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Publication Date

2016-11-01

DOI

10.1016/j.apsoil.2016.05.012

Peer reviewed

Applied Soil Ecology 107 (2016) 48–56

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Land-use change drives abundance and community structure alterations of thaumarchaeal ammonia oxidizers in tropical rainforest soils in Rondônia, Brazil

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ARTICLE INFO

Article history:
Received 23 January 2016
Received in revised form 4 May 2016
Accepted 16 May 2016
Available online xxx

Keywords:
Ammonia-oxidation
Thaumarchaea
Rainforest
Land-use change
Brazil
Nitrosotalea

ABSTRACT

The Amazon Rainforest plays major roles in global carbon and nitrogen cycling. Despite this region's immense importance, deforestation and pasture creation are still occurring at alarming rates. In this study, we investigated the effects of land-use change on aerobic ammonia-oxidizers in primary rainforest, young and old pasture, and secondary forest in Rondônia, Brazil. Forest-to-pasture conversion decreased soil nitrate, phosphorus, and exchangeable acidity contents that recovered to pre-disturbance levels as pastures aged or were abandoned and formed secondary forest. The ammonia-oxidizing community, numerically dominated by thaumarchaea, shifted due to land-use change, both in terms of gene abundance and community structure. However, thaumarchaeal ammonia monooxygenase gene abundances did not correlate with any measured soil physicochemial parameters. Phylogenetic analyses showed that community structural changes in ammonia-oxidizing thaumarchaea are driven by a shift away from primary rainforest, old pasture, and secondary forest clusters to separate clusters for young pasture. Additionally, the nearly complete disappearance in young pasture, old pasture, and secondary forest sites of a thaumarchaeal genus, the *Nitrosotalea*, indicates that land-use change can have long lasting effects on large portions of the thaumarchaeal community. The results of this study can be used as a conceptual foundation for determining how ammonia-oxidizers become altered by land-use change in South American tropical forests.

1. Introduction

Amazônia is a region of forest and savannah inside the Amazon River basin and encompasses an area of approximately $6.7 \times 10^6\,km^2$, nearly equivalent to the size of Australia. The rainforest in Amazônia plays a major role in both regional and global carbon, nitrogen, and water cycles and global temperature patterns. For example, the trapping of CO_2 and the evapotranspiration that occurs in the rainforest aids in regulating the warming of the planet (Bala et al., 2007; Davidson et al., 2012).

Despite this importance, slash-and-burn conversion of Amazonian rainforest to pasture and cropland is still occurring at an alarming rate, exposing this deforestation frontier to the "boom-and-bust" patterns that occur in the timber, cattle, and crop industries (Cardille and Foley, 2003; Houghton et al., 2000; Lapola et al., 2014; Rodrigues et al., 2009). Much work has been done to document the change in macroogranism community structure and alteration of forest-related ecosystem services after the slash-and-burn process and development of cropland (Andrade et al., 2015; Eva et al., 2004; Feigl et al., 2006; Foley et al., 2007). More recent work has been performed to investigate the effects of land-use conversion on microbial communities inhabiting the soil of the rainforests. These microbiological studies have either concentrated on how the structure of microbial communities differs in various Amazonian soil types (Borneman and Triplett, 1997; Kim et al., 2007; Rodrigues et al., 2013) or on the altered edaphic factors

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http://dx.doi.org/10.1016/j.apsoil.2016.05.012
0929-1393/

which affect soil bacterial community structure (Jesus et al., 2009). Some recent studies have focused on how land-use conversion effects specific functional groups of microbes, such as those involved in globally important carbon and nitrogen (N) biogeochemical cycles (Mirza et al., 2014; Navarrete et al., 2011; Paula et al., 2014; Taketani and Tsai, 2010).

Perturbations to the N-cycle can alter the amount of ammonium (NH_4^+) or nitrate (NO_3^-) available for microbial or plant usage as well as the amount of the ozone degrading gas nitric oxide (NO) and the potent greenhouse gas nitrous oxide (N_2O) released into the atmosphere (Levy-Booth et al., 2014; Madigan and Martinko, 2006). There have been numerous studies investigating the effects of deforestation on soil N nutrient availability and on biogeochemical N-cycling dynamics (Cerri et al., 2006; Davidson et al., 2004; de Moraes et al., 1996; Neill et al., 1999, 1997, 1995). The studies by Neill et al. (1999, 1997, 1995) have shown a build up of NH_4^+ and a decrease in NO_3^- in pasture soils compared with primary rainforest soils along with decreases of net and gross nitrification and mineralization rates. These findings indicate that processes centered on the production and oxidation of NH_4^+ in rainforest soil are affected by conversion to pasture.

In order to better understand the impact of land-use change on the taxonomy and phylogeny of specific groups of N-cycling organisms, the objective of this current study was to analyze the changes in the community abundance, structure, and diversity of ammonia-oxidizing bacteria (AOB) and thaumarchaea (AOA) in Western Amazonian soils under different land uses. This current study focuses on phylogeny and gene abundance, instead of repeating rate measurements, in order to complement the work of Rodrigues et al. (2013) who found conversion of forest to pasture significantly increased bacterial OTU richness and diversity and led to biotic homogenization of bacterial OTUs in pasture sites. Based upon the similar levels of functional gene richness and the association of ammonia monoxygenase genes with primary and secondary forest sites as seen by Paula et al. (2014), we hypothesized that the abundances and community structures of the AOB and AOA will not be resistant to land use change from forest to pasture. However, we predict such communities will show resiliency and return to pre-disturbance community abundances and structures when pastures are abandoned and secondary forest is allowed to form. Similar to the studies by Navarrete et al. (2011) and Paula et al. (2014), we investigated the effects of land-use change on ammonia-oxidizing communities in tropical forest soil. However, this current study goes beyond the intial GeoChip, DGGE, and Sanger sequencing findings of Navarrete et al. (2011) and Paula et al. (2014) to determine absolute *amoA* gene copy abundance and to investigate the phylogenetic structure of AOA communities in rainforest soils undergoing land-use disturbance using next-generation sequencing of the *amoA* gene.

