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# Contributions of ammonia-oxidizing archaea and bacteria to nitrification in Oregon forest soils

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ABSTRACT

Ammonia oxidation, the first step of nitrification, is mediated by both ammonia-oxidizing archaea (AOA) and bacteria (AOB); however, the relative contributions of AOA and AOB to soil nitrification are not well understood. In this study we used 1-octyne to discriminate between AOA- and AOB-supported nitrification determined both in soil-water slurries and in unsaturated whole soil at field moisture. Soils were collected from 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) on acidic soils (pH 3.9–5.7) in Oregon, USA. The abundances of AOA and AOB were measured using quantitative PCR by targeting the *amoA* gene, which encodes subunit A of ammonia monooxygenase. Total and AOA-specific (octyne-resistant) nitrification activities in soil slurries were significantly higher at Cascade Head (the most acidic soils, pH < 5) than at either the H.J. Andrews or McDonald Forest, and greater in red alder compared with Douglas-fir soils. The fraction of octyne-resistant nitrification varied among sites (21–74%) and was highest at Cascade Head than at the other two locations. Net nitrification rates of whole soil without $NH_4^+$ amendment ranged from 0.4 to 3.3 mg N $kg^{-1}$ soil $d^{-1}$. Overall, net nitrification rates of whole soil were stimulated 2- to 8-fold by addition of 140 mg $NH_4^+$-N $kg^{-1}$ soil; this was significant for red alder at Cascade Head and the H.J. Andrews. Red alder at Cascade Head was unique in that the majority of $NH_4^+$-stimulated nitrifying activity was octyne-resistant (73%). At all other sites, $NH_4^+$-stimulated nitrification was octyne-sensitive (68–90%). The octyne-sensitive activity—presumably AOB—was affected more by soil pH whereas the octyne-resistant (AOA) activity was more strongly related to N availability.



## 1. 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, N turnover varies between red alder (*Alnus rubra* Bong.) sites and conifer sites (Binckley et al., 1992; Perakis et al., 2012) and a several-fold increase in the rates of N turnover by the inclusion of red alder has been reported (Hart et al., 1997; Boyle et al., 2008). As a result of red alder having the potential to fix 50–300 kg N $ha^{-1}$ $y^{-1}$ (Bottomley and Myrold, 2015), there is an increase in the N capital and N availability of the soil. A negative consequence is that red alder can reduce soil pH, to as low as 3.6–3.9 (Yarwood et al., 2010). The pH decline under red alder probably occurs as a result of increased nitrification (Binkley and Sollins, 1990). Although ammonia-oxidizing bacteria (AOB) have been isolated from soils of pH < 5 (De Boer and Kowalchuk, 2001), it remains equivocal whether or not they can effectively oxidize $NH_3$ at that pH. Heterotrophic nitrification, mediated by heterotrophic bacteria and fungi, has been suggested to be an alternate option (Hynes and Knowles, 1982; Stein, 2011; Zhang et al., 2011). Another possibility is the recent discovery of ammonia-oxidizing *Thaumarchaeota* (AOA) that have the potential for $NH_3$ oxidation and growth on $CO_2$. Recently, an acidophilic AOA has been isolated (Lehtovirta-Morley et al., 2011, 2013), and may represent a lineage of dominant AOA types in acidic soils (Nicol et al., 2008; Gubry-Rangin et al., 2010; Yao et al., 2011; Prosser and Nicol, 2012; Hu et al., 2014). Although no isolates of AOB have been found to grow at

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pH < 5 (De Boer and Kowalchuk, 2001), they exist in acidic soils and their potential for activity has been inferred by detection of AOB mRNA for *amoA* (Nicol et al., 2008). This might be due to residence in pH-neutral microsites (Hankinson and Schmidt, 1984), an ability to use urea and/or other organic N sources (Burton and Prosser, 2001), or through protective aggregation (De Boer et al., 1991; Spieck et al., 1992). In most soils, AOA outnumber AOB based on their *amoA* gene abundances, suggesting the potential for a greater role in nitrification (Leininger et al., 2006; Prosser and Nicol, 2008; Norman and Barrett, 2014). In some soils, however, AOB can be more abundant than AOA (Hu et al., 2014; Petersen et al., 2012; Yarwood et al., 2010). Thus, to date no clear consensus has emerged about the mechanism(s) controlling niche differentiation in acidic soils.

We designed a study using our newly developed assay, which is based upon AOB $NH_3$-oxidizing activity being quickly and irreversibly inactivated by low concentrations of 1-octyne, whereas AOA activity is unaffected by the same range of concentrations (Taylor et al., 2013). The study involved a comparison of the relative contribution of AOA and AOB to soil nitrification in acidic forest soils under different tree types. Three sites spanning a range of soil types and containing replicated stands of Douglas-fir (*Pseudotsuga* menziesii Mirb. Franco) and red alder were selected. The soils varied in pH and organic C and N contents, in part because of the N inputs provided by the red alder-*Frankia* symbiosis. The aims of this study were 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 was hypothesized that N input through $N_2$-fixation by red alder would increase both ammonia oxidizer abundances and nitrification potential, and would affect the relative contributions of AOA and AOB to nitrification.

