Nitrosospira Cluster 8a Plays a Predominant Role in the Nitrification Process of a Subtropical Ultisol under Long-Term Inorganic and Organic Fertilization

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ABSTRACT Long-term effects of inorganic and organic fertilization on nitrification activity (NA) and the abundances and community structures of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) were investigated in an acidic Ultisol. Seven treatments applied annually for 27 years comprised no fertilization (control), inorganic NPK fertilizer (N), inorganic NPK fertilizer plus lime ($CaCO_3$) (NL), inorganic NPK fertilizer plus peanut straw (NPS), inorganic NPK fertilizer plus rice straw (NRS), inorganic NPK fertilizer plus radish (NR), and inorganic NPK fertilizer plus pig manure (NPM). In nonfertilized soil, the abundance of AOA was 1 order of magnitude higher than that of AOB. Fertilization reduced the abundance of AOA but increased that of AOB, especially in the NL treatment. The AOA communities in the control and the N treatments were dominated by the *Nitrososphaera* and B1 clades but shifted to clade A in the NL and NPM treatments. *Nitrosospira* cluster 8a was found to be the most dominant AOB in all treatments. NA was primarily regulated by soil properties, especially soil pH, and the interaction with AOB abundance explained up to 73% of the variance in NA. When NL soils with neutral pH were excluded from the analysis, AOB abundance, especially the relative abundance of *Nitrosospira* cluster 8a, was positively associated with NA. In contrast, there was no association between AOA abundance and NA. Overall, our data suggest that *Nitrosospira* cluster 8a of AOB played an important role in the nitrification process in acidic soil following long-term inorganic and organic fertilization.

IMPORTANCE The nitrification process is an important step in the nitrogen (N) cycle, affecting N availability and N losses to the wider environment. Ammonia oxidation, which is the first and rate-limiting step of nitrification, was widely accepted to be mainly regulated by AOA in acidic soils. However, in this study, nitrification activity was correlated with the abundance of AOB rather than that of AOA in acidic Ultisols. *Nitrosospira* cluster 8a, a phylotype of AOB which preferred warm temperatures, and low soil pH played a predominant role in the nitrification process in the test Ultisols. Our results also showed that long-term application of lime or pig manure rather than plant residues altered the community structure of AOA and AOB. Taken together, our findings contribute new knowledge to the understanding of the nitrification process and ammonia oxidizers in subtropical acidic Ultisol under long-term inorganic and organic fertilization.

KEYWORDS nitrification, AOA, AOB, *Nitrosospira*, long-term fertilization

In order to increase crop yields to meet the food demands of an increasing population, increasing amounts of synthetic fertilizers, including nitrogen (N), have been applied to agroecosystems and have had adverse environmental consequences (1). The process of nitrification is a critical step in the N cycle that affects plant uptake of soil inorganic N, nitrate (NO_3^-) leaching, and nitrous oxide (N_2O) emissions (2). It was previously thought that ammonia (NH_3) oxidation, which is the first and rate-limiting step of nitrification, was mediated by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (3, 4) in the soil. However, recently, complete ammonia oxidizers (comammox) were also discovered; they can complete nitrification in one step on their own (5).

The population sizes of AOA in a range of agricultural soils have been found to be larger than those of AOB, especially in acidic soils (6–8). Generally, AOA showed stronger resistance to low soil pH and higher nitrification activity (NA) in acidic soils (7), whereas in neutral and alkaline agricultural soils, nitrification is dominated by AOB (6, 9). Numerous studies have shown that nitrification activity in acidic soils is positively correlated with AOA abundance (7, 10), and changes of AOA communities could significantly affect nitrification activity (7, 11, 12). However, recent studies of acidic upland soils have found that AOB are associated with potential nitrification rates (13) and act as metabolically active ammonia oxidizers (14–16). Such evidence could indicate that the assumption of AOA dominating nitrification in acidic soils may not hold true for all soil types and that the role of AOB in nitrification in acidic soils should be reevaluated.

The community composition of AOA and AOB plays a key role in the regulation of nitrification activity (7, 11). For example, AOA clade B was found to be actively linked to NH_3 oxidation in Arctic soils, while clade A showed little or no nitrification activity (17). Using $^{13}CO_2$-DNA stable isotope probing, Wang et al. (18) found that the clades *Nitrososphaera* and *Nitrosospira* cluster 3 were the most active AOA and AOB phylotypes, respectively, in an acidic Ultisol. The community structures of both AOA and AOB were strongly shaped by soil variables, especially soil pH and substrate (NH_3) availability (6, 19, 20). AOA clade B and the AOB *Nitrosospira* cluster 2 have been found to be well adapted to low-pH soils, while the AOB *Nitrosomonas* preferred neutral or alkaline soils (17, 21). Other soil properties, such as temperature (22), salinity (23), organic carbon (C) concentration (24), and moisture content (25), have also been found to influence the abundance and composition of soil ammonia-oxidizing communities.

Long-term fertilizer application has been shown to affect AOA and AOB abundances and community structures in different soil ecosystems (26, 27). For example, two long-term studies reported that the application of inorganic fertilizer enhanced AOB abundance in grassland soils after 44 years of fertilization (26) and in calcareous agricultural soils after 31 years of fertilization (28), whereas the application of organic fertilizer promoted the abundance of AOA. In contrast, Peng et al. (29) found that although AOA and AOB community compositions were altered by 35 years of fertilizer application to a salt marsh, abundances remained stable.

Despite the widespread distribution of Ultisols and their importance in biogeochemical processes (30), studies of the effects of long-term fertilizer application on AOA and AOB community compositions and roles in nitrification in subtropical Ultisols are limited (21). Therefore, we evaluated the long-term effects of the application of inorganic and organic fertilizer to a subtropical acidic Ultisol on the abundances and community compositions of AOA and AOB and nitrification to test the hypotheses that nitrification in Ultisols is primarily regulated by AOA and that long-term fertilizer application increases nitrification activities of AOA and AOB and elevates the relative importance of AOB.

