2O-production/denitrification ratio (**paper IV**) which in conjunction with phylogenetic placement of sequencing reads indicated the presence of organisms with only *nosZ* lacking a denitrification pathway. The fact that these organisms decreased when a legume was present might indicate that organisms harbouring *nosZ* Clade II might be subject to different environmental cues than those with the Clade I type, which warrants further investigations.

Future research on the ecology of N_2O-reducing microorganisms can draw from a rich reservoir of methods and from a vast amount of publicly available

sequence data and, despite some limitations, from accompanying metadata. The data mining study in **paper II** gave valuable insights into the modularity of the denitrification pathway and future research should make more use of the readily available data at public databases to gain deeper insights and generate hypotheses to be tested in field studies or laboratory experiments. Moreover, the continued development of microbial ecology theory should be prioritized to be able to place the data generated in sequencing studies into a theoretical framework that greatly could enhance the interpretation of results. This would provide a great advantage, especially when studying all but straight-forward processes as denitrification and N_2O-reduction.

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