Denitrification: From Genes to Ecosystems

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Cover: Montage showing the phylogeny of globally distributed nirS sequences, where tip labels indicate habitat type (see Figure 6, this thesis). Earth photo courtesy of NASA.

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Abstract

Denitrification is a part of the global nitrogen cycle in which fixed nitrogen in the biosphere is returned to the atmosphere, and is mediated by diverse communities of microorganisms. This thesis seeks to gain a greater understanding of the ecology of denitrifying microorganisms by examining the pathway from four different aspects; gene, population, community, and ecosystem. A combination of bioinformatic analysis of denitrification genes in pure cultures and environmental samples as well as experimental work with denitrifying bacterial cultures and soil microcosms was performed to understand the relationship between genes and ecosystems in denitrification.

Analysis of the phylogeny of genes involved in key steps in the denitrificaiton pathway revealed a different evolutionary pattern for each gene, as processes such as horizontal gene transfer, duplication/divergence, and lineage sorting have contributed differentially to the evolution of catalytic genes at each step. However, genetic variation is not easily translated into an extended phenotype for a population of denitrifiers, as the denitrification phenotype of a set of closely related denitrifying *Bacillus* soil isolates was variable depending on pH. Yet, the genetic community structure was shown to be an important factor in determining denitrification rates and end product ratios, as denitrifying communities in soil microcosms showed differential response to altered ratios of organisms with an without the ability to reduce nitrous oxide. Finally, patterns of *nirS* and *nirK* sequences suggested that community assembly of both denitrifier types was largely driven by niche-based processes, as community structure varied among habitats with different salinities. However, *nirS* and *nirK* denitrifiers were not ecologically equivalent, as patterns of phylogenetic clustering among co-existing *nirS* and *nirK* type denitrifying communities along the same environmental gradient were not comparable.

In conclusion, denitrification is a complex ecological function that is regulated by the interaction between gene and environmental factors, and evolutionary processes that underlying the diversification and distribution of denitrification genes may have direct consequences on the denitrification unction in ecosystems.

Keywords: denitrification, phylogeny, microevolution, community assembly, niche, *nirK*, *nirS*, *nosZ*, *Bacillus*, phenotype

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Dedication

To my family, on both sides of the Atlantic…

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List of Publications

I Jones CM, Stres B, Rosenquist M, Hallin S. (2008). Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. *Molecular Biology and Evolution* 25 (9), 1955-1966.

II Jones, CM, Welsh AW, Throbäck I, Dörsch P, Bakken LR, Hallin S. Phenotypic and genotypic heterogeneity among closely related soil-borne denitrifying *Bacillus* isolates harbouring the *nosZ* gene. Manuscript.

III Philippot L, Andert J, Jones CM, Bru D, Hallin S (2010). Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N_2O emissions from soil. *Global Change Biology* DOI: 10.1111/j.1365-2486.2010.02334.x

IV Jones CM, Hallin S (2010). Ecological and evolutionary factors underlying global and local assembly of denitrifier communities. *The ISME Journal* 4, 633-641.

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

I Performed a major part of the data collection, phylogenetic analysis, and writing of the manuscript.

II Performed a major part of the laboratory work, phylogenetic and clustering analysis, and writing of the manuscript.

III Participated in the planning of the experiment, performed the statistical analysis, and a minor part of the lab work and writing of the manuscript.

IV Participated in the planning of the study, performed all data collection, analysis, and a major part of the writing of the manuscript.

In addition to the papers within this thesis, the author has contributed to the following papers within the timeframe of the thesis work:

Hallin S., Jones, C.M., Schloter, M. and Philippot, L. 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *The ISME Journal* 3, 597-605.

Beier, S., Jones, C. M., Mohit, V., Hallin, S. and Bertilsson, S. Global phylogeography of chitinase genes in aquatic metagenomes. Manuscript.

Willing B., Vörös A., Roos S., Jones C., Jansson A. and Lindberg J.E. 2009. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. *Equine Veterinary Journal* 41(9), 908-914.

Abbreviations

AFLP	Amplified fragment length polymorphism
cnorB	Gene encoding cytochrome-*c* nitric oxide reductase variant
nirK	Gene encoding copper nitrite reductase
nirS	Gene encoding cytochrome-*cd1* nitrite reductase
NMS	Non-metric multidimensional scaling
NO_3^-	Nitrate
NO_2^-	Nitrite
NO	Nitric oxide
N_2O	Nitrous Oxide
N_2	Dinitrogen gas
nosZ	Gene encoding nitrous oxide reductase
NRI	Net relatedness index
NTI	Nearest taxa index
qnorB	Gene encoding quinol nitric oxide reductase
UniFrac	Unique Fraction Index

1 Introduction

Denitrification is a facultative respiratory pathway during which nitrate (NO_3^-) is stepwise reduced to nitrous oxide (N_2O) or nitrogen gas (N_2) via nitrite (NO_2^-) and nitric oxide (NO) under oxygen-limited conditions. Each step is coupled to the electron transport chain such that electrons from reductants can be passed on to different nitrogen oxides, allowing for the generation of a proton gradient across the membrane for energy conservation. On the ecosystem scale, denitrification effectively closes the nitrogen cycle by converting soluble nitrogen to N_2, which is returned to the atmosphere and once again made available for nitrogen fixation. Because of its role in regulating nitrogen in ecosystems, the ecology of denitrification has been the topic of innumerable studies. This interest will only continue to grow given the recent concerns over the effect of N_2O emissions from different environments on climate change, as N_2O is both a potent greenhouse gas and a cause of stratospheric ozone depletion.

1.1 Denitrification in terrestrial and aquatic ecosystems

The global nitrogen cycle begins with the fixation of atmospheric nitrogen into ammonium (NH_4^+) through physical, anthropogenic, or microbially mediated processes. The NH_4^+ that is not incorporated into growing plant or microbial biomass can be converted to NO_3^- via nitrification, an aerobic process, or respired to N_2 through anaerobic ammonia oxidation (ANAMMOX). The nitrate produced by nitrification is then used in denitrification, which in addition to ANAMMOX is a major pathway by which fixed nitrogen is returned to the atmosphere. Denitrification can result from chemical reactions of NO_3^-/NO_2^- with metal cations at low pH conditions (Van Cleemput, 1998); however the bulk of denitrification

activity in most ecosystems is mediated by heterotrophic bacteria and archaea. Denitrifying microorganisms are ubiquitous in nature, and thus denitrification occurs in a variety of terrestrial and aquatic ecosystems provided that i) oxygen is limiting, ii) nitrate or nitrite is readily available, and iii) suitable electron donors are also available. Since denitrification also removes excess anthropogenic nitrogen from ecosystems, is considered an 'ecosystem service' in that it provides a direct benefit to humans through the removal of excess pollutants (Hooper *et al.*, 2005).

Denitrification is a key biological driver behind nitrogen loss from terrestrial ecosystems, which account for approximately 22% of the global denitrification activity (Seitzinger *et al.*, 2006). Spatial and temporal heterogeneity are important factors in determining denitrification rates in soil, as denitrification typically occurs in microsites of well-drained soils (Sexstone *et al.*, 1985), or after periodic wetting caused by rainfall or flooding. Denitrification rates in soil depend on a large number of environmental variables, the most important being soil moisture, which determines oxygen availability, available carbon, pH, NO_3^- concentrations, and temperature. In addition to rates, the ratio of denitrification end-products (N_2O/N_2) is also influenced by environmental factors, particularly pH (Simek & Cooper, 2002). Terrestrial ecosystems account for the majority of N_2O emission to the atmosphere, at an estimated 53% of global annual N_2O emissions (IPCC, 2007a). Of the total estimated N_2O emissions from terrestrial ecosystems, approximately 33% is of anthropogenic origin, with more than half of this amount due to agricultural practices. The IPCC 2007 report projects an increase in anthropogenic N_2O emissions of 30-60% in the next 20 years due to increases in fertilizer usage (IPCC, 2007b). In agricultural soil ecosystems, denitrification is a significant source of N-loss after fertilizer application, ranging from 4-33% depending on cropping system (Aulakh *et al.*, 2001; Ryden, 1983) as well as the type of nitrogen amendment (Enwall *et al.*, 2005; Eckard *et al.*, 2003). Denitrification rates in forest ecosystems is typically lower than that observed in agricultural soils, mainly due to the lower nitrogen inputs that forest soils receive (Barton *et al.*, 1999). Since the nitrogen cycle in forest ecosystems is typically of a closed nature, increased atmospheric nitrogen deposition may have adverse effects if levels rise in excess of plant and microbial demand for biomass production, through either leaching of nitrate into the watershed or increased N_2O emissions (Ambus & Zechmeister-Boltenstern, 2007; Aber *et al.*, 1989).

In aquatic ecosystems, coastal shelf sediments account for the majority of total global denitrification activity (46%), while open-ocean oxygen minimum zones (OMZ) account for 14%, the third greatest proportion after terrestrial ecosystems (Seitzinger *et al.*, 2006). Depending on carbon, nitrate, or ammonium availability, denitrification or ANAMMOX can be the dominant processes for nitrogen removal from aquatic systems (Lam *et al.*, 2009; Ward *et al.*, 2009; Rich *et al.*, 2008; Dalsgaard *et al.*, 2005). Natural wetlands and riparian zones remove a substantial proportion of excess NO_3^- through denitrification (Van Cleemput *et al.*, 2007), and NO_3^- removal from water supplies by denitrification is utilized in wastewater treatment plants and engineered wetlands (Gersberg *et al.*, 1983). However, this ecosystem service comes at a cost, as denitrification in aquatic ecosystems accounts for approximately one third of the total planetary N_2O emissions, of which 26% is believed to be anthropogenic due to agricultural run-off, animal waste, and other forms of nitrogen input (Seitzinger *et al.*, 2000).

1.2 The Denitrification Pathway

The complete denitrification pathway is catalyzed by a series of different enzymes, some of which can be functionally redundant (Figure 1). The first step, the conversion of NO_3^- to NO_2^- is catalyzed by either a membrane associated nitrate reductase, encoded by the *narG* gene, or its soluble periplasmic homologue encoded by the *napA* gene. However, the conversion of NO_2^- to NO via nitrite reductases is considered the defining step in denitrification (Shapleigh, 2006; Zumft, 1997). This step is carried out by one of two different NO-forming NO_2^- reductases, encoded by the *nirK* or *nirS* genes. Despite having similar functional roles and localization in the cell, each protein has completely different structural features. The NirK protein is a member of the multi-copper oxidase family, with copper ions as ligands within the catalytic center. By contrast, the NirS protein contains two different heme ligands within the active centers of the enzyme. Previous research has demonstrated the functional redundancy of *nir* types in denitrifiers (Glockner *et al.*, 1993), however to date, no organism has been physiologically characterized with both *nir* types present in the same genome. The reduction of NO to N_2O is carried out by nitric oxide reductase (Nor), a membrane associated protein which may have up to three different variants that are structurally homologous at the catalytic site. The ability to reduce NO to N_2O is not unique to denitrification, as NO is highly toxic and a powerful intracellular signaling compound, and thus microorganisms may possess Nor as a means of detoxification (Zumft,

2005). Finally, the conversion of N_2O to N_2 is carried out by the *nosZ* gene product, thus closing the nitrogen cycle as N_2 can re-enter the biosphere through N-fixation.