RESULTS

Soil nitrification activity. Long-term fertilization increased nitrification activity from 0.94 mg N kg^{-1} dry weight of soil (dws) day^{-1} in the control treatment to 1.06 to 5.35 mg N kg^{-1} dws day^{-1} in the fertilized treatments (Fig. 1). Nitrification activity was
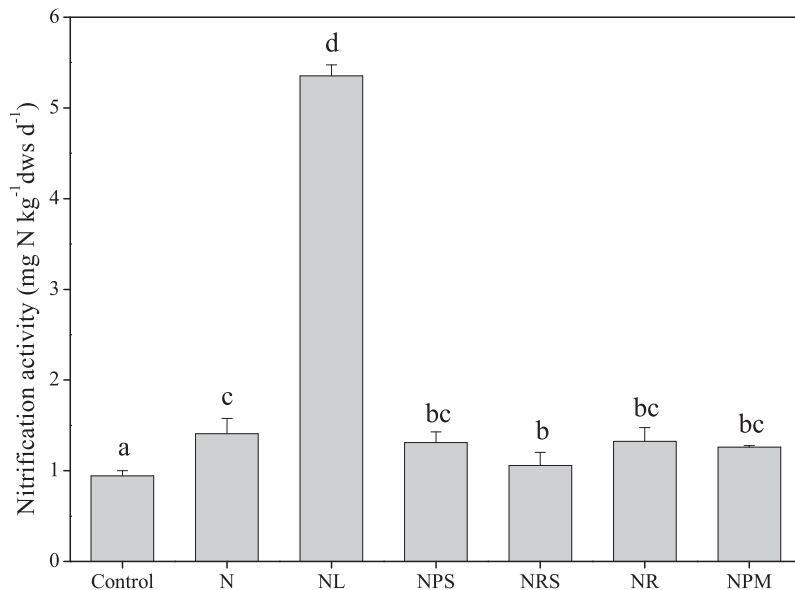
FIG 1 Soil nitrification activity in the different treatments. Vertical bars represent standard errors of the means (SEMs) ($n = 3$). Different letters denote significant differences between treatments ($P < 0.05$).

highest in the inorganic NPK fertilizer plus lime (CaCO$_3$) (NL) treatment, which was significantly higher than those in the other treatments ($P < 0.05$). The application of organic materials in addition to NPK (N) did not increase nitrification activity (Fig. 1). Nitrification activity was correlated with soil pH that ranged from 4.96 to 6.59 ($R = 0.942$; $P < 0.001$) (Table 1; see also Table S1 in the supplemental material).

Abundances of AOA and AOB. The AOA and AOB *amoA* gene abundances ranged from 4.08×10^7 to 6.15×10^7 copies g^{-1} dws and from 4.94×10^6 to 17.15×10^6 copies g^{-1} dws, respectively (Fig. 2). Fertilizer application reduced the AOA *amoA* gene abundance in the NL, inorganic NPK fertilizer plus rice straw (NRS), and inorganic NPK fertilizer plus pig manure (NPM) treatments ($P < 0.05$), and all fertilizer treatments increased the AOB *amoA* gene abundance ($P < 0.05$). The abundance of *amoA* genes for AOA was positively correlated with soil NO$_3^-$-N ($P < 0.01$) and negatively associated with soil pH and available potassium (AK) ($P < 0.01$). In contrast, the abundance of *amoA* genes for AOB was negatively correlated with soil NO$_3^-$-N and dissolved organic nitrogen (DON) ($P < 0.05$) and positively associated with soil pH and AK ($P < 0.01$).

Community diversity and composition of AOA and AOB. After quality filtering, totals of 998,950 and 1,226,551 high-quality sequences were obtained for AOA and AOB, with minima of 28,389 and 30,073 sequences per sample, respectively. There were differences in AOA and AOB community diversity among the treatments ($P < 0.05$) (see Fig. S1 in the supplemental material), where AOA diversity was lowest in the NL and NPM treatments and highest in the N, inorganic NPK fertilizer plus peanut straw (NPS), NRS, and inorganic NPK fertilizer plus radish (NR) treatments. In contrast, AOB community diversity was highest in the NL and NPM treatments (Fig. S1).

Canonical correspondence analysis (CCA) and linear redundancy analysis (RDA) showed that both AOA and AOB communities formed four clusters: control, NL, NPM, and a composite of the other treatments (N, NPS, NRS, and NR) (Fig. 3). AOA and AOB communities in the NL treatment were related to higher soil pH, while the communities in the NPM treatment were associated with soil organic carbon (SOC), dissolved organic carbon (DOC), available phosphorus (AP), and soil total nitrogen (TN). The multivariate regression tree (MRT) analysis of dominant operational taxonomic units (OTUs) (relative abundance of $>0.1\%$) showed that the community composition of AOA and AOB was primarily shaped by soil pH (Fig. S2). Mantel tests confirmed that soil pH was the primary influencing factor in AOA and AOB communities ($P = 0.001$) (Table 2).

TABLE 1 Soil physicochemical variables following application of long-term inorganic and organic fertilizers[a]

| Treatment | Mean value ± SEM | | | | | | | | | | |
	pH (1:5 soil/ H₂O ratio)	SOC concn (g C kg⁻¹)	DOC concn (mg C kg⁻¹)	TN concn (g N kg⁻¹)	NH₄⁺ concn (mg N kg⁻¹)	NO₃⁻ concn (mg N kg⁻¹)	DON concn (mg N kg⁻¹)	AP concn (mg P kg⁻¹)	AK concn (mg K kg⁻¹)	DC_o (10⁻⁶ m² s⁻¹)
Control	$4.96 \pm 0.02a$	$5.69 \pm 0.10a$	$4.27 \pm 0.37a$	$0.59 \pm 0.01a$	$0.54 \pm 0.17ab$	$12.50 \pm 0.39c$	$9.22 \pm 0.37c$	$5.25 \pm 0.54a$	$84.7 \pm 10.38a$	$12.58 \pm 0.39a$
N	$5.20 \pm 0.01b$	$6.44 \pm 0.26b$	$4.38 \pm 0.23a$	$0.70 \pm 0.01b$	$0.66 \pm 0.04b$	$8.60 \pm 0.77ab$	$5.56 \pm 0.24ab$	$15.0 \pm 3.12bc$	$177.6 \pm 23.00b$	$10.71 \pm 0.60b$
NL	$6.59 \pm 0.04d$	$6.32 \pm 0.08b$	$5.33 \pm 0.34a$	$0.72 \pm 0.02b$	$0.56 \pm 0.19ab$	$5.21 \pm 0.81a$	$4.28 \pm 0.75a$	$16.4 \pm 1.82c$	$203.1 \pm 5.62c$	$9.95 \pm 0.19b$
NPS	$5.13 \pm 0.03b$	$6.62 \pm 0.06bc$	$4.35 \pm 0.20a$	$0.78 \pm 0.02c$	$0.69 \pm 0.10b$	$8.71 \pm 0.60ab$	$5.38 \pm 0.57ab$	$10.3 \pm 0.95b$	$178.0 \pm 2.42b$	$9.96 \pm 0.85b$
NRS	$5.16 \pm 0.02b$	$7.13 \pm 0.28c$	$5.53 \pm 0.60a$	$0.82 \pm 0.02cd$	$0.41 \pm 0.13ab$	$8.66 \pm 0.08ab$	$8.90 \pm 2.88bc$	$10.8 \pm 0.52b$	$206.7 \pm 4.16c$	$8.20 \pm 0.55c$
NR	$5.17 \pm 0.02b$	$6.76 \pm 0.29bc$	$5.63 \pm 0.85a$	$0.82 \pm 0.01cd$	$0.25 \pm 0.07a$	$11.6 \pm 2.24bc$	$5.93 \pm 0.46abc$	$11.6 \pm 0.72bc$	$204.8 \pm 0.99c$	$9.70 \pm 0.66bc$
NPM	$5.58 \pm 0.02c$	$8.21 \pm 0.20d$	$12.3 \pm 1.80b$	$0.85 \pm 0.02d$	$0.49 \pm 0.03ab$	$9.56 \pm 0.50bc$	$5.89 \pm 0.45abc$	$157 \pm 8.85d$	$170.8 \pm 3.86b$	$2.81 \pm 0.35d$

