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Nitrification and denitrification are major biological processes transforming nitrogen (N) in soils to plant available N, highly leachable nitrate (NO_3^-) and gaseous N oxides. Although many studies in the past have studied N cycling communities, the effect of increased N inputs on ammonia-oxidizer and denitrifier population dynamics is still under active investigation. The purpose of this study was to examine the variation of ammonia-oxidizer and denitrifier populations in boreal forest soils providing markedly different localized sources of N availability. Here, we sampled along a natural N-availability gradient, characteristic of the inherent variation in N supply and plant productivity in a boreal forest, and in a long-term fertilized site receiving three levels of N fertilizer, of which the highest was terminated over 10 years ago. Quantitative PCR of archaeal (AOA) and bacterial (AOB) ammonia-oxidizer genes (*amoA*) and denitrifier genes (nitrite reductase, *nirS* and *nirK*; nitrous oxide reductase, *nosZ*) was used to quantify the populations. In these boreal soils the abundance of AOB amoA dominated

over AOA *amoA*. The size of the AOB population was significantly affected by fertilization application, whereas AOA was not; however, AOA amoA gene copies did increase in response to the high mineralization rates at the high N site along the natural gradient. Our findings indicate that AOB dominate ammonia oxidation in heavily fertilized soils and AOA may contribute significantly to ammonia oxidation in low N environments controlled by N mineralization. The abundance of *nirK* and *nosZ* responded most to N additions, but *nirS* did not, suggesting *nirK*-bearing denitrifiers dominate these high organic matter containing, well-drained soils. The size of the *nirK* and *nosZ* functional groups increased with increasing N availability at all sites. It was found that differences in gene copies per gram of soil could not be explained directly by increased NO_3^- concentrations but, rather indirectly, through an increase in organic matter substrate quality. In fact, the increased organic matter quality explained the high denitrifier population after fertilization cessation suggesting a resilient effect of fertilization lasting over a decade; whereas, the ammonia-oxidizer community returned to baseline population size after N fertilization was stopped. The community size results along the natural gradient were combined with previously published data on potential nitrification and denitrification rates. It was found that the difference in ammonia-oxidizer and denitrifier populations was positively related to differences in process rates. The study of these functional genes provides insight into the variability of the microbial community populations responsible for regulating important steps in the N cycle.

Ammonia-Oxidizer and Denitrifier Populations in Boreal Forest Soils of Differing Nitrogen Availability

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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.

Marci S. Burton, Author

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INTRODUCTION

Nitrification and denitrification are key microbial processes involved in the transformation of nitrogen (N) in soils. Nitrogen is an essential element for all living organisms. Although nitrogen gas (N_2) makes up about 78% of the atmosphere, in many terrestrial ecosystems, N is the limiting nutrient. The availability of N in the soil is mediated by microorganisms utilizing various forms of N for energy, through oxidation and reduction reactions.

Nitrification is a two-step aerobic process in which ammonia is oxidized to nitrite (NO_2^-) and subsequently NO_2^- is oxidized to nitrate (NO_3^-). Ammonia oxidation is considered the rate-limiting step in nitrification (De Boer and Kowalchuk 2001) and is carried out by both autotrophic ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The key enzyme for autotrophic ammonia oxidation is the membrane bound ammonia mono-oxygenase (AMO). The *amoA* gene encodes the active site-containing subunit for the ammonia mono-oxygenase (Hyman and Wood 1985); therefore, the *amoA* gene is used as a functional marker for AOA and AOB communities (Rotthauwe et al. 1995); (Tourna et al. 2008). The contributions of AOB and AOA to nitrification have been analyzed by correlating the relative abundance of *amoA* genes to nitrification rates in soils (Leininger et al. 2006, Boyle-Yarwood et al. 2008, Petersen et al. 2012, Alves et al. 2013). Most often AOA abundance exceeds AOB

abundance (Leininger et al. 2006, Morales et al. 2010) , including acidic soils (Prosser and Nicol 2012), but sometimes the AOB dominate(Petersen et al. 2012). However, it is not clear which conditions favor one or the other two types of ammonia oxidizers. Thus, linking environmental factors to population dynamics is fundamental to understanding the drivers of nitrification in soils.

Denitrification is the stepwise reduction of NO_3^- to NO_2^-, nitric oxide (NO), nitrous oxide (N_2O), and N_2. Each step is catalyzed by a metalloenzyme: dissimiliatory nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, respectively. The relative activities of these enzymes determines the amounts of intermediary N gases that are released to the atmosphere (Richardson et al. 2009). Functional genes encoding the different enzymes are used as molecular markers to characterize the denitrifier community. In this study, we focused on NO_2^- reduction and N_2O reduction. Nitrite reductase, encoded by the *nirK* and *nirS* genes, distinguishes denitrifiers from other NO_3^- respiring bacteria and is the first step yielding gaseous products (Knowles 1982). Although functionally similar, each enzyme differs in terms of structure and redox active metal cofactor: iron cd_1 type reductase for *nirS*, and a copper reductase for *nirk (Philippot 2002, Richardson et al. 2009)*. The *nosZ* gene codes for N_2O reductase, which is the final step in denitrification, returning N_2 to the atmosphere (Zumft 1997, Philippot 2002).

Ammonia-oxidizing and denitrifying processes in soil are influenced by environmental factors, primarily pH, temperature, moisture and O_2 supply (Wallenstein

et al. 2006, Nicol et al. 2008). Recent studies have linked the abundance and diversity of N cycling microorganisms to potential rates(Hallin et al. 2009, Petersen et al. 2012). However, it is possible that cell numbers of a given functional group would be so strongly correlated with process rates that they become independent of community composition and are driven by community size (Hallin et al. 2009). Petersen et al. (2012) found functional gene abundance to be the most important variable to predict potential N cycling rates

The abundance of ammonia-oxidizing and denitrifying communities vary spatially and temporally through variation of substrate inputs (Philippot et al. 2009, Stopnisek et al. 2010, Levičnik-Höfferle et al. 2012). Anthropogenic sources of fixed N have doubled the biological rate of terrestrial N inputs (Vitousek et al. 1997, Galloway et al. 2003, Canfield et al. 2010) and provide ~ 45% of the total fixed N produced annually on earth (Canfield et al. 2010). This increase in N availability in soils can lead to altered biogeochemical processes, shifting the relative balances of production and consumption of N products, with resulting byproducts such as NO_3^-, NO, and N_2O. Increased NO_3^- can be leached from the system and the gaseous losses can lead to ozone depletion (Chen et al. 2008) and increased global warming potential (Attard et al. 2011, Baggs 2011). It is well recognized that increased N availability is likely to increase nitrification and denitrification rates (Enwall et al. 2005, Wallenstein et al. 2006, Richardson et al. 2009) direct and indirect effects of increased N on community abundance are still an open question. Thus, understanding the factors that influence the net production of these N products by nitrifying and denitrifying bacteria at a soil population level is needed.

Boreal forest ecosystems are generally N limited and the low N availability is reflected in low plant productivity. The productivity of the forest and the N budget differs greatly with landscape position, as N supply and pH generally increase downhill as a result of water transport, resulting in dramatic differences within small geographic areas (Galloway et al. 2003, Högberg et al. 2005). Boreal forests are subject to increased levels of N deposition ranging from 80 to 1 kg N $ha^{-1}yr^{-1}$, in areas far from pollution (Högberg et al. 2006b). Furthermore, N fertilization is used in boreal forests as a management practice to increase forest production and soil C storage (Nohrstedt 2001, Olsson et al. 2005). When excess N is supplied and N becomes non-limiting for plant growth, net nitrification and denitrification can be induced (Vitousek et al. 1997, Galloway et al. 2003). This forest ecosystem provides a unique opportunity to relate microbial community abundance to ecosystem functioning in relation to the effects of increased N availability, related to landscape position and long-term fertilization.

Quantitative PCR of AOB and AOA *amoA* and the denitrification genes, *nirS*, *nirK*, and *nosZ* was used to quantify the soil ammonia-oxidizing and denitrifier communities. To explore the correlation of community abundance and ecosystem function, the abundance data were subjected to statistical analysis using soil parameters, together with the results on potential N cycling rates reported previously from the same sampling location (Högberg et al. 2005). This quantification allowed us to characterize the variability in ammonia-oxidizer and denitrifier abundance in soils that differ in environmental parameters as a function of increased N availability.

The objectives of this study were to examine the variation of ammonia-oxidizing and denitrifying communities: (i) along a natural N-availability gradient in a boreal forest; (ii) in a long-term fertilized forest receiving different levels of N fertilizer; and (iii) the effect of long-term fertilization after the termination of high levels of N fertilization.

The hypotheses were: (i) the abundance of the ammonia oxidizers will increase with increasing N inputs along the natural N gradient and increased fertilization rates; (ii) in this acidic soil, AOA will dominate over AOB; (iii) the denitrifier community abundance will follow the same trends as the ammonia-oxidizing community and increase concurrently with N inputs; (iii) both the ammonia-oxidizing and denitrifying communities will decrease after fertilizer termination.

LITERATURE REVIEW

Introduction

Nitrogen (N) is an essential element for life. Nitrogen takes many forms in soils and transformations among these forms are mediated by microorganisms. Nitrogen gas (N_2) makes up about 78% of the atmosphere; however, in many terrestrial ecosystems N is the limiting nutrient.

Human activities have altered the global N cycle by increasing available N in soils through increased industry and use of synthetic fertilizers. Industrial fixation of atmospheric N into agricultural fertilizers and the production of N oxides from combustion processes are two major sources of increased N deposition (Högberg 2007, Gruber and Galloway 2008, Canfield et al. 2010). Anthropogenic sources of fixed N contribute double the biological rate of terrestrial N_2 fixation (Vitousek et al. 1997, Galloway et al. 2003, Canfield et al. 2010), and provide ~ 45% of the total fixed N produced annually on earth (Canfield et al. 2010). From 1960 to 2000, the use of N fertilizers increased by ~ 800% (Canfield et al. 2010). In fact in 2008 alone, the Haber-Bosch process of ammonium (NH_4^+) production supplied 9.5 x 10^{12} mol N and fossil fuel combustion generated another 1.8 x 10^{12} mol N (Canfield et al. 2010).

Nitrification and denitrification are key microbial processes involved in transforming N in soils. Nitrification converts ammonia to nitrite (NO_2^-) and then nitrate (NO_3^-); and denitrification is the conversion of NO_3^- to N_2. During transformation in the soil a variety of N gases including nitric oxide (NO), and nitrous oxide (N_2O) are produced

as byproducts of microbial activity and can be harmful to the environment. Ninety percent of N fertilizer is NH_4^+-based (Canfield et al. 2010), which nitrifying bacteria can convert to highly mobile NO_3^-, which in turn can be leached from the soil system or converted to gaseous products via denitrification. Additional N inputs have the potential to alter biogeochemical pathways, shifting the relative balances of production and consumption of N products, with resulting byproducts such as NO and N_2O. The response of the nitrifier and denitrifier communities to increased N is likely to be affected by both the effects of increased N, and indirect effects of other environmental parameters. Furthermore, nitrification and denitrification are two important processes determining the fate of N in the soil. Thus, understanding the soil microbial response to increased N availability is essential for ecosystem health.

Nitrification

Nitrification is a two-step aerobic process in which ammonia is oxidized to NO_2^- and subsequently NO_2^- is oxidized to NO_3^-. Nitrification supplies NO_3^- to plants and microbes for assimilation. Alternatively, NO_3^- is converted to gaseous products via denitrification or leached from the soil due to the increased mobility. Two other products of nitrification include N_2O and acidity. The first step in nitrification is ammonia oxidation carried out mostly by autotrophic ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Nitrite-oxidizing bacteria (NOB) complete the two-step process; oxidizing NO_2^- to NO_3^-.

Ammonia Monooxygenase

The key enzyme for autotrophic ammonia oxidation is the membrane bound ammonia mono-oxygenase (AMO) encoded by the functional genes *amoA*, *amoB*, and *amoC*. The *amoA* gene encodes the active site-containing subunit for the AMO (Hyman and Wood, 1985) and is used as a molecular marker in the study of ammonia-oxidizing communities (Rotthauwe et al. 1995, Tourna et al. 2008). Ammonia, the substrate for this enzyme, rather than $NH4^+$, is converted to hydroxylamine and further oxidized to NO_2^- by the hydroxylamine oxidoreductase(Arp et al. 2002).

