Contrasting effects of nitrogen forms and soil pH on ammonia oxidizing microorganisms and their responses to long-term nitrogen fertilization in a typical steppe ecosystem

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ABSTRACT

Ammonia-oxidizing microorganisms, i.e. ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), are the primary agents responsible for soil nitrification. Few studies, however, have evaluated how AOA and AOB responses to long-term N fertilization are affected by soil pH and different forms of nitrogen (N). We examined the effects of soil acidification and different forms of N (NH_4^+ and NO_3^-) on abundances and community structure of AOA and AOB based on a field acid addition experiment and a short-term microcosm N addition experiment. The field acid addition experiment demonstrated that, with decreasing soil pH, AOB abundance decreased while AOA abundance mostly increased except for an extremely low pH treatment. Relationships between soil pH and abundance of ammonia oxidizers in the acid addition experiment conflict with those in the long-term N fertilization experiment, indicating a predominant role of N rather than soil pH on responses of AOB and AOA under N inputs. The short-term N addition experiment confirmed the general positive effect of NH_4^+ on AOB and the negative effect of NO_3^- on AOA which help explain the responses of AOB and AOA abundance to long-term N fertilization. Community structure of ammonia oxidizers, in contrast, showed little response to acid addition and short-term N additions. The positive responses of AOB and not AOA to various N additions revealed a dominant role of AOB in nitrification with broad ranges of soil pH and N availability. We found a negative effect of NO_3^- addition on AOA abundance, which may be a key factor limiting contributions of AOA to nitrification in many soils.

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1. Introduction

Nitrification is a key process in the N cycle that is important to many ecosystem functions, such as soil nutrient supply, NO_3^- pollution, and greenhouse gas emissions (Purkhold et al., 2000). Ammonia-oxidization is the first key step of nitrification and, in natural ecosystems, this is driven by ammonia-oxidizing microorganisms (Kowalchuk and Stephen, 2001). Ammonia-oxidation was

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once considered to be driven mainly by ammonia-oxidizing bacteria (AOB) (Purkhold et al., 2000). Recently, studies of several aquatic environments and soils indicate that ammonia-oxidizing archaea (AOA) also play important roles in nitrification in many ecosystems (Prosser and Nicol, 2008; He et al., 2012). The thaumarchaeal *amoA* gene was found in nearly all ecosystems and outnumbered bacterial *amoA* gene copies in most environments (Leininger et al., 2006). A few AOA strains with ammonia oxidation activity have been isolated from both terrestrial and aquatic environments (Könneke et al., 2005; Tourna et al., 2011; Lehtovirta-Morley et al., 2011). In addition to the evidence from isolated strains, autotrophic ammonia oxidation by archaea in soil was also identified by stable isotope probing of ^{13}C (Zhang et al., 2010, 2012; Lu and Jia, 2013) or using a nitrification inhibitor

(Offre et al., 2009). These findings aroused attention about the contributions of AOB and AOA in nitrification within various ecosystems (Erguder et al., 2009) and the factors controlling abundance, structure and function of AOB and AOA communities.

Nitrogen availability has doubled globally over the last 50 years through industrial and agricultural processes, which is an important driver of widespread changes in biogeochemical cycles, soil acidification, and degradation of ecosystem services worldwide (Phoenix et al., 2012). N inputs into soils are usually accompanied by an increase of N availability and a decrease of soil pH, which tend to have opposite effects on both the abundance and activities of AOB and AOA. Increasing soil pH positively affects AOB, but negatively affects AOA (Nicol et al., 2008). Compared with AOB, AOA have high affinity for reduced N (Martens-Habben et al., 2009). Congruent with the effects of soil pH and N, AOA dominates ammonia oxidation in many acidic soils whether or not N is added (Gubry-Rangin et al., 2010; Yao et al., 2011; He et al., 2012; Isobe et al., 2012; Zhang et al., 2012; Lu and Jia, 2013) or in pH neutral soils without heavy N addition (Di et al., 2010; Verhamme et al., 2011), while AOB were more active than AOA in some non-acidic soils receiving N inputs (Shen et al., 2008; Di et al., 2009; Jia and Conrad, 2009; Xia et al., 2011). Although soil pH and N availability are two important factors controlling abundance, structure and activity of AOB and AOA and may help explain effects of N enrichment on AOB and AOA in some studies, the roles of soil pH and N availability on responses of ammonia oxidizers to N inputs are complex and generally not addressed by previous studies.

