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ORIGINAL ARTICLE

Evidence that Ammonia-Oxidizing Archaea are More Abundant than Ammonia-Oxidizing Bacteria in Semiarid Soils of Northern Arizona, USA

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Abstract Autotrophic ammonia-oxidizing communities, which are responsible for the rate-limiting step of nitrification in most soils, have not been studied extensively in semiarid ecosystems. Abundances of soil archaeal and bacterial *amoA* were measured with real-time polymerase chain reaction along an elevation gradient in northern Arizona. Archaeal *amoA* was the predominant form of *amoA* at all sites; however, ratios of archaeal to bacterial *amoA* ranged from 17 to more than 1,600. Although size of ammonia-oxidizing bacteria populations was correlated with precipitation, temperature, percent sand, and soil C/N, there were no significant relationships between ammonia-oxidizing archaea populations and any of the environmental parameters evaluated in this study. Our results suggest that in these soils, archaea may be the primary ammonia oxidizers, and that ammonia-oxidizing archaea and ammonia-oxidizing bacteria occupy different niches.

Introduction

Nitrification is the biological oxidation of ammonia (NH_3) to nitrate (NO_3^-) and an essential step in the global nitrogen cycle. In soils, nitrification can result in nitrogen loss from an ecosystem. NO_3^- is leached much more readily than NH_3. It also serves as an electron acceptor for denitrification, the reduction of NO_3^- to the gaseous forms NO, N_2O, and N_2. Nitrate leaching can result in groundwater pollution, and N_2O is a potent greenhouse gas. The first and rate-limiting step of nitrification is the oxidation of NH_3 to nitrite (NO_2^-). Extensive research has focused on ammonia-oxidizing microorganisms because their activity impacts agricultural yields, water quality, and global climate change [10].

Two groups of organisms, a monophyletic subset of the β-Proteobacteria and members of the non-extremophilic Crenarchaeota, are responsible for ammonia oxidation in most soils [11, 13]. While a robust body of research has demonstrated that autotrophic ammonia-oxidizing bacteria (AOB) contribute to soil ammonia oxidation, the role of ammonia-oxidizing archaea (AOA) remains unclear. Several lines of evidence suggest that AOA may be important to nitrification. First, metagenomic studies revealed that some crenarchaea contain genes with high similarity to the ammonia monooxygenase genes of AOB [17, 18]. Second, archaeal and bacterial versions of these genes are transcribed in the same soils [11]. Third, a chemoautotrophic crenarchaea that oxidizes NH_3 to NO_2^- aerobically was isolated from an aquarium verifying that archaea capable of ammonia oxidation exist [9].

Ammonia monooxygenase, an enzyme essential for autotrophic ammonia oxidation, is encoded by the genes *amoA*, *B*, and *C* in AOB and AOA [13]. The *amoA* gene codes for the subunit containing the active site, and primer sets have been developed that detect either archaeal *amoA* or bacterial *amoA* in environmental samples [5, 16]. If the number of *amoA* copies per cell is similar in AOB and AOA, population densities of the two groups of ammonia oxidizers can be compared with real-time polymerase chain reaction (PCR) methods. Cultured AOB have two to three copies of the *amoA* gene [14]. Much less is known about the genome structure of AOA, but a study has suggested that marine AOA have one to three copies of *amoA* per cell [19].

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A few studies have used *amoA* as a biomarker to compare the size of AOA and AOB populations. In a time series from the coastal North Sea, bacterial *amoA* was present in equal to or slightly greater numbers than archaeal *amoA* for most of the year, except from November until March when spikes in archaeal *amoA* abundance coincided with decreases in NH_3 and increases in NO_3^-. Bacterial *amoA* increased slightly during this period, but archaeal *amoA* abundance was 1–2 orders of magnitude greater than bacterial *amoA* [19]. In samples taken from a variety of European grassland and agricultural soils, archaeal *amoA* was always more abundant than bacterial *amoA*, although the ratio of archaeal *amoA* to bacterial *amoA* varied from 1.5 to more than 3,000. Increasing soil depth had an impact on this ratio due to declines in bacterial *amoA*; however, this result was not consistent across soil types [11]. To gain a clear picture of what factors regulate ammonia-oxidizing populations, AOA and AOB must be studied in a wide variety of habitats. Results from one system may be specific to the organisms found there.