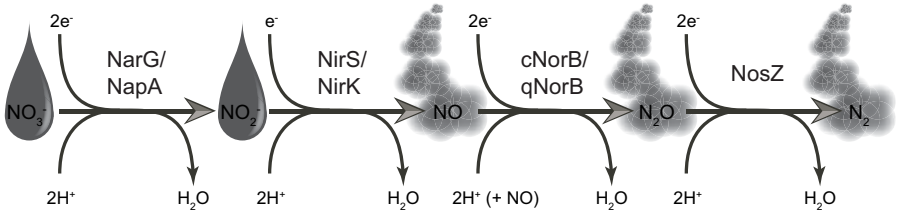

Figure 1. The denitrification pathway, with soluble and gaseous products indicated. Enzymes catalyzing each step are listed above their respective function.

Physiologically, dentrification activity depends on the availability of oxygen, nitrogen oxides, and suitable reductants to drive electron transport. In most denitrifying species, expression of denitrification genes is tightly regulated by oxygen levels due to its status as the preferred electron acceptor in most cases. However, the exact level of anoxia required for denitrification gene expression can differ substantially among organisms (Ka *et al.*, 1997), and denitrification activity can persist in the presence of oxygen in environments that have shifted from anaerobic to aerobic (Morley *et al.*, 2008; Ka *et al.*, 1997).

1.3 Evolutionary aspects of Denitrification

Throughout the course of prokaryotic evolution, a variety of different pathways that allow for respiration in the absence of oxygen evolved before the appearance of atmospheric oxygen, allowing different species to survive in environments where oxygen is permanently or periodically limited (Canfield *et al.*, 2006). Several authors have speculated that denitrification emerged not long after the divergence of the archaeal/eukaryotic and bacterial lineages, prior to the emergence of nitrogen fixation (Klotz & Stein, 2008; Canfield *et al.*, 2006; Capone *et al.*, 2006; Tomiki & Saitou, 2004). This is further supported by geochemical studies on nitrogen isotopes, which suggested that both nitrifiers and denitrifiers had already evolved and were active in oceans prior to oxygen accumulation in the atmosphere (Garvin *et al.*, 2009).

For microbes in rapidly changing environments, ability to use alternative pathways in addition aerobic respiration provides a great level of flexibility

when coping with potentially adverse environmental changes. While the canonical definition of denitrification is the reduction of nitrate to nitrogen gas for the purpose of energy conservation, the reduction of nitrogen oxide species at each step can be carried out separately for the purpose of energy conservation. Thus, denitrification is thought of as a modular pathway where organisms may have different assemblages of denitrification genes depending on their energetic needs or substrate availability (Zumft, 1997). For example, some denitrifying organisms may have truncated pathways, where the end product of nitrate reduction is N_2O rather than N_2 (Zumft & Kroneck, 2007). Genome sequences have revealed that this lack of N_2O reduction is due to a lack of the *nosZ* gene, encoding the nitrous oxide reductase (Philippot, 2002). It has been speculated that the reduction of nitrous oxide to nitrogen gas contributes relatively little to the overall bioenergetics of denitrifying cells, and thus the loss of this step is tolerated (Richardson *et al.*, 2009). However, some organisms (*Wolinella*, *Anaeromyxobacter*) may respire N_2O as a terminal electron acceptor independent of the previous reduction steps (Zumft & Kroneck, 2007). These observations complicate evolutionary studies of the pathway since the gene necessary for each step may have an evolutionary trajectory separate from genes in the other steps. In its capacity as a respiratory pathway, denitrification could be considered a peripheral metabolic pathway, as to date there are no denitrifiers that have been described as obligate denitrifiers, with the possible exception of *Proprionibacterium* (Ritter & Eastburn, 1988).

The ability to denitrify occurs among a large range of organisms from different habitats (Philippot *et al.*, 2007; Figure 1, **paper I**). The most commonly studied denitrifying species are those from the proteobacteria, in which denitrifying representatives can be found in each of the different classes of proteobacteria. Denitrifying species within other bacterial phyla have also been characterized, and the accumulating amount of genomic data has indicated an increasing number of species from different Bacterial phyla as having the genetic capacity to denitrify. Examples of Archeal denitrifying species also exist, including hyperthermophilic (*Pyrobaculum* sp.) and salinophilic (*Haloarcula*, *Halorubrum*, etc.) species. Several species of fungi have also been shown to denitrify (Shoun *et al.*, 1992), with the genes encoding homologs of NirK and Nor located in the mitochondria. Because of the lack of a nitrous oxide reductase, the pathway is truncated after NO reduction to N_2O. Only a few studies have investigated the relative importance of fungal denitrifiers for N_2O emissions from terrestrial environments (Hayatsu *et al.*, 2008).

1.4 Community and Functional Ecology of Denitrifiers

Because of the polyphyletic nature of denitrifying organisms, it is not feasible to link denitrifier activity in the environment to microbial community composition based on species. Closely related species may have very different abilities to denitrify, as made evident by both genetic and phenotypic analysis (Cladera *et al.*, 2006; Cavigelli & Robertson, 2001; Clays-Josserand *et al.*, 1999). To overcome this obstacle, PCR-based assays were developed in the late 1990's using functional genes in the denitrification pathway as molecular markers (Hallin & Lindgren, 1999; Braker *et al.*, 1998; Scala & Kerkhof, 1998). These have been refined and used frequently to determine the community structure or abundance of denitrifiers in various environments (Dandie *et al.*, 2007; Throback *et al.*, 2004; Gregory *et al.*, 2003; Prieme *et al.*, 2002; Braker *et al.*, 2000). This approach incorporates the concept of a 'functional guild', or a group of organisms that have the same role in the ecosystem (Hooper *et al.*, 2005). Thus, it is assumed that organisms within the same functional guild will have one or several marker genes that encode the same ecologically significant trait. This approach largely ignores the species composition of the community, since the phylogeny of different genes within a set of organisms may have varying levels of agreement with the overall phylogeny of the organisms (Doolittle & Papke, 2006).

The composition of denitrifying communities is influenced by a wide range of environmental factors. Salinity and pH can both have a strong influence on denitrifier community diversity (Mosier & Francis, 2010; Santoro *et al.*, 2006; Enwall *et al.*, 2005; Prieme *et al.*, 2002), which is not surprising given that both factors are viewed as 'master-variables' that shape the overall structure of microbial communities (Lozupone & Knight, 2007; Fierer & Jackson, 2006). Nitrate and nitrite concentrations also shape the community structure and abundance of denitrifying organisms, particularly in aquatic ecosystems (Hannig *et al.*, 2006; Liu *et al.*, 2003). In soil ecosystems, the spatial heterogeneity of physical parameters such porosity, nutrient concentrations, and soil organic matter is reflected in the spatial patterns of denitrifier community structure (Enwall *et al.*, 2010; Philippot *et al.*, 2009). Interestingly, the community structure of denitrifiers with *nirS* can differ from that of *nirK* communities along environmental gradients, indicating niche differentiation between the two denitrifying types (Enwall *et al.*, 2010; Smith & Ogram, 2008; Santoro *et al.*, 2006; **paper IV**). Similar spatial structuring of denitrifying communities has also been observed in water

columns with concentration gradients of different nitrogen species (Oakley *et al.*, 2007). However, the perspective that microbial communities are shaped by biogeographical rather than niche based processes is gaining increasing support within microbial ecology (Green & Bohannan, 2006; Martiny *et al.*, 2006), including among functional guilds such as ammonia oxidizers and denitrifiers (Woodcock *et al.*, 2007). Understanding community assembly processes that shape biodiversity provides additional insight into the stability and magnitude of community functioning (Hooper *et al.*, 2005), which may be a critical issue in the management of microbially-mediated nutrient cycling pathways such as denitrification.

1.5 Aim and Outline of Thesis

The aim of this thesis was to examine the denitrification pathway at different scales to better understand the relationship between genetic, functional, and community diversity among denitrifying organisms:

1. **Gene** - Examine the evolutionary relationship between denitrifying genes and the species capable of denitrification.l

2. **Population** - Compare the phenotypic diversity among a set of denitrifying *Bacillus* strains with their genetic diversity.

3. **Community** - Determine the effect of altered composition of denitrifying communities on denitrification rates and end products from soil.

4. **Ecosystem** - Examine the genetic diversity of globally distributed denitrifying communities using nitrite reductase genes to infer the evolutionary/ecological processes that drive the diversification of denitrifying organisms.

1. Gene - At the genetic level, the relative influence of horizontal gene transfer vs. vertical inheritance of genes are of key importance for understanding the processes that lead to the dissemination of traits among microorganisms. In **paper I**, we sought to compare the phylogenies of genes in the denitrification pathway with the species phylogenies based on 16S rRNA genes from the same sets of organisms, available from public sequence databases. We looked at genomic signatures from genomes with

denitrification genes to further examine the relative influences of horizontal gene transfer events with other evolutionary processes such as lineage sorting, vertical inheritance, and duplication/divergence of proteins. We found that the evolution of denitrification genes is quite different between each core gene, ranging from being relatively concordant with species taxonomy (*nosZ*), to being intermediate (*nirS*) or substantially different (*nirK* and *qnorB*) based on statistical test of tree topologies at different taxonomic levels. We additionally observed that the clustering of different species within the same clade in the denitrification gene phylogenies did not always correspond to genomic signatures that indicated horizontal gene transfer, and genomes could have several copies of denitrification genes. These findings highlighted the complicated evolutionary history of denitrification, which have several driving forces in addition to HGT.

2. Population – Since the use of functional genes as markers for denitrification communities in environmental samples pivots on the relationship between genetic diversity and denitrification activity, we tested this link in a set of closely related denitrifying isolates in **paper II**. The denitrification phenotype of set of 14 denitrifying *Bacillus* isolates from agricultural soil was characterized by measuring the production of NO, N_2O, and N_2 over time. We found that phenotypic variation was minimal when isolates were grown at neutral pH, but substantial variation in the production of N_2O was observed at lower pH. After developing PCR primers to target *nosZ* genes in *Bacillus* spp. and closely related Gram-positive bacteria, we found that *nosZ* sequences from all nearly all isolates were identical. A notable exception was one isolates, which had two copies of the *nosZ* gene, a more divergent genome based on genomic fingerprinting using AFLP, and the highest rate of N_2 production among the isolates.

3. Community – The finding of two copies of *nosZ* in an isolate with high N_2 production, as well as the observation of 1/3 of the denitrifier genomes lacking *nosZ* in **paper I,** led us to inquire about how changes in denitrifier community composition effects the ratio of denitrification products, namely N_2O/N_2. In **paper III**, we therefore examined the effects of altering the denitrifying communities in soil microcosms by inoculating them with different amounts of *Agrobacterium tumefaciens*, a denitrifying soil bacterium that has a truncated denitrification pathway due to the lack of a *nosZ* gene. By adding *A. tumefaciens*, we altered the ratio of *nir*/*nos* genes in the denitrifying communities of three different agricultural soils, and then

compared the change in the ratio of denitrification end products (N_2O:N_2) after short incubations under denitrifying conditions. We observed a corresponding increase of potential denitrification activity and N_2O emissions with increasing levels of *A. tumefaciens* inoculum. Additionally, the native communities of two of the three soil communities were capable of serving as sinks for the excess N_2O produced by the introduced non-*nosZ* denitrifiers, as the increase in potential denitrification activity was greater than the increase in N_2O emissions. These findings demonstrated the importance of denitrifier community structure in determining denitrification end-products in soil ecosystems.

