Soil Biology & Biochemistry xxx (2012) 1–13

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

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

Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil

Q1 Chao Ai, Guoqing Liang, Jingwen Sun, Xiubin Wang, Ping He, Wei Zhou*

Ministry of Agriculture Key Laboratory of Plant Nutrition and Fertilizer, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, PR China

ARTICLE INFO

Article history:
Received 20 April 2012
Received in revised form
2 August 2012
Accepted 2 August 2012
Available online xxx

Keywords:
Rhizosphere
Long-term fertilization
Ammonia-oxidizing bacteria
Ammonia-oxidizing archaea
Potential nitrification activity
Population size
Community structure

ABSTRACT

Ammonia oxidation is a critical step in the soil nitrogen (N) cycle and can be affected by the application of mineral fertilizers or organic manure. However, little is known about the rhizosphere effect on the function and structure of ammonia-oxidizing bacterial (AOB) and archaeal (AOA) communities, the most important organisms responsible for ammonia oxidation in agricultural ecosystems. Here, the potential nitrification activity (PNA), population size and composition of AOB and AOA communities in both the rhizosphere and bulk soil from a long-term (31-year) fertilizer field experiment conducted during two seasons (wheat and maize) were investigated using the shaken slurry method, quantitative real-time polymerase chain reaction and denaturing gradient gel electrophoresis. N fertilization greatly enhanced PNA and AOB abundance, while manure application increased AOA abundance. The community structure of AOB exhibited more obvious shifts than that of AOA after long-term fertilization, resulting in more abundant AOB phylotypes similar to *Nitrosospira* clusters 3 and 4 in the N-fertilized treatments. Moreover, PNA was closely correlated with the abundance and community structure of AOB rather than that of AOA among soils during both seasons, indicating that AOB play an active role in ammonia oxidation. Conversely, the PNA and population sizes of AOB and AOA were typically higher in the rhizosphere than the bulk soil, implying a significant rhizosphere effect on ammonia oxidation. Cluster and redundancy analyses further showed that this rhizosphere effect played a more important role in shaping AOA community structure than long-term fertilization. Overall, the results indicate that AOB rather than AOA functionally dominate ammonia oxidation in the calcareous fluvo-aquic soil, and that rhizosphere effect and fertilization regime play different roles in the activity and community structures of AOB and AOA.

1. Introduction

Microbial ammonia oxidation is the first and rate-limiting step of the nitrification process and is therefore believed to play a key role in the global nitrogen cycle by influencing the availability of fertilizer, nitrogen leaching of NO_3^- and NO_2^-, and release of N_2O and N_2 gas (Kowalchuk and Stephen, 2001). The rhizosphere, which is the volume of soil adjacent to and affected by plant roots (Sørensen, 1997), plays an active role in plant growth and soil fertility (Rovira, 1969). Because soil microbes are often limited by energy in soils, root exudates such as organic acids, sugars and amino acids may stimulate the growth of microbial populations capable of influencing biogeochemical cycling of C, N, P, and S (Fontaine and Barot, 2005; Rovira, 1969). Fertilization, which is widely used to enhance soil fertility and crop yield, strongly influences soil biochemical and biological properties. The effects of fertilization on the activity and community structure of soil ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), which are ubiquitous in soils and aquatic environments, has recently been emphasized (Cavagnaro et al., 2008; Shen et al., 2008; Verhamme et al., 2011; Wang et al., 2009). However, most investigations have been conducted on a bulk soil scale or in short-term experiments; therefore, there is still little information available regarding rhizosphere effects on ammonia oxidation in agricultural soils subject to long-term fertilization.

Autotrophic AOB have traditionally been considered the exclusive contributors to ammonia oxidation (Prosser, 1990). However, identification of the key gene responsible for ammonia oxidation (ammonia monooxygenase, *amoA*) in *Crenarchaeota* (Venter et al., 2004) and the isolation of *Nitrosopumilus maritimus* (Könneke

* Corresponding author. Tel.: +86 10 82108671; fax: +86 10 82106225.
E-mail address: wzhou@caas.ac.cn (W. Zhou).

0038-0717/$ – see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.soilbio.2012.08.003

et al., 2005) demonstrated that archaea also have ammonia-oxidizing activity (Francis et al., 2007; Zhang et al., 2010b). Nevertheless, comparative genomic analyses indicate that AOB and AOA may differ greatly in their physiology and metabolic pathways (Park et al., 2010; Walker et al., 2010). These differences imply that environmental factors such as pH, soil nitrogen nutrients, organic C and plant roots may determine the functional importance of both guilds in natural environments, especially anthropogenically disturbed agricultural ecosystems. Jia and Conrad (2009) reported that changes in the activity of ammonia oxidation were coupled with the abundance and community pattern of AOB, but not AOA. In addition, they found that CO_2 applied as a carbon source was mainly assimilated by AOB rather than AOA owing to ammonia oxidation. The results of this and other studies (Glaser et al., 2010; Shen et al., 2008; Wu et al., 2011) seem to suggest that bacteria rather than archaea dominate ammonia oxidation in near-neutral or alkaline agricultural soils. In contrast, AOA play a more important role than AOB in ammonia oxidation in strongly acidic soils (Yao et al., 2011; Zhang et al., 2011). Phylogenetic analyses of the 16S rRNA sequences of AOB have shown that there are at least seven distinct clusters within the β-subclass of proteobacteria (Kowalchuk et al., 2000; Stephen et al., 1996) and that arable soils are dominated by *Nitrosospira* of clusters 2, 3 and 4 (Innerebner et al., 2006; Phillips et al., 2000; Stephen et al., 1996), especially that of cluster 3, which was nearly ubiquitous in soil environments that have been investigated to date (Fierer et al., 2009; Glaser et al., 2010; Shen et al., 2008).

Mineral N fertilizer often leads to a rapid increase in soil potential nitrification activity (PNA) (Chu et al., 2007), which is correlated with soil pH and AOB abundance (Shen et al., 2008; Wu et al., 2011). However, a significant reduction in soil nitrification and abundance of AOB was observed in a Chinese red upland soil following long-term application of inorganic N fertilizer. Fan et al. (2011a) emphasized that the effects of mineral N fertilizer on ammonia oxidizers in soil vary in response to changes in the soil pH induced by fertilization. The effects of inorganic and organic fertilizers on the AOA community are less well studied and appear to be incongruent (Schauss et al., 2009; Shen et al., 2008; Wang et al., 2011), which may in part be due to mixotrophic or heterotrophic metabolism (Walker et al., 2010). Rice plantations have a greater effect on the abundance of the *amoA* gene in the rhizosphere than in the bulk soil, implying a possible rhizosphere effect on the soil nitrification process (Hussain et al., 2011). In another study, increases in AOB community size were commonly stronger in bulk soil than in the rhizosphere following application of $[NH_4]_2SO_4$. Glaser et al. (2010) suggested that there was fierce competition among plants, nitrifiers and other N-assimilating microorganisms for NH_4–N in the rhizosphere. Moreover, suppression of soil nitrification has been found to occur naturally in the rhizosphere via nitrification inhibitors produced by plants (Subbarao et al., 2006, 2007). In the same experimental field tested in this study, rhizosphere effects played an important role in mediation of the degree to which long-term fertilization affects the soil microbial community and extracellular enzyme activities (Ai et al., 2012). However, the specific effects of these factors on the nitrification activity and AOB and AOA communities remain unclear.

Long-term field fertilization experiments may provide profound insight into how anthropogenic disturbances lead to changes in soil properties such as pH, organic C, NH_4^+–N and NO_3^-–N, which in turn influence the function and structure of AOA and AOB communities. The present study was conducted to examine the differences in nitrification activity and AOB and AOA communities between rhizosphere and bulk soil, and how each responds to long-term fertilizations (31-year) during two seasons (wheat and maize). Quantitative real-time polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) were used to estimate AOB and AOA abundance and community structure, respectively. We hypothesized that rhizosphere and bulk soils would have different ammonia oxidizer communities with distinct nitrification activities after long-term fertilization, and that rhizosphere effects would mediate the influence of fertilization on the function and structure of soil AOB and AOA communities.

2. Material and methods

2.1. Field design and sampling

A long-term field fertilizer experiment was initiated in 1979 at Malan Farm (37°55′N, 115°13′E), Hebei Province, China, where wheat-maize rotation is the common cropping system. This region has a temperate and monsoonal type climate with an annual average temperature and precipitation of 12.6 °C and 490 mm, respectively. The experimental field contains calcareous fluvo-aquic soil, which is widespread in the North China Plain. At the beginning of the experiment, the soil had a pH (H_2O) of 7.8, 1.1% organic matter, 1.8 g kg^{-1} total N, and 5.0 and 87.0 mg kg^{-1} of available P and K, respectively. Six treatments (three replicates each) were implemented in 18 plots (12 m × 6.7 m) under a rotation of winter wheat (*Triticum aestivum* L.) and summer maize (*Zea mays* L.) (Ai et al., 2012). Treatments consisted of soil without fertilizer (control, CK), fertilizer N (N), fertilizer N and P (NP), fertilizer N, P and K (NPK), organic manure (M), and organic manure plus fertilizer N, P and K (MNPK). For NPK treatment, fertilizer N, P and K were applied in the form of urea (300 kg N ha^{-1} per year), superphosphate (150 kg P_2O_5 ha^{-1} per year) and potassium chloride (150 kg K_2O ha^{-1} per year), respectively, while no PK or K was applied for the N and NP treatments, respectively. All fertilizer P and K and Manure were applied once as basal dressing during wheat season. Manure and mineral fertilizers were evenly broadcast onto the soil surface and immediately incorporated into the plowed soil (0–20 cm depth) by tillage before sowing. For the N fertilizer, 20% of the urea was used as a basal dressing before sowing wheat, 30% was top-dressed at the reviving stage of wheat, and 50% was top-dressed at the 10-leaf stage of maize. The organic manure (3.75×10^4 kg ha^{-1}) consisted of straw bedding impregnated with liquid and solid horse manure, which had 120 g kg^{-1} organic matter, 5.0 and 2.2 g kg^{-1} total N and P, respectively, and about 50% water content.