The Inner Mongolia grassland occupies a large area of northern China. It is representative of the Eurasian steppe region and important for livestock farming and environmental protection. Increased agriculture and overgrazing in this area has led to higher N inputs due to fertilization and excretion of sheep urine. Our previous study in a typical steppe of Inner Mongolia showed that long-term N fertilization had a positive effect on AOB abundance of AOB but not on AOA. N fertilization significantly decreased soil pH and potential nitrification rate (PNR) (Li et al., 2011). Therefore, responses of AOB and AOA to long-term N fertilization differed from the effects of soil pH on AOB and AOA seen in most previous studies. Although increased soil pH and N availability are usually considered to have positive effects on AOB and negative effects on AOA, the detailed effects of soil pH and N availability have been well studied in only a few controlled experiments (Nicol et al., 2008; Verhamme et al., 2011). The reasons why effects of soil pH on AOB and AOA in the soils of our study differ from those in other soils studied or whether the responses of AOB and AOA to N fertilization were mostly affected by increased N availability are unclear. NH_4^+ and NO_3^- are the two main inorganic N forms in soils that are most closely related to nitrification and usually accumulated with N inputs. However, the effects of NO_3^- have rarely been considered. Although NH_4^+ enrichment can explain the positive effects of N inputs on ammonia oxidizers by a substrate induced mechanism, the effective N form that is responsible for the frequently observed negative effects of N additions on AOA has not been determined.

To isolate the effect of soil pH resulting from reactive N additions under N fertilization, we established a field experiment of acid addition near the site of the N fertilization experiment at the same station (Lan and Bai, 2012). In addition, we conducted a microcosm experiment to distinguish the effects of the two main inorganic N forms (NH_4^+ and NO_3^-) and better understand the mechanisms for the effects of N inputs on ammonia oxidizers. Responses of AOB and AOA abundance and community structure and nitrification activity to soil acidification and different N enrichment were measured and compared with responses to N fertilization. The main question of this study was to ask: are the responses of the AOB and AOA community and nitrification activity to long-term N fertilization, as

we previously observed, due to alteration of soil pH, NH_4^+ or NO_3^- ? We hypothesized that: 1) reduction in soil pH has negative effect on AOB but positive effect on AOA, as previously reported; 2) NH_4^+ has a positive effect on AOB; and 3) either NH_4^+ or NO_3^- has a negative effect on AOA. Based on these hypotheses, we expect that responses of AOB to long-term N fertilization would mainly be determined by NH_4^+ , while responses of AOA to long-term N fertilization would mainly be determined by NH_4^+ or NO_3^- .

2. Materials and methods

2.1. Study site

The field experiments of this study were conducted at the Inner Mongolia Grassland Ecosystem Research Station (IMGERS), Chinese Academy of Sciences (116°42'E, 43°38'N, and 1250 m a.s.l.), which is located in the Xilin River Basin, Inner Mongolia Autonomous Region of China. The mean annual temperature in the study area is 0.3 °C and the mean monthly temperatures ranges from −21.6 °C in January to 19.0 °C in July. The mean annual precipitation is 346 mm, 60–80% of which falls during the growing season from May to August. The soil in the study area is classified as dark chestnut (Calcic Chernozem according to ISSS Working Group RB, 1998). The soil texture is loamy sand, with 80.2% sand, 17.6% silt, and 2.2% clay. The plant community is dominated by perennial rhizomatous grasses (e.g. *Leymus chinensis* Trin.) Tzvel. and perennial bunchgrasses (e.g. *Stipa grandis* P. Smirn.), together accounting for more than 70% of the total community aboveground biomass (Bai et al., 2010).

2.2. Acid addition experiment

The acid addition experiment was started in 2009 near the N fertilization experiment at IMGERS. There were seven acid addition rates (0, 2.76, 5.52, 8.28, 11.04, 13.80, 16.56 mol H⁺ m^{−2}) in the form of a sulfuric acid solution, which was described in detail by Lan and Bai (2012). Soils were sampled in September 2010 at a depth of 0–15 cm. Four replicates were carried out for each treatment. Three soil cores were mixed for each sample, then sieved (2 mm diameter opening), and stored at −20 °C for nucleic acid analysis, or stored at 4 °C and measured for NH_4^+ -N and NO_3^- -N contents and potential nitrification rates within 7 d, or air dried for analysis of chemical properties.