Until recently, ammonia oxidation was thought to be carried out mostly by bacteria (Treusch et al. 2005), that is all cultivated autotrophic ammonia oxidizers belonged to the betaproteobacteria (*Nitrosospira* and *Nitrosomonas*) or gammaproteobacteria (*Nitrosococcus*) (Leininger et al. 2006, Schleper and Nicol 2010). Recent metagenomic studies revealed homologues for the *amoA* and *amoB* bacterial subunits belonging to an archaeon (Treusch et al. 2005). Archaeal ammonia oxidation was confirmed in a subsequent laboratory isolate in which ammonia was used as the sole energy source, demonstrating conversion to NO_2^- and a concurrent increase in cell numbers (Konneke et al. 2005, Prosser and Nicol 2012). No homologous genes encoding for hydroxylamine oxidoreductase were found; however, NO_2^- reductase homologues were present (Schleper and Nicol 2010). Many bacteria and fungi are capable of heterotrophic ammonia oxidation; however, it is not linked to cellular growth (De Boer and Kowalchuk 2001).

AOA and AOB niche differentiation

The initial identification of archaeal AMO functional genes has led to molecular studies targeting the *amoA* gene to examine relative abundances of AOA to AOB ammonia oxidizers (Leininger et al. 2006, Boyle-Yarwood et al. 2008, Schleper and Nicol 2010). Bacterial and archaeal ammonia-oxidizing communities demonstrate ecological differentiation; therefore, linking the environmental factors to population dynamics is fundamental to understanding the drivers of nitrification in soils. Leininger et al. (2006) studied 12 different soils across Europe, including natural and managed ecosystems exhibiting different soil types with a wide range of pH, carbon (C) and N. In all samples archaeal *amoA* dominated over bacterial *amoA*; with AOA:AOB ratios ranging from 1.5 to over 230. However, in an acidic forest soil with high N, AOB outnumbered AOA with an AOA:AOB ratio of 0.42(Boyle-Yarwood et al. 2008). Additionally, AOB were found to be consistently higher than AOA across an N-limited boreal ecosystem including forest, fen, and grasslands (Petersen et al. 2012).

Preferential adaptation of AOA or AOB to an environment has been linked to physiological and environmental characteristics such as ammonia limitation and pH (Prosser and Nicol 2012). Since ammonia is the sole source of energy under aerobic conditions, differences in ammonia affinity, tolerance to ammonia concentrations, and the source of ammonia might be drivers for niche specialization(Prosser and Nicol 2012). One cultivated AOA (*Nitrosopumilus maritimus)* has a higher substrate affinity than AOB which has led to suggestions that AOA might be better adapted to growth in low NH_4^+

soils and AOB in high NH_4^+ soils (Prosser and Nicol 2012). Furthermore, ammonia can inhibit some AOA and AOB at high concentrations (Stopnisek et al. 2010, Prosser and Nicol 2012). Recent studies suggest niche specialization by AOA or AOB based on the N source; organic or inorganic sources of ammonia. There is evidence that the addition of inorganic NH_4^+ to soil does not stimulate archaeal ammonia oxidation, unlike bacterial ammonia oxidation (Stopnisek et al. 2010, Levičnik-Höfferle et al. 2012). Furthermore, Levičnik-Höfferle et al. (2012) demonstrated AOA preferentially utilize ammonia generated from the mineralization of organic N. In fact, oxidation of added organic N, but not added inorganic N, was accompanied by an increase in AOA *amoA* genes (Levičnik-Höfferle et al. 2012).

Soil pH plays an important regulatory role in the availability of ammonia due to the ionization of NH_4^+. Ammonium has a pK_a of 9.24 at 25°C; therefore, at lower pH the ammonia concentration in the soil solution will decrease (Stopnisek et al. 2010). Furthermore, most AOA and all AOB demonstrate little or no growth in a suspended liquid culture at a pH less than 6.5 (Lehtovirta-Morley et al. 2011). However, nitrification rates in acidic soils (pH < 5.5) equal or exceed those of neutral soils (Lehtovirta-Morley et al. 2011). It has also been suggested that the selection of distinct populations of microorganisms can be determinant for nitrification activity rather than whole populations (Alves et al. 2013). For example, *Nitrosotalea devanterra* grows optimally in the pH range 4 to 5 on an inorganic medium containing NH_4^+ and is restricted to the pH range of 4 to 5.5 (Lehtovirta-Morley et al. 2011). The organism has a high NO_2^--sensitivity, which suggests a mechanism for NO_2^- removal is present. The

discovery of this obligatory acidophilic thaumarchaeal ammonia oxidizer provides an explanation for nitrification occurring in acidic soils but to our knowledge acidophilic AOB have not been isolated.

Distinct environmental niches for AOA and AOB have not yet been identified, but a further understanding of the relative activities of both groups is needed for future models of soil nitrification and for development of control strategies to minimize N fertilizer loss and N_2O production.

Denitrification

Denitrification is the microbially mediated stepwise reduction of NO_3^- to NO_2^-, NO, N_2O, and finally N_2. The denitrification pathway is carried out by a wide range of denitrifying bacteria belonging to over 60 genera as well as some archaea and fungi (Philippot 2002, Demaneche et al. 2009). The N oxides are used as terminal electron acceptors when O_2 availability is low and organic C is available as the electron donor.

Four metalloenzymes catalyze each reduction: dissimiliatory NO_3^- reductase; NAR or NAP, NO_2^- reductase; NIR, NO reductase; NOR, and N_2O reductase; NOS. It is the relative activities of these enzymes that determine the amounts of intermediary N gases that are released to the atmosphere. The functional genes that code for these enzymes are used as molecular markers to characterize the denitrifier community. Screening for denitrifying communities is commonly based on the functional genes encoding the enzymes catalyzing the reduction of NO_2^- to gaseous N (*nirS* and *nirK*) and the gene encoding the N_2O reductase (*nosZ*).

Nitrate Reductase

The first step in the denitrification pathway is the reduction of NO_3^- to NO_2^-. The dissimilatory reduction of nitrate is catalyzed by two enzymes (NAR and NAP); however, these enzymes can be found in bacteria that do not denitrify. Therefore the genes *narG* and *napA* are not widely used in denitrifier studies (Wallenstein et al. 2006).

Nitrite Reductase

Nitrite reductase genes were the first to be used in denitrifier diversity studies, (Wallenstein et al. 2006) as the reduction of NO_2^- to NO is the key step in denitrification; it distinguishes denitrifiers from other NO_3^- respiring bacteria and is the first step yielding gaseous products (Knowles 1982).

The reduction of NO_2^- to NO can be catalyzed by two distinctly different NO_2^- reductases: a cytochrome cd_1 reductase and a multi-copper-containing reductase. Although functionally similar each enzyme differs in terms of structure and redox active metal cofactor: iron for *nirS* and copper for *nirK* (Knowles, 1982; Philippot, 2002; Richardson et al., 2009; Zumft, 1997). Several studies have suggested that *nirS* and *nirK* bearing organisms take up different niches in the environment or prefer different habitats. In a waterlogged boreal forest soil *nirS* copy numbers were found to be higher than *nirK*, suggestion *nirS* is better adapted for environments with high water content (Petersen et al., 2012). In a glacial succession study, *nirS* was more abundant in younger soils along a chronosequence; whereas, as soils developed, *nirK* increased. In agreement with this study, *nirK* was more abundant than *nirS* in soils with high organic

matter inputs (Hallin et al., 2009). Quantification of the *nirS* and *nirK* genes in an unfertilized bare fallow soil revealed a significantly higher *nirS*:*nirK* ratio than in treatments with crops (Hallin et al., 2009), suggesting selection for NIR type created by presence or absence of crops.

Nitric Oxide Reductase

Nitric oxide reductase is a periplasmic enzyme, crucial to energy generation. Nitric oxide is a cytotoxin (Philippot, 2002); thus, NO reduction is purported to occur in concerted fashion with NO consumption as a protective mechanism against toxic concentrations (Richardson et al., 2009).

Nitrous Oxide Reductase

Nitrous oxide (N_2O)reduction is the final step in denitrification; returning N_2 to the atmosphere. Nitrous oxide is an obligate intermediary, therefore, N_2O emission by denitrification is the result of imbalance between production and reduction (Henry et al., 2006a). Nitrous oxide production by denitrifiers is due to the regulation of the N_2O reductase activity as well as some denitrifying populations lacking the genes encoding this enzyme (Henry et al. 2006a).

The N_2O reductase (NOS) is a periplasmic enzyme encoded by the functional gene *nosZ*. Of all the denitrifying enzymes, the N_2O reductase is the most sensitive to O_2 (Philippot, 2002). Not only can transient exposure irreversibly damage the catalytic site, but the generation of super oxides can be toxic to the cells (Richardson et al., 2009).

The activity of NOS is also sensitive to pH, being most active at pH<7. Furthermore, it has been shown that some bacteria lack the *nosZ* gene coding for NOS (Richardson et al., 2009). This suggests that the denitrification population does not always carry out the final step of N_2O reduction in synchrony with the initial steps of the pathway, resulting in an increase of N_2O released to the atmosphere, making denitrification another important source of this greenhouse gas.

Nitrous Oxide

As a greenhouse gas, N_2O has 300 times the global warming potential as CO_2 and can persist in the atmosphere for ~120 years. N_2O is susceptible to photolysis with the subsequent formation of NO linked to ozone depletion (Chen et al., 2008). Two thirds of global N_2O emissions come from soil based processes (Chen et al., 2008), mainly nitrification and denitrification (Attard et al., 2011; Baggs, 2011). Better understanding of the factors that influence the net production of this gas by nitrifying and denitrifying bacteria at the soil population level is needed.

Environmental Controls

Denitrification rates and the denitrifier N_2O to N_2 ratio are controlled by pH, N availability, C availability, O_2 availability, and the C-to-N ratio (Wallenstein et al. 2006, Morley and Baggs 2010). Denitrifiers use organic C as the electron donor for energy production; therefore, an adequate supply of C substrate is needed to regulate denitrification rates. In fact, greater C availability often increases the reduction of N_2O to N_2 as C may be limiting for the final reductive step (Morley and Baggs, 2010).

Although NO_3^- supply is known to be a strong control on denitrification rates, a study in an acidic forest soil found little correlation to denitrifier abundance and NO_3^- concentrations (Wallenstein et al., 2006). However, after addition of NO_3^- fertilizers the ratio of N_2O to N_2 can be high due to a preference of NO_3^- over N_2O as an electron acceptor at concentrations greater than 10 mg NO_3^- g^{-1} soil (Richardson et al., 2009). Denitrifiers are facultative anaerobes; that is, as O_2 concentrations lower, denitrifiers use N oxides as alternative electron acceptors. The enzymes involved in denitrification are sensitive to O_2 to varying degrees, NO_3^-, NO_2^-, and NO reductases are generally tolerant of O_2, whereas the N_2O reductase is the most sensitive (Richardson et al., 2009). Due to the location of the active sites of the denitrification enzymes, their operations are sensitive to pH, and typically these enzymes are more active at a pH of 7 or below. In contrast, the NOS is most active at pH greater than 7. It has been suggested that at low pH, free nitrous acid generated from NO_2^-, inhibits NOS and the pH effect could be indirect (Richardson et al., 2009).

Community Abundance

The relationship between soil microbial community structure and ecosystem processes, such as nitrification and denitrification, is a research topic under active investigation. Previous studies have analyzed soil microbial processes by measuring process rates and enzyme activities, ignoring the composition of these communities (Firestone and Tiedje 1979, Wallenstein and Vilgalys 2005). The advent of molecular approaches has allowed for investigation into the community structure of N cycling

functional groups. One approach to investigating the composition of whole soil communities is to analyze the abundance of functional genes in genomic DNA extracted from soil samples via quantitative polymerase chain reaction (qPCR) (Henry et al., 2004, 2006a; Rotthauwe et al., 1995; Throback et al., 2004; Tourna et al., 2008; Wallenstein et al., 2006). Quantitative PCR targets the functional genes encoding for key enzymes catalyzing reactions in nitrification (*amoA*) and denitrification (*nirK*, *nirS*, *nosZ*). By utilizing qPCR the abundance of each gene in soil DNA extracts can be measured and used as a proxy for the community abundance of nitrifiers and denitrifiers. Previous studies on soil microbial communities involved in nitrification and denitrification have related process rates in relation to community composition rather than community size (Rich and Myrold 2004). However, it is possible that cell numbers of a given functional group would be so strongly correlated with process rates that they become independent of community composition and are driven by community size (Hallin et al., 2009). However, Attard et al. (2011) suggests soil environmental conditions rather than abundance and composition drive N cycling. The response of microbial communities to environmental change is an important component of ecosystem response.

