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Evidence for Different Contributions of Archaea and Bacteria to the Ammonia-Oxidizing Potential of Diverse Oregon Soils▽

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A method was developed to determine the contributions of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) to the nitrification potentials (NPs) of soils taken from forest, pasture, cropped, and fallowed (19 years) lands. Soil slurries were exposed to acetylene to irreversibly inactivate ammonia monooxygenase, and upon the removal of acetylene, the recovery of nitrification potential (RNP) was monitored in the presence and absence of bacterial or eukaryotic protein synthesis inhibitors. For unknown reasons, and despite measureable NPs, RNP did not occur consistently in forest soil samples; however, pasture, cropped, and fallowed soil RNPs commenced after lags that ranged from 12 to 30 h after acetylene removal. Cropped soil RNP was completely prevented by the bacterial protein synthesis inhibitor kanamycin (800 μg/ml), whereas a combination of kanamycin plus gentamicin (800 μg/ml each) only partially prevented the RNP (60%) of fallowed soils. Pasture soil RNP was completely insensitive to either kanamycin, gentamicin, or a combination of the two. Unlike cropped soil, pasture and fallowed soil RNPs occurred at both 30°C and 40°C and without supplemental NH_4^+ (≤10 μM NH_4^+ in solution), and pasture soil RNP demonstrated ~50% insensitivity to 100 μM allyl thiourea (ATU). In addition, fallowed and pasture soil RNPs were insensitive to the fungal inhibitors nystatin and azoxystrobin. This combination of properties suggests that neither fungi nor AOB contributed to pasture soil RNP and that AOA were responsible for the RNP of the pasture soils. Both AOA and AOB may contribute to RNP in fallowed soil, while RNP in cropped soils was dominated by AOB.

Until 2005 the majority of ammonia (NH_3) oxidation in natural environments was thought to be carried out by a phylogenetically distinct group of NH_3-oxidizing bacteria (AOB). This nitrification paradigm was challenged by reports that thaumarchaeota carry the genes that encode the enzyme ammonia monooxygenase (AMO) (55); the isolation of "*Candidatus* Nitrosopumilus maritimus" strain SCM1, an obligate autotrophic thaumarchaeal NH_3 oxidizer (35); the detection of archaeal *amoA* gene transcripts in soil (54, 55); and the realization that ammonia-oxidizing archaea (AOA) were widely distributed (19) and often more numerous than AOB in soil (36), marine (40), and hot spring (52, 57) ecosystems. Others have correlated either the AOA (7, 46, 58) or AOB *amoA* gene copy abundance (17, 32) with nitrification activity and detected the expression of AOA *amoA* mRNA (43, 60). To our knowledge there is no published work that has directly distinguished NH_3-oxidizing activity by AOA from NH_3-oxidizing activity by AOB in soils.

As yet, it is unknown what factors determine whether AOA or AOB nitrify in an environment. We hypothesized that AOA and AOB might contribute differentially to nitrification in diverse soils across a landscape with different physical and chemical characteristics, plant community compositions, and management practices. We know that nitrification by AOA and AOB is inhibited by acetylene (28, 30, 46), and we hypothesized that antibiotics that prevent protein synthesis could be used to discriminate between AMO activities of AOA and AOB during the recovery of nitrification potential (RNP) after acetylene exposure that specifically and irreversibly inactivates AMO (28, 30). Upon the removal of acetylene, NH_3 oxidation resumed in *Nitrosomonas europaea* after a 1- to 2-h delay and was prevented by the bacterial protein synthesis and transcriptional inhibitors chloramphenicol and rifampin (28). We hypothesized that our RNP assays should be of relatively short duration (<48 h) and be independent of population growth occurring during the assay. In the presence of the sulfa drug sulfadiazine (SDZ), which inhibits folic acid synthesis, AOB proliferation was completely prevented during a 32-day microcosm study of soil amended with pig manure (50), whereas AOA proliferation was marginally affected or unaffected. Although SDZ clearly prevented the proliferation of AOB to a greater extent than AOA, it could not be discerned whether AOB, AOA, or both contributed to the ammonia oxidation occurring throughout the 32-day incubation. Because the mechanism of protein synthesis in archaea is quite different from that in bacteria and more closely resembles the mechanism of eukaryotic protein synthesis, archaea are insensitive to many of the traditional bacterial protein synthesis inhibitors (6, 16, 37). To our knowledge, there are no antibiotics or protein synthesis inhibitors specifically developed to inhibit archaea. Those that have been successfully used with *Haloferax mediterranei* (31) and *Sulfolobus solfataricus* (2, 9) were identified initially as eukaryotic inhibitors and fortuitously inhibited these two archaea. It remains to be determined how other archaea will respond to these inhibitors.

Although only a few strains of AOA have been cultured or

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enriched (15, 25, 35), a number of potentially discriminating traits have been discovered among these isolates/enrichments that lead us to believe that it might be possible to use these traits, in combination with acetylene inactivation and the recovery of nitrification, to discriminate between AOA and AOB NH_3-oxidizing activities in soil samples. These traits include differences between AOA and AOB in allyl thiourea (ATU) sensitivity (1, 20, 25, 27), a relatively narrow temperature optimum (25°C to 33°C) for all known cultures of AOB versus some AOA (3, 10, 33), and a much lower apparent K_m for NH_3 by "*Candidatus* Nitrosopumilus maritimus" strain SCM1 than that by AOB (39). To evaluate these ideas, we sampled a range of Oregon soils along a transect of different land uses that included forest, pasture, cropped, and nearby fallowed sites.

