Nitrogen loading levels affect abundance and composition of soil ammonia oxidizing prokaryotes in semiarid temperate grassland

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Abstract

Purpose Global nitrogen deposition has profound impact on the terrestrial ecosystem including the semiarid temperate grassland, causing vegetation community shifts and soil acidification. Little is known regarding the effect of nitrogen (N) deposition on the below-ground microbial communities. This study aimed to examine the response of ammonia-oxidizing bacteria (AOB) and archaea (AOA) to added N in semiarid temperate grassland.

Materials and methods We studied the changes of AOB and AOA by using molecular techniques targeting *amoA* genes along a urea fertilization gradient, i.e., 0, 1, 2, 4, 8, 16, 32, 64 g N m⁻² year⁻¹, in a 6-year field experiment of semiarid temperate grassland, Inner Mongolia of China.

Results and discussion AOB community responded to urea–N substrate clearly, and N addition rates 2–4 g N m⁻² year⁻¹

induced an increase in its abundances and the shift of its composition. However, AOA community remained unchanged and the highest N loading at 64 g N m⁻² year⁻¹ even decreased its abundance. Moreover, higher N loading rates (more than 16 g N m⁻² year⁻¹) significantly decreased the diversity of AOB but not AOA, as indicated by the decrease of its Shannon and Evenness indices.

Conclusions The relative long-term nitrogen loading of more than 2–4 g N m⁻² year⁻¹ resulted in diversity loss of AOB in this semiarid temperate grassland. Increasing N loading altered AOB abundance and composition, but AOA showed nonsignificant changes.

Keywords Ammonia oxidizing bacteria • Ammonia oxidizing archaea • *amoA* gene • Inner Mongolia • Nitrification • Nitrogen deposition

1 Introduction

Ammonia oxidation, the first and rate-limiting step of nitrification, was thought to be performed almost exclusively by ammonia-oxidizing bacteria (AOB) since the first isolation of the autotrophic prokaryotes in the late nineteenth century. Recent studies demonstrated the potential for significant ammonia oxidation by ammonia-oxidizing archaea (AOA) containing all three ammonia monooxygenase (AMO) subunits (*amoA*, *amoB*, and *amoC*; Könneke et al. 2005; Treusch et al. 2005), whose ecosystem function was previously unknown. The archaeal *amoA* genes outnumber bacterial *amoA* genes in the ocean and various soils, suggesting a potential role for archaea, in addition to bacteria, in the global nitrogen cycle (Francis et al. 2005; Nicol and Schleper 2006; Prosser and Nicol 2008; Zhang et al.

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2010). Due to their important function in N cycling, the research of the ecology of AOB and AOA has become a subject of intense research over recent years.

Nitrogen accumulation through industrial and agricultural processes has dramatic impacts on the biodiversity and ecosystem function (Tilman et al. 1996, 2006; Bai et al. 2004). A number of studies have shown that the abundance and community structure of AOA and AOB shifted in response to different ammonium concentrations and fertilizers. AOB community composition was significantly different after incubated with 0.3% (wt/wt) and 1% (wt/wt) N-containing fertilizer for 11 weeks (Avrahami and Conrad 2003). In our previous work, a pronounced difference in the community composition of AOA in response to the different fertilization practices was observed in acid soils after continuous fertilization for 16 years, and that the chemical nitrogen fertilizer treatment decreased the abundance of AOA and AOB (He et al. 2007). While in alkaline soils (pH 8.3–8.7), long-term (17 years) fertilization practices had clear influence on the abundance and composition of AOB but not on AOA (Shen et al. 2008). In nitrogen-rich grassland soils, neither AOA abundance nor their activity changed with the applications of an ammonia substrate while the number and activity of AOB increased in response to ammonia additions in microcosms (Di et al. 2009). All these indicate that nitrogen inputs have great impacts on the community, abundance, and activity of AOB and AOA in soils but the influence is different among different soil systems.

