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Title: Contributions of Ammonia-Oxidizing Bacteria and Archaea to Nitrification in Forest Soils.

Abstract approved:

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Prior to 2005, ammonia oxidation, the first step of nitrification, was thought mediated mainly by ammonia-oxidizing bacteria (AOB). However, the discovery of Thaumarchaeota carrying the genes coding for the ammonia monooxygenase (AMO) enzyme led to the discovery that ammonia-oxidizing archaea (AOA) also contribute to nitrification. Despite the uptick in studies on nitrification following the recognition of AOA, the relative importance and the contribution of AOA and AOB in different ecosystems, and the factors controlling their abundance and activity, have not been well understood. Because nitrogen (N) cycling is mediated by microbes and controls the net primary productivity in forests, and nitrification is the rate controlling step in N cycling, it is important to have a better understanding of nitrification in forest ecosystem, especially the abundance and contribution of AOA and AOB in forest soils. The objective of this study was to determine nitrification activity and nitrifier abundance in forest soils. I wanted to know: (i) if the inclusion of red alder influences

the distribution, total nitrification activity, and relative contribution of AOA and AOB to nitrification; (ii) if pH and substrate concentration influence the distribution and activity of ammonia oxidizers, and if so, (iii) what is the relationship between these two factors and nitrification.

The study selected soils from stands of red alder (*Alnus rubra* Bong.) and Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) at three sites in Oregon (Cascade Head, the H.J. Andrews, and McDonald Forest) that had a soil pH range of 3.9 to 5.7. The abundances of AOA and AOB were investigated using quantitative PCR (qPCR) by targeting the *amo*A gene. Nitrification activity was evaluated by nitrification potential in a slurry assay and nitrification rate in whole soil. Activity of AOA and AOB was differentiated by using octyne, which inhibits the AMO enzyme in AOB but not AOA. Nitrification activity and octyne-resistant activity (AOA) were significantly higher at Cascade Head than at the H.J. Andrews and McDonald Forest and greater in red alder compared with Douglas-fir soils. Whole soil nitrification rate was stimulated by high NH_3 concentration (10 mmol kg^{-1} soil) addition but not by low NH_3 concentration (1 mmol kg^{-1} soil). At the high concentration of NH_3, both AOA and AOB responded, and nitrification was highest at McDonald Forest, followed by Cascade Head and the H.J. Andrews. There was strong evidence that soil pH was an important factor controlling AOA but not AOB abundance, and the AOA to AOB ratio decreased with increasing soil pH. Collectively, the data indicated that nitrification was mainly driven by AOA in acidic forest soils ($pH < 5$).

Contributions of Ammonia-Oxidizing Bacteria and Archaea to Nitrification in Forest Soils

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

Xinda Lu, Author

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TABLE OF CONTENTS

Literature Review 1

Introduction 1

Nitrification 2

Ammonia-oxidizing Microorganisms 4

Factors that Influence Nitrification 5

Abundance and Composition of AOA and AOB Communities in Soil 6

AOA and AOB in Acid Soils 8

Acidic Forest Soils of the PNW 10

Contributions of Ammonia-oxidizing Bacteria and Archaea to Nitrification in Forest Soils 13

Abstract 13

Introduction 14

Materials and Methods 16

Results 21

Discussion 29

Conclusion 50

Bibliography 53

LIST OF FIGURES

Figure Page

Nitrification Potentials ……37

Relationship between Nitrification Potential and Soil Properties ...……38

Relationship between Fraction of Octyne-resistant Nitrification Potential to total Nitrification Potential and Soil Properties ..……39

Whole Soil Nitrification Rate without and with high NH_3 Addition….……...……...40

Relationship between Whole Soil Nitrification Rate and Soil Properties ...……41

Relationship between the Whole Soil Octyne-resistant Activity Fraction (without NH_3) of the Total Whole Soil Nitrification Rate and Soil Properties ..…….42

Relationship between the Whole Soil Octyne-resistant Activity Fraction (with high NH_3) of the Total Whole Soil Nitrification Rate and Soil Properties…….43

*amo*A Gene Copy Numbers for AOA and AOB…….……44

Relationship between *amo*A Gene Copy Number and Soil pH and for both AOA and AOB……...45

Relationship between AOA:AOB Ratio and Soil pH …….……46

Relationship between Nitrification Potential and AOA amoA Gene Copy Number…47

LIST OF TABLES

Table Page

Soil Properties at Each Site …….. 48

Details of qPCR Reaction ……. 49

Literature Review

Introduction

Nitrogen (N) is the nutrient that most often limits the productivity of terrestrial ecosystems. This is certainly the case in the conifer-dominated forests of the Pacific Northwest (PNW) of the United States. Although the total amount of N in PNW forest soils can vary widely, organic N is always the dominant form. Because organic N is largely unavailable to plants, microorganisms play a vital role by converting it to inorganic forms (ammonium (NH_4^+) and nitrate (NO_3^-)). Consequently, soil microbes control the availability of N to plants and ultimately plant productivity.

Most types of microorganisms (archaea, bacteria, and fungi) can mineralize organic N into NH_4^+, but only a limited subset of soil microbes participate in nitrification. Nitrification is the microbially mediated process by which ammonium/ammonia (NH_3) is oxidized to nitrate (NO_3^-). Nitrate is not only used by plants, but its mobility in soil makes it susceptible to leaching and the green-house gas, nitrous oxide (N_2O), is associated with its production by nitrification or its reduction by microbial denitrification.

The importance of nitrification in forest soils has been debated for a long time because cultured chemolithoautotrophic ammonia-oxidizing bacteria (AOB) are inefficient under conditions often found in forest soils, such as low pH (De Boer and Kowalchuk, 2001). Because of this, some have suggested that NO_3^- is produced by

heterotrophicbacteria and fungi through a poorly understood process known as heterotrophic nitrification. More recently, the discovery of ammonia-oxidizing archaea (AOA) that are capable of chemolithoautotrophic growth with NH_3 as their major energy source (Tourna et al., 2011), and that some AOA function in acid soils (pH 4.3) (Nicol et al., 2008), has prompted the question of the role they play in forest soils. Because our knowledge of AOA is still limited, it remains unclear how important they are in forest soils compared to AOB, or what factors influence their distribution and contribution to nitrification.

Nitrification

Nitrification is a two-step process in which NH_3 is oxidized to nitrite (NO_2^-) and is subsequently converted to NO_3^- (De Boer and Kowalchuk, 2001). Autotrophic oxidation of NH_3 to NO_2^-, the first and rate-limiting step in nitrification, plays an important role in the global N cycle because it bridges the reduced and oxidized inorganic N pools (Martens-Habbena et al., 2009). Ammonia oxidation is catalyzed by AOA and AOB, which use NH_3 as the principle electron donor for chemolithoautotrophic growth under aerobic conditions (Stahl and Torre, 2012). The energy yield from NH_3 oxidation can be used by these organisms to fix carbon dioxide (CO_2) and grow (Hooper et al., 1997). Nitrite-oxidizing bacteria (NOB) complete the second step, conversion of NO_2^- to NO_3^-.

Besides autotrophic nitrification, NO_2^- or NO_3^- can be produced to a limited extent by heterotrophic nitrification, which is driven by fungi and bacteria and often found in

forest soils. Although the rates of heterotrophic nitrification are low, accounting for less than 5% of the gross rates of N mineralization in mature forest soils (Pedersen et al., 1999), they might be a major source of NO_3^- in these soils. Since the first observation of nitrification driven by a fungus in 1896 (Schimel et al., 1984), many heterotrophic organisms have been reported to oxidize organic N directly to NO_2^- and NO_3^- without passing through NH_4^+ pool (Barraclough and Puri, 1995). However, De Boer and Kowalchuk (2001) found that some heterotrophs can also oxidize inorganic N and they pointed out that unlike autotrophic nitrification, sometimes heterotrophic nitrification was not linked with cell growth. Acetylene was first suggested as a useful inhibitor of autotrophic ammonia oxidation in by Hynes and Knowles (1982) and was shown to irreversible inactivate ammonia mono-oxygenase (AMO) by Hyman and Wood (1985). Since then a low concentration of acetylene has been commonly used in inhibition studies that distinguish autotrophic nitrification, which is acetylene sensitive, from heterotrophic nitrification, which is acetylene insensitive.

Ammonia oxidation is driven by a key enzyme, ammonia monooxygenase (AMO), which oxidizes NH_3 to hydroxylamine. The functional genes *amo*A, *amo*B, and *amo*C encode for subunits of AMO, and *amo*A is commonly used as a molecular marker for AOA and AOB. Ammonia monooxygenase can oxidize not only NH_3 but a wide range of other organic, non-polar compounds (Hooper et al., 1997). Hydroxylamine is further oxidized to NO_2^- by hydroxylamine oxidoreductase in AOB (Arp et al., 2002), but the biochemistry of the hydroxylamine to NO_3^- is still unknown for AOA.

Ammonia-oxidizing Microorganisms

Nitrification is microbially mediated by both autotrophic AOB and AOA. However, for more than a century since their initial isolation, AOB had been viewed exclusively as the major contributor to oxidize NH_3 to NO_2^- (Arp et al., 2007). The AOB can be divided into two monophyletic groups taxonomically: the Betaproteobacteria and the Gammaproteobacteria. All cultured AOB from soil environment belong to the family *Nitrosomonadaceae* of β-proteobacteria, which include the *Nitrosomonas*, *Nitrosospira*, and *Nitrosovibrio* genera (Head et al., 1993; Purkhold et al., 2003). Evidence has been found for the predominance of *Nitrosospira* over *Nitrosomonas* in soils (Horz et al., 2004; Yao et al., 2013).

The first detection of the presence of *amo*A gene in members of the archaea in the ocean (Venter et al., 2004) has triggered a huge number of follow-up studies observing representatives of archaea capable of NH_3 oxidation in both aquatic and terrestrial environments and that AOA usually far more abundant than AOB (Leininger et al., 2006; Offre et al., 2013; Wuchter et al., 2006), indicating AOA as a prominent player in nitrification. Four major lineages have been revealed based on *amo*A gene sequences: *Nitrosopumilus* (marine group 1.1a), *Nitrosophaera* (soil group 1.1b), *Nitrosotalea* (1.1a-associated), and *Nitrosocaldus* (Stahl and Torre, 2012). For chemolithoautotrophic AOA and AOB, NH_3 is their sole source of energy and reductant and CO_2 is their sole carbon (C) source (Arp et al., 2002; Tourna et al., 2011).

Factors that Influence Nitrification

Nitrification is a microbially mediated process, so linking environmental factors to population dynamics is important for understanding the drivers of nitrification in soils. Nitrification is influenced by environmental factors such as pH, NH_4^+ concentration, other nutrients, temperature, etc. Soil pH is considered as the factor that influences nitrification by affecting substrate availablity (Kemmitt et al., 2006), nitrifier community structure (Fierer and Jackson, 2006), and the growth and activity of some microbial functional groups (De Boer and Kowalchuk, 2001). For example, nitrification in acid soil may result from the selection for acidophilic NH_3 oxidizers with the ability to adapt to acid environment by biofilm formation, urease activity, or other unknown methods (Nicol et al., 2008). There is evidence that AOA and AOB have different affinity for NH_3 and their growth is closely related with substrate concentration. For example, AOA grew at low NH_3 concentration while AOB showed growth at only high NH_3 concentration (Verhamme et al., 2011). Other nutrients, such as K and P, are also known to affect nitrification. For example in a temperate forest soil, AOB were the main contributor under high nutrient conditions (Norman and Barrett, 2014). Temperature was reported have direct influence on nitrification rate, for example, a 1.8-fold increase in nitrification potential in a forest soils from 22℃ to 30℃ has been found (Taylor et al., 2010). In the case of acid forest soils, with limiting N and low pH, NH_3-oxidizing populations tend to be more influenced by pH and NH_3 concentration than other factors.

Abundance and Composition of AOA and AOB Communities in Soil

The introduction of molecular techniques has promoted research about soil microbial communities. Previously, studies were restricted to either culturing isolates or measuring process rates without being able to tell the composition of the communities responsible for these processes (Wallenstein and Vilgalys, 2005). One approach to investigating soil microbial communities is by extracting DNA from soil samples and measuring the abundance functional genes using the quantitative polymerase chain reaction (qPCR) (Grüntzig et al., 2001). Ammonia oxidizer communities have most often been assayed by qPCR of the *amo*A gene (Tourna et al., 2008).

