Exploring Denitrifying Communities in the Environment

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Abstract

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Denitrifiers are aerobically respiring bacteria that under anoxic conditions have the ability to switch to anaerobic respiration, so that nitrate and nitrite are stepwise reduced to nitric oxide, nitrous oxide and dinitrogen. Denitrifiers are a very diverse functional group with members from almost all phylogenetic bacterial groups.

One aim of this thesis was to re-evaluate published primers targeting the functional genes, *nirS*, *nirK* and *nosZ*, encoding different enzymes in the denitrification pathway. New primer combinations for *nirS* and *nosZ* were designed, whereas the existing primers for *nirK* still seemed satisfactory. It was possible to PCR-amplify *nirS* genotypes from soil samples using the new *nirS* primer pair. In addition, denaturing gradient gel electrophoresis was introduced as a fingerprinting method for *nirK*- and *nosZ*-denitrifiers.

The methods developed in this initial project were then used to study denitrifying communities in two environments where denitrification is especially important from a human perspective. In the first, methanol and ethanol were added to a wastewater treatment plant with an activated sludge process to increase the denitrification rate. As a result of the additions of external carbon sources the denitrifying communities altered their metabolic function, and specific *nirS*- and *nirK*-denitrifiers developed.

In the second project, the *nirK*-denitrifiers were used as a model community to investigate the toxicity of the heavy metal silver to soil denitrifiers. The use of silver is increasing because of its well-known antimicrobial effects, and this may lead to increased environmental contamination. The addition of silver reduced activity and number of denitrifiers, whereas their diversity increased. Because of this demonstrated sensitivity, it is proposed that denitrifiers are suitable indicator organisms for environmental pollution.

In conclusion, this thesis shows that molecular methods show great potential for investigating diversity of denitrifiers in various environments, much of which is yet to be discovered. However, in order to fully understand the ecology of denitrifiers, methods targeting the active organisms must be developed, and more denitrifiers must be isolated and characterised.

Keywords: denitrifying bacteria, community structure, primer evaluation, denaturing gradient gel electrophoresis, agricultural soil, activated sludge

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Still round the corner there may wait,
a new road or a secret gate.

J.R.R. Tolkien

Contents

Appendix

Papers I-III

This thesis is based on the following papers, which will be referred to by their Roman numerals.

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

II. Hallin, S, Throbäck, I.N., Dicksved, J. and Pell, M. Composition and functioning of denitrifying bacteria in activated sludge processes after addition of methanol or ethanol. (manuscript)

III. Throbäck, I.N., Johansson, M., Rosenquist, M., Pell, M. and Hallin, S. Denitrifying communities in soil are affected strongly by the heavy metal silver. (submitted)

My contributions to the papers included in this thesis have been as follows:

I. Participated in planning the project and performed the major part of the laboratory work and analysis of the results regarding *nirK* and *nirS*, and major part in writing of the manuscript.

II. Performed half of the laboratory work, and participated in analysis of the results and writing of the manuscript.

III. Took part in planning the study and performed the major part of the laboratory work, analysis of the results and writing of the manuscript.

"The earth is a planet, on which macroorganisms are recent additions – highly interesting and extremely complex in ways that most microbes aren't, but in the final analysis relatively unimportant in a global context"
Mark Wheelis

Introduction

It has been suggested that the number of prokaryotic species is higher than that of all other life forms together on the planet (Curtis, Sloan & Scannell, 2002), and that one gram of soil may contain as many as one billion bacteria (Torsvik, Övreås & Thingstad, 2002). Prokaryotes are ubiquitous and hence found everywhere on earth - in soils, in the water of oceans and lakes, as well as in their sediments, on the surface of and inside plants and animals. Even extreme environments like hypothermal vents and hypersaline environments are occupied by prokaryotes. Furthermore, bacteria are key players that perform unique reactions, being responsible for nutrient cycling in various ecosystems, and the degradation of organic and inorganic compounds. However, less than 1% of them have been cultivated and characterised, which means that much genetic and metabolic diversity awaits discovery.

Microbial diversity can be described by richness, evenness, redundancy and functional diversity. Richness means the total number of populations present whereas evenness describes the distribution of individuals among the populations. Redundancy can be defined as the number of populations capable of performing a specific function and high species richness within the functional guilds can be regarded as a safety component in ecosystems. It is assumed that microbial diversity is linked with ecosystem function, and that ecosystems with functional redundancy have an increased ability to withstand perturbations caused by pollutants (Girvan *et al.*, 2005). Degens *et al.* (2001) showed that soil with lower diversity was more sensitive to stress, when two soils with different microbial diversities were exposed to three different factors of stress - decreased pH, increased salinity and heavy metal contamination. It has been proposed that diversity within certain functional guilds, e.g. symbiotic nitrogen fixers and nitrifiers, is more important for ecosystem function than total diversity (Cavigelli & Robertson, 2001).

Denitrifying bacteria are one important functional guild involved in the nitrogen cycle. They live most of their lives as heterotrophic, aerobic bacteria but have the ability to respire anaerobically using nitrogen oxides as electron acceptors, which are reduced to nitrous oxide and dinitrogen. Denitrifiers are one of the most diverse functional groups with members from almost all phylogenetic bacterial groups, and hence contain much genetic and metabolic diversity. Even though they are important from an ecological perspective, there are questions as to whether cultivated denitrifiers are representatives of the group as a whole, and if the molecular tools available to study them are adequate to further assist in our exploration of complex ecosystems.

Aim and outline of the thesis

The objective of this thesis was to evaluate and apply newly developed molecular methods to study denitrifying communities in two environments, agricultural soil and activated sludge processes, where denitrification is of special interest to mankind. Denitrifying bacteria transform bioavailable nitrogen into inert nitrogen gas, which is emitted to the atmosphere. This can both be a blessing and a threat. In agriculture this causes nitrogen losses to the dismay of farmers, whereas in wastewater treatment the aim is to maximise losses in order to reduce the nitrogen load to lakes and oceans.

Molecular methods employed to study denitrifiers in the environment often start with PCR amplification of the functional genes encoding the enzymes in the denitrification pathway. The first primers targeting the functional genes encoding the enzymes involved in denitrification were published at the end of the 90s (Braker, Fesefeldt & Witzel, 1998; Scala & Kerkhof, 1998; Hallin & Lindgren, 1999) and they were based on only a few cultivated laboratory strains. Since then the number of available sequences in the databases has increased hundredfold. Therefore we wanted to re-evaluate these published primers, and if needed, design new and better primers (**I**). New primer combinations for *nirS*, encoding the cd_1 nitrite reductase, and *nosZ*, encoding the nitrous oxide reductase, were designed, whereas the existing primers for *nirK*, encoding the copper nitrite reductase, still seemed adequate. In addition, denaturing gradient gel electrophoresis (DGGE) was successfully applied to fingerprint *nirK* and *nosZ* communities in a variety of environmental samples. Using the new primer set, it was possible to amplify *nirS*-genotypes from in soil. After this initial project, these newly developed methods were used in two parallel studies where effect of external factors on the structure and function of denitrifying communities were assessed. In the first, the effects of adding external carbon sources on denitrifying capacity and community structure were studied in an activated sludge process (**II**). Little is known about denitrifier populations in these systems, even though the efficiency and stability of the nitrogen removal process is highly influenced by their activity. We showed that the addition of ethanol and methanol selected for specific *nirS*- and *nirK*-denitrifiers respectively. However, the effect was more apparent for *nirS*. In the second project, the *nirK*-denitrifiers were used as a model community to assess the effects of the heavy metal silver on soil bacteria (**III**). The use of silver is increasing because of its well-known anti-microbial effects, and this will lead to an increased contamination of silver in natural environments. We determined the toxicity of silver for soil denitrifiers in order to evaluate the likely effects of an increased environmental pollution of silver. The activity and number of denitrifiers were negatively affected whereas diversity increased. Because of their demonstrated sensitivity it is proposed that denitrifiers are a suitable prokaryotic indicator group for environmental pollution.

Denitrification

Denitrification in the nitrogen cycle

Nitrogen is important for all living organisms. The atmosphere contains about 80% dinitrogen gas (N_2), but nitrogen is nevertheless often the limiting factor for plant growth. The reason for this is that the dinitrogen molecule is inert, containing a triple bond that may be the most stable bond that any biological system has to overcome. Biological fixation of dinitrogen is performed by bacteria that are capable of reducing dinitrogen to ammonia (NH_3), since they possess the unique enzyme nitrogenase. NH_3 can then be incorporated into organic nitrogen (Fig. 1). Nitrogen mineralization is the transformation of organic nitrogen to ammonium (NH_4^+). Virtually all microorganisms produce enzymes for mineralization both under aerobic and anaerobic conditions. The mineralised ammonium is assimilated into new cell material or released from the cell. Released ammonia can then be oxidised by nitrification into nitrate (NO_3^-). This reaction occurs in two steps – first, ammonia is oxidised by ammonia oxidising bacteria to nitrite (NO_2^-), and then NO_2^- is further oxidised by nitrite oxidising bacteria to NO_3^-. Both groups of nitrifiers are aerobic lithotrophic bacteria (i.e. they gain energy from the oxidation). Nitrate can be assimilated to organic nitrogen or transformed back to ammonia through dissimilatory nitrate reduction (DNRA). The nitrogen cycle is completed when nitrate is reduced back to dinitrogen through denitrification. In recent years, anaerobic ammonia oxidation (Annamox) was discovered (Jetten, 2001; Devol, 2003). In this process, ammonium is oxidised to dinitrogen anaerobically, using nitrite as electron acceptor.

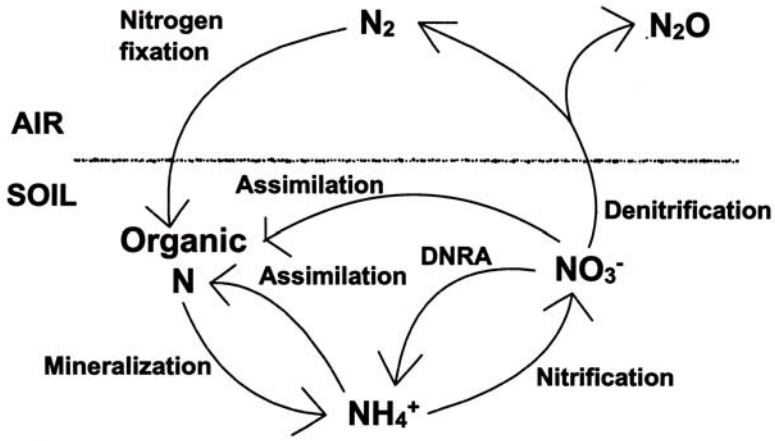

Figure 1. A simplified picture of the nitrogen cycle in soil with focus on the biological reactions.

Denitrification plays several important ecological roles. Large amounts of fertilisers are added to arable land to improve the growth of agricultural crops, but only about half of the applied nitrogen is actually assimilated into biomass. The remaining nitrogen is leached to groundwater, lakes and seas, or emitted to the atmosphere by biological processes, among which denitrification is considered to be the most significant (Einsle & Kroneck, 2004). The gaseous nitrogen oxides are potent greenhouse gases and contribute to the depletion of the stratospheric ozone. It has been estimated that 70% of the total emissions of nitrous oxide originate from microbial denitrification and nitrification in soil (Conrad, 1996). The ability of bacteria to remove nitrate and nitrite from contaminated waters is utilised by municipal and industrial wastewater treatment plants. Moreover, the denitrifying bacteria residing in deeper soil layers have been employed to remediate groundwater from excess nitrate (Weier *et al.*, 1994). Denitrifying bacteria are capable of degrading various organic pollutants, including aromatic and aliphatic hydrocarbons (Holliger & Zehnder, 1996; McNally, Mihelcic & Lueking, 1998), and therefore they may be suitable for bioremediation.