4. Ecosystem – That changes in denitrification product ratios can have a genetic basis is interesting when considering that, in **paper I**, we found that *nirS* type denitrifiers are far more likely to have *nosZ* than are *nirK* type denitrifiers. We therefore inquired in **paper IV** as to whether denitrifiers with different *nir* types are ecologically equivalent, and in place of performing a more localized analysis, we examined the total available set of *nirK* and *nirS* sequences from databases using a phyloecology approach. Phylogenetic trees were constructed for both sequence sets, and available data on habitat type and environmental parameters were also collected. Using a combination of tree-based beta-diversity analysis and examining the extent of phylogenetic clustering and rates of cladogenesis within a community, we found salinity to be an important determinant in denitrifier community structure for both *nir* genes, however saline *nirK* communities were more phylogenetically diverse than *nirS* communities. In addition to examining the global pattern, three case studies in which more detailed environmental information were used to compare *nirS* and *nirK* communties along the same gradient. We observed that the phylogenetic community structure of *nirS* differed from that seen in *nirK* communities, and that nitrate was another important driver of community structure, particularly for *nirS*.

2 Evolutionary influences on the denitrification Pathway

2.1 Diverging evolutionary histories of nitrogen cycling genes

Studies on functional genes in the nitrogen cycle have shown varying levels of agreement between gene and organism phylogenies. Zehr *et al.*, (1997) demonstrated that the phylogeny of the nitrogen fixing gene *nifH* was largely coherent with the 16S rRNA phylogeny. A more recent phylogenetic analysis of *nifDK* genes further illustrated that the evolution of these genes is dominated by vertical inheritance and different patterns of gene gain and loss, which is in some instances obscured by instances of horizontal gene transfer (Hartmann & Barnum, 2010). Purkhold *et al.*, (2000) found a similar pattern of phylogenetic agreement between *amoA* and 16S rRNA in ammonia oxidizing species, and concluded that *amoA* similarities greater than 80% are indicative of previously described species. Ammonia oxidizing bacteria may also possess nitrite and nitric oxide reductases, and previous studies comparing the phylogenies of these genes have shown varying levels of agreement among tree toplogies. Casciotti & Ward (2001) showed that *nirK*, *amoA*, and 16S rRNA genes were largely concordant among *Nitrosomonas*, while a more recent study comparing the phylogenies of 16S rRNA, *amoA*, *nirK*, and *norB* genes among *Nitrosospira* species concluded that that the *nirK* and *norB* gene were likely to have been the result of horizontal gene transfer, as the phylogenies of these two genes were substantially different from both *amoA* and 16S rRNA phylogenies, which were largely concordant to each other (Garbeva *et al.*, 2007). By contrast, Gregory *et al.*, (2003) found that the phylogeny of *narG* genes

among nitrate reducing species was substantially different from the 16S rRNA phylogeny. A similar result was also observed in studies on denitrifying isolates from activated sludge samples in which the phylogeny of *nirS*, *nirK,* and *norB* were found to be largely discordant with 16S rRNA phylogenies (Heylen *et al.*, 2007; Heylen *et al.*, 2006). However, no study had compared the phylogenies of genes encoding the three main steps in the denitrification pathway using a statistically based approach to compare the discordance between gene and species phylogenies. The objective of the work in **paper I** was to examine the level of agreement between gene and species phylogenies for key denitrification genes, and examine the relative influences of different evolutionary processes.

2.2 Phylogeny estimation and hypothesis testing – likelihood and Bayesian techniques

Studies of molecular evolution use a number of different techniques to infer the evolutionary history of a set of macromolecules, yet the underlying procedure is largely the same in that differences in homologous characters are used to estimate evolutionary divergence. This can be broken down into two equally important steps in the reconstruction of molecular phylogenies; i) sequence alignment and ii) tree construction. Literature on the theory and methods for both steps is extensive, as the field of computational biology is growing at an ever increasing rate. However, both steps require careful consideration of the methods chosen based upon the overall research question as well as the accuracy of phylogenetic reconstruction.

2.2.1 Sequence Alignment

It is important to consider the nature of the sequences being analyzed, as it is easy to forget that a string of letters is a representation of real, biological structures. For alignment and analysis of protein coding genes, it is obviously important to align sequences by translated amino acid to keep codon boundries intact. Numerous methods exists for alignment of multiple amino acid sequences, the most widely used being the Clustal series, named for the use of cluster analysis as a heuristic to increase the program speed (Thompson *et al.*, 1994). This method is quite accurate for alignment of amino acid sequences with moderate levels of divergence, as it uses a progressive alignment strategy that first aligns the most similar sequences (based on the cluster analysis) and progressively adds more divergent sequences, optimizing the overall alignment with each new sequence. This

general strategy has been improved upon with the most recent generation of iterative multiple sequence alignment methods, which have both increased speed and alignment accuracy (Katoh *et al.*, 2005; Edgar, 2004). While the resulting amino acid alignments can be used for phylogenetic reconstruction after inspection of alignment quality, the phylogenetic analysis of nucleic acid sequences of protein coding genes require the insertion of gaps that corresponds to the gaps in the amino acid alignment. The Trans-align perl script (Bininda-Emonds, 2005) provides a convenient pipeline for the translation, amino acid alignment, and back-alignment of nucleotide sequences.

Alignment of non-protein coding nucleotide sequences may seem somewhat less complicated in that there are no codon boundries to be considered. However, the stem and loop regions in structural RNA molecules are important to consider for the accuracy of the alignment, as the evolution of nucleotides in stem regions is under different constraints than those in loops regions. To account for secondary structure, the Ribosomal Database Project (RDP; Cole *et al.*, 2003) has long used what are known as Context-Free grammar alignments (Brown, 2000), a generalization of hidden Markov models (HMM; Eddy, 1998). Briefly, this approach takes the secondary structure of RNA into account by generating a statistical model of a manually (and therefore assumed accurate) seed alignment, where each site in the seed alignment is used to calculate the probability of occurrence for each nucleotide, as determined by the consensus secondary structure. Sequences are then aligned to the model rather than in a progressive pair-wise manner. This provides for a more accurate alignment of homologous sites in structural RNA molecules for use in phylogenetic reconstruction.

Hidden Markov model alignments are also used for amino acid sequences, where the secondary structure of the protein is incorporated into the alignment by using a seed alignment that has been aligned according to secondary structure. The HMM can also be used for homology searches through databases, which is the strategy employed by the FUNGENE database to search for functional genes (http://www.fungene.cme.msu.edu). In **paper IV**, this approach was used to align *nirS* and *nirK* sequences using a seed amino acid alignment generated with variant of the MAFFT aligner (MAFFT homologs) that incorporates additional homologous sequences during alignment, leading to an increase in alignment accuracy of distantly related sequence that is comparable to alignment by secondary structure

(Katoh *et al.*, 2005). The resulting alignment can then be checked against the secondary structure of a representative sequence using a graphical alignment editor, such as the ARB phylogeny package (Ludwig *et al.*, 2004), and final alignment is used for generating an HMM using the HMMer software (http://hmmer.janelia.org), which can then be used to efficiently align large numbers of sequences.

2.2.2 Phylogenetic Analysis using Maximum Likelihood

The inference of evolutionary relationships is in essence a hypothetical-deductive approach, in which relationships between sets of sequences are mathematically inferred based on the proportion of shared characteristics. (Kluge, 2003), Phylogenetic models can be broken down into two components: i) the model of character substitution over time, and ii) the tree topology, or clustering of species into clades. A multitude of methods exist for inferring phylogenies, each having their relative strengths and weaknesses. While distance-based and parsimony methods are relatively accurate and have low computational demand, maximum likelihood methods are becoming increasingly common as the combination of efficient algorithms, such as RAxML and PHYML (Stamatakis, 2006; Guindon & Gascuel, 2003), and increasing computing power reduces the computational burden for calculating maximum likelihood phylogenies. The RAxML algorithm is particularly well suited for analyzing thousands of sequences on a computing cluster, and was used in **paper IV.**

Maximum likelihood estimation of molecular phylogenies analyzes a set of sequences in a column-wise manner, seeking out the tree topology and substitution parameters that best explain the evolution of the sequence data. These methods have been shown to be the most robust for dealing with short sequences or incomplete datasets, as low signal-to-noise ratios are tolerated to a greater degree using likelihood than distance based or parsimony methods (Wiens, 2003). However, the real advantage is that it allows for a statistical framework to be incorporated into the analysis, which can then be used to develop and test different hypothesis. The applicability of hypothesis testing in phylogenetic analysis ranges from determining the optimal model of character substitution in a set of sequences, to accepting or rejecting a hypothesis of monophyly for a particular clade. Maximum likelihood analysis requires that a specific model of character substitution be chosen *a priori*, upon which the optimal tree topology and substitution parameters are calculated. The optimal substitution model can be chosen using likelihood ratio tests which compare the gain in likelihood value

relative to the increase in the number of parameters of the substitution model. Likelihood ratio tests can be similarly applied to testing tree topologies, where tests such as the Approximately Unbiased and Shimodaira-Hasegawa test (Shimodaira, 2002) use the same principle to determine if a set of tree topologies are equally probable given the data and substitution model. Since maximum likelihood estimation of phylogeny results in a single optimal tree which may or may not be the 'true' tree, non-parametric bootstrapping can be used to examine node support. Bootstrap probability (or proportion) is defined as the proportion of times a group is obtained from analysis of a set of pseudo-replicates, or randomized versions of the original alignment.

A related approach is Bayesian estimation of phylogenies (Huelsenbeck *et al.*, 2001; Larget & Simon, 1999), in which the phylogeny is determined based on the prior probability of tree topologies and substitution rate parameters. Bayesian analysis uses the same principles as maximum likelihood, however rather than deriving a single maximum likelihood estimate of phylogeny, Bayesian analysis results in a posterior distribution of phylogeny that can be used to examine the uncertainty of any parameter of interest in the phylogeny, as well as assessing node support by summarizing all tree topologies within the posterior distribution in a consensus tree, thereby generating Bayesian posterior probabilities of node support. As a general rule, Bayesian posterior probability values tend to overestimate node support, while bootstrap probabilities underestimate support (Alfaro *et al.*, 2003). Another useful feature of the posterior probability is that it can be readily used for hypothesis testing of any parameter within the phylogeny, including tree topology. This can be performed using topological constraints, in which the probability of particular groupings can be assessed by counting the proportion of trees in the posterior distribution that contain the grouping of interest. This approach was used in **paper I** to compare the phylogenies of 16S rRNA and denitrification genes relative to Bergey's taxonomic classification (Garrity, 2004), which is based on 16S rRNA phylogenies (Figure 2).

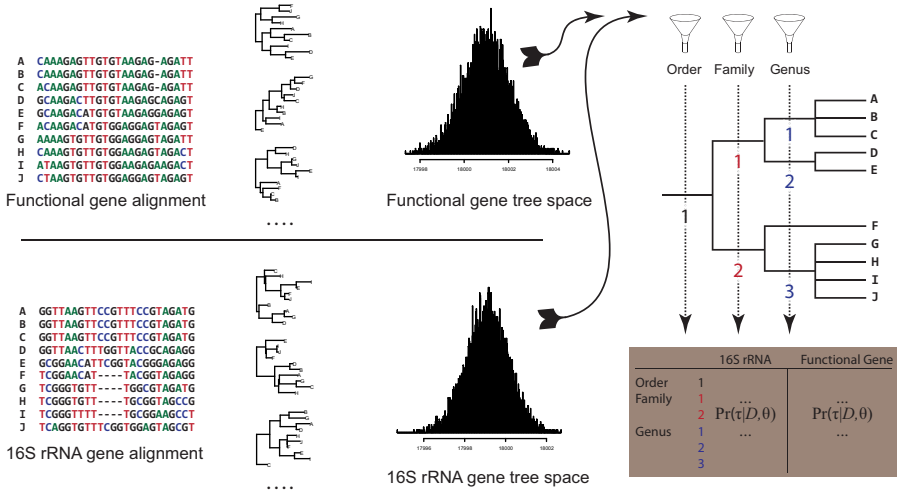

Figure 2. Strategy for hypothesis testing of monophyly using Bayesian posterior probability distributions of tree topologies. Alignments for both the 16S rRNA and functional genes are used to produce discrete distributions of tree topologies from the same set of taxa. These distributions are used to determine the probability of a monophyletic clade corresponding to a taxonomic grouping in the tree topology (τ), given the alignment (D) and substitution model parameters (θ) used to analyze both genes.

