Community Ecology of Denitrifying Bacteria in Arable land

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Abstract

Denitrifying bacteria comprise a functional guild that under anaerobic conditions is able to use nitrogenous compound for respiration. Agricultural soils are often fertilized with nitrogen, and the reduction of nitrate and nitrite to nitric oxide, nitrous oxide and dinitrogen via the denitrification pathway, leads to gaseous emissions from the nitrogenous compounds, a subject of great environmental and economic concern.

This thesis examined denitrifiers, nitrate reducers, ammonia oxidizers and total bacteria, in terms of community structure, abundance and function in different arable soils. Denitrifying bacteria were described using the signature genes that encode the analytic subunits of denitrification enzymes. Appropriate targets and methods for this were evaluated. An assessment was carried out of how these biological factors are affected by different fertilizer and management practices. The spatial distribution of the denitrifying bacteria, and how this was correlated to soil properties, was also explored.

Long-term application of different organic and mineral fertilizers altered both the community structure and function of the different bacterial groups. However, fertilization practices producing the most different community structure were not correlated to the treatments with the most different activities. Thus activities were not coupled to community composition. However, a study with different carbon additions, with and without nitrogen additions, showed that nitrogen addition increased the microbial activity and biomass, and in this case community composition was correlated with potential activity. In long-term fertilization, pH, C:N ratio and organic carbon content were important drivers shaping the community composition of the denitrifiers and total bacteria.

Determination of the structure and size of denitrifying bacteria at field scale revealed significant relationships between denitrifying community composition, size and activity, and soil factors.

These results are important in identifying the resource-based niches for denitrifiers at scales relevant for developing sustainable land management and agricultural practices.

Keywords: agricultural soil, community structure, denitrifying bacteria, DGGE, fertilizer, long-term, microbial ecology, N cycle, T-RFLP.

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Samhällsstrukturen hos denitrifierande bakterier i jordbruksmark

Sammanfattning

Denitrifierande bakterier är en funktionell grupp som under syrefria miljöer kan använda kväveföreningar för respiration. Under respirationsprocessen reducerar bakterierna nitrat och nitrit till kvävemonoxid och lustgas. Eftersom jordbruksmark ofta är gödslat med kväve kan denitrifikationsprocessen leda till kväveemissioner från marken, vilket är en nackdel både för miljön och ekonomin.

Denitrifierande-, nitratreducerande-, ammoniumoxiderande bakterier och det totala bakteriesamhället och har studerats och olika metoder har utvärderats och utvecklats.

En av målsättningarna var att undersöka hur samhällsstrukturen, mängden av bakterier och deras funktion eller aktivitet hos olika bakteriegrupper har påverkas av olika gödningsmedel. Vidare så studerades även hur den rumsliga variationen av denitrifierare var korrelerat till olika markegenskaper.

Långsiktlig tillsättning av olika mineral- och organiska gödningsmedel förändrade både populationssammansättningen och funktionen hos de olika bakteriegrupperna. Behandlingen som påvisade den största skillnaden på samhällsstrukturen var inte korrelerad till behandlingen med störst skillnad i aktivitet vilket visade att aktiviteten hos bakterier inte är kopplat till samhällsstrukturen.

En senare studie med tillsats av olika kolkällor till jordbruksmark, med eller utan kvävetillsats, visade att kvävetillsatsen ökade den mikrobiella aktiviteten och biomassan. Samhällsstrukturen av denitrifierare och totala bakteriesamhället var även påverkad, och i denna studie kopplad till aktiviteten. Långsiktlig tillsats av olika gödningsmedel påverkar markens egenskaper och pH, C/N-kvot och mängden organiskt kol är viktiga faktorer som i sin tur påverkar och formar bakteriesamhället.

I den sista studien undersöktes den rumsliga variationen av samhällsstrukturen och mängden av denitrifierande bakterier i ett jordbruksfält. Vidare studerades om olika markfysikaliska och markkemiska faktorer kunde förklara de denitrifierande bakteriernas rumsliga variation. Resultaten visade att denitrifierare var rumsligt strukturerade och denna var korrelerad till olika markfaktorer.

Slutsatserna i denna avhandling är viktiga för att förstå hur denitrifierande bakterier påverkas av gödningsmedel och markskötsel i större skala. Detta för att vidare kunna utveckla ett hållbart utnyttjande av jordbruksmarken.

For my Family

Contents

List of Publications

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

I Throbäck, I.N., Enwall, K., Jarvis, Å. and Hallin, S. (2004). Reassessing PCR primers targeting *nirK*, *nirS* and *nosZ* genes for molecular diversity surveys of denitrifying bacteria, and the analysis of community structure with DGGE. FEMS Microbiology Ecology 49(3), 401-417.

II Enwall, K., Philippot, L. and Hallin, S. (2005). Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. Applied and Environmental Microbiology 71(12), 8335-8343.

III Enwall, K., Nyberg, K., Bertilsson, S., Cederlund, H., Stenström, J. and Hallin, S. (2006). Long-term impact of fertilization on activity and composition of bacterial communities and metabolic guilds in agricultural soil. Soil Biology and Biochemistry 39(1), 401-417.

IV Enwall, K. and Hallin, S. Comparison of T-RFLP and DGGE techniques to assess denitrifier community composition in soil (submitted).

V Wessén, G., Enwall, K., Jansson, J. and Hallin, S. Soil carbon sequestration and depletion structure bacterial communities and alter soil respiration (manuscript).

VI Enwall, K., Throbäck, I.N., Stenberg, M., Söderström, M. and Hallin, S. Soil-based resources influence field-scale spatial patterns of N-cycling bacterial communities: A case study of denitrifiers (manuscript).

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Abbreviations

DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
napA	gene encoding the periplasmic nitrate reductase
narG	gene encoding the membrane-bound nitrate reductase
N_2	dinitrogen gas
N_2O	nitrous oxide
NH_4^+	ammonium
NH_3	ammonia
nirK	gene encoding the copper-containing nitrite reductase
nirS	gene encoding the cytochrome cd_1 nitrite reductase
NMS	non-metric multidimensional scaling
NO_3^-	nitrate
NO_2^-	nitrite
NO	nitric oxide
norB	gene encoding the cytochrome b subunit of the nitric oxide reductase
norC	gene encoding the cytochrome c subunit of the nitric oxide reductase
nosZ	gene encoding the nitrous oxide reductase
PCR	polymerase chain reaction
qnorB	gene encoding the quinol nitric oxide reductase
RISA	ribosomal intergenic spacer region analysis
RNA	ribonucleic acid
T-RFLP	terminal restriction fragment length polymorphism

Introduction

The microbial world in terrestrial ecosystems is not easy to observe and the diversity is immense. A few grams of soil contain more bacteria than there are people in the world. These bacteria carry out fundamental ecosystem functions, such as nutrient cycling and degradation of pollutants, but the link between microbial diversity and function in soil is not fully understood. An understanding of the ecology of microorganisms and their roles in ecosystems is vital in developing sustainable ecosystem management and guiding the exploitation of ecosystem services. Recently, the advent of molecular tools in microbial ecology has made it easier to study microbial community composition and we are becoming increasingly aware of the significance of specific microorganisms in nature, the generation and maintenance of microbial diversity and the ecological consequences of microbial diversity for ecosystem functioning.

This thesis focuses on the ecology of one specific guild of bacteria in arable soil ecosystems, the denitrifying bacteria. The denitrifiers do not belong to a specific taxonomic group, since denitrification is a trait present among a broad range of genera. Therefore, 16S rRNA, a commonly used molecular marker for analyzing the community structure of bacteria, is not suitable for targeting the denitrifying bacterial community. Instead, the functional genes associated with the denitrification process, such as *nirS*, *nirK* and *nosZ* are used.

Denitrifying bacteria are facultative, which means that they are aerobic bacteria but that they are able to use oxidized nitrogenous compounds for respiration under anaerobic conditions or when oxygen concentration is low. In agricultural soil, the conversion of nitrate and nitrite to the gaseous compounds nitric oxide, nitrous oxide and dinitrogen is of major economic concern, since valuable nitrogen added as fertilizer disappears from the soil through emissions. These gaseous emissions are also of environmental

concern, since nitrous oxide is a potent greenhouse gas and can destroy the stratospheric ozone layer. The activity, numerical amount and composition of denitrifying bacterial communities are largely driven by soil characteristics, particularly energy and nutrient resources. Hence, different soil management practices can have a dramatic impact on the denitrifying community, with associated impacts on ecosystem functions.

Aims and outline of this thesis

The main aims of this thesis were to study the community ecology of denitrifying bacteria in arable land and to examine how structure and function are affected by different fertilization, soil management and cropping regimes.

In this work described in thesis, PCR-based molecular methods were used to study the structure and size of the denitrifying bacterial community in soil. Several primer sets amplifying *nirS*, *nirK* and *nosZ* genes have been published and used in environmental surveys of denitrifiers (e.g. Braker *et al.*, 1998; Hallin & Lindgren, 1999; Scala & Kerkhof, 1999). However, the primers targeting the gene of interest have to be both specific and sufficiently sensitive, and must suit the subsequent community analysis techniques. Therefore, we started by evaluating different primers (**Paper I**). For *nirS* and *nosZ*, new primers were designed, while existing primers were found to adequately amplify the *nirK* gene in a range of bacterial species and variety of environmental samples. With the new *nirS* primers we were able to revise earlier conclusions that *nirS* denitrifiers are preferentially found in marine habitats and to show that soil harbour substantial *nirS* denitrifier diversity. In the same study we also adapted DGGE for the amplified denitrification genes. In addition to the denitrifiers, we also analyzed the ammonia oxidizing bacteria, the nitrate reducing bacteria, and the total bacterial community to study how the activity and composition of different bacterial groups respond to different long-term fertilization regimes (**Papers II** and **III**). The soil in Ultuna long-term soil organic matter experiment has been fertilized with different mineral and organic fertilizers for 50 years. The activity and community composition have responded differently to the fertilizer regimes. pH was the overriding factor for the changes in the community structure, whereas soil organic carbon and nitrogen had an impact on the activity. To allow for higher throughput and standardisation of assay conditions, we developed an alternative genotyping method, T-RFLP, for the primer sets used in the first experiments. The performance of the two genotyping methods, DGGE and T-RFLP, was first assessed for

nosZ (**Paper IV**). To accomplish this, two different soils were sampled, each with two different treatments in three field replicates. Both methods separated the *nosZ* community structure in the two soils, but DGGE had a slightly higher resolution and could separate treatments resulting in small differences in the *nosZ* community composition. In the next study we explored how the denitrifying and total soil bacterial communities were affected by long-term carbon sequestration and depletion (**Paper V**). Once again, soil samples from the Ultuna long-term soil organic matter experiment were used, since different organic amendments in combination with nitrogen fertilization have generated plots with contrasting C:N ratios. Aerobic respiration, respiration under denitrifying conditions and plant growth was controlled by nitrogen fertilization and soil organic carbon content, but the type of organic amendment had no effect on these parameters. However, the type of organic matter added affected community composition, since differences among treatments were mainly explained by differences in soil C:N ratio. In the last study we analyzed spatial patterns of community composition, activity and abundance of the denitrifying bacteria at the landscape scale and how these spatial patterns were related to various soil chemical and physical properties (**Paper VI**). We showed that the spatial distribution of denitrifiers was related to soil habitats in ways that suggest specialization to different resources. Soil structure, which affected soil water-holding capacity, and certain macronutrients were the most important factors explaining denitrifier community structure at the landscape scale.

