Influence of oxic/anoxic fluctuations on ammonia oxidizers and nitrification potential in a wet tropical soil

Jennifer Pett-Ridge1,2, Dorthe G. Petersen2, Erin Nuccio2 & Mary K. Firestone2

1Lawrence Livermore National Laboratory, Livermore, CA, USA and 2University of California, Berkeley, CA, USA

Correspondence: Jennifer Pett-Ridge, Lawrence Livermore National Lab, PO Box 808, L-231, Livermore CA 94551-9900, USA. Tel.: 925 424 2882; fax: 925 422 3160; e-mail: pettridge2@llnl.gov

Present address: Dorthe G. Petersen, Bioscience, Center for Geomicrobiology, Aarhus University, Aarhus, Denmark

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Abstract

Ammonia oxidation is a key process in the global nitrogen cycle. However, in tropical soils, little is known about ammonia-oxidizing microorganisms and how characteristically variable oxygen regimes affect their activity. We investigated the influence of brief anaerobic periods on ammonia oxidation along an elevation, moisture, and oxygen availability gradient in wet tropical soils. Soils from three forest types were incubated for up to 36 weeks in lab microcosms under three regimes: (1) static aerobic; (2) static anaerobic; and (3) fluctuating (aerobic/anaerobic). Nitrification potential was measured in field-fresh soils and incubated soils. The native ammonia-oxidizing community was also characterized, based on diversity assessments (clone libraries) and quantification of the ammonia monooxygenase α-subunit (amoA) gene. These relatively low pH soils appear to be dominated by ammonia-oxidizing archaea (AOA), and AOA communities in the three soil types differed significantly in their ability to oxidize ammonia. Soils from an intermediate elevation, and those incubated with fluctuating redox conditions, tended to have the highest nitrification potential following an influx of oxygen, although all soils retained the capacity to nitrify even after long anoxic periods. Together, these results suggest that wet tropical soil AOA are tolerant of extended periods of anoxia.

Introduction

Microbial ammonia oxidation is the first and often rate-limiting step of nitrification, a fundamental process in global nitrogen cycling. For many years, it was presumed that chemoautotrophic proteobacteria were primarily responsible for this process (Kowalchuk & Stephen, 2001). However ammonia-oxidizing archaea (AOA) contain an archaeal version of the amoA gene that codes for a key subunit of the ammonia monooxygenase enzyme, and it has become clear that members of the archaea also carry out ammonia oxidation (Köneke et al., 2005). Subsequent research has shown that AOA are widely distributed in marine systems (Francis et al., 2005; Wuchter et al., 2006), sediments (Caffrey et al., 2007; Herrmann et al., 2008), and temperate soils (Leininger et al., 2006; He et al., 2007; and references cited by Bouskill et al., 2012; and Pester et al., 2011, 2012).

The ecology of AOA remains under investigation, and there is no obvious causality between the relative abundances of AOA and ammonia-oxidizing bacteria (AOB) and their functional effect on ammonia oxidation rates (Nicol & Schleper, 2006; Prosser & Nicol, 2008). Ecosystems numerically dominated by AOA may be functionally dominated by AOB activity (He et al., 2007; Di et al., 2009; Jia & Conrad, 2009); however, the converse has also been documented (Caffrey et al., 2007; Herrmann et al., 2008; Nicol et al., 2008), and in some cases only AOA are detectable in soil (Stopnišek et al., 2010; Daebeler et al., 2012; Isobe et al., 2012). Such contradictory observations raise interesting questions about the responses of AOA to environmental factors, the possibility of niche differentiation, and the functional equivalency of phylogenetically distinct groups of ammonia oxidizers.

Data gleaned from marine systems indicate AOA may have higher substrate affinity for ammonia (Martens-Habbena et al., 2009) and may thrive under relatively low ammonia conditions in soil (Di et al., 2010). Recent findings show AOA often have a higher affinity for oxygen than AOB (Chen et al., 2008; Jung et al., 2011; Pitcher...
et al., 2011; Kim et al., 2012), and even within the AOA, different ecotypes appear to exist, with some better adapted to suboxic conditions (Gleeson et al., 2010; Molina et al., 2010). Relatively few studies have focused on the effect of variable oxygen regimes as a driving selective force for niche differentiation between AOA and AOB (Bouskill et al., 2012).

Because upland tropical soils characteristically experience fluctuating oxygen availability, we hypothesize that they may represent a selective habitat for ammonia oxidizer adaptation to oxygen regime. Both field and laboratory measurements in tropical soils show substantial gross nitrification rates; indicating that substantial ammonia oxidation occurs despite low pH and limited oxygen availability that can persist for extended periods due to fluctuating redox regimes (Silver et al., 1999; Pett-Ridge et al., 2006). Currently, ammonia oxidizer amoA sequences have been described from only a single tropical forest soil, as part of two separate meta-analyses (Fierer et al., 2009; Pester et al., 2012); in general, little is known about the process rates or physiochemical niches inhabited by tropical soil ammonia oxidizers.

