AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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The objectives of this thesis were to examine the links between soil microbial community composition and function using the nitrogen (N) cycle as a model for these interactions and to assess the impact of environmental factors such as microclimate, vegetation type, and nutrient availability on microbial diversity and N transformations in forest soils. The first study consisted of a reciprocal transfer experiment where soil cores were transferred between high-elevation forest and adjacent meadow environments. It focused on bacterial denitrifying communities by measuring denitrification enzyme activity and community composition as determined by terminal restriction fragment length polymorphism (T-RFLP) profiles targeting the gene for nitrous oxide reductase (*nosZ*). Results from this experiment showed that while transferring meadow soils into forests increased denitrification rate, denitrifying community composition did not appear to change after two years. The second study examined N cycling and microbial community composition in soils from 20-year-old experimental tree plantations with pure stands of Douglas fir (*Pseudotsuga menziesii*) and red alder (*Alnus rubra*) in a high- and a low-productivity forest. ^{15}N isotope dilution was combined with

antibiotics to assess the roles of bacteria and fungi in N mineralization and nitrification. Data showed that nitrification was a major sink for NH_4^+ in all soil types and bacteria were the primary nitrifiers. Increased ammonification following antibiotic additions suggested that organic N may be important for the growth of heterotrophic bacteria and fungi. Results of nitrification potential assays showed that most nitrification was acetylene insensitive (autotrophic). Community composition of ammonia-oxidizing bacteria and archaea were assessed by targeting bacterial and archaeal ammonia-monooxygenase (*amoA*) genes. The composition and population size of ammonia-oxidizing bacteria differed between Douglas fir and red alder and tended to group with *Nitrosospira* clusters 2 and 4. Archaeal *amoA* was only amplified from the high-productivity site and grouped with other archaeal clones from soil and estuary sediments. Environmental factors affected rates of N cycling within two years, but community compositional changes responded more slowly, e.g., nitrifying communities differed between 20-year-old tree stands. This suggests that if environmental changes persist they may lead to changes in microbial community composition.

The Link between Nitrogen Cycling and Soil Microbial Community Composition in Forest Soils of Western Oregon

by
Stephanie A. Boyle

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

Stephanie A. Boyle, Author

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CONTRIBUTION OF AUTHORS

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The Link Between Nitrogen Cycling and Soil Microbial Community Composition in Forest Soils of Western Oregon

General Introduction

Chapter 1

Stephanie A. Boyle

1.1 Linking microbial diversity and function

Everything is everywhere, but the environment selects -Baas Becking, 1934

These famous words of Baas Becking in his 1934 book, Geobiology or Introduction to the Science of the Environment, have often been repeated over the last decade by modern microbial ecologists (de Wit and Bouvier, 2006). With the advent and increasing use of culture-independent methods to study the microbial world, microbiologists now have the means to empirically test if all microbes are indeed everywhere and to begin to better understand the roles that environmental factors play in the selection of organisms. These new molecular techniques are especially important in soils studies where microbial populations inhabiting a 10 g sample of soil can comprise as many as 10^{10} individuals (Sloan et al., 2007).

In soils the use of molecular techniques has fundamentally changed the way that microbial ecologists think about diversity and the organisms that comprise it. In 1977, Martin Alexander listed the nine genera of bacteria significant in soil as: *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Micromonospora*, *Nocardia*, *Pseudomonas*, and *Streptomyces* (Alexander, 1977). We know today that Alexander's list comprises just 2.5 to 3.2% of the population of soil bacteria (Janssen, 2006) -- a striking statistic to say the least. Janssen's (2006) review goes on to explain that of those organisms that comprise the soil majority, only about 11% can be assigned to known genera. Therefore, while molecular techniques have given new insights about the size of

soil microbial diversity, they have also revealed that when we study soil function we are observing the collective activity of the almost 90% unknown majority.

Some researchers may argue that studying microbial diversity is an independent pursuit and not critical to understanding soil function, but to not understand the organisms responsible for biogeochemicial cycling is to keep microbial ecology in the realm of natural history and not push towards a predicative understanding of functionality (Amann, 2000; Bell et al., 2005). Although microbial communities possess some "functional redundancy" (Setälä and McLean, 2004), species composition and diversity does not affect all processes equally (Griffiths et al., 2000; Bell et al., 2005). For example, nitrification and methane oxidation may be more sensitive to harsh environmental perturbation, because these processes are carried out by a specific subset of the microbial community (Griffiths et al., 2000). Before we can accurately predict changes in ecosystem function brought about by natural and anthropogenic disturbance, we must understand the links between microbial community composition and ecosystem function (Zak et al., 1994; Hooper et al., 2005).

Perhaps nowhere are the links between microbial community composition and function more important than in nitrogen (N) cycling, where the majority of transformations are microbially-mediated and where many individual processes such as nitrification and denitrification are carried out by specialized organisms (Zumft, 1999; De Boer and Kowalchuk, 2001; Vitousek et al., 2002). By examining N cycling rates in conjunction with examining microbial community

structure, we can gain new understanding about the roles of microorganisms in ecosystem processes and how environmental factors affect both microbial diversity and function (Zak et al., 2003; Philippot and Hallin, 2005; Cookson et al., 2006).

1.2 N cycling in acidic forest soils

In the conifer-dominated forests of the Pacific Northwest where N is often a limiting nutrient, soil microorganisms play key roles in the N cycle, mediating the availability of N for ecosystem primary productivity. Although the ability of microbes to break down N-containing compounds and recycle small organic and inorganic compounds has been known for sometime, the extent to which microbial diversity affects these processes is not clear. Through the secretion of exoenzymes, soil bacteria and fungi carry out the decomposition of soil organic matter (Schimel and Wientraub, 2003), freeing small N-containing monomers that can be further mineralized or taken up by microbes or plant roots (Schimel and Bennett, 2004). Although the environmental and biological controls on soil organic matter decomposition are not completely known, it has been proposed that the availability of mineral N may regulate decomposition by suppressing enzymes related to lignin degradation, but increasing enzymes such as cellulase (Fog, 1988; Hammel, 1997; Schimel and Weintraub, 2003). Through time N availability may impact microbial community composition (Sinsabaugh et al., 2003). For example, in conifer forest soils where ammonification appears to be tightly coupled to ammonium (NH_4^+) consumption, fungi may have some advantage because of their ability to explore more of the soil matrix and access newly mineralized N.

For many years researchers measuring net N mineralization in forest soils speculated that nitrification, the conversion of ammonia (NH_3) to nitrate (NO_3^-), did not occur in conifer forests. This view changed, however, when Davidson et al. (1991) used isotope dilution to show that although net nitrification was low, gross nitrification rates could be quite high and NH_4^+ may turnover rapidly (~ 1 day). Under low mineralization rates nitrification may be proportionally more important than assimilation as a fate for NH_4^+ (Booth et al., 2005). Both heterotrophic and autotrophic microorganisms have been shown to contribute to nitrification in acidic forest soils (Schimel et al., 1984; Stroo et al., 1986; De Boer et al., 1992; Hart et al., 1997; Pavvolainen and Smolander, 1998); and N mineralization, NH_4^+ availability, and pH have all been cited as factors determining the relative contributions of heterotrophs and autotrophs (Schimel et al., 1984; De Boer et al., 1988; Hart et al., 1997; Pederson et al., 1999). Traditionally, researchers believed that heterotrophic fungi contributed significantly to nitrification in acidic forest ecosystems, because they were better suited to deal with conditions of low pH (Stroo et al., 1986), but only a few heterotrophic bacteria and fungi have been isolated that may be able to efficiently carry out nitrification (Stroo et al., 1986; Brierley and Wood, 2001; De Boer and Kowalchuk, 2001). Although cultured isolates of autotrophic nitrifiers appear to be acid sensitive and cease nitrification at pH < 5, molecular-based studies have demonstrated that genes for ammonia monooxygenase (*amoA*) are present in many acidic environments (De Boer and Kowalchuk, 2001; Burton and Prosser, 2001; Laverman et al., 2001; Mintie et al.,

2003; Nugroho et al., 2006) and that *Nitrosospira* clusters 2 and 4 of the NH_3-oxidizing bacteria may actually have acid-tolerant members (Nugroho et al., 2006). The discovery of crenarchaeota that also possess *amoA* genes (Treusch et al., 2005; Leininger et al., 2006) points to yet a third group of organisms that may be capable of contributing to nitrification in acidic systems.

The NO_3^- produced in soils may have several fates including assimilation, denitrification, and leaching. Both denitrification and leaching lead to a loss of N from the system, however, assimilation of NO_3^- leads to N retention. Many soil bacteria and fungi are capable of NO_3^- assimilation, but pure culture studies have shown that NO_3^- assimilation is often tightly regulated and inactive in the presence of NH_4^+, glutamine, or glutamate (Merrick and Edwards, 1995; Marzluf, 1997). In soils, however, NO_3^- assimilation has been observed even when NH_4^+ is present (Hart et al., 1994), leading some researchers to hypothesize that microsite heterogeneity may play a role in the assimilation of inorganic N (Chen and Stark, 2000). If fungi are able to explore more of the soil matrix and more effectively scavenge for NH_4^+, then bacteria may rely on more mobile NO_3^-. Other researchers have proposed that incorporation of NO_3^- may be more complex and suggest that various species of soil microbes may have different preferences for NH_4^+ and NO_3^- (Bengtson and Bengtsson, 2005).

In addition to assimilation, NO_3^- may be converted to N_2 gas through the process of denitrification. The amount of denitrification in forest soils is typically low (Vermes and Myrold, 1992; Griffiths et al., 1998), but rates can be spatially

variable and higher rates have been observed in the litter layers of some coniferous forests (Laverman et al., 2000) and under tree species such as red alder (Griffiths et al., 1998). Although denitrification rates have been measured extensively (Öquist et al., 2004; Parsons et al., 1991), fewer studies have sought to link denitrification rates to microbial community composition. Studies that have utilized molecular methods to target functional genes have found that denitrifying community composition varies between sites and vegetation types (Prieme et al., 2002; Rich et al., 2003), but connections between denitrifier diversity and function are still not clear (Philippot and Hallin, 2005; Wallenstien et al., 2006b). Bacteria are thought to be the primary denitrifiers in most soil systems, but we also know that some fungi (Shoun et al., 1992; Tanimoto et al., 1992) and archaea (Ichiki et al., 2001) are also capable of denitrification. The extent to which these groups contribute to denitrification is not known, but some evidence suggests that fungi may be important in denitrification in grassland soils (Laughlin and Stevens, 2002; McLain and Martens, 2006).

1.3 Environmental factors altering microbial communities

Researchers have attempted to better understand the links between community composition and N cycling processes by carrying out a number of observational and manipulative experiments. For example, a number of studies have examined differences in microbial diversity and N cycling in forest soils associated with different tree types (Hart et al., 1997; Pennanen et al., 1999; Myers et al., 2001; Leckie et al., 2004; Grayston and Prescott, 2005; Lejon et al., 2005) to

gain a better understanding of natural diversity of forest microorganisms. Other studies have examined compositional changes after disturbances such as fire (Yeager et al., 2005) or reciprocal transfers of soils cores (Clien and Schimel, 1995; Hart and Perry, 1999; Bottomley et al., 2004; Waldrop and Firestone, 2006), and several studies have used fertilizer additions to better understand the impact of increased N deposition on forest soils (Compton et al., 2004; Wallenstien et al., 2006a; Zak et al., 2006; Högberg et al., 2007). They have combined process information with molecular characterizations of the soil microbial community to gain a better understanding of the links between community structure and function. Molecular methods including phospholipids fatty acid (PLFA) profiling as well as DNA-based techniques have expanded our current knowledge of the diversity and abundance of key functional groups.

A number of environmental factors have been found to impact N cycling rates including: climate (Waldrop and Firestone, 2006; Horz et al., 2004, Hart and Perry, 1999), tree type (Grayston and Prescott, 2005; Lejon et al., 2005), understory vegetation (Leckie et al, 2004), and N availability (Sinsabaugh et al, 2003; Högberg et al., 2007). Additionally, research has shown that these changes in N cycling are often accompanied by changes in the microbial community composition, including differences in fungal:bacterial ratios (Pennanen et al., 1999; Myers et al., 2001; Priha et al., 2001a and b; Hackl et al., 2004; Leckie et al., 2004; Grayston and Prescott, 2005; Högberg et al., 2007). The varied contributions of fungi and bacteria to N cycling processes imply that their relative proportions in

the soil matrix could significantly impact the rates at which N is cycled. In general, researchers have observed that increased N availability lowers fungal:bacterial ratios (Leckie et al., 2004; Högberg et al., 2007). Some research suggests that the proportion of mycorrhizal and saprophytic fungi may decrease in soils with high N availability (Avis et al., 2003; Nilsson and Wallander, 2003; Grønli et al., 2005; Sinsabaugh et al., 2005; Wallenstein et al., 2006) and these decreases could impact not only N cycling, but the rates at which soil organic matter is decomposed (Zak et al., 2006). More research is needed to better understand the contributions of fungi, bacteria, and archaea to N cycling processes and the environmental factors that affect their proportions and diversity.

1.4 Thesis objectives

The primary objective of this work was to expand current understanding of the links between microbial community composition and function and the impact that environmental factors have on N cycling processes in conifer-dominated forest soils.

Chapter 2 describes an experiment where soil cores were reciprocally transferred between high elevation meadow and adjacent forest environments. The focus of this study was to better understand how variations in vegetation and microclimate would affect the function and diversity of soil denitrifying bacteria over a two-year period. Open and closed cores were included in the study design to test the hypothesis that vegetation type would impact both denitrification rate and composition. Denitrification potential assays were used to determine process

rates, and the gene for nitrous oxide reductase (*nosZ*) was used to generate community profiles of denitrifying bacteria.

Given that fungal:bacterial ratios have been shown to vary with N availability (Leckie et al., 2004; Högberg et al. 2007) and that bacteria and fungi may impact N cycling in different ways, a study was designed to test the contributions of bacteria and fungi to N cycling in forest soils under Douglas fir (*Pseudotsuga menziesii*) and red alder (*Alnus rubra*). This study took place at two experimental tree plantations, one a high-productivity site located in the Coast Range and the second a lower-productivity site located in the Cascade Mountains. Douglas-fir soils were contrasted with soils supporting N-fixing red alder, because previous work indicated that red alder not only increased rates of nitrification and the concentrations of soil NO_3^- (Hart et al., 1997), but also appeared to lower pH (Rhoades and Binkley, 1992).

Chapter 3 describes an experiment that combined ^{15}N isotope dilution with antibiotics to block bacterial and fungal protein synthesis. Laboratory incubations were used to determine gross and net rates of N mineralization and nitrification. PLFA profiles and quantitative (Q)-PCR techniques were used to determine fungal:bacterial ratios. Although antibiotics have been used in the past to assess fungal:bacterial ratios (Anderson and Domsch, 1973; West, 1986; Velvis, 1997; Lin and Brookes, 1999; Bailey et al., 2003; Wallenstein et al., 2006a), few studies have paired antibiotics with isotope dilution (Laughlin and Stevens, 2002). This experiment tested the hypothesis that the relative contribution of fungi to N

cycling would be greater in low-productivity forests and greater in Douglas-fir soils where N availability would be lower.

Chapter 4 describes an experiment to determine the soil microbial communities responsible for nitrification and to examine the diversity of nitrifying communities by targeting the functional gene NH_3-monooxygenase (*amoA*). Nitrification potential assays with and without acetylene were used to determine the relative amounts of autotrophic and heterotrophic nitrification in each treatment. NH_3-oxidizing bacterial and archaeal communities were then examined by amplifying *amoA* using PCR. In the case of bacterial *amoA*, populations were quantified using Q-PCR and communities examined using T-RFLP profiles and DNA sequencing. Archaeal *amoA* was examined by DNA sequencing to determine how sequences present in these forest soils were related to those found in agricultural soils and estuary sediments (Francis et al., 2005; Leininger et al., 2006).

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Reciprocal Transfer Effects on Denitrifying Community Composition and Activity at Forest and Meadow Sites in the Cascade Mountains of Oregon

Chapter 2

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2.1 Abstract

In order to examine the effects of disturbance, vegetation type, and microclimate on denitrification and denitrifier community composition, experimental plots were established at the H.J. Andrews Experimental Forest in the Cascade Mountains of Oregon. Soil cores were reciprocally transplanted between meadow and forest and samples were collected after one and two years. Denitrifying enzyme activity (DEA) was measured using the acetylene block assay and terminal restriction length polymorphism profiles were generated with *nosZ* primers that target the gene coding for nitrous oxide reductase. Nitrate concentrations, C mineralization, and water content were measured to gain additional insights into soil properties controlling DEA. Meadow soils were significantly higher in DEA than forest soils, and the highest DEA levels were observed in cores transferred from the meadow into the forest. Nitrate concentrations were also different between forest and meadow soils, but did not correlate to DEA. DEA was higher in open versus closed cores, suggesting an association between denitrification and the rhizosphere. Denitrifier communities of undisturbed forest and meadow soils shifted through a four-year period but remained distinct from each other. Similarly, denitrifier communties clustered by vegetation type of origin regardless of manipulation, suggesting that the overall denitrifier communities are well buffered against environmental changes.