Soil microbes and their habitat

Soil is the most heterogeneous ecosystem on Earth. It is a complex medium, with great spatial and temporal variation in soil properties. The spatial heterogeneity is an important characteristic, allowing genotypes to coexist in the soil system, and the heterogeneity occurs at many different scales from soil particles via the plant rhizosphere to field and landscape level. Bacteria, archaea and fungi, as well as plants and soil animals, such as protozoa, all compete for the nutrients present in the soil.

Microbial diversity in soil is enormous, with billions of organisms and thousands of species in one gram of soil (Torsvik *et al.*, 1990). Torsvik *et al.* (2002) showed that soil is more diverse than aquatic or marine environments and most of the organisms present are uncharacterized. It is estimated that less than 1% of the microbial species in the environment have yet been cultivated and characterized (Amann *et al.*, 1995) and in addition to this great species diversity, functions and traits within species are also diverse.

Bacteria are ubiquitous, and through their high range of metabolic activities, they play a central role in ecosystem functioning. The biogeochemical processes transforming nitrogen, carbon, sulphur and phosphorus are highly controlled by bacteria. Soil microorganisms can promote plant growth (Kloepper *et al.*, 1980) and increase soil fertility, e.g. due to nitrogen fixation and mycorrhizae (Jeffries *et al.*, 2003; Watson *et al.*, 2002). Since soil microbial communities are actively involved in soil processes and functions, alterations to the microbial community compositions have the potential to modify the biotic soil properties.

Microbes and terrestrial ecosystem functions

Most ecological theories have developed from studies of plants and animals and not microorganisms, despite the fact that microbes are the most abundant and diverse organisms on Earth and play major roles in biogeochemical processes. A new theoretical foundation for microbial ecology is therefore warranted, to improve or replace established theory (Prosser *et al.*, 2007).

Diversity

To determine biodiversity, information is needed about the number of different species (richness) and the relative abundance of the individual species (evenness). However, different species perform the same functions and similar species perform different functions, so it is important to also consider the redundancy and the functional diversity of the species. A definition of functional diversity is the number of different processes or functions that are performed by a community, while functional redundancy is a measure of the number of species that perform that particular function in different functional groups or guilds (Gaston, 1996).

The role of biodiversity for ecosystem functioning has become a central issue in ecological and environmental sciences during the past decade (Loreau *et al.*, 2001). If many taxa equate to diverse ecosystem functions, diversity can be regarded as a safety net for the soil ecosystem. Thus, high species richness ensures the maintenance of ecosystem functions, since it is more likely that the system will harbour species able to adapt to changing conditions (Yachi & Loreau, 1999). Yin *et al.* (2000) believe that functional redundancy implies that different species occupy similar functional niches, so biodiversity does not have a major impact on ecosystem functioning. However, changes in the microbial diversity do not always correspond to changes in functional redundancy (Atlas *et al.*, 1991). Different studies have shown that microbial communities can change in response to disturbances in the soil (Atlas *et al.*, 1991; McCaig *et al.*, 1999; Ovreas & Torsvik, 1998) and that ecosystems with high biodiversity have an increased ability to withstand perturbation in soil caused by pollutants (Girvan *et al.*, 2005). Before we can predict changes in ecosystem functions, the link between microbial community composition and ecosystem functions has to be understood.

Spatial distribution

Researchers working with microbial ecology in soil systems sometimes feel that the heterogeneous nature of the soil is frustrating, and samples are homogenized to overcome this problem. However, Ettema and Wardle

(2002) claim in their review that it is likely that spatial variability is the key, not the obstacle, to understanding the structure and function of soil biodiversity.

Another difficult issue to take into account when working with microbial ecology is the scale (Levin, 1992; Standing et al., 2007; Woodcock et al., 2007). From a very small soil sample, very large microbial communities are characterized, and further scaled up to represent e.g. a treatment in an incubation trial or field site.

The study of spatial patterns of organisms and processes is known as biogeography. This has been extensively reported for eukaryotes, especially plants, while the interest in the biogeography of prokaryotes arose during the beginning of the 20th century. There are three processes that generate biogeographical patterns; dispersal, speciation and extinction. Due to their small size, bacteria can disperse rather easily over large distances and this may result in 'cosmopolitan behaviour' (Baas Becking, 1934; Finlay, 2004). A famous quote in this regard is that 'everything is everywhere, the environment selects' (Baas Becking, 1934), but this conception has been questioned since the advent of molecular tools. Speciation considers how new species arise and there are different routes for this. Vertical speciation for microbes from a common ancestor is quite indistinct, due to lateral gene transfer and homologous recombinations (Cohan, 2002; Lawrence, 2002). Allopatric speciation occurs when a population is geographically separated and divergence over time makes the former population into two different species. According to Cohan (2002), niche diversification can also lead to speciation, and hence ecological divergence even within the same habitat is sufficient for speciation to occur (sympatric speciation). In general, the speciation rate in bacteria is expected to be high, due to their large population size, fast reproduction rates, genetic promiscuity and their ubiquitous distribution patterns. However, little is known about the magnitude of these processes in nature (Horner-Devine *et al.*, 2004). Bacteria have low extinction rates (Dykhuizen, 1998; Torsvik *et al.*, 2002), and high population and generation sizes. In addition, some bacteria can change their life style by forming spores and thus, survive in harsh environments.

Spatial scaling of microorganisms is an interesting field in microbial ecology and can provide information on how biodiversity is generated and maintained (Green & Bohannan, 2006; Horner-Devine *et al.*, 2004). Multiple forces structure communities and a given microbial community in space and time is most likely the product of dispersal of microbes in combination with habitat selection in varying proportions (Martiny *et al.*,

2006). The few existing reports on field-scale spatial distribution of microorganisms in arable land have adopted a taxa-centred perspective focusing on the total bacterial community, and functional communities or traits have been largely neglected (Franklin & Mills, 2003; Green *et al.*, 2008; Ritz *et al.*, 2004). Characterizing spatial patterns of microorganisms is the first step towards identifying the resource-based habitats of microbial populations and functionally coherent communities. At larger scales, compatible with land management scales, predicted patterns of microbial community distribution would not only provide an insight into the mechanisms shaping microbial communities, but also facilitate our understanding of the relationships between the ecology of microbial communities, microbial processes and ecosystem functioning. This in turn could build the foundation for knowledge-based land management strategies.

Nitrogen cycling in soil

In soil, nitrogen is the most important nutrient for plant growth and therefore primary production is often limited by nitrogen availability. The transformation of nitrogen in the soil or other ecosystems is mediated by processes performed by different groups of organisms (Fig. 1). The atmosphere contains 78% dinitrogen gas (N_2), which has the potential to enter the nitrogen cycle through the action of different groups of organisms that can reduce N_2 to ammonia (NH_3). This biological process is called nitrogen fixation and is carried out by the diazotrophic bacteria. These organisms contain the gene coding for the enzyme nitrogenase, which can break the triple covalent bond of N_2. The nitrogen fixing bacteria are either free-living, such as *Azotobacter* and different cyanobacteria, or symbiotic and in association with plant roots, such as *Rhizobium* and *Frankia* (Brill, 1980).

The mineralization or the immobilization describes the conversion of ammonium to organic nitrogen, primarily as a result of assimilation of ammonium into the microbial biomass.

Ammonia is oxidized to nitrate (NO_3^-) in a two-step process called nitrification. In the first step, NH_3 is oxidized to nitrite (NO_2^-) via hydroxylamine (NH_2OH) by ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA). Most AOB in soil belong to the betaproteobacteria and AOA to the crenarchaea. In the second step, NO_2^- is then further oxidized to NO_3^- by nitrite oxidizing bacteria belong to the proteobacteria (NOB). The first step, ammonia oxidation, is often considered rate limiting. The nitrifiers are aerobic and use oxygen as a terminal electron acceptor, NH_3 and NO_2^- as energy sources and CO_2 as a carbon source (Könneke *et al.*, 2005; Prosser, 1989). A recent publication (Leininger *et al.*, 2006) revealed that AOA are more abundant in soil than AOB, indicating that crenarchaea may be the most abundant ammonia

oxidizing organisms in the soil. Whether AOA are also the most active remains to be determined.

The NO_2^- formed by the NOB can also be used in the oxidation of ammonium (NH_4^+) to N_2 (Jetten, 2001) by anaerobic ammonium oxidation (anammox). This process is performed by anaerobic bacteria, mainly belonging to the *Plantomycetes*. The anammox bacteria can disguise themselves as denitrifiers, since they can produce NH_4^+ by themselves. This occurs via dissimilatory nitrate reduction to ammonium, which is then oxidized with NO_2^- and N_2 is produced (Kartal *et al.*, 2007).

Nitrate can be reduced to N_2 via the denitrification process, which is discussed in the next section, or by dissimilatory nitrate reduction to ammonium, a process abbreviated DNRA. DNRA is a strictly anaerobic two-step process where NO_3^- is reduced to NH_4^+ via NO_2^- (Tiedje, 1988). It is a respiratory process and the capacity for DNRA has been found in facultative and obligate fermentative bacteria (Tiedje, 1988).