Both AOA and AOB are found in terrestrial environments such as agricultural, alpine grassland, and forest soils (Boyle-Yarwood et al., 2008; Leininger et al., 2006; Nicol et al., 2008). Reported AOA *amo*A gene copy numbers in acid soils can range from below detection limit (10^4 g^{-1} soil) (Boyle-Yarwood et al., 2008) to 10^4 to 10^8 g^{-1} dry soil (Yao et al., 2011), whereas AOB *amo*A gene copy numbers in acid soils range from below the detection limit (10^4 g^{-1} soil) (Yao et al., 2011) up to 10^7 g^{-1} dry soil (Boyle-Yarwood et al., 2008). Ratios of AOA to AOB vary from site to site and are also influenced by soil depth. Leininger et al. (2006) were among the first to show that AOA can be numerically dominant over AOB in soils. In an investigation of 12 pristine and agricultural soils, they found that the AOA to AOB ratio ranged from 2 to 200 in top soils, whereas at a depth of 30 cm, the ratio increased to 3000. Other studies reported AOA:AOB ratio ranged from 0.05-0.125 in interior Alaska soils (pH 4.3-4.8), 3-238 in tea orchid soils (pH 3.6-6.3), 0.7-1.8 in agricultural soils (pH 4.5-6), 0.03-

18.3 in forest soils (pH 5.0-6.7), and 119-138 in pasture soils (pH 6.0-6.7) (Boyle-Yarwood et al., 2008; Gubry-Rangin et al., 2010; Norman and Barrett, 2014; Petersen et al., 2012; Yao et al., 2011; Zeglin et al., 2011).

The contributions of AOB and AOA to nitrification have been determined by correlating the relative abundance of *amo*A genes to nitrification rates in soils. Several studies of nitrification in acid soils reported significant positive relationship between nitrification potential and AOA, but not AOB *amo*A abundance, suggesting that nitrification is driven by AOA at low pH (Gubry-Rangin et al., 2010; Nicol et al., 2008; Yao et al., 2011).

Community composition analysis of NH_3 oxidizers has been carried out by terminal restriction fragment length polymorphism (T-RFLP). Samples with different dominant terminal restrict fragments (TRFs) were selected to construct clone libraries and phylogenetic affiliation was assigned to specific TRFs by sequencing analysis. The composition of both AOA and AOB are influenced by environmental factors such as pH, N supply, and vegetation type (Boyle-Yarwood et al., 2008; Nugroho et al., 2005; Yao et al., 2013). All know terrestrial AOB belong to a monophyletic assemblage of *Nitrosospira*, *Nitrosomonas*, and *Nitrosovibrio* in the β-subdivision *Proteobacteria* (Purkhold et al., 2003). Molecular analyses have identified *Nitrosospira* clusters 2, 3, and 4 in acidic soils (Nugroho et al., 2005; Yao et al., 2013). *Nitrosospira* cluster 3 is also often dominant in high N conditions (Kowalchuk et al., 2000). *Nitrosomonas europaea* cluster 7 was found in unfertilised grasslands (Webster et al., 2002). In the

case of AOA, some research has demonstrated that pH is an important factor differentiates AOA as acidophilic, neutrophilic, or alkalinophilic (Gubry-Rangin et al., 2011). Most AOA grow optimally at neutral pH whereas two clusters, lineage B (Group 1.1b) and lineage C (Group 1.1a-associated), dominate acid soils (pH < 5) (Gubry-Rangin et al., 2011). The composition of AOA is also influenced by substrate concentration. *Nitrosopumilus*-like AOA could successfully compete with AOB for substrate because of its low substrate threshold (≤10nM) (Martens-Habbena et al., 2009), making it adapted to life under extreme substrate limitation.

AOA and AOB in Acid Soils

In soils, nitrifiers and nitrification rates are influenced by soil factors and plant community composition (Boyle-Yarwood et al., 2008). Approximately 30% of the soils around the world are acidic (pH < 5.5) (Lehtovirta-Morley et al., 2011) and nitrification in acid soils was first reported in the early 20th century (De Boer and Kowalchuk, 2001). Evidence is accumulating that pH and substrate (NH_3) supply are the two main drivers for niche differentiation among NH_3-oxidizers (Verhamme et al., 2011; Yao et al., 2013). One explanation for the selective effect of pH on NH_3 oxidizer populations is that it influences the chemical form, concentration, and availability of the substrate (Kemmitt et al., 2006). Ammonia availability decreases exponentially with decreasing pH because the pKa of NH_4^+/NH_3 is 9.24 (25℃). It has been suggested that the lack of nitrification activity in some acidic soils is due to AOB sensitivity to low pH (De Boer and Kowalchuk, 2001). For example, Yao et al. (2011) reported AOB *amo*A genes were below detection limit in all soils with a pH < 3.5.

Others have found AOB to be active and capable of growth at low pH, perhaps because AOB resided in pH-neutral microsites (Hankinson and Schmidt, 1984), increased microsite pH through ureolytic activity (Burton and Prosser, 2001), or through formation of protective aggregation (Spieck et al., 1992). In fact, some of the greatest gross nitrification rates have been found in acidic soils (pH < 5.5) (Booth et al., 2005). Moreover, recent data revealed that AOA may represent the predominantly active populations in highly acidic soils (Prosser and Nicol, 2012; Yao et al., 2011), but relationships between soil pH and *amo*A-based AOA and AOB abundance vary from site to site. The abundance of AOB decreased as pH decreased in tea orchard soils (Yao et al., 2011), with similar trend reported in agricultural soils (Nicol et al., 2008); however, in some soils no correlation was found between AOB abundance and pH (O'Sullivan et al., 2013). In the case of AOA, a negative effect of pH on population abundance was reported in agricultural soils (Jia and Conrad, 2009). A recent study revealed that selection of a distinct functional group of microorganisms can be the determinant for nitrification activity rather than the entire community (Alves et al., 2013), for example, the predominant role of the acidophilic archaeal nitrifier (*Nitrosotalea*) in acidic soil (Lu et al., 2012). By contrast, Wang et al. (2014) reported NH_3 oxidation in some acid soils was linked to the *Nitrosophaera*, which grows optimally at neutral pH (Hatzenpichler, 2012). This observation raised suspicions that some unknown AOA phylotypes within the *Nitrosophaera* cluster might be able to survive under acidic conditions.

Ammonia, the sole known energy source under aerobic conditions, influences AOA and AOB through their differences in NH_3 affinity and tolerance of high NH_3 concentration (Prosser and Nicol, 2012). The fact that cultivated AOA have much higher substrate affinity than AOB (Martens-Habbena et al., 2009) has led to suggestions that AOA and AOB will dominate soils with low and high NH_3 concentration, respectively. Microcosm studies showed positive correlations between AOB activity and NH_4^+ concentration (Jia and Conrad, 2009). Ammonia may also inhibit AOA and AOB at high concentrations, and AOA appear to be more sensitive to NH_3, being inhibited by as little as 0.04 μM HN_3 (Prosser and Nicol, 2012). In the case of AOB, high NH_3 concentration was reported to reduce the growth by reducing the pH significantly as a result of NH_3 oxidation (Norman and Barrett, 2014). Nevertheless, certain functional groups can tolerate high NH_3 concentrations; Kowalchuk et al. (2000) reported that members of *Nitrosospira* cluster 3 dominate in early successional soils with relatively high NH_4^+ concentrations and Chu et al., (2007) found *Nitrosospira* cluster 3 can be stimulated by N fertilizer in a long-term experiment.

Acidic Forest Soils of the PNW

Both Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) and red alder (*Alnus rubra* Bong.) are common tree species in Pacific Northwest forests. Nitrogen turnover varies between red alder sites and conifer sites within the Pacific Northwest (Grayston and Prescott, 2005) due to the fact that alder leaves and roots contain higher N

concentration than conifer litter. In fact, the rates of N turnover increase by several-fold by the inclusion of red alder (Hart et al., 1997).

Red alder associates with the actinomycete *Frankia*, forming root nodules that fix N_2 and consequently increases the N capital and N availability in soils. However, the presence of red alder on the more fertile sites can also increase NO_3^- leaching and decrease soil pH (Binkley and Sollins, 1990; Hart et al., 1997). The pH of soil under red alder can be as low as 3.6-3.9 (Binkley and Sollins, 1990; Yarwood et al., 2010), which creates an ideal environment for studying the effect of acidity on nitrification and the behavior of AOA and AOB.

For many years researchers speculated that nitrification did not occur in forest soils because measured rates of net nitrification were low; however, it was subsequently shown that gross nitrification rates can be high even when net nitrification rates are low because of NO_3^- consumption (Davidson et al., 1991). Nitrification potential in forest soils ranged from less than 0.1 to over 14.3 mg N kg^{-1} soil d^{-1} (Vitousek et al., 1982; Wertz et al., 2012). The inclusion of red alder in conifer-dominated forests has been found to increase both gross and net nitrification in soils from both low- and high-productivity sites (Binkley and Sollins, 1990; Boyle et al., 2008; Hart et al., 1997). Previous studies with soils of Cascade Head and the H.J. Andrews showed higher nitrification potential under red alder compared with Douglas-fir, and nitrification potentials as high as 2.7 mg N kg^{-1} soil d^{-1} (Boyle-Yarwood et al., 2008). Community composition analysis of AOA identified *Crenarchaea* groups 1.1a-

associated, 1.1b, 1.1c, and 1.1c-associated in soils from Cascade Head and the H.J. Andrews, and greater abundance of *Crenarchaea* 1.1b was observed under red alder compared to Douglas-fir, suggesting its correlation with tree type (Yarwood et al., 2010). The dominant AOA at McDonald Forest fell into phylogenetic clade (iii), which was different from phylogenetic clades (iv and v) at Cascade Head taxonomically (Zeglin et al., 2011). In the case of AOB, phylogenetic analysis suggested that most sequences belonged to *Nitrosospira* clusters 2 and 4 and few belonged to cluster 1 at Cascade Head and the H.J. Andrews (Boyle-Yarwood et al., 2008), whereas AOB at McDonald Forest fell in the *Nitrosospira* clusters 3a and 4 (Zeglin et al., 2011).

Contributions of Ammonia-oxidizing Bacteria and Archaea to Nitrification in Forest Soils

Abstract

Ammonia oxidation, the first step of nitrification, is mediated by both ammonia-oxidizing archaea (AOA) and bacteria (AOB). However, the relative importance and the contribution of AOA and AOB, and the factors controlling their abundance and activity, have not been well understood. This study determined nitrification activity and nitrifer abundance in soils under stands of red alder (*Alnus rubra* Bong.) and Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) at three sites (Cascade Head, the H.J. Andrews, and McDonald Forest) with a pH range of 3.9-5.7 in Oregon, USA. The abundances of AOA and AOB were investigated using quantitative PCR (qPCR) by targeting the *amo*A gene, which codes for subunit A of ammonia monooxygenase. Total and octyne-resistant nitrification (AOA activity) were significantly higher at Cascade Head than at the H.J. Andrews and McDonald Forest, and greater in red alder compared with Douglas-fir soils. The fraction of octyne-resistant nitrification (AOA activity) varied among sites and was highest at Cascade Head. The whole soil nitrification rate was highest at Cascade Head and was stimulated by high NH_3 concentration (10 mmol kg^{-1} soil) addition. Octyne-resistant whole soil nitrification (AOA activity) was significantly higher at Cascade Head and greater under red alder. There was strong evidence that soil pH was an important factor controlling AOA but not AOB abundance, and the AOA to AOB ratio decreased with increasing soil pH. High nitrification potential indicates that nitrification was mainly driven by AOA in acidic forest soils (pH < 5).