Similarly, little is known about the ecophysiology of ammonia-oxidizing populations that might dominate upland tropical soils where low redox conditions may result from a combination of high biological oxygen demand, ample organic carbon, warm temperatures, and fine textured soils that limit diffusive transport of oxygen (Silver et al., 1999; Pett-Ridge et al., 2006; Liptzin et al., 2011). Most of what is known is derived from studies of AOB. In freshwater sediments, nitrification begins quickly following reintroduction of oxygen into previously anoxic sediments (Jensen et al., 1993; Kowalchuk et al., 1998), and nitrifier populations are known to persist in sites with periodically reduced conditions (Hall & Jeffries, 1984; Smorczewski & Schmidt, 1991; Bodelier et al., 1996). However, the survival mechanisms of these nitrifiers during periods of prolonged anaerobiosis are not well known. It has been suggested that under low-oxygen tension, ammonia oxidizers may use nitrite as a terminal electron acceptor (Poth, 1986; Bock & Koops, 1992) to preserve available oxygen for ammonia monooxygenase, or exploit spatial segregation at oxic-anoxic (Jensen et al., 1993) or rhizosphere interfaces (Christensen & Sørensen, 1986; Laan et al., 1989; Both et al., 1992). Based on evidence from a limited number of culture-based studies and geographic meta-analyses, growth and presence of AOA populations appear to be intriguingly insensitive to oxygen availability (Bouskill et al., 2012; French et al., 2012; Mosier et al., 2012).

In this study, we explored the effects of variable redox regimes on nitrification potential and AOA/AOB communities in tropical soils that occur along a gradient of oxygen availability. We hypothesized that humid tropical forest soils may be marginal environments for ‘classical’ chemolithotrophic nitrifiers (due to low pH and frequently reduced conditions); tropical soils constitute profoundly different environments than many temperate forests and as a result may harbor phylogenetically novel microbial communities (Borneman & Triplett, 1997; Kim et al., 2007). As the upland soils in the Luquillo Experimental Forest (LEF), Puerto Rico, are characterized by substantial gross nitrification rates (Pett-Ridge et al., 2006; Templer et al., 2008), we also hypothesized they might contain redox-tolerant ammonia-oxidizing communities. We used a natural field gradient as well as laboratory manipulations to characterize the natural variability in ammonia oxidizer composition and function, and to estimate the flexibility of these communities’ functional capacities.

Materials and methods

Site description

Soil samples were collected from three forest types (cloud, colorado, tabonuco) along a macro-climate elevation gradient (see Table 1) of approximately 10 km in the Luquillo Experimental Forest, an NSF sponsored Long-Term Ecological Research site in Puerto Rico, USA (18°18'N, 65°50' W). The climate is aseasonal with relative humidity of 98% (Weaver, 1994). Soils are highly weathered clay loam ultisols derived from volcanoclastic sandstone and are rich in ferromagnesium minerals (Beinroth, 1982). The short cloud forest association (cloud), which is also sometimes called ‘Dwarf’ forest, occurs on ridges at the highest elevations in the Luquillo Mountains and consists of dense, epiphyte-rich forest dominated by Tabebuia rigida that average 3 m in height, on frequently saturated soils. A thick humic-rich organic horizon overlays mineral soils with a mean bulk O₂ of 3.0 ± 0.8% and consistently <10% soil O₂ (Silver et al., 1999; Liptzin et al., 2011). The ‘colorado’ forest type is dominated by Cyrilla racemiflora with an average of 40 tree species per hectare (Brown et al., 1983). Soils have an average of 8% organic matter, with little litter accumulation. Bulk O₂ concentrations measured in the upper layers average 13 ± 0.2%, but fluctuate between 0 and 21% on a time scale of days to weeks, rarely remaining below 10% for longer than 1 month (Silver et al., 1999). Tabonuco forest occurs on lower slopes and has well-developed stands where larger trees exceed 30 m in height are dominated by Dacryodes excelsa. For this study, tabonuco soils were collected from sites in the Bisley Research Watersheds that are typically aerated (50% of the time with >10% soil O₂ (Liptzin et al., 2011)) and have little litter layer accumulation. All cores were collected from upper slope.
positions near permanent plots where belowground O₂ has been monitored biweekly for many years (Silver et al., 1999). Additional site details are included in Table 1.

**Redox incubation**

In each forest type, 100 1" diameter intact soil cores were collected from 0 to 10 cm mineral soil depth within a 30 x 6 m plot. Cores were sampled at five random locations within the plot; at each location, sets of immediately adjacent cores were collected and randomly assigned to static and fluctuating redox treatments (see below) and three harvest dates (T₀, T₁.₅ weeks, T₃ weeks). Additional cores from the colorado forest type were collected and incubated for two further harvest points (T₈ weeks, T₃₆ weeks). In the field, cores were gently extruded from the sampler and immediately wrapped in 10 x 10 cm mesh ‘sleeves’, to maintain intact soil structure yet allow gas diffusion into all sides of the core. Placed in airtight bags, cores were kept at ambient temperature (~20 °C) and transported to UC Berkeley within 2 days. Upon arrival, each group of adjacent cores was placed in a sealed airtight jar; five cores per forest type were kept separate for T₀ ‘initial’ analysis. Jar headspace was carefully controlled to estimate the effects of static and fluctuating redox regimes (Pett-Ridge & Firestone, 2005; Pett-Ridge et al., 2006) using the following treatments: (1) ‘static aerobic’ jars constantly flushed with humidified air; (2) ‘static anoxic’ jars flushed with humidified N₂ gas; and (3) ‘fluctuation’ jars where flushing alternated between ambient air and N₂ gas every 4 days. Flushing was regulated at 66 mL min⁻¹, yielding a complete headspace turnover in 8 min. All manipulation of cores destined for the ‘static anoxic’ treatment was performed in an anaerobic glove box. Soils were incubated at 20 °C (to simulate field T) and were harvested at 0, 1.5, and 3 weeks (plus 8 and 36 weeks for colorado soils). Periodic measurements of headspace O₂, N₂O, and CH₄ gas concentrations indicated that N₂ flushing had the desired effect in lowering soil redox, as evidenced by dramatically increased CH₄ and N₂O fluxes, and zero headspace O₂.

