AN ABSTRACT OF THE THESIS OF

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Title: Variation in Communities of Ammonia-Oxidizing and Denitrifying Bacteria in Fennoscandian Boreal Forest Soils.

Abstract approved:

David D. Myrold

The objective of this study was to examine the variation among ammonia-oxidizing and denitrifying communities in soils of a natural Fennoscandian boreal forest and of a forest with a long history of fertilization with different levels of nitrogen (N), and to examine whether there are any changes in the microbial communities after the termination of N fertilizer. The natural forest at Betsele, Sweden, represents the inherent variation in N supply, plant productivity, and contrasting soil microbial communities. The long-term fertilized forest, Norrliden, represents an on-going N fertilization experiment with up to 34 years of annual N loading at four levels of ammonium nitrate additions, of which the highest was terminated 14 years ago. Although many studies in the past have been done with microbial communities related to the N cycle, the effect of N and N fertilizer on the ammonia-oxidizing and denitrifying communities is still unclear. Terminal restriction fragment length polymorphism (T-RFLP), a fingerprinting method, was used to examine the variation

of the ammonia-oxidizer and the denitrifier communities. Functional biomarkers, the *amoA* gene for ammonia-oxidizing bacteria (AOB) and the *nirK*, *nirS* and *nosZ* genes for denitrifying bacteria, were subjected to PCR amplification. Amplification of ammonia-oxidizers was more successful in the soils with a higher level of N. The AOB community could be assessed only within the two fertilized plots, which showed a difference in the communities with different level of N fertilization. The difference in denitrifying communities regarding the nitrite reductase (*nirK* and nirS) and nitrous oxide reductase (*nosZ*) genes were found to be associated with pH and organic matter concentration in the natural forest at Betsele, followed by nitrate and ammonium concentrations. In the case of the fertilized forest, Norrliden, the community difference was driven predominantly by nitrate and ammonium concentrations, and pH. A fertilization effect was found in the *nirK* communities but this was less apparent with the *nirS* communities. The *nosZ* communities showed fertilization effects. The *nirK* and *nirS* communities each represent a part of the denitrifying community whereas the *nosZ* communities gave the entire picture of the denitrifying communities. Nevertheless, the *nirS*, *nirK*, and *nosZ* communities were all significantly correlated. A shift in the *nosZ* community was observed towards the control and less fertilized plots after the cessation of the fertilizer at Norrliden. The study of these functional genes provided insight about the variability of the microbial communities responsible for regulating important steps in the N cycle.

Variation in Communities of Ammonia-Oxidizing and Denitrifying Bacteria in Fennoscandian Boreal Forest Soils

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Rama D. Ghimire

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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CHAPTER ONE

INTRODUCTION

Nitrification and denitrification are two important processes in nitrogen (N) cycling. Nitrogen, the most limiting nutrient in soil, plays a significant role in plant growth as it accounts for on average 6.25% of the dry mass in living organisms. The availability of N in the soil is dependent on the activities of the microorganisms that fix atmospheric N and transform organic N to plant-available inorganic forms. Plants and microorganisms meet their N demand by utilizing ammonium (NH_4^+) and nitrate (NO_3^-) from the soil.

Nitrification is a two-step biological process where NH_4^+ is oxidized to NO_3^- and denitrification reduces NO_3^- all the way to gaseous N through a series of dissimilatory reduction reactions. Both nitrification and denitrification are equally important in N management. During nitrification, NH_4^+ is first converted to nitrite (NO_2^-) and then to NO_3^-. In general, NH_4^+ is bound to the soil matrix and retained in soil but once it converted to NO_3^-, it is more susceptible to leaching. Oxidation of ammonia is a rate-limiting step and is carried out primarily by autotrophic ammonia-oxidizing bacteria (AOB) of the class β Proteobacteria. The three genes *amoA*, *amoB* and *amoC* are responsible for encoding the enzyme ammonia monooxygenase. The *amoA* gene encodes the active-site polypeptide of ammonia monooxygenase (Hyman and Wood, 1985) and is used as a molecular marker for the studies of AOB communities (Rotthauwe et al., 1997; Stephen et al., 1999).

Biological denitrification is an important step of the N cycle where soil N is transferred into atmospheric N. This process is the sequential reduction of NO_3^- to gaseous N_2, where N oxides serve as alternative electron acceptors for the electrochemical gradient across the cytoplasmic membrane under anaerobic conditions. Besides the release of N_2 gas, the concomitant release of NO and N_2O also takes place depending on the environmental condition of the soil. The release of these gases has caused concern because these gases are believed to cause the global warming and destroy the ozone layer (Bouwman et al., 1995).

The stepwise reduction of NO_3^- to N_2 gas takes place with the involvement of several enzymes present in denitrifying bacteria. Four functional genes encode the key enzymes of this process: nitrate reductase (*nar*), nitrite reductase (*nir*), nitric oxide reductase (*nor*), and nitrous oxide reductase (*nosZ*). Denitrifying bacteria are phylogenetically diverse. More than 50 genera, mostly Proteobacteria, with about 130 denitrifying bacteria species contribute to denitrification (Zumft et al., 1992).

Because only less than 1% of naturally occurring microorganisms are cultivable, culture-dependent techniques are not very helpful to characterize these communities. Recent molecular techniques have, however, been used widely to characterize these communities. These new and fast-developing culture-independent DNA technologies have made it easier to study soil microorganisms. The development of primers that target specific regions of genomic DNA for use in PCR amplification followed by cloning and sequencing has allowed researchers to broaden the knowledge about the communities associated with these processes.

The availability and turnover of N varies greatly depending on the geographic location, soil type, and affects the productivity of agricultural land as well as forests. Boreal forests are considered to be N-limited environments and their productivity is restricted by low availability of N (Tamm, 1991; Saari et al., 2004). Depending upon the landscape position within Fennoscandian boreal forests, the productivity of the forest and the N budget differs greatly. Research done at Betsele in northern Sweden along such a landscape gradient showed great variability in N turnover. Forests in the toe-slope position were more productive with higher N mineralization. Högberg (2001) suggested that leaching of N from upslope land and base cations flushed with ground water may increase the productivity downhill. In the upslope position, there was no net N mineralization and NH_4^+ consumption exceeded gross N mineralization (Högberg et al., 2006). The upland recharge was found to have relatively high concentrations of amino acid N and the toe-slope had higher amounts of plant-available NH_4^+ and NO_3^- (Nordin et al., 2001). Not only is there a difference in N availability, there also is a difference in soil chemistry. The upslope soils have low pH which increases as it runs to the toe-slope

Application of N fertilizer in these Fennoscandian boreal forests is a common forestry management tool and has been practiced for a long time. Swedish forests have received fertilizer since the 1960s to increase the forest productivity. The Swedish Forest Research Institute established many N-fertilization experiments even before then (Nohrstedt, 2001) and long-term fertilization research experiments are still on-going. The addition of N fertilizer in N-limited forests has proven effective in

increasing wood production (Pettersson, 1994; Nohrstedt, 2001; Olsson et al., 2005). It has also been reported that N fertilization can contribute to the long-term carbon retention in trees and soil (Nohrstedt, 2001). Although there is an advantage in getting higher productivity, the annual addition of N fertilizer may lead to acidification of soil and increased N leaching. A concern has been raised about N deposition and soil acidification in Swedish forests where a reduction in pH and base cations has been associated with higher weathering rates (Binkley and Högberg, 1997). Along with these concerns, an additional concern is whether long-term fertilization affects the soil microbial community. Does the amount of N fertilizer applied make any difference? Are these microbial communities different from those in the natural forests that differ in N availability?

To answer these questions, I examined the microbial communities related to two key processes in the N cycle: nitrification and denitrification. Terminal restriction fragment length polymorphism (T-RFLP), a culture-independent DNA fingerprinting method (Marsh, 1999; Thies, 2007), was used to examine the variation of the ammonia oxidizer and the denitrifier communities.

The objectives of this study were to examine the variability of ammonia-oxidizing and denitrifying communities: (i) along a N-availability gradient in a natural Fennoscandian boreal forest; (ii) in a long-term fertilized forest receiving different levels of N fertilizer; and (iii) to examine if there are any changes in the microbial communities after the termination of N fertilizer.

CHAPTER TWO

LITERATURE REVIEW

Nitrogen is one of the most important essential nutrients for forest productivity. Boreal forests are generally limited in N and the availability of N varies depending upon landscape position. Similarly, soil chemistry and soil biology, such as microbial processes and activities, differ by landscape position in these forests. The rates of N transformations depend on microbial activity and these processes are affected by substrate availability. In the N cycle, mineralization of organic matter makes N available for plants and microorganisms in the form of NH_4^+, which can then be converted into NO_3^- via nitrification, and subsequently lost as gaseous N via denitrification. Nitrification and denitrification are therefore two crucial steps that determine whether or not N remains in soil.

2.1 Nitrification

Nitrification is a two-step process in which AOB oxidize NH_3 to NO_2^- and nitrite-oxidizing bacteria (NOB) oxidize NO_2^- to NO_3^-. Ammonia oxidation is considered to be the rate-limiting step in the nitrification process (Kowalchuk and Stephen., 2001). It is the process that links mineralization of organic N with denitrification. Nitrification determines the form and mobility of inorganic N in the soil. Even though autotrophic bacteria play a major role in nitrification, heterotrophic bacteria, archaea and some fungi are also involved in this process (Schimel et al.,

1984; Lang and Jagnow, 1986; Papen and Von Berg, 1998; Brierley and Wood, 2001; Leininger et al., 2006).

2.1.1 Ammonia-oxidizing bacteria (AOB) communities

Ammonia oxidizers are mostly obligatory chemolithoautotrophic bacteria. *Nitrosospira*, *Nitrosomonas* and *Nitrosococcus* are three genera responsible for the oxidation of NH_3. *Nitrosospira* and *Nitrosomonas* are in the β subclass of Proteobacteria (Head et al., 1993), whereas *Nitrosococcus* is related to the γ subclass of Proteobacteria (Teske et al., 1994). To date, *Nitrosococcus* have been isolated from mostly marine environments and other saline water bodies (Koops et al., 1990; Ward and Mullan, 2002). *Nitrosospira* and *Nitrosomonas* are the main contributors to NH_3 oxidation in soils.

Even though AOB are tightly clustered together, within each genus there is a large amount of diversity. Past studies have shown that the variability of AOB communities is associated with N availability, type of fertilizer used, pH, and aboveground biomass. Several phylogenetically coherent clusters of *Nitrosospira,* such as Cluster 2, 3 and 4 have been frequently reported from the soil (Bruns et al., 1999; Mendum et al., 1999; Hastings et al., 2000; Kowalchuk et al., 2000b). Clusters 1 and 3 have been found in soils with high NH_4^+ concentrations, Cluster 3 in soils under long-term N fertilization (Kowalchuk et al., 2000a; Avrahami and Conrad, 2003; Chu et al., 2007; Bruns et al, 1999), Cluster 2 in soils with high N deposition, high nitrification rates, or that have been limed (Horz et al., 2004; Nugroho et al., 2005; Nugroho et al., 2006) and Cluster 4 from unfertilized and undisturbed soils with low

NH_4^+ concentrations (Bruns et al, 1999; Kowalchuck et al., 2000a; Mintie et al., 2003). The shifting of these communities from one cluster to another has been noticed upon environmental changes. Increased N deposition, availability of NH_3, and N turnover have been reported to cause shifts in the AOB community (Prosser, 1989; Kowalchuk et al., 2000a; Horz et al., 2004). The types and amounts of fertilizer also affect the AOB community. *Nitrosospira* members were found in both unamended soil and soil amended with pig slurry, independent of the amount of slurry applied, but the members of *Nitrosomonas* were detected only in soil with a higher quantity of pig slurry (Hastings et al., 1997). Mineral N, organic N, phosphorus and potassium also have shown different effects on AOB communities in soil (Avrahami and Conrad, 2003; Avrahami et al., 2003; Chu et al., 2007). The application of N fertilization increased the size of the AOB community in a forest soil (Compton et al., 2004). A reciprocal transfer study between a meadow with high nitrification potential to a forest with low nitrification potential showed a subsequent increase in nitrification potential and NH_3 oxidizers with relatively little change in AOB communities (Bottomley et al., 2004).