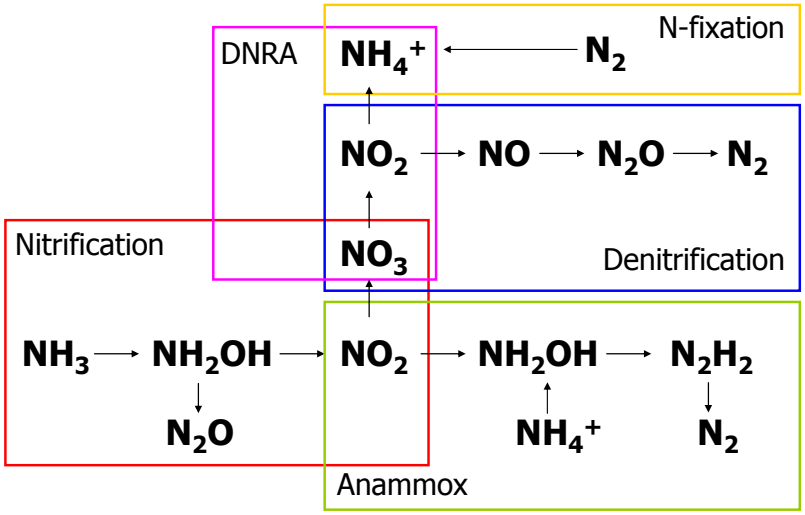

Figure 1. The different microbial processes that contribute to the global N cycle.

Implications of nitrogen cycling for environment and agronomy

Transformations of nitrogen in soil are important for both environmental and agricultural issues in various ways. Thus, processes in the nitrogen cycle can result in nitrogen losses from the soil and only about half of the nitrogen added to the soil by fertilization is assimilated into the biomass, the rest being either leached to the groundwater and surface water or emitted in gaseous form to the atmosphere (Einsle & Kroneck, 2004).

Nitrification is mainly responsible for losses of nitrogen through leaching. Since NH_4^+, the substrate of nitrifiers, is positively charged, it can therefore be adsorbed to the negatively charged clay minerals or soil organic matter and held in a relatively immobile form available for plant roots but partially protected from leaching. On the other hand, NO_3^-, the final product of nitrification, is negatively charged and therefore more mobile, and can easily be leached through the soil to reach the groundwater or watercourses, and eventually to cause eutrophication in lakes and oceans (Vitousek *et al.*, 1997).

Denitrification, nitrification and DNRA are responsible for the gaseous nitrogen emissions and Conrad (1996) estimated that as much as 70% of the total emissions of nitrous oxide (N_2O) originate from these processes. This is of great concern since N_2O is a potent greenhouse gas and a contributor to the destruction of the stratospheric ozone layer (Cicerone, 1987; Rasmussen & Khalil, 1986). The global warming potential of N_2O is almost 300 times higher than that of carbon dioxide (IPCC, 2001) and its atmospheric concentration is increasing by 0.3% annually. However, the denitrification process can also be beneficial, e.g. for removing nitrogen from nitrate-polluted water.

Denitrification

Denitrification is the microbial process in the nitrogen cycle in which the soluble nitrogen forms NO_3^- and NO_2^- are reduced into the gases NO, N_2O and N_2 (Fig. 2). This is a facultative process (Tiedje, 1988). The two major criteria for true denitrification are production of N_2O or N_2 from NO_2^- or NO_3^-, and coupling of these reactions to energy conservation and growth yield (Mahne & Tiedje, 1995). The bacteria use the nitrogen oxides as alternative terminal electron acceptors in respiration when oxygen concentrations are low or absent, even though some organisms are capable of denitrification under aerobic conditions (McDevitt *et al.*, 2000). Most denitrifiers are heterotrophic bacteria, although lithotrophic (*Thiobacillus*) and phototrophic (*Rhodobacter*) denitrifiers also exist.

The denitrification pathway

Seven known enzymes are associated with the different steps in the denitrification pathway (Fig. 2) but not all denitrifying bacteria possess all of these enzymes.

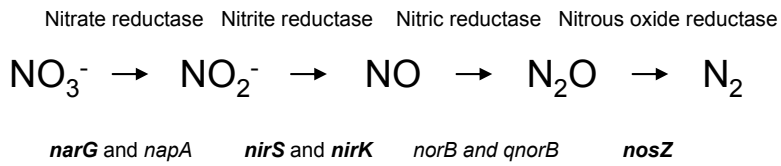

Figure 2. The denitrification pathway and the genes associated with each step. The genes marked in bold type were studied in this thesis.

The first step, where NO_3^- is reduced to NO_2^- is catalyzed by two types of dissimilatory nitrate reductases; a membrane-bound (Nar) and a periplasmic (Nap). These enzymes are also found in nitrate reducing bacteria that are not denitrifying bacteria.

The reduction of NO_2^- to nitric oxide (NO) is a crucial step in denitrification since the product is a gaseous compound. This step is catalyzed either by a copper (NirK) or cytochrome type (NirS) nitrite reductase. A bacterium can contain either NirS or NirK but not both. The two different enzymes are unrelated in terms by evolution but have a similar function.

The Nor enzymes are particularly interesting since they catalyze the formation of a double bond between two nitrogen atoms. The Nor enzymes have a cytochrome *b* or *c* subunit located in the membrane. The NorB and the NorC enzymes are encoded by two variants of the *norB* gene. Another type of enzyme is characterized by instead having quinol dehydrogenase, qNor. However, bacteria that are not denitrifiers may possess this enzyme since it has a detoxifying function, and NO is toxic to all life forms (Zumft, 1997; Zumft, 2005).

The last enzyme in the denitrification pathway is nitrous oxide reductase (Nos), encoded by the *nosZ* gene (Fig. 2). So far only one Nos type has been characterized. The enzyme is located in the periplasm, although membrane-associated variants occur (Hole *et al.*, 1996). It has been speculated that nitrous oxide reductase is more sensitive to oxygen (Knowles, 1982), low temperature and heavy metals (Holtan-Hartwig *et al.*, 2002) than the other enzymes in the denitrification pathway.

Denitrifying bacteria

The ability to denitrify is spread widely within different bacterial phyla and up to 5% of the total amount of bacteria in soil are believed to be capable of denitrifying (Henry *et al.*, 2006; Tiedje, 1988). However, the capacity to denitrify is not restricted solely to bacteria. Different archaea (Zumft, 1997) and fungi (Shoun & Tanimoto, 1991; Usuda *et al.*, 1995) have also been reported to be able to denitrify, although the focus in this thesis is on bacteria. Philippot *et al.* (2007) listed over 130 denitrifying bacterial species within more than 60 genera. The denitrifying bacteria are found in *Alpha-*, *Beta-*, *Gamma-* and *Epsilonproteobacteria* and *Bacteroides* and also among the Gram-positive bacteria *Firmicutes* and *Actinobacteria*.

Closely related bacteria do not necessarily share the denitrifying trait. This means that denitrification can not be deduced from taxonomic affiliation of the organism and that the phylogeny of the denitrification genes does not give an answer to the taxonomic affiliation of the organism.

Analyzing the denitrifying bacteria

Since the ability to denitrify is sporadically distributed within bacteria and cannot be associated with any specific taxonomic group, the use of 16S rRNA genes is not an option for analyzing the denitrifying bacteria. Instead, the denitrifiers are analyzed using functional genes (Braker *et al.*, 1998; Hallin & Lindgren, 1999; Philippot & Hallin, 2006; Scala & Kerkhof, 1999). Functional genes usually have higher sequence polymorphism than the 16S rRNA genes, and can be used as biomarkers to discriminate between closely related but ecologically different populations (Palys *et al.*, 1997).

The use of functional genes instead of 16S rRNA genes has the disadvantage that no information about taxonomic affiliation is gained, provided that there is no congruence between functional genes and 16S rRNA. The lack of taxonomic affiliation for denitrifiers has often been explained by horizontal gene transfer (Delorme *et al.*, 2003; Goregues *et al.*, 2005; Gregory *et al.*, 2003; Heylen *et al.*, 2006; Heylen *et al.*, 2007), but other evolutionary events may have differently influenced the evolution of each denitrification gene (Jones *et al.*, 2008). Recent studies have shown that the *nosZ* gene has the highest level of congruence with taxonomic classification based on 16S rRNA, compared with other denitrification genes (Dandie *et al.*, 2007; Jones *et al.*, 2008).

Methods in microbial ecology for analyzing community structure, abundance and activity

Previously, the most common way to detect environmental bacteria was to study them under the microscope or cultivate them in a medium. Today, with the molecular biological revolution underway and with the amounts of genetic information available rapidly growing, the most common way is based on studying the bacterial DNA or RNA. One approach is to recover the genetic material in an environmental sample directly using metagenomics (Handelsman *et al.*, 1998). This method has been used to sequence entire bacterial communities in different environments such as the picoplankton fraction in the Sargasso Sea (Venter *et al.*, 2004) and an acidophilic biofilm from an iron mine (Tyson *et al.*, 2004). However, most community studies are still based on amplifying the genetic material with polymerase chain reaction (PCR). With this approach the DNA or RNA is first extracted and purified from the environmental sample. Different extraction procedures exist and several commercial kits are available for this purpose. A study comparing three different extraction methods applied to three different soils has shown that the results of the extractions of the bacterial community are dependent on the recovery method used (Martin-Laurent *et al.*, 2001). The purification step is important since coextraction with DNA/RNA of different compounds that inhibit the polymerase activity, e.g. humic acids in soil samples, is a common problem (Tsai & Olson, 1992). The choice of primer for PCR amplification is the next critical step. In **Paper I**, we designed and evaluated different primer sets for amplifying the denitrification genes *nirS*, *nirK* and *nosZ* and demonstrated that the choice of primers is crucial for successful amplification of the target genes in different environmental samples. Following successful PCR amplification, the pool of amplified DNA/RNA fragments is analyzed

further to resolve the different genotypes. Several methods are available to describe and identify the dominant genotypes in the sample but this does not mean that the methods assess the total diversity in the sample, particularly in such diverse communities as those found in soil.

PCR based approaches for assessing community composition

This thesis studied the denitrifiers, nitrate reducers, ammonia oxidizers and total bacteria. Different molecular approaches were applied to unravel the polymorphism of amplicons (Fig. 3) obtained using primers targeting either functional genes *narG*, *nirS*, *nirK* and *nosZ* (**Papers I-II** and **IV-VI**), SSU ribosomal RNA (rRNA) genes (**Papers III** and **V**) or the intergenic spacer region (IGS, **Paper II**).

