Environmental Microbiology (2008) **10**(11), 2956–2965 doi:10.1111/j.1462-2920.2008.01600.x

Community composition of ammonia-oxidizing bacteria and archaea in soils under stands of red alder and Douglas fir in Oregon

Stephanie A. Boyle-Yarwood,[1]* Peter J. Bottomley[1,2] and David D. Myrold[1]
Departments of [1]Crop and Soil Science and [2]Microbiology, Oregon State University, Corvallis, OR, USA.

Summary

This study determined nitrification activity and nitrifier community composition in soils under stands of red alder (*Alnus rubra*) and Douglas fir (*Pseudotsuga menziesii*) at two sites in Oregon. The H.J. Andrews Experimental Forest, located in the Cascade Mountains of Oregon, has low net N mineralization and gross nitrification rates. Cascade Head Experimental Forest, in the Coast Range, has higher net N mineralization and nitrification rates and soil pH is lower. Communities of putative bacterial [ammonia-oxidizing bacteria (AOB)] and archaeal [ammonia-oxidizing archaea (AOA)] ammonia oxidizers were examined by targeting the gene *amoA*, which codes for subunit A of ammonia monooxygenase. Nitrification potential was significantly higher in red alder compared with Douglas-fir soil and greater at Cascade Head than H.J. Andrews. Ammonia-oxidizing bacteria *amoA* genes were amplified from all soils, but AOA *amoA* genes could only be amplified at Cascade Head. Gene copy numbers of AOB and AOA *amoA* were similar at Cascade Head regardless of tree type ($2.3–6.0 \times 10^6$ *amoA* gene copies g^{-1} of soil). DNA sequences of *amoA* revealed that AOB were members of *Nitrosospira* clusters 1, 2 and 4. Ammonia-oxidizing bacteria community composition, determined by terminal restriction fragment length polymorphism (T-RFLP) profiles, varied among sites and between tree types. Many of the AOA *amoA* sequences clustered with environmental clones previously obtained from soil; however, several sequences were more similar to clones previously recovered from marine and estuarine sediments. As with AOB, the AOA community composition differed between red alder and Douglas-fir soils.

Received 23 April, 2007; accepted 29 January, 2008. *For correspondence. E-mail stephanie.yarwood@oregonstate.edu; Tel. (+1) 541 737 1840; Fax (+1) 541 737 0496.

Introduction

Nitrogen availability and turnover vary considerably among different forest types within the Pacific Northwest (Hart *et al*., 1997; Leckie *et al*., 2004; Grayston and Prescott, 2005), and high rates of nitrification have been observed in forested sites where net primary productivity is high and in soils associated with red alder (Hart *et al*., 1997). Although the existence of acetylene-sensitive nitrification and the presence of ammonia-oxidizing bacteria (AOB) in soil at pH < 5 has led to speculation that AOB may be the primary nitrifiers in acidic soils (De Boer and Kowalchuk, 2001; Gieseke *et al*., 2006), questions remain about the ability of AOB to nitrify under these conditions because no cultured isolates can nitrify at pH < 5.

During the last few years, crenarchaeota possessing putative *amoA* genes have been identified in marine and terrestrial environments (Könneke *et al*., 2005; Treusch *et al*., 2005; Leininger *et al*., 2006). Detection and quantification of *amoA* RNA transcripts (Leininger *et al*., 2006) and an increase in transcript number following NH_4^+ additions (Treusch *et al*., 2005) suggest that ammonia-oxidizing archaea (AOA) may function in at least some agricultural and grassland soils (Nicol and Schleper, 2006). Investigations in other soil types are needed to determine if AOA are ubiquitously distributed in soils and if their composition and activities are influenced by soil factors and plant community composition.

In the case of AOB, *Nitrosospira* clusters 2, 3 and 4 have been identified in acidic forest soils (Laverman *et al*., 2001; Mintie *et al*., 2003; Compton *et al*., 2004; Nugroho *et al*., 2005; 2006) and research has linked nitrifier community composition to factors such as vegetation type (Nugroho *et al*., 2005), microclimate (Mintie *et al*., 2003), disturbance (Yeager *et al*., 2005) and temperature (Avrahami *et al*., 2003; Avrahami and Conrad, 2005). The links between AOB diversity and function remain unclear, however (Kowalchuk *et al*., 2000; Laverman *et al*., 2001; Webster *et al*., 2002; 2005; Horz *et al*., 2004; Chu *et al*., 2007). Bottomley and colleagues (2004) found that even when soil was reciprocally transferred from forest to

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd

Table 1. Nitrification potentials.

Site/tree type	–Acetylene (mg of N kg^{-1} of soil day^{-1})	+Acetylene (mg of N kg^{-1} of soil day^{-1})
Cascade Head		
Douglas fir	1.68 ± 0.75	–0.38 ± 0.02
Red alder	3.69 ± 0.57	0.46 ± 0.13
H.J. Andrews		
Douglas fir	0.14 ± 0.10	0.04 ± 0.00
Red alder	1.62 ± 0.18	–0.05 ± 0.03
P-value		
Site	< 0.001	NS
Tree	0.056	NS

Mean ± standard error ($n = 3$). Statistical significance for main effects of site and tree species is shown as a *P*-value (NS = $P > 0.10$); site-by-tree interactions were not significant ($P < 0.05$).

meadow environments and nitrification and AOB populations increased, *amoA* composition did not significantly vary after 2 years. In contrast, Horz and colleagues (2004) reported increased nitrification and a community shift towards *Nitrosospira* cluster 2 following fertilization experiments. The question remains to what extent tree type or ecosystem net primary productivity may influence the population sizes and composition of AOB in forest soils.

A study was designed to compare the AOB and AOA community compositions in soils under different tree species and that express different rates of nitrification. Two experimental tree plantations containing pure stands of Douglas fir (*Pseudotsuga menziesii*) and red alder (*Alnus rubra*) were selected for these experiments. Previous research had indicated differences in gross and net nitrification rates between vegetation types at each site (Boyle *et al*., 2008). The soil at Cascade Head Experimental Forest had gross nitrification rates that were threefold higher than in soils at the H.J. Andrews Experimental Forest. The objectives of the current study were to: (i) measure nitrification potential in soils under differing vegetation types, and (ii) quantify and characterize the AOB and AOA communities and examine correlations between composition and function.