Denitrifying organisms

A variety of microorganisms found in soil, sewage, sediment, and marine and estuarine environments can denitrify. It was previously thought that the ability was restricted to Bacteria, but members of Archaea have also been found to denitrify (Cabello, Roldan & Moreno-Vivian, 2004). Fungi can also perform dissimilatory reduction of nitrate and nitrite (Takaya, 2002), but whether this should be considered as true denitrification is uncertain. Denitrifying species are found in more than 50 bacterial genera (Zumft, 1997), both gram-positive and gram-negative. Most studies have focused on gram-negative organisms, mainly the various subclasses of the *Proteobacteria*, and, hence, very little is known about the gram-positive denitrifiers. Because of the great taxonomical diversity, many denitrifiers fulfil other functions in the nitrogen cycle, such as ammonia oxidation (*Nitrosomonas* and *Nitrosospira*) and nitrogen fixation (*Rhizobium* and *Azospirillum*). Most denitrifiers are organotrophic organisms, but some are lithotrophic (*Nitrosomonas* and *Thiobacillus*) or phototrophic (*Rhodobacter*).

Enzymes in the denitrification pathway

Denitrifying bacteria are primarily aerobic heterotrophic bacteria that have the ability to switch to anaerobic respiration under anoxic conditions, reducing NO_3^- and NO_2^- stepwise to nitric oxide (NO), nitrous oxide (N_2O) and N_2. The different nitrogen oxides serve as electron acceptors instead of oxygen (O_2) in a branch of the electron transport chain in the cell. The process is initiated by a combination of external and internal signals, the dominant ones being low oxygen tension and the presence of nitrate or nitrite. Denitrification can be considered as four partly independent processes, since the enzymes respond differently to the signals (Zumft, 1997). Seven different enzymes involved in the pathway have so far been identified (Fig. 2). Almost all knowledge regarding denitrification enzymes originates from gram-negative bacteria, and *Pseudomonas stutzeri* and *Paracoccus denitrificans* have often been used as model species.

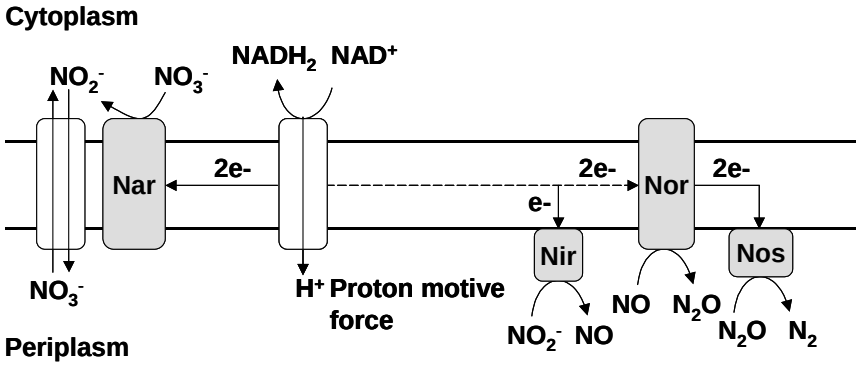

Figure 2. Organisation of the anaerobic electron transfer chain involved in denitrification in *Paracoccus denitrificans.* The enzymes involved in the pathway are nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos). Nar and Nor are membrane-bound enzymes, whereas Nir and Nos are periplasmic (redrawn from Stouthamer, 1991).

Nitrate reductase

The first step in the denitrification process, the reduction of NO_3^- to NO_2^-, is catalysed by the enzyme nitrate reductase. There are two different types of dissimilatory nitrate reductases, the membrane bound (Nar) and the periplasmic (Nap). Both Nar and Nap are molybdenum-dependent enzymes that possess a characteristic cofactor, molybdopterin. The active subunits of Nar and Nap are encoded by the genes *narG* and *napA*, respectively. Denitrifying bacteria possess either one or both enzymes (Carter *et al.*, 1995; Roussel-Delif *et al.*, 2005), and studies on *Paracoccus pantotrophus* and *P. denitrificans* has revealed that Nar is predominantly expressed under anaerobic denitrifying conditions, whilst Nap is also expressed under aerobic conditions (Richardson *et al.*, 2001). The ability to reduce NO_3^- is not restricted to denitrifiers and can also be performed by nitrate reducing bacteria, e.g. *Escherichia coli*.

Nitrite reductase

The next step, reduction of NO_2^- to NO, is specific to denitrifiers, and is considered the key step since a soluble nitrogen oxide is reduced to a gaseous molecule. This reduction is catalysed by a periplasmic nitrite reductase that can either be a copper nitrite reductase (NirK) or a cytochrome cd_1 nitrite reductase (NirS; Zumft, 1997). The two enzymes are equivalents, since the *nirK* gene from *Pseudomonas aureofaciens* has been cloned and expressed in a *P. stutzeri* mutant having a deficient *nirS* gene (Glockner, Jungst & Zumft, 1993). The first three-dimensional structure of NirK showed that it was a trimeric enzyme containing six copper atoms, whereas NirS has been shown to be a homodimer with two different prosthetic groups, heme C and heme D_1. It was previously believed that denitrifiers only have one copy of the gene encoding the nitrite reductase, but

recent studies indicate that this is not always true. Etchebere and Tiedje (2005) isolated a *Thauera* sp. that had two copies of the *nirS* gene, where one showed similarity to the *nirS* of *Thauera mechernichensis* and the other to *nirS* of *P. stutzeri*. One was constitutively expressed whereas the other was positively regulated by nitrate. However, no bacterium has been isolated with both NirS and NirK. It should be noted that there appear to be no relationship between the type of nitrite reductase and taxonomic affiliation.

Nitric oxide reductase

Nitric oxide reductase (Nor) is an integral membrane protein that catalyses the reduction of NO to N_2O. To avoid toxic concentrations, the nitric oxide reductase has a very high affinity for the substrate. Excess NO is toxic to all life, including denitrifiers, and a knockout mutation for Nor has been shown to be lethal (Zumft, 1997). The genes encoding Nir and Nor appear to be clustered in denitrifiers having NirS, and hence, the regulation of NirS and Nor are closely coupled. This coupling does not seem to occur in denitrifiers having NirK (Tosques *et al.*, 1997). There are two different types of Nor: one receiving electrons from cytochrome c or pseudoazurin (cNor) and the other from a quinol pool (qNor). The cNor is a heterodimer encoded by the *norB* and *norC* genes. Genes of the *qnorB* type have also been discovered in a variety of non-denitrifying bacteria such as *Synechocystis* sp., *Neisseria meningitides* and *Mycobacterium avium* (Busch, Friedrich & Cramm, 2002). The nitric oxide reductase appears to be very different in both gram-positive bacteria and *Archaea* (Einsle & Kroneck, 2004).

Nitrous oxide reductase

The last step, the reduction of N_2O to N_2, is catalysed by a nitrous oxide reductase (Nos). The enzyme is a periplasmic homodimeric protein with each monomer containing two characteristic copper centres, and where the catalytic subunit is encoded by the *nosZ* gene. Some denitrifiers lack this enzyme and so their end product is N_2O. This enzyme seems to be the most sensitive to various disturbances like low levels of oxygen (Firestone, Firestone & Tiedje, 1980) and high concentrations of heavy metals (Holtan-Hartwig *et al.*, 2002), which could lead to situations where N_2O is a substantial end product. An alternative to Nos has not yet been characterised in gram-positive denitrifiers.

Molecular tools to study denitrifiers in the environment

Most studies on bacterial community structure are based on targeting and PCR-amplifying specific genes linked to the bacteria of interest. The genes most commonly used are the 16S rRNA genes, which encode the 30S ribosomal subunit. The rRNAs are universally distributed and have the same function in protein synthesis in cells. They can be used as molecular chronometers since they evolve at the same rate as the organisms themselves, and they are also the basis for

modern bacterial taxonomy (Woese, 1987). The 16S rRNA genes contain both conserved and variable regions. The conserved regions can be used to study the total bacterial community, whereas closely related groups, like ammonia oxidising bacteria, can be targeted with the variable regions. However, denitrifying bacteria share a specific function and are not necessarily closely related. Moreover, some strains within a denitrifying genus may even lack this ability. This means that 16S rRNA genes cannot be used to study this functional group in the environment. Instead, the functional genes encoding the denitrification enzymes can be used as markers. Primers are available for all the four steps in the denitrification process, and all genes, except *norB*, have been targeted in environmental studies.

Primers targeting the functional genes

The genes encoding the two nitrate reductases, NarG and NapA have been used as markers to study denitrifiers and nitrate-reducers in soil (Philippot *et al.*, 2002; Mounier *et al.*, 2004; Enwall, Philippot & Hallin, 2005; Sharma *et al.*, 2006), marine (Flanagan *et al.*, 1999) and estuarine sediments (Nogales *et al.*, 2002). The most promising *narG* primers, 1960f:2650r (Philippot *et al.*, 2002), amplify a 690 bp gene fragment in a direct PCR. The other published *narG* primers (Gregory *et al.*, 2000), as well as the *napA* primers (Flanagan *et al.*, 1999), require a nested PCR, which could lead to an increased accumulation of bias. Among the primers for denitrifying genes, the *narG* primers are unique in that they amplify gene fragments from both gram-positive and gram-negative bacteria. However, they target not only denitrifiers but also nitrate reducers.

Denitrifiers can be distinguished from nitrate reducers by targeting genes encoding the nitrite reductases. The first primers targeting the gene encoding the cytochrome cd_1 nitrite reductase, *nirS*, were published in 1998 (Braker, Fesefeldt & Witzel, 1998). Their best primer combination, nirS1F and nirS6R, has successfully been used to study *nirS* in marine (Braker *et al.*, 2000; Braker *et al.*, 2001) and estuarine sediments (Nogales *et al.*, 2002), cyanobacterial bloom (Tuomainen *et al.*, 2003), marine water column (Castro-González *et al.*, 2005) and coastal aquifers (Santoro, Boehm & Francis, 2006). However, they have failed to amplify *nirS* from various soils (Avrahami, Conrad & Braker, 2002; Priemé, Braker & Tiedje, 2002; Wolsing & Prieme, 2004; Sharma *et al.*, 2005). In our primer-evaluation, we concluded that they were inadequate for soil denitrifiers, and that the primer combination, cd3aF:R3cd, was better fitted for this purpose (**paper I**). The primer cd3F was originally designed to quantify *nirS*-denitrifiers in marine sediments (Michotey, Mejean & Bonin, 2000), whereas R3cd was newly designed. It was previously believed that *nirS*-denitrifiers were not commonly found in soil and that there was an environmental separation between *nirS* and *nirK*. We showed that soil contains a substantial amount of different *nirS*-genes and that these could be amplified with primers cd3aF and R3cd (**paper I**). This primer combination has later successfully been used for assessments of the *nirS* community structure in soil (Sharma *et al.*, 2006) and activated sludge (**paper II**).

nirK1F and nirK5R are the most commonly used primers for *nirK*, the gene encoding the copper nitrite reductase (Braker, Fesefeldt & Witzel, 1998). They

have been used to study *nirK* communities in soil (Avrahami, Conrad & Braker, 2002; Priemé, Braker & Tiedje, 2002; Wolsing & Prieme, 2004; Sharma *et al.*, 2005), marine sediment (Braker *et al.*, 2000; Braker *et al.*, 2001), estuarine sediment (Nogales *et al.*, 2002) and cyanobacterial blooms (Tuomainen *et al.*, 2003). The forward primer, nirK1F, lacks an insert of three bases that can be found in some *nirK* sequences and this insert may influence the success of amplification. We found (**paper I**) that the primer pair F1aCu:R3Cu (Hallin & Lindgren, 1999) was better, since it was both more sensitive and specific and did not produce any multiple bands with environmental samples. The reverse primers, nirK5R and R3Cu, target the same region and differ in only one base pair. The community structure in soil (Sharma *et al.*, 2006, **paper I**; **III**) and activated sludge (**paper I**; **II**) has been analysed with this primer combination.