2.2 Keywords

Denitrification, Forest soils, Microbial community composition, N cycling

2.3 Introduction

Denitrification, the process by which mineral N in the forms of nitrate (NO_3^-) and nitrite (NO_2^-) is converted to N_2 gas, represents an important N-cycling transformation. Not only does denitrification lead to a loss of N from soil systems, but incomplete conversion of mineral N to N_2 results in the formation of nitric oxide (NO), which can contribute to ozone formation, and nitrous oxide (N_2O), a greenhouse gas (Öquist et al., 2004; Zumft, 1999). The amounts of N_2O released from forest soils were traditionally thought to be low, approximately 0.2 kg N ha^{-1} yr^{-1} (Bouwman et al., 1995), but a more recent study has shown that denitrification rates can be spatially variable and quite high in the litter layer of coniferous forests (Laverman et al., 2000). Despite typically low NO_3^- concentrations in coniferous forest soils, isotope dilution studies have shown that gross rates of NO_3^- production and consumption can occur at relatively high rates (Davidson et al., 1992; Stark and Hart, 1997). Therefore, under some circumstances denitrification may in fact be an important fate of NO_3^- in these systems. For example, Brooks et al. (1997) showed that denitrification increased during the winter and spring months under snow cover when plant uptake of NO_3^- was low.

Although the rates of denitrification, as well as other N-cycling processes, have been measured extensively (Laverman et al., 2000; Öquist et al., 2004; Parsons et al., 1991), fewer studies have sought to link these rates to the microbial communities. Studies that have utilized molecular methods to target functional

genes have found that denitrifying community composition varies between: cultivated and uncultivated agricultural soils (Stres et al., 2004), riparian and agricultural soils (Rich and Myrold, 2004), wetland and forested uplands (Prieme et al., 2002), and forest and meadow soils (Rich et al., 2003). Temporal studies have shown seasonal shifts in these communities, as well (Mergel et al., 2001; Wolsing and Prieme, 2004). The long-term dynamics and overall environmental effects on these communities are still unknown, however. The goals of this study were to expand our knowledge of denitrifying community dynamics by evaluating the roles of plant community and microclimate on community composition in the Oregon Cascade Mountains where open meadows are interspersed among coniferous forests.

As part of a study designed to more fully understand how the forest and meadow environments might influence N cycling, denitrification and denitrifying community composition were determined in soil cores that had been reciprocally transferred between adjacent meadow and forest sites. In the light of previous work (Rich et al., 2003) we hypothesized that treatments controlling root in-growth would allow us to examine the importance of vegetation type on denitrifying enzyme activity (DEA) and community composition. Likewise we hypothesized that transferring cores between the shaded, wetter forest and the drier meadow would permit us to evaluate the relative impact of forest and meadow microclimate on denitrifying parameters.

2.4 Material and methods

2.4.1 Site description

This study was conducted at the H.J. Andrews Experimental Forest, located in the western Cascade Mountains of Oregon. Two separate sites were selected, Lookout and Carpenter Mountains. The sites were at an elevation of roughly 1500 m and both contain coniferous forest with interspersed meadows. For complete site description and details of vegetation type, refer to Rich et al. (2003) and Bottomley et al. (2004).

At each of the two locations 35 X 35 m grids were established in the forest and adjoining meadow. The organic horizon was removed and a PVC pipe (inner diameter 5 cm) was driven into the mineral soil to a depth of 10 cm. A total of five treatments were included in the study design. In the "remaining" treatment, soils were cored and then left in place. An equal number of soil cores were transferred to the adjacent vegetation type. Transferred and remaining cores were subdivided into open and closed cores to test for root effects on soil processes. Open cores were placed in mesh bags and closed cores remained in the PVC pipe with mesh enclosing the top end. Soil cores were transferred in September 2000 and collected in September of 2001 and 2002. To control for disturbance effects, background cores were taken at the same time of core collection, as well as at the time of plot establishment and again in 2004, so as to also examine temporal variation in denitrifying communities.

2.4.2 Soil Properties

The soil samples were passed through a 4-mm sieve one day after they were collected from the field. A 10-g sample was dried at 105^{o}C overnight and reweighed to determine gravimetric water content. Another 10-g sample was immediately extracted using 2 M KCl and NO_3^- was measured colorimetrically using an autoanalyzer (Astoria-Pacific, Portland, OR). An additional 30-g sample was placed in a 1-L mason jar and incubated at 25^{o}C for 28 days. CO_2 was measured at 7, 14, and 28 days using a gas chromatograph equipped with a thermal conductivity detector. The jars remained sealed throughout the incubation, except for 5-min aerations just following the weekly measurements. Aeration ensured that the soils did not become anaerobic.

2.4.3 Denitrification enzyme activity

Denitrification enzyme activity (DEA) was determined using methods described by Rich et al. (2003). Fresh soil (10 g) was added to a solution containing glucose, NO_3^-, and phosphate buffer. Assay systems were made anaerobic and acetylene was added to block N_2O reductase activity. Production of N_2O was quantified in samples taken at 15 and 75 min using gas chromatography.

2.4.4 DNA extraction and amplification

A Bio101 soil extraction kit (Q-Biogene, Irvine, CA) was used to extract DNA from each soil sample. Briefly, approximately 0.5 g of soil was added to a mini-prep tube containing garnet beads and cells were lysed using physical disruption. The extract was then purified by binding the DNA to a silicon matrix

and rinsing with ethanol containing buffer, before elution with water. Extracted DNA was quantified using a UV spectrophotometer before amplification by PCR.

A primer set targeting a 700-bp region of the *nosZ* gene was used with previously described PCR parameters (Rich et al., 2003). The only modification was an increase from 25 to 30 PCR cycles making it unnecessary to pool products. These PCR products were then purified using a Qiagen DNA clean-up kit (Qiagen, Valencia, CA) in order to eliminate genomic DNA, excess primers, and unused nucleotides.

2.4.5 Restriction and T-RFLP profiles

Products were digested using three restriction enzymes: *cfo*I, *rsa*I, and *msp*I (Promega Corp., Madison, WI). Digests were run according to manufacturers' specifications by incubating the restriction digest for 3 h at 37^{o}C and heat inactivating at 65^{o}C for 15 min.

Restricted samples were submitted to the Oregon State University Center for Genome Research and Biocomputing for analysis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Approximately 1 ng of amplified DNA was submitted for analysis. Samples were re-run if the largest peak in an individual profile was less than 2000 fluorescent units.

2.4.6 Statistical Analysis

DEA and soil properties were analyzed using repeated measures analysis of variance (ANOVA) (Ramsey and Schafer, 2002), included in the SAS software package. Significant interactions between year of sampling, site, and treatments

required that the data be analyzed separately for each year and site. Soil disturbance effects were analyzed by comparing background with open remaining cores. Effects of transferring soil and core type were analyzed using the data from remaining and transferred cores and open and closed cores, respectively (i.e., background cores were excluded). Linear regession analysis was also performed to determine if DEA correlated to NO_3^- concentration, C mineralization, and soil water content. Treatment effects were considered significant if the p-value was < 0.05.

In the case of terminal restriction fragment length polymorphisms (T-RFLP) profiles, length and fluorescence of the terminal restriction fragments (T-RF) were determined using GeneScan version 3.5 and Genotyper version 2.5 software (Applied Biosystems). The community data set was then analyzed using multivariate statistical analysis performed with PC-ORD version 4.27 (B. McCune and J.J.Mefford, PC-ORD for Windows: multivariate analysis of ecological data, 4.01ed, MjM Software, Gleneden Beach, OR, 1999). The total fluorescence was summed for each sample and then used to relativize peaks as a fraction of the total fluorescence. After examining electropherograms of negative controls, peaks contributing less than 1.5% of the total fluorescence in a single profile were excluded and the relative fluorescence recalculated, making the sum of each profile equal to 100% (Rich et al., 2003). Peaks were then aligned by base pairs and any peaks less than two base pairs apart were combined for final fragment identifications. The three enzyme profiles were combined to produce composite T-

RFLP profiles. Differences in sample community profiles were determined using non-metric multidimensional scaling (NMS) (McCune and Grace, 2002) ordination techniques and Sørenson distance measures. Starting from random configurations, 500 iterations were run to produce NMS ordinations with a final instability criterion of 0.0001. Monte Carlo test results were compared against real data to determine the significance of a given solution. Ordinations were constrained to two axes; the ordination with the lowest final stress was chosen for further analysis. Relationships between DEA and denitrifier community composition were explored with the joint plot feature of PC-ORD (McCune and Grace, 2002), which basically correlated the DEA values with the NMS axis scores of the samples.

Multi-response permutation procedures (MRPP) were used to test the strength and statistical significance of group membership in total community profiles. MRPP is a nonparametric method for testing group differences, similar to multivariate analysis of variance (MANOVA). A p-value < 0.05 and an *A*-statistic > 0.1 was considered significant (McCune and Grace, 2002). Indicator species analysis was used to identify possible biomarkers and to identify TRFs responsible for the separation of individual samples into groups.

2.5 Results

2.5.1 Denitrification Enzyme Activity

DEA was an order of magnitude higher in soils originating from the meadow compared to the forest, with values slightly higher in year two than year

one and Lookout higher than Carpenter (Figure 2.1 A and B). Forest samples ranged from undetectable levels for some Carpenter samples to 0.92 μg N_2O-N g^{-1} soil d^{-1} for the Lookout forest open transfer samples in year two. In contrast, meadow samples ranged from 0.60 to 4.38 μg N_2O-N g^{-1} soil d^{-1}. DEA was lower in background compared to open remaining cores at Lookout forest in year one, but this was the only disturbance effect observed. Transfer of open cores from Lookout meadow to forest caused an increase in DEA relative to the cores that remained in place. DEA in open cores was usually higher than closed cores. This core effect was statistically significant for meadow soils at both sites in year one and at Lookout in year two.

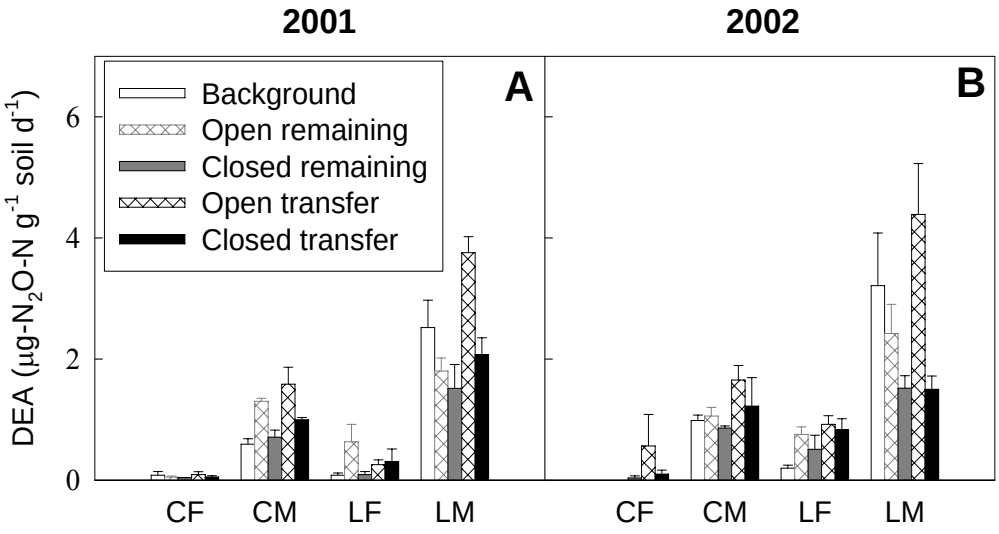

Figure 2.1 Denitrification enzyme activity (DEA). Values are shown in panels A and B, where bars represent the mean values of three field replicates and error bars show the standard error. Original soil location is listed along the bottom: Carpenter forest (CF), Carpenter meadow (CM), Lookout forest (LF), and Lookout meadow (LM). Treatments include: background (B), open remaining (OR), closed remaining (CR), open transfer (OT), and closed transfer (CT). Panel A is the data for year one and B is year two data.

2.5.2 Soil Properties

Nitrate concentrations were an order of magnitude higher between years one and two (Figure 2.2 A and B). Despite the low levels of NO_3^- in year one, statistically significant differences were observed between open and closed, as well as transferred and remaining treatments. Closed cores had more NO_3^- than open cores. Nitrate concentrations were significantly higher at the Carpenter site in cores incubated in the meadow compared to forest, during both years and at the Lookout site in year two.

Carbon mineralization tended to be higher in year two compared to year one (Figure 2.2 C and D). A single disturbance effect was observed in Lookout forest soils during the second year where the open remaining treatment was higher than the background. At Carpenter in year two, C mineralization rates were higher for cores located in the forest compared to the meadow. In year two at Lookout, C mineralization rates were higher in open versus closed cores.

Water contents were significantly higher in cores incubated in the forest compared to meadow-incubated cores (Figure 2.2 E and F). A disturbance effect was observed in Lookout forest during year one, but this was the only case where open remaining cores contained significantly more water than backgrounds. Closed cores incubated in the forest tended to contain more water than open cores. This was significant both years at Carpenter and the second year at Lookout.

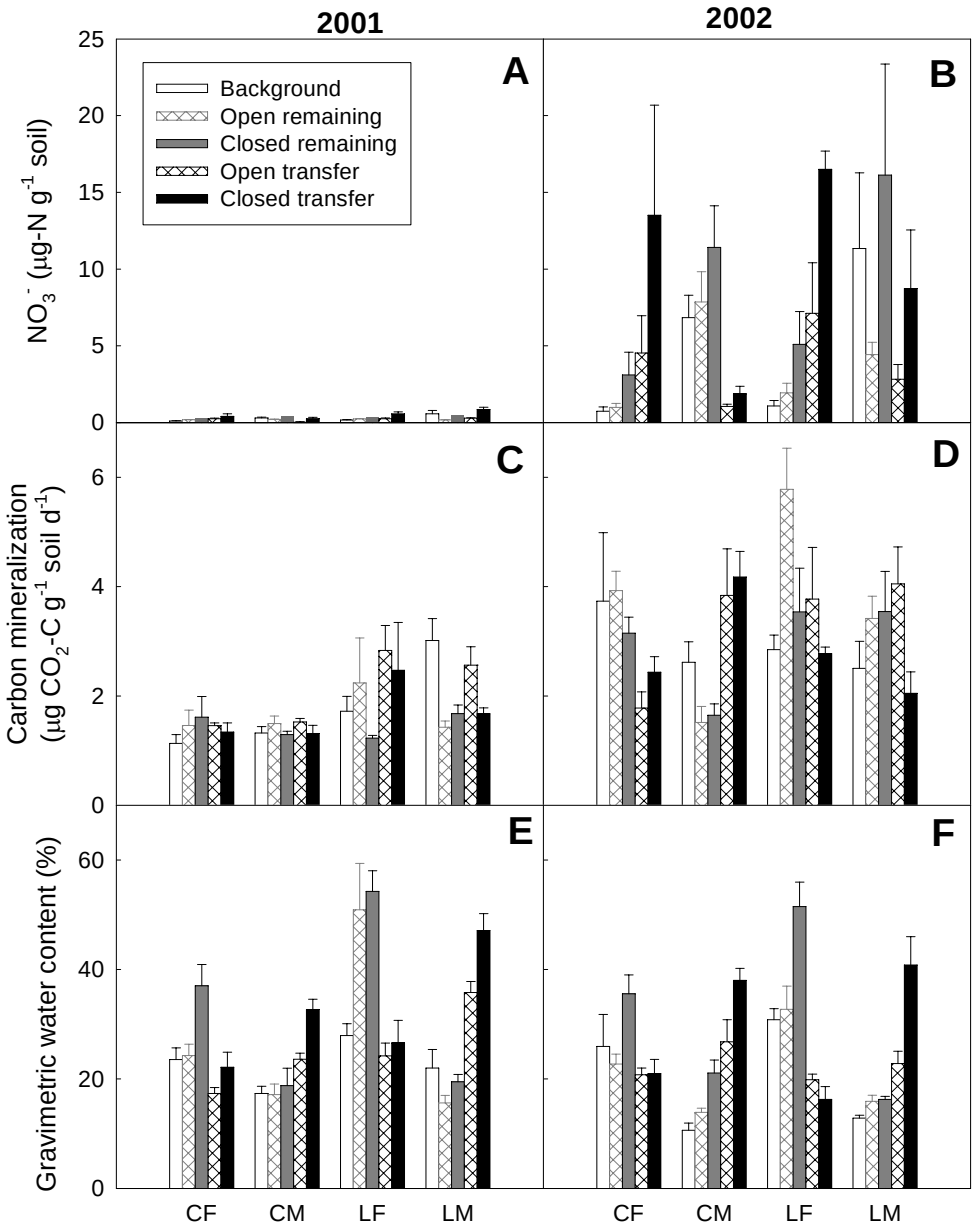

Figure 2.2 Soil properties for each location where soil originated. Bars are an average of three field replicates and error bars are standard errors. Original soil location is listed along the bottom: Carpenter forest (CF), Carpenter meadow (CM), Lookout forest (LF), and Lookout meadow (LM). Treatments include: background (B), open remaining (OR), closed remaining (CR), open transfer (OT), and closed transfer (CT). Panels A and B list NO_3^- concentrations for year one (2001) and two (2002). Panels C and D show C mineralization values, and E and F give water content at the time of collection.