Introduction

Nitrification, the oxidation of ammonia (NH_3) to nitrate (NO_3^-), is mediated by microorganisms and is a key component of the nitrogen (N) cycle. The importance of nitrification in forest soils has been debated for a long time because N turnover and availability limit net primary productivity. In the conifer-dominated forests of the Pacific Northwest of the United States, N turnover varies between red alder (*Alnus rubra* Bong.) sites and conifer sites (Grayston and Prescott, 2005), and a several-fold increase in the rates of N turnover by the inclusion of red alder has been reported (Hart et al., 1997). Red alder has been found to reduce soil pH, which can be as low as 3.6-3.9 (Yarwood et al., 2010). As a result of red alder forming root nodules that fix N_2 there is an increase in the N capital and N availability. The pH decline under red alder has been suggested as a result of proton production by nitrification (Binkley and Sollins, 1990). Although the isolation of ammonia-oxidizing bacteria (AOB) from soil at pH < 5 suggested that AOB might contribute to nitrification in acid forest soils (De Boer and Kowalchuk, 2001), their inefficiency under acidic conditions led to speculation that other microorganisms also play an important role in soil nitrification. Heterotrophic nitrification, mediated by heterotrophic bacteria and fungi, has been indicated as one option. Another is the recent discovery of crenarchaeota (now known as Thaumarchaeota) that possess putative *amo*A genes have the potential for NH_3 oxidation (Könneke et al., 2005; Leininger et al., 2006; Tourna et al., 2011). Evidence has demonstrated that ammonia-oxidizing archaea (AOA) can survive in extreme environments, such as those with low pH. Their high affinity for NH_3 (Martens-

Habbena et al., 2009) suggested that AOA might dominate NH_3 oxidation in oligotrophic environments. Recent data revealed that AOA may be the dominant population in highly acidic soils (Prosser and Nicol, 2012; Yao et al., 2011). In the case of AOB, although no cultured isolates can oxidize NH_3 at pH < 5 (De Boer and Kowalchuk, 2001), they have been found capable of growth at low pH. This might be due to AOB residing in pH-neutral microsites (Hankinson and Schmidt, 1984), increased microsite pH through ureolytic activity (Burton and Prosser, 2001), or through formation of protective aggregation (Spieck et al., 1992). Specific AOA and AOB phylotypes have been found associated with soils of different pH. For example, in terms of AOA, the predominant role of *Nitrosotalea* in acidic soil (Lu et al., 2012) whereas *Nitrosophaera* showed optimal growth at neutral pH (Hatzenpichler, 2012); in the case of AOB, *Nitrosospira* clusters 2, 3, and 4 have been detected in acidic soils (Nugroho et al., 2005; Yao et al., 2013). In most soils, AOA outnumber AOB based on their *amo*A gene abundances, suggesting the potential greater role of archaea in nitrification than AOB (Leininger et al., 2006; Norman and Barrett, 2014; Prosser and Nicol, 2008). In some soils, however, AOB can be more abundant than AOA (Petersen et al., 2012). Thus, there is no clear conclusion about the mechanism(s) controlling niche differentiation.

A study was designed to compare the relative contribution of AOA and AOB in soils under different tree types and determine which factors influence their activity. Three sites containing stands of Douglas-fir (*Pseudotsuga* menziesii Mirb. Franco) and red alder were selected. The aim of this study was to: (i) assess the relative contributions

of AOA and AOB to nitrification; and (ii) determine what factors influence their distribution and contribution to nitrification. It is hypothesized that AOA are responsible for most of the nitrification in acidic forest soils, that N input through N_2-fixation by red alder influences both NH_3 oxidizer abundances and nitrification potential, and that different AOA and AOB phylotypes occupy distinct niche.

Materials and Methods

Site description and soil properties

Soils were collected from three forest sites with plots of either pure stands of Douglas-fir or red alder. The stands were around 30 years old (Radosevich et al., 2006). One site lies within the Cascade Head Experimental Forest, 1.6 km from the Pacific Ocean at an elevation of 330 m. Average precipitation is about 2400 mm y^{-1} and temperatures average 20℃ in July and 10℃ in January. Soil at the site is classified as a Histic Epiaquand (Binkley and Sollins, 1990; Boyle-Yarwood et al., 2008). A second site was located within the H.J. Andrews Experimental Forest, which lies in the Blue River Ranger District of the Willamette National Forest at an elevation of 800 m. Temperatures range from 1℃ in January to 18℃ in July with average precipitation about 2300 mm y^{-1} (Art and Pamela, 1998). Soils in the area are classified as Haplumbrepts (Dyrness, 2001). The third site was located within the McDonald-Dunn Forest, on the eastern foothills of the Coast Range, at 350 m elevation. The soil is classified as the Jory series (Xeric Palehumults). Annual precipitation averages 1066 mm and temperature ranges from 4℃ in January to 17℃ in July (Spragu and Hansen,

1946). All soil samples were collected during the spring of 2013; Cascade Head and H.J. Andrews soils in June and McDonald Forest soil in April. Soil was collected from three field replicate plots of each tree type at each location, kept in separate bags, and stored at 4℃ for future use. Samples (10 g) were removed from each bag for DNA extraction.

Soils spanned a relatively wide range of pH (3.9-5.5), a three-fold range in total C, a four-fold range of total N, and an eight-fold range of NH_4^+ and NO_3^- (Table 1). Soil pH was measured using 2.5 g soil in 15 ml DI water and then converted to a 2:1 ratio. Total C and total N of Cascade Head and H.J. Andrews soils were measured using an isotope ratio mass spectrometer (PDZ Europa, England) (Boyle et al., 2008) and a Leco CNS-2000 Macro Analyzer (St. Joseph, MI, USA) was used to measure total C and total N of McDonald Forest soils (Zeglin et al., 2011). Nitrate (NO_3^-) plus nitrite (NO_2^-) concentrations (referred to simply as NO_3^- afterwards) were determined colorimetrically using SHIMADZU Biospec-1601 analyzer (Kyoto, Japan). Ammonium (NH_4^+) was extracted using 2 M KCl and then measured colorimetrically with the same instrument.

Nitrification potential assay

Nitrification potential assays were used to characterize the potential activity of nitrifiers under conditions of non-limiting NH_3 and O_2 by using a shaken soil-slurry method with 1 mM supplemental NH_4^+ (NH_4Cl) (Taylor et al., 2010). Previous study showed TES buffer did not maintain soil slurry pH as expected and that there was no

significant difference in nitrification potential with or without TES. Therefore, the slurry assay was carried out with deionized (DI) water. Prior to doing the assays, soils were removed from the 4℃ cold room, sieved (4 mm), and 2.5 g of soil (field moist) were added to 150-ml serum bottles loosely capped with black phenolic caps fitted with gray butyl stoppers, and preincubated at room temperature (22±2℃) for 2 d. For the assays, 15 ml DI water with supplemental 1 mM NH_4^+ was added to make soil slurries that were shaken at 200 rpm in a Brunswick (Enfield, CT) orbital constant-temperature shaker maintained at 30℃. Initial, background NO_3^- concentrations were measured by taking samples out of soil slurries after shaking for 15 min on a bench-top orbital shaker at room temperature. At time intervals (24 and 48 h), soil slurry samples (1.0 ml) were transferred by syringe into microcentrifuge tubes and centrifuged for 3 min at 13,000× g. Concentration of NO_3^- in the supernatants was determined immediately. At the beginning of the slurry assays, a set of samples were amended with acetylene (0.02% vol/vol or 8 μM) or octyne (4 μM). Acetylene was used as a negative control to evaluate the possibility of heterotrophic nitrification (it blocks NH_3 oxidation by both AOA and AOB) and also allow evaluation of any NO_3^- consumption. Octyne was used to discriminate nitrification activities of AOA and AOB (Taylor et al., 2013). Nitrification potentials were calculated from NO_3^- accumulation during 24 h for Cascade Head and McDonald Forest soils. Nitrification potential was calculated using NO_3^- accumulation during 24 to 48 h due to lack of significant accumulation during 0 to 24 h in red alder soils from the H.J. Andrews. Nitrification potential was calculated by subtracting NO_3^- accumulation in the

acetylene treatment from values measured without acetylene to focus solely on AOA and AOB activities. The pH of the soil slurries was measured at the end of the incubation.

Whole soil nitrification assay

A whole soil nitrification assay was used to measure nitrifier activity at field moisture, which is more similar to *in situ* conditions than the nitrification potential assay. Soil samples were incubated under three NH_3 levels (0, 1, and 10 μmol g^{-1} soil) achieved by adding anhydrous NH_3 gas to the headspace of 150-ml serum bottles sealed with black phenolic caps fitted with gray butyl stoppers. Prior to the assays, soils were removed from the 4℃ cold room and preincubated field moist at room temperature (22±2℃) for 2 d. Three treatments were imposed at each NH_3 level: (i) positive control (no octyne or acetylene amendment), (ii) acetylene amendment (0.02% vol/vol or 8 μM), and (iii) octyne amendment (4 μM). Positive control and octyne treatment soil samples were incubated at 25℃ for 7 d, whereas the acetylene treatment was incubated at 4℃ to prevent autotrophic nitrification and minimize heterotrophic nitrifier activity. At time intervals (2 and 7 d), NH_4^+ and NO_3^- concentrations were determined. Nitrification rates were calculated from NO_3^- accumulation during the incubation and were calculated by subtracting NO_3^- accumulation in acetylene treatment from values measured without acetylene. The pH at the beginning of whole soil incubation was measured 12 h after NH_3 gas was added, when NH_3 gas fully defused into soil samples and pH came to equilibrium. The final pH of soil samples was measured at the end of the incubation.

Copy numbers of *amo*A genes

DNA was extracted from soil (0.25 g dry weight equivalent) using a MoBio PowerSoil™ DNA isolation kit (MoBio Laboratories Inc, Carlsbad, CA) according to the manufacturer's instructions. DNA extracts were quantified by using a NanoDrop™ ND-1000 UV-Vis Spectrophtometer (NanoDrop Technologies, Wilmington, DE) and diluted to 1 ng μl^{-1}. DNA extracts were stored at -20℃ for future use.

The quantitative polymerase chain reaction (qPCR) was used to estimate AOA and AOB abundance by quantifying *amo*A genes for each group. All qPCR reactions were performed in triplicate by using an ABI PRISM 7500 FAST (Carlsbad, CA) sequence detection system set to read SYBR green fluorescence. Standard curves were constructed using *Nitrosomonas europaea* genomic DNA (AOB *amo*A) or *Nitrososphaera viennensis* genomic DNA (AOA *amo*A). AOB *amo*A genes were amplified using primers *amo*A-1F (5'-GGG GGT TTC TAC TGG TGG T) (Stephen et al., 1998) and *amo*A-2R (5'-CCC CTC KGS AAA GCC TTC TTC) (Rotthauwe et al., 1997). AOA *amo*A genes were amplified using primers CrenamoA23f (5'-ATG GTC TGG CTW AGA CG) (Tourna et al., 2008) and CrenamoA616r (5'-GCC ATC CAT CTG TAT GTC CA) (Tourna et al., 2008). Copy numbers were corrected for initial soil moisture and reported as *amo*A gene copies g^{-1} dry soil. Primers sets, thermal protocols master mix recipes, standard curve r^2, and reaction efficiency are summarized in Table 2.

Statistical Analysis

Treatment differences were evaluated using analysis of variance (ANOVA), followed by the LSD (Least Significant Differences) test. Unless otherwise noted, only significant interactions are discussed. Whole soil nitrification rates were square-root transformed to meet the assumptions of normality and homogeneity of variance. Relationships among variables were examined using linear correlation. All analyses were conducted using StatGraphics Centurion Version 16.1.03 (Statpoint Technologies, INC. Warrenton, Virginia).

Results

Soil properties

McDonald Forest soils had the lowest amount of total C and Cascade Head soils the most (Table 1). There were no significant difference in total C between soils under red alder and Douglas-fir at any of the sites. Total N of Cascade Head soils was significant higher than soils from other two sites, which were not significantly different. Total N was higher in red alder compared to Douglas-fir, but this was significant at only Cascade Head. Soil pH ranged from 3.9 to 5.5 with highly significant difference among soils. Cascade Head soils had the lowest pH while McDonald Forest soils had the highest. At Cascade Head, soils under red alder had significantly lower pH compared to soils under Douglas-fir. This trend also held for soils from other two sites but it was not significantly different. At all sites, NH_4^+ was similar and NO_3^- was

higher under red alder than under Douglas-fir. Cascade Head soils had the highest amount NO_3^-; the H.J. Andrews the lowest.

Nitrification potential

When acetylene was added to soil slurries, no significant NO_3^- accumulation was measured in any of the soils during the first 24 h of incubation; however, from 24 to 48 h there was some acetylene-resistant activity in red alder soils at the H.J. Andrews (2.8 ± 0.4 mg N kg^{-1} soil d^{-1}), which may indicate the presence of a low population of heterotrophic nitrifiers in that soil. Therefore, all nitrification potentials were adjusted by subtracting the NO_3^- values of the acetylene treatment prior to statistical analysis of the nitrification potential data.

Two-way ANOVA indicated that the nitrification potential of soils varied significantly among sites ($p<0.01$) and tree types ($p=0.04$). Cascade Head soils averaged 10.8 mg N kg^{-1} soil d^{-1}, McDonald Forest soils averaged 2.3 mg N kg^{-1} soil d^{-1}, and H.J. Andrews soils averaged 1.2 mg N kg^{-1} soil d^{-1}. Across all sites, nitrification potential was two-fold greater in red alder compared with Douglas-fir soils. No significant NO_3^- accumulation was detected in H.J. Andrews Douglas-fir soils.

The initial pH of soil slurries varied significantly among sites ($p<0.01$) and tree types ($p<0.01$): 4.4-5.4 at Cascade Head, 5.1-5.6 at the H.J. Andrews, and 5.6-6.2 at McDonald Forest. At the end of incubation, pH values of the H.J. Andrews and McDonald Forest soil slurries remained similar to those measured initially, but the pH of Cascade Head soil slurries decreased significantly to 3.9-4.6 ($p=0.02$).