Soil samples were collected during the reproductive stages of wheat and maize in early May 2010 and late August 2010, respectively, when the rhizosphere effects tend to be most pronounced (Cheng et al., 2003). Rhizosphere soil was operationally defined as soil adhering to the total roots after gentle shaking, while bulk soil was defined as unvegetated soil adjacent to the plants. The whole plant with their roots was extracted from soil and, after shaking off the loosely adhering soil, the tightly adhering soil (i.e. rhizosphere soil) was carefully collected. The unvegetated soil cores (5 cm diameter) adjacent to the plants (i.e. bulk soil) were sampled at depth 0–20 cm. In order to obtain the enough rhizosphere soil for multiple assays, twenty plants were randomly selected from each plot, and these rhizosphere soils were pooled to form one composite sample. Correspondingly, one composite bulk soil consisting of twenty cores was taken from each plot. Thus, six composite samples of each treatment were collected per sampling time, and a total of 72 composite samples were taken for two consecutive seasons. The fresh samples were placed immediately on ice and transported to the laboratory. Plant roots were removed by passing the sample through a 2-mm mesh sieve, and aliquots of the samples were then stored at room temperature until chemical

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

analysis, at 4 °C until PNA analysis (within 1 week), or at −20 °C until molecular analysis.

2.2. Chemical analysis

Soil pH was measured with a compound electrode (PE-10, Sartorious, Germany) using a soil to water ratio of 1:2.5. Soil organic C was determined by dichromate oxidation, while total N was measured using a vario MACRO cube element analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). The ammonium N (NH_4^+–N) and nitrate N (NO_3^-–N) contents were determined by extracting the soil with 0.01 M $CaCl_2$ solution (1:10, w/v) for 30 min and then determining the NH_4^+ and NO_3^- concentrations using a flow injection autoanalyzer (FLA star 5000 Analyzer, Foss, Denmark).

2.3. Slurry assay of potential nitrification activity

The PNA was determined via the shaken slurry method described by Hart et al. (1994), which evaluates the maximum nitrate production rate of a soil sample. Briefly, fresh soil samples (15 g) were placed in Erlenmeyer flasks with 100 ml of a 1.5 mM NH_4^+ and 1 mM PO_4^{3-} mixture with the pH adjusted to 7.2. The slurry was then shaken on an orbital shaker at 180 rpm for 24 h at 25 °C to maintain aeration in the dark. Aliquots of 5 ml were subsequently removed using a wide-mouth pipette at 2, 6, 12, 22 and 24 h after the start of the incubation. The aliquots were then centrifuged, and the supernatant was filtered and stored at −20 °C until analysis. The NO_3^-–N concentrations were measured using a flow injection autoanalyzer (FLA star 5000 Analyzer, Foss, Denmark), after which PNA was calculated from the rate of linear regression of nitrate concentrations over time (μg NO_3^-–N g^{-1} h^{-1}).

2.4. DNA extraction, PCR amplification and DGGE analysis

Soil total DNA was extracted using a Fast DNA SPIN Kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA was finally eluted with 100 μl of the DNA elution solution included in the kit. Successful DNA extraction was characterized by electrophoresis on 0.7% (wt/vol) agarose gels.

To amplify specific 16S rRNA of AOB from soils for DGGE, nested PCR was performed (Zhang et al., 2010a). The first PCR was conducted using the AOB-specific primer pair CTO189f and CTO654r, which amplified a 465-bp fragment (Kowalchuk et al., 1997). The product from this round of PCR was then used as the template DNA for a second round of PCR carried out using universal primers (F338-GC and R518) (Muyzer et al., 1993). AOA *amoA* genes were amplified using primers CrenamoA23f and CrenamoA616r (Tourna et al., 2008). PCR mixtures consisted of 12.5 μl 2 × EasyTaq PCR SuperMix (TransGen Biotech, Beijing, China), 0.5 μM of each primer and 1 μl of 10-fold diluted DNA template diluted to a final volume of 25 μl. PCR reactions were performed on a MyCycler Thermal Cycler (Bio-Rad) as previously described for AOB (Zhang et al., 2010a) and AOA (Tourna et al., 2008). All PCR products were electrophoresed on 1.5% (wt/vol) agarose to verify their size and quality.

The PCR amplicons were separated by DGGE using a D-Code universal mutation detection system (Bio-Rad, USA) according to the manufacturer's instructions. Briefly, 20 μl of each PCR product was loaded onto an 8% (wt/vol) polyacrylamide gel (acrylamide:bisacrylamide = 37.5:1) with a denaturant gradient of 35%–60% for AOB and 20%–50% for AOA (100% denaturant contains 7 M urea and 40% deionized formamide). Electrophoresis was then conducted at 60 °C in 1× tris-acetate-EDTA buffer at 75 V for 16 h. After DGGE, the gels were stained with 1:10,000 SYBR green I for 30 min and then scanned with a Bio-Rad image scanner. Band intensity and position data were analyzed using Quantity One (Bio-Rad, USA).

2.5. Cloning, sequencing and phylogenetic analysis

Prominent bands in the DGGE gels were excised and reamplified. For AOA, the purified PCR products were directly sequenced by Sangong Biotech Co., Ltd. (Shanghai, China). Whereas, the purified PCR products of AOB DGGE bands were cloned into the pGM-T vector (Tiangen Biotech, Beijing, China) and transformed into *Escherichia coli* TOP10 (Tiangen Biotech, Beijing, China). The Plasmids of positive colonies were extracted and sequenced. The sequences of the DGGE bands were then compared with those available in the National Center for Biotechnology Information (NCBI) GenBank database using the BLAST algorithm. The nucleotides generated in this study and obtained from the NCBI GenBank database were aligned, and a phylogenetic tree was constructed by the neighbor-joining method using Kimura 2-parameter distance, as implemented in MEGA version 4.0 (Tamura et al., 2007). Bootstrap support (>50%) from 1000 replications is shown at the nodes of the trees.

2.6. Quantitative real-time PCR

Real-time quantification of AOB 16S rRNA in soil samples was performed using primers CTO189f and RT1r and the *Taq* Man Probe TMP1, as described by Hermansson and Lindgren (2001). The PCR mixture was prepared in a total volume of 20 μl using a Premix Ex Taq™ Kit (Perfect Real Time) (TaKaRa, Dalian, China), 0.3 μM of each primer (CTO189f and RT1r), 0.2 μM *Taq* Man probe TMP1, and 1 μl 10-fold diluted extracted DNA. For the *amoA* in the AOA, the 20 μl PCR mixture contained 10 μl of SYBR Green I PCR Mix (TaKaRa, Dalian, China), 0.2 μM each primer (CrenamoA23f and CrenamoA616r) and 1 μl 10-fold diluted extracted DNA. Quantitative real-time PCR for AOB and AOA was carried out in triplicate using an ABI 7500 Real-Time PCR System (Applied Biosystems) under the following thermocycling conditions: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C. The amplification specificity of AOA was confirmed by generating a melting curve. Standard curves ranging from 1×10^2 to 1×10^7 copies were prepared by 10-fold serial dilution of known copy numbers of plasmid DNA possessing the genes of interest. To estimate the population sizes, it was assumed that AOB contains one copy of 16S rRNA per cell (Coci et al., 2010) and AOA carries one copy of the *amoA* gene per cell (Agogué et al., 2008; Hallam et al., 2006).

2.7. Statistical analysis

Statistical analyses were performed using SAS version 8.1. For each variable measured in the rhizosphere or bulk soil, the data were analyzed by one-way ANOVA using Fisher's least significant difference ($P = 0.05$) to compare the treatment means. Two-way ANOVA was used to compare the soil fractions (rhizosphere and bulk soil) and fertilizer treatments. Pearson's correlation analyses were performed to assess the relationships among PNA, soil properties and the abundances of AOB and AOA. Redundancy analysis (RDA) with the Monte Carlo permutation's test (499 permutations) was carried out to determine if the AOB and AOA community structures were correlated with PNA and soil properties, as implemented in Canono for Windows version 4.5.

2.8. Nucleotide sequence accession numbers

The sequences obtained from the DGGE bands in this study were deposited in the GenBank database under accession numbers JQ904470 to JQ904529.

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

3. Results

3.1. Soil chemical properties and potential nitrification activity

Soil pH values, which were significantly lower in the rhizosphere than in bulk soil (Two-way ANOVA, $P < 0.0001$), were not significantly affected by the long-term fertilizer treatments, except for the M and MNPK treatments during maize season, which induced a slight decrease in soil pH (Table 1). Soil organic C, total N and NH_4^+–N tended to be greater in the rhizosphere than in bulk soil, and increased in response to long-term organic fertilization (M and MNPK) during both seasons. In contrast, during wheat season, the NO_3^-–N concentrations in bulk soil, which were generally higher than in the rhizosphere, were markedly enhanced in the N, NP, NPK and MNPK treatments. This trend was not observed during maize season, when the NO_3^-–N concentrations increased markedly in response to organic fertilization and the rhizosphere effect.

The rhizosphere and fertilizer regimes had strong effects on the soil PNA during both the wheat and maize season (Table 2). The PNA ranged from 0.87 to 3.08 μg NO_3^-–N g^{-1} h^{-1}, and was typically higher in the rhizosphere than in bulk soil in all treatments (Fig. 1), indicating a typical rhizosphere effect. Additionally, PNA in the rhizosphere and the bulk soil showed a significant positive correlation during wheat season ($r = 0.917$, $n = 6$, $p < 0.05$) and maize season ($r = 0.807$, $n = 6$, $p = 0.05$). Overall, the PNA showed similar responses to the fertilization regimes during both seasons. Specifically, the PNA was 31%–95% higher in the N, NP, NPK and MNPK treatments than in the control (CK) in both the rhizosphere and bulk soil. Although the PNA in the M treatments was also enhanced, the degree of increase ranged from 4% to 39% was obviously lower than those in the mineral N fertilizer treatments.

3.2. Population sizes of ammonia-oxidizing bacteria and archaea

We used quantitative real-time PCR to determine the population sizes of AOB and AOA. The total AOB, which ranged from 1.9×10^7 to 37.0×10^7 cells g^{-1} soil (Fig. 2a and b), was 13%–778% higher in the rhizosphere than in bulk soil during both seasons. In the bulk soil collected during wheat season, the abundance of AOB in the N, NP, NPK and MNPK treatments were 2.0, 2.4, 5.1 and 1.6 times that of the control, respectively (Fig. 2a). However, the abundance of AOB in treatment M was 43% lower than that of CK. A similar trend was observed in the rhizosphere; however, the levels of AOB in the M treatment also significantly increased when compared with CK. A significant positive correlation was observed between the abundance of AOB during both seasons ($r = 0.783$, $n = 12$, $p < 0.01$) (Fig. 2a and b).

AOA abundance ranged from 43.9×10^7 to 160.5×10^7 cells g^{-1} soil across treatments (Fig. 2c and d). The rhizosphere effects and fertilizer treatments also both had strong effects on AOA abundance (Table 2). In contrast to AOB, the AOA abundance was primarily enhanced by organic fertilization (M and MNPK). Correlation analyses also confirmed that the AOA population size was significantly positively related to soil organic C and total N during both seasons (Table 3), which were greatly increased by long-term organic fertilization (Table 1). Moreover, there was a significant positive correlation between PNA and AOB abundance, whereas no relationship was observed between PNA and AOA abundance (Table 3).

