

Effect of chemical fertilization and green manure on the abundance and community structure of ammonia oxidizers in a paddy soil

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Ammonia oxidization is a critical step in the soil N cycle and can be affected by the fertilization regimes. Chinese milk-vetch (*Astragalus sinicus* L., MV) is a major green manure of rice (*Oryza sativa* L.) fields in southern China, which is recommended as an important agronomic practice to improve soil fertility. Soil chemical properties, abundance and community structures of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) in a MV-rice rotation field under different fertilization regimes were investigated. The field experiment included six treatments: control, without MV and chemical fertilizer (CK); 100% chemical fertilizer (NPK); 18 000 kg MV ha⁻¹ plus 100% chemical fertilizer (NPKM1); 18 000 kg MV ha⁻¹ plus 40% chemical fertilizer (NPKM2); 18 000 kg MV ha⁻¹ alone (MV); and 18 000 kg MV ha⁻¹ plus 40% chemical fertilizer plus straw (NPKMS). Results showed that NPKMS treatment could improve the soil fertility greatly although the application of 60% chemical fertilizer. The abundance of AOB only in the MV treatment had significant difference with the control; AOA were more abundant than AOB in all corresponding treatments. The NPKMS treatment had the highest AOA abundance (1.19×10^8 *amoA* gene copies g⁻¹) and the lowest abundance was recorded in the CK treatment (3.21×10^7 *amoA* gene copies g⁻¹). The abundance of AOA was significantly positively related to total N, available N, NH₄⁺-N, and NO₃⁻-N. The community structure of AOA exhibited little variation among different fertilization regimes, whereas the community structure of AOB was highly responsive. Phylogenetic analysis showed that all AOB sequences were affiliated with *Nitrosospira* or *Nitrosomonas* and all AOA denaturing gradient gel electrophoresis (DGGE) bands belonged to the soil and sediment lineage. These findings could be fundamental to improve our understanding of AOB and AOA in the N cycle in the paddy soil.

Key words: Abundance, ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), *Astragalus sinicus*, community structure, Chinese milk vetch, soil chemical properties.

INTRODUCTION

Appropriate fertilizer application is an important management practice to improve soil fertility. In recent years, due to the rapid population growth and continuous decline in the amount of cultivated land area, the application rate of fertilizer keeps on rising in tropical and subtropical regions of China in order to obtain high crop production. However, the long-term inappropriate fertilization has caused many environmental problems, such as high acidity, low nutrients in cultivated land and unbalanced ecosystem (Chen et al., 2010). Recently, soil quality has gained much attention as a result of environmental issues related to soil degradation and production sustainability under different farming systems (Galantini and Rosell, 2006). Chinese milk vetch (*Astragalus sinicus* L., MV)

is considered as the most popular green manure in paddy field of South China, due to its high N-fixing potential and better growth under wet paddy soil environment. Chinese milk vetch can partly replace N fertilizer and thus decrease application of N fertilizer.

Nitrification is a critical step in the N cycle and has significantly agricultural consequence for the N availability as a plant nutrient. Microbial oxidation of ammonia, the first and rate-limiting step of nitrification, can be performed by both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Both groups have been detected in a wide variety of soil ecosystems (Chen et al., 2008; Di et al., 2009; Strauss et al., 2014). It is important to identify parameter that could impact the activities of AOB and AOA. Some previous studies have investigated the effects of fertilizer types, soil properties and land use on abundance and community structure of ammonia oxidizers in soils. Ai et al. (2013) reported that N fertilization greatly enhanced AOB abundance, while manure application increased AOA abundance. The community structure of AOB exhibited more obvious changes than that of AOA after 31-yr fertilizer field experiment. Xu et al. (2012) studied responses of AOB

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and AOA to different N fertilization rates. They reported that the AOB abundance was more abundant in the N fertilization treatments than the control, AOA abundance decreased with increasing N fertilization rates. Glaser et al. (2010) and Wu et al. (2011) suggested that bacteria rather than archaea dominate ammonia oxidation in near-neutral or alkaline soils and in acidic soils, AOA play a more important role in ammonia oxidization (Zhang et al., 2012). From above literature reviews, it demonstrated that AOB and AOA may prefer to different ecological environments.

In this study, the effects of organic and inorganic fertilizers on the abundance and community structures of AOB and AOA in field fertilization were investigated by using quantitative real-time polymerase chain reaction (qPCR) and denaturing gradient gel electrophoresis (DGGE) respectively.