Soil pH is another important environmental factor that affects AOB because free ammonia (NH_3), rather than the NH_4^+ ion, is utilized by AOB (Suzuki et al., 1974; Stark and Firestone, 1996; Burton and Prosser, 2001). Higher pH shifts the equilibrium towards NH_3, which increases its availability. In acid soils, heterotrophic nitrification by pseudomonads and fungi may be more common than autotrophic nitrification in acid soils (Lang et al., 1986; Stroo et al., 1986; Brierley and Wood,

2001), although some studies have suggested that acid-tolerant NH_3 oxidizers are important contributors to nitrification in N-saturated acid forest soil (De Boer et al., 1992). For example, *Nitrosospira* Cluster 2 was found to be associated with acidic environments (Kowalchuk et al., 2000b; Laverman et al., 2001) and Cluster 10 includes *Nitrosospira* sp. strain AF, which can tolerate a pH as low as 5 (Jiang and Bakken, 1999) and was isolated from an acidic sandy soil at pH 4 (Utaker et al., 1996). In addition to pH, there is different temperature adaptability of the AOB community in a single ecosystem (Stark and Firestone, 1996; Avrahami and Conrad, 2003).

2.2 Denitrification

Another crucial process in the soil N cycle is denitrification, the loss of fixed N through dissimilatory reduction of NO_3^- to NO_2^- to gaseous N. The stepwise dissimilatory reduction process is carried out by facultatively anaerobic microorganisms under anaerobic conditions. Denitrification is important in the loss of N from the soil system and in removing unwanted nitrogenous pollutants from water. Denitrification is carried out by wide range of microorganisms belonging to phylogenetically diverse groups of Bacteria and Archaea (Philippot, 2002), although the majority of denitrifiers isolated from soil are *Pseudomonas* and *Alcaligenes* species (Gamble et al., 1977). These organisms are aerobic and have the capacity to use N oxides in the absence of oxygen.

2.2.1 Denitrifying communities

The genetic diversity of denitrifiers can be conveniently explored by using specific genes as functional markers. Nitrate reductase (*nar*), nitrite reductase (*nir*), nitric oxide reductase (*nor*) and nitrous oxide reductase (*nos*) are the four genes that encode the production of the respective enzymes involved in the denitrification pathway. Genetic diversity of denitrifying communities in activated sludge, waste water treatment systems and sediments have been mostly studied in the past. This study was focused on the variability of denitrifying soil communities using the nitrite reductase and nitrous oxide reductase genes.

2.2.2 *nirK/nirS* community

Nitrite reductase is the defining enzyme in denitrification because it catalyzes the conversion of soluble NO_2^- to a gaseous form of N. This reduction reaction differentiates denitrifiers from nitrate-respiring bacteria (Zumft, 1997). The gene responsible for encoding nitrite reductase is *nir* and is one of the common molecular markers for denitrifier communities (Braker et al., 1998). The *nir* gene is found in two different forms, which are structurally different but functionally and physiologically equivalent (Zumft, 1997): *nirK* translates to a copper-containing nitrite reductase and *nirS* translates to a nitrite reductase containing cytochrome cd_1 (heme c and heme d_1) (Coyne et al., 1989). The *nirK* genes are predominantly found in α-proteobacteria and *nirS* genes in β-proteobacteria, whereas both *nirK* and *nirS* were found equally in γ-proteobacteria (Heylen et al., 2006). Coyne et al. (1989) found that *nirS* predominates the denitrifying bacteria but *nirK* has greater variation. These nitrite reductase genes

are mutually exclusive in a given bacterial species (Braker et al., 1998). Prieme et al. (2002) found higher richness as well as higher diversity of *nirS* gene than *nirK* in soils. You (2005) reported higher percentage of *nirS* gene than *nirK* in an activated sludge.

Previous studies have reported a very high diversity of *nir* sequences with novel clusters from soil and marine environments whose sequences are different from isolated and characterized denitrifiers (Prieme et al., 2002; Throbäck et al., 2004). Another study on *nirK* and *nirS* genes showed a very little overlap in *nirK* gene sequences between the forested upland and wetland soils (Prieme et al., 2002), and showed the presence of many uncharacterized denitrifiers in the environment.

Denitrification rates are controlled by several environmental factors such as NO_3^- availability, oxygen and pH (Davidson et al., 1986; Thomas et al., 1994). Besides these, fertilizer type, NH_4^+ concentrations and external carbon source are other important factors that affect denitrifying activity as well as *nirK* and *nirS* community structure. A shift in the soil *nirK* community was observed during incubation with medium and high concentrations of NH_3 (Avrahami et al., 2002). The denitrifier communities also differed in the type of fertilizer applied: *nirK* communities differed between the mineral fertilizer and the cattle manure (Wolsing and Prieme, 2004). Seasonal and temporal variation also plays an important role in the denitrifying communities. Wolsing and Prieme (2004) were able amplify *nirK* in all three seasons but *nirS* communities were amplified only in March. They also found that there was a significant seasonal shift in the *nirK* communities. Another study that

focused on NO_3^- contaminated groundwater showed functional gene diversity changed with the contaminant gradient: the diversity of *nirK* and *nirS* community were inversely proportional but the diversity indices of communities were not related to a single geochemical characteristic (Yan et al., 2003). The external carbon source affected both *nirK* and *nirS* community composition in activated sludge, where *nirS* communities were most greatly affected (Hallin et al., 2006). Studies done in continental margin sediments suggested that denitrifying communities were affected by the NO_3^- and oxygen levels as well as geographic location and biogeochemical conditions (Liu et al., 2003; Yan et al., 2003; Tiquia et al., 2006). Tiquia et al. (2006) also reported that the nitrite reductase (*nirS*) community differed with respect to sediment depth.

2.2.3 *nosZ* community

The final step in the denitrification pathway is the conversion of N_2O to N_2 gas. The nitrous oxide reductase (*nosZ*) gene is responsible for the completion of denitrification process where incomplete reduction leads to the emission of N_2O gas. N_2O is believed to contribute to global warming and the destruction of the ozone layer. The expression of this gene is regulated by oxygen and N oxides (Cavigelli and Robertson, 2000; Cavigelli and Robertson, 2001). pH and the ratio of NO_3^- to available carbon and NO_3^- availability are other important environmental factors that affect the end product (Tiedje, 1988; Robertson et al., 2000; Schimel and Holland, 2005). The *nosZ* gene is unique to denitrifiers and has been used for detection of overall denitrifying communities in environmental samples (Scala and Kerkhof, 1998).

Fertilization has been shown to influence *nosZ* denitrifier communities. A long-term fertilization study found that the communities with organic fertilizer were different from the communities with inorganic fertilizer (Enwall et al., 2005). In another study, Dambreville et al. (2006) found that *nosZ* community composition was significantly different when amended with pig slurry or ammonium nitrate fertilizer. They also reported that the potential denitrifying activity was higher in soils with pig slurry. Sometimes availability of the substrate itself may not be as important because other environmental conditions also control the amount and activity of these communities. Mergel et al. (2001b) reported that the denitrifier communities decreased with the soil depth even if the NO_3^- concentration was constant throughout the depth. A substantial diversity in *nosZ* composition with respect to oxygen sensitivity has also been found (Cavigelli and Robertson, 2001).

Denitrifier community composition differs depending on habitat. For example, there was a significant difference among agricultural soil, riparian soil and creek sediment in Oregon where the creek sediment community was unique (Rich and Myrold, 2004). Another study found that the denitrifying community from meadow soil was different from forest soil and the denitrifying enzyme activity was significantly higher in meadow soil (Rich et al., 2003). Through a 4-year period after the reciprocal transfer of meadow and forest soil, these communities changed and remained distinct from each other but did not shift towards their new location.. The communities still clustered by vegetation type of origin (Boyle et al., 2006). Other studies also found that plant community composition and disturbance regime affect

denitrifier communities (Cavigelli and Robertson, 2000; Cavigelli and Robertson, 2001; Rich et al., 2003). Higher diversity of denitrifiers containing *nosZ* was reported in the cultivated soil with a difference between cultivated and uncultivated soil (Stres et al., 2004). Similarly, higher denitrifier diversity was observed in agricultural soil compared to that of a successional field (Cavigelli and Robertson, 2000), although N_2O reduction was more active in a successional field than in an agricultural field (Cavigelli and Robertson, 2000; Rich and Myrold, 2004). Shifting of *nosZ* composition was reported after agricultural land use (Stres et al., 2004).

CHAPTER THREE

MATERIALS AND METHODS

Two sites in northern Sweden, Betsele and Norrliden, were studied. These sites were selected because they have been shown to differ in N cycling and overall microbial community structure and therefore serve as a good template to examine the relationships between communities of N-cycling bacteria and their activity.

3.1 Site Description

Betsele is located in the Umeå River Valley (64^{o} 39'N, 18^{o} 39'E, 235 m above the sea level). It represents a natural gradient of forest productivity and soil fertility. The gently sloping site supports a 130-year-old forest with a low-productivity stand of Scots pine (*Pinus sylvestris*) in the upslope position that transitions to a more productive stand of Norway spruce (*Picea abies*) at the bottom of the slope. This gradient occurs over a distance of 90 m. Soil properties (Table 3.1) and ground vegetation change along this gradient starting from a dwarf shrub (DS), progressing through intermediate short herbs (SH), and ending in a tall herb (TH) community. The DS type has *Pinus sylvestris* with ericaceous dwarf shrubs (e.g, *Vaccinum myrtillus* and *Empetrum hermaphroditum*); the SH type has *Picea abies* with *Oxalis acetosella*, *Maianthemum bifolium,* and *Solidago virgaurea;* and the TH type has *Picea abies* with *Aconitum septentrionale, Actaea spicata* and *Rubus idaeus.* The concentration of inorganic N gradually increases along the gradient from DS to TH types; pH increases gradually from DS to TH types. Three transects were established across the vegetation gradient, providing replication for each vegetation type.

Norrliden is located 65 km east of Betsele (64^{o} 21'N, 19^{o} 45'E, 267 m altitude) with similar climate and soils. This site also has a gentle slope and is dominated by a 50-year-old, low-productivity Scots pine forest of the DS type. Ammonium nitrate is applied annually on the 30 x 30 m plots at three different levels. N0 is the control plot without N fertilization, N1 receives 34 kg N ha^{-1}, N2 receives 64 kg N ha^{-1} and N3 received 108 kg N ha^{-1}. The N1 and N2 treatments are still under fertilization, but the application of ammonium nitrate was stopped in 1991 in the N3 treatment. The three replicate plots per treatment were part of a randomized complete block design.

Table 3.1. Soil chemical properties of the mor (F and H) layer at Betsele and Norrliden. The data are means with standard deviations in parentheses (n=3). Data from Högberg et al. (2007).