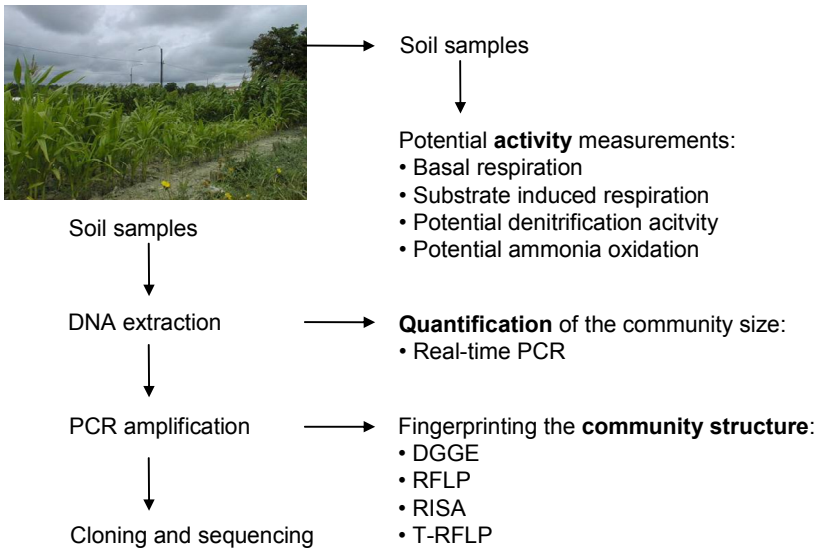

Figure 3. An overview of the different molecular tools and activity measurements used in this thesis for studying the community structure, abundance and activity of soil bacteria.

DGGE

Denaturing gradient gel electrophoresis (DGGE) is a technique in which PCR products are separated based on using polyacrylamide gels with an increasing chemical gradient (Muyzer *et al.*, 1993). The gradient is usually based on urea and formamide and PCR products are loaded on top of the

gel. The double-stranded PCR products migrate through the gel as a voltage is applied. According to the melting behaviour of unique fragments, the migration decreases and eventually stops due to formation of secondary structures and this generates a banding pattern for the sample (Fig. 6; **Papers I-IV**). A GC clamp consisting of 30-40 base pairs of mostly G and C is normally added to one of the primers to avoid complete denaturation of the DNA. For visualization of the banding pattern, the gel has to be stained. There are different staining methods, such as silver staining, ethidium bromide staining and staining with different SYBR dyes. The two latter staining procedures are followed by UV translumination for documentation of the gel patterns.

DGGE is an efficient technique for PCR fragments shorter than 500 base pairs (Myers *et al.*, 1985). The bands of interest can be excised, cloned and sequenced (**Paper I**), which is the great advantage of the DGGE method compared with other methods. The major drawback of DGGE is that sequences amplified from different organisms may have the same melting temperature and migrate to the same position on the gel meaning that a single band can contain a mixture of genotypes. This method has been frequently used in microbial ecology to fingerprint bacterial communities, most often based on the 16S rRNA genes, and recently also for studies of the denitrifying community composition (Hallin *et al.*, 2006; Kjellin *et al.*, 2007; Sharma *et al.*, 2005; Throbäck *et al.*, 2007). DGGE was used in this thesis to analyze the community composition of denitrifiers (**Papers I, II** and **IV**) and of the ammonia oxidizers (**Paper III**).

RFLP

Restriction fragment length polymorphism (RFLP) is a method where amplicons that have been digested with restriction enzymes are separated based on their size on a polyacrylamide gel. RFLP is commonly used to screen clone libraries (see 'Clone library analysis' below), and has been used by a number of researchers when studying denitrifying bacteria (Braker *et al.*, 2000; Hallin *et al.*, 2006; Horn *et al.*, 2006; Scala & Kerkhof, 1999; Sharma *et al.*, 2005; Stres *et al.*, 2004) and more seldom for analyzing community structure in environmental samples (Dambreville *et al.*, 2006; Deiglmayr *et al.*, 2004). In this thesis, RFLP was used for analyzing the community structure of nitrate reducing bacteria in soil, by targeting the functional gene *narG*, and for screening clones in libraries of *narG* and *nosZ* genes (**Paper II**).

T-RFLP

Like RFLP, terminal restriction fragment length polymorphism (T-RFLP) is a method based on sequence-specific restriction digestion. However, when amplifying the extracted DNA, one or both of the primers are fluorescently labelled. The PCR products are digested with restriction enzymes and it is common to use more than one enzyme for each sample to obtain a more complex pattern. Due to the sequence polymorphism, the location of the restriction sites varies and the terminal fragments have different lengths. The terminal fragments are separated either on a gel or capillary sequencer based on length. The resulting electropherogram gives a profile of the microbial community composition and the terminal fragments, and their relative abundance is used in the further analysis (**Papers III-VI**). T-RFLP is widely used for analyzing the community structure of microorganisms in the environment (Clement *et al.*, 1998; Liu *et al.*, 1997). Figure 4 shows the resulting profiles of the terminal fragments of *nosZ* genes in a soil sample after digestion with three different restriction enzymes. T-RFLP analysis has also been commonly used by other researchers for studying the denitrifier community structure in various environments (Avrahami *et al.*, 2002; Braker *et al.*, 2001; Bremer *et al.*, 2007; Scala & Kerkhof, 2000; Wolsing & Prieme, 2004).

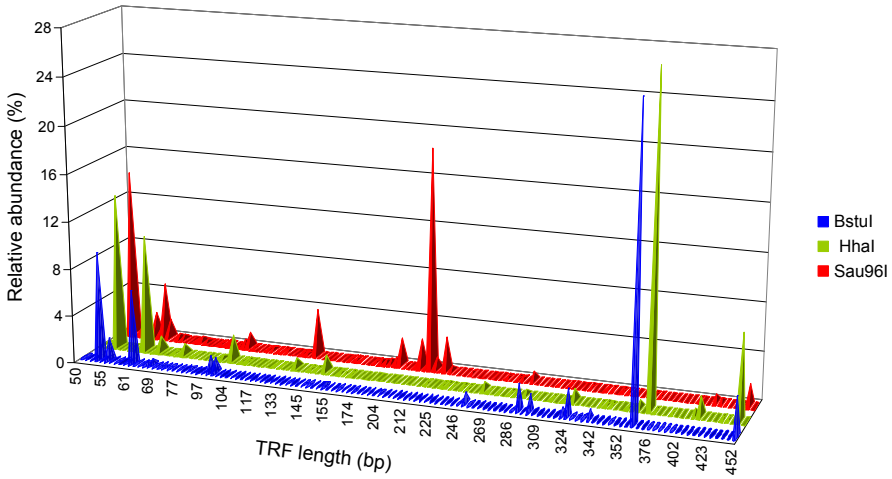

Figure 4. T-RFLP profiles for the *nosZ* gene with three different restriction enzymes; *Bstu*I, *Hha*I and *Sau96*I presented as the length of the terminal fragments and their relative abundance (**Paper VI**).

RISA

Ribosomal intergenic spacer analysis (RISA) is based on the size polymorphism of the 16S-23S rRNA intergenic region between strains (Borneman & Triplett, 1997; Fisher & Triplett, 1999; Jensen *et al.*, 1993). After amplification of this region, the PCR products are separated on a polyacrylamide gel according to size to get a fingerprint of the environmental community (Martin-Laurent *et al.*, 2001; Ranjard *et al.*, 2001; Wertz *et al.*, 2006). High resolution gels can be obtained using an automated sequencing machine. In this thesis, RISA was used to fingerprint the dominant genotypes of the bacterial community composition (**Paper II**).

Clone library analysis

PCR products from environmental samples can be cloned, and the resulting libraries are screening either by RFLP, sequencing or both. RFLP clones can be sorted according to the restriction patterns and can thereby be grouped into clone families (**Paper II**). In addition, excised bands from a DGGE can be cloned and sequenced (**Paper I**). However, cloning and sequencing is very time-consuming, especially when comparing large sets of samples, but it is the method that provides the most information on community composition. In soil, hundreds of clones usually have to be screened to get some coverage of the diversity. The amplified part of the gene of interest is inserted into a plasmid, which is transinfected into a host cell (*E. coli*). Since each cell only harbours one plasmid, the amplicons are separated by plating the modified cells and selecting colonies that have arisen from single cells. Before sequencing, the libraries are often screened using RFLP. Based on the polymorphism of the inserted fragment, different clones generate different restriction patterns and can thereby be divided into clone families. Clone libraries have been used in many studies of denitrifying bacteria in various environments (Hallin *et al.*, 2006; Henry *et al.*, 2008; Prieme *et al.*, 2002; Rösch *et al.*, 2002; Stres *et al.*, 2004; Throbäck *et al.*, 2007). In **Paper II**, small libraries with 48 clones were constructed for *narG* and *nosZ* to verify the fingerprints for all six different treatments. These libraries were screened using RFLP and divided into different clone families (Fig. 5). In the pie charts the dominant, shared or unique sequences in the samples appear distinctly. For each of the families, a few clones were sequenced to identify the genotype/confirm identity of the PCR products (**Paper II**).

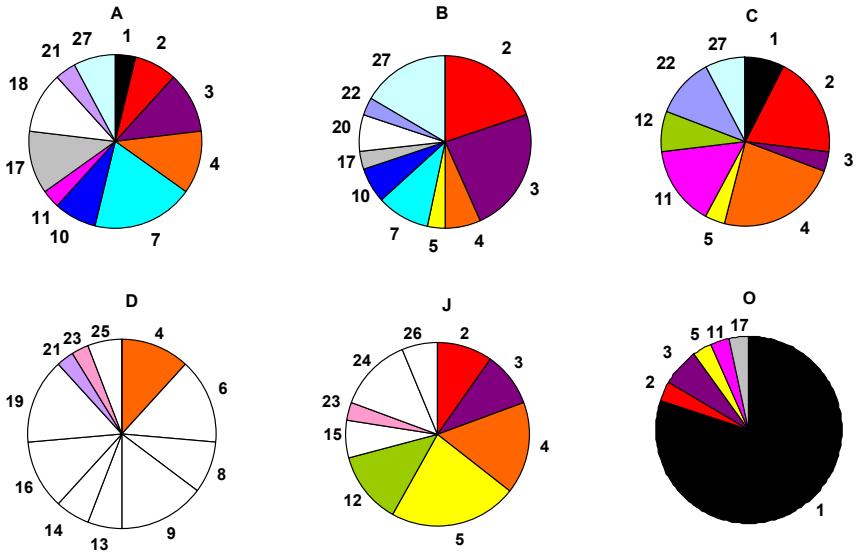

Figure 5. Distribution of *nosZ* clones within RFLP families for each treatment from the Ultuna long-term soil organic matter experiment. a) unfertilized (bare fallow) b) unfertilized c) calcium nitrate ($Ca(NO_3)_2$) d) ammonium sulfate ($(NH_4)_2SO_4$) j) solid cattle manure and o) sewage sludge. The numbers for each slice refer to the RFLP family. White sections indicate unique RFLP families for the treatment. Unpublished data from **Paper II**.