MATERIALS AND METHODS

Chemicals. ATU, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, neomycin trisulfate salt, nystatin, dimethyl sulfoxide (DMSO) (99.5%), actinomycin D, and NH_4Cl were obtained from Sigma (St. Louis, MO). Acetylene was obtained from Airgas (Radnor, PA); cycloheximide (96.4%), kanamycin sulfate, and gentamicin sulfate were obtained from EMD Biosciences, Inc. (La Jolla, CA); and emetine was purchased from TCI America (Portland, OR). The fungicide Abound, with the active ingredient methyl-(*E*)-2-{2-[6-(cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate (azoxystrobin), was obtained from Syngenta Crop Protection, Inc. (Greensboro, NC), and Szechrome NAS reagent was obtained from Polysciences, Inc. (Warrington, PA).

Soils. After the removal of litter or sod, mineral soil samples (0 to 10 cm) were collected from sites along a west-to-east transect of different land uses approximately 6 miles (9.5 km) in length and located 10 miles (16 km) north of Corvallis, OR (44.7°N, 123.3°W). Soils were taken from three sites under mature red alder stands (forest) in the uplands (~350-m elevation) of the MacDonald-Dunn Forest (Jory-Gelderman series, Paleo/Haplohumults [http://soils.usda.gov/technical/classification/tax_keys/], pH 6.0 to 6.3) from three sites under permanent grass pastures situated on a toe slope below the forest (~125-m elevation) on the Oregon State University (OSU) Beef Farm (Dixonville-Gellatly-Witham complex, Argi/Haploxerolls, pH 5.7 to 6.4) and from three cropped fields (under wheat or barley at the time of sampling) and three fallowed fields (unfertilized and uncultivated since 1990 and colonized by volunteer grasses and forbs, which are mowed twice yearly) at the OSU Hyslop Field Research Laboratory (Woodburn series, Argixerolls, pH 5.9 to 6.5) on the Willamette Valley floor (~85-m elevation). Four to five soil samples were recovered from each of the three locations that were spaced between 100 m and ~1,000 m apart in each of the forest, pasture, cropped, and fallowed areas. The samples at each location were composited, thoroughly mixed in the field, and brought to the laboratory, where they were sieved (4.75 mm) and stored at 4°C. Samples of soil (5 to 10 g) were oven dried to determine the water content. The levels of net readily mineralizable nitrogen (N Min) were determined for soils adjusted to ~60% of the water-holding capacity, incubated at 30°C, and sampled at 7, 14, 21, and 30 days, and the accumulations of ammonium (NH_4^+) plus nitrate (NO_3^-) were measured. Physical and chemical properties of the soil samples were determined by standard procedures of the Central Analytical Services Laboratory, Department of Crop and Soil Science, Oregon State University.

NP. Nitrification potential (NP) assays are used primarily to characterize the potential activity of the nitrifying population under conditions of nonlimiting NH_3 and O_2 and neutral pH (51). Soils were removed from 4°C storage and preincubated field moist at room temperature (22°C ± 2°C) for 2 days prior to experimental use. The fallowed and cropped soils from Hyslop Farm had a low water content (≤11%, wt/wt); therefore, deionized water was added (1 ml/15 g soil) before preincubation to achieve a water content that approximates field capacity (18%, wt/wt). For NP assays, 5-g portions of moist soil were added to 50-ml aliquots of 30 mM TES, buffered to pH 7.2 with 10 M KOH, in 150-ml bottles with black phenolic caps fitted with gray butyl stoppers. TES buffer (30 mM) was used because its pK_a is pH 7.4, it did not extract humic compounds from the soil, and its buffering capacity was sufficient to maintain the soil slurries at pH 6.8 to 7.0 for the duration of the assays. Preliminary experiments showed that 1 mM NH_4^+ was a saturating, noninhibiting concentration, and all NP assays included 1 mM supplemental NH_4^+ (as NH_4Cl) unless otherwise stated. Soil slurries were shaken at 200 rpm either in a Brunswick orbital constant-temperature shaker maintained at either 30°C or 40°C or on a bench-top orbital shaker at room temperature (22°C ± 2°C). At time intervals, samples (1.8 ml) of soil slurry were removed into microcentrifuge tubes and centrifuged for 3 min at 13.4 × 10^3 × *g*, and nitrite (NO_2^-) and NO_3^- concentrations in the supernatants were determined immediately or stored at −20°C until the samples could be processed. NO_2^- and NO_3^- concentrations were calculated using the oven-dried weight of the soil used in the assay. Background NO_2^- and NO_3^- concentrations were measured after soil slurries were shaken for 15 min. NP rates were determined as NO_2^- plus NO_3^- accumulations over 24 h. Although background NO_2^- was undetected in any of the soils, NO_2^- accumulated to various fractions of the total NO_2^- plus NO_3^- in actively nitrifying soil slurries. NO_2^- concentrations were determined as described elsewhere (22). NO_3^- concentrations were determined by using either Szechrome reagent according to the manufacturer's instructions or an Astoria Pacific (Clackamas, OR) flow solution autoanalyzer. It was determined that both ATU and cycloheximide interfered with the Szechrome assay, and in those cases, the NO_3^- concentration was always determined by use of an autoanalyzer. In a number of experiments the NH_4^+ concentration was also determined by using the flow solution autoanalyzer. In some cases, high concentrations of background soil NO_3^- were removed prior to NP assays as follows: 5-g portions of moist soils were dispensed into 40-ml centrifuge tubes with ~30 ml of distilled water (dH_2O), vortexed briefly, and centrifuged at 7.5 × 10^3 × *g* for 15 min, and the supernatant was carefully decanted. Soil pellets were resuspended in NP assay buffer and transferred into assay bottles. Preliminary experiments showed that pretreating the soils in this manner led to a minimal loss of NP.