Site	Forest type†	Treatment‡	pH§	C-to-N ratio	NH_4^+-N µg g^{-1} o.m	NO_3^--N µg g^{-1} o.m
Betsele	DS	-	4.0(0.1)	38.1(2.4)	4.6(1.8)	0.9(0.5)
	SH	-	4.6(0.1)	22.9(1.1)	5.2(1.2)	0.7(0.3)
	TH	-	5.3(0.1)	14.9(0.3)	15.9(5.5)	3.4(0.8)
Norrliden	DS	N0	4.1(0.0)	37.5(1.2)	0.5(0.2)	0.7(0.2)
	DS	N1	4.1(0.0)	31.1(1.8)	39.9(32.1)	1.5(0.3)
	DS	N2	4.2(0.0)	27.7(0.6)	88.4(10.1)	7.3(1.8)
	DS	N3	4.1(0.0)	27.2(0.7)	3.3(2.0)	0.6(0.1)

†DS – dwarf shrub, SH – short herb, and TH – tall herb.
‡N0 – control plot; N1, N2, and N3 are the fertilized treatment at three different levels. Fertilization of the N3 treatment stopped in 1991.
§pH soil water ratio 1:3 (v/v)

3.2 Soil Sampling

Soil samples were taken from Betsele on 18 August 2004 and from Norrliden on 25 August 2004 (see Högberg et al., 2007 for details). In short, soil samples of the organic mor-layer F and H horizons were taken with a 0.15-m diameter corer. Three cores were taken from each plot and bulked together representing one composite sample for analysis. The auger was cleaned between treatments, and screening and homogenization were done to the soil samples prior to analysis.

3.3 DNA Extraction

The Powersoil DNA Isolation kit (MO Bio Laboratories, Carlsbad, CA) was used for the extraction of DNA from the soil samples. DNA was extracted according to manufacturer's instructions. Briefly, 0.10 to 0.25 g of soil was used for the lysis of cells, and release of DNA was done by the combination of chemical agents and mechanical shaking. A Bio101 FastPrep instrument (Bio 101, Carlsbad, CA) was used to shake the samples for 45 s. The extracted DNA was bound tightly to the silica at high salt concentrations on spin filters. The bound DNA was washed with ethanol-based wash solution and finally eluted in the tube by using elution buffer. Extracted DNA was quantified by using a NanoDrop^{TM} ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE). DNA extracts were diluted to 25 ng ul^{-1} and stored at -20°C.

3.4 PCR Amplification

PCR amplification was done using different sets of primers for functional genes related to nitrification and denitrification (Table 3.2). The PCR products were

visualized on 1.1% agarose gels stained with ethidium bromide. Purification of PCR products was done to remove remaining genomic DNA and excess primers using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Table 3.2. Primer sequences and positions of different primers used for PCR amplification. Positions described refer to the following organisms: *Nitrosomonas europaea* for *amoA*, *Pseudomonas stutzeri* ZoBell ATCC 14405 (X568) for *nirS*, *Alcaligenes faecalis* S-6 (D13155) for *nirK*, and *Pseudomonas aerugenosa* DSM 5007 (X65277) for *nosZ*.

Target gene	**Primer**	**Position**	**Primer sequence†**	**Reference**
amoA	A189(1)	151-168	GGN GAC TGG GAC TTC TGG	Holmes et al., 1995
	amoA-2R	802-822	CCC CTC KGS AAA GCC TTC TTC	Horz et al., 2000
	amoA-1F	332-349	GGG GGT TTC TAC TGG TGG T	Horz et al., 2000
	amoA-2R	802-822	CCC CTC KGS AAA GCC TTC TTC	Horz et al., 2000
nosZ	Nos661F	303-320	CGG CTG GGG GCT GAC CAA	Scala and Kerkhof, 1998
	nosZ-R	1849-1869	CAT CTG CAG NGC RTG GCA GAA	Kloos et al., 2001
	nosZ-F-1181	1169-1188	CGC TGT TCI TCG ACA GYC AG	Rich et al., 2003
	nosZ-R-1880	1849-1869	ATG TGC AKI GCR TGG CAG AA	Rich et al., 2003
nirS	nirS1F	763-780	CCT AYT GGC CGC CRC ART	Braker et al., 1998
	nirS6R	1638-1653	CGT TGA ACT TRC CGG T	Braker et al., 1998
	cd3aF	916-935	GTS AAC GTS AAG GAR ACS GG	Michotey et al., 2000
	R3cd	1322-1341	GAS TTC GGR TGS GTC TTG A	Throbäck et al., 2004
nirK	nirK1F	526-542	GGM ATG GTK CCS TGG CA	Braker et al., 1998
	nirK5R	1023-1040	GCC TCG ATC AGR TTR TGG	Braker et al., 1998
	FlaCu	568-584	ATC ATG GTS CTG CCG CG	Hallin and Lindergen, 1999
	R3Cu	1021-1040	GCC TCG ATC AGR TTG TGG TT	Hallin and Lindergen, 1999

†S = G+C W = A+T Y = C+T K = G+T R = A+G M = A+C N= A+C+G+T I = Inosine

3.4.1 Amplification of *amoA*

The *amoA* gene encodes for the enzyme ammonia monooxygenase, which oxidizes NH_3 to NO_2^-. Due to the low amplification of the gene, a nested PCR was used in which the amplification of the larger fragment was done by using the primer

set of A189(1):amoA-2R. Touchdown PCR was used for amplification using the first set of primers with an initial denaturation at 94°C for 3 min, followed by 30 cycles at94°C for 30 s, 62 to 52°C with the annealing temperature decreasing of 0.5°C after each cycle until it reached 52°C for 30 s, 72°C for 45 s and the final elongation step at 72°C for 10 min. The PCR product (4 µl) was then re-amplified by using a second set of primers (amoA-1F:amoA-2R) to produce a fragment of ~491 bp. The 5' end of amoA-1F was labeled with 6-FAM (6-carboxyfluorescein) for terminal restriction fragment length polymorphism (T-RFLP) analysis. The second PCR was performed in a total volume of 50 µl for 18 cycles with initial denaturation at 94°C for 5 min followed by 94°C for 30 s, 55°C for 50 s, 72°C for 45 s and final extension for 10 min at 72°C. A solution containing 1X buffer, 2 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 0.064% Bovine serum albumin (BSA), 0.2 µM of each primer, 100 ng of DNA template, and 2.5U *Taq* polymerase (Promega, Madison, WI) was used. Amplification was done using a PTC-100 Programmable Thermocycler (MJ Research, Watertown, MA).

3.4.2 Amplification of *nirK/nirS*

The *nirK* and *nirS* genes encode for the nitrite reductase enzyme, which converts NO_2^- to NO during denitrification. Due to low amplification of *nirK* and *nirS* genes, a nested PCR approach was used. With the nested PCR technique, a ~514-bp fragment for *nirK* was generated by using the primer set of nirK1F and nirK5R, which was then subjected to another PCR by using the primer set of FlaCu and R3Cu to produce a fragment of ~473 bp. The forward primer FlaCu was labeled with 6-FAM

for T-RFLP analysis. Similarly, the primer set nirS1F and nirS6R was used in the first amplification of *nirS*, producing a fragment of ~860 bp, followed by amplification to produce a ~421-bp fragment using 6-FAM-cd3aF and R3cd. In both cases, 4 µl of the first round of PCR product was used for the second nested PCR. For all reactions, DNA was amplified using the total volume of 50 µl with a PCR mixture containing 1X buffer, 2 mM $MgCl_2$, 0.20 mM of each deoxynucleotide triphosphate, 0.20 µM forward and reverse primers, 0.064% BSA, and 0.5 units of *Taq* DNA polymerase. A total of 50 to 100 ng of DNA template was used in each reaction. The PCR amplification of all primer pairs was run with an initial denaturation of the DNA at 94°C for 2 min followed by 94°C for 30 s, annealing at 57°C for 1 min, and 1 min at 72°C for 35 cycles. The final extension was done at 72°C for 10 min. In the case of the primer set nirK1F and nirK5R, the denaturation time was 3 min and the annealing temperature was 56°C.

3.4.3 Amplification of *nosZ*

The *nosZ* gene encodes the nitrous oxide reductase enzyme, which converts N_2O to N_2 gas, the final product during denitrification. PCR amplification of *nosZ* gene targeting a ~700-bp fragment was done using a modified version of the primer set of nosZ-F-1181 and nosZ-R-1880 as described by Rich et al. (2003). Where there was low success in amplification, a nested PCR was done: a larger fragment was generated with the primer set of nos661F and nosZ-R, which was then subjected to another PCR using the primer set of nosZ-F-1181 and nosZ-R-1880 to produce a fragment of ~700 bp. The forward primer was labeled with 6-FAM for T-RFLP

analysis. DNA was amplified using the total volume of 50 µl with a PCR mixture containing 1X buffer, 2 mM $MgCl_2$, 0.20 mM of each deoxynucleotide triphosphate, 0.20 µM forward and reverse primers, 0.064% BSA, and 1.25 units of *Taq* DNA polymerase. The total of 50 to 100 ng of DNA template was used in each reaction. The amplification was done with an initial denaturation of DNA at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 55°C for 1 min, 72°C for 1 min and the final extension period of 10 min at 72°C. The second PCR was done with an initial denaturation of 94°C for 3 min, and 22 cycles at 94°C for 45 s, 56°C for 1 min, and 72°C for 2 min with a final extension at 72°C for 7 min.

3.5 T-RFLP Analysis

Purified DNA fragments of *nirK, nirS* and *nosZ* were restricted with three different restriction enzymes: CfoI (recognition site GCG/C), MspI (recognition site C/CGG), and RsaI (recognition site GT/AC) (Promega Corp., Madison, WI). The restriction was done for 3 hrs at 37°C followed by 15 min of heat inactivation at 65°C. In the case of *amoA*, CfoI, TaqI (recognition site T/CGA) and AluI (recognition site AG/CT) were used under the same conditions except that TaqI was digested at 65°C for 3 hours. The samples were then submitted to The Center for Genome Research and Biotechnology (CGRB), Oregon State University, Corvallis, OR.

An ABI 3100 capillary DNA sequencer was used to analyze fluorescently labeled DNA fragments for T-RFLP analysis. The fragment size was determined on the basis of an internal size marker (MapMarker ROX (35-1000 bp)) using ABI Genescan® software. Genotyper® software was used to analyze the fragments by

categorizing and sorting peaks. The percent relative fluorescence was based on the total peak area covered by all peaks for a soil sample. Fragments with signals less than 1.5% relative fluorescence were considered as noise, so these were excluded from the analysis and percentage of all peaks were recalculated to sum to 100%. The fragments from all the samples of each restriction enzyme were lined up in ascending order and the size difference from each preceding fragment was calculated. A difference of ≥1.5 bp was called a separate fragment for that enzyme (Rich et al., 2003).

3.6 Statistical Analyses

PC-ORD version 5.04 (McCune and Mefford, 1999) was used for multivariate statistical analysis. For statistical analysis, the main matrix contained samples in rows and the fragment areas as "species" in columns. The secondary matrix contained environmental factors such as pH, ammonium concentration, and nitrate concentration. Variation in the species composition was visualized by Non Metric Multidimensional Scaling (NMS), where sample units were ordinated in species space. NMS is an iterative search for minimum stress. The autopilot mode with Sørenson's distance measure was used for the analysis of the matrix, 250 runs of real data starting from random configurations and 250 runs of randomized data with shuffling of the main matrix each time. This setting can run a maximum number of 500 iterations with the instability criteria of 0.00001. The comparison between real and randomized data was done using the Monte Carlo test. Multi-Response Permutation Procedures (MRPP), a non-parametric procedure, was used to test the statistical difference and significance between the groups. The within group homogeneity was described by the

A-value. The A-value is the chance correlated within group agreement. It gives a description of the effect size that is independent of the sample size and ranges from values of 0 to 1. The p-value value evaluates an observed difference among groups due to chance. A p-value <0.05 was considered statistically significant.