Which method to choose?

The specific method to choose for assessing a certain community composition is not an easy decision because all methods have advantages and drawbacks. For the first studies in this thesis, DGGE (**Papers I-III**) was adapted for analyzing the denitrifying bacterial community structure, but for the last two studies (**Papers V** and **VI**), T-RFLP was used as an alternative method capable of higher throughput. Besides, when analyzing many samples, DGGE has the disadvantage that there is a considerable gel-to-gel variation in separation characteristics. To evaluate these two methods applied to denitrifier community, we analyzed with either T-RFLP or DGGE the polymorphisms of *nosZ* PCR products amplified from samples collected in two different soils subjected to different agronomic treatments (**Paper IV**; Fig. 6). Fingerprints were compared using both presence/absence and relative abundance matrices. The results from this study revealed that both DGGE and T-RFLP could distinguish between the two different soils, but that DGGE had a slightly higher resolution and could separate individual treatments.

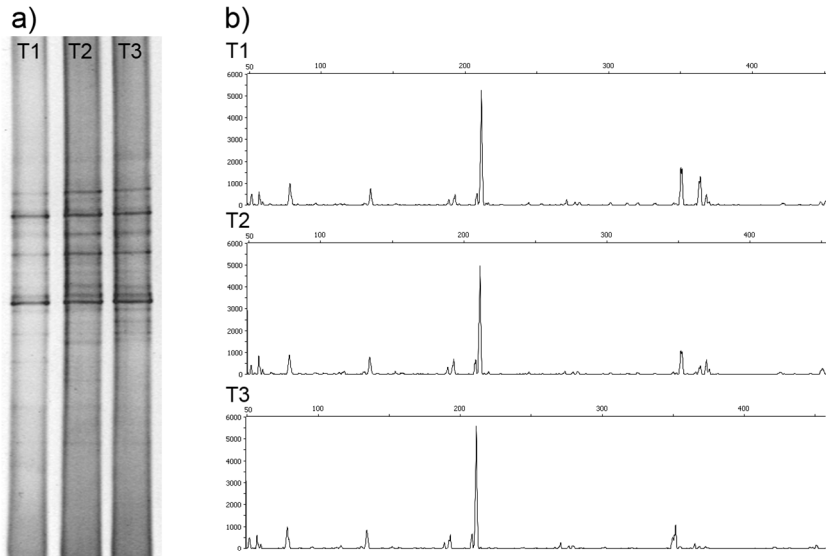

Figure 6. Examples of the *nosZ* gene fingerprints for one triplicate treatment of the Lanna soil obtained by a) DGGE banding patterns and b) T-RFLP electropherograms based on *Sau*96I (**Paper IV**).

In this thesis, we applied two different methods for studying the total bacterial community structure. In **Paper II** we used RISA, while in **Paper III** we used T-RFLP. Although both methods could discriminate between the samples, RISA had a higher resolution. Hartmann *et al.* (2005) compared T-RFLP and RISA by analyzing two different heavy metal contaminated soils, and concluded that both methods gave highly consistent and correlated results.

Quantitative PCR methods for assessing community size

To quantitatively determine the abundance of gene copy numbers in a sample, PCR-based methods have been developed. A genome of a denitrifying bacterium contains only one gene copy, so the gene copy number can be correlated with the number of organisms, with exception of *nar*G (Philippot, 2002). However, this is not the case for 16S rRNA, where the number of the gene copies in one cell can vary between 1 and 15 (Rainey *et al.*, 1996).

There are two PCR different methods available, competitive PCR (cPCR) (HallierSoulier *et al.*, 1996) and real-time PCR (Lee *et al.*, 1993).

Quantification using cPCR is based on the use of an internal DNA standard. The standard is amplified in competition with the target DNA sequence. The relative amount of the two samples is then compared on an agarose gel to estimate the copy number in the sample. Real-time PCR detects the products formed in real time. When double-stranded DNA is formed, a fluorescent signal is emitted and detected after each amplification cycle. The signal can then be related to the standard curves that are included, allowing calculations of the target DNA amount in the sample. The fluorescent signal used in the assay can be from a fluorescent dye, such as SYBR Green, which binds to the DNA during each cycle. Another approach is to use Taq-man probes that bind to the target sequence. The probe is labelled with a reporter dye and a quencher. When the probe is intact, the fluorescence in the reporter dye is inhibited by the quencher. During amplification, the reporter dye is released, and the fluorescence increased.

Quantitative PCR is a useful approach for quantifying microbial communities in the environment and real-time PCR based on SYBR Green is the method most frequently used today. Competitive PCR (Michotey *et al.*, 2000; Qiu *et al.*, 2004) and real-time PCR (Henry *et al.*, 2004; Henry *et al.*, 2006; Kandeler *et al.*, 2006; Throbäck *et al.*, 2007) have been used for quantification of denitrifying bacteria. In **Paper V**, real-time PCR was used to quantify the amount of 16S rRNA and *nosZ* genes, reflecting the total and the denitrifying bacterial communities, respectively. In **Paper VI**, real-time PCR was used to study the spatial distribution of the abundance of the *nirS*, *nirK* and *nosZ* genes in an agricultural soil.

Measuring microbial activity

Community fingerprints provide information about the most abundant genotypes in the total gene pool, independent of whether they are expressed or not. In order to understand the function of the community, it is necessary to measure the microbial activity of the expressed genes. Soil microbiologists have developed many methods to assess both general and specific microbial activities using non-molecular approaches. For example, microbial activity can be measured *in situ* e.g. by measuring greenhouse gas emissions from a field using a chamber technique, or *ex situ* e.g. by measuring specific enzyme activities in soil samples. Measuring microbial activities using potential activities is an *ex situ* method in which soil samples are analyzed in the laboratory under favourable conditions, meaning that the results show the maximal potential of an organism group to perform a certain activity.

In this thesis potential activities were measured in soil incubated in the laboratory under optimal conditions.

Respiration

Basal respiration, or background microbial respiration, is assumed to be a measure of the general soil microbial activity (Anderson, 1982). Carbon dioxide is produced when heterotrophic microorganisms mineralize organic matter. However, CO_2 is produced not only by the mineralization of organic matter, but also by root respiration and soil animals. Hence when measuring basal respiration in soil, it is important to exclude roots from the soil prior to the experimental incubation. In **Papers II-III** and **V**, basal respiration was measured under controlled, plant free conditions. The CO_2 produced was absorbed in an alkali solution and the resulting change in conductivity was measured using an automated respirometer (Nordgren, 1988). Samples were taken every 30 minutes during one week to calculate the respiration rate (Stenberg *et al.*, 1998).

Substrate induced respiration (SIR) is another strategy for measuring potential respiration that is thought to correlate to the microbial biomass (Anderson & Domsch, 1978). After measuring the basal respiration, an easily available substrate, e.g. glucose, is mixed into the soil samples. The CO_2 produced after addition of the substrate is calculated using non-linear regression of the accumulated data. From the curve the growing (*r*) and non-growing (*K*) fraction of the microflora can be estimated according to Stenström *et al.* (2001). There are also other methods available for estimating the microbial biomass, e.g. direct counting of bacteria under the microscope, chloroform fumigation incubation (Jenkinson & Powlson, 1976) and chloroform fumigation extraction (Brookes *et al.*, 1985). Beck *et al.* (1997) made a comparison of these methods using 20 different soils and found that all three methods produced similar results.

The ratio of basal respiration to SIR is termed the metabolic quotient (qCO_2) (Anderson & Domsch, 1985). This ratio shows how efficiently the heterotrophic microorganisms can transform organic carbon into microbial biomass. The ratio can be used as a stress indicator because different stress factors such as heavy metal pollution and nutrient deficiency increase qCO_2 due to a decrease in microbial biomass and an increase in the respiration rate (Jones & Ananyeva, 2001; Kandeler & Eder, 1993; Renella *et al.*, 2005).

Potential denitrification and ammonia oxidation

To assess the activity of the denitrifying microorganisms, potential denitrification activity can be measured (**Papers II, V** and **VI**). The method was first described by Smith and Tiedje (1979) and the method used in this thesis was further developed by Pell *et al.* (1996). Soil samples are incubated under optimal conditions for denitrification, i.e. anaerobic conditions and non-limiting concentrations of electron donors and acceptors. The last step of the denitrification pathway, where nitrous oxide is reduced to dinitrogen gas, is blocked by acetylene addition, which causes an accumulation of nitrous oxide. The nitrous oxide can be analyzed using a gas chromatograph and the potential denitrification activity can be calculated. The method can be used to estimate of the amount of active or readily activated denitrification enzymes (Smith & Tiedje, 1979).

In **Paper III**, the potential ammonia oxidation was assessed using a short incubation method with chlorate to block the nitrite oxidation step (Belser & Mays, 1980). This leads to an accumulation of nitrite, which is easily analyzed using a spectrophotometer. The soil is incubated in aerated slurries buffered with excess ammonium.

Statistical methods in community ecology

When using molecular methods to study microbial community composition in soil, there is often a need for rigorous statistical methods to compare communities in various samples. Choosing an adequate statistical tool to analyze, evaluate and later present the data set is often an overlooked challenge, since there are many different statistical methods to choose from. The methods used throughout this thesis are described below.