When data for both years was used, no significant correlations was found between DEA and soil NO_3^- concentration, C mineralization, or water content; however, a few significant correlations were found for subsets of the data. DEA was positively correlated with C mineralization for meadow soils in year one (r^2=0.45) and DEA was positively correlated with water content of open cores in forest and meadow soils in year one and in meadow soils in year two (r^2 values ranging from 0.46 to 0.69).

2.5.3 Community Composition

A total of 242 peaks were identified with *cfo*I, *rsa*I, and *msp*I containing 83, 64, and 95 peaks, respectively. When the digest profiles were combined, each sample contained an average of 18 peaks.

Vegetation type (defined by soil origin) was an important factor governing denitrifer community composition ($p < 0.001$, $A = 0.12$). Indicator species analysis identified 51 TRFs that contributed to the observed differences in vegetation. Many of these fragments were rare, but 16 of these fragments were examined more closely because they were seen in at least 25% of all generated profiles; among these fragments were *cfoI* 453, *mspI* 73, and *rsaI* 450 (Figure 2.3). Similar to the results reported by Rich et al. (2003), these fragments appeared to be markers for forest or meadow soil. For example, *cfoI* 453 was a minor peak in Lookout forest soils making up less than 5% of the mean relative fluorescence and present in only 14 of the 30 forest profiles. In contrast, *cfoI* 453 was present in all the Lookout

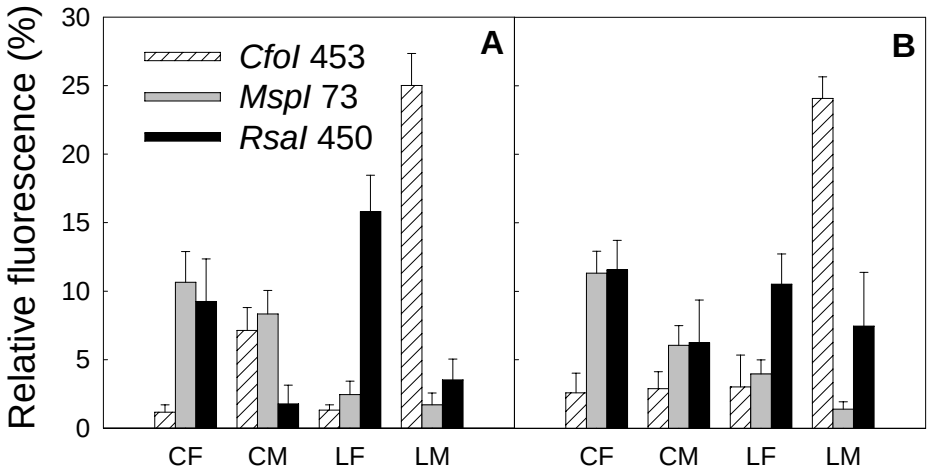

Figure 2.3 Panels A and B show the relative fluorescence of three terminal restriction fragments *cfoI* 453, *mspI* 73, and *rsaI* 450. Bars represent the average of 15 cores and include all treatment types. Error bars are the standard error of the mean. Original soil location is listed along the bottom: Carpenter forest (CF), Carpenter meadow (CM), Lookout forest (LF), and Lookout meadow (LM). Panels A and B are year one and two, respectively.

meadow profiles and made up 25% of the mean relative fluorescence (Figure 2.3). Fragment *rsaI* 450, on the other hand, was more indicative of forest soils.

When stratified by year and site community profiles differed between selected treatments (Table 2.1). For example, in year two cores from Lookout forest transferred to the meadow environment differed from those that remained in place. Further investigation of the community profiles showed that indicator peaks, for forest or meadow soils, varied between the remaining and transferred samples (Figure 2.4). Although not statistically significant in most cases, the meadow-specific *cfoI* 453 fragment consistently made up a slightly higher proportion of the fluorescence in soils transferred to the meadow compared to those forest soils that remained in place. Likewise, an examination of the forest-specific *rsaI* 450

showed that this TRF was more abundant in meadow soils that were moved to the forest (Figure 2.4).

Table 2.1 Differences between denitrifying community composition. P-values with A-statistics in parentheses were calculated using MRPP. A p-value of < 0.05 was considered significant.

Year	Site	Soil origin	Disturbance effect	Transfer effect	Root effect
2001	Carpenter	Forest	----------	0.041 (0.08)	----------
		Meadow	----------	0.005 (0.14)	----------
	Lookout	Forest	< 0.001 (0.28)	----------	----------
		Meadow	----------	----------	----------
2002	Carpenter	Forest	----------	----------	----------
		Meadow	----------	----------	----------
	Lookout	Forest	0.022 (0.43)	0.039 (0.12)	----------
		Meadow	----------	----------	----------

The low *A*-statistic reported for Carpenter forest in year one reflects a relatively high amount of within-group variability associated with denitrifying communities in forest soils (Table 2.1). The heterogeneous nature of these soil communities may have masked additional treatment effects. For instance, no root effects (closed versus open cores) were observed during either year, and disturbance effects were only observed in Lookout forest (Table 2.1). The data were also examined to find possible correlations between the denitrifier community composition and the DEA activity. In year one, forest and meadow communities separated on the NMS ordination and DEA was positively correlated ($r^2 = 0.54$) with these forest community profiles (data not shown). A similar trend was observed in 2002. No correlation was observed between imposed treatments, however.

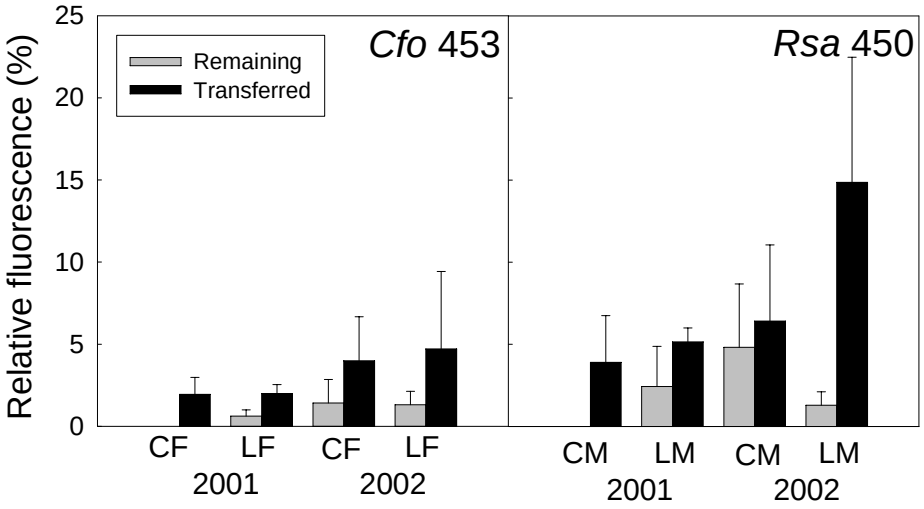

Figure 2.4 Relative fluorescence of two terminal restriction fragments. The abundance of *cfoI* 453, a meadow marker, is compared in soils from Carpenter forest (CF) and Lookout forest (LF), during year one (2001) and year two (2002). The abundance of the forest-specific marker *rsaI* 450 is compared in soils from Carpenter meadow (CM) and Lookout meadow (LM). All bars are the average of six field replicates and error bars represent the standard error of the mean.

2.5.4 Temporal variability in community composition

Denitrifying community composition was compared in background cores from all four locations through a four-year period (Figure 2.5). Similar to the findings in all reciprocal transfer treatments, background communities differed between the two vegetation types ($p = 0.008$, $A = 0.13$), but there was no significant difference between the Carpenter and Lookout site ($p=0.708$, $A=-0.01$). Therefore sites were combined for further statistical analysis. Year zero background samples cluster on the right, year two and four samples are on the left, and year one samples are intermediate. Separation in background communities by year was supported by MRPP ($p < 0.001$, $A = 0.25$).

Because the year zero and year one samples were extracted and then stored before amplification and analysis of the *nosZ* fragment, we investigated the effects of storage. The initial analysis of the year two samples began within a month of field collection, but a subset of these extracts was stored under the same conditions as the year zero and year one extracts. After two years of storage, the year two extracts were re-amplified and new community profiles were generated. A comparison of community profiles from fresh and stored samples indicated no change in the community fingerprint (data not shown). These results indicate that

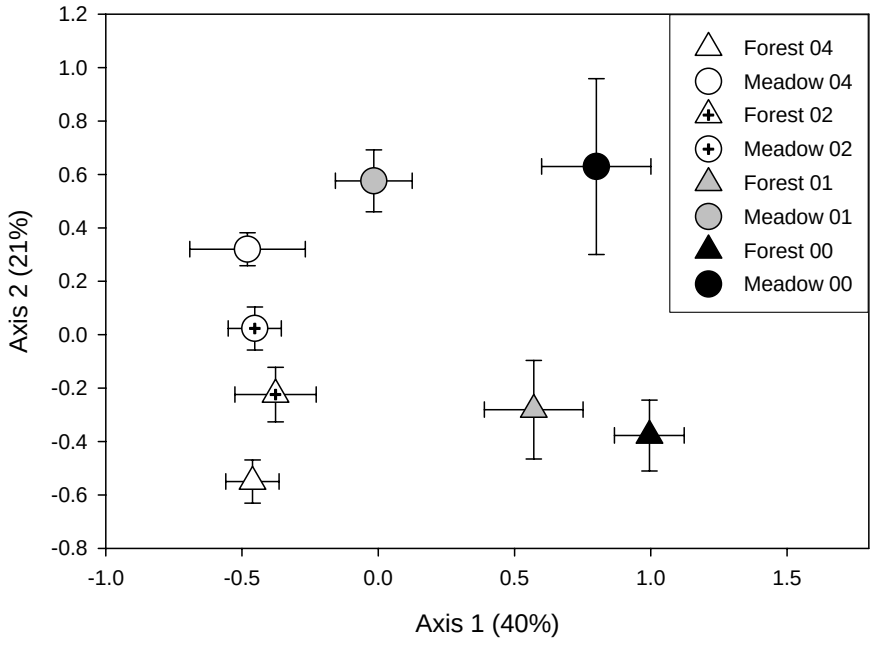

Figure 2.5 All terminal restriction fragment length polymorphism (T-RFLP) profiles of background cores were ordinated using non-metric multidimensional scaling (NMS). The two dimensional solution had a final stress of 11.14 and a final instability of 0.00001. Percentages associated with axis one and two show the percent variance explained along that axis. Points represent the average of all vegetation-specific soil and are the average of six replicates, three replicate cores from each of the two sites. Error bars are the standard error of the mean.

storage did not significantly alter microbial community structure, but that the communities did change over time.

2.6 Discussion

The reciprocal transfer treatments allowed us to examine the extent to which a combination of forest and meadow microclimates and plant community composition influenced denitrifying activity and denitrifier community composition in these environments of the Oregon Cascade Mountains. We found that denitrifier community composition was highly dependent on original vegetation type and was quite resistant to changes brought about by disturbance and transfer of soils. Our data confirmed the hypothesis that root-in-growth affects denitrification activity in meadow soils. We also found that DEA remained low in forest soils transferred to the meadow despite a large increase in rates of nitrification and NO_3^- concentrations (Bottomley et al., 2004). Because differences were observed in specific TRFs of meadow and forest soil and the composition of background communities indicated shifts over time, some members of the denitrifier community may be dynamic and subject to a complex set of biological and abiological controls.

Most studies from forested ecosystems suggested that NO_3^- availability limits DEA (Griffiths et al., 1998; Groffman and Tiedje, 1989; Rich et al., 2003; Robertson and Tiedje, 1984; Vermes and Myrold, 1992). We found DEA to be unrelated to soil NO_3^- concentrations (Figure 2.1 and 2.2), which were highly variable year to year; however, there was a significant correlation (r^2=0.17) of

DEA with the net nitrification rates reported by Bottomley et al. (2004). This correlation was stronger in year one (r^2=0.51) than year two (r^2=0.30), probably because there was more NO_3^- available the second year (Figure 2.2).

We measured soil water content as a proxy for soil aeration but found no overall correlation between soil water content and DEA. Soils that experienced the forest microclimate were always wetter than soils of comparable treatments located in the meadow (Figure 2.2), however, and meadow soils incubated in the forest had higher DEA. Although closed cores were usually wetter than open cores (Figure 2.2), which excluded roots, the effect of water content on DEA was only significant for open cores, suggesting a complex interaction among denitrifiers, roots, and soil water content.

Enhanced DEA in most open cores suggests that a rhizosphere effect may have favored a larger or more active denitrifying population. The influence of roots on denitrifiers is a balance between positive and negative effects. Respiration by roots and enhanced microbial respiration from root-derived C may decrease O_2 availability, thereby favoring denitrifying bacteria (Klemedtsson et al., 1987; Qian et al., 1997). The C supplied by rhizodeposition is known to stimulate microbial respiration and activity (Butler et al., 2004; Kuzyakov and Domanski, 2000; Qian et al., 1997; Schimel and Bennett, 2004), increasing denitrifier activity and possibly population size (Hall et al., 1998; Mounier et al., 2004; Priha et al., 1999; Smart et al., 1997; Smith and Tiedje, 1979). Although significant differences were not observed in the C mineralization during laboratory incubations, we did not

focus upon rhizosphere soil, specifically, and root supplied C may turn over quickly and so would need to be examined *in situ* (Bulter et al., 2004; Kuzyakov and Domanski, 2000). Plant uptake of water and NO_3^- could have a negative impact on denitrifying bacteria; the former by increasing aeration, the latter by direct competition. The open cores in our study were consistently drier than closed cores but this did not have a negative impact on DEA (Figure 2.1 and 2.2), suggesting that conditions conducive to denitrification occur at least some time during the year. Indeed, recent studies indicate that anaerobic microbial processes can occur in wet soil under snow pack during winter and early spring (Brooks et al., 1996). The water content of the open cores likely influenced denitrifiers because, in most cases, DEA was positively correlated to the water content of the open cores. Concentrations of NO_3^- (Figure 2.2) and net nitrification rates (Bottomley et al., 2004) were often lower in open versus closed cores, most likely because of plant uptake of NO_3^-. Because DEA was higher in the open cores, it is likely that denitrifiers compete effectively with other microbial and plant sinks for NO_3^- (Christensen and Tiedje, 1988; Højberg et al., 1996; Murray, 1989). In addition to activity measurements, Mergel et al. (2001), using probes for denitrifying genes, reported an increase in the abundance of *nirS*, *nirK*, and *nosZ* genes in rhizosphere versus bulk soil, further supporting the link between denitrifying bacteria and the rhizosphere.

The clustering of meadow denitrifier community profiles apart from forest profiles lends further support for the coupling of denitrifier communities to plant

root activities (Hall et al., 1998; Rösch et al., 2002; Rich et al., 2003; Smart et al., 1997). Denitrification rates and communities differed between meadow and forest, but treatment-imposed changes of DEA generally did not correlate well with community profiles. For example, although DEA rates increased in meadow soils transferred to the forest (Figure 2.1), this transfer effect was not usually accompanied by a detectable shift in the community composition (Table 2.1). The relatively high diversity and numbers of denitrifiers in soil (Cavigelli and Robertson, 2001; Palumbo et al., 2004; Throbäck et al., 2004) may have obscured changes in active but less numerous groups of denitrifiers: we examined the entire *nosZ*-containing community and cannot determine how many of these TRFs are associated with actively denitrifying organisms. A significant increase in a specific subpopulation of denitrifiers might not be detected against a larger *nosZ*-containing population that remained inactive. Alternatively, the lack of correlation between DEA and denitrifier community composition may have resulted from the stimulation in activity, but not growth of certain denitrifiers. For example, we observed a rhizosphere effect (open versus closed cores) on DEA (Figure 2.1) but not on denitrifier community composition (Table 2.1), similar to the responses observed by Mounier et al. (2004) to root mucilage.