The relationship between the environmental variables from the second matrix were shown using joint plots, where radiating lines indicate the direction and strength of the variables with the ordination scores.

Indicator species analysis was done using the same software; this provides indicator values that combine the relative abundance and relative frequencies of the species in each group. The indicator value ranges from 0 to 100 where 0 is no indication and 100 is perfect indication. A Monte Carlo technique using 10,000 randomizations was used to test the significance of indicator values. The p-value is based on the proportion of randomized trials with an indicator value which is equal to or exceeding the observed indicator value. Outlier analysis was also done. Any samples greater than two standard deviations from the grand mean were not included in subsequent analyses.

The Mantel test, which evaluates the correlation between distance matrices, was used to test the relationship among *nirK*, *nirS* and *nosZ* communities. Sørenson distance with asymptotic approximation method was used to evaluate test statistics.

CHAPTER FOUR

RESULTS

There was variable success in amplifying *amoA*, *nirK*, *nirS* and *nosZ* from the soils at Betsele and Norrliden. Amplification was more successful in forests with relatively high levels of N at both sites and the denitrifier genes amplified better than *amoA*. A nested PCR technique was used to increase amplification success. After the nested PCR, *nirK* and *nirS* genes were more successfully amplified than *nosZ* and *amoA* genes (Table 4.1). Nested PCR sometimes resulted in non-specific amplification of the *nosZ* gene; such samples were excluded from further analysis.

Table 4.1. Amplification success of DNA extracted from soils at Betsele (n=27) and Norrliden (n=36) after nested PCR.

Functional Gene	Betsele PCR amplification	Norrliden PCR amplification
amoA	10	15
nirK	27	30
nirS	27	30
nosZ	26	27

4.1 T-RFLP analysis of *amoA* gene

All of the soil DNA extracts from TH plots at Betsele were amplified successfully. However, only two DNA extracts could be amplified from SH plots and none from DS plots. At Norrliden, amplification was successful for all samples from N2 plots but very few from N0 and N1 plots, and none from N3 plots. Re-extraction of DNA from soil samples followed by amplification was done for those samples without success. Spiking by adding 2 µl DNA from *Nitrosomonas europaea* and use of 16S rRNA primers was also done to check the presence of inhibitors and the

viability of the DNA. Both spiking and 16S rRNA amplification produced the desired products, suggesting that lack of amplification was due to low copy numbers of the *amoA* gene.

Because successful amplification could not be obtained from the all treatments, only a few comparisons could be made. A significant difference was found between the ammonia oxidizing communities from the N1 and N2 treatments at Norrliden (Table 4.2). Some of the TRFs with higher percentage of mean florescence were evaluated showing that CfoI 94, CfoI 131 and AluI 489 were present at both sites at a relatively high percentage. CfoI 86, CfoI 488, AluI 88, AluI 104 and AluI 486 were dominant in N0 treatment at Norrliden whereas AluI 388 was found only at Betsele (Fig 4.1). The restriction digests from enzyme TaqI produced a single major peak, TaqI 279, at both sites, suggesting that *Nitrosomonas* sp. were not present.

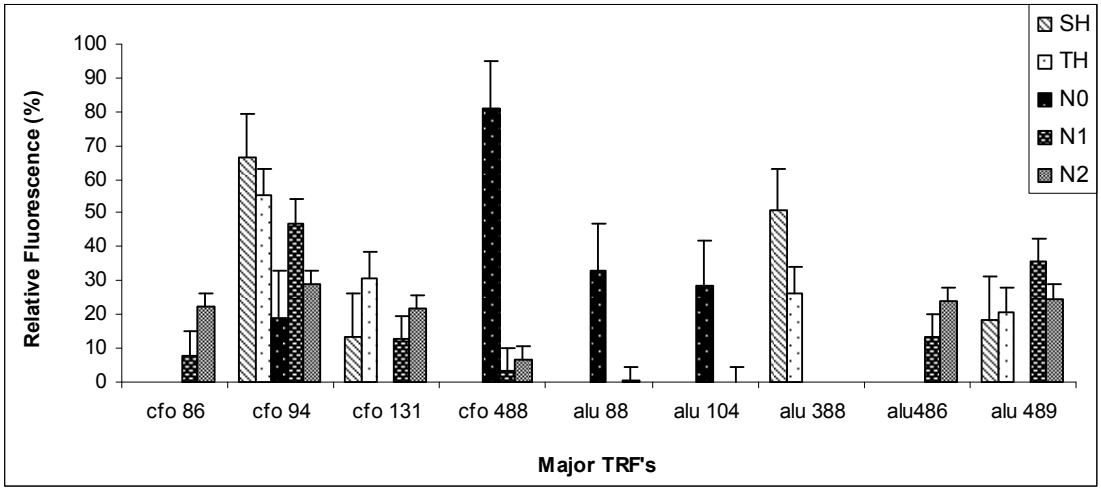

Fig 4.1. Dominant TRFs of the *amoA* gene from Betsele and Norrliden. Bars represent the average of nine samples for the TH type and N2 treatment; other types/treatment had fewer samples. Error bars are the standard error of the mean.

Table 4.2. Differences between ammonia oxidizing and denitrifying communities based on proportional abundance. The p-value and A-value were calculated by using multi-response permutation procedures. The numbers in bold (A-value and p-value) are shown as significant. The A-value measures the chance-correlated within-group agreement; an A-value of about 0.3 represents strong difference between groups. A p-value of < 0.05 was considered significant.

Site	Compared Treatment	*amoA*		*nirK*		*nirS*		*nosZ*	
		A	P	A	P	A	P	A	P
Betsele vs Norrliden		**0.18**	**0.0007**	**0.03**	**0.006**	0.01	0.063	**0.04**	**0.004**
Betsele treatment effect		-	-	**0.15**	**0.001**	**0.07**	**0.037**	**0.31**	**0.001**
Betsele	DS vs SH	-	-	**0.13**	**0.030**	-0.01	0.560	**0.13**	**0.024**
	DS vs TH	-	-	**0.14**	**0.023**	**0.13**	**0.022**	**0.38**	**0.022**
	SH vs TH	-	-	**0.11**	**0.022**	0.04	0.088	**0.27**	**0.021**
Norrliden treatment effect		-	-	**0.11**	**0.024**	**0.12**	**0.005**	**0.19**	**0.0005**
Norrliden	N0 vs N1	-	-	0.01	0.320	0.04	0.126	**0.10**	**0.023**
	N0 vs N2	-	-	**0.19**	**0.023**	-0.01	0.425	**0.19**	**0.025**
	N0 vs N3	-	-	**0.22**	**0.023**	**0.08**	**0.023**	0.06	0.098
	N1 vs N2	**0.11**	**0.028**	0.00	0.600	**0.13**	**0.045**	**0.17**	**0.030**
	N1 vs N3	-	-	0.02	0.188	**0.14**	**0.023**	0.13	0.055
	N2 vs N3	-	-	**0.06**	**0.040**	**0.14**	**0.039**	**0.25**	**0.025**

4.2 T-RFLP analysis of *nirK* gene

All 27 samples from Betsele and 30 of 36 samples from Norrliden were amplified (Table 4.1). A total number of 138 TRFs were produced from restriction of *nirK* amplification products with three restriction enzymes CfoI, MspI and RsaI from Betsele, whereas 214 TRFs were produced from Norrliden.

Non-metric multidimensional scaling (NMS) ordination was used to visualize the separation between the sites as well as treatments. A two-dimensional ordination was created for Betsele with a cumulative variance of 94% (Fig 4.2). The denitrifier communities from the TH forest type were found in the lower right corner of the ordination whereas DS and SH forest types were in the upper half. MRPP of the three plots from Betsele showed significant differences among treatments (Table 4.2). Pairwise comparison of treatments showed that the denitrifying communities of the DS, SH and TH soils were significantly different from each other (Table 4.2). A secondary matrix of environmental variables was imposed on the community ordination by the joint plot method to show the correlations between the environmental variables and the community ordination. Concentrations of NH_4^+, NO_3^- and pH were strongly correlated with the Axis 2 and were also related the TH forest type whereas carbon to nitrogen ratio (C-to-N) was negatively correlated to Axis 1 (r^2 = 0.56).

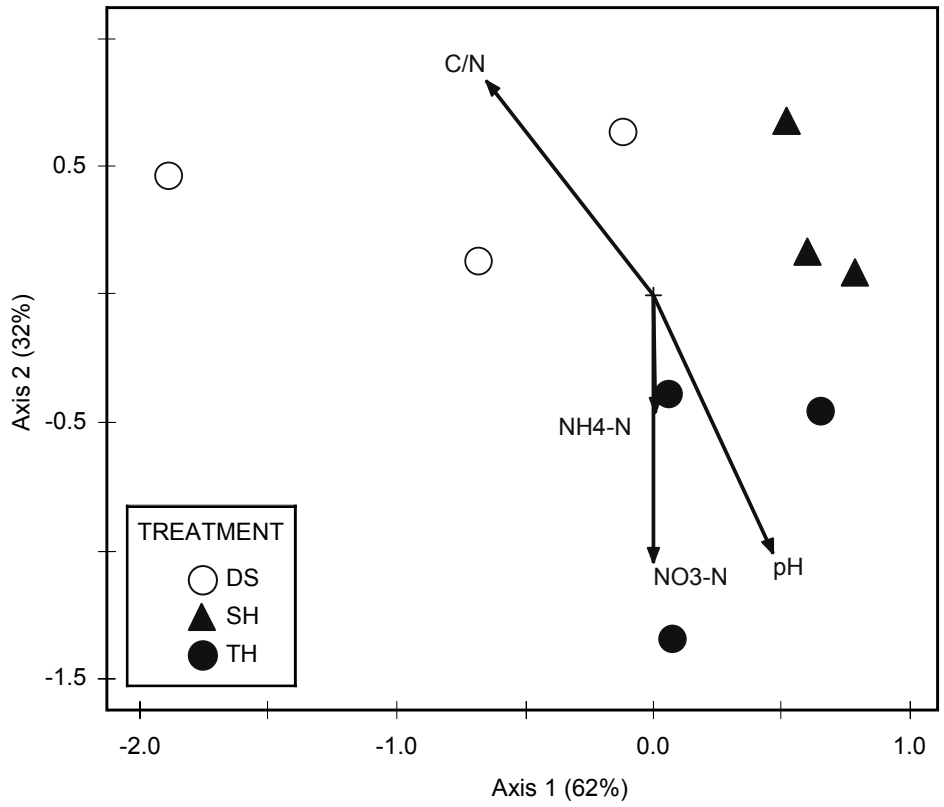

Fig 4.2. NMS ordination of the *nirK* community from Betsele with the final stress of 5.09, final instability of 0.0000 and 56 iterations. The correlation coefficients explained by the axes are in parentheses. Vectors for NO_3-N (r^2=0.69), NH_4-N (r^2=0.30), pH (r^2=0.67) and C-to-N ratio (r^2=0.56).

An NMS ordination of the *nirK* gene of denitrifying communities from the Norrliden site generated a three-dimensional ordination (Fig 4.3) with a cumulative variance explained of 86%. Axes 2 and 3 are shown because the groups can be visualized well on these axes and the percent variances represented by these axes were higher. The N2 plots were distinctly separated from the control N0 plots and N3 plots. Pairwise comparison using MRPP showed that the N0 treatment was significantly

different from the N2 and N3 treatments. Similarly, there was a significant difference between the N2 and N3 treatments; however, the N1 treatment was not significantly different from any of the other treatments (Table 4.2). Soil NO_3^-, NH_4^+ and pH were strongly correlated to the denitrifying communities from N2 plot and to Axis 3 (r^2=0.49, 0.46 and 0.40 respectively), whereas the N0 community were strongly correlated to C-to-N ratio (r^2=0.55).