Ordination methods

Multivariate statistics are well described in the literature for analyzing community diversity patterns, and the use of multivariate analyses is increasing in microbial ecology. Cluster analysis is a good example of a method where relationships between objects are assumed to be discontinuous and where defined categories or groups of objects are assumed. In contrast, ordinations such as principal component analysis (PCA), correspondence analysis (CA) or non-metric multidimensional scaling (NMS), are more useful when the variation between objects is assumed to be continuous (Ramette, 2007).

In **Papers V** and **VI**, non-metric multidimensional scaling (NMS or NMDS) was used to explore similarities and dissimilarities in community

composition between samples. Similar samples cluster close to each other and dissimilar samples far from each other. By using a second matrix with other parameters than those describing the community composition, NMS can also be used to search for the environmental factors or other soil parameters that correlate to the bacterial community structure. The parameters in the second matrix are displayed as vectors fitted with ordination scores and show the direction and strength of the correlation. NMS is very useful when the data are not normally distributed or are in other ways in questionable scales (McCune & Grace, 2002).

Cluster analysis

In **Papers II-IV**, cluster analysis was used to present data from different molecular fingerprinting methods. Cluster analysis is a pair-wise comparison of samples, and different distance measures can be used in combination with the analysis. The unweighted pair group method with arithmetic mean (UPGMA) was used to compare the community patterns for DGGE and T-RFLP. There are different ways to construct the pair-wise similarity by using the different distance measures available (McCune & Grace, 2002). In this thesis, three different distance measures were applied. In **Papers II** and **III**, the gel-based fingerprint matrices, based on binary data of presence and absences of the bands, were compared using dice indices. A dice coefficient is a good option when analyzing binary data, but the coefficient does not take into account species abundance. When analyzing abundance data, a better option is instead to use the Bray-Curtis distance measure. In **Paper IV** we used both Bray-Curtis and Euclidean distance measures for constructing the cluster analysis, and the Bray-Curtis distance measure proved more efficient in discriminating between similar T-RFLP patterns. For the DGGE patterns, both distances measured resulted in the same clustering. The reason for this might be that the Euclidean distance measure is less appropriate for analyzing data sets with many absent fields (Legendre & Legendre, 1998), which is usually the case for T-RFLP profiles. When using either binary or relative data, similar results were obtained for the DGGE according to **Paper IV**. However, for T-RFLP**,** the results were different depending on whether binary or relative data were used. When relative abundance is considered, the amount of noise versus the signal in the data increases and this obscures the results of the analysis. Similarly, Culman *et al.* (2008) recently suggested using binary data from T-RFLP patterns to obtain the most robust analysis.

Geostatistical modelling

Geostatistical modeling (Burrough & McDonnell, 1998; Isaaks & Srivastava, 1989) was initially used in prospecting natural resources and was first applied in the mining industry for mineral exploration (Matheron, 1963). It is also a good tool for describing the spatial distribution of soil properties (White & Zasoski, 1999) and has recently proven useful in precision agriculture (Cassel *et al.*, 2000; Frogbrook *et al.*, 2002). In ecology, geostatistical modelling has been used for mapping spatial patterns of e.g. plants, but is less used for describing the spatial distribution of microbial communities.

Kriging is a geostatistical method where the spatial variation is considered (Krige, 1951). The method interpolates values of the variable in between sampling points and autocorrelation in the measured samples is taken into account. The best model for each variable is sought and the resulting variogram describes the autocorrelation of the kriging and shows the dependence of the distance. A variogram is thus unique for each variable. In **Paper VI**, kriging was used to analyze the spatial variation in different chemical and physical soil parameters and the community structure, activity and abundance of the denitrifying bacteria.

Denitrifying bacterial communities in arable land

Despite the crucial role denitrifying bacteria play in agricultural systems, causing losses of valuable nitrogen as well as contributing to global warming by emitting the greenhouse gas N_2O, we still have limited knowledge about the community ecology of these bacteria (Philippot *et al.*, 2007). A mixture of biotic and abiotic factors affects community composition, abundance and the activity, and better knowledge about the mechanisms shaping these communities and the primary controlling factors is critical for our understanding of the relationships between the ecology of denitrifying bacteria, nitrogen losses and the impact of agriculture on climate change. Hence, this could assist in suitable management of the agro-ecosystems.

The denitrifiers and the ammonia oxidizers have both been suggested as models in microbial community ecology (Kowalchuk & Stephen, 2001; Philippot & Hallin, 2005), since information on functional communities could be a way to link structure with function and may predict specific ecosystem responses. The important role of the denitrifying and ammonia-oxidizing communities in nitrogen cycling and the well-established techniques for studying abundance, composition and activity of these communities provide a good model system for assessing the relationship between spatial patterns of the functional communities, process rates, soil properties and ecosystem functions, e.g. N_2O emissions or nitrate leaching. There is also an emerging interest in revealing and understanding spatial patterns of microbial traits to gain knowledge about bacterial responses to environmental change (Green *et al.*, 2008) and both denitrification and ammonia oxidation are candidate functions for such analyses.

Spatial distribution

The soil system is characterized by spatial and temporal variation in chemical, physical and biological properties, which also contribute to the great heterogeneity and complexity of microbial habitats available in the soil (Torsvik *et al.*, 1996). The soil microorganisms are not randomly distributed in a field, but demonstrate spatial patterns at different scales (Horner-Devine *et al.*, 2004). Different scales of distribution of the organisms have been studied. Grundmann and Debouzie (2000) studied the distribution of *Nitrobacter* at millimetre scale and they suggest that soil aggregates and fine roots contribute to the distribution patterns. Other studies establish spatial dependence on the meter scale (Franklin & Mills, 2003; Ritz *et al.*, 2004). Several studies have focused more on the spatial distribution of microbial activity (Parkin, 1993), and less on the structure and abundance. For spatial patterns of microorganisms, the focus has been on specific species or total bacterial communities and not so much on functional communities (Martiny *et al.*, 2006). In **Paper VI**, the spatial distribution of the community structure, abundance and activity of denitrifying bacteria was explored at the farm scale using the denitrification genes *nirS*, *nirK* and *nosZ* as molecular markers. Fifty soil samples from a 44 ha experimental farm, Logården, were analyzed. The farm is divided into an organic and an integrated crop production system. The spatial patterns of the community composition, size and activity of the genes were mapped by geostatistical modelling. A range of soil physical and chemical properties were analyzed and correlation structures in the data were explored using non-metric multidimensional scaling (NMS).

The denitrifying community parameters of structure, size and activity, were not randomly distributed, but exhibited spatial autocorrelations, i.e. locations close to each other were more similar than locations further apart at the field scale. In spite of the relatively few sampling points over a large area, indicative spatial correlation structures were observed. The results show that the spatial distributions of denitrifying genotypes are related to soil habitat in ways that suggest specialization to different resources. When the distribution of the denitrifiers was analyzed in relation to the different soil properties, the results revealed that soil water-holding capacity and the content of the nutrients P and K were the most important factors influencing the distribution of denitrifiers. The kriged maps showed that all three denitrification genes had different spatial distributions throughout the field, and that the *nirK* community structure was different in the different cropping regimes. The abundance of the denitrifying bacteria revealed a

variable distribution for *nirS*, while both *nirK* and *nosZ* suggested a lower abundance of denitrifiers in the organic system compared with the integrated farming system. To summarize, the denitrifying communities at the different locations were mainly determined by soil structure and nutrient resources, of which some are controlled by farming history. These findings constitute a first step in identifying the resource-based niches for denitrifiers at scales relevant for land management and agricultural practices.

Effects of fertilization

Soil management practices and choice of crop production systems have been shown to affect biological, physical and chemical soil properties. This in turn affects the activity, abundance and structure of microbial communities (Drinkwater *et al.*, 1998; Lockeretz *et al.*, 1981; McCaig *et al.*, 1999; Mäder *et al.*, 2002; Reganold *et al.*, 1993).

Figure 7. The Ultuna long-term soil organic matter experiment, situated in Uppsala. The plots are separated by wooden frames, and two of the field replicates are shown here.

Understanding the effects of long-term management practices is particularly important for developing sustainable agricultural practices. Field studies of long-term experiments are valuable for this purpose. The Ultuna long-term soil organic matter experiment (Fig. 7; Table 1) in Uppsala is an experimental site where different fertilizers and organic amendments have been added since 1956 (Kirchmann *et al.*, 1994). The set-up is a block design with four independent replicate blocks, where each block comprises 15 treatments randomized in plots of 2 m × 2 m separated by wooden

frames down to 50 cm soil depth. The Ultuna experiment is an excellent model system for studying how long-term application of different fertilizers can affect the community structure, activity and abundance of different groups of microorganisms (**Papers II, III** and **V**). Twelve different treatments, using three of the four field replicates, were analyzed in this thesis and pH values and different activity measurements are summarized in Table 1.

Table 1. *The treatments studied in the Ultuna long-term soil organic matter experiment with pH, respiration (μg CO_2-C g^{-1} dw h^{-1}), substrate induced respiration (SIR; μg CO_2-C g^{-1} dw h^{-1}) and potential denitrification (ng N_2O-N g^{-1} dw h^{-1}) rates. Mean values of the triplicate plots with standard deviations (±SD) (**Papers II, III** and **V**).*

Fertilizer regime	pH ($CaCl_2$)	Respiration	SIR	Denitrification activity
Unfertilized (no crop)	5.47 ± 0.16	0.24 ± 0.04	1.53 ± 0.19	1.84 ± 0.12
Unfertilized	5.63 ± 0.05	0.39 ± 0.05	2.51 ± 0.31	2.98 ± 0.40
Calcium nitrate $Ca(NO_3)_2$	6.26 ± 0.04	0.47 ± 0.12	4.89 ± 0.74	4.37 ± 0.14
Ammonium sulphate $(NH_4)SO_4$	3.97 ± 0.14	0.58 ± 0.07	2.28 ± 0.31	3.34 ± 0.57
Solid cattle manure	6.02 ± 0.09	0.73 ± 0.05	7.74 ± 0.54	8.21 ± 1.07
Sewage sludge	4.68 ± 0.03	0.89 ± 0.11	4.21 ± 0.33	6.18 ± 2.16
Straw	5.70 ± 0.10	0.51 ± 0.01	4.61 ± 1.16	5.90 ± 0.51
Straw and $Ca(NO_3)_2$	6.03 ± 0.06	0.56 ± 0.05	7.55 ± 0.98	7.95 ± 1.92
Sawdust	5.73 ± 0.12	0.58 ± 0.06	5.14 ± 0.84	5.50 ± 0.65
Sawdust and $Ca(NO_3)_2$	6.20 ± 0.10	0.71 ± 0.06	8.76 ± 0.77	8.17 ± 1.22
Peat	4.83 ± 0.12	0.72 ± 0.16	4.81 ± 1.17	4.29 ± 0.08
Peat and $Ca(NO_3)_2$	5.47 ± 0.15	0.76 ± 0.15	7.19 ± 0.99	7.57 ± 0.71

Mineral fertilization

Nitrogen fertilization to arable soil stimulates denitrification activity (Bremner, 1997; Mulvaney *et al.*, 1997) and a substantial amount of the added nitrogen is emitted to the atmosphere. Increased input of fertilizer increases N_2O emissions from the soil (Skiba & Smith, 2000). Nitrogen fertilization also increases the activity of the ammonia oxidizing bacteria (Mendum *et al.*, 1999; Phillips *et al.*, 2000). **Papers II** and **III** investigated the effect of long-term fertilization with calcium nitrate and ammonium sulphate on the composition of denitrifying, nitrate reducing, ammonia oxidizing and total bacteria in terms of activity and community composition. The potential denitrification, ammonia oxidation and substrate induced respiration, all displayed a lower activity in the plots amended with ammonium sulphate (Table 1; Figs. 1 and 2 in **Paper II**; Figs. 1 and 3 in **Paper III**).