The last genes in the denitrification chain to be used as a marker were *qnorB* and *cnorB*, encoding the two nitric oxide reductases. Braker and Tiedje (2003) published primers amplifying *cnorB* and *qnorB*, and the best primer combinations were qnorB2F:7R and cnor2F:6R, respectively. These were tested on marine sediments and revealed a great diversity among *norB*. Casciotti and Ward (2005) designed primers for *cnorB*, emphasizing on nitrifiers. The primers were very similar to those previously published. For example, cnorB2F (Braker & Tiedje, 2003) and norB2 (Casciotti & Ward, 2005) targeted almost identical regions with different degrees of degeneracy. Primers targeting *norB* have not yet been applied in environmental studies.

Most published *nosZ* primers appear to be suitable for environmental studies (**paper I**). The primer pair nosZ-F:nosZ6122R successfully amplified *nosZ* from soil, activated sludge and peat (**paper I**), and has been used to study the community structure of *nosZ*-denitrifiers in soil (Enwall, Philippot & Hallin, 2005; Sharma *et al.*, 2006). The forward primer in our combination, nosZ-F, was designed by Kloos *et al*. (2001) to investigate *nosZ* in growth-promoting rhizobia. The combination nosZ-F and nosZ-R has been used in different soils (Rösch, Mergel & Bothe, 2002; Rich *et al.*, 2003; Mounier *et al.*, 2004). The most commonly used primer pair for *nosZ*, nos611F:nos1773R (Scala & Kerkhof, 1998) has successfully amplified *nosZ* from soil (Stres *et al.*, 2004; Horn, Drake & Schramm, 2006), marine sediments (Scala & Kerkhof, 1999; Scala & Kerkhof, 2000), estuarine sediment (Nogales *et al.*, 2002) and earthworm guts (Horn, Drake & Schramm, 2006). These primers were less successful in our re-evaluation: *nosZ* was only amplified from eight of the 28 pure cultures and they were not specific enough as indicated by multiple bands from activated sludge and peat samples (**paper I**).

There are strong indications that many denitrifiers found in environmental samples are very different from those growing in the laboratory. It is therefore necessary to re-evaluate primers on a regular basis. If the same primers are continuously used, there is a great risk that many functional denitrification genes will remain undiscovered. In primer-evaluations it is important to establish certain criteria that the primers must fulfil, and in **paper I** we decided on four criteria: 1) the number of strains that the gene was amplified from, 2) the number of genera that the gene was amplified from, 3) the number of environmental samples that the gene was

amplified from, and 4) the size of the amplicon. Criteria 1-3 are important for all primers used in environmental assessments whereas criterion 4 is important for further applications. One problem with many of the published primers is that they target locations in the same regions of their respective genes and that these regions are often not the most variable. The sequencing of more complete genes will be beneficial for the further design of new primers that amplify the whole gene or at least new, more variable regions. It is also important to remember that only the *narG* primers can amplify sequences from both gram-positive and gram-negative denitrifiers, and that *narG* and *napA* are not exclusively found in denitrifiers. It may not be possible to design primers for *napA*, *nirS*, *nirK* and *norB* that are suitable for both gram-positive and gram-negative denitrifiers, since the corresponding genes appear to be so different. Whether the situation is similar for *nosZ* will not be known until a nitrous oxide reductase-alternative in gram-positives has been characterised. It is, however, important to study gram-positive denitrifiers, since they represent a substantial proportion of this functional group.

Fingerprinting methods versus clone libraries

Denitrifying communities sometimes consist of several hundred different sequences, especially in soil (e.g. Stres *et al.*, 2004; **paper III**). Amplification by PCR of the functional genes from these communities only generates a single gel band containing multiple DNA fragments of equal size. Further analysis by molecular cloning and/or fingerprinting methods, like denaturing gradient gel electrophoresis (DGGE; Muyzer, de Waal & Uitterlinden, 1993; Muyzer & Smalla, 1998) and terminal- restriction fragment length polymorphism (T-RFLP; Marsh, 1999; Osborn, Moore & Timmis, 2000), is required to resolve the polymorphism of the single band (Fig. 3). All the methods described in this chapter are appropriate for assessments of denitrifying communities. The experimental aims and designs will determine which method is the best choice.

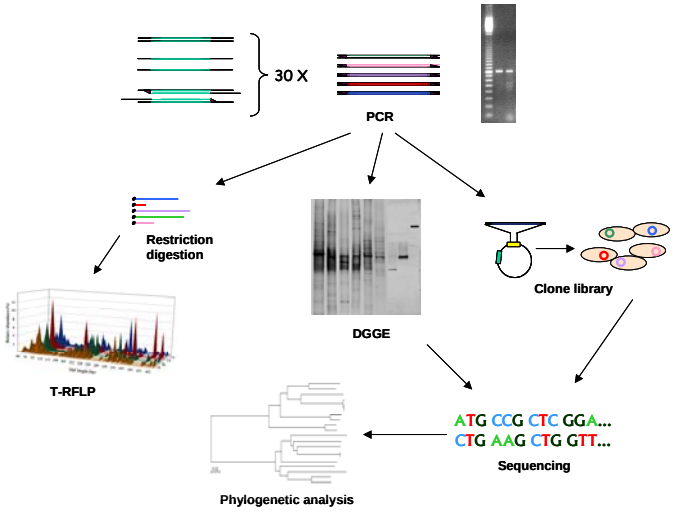

Figure 3. Schematic drawing of the molecular methods that are described in this chapter and that have been used to assess denitrifying community structure. PCR amplification generates a single band, and the polymorphism within that band can then be analysed with molecular cloning, DGGE or T-RFLP. It is only clone libraries and DGGE that can be further sequenced and thereby used for phylogenetic analyses.

Clone libraries

In molecular cloning, amplified DNA fragments representing different denitrifiers are inserted into plasmid vectors. In the cloning process, the fragment is inserted into another gene, whose disruption enables the positive screening of the clone. This gene can, for example, be a suicide gene or encode a colour-product. The plasmids also contain genes encoding antibiotic resistance and once they are transformed into their hosts (e.g. *E. coli*), the bacteria are able to grow on media containing antibiotics. The resulting collection of antibiotic-resistant *E. coli* cells, each containing a unique DNA fragment, is called a clone library. The polymorphism of the clone library can be directly sequenced (**paper III**) or further screened with a fingerprinting method to sort the library and evaluate its polymorphism (**paper II**). Clone libraries have a higher resolution than fingerprinting methods, and hence provide more information. In **paper II**, we analysed the community structure of *nirK*-denitrifiers in a wastewater treatment plant with both clone libraries and DGGE. Even though the two methods gave a similar result, it was evident that the resolution was much higher for the clone libraries. However, they are time-consuming and expensive to analyse, since they need to be fairly large in order to capture enough diversity. It is not uncommon that soil libraries consisting of more than 100 clones, fail to cover the existing diversity (Priemé, Braker & Tiedje, 2002; Stres *et al.*, 2004; **paper III**). One advantage is that clone libraries with enough coverage can be used to estimate and compare the diversity between samples by statistical methods, for example using diversity indices (e.g. Shannon & Weaver, 1949; Simpson, 1949), and rarefaction curves (http://www.uga.edu/strata/software/Software.html; **paper II**; **III;** Fig. 4).

DGGE

With DGGE, DNA fragments of equal length but with different sequences are separated in a polyacrylamide gel with increasing concentrations of the denaturants urea and formamide. DNA fragments can be divided into stretches of DNA with identical smelting properties called melting domains. These are sequence-specific and once a domain reaches its maximum denaturant concentration the base pairs begin to separate and secondary structures are formed. This causes the migration to slow down and eventually stop. A GC-clamp consisting of 30-40 guanine and cytosine bases is often added to one of the primers to avoid complete separation of the two DNA strands. DGGE is a simple and fast method that is suitable for the analysis and comparison of many samples. In contrast to T-RFLP discussed below, DGGE allows the identification of the gene fragments, since the bands on the gel can be excised and directly sequenced. However, sometimes a band may contain more than one sequence (Sekiguchi *et al.*, 2001; Kisand & Wikner, 2003; **paper I**; **II**), and problems arise when multiple sequences form similar secondary structures. These difficulties can be solved if the excised band is re-amplified and then separated on another DGGE, or if the band is cloned. There are restrictions regarding the primers used for DGGE, since a fragment ideally should not be larger than 500 bp. We faced this problem when DGGE was adapted for the analysis of *nosZ* (**paper I**). Several of the published primers were successful in the amplification of the *nosZ* gene fragment from environmental samples, but the amplicon was too large. For best results, DGGE methods must be optimised regarding the gradient concentration and running time for each gene. It can sometimes be difficult to analyse environmental samples with high diversity using DGGE, since a large number of bands is seen as a smear. In addition, only the most abundant populations are visible. Adapting the method to functional genes can be difficult, since they often contain multiple melting domains. This was probably one reason why we failed to optimise DGGE for *nirS* (**paper I**). However, Sharma *et al.* (2006) later managed to optimised DGGE for *nirS*, using the same primer pair, cd3aF:R3cd. DGGE has also been applied for *nirK* (Sharma *et al.*, 2005; **paper II**) and *nosZ* (Enwall, Philippot & Hallin, 2005). The diversity of *nirK*-denitrifiers have been assessed by DGGE using two different primer-combinations, F1aCu:R3Cu (**paper I**; **II;** Fig. 4.) and nirK1F:nirK5R (Sharma *et al.*, 2005).

T-RFLP

In T-RFLP, amplicons from a single agarose gel band are digested with restriction enzymes that recognise and digest specific 4-6 bp long sequences. Different amplicons vary in the number and positions of the restriction sites. One or both of the primers amplifying the sample are marked with a fluorescent tag, such as 5-hexachlorofluorescein. After digestion, the labelled terminals are sorted by molecular size using automated gel or capillary electrophoresis, which results in a digital electropherogram showing a number of different peaks. The number of peaks, and sometimes their relative abundance, is used to determine richness and evenness within a sample. The most important factor for successful T-RFLP is the choice of restriction enzymes. These should preferentially cut the amplicons in a

variable region and not too many times to avoid fragments that are too small for analysis. There are three advantages with T-RFLP compared to DGGE: 1) the resolution is higher, 2) the output is digital, which means that the subsequent analysis is much simpler, and 3) it is possible to analyse samples with higher diversity. However, the method is limited since it only provides a profile for each sample, and does not give information about possible identities within the sample. Databases are available for 16S rRNA genes where bacterial groups, genera or species cleaved *in silico* with various restriction enzymes are used for comparison. Although, the number of database entries for functional denitrification genes is increasing (e.g. Rosch & Bothe, 2005), the available information is still too limited. T-RFLP has been applied for environmental studies of *nirS* (Braker *et al.*, 2001; Wolsing & Prieme, 2004), *nirK* (Avrahami, Conrad & Braker, 2002; Wolsing & Prieme, 2004) and *nosZ* (Scala & Kerkhof, 2000) using the primers nirS1F:nirS6R, nirK1F:nirK5R and nosZ661:nosZ1773R, respectively.