Results

Nitrification potential

Nitrification potential varied significantly between sites and tree types (Table 1). Cascade Head soils averaged 2.69 mg of N kg^{-1} of soil day^{-1} compared with H.J. Andrews soils, which averaged 0.88 mg of N kg^{-1} of soil day^{-1}. Nitrification potential was 2- and 12-fold greater in red alder compared with Douglas-fir soils at Cascade Head and the H.J. Andrews respectively. When acetylene was added to nitrification slurries, NO_3^- did not accumulate in H.J. Andrews soils or in soils from under Douglas fir at Cascade Head. A residual rate of acetylene-insensitive nitrification remained in red alder soil from Cascade Head that was approximately 12% of the control rate (Table 1). At the end of the incubation, pH values of the soil slurries remained similar to those measured in the soils initially (4.6–5.3 at Cascade Head and 5.2–5.8 at H.J. Andrews soils).

Quantitative PCR determination of amoA *gene copy numbers*

Ammonia-oxidizing bacteria *amoA* copy numbers were significantly higher in red alder soils from H.J. Andrews than red alder soils from Cascade Head; at H.J. Andrews copy numbers were significantly higher in soil under red alder compared with Douglas fir (Fig. 1). Ammonia-oxidizing archaea *amoA* copy numbers were below detection limits (5×10^4 copies g^{-1} of soil) in H.J. Andrews soils. At Cascade Head AOA *amoA* copies were higher in Douglas-fir soil compared with red alder soil ($P = 0.07$). The ratios of AOA:AOB copy numbers in Cascade Head Douglas-fir and red alder soils were 1.80 and 0.42 respectively (Fig. 1). Ammonia-oxidizing bacteria *amoA* copy numbers were higher in red alder soils compared with AOA copy numbers ($P = 0.10$). Copy numbers did not correlate with nitrification potential for either gene.

Terminal restriction fragment length polymorphism (T-RFLP) profiles and terminal restriction fragment abundance

Restriction patterns of PCR-amplified AOB *amoA* sequences generated with TaqI indicated that all AOB were members of the genus *Nitrosospira*; all patterns

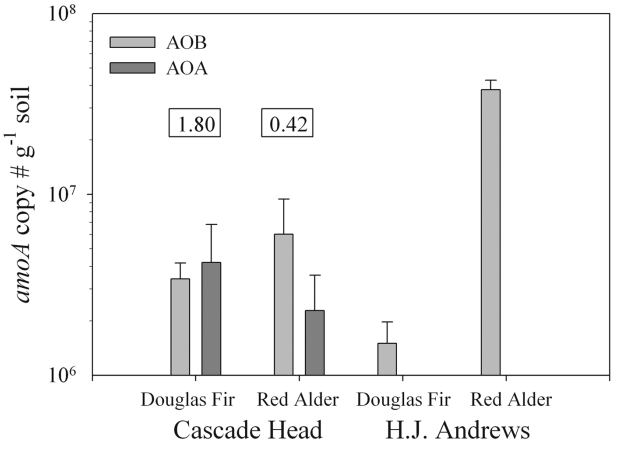

Fig. 1. *amoA* gene copy numbers for AOB and AOA. Bars represent the mean of three field replicates with SE ($n = 3$). The ratio of AOA:AOB is given above the bars for Cascade Head. Ammonia-oxidizing archaea copy numbers at H.J. Andrews were below a detection limit of 5×10^4 copies g^{-1} of soil.

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

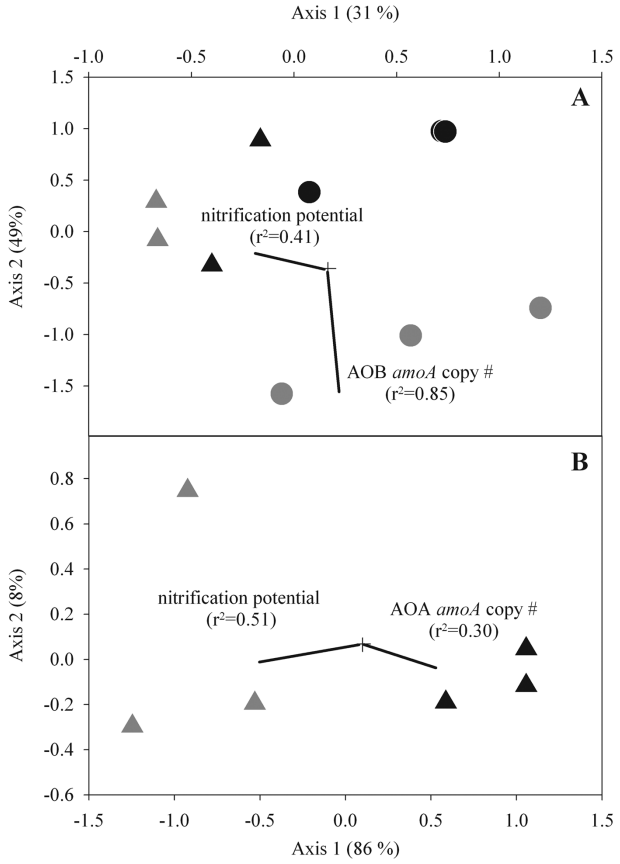

Fig. 2. Non-metric multidimensional scaling (NMS) ordination of T-RFLP profiles for AOB *amoA* (A) and AOA *amoA* communities (B). Cascade Head (triangles), H.J. Andrews (circles), Douglas fir (black symbols) and red alder (grey symbols). There was no amplification of AOA *amoA* in H.J. Andrews soils. Vectors show correlation with nitrification potentials and ammonia oxidizer populations.

contained a single peak at 283 bp (Horz *et al.*, 2000). Community composition determined by AOB *amoA* terminal restriction fragment length polymorphism (T-RFLP) profiles using AluI and CfoI varied by site ($P = 0.02$, *A*-statistic = 0.12) and tree type ($P = 0.03$, *A*-statistic = 0.11) (Fig. 2A). Ammonia-oxidizing bacteria *amoA* community composition correlated with nitrification potential ($r^2 = 0.41$) and AOB *amoA* copies g^{-1} of soil ($r^2 = 0.85$). Indicator species analysis identified specific terminal restriction fragments (T-RFs) that varied based on site and tree type. There were several T-RFs that differed significantly between sites, including AluI 490. AluI 490 was significantly higher in Cascade Head soils than H.J. Andrews soils (Fig. 3A). In the case of tree type CfoI 95 was different ($P = 0.06$) with a higher percentage of total fluorescence in red alder compared with Douglas-fir soils (Fig. 3A).