2.2.3 Genomic Signatures – GC content and Codon usage

Close phylogenetic association of genes from distantly related species is often interpreted as an indicator of horizontal gene transfer. However, a variety of other processes may also lead to discordance between gene and species trees, including lineage sorting, differing patterns of gene gain and loss, or convergent evolution (Lawrence & Ochman, 2002; Maddison, 1997). Analysis of genomic signatures, such as deviations in GC-content and atypical codon usage, examine the pattern of nucleotide usage in a gene relative to the genome of the organism. This provides an additional line of evidence along with phylogenetic inference to further differentiate among possible evolutionary scenarios. However, this is not without limitations; potentially transferred genes from organisms with similar mutational backgrounds will not be detected as nucleotide usage patterns will be quite similar. Also, ancient transfers will be more difficult to detect due to the amelioration of codon usage in the transferred genes to that of the recipient genome (Ragan *et al.*, 2006). The SIGI-HMM (score-based identification of genomic islands using hidden Markov models) algorithm attempts to circumvent these difficulties by incorporating taxon specific differences in codon usage to identify potentially transferred genes (Waack *et al.*, 2006). The results of this analysis can be visualized in combination with changes in

GC-content to identify genomic regions that are likely to have been transferred.

2.3 Modular evolution of the denitrification pathway

Discordant phylogenies of genes involved in the same biogeochemical cycle are observed in other pathways than denitrification. For example, the phylogeny of genes for different sulfur transformation processes in sulfate-reducing prokaryotes have been shown to differ from that of 16S rRNA genes as well as each other among major lineages (Meyer & Kuever, 2007; Zverlov *et al.*, 2005; Boucher *et al.*, 2003), and researchers speculate that the enzymes may have emerged for one physiological function (detoxification) but then evolved to fill another role (respiration; Meyer & Kuever, 2007). This has also been speculated for denitrification genes, particularly the nitric oxide reductase which has been suggested as a precursor to enzymes involved in aerobic respiration (Zumft, 2005; Saraste & Castresana, 1994). A recent paper on genes involved in nitrogen fixation (*nifD/K*) demonstrated that while both genes were generally congruent with 16S rRNA phylogeny, significant statistical support was observed for incongruent tree topologies between all three genes (Hartmann & Barnum, 2010), primarily within the deeper branches of the phylogenies.

Among all denitrification gene phylogenies, the *nosZ* gene showed the greatest level of similarity to the 16S rRNA phylogenies, suggesting that horizontal gene transfer of *nos* gene clusters is constrained by selective factors (**paper I**). This is further substantiated by the observation that of the organisms in which denitrification genes were found in fully sequenced genomes, one third were missing the *nosZ* gene. Since the reduction of nitrous oxide contributes relatively little to the overall bioenergetics of denitrification (Richardson *et al.*, 2009; Zumft & Kroneck, 2007), it may be more prone to loss as its transfer into a new host organism may contribute relatively little to the overall fitness of the organism. Of course, this is entirely dependent on the environmental stresses on the organism, as acquiring nitrous oxide reduction may still impart some level of adaptive advantage. This may be the case with the *nosZ* in *Haloarcula marismortui*, as *nosZ* genes were inserted between the proteobacteria and other more divergent bacteria phyla, rather than clustering with *Pyrobaculum* (Figure 3). It is difficult to determine if this is a result of horizontal gene transfer from bacteria, however this is highly plausible given that gene transfer between archaea and bacteria has been documented previously (Nelson *et al.*, 1999),

and that genes encoding a transposase, recombinase, and a putative plasmid transfer protein are located within 20 kb of the *nos* gene cluster. Another notable clustering is the positioning of *Dechloromonas* and *Magnetospirillum* sequences with the epsilon proteobacteria. This lineage of *nos* is structurally different from other *nos* types by having a C-terminal domain with a single heme-binding site (Simon *et al.*, 2004). However, little other evidence exists for transfer of these genes, as codon usage and GC-content showed this region of the genome for both species to be similar to that of the rest of the genome (Figure 3, **paper I**). This suggests that any transfer of *nos* genes to either species from a possible epsilon-proteobacteria donor species is not recent, as the amelioration of codon usage and GC-content of *nos* gene has removed any signal of atypical nucleotide or codon usage patterns.

A pattern of clustering of sequences from similar taxa was also observed in the *nirS* and *cnorB* phylogenies, which were both comparable to organism phylogeny albeit less so than the *nosZ* phylogeny. The *cnorB* clade is much more coherent with species phylogeny than the *qnorB* clade, however it is difficult to explain this in terms of denitrifying vs. non-denitrifying bacteria since both clades contain representatives of denitrifying species. Since *nosZ*, *nirS*, and *cnorB* are within clusters of accessory genes involved in their assembly, regulation, or electron transport (Zumft, 1997), it could be reasoned that a horizontal gene transfer event must include all genes if an organism is to successfully utilize the newly introduced denitrification genes. This would have to be followed by an increase in fitness for the recipient organisms in order for the transferred genes to become fixed in the population. This is in line with the 'selfish-operon' theory, where genes that are involved in the same process tend to cluster together to facilitate their transfer and subsequent fixation in recipient genomes (Lawrence & Roth, 1996). However, the concordant phylogeny of *nosZ* and its accessory genes (Zumft & Kroneck, 2007) with species phylogeny indicate that transfers of *nosZ* have been infrequent, or have remained largely between close relatives. This is in contrast to *nirK* (Figure 4) and the *qnorB* variant of the nitric oxide reductase, which both typically occur with only one (*nirV* in the case of *nirK*) or no accessory genes, and for which we observed the least agreement with species phylogeny. While it is tempting to explain *nirK* evolution in terms of rampant HGT, the NirK protein has two structural classes which form distinct clades in the phylogeny, with no clear association between *nirK* class and species phylogeny. Additionally, NirK is a member of the multi-copper oxidase proteins, which is known for its ability to shuffle domains to form

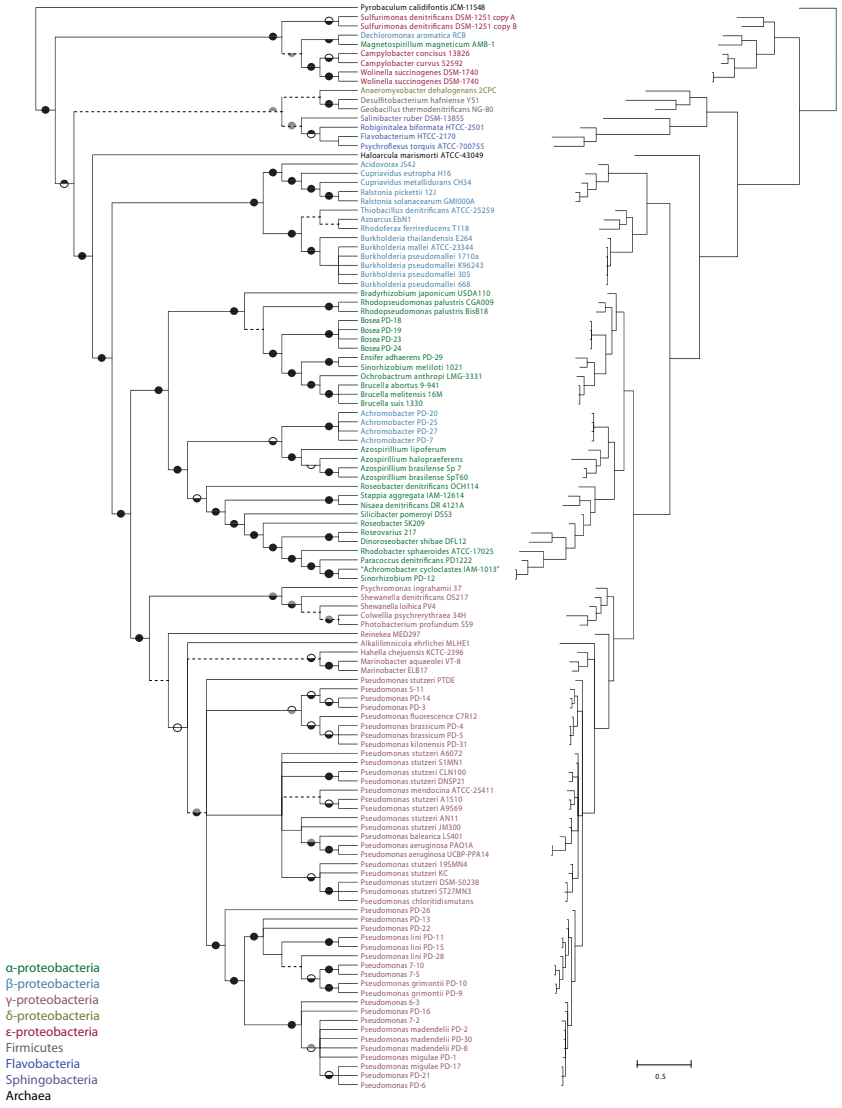

Figure 3. Unrooted phylogeny of partial *nosZ* sequences. Topology and branch lengths are based on Bayesian analysis of amino acids under the WAG+I+Γ model, and topological conflicts among separate MrBayes and PHYML analysis of nucleotide and amino acid data sets are indicated by dashed branches. Branch lengths are shown at right, and support based on all four analyses is represented by symbols at nodes. Bayesian posterior probabilities greater than 0.95 are indicated by the upper half-circles, and likelihood bootstrap probabilities greater than 50% are indicated by lower half-circles. Coloring of symbols represents support from each analysis: gray, supported by amino acid analysis; white, supported by nucleotide analysis; black, supported in both analyses. Isolate names are colored according to taxonomic classification at the class or phylum level.