[a]$n = 3$. Values within the same row followed by different letters indicate significant differences at a P value of <0.05. SOC, soil organic carbon; DOC, dissolved organic carbon; TN, soil total nitrogen; DON, dissolved organic nitrogen; AP, available phosphorus; AK, available potassium; DC_o, effective diffusion coefficient of oxygen.
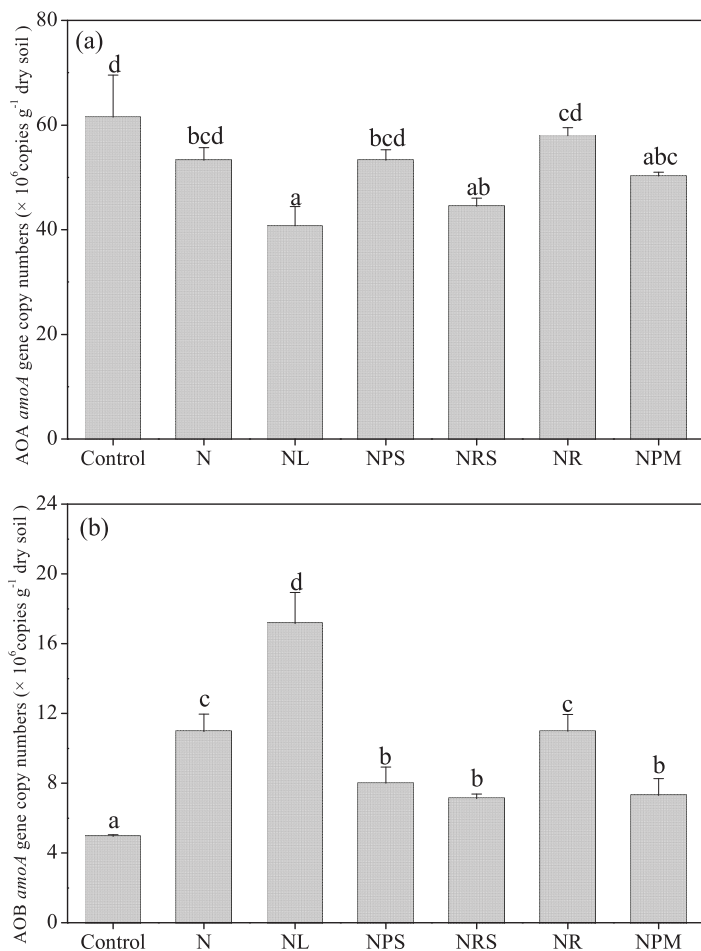
FIG 2 Abundances of *amoA* genes of ammonia-oxidizing archaea (AOA) (a) and ammonia-oxidizing bacteria (AOB) (b) in the treatments. Vertical bars represent SEMs ($n = 3$). Different letters denote significant differences between treatments ($P < 0.05$).

Sequences of dominant AOA and AOB *amoA* OTUs (relative abundance of >0.1%) were taxonomically classified into different phylotypes across the treatments (Fig. 4). Phylogenetic analysis of AOA communities showed that the dominant *amoA* OTU sequences were exclusively from the *Nitrososphaera* cluster (group I.1b lineage) and were distributed across nine robust phylogenetic clades (Fig. 4a and Fig. S3). In untreated soil (control), the *Nitrososphaera* and B1 clades represented 57.94% and 39.61% of AOA *amoA* sequences, respectively, and long-term fertilization reduced these proportions to 3.21 to 50.76% and 0.90 to 27.97%, respectively ($P < 0.05$), with the lowest proportions being recorded for the NL (3.21 and 0.90%, respectively) and NPM (17.28% and 4.12%, respectively) treatments. In contrast, the application of liming (NL) and pig manure (NPM) increased the proportions of clade A from 0.81% in the control soil to 76.80% and 69.23%, respectively ($P < 0.001$). The *Nitrososphaera* and B1 clades were negatively correlated with soil pH ($P < 0.001$), whereas clade A was positively associated (Table S2).

The dominant detected AOB *amoA* OTUs were exclusively affiliated with the *Nitrosospira*-like genus and grouped into *Nitrosospira* clusters 3a.1, 3a.2, 3c, 8a, and 8b; no AOB sequence was affiliated with the *Nitrosomonas* genus (Fig. 4b and Fig. S4). *Nitrosospira* clusters 8a and 3a.2 dominated the unfertilized control soil, accounting for 62.56% and 37.43%, respectively. Long-term fertilization decreased the relative abundance of *Nitrosospira* cluster 3a.2 to 11.29 to 31.56% ($P < 0.05$), and the relative abundance of *Nitrosospira* cluster 3a.1 increased from 0.01% in the control soil to
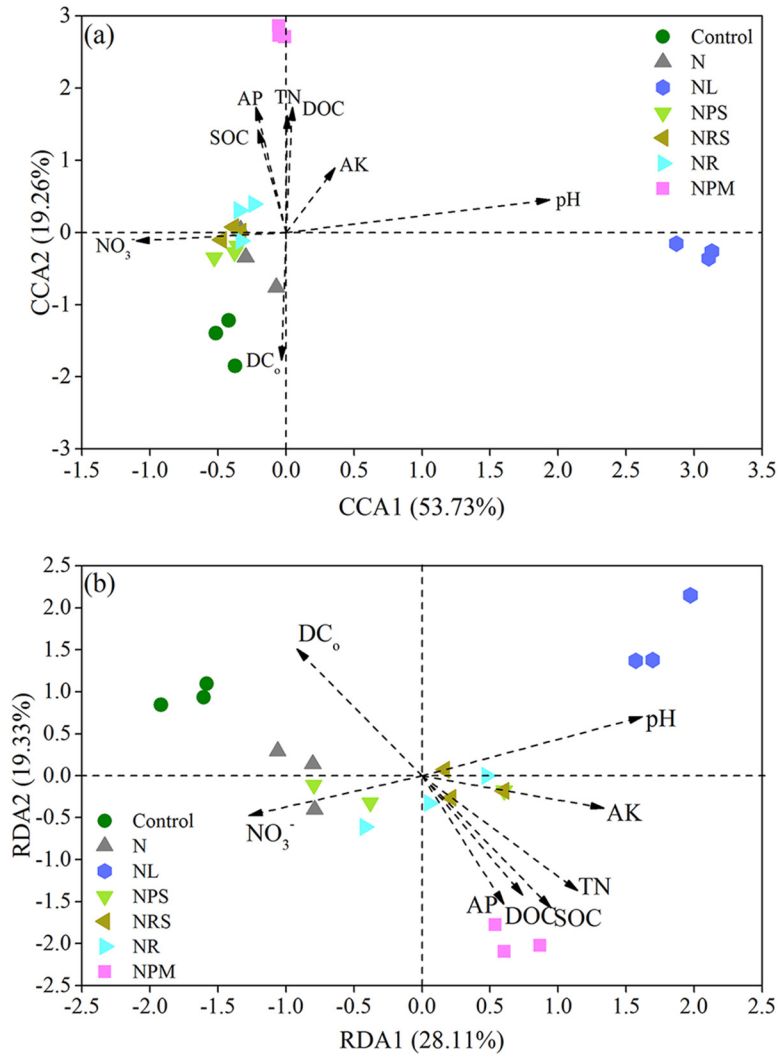
FIG 3 Canonical correlation analysis (CCA) of AOA (a) and redundancy analysis (RDA) of AOB (b) communities in the treatments. The positions and lengths of the arrows indicate the directions and strengths, respectively, of the effects of soil variables on the communities.