Table 2
Two-way ANOVA of soil biological properties in two soil fractions (rhizosphere and bulk soil) and six fertilizer treatments with three replicates each ($n = 36$) during wheat season and maize season.

	Soil fractions (rhizosphere or bulk)		Fertilizer treatments		Soil fractions × fertilizer treatments	
	F	*P*	*F*	*P*	*F*	*P*
Wheat season						
PNA[a]	521.53	<0.0001	86.36	<0.0001	8.13	0.0002
AOB population size	69.12	<0.0001	17.26	<0.0001	4.54	0.0054
AOA population size	3.50	0.0448	40.13	<0.0001	7.85	0.0002
Maize season						
PNA	23.63	<0.0001	8.01	0.0002	0.86	**0.5243**[b]
AOB population size	88.10	<0.0001	5.69	0.0016	4.76	0.0042
AOA population size	96.19	<0.0001	45.17	<0.0001	0.60	**0.7036**

[a] PNA, potential nitrification activity.
[b] No significant effects ($p > 0.05$) are highlighted in bold.

Table 1
Soil pH and nutrient concentrations after long-term fertilization in the rhizosphere and bulk soil during two seasons (wheat and maize season). Data are the means ± S.E., $n = 3$. Different letters indicate significant differences among fertilizer treatments at $p < 0.05$.

Treatments		CK	N	NP	NPK	M	MNPK
Wheat season[a]							
pH	Bulk soil	8.26 ± 0.04 a	8.22 ± 0.03 a	8.25 ± 0.12 a	8.23 ± 0.05 a	8.26 ± 0.04 a	8.10 ± 0.14 a
	Rhizosphere	8.08 ± 0.03 a	8.09 ± 0.08 a	8.14 ± 0.06 a	8.10 ± 0.02 a	8.05 ± 0.06 a	8.12 ± 0.02 a
Total N (g kg^{-1})	Bulk soil	1.45 ± 0.12 b	1.45 ± 0.04 b	1.54 ± 0.07 b	1.44 ± 0.10 b	1.89 ± 0.12 a	1.95 ± 0.15 a
	Rhizosphere	1.76 ± 0.12 c	1.65 ± 0.01 c	1.76 ± 0.05 c	1.83 ± 0.07 c	2.54 ± 0.15 b	2.75 ± 0.13 a
Organic C (g kg^{-1})	Bulk soil	9.54 ± 0.67 b	9.74 ± 0.68 b	9.57 ± 0.64 b	9.77 ± 0.39 b	11.84 ± 0.94 a	11.9 ± 0.32 a
	Rhizosphere	11.03 ± 0.74 b	10.56 ± 0.78 b	10.64 ± 0.33 b	10.76 ± 0.25 b	15.11 ± 0.82 a	15.01 ± 1.1 a
NH_4^+–N (mg kg^{-1})	Bulk soil	1.66 ± 0.28 c	1.55 ± 0.89 c	1.99 ± 0.63 bc	3.24 ± 0.54 ab	4.07 ± 0.43 a	3.73 ± 1.06 a
	Rhizosphere	3.27 ± 1.04 bc	1.93 ± 0.53 c	2.54 ± 0.75 c	2.48 ± 1.25 c	5.53 ± 1.25 ab	6.34 ± 0.44 a
NO_3^-–N (mg kg^{-1})	Bulk soil	10.52 ± 2.95 bc	23.13 ± 3.85 a	17.28 ± 4.94 ab	14.03 ± 5.80 bc	7.21 ± 2.73 c	16.84 ± 5.92 ab
	Rhizosphere	10.42 ± 3.06 bc	15.19 ± 0.48 a	7.71 ± 1.85 bcd	7.49 ± 1.24 cd	6.19 ± 0.97 d	11.73 ± 3.67 ab
Maize season							
pH	Bulk soil	8.27 ± 0.02 a	8.22 ± 0.05 ab	8.23 ± 0.03 ab	8.21 ± 0.01 ab	8.08 ± 0.07 bc	7.94 ± 0.18 c
	Rhizosphere	8.03 ± 0.04 a	8.06 ± 0.04 a	8.00 ± 0.03 a	8.02 ± 0.04 a	7.92 ± 0.03 b	7.79 ± 0.07 c
Total N (g kg^{-1})	Bulk soil	1.42 ± 0.09 c	1.42 ± 0.07 c	1.37 ± 0.04 c	1.43 ± 0.01 c	1.75 ± 0.06 b	1.89 ± 0.09 a
	Rhizosphere	1.54 ± 0.03 c	1.56 ± 0.07 c	1.62 ± 0.06 c	1.57 ± 0.05 c	2.18 ± 0.10 b	2.33 ± 0.14 a
Organic C (g kg^{-1})	Bulk soil	9.59 ± 0.34 b	9.40 ± 0.66 b	9.38 ± 0.17 b	9.55 ± 0.24 b	12.02 ± 0.90 a	12.62 ± 0.02 a
	Rhizosphere	11.13 ± 1.09 b	11.31 ± 0.82 b	12.03 ± 0.53 b	11.67 ± 0.58 b	17.5 ± 0.21 a	18.20 ± 1.15 a
NH_4^+–N (mg kg^{-1})	Bulk soil	1.88 ± 0.05 ab	1.23 ± 0.93 bc	0.72 ± 0.21 c	1.14 ± 0.23 bc	2.28 ± 0.45 a	2.3 ± 0.25 a
	Rhizosphere	3.45 ± 0.53 a	3.39 ± 1.06 a	0.55 ± 0.13 b	1.09 ± 0.83 b	2.83 ± 0.47 a	3.84 ± 1.53 a
NO_3^-–N (mg kg^{-1})	Bulk soil	6.93 ± 1.73 b	8.71 ± 1.90 b	9.97 ± 1.84 b	8.62 ± 1.42 b	41.51 ± 16.65 a	53.23 ± 31.40 a
	Rhizosphere	13.95 ± 7.25 a	11.05 ± 5.23 a	11.81 ± 5.43 a	11.81 ± 6.16 a	15.03 ± 7.66 a	18.49 ± 9.34 a

[a] Wheat season data from Ai et al. (2012).

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

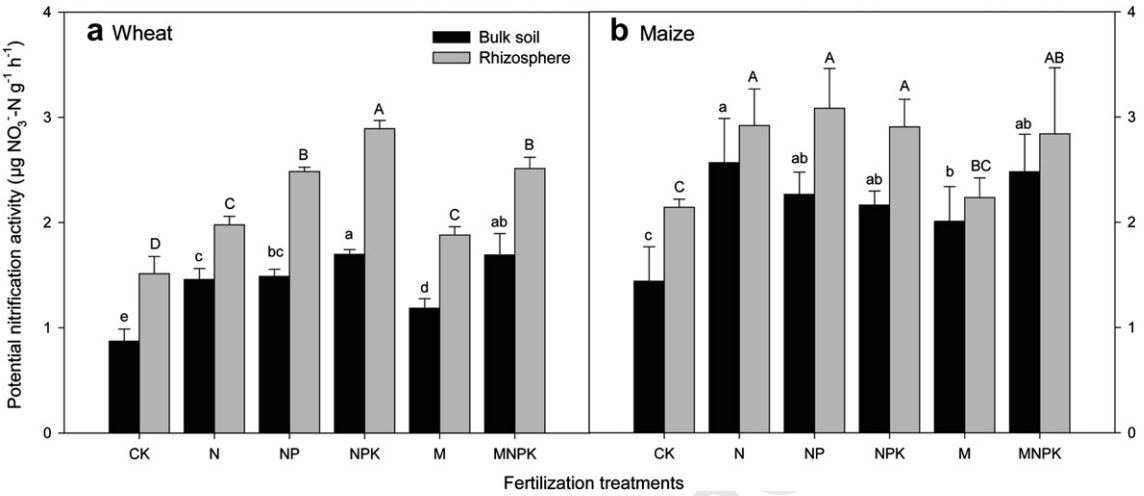

Fig. 1. Potential nitrification activity (PNA) in the rhizosphere and bulk soil under different fertilization treatments. (a) Wheat, PNA during wheat season; (b) Maize, PNA during maize season. Vertical bars represent the standard deviations ($n = 3$) and different letters indicate significant differences among fertilizer treatments in the rhizosphere or bulk soil at $P < 0.05$.

3.3. Community structure of ammonia-oxidizing bacteria and archaea

The community structures of AOA and AOB in soils were characterized by DGGE. The analysis of replicates for each treatment showed good reproducibility of the DGGE banding patterns (data not shown); therefore, the results for only one replicate are shown here (Figs. 3 and 4). During wheat season, mineral N-fertilized treatments (N, NP, NPK and MNPK) in both the rhizosphere and bulk soil resulted in increased numbers of AOB bands in the DGGE