The semiarid temperate steppe of Inner Mongolia in northern China is an integral part of the typical vegetation across the Eurasian continent. The soil here has low fertility and N has been identified to be one of the major limiting factors for biomass and aboveground net primary productivity (Yu et al. 2009). Such ecosystem is therefore likely to be highly sensitive to N input. For the aboveground plant communities, Bai et al. (2010) identified the critical threshold of N loading of below $1.75 \text{ g N m}^{-2} \text{ year}^{-1}$ for N-induced species loss to mature Eurasian grasslands and of $10.5 \text{ g N m}^{-2} \text{ year}^{-1}$ for the changes in aboveground biomass, species richness, and plant functional group composition to both mature and degraded ecosystems. However, the impact of N loading on the important functional microbes mediating N transformations underground, ammonia oxidizers in this area, is unknown. In this study, abundance and community structure AOB and AOA along eight urea–N fertilization gradients in a 6-year experimental field in Inner Mongolia were investigated, aiming to examine the response of AOB and AOA along N addition gradient and give hints on the impact of N addition on the underground microorganisms in this area.

2 Materials and methods

2.1 Experimental site and soil sampling

The experiment was conducted in a semiarid area located in Duolun County (N $42^{\circ}2'29''$, E $116^{\circ}17'20''$), Inner Mongolia Autonomous Region, China. Historically, this area was a pastoral zone, but now it is mainly an agropastoral ecotone. Averaged over the past several decades (1953–2004), the mean annual precipitation was 385.5 mm with peaks in July and August. Mean monthly air temperatures range from -17.5°C in January to 18.9°C in July, with mean annual temperature of 2.1°C . The soil is a chestnut soil with 62.8% sand, 20.3% silt, and 16.95% clay, bulk density 1.3 g cm^{-3} and pH 7.1. The vegetation of this site is dominated by *Stipa krylovii* Roshev, *Agropyron cristatum* (L.) Gaertn, *Cleistogenes squarrosa* (Trin.) Keng, *Potentilla acaulis* (L.), and *Artemisia capillaries* Thunb (Niu and Wan 2008).

The urea–nitrogen fertilization gradient experiment was established in 2003, including eight treatments and each with four replicate plots in a randomized plot design. Each plot was $10 \times 15 \text{ m}^2$, and the distance among them was 1 m. Treatments designated numbers #1–8 were fertilized along a urea gradient respectively (addition rates of 0, 1, 2, 4, 8, 16, 32, $64 \text{ g N m}^{-2} \text{ year}^{-1}$, respectively) in middle July annually. Treatment #1 with nil fertilization was denoted as NF, low fertilization treatments #2–4 were denoted as LF, moderate fertilization treatments #5–6 were denoted as MF, and high fertilization treatments #7–8 were denoted as HF. The soil samples were collected in May 2009. Five soil cores (5 cm in diameter) were taken from the soil surface (0–10 cm depth) in each plot and mixed to form one composite sample. Each sample was placed in a sterile plastic bag, kept on ice, and transported to the laboratory. All samples were passed through a 2.0-mm sieve, stored at 4°C for analysis of soil characteristics and at -80°C for DNA extraction. Soil pH was determined with a soil to water ratio of 1:2.5, and soil organic matter was determined by the $\text{K}_2\text{Cr}_2\text{O}_7$ oxidation-reduction titration method. Nitrate and ammonium were extracted with 2 M KCl and determined by a continuous flow analyzer (SAN++, Skalar, Holland). Total nitrogen was determined using the Dumas method by an element analyser (Vario EL III, Elementar, Germany).