The effects of bacterial and eukaryotic protein synthesis inhibitors, ATU, and fungicides on nitrification were determined by comparing nitrifications in the presence and absence of the individual compounds. During preliminary experiments with the relatively water-insoluble antibiotic actinomycin D, we found that nitrification in soil slurries was inhibited by extremely small volumes of DMSO and other solvents such as methanol, thereby preventing the use of actinomycin D concentrations of >200 μg/ml. Due to their insolubility in aqueous solutions, we did not pursue the use of antibiotics such as rifampin or chloramphenicol or fungicides such as triazoles. The water-soluble bacterial protein synthesis inhibitors neomycin, kanamycin, and gentamicin were utilized. Kanamycin and gentamicin have been proven effective in preventing the growth of the AOB *Nitrosomonas europaea* (13, 21, 56). The effects of the water-soluble eukaryotic protein synthesis inhibitors emetine and cycloheximide were evaluated. A stock of the nitrification inhibitor ATU was mixed immediately before use. The water-soluble fungicide nystatin (1.2 ml/50 ml; 12,000 units) or azoxystrobin (20 μl/50 ml; 13 mg of active ingredient) was used as recommended by the manufacturers.

RNP. For assays of the recovery of nitrification potential (RNP), 5-g portions of moist soil were dispensed into 50-ml aliquots of 30 mM TES buffer (pH 7.2) in 150-ml bottles with black phenolic caps fitted with gray butyl stoppers. Acetylene was added (0.025%, vol/vol, or 0.025 kPa) to the headspace for 6 h. From preliminary experiments it was determined that a 6-h acetylene exposure was sufficient to inactivate all NH_3 oxidation in the soils used in the study. Acetylene inhibition and RNP steps were carried out at 30°C with 1 mM supplemental NH_4^+, unless otherwise indicated. These conditions in the absence of inhibitors were considered to be the standard RNP, designated RNP°. NP controls comprised soil suspensions to which acetylene was not added. An acetylene-containing control was also included to evaluate the possibility of heterotrophic nitrification. The removal of acetylene was achieved by placing the soil slurries under a vacuum (−100 kPa) and degassing. Analysis of headspace samples by gas chromatography with flame ionization detection (FID), as described previously (59), determined that degassing for 5 min under a vacuum was adequate to remove all traces of acetylene from soil slurries.

After degassing, all bottles were incubated with caps loosened to permit aeration. Although RNP varied with soil samples from different land use regimens, replicate soil samples from the same land use regimen responded similarly. In the case of pasture and cropped soil samples, the length of lag time was evaluated by determining the accumulation of NO_2^- plus NO_3^- at 6-h intervals for 24 to 30 h, followed by sampling at less frequent intervals for a total of 48 h. Fallowed soil had a lower NP, showed a longer lag time (~24 to 30 h), and recovered more slowly. The RNPs were compared with the initial NP controls by assessing the amounts of NO_2^- plus NO_3^- that accumulated in the 24-h postlag period relative to the accumulation occurring between 0 and 24 h in the NP. From preliminary experiments we established there were no detrimental effects of degassing the NP controls after 6 h of incubation on the subsequent rate. Aqueous solutions of antibiotics, nitrification inhibitors, or fungicides were added after acetylene was evacuated. Preliminary experiments were carried out with a range of concentrations of the antibiotics to determine their minimum effective concentrations.

Extraction of nucleic acids and Q-PCR. DNA was extracted from 0.25 to 0.3 g dry soil by using a MoBio (Carlsbad, CA) PowerSoil extraction kit, and DNA was quantified by using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo-Scientific, Rockwood, TN). Quantitative PCR (Q-PCR) of the archaeal and bacterial *amoA* genes was performed by using Brilliant II SYBR green master mix (Stratagene, La Jolla, CA) and an ABI 7500 real-time PCR system. Each 25-μl reaction mixture volume included 10 ng template DNA. Primers and thermal cycler protocols for both bacterial (primers amoA_1R and amoA_2F) and archaeal (primers Arch-amoAF and Arch-amoAR) *amoA* genes were described elsewhere previously (41, 48). Standard curves were constructed with 2.0×10^{1} to 2.0×10^{-4} ng *Nitrosomonas europaea* genomic DNA (bacterial *amoA* [efficiency = 91 to 94%; $R^2 = 0.996$]) or 1.25×10^{1} to 1.25×10^{-3} ng of a TOPO plasmid containing the "*Candidatus* Nitrosopumilus maritimus" strain SCM1 *amoBAC* gene insert (archaeal *amoA* [efficiency = 95 to 100%; $R^2 = 0.994$ to 0.995]). Each reaction was run in triplicate. Copy numbers were standardized by the mass of DNA and g dry soil analyzed for each sample and $\log_{10}$ transformed before further analysis.

Statistics. We used one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis using SPSS 11 for Mac to determine the significance of temperature effects within each incubation set. A single-tailed Student's *t* test assuming unequal variance was used to evaluate the effect of supplemental NH_4^+ on NP and to determine the effect of inhibitors and temperature on RNP.