At each harvest point, a randomly selected core was removed from each jar, and soil (approximately 35 g dry weight equivalent) was homogenized in a sealed bag for potential nitrification analysis. A 2 g portion from the field-fresh soil was frozen at −80 °C for molecular community analysis. The remainder was used to estimate soil moisture and potential nitrification rates.

**Nitrification potential assays**

Potential nitrification rates for each core were determined using established procedures (Hart et al., 1994; Norton & Stark, 2011). Specifically, 7 g soil (dry weight equivalent) was added to flasks with 100 mL of 1 mM phosphate buffer at pH 7.2 and 1.5 mM NH₄SO₄. Soil slurries were shaken at 150 r.p.m. at room temperature in aerobic flasks. At five time points (1.5, 4, 8, 12, and 24 h), flasks were firmly shaken to fully homogenize the suspensions, and a 10 mL aliquot was transferred to a centrifuge tube and immediately placed on ice. Five drops of flocculant solution (0.5 M CaCl₂, MgCl₂) were added before centrifuging for 15 min at 3000 r.p.m. at 10 °C. Five milliliters of clear supernatant were decanted and analyzed for NO₃⁻ + NO₂⁻ concentration by colorimetry (Lachat Quik Chem flow injection analyzer, Lachat Instruments, Milwaukee, WI) within 24 h. Nitrification potential rates were calculated from the linear

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Table 1. Site characteristics for tabonuco, colorado, and cloud (dwarf) forest sites in the Luquillo Experimental Forest, PR. Soil moisture (%) and pH values are means of 20 soil cores per forest type measured at the outset of this experiment.

<table>
<thead>
<tr>
<th>Forest type</th>
<th>Life zone</th>
<th>Soil classification*</th>
<th>Elevation (masl)</th>
<th>Mean annual temperature (°C)</th>
<th>Mean annual precipitation (mm)</th>
<th>Soil O₂ (010 cm depth) (%)</th>
<th>Soil C (Mg ha⁻¹)</th>
<th>Bulk density</th>
<th>Mean soil moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloud</td>
<td>Lower montane</td>
<td>Very fine Humic Hapludox</td>
<td>7501050</td>
<td>19</td>
<td>5000</td>
<td>3 ± 0.8</td>
<td>74</td>
<td>0.52</td>
<td>5.4</td>
</tr>
<tr>
<td>Colorado</td>
<td>Lower montane</td>
<td>Urtudo Dark brown loam</td>
<td>600750</td>
<td>21</td>
<td>4500</td>
<td>13 ± 0.2</td>
<td>45</td>
<td>0.55</td>
<td>4.8</td>
</tr>
<tr>
<td>Tabonuco</td>
<td>Subtropical rain</td>
<td>Clay ultisol</td>
<td>300600</td>
<td>25</td>
<td>3500</td>
<td>21 ± 0.0</td>
<td>22</td>
<td>0.60</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Different lowercase letters indicate significant differences in soil moisture between forest types at α = 0.05.

*Huffaker (2002).
†Silver et al. (1999).
‡Liptzin et al. (2011).
slopes of a regression of $\text{NO}_3^- + \text{NO}_2^-$ concentration vs. time.

Acetylene ($\text{C}_2\text{H}_2$) inhibition assays were run at the T0 and T3 weeks harvests in a parallel set of closed flasks to assess the potential for ‘heterotrophic’ nitrification (Hart et al., 1994). For these assays, acetone-free $\text{C}_2\text{H}_2$ was added to sealed flasks at 10 ppmv (10 Pa) at the outset of the assay. Acetylene was purified by passing over 5 N NaOH, 5 N $\text{H}_2\text{SO}_4$, and CaCl$_2$ as per Hyman & Arp (1987). Flasks were later opened to allow subsampling for $\text{NO}_3^- + \text{NO}_2^-$ analysis (as above); however, autotrophic nitrification is thought to be inhibited by exposure to $\text{C}_2\text{H}_2$ for up to 3 days (Berg et al., 1982; Bodelier & Frenzel, 1999). To determine whether methane oxidizers were ‘promiscuously’ oxidizing NH$_4^+$, CH$_4$ was added at the T2 months harvest to the headspace of a replicate set of flasks. As commonly used inhibitors of methane oxidation (difluoromethane, C$_2$H$_2$, CH$_3$F) (Miller et al., 1998), we chose to saturate CH$_4$ oxidizers with high [CH$_4$] based on established K_m values (Conrad, 1995). In this test, CH$_4$ was injected into sealed flasks at the outset and after each sampling point to maintain a headspace concentration of 500–1000 ppmv.

