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Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam

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Summary

The abundance and composition of soil ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) communities under different long-term (17 years) fertilization practices were investigated using real-time polymerase chain reaction and denaturing gradient gel electrophoresis (DGGE). A sandy loam with pH (H_2O) ranging from 8.3 to 8.7 was sampled in years 2006 and 2007, including seven fertilization treatments of control without fertilizers (CK), those with combinations of fertilizer nitrogen (N), phosphorus (P) and potassium (K): NP, NK, PK and NPK, half chemical fertilizers NPK plus half organic manure (1/2OMN) and organic manure (OM). The highest bacterial *amoA* gene copy numbers were found in those treatments receiving N fertilizer. The archaeal *amoA* gene copy numbers ranging from 1.54×10^7 to 4.25×10^7 per gram of dry soil were significantly higher than those of bacterial *amoA* genes, ranging from 1.24×10^5 to 2.79×10^6 per gram of dry soil, which indicated a potential role of AOA in nitrification. Ammonia-oxidizing bacteria abundance had significant correlations with soil pH and potential nitrification rates. Denaturing gradient gel electrophoresis patterns revealed that the fertilization resulted in an obvious change of the AOB community, while no significant change of the AOA community was observed among different treatments. Phylogenetic analysis showed a dominance of *Nitrosospira*-like sequences, while three bands were affiliated with the *Nitrosomonas* genus. All AOA sequences fell within cluster S (soil origin) and cluster M (marine and sediment origin). These results suggest that long-term fertilization had a significant impact on AOB abundance and composition, while minimal on AOA in the alkaline soil.

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Introduction

The oxidation of ammonia to nitrate via nitrite, i.e. nitrification, is of fundamental importance in the global nitrogen (N) cycle. While the general biogeochemistry of autotrophic ammonia-oxidizing bacteria (AOB) is well understood, there have been a number of recent discoveries associated with nitrification. For example, two complementary metagenomic studies of seawater (Venter *et al*., 2004) and soil (Treusch *et al*., 2005) revealed the presence of ammonia monooxygenase (*amoA*) gene derived from uncultivated *Crenarchaeota*, suggesting a potential capacity for ammonia oxidation. The potential of archaeal ammonia oxidation was demonstrated by the isolation of *Nitrosopumilus maritimus* from a marine aquarium and its close phylogenetic relationship to environmental marine crenarchaeal sequences indicates that ammonia-oxidizing archaea (AOA) may be important to the global nitrogen cycle (Könneke *et al*., 2005).

The diversity of AOB has been shown to shift in response to agricultural management practices (Bruns *et al*., 1999; Avrahami and Conrad, 2003). Stephen and colleagues (1996) detected a trend showing domination by *Nitrosospira* cluster 3 in neutral pH agricultural plots towards *Nitrosospira* cluster 2 in acidic soils. Molecular analysis of Deepskye soil in southern Scotland indicated no effect of nitrogen deposition on AOB communities, and the AOB composition contained both *Nitrosomonas europaea* and *Nitrosospira* in year 2002, while only *Nitrosospira* could be detected in year 2003 (Schmidt *et al*., 2007). These studies showed a possible change in abundance and community of AOB in response to the temporal variation and environmental factors.

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Table 1. Chemical properties and potential nitrification rate (PNR) of a sandy loam soil under different fertilization treatments.

Treatment[a]	pH (H_2O)	Organic carbon (g kg^{-1})	NH_4^+–N (mg kg^{-1})	NO_3^-–N (mg kg^{-1})	PNR (μg NO_2^-–N g^{-1} dry soil h^{-1})
CK	8.65 ± 0.05c	4.21 ± 0.26a	6.14 ± 3.58a	6.46 ± 3.25a	0.13 ± 0.03a
NP	8.34 ± 0.04a	5.52 ± 0.17c	4.25 ± 0.40a	33.4 ± 0.8c	2.54 ± 0.55d
NK	8.41 ± 0.09ab	4.30 ± 0.23a	5.35 ± 1.48a	35.2 ± 10.3c	1.54 ± 0.56c
PK	8.51 ± 0.04b	4.86 ± 0.13b	4.74 ± 0.57a	2.36 ± 0.39a	0.09 ± 0.03a
NPK	8.37 ± 0.07a	5.71 ± 0.24c	4.65 ± 0.64a	22.6 ± 2.7b	1.13 ± 0.32b
1/2OMN	8.47 ± 0.05b	7.40 ± 0.46d	5.20 ± 0.45a	10.9 ± 1.1a	0.34 ± 0.08a
OM	8.48 ± 0.01b	9.68 ± 0.67e	5.45 ± 0.43a	4.91 ± 3.63a	0.14 ± 0.08a

a. Treatment: control without fertilization (CK), fertilizers NP (NP), fertilizers NK (NK), fertilizers PK (PK), fertilizers NPK (NPK), half chemical fertilizers NPK plus half organic manure (1/2OMN) and organic manure (OM).
Values are mean ± SD ($n = 4$). Values within the same column followed by the same letter do not differ at $P < 0.05$.

Following the discovery of the archaeal *amoA* gene to be ubiquitous in the ocean, the study on AOA in the terrestrial environment has become more compelling than before. For example, real-time polymerase chain reaction (PCR) analyses showed that transcripts of *amoA*-like genes can be detected in soil and that transcription was probably induced in response to ammonia addition (Treusch *et al*., 2005). A study in the North Sea revealed the abundance of archaeal *amoA* genes was one to two orders of magnitude higher than those of bacterial nitrifiers (Wuchter *et al*., 2006). Similar results were obtained in soil ecosystems, where AOA dominate in numbers over AOB, as much as three orders of magnitude depending on soil type measured (Leininger *et al*., 2006). The pH values of the soil samples used in that study ranged from 5.5 to 7.3, mostly in the neutral pH range. In a previous study, we investigated the abundance and community structure of AOB and AOA in acid soil samples with pH ranging from 3.7 to 6.0, and found that there was a pronounced difference in AOA composition among different fertilization treatments and higher copy numbers of archaeal *amoA* gene than bacterial *amoA* gene (He *et al*., 2007). Phylogenetic analysis of several hundred archaeal *amoA* sequences revealed diverse and distinct AOA communities associated with different habitats and sampling sites, with little overlap between water columns and sediments (Francis *et al*., 2005). Therefore, considering the abundance of AOA in the environment, it is also important to identify the factors that influence the community abundance and structure of AOA in soils.