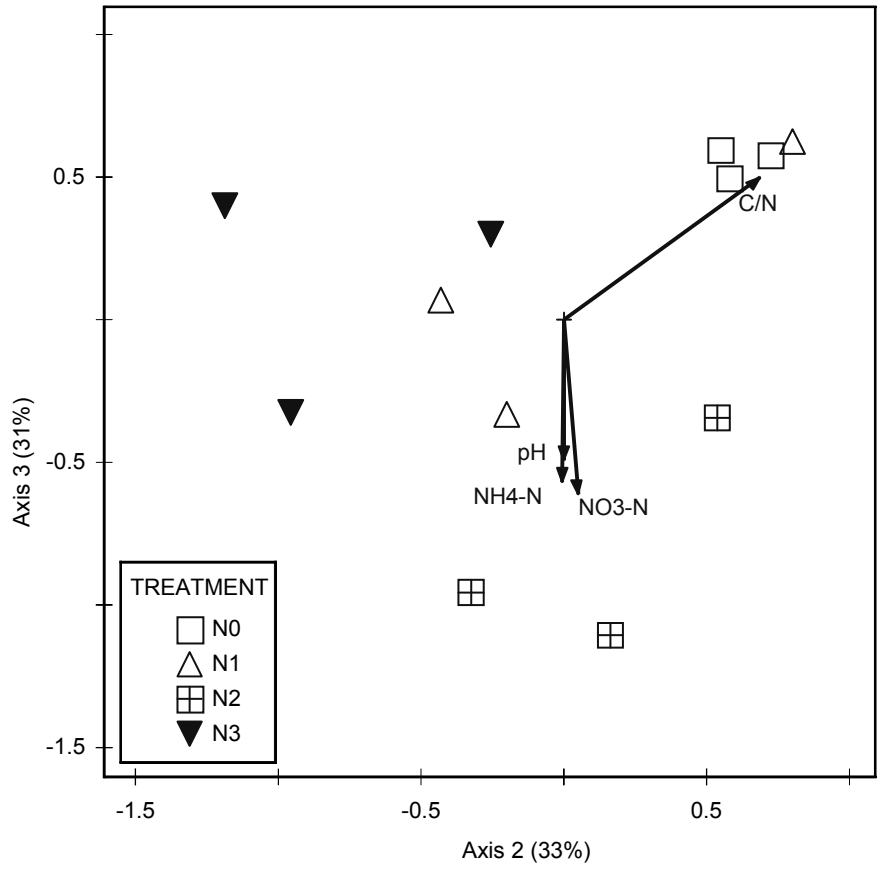

Fig 4.3. NMS ordination of denitrifying communities containing the *nirK* gene from Norrliden, with the final stress of 5.87 and final instability of 0.0000 with 51 iterations. The correlation coefficients explained by the axes are in parentheses. Vectors NO_3-N for (r^2=0.49), NH_4-N (r^2=0.46), pH (r^2=0.40) and C-to-N ratio (r^2=0.55).

Based on the indicator species analysis, 10 TRFs were identified as indicators of the *nirK* community from Betsele, where TRFs Cfo108, Cfo211 and Msp464 were perfect indicators of the TH forest type. Similarly, Msp143 and Rsa143 were perfect indicators of the DS forest type (Table 4.3). In the case of Norrliden, only four TRFs were identified as indicators and none of the TRFs was solely representative of any treatment (Table 4.4).

Table 4.3. List of indicator TRFs identified by indicator species analysis of the *nirK* community at Betsele. The figures in the bold are the representative TRFs of their respective column (treatment).

TRFs	Indicator Value DS	Indicator Value SH	Indicator Value TH	p-value
Cfo108	0	0	**100**	0.0324
Cfo211	0	0	**100**	0.0324
Msp143	**100**	0	0	0.0332
Msp217	7	23	**67**	0.0296
Msp334	3	**76**	9	0.0076
Msp464	0	0	**100**	0.0324
Rsa77	**82**	8	2	0.0332
Rsa102	**87**	1	3	0.0332
Rsa123	**87**	0	4	0.0332
Rsa143	**100**	0	0	0.0332

Table 4.4. List of indicator TRFs identified by indicator species analysis of the *nirK* community at Norrliden. The figures in the bold are the representative TRFs of their respective column (treatment).

TRFs	Indicator Value N0	Indicator Value N1	Indicator Value N2	Indicator Value N3	p-value
Cfo64	4	0	**74**	5	0.0182
Msp129	0	9	1	**83**	0.017
Rsa409	0	1	14	**76**	0.0162
Rsa466	**45**	23	17	15	0.0284

Eight TRFs were found to be major TRFs among the *nirK* denitrifying communities. Cfo60, Rsa466 and Rsa468 were found to be the dominant TRFs with higher mean relative florescence at both Betsele and Norrliden (Fig 4.4). The relative percent fluorescence of Msp60 was significantly higher for Norrliden than Betsele (ANOVA p-value 0.002).

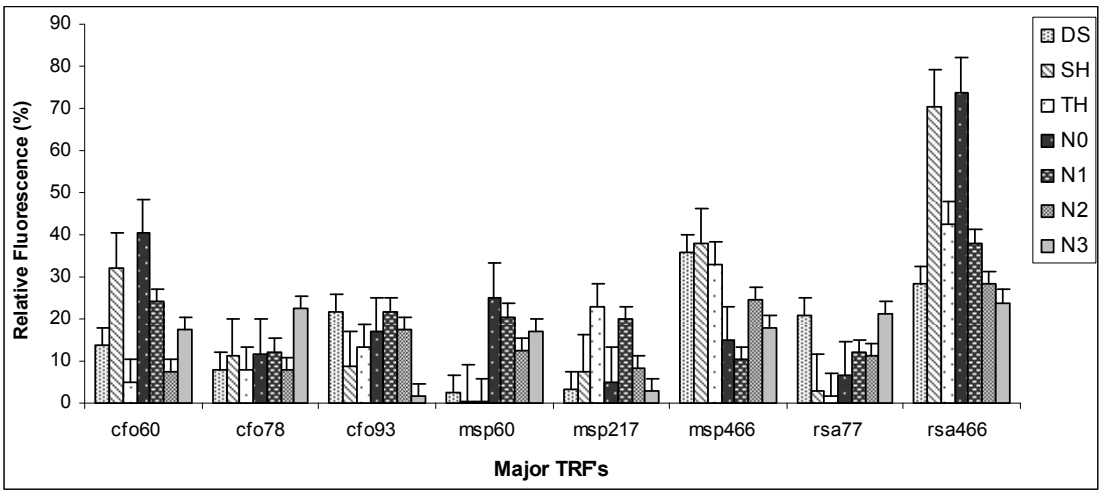

Figure 4.4. Major TRFs of the *nirK* gene generated by T-RFLP profiles from Betsele and Norrliden. Bars represent the average of nine cores from each treatment and error bars are the standard error of the mean.

Based indicator species analyses of the *nirK* communities from Betsele and Norrliden, 16 TRFs were identified as indicator species, of which, seven TRFs were found to be associated with Betsele and nine with Norrliden.

4.3 T-RFLP analysis of *nirS* gene

Greater numbers of TRFs were obtained from T-RFLP profiles by the digestion of three restriction enzymes CfoI, MspI and RsaI in case of *nirS* than that of *nirK* and *nosZ*. The Betsele site had 251 TRFs and 265 TRFs were found at Norrliden. Because so many TRFs were observed, I wanted to check whether these TRFs were

real or just noise. To do this, I compared cut-off values of 1, 1.5, and 2% relative fluorescence. This did not mitigate the problem, so I used the traditional cutoff of 1.5%. Furthermore, I tested the effect of removing one to six rare TRFs prior to MRPP and NMS analysis. Removing up to six rare TRFs strengthened the A-value slightly but the p-value did not change much. Therefore I did not remove rare TRFs for the analysis. This exercise gave insight that the difference in community structure was determined by the major TRFs not the rare TRFs.

A three-dimensional ordination was created by NMS with a cumulative variance explained of 90% for nirS at Betsele (Fig 4.5). Denitrifying community composition of the TH type at Betsele showed a significant difference with DS and a marginal difference with SH; however, there were no significant differences between DS and SH forests types (Table 4.2). The separation of denitrifier community composition of the TH forest type was correlated with by pH (r^2=0.64), NH_4^+ concentration (r^2 = 0.44) and NO_3^- concentration (r^2= 0.39). C-to-N ratio was related mostly to DS and was negatively correlated to Axis 1 (r^2= 0.59) (Fig 4.5).

Ordination of the T-RFLP profiles of the *nirS* gene at Norrliden yielded a two-dimensional solution with a cumulative variance of 86% (Fig 4.6). The ordination showed a distinct separation of the N3 community from the others. The denitrifier

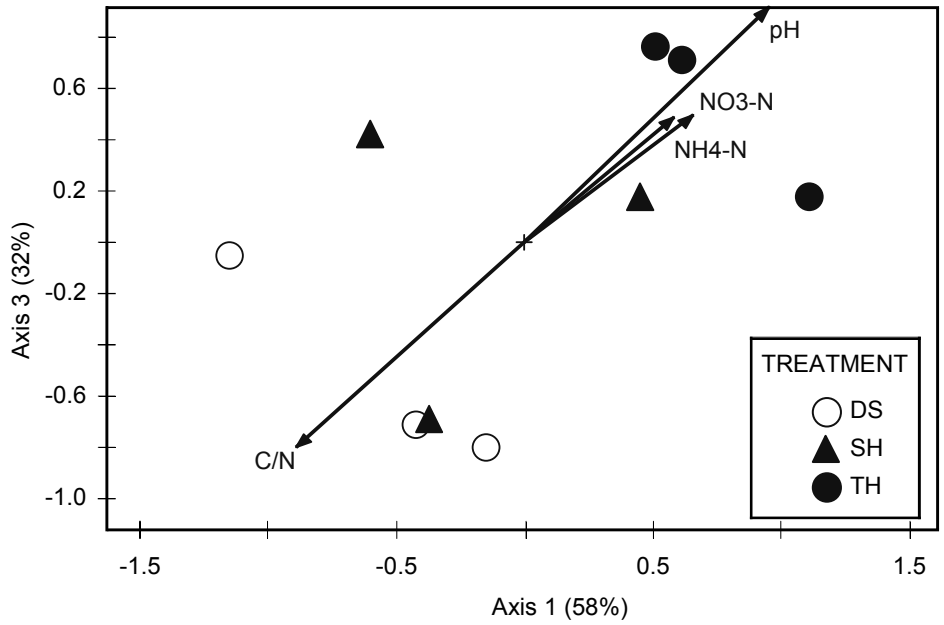

Fig 4.5. NMS ordination of denitrifying communities containing the *nirS* gene based on the TRFs generated by T-RFLP profiles from Betsele with the final stress of 2.60 and the final stability of 0.00000. The percent variances explained by the axes are in the parentheses. Vectors for NO_3-N (r^2=0.39), NH_4-N (r^2=0.44), pH (r^2=0.64), and C-to-N ratio (r^2=0.59).

communities from the N3 treatment showed significant differences with other treatments. The significant difference was also found between treatments N1 and N2 (Table 4.2). NO_3^- concentration (r^2 = 0.41) and pH (r^2 = 0.36) were found to be strongly correlated with N2 plots.