Archaeal *amoA* was amplified from all Cascade Head plots, but was not amplified from any H.J. Andrews plot. Terminal restriction fragment length polymorphism profiles for AOA *amoA* were significantly different between the two tree types ($P = 0.022$, *A*-statistic = 0.43) and correlated with nitrification potential ($r^2 = 0.51$) and AOA *amoA* copies g^{-1} of soil ($r^2 = 0.30$). Indicator species analysis did not identify specific T-RFs that contributed to tree type differences at a *P*-value < 0.05, but several T-RFs were significant at $P = 0.10$, including MspI 442 and RsaI 292. The per cent fluorescence of MspI 442 was greater in red alder soil, whereas RsaI 292 was greater in Douglas-fir soil (Fig. 3B).

AOB and AOA sequences

Ammonia-oxidizing bacteria clones were screened for those T-RFs that contributed to site and tree type differences. Phylogenetic analysis revealed that most sequences belonged to *Nitrosospira* clusters 2 and 4 (Avrahami *et al.*, 2002), with two clones A-36 (AluI 490-CfoI 136) and B-22 (AluI 393-CfoI 167) belonging to cluster 1 (Fig. 4). Of the remaining clones, B-18 (AluI 389-CfoI 167) and B-44 (AluI 167-CfoI 167) were closely related to *Nitrosospira* CT2F (Mintie *et al.*, 2003), a cluster 4 isolate from a higher elevation conifer soil in the H.J. Andrews. Clones C-3 (AluI 490-CfoI 95), C-18 (AluI 490-

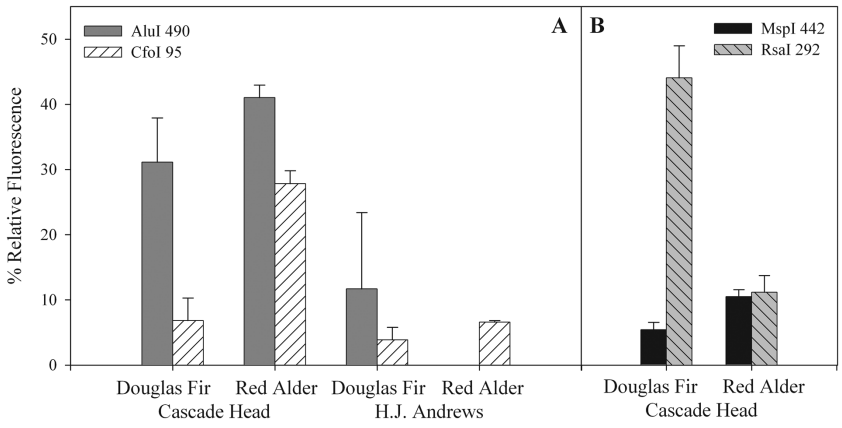

Fig. 3. Relative abundance of selected T-RFs for AOB *amoA* profiles (A) and AOA *amoA* profiles (B). Bars represent the mean of three replicates with SE.

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

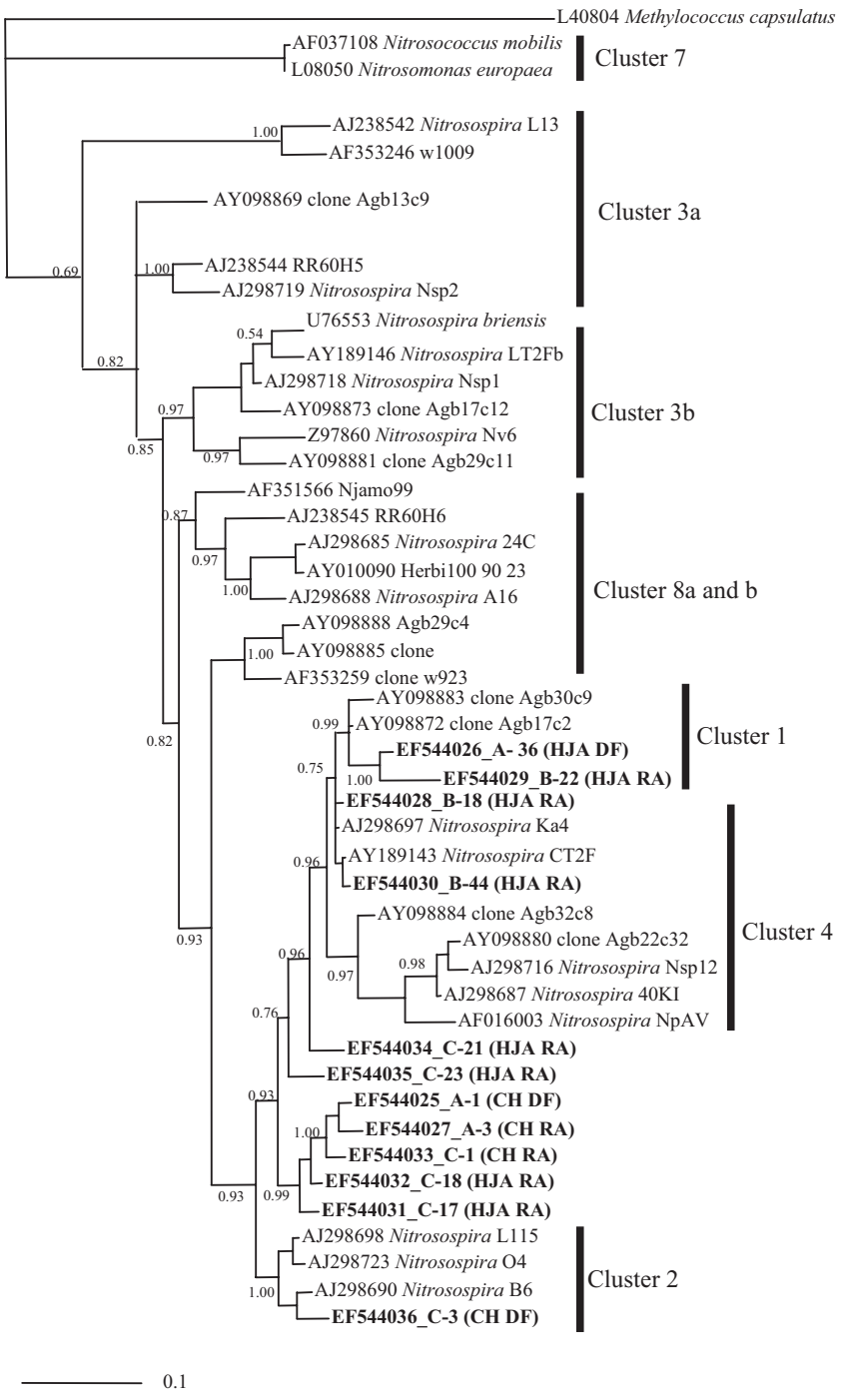

Fig. 4. Bacterial *amoA* tree constructed using Bayesian analysis. Numbers show the probability of clade assignments. Cluster assignments are made based on Avrahami and colleagues (2002). Environmental clones from this study appear in bold with the site, Cascade Head (CH) or H.J. Andrews (HJA), and tree type, Douglas fir (DF) or red alder (RA), appearing in parentheses.