Nitrification in the alkaline soil is different from acid and neutral soils (Sigunga *et al*., 2002), and alkaline soil is widely distributed in northern China. However, knowledge of the interactions between environmental conditions and the communities of AOB and AOA in alkaline arable soil is particularly scarce. In this study, we collected alkaline soil samples from a 17-year fertilization experimental station located in northern China in April 2006 and April 2007, and examined the abundance and composition of AOB and AOA under different fertilization practices. It aimed to assess the relationship between the abundance and composition of AOB and AOA, and the soil characteristics associated with different fertilization regimes using real-time PCR and denaturing gradient gel electrophoresis (DGGE) approaches.

Results

Soil chemical properties and potential nitrification rates

Soil pH values ranged from 8.34 to 8.65 with some changes among the different fertilizer treatments (Table 1). Control without fertilizer (CK) had the highest pH of 8.65, while the lowest pH values were recorded from 8.34 to 8.41 in all the mineral fertilizer treatments receiving mineral nitrogen (N) fertilizer (NP, NK and NPK; P = phosphorus; K = potassium). No significant difference in soil NH_4^+–N content was found among the treatments (Table 1). There was a higher ratio of NO_3^-–N to NH_4^+–N in the mineral N treatments [NP, NK, NPK and half chemical fertilizers NPK plus half organic manure (1/2OMN)] from 7.76 to 2.09, while the ratio in CK was 1.05. The ratio in PK and organic manure (OM) treatments were below 1.0, as 0.50 and 0.90 respectively.

Potential nitrification rates (PNR) provide an independent estimate of the abundance of ammonia oxidizers (Table 1). The highest potential nitrification rates were recorded in NP and 28 times higher than PK. The second and third were in the NK and NPK treatments, respectively, and the lowest were recorded in the other treatments (1/2OMN, OM, CK and PK). Potential nitrification rates had a positive correlation with soil NO_3^-–N content ($r = 0.929$, $n = 7$, $P < 0.01$), but a negative correlation with soil pH ($r = -0.784$, $n = 7$, $P < 0.05$).

Abundance of bacteria in the soil

The real-time PCR assays, targeting bacterial 16S rRNA gene, yielded 3.39×10^9–1.23×10^{10} copy numbers per gram of dry soil in 2006 (Fig. 1). There was no significant

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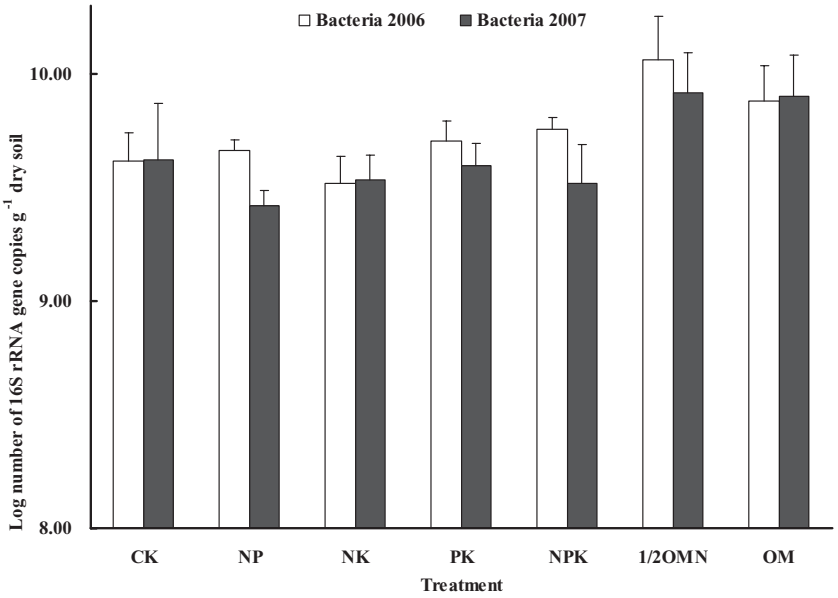

Fig. 1. Bacterial 16S rRNA gene copy numbers in 2006 and 2007 under different fertilization treatments. Treatment: control without fertilization (CK), fertilizers NP (NP), fertilizers NK (NK), fertilizers PK (PK), fertilizers NPK (NPK), half chemical fertilizers NPK plus half organic manure (1/2OMN) and organic manure (OM).

difference in the bacterial 16S rRNA gene copy numbers between years 2006 and 2007. The number of bacterial 16S rRNA gene copies in the 1/2OMN treatment in 2006 was significantly higher than other treatments. However, in 2007 two treatments (1/2OMN and OM) were significantly higher than other treatments. Additionally, there was a significant correlation between soil organic carbon and bacterial 16S rRNA gene copy numbers ($r = 0.769$, $n = 7$, $P < 0.05$).