Population sizes of AOB and AOA in semiarid soils have not yet been reported, and it remains unclear whether environmental factors will impact both ammonia-oxidizing populations in these systems similarly. The goal of this study was to compare the abundances of *amoA* genes, which served as a proxy for population size of AOB and AOA, along an elevation gradient in northern Arizona. Through regression analysis, the influence of environmental factors on the population densities of these two groups was evaluated.

Methods

Soils sampled in this study are from the C. Hart Merriam Elevation Gradient (http://www.mpcer.nau.edu/gradient) located on the San Francisco Peaks in northern Arizona (35°N latitude and 111°W longitude). The regional climate is semiarid, but sites along the 1,064 m gradient differ in temperature, precipitation, dominant vegetation, and soil type (Table 1). Five surface soil cores (4 cm diameter, 10 cm depth) were harvested from randomly chosen locations within 36 m^2 sampling plots placed in open grassy areas at each site in May (post-snowmelt) and August (post-monsoon).

DNA was isolated from 0.5 g of frozen soil with the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) and further purified by ethanol precipitation. Cell lysis was achieved by bead beating for 15 s at setting 5.0 in a FastPrep® Instrument (Q-Bio Gene, Morgan Irvine, CA, USA), replacing step 5 of the MoBio protocol. Concentration and purity of DNA was determined with a BioPhotometer (Eppendorf, Hamburg, Germany), and all extractions were diluted to 5 ng DNA μl^{-1} in Tris-EDTA buffer.

Table 1 Elevation, climate, and soil classifications at the C. Hart Merriam Elevation Gradient sites

Site	Elevation (m)	ADT (°C)	Precipitation (mm)	Soil classification
Great Basin	1556	13.2	140.8	Pachic Udic Argiboroll
Grassland	1760	12.7	215.0	Mollic Eutroboralf
Pinyon-Juniper	2020	10.9	335.0	Calcic Haplustand
Ponderosa	2344	8.6	388.6	Typic Haplustoll
Mixed Conifer	2620	5.7	558.1	Lithic Camborthid

ADT Average daily temperature 2004, *Precip* annual precipitation 2004

Using the primers Arch-amoAF and Arch-amoAR [5], a 635 bp fragment of the archaeal *amoA* gene was amplified from samples harvested at the grassland site in August 2004. PCR reactions contained 0.2 μM primers, 1X PCR buffer, 0.2 mM dNTPs, 2 mM MgCl2, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) in a final volume of 50 μl and were run in a PT-100 thermocycler (MJ research, Waltham, MA, USA). The cycling protocol was 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 60 s at 55°C, 45 s at 72°C, and a final extension of 7 min at 72°C. Quality of PCR products was determined visually on a 0.5X TBE, 1.5% agarose gel. PCR products were cloned with the TOPO TA Cloning® Kit (Invitrogen), sequenced, and analyzed on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). BLAST searches against the GenBank database verified that the PCR products were most closely related to other archaeal *amoA* sequences.

Archaeal *amoA* clones were pooled to create a standard curve of known archaeal *amoA* concentrations. Plasmids were extracted with the Perfect Prep Plasmid Mini Kit (Eppendorf, Hamburg, Germany) and quantified with a BioPhotometer. From the plasmid concentration and the length of the PCR®4-TOPO® vector (Invitrogen) with a 635 bp insert, *amoA* copy concentration was calculated. A series of 1:10 dilutions, ranging from 10^2 to 10^9 copies, was used as a standard curve for quantifying the abundance of archaeal *amoA* in soil DNA extractions. A 491-bp fragment of bacterial *amoA* was amplified from DNA extracted from the grassland site using primers amoA-1F and amoA-2R [16]. PCR conditions and the cycling protocol were identical to those described previously, except the extension step was decreased from 45 to 30 s. PCR products were cloned. Plasmids were extracted, quantified, and diluted to

create a standard curve of known bacterial *amoA* concentration as described for archaeal *amoA*.

Real-time PCRs for both bacterial and archaeal *amoA* were run in an DNA Engine Opticon Real-Time PCR System (MJ Research) under the following conditions: 0.2 μM primers, 1X PCR buffer, 0.2 mM dNTPs, 4 mM $MgCl_2$, 1.5 U of Platinum *Taq* DNA polymerase, and 0.25X sybr green (Invitrogen) in a final volume of 50 μl. Cycling protocols were 2 min at 94°C followed by 40 cycles of 30 s at 94°C, 60 s at 55°C, 45 s at 72°C for archaeal *amoA* or 30 s for bacterial *amoA*, and a final extension of 7 min at 72°C. Purity of PCR products was verified with melting curves. Threshold cycle (C_T) was designated as the point when fluorescence intensity reached four times the standard deviation of baseline fluorescence.