Quantification

Estimation of the number of denitrifiers in a population has often been neglected probably due to a lack of appropriate and sensitive methods. The fingerprinting methods described in the previous section can give a rough estimate of both richness and evenness based on an analysis of the number of peaks and bands, but they cannot be used for quantification. In order to fully understand the function of denitrifying bacteria and their role in the nitrogen cycle, it is necessary to measure not only diversity, but also abundance.

Denitrifying bacteria can be quantified with cultivation-dependent (e.g. most probable number, MPN), or cultivation-independent (PCR-based) methods. The MPN technique depends on qualitative attributes of the microorganisms of interest (Woomer, 1994). For denitrifiers, this is the production of nitrous oxide from a nitrate or nitrite broth in the presence of acetylene (C_2H_2), which inhibits the nitrous oxide reductase. The estimation of the population size is derived from the pattern of positive tubes i.e. nitrous oxide production across a serial dilution followed by mathematical evaluation. The best confirmatory test for denitrifiers is when at least 20% of the nitrogen in the broth has been converted to N_2O in the presence of 0.1 atm C_2H_2 (Tiedje, 1994). The presence of nitrous oxide can be checked by gaschromatography. The MPN technique usually underestimates the number of bacteria by a factor 10^1 to 10^3 (Michotey, Mejean & Bonin, 2000), since it is dependent on cultivation of the organisms.

Two different PCR-based methods have been developed for estimating the population size of denitrifiers: competitive (c-PCR) and real-time PCR. Both are based on ordinary PCR-reactions, with specific denitrifying genes as targets. The gene copy number can be directly correlated with the number of organisms, since except for *narG*, there is only one copy of each gene per genome (Philippot, 2002). In c-PCR the target gene and an internal standard, the so-called competitor, are simultaneously amplified. The two fragments are recognised by the same primers. The initial amount of the target gene is obtained by a comparison between the intensity of the target DNA and the competitor after gel electrophoresis. The

major advantage with c-PCR is that any biases introduced in the amplification will be the same for the target and the competitor. However, c-PCR is more time consuming than real-time PCR, since multiple test dilutions and electrophoresis are needed. Real time-PCR can be performed with the Taq-man or the SYBR Green detection system. In addition to its primers, the Taq-man system requires a nucleotide-probe located in the amplified region. It can be difficult to find enough conserved regions in functional genes. So far, the Taq-man system has only been used to quantify *nirS* in marine systems (Gruntzig *et al.*, 2001) but these primers were too specific and amplified only *nirS* from *P. stutzeri*-strains. The SYBR Green detection system does not require a probe and is more easily adapted for functional genes. SYBR Green is a fluorescent dye that binds to DNA, and during each PCR cycle the fluorescence increases logarithmically. There is a correlation between the initial target gene concentration and the C_t-value, which is the cycle number when the fluorescence becomes higher than the background. Standard curves are created from 10-fold dilution series of a previously quantified gene fragment, and the number of gene copies in the sample can then be calculated from the C_t-value and the standard curves. Real time-PCR has been used to quantify *narG* (López-Gutiérrez *et al.*, 2004) and *nirK* (Henry *et al.*, 2004; **paper III**), whereas c-PCR has been used for quantification of *nirS* (Michotey, Mejean & Bonin, 2000; Cole, Semmens & LaPara, 2004) and *nirK* (Qiu *et al.*, 2004). So far, **paper III** is the only study where quantification of denitrifiers has been compared to their community structure and activity in soil.

Denitrifying communities in different environments

Agricultural soil and municipal wastewater treatment plants represent two different ecosystems, in which denitrifying bacteria are common inhabitants. Both are thought to harbour a highly diverse but poorly understood denitrifying community, and where the identities of the denitrifiers are essentially unknown. However, the denitrifying community appear to be more diverse in soil than in wastewater treatment processes (Fig. 4).

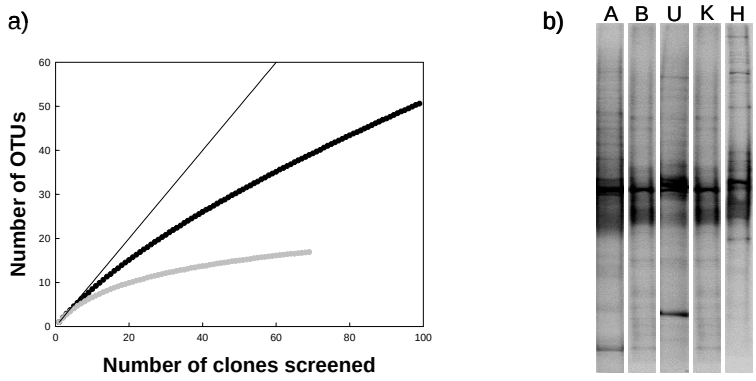

Figure 4. Diversity of denitrifying communities in soil and wastewater treatment processes. a) Rarefaction curves showing the number of OTUs of *nirK*-denitrifiers obtained from soil (•) and wastewater treatment processes (•). The figure is modified from figures 4 in **paper II** and **III**. b) DGGE with samples from activated sludge (K and H) and soil (A, B and U). This is a modified version of figure 2b in **paper I**.

Agricultural soil

Soil is a complex environment, in which biological, chemical and physical properties can vary greatly both temporally and spatially, even on a microscopic scale. A soil aggregate is not homogenous in terms of energy sources, moisture and oxygen contents. The outer zone of the aggregate may be fully aerobic, whereas its inner core, only a short distance away, can retain moisture and remain completely anaerobic. Such gradients create different niches, and this is one reason for the great bacterial diversity in soil. Various physiological types of bacterial communities, with contrasting demands, can thereby co-exist in one soil aggregate.

Typically, denitrification activity in soil displays a patchy pattern. Zones of high denitrification activity, so called 'hot-spots', occur in specific microsites, mainly regulated by the input of moisture, carbon and nitrate (Parkin, 1987; Lensi, Domenach & Abbadie, 1992). The rhizosphere (i.e. the soil influenced by roots) is especially important in providing suitable conditions for denitrifying bacteria (e.g. (Mahmood *et al.*, 1997). Growing plants stimulate denitrification by exuding large amounts of easily available compounds like simple sugars and amino acids. Moreover, root respiration reduces the oxygen tension. However, plant roots can also inhibit denitrification by competing for nitrate and lowering the soil moisture content.

Application of molecular techniques to investigate the community structure of denitrifiers has revealed that agricultural soil harbours an immense diversity (e.g. Mounier *et al.*, 2004; Stres *et al.*, 2004; Enwall, Philippot & Hallin, 2005; **paper I**; **III**), and that each soil has a unique community due to differences in chemical and physical properties (e.g. Stres *et al.*, 2004; Wolsing & Prieme, 2004; **paper I**).

Most *nirK* gene sequences show little similarity to *nirK* from the cultured denitrifiers *Pseudomonas* spp., *Paracoccus* spp., *Alcaligenes* spp., *Rhizobium* spp, and *Rhodobacter* spp. (e.g. Priemé, Braker & Tiedje, 2002; **paper I**; **III**). Sequences of *nosZ* and *nirS* genes are often more similar to those from cultured organisms, and some branch phylogenetically with those from organisms like *Rhizobium*, *Ralstonia*, *Pseudomonas* and *Azospirillum* (Priemé, Braker & Tiedje, 2002; Stres *et al.*, 2004). This could just be a reflection of the primers used in their surveys since other studies have shown the opposite (Enwall, Philippot & Hallin, 2005; **paper I**). The factors that determines the structure of denitrifying communities are not known, but it has been suggested that soil type is one major underlying control (Girvan *et al.*, 2003; Larkin, Honeycutt & Griffin, In press).

Denitrifier communities in agricultural soil seem to undergo annual shifts in composition (Wolsing & Prieme, 2004), and one study showed that the highest number of culturable denitrifiers in a forest soil was found in autumn, winter and early spring (Mergel, Kloos & Bothe, 2001). These shifts probably originate from differences in temperature and soil moisture content during the year. During hot and dry periods, the total microbial activity is low, and especially that of denitrification, since the soil is dry. Cold periods are associated with high emissions of N_2O, and it has been estimated that up to 70% of the annual emissions occur in winter (Wagner-Riddle *et al.*, 1997; Röver, Heinemeyer & Kaiser, 1998). Sharma *et al*. (2006) observed shifts in community structure during freeze-thaw periods and also an increase in the transcript levels of *napA* and *nirS*, which indicated higher denitrification activity. This study supported an earlier hypothesis that the increased emissions of N_2O originate from increased denitrification activity (e.g. Christensen & Tiedje, 1990).

Cultivation of agricultural soil increases the diversity in denitrifier communities because of increased input of available organic carbon, in the form of litter and root exudates. This supports a greater diversity, not only of denitrifiers but also all organotrophic bacteria. Different plant species appear to initiate and maintain different communities (Philippot *et al.*, 2002; Sharma *et al.*, 2005), and this may reflect the fact that different plant species exudes carbon sources that vary not only in amount, but also in availability and degradability. Both inorganic and organic fertilisers have been shown to affect the community structure (Avrahami, Conrad & Braker, 2002; Enwall, Philippot & Hallin, 2005), and it seems that fertiliser type is more important than amount applied (Wolsing & Prieme, 2004). Enwall, Philippot and Hallin (2005) showed that changes in pH as a consequence of fertiliser additions might be the most important determinant in the long-term perspective. Acidity have been shown to be a strong selector for denitrifier populations (Deiglmayr *et al.*, 2004) and the pH optima for communities and isolates appear to reflect the soil pH from where they originate (Cavigelli & Robertson, 2001).

Wastewater treatment plants

A wastewater treatment plant (WWTP) is a complex system combining facets of technology, chemistry and biology. This chapter focuses mainly on the activated

sludge process (ASP), which is the most common type of WWTP. The general features of the biological step in an ASP are often very simple. Growth of microbes, mainly bacteria and protozoa, in special basins, is stimulated in order to degrade organic compounds and sometimes also remove nitrogen and phosphorus. The microbial biomass forms flocs during growth (Fig. 5), although some are also freely suspended in the bulk liquid. The flocs are in intimate contact with soluble nutrients, which are rapidly oxidized or metabolized by the microbial populations in the presence of oxygen. Compressors continuously blow large amounts of air through the water body to accelerate the processing rate of the flocs. In this aerobic environment, carbon and nitrogen end up either fully mineralised or as biomass that settles to the bottom of the clarifier. A fraction of the settled sludge is pumped back to the biological treatment step to ensure that sufficient biomass is maintained in the process. This selects for floc-forming microorganisms since suspended bacteria are washed out. This highly regulated environment represent a specialised ecosystem, one that is probably more selective for specific microorganisms than agricultural soil. Most research on the microbiology of WWTPs is based on the treatment of industrial water even though municipal WWTPs are more common.

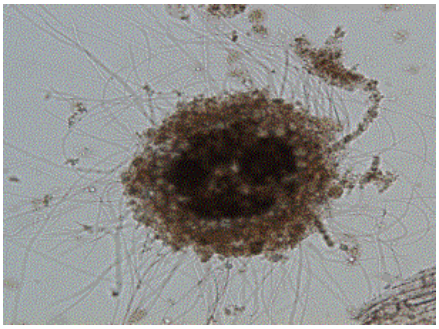

Figure 5. The microbial biomass forms flocs during growth, and the circulation of sludge actively selects for floc-forming micro-organisms.