2. Materials and methods

2.1. Sampling site description, soil processing, and soil chemistry characterization

In order to study the effects of deforestation on soil microbial communities, a chronosequence, or time-series, of primary forest, pasture, and secondary forest was sampled at the Amazon Rainforest Microbial Observatory [ARMO; (Rodrigues et al., 2013)], located at the Fazenda Nova Vida (cattle ranch; 10°10′5″s and 62°49′27″W) in the State of Rondônia, Brazil. This site has been used in numerous studies cataloging the effects of deforestation on soil chemistry, gaseous emissions from soil, and on N-cycling rates in soil (Cerri et al., 2006; Neill et al., 2005, 1999, 1997, 1995; Steudler et al., 1996). The chronosequence used in this study contained a total of four plots. One plot consists of primary, untouched rainforest while another is secondary forest re-growth. The remaining two plots are pastures established by deforestation in 1911 and 2004. These pastures were chosen to determine the effects of deforestation on microbial communities in sites that had undergone recent and long-term (50+ years) disturbance. The 2004 pasture, primary rainforest, and secondary rainforest are approximately one kilometer away from each other. The 1911 pasture is approximately five kilometers away from the other three sites of the chronosequence. Pastures were seeded with the grasses *Brachiaria brizantha* and *Panicum maximum*. The pastures were established using slash-and-burn techniques. None of the pastures have been subjected to chemical fertilization or tillage treatments. However, two years after establishment, pastures were burned in order to control weed growth. Also, larger weeds were removed by hand when found by ranch workers.

In each land use treatment three soil cores were collected along a transect. The first and second cores were separated from each other by a 10 m distance while the first and third cores were separated by a 100 m distance. The soil cores were taken from the top 0–10 cm of the mineral soil profile. Each soil core was placed into sterile bags and mixed by hand. The mixed soil was sieved (2 mm gauge size) to remove large rocks and roots. The soils were transported on ice and stored at −20 °C before analysis. Subsamples of each soil, stored at 4 °C, were also analyzed for pH, total C and N, and other elemental analyses in previous ARMO studies (Mirza et al., 2014; Paula et al., 2014; Rodrigues et al., 2013). Total C and N were determined using a LECO Elemental Analyzer and a Costech ECS140 CHN/S/O elemental analyzer at the Centro de Energia Nuclear na Agricultura, University of Sao Paulo, Brazil, and at the Department of Geosciences, University of Massachusetts-Amherst, USA, respectively. Ammonium (NH_4^+) and nitrate (NO_3^-) contents were determined for the triplicate samples collected per land use by extracting 4.0 g soil with 40 mL of 2.0 M KCl for one hour at room temperature and 200 rpm. After filtering extracts through Whatman GF-A filters and 0.45 μm surfactant-free cellulose acetate syringe filters that were both pre-rinsed with 2.0 M KCl (D.R. Lammel, personal communication), extracts were measured using a Lachat QuikChem 8500 Flow Injection Analysis System (Hach Comp., Loveland, CO). The results for each site are summarized in Table 1.

Table 1
Comparison of soil characteristics closely related to N-cycling between different site treatments.

Site	N	C	C/N	NH_4^+	NO_3^-	pH ($CaCl_2$)
PF	0.173[a]	2.093[a]	12.163[a]	5.7[a]	**3.6[a]**	4.0[a]
	(0.035)	(0.365)	(0.365)	(2.5)	(1.1)	(0.5)
P-2004	0.109[a]	1.483[a]	12.887[a]	11.1[a]	**0.16[b]**	4.7[a]
	(0.070)	(1.143)	(1.712)	(9.5)	(0.3)	(0.3)
P-1911	0.241[a]	3.062[a]	12.916[a]	16.5[a]	**0[b]**	4.9[a]
	(0.060)	(0.522)	(1.538)	(4.3)	(0.0)	(0.1)
SF	0.150[a]	1.740[a]	11.673[a]	5.2[a]	**3.8[a]**	4.3[a]
	(0.000)	(0.030)	(0.006)	(2.5)	(1.5)	(0.5)

Notes:.
[1]Sample abbreviations: PF, pristine forest; P-2004, pasture established in 2004; P-1911, pasture established in 1911; and SF, secondary forest established in 1999.
[2]The units for each of the soil characteristics are as follows: N, nitrogen (%); C, carbon (%); C/N, carbon/nitrogen ratio; NH_4^+ and NO_3^-, μg-N (g dry soil)$^{-1}$.
[3]Sample values that do not have the same letter are significantly different from each other (one-way ANOVA-like Bayesian Comparison, 95% highest density interval of difference between means does not include zero). Soil parameters that showed significant differences are bolded. All data were square-root transformed prior to analysis in order to ensure normality of data and homogeneity of variances.
[4]The values in parentheses indicate the standard deviation of the mean for each soil parameter (n = 3).

2.2. DNA extraction

Total microbial DNA was extracted from each soil sample using the PowerLyzer PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). Triplicate DNA extractions were performed on 0.25 g soil samples from three points per land use for primary forest, 2004 pasture, 1911 pasture, and secondary forest sites. The DNA concentration and quality of each sample pooled per sampling point was determined spectrophotometrically using a Nano-Drop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE).