The lack of significant changes in denitrifying community composition as a whole suggests that these communities may be well buffered to environmental change. Nevertheless, indicator species analysis identified several TRFs that did change in response to treatments and temporal variations were observed. We

observed slight differences in some TRFs when cores were transferred between microenvironments (Figure 2.4). It is possible that the dominant members of the denitrifier community (identified previously to be α-proteobacteria related to *Rhizobiaceae* (Rich et al., 2003)) are more sensitive to perturbation than other community members. On the other hand, these specific changes may foreshadow more significant changes that would have occurred if the treatments had been left in place for a longer period of time. In either case, we may find that differences in the *nosZ* gene translate to enzymatic differences that affect N-oxide scavenging capabilities, O_2 tolerance, or pH tolerance (Šimek et al., 2002).

The observed temporal variability in background cores shows that denitrifier community composition is subject to natural change. Previous studies have shown that microbial communities (Lipson et al., 1999) and N-cycling (Lipson et al., 2002) vary seasonally (Griffiths et al., 1998; Priha and Smolander, 1999); however, to our knowledge no other studies have observed denitrifier communities in any natural environment for more than one year.

These findings highlight the need for long-term ecological studies that examine N transformation and community dynamics both seasonally and over several years. The current study and another recent study (Horz et al., 2004) demonstrates that field manipulations provide insight into the nature of microbial communities and their response to environmental change. Our study examined net production of NO_3^- (Bottomley et al., 2004) and one possible sink for NO_3^- (i.e., denitrification), but could be expanded to other possible sinks, such as microbial

NO_3^- assimilation. These future studies would help to develop models of N-cycling that couple microbial communities and processes in this environment. Additionally, examining seasonal patterns of denitrifying activity, denitrifier community dynamics, and plant root activity may help us to better understand plant and microbe interactions.

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Bacterial and Fungal Contributions to Soil Nitrogen Cycling under Douglas fir and Red alder at two Sites in Oregon

Chapter 3

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3.1 Abstract

A study was conducted at two experimental tree plantations in the Pacific Northwest to assess the role of bacteria and fungi in nitrogen (N) cycling. Soils from red alder (*Alnus rubra*) and Douglas-fir (*Pseudotsuga menziesii*) plots in low- (H.J. Andrews) and high- (Cascade Head) productivity stands were sampled in 2005 and 2006. Relative contributions of fungi and bacteria to microbial biomass were characterized using phospholipid fatty acid (PLFA) profiles and quantitative (Q)-PCR. ^{15}N isotope dilution experiments, using ammonium (NH_4^+) and nitrate (NO_3^-), were paired with antibiotics that block bacterial (bronopol) and fungal (cycloheximide) protein synthesis to determine the relative contribution of bacteria and fungi to net N mineralization and gross rates of ammonification and nitrification. Soil properties and microbial biomass varied significantly between the two experimental sites and to a lesser extent between tree types with fungal:bacterial biomass ratios lower in more N-rich plots. Gross nitrification rates were higher at the high-productivity site and in red alder stands, and this increase led to increased net nitrification, especially in red alder at the high-productivity site. Bronopol treatments significantly decreased NH_4^+ consumption and nitrification rates in all treatments except for the Douglas-fir plots at H.J. Andrews suggesting that prokaryotic nitrifiers are a major sink for mineral NH_4^+ in soils with higher N availability. Cycloheximide enhanced ammonification rates in all treatments without affecting NH_4^+ consumption implying that fungal protein synthesis was a major sink for soil DON in all soils.

3.2 Key Words

Isotope dilution, Bronopol, Cycloheximide, Fungal:bacterial ratios, Nitrification, Ammonification, Nitrogen mineralization

3.3 Introduction

The inclusion of red alder (*Alnus rubra*) in conifer-dominated forest systems has been shown to alter the biogeochemical properties of soils. These changes include a decrease in soil pH (Rhoades and Binkley, 1992), an increase in the cycling of nutrients such as P and Ca (Cole et al., 1978), and an increase in the N status of underlying soils (Tarrant and Trappe, 1971; Van Miegroet et al., 1989; Binkley et al., 1992). In both high- and low-productivity forests, the presence of red alder leads to significantly higher gross rates of N mineralization, N immobilization, and nitrification (Hart et al., 1997). Based on C substrate utilization patterns, it has been speculated that soil microbial communities differ under red alder and Douglas fir (*Pseudotsuga menziesii)* (Selmant et al, 2005), but no studies have examined these communities using molecular techniques.

Fungal:bacterial ratios have been shown to differ in forest ecosystems of different vegetation types (Myers et al., 2001; Priha et al., 2001; Hackl et al., 2004; Grayston and Prescott, 2005) and between sites (Pennanen et al., 1999; Priha, et al., 2001; Leckie et al., 2004). For instance, phospholipid fatty acid (PLFA) profiles showed that fungal:bacterial ratios varied between pure stands of western red cedar (*Thuja plicata*), western hemlock (*Tsuga heterophylla*), Douglas fir, and Sitka spruce (*Picea sitchensis*) (Grayston and Prescott, 2005). Not only does vegetation

type affect microbial community structure, but research has also shown that fungal:bacterial ratios vary in response to forest fertility, with the relative abundance of bacteria increasing in response to increased fertility (Pennanen et al., 1999) and increased N availability (Leckie et al., 2004; Högberg et al. 2007). The proportion of fungi in the microbial community may increase and fungi may be more active in soil with lower nutrient concentrations (Grønli et al., 2005; Wallenstein et al., 2006).

By extension, the relative contributions of fungi and bacteria to ecosystem processes, such as N cycling may also vary in response to vegetation type and nutrient availability. For instance, researchers have hypothesized that the ability of fungi to span microsites and secrete exoenzymes that lead to the depolymerization of N-containing compounds may be key drivers in soil N-cycling (Schimel and Bennett, 2004). Researches have also observed that heterotrophic fungi and autotrophic bacteria may contribute to N cycling in forest soils (Schimel et al., 1984; Hart et al., 1997). Similarly, both bacteria and fungi can assimilate NO_3^- (Merrick and Edwards, 1995; Marzluf, 1997), but in a soil system where fungi are able to explore more of the soil matrix and more effectively scavenge for NH_4^+, heterotrophic bacteria may access NO_3^- (Hart et al., 1994; Chen and Stark, 2000). The relative abundance of these two groups could, therefore, significantly impact N-cycling rates.

Past research has made use of antibiotics that block protein synthesis to assess the relative population sizes (Anderson and Domsch, 1973; West, 1986;

Velvis, 1997; Lin and Brookes, 1999; Bailey et al., 2003; Wallenstein et al., 2006) and functions (Landi et al., 1993) of soil bacteria and fungi, but few studies have applied antibiotics to examine N cycling (Castaldi and Smith, 1998; Laughlin and Stevens, 2002; Tungaraza et al., 2003; Castaldi, 2005; Myrold and Posavatz, 2007). In this work, we assessed bacterial and fungal contributions to N cycling by pairing ^{15}N isotope dilution with antibiotics to block protein synthesis. The study was conducted in low- and high-productivity soils under pure stands of Douglas fir and red alder. We hypothesized that at high-productivity sites, improved soil organic matter quality and availability of nutrients would lead to lower fungal:bacterial ratios and increased bacterial activity in N-cycling processes. In contrast, at low-productivity sites, fungal populations would be higher and their relative contribution to N cycling would increase. We also hypothesized that the presence of red alder would increase gross N-cycling rates, increase nitrification at both experimental sites, and enhance the contributions of bacteria relative to fungi.

3.4 Materials and Methods

3.4.1 Site descriptions and collection.

Two Oregon tree plantations, with differing soil properties (Table 2.1) were selected for this study. Sites were located in the Cascade Head Experimental Forest (Grotta et al., 2004) and the H.J. Andrews Experimental Forest (Radosevich et al., 2006). Cascade Head is located approximately 1.6 km from the Pacific Ocean in the Oregon coastal range at an elevation of 330 m. Soils are classified as Histic Epiaquands (Rhoades and Binkley, 1992) and native vegetation includes

Sitka spruce, western hemlock, and red alder. The H.J. Andrews is located in the Cascade Mountains at an elevation of 800 m and soils are best classified as Haplumbrepts (Dyrness, 2005). Native vegetation at H.J. Andrews includes Douglas fir, western hemlock, and western red cedar.

Tree plantations were established at both sites after clear-cutting in 1985 (Radosevich et al., 2006). The plantations were originally established to test inter- and intra-specific interactions, and included experimental 27 m x 27 m plots differing in the ratios of Douglas fir to red alder. For the purposes of this study, soils were collected from plots containing pure stands of Douglas fir and red alder, which were planted in the summers of 1986 and 1987 with 3 m x 3 m spacing (Grotta et al., 2004).

Soils were sampled one month after red alder leaves emerged, in Spring 2005 and Spring 2006, when the trees were approximately 20 years old. Sampling at Cascade Head was two weeks prior to sampling at H.J. Andrews because of climatic differences. Ten soil cores (3 cm x 10 cm) were taken from each of three replicate plots per treatment. Five cores were placed in each of two sample bags and homogenized. The bags were transported on ice back to the lab and sub-sampled for PLFA and DNA extraction. In 2005 the remaining soil was used in a $^{15}NO_3^-$ isotope dilution experiment and in 2006 the remaining soil was used in a $^{15}NH_4^+$ dilution experiment.

3.4.2 Soil Properties

Background soil properties were determined in 2005 on subsampes of fresh soil. Total soil C and N were analyzed by combustion using an isotope ratio mass spectrometer (IRMS) (PDZ Europa, England) on oven-dried samples. Inorganic N was extracted (20 g of fresh soil with 50 ml of 0.05 M K_2SO_4) and NH_4^+ and NO_3^- concentrations were determined colorimetrically using an autoanalyzer (Astoria Pacific, Portland OR). Dissolved organic N (DON) was determined on unfumigated 0.05 M K_2SO_4 extracts. Soil pH was measured on slurries containing 10 g of soil in 60 ml of DI water using a pH electrode (Orion Research Inc., Beverly, MA). Microbial biomass (MB)-C was measured by drying 2-ml aliquots of unfumigated and fumigated extracts onto tin squares (Bruulsema and Duxbury, 1996) which were then analyzed by IRMS (PDZ Europa, England). Correction factors of 0.38 (Vance et al., 1987; Joergensen, 1996) was used for MB-C.

3.4.3 Phospholipid fatty acid extraction

PLFAs were extracted using a modified Bligh-Dyer technique (Bligh and Dyer, 1959; White and Ringelberg, 1998) as described by Brant et al. (2006). Briefly, soils (2 g dry weight) were incubated overnight in a solution (2:1:0.8) of methanol, chloroform, and phosphate buffer. After filtering, the chloroform phase was separated and phospholipids were purified using Supelclean™ LC-Si solid phase extraction columns (Supelco, Bellefonte, PA). After derivatization, PLFAs were analyzed using an Agilent 6890 gas chromatograph equipped with an HP Ultra™-2 (5% Phenyl-methylpolysiloxane) column and a flame ionization detector.

PLFA concentrations were determined by comparing sample peaks to a 13:0 FAME standard.

Fungal:bacterial ratios were calculated by dividing the mol% of the fungal phospholipid marker 18:2ω6,9 by the sum of mol% values for a set of bacterial phospholipids that included: i15:0, a15:0, i16:0, 16:1ω9, 16:1ω5, 17:1ω9, i17:0, a17:0, cy17:0, 18:1ω7, and cy19:0 (Blume et al., 2002).

3.4.4 DNA extractions and Quantitative-PCR amplification (Q-PCR)

Two, 0.5-g samples of fresh soil were extracted from each plot using an MOBio PowerSoil™ DNA isolation kit (MoBio Laboratories, Carlsbad, CA), according to manufacturer's instructions except that a Bio101 FastPrep instrument was used to lyse cells (Bio 101, Carlsbad, CA). The MOBio bead beating tubes were shaken for 45 sec on the FastPrep. Extracts were quantified using a NanoDrop™ ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and diluted to 25 ng μl^{-1}. The two extracts from each plot were used in a composite template by combining 25 µl of each 25 ng μl^{-1} dilution.

A brilliant SYBR Green™ Q-PCR Core Reagent Kit (Stratagene, Jolla, CA) and an ABI 7500 Sequence detection system (Applied Biosystems, Foster City, CA) was used for all Q-PCR assays. 2 µl of a 1.25 ng μl^{-1} dilution was used with 16S rRNA primers (Eub338 and Eub518) for general bacteria and ITS primers (5.8s and ITS1f) for general fungi as described previously (Fierer et al., 2005). The sequence for ITS1f was as follows: CTTGGTCATTTAGAGGAAGTAA (Fierer personal communication). Each soil DNA extract and standard was run in

triplicate under the following conditions: 50°C 2 min, 95°C 10 min, 40 cycles at 95°C 30s, 53°C 30s, 72°C 30s, and a final disassociation stage of 95°C 15s and 60°C 20s. SYBR green was quantified during the 72°C elongation step. 16S rRNA clones of the bacterium *Pseudomonas aeruginosa* and ITS clones of the fungus *Haematanectria haematocoeca* were plasmid purified and used to generate a standard curve. Plasmid concentrations ranged from 5.0×10^{-1} to 5.0×10^{-7} ng DNA. Standard curves from each run were analyzed to ensure r^2 values > 0.95, efficiency values between 95% and 105%, and disassociation curves containing a single peak.

3.4.5 ^{15}N Isotope dilution experiments

Soils used in the isotope dilution incubations were sieved < 4 mm. The two replicate sample bags from each plot were combined resulting in 12 soil samples. A 5-g sample was removed and dried at 105°C overnight to determine moisture content. The remaining soil was air dried for 24 hours at 25°C to lower water content for isotope and antibiotic addition and then stored in sealed plastic bags at 25°C. A second 5-g sample was dried overnight at 105°C to determine moisture content after air drying.

Laboratory incubations were carried out to determine net N mineralization, microbial biomass C and N, and gross N transformation rates. Aqueous solutions containing $^{15}NO_3^-$ or $^{15}NH_4^+$ and antibiotics to block bacterial or fungal protein synthesis were added to air dried soils, rewetting them to field moisture. $^{15}NO_3^-$ or $^{15}NH_4^+$ solutions (99 atom %) were added at concentrations of 0.6 mg NO_3^- -N kg^{-1}

soil or 0.02 mg NH_4^+-N kg^{-1} soil, to label the soil pools to 2-4 atom % ^{15}N. Bronopol (Shepherd et al., 1988; Bailey et al., 2003) was used to block bacterial protein synthesis and cycloheximide (Velvis, 1997; Castaldi, 2005) was used to block fungal protein synthesis. After preliminary experiments to assess antibiotic effectiveness (data not shown), they were added at a final concentration of 2 g kg^{-1} soil.

Solutions were added, soil samples were thoroughly homogenized, and 35 g soil added to each of six mason jars (475 ml). Jars were sealed and incubated at 25^oC. At time 0 (3 h after addition of solution), half the jars were destructively sampled; the other jars were sampled after an additional 48 h incubation. Portions of soil (20 g) were extracted with 0.05 M K_2SO_4 and additional 10-g portions of soil were chloroform fumigated for 48 h and extracted with 0.05 M K_2SO_4.

Unfumigated soil extracts were used to measure net and gross N transformations. Net rates were determined by measuring NH_4^+ and NO_3^- colorimetrically using an autoanalyzer (Astoria Pacific, Portland OR). Soil extracts were prepared for ^{15}N analysis by sequential diffusion (Hart et al., 1994) and ^{15}N abundance was measured by IRMS (PDZ Europa, England). Net mineralization was calculated as the difference in NH_4^+ and NO_3^- concentrations over the 48 h incubation and nitrification was calculated as the difference in NO_3^- concentrations. Gross rates were calculated using the equations of Kirkham and Bartholomew (1954). When no change in pool sizes occurred during the incubation, the mean concentrations of NH_4^+ and NO_3^- were used in the equation

for equal rates of production and consumption. In other cases, the equation for unequal rates was used.

3.4.6 Statistical analysis

PLFA data were analyzed using ChemStation^{TM} software (Agilent, Palo Alto, CA) and exported to Microsoft Excel (Microsoft, Seattle, WA). Q-PCR data were analyzed using ABI Sequence detection system software version 1.4 (Applied Biosystems, Foster City, CA).

Statistical analyses were performed using S-plus 6.1 (Insightful Corp, Seattle, WA). Repeated measures analysis of variance (ANOVA) was performed on soil property and tracer experiment data (Ramsey and Schafer, 2002). Where interactions occurred data were analyzed separately to check for main effects. Linear regression analysis was also performed to determine if fungal:bacterial ratios measured by PLFA and Q-PCR were correlated. P-values ≤ 0.05 were considered significant, but p-values between 0.05 and 0.10 are reported.