Abundance of AOB and AOA in the soil

Abundance of AOB and AOA were estimated by quantifying their respective *amoA* gene copy numbers. The highest bacterial *amoA* gene copy numbers in 2006 at 2.76×10^6 copies per gram of dry soil were found in the NP treatment, followed by the NK, NPK and 1/2OMN treatments, and the lowest appeared in the CK treatments, with the NP treatment 22.5 times higher than the CK treatment (Fig. 2). The bacterial *amoA* gene copy numbers in treatments receiving N fertilizer were significantly higher than other treatments. No significant differences were found between the CK and PK treatments. In contrast to the abundance of AOB, no significant differences in the archaeal *amoA* gene copy numbers were observed among all the treatments (Fig. 2). The archaeal *amoA* gene copy numbers in different treatments, ranging from 1.54×10^7 to 4.25×10^7 per gram of dry soil, were higher than those of bacterial *amoA* gene copy numbers, ranging between 1.24×10^5 and 2.79×10^6 per gram of dry soil. The ratios of AOA to AOB ranged from 7.75 to over 276 in the treatments, and were significantly higher

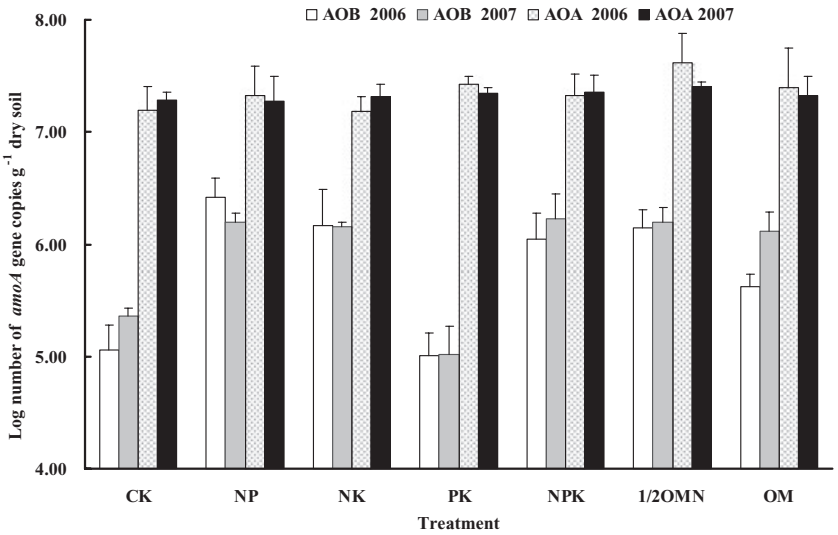

Fig. 2. Abundance of bacterial and archaeal *amoA* genes in 2006 and 2007 under different fertilization treatments. Treatment: control without fertilization (CK), fertilizers NP (NP), fertilizers NK (NK), fertilizers PK (PK), fertilizers NPK (NPK), half chemical fertilizers NPK plus half organic manure (1/2OMN) and organic manure (OM).

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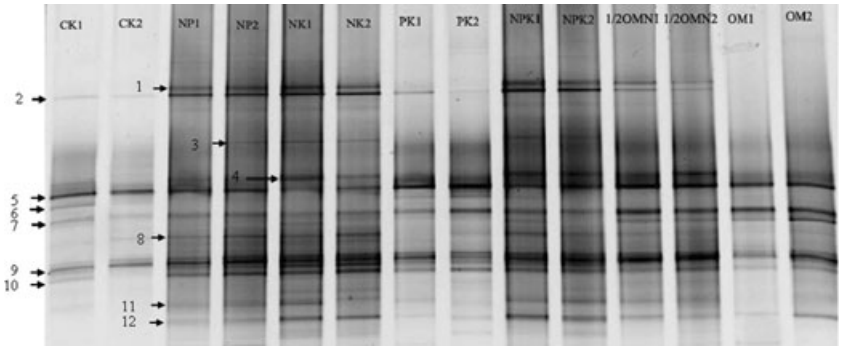

Fig. 3. Denaturing gradient gel electrophoresis profiles of bacterial *amoA* gene under different fertilization treatments. Band position was highlighted with a numbered arrow. Treatment: control without fertilization (CK), fertilizers NP (NP), fertilizers NK (NK), fertilizers PK (PK), fertilizers NPK (NPK), half chemical fertilizers NPK plus half organic manure (1/2OMN) and organic manure (OM).

in the CK and PK treatments (128 and 276 respectively). The data in 2007 showed a significantly positive correlation with those in 2006 for AOB ($r = 0.911$, $n = 7$, $P < 0.01$) and AOA ($r = 0.790$, $n = 7$, $P < 0.05$). In addition, there was no significant difference in the *amoA* gene copy numbers of AOB or AOA in different treatments between the two years.

Genetic profiling of bacterial and archaeal amoA *genes*

The community structure of AOB and AOA were analysed by DGGE. Denaturing gradient gel electrophoresis profiles of three replicates for each treatment indicated good reproducibility and two of three replicates were shown in this study (Figs 3 and 4). Bands with the same mobility in the DGGE gels were marked with the same number and excised for phylogenetic analysis.

Denaturing gradient gel electrophoresis patterns of AOB showed a clear variation with different fertilizer treatments and 12 different bands in total were detected (Fig. 3). Band 3 was unique to all the mineral N fertilizer treatments except in NP1. Those treatments that received N fertilizer (NP, NK, NPK and 1/2OMN) showed a more diverse banding pattern with additional bands 1–2, 8 and 11, than the OM treatments. Although some bands (2, 5, 7, 9, 10) were present in the profiles of most treatments, their intensity was different. For example, band 2 had the highest intensity in those mineral N fertilizer treatments, while slight in the CK and 1/2OMN treatments, disappeared in the OM treatment. Shannon index is commonly used to characterize species diversity in a community. The mineral N fertilizer treatments (NP, NK, NPK) had higher Shannon index values than any other treatments (Table 2). There was a significant correlation between the