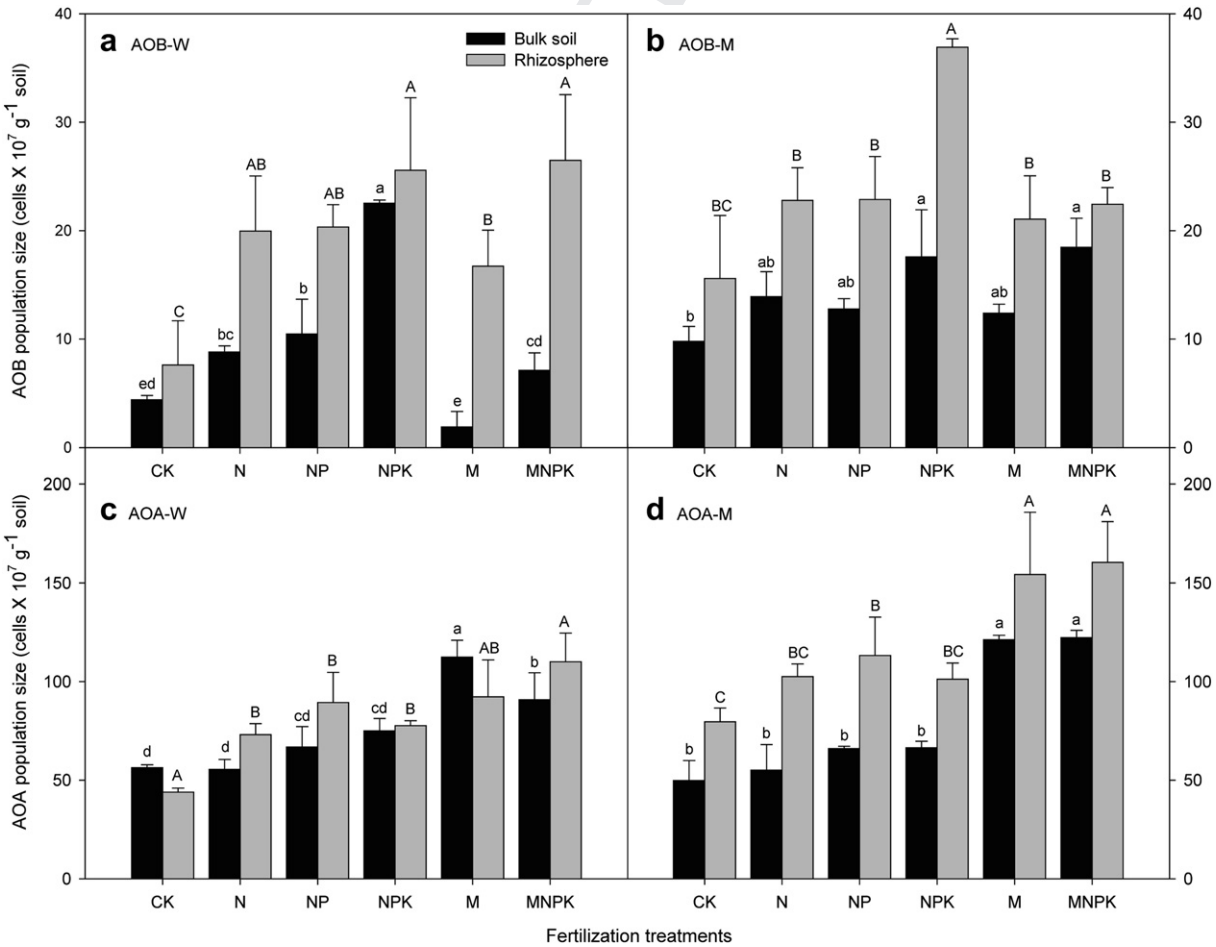

Fig. 2. Abundance of soil ammonia-oxidizing bacteria (AOB) and archaea (AOA) in the rhizosphere and bulk soil under different fertilization treatments. (a) AOB-W, AOB during wheat season; (b) AOB-M, AOB during maize season; (c) AOA-W, AOA during wheat season; (d) AOA-M, AOA during maize season. Vertical bars represent the standard deviations ($n = 3$) and different letters indicate significant differences among fertilizer treatments in the rhizosphere or bulk soil at $P < 0.05$. To estimate the population sizes, it was assumed that AOB contains one copy of 16S rRNA per cell and AOA carries one copy of the *amoA* gene per cell.

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

Table 3
Correlations of soil properties, potential nitrification activity (PNA), and abundance of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA).

	pH	Organic C	Total N	NH_4^+–N	NO_3^-–N	AOA	AOB
Wheat season							
AOB	−0.460	0.280	0.356	0.268	−0.188	0.262	–
AOA	−0.096	0.666*[a]	0.679*	0.705*	−0.408	–	0.262
PNA	−0.604*	0.348	0.450	0.276	−0.284	0.360	0.870**[b]
Maize season							
AOB	−0.541	0.349	0.263	−0.014	−0.099	0.433	–
AOA	−0.925**	0.929**	0.933**	0.512	0.457	–	0.433
PNA	−0.528	0.282	0.231	−0.030	−0.041	0.418	0.731**

[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level.

profile. Specifically, bands 3, 11 and 13 were added when compared with the CK and M treatments (Fig. 3a). These findings indicate that inorganic fertilizer and organic manure had a variety of effects on the soil AOB community. These results were confirmed by Cluster analysis, which revealed that mineral N-fertilized treatments were clearly separate from CK and M treatments (Fig. 3b), whereas no distinct difference was observed between the rhizosphere and bulk soil. A similar variation in the AOB community structure as observed during wheat season was detected during maize season among fertilization regimes (Fig. 3c and d). These findings indicate that the fertilization regime might be a major factor affecting the soil AOB community structure, while the rhizosphere exerted a secondary effect. In contrast to the AOB community, the DGGE profiles of AOA remained largely unchanged for most treatments (Fig. 4). Subtle alterations in the DGGE patterns were detected; however, these were observed between the rhizosphere and bulk soil. For example, the intensities of DGGE band 10 during wheat season (Fig. 4a) and band 7 during maize season (Fig. 4c) appeared higher in the bulk soil than the rhizosphere. Additionally, cluster analysis revealed two major clusters during both seasons, one encompassing patterns derived from almost all rhizosphere samples and another encompassing those from all bulk soil samples (Fig. 4b and d).

The numbered bands in the DGGE profiles were sequenced for phylogenetic analysis. Our definition of the phylogenetic cluster of the 16S rRNA gene in AOB was primarily based on previous studies (Kowalchuk et al., 2000; Stephen et al., 1996). The AOB DGGE profiles corresponding to all treatment groups were dominated by bands 15–17 and 19 (wheat season) and bands 13–16 (maize season), which were affiliated with the *Nitrosospira* cluster 3 lineage (Fig. 5). Interestingly, a high intensity band 11 observed during wheat season, which was only detected in the N-fertilized treatments, fell within the *Nitrosospira* cluster 4 lineage. Moreover, some bands (3, 4, 7 and 8 during wheat season and 2–5 and 7 during maize wheat season) that were affiliated with *Nitrosomonas* cluster 6 were also found in the soil samples; however, they were only detected in the region of DGGE profiles with a low denaturing gradient (Fig. 3). For AOA, 25 DGGE bands corresponding to the archaea *amoA* gene were sequenced. Phylogenetic analysis showed that most DGGE bands belonged to the soil and sediment lineage, while four were associated with the water and sediment lineage (Fig. 6).

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

RDA was conducted to determine the correlation of soil properties with community structures of AOB and AOA (Fig. 7). During wheat season, the first and second axes accounted for 27.3% and 17.6% of the total variation in AOB community structure, respectively (Fig. 7a). PNA ($F = 2.100$, $r = 0.158$, $p = 0.026$), NO_3^-–N content ($F = 2.073$, $r = 0.139$, $p = 0.018$) and NH_4^+–N content ($F = 1.996$, $r = 0.166$, $p = 0.032$) were significantly correlated with AOB structure. During maize season, PNA ($F = 1.845$, $r = 0.136$, $p = 0.048$) and total N ($F = 2.776$, $r = 0.217$, $p = 0.012$) were significantly correlated with the AOB community structure (Fig. 7b). No other investigated soil properties were correlated with the AOB community structure.

In contrast, significant correlations were observed between the AOA community structure and soil pH ($F = 2.004$, $r = 0.167$, $p = 0.012$), NO_3^-–N content ($F = 2.051$, $r = 0.127$, $p = 0.022$) and organic C content ($F = 1.880$, $r = 0.144$, $p = 0.046$) during wheat season, and with soil pH ($F = 2.251$, $r = 0.184$, $p = 0.006$), total N content ($F = 2.535$, $r = 0.179$, $p = 0.002$) and NO_3^-–N content ($F = 2.062$, $r = 0.131$, $p = 0.042$) during maize season (Fig. 7c and d).

4. Discussion

Our results consistently showed that the abundance of AOA was greater than that of AOB in both the rhizosphere and bulk soil, confirming previous reports by Leininger et al. (2006) and Chen et al. (2008). However, the community structure and abundance of AOB were significantly correlated with PNA, while those of AOA were not (Table 3, Fig. 7), suggesting that nitrification is primarily driven by AOB in the calcareous fluvo-aquic soil tested in this study. The predominance of bacterial nitrification is consistent with previous reports for a grassland soil in New Zealand (Di et al., 2009) and an agricultural soil in Germany (Jia and Conrad, 2009), but inconsistent with reports for most acidic soils (Gubry-Rangin et al., 2010; Yao et al., 2011; Zhang et al., 2011). Evolutionary considerations suggest that archaea can be well adapted to extreme conditions, such as highly acidic pH and low ammonia availability (Frijlink et al., 1992; Verhamme et al., 2011), while the opposite responses are detected for bacteria (Nicol et al., 2008). These characteristics may explain why AOB but not AOA play an important role in soil nitrification in this calcareous soil (pH from 7.79 to 8.27) (Table 1) following long-term fertilization.

The rhizosphere effect has been shown to have a strong effect on microbial activities including N transformation processes (Ai et al., 2012; Herman et al., 2006; Kirk and Kronzucker, 2005). In this study, the rhizosphere and bulk soil differed with respect to most soil characteristics (Table 1) and possessed distinct PNAs, which were typically higher in the rhizosphere than the bulk soil (Fig. 1), implying a profound rhizosphere effect on ammonia oxidation. It is well known that the microenvironments in the rhizosphere differ markedly from those in bulk soil owing to interactions among root exudations, plant absorption and rhizosphere microorganisms (Neumann and Römheld, 2002; Sørensen, 1997). The higher resource heterogeneity of rhizodeposition after mineralization may provide a substrate for ammonia oxidizers and stimulate microbial growth (Chen et al., 2008; Malchair et al., 2010). Indeed, this positive impact was confirmed by the fact that AOB and AOA population sizes in the rhizosphere increased equally by approximately 147% and 32%, respectively, when compared with bulk soil (Fig. 2). The predominance of ammonia oxidizers in the rhizosphere is also consistent with previous observations of two paddy soils in China and a cauliflower-planted soil in Germany (Chen et al., 2008; Hussain et al., 2011; Kleineidam et al., 2011). Apparently, the potential existence of natural inhibitors of nitrification (i.e. allelochemicals such as monoterpenes and low-molecular-weight organic acids released by roots) (Subbarao et al., 2007; White, 1994) cannot dramatically impact the predominance of microbial nitrification in the rhizosphere. Although nitrifiers DGGE profiles were dominated by some common bands during both seasons (Figs. 3 and 4), indicating that plant species did not substantially