RESULTS

Soil properties. Due to their close geographic proximity, the soils taken along the landscape transect experience approximately the same precipitation and temperature regimen. Archived soil temperature data from the cropped and fallowed sites show annual maxima and minima of 37.2°C ± 1.5°C and 2.9°C ± 0.9°C, respectively (http://cropandsoil.oregonstate.edu/weather/archive). A suite of chemical and physical properties was determined for the soils (Table 1). While there were only small differences in pHs among the sites, other soil characteristics varied to a greater extent. Notably, high extractable P concentrations in cropped and fallowed soil samples reflect the legacy of P fertilization to promote crop yield, and the high levels of Mg, Na, Ca, and clay in the pasture soils are likely due to the landscape position (toe slope). The cropped soils had the least amount of total C; the adjacent fallowed soils had been out of production for 19 years and had C levels that were 1.9-fold greater than those of the cropped soils. The pasture soils had 2.1-fold-more C than the fallowed soils.

Levels of N that were readily mineralizable over 30 days (N Min) followed a trend similar to that of total C (Table 1). The fallowed soils had a 1.6-fold-greater N Min than the cropped soils, and the pasture soils had a 1.4-fold-greater N Min than the fallowed soils. The highest level of N Min was found in the forest soils and probably reflects the high N input by the N_2-fixing red alder trees at these sites. Q-PCR of *amoA* genes of AOA and AOB demonstrated the presence of both types of NH_3 oxidizers at all sites. Generally, the AOA and AOB *amoA* copy numbers/g soil were within the same order of magnitude, except for pasture soils, where the AOA *amoA* copy number/g soil was 2 orders of magnitude higher than the AOB *amoA* copy number (41.7×10^6 and 0.4×10^6 copies/g soil, respectively).

Properties of soil nitrification potentials (NPs). We conducted an initial screening of soil NPs that revealed that the nitrifier communities possessed different characteristics among the soils and thus provided distinct soil phenotypes for comparing the properties of the recovered NP.

(i) Effects of temperature. The NPs of the soils varied in their responses to temperature (Fig. 1). Pasture soils showed

TABLE 1. Physical and chemical properties of soils from the landscape transect

Soil	Sand concn[a] (g/100 g)	Silt concn[a] (g/100 g)	Clay concn[a] (g/100 g)	pH (SD)[b]	Bray-Pi[a] (mg/kg)	K^+ concn[a] ($cmol_c$/kg)	Mg^{2+} concn[a] ($cmol_c$/kg)	Na^+ concn[a] ($cmol_c$/kg)	Ca^{2+} concn[a] ($cmol_c$/kg)	CEC concn[a] ($cmol_c$/kg)	Total C[a] (g/kg)	Total N[a] (g/kg)	Mean net N Min (mg N/kg) (SD)[c]	AOA *amoA* copy no. (10^6 copies/g soil) (SD)[d]	AOB *amoA* copy no. (10^6 copies/g soil) (SD)[d]
Forest	20	35	45	7.1 (0.1)	9.1	1.1	4.8	0.1	13.6	35.3	55.1	2.9	99.7 (0.9)	6.3 (1.4)	1.9 (0.8)
Pasture	15	35	50	6.7 (0.3)	5.5	0.2	9.3	0.3	18.4	33.9	53.1	3.9	41.5 (17.6)	41.7 (21.2)	0.4 (0.3)
Cropped	11.2	58.8	30	6.5 (0.3)	86.8	0.7	0.7	0.1	7.4	14.2	12.9	0.6	18.4 (8.2)	0.9 (0.7)	0.8 (0.2)
Fallowed	12.5	55	32.5	6.6 (0.1)	103.3	1.1	1.2	0.1	5.4	16.9	25.7	1.7	28.7 (12.7)	3.9 (2.7)	1.0 (0.5)

[a] Particle analysis, total C and N, CEC, and extractable cations were determined by standard methods of the Central Analytical Laboratory. They represent values obtained from a composite sample prepared by mixing individual composite samples from each of three field sites. Bray-Pi is inorganic P extractable in 0.5 M acid (HCl). $cmol_c$ is antimoles of charge of the indicated anion adsorbed to soil.

[b] Values in parentheses are standard deviations of data from three field replicates.

[c] Net N Min is the readily mineralizable N and equals NH_4^+ plus NO_3^- mineralized at 30°C over 30 days. Values in parentheses indicate standard deviations of analytical replicates ($n = 3$) of a composite soil sample prepared by mixing individual composite samples from each of three field sites.

[d] Values in parentheses are standard deviations of triplicate Q-PCRs on each of three field replicate soil samples.

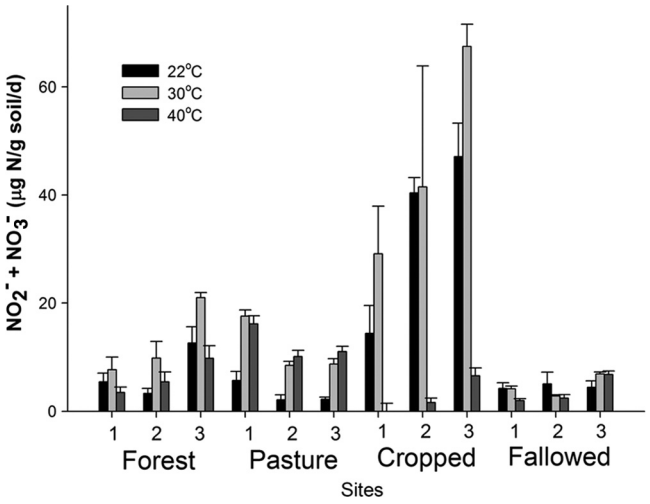

FIG. 1. Temperature responses of the nitrification potentials (NPs) of soil samples taken from three sites each of forest, pasture, cropped, and fallowed land. Mean values and standard deviations are shown ($n = 3$).