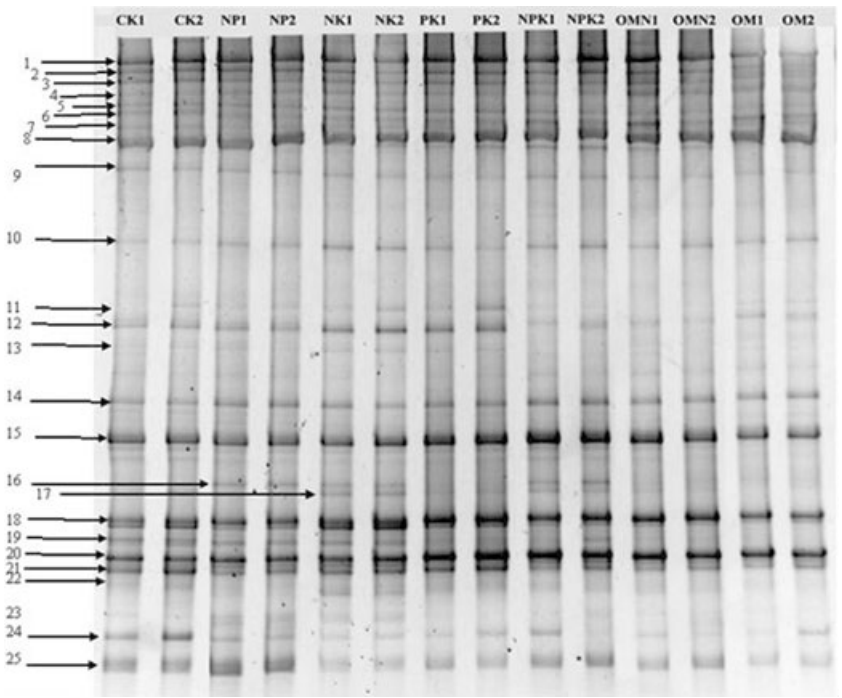

Fig. 4. Denaturing gradient gel electrophoresis profiles of archaeal *amoA* gene under different fertilization treatments. Band position was highlighted with a numbered arrow. Treatment: control without fertilization (CK), fertilizers NP (NP), fertilizers NK (NK), fertilizers PK (PK), fertilizers NPK (NPK), half chemical fertilizers NPK plus half organic manure (1/2OMN) and organic manure (OM).

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Table 2. Diversity properties of AOB and AOA calculated from DGGE band pattern data under different fertilization treatments.

	AOB		AOA	
Treatment	Shannon (*H*)	Evenness (*E*)	Shannon (*H*)	Evenness (*E*)
CK	1.72 ± 0.05a[a]	0.96 ± 0.03	2.99 ± 0.05b	0.95 ± 0.01
NP	2.35 ± 0.02d	0.98 ± 0.01	3.03 ± 0.04b	0.95 ± 0.01
NK	2.45 ± 0.01e	0.98 ± 0.00	3.10 ± 0.02c	0.96 ± 0.01
PK	2.00 ± 0.09b	0.96 ± 0.04	3.04 ± 0.02b	0.97 ± 0.01
NPK	2.34 ± 0.00d	0.98 ± 0.00	3.01 ± 0.04b	0.93 ± 0.01
1/2OMN	2.15 ± 0.02c	0.98 ± 0.01	2.89 ± 0.02a	0.94 ± 0.01
OM	2.00 ± 0.04b	0.96 ± 0.02	2.91 ± 0.02a	0.94 ± 0.01

a. Mean ± SD ($n = 4$). Values within the same column followed by the same letter do not differ at $P < 0.05$.

Shannon index and the copy numbers of AOB ($r = 0.873$, $n = 7$, $P < 0.05$). There were no significant differences in the Evenness among the different treatments.

The DGGE profile of AOA showed a marked difference with the AOB profile and 25 bands were detected in total (Fig. 4). Bands 1–8 appeared at the up part of the gels with higher intensity than other bands. Bands 11 and 13 showed high intensity in NK and PK, but faint in other treatments. Bands 16 and 23 were only detected in the mineral N fertilizer treatments, and band 19 could not be detected in those treatments receiving organic manure. The Shannon diversity of the organic manure treatments (1/2OMN and OM) showed a significantly lower value than other treatments (Table 2).

The sequences at 2% nucleotide cut-offs were used to construct phylogenetic trees with representative sequences retrieved from the GenBank database (Figs 5 and 6). The classification for AOB clusters was tentatively defined in previous studies (Avrahami and Conrad, 2003; He *et al.*, 2007). All the sequenced clones represented *amoA*-like sequences that grouped with *Nitrosospira* and *Nitrosomonas*-like sequences. Band 1 detected in the N fertilizer treatments were affiliated with *Nitrosospira* cluster 3b, indicating that N fertilization resulted in a dominance of cluster 3b. Bands 4 and 6 were grouped in *Nitrosospira* cluster 3c. In addition, bands 5 and 9 detected in all the treatments were affiliated with the genus *Nitrosomonas* and belonged to cluster 7. Interestingly, most of the other DGGE bands were affiliated with the genus *Nitrosospira* cluster 3a. Phylogenetic analysis of archaeal *amoA* gene showed that all selected clones could be divided into two clusters, i.e. cluster S and cluster M (Fig. 6), representing groups dominated by sequences from soil and marine environments respectively (He *et al.*, 2007). The majority of AOA sequences were grouped in cluster S, showing low diversity. Only three DGGE bands, i.e. bands 11–13, were placed in the cluster M.