Nitrogen Limited Forest Soils

Boreal forests offer a unique opportunity to study N cycling processes because of the dramatic differences in productivity within small geographical areas (Petersen et al., 2012). In severely N limited systems, such as boreal forests, low N availability is

reflected in low plant productivity (Galloway et al., 2003; Högberg et al., 2005). The productivity of the forest and the N budget differs greatly with landscape position, as N supply and soil pH generally increase downhill as a result of water transport(Högberg et al. 2006b). Boreal forests are subject to increased levels of N deposition ranging from 80 kg N ha^{-1} yr^{-1} to 1 kg N ha^{-1} yr^{-1}, in areas far from pollution (Högberg et al., 2006). Furthermore, N fertilization is often used as a management practice in these low productivity forests (Nohrstedt, 2001). Thus, in these N limited forests, addition of N could potentially increase forest production and subsequently soil C storage (Nohrstedt, 2001; Olsson et al., 2005). However, when excess N is supplied and N becomes non-limiting for plant growth, net nitrification can be induced and result in possible negative effects such as NO_3^- leaching, N_2O efflux, and acidification (Galloway et al., 2003; Vitousek et al., 1997). It is well recognized that increased N availability is likely to increase nitrification and denitrification rates (Richardson et al., 2009; Wallenstein et al., 2006) but the long-term direct and indirect effects of increased N on community abundance are still an open question.

MATERIALS AND METHODS

Introduction

Two sites, Betsele and Norrliden, located in northern Sweden were studied. These sites represent a natural N availability gradient, Betsele, and a long-term fertilization experiment receiving different levels of N fertilizer, Norrliden. These sites provide an ideal environment to study variation of N-cycling microbial community abundance in response to different N availabilities.

Site Description

Betsele

Betsele is located in northern Sweden (64°39'N, 18°30'E, 235 m altitude). The site consists of three, 90 m transects, 25 – 70 m apart, through a 130-year-old forest. These transects represent a natural N availability gradient as they transition downhill from dwarf shrub (DS), across a short herb (SH), to a tall herb (TH) forest type. Forest productivity increases across the gradient with DS being a low-productivity forest, SH intermediate, and TH highly productive. Along with productivity, pH also increases across the gradient 4.0 to 5.3, respectively (Table 1). The DS type is a *Pinus sylvestris* forest with the field layer dominated by ericaceous dwarf shrubs, e.g., *Empetrum hermaphroditum*. The SH type is densely populated with *Picea abies* with abundance of several short herbs, e.g., *Oxalis acetosella*. The TH type is dominated by *Picea abies* trees and abundant tall herbs, e.g., *Rubus idaeus*. Soils are sandy till soils with many boulders, classified as Haplic Podzol in the FAO-system (Högberg et al., 2005). There is

a slight downhill slope of 2%. Mean annual temperature is 1° C and mean annual precipitation is 570 mm. The site is covered by snow from late October until early May.

Norrliden

Norrliden is located about 65 km from Betsele (64°21'N, 19°45'E, 267 M altitude) in a 50-year-old, low-productivity *Pinus sylvestris* forest of the DS type. The climate here is similar to Betsele and the soil is also classified as a Haplic Podzol or Typic Haplocryod in US Soil Taxonomy (Högberg et al. 2006a).The site consists of four 30 X 30 m plots designated N0-N3 with four differing rates of ammonium nitrate (NH_4NO_3) applied annually, with three replicate plots per treatment. N0 is the unfertilized control, N1 receives 34 kg N ha^{-1}, N2 68 kg N ha^{-1} , and N3 received the highest rate of 108 kg N ha^{-1}. Fertilizer application at the N3 site was stopped ten years prior to sampling, thus N3 represents a site recovering from a high N application.

Soil Sampling

Soils were sampled in 2004 (see Högberg et al., 2005 for details) Briefly, three samples of the organic mor layer (Oe and Oa horizons) were taken at each forest type on a transect at Betsele, or a treatment plot at Norrliden with a 0.15-m auger and bulked together representing one replicate. The corer was cleaned between each forest type and treatment. Soils were passed through a 5-mm sieve and placed into plastic bags. Soil chemical analysis was performed by Högberg et al. (2005), results can be found in Table 1. Soils were stored at -20° C for subsequent molecular techniques. The mean values of the three replicate transects at each forest type (DS, SH, and TH) at

Betsele, and the mean values of the three replicate plots of each treatment (N0 to N3) at Norrliden, were used in statistical analysis, therefore n=3.

DNA Extraction

DNA was extracted from soil samples using the Powersoil DNA Isolation kit (MO Bio Laboratories, Carlsbad, CA), according to the protocol of the manufacturer. In short, about 0.25 g of soil was used per extraction and eluted to 100 µL. Extracted DNA was quantified using a NanoDrop^{TM} ND-1000 UV-Vis Spectrophotometer (ThermoScientific, Rockwood, TN, USA). DNA extracts were diluted to 25 ng μl^{-1} and stored at -20° C for downstream applications.

Quantitative Polymerase Chain Reaction (QPCR)

Quantitative PCR was performed to assess the abundance of the following genes: *amoA* (AOA and AOB), *nirK* and *nirS* (denitrifiers carrying a NO_2^- reductase gene), *nosZ* (denitrifiers carrying the N_2O reductase gene), and the 16S rRNA gene (all bacteria). All qPCR reactions were conducted with an ABI 7500 Real Time PCR System (Foster City, CA, USA). All samples were run in triplicate. Primers specific to genes and detailed qPCR reactions and conditions for each gene are listed below. At the end of each qPCR run, a melting curve was included to confirm the fluorescence signal originated form specific PCR products and not from primer dimers. Furthermore, qPCR products were run on an agarose gel (1.2%) stained with ethidium bromide to check for the correctly sized amplicon. Standard curves based on known quantities of copies were generated to calculate relative gene abundance.

Bacterial *amoA* quantification

Bacterial *amoA* genes were amplified using primers *amoA*_1R and *amoA*_2F (Rotthauwe et al., 1995). Each 25-µl reaction volume included 12.5 µl SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.2 µM of each primer, 0.064% bovine serum albumin (BSA), and 2 µl of diluted DNA template corresponding to 10 ng total DNA.

Thermocycler conditions were 2 min at 50°C, 10 min at 95°C; 40 cycles of 20 s at 95°C for denaturation, 45 s at 55°C for annealing, 60 s at 76°C for extension and data acquisition. The extension and data collection temperature was raised to 76° in order to eliminate false enumeration due to primer dimer interactions at lower temperatures. The increased temperature also resulted in a higher efficiency for the reaction. Standard curves were constructed with *Nitrosomonas europea* genomic DNA. The efficiency ranged from 90-95% and r^2=0.98-0.99.

Archaeal *amoA* quantification

Archaeal *amoA* genes were amplified using primers Cren*amoA*23f and Cren*amoA*616r (Tourna et al., 2008). Each 25-µl reaction volume included 12.5 µl SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.2 µM of each primer, 0.064% BSA, and 2 µl of diluted DNA template corresponding to 10 ng total DNA. Thermocycler conditions were 2 min at 50°C, 10 min at 95°C; 40 cycles of 20 s at 95°C for denaturation, 45 s at 50°C for annealing, 60 s at 76°C for extension and data acquisition. The extension and data collection temperature was raised to 76° in order to

eliminate false enumeration due to primer dimer interactions at lower temperatures. The increased temperature also resulted in a higher efficiency for the reaction. Standard curves were constructed with *Nitrosopumilus maritimus* genomic DNA. The efficiency ranged from 91-92% and r^2=0.98-0.99.

nirK quantification

Amplification of *nirK* genes was conducted using primers nirK876F and nirK1040R (Henry et al. 2004). Each 25-µl reaction volume included 12.5 µl Brilliant II SYBR Master Mix with high ROX (Agilent Technologies, La Jolla, CA, USA), 0.5 µM of each primer, 0.675 µl dimethyl sulphoxide (DMSO), 0.064% BSA, and 2 µl of diluted DNA template corresponding to 5 ng total DNA. Thermocycler conditions were as follows: 2 min at 50°C, 10 min at 95°C; afterwards six touchdown cycles were performed: 30 s at 95°C, 30 s at 63°C progressively decreasing 1 degree per cycle to 58°C, 30 s at 72°C and 15 s at 80°C; followed by 40 cycles: 30 s at 95°C for denaturation, 30 s at 58°C for annealing, 30 s at 72°C for extension and 30 s at 80°C for data acquisition. DMSO was added to the reaction in order to eliminate double bands apparent in standard PCR reactions carried out for quality control. DMSO aids in the denaturation step, teasing apart high GC content areas in the DNA sequence. Standard curves were constructed with a linearized TOPO plasmid containing a *nirK* gene cloned from a soil amplicon. The efficiency ranged from 98-100% and r^2=0.99.

nirS quantification

The *nirS* genes were amplified using the primers nirSCd3aF and nirSR3cd (Throback et al. 2004). Each 25-µl reaction volume included 12.5 µl Brilliant II SYBR Master Mix with high ROX (Agilent Technologies, La Jolla, CA, USA), 0.3 µM of each primer, 0.064% BSA, and 2 µl of diluted DNA template corresponding to 5 ng total DNA. Thermocycler conditions were as follows: 2 min at 50°C, 10 min at 95°C; afterwards six touchdown cycles were performed: 15 s at 95°C, 30 s at 63°C-58°C, 30 s at 72°C and 15 s at 80°C; followed by 40 cycles: 15 s at 95°C for denaturation, 30s at 58°C for annealing, 30 s at 72°C for extension and 30 s at 80°C for data acquisition. Standard curves were constructed with a linearized TOPO plasmid containing a *nirS* gene cloned from a soil amplicon. The efficiency ranged from 94-103% and r^2=0.98-0.99.

nosZ quantification

The *nosZ* genes were amplified using the primers nosZ2F and nosZ2R (Henry et al., 2006a). Each 25-µl reaction volume included 12.5 µl SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.3 µM of each primer, 0.064% BSA, and 2 µl of diluted DNA template corresponding to 5 ng total DNA. Thermocycler conditions were as follows: 2 min at 50°C, 10 min at 95°C; afterwards six touchdown cycles were performed: 15 s at 95°C, 30 s at 65°C progressively decreasing 1 degree per cycle to 60°C, 30 s at 72°C and 30 s at 80°C; followed by 40 cycles: 15 s at 95°C for denaturation, 30 s at 60°C for annealing, 30 s at 72°C for extension and 30 s at 80°C for data acquisition. It is important to note that the use of Brilliant II SYBR Master Mix with high ROX (Agilent Technologies, La Jolla, CA, USA) resulted in positive amplification in the

negative control. We subsequently switched chemistries in order to ensure amplification of the target gene. Standard curves were constructed with a linearized TOPO plasmid containing a *nosZ* gene cloned from a soil amplicon. The efficiency ranged from 98-100% and r^2=0.97-0.99.

16S rRNA quantification

Amplification of 16S rRNA genes was done using primers EUB338 and EUB518 (Fierer et al. 2005). Each 25-µl reaction volume included 12.5 µl Brilliant II SYBR Master Mix with high ROX (Agilent Technologies, La Jolla, CA, USA), 0.5 µM of each primer, 0.064% BSA, and 2 µl of diluted DNA template corresponding to 5 ng total DNA. Thermocycler conditions were as follows: 2 min at 50°C, 10 min at 95°C; 40 cycles: 60 s at 95°C for denaturation, 30 s at 53°C for annealing, 60 s at 72°C for extension and data acquisition. Standard curves were constructed with a linearized TOPO plasmid containing a 16S rRNA gene cloned from a soil amplicon. The efficiency ranged from 101-106% and r^2=0.99.

Statistical analysis

The copy numbers of 16S rRNA, bacterial and archaeal *amoA*, *nirK*, *nirS*, and *nosZ* genes in the soil were calculated on an oven-dry weight basis normalized for extracted DNA mass. Statistical analyses were conducted using R (R Foundation for Statistical Computing). Non-normal data were log transformed. A one-way analysis of variance (ANOVA) was performed to compare gene abundance of forest types and treatments, followed by the LSD (Least Significant Differences) test at $P < 0.05$. The treatment means

and standard deviations presented in figures were calculated from untransformed data. The Pearson product moment correlation coefficient (r) was applied to evaluate relationships between soil environmental factors (C to N ratio, pH, NO_3^-, and NH_4^+) and the abundance of the ammonia-oxidizing and denitrifying genes. T-tests were performed to compare different gene abundance pools by site.