2.3. Quantitative PCR amplification

In order to determine the abundance of ammonia-oxidizer marker genes in soil from the different land uses, quantitative PCR (qPCR) was performed using primers targeting the gene of the α-subunit of the ammonia monooxygenase enzyme (*amoA*) from thaumarchaeal (AOA) and bacterial (AOB) ammonia-oxidizing organisms. For AOA, the primer set *amoA*19F/643R was used (Islam et al., 2008; Leininger et al., 2006; Levy-Booth et al., 2014; Treusch et al., 2005). The standard consisted of purified *amoA* PCR products from an uncultured AOA (GenBank Accession #: EU925228) cloned into and amplified from the pSMARTGC HK vector (Lucigen Corp., Middleton, WI). Gene copy numbers in standard aliquots were determined using the equation from Ritalahti et al. (2006). A 10-fold dilution of the standard was used to generate a standard curve ranging from 1.152×10^9–1.152×10^4 gene copies per reaction. Amplification efficiencies for the 650 bp product ranged from 75.7–86.2% with r^2 values ranging from 0.990-0.996. This primer set was chosen as it provided a higher average efficiency, upwards of 13% for some samples, compared to the 72.5% efficiency obtained with the newer primer set CamoA19F/616R (Pester et al., 2012). For AOB, despite using a range of seven primer sets previously tested *in silico* by Junier et al. (2008), the soil DNA samples did not produce correctly sized products consistently when used in multiple PCRs. Thus, the bacterial *amoA* gene abundance fell below the detection level and could not be quantified in the soil DNA samples (Supplementary Table 1 and Supplementary Materials and methods).

All qPCRs were performed using a MJ Opticon DNA Engine 2 (MJ Research, Waltham, MA) in 20 μL reactions consisting of 2.0 μL of 10X Omni KlenTaq Mutant Reaction Buffer (DNA Polymerase Technologies, St. Louis, MO), 1.6 μL of 2.5 mM each dNTP (GeneScript, Piscataway, NJ), 0.05 μL T4 gene 32 protein (New England Biolabs, Ipswich, MA), 0.50 μL of 5.0 μM forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 1.0 μL of 20X EvaGreen dye (Biotium Inc., Hayward, CA), 0.2 μL of Omni KlenTaq Polymerase, and nuclease-free water. Three biological replicates were tested per land-use type (n = 3) with triplicate DNA extracts measured per biological replicate. The qPCR program consisted of an initial denaturation at 95.0 °C for 5 min, followed by 41 cycles of 95.0 °C for 15 s, 61.4 °C for 30 s, 68.0 °C for 30 s, 83.0 °C for 20 s (to denature potential primer dimers), and a plate fluorescence measurement. After a final extension at 68.0 °C for 5 min and a sample cooling period at 30.0 °C for 5 min, a melting curve from 65.0–90.0 °C (hold 1 s followed by plate fluorescence measurement) was performed in order to ensure product specificity. Samples were also electrophoresed in 1.0% agarose gels to ensure correctly sized product amplification. All samples were also tested for PCR inhibition using the method of Zaprasis et al. (2010), and samples showing inhibition were corrected (Supplementary Materials and methods).

2.4. Pyrosequencing of thaumarchaeal amoA Genes and bacterial 16S rRNA genes

In order to determine what structural shifts may be occurring in the ammonia-oxidizing communities in the soils of the different land-use treatments, the total soil DNA was used in amplification and pyrosequencing of thaumarchaeal *amoA* genes by Molecular Research LP (Shallowater, TX). Briefly, equal concentrations of total soil DNA from each triplicate sample per sample point were combined together to produce one composite DNA sample per land-use type. DNA samples were quantified via Qubit florescent dye measurement (v1.0). The thaumarchaeal *amoA* gene was amplified from each land-use sample using barcoded Arch-amoAF and Arch-amoAR fusion 454 primers (Francis et al., 2005). The genes were amplified using the HotStarTaq Master Mix Kit (Qiagen, Valencia, CA). The program used was an initial denaturation time of 94.0 °C for 3 min followed by 28 cycles of 94.0 °C for 30 s, 53.0 °C for 40 s, and 72.0 °C for 1 min. This was followed by a final elongation at 72.0 °C for 5 min. The different amplicon products were mixed in equal amounts and purified via Agencourt AMPure beads (Beckman Coulter, Inc., Brea, CA). Amplicon products were sequenced using the Roche 454 FLX-Titanium instrument and reagents according to the manufacturer's specifications.

Due to the difficulties in amplifying bacterial *amoA* genes, total soil DNA from the different land-use treatments was used in bacterial 16S rRNA gene pyrosequencing (Supplementary Materials and methods).

2.5. Sequence processing, OTU clustering, and community structure analyses

All initial sequence processing was performed using the Ribosomal Database Project Initial Processing Pipeline (Cole et al., 2014). After eliminating poor quality reads (Q-score < 20, length < 300 bp, max number of Ns = 0, max forward primer distance = 2) and trimming off barcodes and primers, thaumarchaeal *amoA* nucleotide sequences were corrected for frameshift mutations using the FrameBot tool implemented in the FunGene analysis pipeline (Fish et al., 2013; Wang et al., 2013). These corrected nucleotide sequences were aligned in the software program mothur (Schloss et al., 2009) with a reference alignment database consisting of *amoA* sequences of all cultured AOA and select uncultured AOA downloaded from the FunGene Repository (Fish et al., 2013). Chimeras were removed using the implementation of UCHIME (Edgar et al., 2011) in mothur. A distance matrix was made from the aligned sample sequences and was used in clustering to form operational taxonomic units (OTUs) at a cutoff of 85%. This cutoff has recently been recommended by Pester et al. (2012) and is based on the pairwise *amoA* sequence similarity between AOA when compared to a species OTU definition (97%) based on 16S rRNA genes from the same isolates. Bacterial 16S rRNA genes were processed according to a similar pipeline (Supplementary Materials and methods).