Many WWTPs are designed to also remove nitrogen from the wastewater in order to contribute to the efforts to decease eutrophication in lakes and oceans. The simplest systems comprise a basin with a single microbial biomass that performs both nitrification and denitrification. Alternating aerobic and anaerobic zones are created in the basins. Two basic strategies (post- and pre-denitrification) are used to accomplish an effective nitrogen removal (Fig. 6). In post-denitrification, the anaerobic zone is placed after the aerobic, and the organic substrates for denitrification mainly originate from the death and lysis of the aerobic biomass, since the influent organic compounds have already been oxidised by the aerobically respiring biomass. In pre-denitrification, the anaerobic zone is located before the aerobic, so the denitrifiers have access to all the organic substrates in

the incoming wastewater. The nitrate formed by the aerobic nitrification step is re-circulated to the anaerobic zone.

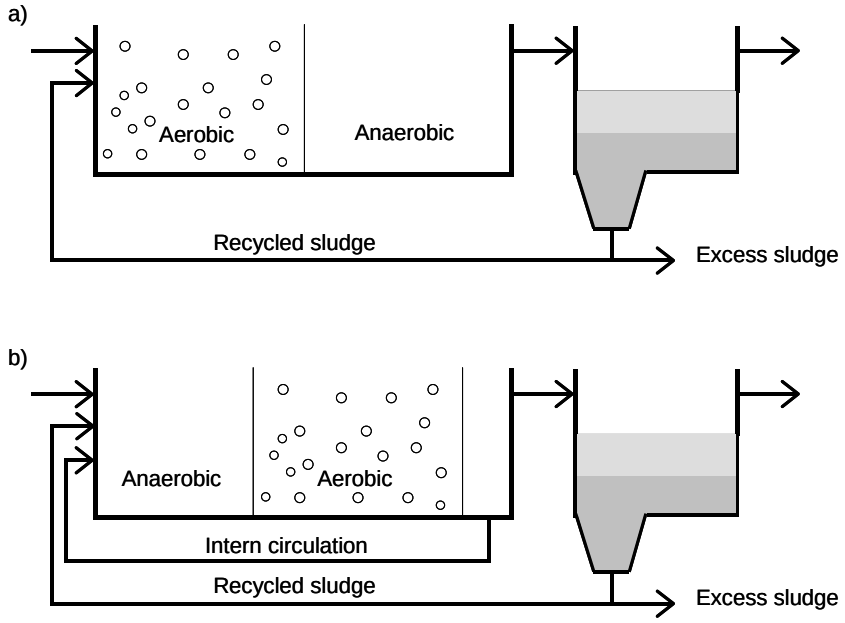

Figure 6. The ASP can have two different strategies to ensure efficient removal of nitrogen, a) post- and b) pre-denitrification.

The community structure of denitrifiers is influenced by the process solutions and different plants appear to have unique communities just like agricultural soils (**paper I**). Functional genes have not previously been used to assess denitrifying populations in ASP treating municipal wastewater. As with soil, the genes appear to be more similar those of previously uncultivated denitrifiers than those of cultivated denitrifiers, although some sequences were distantly related to those from *Rhizobium* spp., *Azospirillum* spp., *Rhodobacter sphaeroides* and *Paracoccus* spp. (**paper I**; **II**). In contrast, Gómez-Villalba *et al*. (In press.) investigated the communities of *nosZ*-denitrifiers in a biofilm reactor treating urban wastewater, and found that most of the clones clustered with *nosZ* sequences from well-known denitrifiers within the β- (*Azospirillum*) and γ-*Proteobacteria (Pseudomonas)*. Sakano *et al*. (2002) obtain a similar result for enriched *nosZ*-denitrifiers in a closed treatment system built for re-cycling of water during long-term space missions, while Tsuneda *et al*. (2005) found that nearly 70% of the *nirS* clones analysed in a sequencing batch reactor process with combined nitrogen and phosphorus removal were similar to *nirS* in *Azoarcus* and *Thauera*.

The major problem with the functional gene approach is that it does not provide any taxonomic information on the denitrifying bacteria. To circumvent this, the

community structure can be characterised with cultivation-based methods or PCR-amplification of 16S rRNA-genes. However, even though the bacteria originate from denitrification hot spots in the WWTP, there is no guarantee that they are denitrifying bacteria. Nevertheless, several studies have indicated that denitrifiers from the families *Comamonadaceae* and *Rhodocyclaceae*, represented by the genera *Azoarcus*, *Paracoccus*, *Hydrogenophaga* and *Acidovorax* (Snaidr *et al.*, 1997; Etchebehere *et al.*, 2001; Juretschko *et al.*, 2002; Hoshino *et al.*, 2005) are predominately found in WWTPs. Interestingly, denitrifiers within these genera are mainly *nirS*-denitrifiers, and assessments based on functional genes also indicate that the *nirS*-communities are more diverse than *nirK*-communities (Yoshie *et al.*, 2004; You, 2005; **paper II**). This phenomena can perhaps be explained by a niche separation, where *nirS* benefits from the higher concentrations of organic nutrients commonly found in wastewater (Cole, Semmens & LaPara, 2004), or because of differences in nitrate requirements between the two genotypes (Yan *et al.*, 2003).

External carbon sources (e.g. acetate, ethanol and methanol) can be added to wastewater treatment processes in order to increase the denitrification rate (e.g. Isaacs & Henze, 1995; Hallin, Rothman & Pell, 1996; Lee & Welander, 1996). The carbon sources have been shown to select for certain denitrifiers in post-denitrification processes. Cultivation of denitrifiers from processes with methanol as an external carbon source have resulted in isolates from mainly the genera *Hyphomicrobium* alone or in combination with *Paracoccus* (e.g. Neef *et al.*, 1996). These results are not surprising as *Hyphomicrobium* spp. are known to utilise C_1-compounds. A recent study based on 16S rRNA identified members from the family *Methylophilales* as the primary denitrifiers in a reactor with methanol as the sole carbon source (Ginige *et al.*, 2004), but this family has not previously been identified as denitrifiers. In contrast, acetate appears to select for denitrifiers commonly found in WWTPs e.g. *Thauera* and *Acidovorax* (Ginige, Keller & Blackall, 2005). The situation in a pre-denitrification process is more complex, since the incoming wastewater contains a complex mixture of various carbon sources and the selective force should therefore be less prominent. However, we showed that addition of ethanol or methanol induced development of unique denitrifying communities, and that the *nirS*-denitrifiers were most affected (**paper II**).

Denitrifiers as indicators of environmental pollution

A large number of anthropogenic compounds, such as heavy metals and organic pollutants, are dispersed in the environment and every year thousands more are introduced. This constitutes a serious threat to ecosystem functions and it is therefore important to assess the risks associated with these compounds. Most chemicals originate from industrial activities, but some are deliberately spread in order to control various pests like fungi and insects. Heavy metals are known for their toxicity to microorganisms, and since they are not degraded they tend to accumulate in the environment. The fate of organic pollutants depends to a great

extent on their chemical structure. Some are easily degraded whereas more recalcitrant compounds will remain in the environment for a long time. Toxicity studies often focus on higher animals and plants, while microorganisms are sometimes neglected. However, since microorganisms are in intimate contact with the pollutants in soil, sediment and water they have potential as sensitive indicators of soil toxicity.

It is difficult to interpret the effects of toxic contaminants on microbial populations in the environment, since any effect may be concealed by functional redundancy. This is especially important if the parameters to be tested are common functions like carbon mineralization. The complexity can be reduced by targeting specific functions or functional groups as indicator organisms (Nannipieri *et al.*, 2003). The ammonia oxidising bacteria is a taxonomically restrained group with members from only three genera. They have often been used as a prokaryotic indicator (e.g. Nielsen *et al.*, 2004; Levén *et al.*, In press; Nyberg *et al.*, In press) as they are easy to study by molecular techniques and are highly sensitive to pollutants (Hicks, Stotzky & Van Voris, 1990; van Beelen & Doelman, 1997; Nyberg, 2006). However, it is important to consider which criteria are most important in the selection of indicator organisms. Denitrifying bacteria are represented by members from most bacterial groups, so disturbances in this function may pose a more serious threat to ecosystems, since many other functions can be affected also. Based on the findings in **paper III** and from results in the literature, we propose that denitrifying bacteria are a suitable prokaryotic indicator group for heavy metal contamination and possibly also for organic pollutants due to their documented sensitivity.

It has been suggested that heavy metal contamination affects processes regulating the nitrogen cycling more than the carbon cycle (Kandeler, Kampichler & Horak, 1996), and that denitrification is more sensitive than aerobic respiration (Bardgett *et al.*, 1994). The effect of heavy metals on denitrification activity is dose-dependent (e.g. McKenney & Vriesacker, 1985; Johansson, Pell & Stenström, 1998; Yin *et al.*, 2003; Lawrence *et al.*, 2004; Vásquez-Murrieta *et al.*, 2005; **paper III**). Different heavy metals differ in their toxicity, and silver seems to be more toxic to heterotrophic bacteria than other heavy metals (Albright & Wilson, 1974; Cornfield, 1977; Johansson, Pell & Stenström, 1998; Brandt, Karlsson & Wennergren, 2005; Murata, Kanao-Koshikawa & Takamatsu, 2005). We showed that silver inhibited the potential denitrification activity and that the denitrifiers failed to recover during 3 months of incubation (**paper III**). Interestingly, the capacity to denitrify per cell deteriorated and this shows that the decrease in activity was not only a result of a decline in the number of denitrifiers. Organic pollutants such as trimethylamine (Eilersen, Henze & Klöft, 1995), monoterpenes (Amaral *et al.*, 1998), petroleum hydrocarbons (Roy & Greer, 2000), aromatic compounds (Siciliano, Roy & Greer, 2000), toluene (Pell & Torstensson, 2002) and pesticides (Bollag & Kurek, 1980; Yeomans & Bremner, 1985a; Yeomans & Bremner, 1985b; Tu, 1994; Tu, 1995) have also been shown to affect denitrification. However, Pell, Stenberg and Torstensson (1998) showed that some pesticides can stimulate denitrification activity, which could be another symptom of stress. They also concluded that, as an indicator, denitrification was almost as sensitive to the pesticides as the ammonia oxidizing bacteria.

Both heavy metals and organic pollutants influence community structure since sensitivity differs between microorganisms. Plasmids carrying genes for resistance to heavy metals are commonly found in bacterial populations (Saxena, Joshi & Srivastava, 2002; Mergeay *et al.*, 2003; Viti, Pace & Giovannetti, 2003), and we saw indications that silver-tolerant denitrifiers were appearing after three months of incubation in soil contaminated with silver (**paper III**). Denitrifiers degrade various organic pollutants and shifts in community structure may occur if these pollutants are used as additional carbon sources. Siciliano, Roy & Greer (2000) found that the relative occurrence of *nirS*-denitrifiers compared to *nirK*-denitrifiers increased in soil contaminated with large concentrations of 2,4,6-trinitrotoluene (TNT). Isolation of a *nirS*-denitrifier, capable of degrading TNT, supports the idea that community shifts can occur due to organic pollutants.

Denitrifying bacteria as models in microbial ecology

Despite recent improvements in methods, much is still unknown in the field of environmental microbiology. The importance of microbial diversity remains a mystery, and links between community composition and activity are not yet established. The study of denitrifying bacteria may help to answer these questions, since denitrification is a facultative trait performed by a heterogeneous bacterial group (Philippot & Hallin, 2005). The fact that so many bacterial groups harbour denitrifiers and that closely related strains have completely different abilities to denitrify, may indicate that the distribution of denitrification genes has not only been influenced by linear evolution but also by horizontal gene transfer (HGT). The uncoupling between 16 rRNA based and functional gene based phylogeny was discussed already when the first primers were published (Braker, Fesefeldt & Witzel, 1998; Hallin & Lindgren, 1999). The hypothesis of uncoupling was recently supported when the phylogeny of *narG* and *nirS*, respectively, was found not be correlated to the phylogeny of 16S rRNA (Gregory *et al.*, 2003; Goregues, Michotey & Bonin, 2005).