## 2. Materials and methods

### 2.1. Site description and soil properties

Soils were collected from three forest sites containing plots of either pure stands of Douglas-fir or red alder. At two of the sites the stands were planted approximately 30 years ago (Radosevich et al., 2006). One site lies within the Cascade Head Experimental Forest (45°01′ N, 123°54′W, 330 m elevation), 1.6 km from the Pacific Ocean, and receives ~2400 mm $y^{-1}$ of precipitation. Soils at the site are classified as Isomesic Fluvudands. A second site was located within the H.J. Andrews Experimental Forest (44°14′ N, 122°11′W, elevation 800 m), along the western slopes of the Cascade Range, and receives ~2300 mm $y^{-1}$ of precipitation. Soils in the area are classified as Typic Haplumbrepts (Dyrness, 2001). The third site was located within the McDonald-Dunn Forest (44°42′ N, 123°20′W, 350 m elevation), on the eastern foothills of the Coast Range. The soils are classified as Xeric Palehumults. Annual precipitation averages ~1070 mm $y^{-1}$. At McDonald Forest, natural stands of red alder are found along streams within a matrix of Douglas-fir; both about 30 years old. All soil samples were collected during the late spring of 2013. Soil was collected from each of three field replicate plots of each tree type at each location, kept in separate bags, and stored at 4 °C and used within six months. Samples (10 g) were removed from each bag and frozen (−20 °C) for DNA extraction at a later date.

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 in deionized water; total C and N were measured by combustion; concentrations of $NH_4^+$ and $NO_3^-$ plus $NO_2^-$ (referred to simply as $NO_3^-$ afterwards) in 2 M KCl extracts were determined colorimetrically (Hood-Nowotny et al., 2010; Kandeler and Gerber, 1988).

### 2.2. Nitrification potential assay

Nitrification potentials of soils were measured using a shaken soil-slurry method with supplemental $NH_4^+$ (1 mM $NH_4Cl$; Taylor et al., 2010). Field moist soil (2.5 g) was added to 150-ml serum bottles loosely capped by phenolic caps fitted with butyl stoppers, and pre-incubated at room temperature (22 ± 2 °C) for 2 d. Deionized water (15 ml) supplemented with 1 mM $NH_4Cl$ was added to each bottle (final concentration ~140 mg $NH_4^+$-N $kg^{-1}$ soil). Bottles were shaken at 200 rpm at 30 °C. Samples of soil slurries (1 ml) were taken after 15 min of shaking and at 24 and 48 h, centrifuged for 3 min at 13,000× g, and $NO_3^-$ determined. At the beginning of the slurry assays, a set of bottles were amended with 2 kPa of either acetylene (6 μM aqueous) or octyne gas (4 μM aqueous) as described by Taylor et al. (2013). Acetylene was used to block $NH_3$ oxidation by AOA and AOB and to evaluate the possibility of acetylene-insensitive heterotrophic nitrification. Octyne was used to block AOB activity while allowing AOA activity to proceed (Taylor et al., 2013). For Cascade Head and McDonald Forest soils, nitrification potentials were calculated from $NO_3^-$ accumulation during the first 24 h. For H.J. Andrews soils, nitrification potentials were calculated from $NO_3^-$ accumulation occurring between 24 and 48 h due to lack of significant accumulation during 0–24 h. Nitrification potentials were calculated by subtracting the $NO_3^-$ accumulation in the acetylene treatment from values measured in the treatments without acetylene to focus primarily on acetylene-sensitive AOA and AOB activities. The pH of the soil slurries was measured at the end of the incubation.

### 2.3. Whole soil nitrification assay

A whole soil assay was used to measure the potential contributions of AOA and AOB to soil nitrification. Soil samples (5 g) were incubated at three different $NH_3$ levels (equivalent to 0, 14, and 140 mg $NH_4^+$ $kg^{-1}$ soil) achieved by adding sufficient anhydrous $NH_3$ gas to the headspace of 150-ml serum bottles sealed by phenolic caps fitted with butyl stoppers. Prior to initiation of the assay, soils were pre-incubated at room temperature (22 ± 2 °C) for 2 d. Three treatments were imposed at each $NH_4^+$ level: (i) positive control (no octyne or acetylene amendment), (ii) acetylene amendment (2 kPa = 6 μM aqueous), and (iii) octyne amendment (2 kPa = 4 μM aqueous). Soil samples were incubated at 25 °C, $NH_4^+$ and $NO_3^-$ concentrations were determined at 2 and 7 d, and nitrification rates were calculated after subtracting $NO_3^-$ accumulation in the acetylene treatment. The pH at the beginning of whole soil incubation was measured 12 h after $NH_3$ gas was added, when $NH_3$ gas had fully defused into soil samples and pH came to equilibrium. The pH of soil samples was measured at the end of the incubation.

### 2.4. Copy numbers of amoA 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 with a NanoDrop™ ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and diluted to 1 ng $\mu l^{-1}$. DNA extracts were stored at −20 °C for future use.