In order to compare the structures of thaumarchaeal ammonia-oxidizing communities between different land-use treatments, OTUs were used in analysis of molecular variance (AMOVA), homogeneity test of molecular variance (HOMOVA), and ∫-libshuff analysis in mothur (Schloss, 2008; Schloss et al., 2009, 2004). AMOVA, a version of traditional analysis of variance, is a non-parametric statistic which determines if the genetic diversity within two communities is different than the diversity that would result from pooling the communities (Schloss, 2008). HOMOVA is a non-parametric version of Bartlett's test of homogeneity of variances. This statistic tests if the genetic diversity in multiple communities is different (Schloss, 2008). Using these tests in conjunction with

∫-libshuff, which compares community structures, enables determination of how two or more communities' structures differ from one another. Prior to performing these analyses, thaumarchaeal *amoA* OTUs for each land-use were subsampled to ensure equal sizes of OTU libraries between land-use treatments. Due to the six comparisons being made between land use treatments, a Bonferroni correction was used to set a significant *p*-value at p = 0.00833 for community structural comparisons.

2.6. Phylogenetic characterizations of thaumarchaeal ammonia-oxidizing communities

Representative OTUs from the chimera screened thaumarchaeal *amoA* dataset, based on the 85% cutoff, were determined using mothur. Sequences for these representative OTUs, which had been quality filtered and corrected for frameshifts using FrameBot, were combined with *amoA* sequences of cultured and uncultured AOA. These sequences were aligned with mothur using the database initially used for OTU clustering. This alignment was used to construct maximum likelihood phylogenetic trees using the Tamura 3-parameter + gamma distribution nucleotide substitution model in the software package MEGA v 6.0 (Tamura et al., 2013). Inferred amino acid based trees showed similar branching topology but a lower degree of phylogenetic separation due to polytomies among some of the subclusters. Thus, only DNA based trees are shown. The thermophilic AOA *Nitrosocaldus yellowstonii* (NCBI Accession #EU239961) was set as the outgroup.

2.7. Statistical analyses of soil physiochemical parameters and archaeal amoA gene copy levels

All soil physiochemical and qPCR data analyses were performed using R implemented through Rstudio (R Development Core Team, 2013; RStudio, 2012). Soil physiochemical parameters were square-root transformed in order to assure normality of data and homogeneity of variances. Due to small sample sizes per land-use type (n = 3) for each soil parameter, a one-factor ANOVA-like Bayesian comparison was used as implemented in the program ANOVAonewayJagsSTZ.R (Kruschke, 2011). For the non-transformed qPCR data (n = 3 biological replicates per land-use with three technical replicates for each biological replicate), a two-factor ANOVA-like Bayesian program (AnovaTwoFactor.R; http://www.indiana.edu/~kruschke/DoingBayesianDataAnalysis/Programs/?C=M;O=D), which makes use of a *t*-distribution as opposed to a normal distribution for comparisons, was used as it provided stronger inferences than the one-factor program. Additionally, in order to determine the interrelatedness of the various soil parameters and qPCR abundances to each other, Spearman correlation coefficients were determined on the non-transformed data using the R package 'psych' (Revelle, 2015). P-values for the correlation coefficients were adjusted using the Bonferroni correction and were considered significant if $p < 0.05$ (Supplementary Table 3).

2.8. Nucleotide sequence accession information

The FASTQ files for the thaumarchaeal *amoA* and bacterial 16S rRNA gene sequences were deposited in the NCBI Sequence Read Archive under SRA Study SRP060575, BioProject ID PRJNA288363.

3. Results

3.1. Soil physiochemical properties

The four different land-use treatments of primary forest (PF), young pasture (P-2004), old pasture (P-1911), and secondary forest (SF) showed statistically different values for three of the 27 measured soil physiochemical properties: NO_3^- content, phosphorus (P) content, and exchangeable acidity (H^+ and Al^{3+} levels combined) (Table 1 and Supplementary Table 2). The change from primary forest to young pasture caused a significant decrease in P content and H + Al, but these properties recovered to primary forest levels in old pasture and secondary forest. Additionally, there was a significant decrease in NO_3^- content between the primary forest and the young and old pasture sites. However, after the abandonment of pasture and conversion back to a secondary forested landscape, NO_3^- content recovered to primary forest levels.

3.2. Quantitative PCR analysis of thaumarchaeal and bacterial amoA genes

Despite numerous efforts (see Supplementary Materials and methods and Supplementary Table 1), consistent amplification of the AOB *amoA* gene was not achieved (data not shown). Successful quantitation of the thaumarchaeal *amoA* gene by qPCR revealed significantly fewer thaumarchaeal *amoA* gene copies per ng total soil DNA in young and old pastures compared to primary and secondary forest (Fig. 1). No difference was seen in thaumarchaeal *amoA* gene copies per unit total soil DNA between primary and secondary forest. When normalizing to gram of dry soil, the old pasture was not significantly different than the primary and secondary forests, and the primary forest and young pasture were not significantly different (Supplementary Fig. 1). Despite the significant decrease in gene copies per unit total soil DNA between the pastures and forests, thaumarchaeal *amoA* gene copies were not correlated significantly with any of the measured soil physiochemical variables (Supplementary Table 3).