Some important issues must be resolved in order to fully understand the ecology of denitrifying bacteria. One of these is the relationship between community structure and function. To do this, we must focus attention on the active denitrifiers within a community. Studies have shown that there need not be a correlation between activity and the community structure of denitrification genes (Rich & Myrold, 2004; Enwall, Philippot & Hallin, 2005). However, it should be emphasised that the methods used in these studies and discussed so far in this thesis, assess denitrification genes that have a potential to be expressed and that they do not represent active denitrifiers. Stable isotope probing (SIP; Radajewski *et al.*, 2002) and bromodeoxyuridine (BrdU) immunocapture (Borneman, 1999; Urbach, Vergin & Giovannoni, 1999) are two methods that target active cells through incorporation of labelled molecules in the DNA of replicating cells. The labelled DNA is then separated from the total DNA before further analysis. SIP

has been used to detect *nosZ* from benzoate-degrading denitrifiers in sediment enrichments (Gallagher *et al.*, 2005), whereas BrdU immunocapture has not, so far, been applied to denitrifiers. However, it has been applied to target other bacterial groups, like ammonia oxidising bacteria (Nyberg, 2006) and actinobacteria (Warnecke *et al.*, 2005) as well as total bacterial communities. Also, mRNA from denitrifying genes represent active denitrifiers, but the extraction of mRNA from environmental samples is complicated, since it is only stable for a few minutes (Rauhut & Klug, 1999). Despite this, reverse transcription of mRNA has been used to analyse *nir* and *nos* in sediment (Nogales *et al.*, 2002), bacterioplancton (Weinbauer *et al.*, 2002) and soil (Sharma *et al.*, 2005; Sharma *et al.*, 2006). The best molecules to analyse would be the active enzymes involved in the denitrification pathway, and promising attempts have been made for one of the enzymes. Cu-nitrite reductase was labelled with monoclonal antibodies and then sorted with flow cytometry (Metz *et al.*, 2003), and the technique was applied to soil and activated sludge.

The major disadvantage in using functional genes as molecular markers is that they give no information about taxonomic affiliation of the denitrifying organisms. In addition, it has become apparent that most of the sequences in environmental clone libraries represent denitrification genes that show little similarity to genes from cultivated denitrifiers. Therefore, more hitherto uncultivated denitrifiers need to be isolated and characterised. A vast majority of environmental bacteria cannot grow on conventional media, perhaps because the conditions in these media differ greatly to natural conditions, for example containing larger concentrations of substrates. Some authors argue that specialised techniques, like gel-droplet incapsulation (Zengler *et al.*, 2002) should be used instead, whereas others suggest that simple solid media can still be employed if multiple carbon sources are used in the enrichments (Joseph *et al.*, 2003; Wawrik *et al.*, 2005). Denitrifiers are normally isolated anaerobically in diluted nutrient broth complemented with NO_3^- (Tiedje, 1994). This method is quite tedious since only about 10% of the isolated anaerobes are true denitrifiers (Gamble, Betlach & Tiedje, 1977; Chèneby *et al.*, 1998). In addition, nutrient broth appears to select for certain organisms like *Pseudomonas* spp., *Paracoccus* spp. and *Bacillus* spp. An experimental design with several chemically different carbon sources would probably increase the potential number of denitrifying genera isolated under anaerobic conditions.

Conclusions

The major findings of this thesis can be summarized as follows:

- Molecular methods are suitable for studies on denitrifier communities, and provide a promising start for understanding the ecology of denitrifiers (**paper I**; **II** and **III**). However, methods focusing on the active organisms are needed in order to get a more complete picture.
- It is necessary to re-evaluate the primers targeting the functional genes involved in the denitrification process on a regular basis. Otherwise, there is great risk that many genotypes will remain undiscovered. This was

especially apparent for *nirS*, since we showed that soil contained a large number of previously uncharacterised *nirS* genotypes, in contrast to what was previously believed (**paper I**).

- For the first time, denitrifying communities were assessed in municipal activated sludge processes based on analysis of the functional denitrification genes. It was shown that most of the genes showed little similarity to previously characterised genes (**paper I** and **II**).
- The heavy metal silver affected various aspects of the denitrification process. The activity of denitrifiers was inhibited and their number decreased, whereas diversity increased. Because of their documented sensitivity to heavy metals, and perhaps also to organic pollutants, it is proposed that denitrifiers are suitable prokaryotic indicators of environmental pollution (**paper III**).

References

Albright, L. J. & Wilson, E. M. 1974. Sub-lethal effects of several metallic salt-organic compound combinations upon heterotropic microflora of a natural water. *Water Research* 8**,** 101-105.

Amaral, J. A., Ekins, A., Richards, S. R. & Knowles, R. 1998. Effect of selected monoterpenes on methane oxidation, denitrification, and aerobic metabolism by bacteria in pure cultures. *Applied and Environmental Microbiology* 64**,** 520-525.

Avrahami, S., Conrad, R. & Braker, G. 2002. Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. *Applied and Environmental Microbiology* 68**,** 5685-5692.

Bardgett, R. D., Speir, T. W., Ross, D. J. & Yeates, G. W. 1994. Impact of pasture contamination by copper, chromium, and arsenic timber preservative on soil microbial properties and nematodes. *Biology and Fertility of Soils* 18**,** 71-79.

Bollag, J.-M. & Kurek, E. J. 1980. Nitrite and nitrous oxide accumulation during denitrification in the presence of pesticide derivates. *Applied and Environmental Microbiology* 39**,** 845-849.

Borneman, J. 1999. Culture-independent identification of microorganisms that respond to specified stimuli. *Applied and Environmental Microbiology* 65**,** 3398-400.

Braker, G., Ayala, H. L., Devol, A. H., Fesefeldt, A. & Tiedje, J. M. 2001. Community structure of denitrifiers, bacteria, Archaea along redox gradients in pacific northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16S rRNA genes. *Applied and Environmental Microbiology* 67**,** 1893-1901.

Braker, G., Fesefeldt, A. & Witzel, K-P. 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology* 64**,** 3769-3775.

Braker, G. & Tiedje, J. M. 2003. Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. *Applied and Environmental Microbiology* 69**,** 3476-3483.

Braker, G., Zhou, J., Wu, L., Devol, A. H. & Tiedje, J. M. 2000. Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. *Applied and Environmental Microbiology* 66**,** 2096-2104.

Brandt, A. P., Karlsson, L. M. & Wennergren, U. 2005. Lettuce grown in silver laden soil at two different activity levels of soil microorganisms. *Journal of Applied Botany and Food Quality* 79**,** 33-37.

Busch, A., Friedrich, B. & Cramm, R. 2002. Characterization of the *norB* gene, encoding nitric oxide reductase, in the nondenitrifying cyanobacterium *Synechocystis* sp. strain PCC6803. *Applied and Environmental Microbiology* 68**,** 668-672.

Cabello, P., Roldan, M. D. & Moreno-Vivian, C. 2004. Nitrate reduction and the nitrogen cycle in archaea. *Microbiology* 150**,** 3527-3546.

Carter, J. P., Hsiao, Y. H., Spiro, S. & Richardson, D. J. 1995. Soil and sediment bacteria capable of aerobic nitrate respiration. *Applied and Environmental Microbiology* 61**,** 2852-2858.

Casciotti, K. L. & Ward, B. B. 2005. Phylogenetic analysis of nitric oxide reductase gene homologues from aerobic ammonia-oxidizing bacteria. *FEMS Microbiology Ecology* 52**,** 197-205.

Castro-González, M., Braker, G., Farias, L. & Ulloa, O. 2005. Communities of *nirS*-type denitrifiers in the water column fo the oxygen minimum zone in the eastern South Pacific. *Environmental Microbiology* 7**,** 1298-1306.

Cavigelli, M. A. & Robertson, G. P. 2001. The functional significance of denitrifier community composition in a terresterial ecosystem. *Ecology* 81**,** 1402-1414.

Chèneby, D., Hartmann, A., Henault, C., Topp, E. & Germon, J.-C. 1998. Diversity of denitrifying microflora and ability to reduce N_2O in two soils. *Biology & Fertility of Soils* 28**,** 19-26.

Christensen, S. & Tiedje, J. M. 1990. Brief and vigorous N_2O production by soil at spring thaw. *Journal of Soil Science* 41**,** 1-4.

Cole, A. C., Semmens, M. J. & LaPara, T. M. 2004. Stratification of activity and bacterial community structure in biofilms grown on membranes transferring oxygen. *Applied and Environmental Microbiology* 70**,** 1982-1989.

Conrad, R. 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O and NO). *Microbiological Reviews* 60**,** 609-640.

Cornfield, A. H. 1977. Effects of addition 12 metals on carbon dioxide release during incubation of an acid sandy soil. *Geoderma* 29**,** 199-203.

Curtis, T. P., Sloan, W. T. & Scannell, J. W. 2002. Estimating prokaryotic diversity and its limits. *Proceedings of the National Academy of Sciences of the U S A* 99**,** 10494-10499.

Degens, B. P., Schipper, L. A., Sparling, G. P. & Duncan, L. C. 2001. Is the microbial community in soil with reduced catabolic diversity less resistant to stress or disturbance? *Soil Biology & Biochemistry* 33**,** 1143-1153.

Deiglmayr, K., Philippot, L., Hartwig, U. A. & Kandelar, E. 2004. Structure and activity of nitrate-reducing community in the rhizospere of *Lolium perenne* and *Trifolium repens* under long-term elevated atmospheric pCO_2. *FEMS Microbiology Ecology* 49**,** 445-454.

Devol, A. H. 2003. Nitrogen cycle-solution to a marine mystery. *Nature* 422.

Eilersen, A. M., Henze, M. & Klöft, L. 1995. Effect of volatile fatty acids and trimethylamine on denitrification in activated sludge. *Water Research* 29**,** 1259-1266.

Einsle, O. & Kroneck, P. M. H. 2004. Structural basis of denitrification. *Biological Chemistry* 385**,** 875-883.

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

Etchebehere, C., Errazquin, I., Barrandeguy, E., Dabert, P., Moletta, R. & Muxi, L. 2001. Evaluation of the denitrifying microbiota of anoxic reactors. *FEMS Microbiology Ecology* 35**,** 259-265.

Etchebehere, C. & Tiedje, J. M. 2005. Presence of two different active *nirS* nitrite reductase genes in a denitrifying *Thauera* sp. from a high-nitrate-removal-rate reactor. *Applied and Environmental Microbiology* 71**,** 5642-5645.

Firestone, M. K., Firestone, R. B. & Tiedje, J. M. 1980. Nitrous oxide from soil denitrification: factors controlling its biological production. *Science* 208**,** 749-751.

Flanagan, D. A., Gregory, L. G., Carter, J. P., Asuman, K.-S., Richardson, D. J. & Spiro, S. 1999. Detection of genes for periplasmic nitrate reductase in nitrate respiring bacteria and in community DNA. *FEMS Microbiology Letters* 177**,** 263-270.

Gallagher, E., McGuinness, L., Young, L. Y. & Kerkhof, L. J. 2005. ^{13}C-Carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. *Applied and Environmental Microbiology* 71**,** 5192-5196.

Gamble, T. N., Betlach, H. R. & Tiedje, J. M. 1977. Numerically dominant denitrifying bacteria from world soils. *Applied and Environmental Microbiology* 33**,** 926-939.

Ginige, M. P., Hugenholtz, P., Daims, H., Wagner, M., Keller, J. & Blackall, L. L. 2004. Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence in situ hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. *Applied and Environmental Microbiology* 70**,** 588-596.