Effect of octyne

In the presence of octyne, all soils produced less NO_3^- (Fig. 1). At Cascade Head, there was no significant difference between the with or without octyne treatments, suggesting the activity was octyne-resistant (AOA dominant). A decrease in nitrification potential was detected for red alder soils from both the H.J. Andrews (p=0.09) and McDonald Forest (p=0.06) when exposed to octyne, suggesting an increased AOB contribution (octyne senstitive). Two-way ANOVA showed that the octyne-resistant nitrification potential (AOA) varied significantly among sites (p<0.01) and tree type (p=0.02), although there was a barely significant interaction (p=0.05). The octyne-resistant nitrification potential at Cascade Head averaged 7.1 mg N kg^{-1} soil d^{-1}, 12-fold greater than that at McDonald Forest and 20-fold greater than at the H.J. Andrews. The fraction of octyne-resistant activity in total activity for all soils ranged from 21 to 78%, indicating the relative contribution of AOA and AOB varied among different soils. To confirm the effect of octyne on NO_3^- accumulation, it was compared to the effect of 100 μM ATU (known to inhibit AOB but not AOA) at two randomly selected soils (Cascade Head and H.J. Andrews red alder soils). The sensitivities of nitrification potential to octyne and ATU were strongly correlated (r^2=0.74, p<0.01), suggesting that AOA were responsible for octyne-resistant activity.

Environmental factors

Nitrification potential was positively correlated with total C (r^2=0.51, p<0.01) and total N (r^2=0.59, p<0.01), and negatively correlated with soil pH (r^2=0.54, p<0.01) and C:N ratio (r^2=0.25, p<0.01) (Fig. 2). The octyne-resistant activity fraction was

positively correlated with total C (r^2=0.22, p=0.05) and total N (r^2=0.48, p<0.01), and negatively correlated with C:N ratio (r^2=0.47, p<0.01) and soil pH (r^2=0.22, p=0.05) (Fig. 3).

Whole soil nitrification assay

When acetylene was added to whole soil, no significant NO_3^- accumulation was detected in soils from McDonald Forest; some accumulation of NO_3^- was measured in soils from Cascade Head (< 3.2 mg N kg^{-1} soil) and the H.J. Andrews (< 5.4 mg N kg^{-1} soil) during the 0 to 2 d interval, but no further significant NO_3^- accumulation occurred between 2 and 7 d. As with nitrification potentials, whole soil nitrification rates were adjusted by subtracting the NO_3^- values of the acetylene-treated samples.

Nitrification rate

Measurable accumulation of NO_3^- was detected within all soils at 7 d (Fig. 4). Three-way ANOVA showed that site (p<0.01), tree type (p<0.01) and NH_3 addition level (p<0.01) affected nitrification rates. Whole soil nitrification rates were greater when NH_3 was added to all soils, however, only the high NH_3 concentration treatment showed significantly greater nitrification rates compared with the no and low NH_3 addition treatments, which were not statistically different.

Focusing on the nitrification rates in whole soil without NH_3 addition, which is similar to *in situ* conditions, two-way ANOVA showed that net NO_3^- accumulation was significantly influenced by site (p=0.04). Cascade Head, had the highest nitrification rate of 2.8 mg N kg^{-1} soil d^{-1}, with McDonald Forest (1.3 mg N kg^{-1} soil d^{-1}), and the

H.J. Andrews (0.9 mg N kg^{-1} soil d^{-1}) being lower. Nitrification rates tended to be higher in red alder soils than in Douglas-fir soils across all sites (p=0.09).

Nitrification rates with high NH_3 addition were about three-fold higher than that of the no NH_3 control. No significant differences in rates were found among sites (p=0.13) but it is interesting that the order of nitrification rates changed relative to the no NH_3 control, with McDonald Forest (7.2 mg N kg^{-1} soil d^{-1}) highest, followed by Cascade Head (5.8 mg N kg^{-1} soil d^{-1}) and H.J. Andrews soils (3.2 mg N kg^{-1} soil d^{-1}). Ammonia-amended nitrification rates were significantly higher in red alder compared to Douglas-fir stands (p=0.03).

After 7 d of incubation, the pH of whole soil was lower than original pH in all treatments, and lowest at Cascade Head. Without NH_3 addition, the final pH was 3.5-4.1 at Cascade Head, 0.6 units lower than original pH; 5.1-5.8 at the H.J. Andrews, 0.5 units lower than original pH, and 5.4-5.8 at McDonald Forest, 0.5 units lower than original pH. With high NH_3 concentration treatment, final pH was 3.6-4.5 at Cascade Head, 0.9 units lower than original pH; 5.1-5.8 at H.J. Andrews, 0.7 units lower than original pH and 5.3-5.6 at McDonald Forest, 0.8 units lower than original pH.

Effect of octyne

In the presence of octyne, all soils showed a decrease in net NO_3^- accumulation, suggesting both AOA (octyne resistant) and AOB (octyne sensitive) contributed to nitrification. Three-way ANOVA showed that octyne-resistant activity varied significantly among sites (p<0.01), tree types (p<0.01), and NH_3 addition (p=0.03).

Nitrate accumulation rate at Cascade Head was 2.72 mg N kg^{-1} soil d^{-1}, which was significantly higher than the rate at the H.J. Andrews and McDonald Forest. No significant difference was observed between the latter two sites. Soils under red alder had a 3-fold higher NO_3^- accumulation rate compared to soils under Douglas-fir across all sites (p<0.01). Octyne-resistent activity at the high concentration of NH_3 addition was significantly higher than the non-amended control, but that of the low NH_3 concentration did not differ from the other two treatments.

The fraction of octyne-resistant activity (percentage of AOA contribution to total nitrification activity) was significantly influenced by site (p<0.01) and tree type (p<0.01) but not by NH_3 additions (p=0.58). Octyne-resistant activity at Cascade Head accounted for 62% of the total activity, significantly higher than the fraction at the H.J. Andrews (13%) and McDonald Forest (19%). Octyne-resistant activity was significantly higher under red alder (45%) than under Douglas-fir (17%) across all three sites.

Environmental factors

When no NH_3 was added into whole soils, the nitrification rate was positively correlated with total C (r^2=0.25, p=0.04) and total N (r^2=0.32, p=0.02), and negatively correlated with C:N ratio (r^2=0.17, p=0.09) and soil pH (r^2=0.21, p=0.06) (Fig. 5). The fraction of octyne-resistant activity showed significant positive correlation with total C (r^2=0.21, p=0.06) and total N (r^2=0.38, p=0.01), and was significantly negatively correlated with C:N ratio (r^2=0.34, p=0.01) and soil pH (r^2=0.51, p<0.01) (Fig. 6).

When treated with high NH_3 concentration addition, no significant correlations were found between nitrification rate and soil properties, but significant correlations were found for the octyne-resistant fraction. This fraction showed positive correlation with total C (r^2=0.46, p<0.01) and total N (r^2=0.64, p<0.01), and negative correlation with C:N ratio (r^2=0.30, p=0.02) and soil pH (r^2=0.66, p<0.01) (Fig. 7).

AOA and AOB amoA gene abundance

Abundances of putative AOA and AOB were assessed by quantifying their *amo*A genes (Fig. 8). Copies of AOA and AOB *amo*A genes were detected in all samples, but varied widely among samples. Across all soils, AOA *amo*A gene abundance ranged from 1×10^4 to 2×10^8 *amo*A genes g^{-1} soil and was more variable than AOB *amo*A gene abundance, which ranged from 4×10^4 to 3×10^7 *amo*A genes g^{-1} soil. There was significant effect of site-by-tree interaction on AOA *amo*A gene abundance (p=0.02). AOA *amo*A copy numbers were higher in soils under red alder compared with Douglas-fir and were significantly higher in Cascade Head soils than H.J. Andrews and McDonald Forest soils but with no significant difference between the latter two sites. Ammonia-oxidizing bacterial *amo*A gene copy numbers at McDonald Forest soils significantly outnumbered copy numbers from H.J. Andrews and Cascade Head soils (p=0.01), and no significant difference was detected between AOB *amo*A gene copy numbers at H.J. Andrews and Cascade Head. Ammonia-oxidizing bacterial *amo*A gene copy numbers were higher in soils under red alder than soils under Douglas-fir from all sites (p=0.03). Ammonia-oxidizing archaeal *amo*A gene copy

number decreased as soil pH increased (r^2=0.53, p<0.01), but there was no correlation between AOB *amo*A gene and pH (Fig. 9).

The AOA:AOB ratio varied significantly across sites (p<0.01). Cascade Head soils were strongly dominated by AOA, whereas McDonald Forest soils were dominated by AOB; H.J. Andrews soils had approximately equal number of AOA and AOB. Tree species also influenced the AOA:AOB ratio, which tended to be higher in soils under red alder than soils under Douglas-fir (p=0.08). The AOA:AOB ratio was negatively correlated with soil pH (r^2=0.73, p<0.01), and there was a sharp increase in the AOA:AOB ratio below pH 4 (Fig. 10).

Linking abundance with activity

Regression analysis showed that nitrification potential was significantly correlated with only AOA *amo*A gene copy number (r^2=0.55, p<0.01) (Fig. 11). Nitrification potential was significantly positively correlated with AOA:AOB ratio (r^2=0.74, p<0.01).The octyne-resistant nitrification potential was significantly correlated with AOA *amo*A gene copy number (r^2=0.80, p<0.01). The fraction of octyne-resistant activity showed significant positive correlation with AOA:AOB ratio (r^2=0.49, p<0.01).

Regression analysis showed there was significant correlation between whole soil nitrification rate and AOA (r^2=0.19, p=0.04) but not with AOB *amo*A gene copy number (p=0.15). Octyne-resistant nitrification rate was significantly correlated with AOA *amo*A copy number with high (r^2=0.44, p<0.01), low (r^2=0.40, p<0.01), or

without (r^2=0.38, p=0.01) NH_3 addition. The fraction of octyne-resistant activity was significantly correlated with AOA:AOB ration with high (r^2=0.68, p<0.01), low (r^2=0.57, p<0.01), or without (r^2=0.40, p<0.01) NH_3 addition.

Discussion

Activity and abundance of ammonia oxidizers

The nitrification rates measured across the three sites fall within the range of less than 0.1 to over 14 mg N kg^{-1} soil d^{-1} that has been reported for forest soils (Vitousek et al., 1982; Wertz et al., 2012). The inclusion of red alder in conifer-dominated forests has been found to increase both gross and net nitrification in soils from both low- and high-productivity sites with a concomitant decrease soil pH (Binkley and Sollins, 1990; Hart et al., 1997). Both slurry and whole soil assays measured greater nitrification activity in red alder compared with Douglas-fir soils across all sites. The higher nitrification activity under red alder agrees with previous findings that nitrification rates are negatively correlated with C:N ratios. Low or no nitrification was found in soils with C:N ratio > 20 (Bengtsson et al., 2003; Ross et al., 2004).

The nitrification potentials in this study showed some difference compared to previous studies at these sites. The nitrification potentials at Cascade Head were four-fold higher than those reported by Boyle-Yarwood et al. (2008), but similar at the H.J. Andrews; the nitrification potentials at McDonald Forest was one-third of that measured by Zeglin et al. (2011). Differences might be explained by a number of factors. First, the different studies used different buffers for the nitrification potential

assays: Boyle-Yarwood et al. (2008) used potassium phosphate (pH 7.2) for soils at Cascade Head and the H.J. Andrews, TES buffer was used to hold pH at 6.8-7.0 with McDonald Forest soils (Taylor et al., 2010; Zeglin et al., 2011), and no buffer was used in the nitrification assays reported here. Non-buffered assays were done because we wanted to measure nitrification potentials at close to native pH of the soil and others have found that the use of buffers in slurry assays affects nitrification rates measured in acidic soils (Killham, 1990). The high populations of both AOA and AOB in these soils suggest that these ammonia oxidizers are adapted to low pH and therefore their activity might be impaired by buffering near neutrality. Actually, high nitrification rates that are inhibited by acetylene (AMO-dependent and presumably autotrophic) were frequently found in other acid soils (De Boer and Kowalchuk, 2001; Gubry-Rangin et al., 2010). Furthermore, a preliminary experiment with TES buffer showed that it was unable to hold the slurry at pH 7 with the slurry pH dropping 1 unit at the end of the 48-h incubation. Second, a higher incubation temperature (30℃) was used for the nitrification potential assays at Cascade Head and H.J. Andrews in this study compared to 25℃ used by Boyle-Yarwood et al. (2008), and it is known that temperature influences nitrifier activity (Taylor et al., 2010). Finally, soils were sampled in different months and in different years, and such intra- and inter-annual variations in environmental conditions could certainly affect the activity of ammonia oxidizers. For example, I sampled Cascade Head and the H.J. Andrews in early summer when soils would have been warmer than the spring samples of Boyle-

Yarwood et al. (2008), and McDonald Forest in spring when soils were moist compared to the drier summer samples of Zeglin et al. (2011).