Because the DS forest at Betsele is comparable to the control (N0) at Norrliden, the experimental design allows us to analyze the community abundance response to the varying range of soil environmental factors found at both sites combined. In fact, due to the hydrochemistry of the slope at Betsele multiple factors are highly correlated and this combined analysis could rule out the effects of confounding factors.

RESULTS

Ammonia-Oxidizing Community

Abundance of the AOA and AOB communities was quantified using the copy numbers of the functional gene *amoA*.

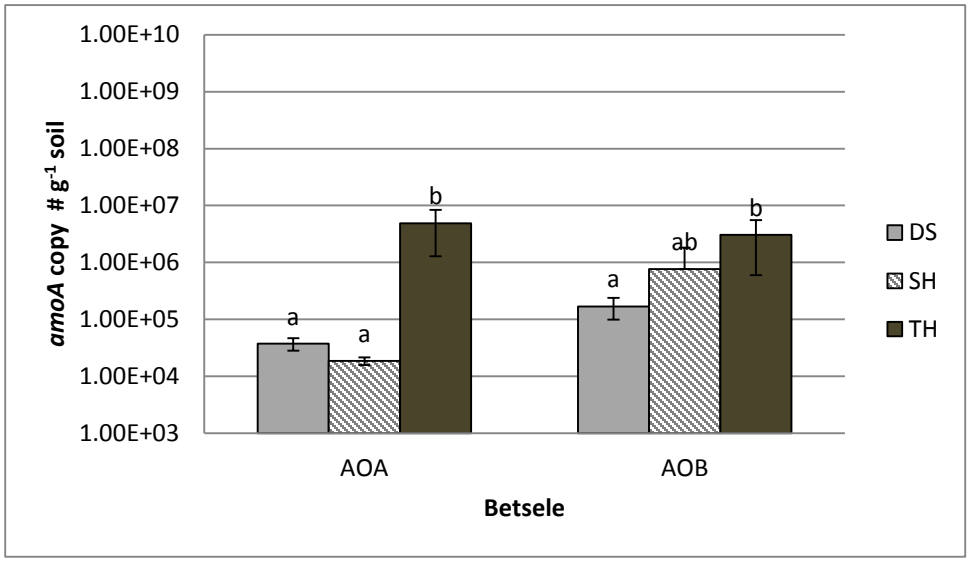

Figure 1 Ammonia oxidizing archaeal (AOA) and bacterial (AOB) *amoA* gene copy numbers at Betsele. Bars represent mean values of *amoA* gene abundance by treatment: dwarf shrub (DS), short herb (SH), tall herb (TH) (n=3). Error bars represent the SDs. Values with the same letter within each type of ammonia oxidizer do not significantly differ according to LSD test (P<0.05).

At Betsele, the mean number of AOA *amoA* genes ranged from 10^4 to 10^6 copies g^{-1} soil. Copy numbers of AOA *amoA* were significantly higher at the TH site than at the SH and DS, whereas, there was no significant difference between DS and SH (Figure 1). Copy numbers of AOB *amoA* increased downhill from DS to TH forest types, ranging from 10^5 to 10^6 copies g^{-1} soil. AOB *amoA* were also higher at the TH forest type than the DS site; however, there was no significant difference between the DS and the SH or the SH and the TH forest types (Figure 1) ($P<0.05$). There was a positive and significant

correlation of AOB *amoA* copy numbers with *nirK*, *nosZ,* and AOA *amoA* copy numbers (P<0.05); whereas, AOB *amoA* did not correlate with abundance of *nirS* or 16S rRNA gene copy numbers (Table 2). AOA *amoA* copy numbers were positively and significantly correlated with *nirK*, *nosZ,* and AOB *amoA* copy numbers, and not significantly correlated to *nirS* and 16S copy numbers (P<0.05). Furthermore, both AOB and AOA *amoA* copy numbers were positively and significantly correlated to pH, NH_4^+, and NO_3^-, but not the C:N ratio (P<0.05) (Table 2).

The ratios of AOA:AOB copy numbers at the DS, SH, and TH forest type were 0.22, 0.02, and 1.59, respectively. Bacterial *amoA* were significantly higher than archaeal *amoA* at the DS and SH forest type; however, there was no difference between AOB and AOA at the TH forest type (P<0.05).

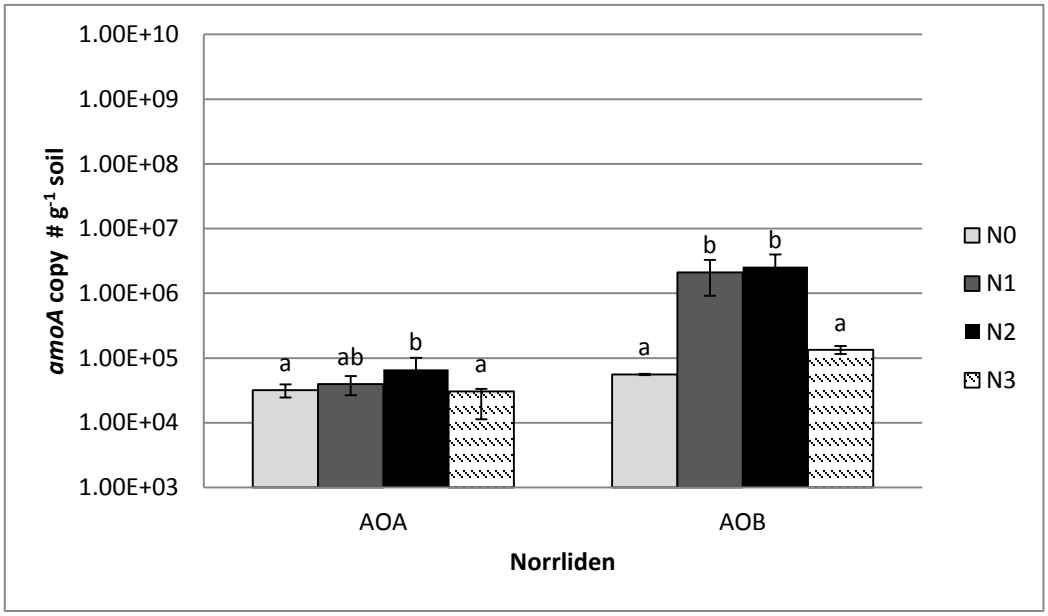

Figure 2. Ammonia oxidizing archaeal (AOA) and bacterial (AOB) *amoA* gene copy numbers at Norrliden. Bars represent mean values of *amoA* gene abundance by treatment: N0 is the control, N1, N2, and N3 denote increasing rate of N load and N3 was terminated in 1991 (n=3). Error bars represent the SDs. Values with the same letter within each type of ammonia oxidizer do not significantly differ according to LSD test (P<0.05).

At Norrliden, the mean number of AOA *amoA* genes was about 10^4 copies g^{-1} soil regardless of treatment. There was a significant difference in the AOA copy numbers in the N2 treatment compared to the control (N0) and N3 treatment; however, there was no significant difference between N1 and N2, and between N1 and N0 treatments (Figure 2) ($P<0.05$). Ammonia-oxidizing bacterial *amoA* copy numbers in the N1 and N2 treatments increased compared to the N0 site. Mean *amoA* copy numbers range from 10^4 to 10^6 copies g^{-1} soil. There was a significant difference between N0 and the N1 and N2 treatments; whereas there was no significant difference between the N3 and N0 (Figure 2) ($P<0.05$). AOB and AOA *amoA* copy numbers were not significantly correlated with any other gene copy numbers ($P<0.05$) (Table 3). AOB *amoA* copy numbers were positively and significantly correlated with NH_4^+ and NO_3^-; whereas, AOA *amoA* copy numbers were positively and significantly correlated with pH and NH_4^+ ($P<0.05$) (Table 3).

The ratios of AOA:AOB at Norrliden were 0.57, 0.02, 0.03, and 0.23 for N0, N1, N2, and N3, respectively. Copy numbers of *amoA* for AOB were significantly higher than AOA in each treatment ($P<0.05$).

When *amoA* copy numbers were analyzed in response to the differing N availabilities and environmental parameters found across both sites, AOB *amoA* had a positive and significant correlation with inorganic N, NH_4^+, NO_3^-, and pH, as well as a negative and significant correlation with the C:N ratio. In contrast, AOA *amoA* was not

significantly correlated to inorganic N; however, there was a positive correlation with pH and a negative correlation to the C:N ratio ($P<0.05$) (Table 4)

Denitrifying Community

To quantify the denitrifying bacterial community, genes coding for the NO_2^- reductase (*nirK* and *nirS*) and N_2O reductase (*nosZ*) were measured.

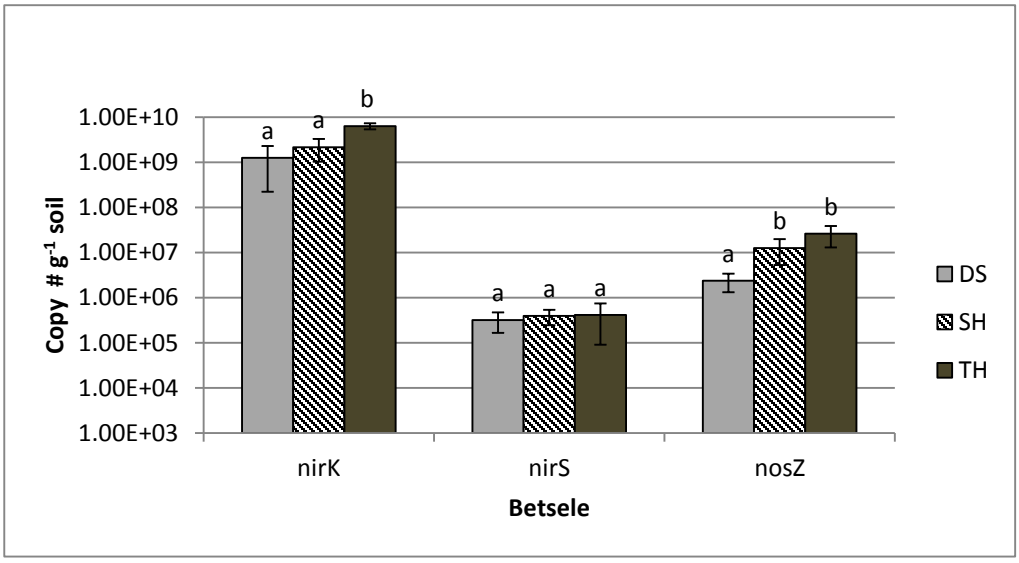

Figure 3. Denitrifier gene copy numbers: NO_2^- reductase (nirK and nirS) and N_2O reductase (nosZ), at Betsele. Bars represent mean values of functional gene abundance by treatment: dwarf shrub (DS), short herb (SH), tall herb (TH) (n=3). Error bars represent the SDs. Values with the same letter within each gene do not significantly differ according to LSD test ($P<0.05$).

At Betsele, NO_2^- reductase was dominated by the *nirK* type; *nirK* gene abundances were over four orders of magnitude higher than *nirS* (Figure 3). Gene abundances of *nirK* increased five-fold along the gradient at Betsele from the DS to TH forest type. Gene abundances of *nirK* were significantly higher at the TH forest type as compared to DS and SH ($P<0.05$). Gene abundances of *nirS* remained stable across the

gradient with no significant difference amongst sites ($P<0.05$). The *nosZ* gene copy numbers showed a comparable pattern to *nirK*, where the lowest number of *nosZ* was at the DS forest type, and the highest at the TH forest type. Copy numbers of *nosZ* at the DS forest type were significantly different than SH and TH; whereas, there was no significant difference between SH and TH. The relative abundance of NO_2^- reductase (*nirK* + *nirS*) genes was significantly higher than the N_2O reductase genes at all forest types ($P<0.05$). Generally, NO_2^- reductase copy numbers were three orders of magnitude higher than N_2O reductase.