CfoI 95) and C-23 (AluI 393-CfoI 95) all contained cut site CfoI 95, the dominant T-RF in red alder soils (Figs 3A and 4).

Twenty archaeal *amoA* sequences were used in a phylogenetic analysis. At 98% similarity the clone library contained 15 unique operational taxonomic units (OTUs). Clones clustered into four main clades. Clones 12C-1, 4B-2, 4C-2, 5C-1 and 9H-1 (CfoI 261-MspI 559-RsaI 292) were 99% similar and grouped closely with other environmental clones from soil (Fig. 5). Clones 12C-2, 4A-1 and 7F-1 (CfoI 559-MspI 258-RsaI 559) were 98% similar and also grouped with soil clones. The remaining AOA sequences including clone 12F-2 (CfoI 559-MspI 442-RsaI 203) were more divergent and tended to cluster with clones recovered from estuarine sediments, marine and wastewater treatment plants (Francis *et al.*, 2005) (Fig. 5).

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

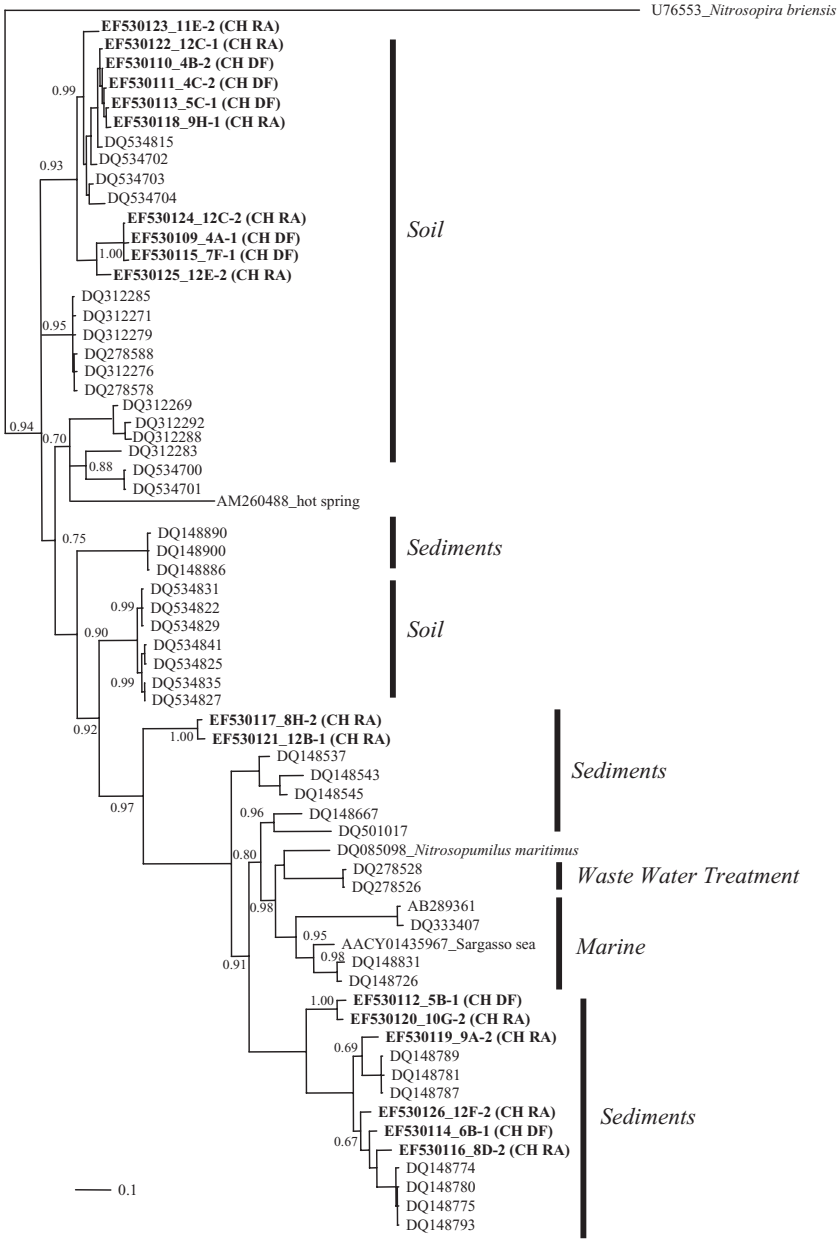

Fig. 5. Archaeal *amoA* tree constructed using Bayesian analysis. Numbers show the probability of clade assignments. Environmental clones from this study appear in bold with the site Cascade Head (CH) and tree type, Douglas fir (DF) or red alder (RA), appearing in parentheses.

Discussion

The presence of red alder in conifer-dominated ecosystems has been shown to increase both gross and net nitrification in soil (Binkley *et al*., 1992; Hart *et al*., 1997; Boyle *et al*., 2008), and reduce soil pH (Rhoades and Binkley, 1992). Data from both experimental sites showed greater nitrification potential in red alder compared with Douglas-fir soils, although the nitrification potential increased to a greater extent under red alder at H.J. Andrews, where red alder may have contributed to a proportionately greater increase in available N. The nitrification potential rates measured in this study, using soil slurries, were similar to the rates of gross nitrification measured in whole soil by Boyle and colleagues (2008) ($r^2 = 0.50$) (Table 1), suggesting that at the time of sampling nitrification conditions may have been close to optimum even at the low pH of whole soil and nitrification potential soil slurries.