Pure culture studies have shown that NH_3 oxidation rates for AOB range from 10^{-15} to 10^{-14} mol cell^{-1} h^{-1} (Jiang and Bakken, 1999). Assuming that there are two *amo*A gene copies per cell (McTavish et al., 1993), the estimated population values calculated by the rates fall within the result obtained by qPCR. For example, the octyne-sensitive nitrification potential measured under Douglas-fir at McDonald Forest could be supported by an active AOB population of 2×10^5 to 2×10^6 cells g^{-1} of soil, which is less than the numbers measured (Fig. 8). The number of AOB commonly observed in agricultural soils is 10^5 to 10^7 g^{-1} soil (De Boer and Kowalchuk, 2001) and 10^4 to 10^7 g^{-1} soil in forest soils (Wertz et al., 2012), which is in the same order of magnitude observed in this study. Copy numbers of AOA *amo*A gene were high in all soil samples (ranging from 3×10^6 to 1×10^8 g^{-1} dry soil), except for Douglas-fir soils from the H.J. Andrews, which had much lower *amo*A copy numbers. The NH_3 oxidation rate of AOA, estimated with *Nitrosopumilus maritimus* (Könneke M et al., 2005), ranges between 0.25 and 0.35×10^{-15} mol cell^{-1} h^{-1}. Assuming that there is one *amo*A gene copy per cell in soil AOA (Santoro et al., 2010), the population density of all soils fall within the qPCR estimate. Ammonia-oxidizing archaeal *amo*A copy numbers from these forest soils were close to those reported in a study of 12 soils from northern to southern Europe, which ranged from 7×10^6 to 1×10^8 g^{-1} dry soil (Leininger et al., 2006).

Relative contribution of AOA and AOB to nitrification

Through the use of octyne as a selective inhibitor of AOB, the activities of AOA and AOB to nitrification rates could be determined and compared to their population sizes. A significant positive relationship was found between AOA *amo*A gene copy number and octyne-resistant activity in both nitrification potential and whole soil assays. Based on the slope of this relationship, a nitrifying rate of 2×10^{-16} mol cell^{-1} g^{-1} soil could be calculated, which is the same magnitude as reported NH_3 oxidation rate of AOA in pure culture (discussed above). The rate allows the calculation of theoretical nitrification activity contributed by AOA. Compared with measured octyne-resistant activity, at least 80% of the AOA population detected by qPCR was active in oxidizing NH_3 across all sites. However, in the case of AOB, no significant correlation was found between their abundance and octyne-sensitive activity. The lack of a significant positive correlation is most likely because not all AOB were physiologically active and the proportion of those that were varied among samples. Comparing actual measured octyne-sensitive nitrification rate to calculated optimal rate, <50% of AOB might be active in nitrification.

Previous studies at these sites demonstrated that the dominant archaeal *amo*A sequences fell into clades iv (group 1.1b) and v (group 1.1a) at Cascade Head (Boyle-Yarwood et al., 2008) and into clade iii (group 1.1b) at McDonald Forest (Zeglin et al., 2011). (No AOA *amo*A genes were detected by Boyle-Yarwood et al. (2008) in H.J. Andrews soils, possibly because the primers used at that time did not capture the full diversity of AOA *amo*A genes.) Furthermore, an interesting pattern emerges when

comparing the archaeal *amo*A gene phylogenetic composition from different sites because it has been mentioned that functional groups of microorganisms can be determinant for nitrification activity rather than whole populations (Alves et al., 2013). For example, the same 'clade iv' was also found closely related to sequences associated with glacier foreland soils (pH < 5) and 'clade iii' was also found in neutral Scottish agricultural soil (pH 6.9) (Nicol et al., 2008), which raise the concern that pH plays more important role in determining AOA phenotype than other factors such as soil type, vegetation, temperature or moisture.

The AOB *amo*A gene was detected in all soils, which is consistent with previous studies as these sites; however, AOB *amo*A gene copy number was higher at H.J. Andrews and lower at McDonald Forest in our previous study (Boyle-Yarwood et al., 2008; Zeglin et al., 2011). Given the fact that we used the same primers for AOB qPCR, it might be that other factors, such as sampling time, influenced AOB community abundance. Previous T-RFLP data showed that most AOB sequences of soils from Cascade Head and the H.J. Andrews belonged to *Nitrosospira* clusters 2 and 4 (Boyle-Yarwood et al., 2008), whereas *Nitrosospira* cluster 3 dominated at McDonald Forest (Zeglin et al., 2011). Molecular analysis revealed that *Nitrosospira* cluster 2 dominated in acid soils; *Nitrosospira* cluster 3 was dominant in neutral arable soils (Laverman et al., 2001; Stephen et al., 1998). From this perspective, pH shapes AOB structure.

Niche differentiation

Regression analysis showed a significant positive relationship between nitrification potential and archaeal but not bacterial *amo*A gene abundance, suggesting that nitrification in these acidic soils is dominated by AOA. This finding is consistent with other reports observing significant relationships between NO_3^- accumulation and AOA abundance (Gubry-Rangin et al., 2010; Yao et al., 2011). The abundance of AOB *amo*A genes has been correlated with nitrification potential in agricultural soils receiving long-term fertilization (He et al., 2007) or with net nitrification (Jia and Conrad, 2009). Several reasons for the difference in the relative contribution of AOA and AOB to nitrification have been given, with strong evidence for both pH selection and substrate limitation.

Soil pH is a major factor controlling the niche differentiation of AOA and AOB. The relative abundance of archaeal and bacterial *amo*A genes correlated strongly with soil pH. As observed previously for other soils (Nicol et al., 2008), AOA outnumbered AOB in soils with pH < 5, i.e., Cascade Head soils. In the case of soils from the H.J. Andrews and McDonald Forest, with soil pH > 5, AOB showed roughly same or higher *amo*A gene abundance than AOA. The AOA *amo*A gene abundance decreased significantly with higher pH, indicating that pH was an important factor controlling AOA abundance in soil and that AOA were favored at low pH. This observation was consistent with other reports of higher AOA abundance in acid soils (Gubry-Rangin et al., 2010). However, no significant correlation between AOB and pH was observed in this study. Ammonia-oxidizing bacteria were present in soils from all the three sites,

even the highly acidic (pH < 5) Cascade Head soils, and octyne-sensitive activity occurred in all soils, suggesting that AOB contribute to nitrification even at low pH. This may be due to microsites with higher pH (Spieck et al., 1992). Nitrification potential was significant higher than whole soil nitrification rate at Cascade Head (p=0.01) but lower at H.J. Andrews and McDonald Forest, possibly indicating different microsites in these sites, although no significant difference was noticed between nitrification potential and whole soil nitrification rate across all sites (p=0.49). This difference between the slurry assay and whole soil incubation, identifies a potential shortcoming of the slurry assay, at least in some soils. For example, the McDonald Forest soil responded differently to NH_4^+ addition in slurry compared with an equivalent amount of NH_3 added to whole soil, which may suggest that the destruction of microsites in shaken soils may expose ammonia oxidizers to unfavorable pH, reducing their nitrification ability.

High NH_3 concentrations stimulated nitrification potentials, octyne-resistant nitrification potentials, and octyne-sensitive nitrification potentials in all soils, suggesting that both AOA and AOB were substrate limited in these forest soils. Laboratory studies have commonly demonstrated similar responses of AOB to substrate addition by either monitoring either growth or CO_2 fixation (Jia and Conrad, 2009; Verhamme et al., 2011). Because AOA have been shown to have a much higher affinity for NH_3 compared to AOB (Martens-Habbena et al., 2009), AOA are thought to outcompete AOB when NH_3 is limiting and dominant in NH_3-limited environments. Therefore, AOA often show no response to NH_4^+ addition (Norman and Barrett, 2014),

although exceptions exist (Verhamme et al., 2011). The positive response to NH_4^+ additions in these forest soils is likely related to their low pH, particularly at Cascade Head. In such soils, AOA activity may be tightly linked to organic N mineralization, as suggested by a study showing AOA activity being stimulated by addition of mineralizable N: urea, glutamate, or yeast extract (Levičnik-Höfferle et al., 2012).

The distribution and relative contribution of AOA and AOB to nitrification in forest soils remains a topic of debate. This study has provided evidence that AOA outnumber AOB in acidic forest soil, and that AOA might contribute to a great extent to nitrification in acid forest soils. An increased AOB contribution to nitrification in less acidic soils was shown and their distribution and activity ranged from acidic to neutral soils. The study also demonstrated soil pH and substrate concentration are the key factors that differentiate niches, and pH might influence the distribution and activity of NH_3 oxidizers to different extents.

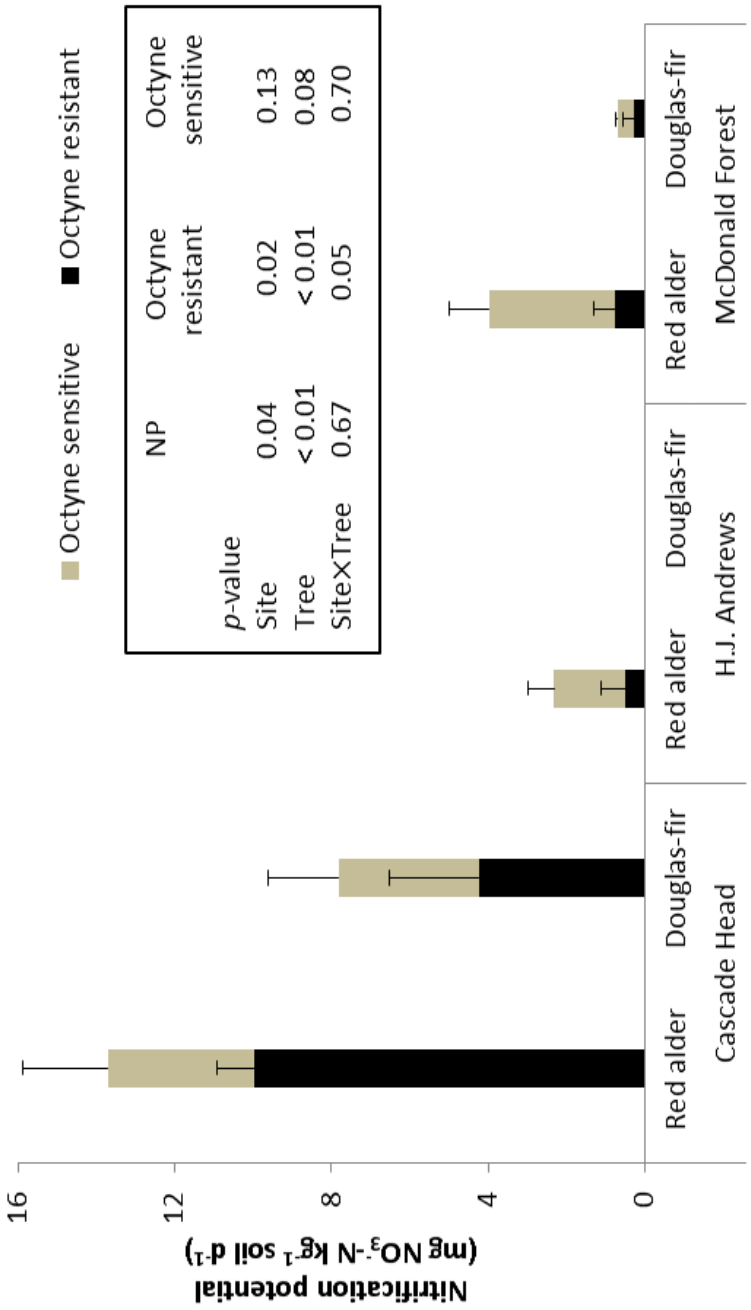

Fig. 1. Nitrification potentials. Bars represent the mean of three field replicates with SE (n=3).