3.5 Results

3.5.1 Soil Properties

Soil properties including soil C and N varied significantly between the two experimental sites; in particular, N content was substantially higher in Cascade Head than H.J. Andrews soils. Although soil C did not vary between Douglas-fir and red alder, N varied ($p = 0.09$) with red alder soils containing an average of 6.3 g N kg^{-1} soil across both sites and Douglas fir soils containing an average of 4.7 g N kg^{-1} soil (Table 3.1). C:N ratios and NH_4^+ concentrations varied between sites,

Table 3.1 Background soil characteristics Douglas fir (DF) and red alder (RA) soils at both experimental sites. The SE is shown to the right of each value (n = 3). Letters within a row represent statistically significant effects.

	Cascade Head		HJ Andrews	
	DF	**RA**	**DF**	**RA**
Total C *(g kg^{-1} soil)*	128 ± 4^{b}	144 ± 18^{b}	90 ± 13^{a}	82 ± 21^{a}
Total N *(g kg^{-1} soil)*	6.7 ± 0.2^{b}	9.2 ± 1.6^{b}	2.7 ± 0.1^{a}	3.4 ± 0.3^{a}
C:N ratio	19.2 ± 1.0^{a}	17.1 ± 3.9^{a}	33.2 ± 3.5^{b}	24.0 ± 4.3^{b}
NH_4^+ *(mg N kg^{-1} soil)*	4.5 ± 0.6^{b}	3.4 ± 0.8^{b}	1.0 ± 0.4^{a}	2.6 ± 0.8^{a}
NO_3^- *(mg N kg^{-1} soil)*	4.4 ± 1.7^{c}	8.0 ± 2.2^{d}	0.3 ± 0.1^{a}	3.2 ± 0.7^{b}
DON *(mg N kg^{-1} soil)*	12.7 ± 1.0^{c}	17.8 ± 1.5^{d}	5.2 ± 0.5^{a}	8.3 ± 1.0^{b}
pH	4.1 ± 0.1^{b}	3.6 ± 0.0^{a}	5.0 ± 0.1^{c}	5.1 ± 0.1^{c}
MB-C *(mg C kg^{-1} soil)*	665 ± 62^{b}	820 ± 123^{b}	468 ± 41^{a}	435 ± 4^{a}

but no vegetation differences were observed. NO_3^- concentrations varied between red alder (5.6 mg N kg^{-1} soil) and Douglas-fir (2.4 mg N kg^{-1} soil) across both sites, and were significantly higher in Cascade Head (6.2 mg N kg^{-1} soil) than H.J. Andrews soils (1.8 mg N kg^{-1} soil). DON varied both between sites and between vegetation types, with red alder averaging 13 mg N kg^{-1} soil compared to 8.9 mg N kg^{-1} soil in Douglas-fir plots. Soil pH was significantly lower at Cascade Head (pH 3.8) compared to H.J. Andrews (pH 5.1). At Cascade Head the pH of red alder soils was lower than Douglas-fir soils, but no vegetation effect was observed at H.J. Andrews (Table 3.1). In 2006, Cascade Head soils contained significantly

more MB-C in comparison to H.J. Andrews soils (Table 3.1). Red alder plots at H.J. Andrews contained the lowest MB with 435 mg C kg^{-1} soil (Table 3.1).

3.5.2 Fungal:Bacterial ratios

The PLFA 18:2ω6,9, a fungal marker, varied significantly between sites with more fungal PLFA in H.J. Andrews soils and more in Douglas-fir compared to red alder soils ($p = 0.06$) (Table 3.2). Significant interactions were observed in the amount of bacterial PLFAs, with Cascade Head Douglas-fir soils containing the highest amount of bacterial PLFA and H.J. Andrews Douglas-fir soils containing the lowest. PLFA-derived fungal:bacterial ratios differed between the two sites, with a mean of 0.05 at Cascade Head and a mean of 0.15 at H.J. Andrews, but did not vary by tree type.

Fungal ITS copy numbers ranged almost four-fold, with the lowest number in Cascade Head Douglas-fir plots (2.8×10^8 copies g^{-1} soil) and the highest in Douglas-fir soils at H.J. Andrews (9.9×10^8 copies g^{-1} soil) (Table 3.2). Cascade Head soil had twice of the bacterial 16S rRNA genes as H.J. Andrews soils. As with fungal:bacterial ratios determined by PLFA extraction, ratios calculated from fungal and bacterial gene copies were significantly higher in H.J. Andrews compared to Cascade Head soils. No variation in tree type was observed. Ratios determined by PLFA and by Q-PCR were highly correlated ($r^2 = 0.86$).

Table 3.2 Fungal:bacterial ratios of microbial biomass. Values are given for each site and for both Douglas fir (DF) and red alder (RA) soils. The SE is shown to the right of each value (n = 3). Letters represent statistically significant main effects.

	PLFA			Q-PCR		
	Fungi	**Bacteria** *mol %*	**F:B ratio**	**Fungi**	**Bacteria** *gene copies g^{-1} soil*	**F:B ratio**
Cascade Head						
DF	2.3 ± 0.3^{a}	50.1 ± 1.0^{d}	0.043 ± 0.006^{a}	$2.8 \pm 0.56 \times 10^{8\,a}$	$2.8 \pm 0.31 \times 10^{10\,b}$	0.010 ± 0.001^{a}
RA	1.9 ± 0.2^{a}	41.3 ± 1.8^{b}	0.043 ± 0.003^{a}	$3.9 \pm 0.85 \times 10^{8\,a}$	$3.8 \pm 0.73 \times 10^{10\,b}$	0.011 ± 0.002^{a}
HJ. Andrews						
DF	7.5 ± 1.5^{b}	38.8 ± 1.7^{a}	0.197 ± 0.046^{b}	$9.9 \pm 3.2 \times 10^{8\,a}$	$1.8 \pm 0.08 \times 10^{10\,a}$	0.053 ± 0.010^{b}
RA	4.5 ± 0.3^{b}	46.5 ± 0.4^{c}	0.097 ± 0.007^{b}	$3.2 \pm 0.67 \times 10^{8a}$	$1.7 \pm 0.39 \times 10^{10\,a}$	0.027 ± 0.009^{b}

3.5.3 Net N-cycling rates

Net N mineralization in water controls was similar for both years, higher in Cascade Head than H.J. Andrews soils, and higher in red alder compared to Douglas-fir soils ($p = 0.08$) (Figure 3.1 A and B). When bronopol treatments were compared to water controls a site-by-tree-by-bronopol interaction occurred. Bronopol did not affect net N mineralization except in Cascade Head red alder soils ($p = 0.06$) during 2005 and 2006 and in H.J. Andrews Douglas-fir soils in 2006. In both cases bronopol increased net N mineralization (Figure 3.1 A and B). Cycloheximide also increased net N mineralization and this increase was significant at Cascade Head in 2005 and at H.J. Andrews in 2006.

Net nitrification varied by site and vegetation type during each year. In general, net nitrification was significantly higher in soils at Cascade Head and in red alder compared to Douglas-fir soils. Cycloheximide additions had no effect on net nitrification for either site or tree type in either year (Figure 3.1 C and D). In bronopol treatments a significant site-by-bronopol interaction occurred. Bronopol had no effect on soils from the H.J. Andrews during either year; however, net nitrification decreased in both tree types at Cascade Head. The decrease was significant in 2005 when net nitrification rates were higher (Figure 3.1 C).

3.5.4 Gross N-cycling rates

There were no significant differences in gross ammonification rates between sites or tree types in water treatments. In Cascade Head soils ammonification rates increased significantly in response to bronopol additions, but

no significant change occurred in H.J. Andrews soils (Table 3.3). Cycloheximide significantly increased ammonification in soils at both sites and tree types. Gross NH_4^+ consumption did not vary based on site or tree type in water controls, but bronopol caused significant decreases in gross NH_4^+ consumption across all treatments except for H.J. Andrews Douglas-fir soils, with control samples

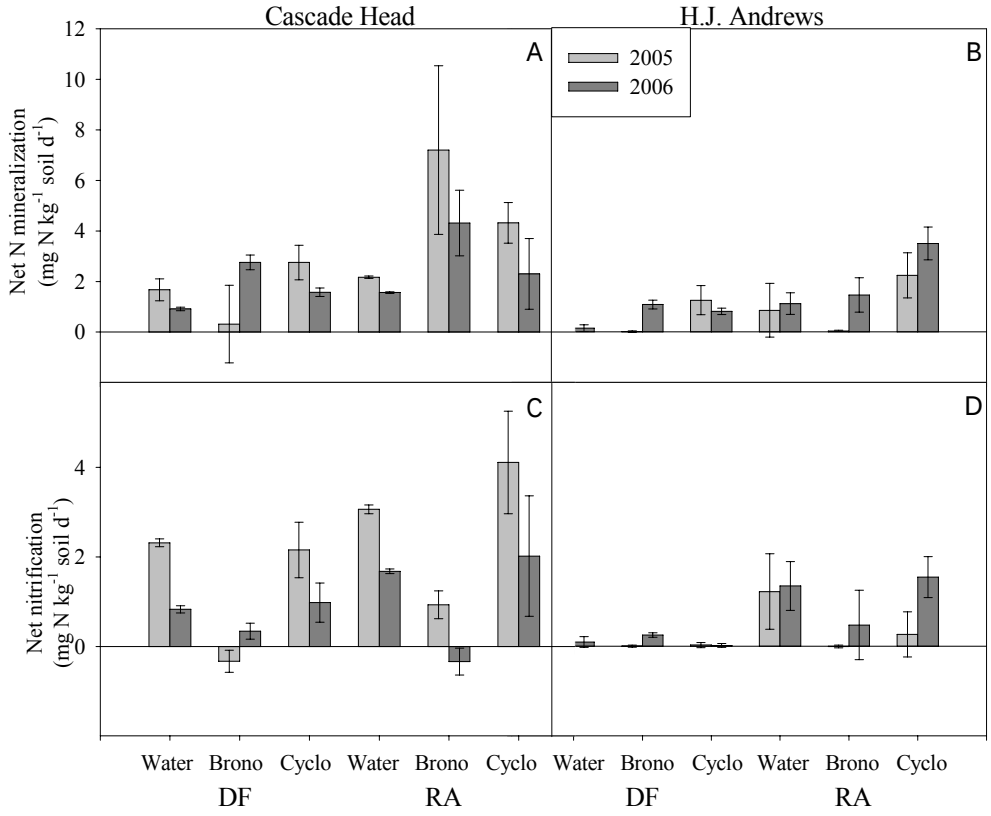

Figure 3.1 Net N mineralization and net nitrification during ^{15}N tracer experiments for 2005 and 2006. Panels A and B show the net change in $NH_4^+ + NO_3^-$ concentrations during the 48 h tracer incubations for both Douglas fir (DF) and red alder (RA) across all three antibiotic treatments: control (water), bronopol (brono), and cycloheximide (cyclo). Panels C and D show net change in NO_3^- concentrations. Panels A and C represent data from Cascade Head and panels B and D show H.J. Andrews results. Each bar is the mean of three field replicates with error bars showing SE (n=3).

averaging 0.73 mg N kg^{-1} soil day^{-1} and bronopol averaging -0.01 mg N kg^{-1} soil day^{-1} (Table 3.3). Cycloheximide had no statistically significant effect compared to water controls but consistently increased NH_4^+ consumption.

Gross nitrification in water controls was significantly higher in Cascade Head soils (2.91 mg N kg^{-1} soil d^{-1}) compared to H.J. Andrews soils (1.00 mg N kg^{-1} soil d^{-1}) (Table 3.3), but did not vary significantly between Douglas-fir and red alder soils. Bronopol blocked gross nitrification in Cascade Head and H.J. Andrews soils ($p = 0.09$) and cycloheximide consistently, but not significantly, decreased nitrification. NO_3^- consumption did not vary between sites or tree types in water controls. Bronopol significantly decreased NO_3^- consumption with a decrease of 81%. Cylcoheximide had no effect on NO_3^- consumption at either site or between tree types (Table 3.3).

3.6 Discussion

Previously, researchers at Cascade Head Experimental Forest reported significant differences in soil N cycling (Hart et al., 1997; Binkley et al., 1992) and C substrate utilization (Selmants et al., 2005) in the presence of red alder compared to Douglas-fir in tree stands older than 50 years. Based upon these findings, we hypothesized that increased N inputs from red alder would lead to significant variations in soil properties and microbial biomass, but we found Douglas-fir and red alder soils to be surprisingly similar, especially at Cascade Head. The strong similarity between Douglas-fir and red alder soils at Cascade Head may have been due to the inherently high soil nutrient status of coastal

Table 3.3 Gross N-cycling rates for Douglas fir (DF) and red alder (RA) soils at Cascade Head and H.J. Andrews. The rates represent the mean of three experimental plots with SE (n=3). (*) Denote significant effects caused by antibiotic additions, p-value ≤ 0.05.

	Treatment	Ammonification	NH_4^+ consumption	Nitrification	NO_3^- Consumption
			mg-N k g^{-1} soil d^{-1}		
Cascade Head					
DF	Control	0.84 ± 0.41	0.84 ± 0.41	2.71 ± 0.12	0.21 ± 0.15
	Bronopol	2.10 ± 0.20*	-0.04 ± 0.11*	-0.20 ± 0.23*	0.13 ± 0.06*
	Cycloheximide	1.26 ± 0.55*	1.26 ± 0.55	1.73 ± 0.46	-0.30 ± 0.88
RA	Control	0.49 ± 0.14	0.49 ± 0.14	3.11 ± 0.04	0.04 ± 0.07
	Bronopol	4.24 ± 0.96*	-0.27 ± 0.25*	0.17 ± 0.11*	-0.77 ± 0.33*
	Cycloheximide	1.27 ± 0.05*	0.99 ± 0.05	2.97 ± 1.02	-1.14 ± 0.53
HJ Andrews					
DF	Control	0.46 ± 0.32	0. 46 ± 0.32	0.59 ± 0.11	0.59 ± 0.11
	Bronopol	1.19 ± 0.81	0.53 ± 0.71*	0.04 ± 0.03	0.04 ± 0.03*
	Cycloheximide	0.81 ± 0.13*	0.81 ± 0.13	0.42 ± 0.04	0.42 ± 0.04
RA	Control	1.20 ± 0.39	1.20 ± 0.39	1.22 ± 0.71	1.22 ± 0.71
	Bronopol	0.50 ± 0.45	-0.36 ± 0.61*	0.11 ± 0.25	0.11 ± 0.25*
	Cycloheximide	3.39 ± 1.09*	1.44 ± 1.25	0.45 ± 0.55	0.45 ± 0.55

andisols. Our work and other research in the Oregon Coast Range (Perakis et al., 2006) has shown that even pure stands of Douglas fir contain soil N values exceeding the threshold for N limitation, and Radosevich et al. (2006) found that mixed stands of Douglas fir and red alder at Cascade Head did not increase Douglas fir production over monocultures.

Although we observed few significant differences in soil properties or fungal:bacterial ratios, NO_3^- and DON concentrations were higher in red alder soils. We also found that net and gross nitrification increased in the presence of red alder in agreement with previous research (Hart et al., 1997). These results show that the presence of red alder does affect some N-cycling processes and may also impact sub-populations of the microbial community such as those involved in nitrification (Boyle et al., in preparation).

A number of factors including landscape position and under-story vegetation have been shown to alter soil pH, microbial community composition, and N cycling at different forest sites with the same dominant tree species (Prescott et al., 2000; Bengston et al., 2006). H.J. Andrews soils contained lower concentrations of organic and mineral N (Table 3.1), and production and consumption of mineral N appeared to be tightly coupled (Figure 3.1 and Table 3.3). Availability of N may have limited microbial biomass, (Table 3.1) and in agreement with our hypothesis, provided a competitive advantage for fungi in these N-limited soils (Table 3.3). Our findings support other research that found relative fungal biomass to decrease with increasing soil fertility and N availability

(Bardgett et al., 1996; Myers et al., 2001; Pennanen et al., 2001; Grayston and Prescott, 2005; Högberg et al., 2007). Nilsson and Wallander (2003) found that ectomycorrhizal (EM) fungal biomass decreased when N fertilizer was added under Norway spruce (*Picea abies*) and other researchers have suggested that increased N availability may lead to a decrease in decomposer fungi (Hammel, 1997; Sinsabaugh et al., 2005). We observed a decrease in fungal PLFA in Cascade Head soils compared to H.J. Andrews soils and an increase in 16S rRNA copy number. Both PLFA and Q-PCR fungal:bacterial ratios were lower in Cascade Head soils, suggesting that high nutrient availability may have decreased the fungal contribution to microbial biomass.