Based on pure culture studies of AOB that determined rates of NH_3 oxidation to be between 1 and 10×10^{-15} mol cell^{-1} h^{-1} (Belser and Schmidt, 1978; Jiang and Bakken, 1999), the nitrification potentials measured in these soils could be supported by AOB population densities as low as $0.4–4.0 \times 10^5$ cells g^{-1} of soil in H.J. Andrews Douglas-fir soil and as high as $0.1–1.1 \times 10^{-7}$ cells g^{-1} of soil in

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

Cascade Head red alder soil. If we assume three copies of *amoA* cell^{-1} (J. Norton, unpublished), these population values fall within estimates made by quantitative PCR (Q-PCR) (Fig. 1) and also agree with values reported elsewhere (Okano *et al*., 2004; Leininger *et al*., 2006). In Douglas-fir soils, differences in gene copy numbers between sites correlated with nitrification potential (Table 1, Fig. 1), but no correlation was found for red alder soil. Furthermore, in Cascade Head red alder soils, the population densities (2.0×10^6 cells g^{-1} of soil) were at the lower limit of the range that we predicted based on nitrification potentials, suggesting that if AOB are solely responsible for nitrification in these soils they are oxidizing NH_3 close to the highest rate documented for pure cultures (10×10^{-15} mol cell^{-1} h^{-1}). Although it is possible to account for all nitrification activity with measured AOB populations these findings provide circumstantial evidence that another population of organisms may be contributing to NH_3 oxidation.

The AOA : AOB ratios measured at Cascade Head (Fig. 2) tended to be lower (1.8 and 0.42) than those reported previously in European soils (1.5–232) (Leininger *et al*., 2006), perhaps because the soils at Cascade Head are forested, more acidic (3.6–4.1), and contain higher concentrations of organic nitrogen (Boyle *et al*., 2008). To the authors' knowledge there are no published reports of rates of NH_3 oxidation per cell by cultures of AOA. Using data presented by Könneke and colleagues (2005) we calculated that the rate of NH_3 oxidation by *Nitrosopumilus maritimus* ranges between 0.25 and 0.35×10^{-15} mol cell^{-1} h^{-1}, if we assume that soil AOA possess one copy of *amoA* cell^{-1} like *N. maritimus* (Stahl and Richardson, 2007). The AOA population density estimates made at Cascade Head of approximately 10^7 cells g^{-1} of soil could support the nitrification potentials measured at the site. Further research is needed to determine the relative contribution of AOA and AOB to nitrification in these soils.

Ammonia-oxidizing bacteria composition determined by T-RFLP profiles varied by site and tree type, suggesting that complex changes brought about by differing climate, soil and vegetation types affected the diversity of AOB. Terminal restriction fragment length polymorphism profiles also correlated with AOB copy number, but correlated to a lesser extent with nitrification potential. These results could be due to T-RFLP profiles being based on total AOB community DNA, which encompasses both active and inactive members of the oxidizer community. Terminal restriction fragment CfoI 95 was significantly higher in red alder compared with Douglas-fir soils (Fig. 3), and at least one clone (C-3) that contained CfoI 95 appeared to belong to *Nitrosospira* cluster 2. Previously, researchers identified *Nitrosospira* cluster 2 in chronic N-amendment plots (Compton *et al*., 2004) and acidic Scots pine forests (Nugroho *et al*., 2005). Although Nugroho and colleagues (2007) suggested that cluster 2 may survive in low pH soils through urea-hydrolysis, urease is not unique to *Nitrosospira* cluster 2 and is widely, albeit non-uniformly distributed among AOB (Koops and Pommerening-Röser, 2001; Koops *et al*., 2003). Further work is needed to understand why *Nitrosospira* cluster 2 thrives in this acidic, high N environment. It is also worth mentioning that two of the clones recovered from the H.J. Andrews also grouped with *Nitrosospira* cluster 1, a group found predominately in marine systems. Because no isolates exist for *Nitrosospira* cluster 1, their role in NH_3 oxidation remains speculative.

We believe our data are the first to show the presence of AOA *amoA* in forest soils under both coniferous and deciduous tree species. Archaeal *amoA* did not amplify even after several attempts in H.J. Andrews soils with different primer sets (Francis *et al*., 2005; Treusch *et al*., 2005) and under different conditions, suggesting that AOA may exist at levels below our current amplification ability. Alternatively, if present, AOA in H.J. Andrews soils may be sufficiently different so as not to amplify with current primers. Given that we were able to recover sequences from Cascade Head that grouped with a variety of other known sequences, H.J. Andrew AOA could be significantly different from those in the current public database. Ammonia-oxidizing archaea *amoA* consistently amplified in all Cascade Head soils, and T-RFLP profiles from putative AOA *amoA* genes at Cascade Head differed by tree type (Fig. 2B), suggesting that AOA community composition, like AOB composition, may be impacted by changes brought about by the presence of red alder. Terminal restriction fragment RsaI 292 was higher in Douglas-fir soil (Fig. 3B) and was found in a group of clones that clustered with other AOA *amoA* soil clones, whereas MspI 442 was greater in red alder soil and was observed in sequences that clustered with sediments. As more sequences of AOA *amoA* are collected and made available, it will be interesting to see if AOA sequences continue to cluster based on environment or if other factors such as vegetation type or N availability will shed new light on the factors influencing their community composition and activity. Our unpublished findings also indicated that archaeal communities based on 16S rRNA sequences differed between Cascade Head and H.J. Andrews soils and between tree types. Other research has also shown that archaeal populations may vary between soil types and plant communities (Jurgens *et al*., 1997; Ochsenreiter *et al*., 2003; Nicol *et al*., 2007). Further research is needed to determine if differences in AOA distribution, population size and community composition contribute to the increased rates of NH_3 oxidation that we observed in soils at Cascade Head and in the presence of red alder.