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

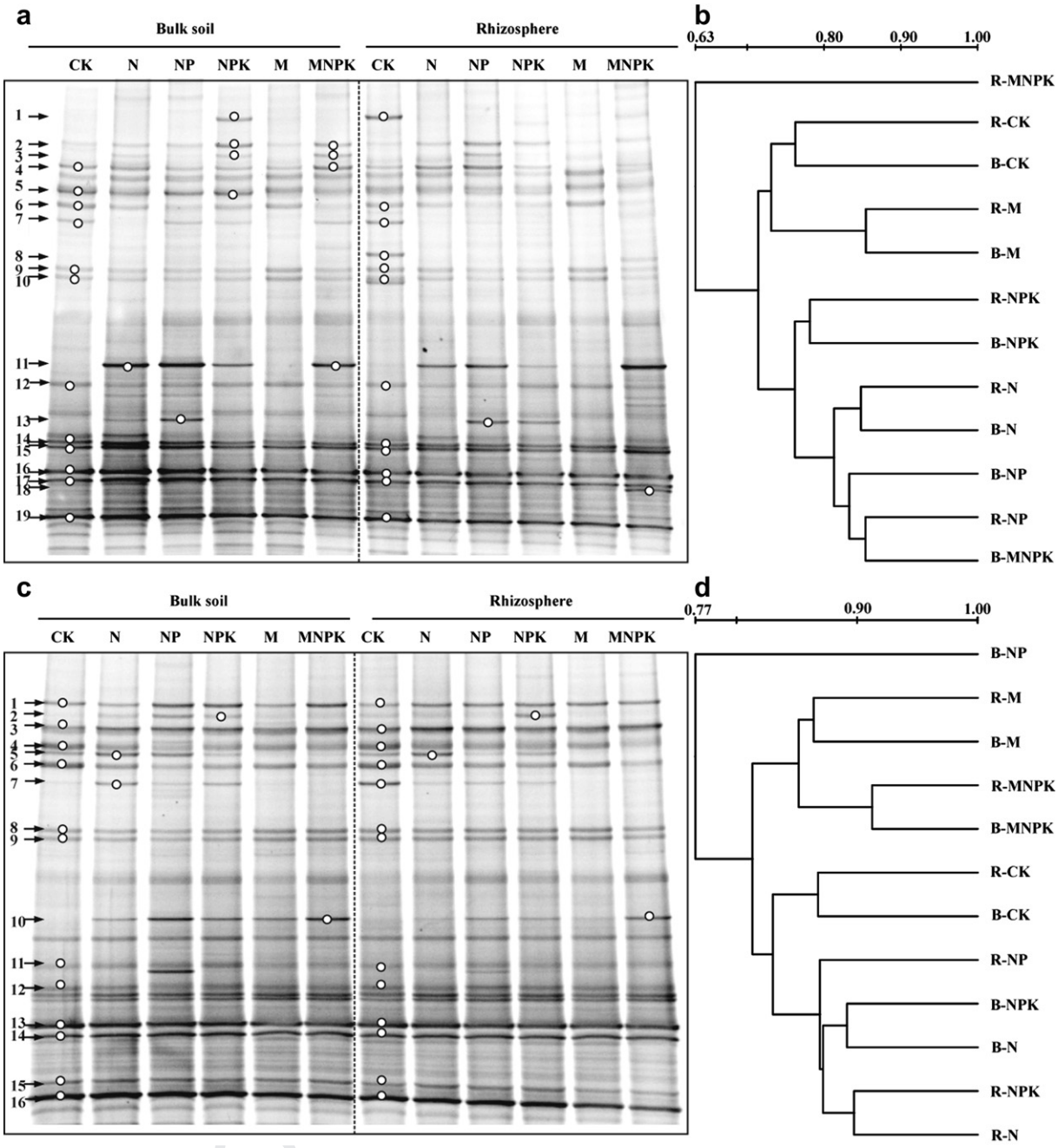

Fig. 3. DGGE analysis of soil ammonia-oxidizing bacteria (AOB) during wheat season (a) and maize season (c) after different fertilization treatments, and similarity dendrograms (UPGMA, Dice coefficient of similarity) of AOB banding patterns calculated from DGGE patterns obtained during wheat season (b) and maize season (d). Arrows indicate DGGE bands for sequencing. R- and B- indicate rhizosphere and bulk soil, respectively.

alter the community structure of ammonia nitrifiers (Fan et al., 2011b), the subtle differences in AOB DGGE patterns between wheat and maize season were also detected. For example, AOB DGGE pattern of rhizosphere soil in MNPK treatment during wheat season was clearly separated from that of other treatment, but which did not happen during maize season (Fig. 3). C3 and C4 plant species are usually different from each other in the quality and quantity of root exudates (Kuzyakov, 2002). Some studies indicated that more carbon is released as respiration from the roots of wheat than from those of maize (Liljeroth et al., 1994). Thus, the different patterns of organic-N mineralization and distributions of N source between two seasons possibly contribute to the partial changes of AOB community composition. In addition, soil nitrification and ammonia oxidizers are frequently influenced by the seasonal factors. Temperature has been regarded as one of the most important environmental factor responsible for microbial nitrification (Avrahami and Conrad, 2003; Fierer et al., 2009).

Long-term applications of different fertilizers had a significant impact on the soil nitrification process (Table 2), and AOB and AOA responded to fertilizers in different manners (Figs. 2–4). N fertilizer selectively stimulated the growth of AOB in both the rhizosphere and bulk soil, as indicated by an average increase in AOB abundance of 33% in the N-fertilized treatments (N, NP, NPK and MNPK) when compared with CK (Fig. 2). Meanwhile, PNA was significantly

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

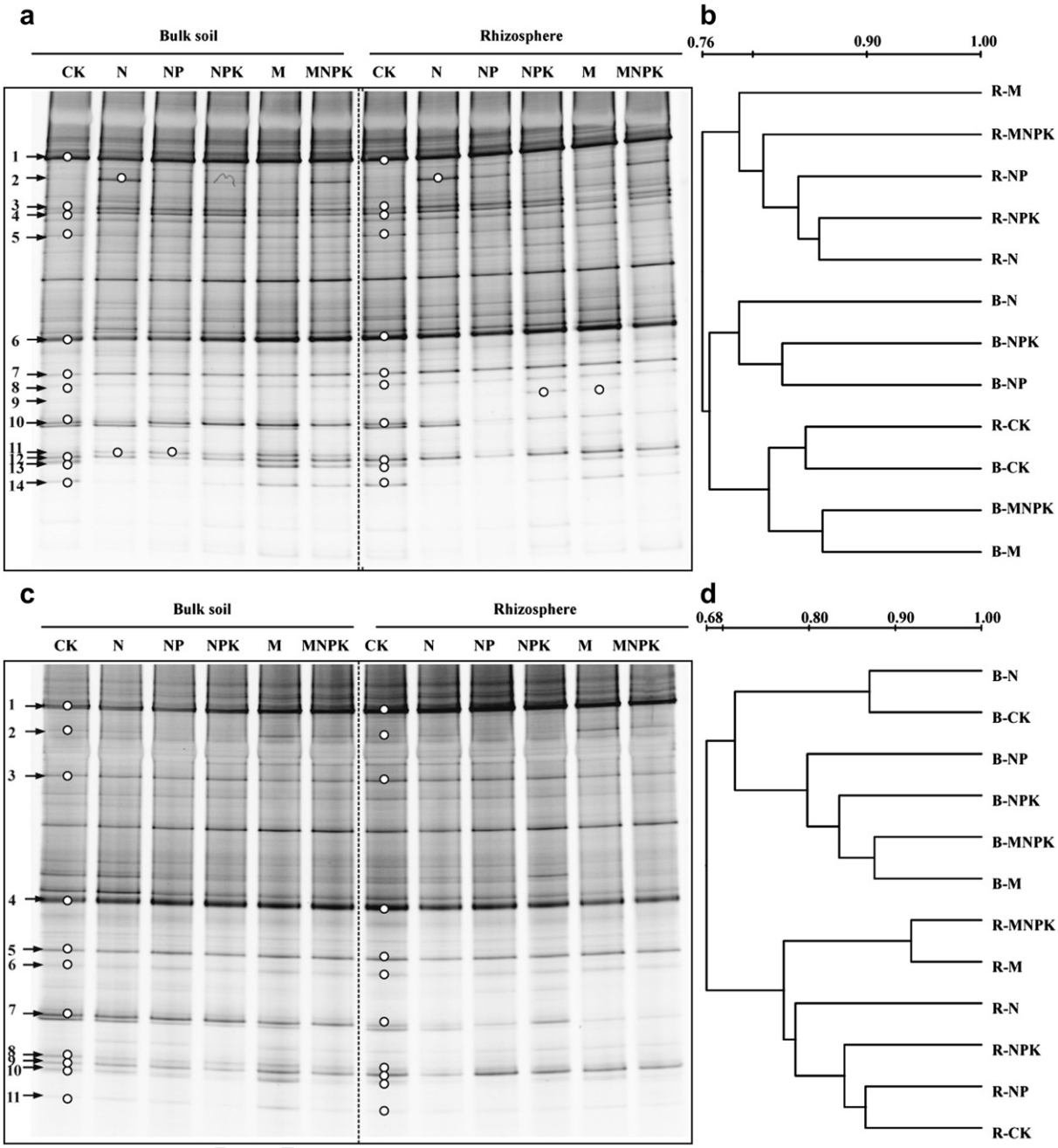

Fig. 4. DGGE analysis of soil ammonia-oxidizing archaea (AOA) during wheat season (a) and maize season (c) after different fertilization treatments, and similarity dendrograms (UPGMA, Dice coefficient of similarity) of AOA banding patterns calculated from DGGE patterns obtained during wheat season (b) and maize season (d). Arrows indicate DGGE bands for sequencing. R- and B- indicate rhizosphere and bulk soil, respectively.

correlated with AOB abundance (Table 3). This N-induced stimulation of AOB is consistent with previous observations in an alkaline sandy loam (Chu et al., 2008; Shen et al., 2008) and a paddy soil (Wu et al., 2011), where soil pH is not strongly affected by application of N fertilizer; however, it is inconsistent with the results observed for a Chinese upland red soil in which acidification occurred in response to N-fertilized treatments (He et al., 2007). In this study, the pH of calcareous soil was not drastically impacted by N fertilizer (Table 1), possibly owing to strong carbonate buffering (Glaser et al., 2010). Soil pH is known to have a considerable effect on the activities of AOB and other microbial processes that they mediate (Frijlink et al., 1992). Nicol et al. (2008) reported that bacterial *amoA* gene copies showed no obvious trend with decreasing soil pH, while their transcript numbers were typically decreased. This sensitivity of AOB to decreasing pH can be attributed to the dependence of monooxygenase on NH_3, which would be ionized exponentially to NH_4^+ with decreasing pH (De Boer and Kowalchuk, 2001). When compared with AOB, the abundance of AOA was selectively enhanced by organic manure (Fig. 2). Highly labile soil organic matter, such as straw and root exudates, may stimulate growth of the AOA community (Chen et al., 2008; Wessén et al., 2010). Positive correlations between AOA abundance and organic C and total N were also observed in the present study (Table 3). These findings further support the idea that AOA have