36.33% in the NL soil ($P < 0.001$) with an increase in soil pH. The relative abundance of *Nitrosospira* cluster 8a increased from 62.56% in the control treatment to 66.13 to 87.44% in fertilization treatments, except for NL, in which it decreased to 33.35%, and was negatively correlated with soil pH (Table S3). The relative abundance of *Nitrosospira*

TABLE 2 Mantel test correlations between community structure of ammonia-oxidizing archaea or ammonia-oxidizing bacteria and soil physicochemical properties[a]

Soil variable	AOA		AOB	
	R	*P*	*R*	*P*
Soil pH	**0.896**	**0.001**	**0.901**	**0.001**
SOC	**0.261**	**0.036**	0.111	0.196
DOC	0.208	0.092	−0.002	0.374
TN	**0.200**	**0.044**	0.185	0.054
DON	0.110	0.209	0.058	0.250
NH_4^+-N	0.060	0.280	0.023	0.379
NO_3^--N	**0.406**	**0.002**	**0.435**	**0.001**
AP	**0.289**	**0.042**	0.018	0.393
AK	0.147	0.140	0.098	0.228
DC_o	**0.278**	**0.046**	0.026	0.386

[a]Values in boldface type indicate significant effects ($P < 0.05$).
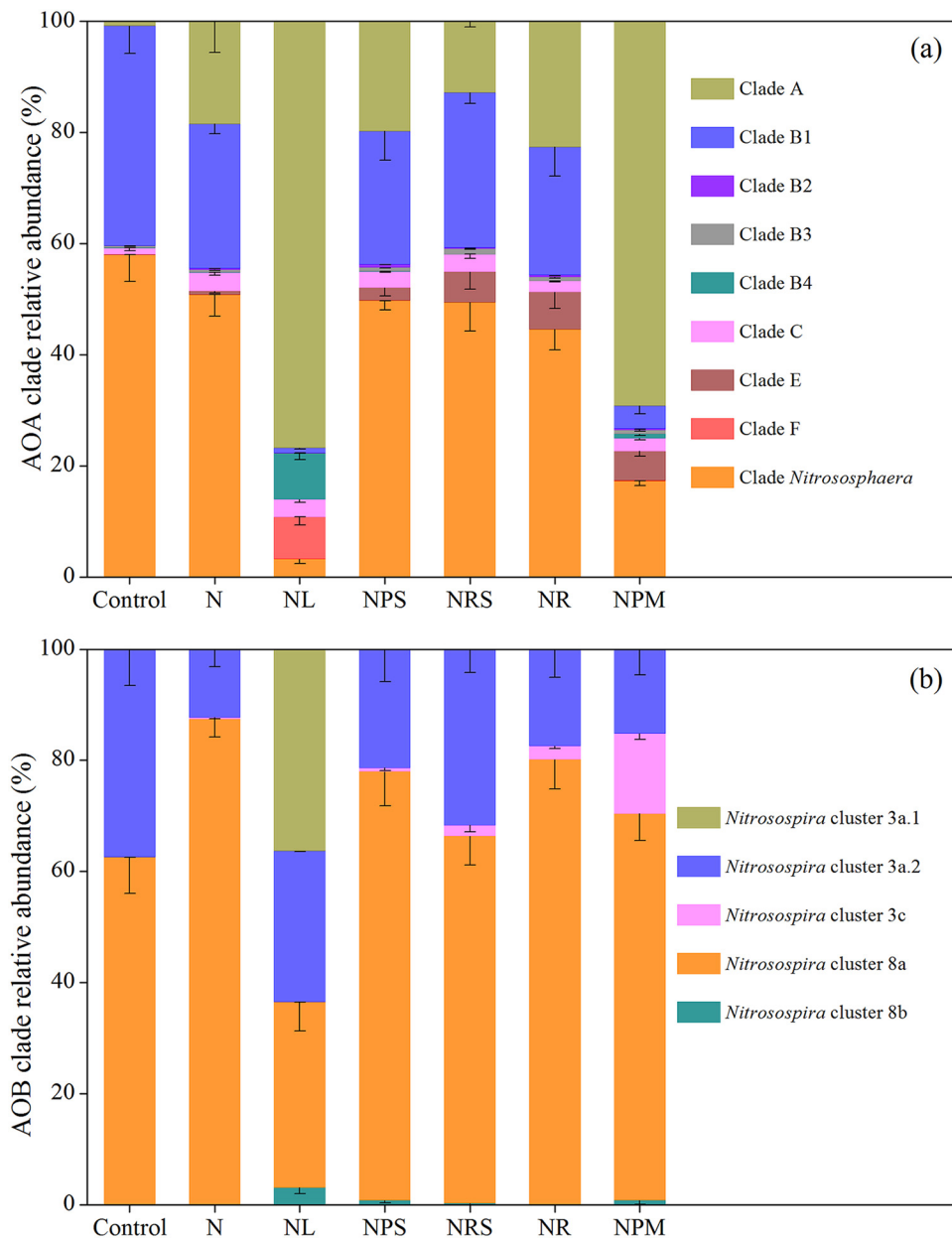
FIG 4 Community compositions of AOA (a) and AOB (b) based on *amoA* gene sequences in the treatments. Error bars represent SEMs (*n* = 3).

cluster 3c was highest in the NPM treatment (*P* < 0.001) and was positively correlated with SOC, DOC, AP, and TN but negatively associated with DC$_o$ (effective diffusion coefficient of oxygen) (*P* < 0.001).