3.3. AOA and AOB community characterizations and phylogenetic analyses

Pyrosequencing of the *amoA* gene (for thaumarchaea) and of the 16S rRNA gene (total bacteria) was performed for total soil DNA samples from each land use. A total of 51,553 thaumarchaeal *amoA* and 148,446 bacterial 16S rRNA quality filtered and chimera checked sequences were generated. Bacterial 16S rRNA reads (OTU data summarized in Supplementary Table 4) were classified using mothur (Schloss et al., 2009), and all reads associated within the family Nitrosomonadaceae were harvested from the dataset. No sequences from the genus *Nitrosococcus* were found. A total of between 9 and 13 thaumarchaeal *amoA* OTUs were found (Table 2). Both Good's coverage (Good, 1953) and Boneh estimators (Boneh et al., 1998) indicated that both communities were thoroughly sampled and that the potential to find additional OTUs would be minimal (Table 2). As primary forest was converted to pasture, the diversity of the AOA community increased as seen by both the inverse Simpson and the Shannon index. However, as pastures age or were abandoned to form secondary forest, the diversity levels decreased to approximately pre-disturbance levels.

Initial 16S rRNA sequence characterization using the naïve Bayesian classifier developed by Wang et al. (2007) had identified all of the retrieved Nitrosomondaecae sequences as 'unclassified'. In order to determine how related these sequences may be to known AOB, maximum likelihood phylogenetic analysis was performed. Despite being classified as belonging to the Nitrosomondaecae family, none of the sequences showed a close association with known ammonia-oxidizers (Supplementary Materials and methods and Supplementary Fig. 3). Concerning the AOA, maximum likelihood phylogenetic analysis of thaumarchaeal *amoA* nucleotide sequences indicated that most of the sequences form clusters which were related to the genus

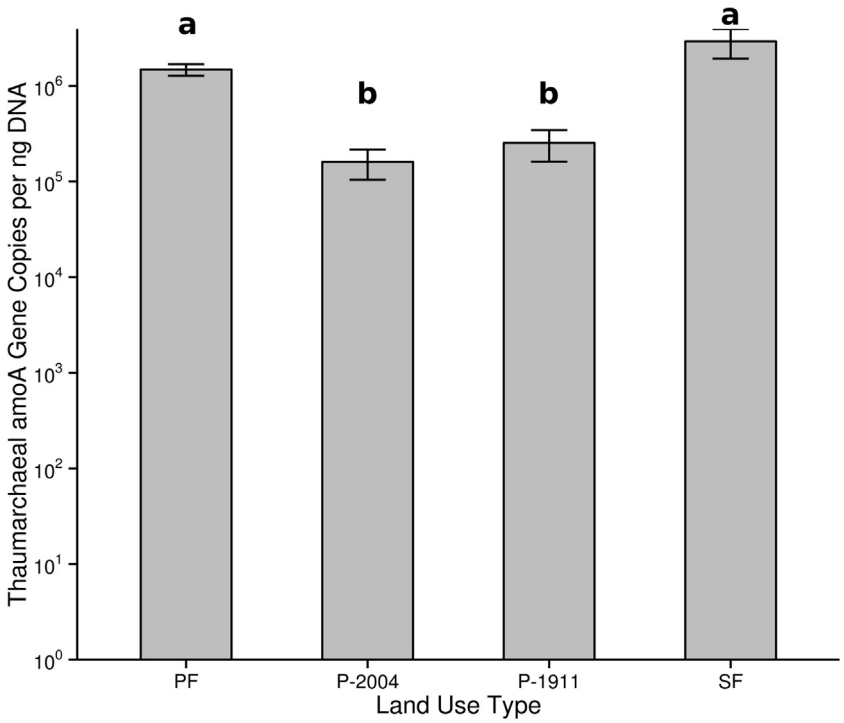


Fig. 1. Abundance of AOA *amoA* genes per ng of DNA extracted from the different land-use plot samples: PF, primary forest; P-2004, pasture established in 2004; P-1911, pasture established in 1911; and SF, secondary forest. Bars represent means ± standard error of the mean (n = 3 biological replicates for each land use type with 3 technical replicates per biological replicate). Land-use plots which do not share the same letter are significantly different (ANOVA-like Bayesian comparison, 95% highest density interval for difference between means does not include zero). Note that the error bars for each sample are of uneven length due to the logarithmic scale of the y-axis.

Table 2
Community characteristics of putative AOA under the different land-use treatments.

Land Use	Obs. OTUs	Good's Coverage	Shannon	Inv. Simpson	Boneh
PF	10.0	0.999	0.675	1.617	1.151
P-2004	13.0	0.999	1.283	3.296	1.840
P-1911	9.0	0.999	0.140	1.061	1.380
SF	12.0	0.999	0.143	1.055	1.261

Notes:.
[1]Each site for AOA was normalized to 5635 sequences per library. The species cutoff used was 85% as detailed in the Section 2.5 of the Materials and methods.
[2]The Boneh calculator estimates the number of additional OTUs that could be observed with more sampling.
[3]Single composite DNA samples for each land-use were sequenced. See Section 2.4 of the Materials and methods for sample preparation details.

Nitrososphaera (Fig. 2 and Supplementary Fig. 2). Specifically, cluster #3 was closely related to *N. viennensis* and *N. evergladensis*. The young pasture sequences cluster almost exclusively from the primary forest, secondary forest, and old pasture sequences, as seen by clusters 2, 3, 5, and 6. In addition to the numerically dominant clusters of sequences related to the *Nitrososphaera*, another cluster was most closely related to *Nitrosotalea devanaterra* and *Nitrosotalea.* sp. *Nd2* (Fig. 2 and Supplementary Fig. 2). This cluster consisted almost completely of primary forest sequences (94.9% PF, 0.7% P-2004, and 4.4% SF).