MATERIALS AND METHODS

Field design and sample collection

A field fertilizer experiment was established in 2009 at Baisha Experimental Station (119°04'10" E, 26°13'31" N), Minhou County, Fuzhou, Fujian Province, China. This region has a subtropical monsoonal climate with an annual average temperature of 19.5 °C and annual average precipitation of 1350 mm. The experiment field is red soil, which is widespread in the South China. The soil is classified as typic Hapli-Stagnic Anthrosols (USDA). At the beginning of the experiment, the soil had a pH (1:2.5) 5.26, 31.41 g kg⁻¹ soil organic matter (SOM), 1.23 g kg⁻¹ total N, and 13.51 and 85.48 mg kg⁻¹ of available P and K, respectively. Six treatments (three replicates each) were implemented in 18 plots (15 m²) under a MV-rice (*Oryza sativa* L.) rotation; MV seeds were sown before the rice harvest and MV plants were ploughed into soil at full blooming stage in the following April. Treatments consisted of soil in absence of MV and chemical fertilizer (control, CK); 100% chemical fertilizer N, P, and K (NPK); 18 000 kg MV ha⁻¹ plus 100% chemical fertilizer N, P, and K (NPKM1), 18 000 kg MV ha⁻¹ plus 60% chemical fertilizer N, P, and K (NPKM2), 18 000 kg MV ha⁻¹ alone (MV), 18 000 kg MV ha⁻¹ plus 40% chemical fertilizer N, P, and K and straw (NPKMS). For NPK treatment, fertilizer N, P, and K were applied in the form of urea (135 kg N ha⁻¹ yr⁻¹), superphosphate (54 kg P₂O₅ ha⁻¹ yr⁻¹), and potassium chloride (94.5 kg K₂O ha⁻¹ yr⁻¹). Chinese MV (18 000 kg ha⁻¹) contains 101 g kg⁻¹ OM, 3.7, 1.1, and 2.8 g kg⁻¹ N, P, and K, respectively, and 86.2% water.

Surface soil samples were collected at 0-20 cm depth on October 2012 (after the harvest of rice). For each plot, five soil cores were taken and mixed to one composite sample. The fresh samples were placed immediately on ice and transported to the laboratory. Aliquots of the soil samples were then stored at -80 °C refrigerator until molecular analysis, or 4 °C until chemical analysis.

Soil chemical analysis

Soil pH was measured with a soil to water ratio of 1:2.5. Soil organic matter was determined by the K₂Cr₂O₇ oxidation method. Total N content (TN) was measured by Kjeldahl digestion analysis. Available N was determined by NaOH hydrolyzable method. The ammonium N (NH₄⁺-N) was determined by extracting the soil with 2 M KCl solution and then detecting by indophenol blue colorimetric method. The nitrate N (NO₃⁻-N) was determined by dual wavelength spectrophotometry method (Huang et al., 2009).

DNA extraction and real-time PCR

Soil total DNA was extracted according to the method described by Zhou et al. (1996) with a modification. The weight of soil samples was decreased to 1 g, the amount of DNA extraction buffer and proteinase K were also decreased to 1/5 of the initial amount. Before the supernatants from the three cycles of extractions were extracted with an equal volume of chloroform-isoamyl alcohol, the supernatants were first extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The pellet of crude nucleic acids was finally resuspended in 100 µL TE buffer. Successful DNA extraction was characterized by electrophoresis on 1% (w/v) agarose gel.

The abundance of bacterial and archaeal *amoA* genes was determined by quantitative polymerase chain reaction (qPCR) using the primers *amoA1-F/amoA2-R* (Rotthauwe et al., 1997) and *CrenamoA23f/CrenamoA616r* (Tourna et al., 2008), respectively. Each reaction contained final 12.5 µL Fast Start SYBR Green Master Mix (Takara, Dalian, China), 0.1 µL of each primer (5 pmol µL⁻¹), 0.2 µL bovine serum albumin (20 mg mL⁻¹, BSA), and 1 µL 10-fold diluted DNA template in a final volume of 25 µL. Real-time PCR was conducted using thermal cycler (CFX96, Bio-Rad Laboratories, Hercules, California, USA) with the following conditions for ammonia-oxidizing bacteria (AOB): initial denaturation for 10 min at 95 °C, amplification for 38 cycles of 30 s at 95 °C, 40 s at 57 °C and 45 s at 72 °C and the following conditions for ammonia-oxidizing archaea (AOA): initial denaturation for 10 min at 95 °C, amplification for 38 cycles of 30 s at 95 °C, 45 s at 58 °C and 50 s at 72 °C. Standard curve was generated by 10-fold serial dilution of plasmid DNA amplified from soil using the qPCR primers. PCR efficiency and linearity (r²) for AOB and AOA were 96.7%, 0.999 and 100.4%, 0.999, respectively.

Denaturing gradient gel electrophoresis (DGGE) analysis

To amplify AOB from soils for DGGE, nested PCR was performed (Zhang et al., 2010). The first PCR was conducted using the AOB-specific primer pair CTO189f and CTO654r which amplifies a 465-bp fragment (Kowalchuk et al., 1997). The PCR product was 50-fold diluted and then used as the template DNA for a second

round of PCR using universal primers (F338GC and R518) (Muyzer et al., 1993). AOA *amoA* genes were amplified using primers CrenamoA23f/CrenamoA616r (Tourna et al., 2008). PCR mixtures consisted of 12.5 μL $2 \times$ Taq mix (Tiangen, Beijing, China), 1 μL of 5 pmol μL^{-1} of each primer, 20 ng DNA template in a final volume of 25 μL . PCR amplification was run on a T1 thermal cycler (Biometra, Goettingen, Germany) as previously described for AOB (Zhang et al., 2010) and AOA (Tourna et al., 2008) using the PCR conditions presented in Table 1. All PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose to verify their size and quality.