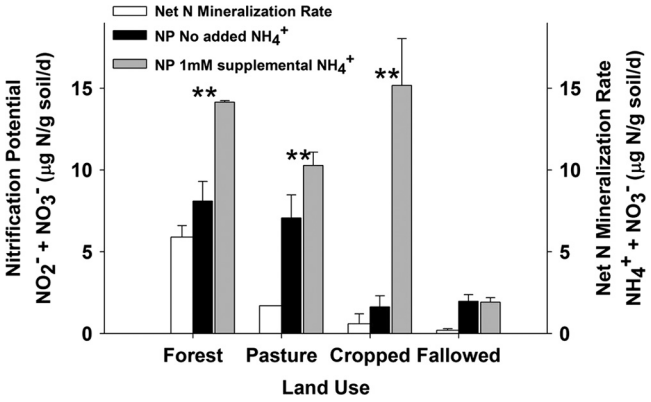

FIG. 2. Comparison of whole-soil rates of net N mineralization and the NPs of soil slurries with and without supplemental NH_4^+. The net N mineralization rate equals NH_4^+ plus NO_3^- mineralized at 30°C over the first 7 days of the N Min study. Mean values and standard deviations are shown for three analytical replicates of a composite soil sample prepared from individual composite samples recovered from each of three field sites under the same land use conditions. ** indicates that values for NP plus 1 mM supplemental NH_4^+ are significantly different from values for NP without added NH_4^+ ($P < 0.01$).

the greatest relative increase in NP at between 22°C and 30°C (3.5-fold), and NPs at 30°C and 40°C were significantly greater ($P \leq 0.01$) than those at 22°C. Forest and cropped soils also showed increases in NPs between 22°C and 30°C, 1.8- and 1.4-fold, respectively, but showed significantly lower ($P \leq 0.01$) levels of activity at 40°C than at 30°C. Cropped soils had only a small fraction (0.06) of their 30°C NP activity at 40°C, while forest soil showed approximately one-half of the 30°C activity at 40°C. At 22°C the NPs of fallowed soils were similar to those of pasture soils but did not increase significantly when incubated at 30°C. The response of fallowed soil NPs to 40°C was mixed: in two cases, the activity at 40°C was less than the NP at 30°C, whereas in one case, the activities were the same.

(ii) Effects of supplemental NH_4^+. The NPs of all soils produced at 30°C were greater than the rates of net N mineralization over the first 7 days of the N Min study of the same soils (Fig. 2). When NPs were done without supplemental NH_4^+, the accumulation of NO_2^- plus NO_3^- was significantly lower ($P \leq 0.01$) in the forest, pasture, and cropped soils than in the presence of 1 mM supplemental NH_4^+. However, fallowed soils produced the same NP with or without supplemental NH_4^+ ($P = 0.4$). Whereas cropped soils demonstrated only 11% of the plus NH_4^+ rate in the absence of supplemental NH_4^+, forest and pasture soils supported NPs of 57 and 69%, respectively, of their rate with NH_4^+. In all cases, during the 24-h time course, the concentrations of NH_4^+ in NP assay solutions without supplemental NH_4^+ were below the limit of accurate detection by the autoanalyzer ($\leq$10 μM). Subsequently, NH_4^+ began to accumulate as N mineralization responded to the incubation at 30°C.

(iii) Effect of ATU. The NPs of soils were measured in the presence of the nitrification inhibitor allyl thiourea (ATU). In the presence of 100 μM ATU, virtually all (92 to 95%) of the NPs of forest, cropped, and fallowed soils were inhibited, whereas pasture soils maintained 53% ± 6% of their NO_2^--plus-NO_3^--producing activity. At 40°C, pasture soils retained all of their NP activity at concentrations of $\leq$1 mM ATU (data not shown). The ATU insensitivity of pasture soil NPs was not due to soil concentrations of Cu^{2+} exceeding the chelating capacity of ATU, as the extractable Cu^{2+} level in 30 mM TES buffer was determined to be below the limits of analytical detection (<0.08 μM) (data not shown), and thus, the ATU level was far in excess of Cu^{2+} levels. Mg^{2+} and Ca^{2+} are divalent cations that could also potentially interfere with the Cu-chelating ability of ATU. The Ca^{2+} and Mg^{2+} concentrations in pasture soil slurry solutions were determined to be 0.73 and 0.48 mM, respectively. We obtained no evidence that the ATU sensitivity of cropped soil slurries could be reduced by raising their Ca^{2+} and Mg^{2+} concentrations to pasture soil slurry levels (data not shown).

Properties of acetylene inactivation and RNP. NPs of all soils were completely inhibited by exposure to 0.025% (vol/vol) headspace acetylene (8 μM aqueous) for 6 h (Fig. 3), indicating that the oxidation of NH_3 in the soils was mediated by a monooxygenase(s) and that heterotrophic nitrification was negligible. After acetylene was removed and slurries were further incubated at 30°C, a significant accumulation of NO_2^- plus NO_3^- could be detected in pasture, cropped, and fallowed soils 12, 18, and 24 to 30 h, respectively, after acetylene removal (Fig. 3). During the 24-h time interval immediately following the acetylene-induced lag, pasture, cropped, and fallowed soils regained 100% ± 39%, 57% ± 36%, and 82% ± 30%, respectively, of the NO_2^- plus NO_3^- produced by their respective NP controls. For unknown reasons, soil slurries from the three forest sites did not consistently demonstrate recovered nitrification potential (RNP) regardless of the incubation temperature (data not shown). The RNP of forest soils did not consistently recover even when time of exposure of slurries to acetylene was reduced to 1 h or when slurries were supplemented with either 5 mM NH_4^+, trace elements (including Cu^{2+}) from standard AOB growth medium (44), or 20 mg/liter of organic supplements, yeast extract, or tryptic soy broth.