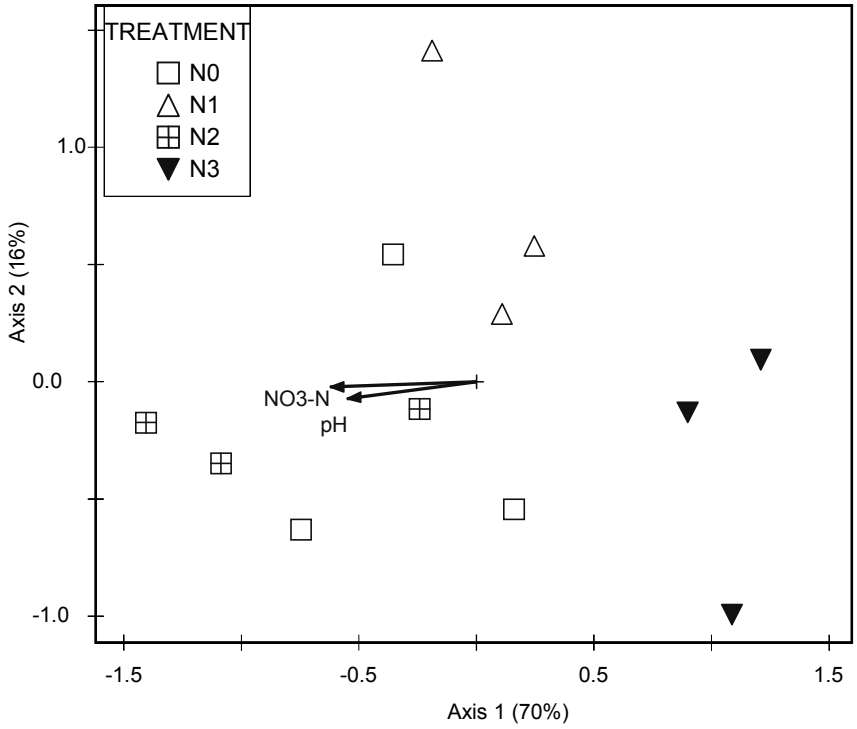

Fig 4.6. NMS Ordination of denitrifying communities containing the *nirS* gene from Norrliden with the final stress of 4.82 with final instability of 0.00000. The percent variances explained by the axes are in the parentheses. Vectors NO_3-N (r^2=0.41), and pH (r^2=0.36).

Indicator species analysis identified seven indicator TRFs from Betsele, of which five TRFs represented DS forest type (Table 4.5). In case of Norrliden, 13 TRFs were found as indicator species and Msp121 and Msp149 were perfect indicators of N1 and N2, respectively (Table 4.6).

Table 4.5. List of indicator TRFs identified for the *nirS* community at Betsele by indicator species analysis. The figures in the bold are the representative TRFs of their respective column (treatment).

TRFs	Indicator Value DS	Indicator Value SH	Indicator Value TH	p-value
Cfo66	11	**84**	0	0.0332
Cfo191	**68**	29	2	0.0332
Msp52	**77**	4	3	0.0384
Rsa54	**63**	35	1	0.0384
Rsa62	**79**	14	0	0.0384
Rsa217	**76**	16	0	0.0384
Rsa405	19	24	**57**	0.0344

Table 4.6. List of indicator TRFs identified for the *nirS* community at Norrliden. The figures in the bold are the representative TRFs of their respective column (treatment).

TRFs	Indicator Value N0	Indicator Value N1	Indicator Value N2	Indicator Value N3	p-value
Cfo89	28	0	**72**	0	0.0376
Cfo114	0	**91**	3	0	0.0196
Cfo123	17	0	3	**66**	0.038
Cfo192	17	3	**67**	2	0.0486
Cfo219	6	0	**71**	3	0.0374
Msp56	9	5	**62**	2	0.0366
Msp86	0	4	0	**89**	0.0194
Msp87	12	0	**68**	9	0.0382
Msp121	0	**100**	0	0	0.0196
Msp149	0	0	**100**	0	0.0184
Msp222	3	3	0	**92**	0.0194
Msp404	19	**72**	8	0	0.0192
Rsa293	**82**	0	6	0	0.0364

Eleven TRFs were identified as dominant TRFs with high mean relative fluorescence from Betsele and Norrliden (Fig 4.7). The major TRFs, Cfo228, Msp175, Msp220, and Rsa114 were present at a higher percentage at Norrliden, whereas Cfo192 and Msp218 were higher at Betsele.

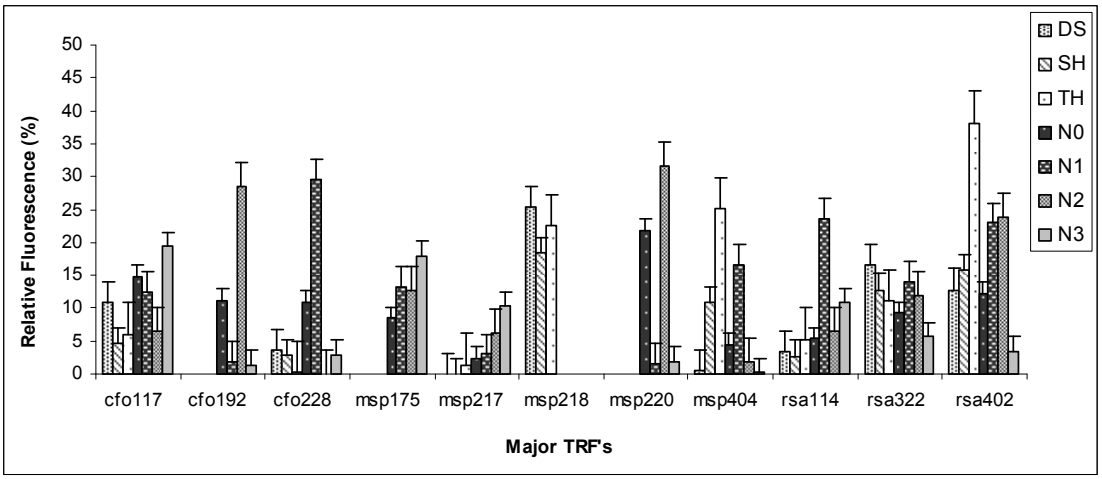

Fig 4.7. Major TRFs of the *nirS* communities from Betsele and Norrliden. Bars represent the average of 9 cores from each treatment and error bars are the standard error of the mean.

4.4 T-RFLP analysis of *nosZ* gene

Restriction digests using CfoI, MspI and RsaI produced 154 fragments from the amplification of the *nosZ* gene from Betsele and 187 from Norrliden. The T-RFLP profiles of *nosZ* gene from Betsele produced a two-dimensional solution by NMS, where the denitrifying communities clustered by forest type (Fig 4.8). MRPP showed that there was a significant treatment effect at Betsele (Table 4.2). Pairwise comparisons showed that the denitrifying communities containing the *nosZ* gene from the DS forest type was significantly different than the SH and TH forest types. Similarly, there was a significant difference between the SH and TH forest types (Table 4.2). From the joint plot, pH was highly correlated to the TH forest type (r^2 =

0.87) as well as Axis 1. Concentrations of NH_4^+ and NO_3^- were also correlated to the TH type ($r^2 = 0.41$ and $r^2 = 0.36$ respectively). The denitrifying community of the DS forest type was correlated to C-to-N ratio ($r^2 = 0.86$), which was negatively related to Axis 1 (Fig 4.8).

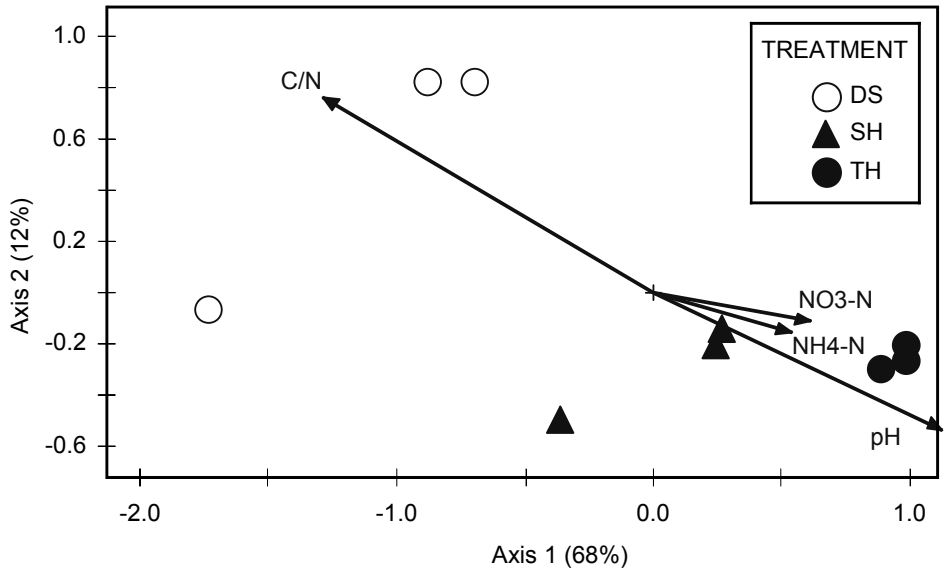

Fig 4.8. NMS ordination of TRFs generated from the *nosZ* gene at Betsele with the final stress of 0.0001 with final instability of 0.00001. The percent variances explained by the axes are in the parentheses. Vectors for NO_3-N (r^2=0.41), NH_4-N (r^2=0.36), pH (r^2=0.87) and C-to-N ratio (r^2=0.86).

Analysis of the *nosZ* gene at Norrliden generated a three-dimensional ordination with a cumulative variance explained of 80% (Fig 4.9). The denitrifying communities of the N2 plots were separated distinctly in the ordination on the lower right corner whereas the N3 plots were nestled between the N0 and N1 plots. There were significant treatment effects in denitrifying communities at Norrliden. Pairwise comparison using MRPP showed that the control N0 treatment was significantly

different from the N1 and N2 treatments. Similarly, the N1 treatment differed significantly from the N2 treatment. There was a significant difference between N2 and N3 communities and marginal differences between the N0 and N3 and the N1 and N3 treatments (Table 4.2). Those communities from the N2 treatment were related to NO_3^- and NH_4^+ concentration, and pH, and were negatively correlated to axis 3 (r^2 = 0.46, 0.43 and 0.37 respectively) while C-to-N ratio was negatively correlated to axis 2 (r^2 = 0.26).

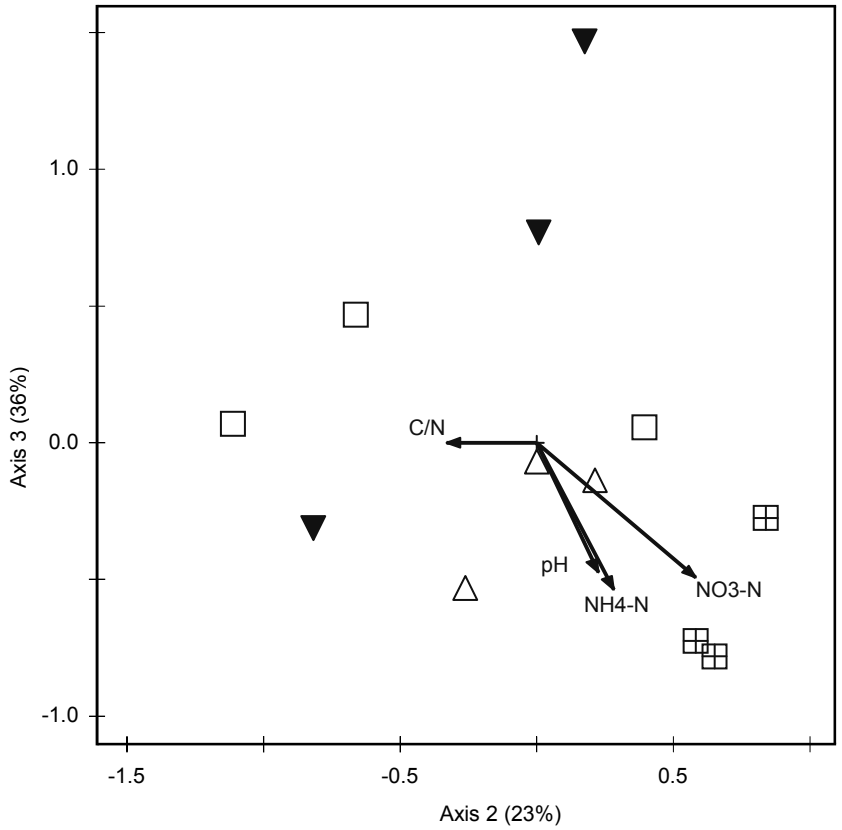

Fig 4.9. NMS ordination of TRFs generated by T-RFLP profiles of denitrifying communities containing the *nosZ* gene at Norrliden with the final stress of 5.68 with final instability of 0.00000. The percent variances explained by the axes are in the parentheses. Vectors for NO_3-N (r^2=0.46), NH_4-N (r^2=0.43), pH (r^2=0.37) and C-to-N ratio (r^2=0.26).