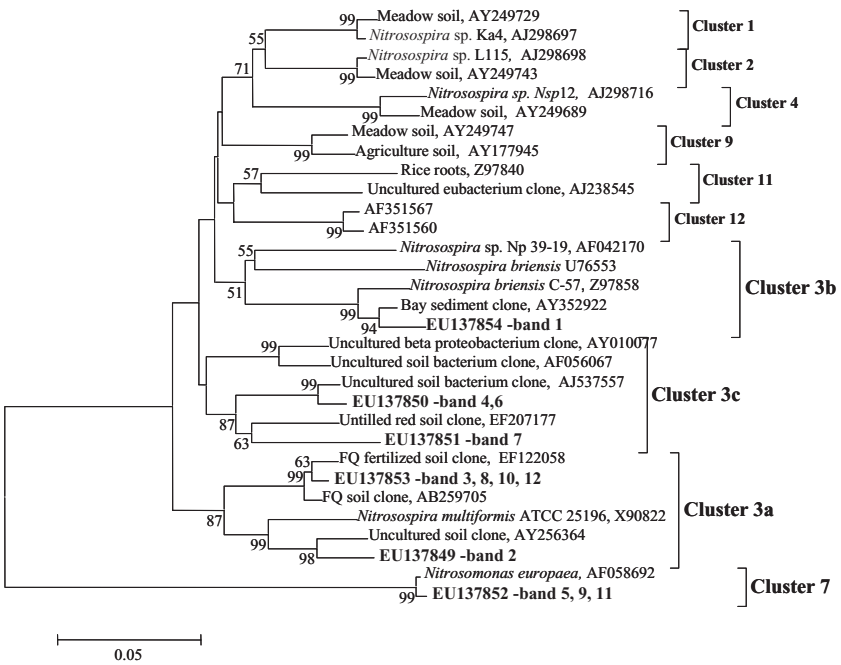

Fig. 5. Phylogenetic relationships among bacterial *amoA* sequences retrieved from the Fengqiu sandy loam. Designation of the clones in bold includes the following information: accession number in the GenBank with DGGE band position. Bootstrap values (> 50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence.

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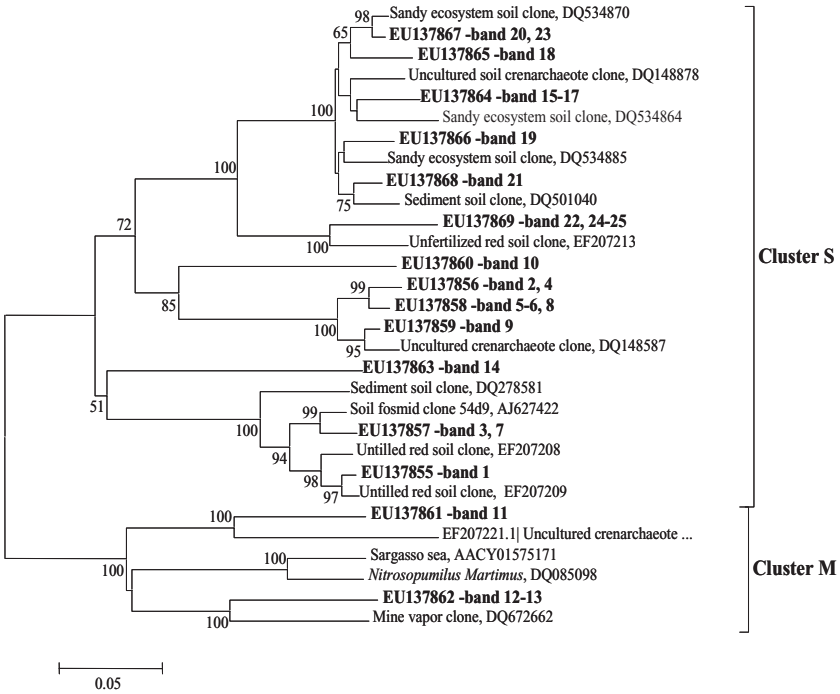

Fig. 6. Phylogenetic relationships among archaeal *amoA* sequences retrieved from the Fengqiu sandy loam. Designation of the clones in bold includes the following information: accession number in the GenBank with DGGE band position. Bootstrap values (> 50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence.

Discussion

In this study, we applied real-time PCR to quantify 16S rRNA genes and *amoA* genes using the same quantification conditions as in a previous study to compare the effect of fertilization practices on an alkaline soil. The copy numbers of bacterial and archaeal *amoA* genes measured in this study were both similar to those measured in another arable soil (He *et al.*, 2007). High ratios of AOA to AOB were found in the alkaline soil, providing evidence of their potential importance in nitrification.

Soil pH had a significantly negative correlation with the bacterial *amoA* gene copy numbers ($r = 0.841$, $n = 7$, $P < 0.05$), indicating pH was an important factor in controlling AOB abundance in the soil. The process of ammonia oxidation due to long-term fertilizer inputs leads to a net increased concentration of protons in the environment, which contributes to a lowering of the soil pH (Kowalchuk and Stephen, 2001; Pernes-Debuyser and Tessier, 2004; Enwall *et al.*, 2005). The sampled soil originating from alluvial sediments of the Yellow River was alkaline (pH 8.65 in CK). The long-term fertilizer application had resulted in a decrease in the soil pH, with the treatments of chemical N application decreasing to 8.3–8.4 and the OM and PK treatments to 8.4–8.5. Although only 0.3 pH units decreased between the treatments of CK and NP, the abundance of AOB increased 22.5-folds. The reduction in pH may have increased AOB growth and abundance. However, no significant correlation was observed between AOA and pH in this study, inconsistent with the result that soil pH played a more important role in AOA populations as found in acid soil (He *et al.*, 2007). The reason for this inconsistency may be due to the different soil types and the pH variation ranges. Girvan and colleagues (2003) proposed that soil type is the primary determinant of the bacterial community composition in arable soils, while there is no available information about the relationship of soil type and AOA abundance.