2.2 Soil DNA extraction

Soil DNA was extracted from 500 mg subsample of each sample using the Fast DNA[®] SPIN Kit for Soil (Q BIOgene Inc., Carlsbad, CA, USA). The extracted DNA was checked on a 1% agarose gel and the concentration was estimated using a Nanodrop[®]ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

2.3 Quantification of *amoA* genes by real-time PCR

Primer pairs *amoA1F/amoA2R* (Rotthauwe et al. 1997) and *Arch-amoAF/Arch-amoAR* (Francis et al. 2005) were used for real-time polymerase chain reaction (PCR) quantification of the bacterial and archaeal *amoA* genes, respectively. The 25- μ L reaction volume contained 12.5 μ L SYBR® Premix Ex Taq™ (TaKaRa Bio Inc.), 0.4 mg mL⁻¹ bovine serum albumin, 400 nM of each AOB primer or 200 nM of each AOA primer, and 2 μ L of tenfold diluted extracted DNA (1–10 ng) as template. Amplifications were performed on an iCycler iQ 5 thermocycler (Bio-Rad) as follows: 95°C for 1 min, followed by 35 cycles of 10 s at 95°C, 30 s at 55°C for AOB or 53°C for AOA, 1 min at 72°C and plate read at 83°C (Zhang et al. 2009).

PCR products amplified from extracted DNA with the primers for real-time PCR assays were gel-purified and ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and the resulting ligation products were transformed into *Escherichia coli* JM109 competent cells following the manufacturer's instructions. After reamplification with the vector-specific primers T7 and SP6, the positive clones were selected to extract plasmid DNA using a MiniBEST Plasmid Purification Kit (TaKaRa Bio Inc.) and used as *amoA* gene standards. The plasmid DNA concentration was determined on a Nanodrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies), and *amoA* gene copy number was calculated directly from the concentration of the extracted plasmid DNA. Tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to real-time PCR in triplicate to generate an external standard curve. PCR efficiency and correlation coefficients for standard curves were 81.3% and $r^2=0.996$ for AOB, and 101.4% and $r^2=0.996$ for AOA.

2.4 T-RFLP analysis

As a rapid screening for the diversity of AOB and AOA, terminal restriction fragment length polymorphism (T-RFLP) analysis of *amoA* gene fragments were performed. The fragments of the *amoA* genes were amplified with the same primers for the above real-time PCR assays. The 5' terminal of forward primer was labeled with 6-carboxyfluorescein. The PCR reaction was the same as described for the above real-time PCR assays excepting that SYBR® Premix Ex Taq™ was replaced with the Premix Ex Taq™ (TaKaRa Bio Inc.). The labeled PCR product was purified by Wizard® SV Gel and PCR Clean-Up System (Promega Corporation) and digested at 37°C for 3 h by endonuclease *Mbo* I (*Sau*3A I) (TaKaRa Bio Inc.; Ying et al. 2010). After purification, the digestion product of each sample was mixed with deionized formamide and internal standard GeneScan™ 1000 ROX™ Size Standard (Applied

Biosystems). The mixture was denatured at 95°C for 3 min and placed on ice immediately. The DNA fragments were then size-separated by capillary electrophoresis (3130xl Genetic Analyzer, Applied Biosystems).

The relative abundance of individual terminal restriction fragment (T-RF) was calculated as the percentage of total peak height in a given T-RFLP profile. Only those T-RFs with the relative abundance of >0.05% were considered in analyses. The Shannon diversity index H and Evenness index E were used to calculate the T-RF pattern diversity of AOB and AOA based on the following equations:

$$H = - \sum_{i=1}^S p_i \ln p_i$$

$$E = H/H_{\max} = H/\ln S$$

where, S is the total number of T-RF types in the sample, and P_i is the percentage of i th T-RF type.

2.5 Cloning and sequencing

Each one clone library of AOB and AOA *amoA* gene retrieved from treatment #5 applied 8 g N m⁻² year⁻¹ was created. DNA extracts from four plots of this treatment were mixed and served as the template of PCR. PCR amplification and cloning was performed as described above. The PCR products of bacterial and archaeal *amoA* genes amplified with the primers for the real-time PCR assays were purified and cloned, as described above. Approximately 130 randomly selected clones from each clone library were sequenced.