The quantitative polymerase chain reaction (qPCR) was used to determine AOA and AOB abundance by quantifying the number of *amoA* gene copies for each group. All qPCR reactions were performed in triplicate by using an ABI PRISM 7500 FAST (Carlsbad, CA)



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**Table 1**
Properties of Douglas-fir and red alder soils at each experimental site. Data are mean ± standard error (n = 3); superscripts denote differences based on two-way ANOVA ($p < 0.05$, Fisher's least significant difference (LSD) procedure).

| Site | Tree species | Total C | Total N | C:N | $NH_4^+$ | $NO_3^-$ | pH | Water content |
|---|---|---|---|---|---|---|---|---|
| | | (g $kg^{-1}$ soil)[a] | | | (mg N $kg^{-1}$ soil) | | | (kg $kg^{-1}$ soil) |
| Cascade Head | Red alder | 144 ± 10.4$^a$ | 9.2 ± 0.9$^a$ | 15.7 ± 0.5$^a$ | 1.5 ± 0.6$^{ab}$ | 25.3 ± 2.6$^a$ | 3.9 ± 0.0$^a$ | 1.24 ± 0.07$^a$ |
| | Douglas-fir | 128 ± 2.3$^a$ | 6.7 ± 0.1$^b$ | 19.1 ± 0.0$^a$ | 0.5 ± 0.2$^a$ | 9.9 ± 0.9$^b$ | 4.7 ± 0.2$^b$ | 0.97 ± 0.09$^{abc}$ |
| H.J. Andrews | Red alder | 82 ± 12.1$^b$ | 3.4 ± 0.2$^c$ | 23.8 ± 2.4$^b$ | 1.2 ± 0.6$^a$ | 7.5 ± 1.0$^{bc}$ | 4.8 ± 0.1$^b$ | 0.55 ± 0.03$^{bc}$ |
| | Douglas-fir | 90 ± 7.5$^b$ | 2.7 ± 0.1$^c$ | 33.2 ± 2.1$^c$ | 2.3 ± 1.3$^{ab}$ | 3.3 ± 0.5$^c$ | 5.0 ± 0.1$^{bc}$ | 0.60 ± 0.04$^{bc}$ |
| McDonald Forest | Red alder | 52 ± 9.4$^c$ | 2.8 ± 0.5$^c$ | 18.6 ± 0.2$^a$ | 1.8 ± 0.5$^{ab}$ | 10.2 ± 2.4$^b$ | 5.2 ± 0.1$^{cd}$ | 1.08 ± 0.36$^{ab}$ |
| | Douglas-fir | 53 ± 1.5$^c$ | 2.7 ± 0.1$^c$ | 19.6 ± 0.8$^a$ | 4.1 ± 1.4$^b$ | 3.6 ± 0.6$^c$ | 5.5 ± 0.2$^d$ | 0.44 ± 0.23$^c$ |

[a] Total C and total N data are modified from Yarwood et al. (2008) and Zeglin et al. (2011).

sequence detection system set to read SYBR green fluorescence. Standard curves were constructed using *Nitrosomonas europaea* genomic DNA (AOB *amoA*) or *Nitrososphaera viennensis* genomic DNA (AOA *amoA*). AOB *amoA* genes were amplified using primers *amoA*-1F (5′-GGG GGT TTC TAC TGG TGG T; Stephen et al., 1998) and *amoA*-2R (5′-CCC CTC KGS AAA GCC TTC TTC; Rotthauwe et al., 1997). AOA *amoA* 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 are reported as *amoA* gene copies $g^{-1}$ dry soil. Primers sets, thermal protocols master mix recipes, standard curve $r^2$, and reaction efficiency are summarized in Supplemental Material (Table S1).

### 2.5. Statistical analysis

Treatment differences were evaluated using analysis of variance (ANOVA), followed by the Fisher's least significant differences (LSD) test. Unless otherwise noted, only significant ($p < 0.05$) interactions are discussed. To meet the assumptions of normality and homogeneity of variance, whole soil nitrification rates were square-root transformed and *amoA* gene copy numbers were log transformed. Relationships among variables were examined by correlation analysis. Analyses were done using StatGraphics Centurion Version 16.1.03 (Statpoint Technologies, Inc,. Warrenton, VA) or SigmaPlot 12.3 (Systat Software, Inc., San Jose, CA).

## 3. Results

### 3.1. Soil properties

McDonald Forest soils contained the lowest amount of total C and Cascade Head soils the most (Table 1). There was 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 significantly higher than soils from other two sites, which were not significantly different. Total N was consistently higher in red alder compared to Douglas-fir, but this was statistically significant only at Cascade Head. Soil pH ranged from 3.9 to 5.5 with highly significant difference among soils. Cascade Head soils had the lowest pH; McDonald Forest soils had the highest pH values. At Cascade Head, soils under red alder had significantly lower pH than soils under Douglas-fir. This trend also held for soils from the other two sites but differences were not significant. There was little difference in extractable $NH_4^+$ among sites or between tree species. Cascade Head soils had more extractable $NO_3^-$ than the other two sites, but at all sites $NO_3^-$ was higher under red alder than under Douglas-fir. Most soil properties, except for C:N ratio, were highly correlated with each other; for example, pH was inversely correlated with total N (r = −0.83) but not to C:N ratio (r = 0.26). Thus, relationships between nitrification rates and soil properties will focus on C:N ratios (an index of N availability) and pH.

### 3.2. Nitrification potentials of soil slurries

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. To eliminate any contribution from heterotrophic nitrification, all nitrification potentials were adjusted by subtracting the $NO_3^-$ values of the acetylene treatment prior to statistical analysis of the nitrification potential data.