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

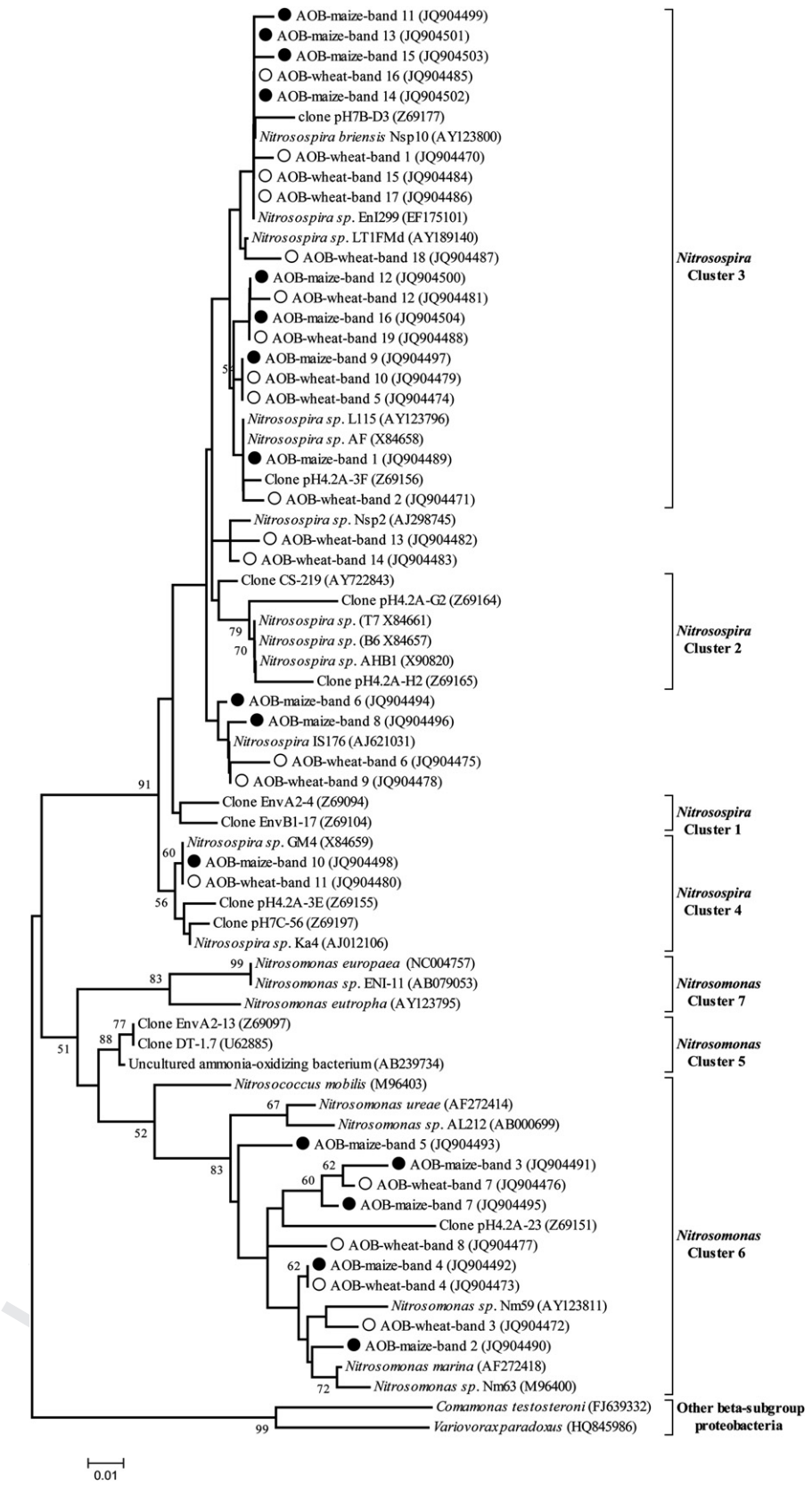

Fig. 5. Neighbor-joining tree with Kimura 2-parameter substitution of partial 16S rRNA gene sequences of AOB retrieved from the DGGE bands in this study and from the NCBI GenBank database. Bootstrap values (>50%) are indicated at branch points. The scale bar represents 1% sequence divergence. Sample sequences from this study are depicted by open (wheat season) and closed (maize season) circles.

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

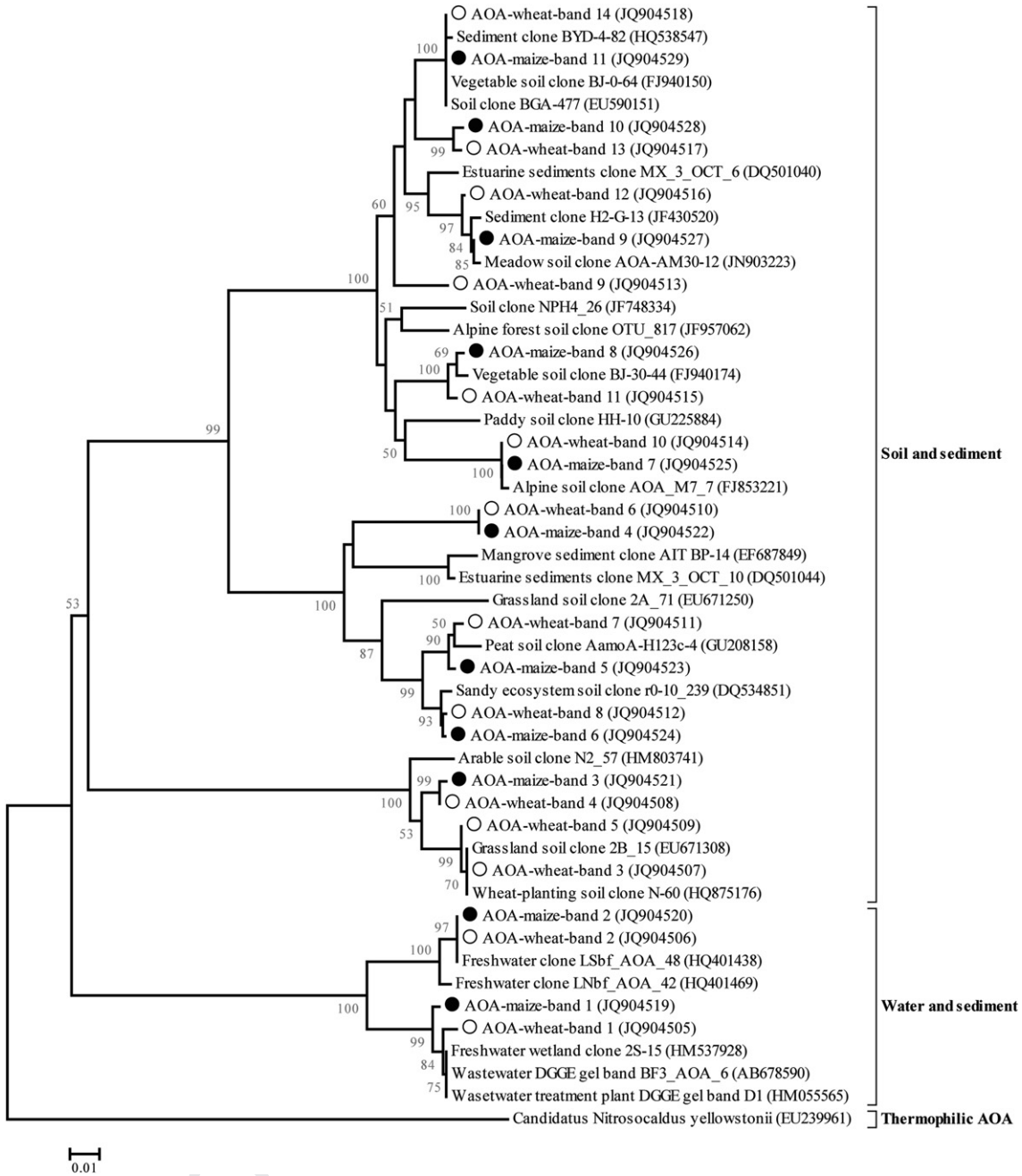

Fig. 6. Neighbor-joining tree with Kimura 2-parameter substitution of archaeal *amoA* gene sequences retrieved from the DGGE bands in this study and from the NCBI GenBank database. Bootstrap values (>50%) are indicated at branch points. The scale bar represents 1% sequence divergence. Sample sequences from this study are depicted by open (wheat season) and closed (maize season) circles.

alternative growth strategies for mixotrophic or heterotrophic growth (Walker et al., 2010).

DGGE analysis revealed that the community structure of AOB was more sensitive to various fertilization regimes than that of AOA (Figs. 3 and 4). The differences in the AOB community structure among treatments were correlated with the soil nitrogen level (NH_4^+–N and NO_3^-–N) and PNA during wheat season (Fig. 7a). Cluster analysis also clearly demonstrated that mineral N-fertilized treatments were clearly separated from CK and manure treatments, whereas most paired-samples (bulk soil and rhizosphere) clustered together (Fig. 3b and d). Thus, at this site, the increased nitrification function could primarily be ascribed to the availability of substrate (NH_3) derived from inorganic N fertilizer, while the rhizosphere effect on AOB community structure was secondary. Long-term applications of N fertilizers often result in increased diversity of AOB communities (Fig. 3), especially within clades affiliated with *Nitrosospira* cluster 3 (Chu et al., 2007; Shen et al., 2008; Wu et al., 2011). Interestingly, in this study, we detected a strong DGGE band affiliated with *Nitrosospira* cluster 4, which was found almost exclusively in N-fertilized treatments. Avrahami and Conrad (2003) speculated that *Nitrosospira* cluster 4 is restricted in cold temperatures because it is commonly detected in temperate soils. However, in a recent study, *Nitrosospira* cluster 4 was also found in a subtropical paddy soil after long-term fertilization (Wu et al., 2011), implying wide ecological diversity within this lineage. Although *Nitrosospira* sp. dominates the AOB community in arable