Factors affecting nitrification activity. Variance partitioning was used to evaluate the relative contributions of different groups of variables to the variation of nitrification activity. The soil physicochemical properties and the abundances of AOA and AOB were separated into three groups of explanatory variables and used to explain each fraction of nitrification activity. Soil properties and their interaction with the AOB *amoA* gene abundance were found to explain up to 73% of the variance in nitrification activity (Fig. 5), and nitrification activity was positively associated with AOB *amoA* gene abundance (*P* < 0.001) (Fig. 6) and negatively associated with AOA *amoA* gene abundance (*P* = 0.005) (Fig. 6). When the NL treatment with neutral soil pH (6.59) was excluded from the analysis, nitrification activity continued to be positively associated with AOB *amoA* gene
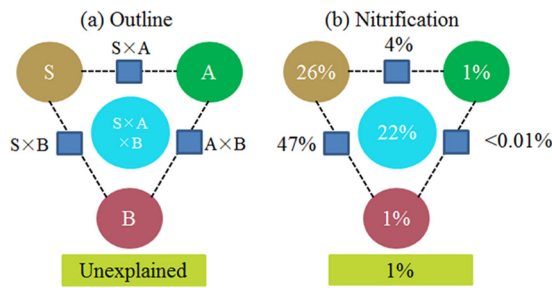
FIG 5 Percent variation in nitrification activity partitioned into soil physicochemical properties (S), abundance of AOA *amoA* genes (A), and abundance of AOB *amoA* genes (B). (a) Model scheme; (b) analysis.

abundance (*P* = 0.007) (see Fig. S5 in the supplemental material), but there was a lack of an association with AOA *amoA* gene abundance; among the AOB communities, there was a positive association with *Nitrosospira* cluster 8a (Fig. 7).

DISCUSSION

Effect of fertilizer on AOA and AOB. We found that the abundance of *amoA* genes for AOA was 1 order of magnitude higher than that for AOB in unfertilized soil, supporting previous reports of much higher abundances of AOA than their bacterial counterparts in acidic soils (6, 8, 18). The availability of ammonia (NH_3) in acidic soils is generally low, due to the strong ionization of NH_3 to NH_4^+, and subsequently decreases
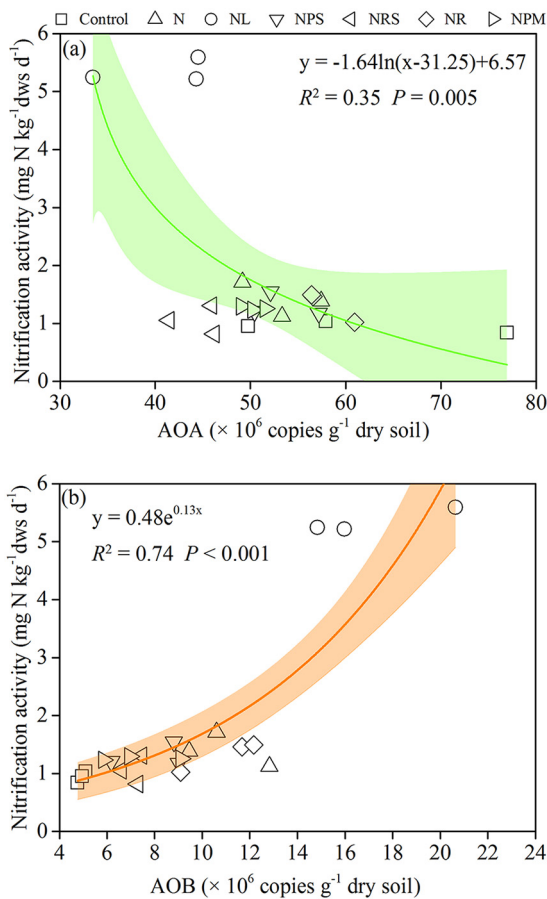
FIG 6 Association between nitrification activity and AOA (a) and AOB (b) *amoA* gene abundances in the treatments. The shaded area indicates the 95% confidence interval of the regression models.
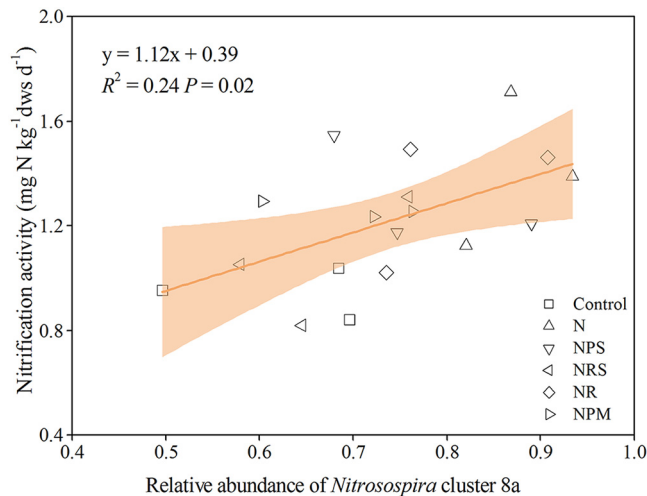
FIG 7 Association between nitrification activity and relative abundance of *Nitrosospira* cluster 8a in the treatments, excluding NL. The shaded area indicates the 95% confidence interval of the regression models.

NH_3 diffusion into microbial cells (31). However, genomic analysis suggested that AOA genes putatively encode pH homeostasis and high-affinity NH_3 acquisition systems (32) and thus could play a crucial role in nitrification in acidic soils where pH and NH_3 availability are particularly low (21). In contrast, AOB, which were also adapted to low soil pH (21), had a far lower substrate affinity than that of AOA (33), which suggests that AOA outcompeted AOB for substrates in the control soil. Our experiment showed that the long-term application of liming (NL) reduced AOA abundance, possibly because the increased soil pH reduced the competitive ability of AOA in soils (6, 34). In contrast, the abundance of AOB has been found to increase with soil pH (8, 34, 35). Intriguingly, the application of organic materials in our experiment had no apparent effect on the abundance of AOA compared to N, despite previous work showing benefits to AOA of mineralization of manure or plant residues in the absence of the addition of inorganic fertilizer (28, 36, 37). The reasons for this lack of an effect of organic fertilizer on AOA abundance may be due to the possible suppression of AOA growth in the treatments with N and N plus organic material due to high concentrations of NH_4^+ immediately after fertilization (38, 39), which overrode the possible stimulation effect induced by organic material amendment, or higher rates of turnover of the labile fraction of amended organic materials in our experimental soils due to higher temperature and precipitation than in previous studies (28, 36). The labile fraction of amended organic materials may have been quickly mineralized before soil ammonia decreased to a low level following fertilization; however, the remaining recalcitrant fraction of the organic material may not have stimulated AOA growth (37) under low levels of ammonia.