These observed variations in fungal:bacterial ratios highlight the need to better understand the contributions of fungi and bacteria to N-cycling processes. Although using antibiotics comes with inherent difficulties, pairing them with ^{15}N isotope dilution techniques provided evidence for different roles of fungi and bacteria in soil N cycle under different vegetation types and nutrient status.

The high sensitivity of gross nitrification rates to bronopol combined with insensitivity to cycloheximide suggest that bacteria were primarily responsible for nitrification in all soil types and represent the primary sink for NH_4^+. When bacterial protein synthesis was blocked, gross nitrification significantly decreased at Cascade Head and was also reduced at the H.J. Andrews (Table 3.3). Bronopol also decreased gross NH_4^+ consumption rates in most soil types except for Douglas-fir soils at H.J. Andrews. These data suggest that bacteria are responsible

for the majority of nitrification in soils at both sites (Table 3.3). We do not know the potential contributions of archaea in these processes however, because although archaea are insensitive to cycloheximide (Madigan et al., 2003; Briones et al., 2005), to our knowledge the effect of bronopol on archaea has not been tested. We were able to amplify archaeal *amoA* from Cascade Head, so it will be important to determine their contribution in future studies (Boyle et al., in preparation).

Heterotrophic bacteria and fungi in forest systems may primarily rely on organic-N for building biomass (Fierer et al., 2001; Schimel and Bennett, 2004). Through the excretion of extracellular enzymes these organisms mediate the decomposition of complex polymers into N-containing monomers. Presumably, even after antibiotics were added, extracellular enzymes continued to breakdown polymeric N which was taken up by the cells, mineralized, and released as NH_4^+. At Cascade Head both bronopol and cycloheximide stimulated ammonification, but in H.J. Andrews soils only cycloheximide increased ammonification further implying that at this site fungi were primarily responsible for DON assimilation. Cycloheximide results support the idea that fungal communities in H.J. Andrews soils rely on organic-N, because neither NH_4^+ nor NO_3^- consumption decreased when the antibiotic was added, and in fact there was a trend for NH_4^+ consumption to consistently increase. Selemants et al. (2005) found that microbial populations in red alder soils showed enhanced utilization of N-rich amino acids compared to

communities in Douglas-fir. Future studies using ^{15}N-amino acids would help us to better understand the role of organic-N in these systems.

Although it has been widely reported that NO_3^- assimilation may play an important role in N retention within temperate forests (Davidson et al., 1992; Hart et al., 1994; Hart et al., 1997), we did not observe enrichment of the MB-N pool when NO_3^- was added (data not shown). At Cascade Head, NO_3^- consumption was not observed in red alder soils and was low in Douglas-fir soils (Table 3.3). Presumably these results to some extent reflect *in situ* NH_4^+-rich conditions, which may repress NO_3^- assimilation and explain the high NO_3^- concentrations observed in background soils (Table 3.1) (Rice and Tiedje, 1989). In spite of measurable consumption rates at H.J. Andrew's treatments, we did not observe abiotic fixation, dissimilatory NO_3^- reduction to ammonia (DNRA), or enrichment of the MB-N pool (data not shown). Our ability to trace ^{15}N assimilation into biomass may have been hindered by the low concentrations of ^{15}N we used in our experiments and by a fast turnover of N in the microbial biomass (Herman et al., 2006) which could have diluted the pool too quickly to be measured. Remarkably, in Douglas-fir soils at the H.J. Andrews bronopol had no effect on NH_4^+ consumption but stopped NO_3^- consumption implying that in these low N systems fungi may assimilate NH_4^+ while bacteria assimilate NO_3^-. Alternatively, bacterial communities within H.J. Andrews soils may have denitrified in anaerobic microsites, explaining disappearance of NO_3^- and the decrease in consumption when bacterial protein synthesis was blocked. Previously, unamended denitrification potential assays

estimated that 0.17 mg N kg^{-1} soil d^{-1} was produced in H.J. Andrews Douglas-fir plots and 2.5 mg N kg^{-1} soil d^{-1} was observed in coastal red alder (Vermes and Myrold, 1992), suggesting that this process could account for NO_3^- consumption observed here.

In conclusion, our data showed that fungal:bacterial ratios were significantly lower in high- compared to low-productivity sites. The presence of red alder increased gross and net nitrification at both sites, but in all treatments these processes appeared to be mediated by bacteria. These results also suggest that the main sink for NH_4^+ in these soils may be autotrophic nitrifiers. In most cases, the addition of antibiotics significantly increased ammonification suggesting that when protein synthesis was blocked excess DON was mineralized. These observations highlight the importance of organic N to heterotrophic forest communities and emphasize the need for future investigations of the dynamics of DON pools.

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Community Composition and Function of Ammonia-oxidizing Bacteria and Archaea in Acidic Forest Soils of the Pacific Northwest

Chapter 4

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4.1 Abstract

A study was conducted at two experimental tree plantations to determine nitrification activity and nitrifier community composition in acidic forest soils in the presence of red alder (*Alnus rubra*) and Douglas-fir (*Pseudotsuga menziesii*). One site was located in the Cascade Mountains of Oregon where previous research had shown low net N mineralization and gross nitrification and the second was located in the high-productivity Coast Range where rates of N mineralization and nitrification were higher. Nitrification potential assays in the presence and absence of acetylene were used to determine the contribution of heterotrophic and autotrophic nitrification. Communities of both bacterial and archaeal ammonia-oxidizers were examined by targeting bacterial *amoA* and putative archaeal *amoA*. Nitrification potential was significantly higher in red alder compared to Douglas-fir and greater in the high-productivity forest. Autotrophic nitrification dominated at both sites, with the only evidence of heterotrophic nitrification in the high-productivity red alder soils. Ammonia-oxidizing bacteria (AOB) were amplified from all soils, but ammonia-oxidizing archaea (AOA) were only amplified at the high-productivity site. AOB T-RFLP profiles and gene copy numbers varied based on tree type. DNA sequences revealed that AOB were members of *Nitrosospira* Cluster 2 and 4. Despite high N availability no *Nitrosospira* cluster 3 were detected. AOA sequences clustered with other environmental clones from both soils and sediments.

4.2 Keywords

Ammonia-oxidizing bacteria, Ammonia-oxidizing archaea, Forest soils, Nitrification

4.3 Introduction

Both heterotrophic and autotrophic nitrification have been observed in acidic forest soils (Schimel et al., 1984; Stroo et al., 1986; De Boer et al., 1992; Hart et al., 1997; Pavvolainen and Smolander, 1998; Brierley and Wood, 2001); where N mineralization, ammonium (NH_4^+) availability, and pH have all been cited as factors determining their relative contributions (Schimel et al., 1984; De Boer et al., 1988; Hart et al., 1997; Pederson et al., 1999). Both heterotrophic fungi and bacteria capable of nitrification have been isolated (Stroo et al., 1986; Brierley and Wood, 2001), but these organisms nitrify at rates significantly lower than chemolithoautotrophic bacteria and may only do so under conditions of high C availability. Currently, the diversity and activity of these organisms is not well known and factors determining their contribution remain unclear (De Boer and Kowlchuk, 2001).

Although some researchers report significant heterotrophic nitrification (Stroo et al., 1986; Barraclough and Puri, 1995; Hart et al., 1997; Pederson et al., 1999), others have found autotrophic nitrification to be dominant in acidic soils (De Boer et al., 1992; Pennington and Ellis, 1993; Pavvolainen and Smolander, 1998; Zhu and Carreiro, 1999). The evidence for acetylene-sensitive nitrification and the presence of ammonia-oxidizing bacteria (AOB) even at pH < 5 has lead to

speculation that AOB may be the primary nitrifiers in acidic systems (De Boer and Kowlchuk, 2001; Gieseke et al., 2006). Three mechanisms have been proposed to explain AOB activity in acidic soils. At least some AOB may survive in neutral microsites, such as those present in limed agricultural soils (Prosser 1989), although this mechanism does not explain the prevalence of AOB in unamended soils or the long-term survival of AOB which acidify their surroundings. Alternatively, AOB may couple ammonia (NH_3) oxidation to urea hydrolysis (Allison and Prosser, 1991; Burton and Prosser, 2001, Nugroho et al., 2007) or may use membrane bound NH_4^+ transporters (Gieseke et al., 2006). These two latter mechanisms may help explain studies where both acid-sensitive and acid-tolerant NH_3 oxidation have been observed (De Boer et al., 1992).

Members of the genus *Nitrosomonas* were the first AOB isolated (Belser and Schmidt 1978), but work in soils has established the dominance of *Nitrosospira* (Horz et al., 2000). *Nitrosospira* clusters 2, 3, and 4 have been identified in acidic forest soils (Laverman et al., 2001; Mintie et al., 2003; Compton et al., 2004; Nugroho et al., 2005; Nugroho et al., 2006), where nitrifier community composition has been linked to factors such as vegetation type (Nugroho et al., 2005), microclimate (Mintie et al., 2003), disturbance (Yeager et al., 2005), and temperature (Avrahami et al., 2003; Avrahami and Conrad, 2005).

The links between AOB diversity and function remain unclear, however (Kowalchuk et al., 2000; Laverman et al., 2001; Webster et al., 2002; Horz et al., 2004; Webster et al, 2005; Chu et al., 2007). Bottomley et al. (2004) found that

even when soil was reciprocally transferred from forest to meadow environments and nitrification and AOB populations increased, *amoA* composition did not significantly vary after two years. In contrast, fertilization experiments have lead to shifts in AOB composition in as short as 16 weeks (Avrahami et al., 2003), and Horz et al. (2004) observed increased nitrification and a community shift towards *Nitrosospira* cluster 2.

Within the last three years, crenarchaeota in marine and terrestrial environments have been identified that possess putative *amoA* genes (Könneke et al., 2005; Treusch et al., 2005; Leininger et al., 2006). As yet a terrestrial isolate does not exist, but detection and quantification of *amoA* RNA transcripts (Leininger et al., 2006) and an increase in transcript number with NH_4^+ additions (Treusch et al., 2005) suggest that ammonia-oxidizing archaea (AOA) may exist and function in at least some soil systems (Nicol and Schleper, 2006). Investigations in other soil types are needed to determine the ubiquity of AOA and their contribution to nitrification.

A study was designed to determine which microorganisms were responsible for nitrification in Pacific Northwest forest soils and to gain a better understanding of the possible links between nitrifier diversity and function. Two experimental tree plantations with pure stands of Douglas fir (*Pseudotsuga menziesii*) and red alder (*Alnus rubra*) were chosen for these experiments. In addition to the two sites varying in a number of soil properties, previous research had indicated differences in gross and net nitrification between sites and vegetation

types (Boyle et al., in preparation). These experimental plots allowed us to address three main objectives: 1) determine the relative contributions of heterotrophic and autotrophic nitrification in soils with differing vegetation types and nutrient availability, 2) characterize the AOB community and examine correlations between composition and function, and 3) discover if AOA are present in acidic forest soils and if so determine their relation to previously described sequences.

4.4 Materials and methods

4.4.1 Site description and sample collection

Soils were sampled in two experimental tree plantations where trees were approximately 20 years old (Radosevich et al., 2006). The first site was located in Cascade Head Experimental Forest 1.6 km from the Pacific Ocean at an elevation of 330m. The weathering of basalt headlands has created a Histic Epiaquand (Rhoades and Binkley, 1992) with high soil fertility and a pH of 4. The second site was located in the H.J. Andrews Experimental Forest in the Cascade Mountains at an elevation of 800 m. Here soils are best classified as a Haplumbrept (Dyrness, 2005). H.J. Andrews soils had pH 5 and previous research had shown that the concentrations of soil N, NH_4^+, and NO_3^- were significantly lower in comparison to Cascade Head (Boyle et al., in preparation).

Plots measured 27 m x 27 m and contained either pure stands of Douglas fir or red alder. Soil samples were collected in the spring of 2005 and 2006, one month after red alder leaves emerged. Ten soil cores (3 cm x 10 cm) were taken

from each of three replicate plots per treatment. Five cores were placed in each of two sample bags and homogenized. Samples were transported on ice to the lab. A more complete site and sampling description can be found in Boyle et al. (in preparation).

4.4.2 Nitrification potential assay

In 2006 fresh soils were sieved to 4 mm. Nitrification potentials were determined using a shaken soil-slurry method (Mintie et al., 2003). After sieving, portions of soil (15 g dry-weight equivalent) from each plot were extracted with 135 ml of 1.0 mM potassium phosphate buffer (pH 7.2) in order to remove background NO_3^-. The slurries were then centrifuged (6,000 g; 10 min) to pellet soil particles and the buffer was decanted. The soil pellet was resuspended in 135 ml of 1.0 mM phosphate buffer (pH 7.2) containing 1.5 mM NH_4^+. Aliquots of the soil suspension (20 ml) were placed into six 160-ml serum vials, taking care to keep the slurry well mixed. The remaining slurry was centrifuged to pellet soil particles and filtered using a 2-µm filter. This filtrate was used to establish time 0 NO_3^- concentrations. The six serum vials were crimp-sealed using butyl rubber stoppers and acetylene was added to three of the vials (10.8 kPa). All vials were shaken at 25^{o}C for 30 h, centrifuged to remove soil particles, and filtered (2 µm). NO_3^- was measured in the extracts using an autoanalyzer (Astoria Pacific, Portland OR).

4.4.3 DNA extractions and PCR amplification

DNA was extracted from soil (0.5 g) using an MOBio PowerSoil™ DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions, with the modification that a Bio101 FastPrep instrument was used to lyse cells (Bio 101, Carlsbad, CA). The MOBio bead beating tubes were shaken for 45 sec using the FastPrep instrument. Extracts were quantified using a NanoDrop™ ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and diluted to 25 ng μl^{-1}. Two extracts from each plot were used in a composite template by combining 25 µl of each 25 ng μl^{-1} dilution.

Approximately 100 ng of DNA was used in each conventional PCR reaction. AOB *amoA* genes were amplified using primers *amoA*-1F(5′-GGG GGT TTC TAC TGG TGG T) and *amoA*-2R(5′-CCC CTC KGS AAA GCC TTC TTC) (Horz et al., 2000), under conditions described previously (Mintie et al., 2003) using a PTC-100 Programmable Thermocycler (MJ Research Inc., Watertown, MA) . Archaeal *amoA* was amplified using primers *amo*111F (5′-TTY TAY ACH GAY TGG GCH TGG ACA TC) and *amo*643R (5′-TCC CAC TTW GAC CAR GCG GCC ATC CA) (Treusch et al., 2005) in a 50-µl reaction mix containing: 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM Primer, 0.064% BSA. Archaeal *amoA* was amplified using the following conditions: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, ending with an extension step of 72°C for 7 min.

4.4.4 T-RFLP and Q-PCR

DNA extracts from 2005 were used to generate terminal restriction fragment length polymorphism (T-RFLP) profiles (Horz et al., 2000; Mintie et al. 2003). Primer *amoA*-1F was labeled with a 6-fam fluorophore and used in PCR reactions. The resulting products were cleaned using a Qiaquick™ PCR Purification kit (Qiagen Inc., Valencia, CA) and restricted using three enzymes. *CfoI* and *aluI* (Promega, Madison, WI) were added to PCR products, incubated for 2 h at 37°C, and then placed in a 65°C water bath for 15 min. *TaqI* was added to PCR products and incubated for 2 h at 65°C. Restriction products were column purified and profiles were analyzed by Oregon State University's Center of Genome Research and Biotechnology using an ABI 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

AOB *amoA* copy number was determined for both 2005 and 2006 using primers *amoA*-1F and *amoA*-2R in combination with the Brilliant SYBR Green™ Q-PCR Core Reagent Kit (Stratagene, Jolla, CA) (Horz et al., 2004) and an ABI 7500 Sequence detection system (Applied Biosystems, Foster City, CA). 2 µl of a 1.25 ng μl^{-1} dilution was used for each reaction. Each soil extract was run in triplicate. PCR conditions were as follows: 50°C 2 min, 95°C 10 min, 40 cycles at 95°C 30s, 53°C 30s, 72°C 1 min, and a final disassociation stage of 95°C 15s, 60°C 20s. SYBR green was quantified during the 72°C elongation step. Genomic DNA from *N. europaea* at a range of 5.0×10^{-1} to 5.0×10^{-7} ng DNA per reaction was used to generate a standard curve. Standard curves from each run were analyzed

to verify r^2 values > 0.95, efficiency values between 95% and 105%, and disassociation curves containing a single peak.