DGGE was carried out using the DCode Universal Mutation Detection System (Bio-Rad) according to the manufacturer's instructions. A sample of 30 μL of each PCR product was loaded onto an 8% (w/v) polyacrylamide gel (acrylamide:bisacrylamide 37.5:1) with a denaturant gradient of 35%-60% for AOB and 20%-45% for AOA (100% denaturant contains 7 M urea and 40% deionized formamide). The electrophoresis was performed in $1 \times$ TAE buffer at 60 $^{\circ}\text{C}$, 120 V for 6 h. After staining with SYBR Green I (1:10 000) for 30 min, gels were scanned with a BioSpectrum 410 imaging system (UVP, Upland, California, USA). Band numbers and relative intensity were analyzed using Quantity One analysis software (Bio-Rad).

Cloning, sequencing, and phylogenetic analysis

Dominant bands in the DGGE gels were excised, and each excised piece was washed twice with 1 mL of sterilized distilled water, and suspended into 30 μL sterilized TE buffer (pH 8.0) and kept overnight at 4 $^{\circ}\text{C}$. The DNA fragments were reamplified and PCR products were purified by gel extraction kit SK8132 (Sangon, Shanghai, China). The purified PCR products were ligated to the pMD-19T vector (Takara, Dalian, China) and inserted into *E. coli* DH 5 α competent cells. Clones with the correct insert were sent to be sequenced. The nucleotide sequences were compared with those available in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) program (National Library of Medicine, NCBI, Bethesda, Maryland, USA). The nucleotides obtained from the NCBI GenBank database were aligned and a phylogenetic tree was constructed by the Neighbor-joining method implemented in MEGA version 5.0 (Tamura et al., 2011).

Table 1. Soil chemical properties under different fertilizer treatments.

Treatment	Organic matter	Total N	Available N	NH ₄ ⁺ -N	NO ₃ ⁻ -N	pH
	g kg ⁻¹		mg g ⁻¹			
CK	24.69 \pm 1.44b	1.40 \pm 0.13c	97.00 \pm 5.29d	4.64 \pm 0.51b	1.86 \pm 0.11c	5.16 \pm 0.03a
NPK	24.94 \pm 1.73b	1.47 \pm 0.04bc	139.22 \pm 8.30c	4.87 \pm 0.31ab	2.28 \pm 0.29bc	5.13 \pm 0.02ab
NPKM1	26.40 \pm 1.43ab	1.60 \pm 0.15ab	161.33 \pm 15.13b	5.96 \pm 1.26a	3.26 \pm 0.44a	5.10 \pm 0.03b
NPKM2	26.22 \pm 1.19ab	1.58 \pm 0.04ab	134.19 \pm 10.87c	5.10 \pm 0.48ab	2.39 \pm 0.68bc	5.10 \pm 0.05ab
MV	27.85 \pm 4.42a	1.65 \pm 0.06a	147.76 \pm 3.99bc	4.67 \pm 0.32b	2.75 \pm 0.53ab	5.11 \pm 0.03ab
NPKMS	26.84 \pm 0.72ab	1.67 \pm 0.06a	283.97 \pm 15.70a	5.60 \pm 0.50ab	2.70 \pm 0.27ab	5.08 \pm 0.02b

Data are means \pm SD, n = 3. Values within the same column followed by the same letter do not differ at $P < 0.05$. CK: Control without milk vetch (MV) and fertilizer; NPK: mineral NPK fertilizer; NPKM1: 18 000 kg MV ha⁻¹ plus 100% NPK; NPKM2: 18 000 kg MV ha⁻¹ plus 60% NPK; MV: 18 000 kg MV ha⁻¹; NPKMS: 18 000 kg MV ha⁻¹ plus 40% NPK and straw.

Data analysis

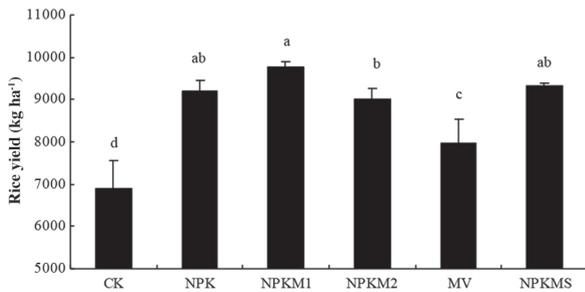
One-way ANOVA for bacterial and archaeal *amoA* gene copy number was performed using the software package SPSS for Windows version 16.0 (SPSS, Chicago, Illinois, USA). Comparison of mean values for different treatments was made using Duncan test. DGGE profiles were analyzed with Quantity One software (version 4.5, Bio-Rad) as described previously (Zhang et al., 2011). Redundancy analysis (RDA) was carried out using Canoco version 4.5 (Centre for Biometry, Wageningen, The Netherlands) to determine the multivariate relationships between community structures of AOB and AOA and soil properties.