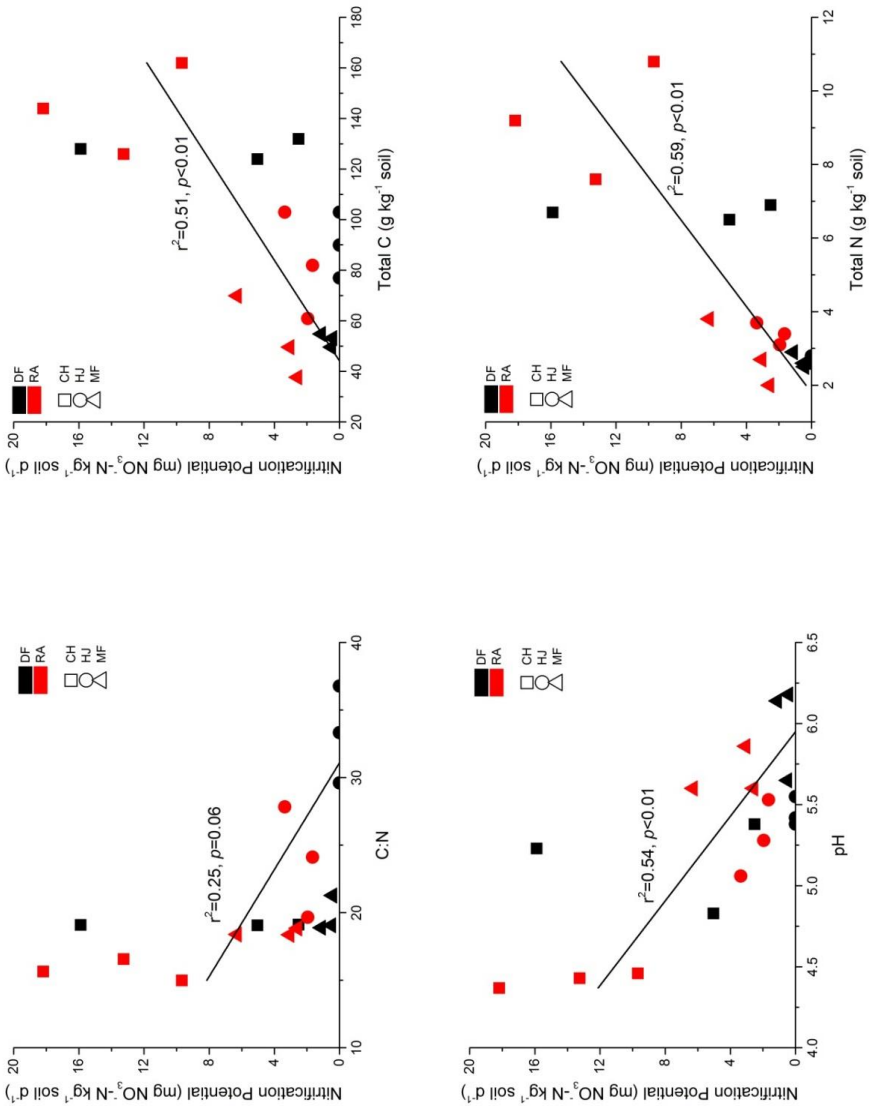

Fig. 2. Relationship between nitrification potential and soil properties.

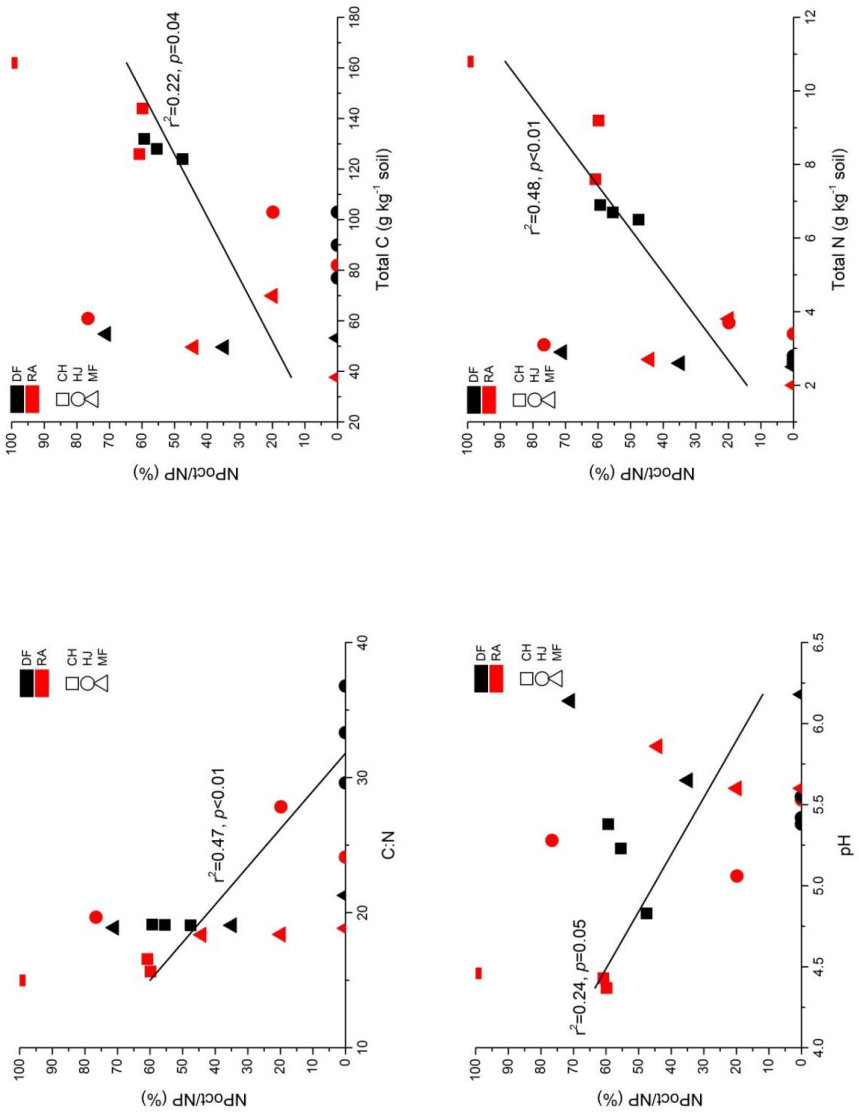

Fig. 3. Relationship between fraction of octyne-resistant nitrification potential (NP_{oct}) to total nitrification potential (NP) and soil properties.

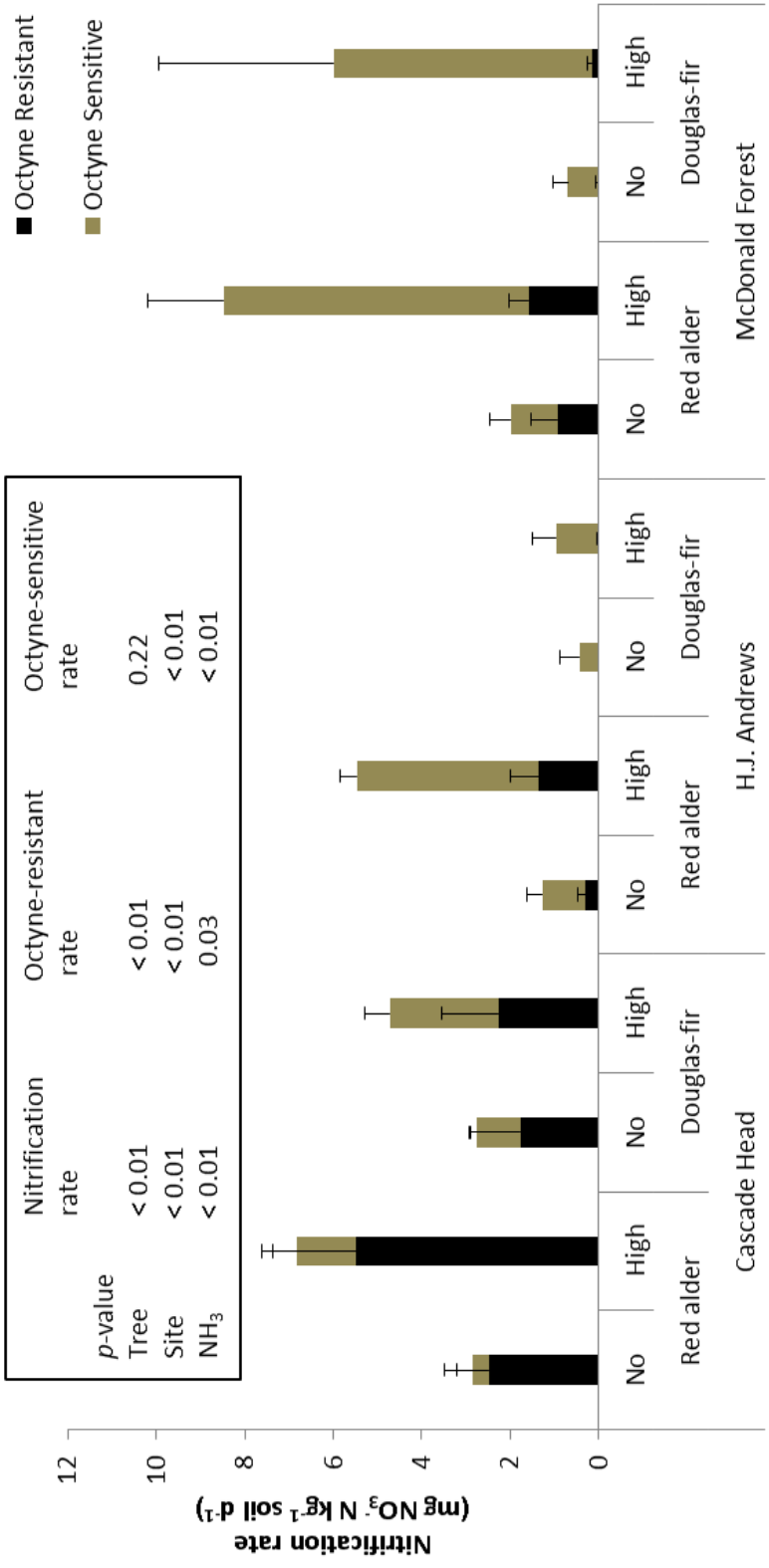

Fig. 4. Whole soil nitrification rate without (No) and with high NH_3 (High) addition. Bars represent the mean of three field replicates with SE (n=3). Only high NH_3 addition treatment showed significant effect of Site×Tree interaction (p<0.01).

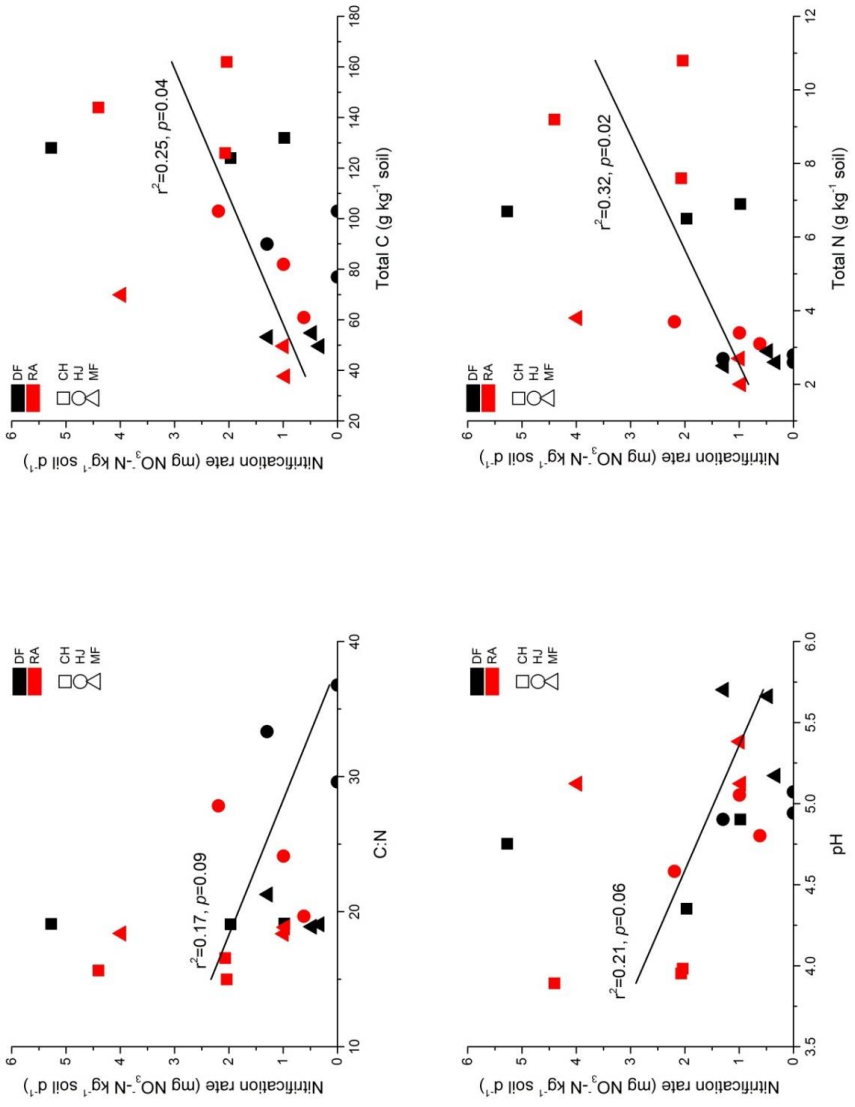

Fig. 5. Relationship between whole soil nitrification rate (without NH_3) and soil properties.