**Molecular assays**

Soil community DNA was extracted in triplicate from 0.25 g of three field-fresh soil samples (‘initial’) per forest type using the MoBio PowerSoil kit and the manufacturer’s guidelines, except that bead beating was used for cell lysis (30 s at 5.5 m s$^{-1}$) (BIO–101 bead beater, Savant), and extracted DNA was eluted with 50 µL elution buffer. The total amount of DNA was quantified spectrophotometrically using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). Quantitative PCR (qPCR) using a SYBR Green I approach was performed to assess the abundance of 16S rRNA genes (total bacterial and archaeal gene copy numbers) and amoA genes (bacterial and archaeal ammonia oxidizers). Total bacterial and archaeal 16S rRNA genes as well as archaeal (AOA) and bacterial (AOB) amoA genes were amplified using previously published primers (primers and PCR conditions are described in Supporting Information, Table S1). Samples were run in triplicate, and all qPCR reactions were conducted on an iCycler thermal cycler equipped with an optical module (Bio-Rad). Single-qPCR reactions were prepared in a total volume of 20 µL including 10 µL of iQ SYBR Green super mix (Bio-Rad), 4 µL of forward and reverse primers (3 µM) (Sigma-Aldrich), 1 µL PCR grade MQ-water (MP Biomedicals), and 1 µL of template DNA (2 ng µL$^{-1}$). The presence of PCR inhibitors like co-extracted humic substances was tested by mixing known amounts of standard DNA in soil DNA extracts prior to qPCR and comparing the quantification of standard DNA with and without DNA extracted from soil. We detected no inhibition of the qPCR assay with template DNA concentrations below 10 ng µL$^{-1}$. At the end of each qPCR run, a melting curve was conducted from 55 to 99 °C with an increase of 0.5 °C every 10 s, and purity of the amplified fragment was checked by the observation of a single melting peak. Also an agarose gel (1%) was run with the qPCR products to check for correctly sized amplicons. The abundances of amoA genes were expressed relative to the total number of bacterial plus archaeal 16S rRNA genes as well as in absolute values (gene copies per gram soil) across the three sites. The average amount of DNA extracted was not significantly different between the three sites ($P > 0.1$).

**Cloning**

DNA extracts from four replicate cores taken from different locations within the 30 × 6 m plots (described above) were composited to generate a representative sample for each forest type. The archaeal amoA gene was cloned from the three soil types using the TOPO TA Cloning Kit with the PCR2.1–TOPO vector (Invitrogen) following the manufacturer’s instructions. The qPCR thermocycling program described above was used for the PCR prior to cloning. The mastermix was composed of 0.6 pmol µL$^{-1}$ Arch-amoA and Arch-amoAr, 0.03 U µL$^{-1}$ ExTaq (Takara), 0.8 mM dNTPs, 0.8 mg mL$^{-1}$ BSA, and 1× buffer. Approximately 30 clones from each site were selected for Sanger sequencing. We found it extremely difficult to amplify bacterial amoA, and we were not able to clone AOB from these soils. Our amplification efforts included using the amoA gene-based primers amoA-1F (5’GGGGTTTTCTACTGTTGTT) and amoA-2R (5’CCC CTCKGSAAGCCTTCCTTC; K _ G or T; S _ G or C) (Rotthauwe et al., 1997; Mendum et al., 1999; Stephen et al., 1999; Mintie et al., 2003), step-down PCR routines, and various dilutions, but all were unsuccessful. We also attempted to use the ammonia oxidizer specific ‘CTO’ primer sets bAOBF-bAOBr (McCag et al., 1994) and TO189f-CTO654r (Kowalchuk et al., 1997) (using protocols outlined in Kowalchuk et al., 1997; Bruns et al., 1999; Mendum et al., 1999; Nicolaisen & Ramsing, 2002; Webster et al., 2002). In all cases, *Nitrosonomas europaea* positive controls produced successful products, but AOB in these Puerto Rican soil samples never amplified.

**Archaeal amoA phylogeny**

To evaluate the phylogenetic relationships of our Puerto Rican soil amoA sequences, a global archaeal amoA tree
was assembled from sequences retrieved from the Genbank Nucleotide database (as of January 9, 2011). We retrieved 15260 amoA sequences longer than 500 bp, and from this master list, removed bacterial sequences, sequences from unpublished studies, sequences that did not state an isolation source, sequences that contained the ambiguous base ‘N’, and duplicate sequences. We aligned 5005 unique sequences with a length of 538 bp using MUSCLE (Edgar, 2004) and edited the sequences using Geneious (Biomatters Ltd). To map phylogenetic clusters on the global amoA tree, 53 phylogenetic reference sequences were aligned with our 5005 sequence database prior to tree analysis. The reference sequences were obtained from the ARB database created by Pester et al. (2012) (see Table S2 for GenBank accession numbers; obtained from the ARB database created by Pester base prior to tree analysis. The reference sequences were aligned with our 5005 sequence data base prior to tree analysis. The reference sequences were obtained from the ARB database created by Pester et al. (2012) (see Table S2 for GenBank accession numbers; within the Pester et al. (2012) database, the sequences are listed under the saved Species Selections entitled ‘cluster_representatives’).

We used the program CONSEL (Shimodaira & Hasegawa, 2001) to evaluate five maximum likelihood trees created by FastTree (Price et al., 2010) using the generalized time reversible model. The trees evaluated had the following parameters: (1) FastTree with 20 categories; (2) FastTree with 25 categories; (3) FastTree with a slow initial topology search (20 categories); (4) FastTree supplied with an initial starting topology created by RAxML (Stamatakis, 2006; Stamatakis et al., 2008); (5) FastTree supplied with an initial starting topology created by RAxML with no outgroup (ungapped alignment), outgroup then added to the tree using the ARB parsimony tool (Ludwig et al., 2004), and branch lengths refined using the FastTree GAMMA20 tool. Based on CONSEL analysis, we selected tree #5 for the global AOA tree topology. FastTree branch support values were calculated by the Shimodaira–Hasegawa test implemented in FastTree with 1000 replications (Shimodaira & Hasegawa, 1999). The RAxML trees provided as starting trees for FastTree were resampled 500 times using the fast bootstrapping tool (GTRCAT for bootstrapping, GTRGAMMA for final tree inference) implemented by the CIPRES Science Gateway (Miller et al., 2010).