Significantly higher copy numbers of bacterial *amoA* gene in the N fertilizer treatments were found compared with the OM and CK treatments in this study, indicating N input could be another key factor for AOB abundance in the natural soil environment. The increasing bioavailability of carbon in the treatments amended with organic manures will naturally promote the growth of heterotrophic bacteria, which could decrease the amount of available ammonia to AOB (Fauci and Dick, 1994; Shi and Norton, 2000). A higher ratio of nitrate to ammonium in those mineral N fertilizer treatments also indicated their preference for these conditions due to a significant correlation between nitrate content and AOB ($r = 0.805$, $n = 7$, $P < 0.05$). The proportion of AOB to total bacteria detected in the mineral N fertilizer treatments, ranging from 0.012% to 0.060%, was significantly higher than those in the CK treatment (0.003%). Although AOB varied significantly in the abundance with different treatments, they were just a small fraction of the total bacterial population. However, this small fraction changed sensitively in response to the N fertilizer application, providing a potential indicator of such change. Therefore, AOB were more sensitive than

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total bacteria as an indicator to index nitrogen cycling in the soil (Kowalchuk and Stephen, 2001). There was a significant correlation between PNR and AOB abundance ($r = 0.773$, $n = 7$, $P < 0.05$), but not with the AOA. This suggested that AOB abundance could be predicted by PNR with high confidence (Bernhard *et al*., 2007).

No significant differences in AOA abundance were observed among the different treatments, suggesting that N fertilizer input may not be a substantial factor for the AOA community in the alkaline soil or the amount of ammonia required for detectable changes in AOA abundance will be significantly greater than for the less abundant AOB. Previous studies demonstrated great abundances of AOA in marine environment which have an alkaline pH (Wuchter *et al*., 2006; Mincer *et al*., 2007; Coolen *et al*., 2007). A study on the Black Sea has demonstrated an abundance of putative nitrifying archaea at very low oxygen levels and might form an important source of nitrite for the anammox reaction (Coolen *et al*., 2007). The higher numbers of AOA detected in soil or marine environment may play a potential role in the global nitrogen cycle. In estuarine sediments from Bahía del Tóbari (Mexico), AOA communities from the interior of the estuary to the mouths of the estuary showed spatial structuring, and the distribution of these two archaeal *amoA* ecotypes was associated with group 1.1a and 1.1b *Crenarchaeota* (Beman and Francis, 2006). These findings represent the first detailed examination of archaeal *amoA* diversity in estuarine sediments, and reflected their potential activity in nitrification.

The difference in the AOB abundance we observed may be due to the shifts of community composition or community density. Polymerase chain reaction-DGGE approaches have been used to analyse the community structure of AOB and AOA (Kowalchuk *et al*., 1997; Freitag and Prosser, 2003; Wuchter *et al*., 2006). There was a significantly positive correlation between AOB abundance and Shannon index ($r = 0.873$, $n = 7$, $P < 0.05$), which implied that the change of AOB abundance could be partly due to the shifts of the community composition. Moreover, the DGGE patterns of the N fertilizer treatments showed an increase in the diversity of the AOB community. Similar results have been reported by Chu and colleagues (2007) who used a modified primer pair for PCR-DGGE analysis in the same soil. Addition of N fertilizer showed no evidence for a community shift of AOB after 4 weeks (Avrahami *et al*., 2002) or 6 weeks of incubation (Mendum *et al*., 1999), but a shift was observed after 16 weeks (Avrahami and Conrad, 2003). Horz and colleagues (2004) also reported a significant change in the structure of AOB after increased nitrogen deposition for 2 years. Obviously, AOB community composition can be consistently altered by fertilization, especially N fertilizer application, and affects the potential ammonia oxidation. There was no difference in the AOA DGGE patterns among the different fertilization treatments, indicating no differences in AOA community structure in this study. However, a pronounced difference in AOA composition was observed in acid soil (He *et al*., 2007). It is likely therefore that the changes of AOA abundance and community in the alkaline soil are not as sensitive as in the acid soil, due to its less pH variation than the acid soil under the different fertilization treatments.

The phylogeny of the *amoA* gene was found to correspond largely to the phylogeny of the 16S rRNA gene in AOB (Purkhold *et al*., 2000; Aakra *et al*., 2001; Avrahami *et al*., 2003). Phylogenetic analysis of bacterial *amoA* sequences suggested that the soil AOB was dominated by *Nitrosospira*-like sequences, with *Nitrosomonas*-like sequences also present. Most sequences in this study were affiliated with *Nitrosospira* cluster 3, which has been detected as the dominant ammonia-oxidizer group in a number of arable soils (Phillips *et al*., 2000; He *et al*., 2007). However, the presence of *Nitrosomonas*-like sequences in this study is inconsistent with some previous studies that only *Nitrosospira*-like sequences are detected (Bruns *et al*., 1999; Webster *et al*., 2002; Chu *et al*., 2007). *Nitrosomonas* species have been detected in manure-treated wetland (Ibekwe *et al*., 2003), enrichment cultures (Stephen *et al*., 1996), wastewater treatment systems (Logemann *et al*., 1998) and limed acid forest soil (Carnol *et al*., 2002). These results provide further evidence for their preference for high-ammonia and high-pH environments (Kowalchuk and Stephen, 2001). The majority of the archaeal *amoA* sequences from this study were placed within cluster S. In addition, sequences affiliated with cluster M were also obtained in all treatments, suggesting that the soil sequences may have a similar original with marine sequences.

This study demonstrated statistically significantly higher copy numbers of bacterial *amoA* gene in the N mineral fertilizer treatments than in the CK treatment, and DGGE patterns also showed the community shifts among the different fertilization treatments. These results are consistent with a number of studies that AOB provide a good index in response to the varying environment (Kowalchuk and Stephen, 2001). The higher numbers of archaeal *amoA* genes than bacterial *amoA* genes among the treatments indicate that AOA may play a potential role in the global nitrogen cycle (Nicol and Schleper, 2006; Francis *et al*., 2007). Intriguingly, the abundance and diversity of AOA showed no significant difference under different fertilization treatments. It remains to be shown whether the higher numbers of AOA in soil also contribute to the nitrification. Thus, we hypothesize that soil type is the key factor in community change for AOA, and further studies should be performed to determine how soil type or other characteristics affect the activity and structure of AOA.