At Betsele, *nirK* copy numbers had a positive and significant correlation with *nosZ*, 16S rRNA, and AOA and AOB *amoA* copy numbers, but not *nirS*. Furthermore *nirK* was positively and significantly correlated with pH; whereas a negative and significant correlation with the C:N ratio was observed ($P<0.05$) (Table 2). Copy numbers of *nirS* positively and significantly correlated with *nosZ* and NH_4^+ ($P<0.05$) (Table 2). Copy numbers of n*osZ* had a positive and significant correlation with *nirK*, *nirS*, and AOA and AOB *amoA* gene copy numbers. Additionally, *nosZ* was positively and significantly correlated to pH and NH_4^+, and negatively correlated to the C:N ratio ($P<0.05$) (Table 2).

There were no significant differences in 16S rRNA gene copy numbers between forest types at Betsele ($P<0.05$) (Table 5). At Norrliden, 16S rRNA gene copy numbers at N1 and N3 were significantly different from the control; however, there was no difference between N2 and the control ($P<0.05$) (Table 5).

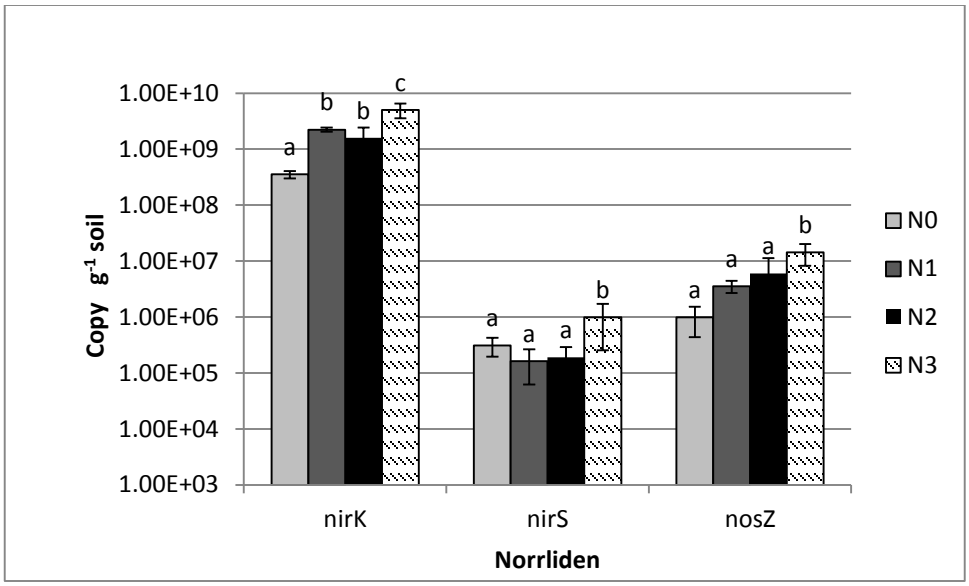

Figure 4. Denitrifier gene copy numbers: nitrite reductase (nirK and nirS) and nitrous oxide reductase (nosZ), at Norrliden. Bars represent mean values of functional gene abundance by treatment: N0 is the control, N1, N2, and N3 denote increasing rate of N load and N3 was terminated in 1991(n=3). Error bars represent the SDs. Values with the same letter within each gene do not significantly differ according to LSD test (P<0.05).

Abundance of *nirK* gene copy numbers was higher than *nirS* for all treatments at Norrliden (Figure 4). The abundance of *nirK* at N0 was significantly different than the N1, N2, and N3 sites ($P<0.05$). Similarly, there was a significant difference between the N3 and the N1, N2, and N0 sites. However, there was no difference between N1 and N2. For *nirS* abundance, the only significant difference was between the N3 treatments as compared to the N0, N1, and N2 treatments ($P<0.05$). The *nosZ* showed a comparable pattern to the *nirK* gene abundance: the N0 differed from the N1, N2, and N3 treatments and the N3 was significantly different from the N0, N1, and N2. The N1 and N2 treatments did not significantly differ in *nosZ* gene copy number. The NO_2^- reductase (*nirK* + *nirS*) was significantly higher than the N_2O reductase (*nosZ*), generally two to three orders of magnitude higher (Figure 4). Copy number of *nirK* had a positive

and significant correlation with *nirS*, *nosZ*, and 16S rRNA gene copy numbers (Table 3). Furthermore, *nirK* showed a negative and significant correlation with the C:N ratio ($P<0.05$) (Table 3). Copy numbers of *nirS* positively and significantly correlated with *nirK*, *nosZ*, and 16S gene copy numbers. Copy numbers of n*osZ* had a positive and significant correlation with *nirK*, *nirS*, and 16S gene copy numbers. Additionally, *nosZ* was negatively correlated with the C:N ratio ($P<0.05$) (Table 2).

Pearson product moment correlation coefficient analysis of the full data set showed both *nirK* and *nosZ* had a negative and significant correlation with the C:N ratio and a positive and significant relationship with pH, as well as positive correlation with each other. Furthermore, the denitrifier gene abundances across the full data set showed positive correlations with each other and with the 16S rRNA gene (Table 4).

Table 1. Soil chemical properties of the mor (Oe and Oa) layer at Betsele and Norrliden. The data are means with standard deviations in parenthesis (n=3). Data revised from Högberg et al. (2006).

Site	Forest Type	Treatment	pH	C:N ratio	NH_4^+- N ($\mu g\ g^{-1}$ soil)	NO_3^- - N ($\mu g\ g^{-1}$ soil)	O.M. ($g\ g^{-1}$ soil)
Betsele	DS	-	4.0 (0.2)	38.1 (2.4)	3.8 (2.5)	0.7 (0.8)	0.83 (0.06)
	SH	-	4.6 (0.1)	22.9 (1.1)	2.8 (0.9)	0.4 (0.2)	0.54 (0.05)
	TH	-	5.3 (0.1)	14.9 (0.3)	7.8 (7.3)	1.6 (1.0)	0.44 (0.16)
Site	Forest Type	Treatment	pH	C:N ratio	NH_4-N ($\mu g\ g^{-1}$ soil)	NO_3-N ($\mu g\ g^{-1}$ soil)	O.M. ($g\ g^{-1}$ soil)
Norrliden	DS	N0	4.1 (0.1)	37.5 (1.2)	0.3 (0.2)	0.4 (0.2)	0.60 (0.09)
	DS	N1	4.1 (0.1)	31.1 (1.8)	27.0 (38.0)	1.0 (0.4)	0.64 (0.1)
	DS	N2	4.2 (0.1)	27.7 (0.6)	54.8 (7.2)	4.5 (1.6)	0.63 (0.05)
	DS	N3	4.1 (0.1)	27.2 (0.7)	2.4 (2.6)	0.4 (0.1)	0.67 (0.07)

DS = dwarf shrub, SH = short herb, TH = tall herb, N0 = control plot, N1, N2, N3 are the fertilized plots at three levels. N3 fertilization stopped in 1991.

Table 2. Correlation table of measured variables at Betsele

Betsele						
	nirK	*nirS*	*nosZ*	16S	AOA *amoA*	AOB *amoA*
nirK	1.00					
nirS	0.32 (0.3947)	1.00				
nosZ	**0.83** (0.0058)	**0.69** (0.0405)	1.00			
16S	**0.69** (0.0390)	0.64 (0.0624)	0.62 (0.0737)	1.00		
AOA *amoA*	**0.79** (0.0113)	0.56 (0.1142)	**0.89** (0.0014)	0.57 (0.1100)	1.00	
AOB *amoA*	**0.68** (0.0421)	0.50 (0.1732)	**0.82** (0.0063)	0.40 (0.2802)	**0.94** (0.0002)	1.00
C:N	**-0.84** (0.0046)	0.66 (0.4668)	**-0.78** (0.0124)	-0.49 (0.1811)	-0.60 (0.0904)	-0.60 (0.0843)
pH	**0.90** (0.0008)	0.33 (0.3797)	**0.85** (0.0039)	0.57 (0.1078)	**0.73** (0.0261)	**0.72** (0.0299)
NH_4^+	0.51 (0.1641)	**0.77** (0.0156)	**0.74** (0.0223)	**0.67** (0.0493)	**0.85** (0.0037)	**0.82** (0.0073)
NO_3^-	0.66 (0.0522)	0.56 (0.1170)	0.66 (0.0537)	**0.68** (0.0460)	**0.85** (0.0034)	**0.82** (0.0066)
OM	**-0.67** (0.0463)	0.10 (0.7888)	-0.50 (0.1721)	-0.27 (0.4892)	-0.27 (0.4744)	-0.29 (0.4505)

The strength of the linear relationship between two variables is described by the Pearson moment correlation coefficient, r. Negative numbers correspond to negative correlations. P-values are given in parentheses and significant values are written in bold.

Variables in the table are *nirK*/*S* = number of nitrite reductase gene copies, *nosZ* = number of N_2O reductase gene copies, 16S = number of 16S rRNA gene copies, AOB = number of bacterial *amoA* gene copies, AOA = number of archaeal *amoA* gene copies, C:N = the C to N ratio, $NH_4^+$and NO_3^- (μg N g^{-1} soil), OM = organic matter (g g^{-1} soil).

Table 3. Correlation table of measured variables at Norrliden

Norrliden						
	nirK	*nirS*	*nosZ*	16S	AOA *amoA*	AOB amoA
nirK	1.00					
nirS	**0.77** 0.0036	1.00				
nosZ	**0.79** 0.0022	**0.76** 0.0038	1.00			
16S	**0.76** 0.0038	**0.74** 0.0061	**0.63** 0.0266	1.00		
AOA *amoA*	-0.29 0.3538	-0.33 0.2967	-0.24 0.4593	-0.16 0.6219	1.00	
AOB *amoA*	0.59 0.5936	-0.41 0.1899	-0.01 0.9644	-0.02 0.9414	0.34 0.2753	1.00
C:N	**-0.67** 0.0161	-0.34 0.2861	**-0.62** 0.0310	-0.54 0.0689	-0.26 0.4080	-0.26 0.4187
pH	-0.28 0.3864	-0.23 0.4770	0.00 0.9954	-0.28 0.3695	**0.63** 0.0276	0.20 0.5364
NH_4^+	-0.21 0.5174	-0.04 0.2571	-0.04 0.8945	-0.02 0.9434	**0.69** 0.0132	**0.79** 0.0020
NO_3^-	-0.23 0.4818	-0.34 0.2829	0.07 0.8399	-0.18 0.5673	0.5 0.0984	**0.79** 0.0021
OM	0.43 (0.162)	0.49 (0.1038)	0.36 (0.2560)	0.52 (0.0824)	0.04 (0.9114)	0.14 (0.6668)

The strength of the linear relationship between two variables is described by the Pearson moment correlation coefficient, r. Negative numbers correspond to negative correlations. P-values are given in parentheses and significant values are written in bold.

Variables in the table are *nirK*/*S* = number of nitrite reductase gene copies, *nosZ* = number of N_2O reductase genes, 16S = number of 16S rRNA gene copies, AOB = number of bacterial *amoA* gene copies, AOA = number of archaeal *amoA* gene copies, C:N = the C to N ratio, $NH_4^+$and NO_3^- (µg N g^{-1} soil), OM = organic matter (g g^{-1} soil).

Table 4. Correlation table of measured variables at Betsele and Norrliden

Betsele and Norrliden						
	nirK	*nirS*	*nosZ*	16S	AOA *amoA*	AOB *amoA*
nirK	1.00					
nirS	**0.54** (0.0118)	1.00				
nosZ	**0.80** (<0.0001)	**0.50** (0.0218)	1.00			
16S	**0.73** (0.0002)	**0.56** (0.0085)	**0.67** (0.0009)	1.00		
AOA *amoA*	**0.61** (0.0031)	0.15 (0.5064)	**0.80** (0.0196)	0.50 (0.3346)	1.00	
AOB *amoA*	0.31 (0.1775)	-0.13 (0.5752)	**0.50** (0.0196)	0.22 (0.3346)	**0.66** (0.0011)	1.00
C:N	**-0.77** (<0.0001)	-0.20 (0.3910)	**-0.78** (<0.0001)	**-0.56** (0.0080)	**-0.59** (0.0045)	**-0.46** (0.0350)
pH	**0.64** (0.0017)	0.25 (0.8643)	**0.77** (<0.0001)	**0.51** (0.0169)	**0.77** (<0.0001)	**0.48** (0.0271)
NH_4^+	-0.16 (0.4909)	0.04 (0.25660	-0.08 (0.7250)	-0.11 (0.6234)	-0.04 (0.8664)	**0.55** (0.0099)
NO_3^-	-0.02 (0.9288)	-0.26 (0.3461)	0.12 (0.5995)	-0.05 (0.8189)	0.18 (0.4300)	**0.68** (0.0007)
Inorganic N	-0.15 (0.5091)	-0.26 (0.2544)	-0.07 (0.7639)	-0.11 (0.6310)	-0.03 (0.9132)	**0.57** (0.0074)
OM	-0.37 (0.1016)	0.22 (0.3476)	-0.35 (0.1238)	-0.08 (0.7339)	-0.28 (0.2269)	-0.17 (0.4696)

The strength of the linear relationship between two variables is described by the Pearson moment correlation coefficient, r. Negative numbers correspond to negative correlations. P-values are given in parentheses and significant values are written in bold.