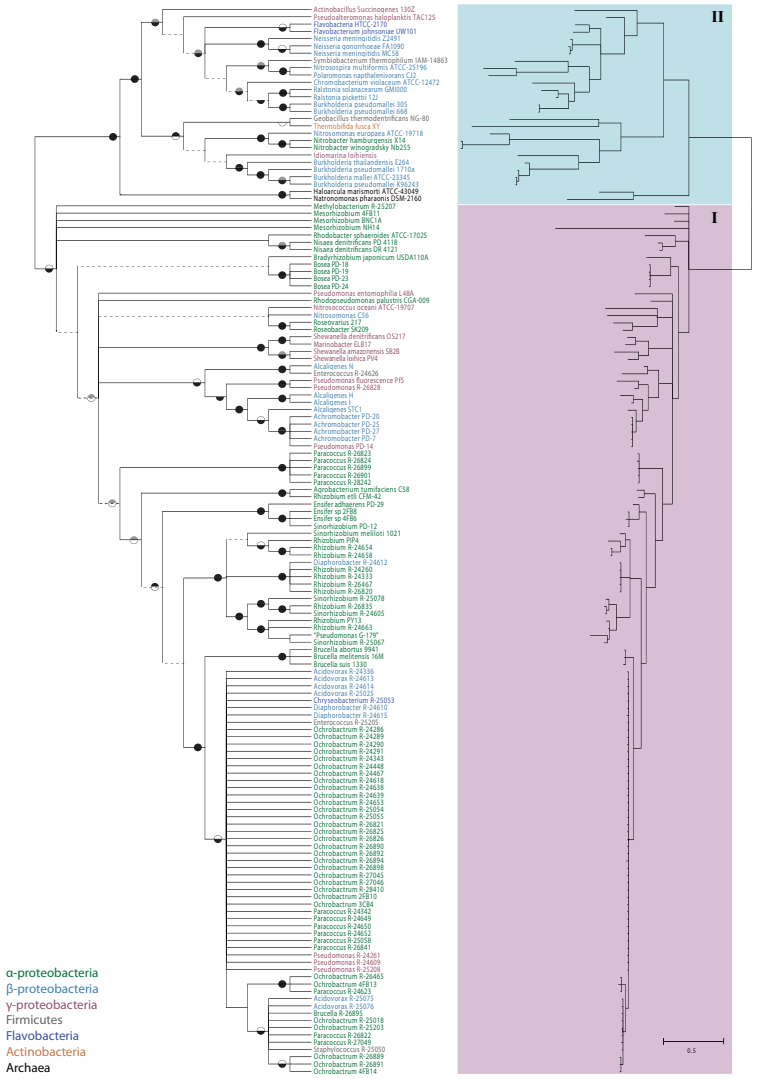

Figure 4. Unrooted phylogeny of partial *nirK* sequences. Topology and branch lengths are based on Bayesian analysis of amino acids under the WAG+I+Γ model, and topological conflicts among separate MrBayes and PHYML analysis of nucleotide and amino acid data sets are indicated by dashed branches. Branch lengths are shown at right, and support based on all four analyses is represented by symbols at nodes. Bayesian posterior probabilities greater than 0.95 are indicated by the upper half-circles, and likelihood bootstrap probabilities greater than 50% are indicated by lower half-circles. Coloring of symbols represents support from each analysis: gray, supported by amino acid analysis; white, supported by nucleotide analysis; black, supported in both analyses. Isolate names are colored according to taxonomic classification at the class or phylum level. Groupings of *nirK* structural variants are indicated at right.

new functions (Murphy *et al.*, 1997). This, coupled with possibility of secondary function for *nirK* in different organisms, i.e. the reduction of selenite for detoxification (Basaglia *et al.*, 2007), leads one to speculate n the influence of environmental stress on protein evolution.

The network analysis of organisms with the full denitrification pathway demonstrated how the discordance in gene phylogenies of the denitrification genes was lineage dependent, as several taxonomic groups showed a tree-like branching pattern, whereas others were more networked (Figure 2, **paper I**). The similarity in phylogenetic signal may be due to co-evolution of interacting proteins (Shi *et al.*, 2003), particularly for organisms in which respiration via denitrification is an important trait in their respective environment. The close association of denitrification genes may be particularly critical for host-associated alpha-proteobacteria species (i.e. *Brucella*, *Bradyrhizobia*), which would require mechanisms to survive in micro-oxic conditions. However, the actual physical location of the genes in the genome relative to each other is not necessarily reflected in the phylogeny, as genes with closely related sequences from the same two species may be localized differentially relative to each other in each genome.

2.4 Future Directions

The different evolutionary histories of the core denitrification genes illustrates the modular nature of the pathway, as each step has been subject to different frequencies of horizontal gene transfer, lineage sorting, or other evolutionary processes. However, the question remains as to what drives the retention and loss of denitrification genes among lineages. Since even closely related organisms can have differing assemblages of denitrification genes, the gain or loss of a gene must be in accordance the change in fitness to the organism. This is also true for gene duplications, which may also have a significant role in the diversification of denitrification genes in light of evidence that bacteria can transiently duplicate sections of their chromosome for the purpose of increasing gene dosage in response to stress (Andersson & Hughes, 2009; Bergthorsson *et al.*, 2007). Thus, long term evolutionary experiments (Elena & Lenski, 2003) could be used to examine the effects of environmental stresses on the evolution of denitrifying organisms. Comparative genomics of denitrifying organisms, coupled with analysis of gene expression through relative codon usage, could also provide further insight into possible reasons for genomes to retain or expunge denitrification genes.

3 Denitrifier Diversity at the population level - physiological vs. genetic diversity

3.1 Population – the unit of evolution

The evolutionary patterns observed in **paper I** occur over a wide range of species, from which each sequence is representative of an individual. However, the evolutionary processes that underlie diversification act on populations of individuals. A population is defined as a group of individuals within the same species that is generally restricted to a geographic location (Berryman, 2002). By contrast, a community is a collection of species 'occuring within the same place and time' (Schaefer, 2006; Fauth *et al.*, 1996). While delineating populations based on spatial arrangements is complicated by scaling issues (Schaefer, 2006; Berryman, 2002; Camus & De Ciencias, 2002), it is generally agreed that the population can be considered the fundamental unit of evolution (Futuyma, 1998). Selection acts on the variation found within a population, where the individuals with phenotypic characteristics that convey the highest level of fitness, based on biotic (e.g. competition) or abiotic factors, are selected for over time (Mayr, 1997). In turn, it is the genotype that manifests itself in the phenotype under selection, thus the fingerprint of selective processes can be found through analysis of genotypic differences. It is these differences that are examined when using genetic tools to assess the diversity of microbial communities in the environment. However, these tools do not provide any insight on the level of phenotypic diversity among populations, the entity upon which selection is acting. Recent reviews on the status of community ecology have argued that the potential for genetic variation to affect ecosystem-scale

processes depends on the level of correlation between genetic and phenotypic variation (Hughes *et al.*, 2008; McGill *et al.*, 2006). Thus, finding a link between genotypic and phenotypic variation is likely to be a necessary direction for microbial ecologists.

3.2 Microvariation in Populations

Establishing a link between genetic and phenotypic variation is a non-trivial task, particularly in the light of micro-evolutionary processes. The term 'microevolution' describes evolutionary processes that occur among individuals within a species (Futuyma, 1998). In bacterial population, the genomes can evolve by a variety of processes ranging from single nucleotide changes, to large scale chromosomal rearrangements, recombination, and horizontal gene transfer (Feil, 2004). These processes have significant implications for clonally reproducing bacterial populations, as they provide the basis for the evolution of new phenotypes within a population to cope with environmental heterogeneity; for example, the formation of different colony morphologies within a single population of *Pseduomonas* in response to oxygen availability (Rainey & Travisano, 1998), or antibiotic resistance (Boles *et al.*, 2004). This ties directly into bacterial populations in ecosystems, which are subject to periodic selection that purge populations of genetic diversity (Acinas *et al.*, 2004; Majewski & Cohan, 1999), leading to the formation of ecologically meaningful groups. This principle has been coined the 'ecotype' model of bacterial speciation (Cohan, 2002), where species definitions of bacteria are based on the ecological context under which genomic changes occur. Given the argument by Mayr (1997) that selection acts directly on phenotype, this would imply that changes in phenotype within a population should correspond to detectable changes in genotype. This may have implications for correlating genetic diversity of functional communities with changes in ecosystem-scale processes that are mediated by microorganisms. For example, (Lennon & Martiny, 2008) found that the response of *Synechococcus* species to viral predation had a significant effect on ecosystem-scale processes (C:N and N:P ratios), the effect of which was diminished over time in the microcosms due to rapid diversification of *Synechococcus* in response to viral predation.

While denitrifying communities can be quite diverse, the temporal and spatial dependence of denitrification activity in soil may result from a handful of denitrifying species that periodically dominate under conditions optimal for their growth via denitrification (Wolsing & Prieme, 2004), or

when soil specific soil environments, i.e. bulk soil vs. rhizosphere, select for a constrained group of species (Cheneby *et al.*, 2004). Previous studies have demonstrated phenotypic diversity among closely related soil bacterial isolates involved in nitrogen cycling. Jiang & Bakken (1999) demonstrated physiological differences among *Nitrosospira* species, particularly regarding differences in ammonium oxidizing activity in response to pH and temperature, that corresponded to the organismal phylogeny (based on 16S rRNA sequences). Similarity between denitrifying phenotype and organismal phylogeny has also been observed among *Bradyrhizobium* isolates (Sameshima-Saito *et al.*, 2006). However, studies on denitrifying *Pseudomonas* strains (Clays-Josserand *et al.*, 1999) as well as more diverse denitrifying populations (Cavigelli & Robertson, 2001) from soils showed no correlation between phenotype and evolutionary relationships. Thus, the objective of the study in **paper II** was to compare the denitrification kinetics, as a measure of phenotype, to the genetic variation as measured by both phylotype (16S rRNA and AFLP) as well as the *nosZ* gene among a set of denitrifying *Bacillus* isolates from soils in which long-term fertilizer application of ammonium sulfate resulted in an enrichment of species belonging to the *Firmicutes* (Wessén *et al.*, 2010).

3.3 Genetic and Phenotypic Characterization of Cultivated Denitrifiers

3.3.1 Culture based studies

Traditional culture-based studies provide the best (if not only) means in establishing a link between genetic and phenotypic variation. The denitrification phenotype was measured using a robotic gas sampling apparatus (Figure 5, Molstad *et al.*, 2007), which allowed for the monitoring of O_2, CO_2, and gaseous denitrification products over time. Previous research using this apparatus has demonstrated that different denitrifying species regulate the expression of denitrification genes differentially in response to O_2, NO_3, NO_2, and NO concentrations, as well as other parameters such as pH and temperature (Bergaust *et al.*, 2010; Liu *et al.*, 2010; Bergaust *et al.*, 2008; Morley *et al.*, 2008). Comparisons of isolates were made at two different pH levels, 6.0 and 7.0. Preliminary experiments showed that several isolates did not grow adequately below pH 6.0, which is not surprising given that the original species description of *Bacillus drentensis* and its close relative states the optimal pH for ranging between 7.0 and 8.0, while minimum growth was observed at between 4.0 and 6.0 (Heyrman, 2004). The effect of pH on denitrification rates in soil communities as well

as pure cultures has been well documented in the literature, specifically regarding the ratio of denitrification end products N_2:N_2O (Simek & Cooper, 2002; Thomsen *et al.*, 1994). Low pH levels result in increased levels of N_2O emissions from soils, most likely due to an inhibitory effect of pH on nitrous oxide reductase activity (Bergaust *et al.*, 2010). Studies on pure cultures of denitrifying bacteria have found that the transcription of *nosZ* is more sensitive to low pH than other enzymes in the denitrification pathway (Thomsen *et al.*, 1994). An alternative explanation has been provided by Liu *et al.*, (2010) who suggested that increased N_2O results from the failure of the *nosZ* enzyme to assemble into a functional protein.

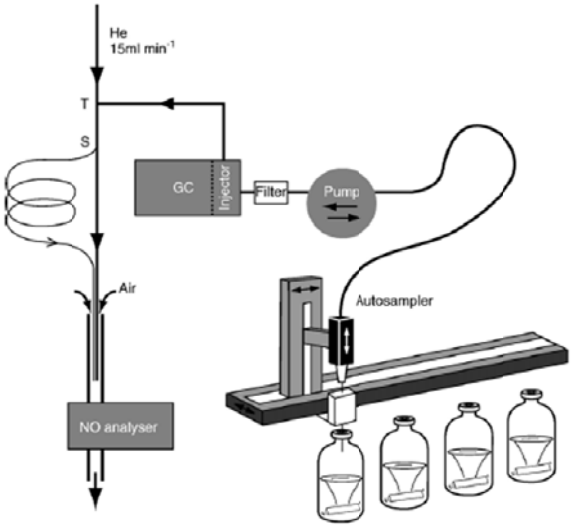

Figure 5. Schematic overview of robotic gas sampling apparatus. Figure reproduced with permisson from Mølstad and Bakken, 2007.

While increased N_2O emissions from soil may also be due to the ratio of denitrifiers with and without the *nosZ* gene (**paper III**), pH appears to be one of the more significant determinants of denitrification product ratios, along with being a master variable for determining community composition of denitrifiers (Cuhel *et al.*, 2010; Enwall *et al.*, 2005) and bacteria in general in terrestrial ecosystems (Fierer & Jackson, 2006).