A large circular tree image was created using the Interactive Tree of Life (Letunic & Bork, 2007) and rooted on the bacterial amoA sequence from N. europaea. The designations ‘soil’, ‘water’, and ‘sediment’ were determined using metadata from the Genbank files; the original references were consulted directly where clarification was needed on the source location of samples. Weighted UniFrac analyses were used to determine whether the soil, water, and sediment clusters formed distinct groups within the global amoA tree using mother (Schloss et al., 2009). Sequences isolated from other types of environments were listed as ‘unclassified’ in the UniFrac analysis and were left uncolored on the global tree (e.g. sequences isolated from corals, sponges, wastewater treatment plants, speleothem biofabrics from vapor caves, and microbial mats).

Smaller trees illustrating the detailed phylogeny of our Puerto Rican soil clones were pruned from the global amoA tree and modified in ARB (Ludwig et al., 2004). Subcluster designations for the smaller trees were determined by inserting the Puerto Rican amoA sequences into the Pester et al. (2012) consensus tree for the archaeal amoA phylogeny using the parsimony tool in ARB (Ludwig et al., 2004). To determine whether the Puerto Rican sequences grouped with amoA sequences from other somewhat acidic environments, the Puerto Rican amoA sequences were affiliated with the pH database from Gubry-Rangin et al. (2011) using the methods described therein. Weighted UniFrac scores and rarefaction curves were calculated for the Puerto Rico archaeal amoA sequences using mothur (Schloss et al., 2009). The Phylip distance matrix needed to create the rarefaction curves was created by SplitsTree4 (Huson & Bryant, 2006). DNA percent identities were calculated with CLUSTALX 2.1 (Larkin et al., 2007).

Statistical analyses

Analysis of variance (ANOVA, JMP, Version 7. SAS Institute Inc., Cary, NC) was used to test for significant changes in nitrification rates across treatments, incubation time and site (P < 0.05), using the Tukey–Kramer Honestly Significant Difference (HSD) as a multiple means comparison test. ANOVA was also used to test for difference in AOA and AOB abundance between sites. Homogeneity of variance was tested using Cochran’s test, normal distribution with Shapiro–Wilk W test (Zar, 1996). Reported means are of untransformed data and are presented ± one standard error. Outliers were tested at the 5% level according to Dixon’s Q test (Dixon & Massey, 1983); only one outlier was excluded from the entire data set (from the relative AOA gene abundance). Total nitrification was compared with heterotrophic nitrification with the Student’s t-test. Correlation analyses were performed between archaeal amoA gene abundance, potential nitrification, pH, soil moisture, soil carbon content, mean annual precipitation (MAT), mean annual precipitation (MAP), and oxygen content. Correlation statistics, including the Pearson’s product–moment correlation coefficient (r), are reported in Table S3.

Nucleotide sequence accession numbers

The archaeal amoA gene sequences determined in this experiment have been deposited in GenBank under accession numbers JX885370–JX885454.
Results

Potential nitrification

At the time of sample collection, soil moisture significantly increased with elevation along this macro-climate gradient (Table 1), although values were at the lower end of what has been previously reported for these sites (McGroddy & Silver, 2000).

Nitrification potential measured on field-fresh soil cores was significantly higher in the mid-elevation colorado forest relative to both the cloud forest and low-elevation tabonuco forest (Fig. 1) \((P = 0.01)\). Nitrification potential rates were also affected by redox incubation treatments. In all three forest types, soils incubated under a fluctuating redox regime tended to exhibit a higher nitrification capacity (Fig. 2) relative to soils kept in static \(N_2\) or \(O_2\) treatments; evidence of redox treatment effects on nitrification potential were measured at the \(T_{1.5}\) weeks harvest point (colorado soil \((P = 0.1)\), tabonuco soil \((P = 0.03)\), and at the \(T_{3}\) weeks harvest point \((P < 0.05\) in all three soils). In both the low and mid-elevation colorado and tabonuco forest soils, soils exposed to fluctuating redox had significantly higher nitrification potential rates than anoxic soils, with intermediate rates in consistently aerobic soils. In the mid-elevation colorado soils, where the incubation regimes were applied for additional weeks \((T_{8} \text{ weeks} \text{ and } T_{36} \text{ weeks})\), all soils retained the capacity to nitrify and the elevated nitrification capacity of the fluctuation treated soils persisted (Fig. 2). Rates of heterotrophic potential nitrification measured on field-fresh soils were substantial in all samples and ranged from 37% of total nitrification in colorado soils \((1.58 \pm 0.25 \mu g N \text{ g}^{-1} \text{ day}^{-1})\) to 52% in cloud forest soils \((0.96 \pm 0.31 \mu g N \text{ g}^{-1} \text{ day}^{-1})\). In nitrification potential assays where we added high concentrations of \(CH_4\) to assess whether methane oxidizers were ‘promiscuously’ oxidizing \(NH_4\), we did not measure any significant effect on potential nitrification rates.