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Furthermore, the long-term fertilization practices had a significant impact on soil conditions, and their effects on the ammonia oxidizers (AOB and AOA) provide an indicator for assessing soil sustainable management.

Experimental procedures

Study site and sampling

The long-term field experiment was located in the Fengqiu State Key Experimental Station for Ecological Agriculture (35°00′N, 114°24′E), Henan province, northern China. The area had an annual precipitation of 605 mm and a mean annual temperature of 13.9°C. The soil was a sandy loam (clay 11%, silt 72% and sand 17%) derived from alluvial sediments of the Yellow River and classified as aquic inceptisol. The fertilization experiment was established in September 1989 with a wheat–maize rotation system including seven treatments with four replicates (plot area 9.5×5 m^2) for each treatment in a randomized plot design (Meng *et al.*, 2005). The seven treatments were control without fertilizer (CK); those with combinations of fertilizer nitrogen (N), phosphorus (P) and potassium (K): NP, NK, PK, NPK; half chemical fertilizers NPK plus half organic manure (1/2OMN); and organic manure (OM). Fertilizers N, P and K were applied in the form of urea (300 kg N ha^{-1} per year), super phosphate (150 kg P_2O_5 ha^{-1} per year) and potassium sulfate (300 kg K_2O ha^{-1} per year). The organic manure was a composted mixture of wheat straw, oil cake and cotton cake in a ratio of 100:40:45 (Meng *et al.*, 2005). Soil samples were collected from 0 to 20 cm surface soil in April 2006 and April 2007 by taking 12 soil cores (*c.* 5 cm diameter) from each plot and mixing them to form one composite sample. All samples were passed through a 2.0 mm sieve and subsamples were stored at –80°C for DNA extraction.

Soil chemical analysis and potential nitrification rate

Soil pH was determined with a soil to distilled water ratio of 1:2.5, and soil organic carbon was determined by $K_2Cr_2O_7$ oxidation-reduction titration method. Nitrate and ammonium were extracted with 2 M KCl and determined by a Continuous Flow Analyser (SAN++, Skalar, Holand). Potential nitrification rates were measured using the chlorate inhibition method (Kurola *et al.*, 2005). Briefly, 5.0 g of fresh soil was added to 50 ml centrifuge tubes containing 20 ml of phosphate buffer solution (PBS) (g l^{-1}: NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 0.2; NaH_2PO_4, 0.2; pH 7.4) with 1 mM $(NH_4)_2SO_4$. Potassium chlorate with a final concentration of 10 mM was added to the tubes to inhibit nitrite oxidation. The suspension was incubated in a dark incubator at 25°C for 24 h, after that nitrite was extracted with 5 ml of 2 M KCl and determined spectrophotometrically at 540 nm with *N*-(1-naphthyl) ethylenediamine dihydrochloride.

DNA extraction

Nucleic acids were extracted from 0.5 g of fresh soil using a MoBio UltraClean™ soil DNA isolation kit (San Diego, CA) according to the manufacturer's protocol with a minor modification (He *et al.*, 2007). DNA was eluted with 50 μl of solution S5 (MoBio Laboratories, cat. No. 12800-100) and stored at –20°C before use.

Real-time PCR assay and PCR amplification for DGGE

Real-time PCR was performed on an iCycler iQ 5 thermocycler (Bio-Rad). For all real-time PCR assays, *Taq*Man probes were labelled at the 5′ end with the reporter dye FAM (6-carboxy-fluorescein) and at the 3′ end with the quencher dye TAMRA (6-carboxy-tetramenthylrhodamine) (Takara Bio, Otsu, Shiga, Japan). Primer and *Taq*Man probe concentrations were optimized with the final concentrations listed in Table 3. The DNA was diluted 10-fold and bovine serum albumin (BSA) was added to reduce the interference of humic acid in the PCR (He *et al.*, 2005). Bacterial *amoA* genes were quantified using the primers A189/amoA-2R′ and the probe A337 (Okano *et al.*, 2004) with HotStarTaq DNA Polymerase (Qiagen, Valencia, CA) in a 25 μl reaction mixture containing 1× PCR buffer, 2.0 mM $MgCl_2$, 200 μM of each dNTP, 2.5 U HotStarTaq DNA polymerase. Quantification of bacterial 16S rRNA gene using primers BACT1369F/PROK1541R and probe TM1389F (Table 3, Suzuki *et al.*, 2000) was performed using iQ™ Supermix (Bio-Rad). Primers Arch-amoAF/Arch-amoAR (Francis *et al.*, 2005) was used for quantification of archaeal *amoA* gene with SYBR® Premix Ex Taq™ (TaKaRa).

Real-time PCR assays were performed with protocols for each target group as shown in Table 3. For bacterial *amoA* gene assay, an initial procedure at 95°C for 15 min was used to activate the HotStarTaq DNA Polymerase activity. In order to obtain lower background fluorescence signal in the negative controls due to host DNA carryover in the cloned DNA polymerase preparations (Suzuki *et al.*, 2000), 35 cycles were used for the bacterial 16S rRNA amplification. Assays for the other two target groups were run using 40 cycles. Assay for the archaeal *amoA* gene was performed with a four-step thermoprofile, and fluorescence intensity was measured at 83°C (above the melting point of primer dimers). Following the four temperature steps, a melting curve analysis was performed to confirm PCR product specificity after amplification by measuring fluorescence continuously as the temperature increased from 55°C to 95°C. Data analysis was carried out with iCycler software (version 1.0.1384.0 CR). The parameter Ct (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

Standard curves for real-time PCR assays were developed as described previously (He *et al.*, 2007). Briefly, the bacterial *amoA* gene, bacterial 16S rRNA gene and archaeal *amoA* gene were PCR-amplified from extracted DNA with the primers A189/amoA-2R′, 27F/1492R (Lane, 1991) and arch-amoAF/arch-amoAR, respectively, and the PCR products were cloned into the pGEM-T Easy Vector (Promega). Plasmids used as standards for quantitative analyses were extracted from the correct insert clones of each target gene. The plasmid DNA concentration was determined on a Nanodrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies) and the copy numbers of target genes were calculated directly from the concentration of the extracted plasmid DNA. Ten-fold serial dilutions of a known copy

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Table 3. Primers, probes and PCR conditions used for the PCR amplification.