3.3.2 Genetic Characterization

As stated earlier, the 16S rRNA gene is the standard gene for delineating evolutionary relationships among bacterial species due to its status as a 'house-keeping' gene (Woese, 1987). Since the 16S rRNA gene is not

optimal for delineating *Bacillus* species (Yamada *et al.*, 1999), we used amplified fragment length polymorphism (AFLP) to further differentiate fine scale variation among the isolates. An additional measure of genetic divergence was the analysis of the *nosZ* genes, for which new primers were designed based on *nosZ* sequences from the genomes of Gram-positive organisms. At the time of primer design (February, 2009), *nosZ* sequences from only two species were available; *Geobacillus denitrificans* and *Desulfitobacterium hafniense*, which both clustered together in a single well-supported clade in the analysis performed in **paper I**.

3.4 Potential Importance of Microvariation

Of the 85 denitrifying isolates screened in this study, more than 50% were *Bacillus* sp. within the *drentensis/niacini* species group, also originally isolated from soils (Heyrman, 2004; Nagel & Andreesen, 1991). While this overrepresentation of Bacillus species may be due to the culturing procedure, previous studies on agricultural soils using the same methodology resulted in cultivation of *Streptomyces* and *Agrobacterium* sp. as the dominant species in bulk and rhizosphere soils (Cheneby *et al.*, 2004). Thus, it is likely that the dominance of *Bacillus* species reflects the extant microbial community in this soil, which is further supported by the significant increase in *Firmicutes* observed by Wessen *et al.*, (2010) in the same soil. Among a randomly selected set of 14 *Bacillus* isolates, little variation in denitrification phenotype was observed at pH 7 (Figure 2, **paper II**), and *nosZ* sequences for most isolates were nearly identical (Figure 5, **paper II**). However, isolate ULT-42 was found to have two divergent copies of the *nosZ* gene, as well as the highest level of N_2 production at pH 7. This corresponded to results of the AFLP analysis, in which ULT-42 had the most divergent genome of all *Bacillus* isolates (Figure 3, **paper II**). However, the pattern of genome divergence determined using AFLP was not reflected in the analysis of 16S rRNA sequences, as ULT-42 did not group separately from the rest of the isolates in the 16S rRNA phylogeny (Figure 1, **paper II**). It not possible to determine if the divergent copies of *nosZ* are due HGT, since both copies are still more similar to each other than any *nosZ* sequence in the database. Regardless, the divergent AFLP pattern among isolates with highly similar 16S rRNA sequences implies that some type of genome rearrangement may have occurred. A similar pattern of genomic rearrangement affecting *nosZ* was seen among *Azospirillum* isolates and attributed to phase variation (Vial *et al.*, 2006), in which isolates can gain

and lose certain traits within a short period of time, depending on environmental factors.

Previous research has demonstrated that microdiversity among 16S rRNA genes may have ecological significance among bacterial populations, as microvariation in 16S rRNA sequences may denote closely related lineages due to differences in the peripheral gene content of a genome (Acinas *et al.*, 2004). This may prevent selective sweeps, as different assemblages of peripheral genes may provide only a periodic fitness advantage that is not competitive enough to remove other competing genomes. While we could not match denitrification phenotype to 16S rRNA phylogeny, the duplicate copies of *nosZ* in combination with a distinct AFLP profile and phenotype suggest that genetic variation within species may lead to differences in denitrifcation phenotype. However, the influence of environmental factors may mask this effect, as the production of N_2O was highly variable among the *Bacillus* isolates when measured at pH 6, and isolate ULT-42 did not produce higher levels of N_2 (Figure 3 and 4, **paper II**). The extent of phenotypic variation may not be apparent unless organisms are under stressful conditions (Badyaev, 2005). Since the optimal pH for growth of *B. drentensis* and its close relatives is in the range of 7-8, it is possible that a pH of 6.0 presents a stressful environment for the bacterium, which in part may be due to inhibition of NosZ activity. It is likely that the variation in phenotype among the isolates at lower pH is influenced by other genetic factors, such as regulatory binding sites (Rodionov *et al.*, 2005) or variation among other denitrification genes, which we did not investigate here.

3.5 Future directions

Culture-based studies on the Gram-positive denitrifying bacteria are rare, despite evidence of their abundance in agricultural soils (Cheneby *et al.*, 2004; Cavigelli & Robertson, 2001). Thus, the results in **paper II** provide some characterization of denitrification activity as well as molecular probes for targeting understudied *nosZ* genotypes from environmental samples. However, determining which environmental parameters strongly influence denitrification gene content and protein evolution within a population of denitrifying species may be best analyzed using an experimental evolutionary approach, in which denitrifying cultures are subjected to different selection regimes over many generations. This is particularly pertinent given the finding that closely related organisms can have different numbers of denitrification genes copies (Etchebehere & Tiedje, 2005), which may

provide a competitive advantage under some conditions. For example, an organism with two *nosZ* copies may have an advantage if NO_3^- is limiting but N_2O is abundant, whereas a species lacking the *nosZ* gene may be more specialized in consuming other denitrification intermediates. Also, if a denitrifying culture is subjected to different regimes of oxgyen availability (i.e. always oxygenated vs. never oxygenated vs. fluctuating oxgyen concentrations), how would this effects the genetic underpinnings of the denitrification phenotype, for both regulatory and structural genes? This sets the stage for a battery of experiments analyzing competition between organisms with different denitrification gene copy numbers, as well the regulation of multiple copies of denitrification genes in response to different environmental stimuli.

4 Denitrification response to altered community structure

4.1 Does Structure Matter?

Environmental parameters have a strong influence on denitrification activity in soils, as factors such as water-filled pore space, pH, and electron-donor and nitrate availability all have a significant effect on denitrification activity in soils. This also is true for the ratio of denitrification end-products, as each step in the pathway may be differentially regulated or influenced by different environmental parameters (Van Spanning *et al.*, 2007). Additionally, the influence of pH on nitrous oxide reductase activity demonstrated in **paper II** has been observed in previous studies, where lower pH inhibited *nosZ* activity leading to higher N_2O production (Bergaust *et al.*, 2010; Thomsen *et al.*, 1994). This effect of pH has been widely documented in soils, thus strategies for mitigating N_2O emissions from agricultural ecosystems has largely centered on managing physiological parameters that most influence denitrification end-product ratios (Richardson *et al.*, 2009; Simek & Cooper, 2002). However, such approaches may present an oversimplified solution since, as we observed in **paper I**, a large proportion of denitrifying species exists that utilize a partial denitrification pathway during anaerobiosis. This implies that the end-product ratios of denitrification from soil ecosystems may have both a physiological and a genetic basis. Factors that determine the composition of denitrifier communities in soils, environmental or otherwise, may favor the dominance of one type of denitrifier over the other.

Previous work investigating denitrifier diversity in agricultural soils have been inconclusive regarding the link between the community composition

and diversity of denitrifying organisms with dentrification activity and product molar ratios. Chèneby *et al.*, (1998) found differences in denitrifier community composition between two soils with opposite molar ratios of N_2O and N_2, however the soil with the highest N_2O emissions had the greatest proportion of organisms with capable of denitrification to N_2, and no correlation was found between the presence of *nosZ* and a species ability to reduce N_2O. Other studies have reported similar patterns of uncoupled relationships between changes in denitrification activity and *nosZ* community structure from both agricultural and natural soil ecosystems (Boyle *et al.*, 2006; Enwall *et al.*, 2005; Rich *et al.*, 2004). However, several studies have shown the contrary; Rich *et al.*, (2003) found that differences in *nosZ* communities were correlated with denitrification activity, whereas Cavigelli & Robertson (2000) observed a strong effect of community composition on denitrification rates and product ratios, which in a subsequent study was attributed to physiological diversity among denitrifying isolates in the regulation of nitrous oxide reductase activity in response to oxygen (Cavigelli & Robertson, 2001). Denitrifier abundance also can influence denitrification rates (Hallin *et al.*, 2009) and end product ratio ($N_2O/N_2O + N_2$; Philippot *et al.*, 2009).

The production of N_2O from soil ecosystems has been described using the 'leaky-pipe' conceptual model (Firestone & Davidson, 1989), where N_2O (and NO) emissions from soil are regulated by i) the flow of nitrogen through the 'pipe', ii) the rate of nitrification/denitrification occurring within the soil microbial community, i.e. the amount 'leaking' through the holes, and iii) the soil physical parameters that control the diffusion of gases through the matrix. This in turn controls the rate at which they are consumed by the microbial community. Thus, much weight is placed on the importance of soil properties in determining N-cycling activity, and the role of the microbial community itself is thought of as a 'transducer' through which soil parameters act as proximate controls that regulate denitrification activity (Wallenstein *et al.*, 2006). This idea is grounded in the discordant results observed across field studies, as reported above, which show varying levels of correlation between denitrifier community structure and activity. However, the potentially large proportion of organisms that lack the *nosZ* gene implies that studies in which denitrifier communities were examined using the *nosZ* gene only look at two-thirds of the whole picture, and organisms that lack *nosZ* may also be playing an important role in ecosystem-scale denitrification processes. We hypothesized that the proportion of denitrifiers with and without *nosZ* will have a significant

effect on the denitrification end product molar ratios, demonstrating that differences in N_2O emissions among ecosystems can have a genetic basis in addition to the effects of environmental factors on the physiology of extant denitrifying communities.

4.2 Microcosms – linking denitrifier function and community structure.

The problem of conflicting results illustrated above is inherent in most field studies, as issues of spatial and temporal scale and heterogeneity make it difficult to resolve underlying causal mechanisms (Jessup *et al.*, 2004; Drake *et al.*, 1996). Manipulative microcosm experiments offer more mechanistic explanations of ecological phenomena (Drenner & Mazumder, 1999), as spatial and temporal variability can be controlled and the complexity inherent in a natural system is reduced so that only specific factors of interest are being compared. However, reduction of ecological complexity in itself is problematic, as factors that prove to be critical in a laboratory set-up may have little bearing at larger scales (Carpenter, 1996). Despite this drawback, microcosms for both aquatic and soil ecosystems have provided valuable insight into the ecology of denitrification through manipulation of environmental parameters such as temperature, water content, salinity, and carbon or other nutrient conditions (Stres *et al.*, 2008; Philippot *et al.*, 2007; Throback *et al.*, 2007). Microcosms also facilitate the use of more sensitive techniques for measuring denitrification activity, such as stable isotope labeling (Baggs, 2008).

To test the hypothesis of whether or not denitrifier community structure is relevant to denitrification activity and product ratios, we alter the microbial community directly. Soils from three different locations with different abiotic properties (Table 1, **paper III**) were used to create soil microcosms that were inoculated with serial dilutions of *Agrobacterium tumefaciens* C58, a denitrifying soil bacterium from the alpha-proteobacteria that has a truncated denitrification pathway due to the lack of a *nosZ* gene (Wood *et al.*, 2001). *A. tumefaciens* cells were added such that the highest inoculation level was equivalent to the indigenous nitrous oxide reducing community, as determined using qPCR of *nosZ* genes prior to inoculation. The soils were chosen under the assumption that the native microbial communities in each soil is unique, based on previous knowledge of mineral properties and different microbial activities including denitrification rates (Stenberg *et al.*,

1998). This approach allowed for a comparison of the response of different denitrifier communities to altered *nosZ*:non-*nosZ* ratios, as all microcosms were incubated under high water content and non-limiting NO_3^- concentrations. The abundance of denitrification genes (*nirK*, *nirS*, and *nosZ*) as well as the inoculated strain was measured using quantitative PCR, and N_2O emissions were compared to denitrification potential by analyzing microcosms with and without acetylene, which inhibits the nitrous oxide reductase. Since we were only interested in examining the effect of altered ratios of *nosZ*:non-*nosZ* denitrifying organisms, microcosms were incubated overnight (20 hours) to avoid possible species interactions that may occur during longer incubation times. Such interactions may also have an effect on the denitrification product ratios in the field, however exploring this level of complexity was beyond the scope of this experiment.