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

Experimental procedures

Site description and sample collection

Soils were sampled in two experimental tree plantations where trees were approximately 20 years old (Radosevich *et al*., 2006). Plots measured 27 m × 27 m and contained either pure stands of Douglas fir or red alder. The first site was located in Cascade Head Experimental Forest, 1.6 km from the Pacific Ocean, at an elevation of 330 m. The weathering of basaltic headlands has created a Histic Epiaquand (Rhoades and Binkley, 1992) with high soil fertility and pH 4. The second site was located in the H.J. Andrews Experimental Forest in the Cascade Mountains at an elevation of 800 m. The soil is best classified as a Haplumbrept (Dyrness, 2005) with pH 5. Concentrations of soil N, NH_4^+ and NO_3^- are significantly lower at H.J. Andrews in comparison with Cascade Head (Boyle *et al*., 2008). Soil NO_3^- concentrations averaged 6.2 mg kg^{-1} of soil at Cascade Head and 1.8 mg kg^{-1} of soil at H.J. Andrews. Red alder soils had significantly higher NO_3^- concentrations (Boyle *et al*., 2008), with the highest NO_3^- concentrations in Cascade Head red alder soil (8.0 mg kg^{-1} of soil) and the lowest in H.J. Andrews Douglas-fir soil (0.3 mg kg^{-1} of soil). Soil samples were collected in the spring of 2006, 1 month after red alder leaves emerged. Ten soil cores (3 cm × 10 cm) were taken from each of three replicate plots per treatment. Five cores were placed into each of two bags per plot and homogenized. Samples were transported on ice to the lab, where 1 g of samples were removed from each bag for DNA extraction. The remaining soil from each plot was homogenized and used in nitrification potential assays.

Nitrification potential assay

Nitrification potentials were determined on fresh soils using a shaken soil-slurry method (Hart *et al*., 1994). After sieving (4 mm), portions of soil (15 g of dry-weight equivalent) from each plot were extracted with 135 ml of 1.0 mM potassium phosphate buffer (pH 7.2) in order to remove background NO_3^-. The slurries were then centrifuged (6000 *g*; 10 min) to pellet soil particles and the buffer was decanted. The soil pellet was re-suspended in 135 ml of 1.0 mM phosphate buffer (pH 7.2) containing 1.5 mM NH_4^+. Aliquots of the soil suspension (20 ml) were placed into six, 160 ml serum vials, taking care to keep the slurry well mixed. The remaining slurry was centrifuged to pellet soil particles and filtered (2 μm). This filtrate was used to establish initial NO_3^- concentrations. The six serum vials were crimp-sealed using butyl rubber stoppers and acetylene was added to three of the vials (10.8 kPa). All vials were shaken at 25°C for 30 h, centrifuged to remove soil particles, and filtered (2 μm). Concentrations of NO_3^- in the filtrates were measured using an autoanalyser (Astoria Pacific, Portland, OR). The pH of the soil slurries was measured at the end of the incubation.

DNA extractions and PCR amplification

DNA was extracted from soil (0.25 g) using an MOBio PowerSoil™ DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions, with the modification that a Bio101 FastPrep™ instrument was used to lyse cells (MP Biomedicals, Solon, OH). The MOBio bead beating tubes were shaken for 45 s using the FastPrep instrument. Extracts were quantified using a NanoDrop™ ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and diluted to 25 ng μl^{-1}. Two extracts from each plot were used as a composite template by combining 25 μl of each 25 ng μl^{-1} dilution.

Approximately 100 ng of DNA was used in each conventional PCR reaction. Ammonia-oxidizing bacteria *amoA* genes were amplified using primers *amoA*-1F (5′-GGG GGT TTC TAC TGG TGG T) and *amoA*-2R (5′-CCC CTC KGS AAA GCC TTC TTC) (Horz *et al*., 2000), under conditions described previously (Mintie *et al*., 2003) using a PTC-100 Programmable Thermocycler (MJ Research, Watertown, MA). Archaeal *amoA* was amplified using primers *amo*111F (5′-TTY TAY ACH GAY TGG GCH TGG ACA TC) and *amo*643R (5′-TCC CAC TTW GAC CAR GCG GCC ATC CA) (Treusch *et al*., 2005) in a 50 μl reaction mix containing: 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μ Primer, 0.064% BSA. Archaeal *amoA* was amplified using the following conditions: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, ending with an extension step of 72°C for 7 min. Additional PCR conditions were tested with DNA extracts from H.J. Andrews in an attempt to achieve amplification. A DNA Engine™ thermocycler (Bio-Rad Laboratories, Hercules, CA) with a gradient block was used to test annealing temperatures from 45°C to 55°C and cycle numbers of 30, 35 and 40. Although these less stringent conditions resulted in non-specific amplification in Cascade Head soils, no amplification of the correct size fragment was observed in H.J. Andrews soils.

T-RFLP and Q-PCR

Terminal restriction fragment length polymorphism profiles were generated using primers *amoA*-1F (Horz *et al*., 2000; Mintie *et al*., 2003) and *amo*111F (Treusch *et al*., 2005) labelled with a 6-FAM fluorophore. The resulting products were cleaned using a Qiaquick™ PCR Purification kit (Qiagen, Valencia, CA) and restricted. Ammonia-oxidizing bacteria PCR products were restricted using AluI, CfoI and TaqI, and AOA PCR products were restricted with CfoI, MspI and RsaI. Restriction products were column-purified and profiles were analysed by the Oregon State University Center of Genome Research and Biocomputing using an ABI 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Ammonia-oxidizing bacteria and AOA *amoA* copy numbers were determined using methods described previously (Leininger *et al*., 2006). Briefly, primers *amoA*-1F and *amoA*-2R in combination with the Brilliant SYBR Green™ Q-PCR Core Reagent Kit (Stratagene, La Jolla, CA) were used in AOB quantification and primers *amo*196F and *amo*277R with the probe *amo*247 labelled with 6-FAM (Treusch *et al*., 2005) were used in AOA quantification. All assays were run on an ABI 7500 Sequence detection system (Applied Biosystems, Foster City, CA). Each reaction contained 2 μl of a 1.25 ng μl^{-1} dilution and was run in triplicate. Genomic DNA from *Nitrosomonas europaea* at a range of 5.0×10^{-1} to 5.0×10^{-7} ng of DNA per reaction was used to generate a

© 2008 The Authors

standard curve for AOB assays. Plasmid DNA from an AOA environmental clone at a range of 5.0×10^{-1} to 5.0×10^{-5} ng of DNA per reaction was used to generate AOA standard curves. Standard curves from each run were analysed to verify r^2 values > 0.95 and efficiency values between 95% and 105%. Detection limits for AOB and AOA *amoA* Q-PCR assays were 3×10^3 copies g^{-1} of soil and 5×10^4 copies g^{-1} of soil respectively.

Cloning and sequencing

Clones were generated using a Topo TA cloning™ kit for sequencing (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Ammonia-oxidizing bacteria clones were screened by PCR amplification and T-RFLP profiles. A total of 30 AOA clones and 24 AOB clones were sequenced by the High Throughput Genomics Unit (Department of Genome Science, University of Washington, Seattle, WA).