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

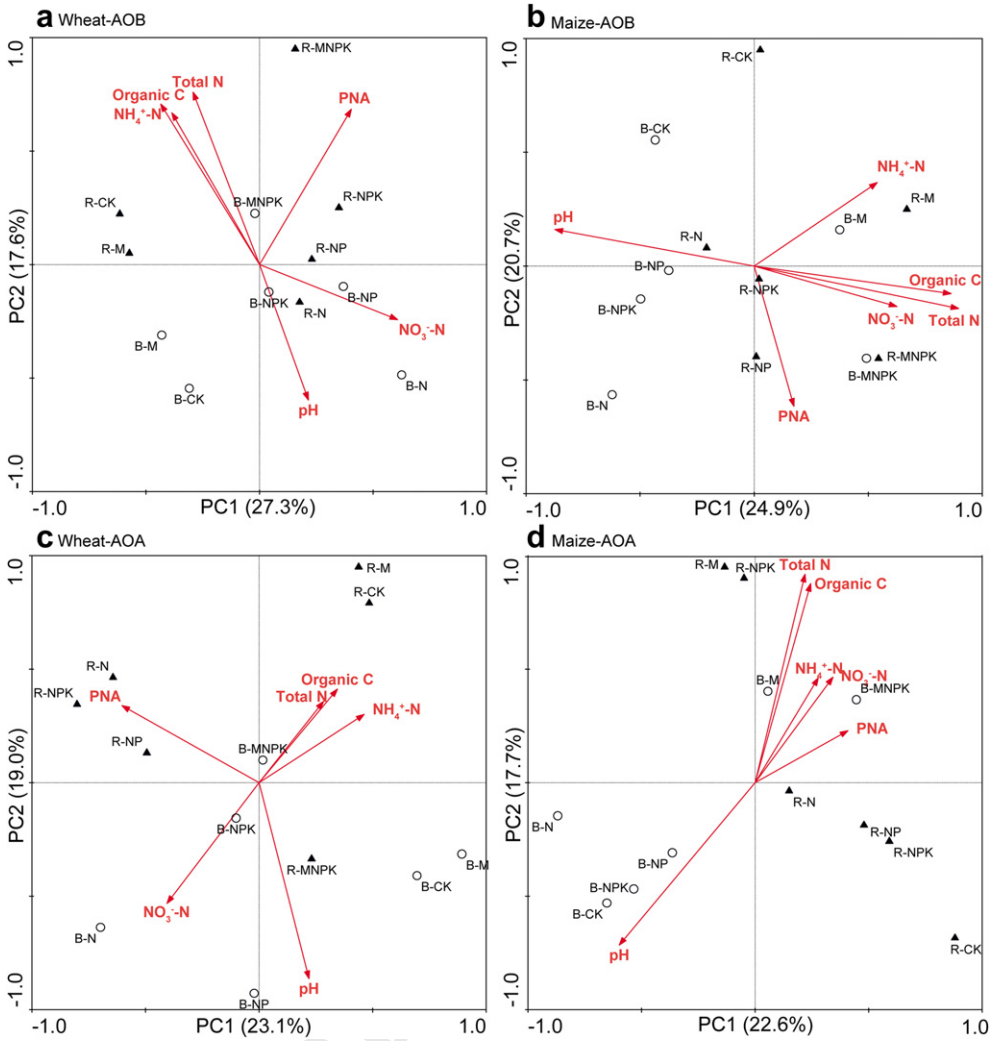

Fig. 7. Correlations of soil properties with community structure of ammonia-oxidizing bacteria (AOB) during wheat season (a) and maize season (b), and with community structure of ammonia-oxidizing archaea (AOA) during wheat season (c) and maize season (d) as determined by redundancy analysis (RDA). R- (filled triangle) and B- (open circles) represent rhizosphere and bulk soil, respectively.

soil (Fig. 5) (Innerebner et al., 2006; Phillips et al., 2000; Stephen et al., 1996), *Nitrosomonas*-like sequences were also present in this study (Figs. 3 and 5). This finding is in contrast to the results of some previous studies in which only *Nitrosospira* sp. were detected (He et al., 2007; Wu et al., 2011). *Nitrosomonas*-like lineages have been observed in manure-treated soil (Fan et al., 2011a), organic matter-rich wastewater (Zhang et al., 2010a) and alkaline calcareous soil (Shen et al., 2008), which provides further evidence of their preference for high-ammonia and high-pH environments (Kowalchuk and Stephen, 2001).

Despite the AOA community structure being less susceptible to long-term fertilization and not significantly relative to PNA, significant correlations between the AOA community structure and soil pH, organic C, total N and NO_3^-–N were still observed during both seasons (Fig. 7c and d). Custer analysis also showed that the AOA communities in the rhizosphere were clearly separated from those in the bulk soil, indicating that rhizosphere effect plays a more important role in shaping the AOA community than long-term fertilization in the fluvo-aquic soil investigated in this study. The decreasing pH (Yao et al., 2011) and abundant root exudates (Herrmann et al., 2008) in the rhizosphere could greatly favor the growth of AOA. These results are supported by the observation that AOA abundance was negatively correlated with pH (Nicol et al., 2008), but positively correlated with organic C and total N (Table 3). Chen et al. (2008) reported that the AOA community could be particularly responsive to carbon dioxide and oxygen released by rice roots into the rhizosphere. It should be noted that the potential nitrification activity in current study was determined by the shaken slurry method of Hart et al. (1994), which involves adjustment of the liquid medium pH to 7.2. This adjustment possibly changes the actual activity of indigenous communities in soils with different pH values. Recent studies demonstrated that measurement of potential nitrification of acidic soils without adjusting pH produced similar trends but significantly lower rates compared to the results with adjusting pH (Xue et al., 2009; Yao et al., 2011). Furthermore, PNR represents the NH_3 oxidation activity in soil incubated with an NH_4^+ substrate within 24 h, during which the activity of cultured AOA isolates could be hardly observed in media even under optimal conditions (Könneke et al., 2005; Park et al., 2010). Thus, the PNR assay possibly introduced a bias toward the positive correlation between PNR and the abundant of AOB rather than that of AOA (Wu et al., 2011). Nevertheless, we cannot exclude the possibility that AOA might have contributed to soil nitrification and other microbial processes, even

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

though PNA was significantly correlated with the community structure and abundance of AOB rather than AOA in this study, because the specific metabolic traits, lifestyles and ecological functions of AOA in complex terrestrial ecosystems are still unclear (Glaser et al., 2010).

5. Conclusions

The results of this study demonstrated that rhizosphere effect and fertilization regimes play different effects in the activity and community structure of AOB and AOA in fluvo-aquic soil. Long-term (31-year) applications of N fertilizers increased the PNA and AOB population size and diversity, whereas organic manure significantly enhanced the AOA population size. The high sensitivity of the AOB community to fertilization regimes, as well as the significant correlations between PNA and the abundance and community structure of AOB, but not those of AOA, suggested that ammonia oxidation is mainly driven by AOB in this calcareous soil. The PNA and population sizes of AOB and AOA were typically higher in the rhizosphere than bulk soil, indicating a profound rhizosphere effect on ammonia oxidation. Rhizosphere effect, which usually leads to high organic matter content and decreasing pH, played an important role in shaping the AOA community. However, the ecological functions of AOA in this calcareous fluvo-aquic soil are still unknown.

Acknowledgments

This research was financially supported by the National Natural Science Foundation of China and the National Basic Research Program of China. We are grateful to two anonymous reviewers for their constructive comments and suggestions on this paper.

References

Agogué, H., Brink, M., Dinasquet, J., Herndl, G.J., 2008. Major gradients in putatively nitrifying and non-nitrifying Archaea in the deep North Atlantic. Nature 456, 788–791.

Ai, C., Liang, G., Sun, J., Wang, X., Zhou, W., 2012. Responses of extracellular enzyme activities and microbial community in both the rhizosphere and bulk soil to long-term fertilization practices in a fluvo-aquic soil. Geoderma 173–174, 330–338.

Avrahami, S., Conrad, R., 2003. Patterns of community change among ammonia oxidizers in meadow soils upon long-term incubation at different temperatures. Applied and Environmental Microbiology 69, 6152–6164.

Cavagnaro, T., Jackson, L., Hristova, K., Scow, K., 2008. Short-term population dynamics of ammonia oxidizing bacteria in an agricultural soil. Applied Soil Ecology 40, 13–18.

Chen, X.P., Zhu, Y.G., Xia, Y., Shen, J.P., He, J.Z., 2008. Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? Environmental Microbiology 10, 1978–1987.

Cheng, W., Johnson, D., Fu, S., 2003. Rhizosphere effects on decomposition: controls of plant species, phenology, and fertilization. Soil Science Society of America Journal 67, 1418–1427.

Chu, H., Fujii, T., Morimoto, S., Lin, X., Yagi, K., 2008. Population size and specific nitrification potential of soil ammonia-oxidizing bacteria under long-term fertilizer management. Soil Biology and Biochemistry 40, 1960–1963.

Chu, H., Fujii, T., Morimoto, S., Lin, X., Yagi, K., Hu, J., Zhang, J., 2007. Community structure of ammonia-oxidizing bacteria under long-term application of mineral fertilizer and organic manure in a sandy loam soil. Applied and Environmental Microbiology 73, 485–491.

Coci, M., Nicol, G., Pilloni, G., Schmid, M., Kamst-van Agterveld, M., Bodelier, P., Laanbroek, H., 2010. Quantitative assessment of ammonia-oxidizing bacterial communities in the epiphyton of submerged macrophytes in shallow lakes. Applied and Environmental Microbiology 76, 1813–1821.

De Boer, W., Kowalchuk, G., 2001. Nitrification in acid soils: micro-organisms and mechanisms. Soil Biology and Biochemistry 33, 853–866.

Di, H., Cameron, K., Shen, J., Winefield, C., O'Callaghan, M., Bowatte, S., He, J., 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nature Geoscience 2, 621–624.

Fan, F., Yang, Q., Li, Z., Wei, D., Cui, X.a., Liang, Y., 2011a. Impacts of organic and inorganic fertilizers on nitrification in a cold climate soil are linked to the bacterial ammonia oxidizer community. Microbial Ecology 62, 982–990.

Fan, F., Zhang, F., Lu, Y., 2011b. Linking plant identity and interspecific competition to soil nitrogen cycling through ammonia oxidizer communities. Soil Biology and Biochemistry 43, 46–54.

Fierer, N., Carney, K.M., Horner-Devine, M.C., Megonigal, J.P., 2009. The biogeography of ammonia-oxidizing bacterial communities in soil. Microbial Ecology 58, 435–445.

Fontaine, S., Barot, S., 2005. Size and functional diversity of microbe populations control plant persistence and long-term soil carbon accumulation. Ecology Letters 8, 1075–1087.

Francis, C.A., Beman, J.M., Kuypers, M.M.M., 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. The ISME Journal 1, 19–27.

Frijlink, M.J., Abee, T., Laanbroek, H.J., Boer, W., Konings, W.N., 1992. The bioenergetics of ammonia and hydroxylamine oxidation in Nitrosomonas europaea at acid and alkaline pH. Archives of Microbiology 157, 194–199.