4.3 Importance of community structure

In our soil microcosms, the addition of *A. tumefaciens* resulted in significantly higher potential denitrification rates and N_2O emssions for all soils tested (Figure 3a-b, **paper III**). This demonstrates that the denitrification community 'transducer' also has a genetic component, such that the proportion of organisms with and without *nosZ* can influence denitrification product ratios. The importance of community structure is further supported by the observation of two soils acting as N_2O sinks based on comparionson of potential N_2O emissions and denitrification rates. The Lövsta and Ulleråker soils showed no significant effect of *A. tumefaciens* inoculation on N_2O/N_2O+N_2 ratios (Figure 4, **paper III**), indicating that the indigenous soil communities in both soils were able to consume the extra N_2O produced by the inoculum. This consumption of N_2O has been observed for a variety of terrestrial ecosystems (Chapuis-Lardy *et al.*, 2007), however the exact mechanism remains unknown. Previous work has shown that N_2O consumption is increased when nitrate concentrations become limiting (Rosenkranz *et al.*, 2006). However, the short incubation times in combination with the non-limiting nitrate concentrations suggests that other factors besides nitrate limitation may also be important. Cavegelli and Robertson (2001) demonstrated an effect of denitrifier community structure on N_2O consumption, where each community was differentially regulated by environmental factors such as pH and oxygen availability. A similar observation was made by Cheneby *et al.*, (2004), where an increased number of *Agrobacterium*-related strains that did not exhibit N_2O reductase

activity were observed in the rhizosphere of maize roots in comparison to the bulk soils. Another possibility is the existence of species that are able to use N_2O as a terminal electron acceptor, but not NO; for example, *Wolinella succogenes* (epsilon-proteobacteria) is capable of growth via nitrate reduction, respiatory nitrite ammonification, and nitrous oxide reduction but not NO reduction, which results in N_2O production (Payne *et al.*, 1982). Analysis of *nos* gene clusters showed that this species had a novel *nosZ* protein assembly (Simon *et al.*, 2004), which potentially encoded a complete electron transport chain capable of generating a proton motive force. Similar *nos* gene clusters have been also been observed in soil bacteria, including *Anaeromyxobacter* (Zumft & Bothe, 2007).

4.4 Future directions – Observation vs. Manipulation

Manipulative experiments will always be useful for verifying or falsifying correlations observed in field studies, as ambiguous trends can be supported or falsified through direct manipulation of important variables that are not easily manipulated in the field. Experiments that elucidate the mechanisms underlying N_2O consumption are necessary to better understand the potential for soil ecosystems to act as N_2O sinks. Given the rapid increase in genome sequences from novel organisms, additional inoculation experiments with potential N_2O consuming organisms would verify if enrichment of these organisms result in ecosystems acting as N_2O sinks. Even more simplified experiments involving co-culturing of ecologically equivalent *nosZ* and non-*nosZ* organisms could provided additional insight into the mechanisms behind the dynamics of N_2O:N_2 ratios in microbial communities, similar to previous experiments examining denitrifier dynamics in chemostats with mixed denitrifying species (Seitzinger *et al.*, 2006; Van Cleemput, 1998).

5 Global Patterns of Denitrifier Diversity

5.1 Shaping Denitrifier Community Structure

In **paper III**, it was demonstrated that the composition of denitrifying communities can have an effect on the performance of denitrification processes in soil, which in turn is based on the genetic content of the denitrifying populations in the ecosystem. Another interesting aspect of distribution of denitrifying genes is the exclusivity between NirK and NirS enzymes that perform dissimilatory nitrite reduction. Unlike the nitrate or nitric oxide reductases, both nitrite reductases are structurally unrelated with no common catalytic domains or accessory proteins (Philippot, 2002). However, genetic studies have shown that they are functionally equivalent, as the non-denitrifying phenotype of a *Pseudomonas* mutant lacking *nirS* is restored to a denitrifying phenotype upon insertion of the *nirK* (Glockner *et al.*, 1993). This implies that the possession of both *nir* types in the same genome confers some type of fitness cost despite similarity in function, and leads to the question as to whether communities of denitrifiers with one or the other *nir* type are ecologically equivalent.

Studies in which the community structure of denitrifiers with *nirK* or *nirS* were compared along environmental gradients have shown that the communities of different *nir* types can change differentially along environmental gradients (Mosier & Francis, 2010; Smith & Ogram, 2008; Santoro *et al.*, 2006), and recent studies using geostatistical approaches have demonstrated differentiation of denitrifier communities with different *nir* genes in accordance with spatial variation of environmental parameters (Enwall *et al.*, 2010; Philippot *et al.*, 2009). These findings suggest that niche

driven processes, or 'assembly rules', may be important factor in determining the community composition of denitrifiers with different *nir* types. Under niche assembly rules, the presence or absence of species in a community is determined by the ecological niches that can be exploited by each species in a community. Thus, shared traits among related organisms that allow them to persist in a particular habitat may lead to communities of organisms that are more related to each other than expected by chance, also referred to as 'habitat filtering'. At the same time, competition among organisms for a shared resource may lead to communities that are less related than expected by chance, due to competitive exclusion for resources among closely related organisms or convergent evolution of traits important for persistence in the environment (Horner-Devine & Bohannan, 2006).

In contrast, neutral community models assume that communities are random assemblages of ecologically equivalent species that are thrown together by stochastic factors such as speciation, dispersal/migration and colonization of species from a larger, global pool of species (Hubbell, 2001). A growing body of literature exists that emphasizes the influence of neutral processes in shaping microbial communities. For example, *Sulfolobus* communities in different hot springs have been shown to exhibit biogeographical distributions irrelevant of environmental variability among the springs (Whitaker *et al.*, 2003). Neutral community assembly has also been used to describe the diversity of functional guilds such as ammonia oxidizer and denitrifier communities (Sloan *et al.*, 2006). The authors of this study found that the frequency and relative abundance of *amoA* and *nirS* sequences in clone libraries from estuarine sediments did not deviate from that predicted using a neutral community model.

Recent developments in community ecology have led to a reconciliation of these two perspectives on community assembly processes, through the development of phylogeny-based measures of community structure (Cavender-Bares *et al.*, 2009; Graham & Fine, 2008; Webb *et al.*, 2002). These statistical methods provide a means for comparing the relative importance of neutral and niche-based assembly processes at different scales (Kembel & Hubbell, 2006). To compare the ecological equivalency of *nirS* and *nirK* denitrifiers, a meta-study approach was used in **paper IV** where a global comparison of publically available *nir* sequences, obtained from PCR based studies in different aquatic and terrestrial environments, was performed to determine if i) overall patterns community structure could be correlated with environmental variables, ii) if these correlations differed

between the two *nir* genes, and iii) if such differences in community structure could be attributed to underlying differences in the assembly rules of both types of denitrifier communities. These questions were explored by using phylogeny based measures of community diversity at two different scales. First, the complete, or global, set of *nirS* and *nirK* sequences were compared across all habitats by measuring the level of phylogenetic clustering within each community, as well as the difference in community structure among all sites. We also re-examined sequence from three studies in which *nirS* and *nirK* sequence were sampled along gradients of salinity in a coastal aquifer (Santoro *et al.*, 2006), a water column in the Black sea suboxic zone (Oakley *et al.*, 2007), and a soil restoration chronosequence (Smith & Ogram, 2008).

5.2 Phyloecology - Merging of Ecology and Evolution

Classical analysis of ecological communities uses matrices of species abundance across different sites to determine the diversity of communities within sites (alpha-diversity), or the change in diversity across sites (beta-diversity). However, this approach disregards evolutionary processes, such as speciation or extinction, which also shape the structure of ecological communities (Ricklefs, 1987). An initial step towards using species relatedness in evaluation of biological diversity was taken by Faith (1992). Faith developed a measure of phylogenetic diversity (PD) based on internodal distances between bee species in different reserves, which could be applied to conservation practice to preserve diversity. This approach also circumvented the issue of defining taxonomic units, as diversity was determined by evolutionary divergence rather than the number of different taxa. These concepts were further developed by Webb *et al.*, (2002) to help differentiate between underlying processes in community assembly, in an effort to alleviate the controversy between niche-based and neutral perspectives on community assembly. These approaches have recently gained significant attention in microbial ecology.

Patterns of phylogenetic community clustering are used to infer underlying processes in assembly of communities, under the assumption that significant community structure is due to niche-driven processes, such as environmental filtering or competition (Webb *et al.*, 2002), whereas random patterns indicate the influence of netrual processes (Kembel & Hubbell, 2006). The influence of niche-driven vs. neutral assembly rules can be inferred using the net relatedness and nearest taxa indices (NRI and NTI,

respectively; Webb *et al.*, 2002). Both metrics determine the degree to which taxa cluster within a given location, relative to the entire phylogeny. The NRI metric is derived from the sum of the branches between all pairwise associations of taxa within a community, while NTI quantifies the average branch length between nearest co-occuring taxa in a community (Webb, 2000). These metrics indicate if species within a community are more related or divergent than one would expect by chance given the total phylogeny of species across all communities, based on either the full depth of the phylogeny (NRI), or the clustering at the tips of the tree (NTI). Both metrics increase with increasing levels of phylogenetic clustering, and become negative when taxa are dispersed throughout the phylogeny. However, NTI is more sensitive to clustering at the tips, while NRI takes the deeper nodes of the phylogeny into account. The significance of the community structure can then be tested based on different permutation tests, typically an iterative shuffling of the taxa and environmental associations, using adequate null models for differentiating non-random community structure from that which may occur purely by chance given the larger species pool. Choice of the proper null model is an important consideration, as detection of significant community structure is dependent on the method of randomization (Kembel, 2009).

Another way in which phylogenies can be used to examine historical processes in shaping community structure is the analysis of diversification over time (Martin, 2002). This type of analysis provides information on the rate of diversification among lineages within a given community, primarily through the use of lineage through time plots and the tree shape parameter, γ. A positive γ-value indicates an increasing rate of diversification within the tree (the internal nodes are drawn towards the tips), whereas a negative value indicates a decrease in the rate of diversification. This approach is analogous to using lineage through time plots, where communities with older and thus more divergent lineages ($\gamma < 0$) can be differentiated from communities in which more rapid diversification is taking place ($\gamma > 0$). This method examines the phylogenetic structure of a community from a slightly different viewpoint than the NRI and NTI metric, in that the rate of cladogenesis has an implicit time assumption. Thus, a large number of deep internal nodes ($\gamma < 0$) would suggest a relatively stable evolutionary course throughout the history of the lineages within the communities, while adaptive radiation events are more likely to be detected by positive gamma values.