Sequences were subject to homology analysis using the software DNAMAN version 4.0 (Lynnon Biosoft). Sequences of chimeric origin were checked by partial treeing analysis and compared with GenBank sequences using BLASTN search. For sequence types that exhibited >98% identity to each other, only one representative was used for construction of the phylogenetic tree. The GenBank sequences most similar to the clone sequences in this study and reference sequences for defining clusters were included in the phylogenetic tree construction. Phylogenetic analysis based on nucleotide sequences was performed using MEGA version 4.0 (Tamura et al. 2007) and a neighbor-joining tree was constructed using Kimura two-parameter distance with 1,000 replicates to produce bootstrap values.

2.6 Statistical analyses

The abundance of *amoA* gene data was log-transformed to provide variance homogeneity. All statistical analyses were performed using SPSS version 13.0 and one-way analysis

of variance followed by S-N-K-test was used to check for quantitative differences between treatments. Bivariate correlations were carried out to link different parameters. $P < 0.05$ was considered to be significant.

2.7 Nucleotide sequence accession numbers

The sequences determined in this study were deposited in the GenBank database and assigned accession numbers GU931340 to GU931357 affiliated with AOB and GU931358 to GU931380 affiliated with AOA.

3 Results

3.1 Soil chemical properties

Soil pH, compared to the NF and LF treatments (#1–4) ranging from 6.95 to 6.83, decreased significantly in MF and HF treatments (#5–8), with the lowest value of 5.42 ± 0.38 in treatment #8 (Table 1). Soil NH_4^+ -N concentration was relatively low (1.69 – 2.66 mg kg^{-1} soil) in treatments #1–6, but significantly accumulated (5.14 – 18.0 mg kg^{-1} soil) in the HF treatments (#7–8). NO_3^- -N concentration in soils (0.61 – 11.4 mg kg^{-1} soil) showed a rising trend as N loading rates increased with a significantly positive correlation with NH_4^+ -N concentration ($r = 0.680$, $n = 32$, $P < 0.01$, two-tailed). Organic matter in these eight treatments varied between 39.5 and 47.1 g kg^{-1} soil and total nitrogen varied between 2.6 and 3.1 g kg^{-1} soil without significant differences.

3.2 Abundance of AOB and AOA

The AOB *amoA* gene copy numbers did not change too much in treatments #1–3, but increased 4.9-, 6.9-, 11.7-,

17.6-, and 21.2-fold in treatments #4–8 compared to the average abundance of treatments #1–3, respectively (Fig. 1). In contrast, AOA *amoA* gene copy numbers, ranging from 2.8×10^8 to 7.8×10^8 , remained largely unchanged irrespective of the urea-N addition levels, excepting the treatment #8 which received the highest N addition had significantly lower *amoA* gene copy numbers (see Fig. 1). The AOB *amoA* gene copy numbers were lower than that of AOA in NF and LF, similar to AOA in MF, but greater than AOA in HF (see Fig. 1). The ratio of AOA to AOB decreased from 21.9 in the lowest N loading treatment #2 to 0.2 in the highest N loading treatment #8, and negatively correlated with the N loading rates ($r = -0.737$, $n = 32$, $P < 0.01$, two-tailed).

3.3 Community structure of AOB and AOA

The digestion by enzymes *Mbo* I produced 6–8 T-RFs of AOB and 7–9 T-RFs of AOA *amoA* gene in the T-RFLP profiles of all samples (Fig. 2). In all treatments, AOB profiles were dominated by 109 and 280 bp T-RFs and the relative abundance of the two T-RFs changed dramatically along the N gradients. The relative abundance of 109 bp T-RF increased distinctly from 41.4% to 89.8%, while T-RF 280 bp decreased from 47.1% to 7.9% as the N addition rate increased from $1 \text{ g N m}^{-2} \text{ year}^{-1}$ to $64 \text{ g N m}^{-2} \text{ year}^{-1}$, indicating clear community shift of AOB occurred in response to the different N gradients. T-RFLP profiles of AOA throughout the samples were codominated by 73, 329, 421, and 443 bp T-RFs with the relative abundance ranging between 10.3% and 32.5%, and showed no distinct variation among different treatments.