Target group	Primer and probe	Sequence (5′–3′)	Length of amplicon (bp)	Concentration (nM)	Thermal profile	Reference
AOB	Primer A189	GGHGACTGGGAYTTCTGG	670	100	15 min at 95°C, followed by 40 cycles of 60 s at 94°C, 45 s at 57°C and 45 s at 72°C	Okano *et al.* (2004)
	Primer amoA-2R′	CCTCKGSAAAGCCTTCTTC		100		
	Probe A337	TTCTACTGGTGGTCRCACTACCCCATCAACT		120		
	Primer amoA1F[a]	GGGGTTTCTACTGGTGGT	490	100	5 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 55°C and 1 min at 72°C	Rotthauwe *et al.* (1997)
	Primer amoA2R	CCCCTCKGSAAAGCCTTCTTC		100		
AOA	Primer Arch-amoAF	STAATGGTCTGGCTTAGACG	635	100	94°C for 2 min followed by 40 cycles of 45 s at 94°C, 1 min at 53°C, 45 s at 68°C, plate read at 83°C	Francis *et al.* (2005)
	Primer Arch-amoAR[a]	GCGGCCATCCATCTGTATGT		100		
Bacteria	Primer BACT1369F	CGGTGAATACGTTCYCGG	170	1000	10 s at 95°C, followed by 35 cycles of 15 s at 95°C, 1 min at 56°C	Suzuki *et al.* (2000)
	Primer PROK1541R	AAGGAGGTGATCCRGCCGCA		500		
	Probe TM1389F	CTTGTACACACCGCCCGTC		500		

a. Forty-base pair GC clamp (cgcccgccgcgcccgcgcccggcccgcgccccgcccg) was attached to the 5′ end of the primers.

number of the plasmid DNA were subjected to real-time PCR assay in triplicate to generate an external standard curve as the previous study (He *et al.*, 2007).

For DGGE analysis, the primers used for bacterial and archaeal *amoA* gene amplification were amoA1F-GC/amoA2R and arch-amoAF/arch-amoAR-GC (Table 3) respectively. Amplification was performed in 50 μl reaction mixtures including 1× PCR buffer, 3.0 mM $MgCl_2$, 400 μM each dNTP, 2.5 U Taq DNA polymerase (TaKaRa) plus primers and BSA. The thermal profile used for amplification of archaeal *amoA* gene was the same as used for the bacterial *amoA* gene amplification as listed in Table 3 and 35 cycles were used.

Denaturing gradient gel electrophoresis analysis

Denaturing gradient gel electrophoresis analysis of *amoA* PCR products was performed with the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA). Polymerase chain reaction products of bacterial and archaeal *amoA* genes were loaded onto polyacrylamide gradient gels with a denaturing gradient of 40–65% and 20–45% (100% denaturant contains 7 M urea and 40% formamide) respectively. Electrophoreses were run at 120 V for 8 h and 6 h for AOB and AOA respectively. The gels were stained with 1:10000 SYBR Gold Nucleic Acid Gel Stain (Invitrogen 20 Molecular Probes, Eugene, USA) for 30 min according to the manufacturer's instructions, scanned by a GBOX/HR-E-M (Syngene, UK) and analysed using the software Quantity One (Bio-Rad Laboratories).

Cloning and sequencing

Numbered bands in the DGGE gel and other bands with the same mobility were excised for clone and sequencing analysis. Initial experiment found that the bands with the same mobility in a DGGE gel had highly similar (> 98%) nucleotide sequences. The dominant bands in the DGGE gel were excised and suspended in 50 μl of Tris-HCl buffer (pH 8.0) for 12 h, and reamplified with the primers amoA1F/amoA2R and arch-amoAF/arch-amoAR. The purified reamplified PCR products were ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and the resulting ligation mix transformed into *Escherichia coli* JM109 competent cells following the instructions of the manufacturer. The positive clones were amplified using the above primers with GC clamp and checked by DGGE. The correct one was selected for sequencing.

Phylogenetic analysis

The sequences were aligned with BLAST search program and similarity analysis was performed with DNAStar and DNAMAN version 4.0. Phylogenetic analyses were conducted using MEGA version 3.0 (Kumar *et al.*, 2004; He *et al.*, 2006) and the neighbour-joining tree was constructed using Kimura 2-parameter distance with 1000 replicates to produce Bootstrap values. UPGMA algorithms were used to cluster the DGGE patterns. The Shannon diversity index *H* and Evenness index *E* were used to calculate the band

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pattern diversity of AOB and AOA based on the following equations:

$$H = -\sum_{i=1}^{S} p_i \ln p_i = -\sum_{i=1}^{S} (N_i/N)\ln(N_i/N) \quad E = H/H_{\max} = H/\ln S$$

where N_i is the abundance of the *i*th ribotype, N is the total abundance of all ribotypes in the sample (lane of DGGE gels) and S is the number of ribotypes.

Statistical analysis

Copy numbers were log-transformed as needed to normalize the distributions prior to statistical analysis. All statistical analyses were performed using SPSS version 11.5, and one-way analysis of variance (ANOVA) followed by S-N-K-test was used to check for quantitative differences between treatments. $P < 0.05$ was considered to be statistically significant.

Sequence accession numbers

All *amoA* gene sequences have been deposited in the GenBank nucleotide sequence database under Accession No. EU137849 to EU137854 for AOB and EU137855 to EU137869 for AOA.

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