Several soil characteristics predicted to affect the size of ammonia-oxidizing populations were measured. NH_4^+ and NO_3^- concentrations of filtered 2 M KCl soil extractions were quantified by automated spectrophotometric analysis on a QuikChem® 8500 FIA System (Lachat Instruments, Loveland, CO, USA). The gravimetric water content of each soil sample was determined by calculating mass loss after drying 5 g of soil at 100°C for 24 h. Temperature and precipitation data were collected by weather stations located at each elevation gradient site. Soil pH was measured in water as described in Hendershot, Lalande, and Duquette (1993) [7]. Sieves, 2 and 0.05 mm, were used to determine soil texture. Soil carbon and nitrogen were measured with a Carlo Erba NC2100 Elemental Analyzer at the Colorado Plateau Stable Isotope Laboratory.

Results of the real-time PCRs were expressed in relative abundance, *amoA* copies per nanogram of DNA. These values were multiplied by the amount of DNA extracted from the soil sample to determine absolute abundance, *amoA* copies per gram of dry soil. To compare *amoA* copies per gram of soil among sites, it is necessary to assume DNA extraction efficiencies are similar for all soils. The benefit of absolute gene abundances is that they reflect differences among the sites in total microbial community size, which are substantial along the elevation gradient. This perspective is absent when relative abundances are compared. JMP 6.0 (SAS Institute, Cary, NC, USA) was used to run analyses of variance on *amoA* abundance by site, *t* tests to determine if there were differences in *amoA* abundances between sampling dates, and linear regressions of log transformed *amoA* abundances versus environmental factors.

Results

There were linear relationships between target gene copy number and C_T values for bacterial *amoA* ($y=-3.744x+46.114$; $R^2=0.998$) and archaeal *amoA* ($y=-3.817x+45.121$; $R^2=0.999$) over a range of seven orders of magnitude with the real-time PCR protocols used in this study. The two primer sets had similar amplification efficiencies, 82.82% for archaeal *amoA* and 84.51% for bacterial *amoA*. Detection limits were ~130 archaeal *amoA* copies and ~200 bacterial *amoA* copies (Fig. 1).

Archaeal *amoA* was more abundant than bacterial *amoA* at all sites along the C. Hart Merriam Elevation Gradient on both sampling dates (Fig. 2). The greatest differences in archaeal and bacterial *amoA* copies were found at the pinyon-juniper site in May and the grassland site in August where archaeal *amoA* was over three orders of magnitude more abundant than bacterial *amoA*. Mean archaeal *amoA* to bacterial *amoA* ratios were lowest at the ponderosa site, being 17 in August and 33 in May.

There were significant differences in bacterial *amoA* abundance among elevation gradient sites, regardless of whether the data was expressed in copies per nanogram of DNA (May, $F=5.299$, $p<0.01$; August, $F=5.650$, $p<0.01$) or copies per gram of soil (May, $F=6.826$, $p<0.01$; August, $F=13.187$, $p<0.001$; Fig. 2). Archaeal *amoA* abundance also differed among elevation gradient sites when data was expressed in copies per nanogram of DNA (May, $F=6.713$, $p<0.01$; August, $F=5.443$, $p<0.01$) and copies per gram of soil (May, $F=10.505$, $p<0.001$; August, $F=22.971$, $p<0.001$; Fig. 2). Bacterial *amoA* abundance did not differ between the two sampling dates at any site except the pinyon-juniper where abundance was greater in August than in May (copies per nanogram of DNA, $t=3.446$, $p<0.05$; copies per gram of soil, $t=4.095$, $p<0.01$). Archaeal *amoA* abundance was greater in May than August at the great basin (copies per nanogram of DNA, $t=3.102$, $p<0.05$; copies per gram of soil, $t=4.409$, $p<0.01$) and pinyon-juniper sites (copies per nanogram of DNA, $t=6.216$, $p<0.001$; copies per gram of soil, $t=4.987$, $p<0.001$). At the ponderosa and mixed conifer sites, archaeal *amoA* abundance was greater in May than August when expressed in copies per nanogram of DNA (ponderosa, $t=2.638$, $p<0.05$; mixed conifer, $t=2.640$, $p<0.05$). However, seasonal differences in archaeal *amoA* abundance at the two highest elevation sites were not significant when abundances were expressed in copies per gram of soil. At the grassland site, archaeal *amoA* did not differ between sampling dates.