While metrics of phylogenetic clustering are useful for differentiating between niche and neutral processes, they are also sensitive to taxonomic and spatial scale, and communities under neutral assembly may still exhibit significant patterns of clustering or overdispersion (Kraft *et al.*, 2007; Kembel & Hubbell, 2006). However, additional evidence for either process may be provided through metrics of phylogenetic beta-diversity, which allow for explicit comparison of communities across environmental or geographic continuums (Graham & Fine, 2008). Several metrics exist that analyze the beta-diversity in communities using phylogenetic information. The most well known is the Unique Fraction (UniFrac) index (Lozupone & Knight, 2005). This metric describes the proportion of the total branch length of a tree that is shared by two communities. Thus, if most of the nodes within a tree have descendants from both communities, the beta-diversity is low between the two communities as there is little divergence within the tree that is specific to one community or the other. Conversely, if the sequences have evolved to such a degree that lineages in either community are distinct to their respective habitat, the beta-diversity is high between the two. The resulting pair-wise comparison between all communities can then be used as input for a variety of different clustering or ordination procedures, as well as permutation-based multivariate analysis of variance (MANOVA) tests to determine the significance of the observed differences in community structure in response to differences in environment (Hamady *et al.*, 2010).

5.3 Influence of habitat on denitrifier communities.

Denitrifiers with both *nirS* and *nirK* are found in a wide variety of habitats, however it is clear the *nirS* data set is dominated by representatives from aquatic environments, whereas soil denitrifiers dominate the *nirK* dataset (Figure 6). This skew in habitat may be due to primer bias, as unique *nirK* or *nirS* sequences in aquatic and soil habitats may not be detected. Despite the potential influence of primer selection among different studies, UniFrac analysis followed by permutational MANOVA of both datasets demonstrated a significant effect of habitat type on community relatedness for both *nirS* and *nirK* denitrifying communities. This was largely due to differences in habitat salinity, as saline and non-saline habitats grouped separately in ordinations of UniFrac distance matrices, regardless of the

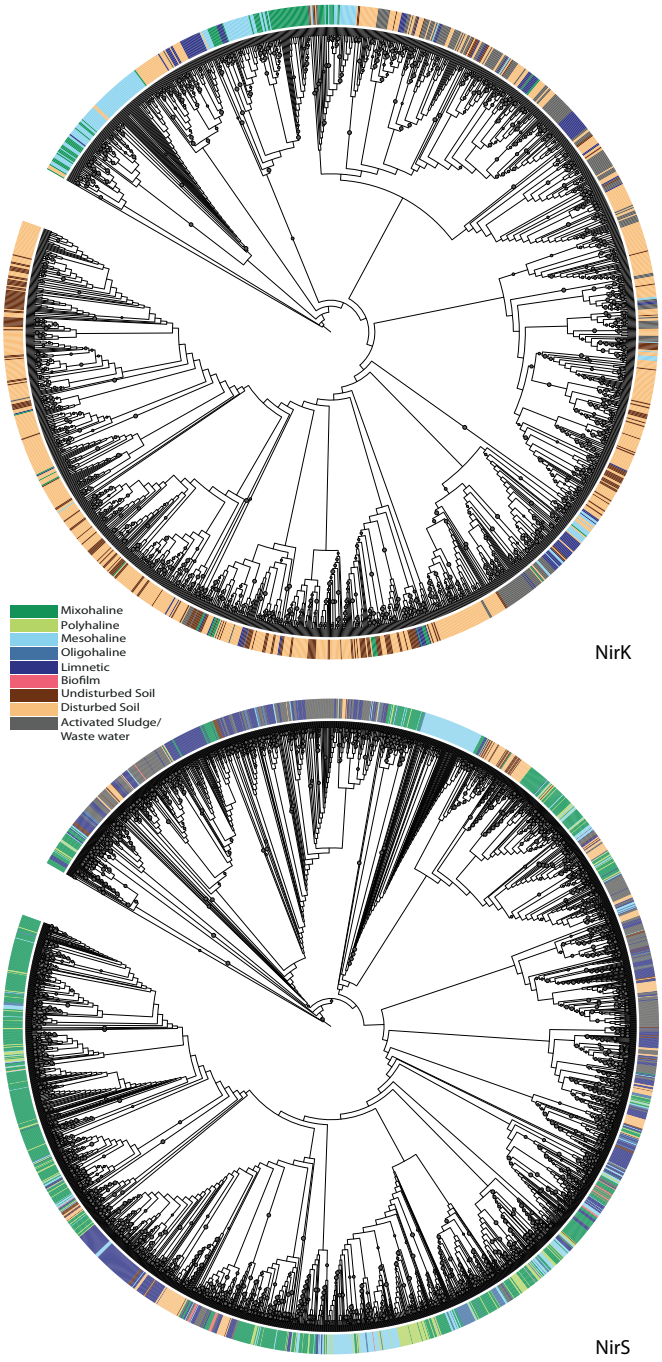

Figure 6. Maximum likelihood phylogenies of *nirK* and *nirS* nucleotide sequences from the FUNGENE database (http://fungene.cme.msu.edu). Filled circles indicate branches with > 50% bootstrap support, and tip label colors indicate habitat of origin.

geographic location of the sampling sites (Figure 1a-b, **paper IV**). These results imply that niche-driven processes are equally important for both denitrifier types, which was further substantiated by significant patterns of phylogenetic clustering in a majority of communities in each data set using NRI and NTI metrics. The main exception to this trend was the saline *nirK* communities, which were either non-clustered or significantly over-dispersed (Figure 2a-b, **paper IV**). Salinity has been previously indicated as a driving force in shaping microbial communities (Lozupone & Knight, 2007), as well as communities of chitin degrading micro-organisms, based on analysis of chitinase sequences from aquatic metagenomic datasets (Beier *et al.*, manuscript).

The task of differentiating niche-driven and neutral processes is non-trivial for the complete *nirS* and *nirK* dataset, as significant clustering of communities (as determined by NRI and NTI type metrics) analyzed at a global scale may be due to biogeographical processes as well as habitat specialization, which is of lesser importance as the spatial scale of the analysis increases (Cavender-Bares *et al.*, 2009). This issue was addressed by using UniFrac to determine that habitat type had a significant effect on the difference in phylogenetic community structure among all sites, as well as by comparing *nirS* and *nirK* communities at smaller spatial scales in the three case studies. We observed that besides salinity, nitrate concentrations also played a strong role in shaping denitrifier communities in all three case studies (Table 2, **paper IV**). Interestingly, the extent of phylogenetic clustering of communities along each gradient differed between *nirS* and *nirK*, implying that each denitrifier community reacts differentially to changes in environmental parameters. Phlyogenetic clustering of *nirS* communities in both the soil chronosequence and the Black sea studies were positively correlated with nitrate concentration, while *nirK* communities show no significant response. However, this is in contrast with the results from the pacific coastal aquifer, where the presence of a salinity gradient may have a confounding effect.

Identifying community assembly rules with absolute certainty using statistical methods is difficult given the scale of the analysis, as well as the overall uncertainty inherent in statistical analysis of phylogenetic community structure (Kembel, 2009; Kembel & Hubbell, 2006). However, given the strong influence of environmental parameters such as pH and salinity observed in previous studies of microbial communities at large geographical scales, it is not overly surprising that it should shape denitrifying organisms

as well. It is interesting to see this pattern reflected in the functional genes of what may be considered a peripherally important metabolic pathway, given the facultative nature of denitrification. This lends further support to the idea that, while horizontal gene transfer is an important process in microbial evolution, it is constrained by the overall environmental parameters that shape microbial communities. Regardless, that co-existing *nirS* and *nirK* communities did not show equivalent patterns of phylogentic clustering lends further support to the hypothesis that *nirS* and *nirK* denitrifiers are not ecologically equivalent, and each community may be under different assembly rules that are in part determined by habitat.

5.4 Future Directions

The hypotheses raised in this study could be tested through a series of microcosm experiments similar to those used in **paper III**, in which denitrifiers with either *nirS* or *nirK* compete under a variety of environmental conditions. Experimental approaches have been used to test the importance of niche-driven vs. neutral assembly in plant communities (Fargione *et al.*, 2003), which circumvents some of the conceptual difficulties of inferring community assembly rules from patterns of phylogenetic community clustering illustrated above. A manipulative microcosm approach was recently used by (Salles *et al.*, 2009), in which the range of resources used by each species in a community, termed 'niche-breadth' was used to describe the overall denitrifying community and predict the overall rates of denitrification for different community assemblages. While the authors found a significant positive correlation between species richness and denitrication rates, no distinction was made among denitrifying species with either *nir* gene. This, in addition to the observation of niche differentiation among *nir* types in field studies, warrants further experimental work.

6 Conclusions and Perspectives

The denitrifying bacterial community is an attractive model system to provide general insights into the concept of diversity and the functional role of diversity in soil ecosystems due to the high degree of denitrifying bacterial diversity, its integral role in the N-cycle, and the fact that it is a facultative trait in bacteria (Philippot & Hallin, 2005). Ecosystem-scale processes that are mediated by microorganisms are inextricably tied to the underlying genetic diversity within the functional guild responsible for that process, and thus understanding the effects of changes in biodiversity on ecosystem functioning is a top priority in ecology. A recent consensus on the current state of knowledge about the relationship between ecosystem functioning and biodiversity (Hooper *et al.*, 2005) has specifically indicated a need for greater understanding of the relationship between taxonomic diversity, functional diversity, and community structure to better identify the effects of altered biodiversity on ecosystem functioning. Given increasing probability that ecosystems will be described in large part by their genetic content, conceptual developments are underway that seek to identify an 'ecosystem phenotype', in which evolutionary processes among interacting species are analyzed in relation to their effect on the ecosystem (Whitham *et al.*, 2003). However, application of this approach to denitrifying communities must account for the following:

- Denitrification is a modular pathway in which each step has its own evolutionary history (**paper I**), where processes such as horizontal gene transfer, lineage sorting, and gene duplication differentially influence each gene. The degree to which denitrification gene phylogenies agree with each other as well as organismal phylogeny is lineage specific.

- The genetic variation of denitrifying isolates is not necessarily reflected in the phenotypic variation when compared at different environmental conditions (**paper II**). This may partly explain the variable results seen among field studies attempting to link denitrifier community structure with activity. However, genomic rearrangements can have an influence on the denitrification phenotype, particularly regarding the gain/loss of *nosZ* among the genomes of denitrifying prokaryotes

- The gain/loss of *nosZ* among denitrifying organisms can influence the ratio of denitrification end products from denitrifying communities in soils (**paper III**). In addition to environmental parameters, this can determine if soils act as a source or sink for N_2O emissions.

- Denitrifying community assembly is largely niche driven, however the diversity of denitrifiers with different *nir* types may be differentially affected by environmental constraints, as indicated by the difference in phylogenetic clustering of *nirS* and *nirK* sequences along both nitrate and salinity gradients (**paper IV**).

Since the science of ecosystem-scale genomics is still in its infancy, particularly regarding the functional links between the genomic parts of an ecosystem (Raes & Bork, 2008), there is still place for continuing development of current molecular probe based techniques for capturing the true extent of diversity and ecological dynamics among denitrifying prokaryotes. For example, redesign of primers targeting the divergent clades of functional genes using the growing amount of genomic data (Bartossek *et al.*, 2010; **paper II**) may be useful for more accurate characterization of the currently under-sampled denitrifier diversity in environmental samples, such as Gram-positive denitrifiers or species that contribute to N_2O consumption in different ecosystems. Genomic data may also be used in comparative studies among organisms sampled from different environments to generate hypotheses about the underlying evolutionary mechanisms the drive the gain and loss of denitrification genes. For example, what is the effect of periodic vs. constant expression of genes on their continuing maintenance in the genome? Such questions could be probed using the *nos* operon as a model system, and inferences made using bioinformatics tools can be used to inform both laboratory and field experiments in testing the relationship between genes, communities, and ecosystems.

'Community genetics requires and promotes an integrative approach, from genes to ecosystems, that is necessary for the marriage of ecology and genetics. Few studies span from genes to ecosystems, but such integration is probably essential for understanding the natural world.'

Whitham *et al.*, 2003.

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