#### 3.2.1. Nitrification potentials of soil slurries

Two-way ANOVA showed that the nitrification potentials varied significantly among sites and between tree types (Fig. 1). Cascade Head soils averaged 11.0 mg N $kg^{-1}$ soil $d^{-1}$, which was significantly higher than McDonald Forest (2.4 mg N $kg^{-1}$ soil $d^{-1}$) and H.J. Andrews (1.2 mg N $kg^{-1}$ soil $d^{-1}$) soils. Across all sites, nitrification potential was two-fold greater in red alder soils 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 the nitrification potential incubations, 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

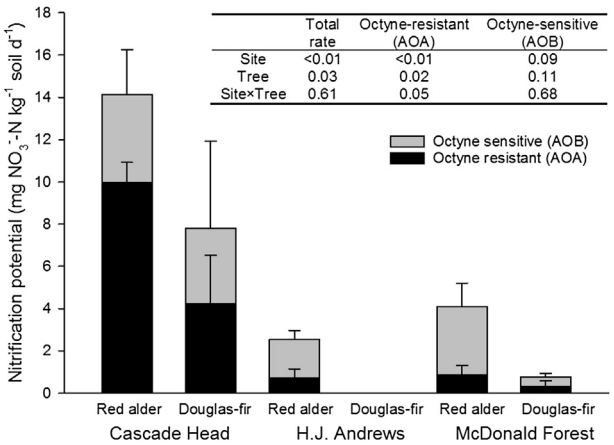


**Fig. 1.** Potential nitrification activities of octyne-resistant (archaeal) and octyne-sensitive (bacteria) ammonia oxidizers in short-term soil slurry assays supplemented with 1 mM $NH_4^+$ (~140 mg $NH_4^+$-N $kg^{-1}$ soil). Data are means ± standard errors (n = 3). No potential nitrification activity was found for the Douglas-fir soil at the H.J. Andrews. Two-way ANOVA table shows the statistical significance (p-value) of site, tree type, and their interaction.

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had decreased significantly to pH 3.9–4.6 ($p = 0.02$), presumably due to greater $H^+$ associated with higher nitrification activities and differential buffering among the soils.

#### 3.2.2. Effect of octyne on nitrification potentials of soil slurries

Two-way ANOVA showed that the octyne-resistant nitrification potential varied significantly among sites and tree type, although there was a barely significant interaction (Fig. 1). The octyne-resistant nitrification potential at Cascade Head averaged 7.1 mg N $kg^{-1}$ soil $d^{-1}$, 12-fold greater than at McDonald Forest and 20-fold greater than at the H.J. Andrews. The fraction of the nitrification potential that was octyne-resistant ranged from 21 to 74%, indicating that the relative contribution of AOA varied among soils. At Cascade Head, nitrification potentials with or without octyne were not significantly different, suggesting that the high potential nitrification activity was dominated by AOA. A decrease in nitrification potential was detected at McDonald Forest for Douglas-fir ($p = 0.05$) and red alder ($p = 0.08$) soils when exposed to octyne, indicating an increased AOB contribution (octyne senstitive). The effect of octyne on nitrification potentials was compared to the effect of 100 μM ATU (1-allyl-2-thiourea; 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 ($r^2 = 0.74$, $p < 0.01$), adding further evidence to suggest that AOA were responsible for the octyne-resistant potential nitrification activity.

#### 3.2.3. Environmental factors

Nitrification potentials decreased significantly in a curvilinear manner with both soil pH ($r^2 = 0.60$) and C:N ratio ($r^2 = 0.51$; Fig. 2a,b). Similar trends were found for the fraction of octyne-resistant activity but with a weaker relationship for pH ($r^2 = 0.26$) than C:N ratio ($r^2 = 0.59$; Fig. 2c,d).

### 3.3. 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 0–2 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.

#### 3.3.1. Nitrification rate

Measurable accumulation of $NO_3^-$ was detected within all soils at 7 d. Three-way ANOVA showed that site, tree type, and $NH_3$ addition level (Fig. 3) affected nitrification rates. Whole soil nitrification rates of all soils were greater when $NH_3$ was added, 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 (Fig. 3). 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 (Fig. 3). 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 the 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.

#### 3.3.2. Effect of octyne

Three-way ANOVA showed that octyne-resistant activity varied significantly among sites, tree types, and $NH_3$ addition (Fig. 3). 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 three-fold higher $NO_3^-$ accumulation rate compared to soils under Douglas-fir across all sites ($p < 0.01$). Octyne-resistant 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 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 nitrifying 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%).

#### 3.3.3. Environmental factors

In non-amended whole soils, the nitrification rate was positively, linearly correlated with total N ($r^2 = 0.32$) but not with pH or C:N ratio (Suppl. Fig. 1a,b). The fraction of octyne-resistant activity showed a linear positive correlation with total N ($r^2 = 0.38$), and negative curvilinear correlations with soil pH ($r^2 = 0.52$) and C:N ratio ($r^2 = 0.47$; Suppl. Fig. 1c,d). In soils amended with a high concentration of $NH_3$, 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 linear correlation with total N ($r^2 = 0.64$), negative curvilinear correlations with soil pH ($r^2 = 0.75$) and C:N ratio ($r^2 = 0.49$; Fig. 4).