Variables in the table are *nirK*/*S* = number of nitrite reductase gene copies, *nosZ* = number of N_2O reductase gene copies, 16S = number of 16S rRNA gene copies, AOB = number of bacterial amoA gene copies, AOA = number of archaeal *amoA* gene copies, C:N = the C to N ratio, $NH_4^+$and NO_3^- (µg N g^{-1} soil), inorganic N (µg g^{-1} soil), OM = organic matter (g g^{-1} soil)

Table 5. 16S rRNA Gene copy numbers.

Site	Forest Type	Treatment	Gene copy numbers (g^{-1} of soil)	
			16S	SD
Betsele	DS	-	3.92E+09[a]	2.37E+09
	SH	-	3.34E+09[a]	1.53E+09
	TH	-	5.95E+09[a]	1.37E+09
Site	Forest Type	Treatment	Gene copy numbers (g^{-1} of soil)	
			16S	SD
Norrliden	DS	N0	1.28E+09[b]	1.16E+08
	DS	N1	3.63E+09[a]	1.51E+08
	DS	N2	2.29E+09[ab]	5.89E+08
	DS	N3	3.96E+09[a]	2.73E+09

The data are means and standard deviations (n=3). DS = dwarf shrub, SH = short herb, TH = tall herb, N0 = control plot, N1, N2, N3 are the fertilized plots at three levels. N3 fertilization stopped in 1991. Values with the same letter do not significantly differ according to LSD test ($P<0.05$).

DISCUSSION

Ammonia-Oxidizer Community

Both bacterial and archaeal *amoA* genes were detected in the acidic forest soils investigated in our study. An important result of this study is that in these acidic soils bacterial *amoA* predominates over archaeal *amoA* at all sites, except for the TH at Betsele, where no significant difference was found between AOB and AOA *amoA* copy numbers. Many previous studies have shown that archaeal *amoA* are more abundant than bacterial *amoA* in a variety of soil systems (Leininger et al. 2006, Jia and Conrad 2009, Zeglin et al. 2011) (Jia and Conrad, 2009; Leininger et al., 2006; Zeglin et al., 2011). However, in soil systems with similarities to our sites bacterial genes have been found to be higher or equal to archaeal. For example, Boyle-Yarwood et al. (2008) observed higher bacterial *amoA* than archaeal *amoA* in a forested soil system in Oregon. Similarly, in a boreal forest study across multiple plant communities, bacterial *amoA* consistently outnumbered archaeal *amoA* (Petersen et al., 2012). This suggests that AOB may be better adapted to forest soils than AOA. Also, bacterial *amoA* were found to be higher than archaeal *amoA* genes in Antarctic soils (Jung et al. 2011), suggesting a bacterial *amoA* preference for cooler climates, such as boreal forests. Alternatively, there may be archaeal and bacterial ammonia oxidizers in this highly organic, low pH soil, that cannot be targeted with the primers used in this and many other studies (Leininger et al., 2006; Levičnik-Höfferle et al., 2012; Tourna et al., 2008). It is also important to note that the mere existence of bacterial or archaeal *amoA* does not indicate activity.

At Betsele the natural forest gradient provides a range in N availability and pH; increasing from the DS to SH to TH forest types (Högberg et al., 2006). Here, we observed the bacterial and archaeal *amoA* abundance to be highest at the TH type. AOA *amoA* gene abundance at the TH type ranged over 100-fold higher than the DS and SH types. AOB *amoA* gene abundance was 10-fold higher in the TH than the DS and SH sites, although the difference was not statistically significant at the SH type (Figure 1). The smaller ammonia-oxidizer community measured at the DS and SH forest types may be due to the low NH_4^+ concentrations and low pH present in the soil, limiting the availability of ammonia. Consistent with the ammonia-oxidizer abundance, Högberg et al. (2005) reported no gross nitrification for the DS and SH forest types, and that rates of gross nitrification in the TH forest type were among the higher reported for boreal soils worldwide (Högberg et al., 2005).

In the fertilized forest at Norrliden, the abundance of the AOB community was highest at the N1 and N2 treatments, which are still under fertilization. The AOB abundance at N1 and N2 was over ten-fold greater than in the plots that are not currently fertilized, N0 and N3 (Figure 2). This result is supported by previous studies where AOB population size showed a significant increase in response to NH_4^+ additions in contrast to unfertilized soil (Di et al. 2009, Levičnik-Höfferle et al. 2012). These data imply that the AOB community increased in response to the increased fertilizer; however, there was no significant difference between N1 and N2. This observation suggests that the addition of fertilizer stimulates AOB growth; however, at the higher levels of NH_4NO_3 application, AOB may reach a threshold and after that addition or

more fertilizer may not be as important. Similarly, in microcosms receiving low, medium, and high NH_4^+ concentrations, Verhamme et al. (2011) observed AOB abundance reached a plateau in soils receiving high NH_4^+ concentrations. The data suggest that the AOB community may have increased at N3, but after cessation of fertilization the AOB community abundance levels are near that of the control treatment. The AOA abundance was unresponsive to fertilization, with less than a two-fold increase at the N2 treatment. It seems AOA abundance is less influenced by high N supply than AOB abundance. This observation has been found in previous studies, where AOA abundance has been shown to have little to no response to N fertilization (Jia and Conrad, 2009; Levičnik-Höfferle et al., 2012; Zeglin et al., 2011).

When *amoA* abundance is correlated to the range of N availabilities found across both sites, AOB abundance is highly correlated to inorganic N; whereas, AOA was not (Table 4). These data seem to indicate that AOA are less influenced by addition of inorganic fertilizer than AOB, which is consistent with previous studies (Di et al., 2009; Jia and Conrad, 2009; Stopnisek et al., 2010). This hypothesis is also supported by Levičnik-Höfferle et al. (2012), who reported the nitrification rate from microcosms containing acidic forest dominated by archaea to be unaffected by added inorganic NH_4^+. However, the nitrification rate was significantly stimulated by the addition of organic N and was accompanied by increased abundance of the AOA *amoA* gene. Furthermore, NH_4^+ that accumulated when mineralization exceeded nitrification was oxidized, whereas added NH_4^+ was not (Levičnik-Höfferle et al., 2012). This may help explain the increase in abundance of AOA at the TH forest type, the only forest type

where gross N mineralization rates exceeded gross NH_4^+ consumption (assimilation and nitrification) (Högberg et al., 2005). Mineralization control potentially involves effects of a slow release of NH_4^+ rather than high concentrations following inorganic N fertilization (Levičnik-Höfferle et al., 2012). In fact, in many unfertilized soils, nitrification rate is controlled by the rate of NH_4^+ release following the mineralization of organic matter (Booth et al. 2005). Thus, AOA and AOB could compete for the same resources under similar environmental conditions with limiting amounts of NH_4^+. However, recent studies demonstrated that plant-induced organic substrates increased archaeal *amoA* transcripts (Chen et al., 2008). Similarly, the archaeal *amoA* population was found to be up to 8000-fold more abundant than bacteria *amoA* in rhizosphere soil (Herrmann et al. 2008), suggesting archaeal growth can occur in the presence of root exudates. Accordingly, it is suggested that AOA possibly grow by heterotrophy or mixotrophy. Thus, along with NH_4^+ availability, it is possible that the high archaeal *amoA* abundance at the highly productive TH forest type is supported by this hypothesis.

Denitrifier Community

We found distinct patterns amongst the denitrifier genes across both study sites. At Betsele, *nirK* and *nosZ* copy numbers increased along the transect, from the DS forest type to the TH forest type, whereas *nirS* remained unchanged across all forest types. At Norrliden, *nirK* and *nosZ* increased in relation to the increased fertilizer application rate; however, there was no difference between the N1 and N2 treatment. The *nirS* copy numbers remained unchanged, except for an increase at the N3 treatment. In contrast

to the *amoA* genes, all denitrifier gene abundances remained high at the N3 site after cessation of fertilizer application. The total bacterial community, as measured by the 16S rRNA gene copy numbers, did not differ along the transect at Betsele and only slight differences were measured between N0 and the fertilized plots at Norrliden (Table 5).

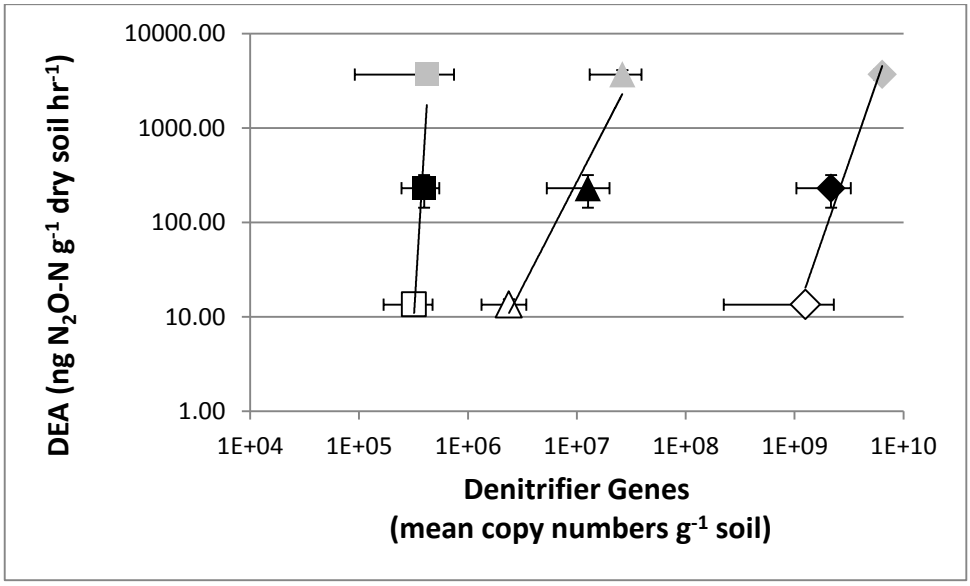

Figure 5. Denitrification enzyme assay vs. Denitrifier gene copy numbers. Gene copy numbers are mean copy numbers per gram of soil *nirK* = diamonds, *nirS* = squares, *nosZ* = triangles. Denitrification enzyme assay are mean rates by forest type dwarf shrub = white, short herb = solid, and tall herb = gray. Error bars represents SE. DEA data provided by Högberg et al., (2005).

Potential denitrification rates at Betsele, as measured by Högberg et al. (2005), were positively correlated to the *nirK* and *nosZ* denitrifier gene abundances (r^2=0.96 and 0.95, respectively), with rates 16-fold and 230-fold higher at the TH forest type than the DS and SH, respectively (Figure 5). These results are consistent with recent studies showing that denitrification enzyme activity was correlated with the size of the denitrifier community estimated by the quantification of *nirK* genes (Throback et al., 2004) or *nosZ* genes (Hallin et al., 2009). These results suggest the size of the

community could predict corresponding process rates. Similarly, Petersen et al. (2012) found functional gene abundance was the most important variable to predict potential rates. However, the size of the gene pool might not reflect the activity of the enzymes in situ due to environmental constraints.

Because most denitrifiers are heterotrophic, C and N are suggested to be the most important factors determining community size (Mosier and Francis 2010). Interestingly, no correlation was found between NO_3^- concentrations and the denitrification genes. This result is consistent with previous studies, where the NO_3^- content of soils could not explain the abundance of denitrifier genes (Attard et al., 2011; Morales et al., 2010; Throback et al., 2004). The lack of agreement between denitrifier abundance and NO_3^- concentrations suggests that NO_3^- availability might contribute more to NO_3^- reducing activities rather than the denitrifier abundance. Thus, this is consistent with the concept that denitrifier abundance is controlled by factors other than NO_3^- supply (Wallenstein et al., 2006).