The effect of mineral fertilization on community structure of the denitrifying bacteria showed small differences after 7 years of ammonium nitrate addition (Dambreville *et al.*, 2006). In another field trial, fertilization with nitrogen gave rise to small differences in the community structure that could be explained by the fertilizer (Wolsing & Prieme, 2004). In an incubation study with different ammonia concentrations, Avrahami *et al.* (2002) did not observe any changes in the community structure of the ammonia oxidizing bacteria. However, the community structure of *nirK* denitrifiers was altered by increasing concentrations of ammonium. Other studies of ammonia oxidizing bacteria have shown that mineral fertilization does not affect the bacterial community composition (Mendum *et al.*, 1999; Phillips *et al.*, 2000). The two mineral fertilizers generated the most different community structures of all the bacterial groups studied (Fig. 3 in **Paper II**; Figs. 4 and 6 in **Paper III**). The numbers of bands or peaks were lower in the plots with ammonium sulphate addition than in the plots amended with calcium nitrate. Nevertheless, the diversity of AOB in the ammonium sulphate treatment was extremely low, with only one band in the DGGE. This was probably an effect of the lower pH in the plots with ammonium sulphate compared with those with calcium nitrate addition (Table 1; Table 1 in **Papers II** and **III**). Soil pH is a well-known factor controlling growth and activity of AOB (Prosser & Embley, 2002).

Organic fertilization

Organic fertilization leads to an increase in soil organic matter, which has soil benefits such as improvement of the soil structure and increased water-holding capacity (Debosz *et al.*, 2002; Marinari *et al.*, 2000). Organic fertilizers, such as swine and cow manure, but also sewage sludge, are known to promote microbial activity in general (e.g. Ritz *et al.*, 1997; Simek *et al.*, 1999). Several studies have shown that the bacterial community structure is also affected (Larkin *et al.*, 2006; Peacock *et al.*, 2001; Stark *et al.*, 2008). Organic fertilizers also increases denitrification rates (Rochette *et al.*, 2000), but less is known about the impact on the community of these bacteria. However, some studies have shown that organic fertilization affects the community structure of denitrifying bacteria (Dambreville *et al.*, 2006; Wolsing & Prieme, 2004) and of AOB (Larkin *et al.*, 2006). In an incubation study by Nyberg *et al.* (2006), different organic fertilizers did not affect the composition of the ammonia oxidizing bacteria.

Papers II and **III** compared community structure and activity of the nitrogen cycling and total bacteria in response to addition of two different organic fertilizers, solid cattle manure and sewage sludge. The community

structure was affected by the organic fertilizer type (Fig. 3 in **Paper II**; Figs. 4 and 6 in **Paper III**). While the potential denitrification activity was not significantly different between the two organically fertilized samples, the potential ammonia oxidization was much lower in the plots that had received sewage sludge. The latter effect was also observed for the substrate induced respiration (Table 1; Figs. 1 and 2 in **Paper II**; Figs. 1 and 3 in **Paper III**). Sewage sludge can contain elevated concentrations of heavy metals. Heavy metals commonly found in sewage sludge, such as Cd, Cu, Zn and Ag, have been shown to exert strong effects on ammonia oxidation (e.g. Stephen *et al.*, 1999) and microbial activity in general (e.g. Fliessbach *et al.*, 1994; Kandeler *et al.*, 1996), but also on denitrification (Holtan-Hartwig *et al.*, 2002; Throbäck *et al.*, 2007).

Paper V examined the effects of different carbon amendments affecting soil organic carbon content on both denitrifying and total bacteria using soil samples from the Ultuna long-term soil organic matter experiment; three different organic amendments (peat, sawdust and straw), with or without nitrogen addition, and an unfertilized control. After almost 50 years of the same applications, the C:N ratio had increased in all the plots with organic amendment. The highest C:N ratio was observed with peat (~18) followed by sawdust (~13) and straw (~11). The lowest C:N ratio was recorded in the unfertilized control plots (~10). The respiration and denitrification activity were significantly higher in the plots with organic matter amendments, and when combined with nitrogen fertilization the potential activity was even higher. In contrast to the microbial activity, the type of organic amendment had a strong impact on the community composition. Thus, the composition of denitrifying and total microbial communities in soil varied depending on the treatment, with the unfertilized and peat-amended plots being the most different (Fig. 3 in **Paper V**). Interestingly, the community structure of the denitrifying bacteria was less affected by the different amendments than that of the total bacteria. It was concluded that the main drivers for shaping the bacterial and denitrifying communities were C:N ratio and organic carbon content, while for the denitrifiers pH was also an important factor. Similarly, variations in C:N ratio have been shown to discriminate between bacterial community composition in soil treated with straw or coniferous compost (Lejon *et al.*, 2007). For the total bacterial community in our study there was also a separation of samples from plots with or without nitrogen fertilization. Since nitrogen fertilization increase plant production, easily available plant-derived carbon is likely to affect the bacterial community in these plots. Distinct carbon preferences between Gram-negative and Gram-positive bacteria in soil have been shown, with the Gram-positives using more soil

organic matter as carbon source and Gram-negative bacteria using plant-derived carbon source (Kramer & Gleixner, 2008).

Mineral versus organic fertilization

Organic fertilizers promote denitrification rates even more than application of inorganic fertilizers. Dambreville *et al.* (2006) showed that 7 years of application of composted pig manure or ammonium nitrate affected both the structure and the activity of *narG* and *nosZ*. Wolsing and Prieme (2004) studied the variation in denitrifying bacteria receiving mineral fertilizer and cattle manure and observed a difference in the community structure between the samples treated with mineral fertilizer and cattle manure. Peacock *et al.* (2001) showed application of diary cow manure and ammonium nitrate over a 5-year period resulted in an increase in carbon and nitrogen and in microbial biomass, and brought about a change in the total microbial community.

In **Papers II** and **III**, changes in community structure were correlated to the changes in the pH, with the most deviating community structures arising in the plots with the lowest pH, resulting from amendment with ammonium sulphate and sewage sludge. These results are in agreement with a more recent study, conducted on the same field site, where the community structure of arbuscular mycorrhizal fungi and total bacteria as an effect of mineral versus different organic fertilizers was compared. It was demonstrated that the fertilizer type significantly affected the community structure and that the changes in the microbial community composition were mainly correlated with pH changes (Toljander *et al.,* 2008). Our two studies concerning the long-term effect of different fertilizer regimes on functional groups (**Papers II** and **III**) were evaluated by combining DGGE fingerprints of the denitrifying bacteria and the ammonia oxidizing bacteria, and the RFLP pattern for the nitrate reducing bacteria, and were analyzed by a multivariate approach. The total communities were compared with an ordination based on RISA pattern and T-RFLP pattern of the total bacterial community (**Paper II** and **III**). The data were jointly analysed using non-metric multidimensional scaling (NMS). The fingerprints were analyzed for correlation to different environmental variables, activities and crop yield (Fig. 8) in order to identify factors that were most important for separating the communities in the different treatments.

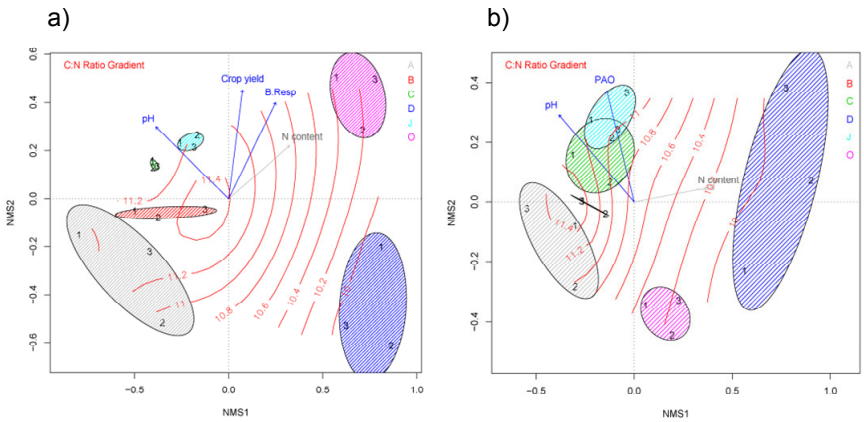

Figure 8. Joint NMS analysis of the six treatments in **Papers II** and **III**. Unfertilized (bare fallow; grey), unfertilized (red), calcium nitrate ($Ca(NO_3)_2$; green), ammonium sulphate ($(NH_4)_2SO_4$; blue), solid cattle manure (turquoise) and sewage sludge (pink). a) The denitrifying bacteria, the nitrate reducing bacteria and the ammonia oxidizing bacteria combined (stress value 8.56). b) Total bacterial communities analyzed with RISA and T-RFLP (stress value 11.71).