To determine what environmental factors influenced the abundance of archaeal and bacterial *amoA* in northern Arizona soils, a series of linear regressions were performed. Archaeal *amoA* abundance was not significantly related to any of the measured environmental variables, regardless of whether relative or absolute gene abundances were considered (Table 2). In contrast, regressions of precipitation, air temperature, and soil C/N against absolute bacterial *amoA* abundance (in copies per gram

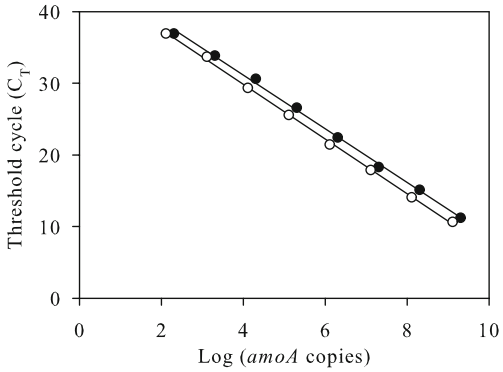

Figure 1 Standard curves developed for real-time PCR assays of bacterial *amoA* (*open circles*, R^2=0.998) and archaeal *amoA* (*filled circles*, R^2=0.999)

of soil) were all highly significant (Table 2, Fig. 3). In a multivariate analysis, these variables together accounted for nearly 80% of the variation found in bacterial *amoA* copies per gram of soil. There was a less significant relationship between bacterial *amoA* copies per gram of soil and percent sand by mass (Table 2). Soil C/N was the only measured parameter to be correlated with both relative and absolute bacterial *amoA* abundance (Table 2).

Discussion

Archaeal *amoA* was more abundant than bacterial *amoA* in all soils analyzed in this study, regardless of whether samples were taken during the post-snowmelt or post-monsoon seasons. Other studies have shown that in grassland and agricultural soils from Europe, as well as in the North Sea, archaeal *amoA* was more abundant than bacterial *amoA* [11, 19]. Our results from semiarid soils in northern Arizona add to the growing list of ecosystems where archaea appear to be the predominant ammonia oxidizer. This conclusion relies on the assumption that the number of *amoA* copies is similar in the genomes of AOA and AOB. An alternative explanation is that the genomes of AOA contain many more *amoA* copies than the genomes of AOB and differences in *amoA* copies do not reflect differences in cell densities. However it seems unlikely that AOA would have many more than the two to three *amoA* copies found in cultured AOB [14]. It is also possible that higher abundances of archaeal *amoA* were measured because AOA are more susceptible to lysis than AOB. *AmoA* abundance, expressed in copies per nanogram of DNA, can be interpreted as the relative abundance of AOB or AOA in a DNA extraction. While this is a useful measurement, it does not provide any information about the population densities of ammonia oxidizers in soil. By multiplying *amoA* copies per nanogram of DNA in an individual sample by the total amount of DNA extracted from that sample, *amoA* copies per gram of soil can be estimated. Recovery of DNA from soil may be influenced by soil type and microbial community composition [20]. Therefore, our estimates of *amoA* copies per gram of soil, that assume equal extraction efficiencies, could be biased