Statistical and phylogenetic analysis

Univariate statistical analyses were performed using SAS 9.1 (SAS Institute, Cary, NC). Nitrification potential was analysed by two-way analysis of variance (ANOVA) (Ramsey and Schafer, 2002). Where interactions occurred, data were analysed separately and contrasts were used to test specific treatment differences. Linear regression analysis was also performed to determine if population size or composition correlated to nitrification activity. *P*-values ≤ 0.05 were considered significant.

Quantitative PCR data were analysed using ABI Sequence detection system software version 1.4 (Applied Biosystems, Foster City, CA) and T-RFLP profiles were analysed using GenoTyper version 3.7 (Applied Biosystems, Foster City, CA). Terminal restriction fragment length polymorphism profiles were further analysed according to methods described previously (Boyle *et al*., 2006) and compositional differences were investigated using PC-ORD Multivariate Analysis of Ecological Data version 5.0 (MjM software, Gleneden Beach, OR). Non-metric multidimensional scaling (NMS) was used to evaluate community compositional variation and multiresponse permutation procedures (MRPP) were used to determine differences between site and tree type (McCune and Grace, 2002).

DNA sequences were aligned using CLUSTALX version 1.81 (Thompson *et al*., 1997) and alignments were edited using Bioedit sequence alignment editor version 7.0.5 (Hall, 1999). All sequences were analysed using Mallard Version 1.02 (Ashelford *et al*., 2006) to ensure that no chimeras or other sequencing anomalies occurred. Phylogenetic trees were constructed using Mr Bayes Version 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and confirmed using Phylip Version 3.2 (Felsenstein, 1989). Mr Bayes was run using an omega variation model (M3), a codon model that calculates the likely rate of variation at each site. The model was run for 1 million generations to ensure convergence at a stable value (Hall, 2001). Terminal restriction cut sites were determined for each sequence using Bioedit alignment editor and confirmed using Webcutter 2.0 (Heiman, 1997).

Acknowledgements

Support was provided by grants from the USDA NRICGP, the National Science Foundation IGERT Program and the H.J. Andrews LTER fund. Thanks go to Dr Rockie Yarwood and Elizabeth Brewer for assistance in sample collection and preparation, to Oregon State University's Center for Genome Research and Biocomputing for use of Q-PCR facilities and genotyping analysis, and to the University of Washington High Throughput Genomic Sequencing Unit for sequence analysis.

References

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., and Weightman, A.J. (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl Environ Microbiol* **72:** 5734–5741.

Avrahami, S., and Conrad, R. (2005) Cold-temperature climate: a factor for selection of ammonia oxidizers in upland soil? *Can J Microbiol* **51:** 709–714.

Avrahami, S., Conrad, R., and Braker, G. (2002) Effect of soil ammonium concentration on N_2O release and on community structure of ammonia oxidizers and denitrifiers. *Appl Environ Microbiol* **68:** 5685–5692.

Avrahami, S., Liesack, W., and Conrad, R. (2003) Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. *Environ Microbiol* **5:** 691–705.

Belser, L.W., and Schmidt, E.L. (1978) Diversity in the amonia-oxidizing nitrifier population of a soil. *Appl Environ Microbiol* **36:** 584–588.

Binkley, D., Sollins, P., Bell, R., Sachs, D., and Myrold, D. (1992) Biogeochemistry of adjacent conifer and alder-conifer stands. *Ecology* **73:** 2022–2033.

Bottomley, P.J., Taylor, A.E., Boyle, S.A., McMahon, S.K., Rich, J.J., Cromack, K., Jr, and Myrold, D.D. (2004) Responses of nitrification and ammonia-oxidizing bacteria to reciprocal transfers of soil between adjacent coniferous forest and meadow vegetation in the Cascade Mountains of Oregon. *Microb Ecol* **48:** 500–508.

Boyle, S.A., Rich, J.J., Bottomley, P.J., Cromack, K., and Myrold, D.D. (2006) Reciprocal transfer effects on denitrifying community composition and activity at forest and meadow sites in the Cascade Mountains of Oregon. *Soil Biol Biochem* **38:** 870–878.

Boyle, S.A., Yarwood, R.R., Bottomley, P.J., and Myrold, D.D. (2008) Bacterial and fungal contributions to soil nitrogen cycling under Douglas-fir and red alder at two sites in Oregon. *Soil Biol Biochem* **40:** 443–451.

Chu, H., Fujii, T., Morimoto, S., Lin, X., Yagi, K., Hu, J., and Zhang, J. (2007) Community structure of ammonia-oxidizing bacteria under long-term application of mineral fertilizer and organic manure in sandy loam soil. *Appl Environ Microbiol* **73:** 485–491.

Compton, J.E., Watrud, L.S., Porteous, L.A., and Degrood, S. (2004) Response of soil microbial biomass and community composition to chronic nitrogen additions at Harvard Forest. *For Ecol Manage* **196:** 143–158.

De Boer, W., and Kowalchuk, G.A. (2001) Nitrification in acid

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

soils: micro-organisms and mechanisms. *Soil Biol Biochem* **33:** 853–866.

Dyrness, C. (2005) *Soil Descriptions and Data for Soil Profiles in the Andrews Experimental Forest, Selected Reference Stands, Research Natural Areas, and National Parks: Long-Term Ecological Research.* Corvallis, OR: Forest Science Data Bank: SP001. [WWW document]. URL http://www.fsl.orst.edu/lter/data/abstract.cfm?dbcode=SP001.

Felsenstein, J. (1989) *HYLIP – Phylogenetic Inference Package* (Version 3.2). *Cladistics* **5:** 164–166.

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., and Oakley, B.B. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* **41:** 14683–14688.

Gieseke, A., Tarre, S., Green, M., and de Beer, D. (2006) Nitrification in a biofilm at low pH values: role of *in situ* microenvironments and acid tolerance. *Appl Environ Microbiol* **72:** 4283–4292.

Grayston, S.J., and Prescott, C.E. (2005) Microbial communities in forest floors under four tree species in coastal British Columbia. *Soil Biol Biochem* **37:** 1157–1167.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41:** 95–98.

Hall, B.G. (2001) *Phylogenetic Trees Made Easy*. Sunderland, MA, USA: Sinauer Associates.

Hart, S.C., Stark, J.M., Davidson, E.A., and Firestone, M.K. (1994) Nitrogen mineralization, immobilization, and nitrification. In *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*. Weaver, R.W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A., and Wollum, A. (eds). Madison, NJ, USA: Soil Science Society of America, pp. 985–1018.