Seventeen TRFs from Betsele were identified as indicator species, with Cfo177, Cfo265, Msp265 and Rsa637 found to be perfect indicators of the TH forest type. Similarly, Cfo450 and Rsa480 were found only in the DS forest type and Msp201 and Rsa446 only in the SH forest (Table 4.7). Likewise, eight TRFs from Norrliden were found as indicator species (Table 4.8).

Table 4.7. Indicator TRFs of the *nosZ* gene community at Betsele. The figures in the bold are the representative TRFs of their respective column (treatment).

TRFs	Indicator Value DS	Indicator Value SH	Indicator Value TH	p-value
Cfo137	0	15	**78**	0.022
Cfo177	0	0	**100**	0.0368
Cfo265	0	0	**100**	0.0368
Cfo279	30	1	**67**	0.0368
Cfo354	0	2	**93**	0.0368
Cfo450	**100**	0	0	0.0354
Cfo475	0	2	**95**	0.0368
Msp107	0	2	**95**	0.0368
Msp191	**94**	2	0	0.0354
Msp201	0	**100**	0	0.041
Msp246	6	30	**61**	0.0326
Msp265	0	0	**100**	0.0368
Msp476	0	4	**89**	0.0368
Msp697	**60**	25	15	0.0354
Rsa446	0	**100**	0	0.041
Rsa480	**100**	0	0	0.0354
Rsa637	0	0	**100**	0.0368

Table 4.8. Indicator TRFs of the denitrifying communities related to the *nosZ* gene at Norrliden. The figures in the bold are the representative TRFs of their respective column (treatment).

TRFs	Indicator Value N0	Indicator Value N1	Indicator Value N2	Indicator value N3	p-value
Cfo79	0	29	**71**	0	0.0148
Cfo141	0	7	**78**	0	0.0184
Cfo346	0	0	**100**	0	0.0184
Cfo351	2	**52**	19	14	0.0246
Msp201	0	0	3	**90**	0.0372
Rsa262	0	0	**100**	0	0.0184
Rsa312	0	0	0	**100**	0.019
Rsa480	**99**	0	0	0	0.0176

Twelve major TRFs with higher mean florescence from the restriction digest of *nosZ* gene were observed. Cfo79, Cfo351, Msp218, and Rsa669 were found to be dominant in fertilized forest at Norrliden whereas Cfo354, Cfo698, Rsa664 were associated with Betsele. Msp52, Msp105 and Msp107, Rsa697 were found at both sites (Fig 4.10)

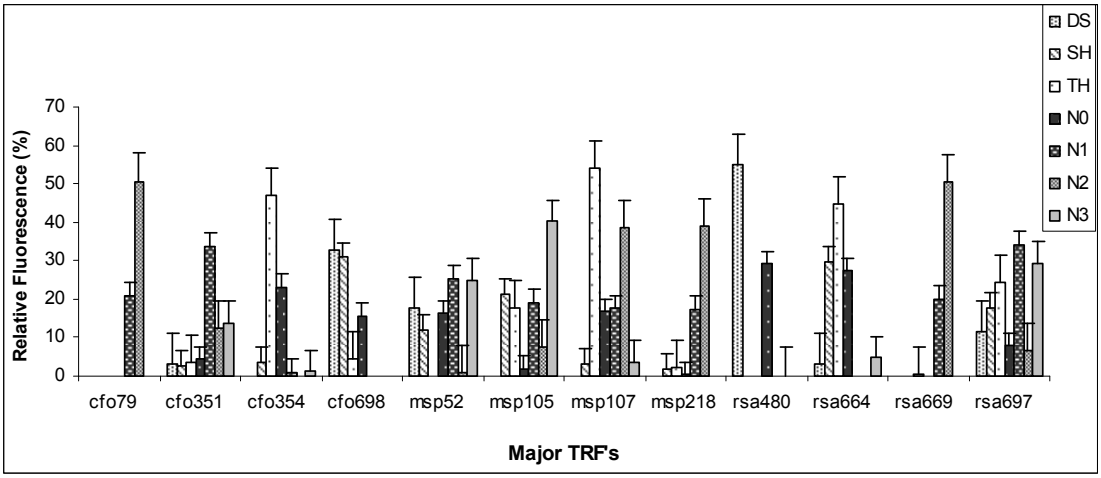

Fig 4.10. Dominant TRFs from the T-RFLP profiles of the *nosZ* gene at Betsele and Norrliden. Bars represent the average of nine cores from each treatment and error bars are the standard error of the mean.

Indicator species analysis identified 13 statistically significant TRFs of *nosZ* communities at Betsele and Norrliden. Msp697 was found to be a perfect indicator of Betsele.

A Mantel test was done to evaluate the correlation among distance matrices by using Sørenson distance and the method for evaluating test statistic was asymptotic approximation, where *nirS*, *nirK* and *nosZ* communities showed a positive association with each other (Table 4.9).

Table 4.9. Mantel test result for association among distance matrices based on Mantel`s asymptotic approximation method and Sørenson distance.

Treatment	Mantel statistic (r)†	t-statistic (t)‡	p-value
nirK and *nirS*	0.192	2.251	0.024
nirK and *nosZ*	0.207	2.353	0.018
nirS and *nosZ*	0.333	4.361	<0.001

† r is the standard Mantel statistic and ranges from -1 to 1.
‡ A t-statistic greater than 0 indicates the positive association between the matrices and less than 0 indicates negative association (McCune and Grace, 2002).

The T-RFLP profiles of all four functional genes were also evaluated based on the presence and absence of each TRF. The results obtained using presence/absence versus proportional abundance were similar for *amoA* and *nosZ*, but less so for *nirK* and *nirS* genes. In general, proportional abundance was more sensitive in detecting treatment differences that using just presence and absence (Table 4.10).

Table 4.10. Differences between ammonia oxidizing and denitrifying communities based on presence and absence. The p-value and A-value were calculated by using multi-response permutation procedures. The numbers in bold (A- value and p-value) are shown as significant. The A-value measures the chance-correlated within-group agreement; A p-value of < 0.05 was considered significant.

Site	Compared Treatment	***amoA***		***nirK***		***nirS***		***nosZ***	
		A	P	A	P	A	P	A	P
Betsele vs. Norrliden		**0.22**	**0.0004**	0.01	0.404	**0.12**	**0.0003**	0.02	0.166
Betsele treatment effect		-	-	**0.19**	**0.003**	**0.07**	**0.037**	**0.25**	**0.001**
Betsele	DS vs SH	-	-	0.14	0.052	-0.01	0.542	**0.12**	**0.023**
	DS vs TH	-	-	**0.20**	**0.028**	**0.13**	**0.023**	**0.32**	**0.021**
	SH vs TH	-	-	**0.12**	**0.024**	0.05	0.051	**0.21**	**0.025**
Norrliden treatment effect		-	-	0.05	0.083	**0.085**	**0.001**	**0.13**	**0.0005**
Norrliden	N0 vs N1	-	-	-0.02	0.789	**0.05**	**0.032**	**0.05**	**0.039**
	N0 vs N2	-	-	**0.13**	**0.030**	0.04	0.091	**0.15**	**0.023**
	N0 vs N3	-	-	**0.08**	**0.038**	0.03	0.178	**0.07**	**0.030**
	N1 vs N2	**0.10**	**0.023**	0.02	0.224	**0.08**	**0.034**	0.04	0.078
	N1 vs N3	-	-	-0.01	0.657	0.063	0.052	**0.10**	**0.024**
	N2 vs N3	-	-	0.02	0.129	**0.10**	**0.041**	**0.17**	**0.023**

CHAPTER FIVE

DISCUSSION

The main focus of this study was to investigate the variation in ammonia-oxidizer and denitrifier communities in an unmanaged forest with a natural gradient of N and pH, and in a forest subjected to long-term N fertilization. The investigation was done using the T-RFLP fingerprinting technique with the functional genes *amoA*, *nirK*, *nirS* and *nosZ*.

Amplification of the *amoA* gene was more successful in the forests with relatively high levels of N even after nested PCR. The natural forest gradient at Betsele varies widely in N availability and pH. Nitrogen and pH increase along the gradient from DS to SH to TH forest types (Högberg et al., 2006). The low amplification rate from DS and SH forest types might be due to the low NH_4^+ concentration present in the soil, which may limit the size of the ammonia-oxidizer community. This hypothesis is also supported by Högberg et al. (2006), who reported no gross nitrification in the DS and SH forest types. Along with low N availability, the low pH of the DS and SH forest types may limit the availability of NH_3 or otherwise make it a less favorable environment for AOB. This is consistent with very low numbers autotrophic nitrifiers based on MPN counts have been reported in acid soils of boreal forests (Martikainen, 1985; Klemendtsson et al., 1999), although other studies have reported ammonia-oxidizers and nitrification from low pH soils (Robertson, 1982; Lang and Jagnow, 1986; Killham, 1990; Papen and Von Berg, 1998). Even if nitrification is occurring in the soil, this study focused only on the AOB

community and recent studies have found that the archaeal ammonia-oxidizers are also active in soil and archaeal *amoA* gene copies were found up to 3000-fold more abundant than bacterial *amoA* gene copies in a survey of several soils (Leininger et al., 2006).

There were similar results in the fertilized forest at Norrlidin, where amplification of *amoA* was most successful for N1 and N2 treatments, which are still under fertilization, but below our detection limit for AOB in unfertilized plots. This result is supported by many previous studies where AOB population size was significantly greater in fertilized soils than in unfertilized soil (Mendum et al., 1999; Hermansson and Lindgren, 2001; Okano et al., 2004). Another study on N fertilization done by Wallenstein (2004) also found an increase in abundance of *amoA* on the strongly fertilized plots at Harvest Forest.

The AOB communities were significantly different from each other in the N1 and N2 plots at Norrliden. The difference in communities between these two plots might be due to the amount of fertilizer applied. Avrahami and Conrad (2003) found a shift in the AOB community with fertilization and identified NH_4^+ concentration as the selective factor. Other studies have found that the type of fertilization can cause changes in AOB communities (Hastings et al., 1997; Avrahami and Conrad, 2003; Avrahami et al., 2003; Chu et al., 2006). It has also been found that the fertilization can lead to changes in *Nitrosospira* communities (Prosser, 2007). Fertilization can also affect soil pH, which has selective effects on AOB structure as it controls the availability of NH_3 in the soil solution.