RESULTS

Effect of different fertilization regimes on soil chemical properties and rice yield

The highest soil organic matter (SOM) content was in the MV treatment (Table 1), CK and NPK treatments had the lowest SOM. SOM only in the MV treatment was significantly higher than in the NPK treatment. SOM in treatments including NPKM1, NPKM2, and NPKMS were not different with the NPK treatment. CK and NPK treatments had the lowest total N and TN only in the MV and NPKMS treatments were significantly higher than in the NPK treatment. The highest available N was recorded in the NPKMS treatment and the lowest was recorded in the CK treatment. AN only in the NPKM1 and NPKMS treatments were significantly higher than in the NPK treatment. The NPKM1 treatment had the highest NH₄⁺-N and there were no significant differences among other treatments. The highest content of NO₃⁻-N was also observed in the NPKM1 treatment and the lowest was in the CK treatment. The CK treatment had the highest pH and fertilizer application could decrease soil pH. Fertilization regimes had no significant effect on the soil pH. From above results, it demonstrated that NPKMS fertilizer regime could maintain soil fertility although application of 40% chemical fertilizer.

Fertilization regimes had a significant effect on the rice yield (Figure 1). The NPKM1 treatment had the highest rice yield, around 9780 kg ha⁻¹. The NPKMS and NPK treatment had the second and third highest yield, 9340 and 9190 kg ha⁻¹ respectively. There was no significant



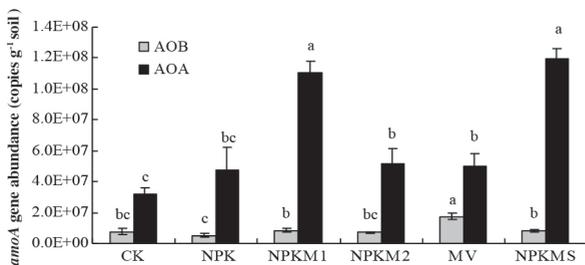
Vertical bars represent the standard deviations ($n = 3$) and different letters indicate significant differences among fertilizer treatments ($P < 0.05$). CK: Control without milk vetch (MV) and fertilizer; NPK: mineral NPK fertilizer; NPKM1: 18 000 kg MV ha⁻¹ plus 100% NPK; NPKM2: 18 000 kg MV ha⁻¹ plus 60% NPK; MV: 18 000 kg MV ha⁻¹; NPKMS: 18 000 kg MV ha⁻¹ plus 40% NPK and straw.

Figure 1. Rice yield under different fertilization treatments.

difference between NPKM1 and NPKMS treatments. The lowest yield was recorded in the CK treatment, around 6890 kg ha⁻¹. In view of the chemical properties and rice yield, the NPKMS treatment could be recommended as the best fertilization regime.

Effect of different fertilization regimes on abundance of AOB and AOA

Mean AOB abundance ranged from 5.22×10^6 to 1.76×10^7 copies g⁻¹ dry soil under different treatments (Figure 2). The highest AOB population size was found in the MV treatment, followed by the NPKM1, NPKMS, CK, NPKM2, and NPK treatments. The lowest AOB population size appeared in the NPK treatment, with the MV treatment 3.36 times higher than the NPK treatment. The population sizes of AOB in MV treatment and MV plus mineral fertilizer treatments with the exception of the NPKM2 were significantly higher than in the NPK treatment. However, mean AOA abundance ranged from 3.21×10^7 to 1.19×10^8 copies g⁻¹ dry soil under different treatments. The population sizes of AOA in all fertilizer treatments were higher than that in the control soil. The abundance of AOA in NPKM1 and NPKMS treatments were significantly higher than in the NPK treatment.



Vertical bars represent the standard deviations ($n = 3$) and different letters indicate significant differences among fertilizer treatments ($P < 0.05$). CK: Control without milk vetch (MV) and fertilizer; NPK: mineral NPK fertilizer; NPKM1: 18 000 kg MV ha⁻¹ plus 100% NPK; NPKM2: 18 000 kg MV ha⁻¹ plus 60% NPK; MV: 18 000 kg MV ha⁻¹; NPKMS: 18 000 kg MV ha⁻¹ plus 40% NPK and straw.

Figure 2. Abundance of soil ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) under different fertilization.

Correlation analysis confirmed that the AOB population size was significantly positively related to SOM and total N contents at $P < 0.01$ and $P < 0.05$, respectively (Table 2). Available N, NH₄⁺-N, NO₃⁻-N, and rice yield were significantly positively related to AOA abundance ($P < 0.01$), and there was a significantly positive correlation between total N and AOA abundance ($P < 0.05$). Soil pH was negatively related to AOA abundance ($P < 0.05$).

Effect of different fertilization regimes on community structure of AOB and AOA

The community structures of AOB and AOA in soils were characterized by DGGE. All fertilizer treatments resulted in increased numbers of AOB bands in the DGGE profiles (Figure 3a). Specifically, band 20 was only detected in the NPK treatment. Bands 18, 19, and 21 were added in comparison with the CK treatment. This result was confirmed by Cluster analysis, which showed that fertilizer treatments were clearly separated from the CK treatment (Figure 4a). These findings indicate that the fertilization regimes might play a major role in shaping the soil AOB community structure.