2.3. N addition experiment

Soil used for the microcosm study was sampled from a fenced area that was undisturbed since 1979. This area is located near the N fertilization experimental sites within the same plant community. Three replicates were performed with 100 g soil incubated in a 500 mL flask. NH_4Cl or CaNO_3 were added to give a gradient of N concentrations of 0, 5, 10, 20, 40 and 80 mg N per kg^{−1} soil. Soils were incubated with 60% WPFS (water filled pore space) at 30 °C. NH_4^+ -N, NO_3^- -N and abundance of AOB and AOA were measured after 7, 14, and 28 d of incubation. The highest amounts of AOB and AOA were observed at 14 d for most samples, so the values at 7 d were subtracted from those at 14 d to calculate the growth rate of ammonia oxidizers and the NO_3^- accumulation rate. Community structures of AOB and AOA were also measured after 14 d of incubation.

2.4. Soil chemical properties and potential nitrification rate

Soil pH was measured with water extraction (soil: H₂O ratio = 1: 2). NH_4^+ and NO_3^- -N were extracted by 2 M KCl within 2 d after

sampling and determined with a Continuous Flow Analyser (SAN⁺⁺, Skalar, Holland). To measure potential nitrification rate, a chlorate inhibition method (Kurola et al., 2005) was modified by replacing the phosphate buffer solution with water to obtain a pH close to the original soil.

2.5. DNA extraction and real-time PCR

DNA was extracted from 0.5 g of fresh soil using the FastDNA spin kit for soil (MP Biomedicals, Cleveland, OH, USA) according to manufacturer's instructions.

Real-time PCR was conducted on an iCycler iQ 5 thermocycler (Bio-Rad). The archaeal *amoA* gene was amplified by primers Arch-amoAF/Arch-amoAR (Francis et al., 2005) and the bacterial *amoA* genes were quantified using primers amoA-1F and amoA-2R (Horz et al., 2004) with SYBR[®] Premix Ex Taq[™] (TaKaRa). A plasmid inserted with the target gene was used to form the standard curve for each gene. Copy number of plasmids was calculated from DNA concentrations measured by Nanodrop[®] ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). In order to address inhibition of PCR from soils, the extracted DNA samples were diluted 10 times to make templates and BSA (Bovine Serum Albumin) was added to PCR mixtures at a final concentration of 0.2 mg/ml. The 2 μ L DNA templates and 0.5 μ L primers (10 μ M) were included in each PCR reaction (25 μ L). Standard curves with template concentrations of 10^2 – 10^5 gene copies/ μ L for AOB and 10^3 – 10^7 gene copies/ μ L for AOA were used to calculate AOB and AOA abundance. All standard curves possessed $R^2 > 0.995$ and reaction efficiencies within $100\% \pm 5\%$.

2.6. Clone libraries and phylogenetic analysis

To construct the clone libraries, archaeal and bacterial *amoA* genes were amplified with the same primers for real-time PCR analysis. The fragments were ligated into pGEM[®]-T easy vector (Promega) and positive clones were selected randomly and sequenced. Nearly 40 clones were selected from each treatment and sequenced. AOB sequences were separated into different OTUs with 95% similarity by UPARSE (<http://drive5.com/uparse>) and one representative sequence of each OTU was used to construct the phylogenetic tree. Sequences were aligned by Clustal W and neighbor-joining phylogenetic trees were constructed (bootstrap, 500 replicates) using Kimura 2-parameter distance (complete deletion) with MEGA version 3.0 (Kumar et al., 2004).

2.7. T-RFLP assay

Primers for T-RFLP analysis of archaeal *amoA* gene were the same as used for real-time PCR except that the forward primer was labeled by 6-FAM. The amplified fragments were digested by *Mbo* I and purified by ethanol precipitation and dissolved in distilled water to be analyzed with ABI 3700 capillary DNA sequencer. Data were analyzed with GeneMapper V4.0. Terminal restriction fragments (T-RFs) under 50 bp were ignored and the remained T-RFs with relative fluorescence less than 1% were also neglected.

2.8. Statistical analysis

Statistical analyses were performed using SPSS Version 16.0 (SPSS Inc., Chicago, USA, 2007). Linear regression models were used with soil pH and N availability as continuous variables to explore how changes in soil pH and N forms affect the abundance and growth rate of AOA and AOB, potential nitrification rate, and nitrate accumulation rate. One-way ANOVA analysis (LSD test) was used to determine differences between treatments. Tests with

$P < 0.05$ were considered significant. Principal components analysis (PCA) was performed with the subroutine "prcomp" in the statistical program R i386 2.15.2 (<http://www.r-project.org>) to compare the community structure of ammonia oxidizers in different treatments and experiments.