The time delay in the RNP of the soils indicated that *de novo*

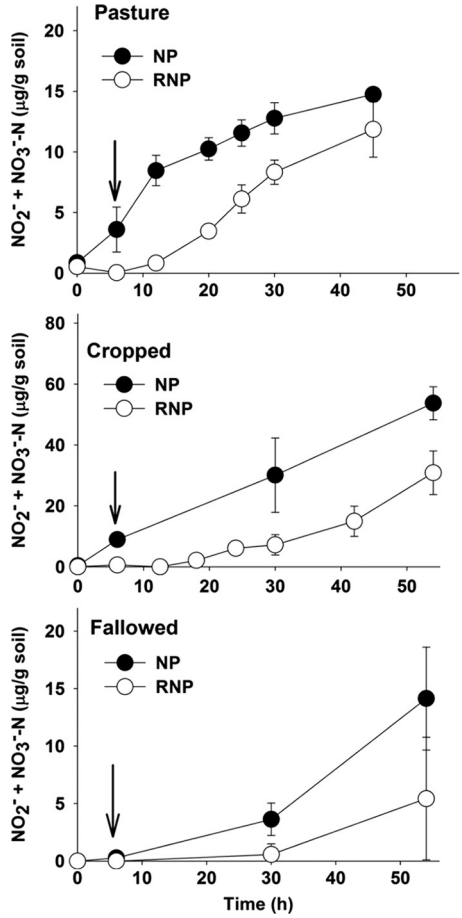

FIG. 3. Comparison of the time courses of NPs and recovered nitrification potentials (RNPs) from representative examples of pasture, cropped, and fallowed soils at 30°C. Error bars indicate the standard deviations of data from three analytical replicates. RNPs were treated with 0.025% (vol/vol) acetylene at time zero. Arrows indicate the time at which acetylene was removed (6 h).

protein synthesis might be required to replace irreversibly inactivated AMO before the resumption of NO_2^- and NO_3^- production could occur. Indeed, the RNPs of cropped soils were 90% ± 10% and 80% ± 0% prevented by 800 μg/ml kanamycin and gentamicin, respectively (Table 2). The antibiotics did not have an effect on the non-acetylene-treated NP control of cropped soil for 18 h. After 18 h, the production of NO_2^- and NO_3^- ceased (data not shown), indicating that the antibiotics were not inhibitors of nitrification *per se* and were likely preventing *de novo* protein synthesis. In the fallowed soils, only fractions of RNP were prevented by either kanamycin or gentamicin (50% and 10%, respectively), but a combination of both antibiotics had a greater effect (60% ± 30%). In complete contrast, and over a wide range of concentrations (≤800 μg/ml), the bacterial protein synthesis inhibitors neomycin (data not shown) and kanamycin and gentamicin, singly or in combination, had virtually no effect on the RNP of pasture soils (Table 2). In addition, kanamycin had no effect on the NP of pasture soils over 0 to 48 h of incubation (data not shown). Similar results were obtained with samples of pasture soil taken at other times of the year (May and December) and from another series of three pasture sites located one-half mile further upslope (data not shown).

TABLE 2. Sensitivity of RNPs to bacterial protein synthesis inhibitors

RNP treatment	Mean RNP^+/RNP^o ratio (SD)[a]		
	Pasture soil	Cropped soil	Fallowed soil
Kanamycin	0.9 (0.2)	0.1 (0.1)**	0.5 (0.1)**
Gentamicin	1.0 (0.2)	0.2 (0.0)**	0.9 (0.2)
Kanamycin + gentamicin	1.0 (0.3)	0.0 (0)**	0.4 (0.3)**

[a] RNP^+ indicates plus antibiotic treatment. RNP^o indicates standard conditions minus antibiotic. Standard deviations of the means of data from three field replicates are shown in parentheses. **, RNP^o significantly different from RNP^+ ($P < 0.01$).

TABLE 3. Comparison of soil RNPs under selective versus standard conditions

RNP comparison	Mean $RNP_{selective}/RNP^o$ ratio (SD)[a]		
	Pasture soil	Cropped soil	Fallowed soil
40°C vs 30°C[b]	1.0 (0.1)[e]	0.0 (0)**	0.9 (0.1)
+ or − 100 μM ATU[c]	0.5 (0.2)**	0.0 (0)**	0.0 (0)**
+ or − NH_4^+[d]	0.8 (0.2)	0.1 (0.1)**	0.4 (0.2)**

[a] $RNP_{selective}$ represents the specific treatment indicated. RNP^o indicates standard conditions. **, RNP^o significantly different from $RNP_{selective}$ ($P < 0.01$).
[b] Performed with 1 mM supplemental NH_4^+.
[c] Performed with a 30°C incubation temperature with 1 mM supplemental NH_4^+.
[d] Performed with a 30°C incubation temperature.
[e] Standard deviations of the means of data from three field replicates are shown in parentheses.