The predominant bands in the AOB DGGE profile were sequenced for phylogenetic analysis (Figure 5). The AOB DGGE profile corresponding to all treatments was dominated by bands 1, 7-14, and 17, which were affiliated with the *Nitrosospira*. Band 15 affiliated with *Nitromonas* was also found in all soil samples. Bands 18, 19, and 21 detected in the fertilizer treatments were all affiliated with *Nitromonas*.

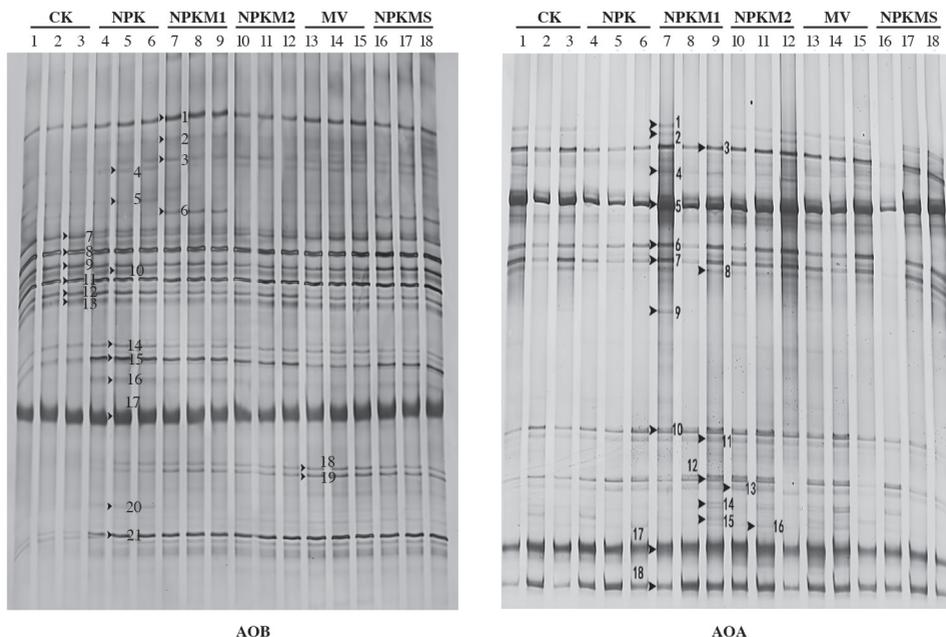
In contrast to the AOB community, the DGGE profiles of AOA remained largely unchanged for most treatments (Figure 3b). Cluster analysis revealed that all treatments were not separated from each other (Figure 4b). Phylogenetic analysis showed that all sequences of these bands were very similar to *amoA* gene clone from soil and sediment lineage, especially from acidic paddy soil (Figure 6).

The Shannon index of AOB in fertilizer treatments were significantly higher than those in the CK treatment (Table 3). Among fertilization treatments, NPKM1 treatment had the highest Shannon index and NPKM2 treatment had the lowest Shannon index. There were no significant differences in other fertilizer treatments. The NPK and MV treatments had the highest richness index, and the lowest richness index was observed in the CK treatment. In contrast, the NPK treatment had the lowest Shannon and richness indices of AOA. Fertilization regimes had no significant effect on the diversity of AOA compared to the CK treatment.

Table 2. Correlations of soil properties, yield and abundance of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA).

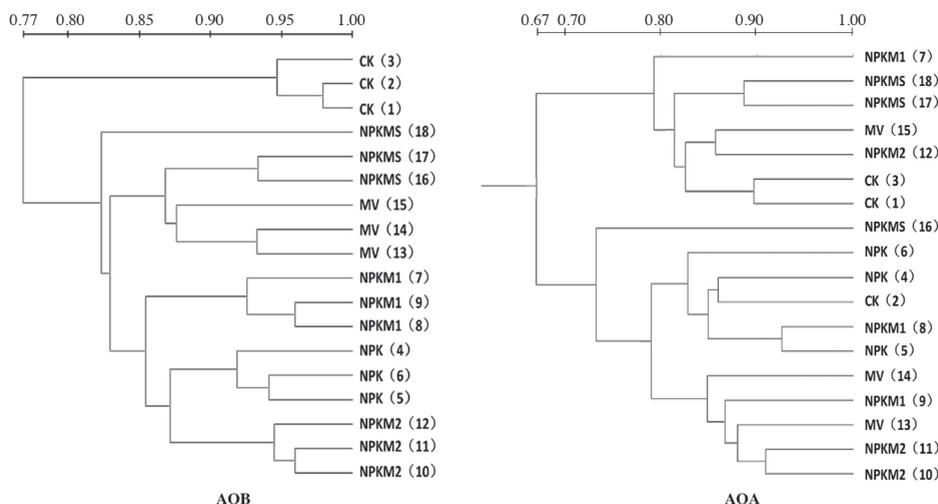
	Organic matter	Total N	Available N	NH ₄ ⁺ -N	NO ₃ ⁻ -N	pH	Yield
AOB	0.627**	0.53*	0.024	-0.233	0.3	-0.059	-0.266
AOA	0.308	0.511*	0.785**	0.625**	0.637**	-0.577*	0.703**

*, **Significant at the 0.05 and 0.01 probability level, respectively.