Previous studies have found a correlation between denitrifier genes and organic matter (OM) (Kandeler et al. 2006, Attard et al. 2011, Dandie et al. 2011), suggesting that the increase of OM, and therefore C substrate stimulates the growth of the denitrifying community. Contrastingly, in our results, the *nirK*, *nirS,* and *nosZ* copy numbers were not significantly related to OM content, except at Betsele where there was a significant negative correlation between *nirK* and OM. However, in our study, samples were taken from the mor layer (Oe and Oa) and it is possible the decrease in

OM was due to the mixing of the mineral surface with the organic layer. Moreover, Högberg et al. (2005) measured total C along the transect at Betsele and found an increase in total C from the DS to the TH forest types. Furthermore, the increase in the *nirK* and *nosZ* copy numbers was correlated to the decrease in the C:N ratio at both sites. These data suggest that not only the amount of the C substrate, but also the organic matter quality, has distinct effects on the denitrifier community abundance. As N availability increases, the trees take up more N, resulting in leaf litter with a lower C:N ratio. Organic matter inputs with low C:N ratios would promote mineralization; whereas, high C:N ratios may cause immobilization of soil N (Högberg et al., 2005). Furthermore, organic matter with lower C:N ratios provide a more readily available C source (Frimpong and Baggs 2010). A study in a temperate forest soil showed a significant increase in denitrifier gene abundance with the addition of a low C:N ratio plant residue, as compared to a high C:N ratio addition (Su et al. 2010) . These results are supported by previous studies where an increase in soil organic matter quality resulted in an increase in denitrifier abundance (Chen et al. 2010, Miller et al. 2012). Together, this evidence suggests that the increased N availability at Betsele and Norrliden does not directly affect denitrifier abundance, but indirectly through environmental controls, such as increased organic matter quality.

Increased organic matter quality may help to explain the increased abundance of denitrifier genes at the N3 treatment. Our initial hypothesis was that after cessation of high levels of fertilizer application the denitrifier abundance would return to that of the control site. However, the high denitrifier gene copy numbers present 10 years after

fertilization application stopped suggests resilience of treatment effects. This result is consistent with previous findings from Morales et al.(2010), showing that denitrifying communities do not readily return to native populations following agricultural fertilization and soil nutrient levels require decades or more to recover from agriculture. The long-term effects of fertilization at the N3 site have changed soil properties, decreased the C:N ratio of the organic matter, therefore increasing the C substrate quality and community abundance.

Several studies have reported that pH strongly affected the denitrifying community composition (Hallin et al., 2009) and abundance (Kandeler et al., 2006) in soils. In fact, Bru et al. (2010) found pH to be the main driver for spatial variation in denitrifier abundances. Although there was no significant difference in pH at Norrliden, an increase at Betsele was correlated to an increase in the *nirK* and *nosZ* genes. This correlation was significant across the entire data set when both sites were correlated to pH. However, there are multiple factors at Betsele that co-vary due to the hydrochemistry of the slope. Therefore, the correlation between pH and denitrifiers might not represent a direct causal relationship, as confounding factors cannot be ruled out. However, an increase of denitrifier abundance at the TH forest type due to the increase of pH (5.3) is plausible. In fact, in a recent study of an acidic forest organic litter horizon, the amount of *nirK* denitrifiers rapidly decreased below a pH of 5 (Bárta et al., 2010). This effect was attributed to the lower amounts of dissolved organic C and dissolved organic N found in highly acidic soils (Bárta et al. 2010), and may explain the pH correlation in our study,

Several lines of evidence suggest that *nirK*-type denitrifiers may be more important to denitrification in this study than *nirS*-type, including the fact that potential denitrification rates were positively correlated with *nirK* abundance (Figure 5), and copy numbers of *nirK* were more than three orders of magnitude higher than *nirS* abundance at every forest type and treatment (Figure 3 and Figure 4) Previous studies have observed high *nirK* to *nirS* ratios (Bárta et al., 2010; Chen et al., 2010; Dandie et al., 2011); however, higher ratios of *nirS* to *nirK* have also been reported (Attard et al., 2011; Kandeler et al., 2006), including a survey of boreal forest soils (Petersen et al., 2012). Because NO_2^- reduction is functionally equivalent between the copper NO_2^- reductase (*nirK*) and the cytochrome cd_1 NO_2^- reductase (*nirS*), and denitrifying bacteria possess only one type of NIR, these conflicting results suggest niche differentiation of *nirK* and *nirS* denitrifiers. However, the drivers of niche differentiation between *nirK* and *nirS* denitrifiers are not clear. It is suggested that *nirS*-type denitrifiers dominate younger soils, and as soils develop the *nirS* to *nirK* ratio decreases (Kandeler et al., 2006). The most important factor related to the increase in *nirK* was found to be the increase of organic matter as soils developed (Kandeler et al., 2006). Similarly, Hallin et al. (2009) suggested the presence of plants indicated selection for *nirK* over *nirS*. Thus, the well-developed, highly organic soils used in this study could be an ideal habitat for *nirK*-type denitrifiers. Contrastingly, in a boreal study similar to this one, using the same primers, Petersen et al. (2012) found *nirS* to dominate across all plant types, except a bog birch site dominated by shrubs. The dominance of *nirS* was attributed to the adaptation of *nirS* to poorly-drained static wet environments. In contrast, the Swedish

boreal soils are well drained and therefore drier than the boreal soils dominated with *nirS*. This observation is consistent with the hypothesis of Petersen et al. (2012) that drier soils are likely to be dominated by *nirK*. Moreover, these data imply that there is not one main driver of ecosystem differentiation and that it is more likely a combination of factors. Further study is needed to investigate the drivers affecting niche differentiation of *nirK* and *nirS* denitrifiers.

It is apparent in this study that *nirK*-type denitrifiers were more sensitive to land change than the *nirS*-types; as the *nirK* copy numbers increased along the gradient at Betsele, and with fertilization amount at Norrliden but *nirS* copy numbers did not. This observation is supported by previous work where *nirK*, but not *nirS* increased in response to denitrification-inducing conditions (Yoshida et al. 2010, Yuan et al. 2012) . Together, this evidence suggests that denitrifiers bearing *nirK* play a greater role in N removal from this system compared with denitrifiers bearing *nirS*.

Across all sites the total NO_2^- reducing abundance (*nirK* + *nirS*) outnumbered the *nosZ* abundance by over three orders of magnitude. A higher abundance of the nir genes than the *nosZ* has been observed in several studies (Henry et al. 2006b, Philippot et al. 2009, Bru et al. 2010, Cuhel et al. 2010) (Bru et al., 2010; Cuhel et al., 2010; Henry et al., 2006b; Philippot et al., 2009). The apparent difference between the copy numbers of the NO_2^- reducing and N_2O reducing functional groups suggest a decrease in the proportion of bacteria possessing *nosZ*. Denitrifiers with this truncated pathway, such as *Agrobacterium tumefaciens* have previously been observed (Richardson et al.,

2009). Philippot et al. (2009) suggested the loss of *nosZ* might be a consequence of lower activity of the N_2O reductase; the bacteria losing a functional trait which is no longer needed. It is documented that complete denitrifiers exhibit inhibition of N_2O respiration in the presence of NO_3^- (Sanford et al. 2012), as NO_3^- is more energetically favorable (Richardson et al., 2009). Furthermore, N_2O reductase activity is inhibited in low pH soils, such as the soils in this study. This seems to indicate a smaller N_2O-reducing community than a NO_2^--reducing community; which could lead to higher rates of N_2O released to the atmosphere. However, recent studies suggest that a large group of environmental *nosZ* genes is unrecognized by commonly used primers (Jones et al. 2012, Sanford et al. 2012), including the ones used in this study. In fact, Jones et al. (2010) demonstrated with qPCR that this unaccounted for *nosZ* was most often as abundant as the other typical *nosZ*. Therefore this evidence suggests that we may not be accounting for the full scale of the denitrifying community. Moreover, the *nirK* genes measured in this study were unusually high numbers (10^8 to 10^9 copies per gram of soil). Other studies report *nirK* genes with lower magnitudes 10^5 to 10^6 (Attard et al., 2011; Henry et al., 2004; Kandeler et al., 2006), which suggests the possibility of non-specific priming. However, multiple quality control measures were used in order to confirm reported copy numbers: efficiency ranged from 98-100%, r^2=0.99, and dissociation curves showed a consistent melting peak. However, qPCR products ran on an agarose gel resulted in multiple bands in some samples but the copy numbers were comparable to samples with one peak. Moreover, similar high abundances of *nirK* genes have also been reported (Chen et al., 2010; Dandie et al., 2011). Both of these studies suggest an

ecological differentiation based on high organic matter soils which is consistent with the soils in this study. Again, it is important to note that gene presence does not distinguish between active and dormant denitrifier organisms. Future research including measurements of functional gene expression combined with community analysis would help to relate this microbial abundance data to denitrifier activity.

CONCLUSION

The abundance of AOB and AOA *amoA* genes and *nirK*, *nirS* and *nosZ* denitrifier genes showed varying patterns in response to soil N availability. This study demonstrates the potential influence of increased N inputs on population size and the N cycling processes they mediate.

Quantification of *amoA* revealed a dominance of AOB over AOA in these boreal forest soils. The AOB and AOA populations at Betsele showed a positive relationship to previously published nitrification rates. At Norrliden, the size of the AOB population was greatly influenced by addition of fertilizer, whereas AOA populations were less responsive. However, there was a significant increase of AOA at Betsele at the TH forest type, where N mineralization rates exceeded consumption. These findings suggest different growth responses to NH_4^+ concentration; bacteria dominate ammonia oxidation in heavily fertilized soils and archaea may contribute significantly to ammonia oxidation at low- NH_4^+ environments where mineralization exceeds consumption. Although further research on AOA metabolism is needed, these differences provide implications for niche specialization of AOA and AOB, based on tolerance of high ammonia concentrations and NH_4^+ source.

The *nirK* and *nosZ* denitrifier genes were more responsive to increased N inputs than *nirS*. In fact across all sites *nirK* dominated over *nirS* abundance, suggesting a niche specialization of *nirK* in these highly organic, well drained, forest soils. The increase in *nirK* and *nosZ* functional groups could not be explained directly by increased NO_3^-

concentrations; however, there was a strong correlation to the decrease in the C:N ratio. This relationship suggests an indirect effect of increased N availability as organic matter quality increased with N availability. In our study, the total *nirS* and *nirK* significantly outnumbered *nosZ*, suggesting a truncated denitrifier population. However, this apparent difference could be due to inadequate *nosZ* primers. Future research using primers designed to capture the full *nosZ* community is essential to understanding denitrifier population dynamics in the regulation of N oxides.

At the N3 sites, the AOB population size returned to control size populations after fertilization stopped. In contrast, the denitrifier population remained high after over a decade. Differences in the population response of ammonia-oxidizers and denitrifiers at the N3 site, in response to fertilizer cessation, reflect the different growth requirements of the two populations. Ammonia oxidizers depend on NH_4^+ for energy generation; whereas, most denitrifiers are heterotrophs and require a C substrate for energy when using NO_3^- as an electron acceptor. The direct energy source for AOB reduced when fertilization stopped; whereas, soil organic matter quality remained high. The N3 site demonstrates the long-term effects of fertilization on the denitrifier community, providing increased organic matter quality, resulting in increased population size.

This work focused on the quantification of functional genes as a proxy for population size; however the mere existence of the gene does not reflect activity.

Therefore, ongoing research measuring gene expression and community composition will enhance the study of these N cycling processes.

BIBLIOGRAPHY

Alves, R. J. E., W. Wanek, A. Zappe, A. Richter, M. M. Svenning, C. Schleper, and T. Urich. 2013. Nitrification rates in Arctic soils are associated with functionally distinct populations of ammonia-oxidizing archaea. The ISME Journal.

Arp, D., L. Sayavedra-Soto, and N. Hommes. 2002. Molecular biology and biochemistry of ammonia oxidation by Nitrosomonas europaea. Archives of Microbiology **178**:250-255.

Attard, E., S. Recous, A. Chabbi, C. De Berranger, N. Guillaumaud, J. Labreuche, L. Philippot, B. Schmid, and X. Le Roux. 2011. Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. Global Change Biology **17**:1975-1989.

Baggs, E. M. 2011. Soil microbial sources of nitrous oxide: recent advances in knowledge, emerging challenges and future direction. Current Opinion in Environmental Sustainability **3**:321-327.

Bárta, J., T. Melichová, D. Vaněk, T. Picek, and H. Šantrůčková. 2010. Effect of pH and dissolved organic matter on the abundance of *nirK* and *nirS* denitrifiers in spruce forest soil. Biogeochemistry **101**:123-132.