Shannon index (H) and Evenness index (E) are commonly used to characterize species diversity in a community. H and E values of AOB in MF and HF (N addition rates $\geq 16 \text{ g N m}^{-2} \text{ year}^{-1}$) were significantly lower than

Table 1 Chemical properties of a semiarid temperate grassland soil under different amounts of urea fertilization

N loading level	Treatment no.	N addition rate ($\text{g N m}^{-2} \text{ year}^{-1}$)	pH (H_2O)	Organic matter (g kg^{-1})	Total N (g kg^{-1})	NH_4^+ -N (mg kg^{-1})	NO_3^- -N (mg kg^{-1})
NF	#1	0	6.91 ± 0.16 c	43.4 ± 12.1 a	3.0 ± 0.2 ab	1.77 ± 0.26 a	0.79 ± 0.44 a
LF	#2	1	6.95 ± 0.12 c	44.4 ± 12.9 a	2.9 ± 0.1 ab	1.69 ± 0.76 a	0.61 ± 0.25 a
	#3	2	6.91 ± 0.13 c	42.3 ± 10.8 a	2.8 ± 0.3 ab	2.18 ± 0.74 a	1.20 ± 0.78 a
	#4	4	6.83 ± 0.15 c	40.5 ± 14.4 a	2.6 ± 0.2 a	2.14 ± 1.00 a	1.66 ± 1.53 a
MF	#5	8	6.35 ± 0.27 b	46.5 ± 15.1 a	3.1 ± 0.3 b	2.28 ± 0.62 a	2.92 ± 1.66 a
	#6	16	6.17 ± 0.19 b	47.1 ± 15.1 a	3.0 ± 0.2 ab	2.66 ± 0.55 a	3.99 ± 1.80 a
HF	#7	32	5.63 ± 0.15 a	39.5 ± 14.5 a	2.9 ± 0.2 ab	5.14 ± 2.32 b	11.1 ± 2.63 b
	#8	64	5.42 ± 0.38 a	41.0 ± 11.1 a	2.8 ± 0.2 ab	18.0 ± 2.44 c	11.4 ± 3.03 b

Treatments #1–8 were fertilized with 0, 1, 2, 4, 8, 16, 32, and $64 \text{ g N m}^{-2} \text{ year}^{-1}$, respectively. Four N loading levels were: NF nil fertilizer, LF low fertilizer, MF moderate fertilizer, HF high fertilizer. Mean $\pm$ SD ($n = 4$). Values within the same column followed by the same letter do not differ at $P < 0.05$

Fig. 1 Abundance of bacterial and archaeal *amoA* genes. **a** Treatments #1–8 were fertilized with 0, 1, 2, 4, 8, 16, 32, and 64 g N m⁻² year⁻¹, respectively. **b** Error bars indicate SDs ($n=4$). Values followed by the same letter (AOB small letter, AOA capital letter) do not differ at $P<0.05$. **c** Ratio of AOA to AOB *amoA* gene copies are shown in boxes above the chart

those of NF and LF (Table 2), while the values of AOA showed no significant change among different treatments, indicating high N addition have significant effect on the diversity of AOB but not on AOA.