We examined the properties of the RNP from each soil sample to determine to what extent they matched those of the NPs (Table 3). Patterns of temperature response and ATU sensitivity were similar to those demonstrated for the NP controls. The RNPs of pasture and fallowed soils occurred at both 30°C and 40°C, while in cropped soils, RNP did not occur at 40°C. Whereas RNPs of both cropped and fallowed soils were prevented by 100 μM ATU, the RNP of pasture soil was inhibited by only ~50%, and at 40°C, the RNP of pasture soil was completely insensitive to ATU concentrations of ≤1 mM (data not shown). Furthermore, cropped soil RNP did not occur in the absence of supplemental NH_4^+, whereas RNPs of both pasture and fallowed soils occurred in the absence of supplemental NH_4^+ (80% ± 20% and 40% ± 20% of their RNP controls, respectively) at soil solution concentrations of NH_4^+ too low to be accurately measured by the autoanalyzer (≤10 μM). All of the RNPs were completely inactivated by the addition of acetylene (data not shown).

Because of the complete insensitivity of the RNP of pasture soil to bacterial protein synthesis inhibitors, we evaluated the effects of eukaryotic protein synthesis inhibitors and especially ones that have been reported to be effective on archaea. No inhibition of RNPs was observed for pasture soil slurries treated with the eukaryotic transcriptional inhibitor actinomycin D (200 μg/ml) (data not shown), and the protein synthesis inhibitor emetine (800 μg/ml) had no effect on pasture, fallow, or cropped soils (data not shown). Furthermore, neither of the fungicides nystatin and azoxystrobin had an effect on the RNP of pasture or fallowed soils (data not shown). Despite the

ineffectiveness of the above-mentioned compounds, an unpublished observation from our laboratory has shown that the growth of the AOA "*Candidatus* Nitrosopumilus maritimus" strain SCM1 is strongly inhibited by 200 μg/ml of the eukaryotic protein synthesis inhibitor cycloheximide, while the growth of *Nitrosomonas europaea* is insensitive (N. Vajrala, personal communication). We obtained reproducible evidence for a negative effect of 600 to 800 μg/ml cycloheximide on the RNP of pasture soil (0.6 ± 0.2 of their RNP controls); however, cycloheximide had a similarly negative effect on the RNP of cropped and fallowed soils as well (0.5 ± 0.4 and 0.3 ± 0.1 of their RNP controls, respectively). A wide range of cycloheximide concentrations (25 to 600 μg/ml) was shown to have no immediate effect (0 to 12 h) on the NPs of either pasture or cropped soils, suggesting that its effect on the RNP was not due to some direct inhibition of NH_3 oxidation metabolism. However, because the eukaryotic growth inhibitors emetine and actinomycin D had no effect on the pasture soil RNP, it remains unclear how cycloheximide is influencing the RNP and needs further investigation.

DISCUSSION

In this study, we exploited the need for the *de novo* protein synthesis of AMO after irreversible acetylene inactivation to investigate the potential contributions of AOB and AOA to the nitrification potentials of various soils. In targeting AMO, our RNP assays rely on the resynthesis of just one specific enzyme rather than an increase in *amoA* gene abundance (as a proxy for population increase), allowing our assays to be short (~2 days). By showing that the NPs of different soils had distinct features and that the properties of the RNPs were very similar to the properties the NPs, it seems reasonable to believe that the organisms contributing to the RNPs are indeed those participating in NH_3 oxidation in the original soil slurries. Because the NP capacities of the soils were determined with a well-buffered and well-aerated system, we readily concede that our results should not be extrapolated to *in situ* nitrification at this time. Nonetheless, several interesting points have arisen from this work that are worthy of comment and further study.

The highest NP was found in the cropped soil, where the AOA and AOB *amoA* gene copy abundances were approximately equal. However, we demonstrated that all cropped soil RNP was kanamycin sensitive, suggesting that the nitrification potential could be attributed exclusively to AOB. This observation coincides with the work of Jia and Conrad (32), who concluded that AOB were more important to nitrification in an agricultural cropped soil than AOA. In contrast, the RNP in pasture soils was totally insensitive to the bacterial protein synthesis inhibitors kanamycin, gentamicin, and neomycin at high concentrations, indicating that those nitrification potentials were not mediated by AOB. The short duration of the RNP and the high starting concentration of antibiotics in the assay reduce the possibility that the insensitivity to kanamycin and gentamicin is due to their rapid degradation. Since all of the NH_3-oxidizing activity in pasture soil was inactivated with acetylene, and recovery was insensitive to the fungicides nystatin and azoxystrobin, heterotrophic nitrification was unlikely to be the source of NO_2^- and NO_3^-. Although the possibility exists that some fraction of the NP is due to acetylene-sensitive monooxygenases of hydrocarbon-oxidizing bacteria, in that case the RNP should still be sensitive to bacterial protein synthesis inhibitors (14, 23).

To obtain unequivocal evidence for the involvement of AOA in pasture soil nitrification using our approach will require the identification of an antibiotic that selectively prevents protein synthesis in archaea. Although actinomycin D has been shown to effectively prevent transcription in some archaea (2, 9, 31), it did not prevent RNP in the pasture soils, at least at the limit of its water solubility. A number of other eukaryotic inhibitors have proven to be effective on the crenarchaeote *Sulfolobus solfataricus* (9), but they could not be used because of their poor solubility in water and the extreme sensitivity of nitrification and RNP in our soils to the solvents DMSO and methanol, which are routinely used to dissolve these compounds. Despite the ability of cycloheximide to inhibit the growth of "*Candidatus* Nitrosopumilus maritimus" and to prevent it from recovering from acetylene inactivation (N. Vajrala, unpublished observation), it is unknown if this experimental result with "*Candidatus* Nitrosopumilus maritimus" can be extended to soil AOA since most 16S rRNA and *amoA* gene sequences from soil environments are phylogenetically distinct and group separately from those retrieved from marine environments (36, 43, 54). In addition, we remain uncertain about cycloheximide's mode of inhibition and skeptical of its selectivity. For example, because cycloheximide only partially inhibited kanamycin-resistant RNP in the pasture soils, some of the AOA must be cycloheximide resistant. Furthermore, cycloheximide also partially inhibited the RNP in the cropped soil, whereas all other evidence suggested that AOB are completely responsible for the RNP.