Ginige, M. P., Keller, J. & Blackall, L. L. 2005. Investigation of an Acetate-Fed Denitrifying Microbial Community by Stable Isotope Probing, Full-Cycle rRNA Analysis, and Fluorescent In Situ Hybridization-Microautoradiography. *Applied and Environmental Microbiology* 71**,** 8683-91.

Girvan, M. S., Bullimore, J., Pretty, J. N., Osborn, A. M. & Ball, A. S. 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. *Applied and Environmental Microbiology* 69**,** 1800-1809.

Girvan, M. S., Campbell, C. D., Killham, K. S., Prosser, J. I. & Glover, L. A. 2005. Bacterial diversity promotes community stability and functional resilience after pertubations. *Environmental Microbiology* 7**,** 301-313.

Glockner, A. B., Jungst, A. & Zumft, W. G. 1993. Copper-containing nitrite reductase from *Pseudomonas aureofaciens* is functional in a mutationally cytochrome cd1-free background (NirS-) of *Pseudomonas stutzeri*. *Archives of Microbiology* 160**,** 18-26.

Gómez-Villalba, B., Calvo, C., Vilchez, R., Gonzalez-Carcedo, S. & Rodelas, B. In press. TGGE analysis of the diversity of amminia-oxidizing bacteria and denitrifying bacteria in submerged filter biofilms for the treatment of urban wastewater. *Applied and Environmental Biotechology*.

Goregues, C. M., Michotey, V. D. & Bonin, P. 2005. Molecular, biochemical and physiological approaches for understanding the ecology of denitrification. *Microbial Ecology* 49**,** 198-208.

Gregory, I. R., Karakas-Sen, A., Richardson, D. J. & Spiro, S. 2000. Detection of genes for membrane-bound nitrate reductase in nitrate-respiring bacteria and in community DNA. *FEMS Microbiology Letters* 183**,** 275-279.

Gregory, L. G., Bond, P. L., Richards, S. R. & Spiro, S. 2003. Characterization of a nitrate-respiring bacterial community using the nitrate reductase gene (*narG*) as a functional marker. *Microbiology* 149**,** 229-237.

Gruntzig, V., Nold, S. C., Zhou, J. & Tiedje, J. M. 2001. Pseudomonas stutzeri nitrite reductase gene abundance in environmental samples measured by real-time PCR. *Applied and Environmental Microbiology* 67**,** 760-768.

Hallin, S. & Lindgren, P.-E. 1999. PCR detection of genes encoding nitrite reductase in denitrifing bacteria. *Applied and Environmental Microbiology* 65**,** 1652-1657.

Hallin, S., Rothman, M. & Pell, M. 1996. Adaptation of denitrifying bacteria to acetate and methanol in activated sludge. *Water Research* 30**,** 1445-1450.

Henry, S., Baudoin, E., López-Gutiérrez, J. C., Martin-Laurent, F., Brauman, A. & Philippot, L. 2004. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *Journal of Microbiological Methods* 59**,** 327-335.

Hicks, R. J., Stotzky, G. & Van Voris, P. 1990. Review and evaluation of the effects of xenobiotic chemicals on microorganisms in soil. *Advances in Applied Microbiology* 35**,** 195-253.

Holliger, C. & Zehnder, A. J. B. 1996. Anaerobic biodegradation of hydrocarbons. *Current Opinion in Microbiology* 7**,** 326-330.

Holtan-Hartwig, L., Bechmann, M., Risnes Höyås, T., Linjordet, R. & Bakken, L. R. 2002. Heavy metal tolerance of soil denitrifying communities: N_2O dynamics. *Soil Biolog & Biochemistry* 34**,** 1181-1190.

Horn, M. A., Drake, H. L. & Schramm, A. 2006. Nitrous oxide reductase genes (*nosZ*) of denitrifying microbial populations in soil amd the earthworm gut are phylogenetically similar. *Applied and Environmental Microbiology* 72**,** 1019-1026.

Hoshino, T., Terahara, T., Tsuneda, S., Hirata, A. & Inamori, Y. 2005. Molecular analysis of microbial population transition associated with the start of denitrification in a wastewater treatment process. *Journal of Applied Microbiology* 99**,** 1165-1175.

Isaacs, S. H. & Henze, M. 1995. Controlled carbon source addition to an alternating nitrification-denitrification wastewater treatment process including biological P removal. *Water Research* 29**,** 77-89.

Jetten, M. 2001. New pathways for ammonia conversions in soil and aquatic systems. *Plant and Soil* 230**,** 9-19.

Johansson, M., Pell, M. & Stenström, J. 1998. Kinetics of substrate-induced respiration (SIR) and denitrification: Applications to a soil amended with silver. *Ambio* 27**,** 40-44.

Joseph, S. J., Hugenholtz, P., Sangwan, P., Osborne, C. A. & Janssen, P. H. 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. *Applied and Environmental Microbiology* 69**,** 7210-7215.

Juretschko, S., Loy, A., Lehner, A. & Wagner, M. 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Systematic and Applied Microbiology* 25**,** 84-99.

Kandeler, E., Kampichler, C. & Horak, O. 1996. Influence of heavy metals on the functional diversity of soil microbial communities. *Biology and Fertility of Soils* 23**,** 299-306.

Kisand, V. & Wikner, J. 2003. Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. *Journal of Microbiological Methods* 54**,** 183-191.

Kloos, K., Mergel, A., Rosch, C. & Bothe, H. 2001. Denitrification within the genus *Azospirillum* and other associative bacteria. *Australian Journal of Plant Physiology* 28**,** 991-998.

Larkin, R. P., Honeycutt, C. W. & Griffin, T. S. In press. Effect on swine and dairy manure amendments on microbial communities in three soils as influenced by environmental conditions. *Biology and Fertility of Soils*.

Lawrence, J. R., Chenier, M. R., Roy, R., Beaumier, D., Fortin, N., Swerhone, G. D. W., Neu, T. R. & Greer, C. W. 2004. Microscale and molecular assessment of impacts of nickel, nutrients, and oxygen level on structure and function of river biofilm communities. *Applied and Environmental Microbiology* 70**,** 4326-4339.

Lee, N. M. & Welander, T. 1996. The effect of different carbon sources on respiratory denitrification in biological wastewater treatment. *Journal of Fermentation and Bioengineering* 82**,** 277-285.

Lensi, R., Domenach, A. M. & Abbadie, L. 1992. Field study of nitrification and denitrification in a wet savanna of West Africa (Lamto, Côte d'Ivoire). *Plant and Soil* 147**,** 107-113.

Levén, L., Nyberg, K., Korkea-aho, L. & Schnürer, A. In press. Phenols in anaerobic digestion processes and inhibition of ammonia oxidising bacteria (AOB) in soil. *Science of the Total Environment.*

López-Gutiérrez, J. C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G. & Philippot, L. 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. *Journal of Microbiological Methods* 57**,** 399-407.

Mahmood, T., Ali, R., Malik, K. A. & Shamsi, S. R. A. 1997. Denitrification with and without plant (*Zea amys* L.) under irrigated field conditions. *Biology and Fertility of Soils* 24**,** 323-328.

Marsh, T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Current Opinion in Microbiology* 2**,** 323-327.

McKenney, D. J. & Vriesacker, J. R. 1985. Effect of cadmium contamination on denitrification processes in Brookston clay and Fox sandy loam. *Environmental Pollution (Series A)* 38**,** 221-233.

McNally, D. L., Mihelcic, J. R. & Lueking, D. R. 1998. Biodegradation of three- and four-ring polycyclic aromatic hydrocarbons under aerobic and denitrifying conditions. *Environmental Science and Technology* 32**,** 2633-2639.

Mergeay, M., Monchy, S., Vallaeys, T., Auquier, V., Benotmane, A., Bertin, P., Taghavi, S., Dunn, J., van der Lelie, D. & Wattiez, R. 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS Microbiological Reviews* 27**,** 385-410.

Mergel, A., Kloos, K. & Bothe, H. 2001. Seasonal fluctuations in the population of denitrifying and N_2-fixing bacteria in an acid soil of a Norway spruce forest. *Plant and Soil* 230**,** 145-160.

Metz, S., Beisker, W., Hartmann, A. & Schloter, M. 2003. Detection methods for the expression of the dissimilatory copper-containing nitrite reductase gene (*DnirK*) in environmental samples. *Journal of Microbiological Methods* 55**,** 41-50.

Michotey, V., Mejean, V. & Bonin, P. 2000. Comparison of methods for quantification of cytochrome cd_1-denitrifying bacteria in marine samples. *Applied and Environmental Microbiology* 66**,** 1564-1571.

Mounier, E., Hallet, S., Cheneby, D., Benizri, E., Gruet, Y., Nguyen, C., Piutti, S., Robin, C., Slezack-Deschaumes, S., Martin-Laurent, F., Germon, J. C. & Philippot, L. 2004. Influence of maize mucilage on the diversity and activity of the denitrifying community. *Environmental Microbiology* 6**,** 301-312.

Murata, T., Kanao-Koshikawa, M. & Takamatsu, T. 2005. Effects of Pb, Cu, Sb, In and Ag contamination on the proliferation of soil bacterial colonies, soil dehydrogenase activity, and phospholipid fatty acid profiles of soil microbial communities. *Water Air and Soil Pollution* 164**,** 103-118.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59**,** 695-700.

Muyzer, G. & Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* 73**,** 127-141.

Nannipieri, P., Ascher, J., Ceccherini, M., Landi, M. T., Pietramellara, G. & Renella, G. 2003. Microbial diversity and soil functions. *European Journal of Soil Sciences* 54**,** 655-670.

Neef, A., Zaglauer, A., Meier, H., Amann, R., Lemmer, H. & Schleifer, K. H. 1996. Population analysis in a denitrifying sand filter: conventional and in situ identification of

Paracoccus spp. in a methanol-fed biofilm. *Applied and Environmental Microbiology* 62**,** 4329-4339.

Nielsen, K. B., Brandt, K. K., Jacobsen, A. M., Mortensen, G. K. & Sorensen, J. 2004. Influence of soil moisture on linear alkylbenzene sulfonate-induced toxicity in ammonia-oxidising bacteria. *Environmental Toxicology and Chemistry* 23**,** 363-370.

Nogales, B., Timmis, K. N., Nedwell, D. B. & Osborn, A. M. 2002. Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. *Applied and Environmental Microbiology* 68**,** 5017-5025.

Nyberg, K. (2006) Impact of organic waste residues on structure and function of soil bacterial communities. *Swedish University of Agricultural Sciences, Department of Microbiology, Doctoral thesis 2006:20.* 1-36. ISBN 91-576-7069-2

Nyberg, K., Schnurer, A., Sundh, I., Jarvis, Å. & Hallin, S. In press. Ammonia-oxidising communities in agricultural soil incubated with organic waste residues. *Biology and Fertility of Soils.*

Osborn, A. M., Moore, E. R. & Timmis, K. N. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology* 2**,** 39-50.

Parkin, T. B. 1987. Soil microsites as a source of denitrification variability. *Soil Science Society of American Journal* 51**,** 1194-1199.

Pell, M., Stenberg, B. & Torstensson, L. 1998. Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. *Ambio* 27**,** 24-28.

Pell, M. & Torstensson, L. 2002. Toxicity testing in soil, use of microbial and enzymatic tests. *Bitton E (ed) Encyclopedia of Environmental Microbiology* Vol 6**,** 3155-3168.

Philippot, L. 2002. Denitrifying genes in bacterial and Archaeal genomes. *Biochimica et Biophysica Acta* 1577**,** 355-376.

Philippot, L. & Hallin, S. 2005. Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. *Current Opinion in Microbiology* 8**,** 234-239.