Relative abundance of AOA and bacteria

The relative abundance of AOA \((amoA\) gene copies/total 16S rRNA gene copies from bacteria + archaea) and the absolute abundance of AOA \((amoA\) gene copies/g soil) were both higher in the cloud and colorado forest compared with low-elevation tabonuco soils \((P < 0.05\) for the absolute abundance; Table 2). We found no significant correlation between gene copy numbers and potential nitrification rates measured in the same field-fresh soils when examined across all three forest/soil types (Fig. 1). Both relative and absolute archaeal \(amoA\) gene abundances correlated positively with soil \(pH\), soil carbon content, mean annual precipitation (MAP) and negatively with mean annual temperature (MAT) and oxygen content \((P < 0.05\) (Table S3). Of the environmental parameters we measured, the only variable that correlated significantly with nitrification potential rates was \(H^+\) ion
concentration (pH expressed on a linear scale, so that a linear-linear correlation could be performed), at slightly less than the 95% confidence level \((P = 0.083, R^2 = -0.6)\). The distribution of total archaea (16S rRNA gene copies relative to total extracted DNA) exhibited a significant pattern across the gradient with the highest relative abundance in the high-elevation cloud forest soils \((P = 0.0018)\). No such pattern was found for the relative
abundance of total bacteria \( (P = 0.2211) \) (Table 2). Bacterial amoA genes could not be detected in both the qPCR and PCR assays, presumably because of low abundance of bacterial ammonia oxidizers.

**Clone libraries**

Overall, we observed 52 unique archaeal amoA sequences in the three Puerto Rican soils, which formed three distinct OTUs at the 85% identity level and 14 distinct OTUs at the 98% identity level. At the 85% level, the sequences group with the Nitrosotalea subcluster 1.1 (cloud forest), Nitrososphaera subcluster 7.2 (colorado and tabonuco forests), and one singleton that was not similar to anything in the database (Genbank accession: JX885389). Rarefaction analysis indicates that the number of OTUs detected plateau at the 98% identity level, suggesting that the archaeal amoA diversity was sufficiently sampled in these soils (Fig. S1). When placed in context of the global amoA tree, all of the Puerto Rican soil sequences cluster with sequences from other acidic environments; the colorado forest and tabonuco forest sequences grouped within the acidophilic cluster C11, while the cloud forest sequences groups within acidophilic cluster C14 (clusters from Gubry-Rangin et al. (2011), personal communication C. Gubry-Rangin).

Unifrac analysis indicates that the AOA community in the cloud forest soils was significantly different than the colorado and tabonuco AOA communities (cloud-colorado: \( D = 0.783, P < 0.001 \); cloud-tabonuco: \( D = 0.786, P < 0.001 \)). The cloud forest amoA sequences group within the Nitrosotalea subcluster 1.1, and were 90–95% similar to an acidophilic archaeal ammonia oxidizer, Nitrosotalea devanaterra (Fig. 3b), isolated from an agricultural soil. The Nitrosotalea cluster was previously referred to as AOA group I.1a (Lehtovirta-Morley et al., 2011). The cloud forest amoA sequences were most closely related to AOA sequences found in European lake sediments (96–99%) (Herrmann et al., 2009), and were also 88–93% similar to an amoA sequence from a Costa Rican rain forest soil identified by Pester et al. (2012).

The colorado and tabonuco soil archaeal ammonia oxidizers, located 1 km apart from the cloud forest site, group with the amoA lineage Nitrososphaera subcluster 7.2 (Fig. 3c). This Nitrososphaera cluster was previously referred to as AOA group I.1b (DeLong, 1998). While the colorado and tabonuco AOA communities have a substantial degree of overlap, as shown by a low Unifrac distance measure, they were statistically different (colorado-tabonuco: \( D = 0.127, P < 0.001 \)). The colorado soil sequences split into two main groups: the first was approximately 94% similar to sequences from European lake rhizosphere sediment (Herrmann et al., 2009) and the second group was 97% similar to deep sediment (Dang et al., 2009). The tabonuco sequences were 95–97% similar to sequences found in deep-sea sediments and 93–95% similar to forest peat soil (Stopnišek et al., 2010). The colorado and tabonuco soil amoA genes were 91–97% and 91–93% similar, respectively, to the indicator amoA from a Costa Rican arable soil identified by Pester et al. (2012).

**Global archaeal amoA analysis**

Within the global amoA tree, the soil, water, and sediment groups formed distinct groups by Unifrac analysis. The Unifrac D metric measures the difference between two environments in terms of the tree branch length that is unique to one environment relative to another environment. Where \( D = 1 \), this indicates that the two environments have completely different taxa; \( D = 0 \) indicates that all taxa are present in both environments (Lozupone & Knight, 2005). The soil and water clusters in the global amoA tree were almost completely distinct (soil-water: \( D = 0.96, P < 0.001 \)). The sediment cluster was also distinct from the soil and water clusters, but there were more overlaps between sediment-derived amoA sequences, resulting in lower D scores (sediment-water: \( D = 0.81, P < 0.001 \); sediment-soil: 0.57, \( P < 0.001 \)). Branch splits in the global amoA tree were well supported according to the Shimodaira–Hasegawa test (Fig. S2). Two Nitrososphaera marker sequences determined by Pester et al. (2012) clustered with the Nitrospumilus section of the tree (GenBank accession numbers FN562532 and DQ304868).

**Discussion**

Relatively few studies have explored whether tropical soil microbial communities are phylogenetically or physiologically distinct from those in temperate or arctic soils. There is reason to expect that tropical communities might be unique, as wet tropical forest soils are characterized by highly dynamic soil \( O_2 \), low pH, and ample availability of organic C, a combination which likely imposes unique selective pressure on soil microbial communities. How these edaphic regulators affect the balance of AOB/AOA activity and abundance in tropical soils has not been explored previously. Classically defined chemolithotrophic ammonia-oxidizing communities would seem to be particularly challenged by variable redox conditions, as ammonia monoxygenase requires molecular oxygen. Yet, the substantial rates of gross nitrification in Puerto Rican and Costa Rican soils imply that tropical ammonia oxidizers must be resilient to anoxic exposure (Pett-Ridge & Firestone, 2005; Silver et al., 2005; Pett-Ridge et al., 2006; Templer et al., 2008). Using both a natural gradient
of oxygen availability, and laboratory incubations designed to push soil microbial communities to experience atypical redox extremes, we explored the abundance and functional capacity of tropical soil ammonia oxidizers.