As has been reported in previous studies (6, 40), we found that pH was the primary driver for the shift of AOA community structure. Although the soil pH varied from 4.96 to 6.59, AOA sequences were within the *Nitrososphaera* cluster, which was recognized as a neutrophilic or alkaline, rather than acidic, archaeon (40). The *Nitrososphaera* cluster has been found to be metabolically active in acidic Ultisols using a DNA-SIP technique (18) and dominant in fertilized acidic soils (8). In this study, we found that dominant members of AOA were from the clades *Nitrososphaera* and B1 in the more acidic control soil and from clade A in the higher-pH NL and NPM soils, indicating the possibility of the occurrence of the *Nitrososphaera* cluster in neutrophilic or acidic soils, depending on its specific sublineages. Our results suggested that the phylogeny and characteristics of the *Nitrososphaera* cluster were more complicated than previously understood, so we suggest that phylogenetic analysis of AOA communities should

focus on the sublineages of the *Nitrososphaera* cluster in soils, where they are known to be dominant ammonia oxidizers (8, 17).

Previous studies have suggested that NH_4^+ is the main factor affecting AOB composition (20); however, in our study, Mantel tests showed that soil pH rather than NH_4^+ shaped AOB communities. The weak effect of NH_4^+ on AOB community structure in this study may be due to the particularly low NH_4^+ content (<0.7 mg N kg^{-1}) caused by intensive crop uptake and/or N losses in summer (41). We found that *Nitrosospira* was exclusively dominant in the AOB communities, probably because *Nitrosospira* generally outcompeted *Nitrosomonas*, which was previously reported for acidic soils (8, 21, 42). In contrast to previous studies that found that *Nitrosospira* cluster 2 dominated in acidic soils (21, 43), we found a dominance of *Nitrosospira* cluster 8a. Generally, *Nitrosospira* cluster 2 is restricted to cold temperate soils (44), whereas *Nitrosospira* cluster 8a, which is also referred to as clusters 10 and 11 (45, 46), tends to be dominant (relative abundance of $>90\%$) in soils that experience long periods of warm temperatures (47), with optimum growth temperatures of 31°C to 33°C (48), and is active in extremely acidic soils (49). It is likely that low soil pH and, more importantly, long periods of warm temperatures at this subtropical study site selected for *Nitrosospira* cluster 8a rather than cluster 2. We found that the long-term application of lime increased the relative abundance of *Nitrosospira* cluster 3a.1, reflecting the known preference for neutral and alkaline soils (19, 21). Likewise, Pommerening-Röser and Koops (50) found that the optimum pH for *Nitrosospira* cluster 3a.1 was 6.0 under urea addition, and Zhang et al. (51) noted a preference for lower-altitude soils with higher temperatures in the Mount Everest region. It appears that high annual temperatures may have enhanced the suitability of the neutral soil pH of the NL treatment soil for *Nitrosospira* cluster 3a.1. In contrast, we found that the abundance of *Nitrosospira* cluster 3c increased with the application of pig manure, which also led to the high levels of SOC. Since cluster 3c has been found to be dominant in an upland Ultisol with an SOC content of >13.6 g C kg^{-1} (9) and a Mollisol with an SOC content of >22.8 g C kg^{-1} (52), we postulate that this cluster responded to the high SOC content.

Contribution of AOA and AOB to nitrification activity. We used the increase in the rate of the soil NO_3^- concentration to assess nitrification activity (18), and soil pH and substrate availability (usually NH_3) have been suggested to be the two key factors affecting nitrification activity in acidic soils (21, 53). We found that the application of lime increased the abundance of AOB and substantially enhanced nitrification activity. While this increase in nitrification activity may be a result of reduced ionization of NH_3 to NH_4^+ and a rise in the associated substrate availability under the increased-pH conditions and increased abundance of AOB, maximum rates of nitrification by AOB have been shown to be 20-fold higher than those by AOA (33).

Previous studies have suggested that nitrification in acidic soils is primarily affected by AOA (7, 10, 54). However, in contrast to previous findings that showed that nitrification rates in acidic soils were closely correlated with AOA abundance (21), we found a negative and a lack of association between AOA abundance and nitrification activity with all treatments and with all treatments except NL (neutral soil pH), respectively. This may be due to a dominance of AOA-mediated nitrification processes in previous studies of fertilizer addition that benefited AOA abundance as a result of reduced soil pH (6). For example, AOA played a key role in nitrification in 50-year-old tea orchard soil that received 450 kg N ha^{-1} $year^{-1}$ and led to a reduction in the soil pH from 5.16 to 4.01 (7, 55). In that study, long-term fertilization slightly increased the soil pH due to the addition of P fertilizer as calcium magnesium phosphate (56), and we found that the dominant AOA clades shifted from the *Nitrososphaera* and B1 clades in the control and N treatments to clade A in the NL and NPM treatments. Alves et al. (17) noted that the *Nitrososphaera* and B1 clades were actively linked to NH_3 oxidation, and in contrast, clade A elicited little or no nitrification activity. Our results suggest that AOA may have played a minor role in nitrification in the experimental Ultisol, and the

contribution of AOA to nitrification activity may have been reduced in the NL and NPM soils.

The abundance of AOB in our study was more closely coupled with nitrification activity than AOA, differing from most previous findings for acidic soils (27, 57). Although similar correlations have been reported for some acidic soils (13, 58), Li et al. (21) argued that those studies used atypical acidic soils (pH >6), as were used in our study. However, when the NL soil (neutral pH) was excluded from the analysis, AOB abundance remained weakly correlated with nitrification activity, indicating that nitrification was primarily affected by AOB in soils under long-term N fertilization. It is likely that the increased substrate availability by long-term fertilization favored AOB, due to its much lower substrate affinity than that of AOA (33). He et al. (59) suggested that AOA may play more-important roles in ammonia oxidation than AOB from acid soils, due to the reduced availability of ammonia, and they highlighted that a wide range of AOB members could also contribute to nitrification in acidic environments and that more investigations were required. Using DNA-SIP, Wang et al. (18) found that AOA played a key role in nitrification under long-term fertilization in Ultisols; however, they also suggested that DNA-SIP relied on cell proliferation, and it is likely that AOB conducted ammonia oxidation without cell division and that RNA-SIP might be more helpful. Furthermore, the correlation of *Nitrosospira* cluster 8a with nitrification activity is weakly positive and at best suggests an association when NL treatment was excluded from the analysis, indicating that cluster 8a may play a key role in nitrification in acidic Ultisols. This study suggests that a specific phylotype of AOB has been associated with nitrification activity in acidic Ultisols, and it is possibly supported by *Nitrosospira* cluster 8a being the dominant AOB phylotype in the test soil and favored by warm temperatures and low soil pH in the study soils. Thus, *Nitrosospira* cluster 8a may play a key role in nitrification in tropical and subtropical acidic Ultisols. DNA/RNA-SIP experiments would be helpful to directly identify the active player in nitrification in further investigations.