4.4.5 Cloning and Sequencing

Clones were generated using a Topo TA cloning™ kit for sequencing (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, 4 µl of fresh PCR product reaction was ligated and transformed into One Shot™ competent *E. coli* (Invitrogen, Carlsbad, CA). Transformants were spread onto plates containing Luria-Bertani medium and grown at 37°C overnight. PCR was used to test for the presence of the insert. In the case of AOB clones, *amoA*-1F primer was used in combination with the T7 promoter primer. Primers *amoA*-111F and T7 promoter were used to screen AOA clones. Approximately 96 clones were screened for each of the two genes. Those clones containing inserts were plasmid purified using QIAprep™ Spin Miniprep kit (Qiagen, Carlsbad, CA). AOA clones containing inserts were then sequenced. AOB clones were further screened by PCR amplification and T-RFLP profiles. A total of 30 AOA clones and 24 AOB clones were sequenced by the High Throughput Genomics Unit (Dept. of Genome Science, University of Washington, Seattle, WA).

4.4.6 Statistical and Phylogenetic Analysis

Univariate statistical analyses were performed using S-plus 6.1 (Insightful Corp, Seattle, WA). Repeated measures analysis of variance (ANOVA) was performed on nitrification potential and Q-PCR values to test for main effects of site and tree type (Ramsey and Schafer, 2002). Where interactions occurred data

were analyzed separately. Linear regression analysis was also performed to determine if population size or composition correlated to nitrification activity. P-values ≤ 0.05 were considered significant.

Q-PCR data were analyzed using ABI Sequence detection system software version 1.4 (Applied Biosystems, Foster City, CA) and T-RFLP profiles were analyzed using GenoTyper version 3.7 (Applied Biosystems, Foster City, CA). T-RFLP profiles were further analyzed according to methods described previously (Boyle et al., 2006) and compositional differences were investigated using PC-ORD Multivariate Analysis of Ecological Data version 4.06 (MjM software, Gleneden Beach, OR). Non-metric multidimensional scaling (NMS) was used to test community compositional variation and multi-response permutation procedures (MRPP) were used to determine differences between site and tree type (McCune and Grace, 2002).

DNA sequences were aligned using ClustalX version 1.81 (Thompson et al., 1997) and alignments were edited using Bioedit sequence alignment editor version 7.0.5 (Hall, 1999). All sequences were analyzed using Mallard Version 1.02 (Cardiff University, Boston, MA) to ensure that no chimeras or other sequencing anomalies occurred. Phylogenetic trees were constructed using Mr. Bayes Version 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and confirmed using Phylip Version 3.2 (Felsenstein, 1989). Mr. Bayes was run using an omega variation model (M3), a codon model that calculates the likely rate of variation at each site. The model was run for 1 million generations to

ensure convergence at a stable value (Hall, 2001). AOA sequences were further analyzed by calculating richness estimates and examining the numbers of operational taxonomic units (OTUs) using the DOTUR program (Schloss and Handelsman, 2005)

4.5 Results

4.5.1 Nitrification potential

Nitrification potential varied significantly between sites and tree types (Table 4.1). Cascade Head averaged 2.69 mg N kg^{-1} soil d^{-1} compared to H.J. Andrews soils which averaged 0.88 mg N kg^{-1} soil d^{-1}. Nitrification potential was 2- and 12-fold greater in red alder compared to Douglas-fir soils at Cascade Head and the H.J. Andrews, respectively. When acetylene was added to nitrification slurries, no NO_3^- accumulated in soils from H.J. Andrews or in Douglas-fir soils from Cascade Head. In Cascade Head red alder soils nitrification decreased by approximately 88% to 0.46 mg N kg^{-1} soil d^{-1} (Table 4.1).

Table 4.1 Nitrification potentials of soils collected in spring 2006. Mean ± standard error (n=3).

Site / Tree Type	-Acetylene	+Acetylene
	mg N kg^{-1} soil d^{-1}	
Cascade Head		
Douglas fir	1.68 ± 0.75	-0.38 ± 0.02
Red alder	3.69 ± 0.57	0.46 ± 0.13
HJ Andrews		
Douglas fir	0.13 ± 0.10	0.04 ± 0.00
Red alder	1.62 ± 0.18	-0.05 ± 0.03

4.5.2 Bacterial amoA gene copy numbers

Bacterial *amoA* gene copy numbers did not vary between the sampling years 2005 and 2006 (Figure 4.1), therefore both years were pooled for further analysis. A significant site by tree interaction was observed, so data were run separately to look for main effects. The only tree effect was at the H.J. Andrews, where red alder soil (2.9 x 10^7 *amoA* genes g^{-1} soil) contained significantly more copies than Douglas-fir soil (1.9 x 10^6 *amoA* genes g^{-1} soil) (Figure 4.1). Gene copy numbers did not correlate with nitrification potential or pH, but did correlate with net nitrification ($r^2 = 0.38$) and to a lesser extent with net N mineralization ($r^2 = 0.30$) and gross nitrification ($r^2 = 0.31$) (Boyle et al., in preparation).

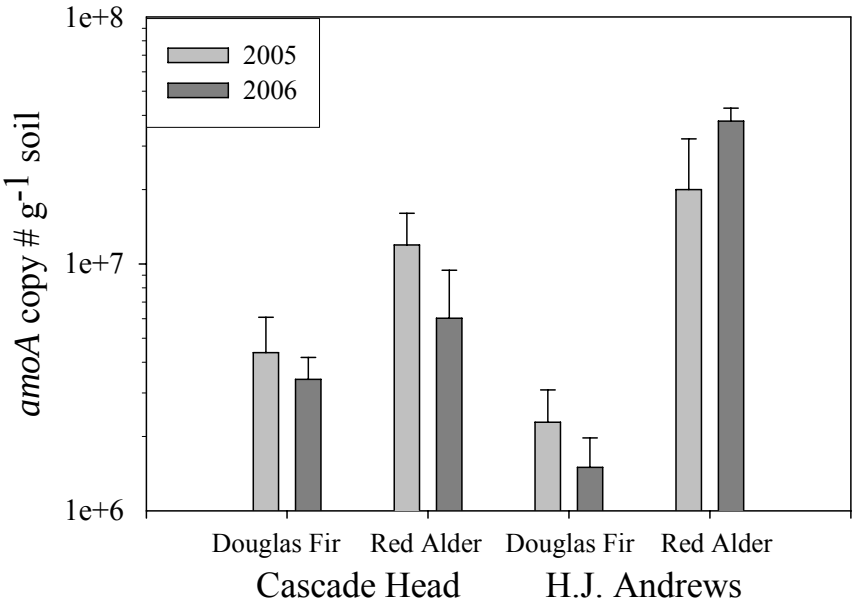

Figure 4.1 Bacterial *amoA* gene copy numbers for 2005 and 2006. Bars represent the mean of three field replicates with SE (n=3).

4.5.3 AOB T-RFLP profiles and T-RF fragment abundance

Restriction patterns generated with *taqI* indicated that all AOB were members of the genus *Nitrosospira*; all patterns contained a single peak at 283 bp (Horz et al., 2000). Community composition determined by bacterial *amoA* T-RFLP profiles using *aluI* and *cfoI* varied by tree type, but did not vary between sites (Figure 4.2). Gross ammonification (r^2=0.68), nitrification potential (r^2=0.60), *amoA* gene copy numbers (r^2=0.56), and net mineralization (r^2=0.50) all correlated with composition (Boyle et al., in preparation). Indicator species analysis was used to identify specific T-RFs that varied based on tree type. Three peaks significantly varied between Douglas-fir and red alder: *aluI* 393, *cfoI* 100, and *cfoI* 297 (Figure 4.3). *AluI* 393 was significantly higher in Douglas-fir compared to red alder soils. *CfoI* 100 and *cfoI* 297 both had significant tree-by-site interactions. In the case of *cfoI* 100, there was no tree difference at Cascade Head, but at H.J. Andrews red alder was higher than Douglas-fir. *CfoI* 297 did not vary at Cascade Head, but at the H.J. Andrews Douglas-fir was higher than red alder (Figure 4.3).

AOB and AOA sequences

AOB clones were screened for those T-RFs that contributed to vegetation differences and for peaks such as *cfoI* 136 that did not appear to have a known match in Genbank. Phylogenetic analysis revealed that most sequences belonged to *Nitrosospira* cluster 2 and 4 (Avrahami et al., 2002), with two clones A-36 (*aluI* 490-*cfoI* 136) and B-22 (*aluI* 393-*cfoI* 67) belonging to cluster 1 (Figure 4.4). Of

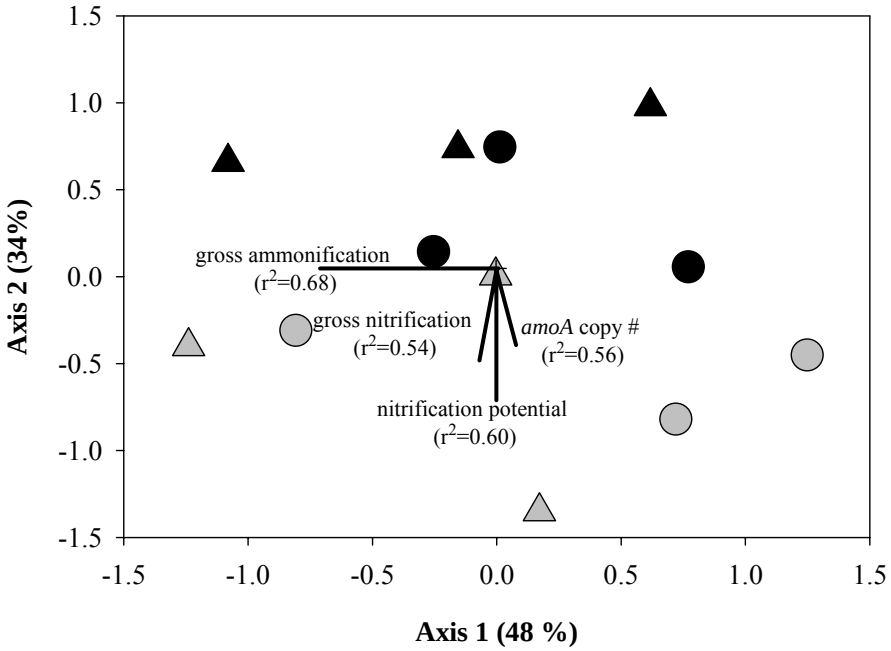

Figure 4.2 Non-metric multidimensional scaling (NMS) ordination of T-RFLP profiles for ammonia-oxidizing bacterial communities in: Cascade Head Douglas fir (▲), Cascade Head red alder (△), H.J. Andrews Douglas fir (●), H.J. Andrews red alder (○). Vectors show correlation with other environmental variables.

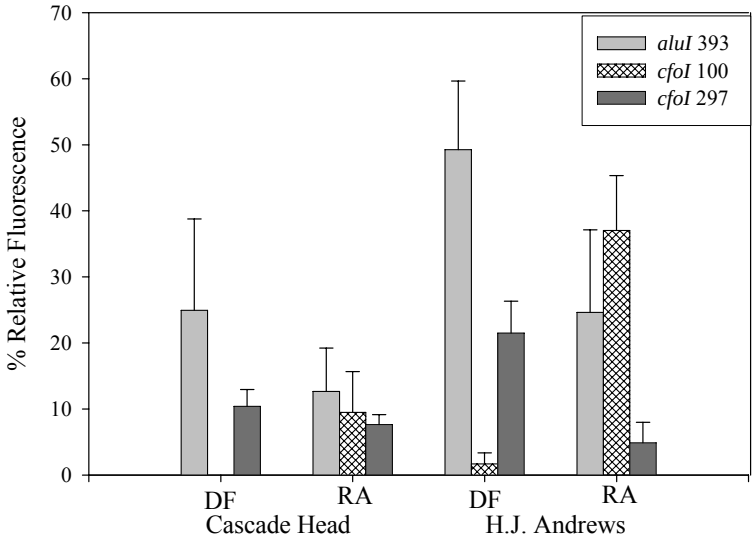

Figure 4.3 Relative abundance of selected T-RFs. Bars represent the mean of three replicates with SE (n=3).

the remaining clones B-18 (*aluI* 393-*cfoI* 167) and B-44 (*aluI* 167-*cfoI* 67) were closely related to *Nitrosospira* CT2F (Mintie et al., 2004), an isolate from a higher elevation conifer soil in the H.J. Andrews Experimental Forest. Clones C-3 (*aluI* 490-*cfoI* 100), C-18 (*aluI* 490-*cfoI* 100), and C-23 (*aluI* 393-*cfoI* 100) all contained cut site *cfoI* 100, the dominant T-RF in red alder soils (Figure 4.4).

Archeal *amoA* was amplified in all Cascade Head plots, but was not amplified in H.J. Andrews soils. Twenty archaeal *amoA* sequences were used in phylogenetic analysis. At 98% similarity the clone library contained 15 unique OTUs. Clones clustered into four main clades. Clones 12C-2, 4B-2, 4C-2, 5C-1, and 9H-1 were 99% similar and grouped closely with other environmental clones from soil (Figure 4.5). Clones 12C-2, 4A-1, and 7F-1 were 98% similar and also grouped with soil clones. Remaining AOA sequences were more divergent and tended to cluster with clones recovered from estuary sediments (Francis et al., 2005) (Figure 4.5). The limited sequencing here did not reveal differences in the diversity of AOA between Douglas-fir and red alder soils as calculated by Shannon's or Simpson's diversity index (data not shown). The Choa1 richness estimator (Chao, 1984) for the entire AOA library was 13 to 57 OTUs at a 5% cutoff.

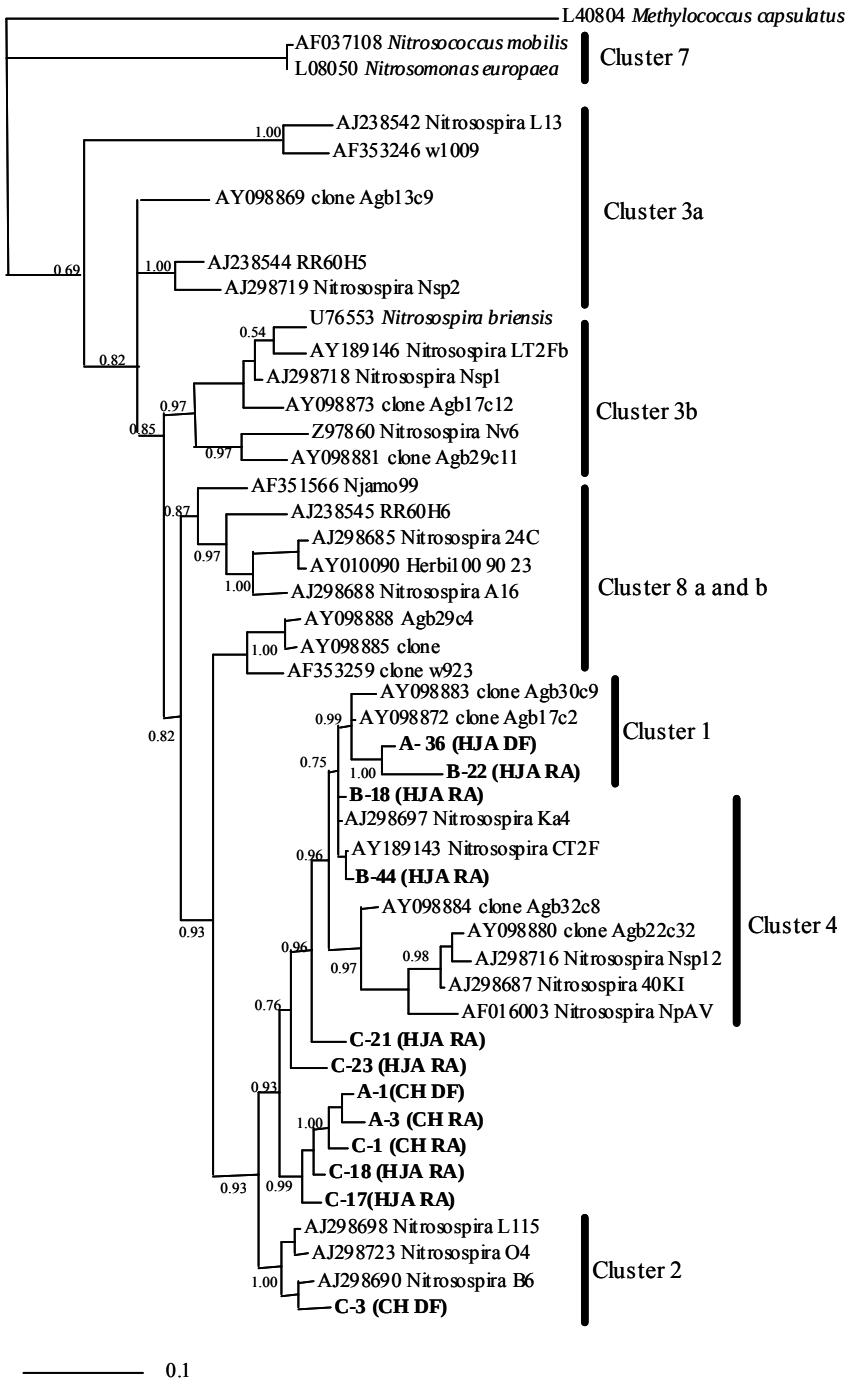

Figure 4.4 Bacterial *amoA* tree constructed using Bayesian analysis. Numbers show the probability of clade assignments. Cluster assignments are made based on Avrahami et al. (2002). Environmental clones from this study appear in bold with the site Cascade Head (CH) or H.J. Andrews (HJA) and tree type Douglas fir (DF) or red alder (RA) appearing in parentheses.