In the series of tropical soils from which we sequenced amoA genes, AOA strongly outnumber AOB. While total bacterial 16S rRNA genes far outnumbered archaeal 16S rRNA genes, in the wet tropical soils we examined we were only able to detect AOA amoA genes. Our repeated inability to amplify and clone AOB from these soils could be because currently available primer sets are not good matches for Puerto Rican soil AOB communities; however, multiple other experiments (using shotgun sequencing, rRNA oligonucleotide microarrays, and transcript sequencing) have also failed to detect AOB 16S or amoA genes and are further evidence of their scarcity in the LEF soils (E. Dubinsky and E. Brodie pers. commun.).

One plausible explanation for the dominance of AOA in the LEF soils is the relatively low soil pH (3.9–5.4); multiple studies suggest some specific groups of AOA may prefer pH niches below pH 5.5 (Nicol et al., 2008; Gubry-Rangin et al., 2010; Yao et al., 2011; Prosser and Nicol, 2012). We note that the obligate acidophile *N. devanaterra* (Lehtovirta-Morley et al., 2011) is closely related to the AOA we identified in the upper-elevation cloud forest soils which have a mean pH of 5.4. In addition, the LEF soil sequences are affiliated with the acidophilic clusters C11 and C14 from Gubry-Rangin et al. (2010), indicating that the LEF’s AOA are related to ammonia oxidizers from other acidic environments. Finally, our study is not alone in finding bacterial amoA genes conspicuously absent; at least two other studies examining acidic soil have found a similar pattern (Stopnišek et al., 2010; Levičník-Höfferle et al., 2012).

Our results also suggest that heterotrophic nitrifiers, which are thought to be more tolerant of acidic conditions, may play a significant role in the LEF soils. Heterotrophic nitrification potential, which we measured via acetylene inhibition of autotrophic nitrification, was highest in the low-elevation tabonuco forest soils, where pH is particularly low, but heterotrophic nitrification was substantial in all three soil types. Although not well understood (Pedersen et al., 1999), heterotrophic nitrification can be significant in temperate acidic forest soils (Schimel et al., 1984) and has been attributed to a small group of bacteria and eukaryotes (fungi) (Stein, 2011), but has not been investigated in tropical soils. It is not clear whether AOA are completely inhibited by C2H2 in the assay for heterotrophic nitrification (Gubry-Rangin et al., 2010). In some studies, AOA are clearly inhibited by C2H2 (Offre et al., 2009; Taylor et al., 2010), but the contrary has also been shown (Jia & Conrad, 2009). It is intriguing to consider that the operationally defined assay we used to measure heterotrophic nitrification may include the activity of some AOA, particularly taxa which might depend on heterotrophic and/or mixotrophic catabolism (Jia & Conrad, 2009; Pester et al., 2011).

While little is known about the ecological preference of heterotrophic AOA, in the LEF’s tropical soils, high amoA copy number was significantly correlated with ample organic carbon content. However, multiple environmental controllers co-vary in these systems (high precipitation, soil moisture, and C content correspond with low soil O2, pH, and temperature) and the proximal controllers of AOA activity and abundance are likely more complex than simply pH or soil carbon availability. We conclude that ammonia oxidation in the LEF soils is certainly not due to the activity of promiscuous methanotrophs, but may well be credited to a combination of both autotrophic and heterotrophic AOA as well as chemoorganotrophic bacteria and even fungi.

In addition to low pH, tropical nitrifiers must cope with the fluctuating oxygen availability characteristic of tropical soils. This is a third factor that may favor AOA relative to AOB in the LEF soils and may also be driving the distinction between the LEF’s *Nitrosotalea*-like cloud forest vs. *Nitrososphaera*-like colorado and tabonuco AOA communities. It has also been proposed that the pathway of ammonia oxidation in AOA may be distinct from the AOB pathway, theoretically requiring only 0.5 O2 per NH3 oxidized (Walker et al., 2010). This hypothesized lower oxygen demand could potentially explain why AOA seem to persist more readily under low-oxygen conditions (Schleper & Nicol, 2010). Both environmental and culture-based studies have shown that AOA can occur at oxic-anoxic interfaces (Francis et al., 2005; Bouskill et al., 2012) and have a low Km for O2 (Martens-Habbena et al., 2009; Jung et al., 2011). Our results from the LEF soils suggest these tropical soil AOA not only tolerate periods of low oxygen but (as has also been documented in waste water treatment plants) can numerically and functionally outcompete AOB in alternating aerobic/anaerobic conditions (Park et al., 2006).

In this study, we hypothesized that soil nitrification potential would mirror amoA gene abundance across the three sites. In the low elevation and dramatic redox fluctuating sites (colorado and tabonuco) with apparently similar communities (*Nitrososphaera*-like), higher gene copy numbers correspond to higher rates of potential nitrification (Fig. 1). However, in the cloud forest with its distinct *Nitrosotalea*-like AOA community, this relationship was not apparent. We hypothesize that these differences between sites may be caused by different pH sensitivities, ammonia affinities, carbon substrate preferences or oxygen tolerances of the *Nitrosotalea*-like vs. *Nitrososphaera*-like AOA groups.
These observations raise the question of whether habitat, for example, the combination of edaphic factors (O₂, moisture, temperature, pH, soil C) can regulate the size and composition of the ammonia-oxidizing community, but not necessarily its functional potential. In the colorado soils, persistent high-amplitude redox fluctuations may have led to a community that is poised to quickly oxidize ammonia whenever it becomes available. Because of the oscillation between aerobic and anaerobic conditions that routinely occurs in the colorado soils, NH₃ is constantly regenerated via dissimilatory nitrate reduction to ammonia (DNRA) (Templer et al., 2008), and with a subsequent influx of O₂, this NH₃ becomes re-available to AOA communities. Previous redox fluctuation experiments with the LEF colorado soils showed elevated NH₃ pools and higher gross N mineralization rates immediately following periods of anaerobiosis (Pett-Ridge et al., 2006).