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S. & Germon, J. C. 2002. Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. *Applied and Environmental Microbiology* 68**,** 6121-6128.

Priemé, A., Braker, G. & Tiedje, J. M. 2002. Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetlands soils. *Applied and Environmental Microbiology* 68**,** 1893-1900.

Qiu, X. Y., Hurt, R. A., Wu, L. Y., Chen, C. H., Tiedje, J. M. & Zhou, J. Z. 2004. Detection and quantification of copper-denitrifying bacteria by quantitative competitive PCR. *Journal of Microbiological Methods* 59**,** 199-210.

Radajewski, S., Webster, G., Reay, D. S., Morris, S. A., Ineson, P., Nedwell, D. B., Prosser, J. I. & Murrell, J. C. 2002. Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing. *Microbiology* 148**,** 2331-2342.

Rauhut, R. & Klug, G. 1999. mRNA degradation in bacteria. *FEMS Microbiology Reviews* 23**,** 353-370.

Rich, J. J., Heichen, R. S., Bottomley, P. J., Cromack, K., Jr. & Myrold, D. D. 2003. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. *Applied and Environmental Microbiology* 69**,** 5974-5982.

Rich, J. J. & Myrold, D. D. 2004. Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. *Soil Biology & Biochemistry* 36**,** 1431-1441.

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S. & Taylor, C. J. 2001. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cellular and Molecular Life Science* 58**,** 165-178.

Rosch, C. & Bothe, H. 2005. Improved assessment of denitrifying, N_2-fixing, and total-community bacteria by terminal restriction fragment length polymorphism analysis using multiple restriction enzymes. *Applied and Environmental Microbiology* 71**,** 2026-35.

Roussel-Delif, L., Tarnawski, S., Hamelin, J., Philippot, L., Aragno, M. & Fromin, N. 2005. Frequency and diversity of nitrate reductase genes among nitrate-dissimilating *Pseudomonas* in the rhizosphere of perennial grasses grown in field conditions. *Microbial Ecology* 49**,** 63-72.

Roy, R. & Greer, C. W. 2000. Hexadecane mineralization and denitrification in two diesel fuel-contaminated soils. *FEMS Microbiology Ecology* 32**,** 17-23.

Rösch, C., Mergel, A. & Bothe, H. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Applied and Environmental Microbiology* 68**,** 3818-3829.

Röver, M., Heinemeyer, O. & Kaiser, E. A. 1998. Microbial induced nitrous oxide emsission from an arable soil during winter. *Soil Biology & Biochemistry* 30**,** 1859-1865.

Sakano, Y., Pickering, K. D., Strom, P. F. & Kerkhof, L. J. 2002. Spatial distribution of total, ammonia-oxidizing, and denitrifying bacteria in biological wastewater treatment reactors for bioregenerative life support. *Applied and Environmental Microbiology* 68**,** 2285-2293.

Santoro, A. E., Boehm, A. B. & Francis, C. A. 2006. Denitrifier community composition along a nitrate and salinity gradient in a coastal aquifer. *Applied and Environmental Microbiology* 72**,** 2102-2109.

Saxena, D., Joshi, N. & Srivastava, S. 2002. Mechanism for copper resistance in a copper mine isolate *Pseudomonas putida* S4. *Current Microbiology* 45**,** 410-414.

Scala, D. J. & Kerkhof, L. J. 1998. Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. *FEMS Microbiology Letters* 162**,** 61-68.

Scala, D. J. & Kerkhof, L. J. 1999. Diversity of nitrious oxide reductase (*nosZ)* genes in continental shelf sediments. *Applied and Environmental Microbiology* 65**,** 1681-1687.

Scala, D. J. & Kerkhof, L. J. 2000. Horizontal heterogeneity of denitrifying bacterial communities in marine sediments by terminal restriction fragment length polymorphism analysis. *Applied and Environmental Microbiology* 66**,** 1980-1986.

Sekiguchi, H., Tomioka, N., Nakahara, T. & YUchiyama, H. 2001. A single band does not always represent single bacterial strains in denaturing gradient gel elctrophoresis analysis. *Biotechnology Letters* 23**,** 1205-1208.

Shannon, C. E. & Weaver, W. 1949. The mathematical theory of communication. *University of Illinois Press, Urbana*.

Sharma, S., Aneja, M. K., Mayer, J., Munch, J. C. & Schloter, M. 2005. Diversity of transcripts of nitrite reductase genes (*nirK* and *nirS*) in rhizospheres of grain legumes. *Applied and Environmental Microbiology* 71**,** 2001-2007.

Sharma, S., Szele, Z., Schilling, R., Munch, J. C. & Schloter, M. 2006. Influence of freeze-thaw stress on the structure and function of microbial communities and denitrifying populations in soil. *Applied and Environmental Microbiology* 72**,** 2148-2154.

Siciliano, S. D., Roy, R. & Greer, C. W. 2000. Reduction in denitrification activity in field soils exposed to longterm contamination by 2,4,6-trinitrotoluene (TNT). *FEMS Microbiology Ecology* 32**,** 61-68.

Simpson, G. H. 1949. Measurement of diversity. *Nature* 163**,** 688.

Snaidr, J., Amann, R., Huber, I., Ludwig, W. & Schleifer, K. H. 1997. Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Applied and Environmental Microbiology* 63**,** 2884-2896.

Stouthamer, A. H. 1991. Metabolic regulation including anaerobic metabolism in *Paracoccus denitrificans*. *Journal of Bioenergetics and Biomembranes* 23**,** 163-185.

Stres, B., Mahne, I., Avgustin, G. & Tiedje, J. M. 2004. Nitrous oxide reductase (*nosZ*) gene fragments differ between native and cultivated Michigan soils. *Applied and Environmental Microbiology* 70**,** 301-309.

Takaya, N. 2002. Dissimilatory nitrate reduction metabolisms and their control in fungi. *Journal of Biosciences and Bioengineering* 94**,** 506-10.

Tiedje, J. M. (1994) In *Methods of soil analysis, part 2*.(Eds, Weaver, R. W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A. and Wollum, A.) Soil Science Society of America, Inc., Madison, WI, pp. 245-267.

Torsvik, V., Övreås, L. & Thingstad, T. F. 2002. Prokaryotic diversity - magnitude, dynamics, and controlling factors. *Science* 296**,** 1064-1066.

Tosques, I. E., Kwiatkowski, A. V., Shi, J. & Shapleigh, J. P. 1997. Characterisation and regulation of the gene encoding nitrite reductase in *Rhodobacter sphaeroides* 2.4.3. *Journal of bacteriology* 179**,** 1090-1095.

Tsuneda, S., Miyauchi, R., Ohno, T. & Hirata, A. 2005. Characterization of denitrifying polyphosphate-accumulating organisms in activated sludge based on nitrite reductase gene. *Journal of Biosciences and Bioengineering* 99**,** 403-407.

Tu, C. M. 1994. Effect of some insecticides on microbial activities in sandy soil. *Journal of Environmental Sciences and Health B* 29**,** 281-292.

Tu, C. M. 1995. Effect of five insecticides on microbial and enzymatic activities in sandy soil. *Journal of Environmental Sciences and Health B* 30**,** 289-306.

Tuomainen, J., Hietanen, S., Kuparinen, J., Martikainen, P. & Servomaa, K. 2003. Baltic sea cyanobacterial bloom contains denitrification and nitrification genes, but has negligible denitrification activity. *FEMS Microbiology Ecology* 45**,** 83-96.

Urbach, E., Vergin, K. L. & Giovannoni, S. J. 1999. Immunochemical detection and isolation of DNA from metabolically active bacteria. *Applied and Environmental Microbiology* 65**,** 1207-1213.

Wagner-Riddle, C., Thurtell, G. W., Kidd, G. K., Beauchamp, E. G. & Sweetman, R. 1997. Estimates of nitrous oxide emissions from agricultural fields over 28 months. *Canadian Journal of Soil Sciences* 77**,** 135-144.

van Beelen, P. & Doelman, P. 1997. Significance and application of microbial toxicity tests in assessing ecotoxicological risks in contaminants in soil and sediment. *Chemosphere* 34**,** 455-499.

Warnecke, F., Sommaruga, R., Sekar, R., Hofer, J. S. & Pernthaler, J. 2005. Abundances, identity, and growth state of actinobacteria in mountain lakes of different UV transparency. *Applied and Environmental Microbiology* 71**,** 5551-5559.

Vásquez-Murrieta, M. S., Cruz-Mondragón, C., Trujillo-Tapia, N., Herrerra-Arreola, G., Govaerts, B., Van Cleemput, O. & Dendooven, L. 2005. Nitrous oxide production of heavy metal contaminated soil. *Soil Biology & Biochemistry*.

Wawrik, B., Kerkhof, L. J., Kukor, J. & Zylstra, G. 2005. Effect of different carbon sources on community compostition of bacterial enrichments from soil. *Applied and Environmental Microbiology* 71**,** 6776-6783.

Weier, K. L., Doran, J. W., Mosier, A. R., Power, J. F. & Peterson, T. A. 1994. Potential for bioremediation of high nitrate irrigation water via denitrification. *Journal of Environmental Quality* 23**,** 105-110.

Weinbauer, M. G., Fritz, I., Wenderoth, D. F. & Hofle, M. G. 2002. Simultaneous extraction from bacterioplankton of total RNA and DNA suitable for quantitative structure and function analyses. *Applied and Environmental Microbiology* 68**,** 1082-1087.

Viti, C., Pace, A. & Giovannetti, L. 2003. Characterisation of Cr(VI)-resistant bacteria isolated from chromium-contaminated soil by tannery activity. *Current Microbiology* 46**,** 1-5.

Woese, C. R. 1987. Bacterial evolution. *Microbiology Reviews* 51**,** 221-271.

Wolsing, M. & Prieme, A. 2004. Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of *nir* gene fragments. *FEMS Microbiology Ecology* 48**,** 261-271.

Woomer, P. (1994) In *Methods of soil analysis, part 2.*(Eds, Weaver, R. W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A. and Wollum, A.) Soil Sciency Society of America., Madison, WI, pp. 59-79.

Yan, T., Fields, M. W., Wu, L., Zu, Y., Tiedje, J. M. & Zhou, J. 2003. Molecular diversity and characterization of nitrite reductase gene fragments (*nirK* and *nirS*) from nitrate- and uranium-contaminated groundwater. *Environmental Microbiology* 5**,** 13-24.

Yeomans, J. C. & Bremner, J. M. 1985a. Denitrification in soil: effects of herbicides. *Soil Biology & Biochemistry* 17**,** 447-452.

Yeomans, J. C. & Bremner, J. M. 1985b. Denitrification in soil: effects of insecticides and fungicides. *Soil Biology & Biochemistry* 4**,** 453-456.

Yin, S., Yang, L., Yin, B. & Mei, L. 2003. Nitrification and denitrification activities of zinc-treated soils worked by the earthworm *Pheretima* sp. *Biology and Fertility of Soils* 38**,** 176-180.

Yoshie, S., Noda, N., Tsuneda, S., Hirata, A. & Inamori, Y. 2004. Salinity decreases nitrite reductase gene diversity in denitrifying bacteria of wastewater treatment systems. *Applied and Environmental Microbiology* 70**,** 3152-3157.

You, S. J. 2005. Identification of denitrifying bacteria diversity in an activated sludge system by using nitrite reductase genes. *Biotechnology Letters* 27**,** 1477-1482.

Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E. J., Short, J. M. & Keller, M. 2002. Cultivating the uncultured. *Proceedings of the National Academy of Sciences of the U S A* 99**,** 15681-15686.

Zumft, W. G. 1997. Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* 61**,** 533-616.

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