Booth, M. S., J. M. Stark, and E. Rastetter. 2005. Controls on nitrogen cycling in terrestrial ecosystems: a synthetic analysis of literature data. Ecological monographs **75**:139-157.

Boyle-Yarwood, S. A., P. J. Bottomley, and D. D. Myrold. 2008. Community composition of ammonia-oxidizing bacteria and archaea in soils under stands of red alder and Douglas fir in Oregon. Environmental Microbiology **10**:2956-2965.

Bru, D., A. Ramette, N. P. A. Saby, S. Dequiedt, L. Ranjard, C. Jolivet, D. Arrouays, and L. Philippot. 2010. Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. The ISME Journal **5**:532-542.

Canfield, D. E., A. N. Glazer, and P. G. Falkowski. 2010. The evolution and future of earth's nitrogen cycle. Science **330**:192-196.

Chen, D., Y. Li, P. Grace, and A. R. Mosier. 2008. N_2O emissions from agricultural lands: a synthesis of simulation approaches. Plant and Soil **309**:169-189.

Chen, Z., X. Q. Luo, R. G. Hu, M. N. Wu, J. S. Wu, and W. X. Wei. 2010. Impact of Long-Term Fertilization on the composition of denitrifier communities based on nitrite reductase analyses in a paddy soil. Microbial Ecology **60**:850-861.

Cuhel, J., M. Simek, R. J. Laughlin, D. Bru, D. Cheneby, C. J. Watson, and L. Philippot. 2010. Insights into the effect of soil pH on N_2O and N_2 emissions and denitrifier community size and activity. Appl Environ Microbiol **76**:1870-1878.

Dandie, C. E., S. Wertz, C. L. Leclair, C. Goyer, D. L. Burton, C. L. Patten, B. J. Zebarth, and J. T. Trevors. 2011. Abundance, diversity and functional gene expression of denitrifier communities in adjacent riparian and agricultural zones. FEMS Microbiology Ecology **77**:69-82.

De Boer, W. and G. A. Kowalchuk. 2001. Nitrification in acid soils: micro-organisms and mechanisms. Soil Biology and Biochemistry **33**:853-866.

Demaneche, S., L. Philippot, M. M. David, E. Navarro, T. M. Vogel, and P. Simonet. 2009. Characterization of denitrification gene clusters of soil bacteria via a metagenomic approach. Appl Environ Microbiol **75**:534-537.

Di, H. J., K. C. Cameron, J. P. Shen, C. S. Winefield, M. O'Callaghan, S. Bowatte, and J. Z. He. 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nature Geoscience **2**:621-624.

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

Fierer, N., J. A. Jackson, R. Vilgalys, and R. B. Jackson. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Applied and Environmental Microbiology **71**:4117-4120.

Firestone, M. K. and J. M. Tiedje. 1979. Temporal change in nitrous oxide and dinitrogen from denitrification following onset of anaerobiosis. Applied and Environmental Microbiology **38**:673-679.

Frimpong, K. A. and E. M. Baggs. 2010. Do combined applications of crop residues and inorganic fertilizer lower emission of N_2O from soil? Soil Use and Management **26**:412-424.

Galloway, J. N., J. D. Aber, J. W. Erisman, S. P. Seitzinger, R. W. Howarth, E. B. Cowling, and B. J. Cosby. 2003. The nitrogen cascade. BioScience **53**.

Gruber, N. and J. N. Galloway. 2008. An Earth-system perspective of the global nitrogen cycle. Nature **451**:293-296.

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

Henry, S., E. Baudoin, J. C. Lopez-Gutierrez, F. Martin-Laurent, A. Brauman, and L. Philippot. 2004. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. J Microbiol Methods **59**:327-335.

Henry, S., D. Bru, B. Stres, S. Hallet, and L. Philippot. 2006. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. Appl Environ Microbiol **72**:5181-5189.

Herrmann, M., A. M. Saunders, and A. Schramm. 2008. Archaea dominate the ammonia-oxidizingcommunity in ther hizosphere of the freshwater macrophyte *Littorella uniflora*. Applied and Environmental Microbiology **74**:3279-3283.

Högberg, M. N., P. Högberg, and D. D. Myrold. 2006a. Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three? Oecologia **150**:590-601.

Högberg, M. N., D. D. Myrold, R. Giesler, and P. Högberg. 2005. Contrasting patterns of soil N-cycling in model ecosystems of Fennoscandian boreal forests. Oecologia **147**:96-107.

Högberg, P. 2007. Environmental science: Nitrogen impacts on forest carbon. Nature **447**:781-782.

Högberg, P., H. Fan, M. Quist, D. Binkley, and C. O. Tamm. 2006b. Tree growth and soil acidification in response to 30 years of experimental nitrogen loading on boreal forest. Global Change Biology **12**:489-499.

Hyman, M. R. and P. M. Wood. 1985. Suicidal Inactiviation and Labeling of Ammonia Mono-oxygenase by Acetylene. Biochemical Journal **227**:719-725.

Jia, Z. and R. Conrad. 2009. Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. Environmental Microbiology **11**:1658-1671.

Jones, C. M., D. R. H. Graf, D. Bru, L. Philippot, and S. Hallin. 2012. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. The ISME Journal.

Jung, J., J. Yeom, J. Kim, J. Han, H. S. Lim, H. Park, S. Hyun, and W. Park. 2011. Change in gene abundance in the nitrogen biogeochemical cycle with temperature and nitrogen addition in Antarctic soils. Research in Microbiology **162**:1018-1026.

Kandeler, E., K. Deiglmayr, D. Tscherko, D. Bru, and L. Philippot. 2006. Abundance of *narG, nirS*, *nirK*, and *nosZ* Genes of Denitrifying Bacteria during Primary Successions of a Glacier Foreland. Applied and Environmental Microbiology **72**:5957-5962.

Knowles, R. 1982. Denitrification. Microbiological Reviews **46**:43-70.

Konneke, M., A. E. Bernhard, J. R. de la Torre, C. B. Walker, J. B. Waterbury, and D. A. Stahl. 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature **437**:543-546.

Lehtovirta-Morley, L. E., K. Stoecker, A. Vilcinskas, J. I. Prosser, and G. W. Nicol. 2011. Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. Proceedings of the National Academy of Sciences **108**:15892-15897.

Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G. W. Nicol, J. I. Prosser, S. C. Schuster, and C. Schleper. 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature **442**:806-809.

Levičnik-Höfferle, Š., G. W. Nicol, L. Ausec, I. Mandić-Mulec, and J. I. Prosser. 2012. Stimulation of thaumarchaeal ammonia oxidation by ammonia derived from organic nitrogen but not added inorganic nitrogen. FEMS Microbiology Ecology **80**:114-123.

Miller, M. N., C. E. Dandie, B. J. Zebarth, D. L. Burton, C. Goyer, and J. T. Trevors. 2012. Influence of carbon amendments on soil denitrifier abundance in soil microcosms. Geoderma **170**:48-55.

Morales, S. E., T. Cosart, and W. E. Holben. 2010. Bacterial gene abundances as indicators of greenhouse gas emission in soils. ISME J **4**:799-808.

Morley, N. and E. M. Baggs. 2010. Carbon and oxygen controls on N_2O and N_2 production during nitrate reduction. Soil Biology and Biochemistry **42**:1864-1871.

Mosier, A. C. and C. A. Francis. 2010. Denitrifier abundance and activity across the San Francisco Bay estuary: Estuarine denitrifier abundance and activity. Environmental Microbiology Reports **2**:667-676.

Nicol, G. W., S. Leininger, C. Schleper, and J. I. Prosser. 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environmental Microbiology **10**:2966-2978.

Nohrstedt, H.-Ö. 2001. Response of coniferous forest ecosystems on mineral soils to nutrient additions: A review of swedish experiences. Scandinavian Journal of Forest Research **16**:555-573.

Olsson, P., S. Linder, R. Giesler, and P. Högberg. 2005. Fertilization of boreal forest reduces both autotrophic and heterotrophic soil respiration. Global Change Biology **11**:1745-1753.

Petersen, D. G., S. J. Blazewicz, M. Firestone, D. J. Herman, M. Turetsky, and M. Waldrop. 2012. Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. Environmental Microbiology **14**:993-1008.

Philippot, L. 2002. Denitrifying genes in bacterial and archaeal genomes. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression **1577**:355-376.

Philippot, L., J. Čuhel, N. P. A. Saby, D. Chèneby, A. Chroňáková, D. Bru, D. Arrouays, F. Martin-Laurent, and M. Šimek. 2009. Mapping field-scale spatial patterns of size and activity of the denitrifier community. Environmental Microbiology **11**:1518-1526.

Prosser, J. I. and G. W. Nicol. 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends in Microbiology **20**:523-531.

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

Richardson, D., H. Felgate, N. Watmough, A. Thomson, and E. Baggs. 2009. Mitigating release of the potent greenhouse gas N_2O from the nitrogen cycle – could enzymic regulation hold the key? Trends in Biotechnology **27**:388-397.

Rotthauwe, J.-h., W. De Boer, and W. Liesack. 1995. Comparative analysis of gene sequences encoding ammonia monooxygenase of *Nitrosospira sp. AHB1* and *Nitrosolobus multiformis C-71*. FEMS Microbiology Letters **133**:131-135.

Sanford, R. A., D. D. Wagner, Q. Wu, J. C. Chee-Sanford, S. H. Thomas, C. Cruz-García, G. Rodríguez, A. Massol-Deyá, K. K. Krishnani, K. M. Ritalahti, S. Nissen, K. T. Konstantinidis, and F. E. Löffler. 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proceedings of the National Academy of Sciences **109**:19709-19714.

Schleper, C. and G. W. Nicol. 2010. Ammonia-Oxidising Archaea – Physiology, Ecology and Evolution. Pages 1-41 *in* K. P. Robert, editor. Advances in Microbial Physiology. Academic Press.

Stopnisek, N., C. Gubry-Rangin, S. Hofferle, G. W. Nicol, I. Mandic-Mulec, and J. I. Prosser. 2010. Thaumarchaeal ammonia oxidation in an acidic forest peat soil Is not Influenced by ammonium amendment. Applied and Environmental Microbiology **76**:7626-7634.

Su, M., K. Kleineidam, and M. Schloter. 2010. Influence of different litter quality on the abundance of genes involved in nitrification and denitrification after freezing and thawing of an arable soil. Biology and Fertility of Soils **46**:537-541.

Throback, I. N., K. Enwall, A. Jarvis, and S. Hallin. 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol Ecol **49**:401-417.

Tourna, M., T. E. Freitag, G. W. Nicol, and J. I. Prosser. 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environmental Microbiology **10**:1357-1364.

Treusch, A. H., S. Leininger, A. Kletzin, S. C. Schuster, H.-P. Klenk, and C. Schleper. 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environmental Microbiology **7**:1985-1995.

Verhamme, D. T., J. I. Prosser, and G. W. Nicol. 2011. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. The ISME Journal **5**:1067-1071.

Vitousek, P. M., J. D. Aber, R. W. Howarth, G. E. Likens, P. A. Matson, D. W. Schindler, W. H. Schlesinger, and D. Tilman. 1997. Human alteration of the global nitrogen cycle: Sources and consequences. Ecological Applications **7**:737-750.

Wallenstein, M. D., D. D. Myrold, M. Firestone, and M. Voytek. 2006. Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. Ecological Applications **16**:2143-2152.

Wallenstein, M. D. and R. J. Vilgalys. 2005. Quantitative analyses of nitrogen cycling genes in soils. Pedobiologia **49**:665-672.

Yoshida, M., S. Ishii, S. Otsuka, and K. Senoo. 2010. *nirK*-harboring denitrifiers are more responsive to denitrification-inducing conditions in rice paddy soil than nirS-harboring bacteria. Microbes and Environments **25**:45-48.

Yuan, Q., P. Liu, and Y. Lu. 2012. Differential responses of *nirK*- and *nirS*-carrying bacteria to denitrifying conditions in the anoxic rice field soil: *nirK* and *nirS* denitrifying bacteria in rice field soil. Environmental Microbiology Reports **4**:113-122.

Zeglin, L. H., A. E. Taylor, D. D. Myrold, and P. J. Bottomley. 2011. Bacterial and archaeal *amoA* gene distribution covaries with soil nitrification properties across a range of land uses. Environmental Microbiology Reports **3**:717-726.

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