CK: Control without milk vetch (MV) and fertilizer; NPK: mineral NPK fertilizer; NPKM1: 18 000 kg MV ha⁻¹ plus 100% NPK; NPKM2: 18 000 kg MV ha⁻¹ plus 60% NPK; MV: 18 000 kg MV ha⁻¹; NPKMS: 18 000 kg MV ha⁻¹ plus 40% NPK and straw.

Figure 3. DGGE analysis of soil ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) under different fertilization.



CK: Control without milk vetch (MV) and fertilizer; NPK: mineral NPK fertilizer; NPKM1: 18 000 kg MV ha⁻¹ plus 100% NPK; NPKM2: 18 000 kg MV ha⁻¹ plus 60% NPK; MV: 18 000 kg MV ha⁻¹ alone; NPKMS: 18 000 kg MV ha⁻¹ plus 40% NPK and straw.

Figure 4. Similarity dendrograms (UPGMA) of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) banding patterns calculated from DGGE patterns.

Correlations of soil properties with community structures of ammonia-oxidizing bacteria and archaea

RDA was performed to determine the correlations of soil properties with community structures of AOB and AOA. The first and second axes explained 27.0% and 8.1%, respectively, of the variance in AOB community structure (Figure 7a). Available N ($F = 3.850$, $r = 0.194$, $P = 0.004$), soil pH ($F = 3.191$, $r = 0.166$, $P = 0.002$) and NH₄⁺-N

content ($F = 2.628$, $r = 0.141$, $P = 0.044$) significantly correlated with AOB structure. The rest of the investigated soil properties did not correlate with AOB community structure ($P > 0.05$).

The first and second axes explained 12.9% and 8.5%, respectively, of the variance in AOA community structure (Figure 7b). However, all soil properties did not significantly correlate with community structure of AOA ($P > 0.05$).

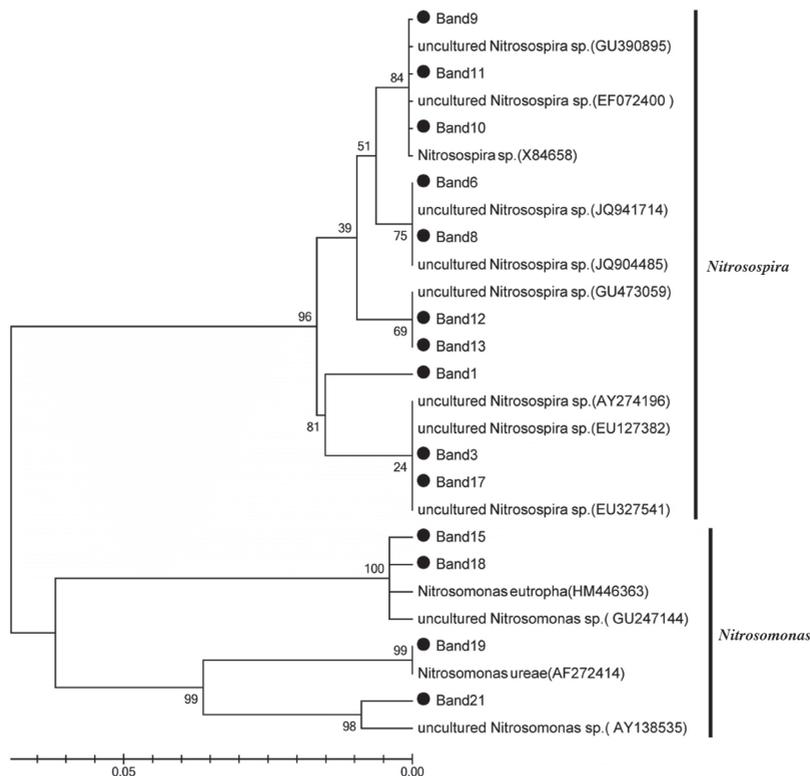


Figure 5. Neighbor-joining tree of soil ammonia-oxidizing bacteria (AOB) retrieved from the DGGE bands and from the NCBI GenBank database.