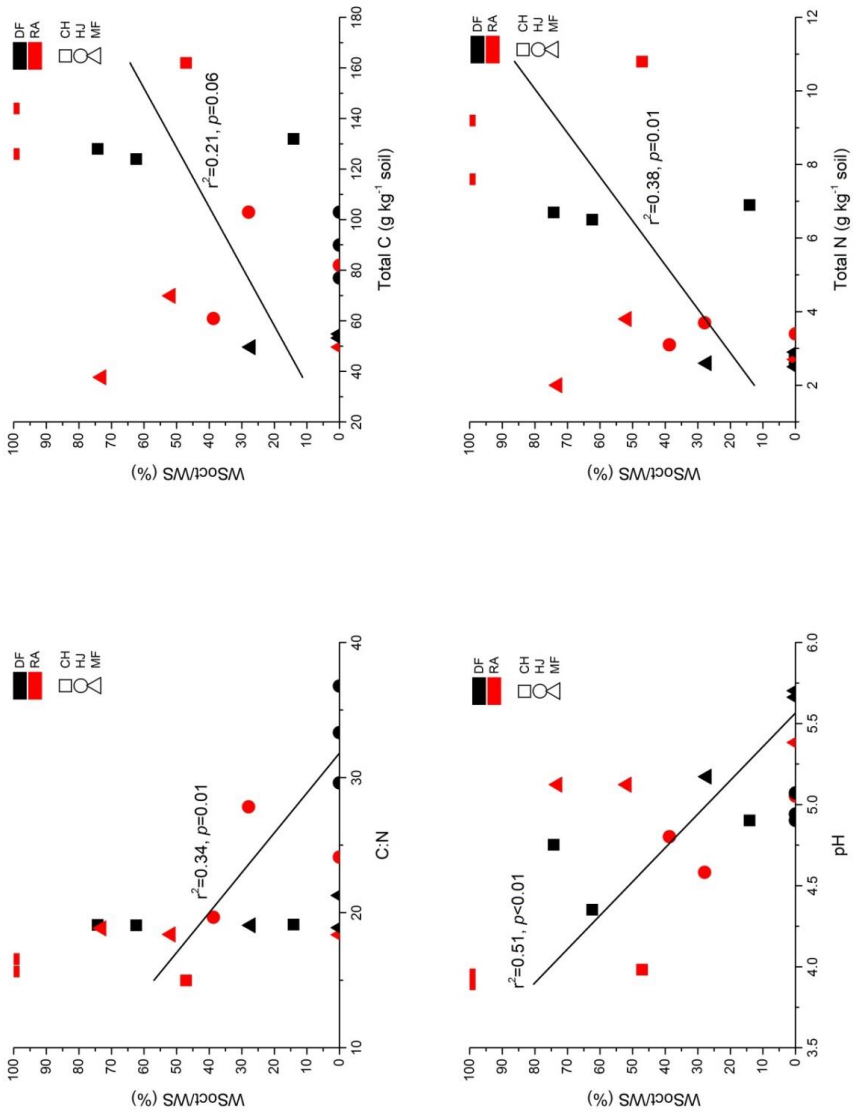

Fig. 6. Relationship between the whole soil octyne-resistant (WS_{oct}) activity fraction (without NH_3) of the total whole soil (WS) nitrification rate and soil properties.

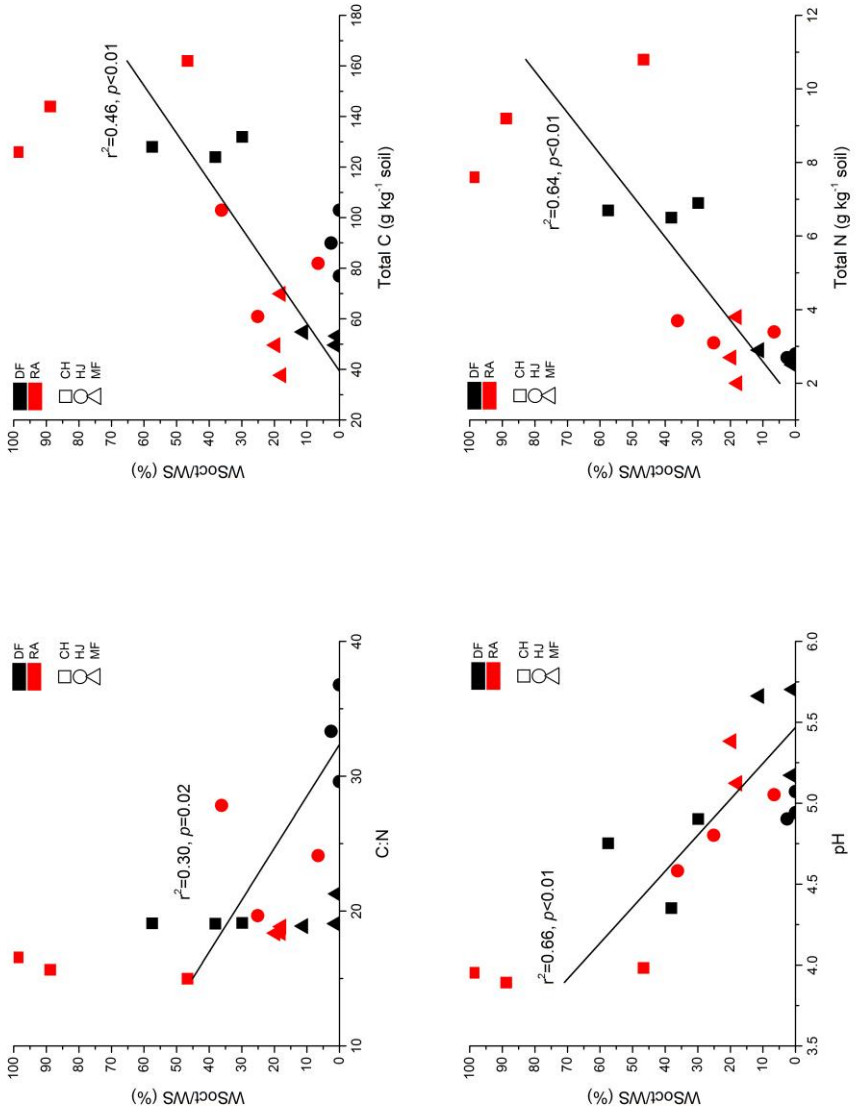

Fig. 7. Relationship between the whole soil octyne-resistant (WSoct) activity fraction (with high NH_3) of the whole soil (WS) nitrification rate and soil properties.

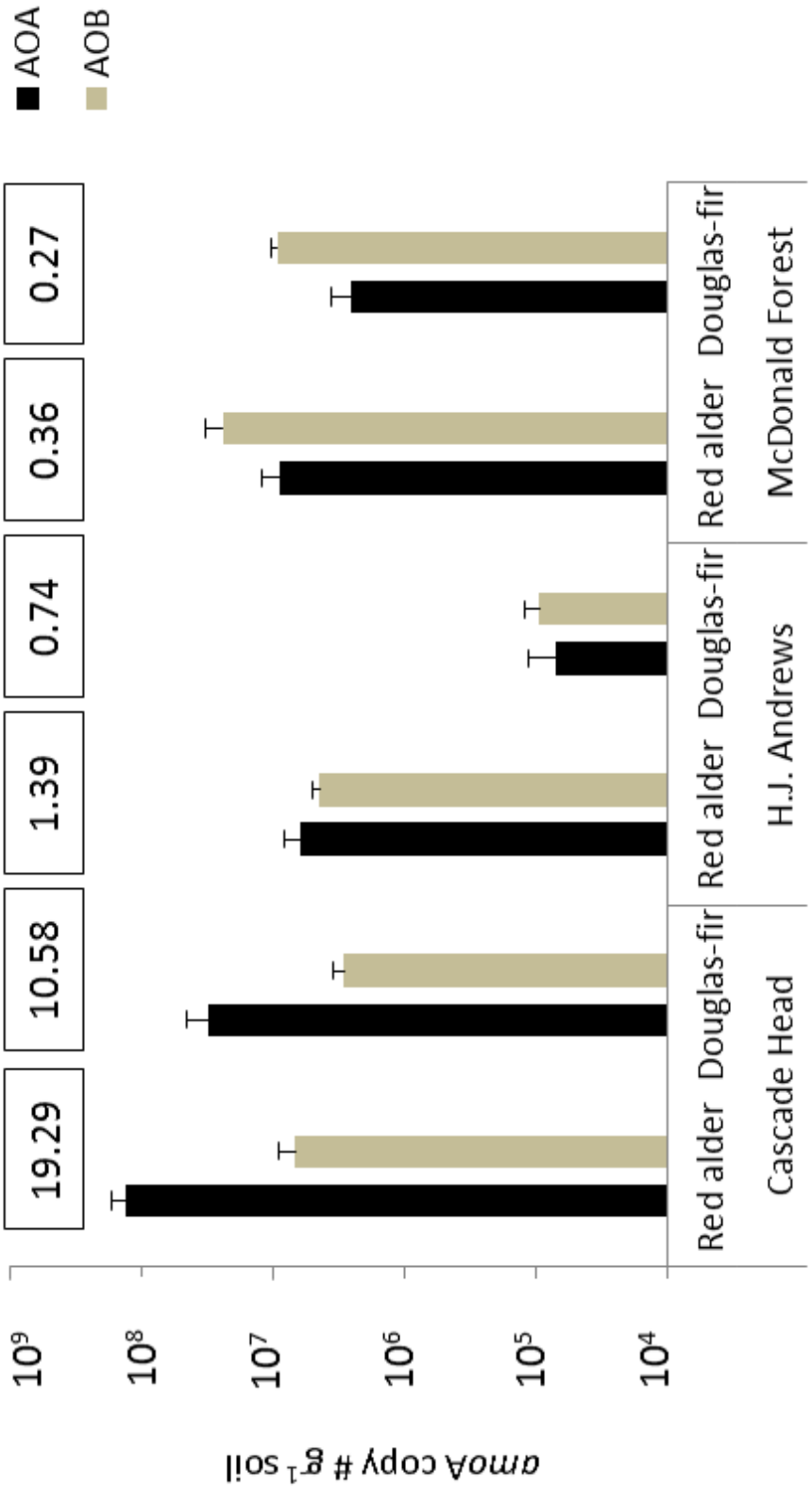

Fig.8 *amoA* gene copy numbers for AOA and AOB. Bars represent the mean of three field replicates with SE (n=3). Ratio of AOA to AOB *amoA* copies are shown above the bars.

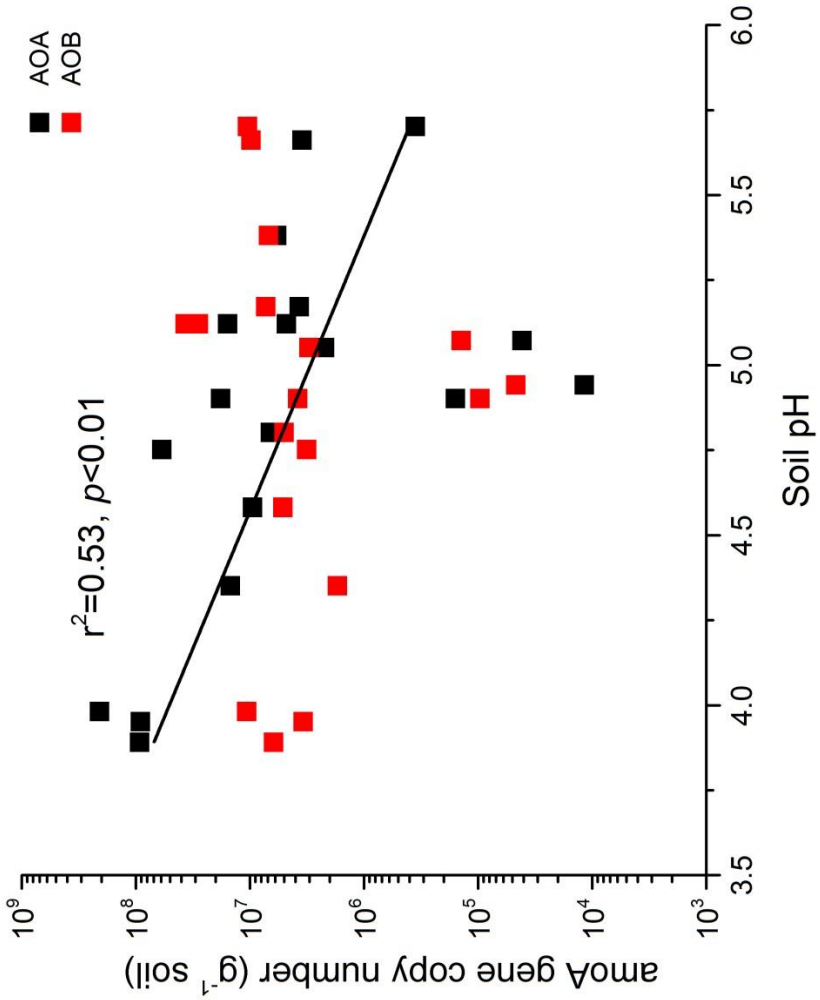

Fig. 9. Relationship between *amo*A gene copy number and soil pH of both AOA and AOB. Line represents a significant linear regression of AOA *amo*A gene number and soil pH with equation ($y=4.18\times10^{8}$-$7.29\times10^{7}x$).