Hart, S.C., Binkley, D., and Perry, D.A. (1997) Influence of red alder on soil nitrogen transformations in two conifer forests of contrasting productivity. *Soil Biol Biochem* **29:** 1111–1123.

Heiman, M. (1997) *Webcutter 2.0*. [WWW document]. URL http://rna.lundberg.gu.se/cutter2/.

Horz, H.P., Rotthauwe, J.H., Lukow, T., and Liesack, W. (2000) Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. *J Microbiol Methods* **39:** 197–204.

Horz, H.P., Barbrook, A., Field, C.B., and Bohannan, B.J.M. (2004) Ammonia-oxidizing bacteria respond to multifactorial global change. *Proc Natl Acad Sci USA* **101:** 15136–15141.

Huelsenbeck, J.P., and Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17:** 754–755.

Jiang, Q.Q., and Bakken, L.R. (1999) Comparison of *Nitrosospira* strains isolated from terrestrial environments. *FEMS Microbiol Ecol* **30:** 171–186.

Jurgens, G., Lindstrom, K., and Sanno, A. (1997) Novel group within the kingdom *Crenarchaeota* from boreal forest soil. *Appl Environ Microbiol* **63:** 803–805.

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

Koops, H.-P., and Pommerening-Röser, A. (2001) Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. *FEMS Microbiol Ecol* **37:** 1–9.

Koops, H.-P., Purkhold, U., Pommerening-Röser, A., Timmermann, G., and Wagner, M. (2003) The lithotrophic ammonia-oxidizing bacteria. In *The Prokaryotes*. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds). New York, NY, USA: Springer-Verlag, pp. 778–811.

Kowalchuk, G.A., Stienstra, A.W., Heilig, G.H.J., Stephen, J.R., and Woldendorp, J.W. (2000) Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. *Environ Microbiol* **2:** 99–110.

Laverman, A.M., Speksnijder, A.G.C.L., Braster, M., Kowalchuk, G.A., Verhoef, H.A., and van Verseveld, H.W. (2001) Spatiotemporal stability of an ammonia-oxidizing community in a nitrogen-saturated forest soil. *Microb Ecol* **42:** 35–45.

Leckie, S.E., Prescott, C.E., Grayston, S.J., Neufeld, J.D., and Mohn, W.W. (2004) Characterization of humus microbial communities in adjacent forest types that differ in nitrogen availability. *Microb Ecol* **48:** 29–40.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., *et al.* (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442:** 806–809.

McCune, B., and Grace, J.B. (2002) *Analysis of Ecological Communities*. Gleneden Beach, OR, USA: MjM Software.

Mintie, A.T., Heichen, R.S., Cromack, K., Myrold, D.D., and Bottomley, P.J. (2003) Ammonia-oxidizing bacteria along meadow-to-forest transects in the Oregon Cascade Mountains. *Appl Environ Microbiol* **69:** 3129–3136.

Nicol, G.W., and Schleper, C. (2006) Ammonia-oxidising *Crenarchaeota*: important players in the nitrogen cycle? *Trends Microbiol* **14:** 207–212.

Nicol, G.W., Campbell, C.D., Chapman, S.J., and Prosser, J.I. (2007) Afforestation of moorland leads to changes in crenarchaeal community structure. *FEMS Microbiol Ecol* **60:** 51–59.

Nugroho, R.A., Röling, W.F.M., Laverman, A.M., Zoomer, H.R., and Verhoef, H.A. (2005) Presence of *Nitrosospira* cluster 2 bacteria corresponds to N transformation rates in nine acid Scots pine forest soils. *FEMS Microbiol Ecol* **53:** 473–481.

Nugroho, R.A., Röling, W.F.M., Laverman, A.M., and Verhoef, H.A. (2006) Net nitrification rate and presence of *Nitrosospira* cluster 2 in acid coniferous forest soils appear to be tree species specific. *Soil Biol Biochem* **38:** 1166–1171.

Nugroho, R.A., Röling, W.F.M., Laverman, A.M., and Verhoef, H.A. (2007) Low nitrification rates in acid Scots pine forest soils are due to pH-related factors. *Microb Ecol* **53:** 89–97.

Ochsenreiter, T., Selezi, D., Quaiser, A., Bonch-Osmolovskaya, L., and Schleper, C. (2003) Diversity and abundance of *Crenarchaeota* in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environ Microbiol* **5:** 787–797.

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

Okano, Y., Hristova, K.R., Leutenegger, C.M., Jackson, L.E., Denison, R.F., Gebeyesus, B., *et al.* (2004) Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl Environ Microbiol* **70:** 1008–1016.

Radosevich, S.R., Hibbs, D.E., and Ghersa, C.M. (2006) Effects of species mixtures on growth and stand development of Douglas-fir and red alder. *Can J For Res* **36:** 768–782.

Ramsey, F.L., and Schafer, D.W. (2002) The analysis of variance for Two-way Classification. In *The Statistical Sleuth*. Crockett, C., and Jenkins, J. (eds). Pacific Grove, CA, USA: Duxbury Thomson Learning, pp. 374–400.

Rhoades, C.C., and Binkley, D. (1992) Spatial extent of impact of red alder on soil chemistry of adjacent conifer stands. *Can J For Res* **22:** 1434–1437.

Ronquist, F., and Huelsenbeck, J.P. (2003) Mr. Bayes 3: Bayesian Phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Stahl, D., and Richardson, P. (2007) *DOE Joint Genome Institute: Nitrosopumilus maritimus SCMI.* [WWW document]. URL http://genome.ornl.gov/microbial/nmar.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997) The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24:** 4876–4882.

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

Webster, G., Embley, T.M., and Prosser, J.I. (2002) Grassland management regimens reduce small-scale heterogeneity and species diversity of β-Proteobacterial ammonia oxidizer populations. *Appl Environ Microbiol* **68:** 20–30.

Webster, G., Embley, T.M., Freitag, T.E., Smith, Z., and Prosser, J.I. (2005) Links between ammonia oxidizer species composition, functional diversity and nitrification kinetics in grassland soils. *Environ Microbiol* **7:** 676–684.

Yeager, C.M., Northup, D.E., Grow, C.C., Barns, S.M., and Kuske, C.R. (2005) Changes in nitrogen-fixing and ammonia-oxidizing bacterial communities in soil of a mixed conifer forest after wildfire. *Appl Environ Microbiol* **71:** 2713–2722.

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,