To characterize the *amoA* gene diversity based on the T-RFLP, 127 AOB clones and 124 AOA clones of *amoA*

genes retrieved from moderate N addition treatment #5 which contained all AOB and AOA T-RF types presented in all treatments were sequenced. Phylogenetic analysis showed that the majority of the AOB sequences were affiliated in the *Nitrosospora* clusters 3a.1 and 3a.2 with some in *Nitrosospora* cluster 9 and some in *Nitrosomonas*

Fig. 2 Relative abundance of T-RFs of bacterial and archaeal *amoA* gene restricted by *Mbo* I (*Sau*3A I). **a** Treatments #1–8 were fertilized with 0, 1, 2, 4, 8, 16, 32, and 64 g N m⁻² year⁻¹, respectively. **b** Error bars indicate SDs ($n=4$)

Table 2 Shannon index (*H*) and Evenness index (*E*) of AOB and AOA calculated from T-RFLP data

N loading level	Treatment no.	N addition rate (g N m ⁻² year ⁻¹)	AOB		AOA	
			Shannon (<i>H</i>)	Evenness (<i>E</i>)	Shannon (<i>H</i>)	Evenness (<i>E</i>)
NF	#1	0	0.98±0.15 b	0.47±0.07 b	1.74±0.17 a	0.79±0.08 a
LF	#2	1	1.11±0.01 b	0.53±0.01 b	1.76±0.04 a	0.80±0.02 a
	#3	2	1.02±0.07 b	0.49±0.03 b	1.64±0.21 a	0.75±0.09 a
	#4	4	1.01±0.27 b	0.48±0.13 b	1.64±0.14 a	0.74±0.06 a
	#5	8	0.84±0.18 b	0.41±0.09 b	1.76±0.14 a	0.80±0.07 a
MF	#6	16	0.61±0.10 a	0.29±0.05 a	1.66±0.10 a	0.75±0.04 a
	#7	32	0.57±0.05 a	0.27±0.02 a	1.66±0.16 a	0.76±0.07 a
HF	#8	64	0.40±0.14 a	0.19±0.06 a	1.52±0.27 a	0.69±0.12 a

Treatments #1–8 were fertilized with 0, 1, 2, 4, 8, 16, 32, and 64 g N m⁻² year⁻¹, respectively. Four N loading levels were: *NF* nil fertilizer, *LF* low fertilizer, *MF* moderate fertilizer, *HF* high fertilizer. Mean ± SD (*n*=4). Values within the same column followed by the same letter do not differ at *P*<0.05

clusters 6 and 8 (Avrahami et al. 2002; Avrahami and Conrad 2003; Fig. 3). All AOB sequences could be classified as the same T-RF types as detected in AOB T-RFLP analysis by *Mbo* I (*Sau*3A I) digestion. Out of 127 AOB sequences, 51% of sequences corresponded to 109 bp T-RF and 41% of sequences corresponded to 280 bp T-RF, indicating the two T-RFs dominated AOB community coinciding with the AOB T-RFLP profile. The T-RF 109 bp (in total 65 sequences) was represented by clusters 3a.1, 3a.2, and 9 with varied proportions of 60%, 37%, and 3%, respectively. Similarly, the T-RF 280 bp (in total 52) was represented by clusters 3a.1, 3a.2, but cluster 3a.1 accounted for the absolute majority of this T-RF with a proportion of 98%. All these data imply that AOB community in all treatments is dominated by cluster 3a.1, followed by cluster 3a.2.

All AOA clones identified fell within the soil and sediment lineage and grouped into three distinct clusters, designated as soil and sediment clusters 1, 2, and 3 as defined in previous study (Zhang et al. 2009; Fig. 4). The main T-RF types 73, 169, 329, 421, and 443 bp in AOA T-RFLP profiles were identified in AOA sequences with the percentage ranging between 4.8% and 51%. The sequence corresponded to the 73 bp T-RF fell within all three clusters and 329 bp T-RF within clusters 1 and 2. The other detected phylotypes of 169, 421, and 443 bp only existed in clusters 3, 1, and 2, respectively.