Amplification of denitrifier communities with *nirK* and *nirS* was more successful than it was for AOB communities after the nested PCR. I found that *nirK* community composition differed among all forest types at Betsele. The separation of these communities might be associated with concentrations of NO_3^-, NH_4^+, pH and C-to-N ratio. The DS forest type was strongly correlated with C-to-N ratio suggesting little or no mineralization and making less NH_4^+ available for nitrification and less NO_3^- available for denitrification. Nordin et al. (2001) also found higher concentrations of amino acids in DS than inorganic N. The community difference in the DS type may be driven by higher organic matter along with high C-to-N ratio. The denitrifying communities from TH forest type differed from DS and SH types and were highly correlated with pH and NO_3^- and NH_4^+ concentrations. Similarly, the N2 treatment at Norrliden, which got the highest amount of ammonium nitrate fertilizer, was separated from rest of the treatments at Norrliden. These communities from fertilized plots were also strongly correlated with pH and NO_3^- and NH_4^+ concentrations, and seemed to be driven by these three soil factors. In contrast with fertilized plots, the control plot was associated with C-to-N ratio. Previous studies have also reported changes in *nirK* and *nirS* communities in response to NO_3^- gradients and found that the diversity indices of communities were inversely proportional to NO_3^- concentration (Yan et al., 2003; Wallenstein et al., 2006). The change in community structure and abundance also may be due to changes in carbon availability and other geochemical characteristics of the environment. Wallenstein et al. (2006) suggested a direct effect of increased N on denitrifier community structure

and indirect effects by other "distal controls", which could be important in controlling denitrifying communities over the long term.

In other studies, it has been reported that the *nirK* community composition changed after the application of NH_4^+ at different concentrations (Avrahami et al., 2002). Similarly, Qiu et al. (2004) also found the correlation between the NH_4^+ content and *nirK* copy numbers. NH_4^+ concentration may serve to increase of supply of NO_3^- by nitrification. Along with NO_3^- and NH_4^+ concentration, pH is another environmental factor that was correlated with the TH communities at Betsele and the N2 treatment at Norrliden. Previous research suggested that there is no direct control of pH on denitrification but that it indirectly controls the carbon availability to denitrifiers in long-term acid sites (Koskinen and Keeney, 1982; Simek and Cooper, 2002). In another study, Parkin et al. (1985) found the adaptation of denitrifying population at low pH after a long-term exposure suggesting a direct effect of pH on selecting denitrifying population and indirect effects on population size. But further study is needed to find out the exact environmental factors responsible for shifting of these communities in controlled environment.

At Norrliden, the control plot differed from the fertilized plots; however, there were no significant differences among the fertilized plots suggesting that the difference in community may be due to fertilization addition. This result agrees with Wolsing and Prieme, (2004), who found a significant difference between mineral fertilized plots, a cattle manure plot and the control plot, showing the fertilizer type as determining factor for the denitrifying community than the fertilizer amount.

Avrahami et al. (2002) also showed that addition of NH_4^+ from low (6.5 μg NH_4^+-N g^{-1}) to medium (58 μg NH_4^+ -N g^{-1}) amounts caused a shift in *nirK* community structure but the same shift was noticed after the addition of high (395 μg NH_4^+-N g^{-1}) ammonium additions to a silt soil. This suggests that a shift in *nirK* community structure occurs once a threshold is reached and after that addition of more fertilizer may not be as important.

The *nirS* community composition also differed with the addition of fertilizer but the difference were more clear in the *nirK* gene, which might be due to greater variability of the nirK gene. The control plots did not differ significantly from the fertilized plots but the treatment plots differed from each other at Norrliden. In case of Betsele, the TH forest type differed significantly from DS and SH forest types. pH was found to be the strongest environmental factor associated with *nirS* communities of the TH forest type, followed by NH_4^+ and NO_3^-concentrations. Similar to the *nirK* community, the separation of the DS forest type might be due to high organic matter and higher C-to-N ratio. NO_3^- along with pH and NH_4^+ concentration were found to be the major factors to differentiate the composition of *nirS* community of the N2 treatment at Norrliden. As stated earlier, a controlled study is needed to find out the determining factors that are responsible for the shift in denitrifying communities.

When denitrifiers were assessed by *nosZ* composition, there were significant treatment effects at both sites. pH was strongly correlated with the TH forest type and C-to-N ratio was strongly correlated with the DS forest type. The low pH and high C-to-N ratio of the DS forest type might alter the nutrient availability, which could

influence the composition of the denitrifier community. Similar to our result, Prieme et al. (2002) and Enwall et al. (2005) reported pH as the strong influencing factor for the denitrifying communities in forested and well as agricultural soil, whereas Kandeler et al. (2006) found the amount of organic substances being an important factor that influences the *nirK* and *nosZ* communities. NO_3^- and NH_4^+ concentrations were also found to be associated with the *nosZ* community of the TH forest type. Besides these factors, the aboveground vegetation changes when it runs from DS to TH forest types at Betsele, which might also contribute to the difference in community structure. Previous studies have also reported the clustering of *nosZ* communities with respect to vegetation and site (Rich et al., 2003). Interestingly, the *nosZ* community composition showed marginal difference between the N0 and N3 treatments and the N1 and N3 treatments, suggesting the denitrifying community may be shifting back towards the control plots after the cessation of fertilizer. In another study at Norrliden, Högberg et al. (2007) found similar results with overall microbial community composition where the N3 treatment was intermediate to that of N0 and N1 treatments. They also reported in another study that soil pH and extractable inorganic N of N3 plots similar or approaching to N0 plots (Högberg et al., 2006). The N2 communities were significantly different from the other treatments. The shift in these N2 communities might be attributed to high NO_3^- and NH_4^+ concentrations.

As we know, denitrifiers have either the *nirK* or *nirS* gene for nitrite reductase, which is consistent with the different patterns observed with the *nirK* and *nirS* communities. Denitrifiers contain either the *nirK* or *nirS* gene along with *nosZ* gene. It

is therefore interesting that *nirK* denitrifiers differed with respect to the fertilizer and *nirS* denitrifiers with respect to fertilizer amount but the *nosZ* community composition differed by both the addition of fertilizer and by the amount of fertilizer applied. Moreover, the Mantel test suggested that all these denitrifying communities were positively associated with each other.

To examine whether the presence of the fragments from T-RFLP profiles show similar differences in communities, I ran the MRPP test after transforming the matrices in binary data. The presence and absence of these TRFs contribute to some extent to community differences. The MRPP result showed similar results to those based on the proportional abundance but the test statistics were not as strong.

Total numbers of TRFs were higher in case of *nirS* than *nirK* suggesting higher diversity in the *nirS* community. A similar result was reported by Prieme et al. (2002) where RFLP analysis showed lower diversity of *nirK* than *nirS* clones. Because many TRFs were generated by T-RFLP analysis, the validity of the TRFs was checked by taking rare species out, resulting in no change in the A-value and p-value during MRPP. This shows that the differences in community composition were contributed by dominant TRFs for all three denitrifying genes. Moreover, the number of TRFs and the population density of denitrifying bacteria may vary seasonally (Wolsing and Prieme, 2004; Mergel, et al., 2001a).

The *nosZ* TRFs were compared with the *nosZ* gene sequence table developed by Rich (2004; http://cropandsoil.oregonstate.edu/HJA_mo/results/nors/table.htm), who sequenced the 700-bp *nosZ* fragment and listed the restriction sites by CfoI, MspI

and RsaI enzymes. None of the TRFs reported in the table exactly matched with our TRFs but they were close in size; for example, I found Cfo354 bp, Msp107 bp, Msp218 bp, Rsa664 bp, Rsa669 bp, and Rsa697 bp where he reported Cfo357 bp, Msp111 bp, Msp112 bp, Msp222 bp, Rsa666 bp, Rsa672, and Rsa700bp. Likewise, the T-RFLP profile generated by the *amoA* gene produced a single major peak Taq279, which is similar to Taq283 bp fragment found by others, suggesting the presence of *Nitrosopira* and/or *Nitrosospira* like organisms (Horz et al., 2000; Mintie et al., 2003). Similarly, Alu388 bp and Alu489 bp were close to the fragment size found by Mintie (2002) for *Nitrosospira* Cluster 4 (Alu390), cluster 3 (Alu491). Our Alu489 bp/Cfo131 bp may resemble to that of uncultured AOBs, which had Alu491 bp/Cfo135 bp.

Because there was consistency of having TRFs 2 to 4 bp smaller than Rich (2004) and Mintie (2002) found, it can be concluded that fragments here represent similar fragments to those they found. The difference might be due to a systematic difference in how the sizes of the fragments were estimated. Only cloning and sequencing would help to verify these TRFs, and further research is needed to identify these organisms and their relationship with environmental factors.

CHAPTER SIX

CONCLUSION

The *amoA* gene of AOB and the *nirK*, *nirS* and *nosZ* genes of denitrifiers showed different patterns in response to soil N availability, and provided insight about those microorganisms responsible for regulating important steps in the N cycle. Even though many studies in the past have been done with microbial communities related to the N cycle, multiple functional genes are seldom included in the same study.

Amplification of AOB required the use of nested PCR and was more successful in the soils with relatively high levels of N, suggesting that N availability strongly influences the abundance of AOB. For example, amplification success at Norrliden was found only in those plots with N fertilization. Communities of AOB in fertilized plots varied significantly at different levels of N fertilization, suggesting that a differential response of AOB ecotypes to N availability.

Nested PCR was also required for effective amplification of the *nirK*, *nirS*, and *nosZ* genes associated with denitrifying bacteria; however, it was much more successful than amplification of the *amoA* gene of AOB. Thus, the abundance of denitrifiers was likely much higher than that of AOB. Differences in the size of these two functional groups of bacteria reflect their different growth requirements. AOB are chemolithoautotrophic and depend on NH_4^+ concentration for energy generation; in contrast, denitrification is performed by facultatively anaerobic heterotrophs that can use a wide range of carbon sources for energy.

Similar patterns in denitrifier community composition were observed at Betsele and Norrliden using *nirK*, *nirS*, and *nosZ* genes but these patterns were clearer and more significant differences were seen among treatments with *nosZ*. This may be because *nosZ* captures the diversity of the entire denitrifier community, whereas the *nir* genes are present in two, different sub-groups of the denitrifier community. In comparing the *nirK* and *nirS* communities, a greater number of TRFs were observed for *nirS* compared to *nirK*, suggesting greater richness of the denitrifiers containing *nirS*. This may be because *nirS* is often more prevalent than *nirK* in soil denitrifiers. The *nirK* gene was slightly better at detecting treatment differences than was *nirS*, however. This result may reflect greater variability of the *nirK* gene, and possibly denitrifier taxa, or that perhaps denitrifiers containing *nirK* were more sensitive to N availability or other environmental factors that varied among treatments.

The difference in denitrifying communities based on *nir* and *nosZ* genes was found to be associated with pH and organic matter concentration in the natural forest at Betsele, followed by NO_3^- and NH_4^+ concentrations. Similarly, in the fertilized forest at Norrliden, the community difference was driven predominantly by pH and NO_3^- and NH_4^+ concentrations. Although the different denitrification genes varied in the strength of their response to N availability, it is interesting that the *nosZ* community, and to a lesser extent the *nirK* community, displayed a linear response to increasing rates of N fertilization and a shift in community composition back towards the control plot after the cessation of the fertilizer.

Because many soil bacteria are notoriously difficult to culture, T-RFLP is a convenient tool for assessing their community structure. It does not, however, distinguish between active or dormant organisms. Consequently, one can only examine correlations between microbial community structure and activity. Further research that measures gene expression in situ is needed to solidify those links between structure and function that are suggested by T-RFLP analysis.

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