Lastly, AOA community structures and genetic diversity in the four land-use treatments were determined using AMOVA, HOMOVA, and ∫-libshuff (Schloss, 2008; Schloss et al., 2009, 2004). We found that both the community structures and genetic diversity for the AOA were significantly different for all pairwise comparisons between forested and pastured sites for all of the metrics used (Table 3; $p < 0.001$).

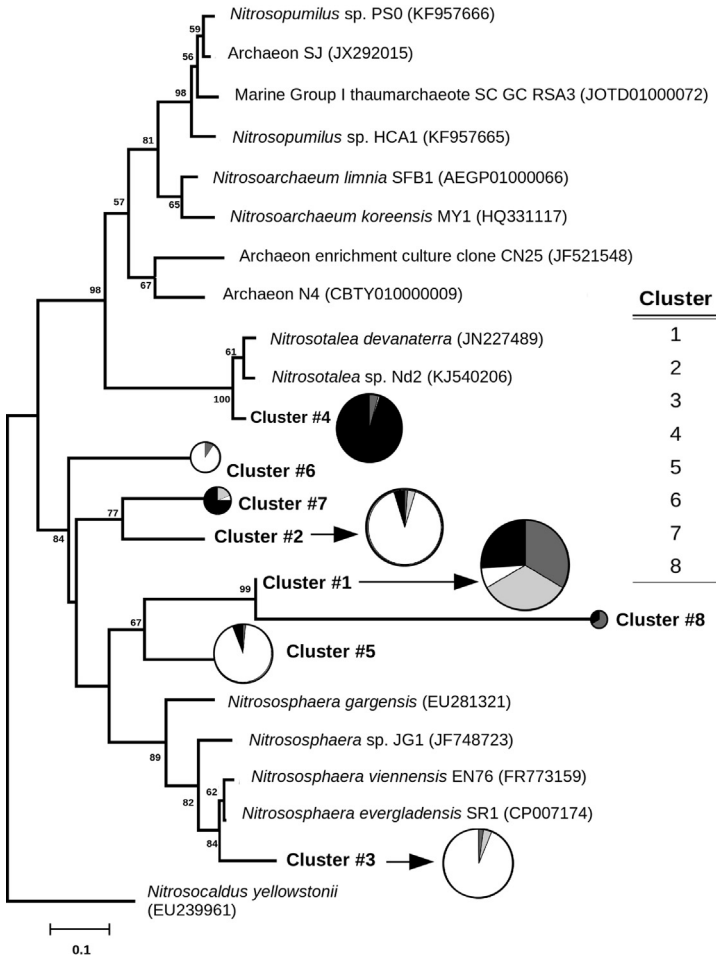
4. Discussion

4.1. Summary

In this study we investigated the effects of land-use change on ammonia-oxidizing soil microbial communities in primary rainforest, young and old pasture, and secondary forest in Rondônia, Brazil, using quantitation and next-generation sequencing of the *amoA* functional gene. The forest-to-pasture conversion common in this region of the Amazonian rainforest caused decreases in soil NO_3^-, P, and exchangeable acidity that appears to recover to pre-disturbance levels as pastures age or are abandoned to form secondary forest. Similar to findings from a GeoChip analysis of functional genes for this sampling site (Paula et al., 2014), the ammonia-oxidizer community, dominated by archaea, has a large proportion of its diversity present in pasture sites. Phylogenetic analyses showed that structural changes in the AOA are driven by a shift away from primary/secondary forest and old pasture clusters to large, separate clusters for young pastures. Additionally, the nearly complete disappearance of a whole genus of thaumarchaea, the *Nitrosotalea*, indicates that land-use change can have substantial and long lasting effects on large portions of the AOA community. While this study lacked replication of entire chronosequences due to difficulty in finding well-documented chronosequence sites in the Amazon rainforest, the findings of this study may indicate a pattern of thaumarchaeal ammonia oxidizer community shifts in response to land-use change.

4.2. Soil physiochemical properties altered by land-use change

The depletion of NO_3^- in pasture soils measured in this study is very similar to results seen by Neill et al. (1999, 1995) in primary forest and pasture sites located on the sites where the current study was conducted. The lack of significant differences in NH_4^+



Cluster	# of Sequences	PF (%)	P-2004 (%)	P-1911 (%)	SF (%)
1	16410	26.0	7.3	33.3	33.4
2	2541	4.8	90.4	3.4	1.3
3	1762	0.1	93.7	4.0	2.2
4	1264	94.9	0.7	0	4.4
5	498	6.0	92.4	0	1.6
6	21	0	90.5	0	9.5
7	16	75.0	6.3	18.8	0
8	6	33.3	0	0	66.7

Fig. 2. (a) Maximum likelihood phylogenetic tree of the representative OTU AOA *amoA* gene sequences from all of the land-use treatments. The phylogeny was generated using 363 nucleotide positions of the *amoA* gene. The Tamura 3-parameter + gamma distribution nucleotide substitution model was used. Bootstrap scores greater than 50% are displayed. The bootstrap scores were calculated from 500 replications. Sequence libraries were rarefied to 5635 sequences for each land-use treatment. The pie charts depict the proportion of each cluster's sequence composition that can be attributed to a specific land-use treatment (primary forest (PF)—black, young pasture (P-2004)—white, old pasture (P-1911)—light gray, and secondary forest (SF)—dark gray). The size of the pie charts is the log-transformed value of the total number of sequences in that particular cluster. The tree was made using *Nitrosocaldus yellowstoni* as an outgroup. (b) The inset table shows the total number of sequences in each cluster and the percentage attributable to each land-use treatment. The scale bar represents 10 substitutions per 100 bases. The numbers in parentheses next to reference sequences are the GenBank accession numbers for those references. Bars within each cluster were collapsed.

Table 3
Comparison of community structures of putative AOA in soils under different land use treatments.