Overall, our results show that the abundance of AOA was higher than that of AOB in the unfertilized control soil; however, long-term fertilization, especially the application of lime, reduced AOA abundance and increased AOB abundance. The dominant members of the AOA were the *Nitrososphaera* and B1 clades for all treatments, except for NL and NPM, in which clade A was dominant. In contrast, *Nitrosospira* cluster 8a was the dominant phylotype of AOB in the treatments, while the addition of lime and pig manure increased the relative abundances of *Nitrosospira* clusters 3a.1 and 3c, respectively. Nitrification activity was primarily regulated by the interaction of soil pH and AOB abundance in fertilized soils. When NL treatment was excluded from the analysis, nitrification activity was weakly positive and at best correlated with *Nitrosospira* cluster 8a. Our findings indicated that AOB rather than AOA played a key role in nitrification in the test Ultisols.

MATERIALS AND METHODS

Study site and soil sampling. The field experiment was established in April 1988 at the Yingtan Red Soil Ecology Experimental Station, Chinese Academy of Sciences, Yujiang, China (28°15′20″N, 116°55′30″E), in a cropping system of continuous summer peanut followed by winter fallow. The area has a typical subtropical monsoon climate, with an annual average temperature of 17.6°C and precipitation of 1,795 mm. The soil, derived from quaternary red clay, is classified as a Typic Plinthudult (Ultisol) based on U.S. Department of Agriculture soil taxonomy, comprising 25.6% sand, 33.2% silt, and 41.2% clay.

Three replicates of seven treatments were arranged in a randomized block design in 34.6-m² plots, where the treatments consisted of no fertilizer (control), inorganic NPK fertilizer (N), inorganic NPK fertilizer plus lime ($CaCO_3$) (NL), inorganic NPK fertilizer plus peanut straw (NPS), inorganic NPK fertilizer plus rice straw (NRS), inorganic NPK fertilizer plus radish residues (NR), and inorganic NPK fertilizer plus pig manure (NPM). Annual application of NPK fertilizer in the N treatment comprised 120 kg N ha^{-1} as urea, 30 kg P ha^{-1} as calcium magnesium phosphate, and 90 kg K ha^{-1} as potassium chloride. In the NPS, NRS, NR, and NPM treatments, 30% of the inorganic N fertilizer was replaced by organic N. Pig manure was stockpiled on a concrete slab for 3 months before application. All fertilizer treatments received the same total N, P, and K; however, as the amounts of P and K in the organic material were generally smaller than the prescribed doses, the organic fertilizer was supplemented accordingly with calcium magnesium phosphate and potassium chloride, depending on the P and K contents of the organic materials. For NL, 1,500 kg ha^{-1} $CaCO_3$ was applied annually. Prior to seed drilling, fertilizers were spread evenly onto the

soil surface by hand and immediately incorporated into the top 20 cm of soil by plowing. Each year on 10 April, peanut (cv. Ganhua 5) was sown manually by placing two seeds per hole to give 20-cm plant spacing in rows 30 cm apart. Field management practices were the same for all treatments during the experimental period.

On 13 December 2015, 10 tillage layer (0 to 20 cm) soil samples were collected at random from each plot by using a 10-cm-diameter auger and mixed to form a single, composite sample. Samples were transported to the laboratory in a constant-temperature box containing ice, within 2 days. Visible stones and plant residues were carefully removed by using forceps, and soil was gently separated along natural breakpoints, passed through an 8-mm sieve, and then divided into three subsamples, which were air dried for physicochemical analysis, stored at 4°C to measure nitrification activity for no more than 3 days, and sieved to <2 mm and immediately stored at −80°C for subsequent DNA extraction.

Analysis of physicochemical properties. Soil pH was measured by using a glass electrode with a 1:5 soil-to-water ratio. Concentrations of soil organic C (SOC) and total N (TN) were determined by using the wet oxidation redox titration and micro-Kjeldahl methods, respectively. Dissolved organic C (DOC) was extracted by incubating 10 g fresh soil (on an oven-dried basis) with 50 ml of deionized water for 30 min, followed by shaking (end-over-end) at 25°C, and the samples were then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was filtered through a 0.45-μm membrane filter (Whatman, Clifton, NJ, USA), and the DOC content was quantified by using a Shimadzu C analyzer (TOC Vcph; Shimadzu, Kyoto, Japan). NH_4^+-N, NO_3^--N, and dissolved total N (DTN) were extracted by using 2 M KCl; the content was measured by using a continuous-flow analyzer (San^{++}; Skalar, Holland); and dissolved organic N (DON) was calculated as DTN − NH_4^+-N − NO_3^--N. Soil available K (AK) was extracted by using 1 M ammonium acetate and measured by using flame photometry (catalog no. FP640; INASA, China). Soil available P (AP) was extracted by using 0.0125 M H_2SO_4 in 0.05 M HCl, and its concentration was determined by using the molybdenum blue method.

Six undisturbed soil cores collected by using a 100-cm³ cylinder were sampled from each plot and used to measure water retention curves with a ceramic pressure plate in a pressure chamber at equilibrium matrix potentials of −0.1, −0.2, −1, −3.5, −6, −10, −33, −50, −100, −200, −500, and −1,500 kPa. The effective diffusion coefficient of oxygen in the soil (DC_o) (square meters per second) was calculated as $DC_o = 1/N^2 \times |D_{ao} \times Q_a^p + KH \times D_{wo} \times Q_w^p|$ (60), where N is soil porosity; D_{ao} is the free diffusion coefficient of oxygen in air at 20°C (1.8×10^{-5} m² s^{-1}); KH is Henry's equilibrium constant at 20°C (0.03); D_{wo} is the free diffusion coefficient of oxygen in water at 20°C (2.2×10^{-9} m² s^{-1}); Q_a and Q_w are the proportions of soil porosity occupied by air and water, respectively ($Q_a + Q_w = 1$); and p is the power constant (3.4). Soil porosity (N) was calculated as $N = 1 − \rho/\rho_0$, where ρ is soil bulk density (grams per cubic centimeter) and ρ_0 is soil particle density (grams per cubic centimeter). The proportion of soil porosity occupied by water (Q_w) was calculated as $Q_w = \rho \times w/N$, where w is the soil gravimetric moisture content (cubic centimeter per gram).