DISCUSSION

Bacterial *amoA* gene copy numbers ranged from 5.22×10^6 to 1.76×10^7 per gram of dry soil. Compared with AOB, the archaeal *amoA* copy numbers were more abundant, ranging from 3.21×10^7 to 1.19×10^8 per gram of dry soil in our study. The result was consistent with some previous studies that also demonstrated AOA were more abundant than AOB in agricultural soils (He et al., 2007; Mincer et al., 2007; Ying et al., 2010). The abundance of AOB and AOA were higher in the MV or MV plus mineral fertilizer treatments than in the NPK treatment. Higher level of AOB and AOA in the MV or MV plus mineral fertilizer treatments can be associated with the highest C bioavailability, but this could not explain the abundance of AOB in the CK treatment. Furthermore, there was no significant difference in the population size of AOB among fertilizer treatments with the exception of the MV treatment and CK. This was inconsistent with previous studies that showed the population size of AOB increased significantly after fertilization (Wu et al., 2011; Ai et al., 2013; Strauss et al., 2014). Compared with AOB, the population size of AOA responded to fertilizers in a different manner (Figure 2). The abundance of AOA was significantly higher than CK treatment after fertilizer application, however, there was no significant difference in the population size of AOA between the NPK and

CK treatments. These results were consistent with the study conducted by Ai et al. (2013), which showed that fertilizer application could increase the AOA abundance in the rhizosphere and bulk soil during maize season. Positive correlations between AOA abundance and total N, available N, $\text{NH}_4^+\text{-N}$, and $\text{NO}_3^-\text{-N}$ were observed in the present study (Table 2). These results indicated that N condition may be a key factor affecting the population size of AOA in the acidic soil. Positive correlations between AOA abundance and organic C and total N observed by Ai et al. (2013) support the idea that AOA have alternative growth strategies for mixotrophic and heterotrophic growth (Walker et al., 2010). However, there was no correlation between AOA abundance and OM which support the idea that AOA may have autotrophic growth strategies. In our study, soil pH was negatively related to AOA abundance, this result was also observed by Nicol et al. (2008).

DGGE analysis revealed that the community structure of AOB was highly sensitive to fertilization regimes. Cluster analysis demonstrated that fertilization treatments were clearly separated from CK treatment (Figure 4a). Diversity indices analysis also demonstrated that application of fertilizer could increase the community diversity of AOB (Table 3). Chu et al. (2007) also reported that the AOB community structure was more diverse in N-fertilized treatments than in the control. Many previous

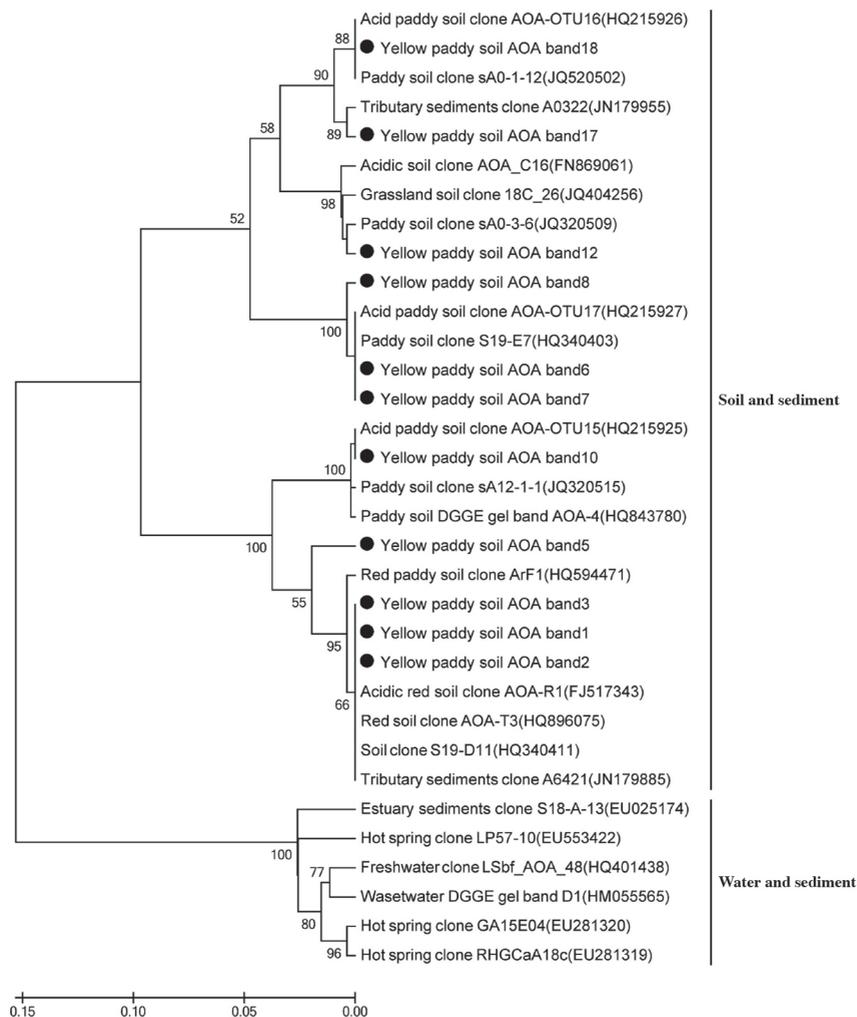


Figure 6. Neighbor-joining tree of soil ammonia-oxidizing archaea (AOA) retrieved from the DGGE bands and from the NCBI GenBank database.

studies have demonstrated that the predominance of *Nitrosospira* over *Nitrosomonas* in AOB community in terrestrial ecosystems (Innerebner et al., 2006; He et al., 2007; Ai et al., 2013). This study also revealed that the sequences related to *Nitrosospira* predominant in the