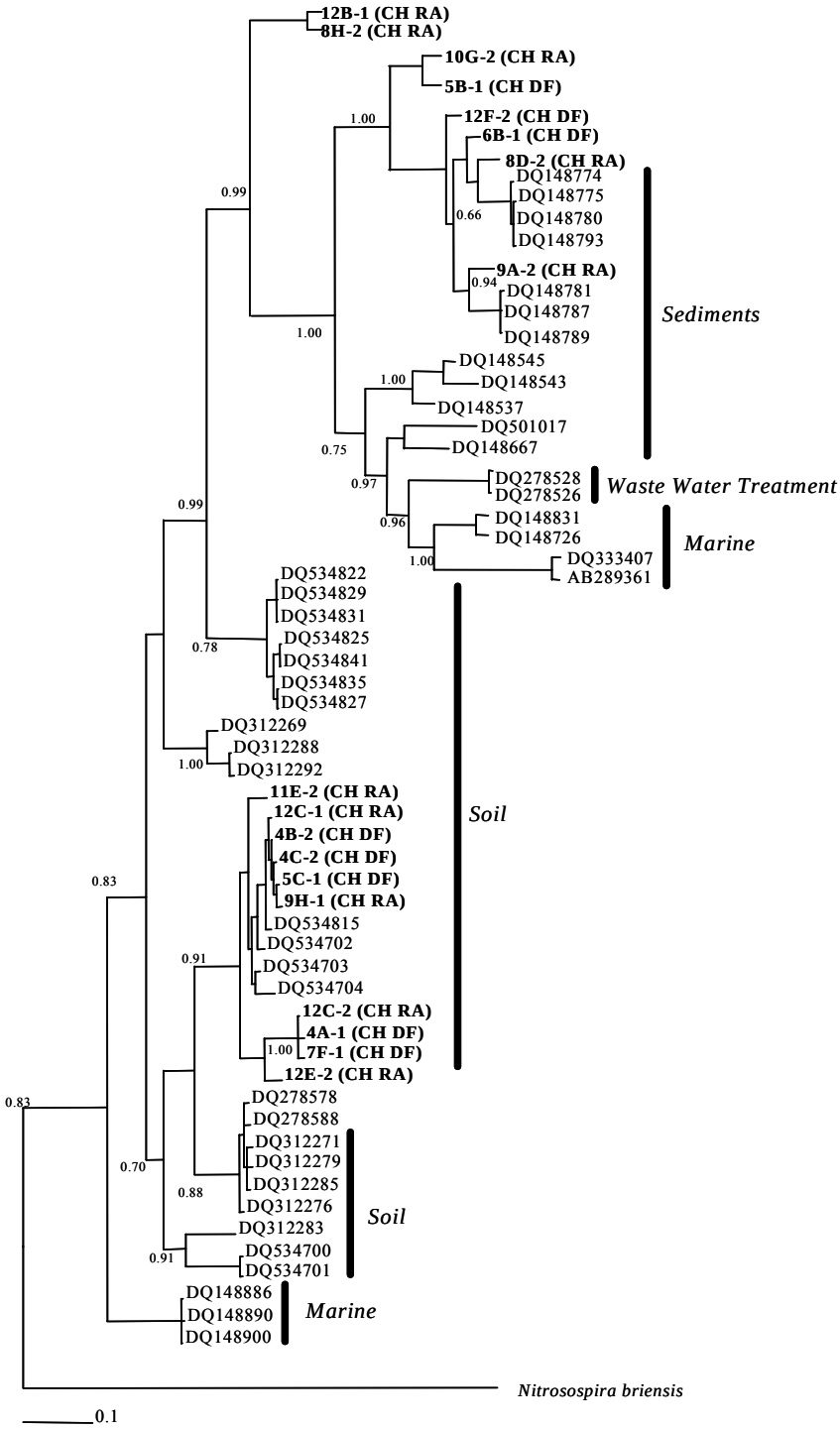

Figure 4.5 Archaeal *amoA* tree constructed using Bayesian analysis. Numbers show the probability of clade assignments. Environmental clones from this study appear in bold with the site Cascade Head (CH) and tree type Douglas fir (DF) or red alder (RA) appearing in parentheses.

4.6 Discussion

The presence of red alder in conifer-dominated ecosystems has been shown to increase both gross and net nitrification (Binkley et al., 1992; Hart et al., 1997; Boyle et al., in preparation), and through this process lower soil pH (Rhoades and Binkley 1992). Data from both experimental sites showed increased nitrification potential in red alder compared to Douglas-fir soils, although the rates of nitrification increased to a greater extent in the low-productivity forest where red alder may have led to a proportionately greater increase in available N. Previously, ^{15}N tracer experiments showed that nitrification was a significant sink for NH_4^+ in all treatments (Boyle et al., in preparation), supporting the observation made by Booth et al. (2005) that at low N mineralizing conditions nitrification is a proportionally more important NH_4^+ fate. Comparisons to isotope dilution experiments showed that gross rates of nitrification (Boyle et al., in preparation) closely agreed with nitrification potential measurements (r^2= 0.5) (Table 4.1), suggesting that at the time of sampling nitrification conditions may have been close to optimum and rates were high.

Although some research has shown significant heterotrophic nitrification in similar soil types (Hart et al., 1997), our data indicated that autotrophic nitrification dominated at both experimental sites and under both vegetation types. The only observed heterotrophic nitrification occurred in red alder soils from Cascade Head (Table 4.1), where approximately one-quarter of the nitrification potential was acetylene-insensitive. Cascade Head red alder soils had the highest

soil C (144 g kg^{-1} of soil), lowest C:N ratio (17), and lowest pH (<4) in comparison to the other site and vegetation type (Boyle et al. in preparation). Although further research is need, these soil properties may help to create favorable conditions for heterotrophic nitrification, by supporting a large microbial biomass and containing ready supplies of labile C, DON, and NH_4^+ (De Boer and Kowalchuk, 2001).

Of the AOB observed, *Nitrosospirads* from clusters 1, 2, and 4 were most prevalent at both sites and under both vegetation types. This is in agreement with other studies that have observed these clusters in similar environments (Laverman et al., 2001; Mintie et al., 2003; Nugroho et al., 2005). Consistent with previous studies, we observed T-RFs associated with cluster 4 clones to be more abundant in Douglas-fir soils where net N mineralization was low (Mintie et al., 2003). Clones B-18 and B-44 which clustered close to *Nitrosospira* CTF2, a cluster 4 isolate from the H.J. Andrews, (Mintie et al., 2003) support work that has observed AOB communities to be stable in space and time (Laverman et al., 2001).

AOB composition determined by T-RFLP profiles changed in response to tree type, suggesting that complex changes brought about by differing vegetation types affected the diversity of AOB. T-RF *cfoI* 100 was significantly higher in red alder compared to Douglas-fir (Figure 4.3), and at least one clone (C-3), that contained *cfoI* 100, appeared to belong to cluster 2. Previously, researchers identified *Nitrosospira* cluster 2 in chronic N-amendment plots (Compton et al., 2004) and acidic Scots pine forests (Nugroho et al., 2005). Nugroho et al. (2007)

suggested that cluster 2 may survive in low pH soils through urea-hydrolysis. Therefore, the prevalence of *Nitrosospira* cluster 2 may be a result of lower pH and increased N availability in red alder soils. Our clone library also included a number of sequences that appeared intermediate to members of *Nitrosospira* cluster 2 and 4. Of these, two clones contained the T-RF *cfoI* 100. Further research is needed to determine what role these AOB play in the nitrification processes and their environmental distribution.

Based on pure culture studies that have determined rates of NH_3 oxidation to be 1-10 x 10^{-15} mol $cell^{-1}$ h^{-1} (Jiang and Bakken, 1999) AOB population density in these soils should have been10^6 to 10^7 cells g^{-1} soil. These numbers agree well with our Q-PCR values (Figure 4.1) and also agree with population sizes of AOB determined by Q-PCR reported elsewhere (Okano et al., 2004; Leininger et al., 2006). In Douglas-fir soils, differences in gene copy numbers between sites correlated to an increase in nitrification potential (Table 4.1, Figure 4.1), but no correlation was found for red alder soils. In fact, Cascade Head red alder contained lower gene copy numbers than estimated by nitrification rates. Even taking into account heterotrophic nitrification, measured AOB copy numbers still appear low in comparison to rates suggesting that several groups may contribute to nitrification including heterotrophs, AOB, and perhaps AOA.

We believe our data are the first to show the presence of AOA *amoA* in forest soils under both coniferous and deciduous tree species. Archaeal *amoA* did not amplify even after several attempts in H.J. Andrews soils, suggesting that if

present these organisms may exist at levels below our current amplification ability or be sufficiently different not to amplify with the primers of Treusch et al. (2005). In contrast, AOA *amoA* consistently amplified in all Cascade Head soils. These results may highlight a fundamental difference in archaeal diversity between the two soil types. Previous work showed that both fungal and bacterial communities differed in Cascade Head soils compared to H.J. Andrews (Boyle et al., in preparation), and other research has shown that archaeal populations may vary between soil types (Jurgens et al., 1997; Ochsenreiter et al., 2003; Leininger et al., 2006; Nicol et al., 2007). Future research is needed to determine if crenarchaeota diversity and population sizes differ between Cascade Head and the H.J. Andrews as well.

AOA *amoA* genes recovered from Cascade Head tended to group with other environmental clones from soils and sediments; findings that are consistent with previous work where sequences clustered generally based upon environment (Francis et al., 2005). Our Choa1 diversity estimator (13 to 57 OTUs) was also comparable to Francis et al. (2005) that estimated 11 to 48 OTUs per sample for sediments. Although archaeal *amoA* genes are present, it is not clear to what extent these organisms are involved in nitrification. Further investigations targeting mRNA (Leininger et al., 2006) and isolation of AOA from these soils may help to answer this question.

Our data show that nitrification was predominately acetylene sensitive and that populations of AOB are responsible for the bulk of nitrification in soils at both

sites. Results also indicate that vegetation type altered AOB community composition with red alder soils having increased populations of *Nitrosospira* cluster 2 and another group of *Nitrosospira* that do not fit within previously described clusters. Rates of nitrification in Cascade Head red alder soils and the presence of AOA *amoA* provide some of the first clues that archaea may contribute to nitrification in acidic forest soils.

4.7 Acknowledgements

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General Conclusions

Chapter 5

Stephanie A. Boyle

In the past decade, molecular techniques have provided new information about the relationships between the community composition and functional capabilities of soil microbes. These methods have provided an opportunity to examine the impact of environmental factors including microclimate, vegetation type, and nutrient availability on soil microbial community composition. Researchers have observed that microbial community structure may be dependent on the ecosystem in which it exists (Hughes Martiny et al., 2006) and speculate that community composition may in turn strongly influence ecosystem function. Our work in the meadow and forest environments supports the hypothesis of a non-random distribution of microorganisms influenced by variations in vegetation and microclimate. Research conducted in the Cascade Mountains, showed that microbial community composition varied between forest and adjacent meadow soils (Mintie et al., 2003; Rich et al., 2003; Bottomley et al., 2004; Bottomley et al., 2006; Boyle et al., 2006). Differences in native meadow and forest communities were observed in both general composition of bacteria and fungi, as well as in functional communities of denitrifiers and ammonia-oxidizing bacteria (AOB).

When soil cores were reciprocally transferred between meadow and forest general community profiles of bacteria and fungi changed in response (Bottomley et al., 2006); however, the composition of AOB (Bottomley et al., 2005) and denitrifiers (Table 2.1) did not significantly change even when cores had been in a new environment for two years. Surprisingly, while there was little evidence for shifts in AOB and denitrifier communities, nitrification potential (Bottomley et al.,

2004) and denitrification enzyme activity (DEA) (Figure 2.1) were significantly different under some treatments. For example, when meadow soil was transferred to the forest DEA increased by an order of magnitude. Sinsabaugh et al. (2005) states that while initial responses to changing environmental factors are probably at the biochemical or organism level, given enough time these factors may lead to change in the microbial community composition. These results suggest that while the diversity of denitrifying bacteria was shaped by the environment in which they originated (i.e. forest or meadow), this community may have been well buffered against changes brought about by disturbance or microclimate variations. Furthermore, the increase in denitrification potential implies that communities may be able to acclimate to a change of environmental conditions thereby altering function in the absence of community compositional changes. Although denitrifying community composition did not appear to change in a two-year period, it is possible that given more time community composition would have changed.

Although AOB community composition did not change in response to the reciprocal transfer, AOB composition did vary between red alder soils and Douglas-fir soils in the 20-year-old tree plantations. Soil properties such as C:N ratio and soil microbial biomass C and N did not appear to differ between the two tree types, but net and gross nitrification was significantly higher in red alder soil (Figure 3.1 and Table 3.3). Furthermore, both nitrification potential and gross nitrification were highly correlated to differences in community composition (Figure 4.2) suggesting a link between AOB community composition and function.

AmoA gene copy numbers were not correlated to nitrification rates, however. In red alder soils at Cascade Head there was some suggestion that heterotrophs and possibly creanarcheota may have contributed to nitrification activity. This work highlights a need to expand the current DNA-based approaches described here to include research targeting mRNA. Although the isolation of mRNA from soils can be difficult because of fast turnover, targeting mRNA would help to bridge the gap between the composition and functional community in both AOB and denitrifying communities (Philippot and Hallin, 2005).

In addition to finding some inconsistencies between microbial community composition and function, work with the H.J. Andrews meadow soils also showed the uncoupling of nitrification and nitrate (NO_3^-) consumption. In contrast to forest soils where inorganic N was at low concentrations and appeared to be tightly regulated, meadow soils had significantly higher net nitrification rates. When forest soil was moved into the meadow net nitrification also increased in these cores (Bottomley et al., 2004). The increase in net nitrification did not result in an increase in DEA (Figure 2.1), however, and significantly higher concentrations of NO_3^- were observed (Figure 2.2). Because the coring process severed plant roots it may have eliminated a major sink for NO_3^- and resulted in NO_3^- accumulation. In meadow soils where only net nitrification was measured it is hard to speculate about the amount of NO_3^- actually consumed and its fate.

Similar to the forest soils in the high elevation reciprocal transfer study, H.J. Andrews soils at lower elevations also showed a tight coupling between

nitrification and NO_3^- consumption as revealed by ^{15}N isotope dilution experiments (Table 3.3). Nitrification rates tended to be lower in Douglas-fir soils than in red alder soils. Regardless of tree type, however, Cascade Head soils had significantly higher rates of net and gross nitrification, and NO_3^- consumption was lower than in H.J. Andrews soils (Figure 3.1, Table 3.3). The ^{15}N isotope dilution experiments were laboratory incubations in the absence of plant roots, but concentrations of soil NO_3^- were also higher at Cascade Head and previous work has reported leaching of NO_3^- at these coastal sites (Rhoades and Binkley, 1992), suggesting that net nitrification typically exceeds NO_3^- consumption.

When antibiotics were used to block protein synthesis, in most cases ammonification increased (Table 3.3) suggesting that heterotrophic fungi and bacteria may primarily access organic N and when antibiotics blocked protein synthesis excess N was mineralized. Both bacterial and fungal antibiotics led to increased ammonification at Cascade Head, but in H.J. Andrews soils ammonification only increased when cycloheximide was added, suggesting that in more N-limited systems fungi may play a more significant role in the decomposition of N-containing compounds. Phospholipid fatty acid profiles and Q-PCR also indicated that the fungal:bacterial ratios were higher in soils with lower N concentrations adding further support for the advantage of fungi in low-N soils. The implied dependence of heterotrophic microorganisms on organic N support the N-cycling model proposed by Schimel and Bennett (2004). In situations of low N availability, heterotrophic microbes may rely more on organic

N. In N rich microsites, mineralizing conditions may lead to release of NH_4^+ that in turn supports nitrifying communities. N availability was significantly higher in Cascade Head soils than in H.J. Andrews soils, yet the net N mineralization observed was low compared to other systems (Booth et al., 2005). Results of this study suggest that in our soils nitrification was a more important fate for NH_4^+ than assimilation. These data highlight the importance of organic N in understanding N cycling in forest soils. Future work is needed to better understand the turnover of organic N and the environmental controls that impact its cycling.

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