4 Discussion

The abundance of AOB was markedly lower than that of AOA in this low-fertility semiarid temperate grassland soil, which was consistent with studies in other ecosystems (Leininger et al. 2006; He et al. 2007; Nicol et al. 2008;

Shen et al. 2008). However, it was AOB that more sensitively responded to urea-N substrate addition and more than 2 g N m⁻² year⁻¹ addition rates had induced significant increase in abundance and community composition shift, whereas the abundance and composition of AOA remained constant except that the highest N addition rate (64 g N m⁻² year) decreased its abundance. As a result, the relative abundance of AOA and AOB shifted drastically. The ratio of AOA to AOB in this study decreased from a high of 21.9 at low N loading rate to a low of 0.2 at the highest N loading rate, well in line with the report for nitrogen-rich grassland soils in New Zealand with ratios varying from 10.7 to 0.2 (Di et al. 2009). The constantly low ammonia concentration corresponding to the increasing nitrate concentration along N loading gradients indicated that active nitrification mediated by soil ammonia oxidizing prokaryotes was going on, and increasing AOB population induced by N addition may make an increasing contribution, which is also in agreement with those found that AOB is mainly driver for nitrification in nitrogen-rich grassland soils in New Zealand (Di et al. 2009). More recent evidences have demonstrated that AOA had high affinity for ammonium and were adapted to low ammonia concentrations (Hatzenpichler et al. 2008; Martens-Habbena et al. 2009; Schleper, 2010). AOA growth was inhibited by the high dose of urine-N substrate in grazed grassland soils in New Zealand was observed recently by Di et al. (2010). All these provide good explanations for our observations that high N addition did stimulate the growth of AOB but not AOA, and that AOB may be responsible for the active nitrification in this soil. Significant decrease in the Shannon and Evenness index values of AOB in higher nitrogen loading treatments (more than 16 g N m⁻² year⁻¹) was observed in this study. The results implied the potential risk of diversity loss in microorganisms caused by nitrogen

Fig. 3 Neighbor-joining phylogenetic tree of bacterial *amoA* sequences (465-bp fragment) retrieved from a semiarid temperate grassland soil in Northern China. Designation of the clones in **bold** includes the information: accession number in the GenBank, following with the T-RF length digested by *Mbo* I (*Sau*3A I) gained from in silico analysis

and the number of clones from treatment #5 with similarities above 98% to the listed sequence in the *parentheses*. Bootstrap values (>50) are indicated at branch points. The scale bar represents 5% estimated sequence divergence

Fig. 4 Neighbor-joining phylogenetic tree of archaeal *amoA* sequences (594-bp fragment) retrieved from a semiarid temperate grassland soil in Northern China. Designation of the clones in **bold** includes the information: accession number in the GenBank, following with the T-RF length digested by *Mbo* I (*Sau*3A I) gained from in silico analysis

and the number of clones from treatment #5 with similarities above 98% to the listed sequence in the *parentheses*. Bootstrap values (>50) are indicated at branch points. The *scale bar* represents 5% estimated sequence divergence

addition presented in N-limited grassland ecosystem, coinciding with what has been observed in macroorganisms (Aber et al. 1998; Bai et al. 2010). The N deposition rate in this area was estimated to be $2 \text{ g N m}^{-2} \text{ year}^{-1}$ (Zhang et al. 2008). Closely to the threshold that N-induced AOB community change happened, $2\text{--}4 \text{ g N m}^{-2} \text{ year}^{-1}$ in this study, while the threshold for plants in this region was found to be approximately $10.5 \text{ g N m}^{-2} \text{ year}^{-1}$ (Bai et al. 2010). This also implied that AOB was a potential sensitive indicator for assessing N addition effect.