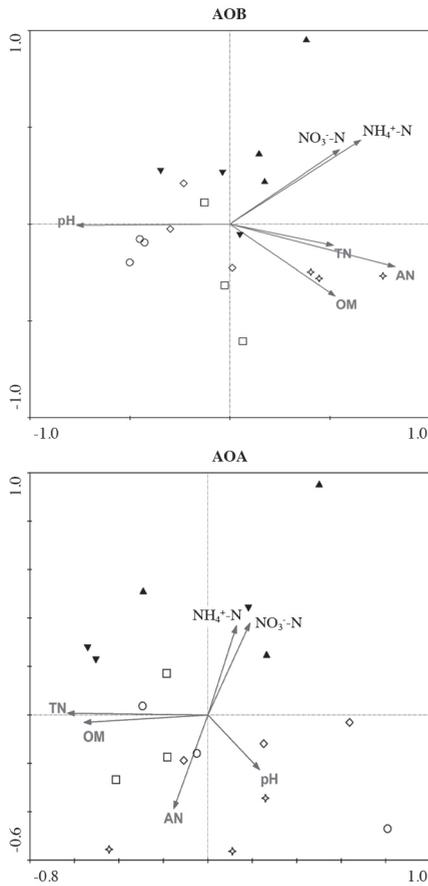
paddy soil. These findings are inconsistent with the results of some previous studies in which only *Nitrosospira* and not *Nitrosomonas* spp. were detected in soil (Chu et al., 2007; Wu et al., 2011). It is likely caused by the differences in soil type which is the primary determinants of the composition of the bacterial communities in arable soils (Girvan et al., 2003). In this study, the differences in the AOB community structure among treatments were correlated with the soil pH (Figure 7a), indicating that pH plays an important role in shaping the AOB community structure. Unlike AOB, we observed that community structure of AOA exhibited no obvious change which is consistent by Shen et al. (2011) and Wang et al. (2009). Fan et al. (2011) also reported that the community structure of AOA in soil exhibited little variation among fertilization treatments. Cluster analysis showed that the CK treatment could not be clearly separated from the fertilized treatments (Figure 4b). Diversity analysis demonstrated that there

Table 3. Diversity indices of soil ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA).

Treatment	AOB		AOA	
	Shannon (H)	Richness (S)	Shannon (H)	Richness (S)
CK	2.42 ± 0.02d	13.00 ± 0.00d	2.54 ± 0.00abc	15.33 ± 0.58ab
NPK	2.87 ± 0.02bc	20.67 ± 0.58a	2.40 ± 0.10c	13.00 ± 1.00b
NPKM1	2.96 ± 0.02a	20.00 ± 0.00b	2.69 ± 0.30ab	17.67 ± 4.04a
NPKM2	2.83 ± 0.01c	19.00 ± 0.00c	2.80 ± 0.05a	19.00 ± 1.00a
MV	2.88 ± 0.04b	20.67 ± 0.58a	2.71 ± 0.03a	17.67 ± 1.15a
NPKMS	2.87 ± 0.08bc	20.00 ± 0.00b	2.41 ± 0.18bc	13.00 ± 2.00b

Data are means ± SD, n = 3. Values within the same column followed by the same letter do not differ at P < 0.05.
 CK: Control without milk vetch (MV) and fertilizer; NPK: mineral NPK fertilizer; NPKM1: 18 000 kg MV ha⁻¹ plus 100% NPK; NPKM2: 18 000 kg MV ha⁻¹ plus 60% NPK; MV: 18 000 kg MV ha⁻¹; NPKMS: 18 000 kg MV ha⁻¹ plus 40% NPK and straw.

were no significant differences in Shannon and richness indices between fertilized treatments and the control (Table 3). Above results demonstrated that AOB and AOA responded to fertilizer regimes in a different way.



○CK: Control without milk vetch (MV) and fertilizer; ◇NPK: mineral NPK fertilizer; ▲NPKM1: 18000 kg MV ha⁻¹ plus 100% NPK; ▼NPKM2: 18000 kg MV ha⁻¹ plus 60% NPK; □MV: 18000 kg MV ha⁻¹ alone; †NPKMS: 18000 kg MV ha⁻¹ plus 40% NPK and straw.

Figure 7. Correlations of soil properties with community structure of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) as determined by redundancy analysis (RDA).

CONCLUSIONS

This study demonstrated that fertilizer regimes had impacts on the soil properties, the activities and community structures of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The NPKMS treatment was recommended as a better fertilizer practice with higher soil fertility, rice yield and application of less chemical fertilizer. Chemical fertilizer plus green manure increased the population size of AOA significantly, whereas fertilization regimes had little effects on AOB abundance. Fertilizer application had little impact on the community structure of AOA. In contrast, the community structure of AOB was highly sensitive to fertilization

regimes and the application of fertilizer could increase the diversity of AOB. However, the mechanisms which drive the response of AOB and AOA to different fertilization regimes are still unknown and need further study.

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