Comparison	AMOVA		HOMOVA		∫-libshuff	
	F-score	P-value	B	P-value	ΔC_{XY}	P-value
PF ↔ P-2004 ↔ P-1911 ↔ SF	568.2	<0.001	747.9	<0.001	NA	NA
PF ↔ P-2004	226.5	<0.001	15.0	<0.001	0.0063	<0.001
PF ↔ P-1911	1070.9	<0.001	639.2	<0.001	0.0201	<0.001
PF ↔ SF	133.8	<0.001	189.9	<0.001	0.0034	<0.001
P-2004 ↔ P-1911	1007.8	<0.001	426.6	<0.001	0.0211	<0.001
P-2004 ↔ SF	320.4	<0.001	107.9	<0.001	0.0202	<0.001
P-1911 ↔ SF	1430.0	<0.001	56.2	<0.001	0.0156	<0.001

Notes:.
[1]AOA libraries were subsampled as indicated in Table 2 before performing AMOVA, HOMOVA, and ∫-libshuff analysis.
[2]In order to account for the multiple comparisons, a Bonferroni correction was applied. Thus, with an $\alpha = 0.05$, only $p < 0.00833$ is considered significant.
[3]∫-Libshuff analysis performed the reverse comparison for each land-use combination. All of these reverse comparisons also had $p < 0.001$ except the SF ↔ PF which had a $p = 0.0868$.

content between the different land-uses, in contrast to what had been found by Neill and colleagues, may be due to the high within-treatment variation of the three replicate samples rather than due to a true lack of difference between the land-use treatments. Neill and colleagues also documented a decrease both in net and gross N-mineralization and nitrification in pastures of increasing age compared to primary forest. While the mechanisms of such a decrease in N-cycling rates are not completely clear, the root systems of the *Bracharia humidicola* and *Panicum maximum* grasses planted in Brazilian pastures have been shown to secrete the potent biological nitrification inhibitor brachialactone in response to NH_4^+ being present in the root environment (Gopalakrishnan et al., 2009; Subbarao et al., 2009). This inhibitor has been shown to decrease both thaumarchaeal and bacterial *amoA* gene abundance in soil planted with different strains of *Bracharia* and *Panicum* grasses (Subbarao et al., 2012, 2009). In addition to *B. humidicola*, *B. brizantha* has been shown to secrete brachialactone, albeit to a lesser degree than *P. maximum* (Subbarao et al., 2012). This ability to produce brachialactone could provide a mechanistic explanation of NO_3^- depletion and nitrification inhibition seen both in this study as well as by Neill et al., (1999, 1995). However, more work is

needed to develop methods to extract and measure brachialactone exudates directly from pasture soils in order to quickly confirm if soils have the potential for biological nitrification inhibition.

4.3. *Thaumarchaeal* amoA *gene copy number response to land-use alteration*

The values of the thaumarchaeal *amoA* gene numbers per unit total soil DNA and per g dry soil illustrate the high abundance of AOA in these tropical soils. The large difference in thaumarchaeal *amoA* gene abundance per unit total soil DNA in young and old pastures compared to primary and secondary forest reflects the effect of major landscape changes on members of the nitrifying communities. The drop in thaumarchaeal *amoA* gene abundance in young and old pastures is consistent with what was found by Subbarao et al. (2012, 2009) in pastures planted with *P. maximum* and *Bracharia* spp. in relation to non-planted, control soil plots. The recovery of thaumarchaeal *amoA* gene abundance in secondary forest also illustrates the resiliency, or ability to recover from disturbance, of thaumarchaeal populations in the face of land-use change. One potential effect of a recovery of *amoA* gene abundance may be a phylogenetic shift in thaumarchaeal communities once the stress of a monoculture of pasture grass is replaced by a secondary forest (Fig. 2). Given the potential of the grasses to secrete brachialactone in order to compete for NH_4^+, the persistence of AOA in the pastures is intriguing. It has been documented in previous studies that thaumarchaea may possess and even express the *amoA* gene and yet not be involved in ammonia-oxidation. For example, Mussmann et al. (2011) investigated nitrifying communities in wastewater treatment plants and found that thaumarchaeal *amoA* genes outnumbered AOB *amoA* genes by 10,000-fold. However, the transcription of thaumarchaeal *amoA* genes and production of crenarchaeol, a membrane lipid found in the thaumarchaea, was independent of the addition of ammonium to the water (Mussmann et al., 2011). Additionally, Palatinszky and colleagues found that *Nitrososphaera gargensis*, a AOA isolated from hot springs, was able obtain ammonium through the oxidation of cyanate when cyanate was supplied as the sole source of energy and reductant (Palatinszky et al., 2015). They also found that other thaumarchaea, which do not encode the genes for the cyanase enzyme needed to degrade cyanate, can form syntrophic interactions with nitrite-oxidizing bacteria (NOB) which posses cyanase-encoding genes. After the NOB supply ammonium to the AOA, the AOA in turn can produce nitrite through nitrification which can be fed back to the NOB (Palatinszky et al., 2015). Lastly, thaumarchaeal amoA is a copper-containing membrane monooxygenase that potentially may be able to oxidize substrates other than ammonium, such as methane, even though this has not yet been documented (Hatzenpichler, 2012). Future studies need to be performed that determine brachialactone concentrations and nitrification rates in old pasture soils (>50 years old) in order to see if decreased nitrification rates can be correlated to brachialactone concentrations in soil.