Glaser, K., Hackl, E., Inselsbacher, E., Strauss, J., Wanek, W., Zechmeister-Boltenstern, S., Sessitsch, A., 2010. Dynamics of ammonia-oxidizing communities in barley-planted bulk soil and rhizosphere following nitrate and ammonium fertilizer amendment. FEMS Microbiology Ecology 74, 575–591.

Gubry-Rangin, C., Nicol, G.W., Prosser, J.I., 2010. Archaea rather than bacteria control nitrification in two agricultural acidic soils. FEMS Microbiology Ecology 74, 566–574.

Hallam, S.J., Konstantinidis, K.T., Putnam, N., Schleper, C., Watanabe, Y., Sugahara, J., Preston, C., De La Torre, J., Richardson, P.M., DeLong, E.F., 2006. Genomic analysis of the uncultivated marine crenarchaeote *Cenarchaeum symbiosum*. Proceedings of the National Academy of Sciences 103, 18296–18301.

Hart, S.C., Stark, J.M., Davidson, E.A., Firestone, M.K., 1994. Nitrogen mineralization, immobilisation and nitrification. In: Weaver, R.W., Angle, J.S., Bottomley, P.S. (Eds.), Methods of Soil Analysis. Part 2. Microbial and Biogeochemical Properties. Soil Science Society of America, Madison, pp. 985–1018.

He, J., Shen, J., Zhang, L., Zhu, Y., Zheng, Y., Xu, M., Di, H., 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environmental Microbiology 9, 2364–2374.

Herman, D., Jaeger, K., Schwartz, C., Firestone, E., 2006. Root influence on nitrogen mineralization and nitrification in rhizosphere soil. Soil Science Society of America Journal 70, 1504–1511.

Hermansson, A., Lindgren, P.E., 2001. Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. Applied and Environmental Microbiology 67, 972–976.

Herrmann, M., Saunders, A.M., Schramm, A., 2008. Archaea dominate the ammonia-oxidizing community in the rhizosphere of the freshwater macrophyte *Littorella uniflora*. Applied and Environmental Microbiology 74, 3279–3283.

Hussain, Q., Liu, Y., Jin, Z., Zhang, A., Pan, G., Li, L., Crowley, D., Zhang, X., Song, X., Cui, L., 2011. Temporal dynamics of ammonia oxidizer (amoA) and denitrifier (nirK) communities in the rhizosphere of a rice ecosystem from Tai Lake region, China. Applied Soil Ecology 48, 210–218.

Innerebner, G., Knapp, B., Vasara, T., Romantschuk, M., Insam, H., 2006. Traceability of ammonia-oxidizing bacteria in compost-treated soils. Soil Biology and Biochemistry 38, 1092–1100.

Jia, Z., Conrad, R., 2009. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environmental Microbiology 11, 1658–1671.

Könneke, M., Bernhard, A.E., José, R., Walker, C.B., Waterbury, J.B., Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543–546.

Kirk, G., Kronzucker, H., 2005. The potential for nitrification and nitrate uptake in the rhizosphere of wetland plants: a modelling study. Annals of Botany 96, 639–646.

Kleineidam, K., Kosmrlj, K., Kublik, S., Palmer, I., Pfab, H., Ruser, R., Fiedler, S., Schloter, M., 2011. Influence of the nitrification inhibitor 3, 4-dimethylpyrazole phosphate (DMPP) on ammonia-oxidizing bacteria and archaea in rhizosphere and bulk soil. Chemosphere 84, 182–186.

Kowalchuk, G.A., Stephen, J.R., 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annual Reviews in Microbiology 55, 485–529.

Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M., Woldendorp, J.W., 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Applied and Environmental Microbiology 63, 1489–1497.

Kowalchuk, G.A., Stienstra, A.W., Heilig, G.H.J., Stephen, J.R., Woldendorp, J.W., 2000. Molecular analysis of ammonia-oxidising bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). FEMS Microbiology Ecology 31, 207–215.

Kuzyakov, Y., 2002. Review: factors affecting rhizosphere priming effects. Journal of Plant Nutrition and Soil Science 165, 382–396.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G., Prosser, J., Schuster, S., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809.

Liljeroth, E., Kuikman, P., Veen, J.A., 1994. Carbon translocation to the rhizosphere of maize and wheat and influence on the turnover of native soil organic matter at different soil nitrogen levels. Plant and Soil 161, 233–240.

Malchair, S., De Boeck, H.J., Lemmens, C.M.H.M., Merckx, R., Nijs, I., Ceulemans, R., Carnol, M., 2010. Do climate warming and plant species richness affect potential nitrification, basal respiration and ammonia-oxidizing bacteria in experimental grasslands? Soil Biology and Biochemistry 42, 1944–1951.

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695–700.

Neumann, G., Römheld, V., 2002. Root-induced changes in the availability of nutrients in the rhizosphere. In: Waisel, Y., Eshel, A., Kafkafi, U. (Eds.), Plant Roots: the Hidden Half, third ed. Marcel Dekker, Inc., New York, pp. 617–649.

Nicol, G.W., Leininger, S., Schleper, C., Prosser, J.I., 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environmental Microbiology 10, 2966–2978.

Park, B.J., Park, S.J., Yoon, D.N., Schouten, S., Damsté, J.S.S., Rhee, S.K., 2010. Cultivation of autotrophic ammonia-oxidizing archaea from marine sediments in coculture with sulfur-oxidizing bacteria. Applied and Environmental Microbiology 76, 7575–7587.

Phillips, C.J., Harris, D., Dollhopf, S.L., Gross, K.L., Prosser, J.I., Paul, E.A., 2000. Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. Applied and Environmental Microbiology 66, 5410–5418.

Prosser, J., 1990. Autotrophic nitrification in bacteria. Advances in Microbial Physiology 30, 125–181.

Rovira, A., 1969. Plant root exudates. The Botanical Review 35, 35–57.

Sørensen, J., 1997. The rhizosphere as a habitat for soil microorganisms. In: van Elsas, J.D., Trevors, J.T., Wellington, E.M.K. (Eds.), Modern Soil Microbiology. Marcel Dekker, New York, pp. 21–45.

Schauss, K., Focks, A., Leininger, S., Kotzerke, A., Heuer, H., Thiele-Bruhn, S., Sharma, S., Wilke, B.M., Matthies, M., Smalla, K., 2009. Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. Environmental Microbiology 11, 446–456.

Shen, J., Zhang, L., Zhu, Y., Zhang, J., He, J., 2008. Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. Environmental Microbiology 10, 1601–1611.

Stephen, J.R., McCaig, A.E., Smith, Z., Prosser, J.I., Embley, T.M., 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. Applied and Environmental Microbiology 62, 4147–4154.

Subbarao, G., Ito, O., Sahrawat, K., Berry, W., Nakahara, K., Ishikawa, T., Watanabe, T., Suenaga, K., Rondon, M., Rao, I., 2006. Scope and strategies for regulation of nitrification in agricultural systems-challenges and opportunities. Critical Reviews in Plant Sciences 25, 303–335.

Subbarao, G., Rondon, M., Ito, O., Ishikawa, T., Rao, I., Nakahara, K., Lascano, C., Berry, W., 2007. Biological nitrification inhibition (BNI) – is it a widespread phenomenon? Plant and Soil 294, 5–18.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596–1599.

Tourna, M., Freitag, T.E., Nicol, G.W., Prosser, J.I., 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environmental Microbiology 10, 1357–1364.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D., Paulsen, I., Nelson, K.E., Nelson, W., 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science 304, 66–74.

Verhamme, D.T., Prosser, J.I., Nicol, G.W., 2011. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. The ISME Journal 5, 1067–1071.

Walker, C., De La Torre, J., Klotz, M., Urakawa, H., Pinel, N., Arp, D., Brochier-Armanet, C., Chain, P., Chan, P., Gollabgir, A., 2010. *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proceedings of the National Academy of Sciences 107, 8818–8823.

Wang, S., Wang, Y., Feng, X., Zhai, L., Zhu, G., 2011. Quantitative analyses of ammonia-oxidizing Archaea and bacteria in the sediments of four nitrogen-rich wetlands in China. Applied Microbiology and Biotechnology 90, 779–787.

Wang, Y., Ke, X., Wu, L., Lu, Y., 2009. Community composition of ammonia-oxidizing bacteria and archaea in rice field soil as affected by nitrogen fertilization. Systematic and Applied Microbiology 32, 27–36.

Wessén, E., Nyberg, K., Jansson, J.K., Hallin, S., 2010. Responses of bacterial and archaeal ammonia oxidizers to soil organic and fertilizer amendments under long-term management. Applied Soil Ecology 45, 193–200.

White, C.S., 1994. Monoterpenes: their effects on ecosystem nutrient cycling. Journal of Chemical Ecology 20, 1381–1406.

Wu, Y., Lu, L., Wang, B., Lin, J., Cai, Z., Yan, X., Jia, Z., 2011. Long-term field fertilization significantly alters community structure of ammonia-oxidizing bacteria rather than archaea in a paddy soil. Soil Science Society of America Journal 75, 1405–1413.

Xue, D., Gao, Y., Yao, H., Huang, C., 2009. Nitrification potentials of Chinese tea orchard soils and their adjacent wasteland and forest soils. Journal of Environmental Sciences 21, 1225–1229.

Yao, H., Gao, Y., Nicol, G.W., Campbell, C.D., Prosser, J.I., Zhang, L., Han, W., Singh, B.K., 2011. Links between ammonia oxidizer community structure, abundance, and nitrification potential in acidic soils. Applied and Environmental Microbiology 77, 4618–4625.

Zhang, B., Sun, B., Ji, M., Liu, H., Liu, X., 2010a. Quantification and comparison of ammonia-oxidizing bacterial communities in MBRs treating various types of wastewater. Bioresource Technology 101, 3054–3059.

Zhang, L.M., Hu, H.W., Shen, J.P., He, J.Z., 2011. Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. The ISME Journal. http://dx.doi.org/10.1038/ismej.2011.168.

Zhang, L.M., Offre, P.R., He, J.Z., Verhamme, D.T., Nicol, G.W., Prosser, J.I., 2010b. Autotrophic ammonia oxidation by soil thaumarchaea. Proceedings of the National Academy of Sciences 107, 17240–17245.

Please cite this article in press as: Ai, C., et al., Different roles of rhizosphere effect and long-term fertilization in the activity and community structure of ammonia oxidizers in a calcareous fluvo-aquic soil, Soil Biology & Biochemistry (2012), http://dx.doi.org/10.1016/j.soilbio.2012.08.003