Both culture-dependent and independent techniques have suggested the predominance of *Nitrosospira* over *Nitrosomonas* in AOB communities in terrestrial ecosystems (Kowalchuk and Stephen 2001; Avrahami and Conrad 2003), whereas *Nitrosomonas* clusters 7 and 8 were often detected in high N environments, such as wastewater treatment plants (Geets et al. 2006), manure-treated wetland (Ibekwe et al. 2003), and enrichment cultures (Stephen et al. 1996). Our study revealed that the AOB community was dominated by T-RFs 109 and 280 bp in T-RFLP profiles and the two T-RFs were characterized mainly by *Nitrosospira* Cluster 3a.1 based on cloning and sequencing analysis for treatment #5. AOB T-RFLP analysis showed that the relative abundance of T-RF 280 bp decreased distinctly but that of T-RF 109 bp increased along the increasing N loading gradient. Combining the sequencing results that T-RF 280 bp was represented by 98% of *Nitrosospira* cluster 3a.1 and 2% by cluster 3a.2 and T-RF 109 was represented by 60% of cluster 3a.1, 37% of cluster 3a.2, and 3% of cluster 9 in treatment #5, we can deduce that high N loading decreased the proportion of *Nitrosospira* cluster 3a.1 and the increase in the relative abundance of T-RF 109 may be caused by the increase in the proportion of cluster 3a.2. All these results demonstrated the AOB community composition and the AOB *amoA* gene abundance can be consistently altered by the relative long-term N fertilizer application. All archaeal *amoA* sequences from this study fell into soil and sediment lineage, suggesting that AOA in this semiarid temperate grassland were distinct from aquatic environments, which was consistent with the results observed in some alkaline soils (Shen et al. 2008; Zhang et al. 2009). Unlike AOB, the AOA community composition was much more stable among the treatments possibly due to their insensitivity to environmental gradients and slow growth (Könneke et al. 2005).

Long-term of 25 years N fertilizer application could result in soil acidification (McAndrew and Malhi 1992), and in the present study, soil pH significantly decreased (approximately from 7.0 to 5.5) with the nitrogen loading increase. The reduction in pH may be another factor inducing the community shift and growth of AOB, but AOA did not show significant change. It was opposite to

the changes of AOB and AOA communities in soils amended into a gradient of pH values (approximately 4.9–7.5) by adding either lime or aluminum sulfate (Nicol et al. 2008). Moreover, it was also inconsistent with the result that soil pH (approximately 3.7–5.8) played a more important role in AOA populations as found in a Chinese upland red soil under a long-term of 16-year fertilizer application (He et al. 2007), but similar trend was found in an alkaline sandy loam (pH around 8.5; Shen et al. 2008). The reason for this inconsistency might be the active AOA group inhabiting in these soils were different. Another possibility is that the effect of high urea-N substrate addition on AOB and AOA predominated over the pH effect. 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 shifts was observed after 16 weeks (Avrahami and Conrad 2003) and 2 years (Horz et al. 2004). Our study provided convincing field experiment evidence for the effect of the relative long term of 6 years N loading levels on the soil ammonia oxidizing prokaryotes in the semiarid temperate grassland soils.

5 Conclusions

In conclusion, AOB and AOA abundance and community structure under relative long-term N loading gradients in semiarid grassland were monitored by combining the quantitative PCR, T-RFLP, and sequencing methods. The archaeal *amoA* gene had a higher copy number than the bacterial *amoA* gene in N-limited grassland soil in natural conditions, with all AOB sequences affiliating to *Nitrosospira*- or *Nitrosomonas*-like species and all AOA sequences affiliating to soil and sediment lineage. Both abundance and composition of AOB were altered by the increasing N loading, but AOA showed nonsignificant changes in abundance and community composition. The relative long-term nitrogen loading of more than $2\text{--}4 \text{ g N m}^{-2} \text{ year}^{-1}$ resulted in a diversity loss of AOB in this study, which is consistent with the previous findings in macroorganisms indicating that the soil ammonia oxidizing prokaryotes are sensitive in response to the N input in soil and can be used as a effective indicator for assessing ecosystem functions and soil sustainable management.

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