Soil physicochemical properties are presented in Table 1.

Nitrification activity. Nitrification activity (NA) was determined according to a modified version of the method used by Ma et al. (61), where 20 g fresh soil (on an oven-dried basis) was placed into a 125-ml glass jar, to which 50 mg N kg^{-1} as $(NH_4)_2SO_4$ was added, and soil moisture was adjusted to a 60% water-holding capacity with deionized water. The jars were incubated in darkness at 25°C, and 0, 1, 4, and 10 days after the addition of the substrate, three replicates for each treatment were destructively sampled to determine the nitrate concentration, where soil nitrate was extracted by using 2.0 M KCl and measured with a continuous-flow analyzer (San^{++}; Skalar, Holland). NA was calculated as the linear accumulation of nitrate over the incubation period.

DNA extraction and real-time quantitative PCR. Soil DNA was extracted from 0.5 g fresh soil (on an oven-dried basis) by using the Fast DNA spin kit for soil (MP Biomedicals, CA, USA) and subsequently purified by using a PowerClean DNA cleanup kit (Mobio, CA, USA) according to the manufacturer's protocols. The quality and concentration of the extracted DNA were measured by using gel electrophoresis (0.8% agarose) and a spectrophotometer (NanoDrop Technologies), and the DNA was stored at −20°C prior to additional analyses.

Real-time quantitative PCR (qPCR) to identify and quantify archaeal and bacterial *amoA* genes was done by using a CFX96 optical real-time detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). A standard curve was generated by using plasmid DNA from one representative clone containing each target gene. Each reaction mixture (25 μl) comprised 12.5 μl SYBR qPCR master mix (Vazyme), 0.25 μl of each primer, and 1 μl of the DNA template containing approximately 1 to 10 ng of DNA. A negative control was always run with sterilized distilled water as the template instead of a DNA sample. A serial dilution of the DNA template was also used to determine whether the PCR had been inhibited during amplification. Details of gene-specific primers and thermal conditions are presented in Table 3. Amplifications resulted in single peaks, and efficiencies were 90.1 to 101.7%, with R^2 values of between 0.992 and 0.999.

High-throughput sequencing and bioinformatics analysis. We used primer sets Arch-amoA26F/ Arch-amoA417R (62) and amoA-1F/amoA-2R (63) for AOA and AOB *amoA* gene amplification, respectively, where the PCR program and reaction composition of AOA and AOB were described previously by Park et al. (62) and Lin et al. (64), respectively. A unique sample-identifying barcode was added to the forward primer in PCR amplification, triplicate PCR amplifications were conducted and pooled for each sample, and PCR products were subsequently purified by using a Qiagen gel extraction kit. Sequencing libraries were generated by using the TruSeq Nano DNA LT library prep kit (Illumina, USA) according to the manufacturer's recommendations, and library quality was assessed on the Qubit 2.0 fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 systems. Finally, high-throughput sequencing of AOA and AOB *amoA* genes was carried out by using Illumina MiSeq, and 300-bp paired-end reads were generated.

TABLE 3 PCR primers and thermal cycling conditions used for gene quantification

Gene	Primer	Sequence	Amplicon size (bp)	Thermal conditions	Reference
Archaeal *amoA*	Arch-*amoA*F	STAATGGTCTGGCTTAGACG	635	95°C for 3 min and 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s + plate read; melt curve, 65.0°C–95.0°C; increment, 0.5°C, 5 s, + plate read	75
	Arch-*amoA*R	GCGGCCATCCATCTGTATGT			
Bacterial *amoA*	*amoA*-1F	GGGGTTTCTACTGGTGGT	491	95°C for 3 min and 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s + plate read; melt curve, 65.0°C–95.0°C; increment, 0.5°C, 5 s, + plate read	63
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC			

Pairs of reads were merged in FLASH version 1.2.7 (65), where forward and reverse reads had overlapping base lengths of ≥10 bp and base mismatches were prevented. Sequence data were processed by using Quantitative Insights into Microbial Ecology (QIIME) v1.8.0 (66), and low-quality sequences that had quality scores of <20, contained ambiguous nucleotides, or did not match the primer or barcode were removed. Barcode and primer sequences were deleted after sample sequences were sorted according to the barcodes. Next, the sequences were compared with sequences in the reference database (67) by using the UCHIME algorithm to detect chimera sequences that were subsequently removed. The remaining high-quality sequences at 97% similarity were assigned to the same OTUs by using Uparse v7.0.1001 software (68), and the most abundant sequence from each OTU was selected as its representative sequence. The representative OTUs were taxonomically classified from the construction of neighbor-joining phylogenetic trees in MEGA 6 using representative sequences of the AOA or AOB *amoA* genes, together with taxonomically determined reference sequences from GenBank. We used nomenclature for AOA *amoA* clusters as defined by Alves et al. (17) and Zhang et al. (8) and for AOB *amoA* clusters as defined by Avrahami et al. (69) and He et al. (9). Heterogeneity in the number of sequences per sample was removed by rarefying sequences prior to calculation of Chao1 (70) and Shannon (71) diversity indices in QIIME.

Data analysis and statistics. Variances in nitrification activity, *amoA* gene copy numbers, alpha diversity, and relative abundances of AOA and AOB clades were tested by using one-way analysis of variance (ANOVA) in SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). All data were checked for normality and homogeneity of variance (Levene's test) prior to testing for treatment differences; data were ln transformed to meet the assumptions of the ANOVA where necessary. Least-significant-differences (LSD) tests at a P value of 0.05 were used to test for differences among treatments. Associations between soil physicochemical variables, AOA or AOB *amoA* gene copy numbers, and nitrification activity were tested by using Pearson's correlation coefficient.

The following analyses were computed in R (version 3.4.1). A multivariate regression tree (MRT) was built to identify the most important abiotic factors for AOA and AOB community compositions using the mvpart package (72). Correlations between soil physicochemical variables and AOA or AOB community composition were calculated in the vegan package (73) by using a Mantel test. As best-fit models, unimodal canonical correspondence analysis (CCA) and linear redundancy analysis (RDA) were conducted for AOA and AOB communities, respectively, and only environmental variables with a VIF of <20 were selected for analysis. Variance partitioning of nitrification activity was analyzed by using the varpart function in the vegan package, as described previously by Domeignoz-Horta et al. (74).

Accession number(s). Sequences have been deposited in the DNA Data Bank of Japan under accession no. DRA006528 and DRA006529.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM .01031-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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