### 3.4. AOA and AOB amoA gene abundance

Copies of AOA and AOB *amoA* genes were detected in all soil samples, but copy numbers varied widely among samples. Across all soils, AOA *amoA* gene abundance ranged from $1 \times 10^4$ to $2 \times 10^8$ *amoA* genes $g^{-1}$ soil and AOB *amoA* gene abundance ranged from $4 \times 10^4$ to $3 \times 10^7$ *amoA* genes $g^{-1}$ soil (Fig. 5). The site-by-tree interaction was significant for AOA ($p = 0.02$) and AOB ($p < 0.01$) *amoA* gene abundances. Abundances of both AOA and AOB *amoA* genes were higher in red alder than Douglas-fir soils, but this difference was statistically significant only at the H.J Andrews. Ammonia-oxidizing archaeal *amoA* gene copy numbers were significantly higher under both tree types at Cascade Head than the other two sites, and higher at McDonald Forest than at the H.J. Andrews under Douglas-fir. Abundances of AOB *amoA* genes were significantly higher under both tree types at McDonald Forest than

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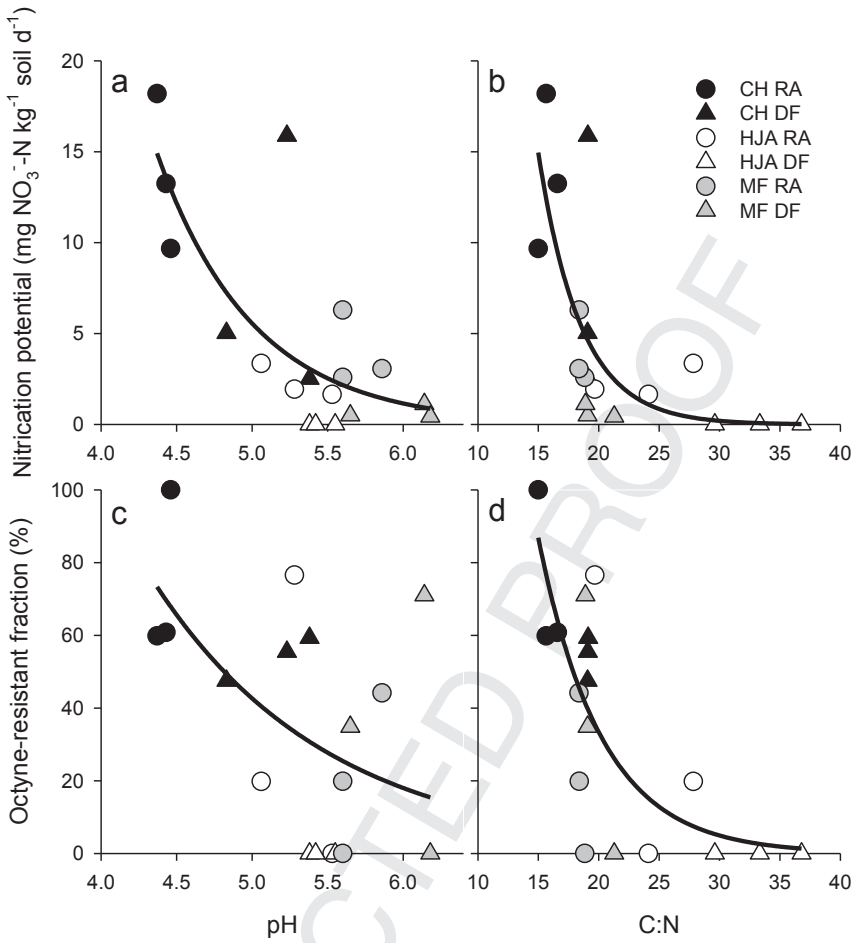


**Fig. 2.** Relationship between total nitrification potential (a, b) or octyne-resistant (AOA) fraction of the nitrification potential (c, d) and soil slurry pH (a, c) and C:N ratio (b, d). Soils are identified by site (CH—Cascade Head, black; HJA—H.J. Andrews, white; MF—McDonald Forest, gray) and tree type (DF—Douglas-fir, triangle; RA—red alder, circle). Lines represent best fit, exponential regressions; all are significant at $p < 0.05$.

the other two sites; and higher at Cascade Head than at the H.J. Andrews under Douglas-fir. The AOA:AOB ratio varied significantly across sites ($p < 0.01$; Fig. 5). Cascade Head soils were strongly dominated by AOA, whereas McDonald Forest soils were dominated by AOB; H.J. Andrews soils contained approximately equal numbers 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 ($p = 0.08$).

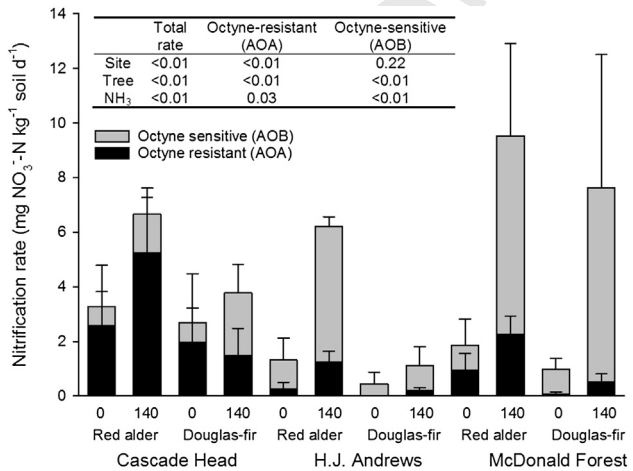


**Fig. 3.** Nitrification activities of octyne-resistant (archaeal) and octyne-sensitive (bacteria) ammonia oxidizers in whole soil assays supplemented with $NH_3$ equivalent to 0 or 140 mg $NH_4^+$-N $kg^{-1}$ soil. Data are means ± standard errors (n = 3). Three-way ANOVA table shows the statistical significance (p-value) of site, tree type, and $NH_3$ addition; interactions were not significant ($p > 0.05$).