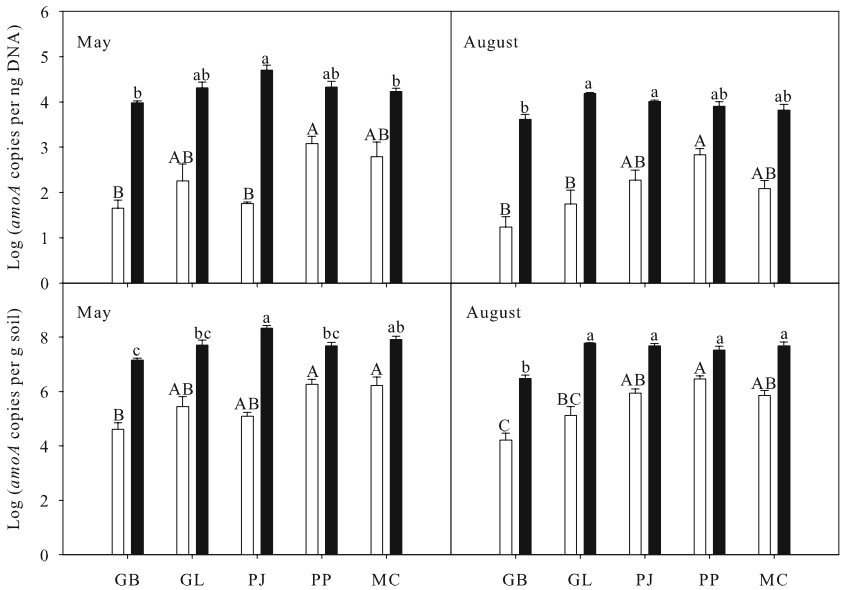

Figure 2 Abundance of bacterial *amoA* (*open bars*) and archaeal *amoA* (*filled bars*), expressed in copies per nanogram of DNA and copies per gram of soil, at the C. Hart Merriam Elevation Gradient sites in May and August 2004. *Capital letters* refer to significant differences determined by Tukey's HSD (p<0.05) in bacterial *amoA* abundance among sites at one sampling date, *lowercase letters* denote significant differences in archaeal *amoA* abundance. Site names are abbreviated: *GB* great basin, *GL* grassland, *PJ* pinyon-juniper, *PP* ponderosa pine, *MC* mixed conifer

Table 2 Correlation coefficients (r) for linear regressions of soil bacterial and archaeal *amoA* gene abundances (log transformed) vs environmental factors on the C. Hart Merriam Elevation Gradient in northern Arizona

Environmental factor	Archaeal *amoA* (copies per nanogram of DNA)	Archaeal *amoA* (copies per gram of soil)	Bacterial *amoA* (copies per nanogram of DNA)	Bacterial *amoA* (copies per gram of soil)
NH_4^+ [a]	−0.026 (0.94)	0.337 (0.34)	0.385 (0.27)	0.446 (0.20)
NO_3^- [a]	−0.323 (0.36)	−0.466 (0.17)	−0.518 (0.13)	−0.609 (0.06)
Soil water content	0.013 (0.97)	0.351 (0.32)	0.422 (0.22)	0.448 (0.19)
Soil pH	0.518 (0.13)	0.602 (0.07)	0.383 (0.27)	0.344 (0.33)
Precipitation (mm)[b]	0.137 (0.71)	0.535 (0.11)	0.563 (0.90)	*0.784 (<0.01)*
Mean daily air temperature (°C)[b]	−0.066 (0.86)	−0.593 (0.07)	−0.440 (0.20)	*−0.795 (<0.01)*
Rocks (% by mass)	0.429 (0.22)	0.229 (0.53)	−0.162 (0.66)	−0.164 (0.65)
Sand (% by mass)	−0.008 (0.98)	0.077 (0.83)	0.463 (0.18)	*0.655 (0.04)*
Silt (% by mass)	−0.529 (0.12)	−0.440 (0.20)	−0.183 (0.61)	−0.210 (0.56)
Soil carbon (% by mass)	−0.146 (0.69)	0.193 (0.59)	0.197 (0.59)	0.278 (0.44)
Soil nitrogen (% by mass)	0.207 (0.56)	0.094 (0.80)	0.068 (0.85)	0.135 (0.71)
Soil C/N	0.130 (0.72)	0.257 (0.47)	*0.688 (0.02)*	*0.811 (<0.01)*

P values are given in parentheses and relationships with $p<0.05$ are in italics
[a] 2 M KCl extractable in mg N kg^{-1} dry soil
[b] 2004

toward sites with higher DNA extraction efficiencies. This assumption does not affect the comparison of archaeal versus bacterial *amoA* copies within a site.