4.4. *Ammonia-oxidzier community structure and phylogenetic characterizations*

The phylogenetic affiliation of Nitrosomondaceae sequences in the bacterial 16S rRNA to non ammonia-oxidizing organisms and poorly classified beta-proteobacteria indicate that the AOB community may be quite small, or non-existent, in these soils. This lack of phylogenetic affiliation of the 16S rRNA sequences with known ammonia- oxidizers may also explain the lack of bacterial *amoA* gene amplification from the soils despite the use of multiple primer sets (Supplementary Materials and methods and Supplementary Table 1). The small size of the AOB community is not surprising as studies have suggested that AOA dominate over AOB (Leininger et al., 2006) and that AOA and AOB grow in differing niches of soil with AOB preferring habitats with higher pH and AOA thriving under lower pH (Nicol et al., 2008). Lastly, low levels of NH_4^+, as seen in our soils, have been documented to enrich for AOA over AOB in culturing experiments (Verhamme et al., 2011). This low soil pH, low NH_4^+ concentration, and any potential brachialactone production by the *Bracharia* and *Panicum* grasses in the pastures could cause AOB to be in extremely low abundance. Ongoing metatranscriptomic analyses of forest and pasture sites from the same sampling location will help to elucidate if AOB are contributing to *amoA* expression in these soils despite their low abundance.

The alterations to the AOA community structure and to the phylogenetic clustering illustrates the varying disruptions that land-use change can have on soil thaumarchaea. The two main genera of isolated soil AOA, *Nitrososphaera* and *Nitrosotalea*, dominate our soil system with *Nitrososphaera* spp. being numerically greater (Fig. 2 and Supplementary Fig. 2). This dominance of *Nitrososphaera* is unexpected given the very low pH of our soils compared to the neutral pH used for enrichment and growth of the cultured isolates *Nitrososphaera gargensis* and *N. viennensis* EN76 (Hatzenpichler, 2012; Hatzenpichler et al., 2008; Tourna et al., 2011). Other studies in acidic, subtropical forest and agricultural soil in Hunan, China, found more sequences related to the group I.1a and I.1a-associated clades (*Nitrosopumilus* and *Nitrosotalea* cluster, respectively) than in the group I.1b (*Nitrososphaera* cluster) (Shen et al., 2013; Ying et al., 2010). It is possible that yet uncharacterized acidophilic isolates of the genus *Nitrososphaera* thrive in our soils and dominate ammonia-oxidation or that various carbon substrates, which have recently been implicated in mixotrophic growth for some AOA isolates (Hatzenpichler et al., 2008; Qin et al., 2014; Tourna et al., 2011), may be present in the rhizospheres of forest trees and pasture grasses. Such mixotrophy, as opposed to autotrophy and dependence on NH_4^+, could allow thaumarchaea to grow in pasture soils containing biological nitrification inhibitors.

The presence of the *Nitrosotalea* cluster (#4), predominantly in primary forests, presents a troubling phenomenon that could be common in other South American tropical sites undergoing land use change. Recent work by Lehtovirta-Morley et al. (2014) on the physiology of two *Nitrosotalea* isolates, *N. devanaterra* Nd1 and *N.* sp Nd2, indicated that these acidophilic archaeal ammonia-oxidiers have optimal growth temperatures of 25 °C and 35 °C, respectively. Additionally, the specific growth rate of *N. devanaterra* Nd1 was found to be significantly decreased in the presence of pyruvate, citrate, α-ketoglutarate, succinate, fumarate, and malate compared to inorganically grown controls (Lehtovirta-Morley et al., 2014). While soil temperature and organic acid content were not measured in the soils of this study, it is possible that land-use change from pristine forest to pasture caused physiochemical alterations that could have been detrimental to *Nitrosotalea* species. The near disappearance of this genus may be indicative of microbial taxon elimination by land use change, and the potential contribution of these thaumarchaea to overall ammonia oxidation rates may be significant as seen in Chinese subtropical soils (Shen et al., 2013; Ying et al., 2010). Future work will determine how ammonia-oxidation rates for secondary forests differ from those of primary forests and pastures of varying age and how these rates correlate with community composition changes.

In summary, the results of this study illustrate the drastic changes that occur to thaumarchaeal ammonia-oxidizers in tropical soils of Amazônia during the process of land-use change. Our initial hypothesis was supported only partially. While thaumarchaeal *amoA* gene abundances in secondary forest recovered to primary forest levels after pasture abandonment,

thaumarchaeal *amoA* community structures do not return to a pre-disturbance state as the thaumarchaeal *amoA* secondary forest community lacks organisms of the genus *Nitrosotalea*. Also, AOB were not found either by qPCR of bacterial *amoA* genes or by bacterial 16S rRNA gene pyrosequencing, in contrast to other studies in acidic (sub)tropical soils (Shen et al., 2013; Ying et al., 2010). Such findings indicate that land-use change may permanently alter the community structures of ammonia-oxidizing organisms in tropical soils.

Acknowledgements

This work was funded by USDA/NIFA CSREES grant # 2009-35319-05186 and a NSF Dimensions grant # 1442183. The authors would like to thank the Amazon Rainforest Microbial Observatory Group members for assistance in soil sampling and soil chemistry determinations, Wagner Piccinini and Siu Mui Tsai of the University of São Paulo for logistical and field sampling support, and the owners and staff of Agropecuaria Nova Vida for logistical support and permission to work on their property. Additionally, the authors are indebted to Dr. Sarah Weis for assistance in NH_4^+ and NO_3^- determination and to Daniel Yip and Dr. John Quensen for bacterial 16S rRNA amplification with barcoded primers. The authors also thank Dr. Anne Bernhard for providing us with the plasmid containing the archaeal *amoA* gene used for setting up qPCR standards. Lastly, the authors would like to thank Drs. Burcu Ünal, Kyunghwa Baek, Daniel Lammel, and Heather Reed for useful discussions concerning the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2016.05.012.

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