Ammonia-oxidizing archaeal *amoA* gene copy numbers decreased significantly as soil pH increased ($r^2 = 0.32$), but there was no correlation between AOB *amoA* gene copy numbers and pH (Fig. 6a). Both AOA ($r^2 = 0.68$) and AOB ($r^2 = 0.63$) *amoA* gene copies numbers significantly decreased as soil C:N ratio increased (Fig. 6b).

### 3.5. Linking abundance with activity

Regression analysis showed that nitrification potential was linearly and positively correlated with AOA ($r^2 = 0.53$) but not AOB ($r^2 < 0.01$) *amoA* gene copy numbers, and was not improved if gene copy numbers of both groups of ammonia oxidizers were included in a multiple regression analysis. The octyne-resistant nitrification potential was even more highly correlated with AOA *amoA* gene copy numbers (Fig. 7; $r^2 = 0.80$) whereas octyne-sensitive nitrification potential was not correlated with AOB *amoA* gene copy numbers ($r^2 = 0.05$).

Nitrification rates of either non-amended or $NH_4^+$-amended whole soils were not significantly correlated with AOA or AOB *amoA* gene copy numbers, either individually or in a multiple regression approach; however, the octyne-resistant nitrification rate of whole soil was significantly correlated with AOA *amoA* copy numbers in both $NH_4^+$-amended ($r^2 = 0.88$) or non-amended ($r^2 = 0.73$) whole soils. No correlation between octyne-sensitive nitrification and

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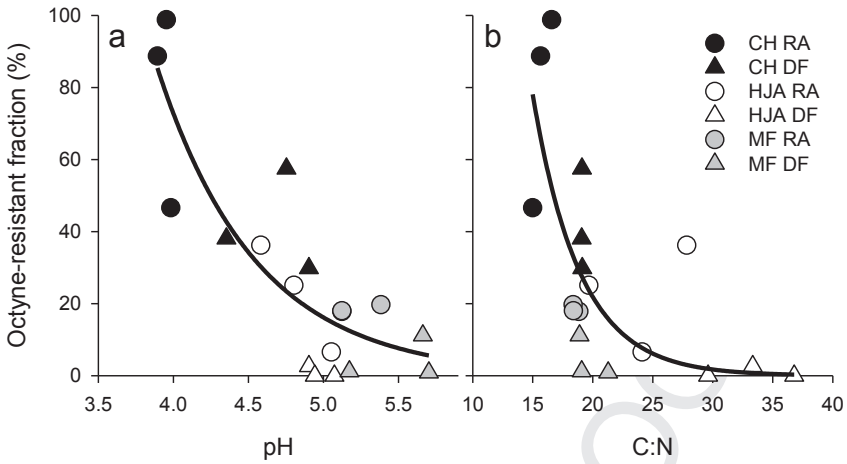


**Fig. 4.** Relationship between the octyne-resistant (AOA) fraction of nitrification in whole soil supplemented with $NH_3$ (equivalent to140 mg $NH_4^+$-N $kg^{-1}$ soil) and soil pH (a) and C:N ratio (b). Soils are identified by site (CH—Cascade Head, black; HJA—H.J. Andrews, white; MF—McDonald Forest, gray) and tree type (DF—Douglas-fir, triangle; RA—red alder, circle). Lines represent best fit, exponential regressions; all are significant at $p < 0.05$.

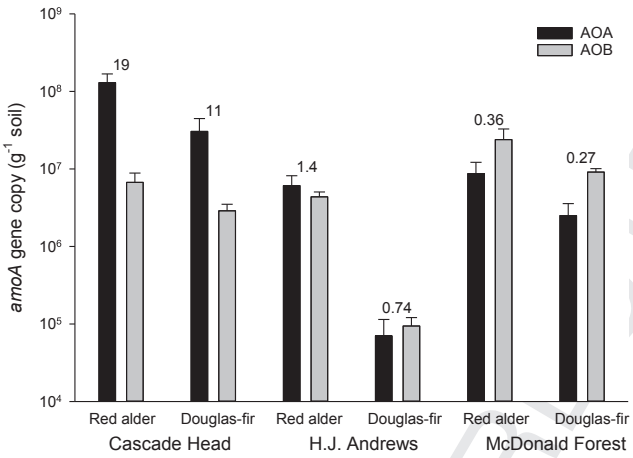


**Fig. 5.** Abundance of ammonia-oxidizing archaea (AOA) and bacteria (AOB) based on copy numbers of their *amoA* genes. Data are means ± standard error (n = 3). Number above a set of bars is the AOA:AOB ratio.

AOB *amoA* gene copy numbers was found in either non-amended or $NH_4^+$-amended whole soils.