Absolute bacterial *amoA* abundance, expressed in copies per gram of soil, was greatest at the sites with lower air temperatures and higher annual precipitation. While the individual effects of each of these climate variables cannot be determined from this study, there are several mechanisms by which climate could influence AOB populations. Temperature has been shown to directly impact AOB. Nitrifying activity in soil has an optimum temperature of 35°C, potential nitrification is highest when soils are incubated at moderate temperatures, and AOB community structure can be affected by incubation temperature [2, 3]. AOB growth may be limited in the lower elevation soils, which experience extremely high temperatures during the summer and are not shaded due to very sparse plant cover. These conditions will also result in decreased soil moisture, another factor known to be important in regulating AOB population size [3, 6, 8]. The role of soil moisture in determining bacterial *amoA* abundance in northern Arizona soils is supported by a positive relationship with precipitation. However, the regression of bacterial *amoA* abundance and soil moisture measured at the time of sampling was not significant. Soil moisture fluctuates dramatically in pulse-driven systems like those found in the southwestern USA [1]. It has been suggested that bacterial communities may be adapted to the moisture regime that they experience in the environment [4]. Populations of AOB, which have shown some resistance to desiccation, are likely to change on much slower time scales than soil moisture will fluctuate [3, 6]. The lack of significant relationships between archaeal *amoA* and temperature or precipitation suggests that AOA and AOB differ in their resistance to desiccation.

As the energy source for ammonia oxidizers, NH_4^+ availability is likely to impact AOB and AOA populations. We did not observe significant relationships between bacterial or archaeal *amoA* abundance and NH_4^+ concentration at the time of sampling. The frequent soil moisture fluctuations described previously result in pulses of

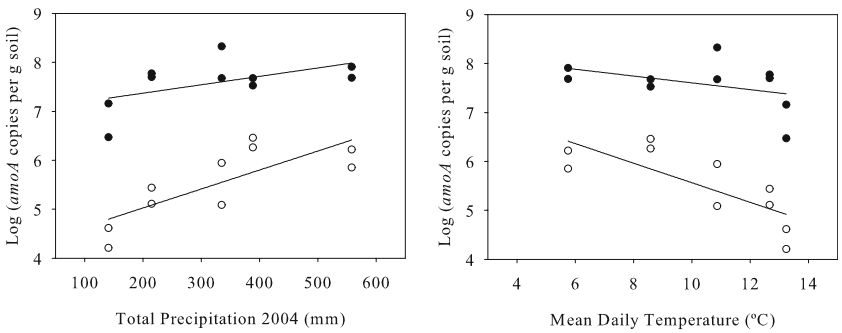

Figure 3 Linear regressions of bacterial *amoA* (*open circles*) and archaeal *amoA* (*filled circles*) with 2004 cumulative precipitation and average daily temperature

nitrogen mineralization after precipitation events followed by periods of immobilization [1]. Studies utilizing molecular techniques in agricultural soils have suggested that AOB populations exhibit long-term, rather than short-term responses to changes in NH_4^+. In soils that had been fertilized for more than 100 years there was no change in the abundance of AOB 3 days after fertilization and in a microcosm study abundance of AOB did not peak until 7 days after addition of ammonium [12, 15]. Due to slow-growth rates and the ability to maintain stable population density during periods of low NH_4^+ availability, AOB abundance was greater in fertilized than control soils even eight and twelve months after NH_4^+ treatment [12, 15]. Frequent changes in the nitrogen mineralization/immobilization status of semiarid soils, and long response times of AOB to both favorable and unfavorable conditions, may explain the absence of a relationship between NH_4^+ availability and AOA or AOB abundance. NO_3^-, the product of nitrification, was not significantly related to bacterial or archaeal *amoA* abundance in these soils. This is not surprising as both plants and microbes can utilize NO_3^- as a nitrogen source, and thus net changes in the NO_3^- pool do not solely reflect nitrifying activity.

The significant positive relationship between bacterial *amoA* abundance and soil C/N was an unexpected result. Under high C/N nitrogen demand by heterotrophs is high, and slow-growing AOB and presumably AOA face competition for available NH_4^+ and oxygen. As a result, a negative relationship between AOB and soil C/N was expected. It is possible that a substantial portion of carbon may be in a recalcitrant form, making the C/N of bioavailable compounds lower than that of the total soil. Alternatively, soil C/N may covary with another environmental factor, such as net primary productivity, that impacts bacterial *amoA* abundance.

While our results, as well as those from other environments, suggest that AOA may be the predominant ammonia oxidizers, it is important to recognize that the contribution of AOA to ammonia oxidation rates has not yet been quantified. The lack of significant relationships between archaeal *amoA* abundance and environmental parameters indicates that there is much we do not yet understand about AOA. To gain a clear understanding of what factors regulate AOA and AOB populations, future studies should manipulate environmental parameters and use techniques that allow growth of ammonia-oxidizing organisms to be monitored, such as stable isotope probing.

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