The pH and C:N ratio correlated well with the differences in community structure between soils treated with ammonium sulphate and sewage sludge from other treatments for both functional and total communities. Nitrogen content in the crop was also highly correlated with separation of these low pH samples in the functional communities, whereas the total crop yield coincided with community patterns in the manure and sewage sludge treatment. As reported in **Papers II** and **III**, field plots fertilized with cattle manure or calcium nitrate and the unfertilized plots with and without crop demonstrated minimal variation in community structure, whereas the potential activities differed significantly among these treatments. Hence, no clear relationship between the soil microbial communities and the potential activities could be demonstrated.

Soil properties controlling community structure, size and function of denitrifying bacteria

The composition and diversity of soil bacteria can be influenced by a wide variety of biotic and abiotic factors (Buckley & Schmidt, 2002) and long-term fertilization changes different soil properties such as pH, soil organic carbon and nitrogen content (**Papers II, III** and **V**). This section provides a summary of the different soil properties that affected the community

structure, abundance and potential activities of denitrifying bacteria in the different studies in this thesis.

Soil type

Soil type is a very important factor in determining the community structure of bacteria in general (Girvan *et al.*, 2003). In **Paper I**, different soils and environmental samples were compared and the DGGE analysis revealed a distinction of the community structure between the samples, with soil type as the driving factor. Sessitsch *et al.* (2001) studied how soil particle size affected the community structure of 16S rRNA genes in different plots from the Ultuna long-term soil organic matter experiment. Their results showed that the community structure was affected to a greater extent by the particle size fraction than the fertilizer regime, although they observed a unique bacterial community in the soil amended with sewage sludge. In **Paper VI**, the water-holding capacity of the soil was an important soil property for the distribution of all three denitrification genes.

pH

In **Papers II** and **III**, pH seemed to be the most important factor in determining the bacterial community composition. In a study by Deiglmayr *et al.* (2004), the *narG* RFLP fingerprints revealed that the structure was primarily affected by pH and time of year. Furthermore, Fierer and Jackson (2006) suggested that microbial biogeography is controlled primarily by soil variables, and that any differences can largely be explained by pH. However, the potential denitrifiction activity in **Paper II** was not correlated with the pH. In **Paper V**, the pH was more similar between the different soil samples. Hence, pH was no longer a main factor controlling the community structure.

Carbon

In **Paper V**, other factors controlled the community composition of the denitrifying and total bacteria, such C:N ratio and organic carbon content. The structure of both the denitrifying and the total bacterial communities was correlated to differences in the potential denitrification activity and substrate induced respiration. Denitrification is generally correlated to the content of organic carbon in the soil (Burford & Bremner, 1975), and in **Paper II** a neat correlation was obtained between the potential denitrification activity and the organic carbon content. However in **Paper II**, there were no differences in the community structure of denitrifying

bacteria in the unfertilized plots and in the plots fertilized with cattle manure, where the organic carbon content had doubled amount.

Nitrogen

Avrahami *et al.* (2002) observed a community shift for *nirK* denitrifier population in an incubation trial with medium and high ammonia concentrations. They concluded that the shift in community structure for the denitrifying bacteria was probably due to the increased supply of oxidized nitrogen through nitrification. In **Paper V**, three different organic fertilizers were studied, with or without nitrogen addition. The addition of nitrogen affected the community structure of the denitrifying and total bacteria, stimulated the primary production and was also responsible for increased respiration and potential denitrification activity. However, in **Paper II**, long-term addition of nitrogen in the plots with manure and calcium nitrate did not changed the community composition of the denitrifiers compared with the control plots with no nitrogen.

Other compounds

In the study of spatial distribution of the denitrifiers, the permutation test of the vector fit (Table 1 in **Paper VI**) showed that the content of the soil nutrients phosphorus and potassium was significant for the denitrifying community structure, independent of denitrification enzyme studied. The sewage sludge added to the plots in **Paper II** contained heavy metals and other pollutants that may have contributed to the shift in the community structure of the denitrifying bacteria.

Linking structure to function

Several attempts have been made to try to link the community structure of the denitrifying bacteria to their actual activity, with contrasting results. In **Papers II** and **III**, we were unable to relate the community structure of the denitrifying and total bacteria to their potential activities. A study by Rich and Myrold (2004) examined the denitrifying bacteria from agricultural soil, riparian soil and creek sediment together with activity measurements and found no correlation between the activity and the community structure in any of the soils. However, in **Paper III** a weak correlation was found between structure and community composition for the ammonia oxidizing bacteria, while in **Paper V**, there was a correlation between the structure of the denitrifiers and the total bacterial community and their functions. Cavigelli and Robertson (2001) studied a conventionally tilled agricultural

field and a successional field that had never been tilled and found that the denitrifier community composition was different between the two sites and that the differences in structure could potentially influence *in situ* N_2O production. Later, Rich *et al.* (2003) found the community composition of denitrifiers to be strongly correlated with the process rate and vegetation type.

A possible explanation for the lack of correlation between structure and activity in **Papers II** and **III** might be that different soil properties affect community composition. In **Paper I**, soil type was the most important driver for changes in community composition. In the subsequent studies (**Papers II** and **III**), the soil factor was removed by exploring the Ultuna long-term soil organic matter experiment where the soil type was the same in the beginning of the experiment in 1956, even though different fertilizer regimes have altered soil properties such as pH, organic carbon and nitrogen. In **Papers II** and **III**, pH was the driver shaping the community structure of the nitrogen cycling and total bacterial communities. In **Paper V**, the pH was more uniform between the different soil samples. Hence pH was not longer the main factor controlling the community structure. This study showed that other factors could also influence the community structure of the denitrifying and total bacterial communities, e.g. C:N ratio and organic carbon content. Sequentially eliminating or accounting for the apparently strongest factors affecting community structure (soil type, pH) enabled us to identify other potentially important factors and to link community structure to function at a fairly detailed level. The reasons why it was difficult to detect any link between community composition and activity might be that we studied the total amount of DNA and not only the expressed part, so we did not obtain any information about the actual active population. Methodological biases might also have concealed the correlations between structure and function.

Conclusions

:: Evaluation of the primers in **Paper I** revealed that organisms containing *nirS* were not only found in marine ecosystems, but were also common in soil, in contrast to what previous studies had shown. This highlights the importance of reassessing the primers used for molecular studies as databases with relevant sequence information expand, in order to infer the ecology of the microorganisms.

:: DGGE and T-RFLP both proved to be suitable methods for analyzing denitrifying bacterial community composition in soil. However, DGGE was able to discriminate between smaller differences (**Paper IV**).

:: Long-term fertilization and organic amendments affect soil properties such as pH, organic carbon and nitrogen content. This in turns affects microbial process rates, community composition and the abundance of N-cycling communities, as well as the total bacterial community (**Papers II, III** and **V**).

:: pH is a strong driver for changing bacterial the community composition in the long-term (**Papers II** and **III**). When the pH effect is less pronounced, other properties driving the community structure include C:N ratio, organic carbon content and water-holding capacity (**Papers III** and **VI**).

:: No link was found between structure and function for the bacterial communities studied in **Papers II** and **III**. However, without the pH effect, denitrifying communities were correlated to denitrification activity and total bacteria communities with respiration activity (**Paper V**).

:: Denitrifier community structure and abundance exhibited spatial patterns at the field scale, and physical parameters seemed critical for shaping the community structure of the denitrifying bacteria (**Paper VI**).

Challenges for the future

There are several exciting directions to continue this work. First, work on the links between community structure and function is still in its infancy and more research is needed to understand these links. One important step would be to target the active denitrifying population, since the presence of a denitrification gene does not imply that the organism is active and there are several methods available for this purpose. When targeting the active population it is possible to explore if there are certain groups of bacteria active under e.g. a given condition and over different time frames. In addition, with all new sequencing methods available it is possible to screen a large number of samples to study the dominant versus rare community members and their relative contribution for ecosystem functioning. Furthermore, nitrous oxide emissions from agricultural soil exhibit highly seasonal fluctuations. It would be interesting to study how the activity of the denitrifiers shifts during the season and to correlate the active population to *in situ* measurement of nitrous oxide in the field scale.

We need to deepen our understanding of what factors determine the activity, abundance and community composition of the denitrifying bacteria to build the foundation for knowledge-based land management strategies. I look forward to follow the continuous research development within this field.

Unfortunately, the number of clones analyzed is typically small (ten to hundreds) compared to the number of individual microbes being analyzed (billions or trillions). This is like randomly sampling a bus load of people and then trying to infer the diversity of all people in the world. You would not expect to find many Lithuanians.

Curtis & Sloan, in 'Exploring microbial diversity - a vast below', 2005. Science.

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Tack till **alla kompisar** och deras familjer som inte har med mikrobiologen att göra. Fast vi kanske inte träffar varandra så ofta så betyder ni väldigt mycket för mig.
Kristina, min äldsta barndomskamrat. Det känns tryggt att ha dig som vän genom livet. Mina gymnasiekompisar **Viktoria** och **Karolina**. Raringar från Viken, gott att ha. Botaniktjejerna **Anna** och **Mia**, för god mat och vackra blommor! Två riktigt viktiga saker i livet. Fler Ultunatjejer: **Sara** och min gamla sambo **Lulu.** Kanske lyckas jag ta mig ifrån Uppsala nu och hälsa på er. **Vinbrudarna** för gott vin, fantastisk mat och trevligt sällskap.

Jag vill tacka mina föräldrar **Hanno** och **Christina** för att ni alltid ställer upp och finns till närhelst jag behöver er. Ett extra tack till min mamma som uppfostrade mig enligt livsfilosofin ”huvudsaken är att du är lycklig”. Det är rätt så skönt att växa upp med det kravet.

Min brorsa **Anders**. Vi är ganska lika du och jag och det gör det hela mycket spännande. Tack även till min svägerska **Jenny** och barnen **Frida** och **Gustav**.

Min allra käraste syster **Eva**. Du är så klok och så bra och så bäst att jag inte riktigt fattar att vi är systrar. Tack för att du gifte dig med **Thomas** i augusti och fick mig att ändra fokus för en vecka. Lycka till yngsta herr och fru Enwall!

Jonas. Min matlådeman, musiknisse och filmfixare. Jag vill leva med dig och våra barn resten av mitt liv! När **Alice** kom blev livet helt, allt föll på plats. Det var så här det skulle vara. En familj, som ändrar ens fokus och får en att inse att ha kafferep med gosedjuren och plocka höstlöv är det bästa som finns i hela världen.