## 4. Discussion

### 4.1. Activity and abundance of ammonia oxidizers

The nitrification rates measured across the three sites fall within the range of about 0.1–14 mg N $kg^{-1}$ soil $d^{-1}$ that has been reported for forest soils worldwide (Vitousek et al., 1982; Wertz et al., 2012). The inclusion of red alder in conifer-dominated forests of the Pacific Northwest, with its large N input from $N_2$ fixation, 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; Boyle et al., 2008). This combination of shifts in soil pH and N availability raises questions about what kinds of ammonia oxidizers might contribute to soil nitrification, such as AOA versus AOB, and the possibility that different phylotypes of each might contribute depending on the severity of the acidity. Both nitrification potential and whole soil nitrification assays showed a strong inverse relationship between

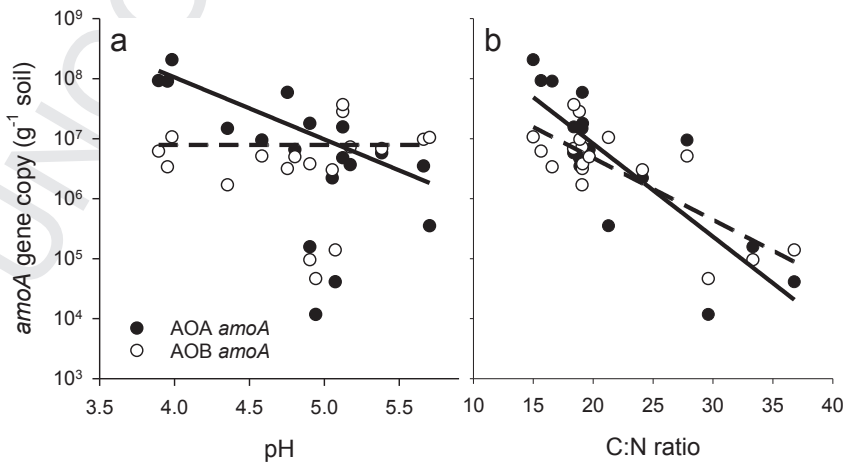


**Fig. 6.** Relationship between ammonia-oxidizing archaea (AOA) or bacteria (AOB), based on copy numbers of their *amoA* genes, and soil pH (a) and C:N ratio (b). Copy numbers of AOA *amoA* genes (black circles, solid lines) were significantly correlated with soil pH ($R^2 = 0.32$, $p = 0.01$) and C:N ratio ($R^2 = 0.68$, $p < 0.01$), whereas AOB *amoA* gene copy numbers (white circles, dashed lines) were correlated with only soil C:N ratio ($R^2 = 0.63$, $p < 0.01$) but not pH ($p = 0.81$).



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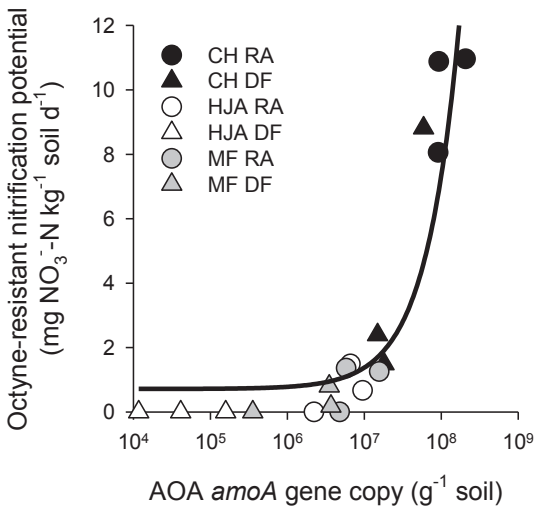


**Fig. 7.** The rate of octyne-resistant (AOA) nitrification potential is strongly correlated with AOA *amoA* gene copy numbers. Soils are identified by site (CH—Cascade Head, black; HJA—H.J. Andrews, white; MF—McDonald Forest, gray) and tree type (DF—Douglas-fir, triangle; RA—red alder, circle). Line represents exponential regression ($R^2 = 0.80$, $p < 0.01$).

nitrification rate and soil C:N ratio, with nitrification rate sharply increasing below a threshold of C:N around 20. This finding is consistent with earlier findings (Aber et al., 2003; Bengtsson et al., 2003; Ross et al., 2004), and a similar trend has been reported in several European sites with a threshold of C:N ratio 24–27 (Emmett et al., 1998; Gundersen et al., 1998).

Regression analysis showed soil pH to have less of an influence on the abundance of ammonia oxidizers than soil C:N ratio, which suggests that production of $NH_4^+$ may be a greater determinant of the carrying capacity of ammonia oxidizers than soil acidity. This is clearest for AOA, which were present in highest abundance in the soil with the lowest pH but also lowest C:N ratio (Cascade Head), consistent with the idea that AOA activity may be tightly linked to organic N mineralization (Levičnik-Höfferle et al., 2012). Ammonia-oxidizing bacteria, on the other hand, were most dominant in the soils of the highest pH but intermediate C:N ratio (McDonald Forest), consistent with the generalization that AOB are less tolerant of acidity than AOA (Gubry-Rangin, 2011; Hu et al., 2014; Zhang et al., 2012).