The pasture soils contained an AOA *amoA* gene copy abundance that was 100 times greater than that of AOB, which resembled some of the values reported previously for AOA *amoA* gene abundances (36). Subsequent soil samples taken from other locations at the pasture site and at different times of the year have also yielded high AOA-to-AOB ratios and insensitivity to kanamycin and gentamicin. In addition to resistance to bacterial protein synthesis inhibitors, RNPs of pasture soil also showed other properties that are atypical of soil AOB, such as robust recovery at 40°C. The predominant AOB in the pasture soils belong to the *Nitrosospira* lineage (L. H. Zeglin et al., manuscript in preparation). The growth profiles of four strains of *Nitrosospira* isolated from terrestrial environments were shown to be optimum between 25°C and 33°C; none demonstrated NO_2^- production at 40°C (33). In another study, soils dominated by natural populations of *Nitrosospira* showed maximum nitrification activity between 10°C and 25°C and showed no activity at 37°C (4). However, there have been reports of soils collected in the western United States (38, 53) and Australia (42) showing vigorous nitrification activity at 35°C and 40°C, and in a California grassland soil sample, the increase in nitrification potential with temperature was accompanied by a decrease in AOB abundance (3). In this context, an AOA, "*Candidatus* Nitrososphaera gargensis," was recently enriched at 46°C from a hot spring (25). The pasture soils also retained much of their NP and RNP activities in the presence of 100 μM ATU. In a previous study (25), "*Candidatus* Nitrososphaera gargensis" maintained some of its ability to oxi-

dize NH_3 in the presence of 100 μM ATU (26), a concentration which completely inhibits AOB by reversibly chelating Cu^{2+} in AMO (1, 5, 20, 26, 27). The complete insensitivity of the pasture soil RNP at 40°C to ≤1 mM ATU, while showing only partial resistance at 30°C, could be explained by the existence of multiple AOA phylotypes that demonstrate variable sensitivity to ATU and that only the type that recovers at 40°C is completely ATU resistant. Finally, the ability of the RNP of pasture soils to occur in the presence of <10 μM NH_4^+, whereas the RNP in cropped soils is dependent upon supplemental 1 mM NH_4^+, is consistent with the idea that AOA might possess a much higher affinity for NH_4^+ plus NH_3 than known cultured AOB. Unfortunately, to date, a K_s value has been reported only for the marine archaeon "*Candidatus* Nitrosopumilus maritimus" (39).

Although the forest soil had a greater NP than the pasture or fallowed soils, it did not consistently demonstrate RNP. Similar observations of other soils have been made where a <1- to 8-day delay occurred before significant NO_2^- or NO_3^- accumulation after acetylene exposure (8, 11, 34). Extended delays before NO_2^- or NO_3^- accumulation could indicate that the forest soil-borne NH_3 oxidizers are sufficiently depleted of energy reserves to hinder the rapid *de novo* protein synthesis of AMO. Indeed, although cultures of *Nitrosospira briensis* starved for ≤7 days were able to recover NH_3 oxidation activity within 10 min after the addition of fresh NH_4^+, recovery was seriously delayed if they were pretreated with acetylene immediately prior to the introduction of NH_4^+ (12). If this is the case, RNP in the forest soils might be promoted by first allowing NP to proceed in the presence of 1 mM NH_4^+, replenishing cellular energy reserves before acetylene inactivation. An alternate explanation for inconsistent RNP in forest soils might be that the co-oxidation of NH_3 by hydrocarbon-oxidizing bacteria was responsible for most of the NP (24, 29, 45) and that the RNP could not occur because correct substrates were not present to induce the expression of the specific monooxygenase genes (18, 47, 49). This hypothesis could be evaluated by the addition of small concentrations of hydrocarbons or their oxidized products after the evacuation of acetylene and monitoring RNPs.

It remains unknown what factors might selectively promote nitrification activity by AOA or AOB in a particular soil environment. The sensitivity of AOA to high NH_4^+ concentrations (25, 39) might explain the dominance of nitrification by AOB in the N-fertilized cropped soils (160 kg urea-N/ha). Interestingly, the adjacent fallowed soils, which had not received inorganic N fertilizer for 19 years, demonstrated only partial sensitivity to kanamycin and gentamicin and indicated that a shift had occurred from the AOB-dominated nitrification observed for the cropped soils to a combination of AOB and AOA. This shift could indicate that in the absence of annual additions of N fertilizer, NH_3 oxidizers need to adapt to a low, albeit steady, supply of NH_4^+ from the mineralization of organic N and must compete for NH_4^+ with plants and microbial heterotrophs. This probably favors both AOB and AOA types with higher NH_4^+ affinities. A similar situation is likely to be the cause in the pasture soil, where both NP and RNP occurred regardless of supplemental NH_4^+. With the RNP method in hand, it may be possible to identify the factors controlling the relative contributions of AOA and AOB to nitrification across soil landscapes and provide physiological insights into the AOA and AOB active in different soils.

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