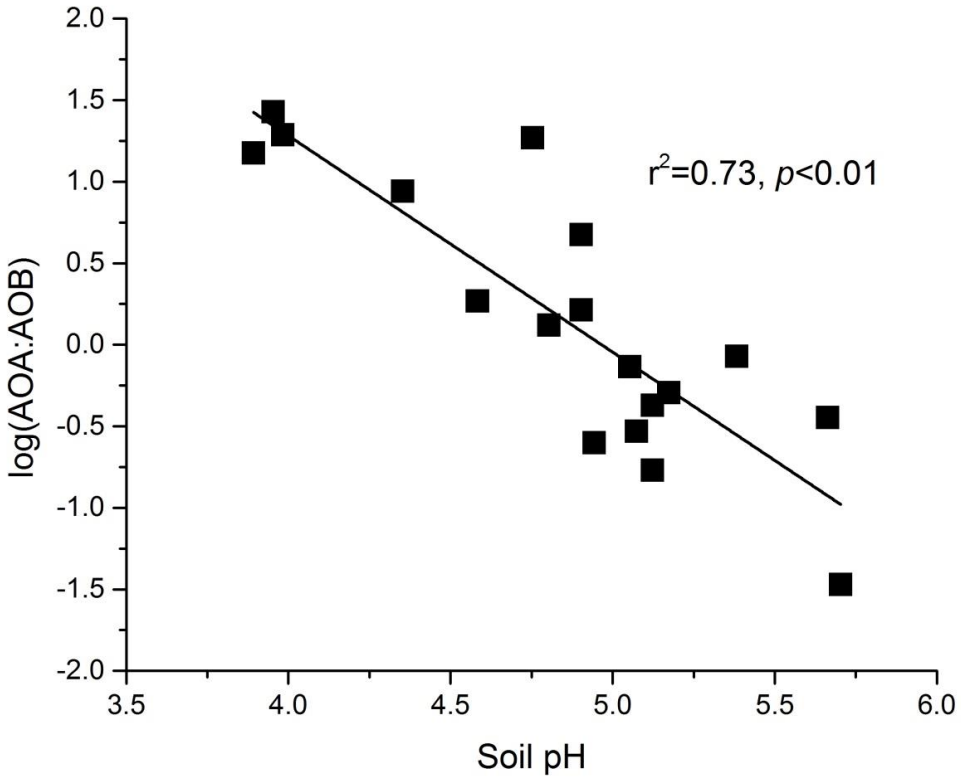

Fig. 10. Relationship between AOA:AOB ratio and soil pH. Line represents a significant linear regression of log(AOA:AOB) and soil pH with equation (y=6.59-1.33x).

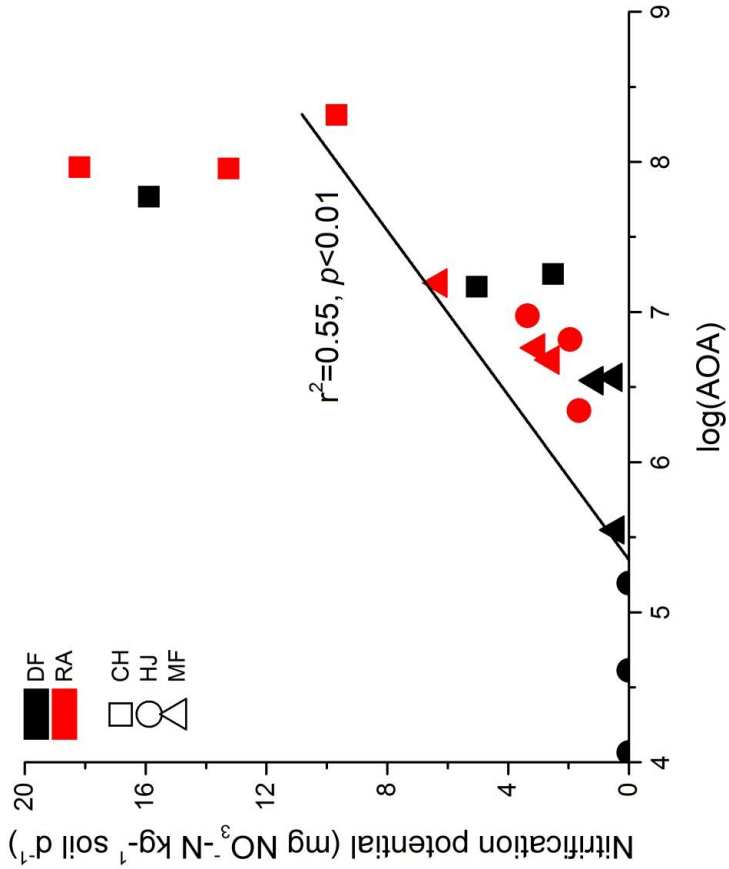

Fig. 11. Relationship between nitrification potential and AOA *amoA* gene copy number.

Table 1. Properties of Douglas-fir and red alder soils at each experimental site. Data are mean ± standard error and superscripts denote differences based on two-way ANOVA ($p < 0.05$, Fisher's least significant difference (LSD) procedure).

	Cascade Head		HJ Andrews		McDonald Forest	
Soil characteristics	Red alder	Douglas-fir	Red alder	Douglas-fir	Red alder	Douglas-fir
Total C (g kg^{-1} soil)[1]	144 ± 10.4^{a}	128 ± 2.3^{a}	82 ± 12.1^{b}	90 ± 7.5^{b}	52 ± 9.4^{c}	53 ± 1.5^{c}
Total N (g kg^{-1} soil)[1]	9.2 ± 0.9^{a}	6.7 ± 0.1^{b}	3.4 ± 0.2^{c}	2.7 ± 0.1^{c}	2.8 ± 0.5^{c}	2.7 ± 0.1^{c}
C:N	15.7 ± 0.5^{a}	19.1 ± 0.0^{a}	23.8 ± 2.4^{b}	33.2 ± 2.1^{c}	18.6 ± 0.2^{a}	19.6 ± 0.8^{a}
NH_4^+ (mg N kg^{-1} soil)	1.5 ± 0.6^{ab}	0.5 ± 0.2^{a}	1.2 ± 0.6^{a}	2.3 ± 1.3^{ab}	1.8 ± 0.5^{ab}	4.1 ± 1.4^{b}
NO_3^- (mg N kg^{-1} soil)	25.3 ± 2.6^{a}	9.9 ± 0.9^{b}	7.5 ± 1.0^{bc}	3.3 ± 0.5^{c}	10.2 ± 2.4^{b}	3.6 ± 0.6^{c}
pH	3.9 ± 0.0^{a}	4.7 ± 0.2^{b}	4.8 ± 0.1^{b}	5.0 ± 0.1^{bc}	5.2 ± 0.1^{cd}	5.5 ± 0.2^{d}
Water content (%)	123.7 ± 7.0^{a}	97.3 ± 8.7^{abc}	55.2 ± 3.1^{bc}	59.9 ± 4.2^{bc}	107.6 ± 36.4^{ab}	44.0 ± 23.2^{c}

1. Total C and total N data are modified from Zeglin *et al*. (2010) and Yarwood *et al*. (2008).

Table 2. Details of qPCR reactions used in this experiment

Gene amplified	AOA *amo* A	AOB *amo* A
Forward Primer	CrenamoA23f (Tourna et al., 2008)	*amo* A-1F (Stephen et al., 1998)
Reverse Primer	CrenamoA616r (Tourna et al., 2008)	*amo* A-2R (Rotthauwe et al., 1997)
Thermal Protocol	Enzyme Activation: 10 min at 95℃	Enzyme Activation: 10 min at 95℃
	40 cycles:	40 cycles:
	20 s at 95℃, 30 s at 58℃, 1 min at 72 ℃	20 s at 95℃, 30 s at 58℃, 1 min at 72 ℃
	Melt curve: 60℃- 95℃	Melt curve: 60℃- 95℃
Master Mix Recipe	5 μL Power SYBR Green PCR Master Mix (Carlsbad, CA)	5 μL Power SYBR Green PCR Master Mix (Carlsbad, CA)
	1.5 μM forward primer	0.5 μM forward primer
	1.5 μM reverse primer	0.5 μM reverse primer
	1 μL template DNA	1 μL template DNA
	Nuclease free water to 10 μL	Nuclease free water to 10 μL
Standard curve r^2	0.920- 0.998	0.979- 0.998
Reaction efficiency	86.9%- 100.7%	93.8%- 94.2%

Conclusion

We evaluated nitrification activity by measuring nitrification potential rates in soil slurries and nitrification rates in whole soil. Both of nitrification potential and whole soil nitrification rates were influenced by location and tree type. Rates were higher at sites that had more soil organic N and across all sites, red alder soils showed higher nitrification rates than Douglas-fir, indicating that an increase in N capital stimulates nitrification activity. Across all sites, higher NH_3 oxidizer abundance was detected under red alder than Douglas-fir. These results support previous findings that red alder positively influenced NH_3 oxidizer populations and consequently altered nitrification rate. Furthermore, a significant positive relationship was found between nitrification potential and AOA, but not AOB, *amo*A gene copy numbers, suggestion the important role of AOA in nitrification in forest soils.

The observation that NH_3 stimulated nitrification in whole soil for both octyne-resistant (AOA) and octyne-sensitive (AOB) activities revealed the effect of substrate on nitrification in these forest soils. Nitrification rates were about three-fold higher at the high NH_3 addition level compared to that without NH_3 addition. It is well accepted that AOB are often responsive to additions of NH_3, but AOA isolates have been shown to reach maximum levels of NH_3 oxidation under very low substrate concentrations (Martens-Habbena et al., 2009). Apparently in these acidic soils, AOA responded to additional substrate. It would be insightful to evaluate the effect of NH_3 addition on the growth of AOA and AOB in these acidic forest soils by measuring changes in their *amo*A gene copy number following supplementation with additional NH_3.

My study demonstrated the dominant role of AOA in acidic forest soils (pH < 5) in the Pacific Northwest. The evidence supporting AOA dominance of nitrification in acid soils are: (i) their high abundance in acid soils (Cascade Head) as demonstrated using qPCR; (ii) the strong positive correlation between octyne-resistant activity and their abundance; (iii) octyne-resistant activity accounted for over 50% in soils with pH < 5. Although AOA dominated nitrification in acid soils, AOB also showed some activity, which indicates the ability of some AOB to adapt to low pH conditions. At soil pH > 5, AOB contributed more that AOA to nitrification. Copies of AOB *amo*A genes were high across all sites; however, no correlation was found between soil pH and AOB *amo*A gene copy numbers, indicating that pH might affect their activity but not their abundance. A relatively large population of AOA was also found in McDonald Forest soils (pH > 5) but octyne-resistant activity (AOA) accounted for only 30% of nitrification activity, indicating higher pH might affect their activity more than their abundance.

The differential effect of pH on AOA and AOB abundance and activity may indicate diversity within their respective communities. For example, different taxa within the AOA (and AOB) respond differently to environmental conditions at different sites, such as NH_4^+ concentration or pH. This is supported by previous studies at these sites. The dominant archaeal *amo*A sequences belonged to clade iv (group 1.1b) and v (group 1.1a) at Cascade Head (Boyle-Yarwood et al., 2008) and to clade iii (group 1.1b) at McDonald Forest (Zeglin et al., 2011) (no data is available for the H.J. Andrews); most AOB sequences of soils at Cascade Head and the H.J. Andrews

belonged to *Nitrosospira* cluster 2 and 4, with a few in cluster 1 (Boyle-Yarwood et al., 2008), whereas *Nitrosospira* cluster 3a dominated McDonald Forest soils (Zeglin et al., 2011). It would be insightful if more comprehensive sequencing of AOA and AOB *amo*A genes was done at these sites using primer sets now available and particularly to see how different groups of AOA and AOB would respond to altered environmental conditions, such as pH, C:N ratios, etc., which would provide insights into their physiology.

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