3. Results

3.1. Relationship of AOB and AOA abundance with soil pH and N availability under different N enrichment and soil acidification treatments

In the long-term N fertilization plots, AOA and AOB abundance and PNR showed significant correlations to soil pH, NH_4^+ -N and NO_3^- -N (Fig. 1). In general, AOA and PNR increased with increasing soil pH (Fig. 1a, d) and decreased with increasing NH_4^+ -N (Fig. 1b, e) and NO_3^- -N (Fig. 1c, f), while AOB abundance (Fig. 1a, b, c) showed trends opposite to those of AOA.

Acid addition treatments led to a soil pH range of 7.3–5.0, which is close to the range produced by N fertilization (Table 1). NH_4^+ -N increased while NO_3^- -N changed little with the decrease of soil pH. AOB abundance decreased with reduction in soil pH (Fig. 2a). AOA abundance increased with decreasing soil pH in most treatments, but it decreased significantly at the highest acid addition rate (pH = 5.0) (Fig. 2b). PNR correlated positively with soil pH as also happened in the long-term N fertilization experiment (Fig. 2c).

In the short-term NH_4^+ addition treatments, with increased NH_4^+ added, AOB growth rate initially increased with addition of 0–40 mg N kg^{-1} soil ($r^2 = 0.95$, $P = 0.0046$ for linear regression), and then decreased at the highest addition rate of 80 mg N kg^{-1} soil (Fig. 3a). Also, AOB growth rate at the addition rate of 80 mg N kg^{-1} soil was significantly lower than that at the addition rate of 40 mg N kg^{-1} soil. AOA growth rate generally decreased with the rate of NH_4^+ addition (Fig. 3b), but only a nearly significant relationship was observed between them ($r^2 = 0.61$, $P = 0.0655$). Nitrate accumulation rate under NH_4^+ addition (Fig. 3c) showed a similar trend to AOB growth (Fig. 3a) and correlated significantly with AOB growth rate ($r^2 = 0.96$, $P = 0.0080$). No relationship between NO_3^- addition rate and AOB growth rate was found (Fig. 3a). NO_3^- addition rate showed significantly negative effect on AOA growth (Fig. 3e) and positive effect on NO_3^- accumulation (Fig. 3f).

In order to better analyze the relationship between AOA growth and the real NO_3^- concentration to explore a potential product inhibition mechanism, and also to learn whether NO_3^- -N is responsible for the negative effect of NH_4^+ addition on AOA, we conducted regression analysis between AOA growth rate and the average NO_3^- -N concentration in a single experiment or using data combined from the NH_4^+ addition and NO_3^- addition treatments. A significant negative linear relationship between AOA growth rate (log-transformed) and NO_3^- -N concentration (log-transformed) was observed, based on data from the NO_3^- addition experiment ($r^2 = 0.84$, $P < 0.0001$), NH_4^+ addition experiment ($r^2 = 0.36$, $P = 0.0089$), and pooled of both experiments ($r^2 = 0.63$, $P < 0.0001$) (Fig. 4). In contrast, negative relationship between AOA (log-transformed) and NH_4^+ -N (log-transformed) was only observed in the NH_4^+ addition experiment ($r^2 = 0.40$, $P = 0.0049$).

3.2. Community structure of AOB and AOA with N enrichment and soil acidification treatments

AOA and AOB community structure in the control and the highest N treatments for the N fertilization plots have previously been studied using clone libraries and sequencing (Li et al., 2011). In this study, AOB and AOA community structure in other treatments of all the three experiments were supplemented except that the

Fig. 1. Regression analysis of AOA, AOB abundance (a, b, c) and potential nitrification rate (d, e, f) against soil pH, NH₄⁺ and NO₃⁻ under long-term N fertilization treatments. Bars indicate standard errors.

AOB community structures in the NO₃⁻ addition treatments were not measured because no significant effect of NO₃⁻ addition on AOB abundance was observed.