Soils incubated under a fluctuating redox regime almost always had higher nitrification potential rates (near or equivalent to the rates measured in field-fresh soils) relative to soils incubated in either static N₂ or O₂ treatments. In the colorado soils, nitrification capacity persisted even after up to 36 weeks of anoxia, indicating that the nitrifiers indigenous to these humid tropical soils have evolved a mechanism that allows them to tolerate prolonged anoxic periods. These results, along with the differences we measured in the natural capacity of the tabonuco vs. colorado vs. cloud forest soils, suggest that a highly dynamic environment may engender nitrifying populations that are optimized to tolerate temporary anoxia and oxidize ammonia faster than the taxa indigenous to consistently anaerobic or aerobic soils. It would be interesting to test whether this capacity is a characteristic separating the Nitrosotalea vs. Nitrososphaera clusters that distinguish the cloud vs. colorado/tabonuco LEF AOA communities.

In general, potential nitrification assays are done in well-mixed aerobic slurries and incubated at neutral pH; while the measured rates are commonly presumed to reflect the Vmax of the indigenous community, there can be problems with this assumption (Hart et al., 1994). In our study, as the potential nitrification rates were measured at pH of 7.2, in well-mixed aerobic assays, the rates determined may not reflect in situ potentials for the field soils with lower pH values and varying oxygen availabilities. However, the use of a standard procedure makes comparisons with other studies more appropriate. The higher potential nitrification rates in the tabonuco and colorado soils previously incubated under fluctuating conditions could have resulted from increased numbers of nitrifiers. Also, previous work has shown higher gross N mineralization rates under fluctuating redox conditions (Silver et al., 2001; Pett-Ridge et al., 2006); hence, ammonium availability may be greater with redox oscillation.

With a global analysis of archaeal amoA gene sequences, we determined that the soil environmental sequences were mostly comprised of the Nitrososphaera and Nitrosotalea clusters (containing the LEF AOA communities). The environmental sequences from water, on the other hand, appeared primarily restricted to the Nitrosopumilus cluster, although only three of the marker sequences analyzed fell into this category. The sediment environmental sequences were the most varied and had members from Nitrosopumilus, Nitrosotalea, and Nitrososphaera lineages. These results differ slightly from those of Pester et al. (2012), mainly because the environmental categories in Pester et al. (2012) did not include a sediment category for freshwater and marine environments. Also, two of the Nitrososphaera marker sequences consistently clustered with the Nitrosopumilus clusters in our tree analyses. This suggests that the phylogenetic clusters determined by Pester et al. (2012) may need to be refined. Our observation of distinct water, sediment, and soil/sediment clusters confirms the pattern originally observed by Francis et al. (2005).

While AOA in the LEF soils may have evolved strategies to tolerate extended periods of low oxygen, their amoA sequences suggest they are fairly closely related to existing sediment AOA clones. Within the global tree, the tropical LEF soils harbor two distinct lineages of amoA, Nitrososphaera subcluster 7.2 and Nitrosotalea subcluster 1.1. Interestingly, even though the LEF sites are only separated by ~1 km, each soil is dominated by a particular lineage, where Nitrososphaera 7.2 dominates the cloud forest and Nitrosotalea 1.1 dominates the colorado and tabonuco forests. This suggests that the environmental conditions present at these sites are more important for selecting AOA composition than biogeographic distance alone.

The majority of the archaeal amoA sequences described in this study clustered with sequences from acidic, water-saturated environments with high organic content from freshwater sediments, deep-sea sediments, and other tropical soils (Dang et al., 2009; Herrmann et al., 2009; Pester et al., 2012). Interestingly, the one other published upland tropical forest amoA sequence (from a pH 4.6 Costa Rican soil) falls into the same cluster as those found in the current study, Nitrosotalea subcluster 1.1 and Nitrososphaera subcluster 7.2. (Pester et al., 2012). It is tempting to speculate that the conditions found in tropical soils (low pH and frequently low O₂) may select for these specific lineages of ammonia oxidizers, although much more molecular microbial diversity data for tropical soils and additional characterization of soil AOA isolates are needed to assess the ubiquity of this pattern.
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References


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Redox fluctuation shapes tropical soil AOA community

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Conditions used for quantitative PCR of group specific one primers.

**Table S2.** Reference sequences and associated Pester et al. (2012) phylogeny used to overlay amoA phylogenetic clusters on the global AOA tree.

**Table S3.** Correlation statistics for archaeal amoA gene abundances (g⁻¹ soil and /total bacteria+archaea) and environmental variables

**Fig. S1.** Rarefaction analysis of unique and 98% identity sequenced amoA clones from three forest types in the Luquillo Experimental Forest, Puerto Rico: high-elevation cloud forest, mid-elevation colorado forest and low-elevation tabonuco forest.

**Fig. S2.** Global amoA maximum likelihood tree with branches colored by confidence scores for each split (green > 75% support, yellow > 50% support, red < 50% support).