### 4.2. Relative contribution of AOA and AOB to nitrification

Although we have evidence to suggest that 1-octyne is a potentially selective inhibitor of AOB activity in agricultural soils (Taylor et al., 2013), that concept was not tested on acidic forest soils. In this current study, we showed that a positive relationship exists between AOA *amoA* gene copy abundance and octyne-resistant nitrifying activity in both slurries (Fig. 7) and whole soil assays (data not shown). Based on the slope of this relationship, an $NH_3$ oxidation rate of approximately $2 \times 10^{-16}$ mol cell$^{-1}$ h$^{-1}$ could be calculated. To place this value into context, the per-cell rate of $NH_3$ oxidation measured in the marine AOA, *Nitrosopumilus maritimus*, ranges between 2.5 and $3.5 \times 10^{-16}$ mol cell$^{-1}$ h$^{-1}$ (Könneke et al., 2005). From published data we calculated a per-cell rate of $1–2 \times 10^{-16}$ mol cell$^{-1}$ h$^{-1}$ for the soil AOA *Nitrososphaera viennensis* grown at circumneutral pH (Tourna et al., 2011); however, per-cell rates calculated from two different data sets for the acidophilic soil AOA, *Nitrosotalea devanaterra* (grown at pH 4.5), provided somewhat lower estimates (~$0.2–1 \times 10^{-16}$ mol cell$^{-1}$ h$^{-1}$; Lehtovirta-Morley et al., 2011, 2013). Clearly, the qPCR based estimates of AOA populations found in the Oregon acidic forest soils could account for the octyne-resistant activity measured, suggesting that most of the AOA in these acidic forest soils were active. By contrast, no significant correlation was found between AOB abundance and octyne-sensitive activity, perhaps because not all measured AOB were physiologically active at the low pH of these soils, that the proportion of those that were active varied among the soils, or that some other type octyne-sensitive microorganisms may contribute to the measured activity. Despite these caveats, the per-cell rate for AOB obtained from averaging across all soils is about $6 \times 10^{-15}$ mol cell$^{-1}$ h$^{-1}$, which is similar to that calculated for soil AOB (Jiang and Bakken, 1999; Taylor and Bottomley, 2006).

Measuring *amoA* gene expression might be a complementary, or even alternative, approach to the selective inhibitor method that we used. For example, both potential $NH_3$-oxidizing activity and *amoA* mRNA increased following the resuscitation of starved cultures of an AOB, but the authors cautioned against making direct correlations because *amoA* mRNA persisted during starvation (Bollmann et al., 2005). In soil, AOA and AOB *amoA* transcripts were observed to increase following wetting of dried soils, however, $NH_3$-oxidizing activities of the two groups were not measured (Placella and Firestone, 2013). In the future, a comparison of AOA and AOB $NH_3$-oxidizing activity and their expression of *amo*A genes would be worthwhile as it is important to determine whether there is a direct relationship between transcript numbers and activity under a range of soil and environmental conditions.

### 4.3. Niche differentiation

Although considerable evidence now suggests that AOA oxidize $NH_3$ in acidic soils (Nicol et al., 2008; Gubry-Rangin et al., 2010; Stopnisek et al., 2010; Yao et al., 2011; Zhang et al., 2011), it remains a fact that in all of these studies, except one (Stopnisek et al., 2010), the soils also contained populations of AOB. In our study we detected AOB in all soils, and found that $NH_4^+$-stimulated activity was octyne sensitive in all soils except red alder at Cascade Head. As mentioned in the introduction, various hypotheses have been put forward through the years to invoke why generally acid-sensitive AOB might persist and can be locally active in acidic forest soils, including protective microsites. In this context, octyne-sensitive nitrification rates in soils from McDonald Forest and the H.J. Andrews were lower in disaggregated soil slurries than in whole soils amended with the equivalent amount of $NH_4^+$.

By contrast, Cascade Head soils, particularly under red alder, were of lower pH and where dominated by AOA activity. There is precedent in the literature for small pH differences among acidic soils affecting AOA composition. Yao et al. (2011) in a survey of about 20 acidic tea soils from China reported that some types of AOA *amoA* sequences were restricted to soils of pH < 4.4, whereas others were found only in soils with pH > 5. In a survey of AOA phylotypes among a large number of soils Gubry-Rangin et al. (2011) separated the soils above and below pH 5, and found some AOA types restricted to soils of pH < 5. Furthermore, Lehtovirta-Morley et al. (2011) showed that $NH_3$ oxidation by the acidophilic AOA *Nitrosotalea devanaterra* was optimum between pH 4–5, and dropped off sharply above pH 5, implying that this type of AOA might prefer to occupy soil of pH < 5. Boyle-Yarwood et al. (2008) found that AOA communities were different in soils under red alder and Douglas-fir at Cascade Head also supporting the hypothesis that small difference in the level of soil acidity might be sufficient to invoke these shifts. Nonetheless, a tree type effect that is independent of pH cannot be ruled out in that study. It is possible that AOA phylotypes exist in the acidic forest soils that are more sensitive to inhibition by octyne, and whose contribution to soil nitrifying activity is affected by pH.

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Interestingly, although AOA are known to have a high affinity for $NH_3$, addition of high concentrations of $NH_3$ stimulated both octyne-resistant and octyne-sensitive nitrification, suggesting that both AOA and AOB were substrate limited in these forest soils. The response of AOB to added $NH_4^+$ is common, but not all studies have found AOA to be responsive (Verhamme et al., 2011; Levičnik-Höfferle et al., 2012; Taylor et al., 2013). The positive response to $NH_3$ additions in these forest soils is likely related to their low pH and accompanying lower soil solution $NH_3$ concentration, 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).

## 5. Summary

The distribution and relative contribution of AOA and AOB to nitrification in forest soils remains a topic of debate. This study demonstrated that soil pH, substrate concentration, and C:N ratio are all key factors that differentiate niches occupied by AOA and AOB. The activity of both AOA and AOB responded the addition of substrate but AOA activity was more closely linked to N availability, as indicated by soil C:N ratio, whereas AOB activity was affected more by soil pH.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.02.034.

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