Due to the high similarities of AOB sequences in most soils, sequencing of clone libraries was used to investigate the AOB community structure in treatments with large variations of AOB abundance including the N fertilization, acidification, and NH₄⁺

treatments. Phylogenetic analysis showed that most sequences were affiliated with genus *Nitrosospora* (Supplementary Fig. S1). Sequences were separated into operational taxonomic units (OTUs) at a similarity level of 95%. Some phylogenetic lineages were named according to the classification by Avrahami and Conrad (2003) and Ying et al. (2010). PCA analysis based on proportions of different OTUs showed clustering of AOB community related to different

Table 1
Soil chemical properties with acid addition treatments.

Acid addition rate (mol H m ⁻²)	pH	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)
0	7.3 ± 0.1	5.33 ± 1.98	3.43 ± 0.93
2.76	6.3 ± 0.4	6.95 ± 0.98	2.38 ± 0.69
5.52	6.5 ± 0.4	7.87 ± 2.19	3.52 ± 1.77
8.28	5.8 ± 0.2	10.21 ± 1.58	2.58 ± 0.95
11.04	5.4 ± 0.4	10.95 ± 3.97	2.47 ± 1.96
13.80	5.3 ± 0.7	22.76 ± 10.89	1.67 ± 0.52
16.56	5.0 ± 0.7	18.85 ± 7.09	2.42 ± 1.46

Fig. 2. Effect of soil pH on abundance of AOB (a) and AOA (b) and potential nitrification rate (c) based on acid addition treatments. Bars indicate standard errors.

experiments (Fig. 5). Different treatments of the N fertilization experiment were distributed along PC2 in order of N fertilization

rate mainly due to the increase of OTU20 and decrease of OTU24 with increase of N fertilization rate. PC2 correlated significantly with soil pH ($r = 0.967$, $P = 0.002$), NH₄⁺ ($r = -0.862$, $P = 0.027$) and NO₃⁻ ($r = -0.847$, $P = 0.033$) in the N fertilization experiment. Significant correlation was also found between PC1 and soil pH in the N fertilization experiment ($r = -0.846$, $P = 0.034$). In contrast, no regular distribution of OTUs with acid addition rate or NH₄⁺ addition rate was found.

T-RFLP analysis and clone libraries were combined to investigate community structure of AOA in different treatments. PCA analysis, based on the relative proportions of different T-RFs, showed that AOA community structure clustered mainly by different experiments (Fig. 6). AOA communities in the N fertilization plots, the acidification plots, and the microcosm experiments are characterized by a higher proportion of T-RF 330, T-RF 445 and T-RF 420 respectively. Distances between treatments from the N fertilization experiment are greater than those from each other experiment. No significant correlations of PC1 and PC2 with soil pH or N availability were found in different experiments with $p < 0.05$ except the negative correlations of PC2 with NH₄⁺-N ($r = -0.855$, $p = 0.030$) and NO₃⁻-N ($r = -0.817$, $p = 0.047$) for the N fertilization experiment. Phylogenetic analysis of archaeal *amoA* sequences from some treatments including N0, N28, A0, A11.04 and A16.56 treatments showed that all sequences belong to the 1.1b group which is mainly composed of sequences from terrestrial environments (Supplementary Fig. S2). The calculated sizes of T-RF of major lineages are congruent with the T-RFLP results.

4. Discussion

The acid addition experiment showed that soil pH reduction have generally positive effect on AOA (within a pH range of 5.3–7.3) and negative effect on AOB (within a pH range of 5.0–7.3) as previously reported (Nicol et al., 2008). However, a negative effect of soil pH reduction on AOA was also observed at the highest acid addition rate with soil pH near 5.0, indicating a detrimental effect of extremely low soil pH on AOA, which has not been reported before. The relationships between abundance of ammonia oxidizers and soil pH in the acid addition plots conflict with those in the N fertilization plots. NH₃ has been considered to be the effective N form by which soil pH affects AOB and AOA (Lehtovirta-Morley et al., 2011; Hatzenpichler, 2012). But the calculated NH₃ concentration in the long-term N fertilization experiment did not show a positive correlation with AOB or a negative correlation with AOA (data not shown). Therefore, variations in AOB and AOA under N fertilization cannot be explained by NH₃ content. The results from this study indicate that soil pH is not likely to be the key factor determining numbers of ammonia oxidizers under long-term N fertilization.

Based on the microcosm experiment, we found a generally positive effect of NH₄⁺ on AOB as expected. Compared with the positive responses of AOA, but not AOB, to low ammonium addition in a Scotland soil (Verhamme et al., 2011), the short-term N

Fig. 3. Effects of NH_4^+ (a, b, c) and NO_3^- additions (d, e, f) on AOB growth, AOA growth and NO_3^- accumulation based on microcosm incubations. Bars indicate standard errors.

additions in this study revealed positive responses of AOB but not AOA to NH_4^+ increase even at low addition rates. This indicates an important role of AOB but not AOA in ammonia oxidation in these soils with broad N availability, which was further supported by the positive relationship between AOB and NO_3^- accumulation. However, a negative effect of excessive NH_4^+ was observed at the highest N addition rate (e.g. 80 mg N kg⁻¹ soil). The negative effect of high NH_4^+ concentration on AOB was also observed by Norman and Barrett (2014) and was considered to be affected by soil pH. Yet, according to our results, NH_4^+ concentration rather than soil pH played a dominant role in controlling responses of AOB abundance to long-term N fertilization even when soil pH decreased greatly. Thus, it is likely that the high NH_4^+ concentration alone has a

negative effect on AOB.

A strong negative relationship between AOA growth and NO_3^- rather than NH_4^+ was observed in the microcosm experiment, indicating that NO_3^- is the effective N form that had negative effect on AOA. Although NH_4^+ addition showed a negative effect on AOA to some extent, it is likely due to the effect of NO_3^- -N according to the negative relationship between AOA growth and NO_3^- -N. The negative effect of N enrichment on AOA has been frequently reported (Di et al., 2009, 2010; Verhamme et al., 2011), yet the effective N form remains unclear and has rarely been attributed to nitrate. NO_3^- is a major inorganic N form in soils and acts as the end product of nitrification. Thus, the negative effect of NO_3^- on AOA found in this study is probably widespread based on a product inhibition

Fig. 4. Regression analysis of AOA growth rate and NO_3^- concentration based on combined data from the NH_4^+ and the NO_3^- addition treatments.

mechanism. It was unexpected that NO_3^- concentrations had a positive effect on NO_3^- accumulation rate. This is unlikely to be caused by ammonia oxidization, which is supported by the non-significant or negative responses of AOB and AOA growth rates to N addition. The measured NO_3^- concentrations were higher at 14 d than 7 d in all treatments and were lower than the concentrations added at all incubation times. Thus, it is probable that NO_3^- accumulation rate in the nitrate treatments is mainly determined by N uptake during the early stage of incubation and N mineralization at, or near, 14 d after incubation by soil organisms such as heterotrophic microbes.

The positive effect of NH_4^+ on AOB and the negative effect of NO_3^- on AOA are congruent with the relationships between ammonia oxidizers and N availability under long-term N fertilization, supporting their dominant roles in determining responses of AOB and

AOA abundance to long-term N fertilization. In contrast, the response of potential nitrification rate under long-term N fertilization can be well explained by the effect of soil pH rather than NH_4^+ or NO_3^- .

Although soil acidification and N enrichment had significant effects on AOA abundance in our experimental soils, they showed little effect on AOA community structure which was dominated by the 1.1b soil group in all treatments. Nicol et al. (2008) reported the positive responses of AOA to decreases in soil pH accompanied by increases in the proportion of the 1.1a associated group. Responses of AOA abundance and community structure to acid addition in this study revealed positive responses of the 1.1b group to soil acidification without the presence of the 1.1a associated group even at the lowest soil pH near 5. These results clearly suggest that low soil pH has a general negative effect on diverse AOA groups including both the 1.1a associated and the 1.1b groups. A negative effect of soil acidification on AOA was also observed at the highest acid addition rate (soil pH near 5) in this study, suggesting that the 1.1b group is not adapted to extremely acidic soils. Therefore, domination of the 1.1a/1.1a associated groups in extremely acidic soils (pH < 5) as previously reported (Gubry-Rangin et al., 2011) is likely associated with reduced competition from the 1.1b group. The AOA dominated nitrification in soils has been ascribed to the 1.1a/1.1a associated group in most previous studies. However, ammonia oxidization by the 1.1b group has recently been found in some acid soils (Wang et al., 2014; Jiang et al., 2015). The niche separations of the 1.1a/1.1a associated groups and the 1.1b group as well as their function in various ecosystems need further studies in the future.

In contrast to the great variations in AOB community structure with long-term N fertilization, AOB community structure changed little by acid or short-term N addition, indicating that responses of AOB community structure to long-term N fertilization cannot be simply explained by soil pH or N availability. This does not mean that soil pH and N availability had no effect on AOB community structure under long-term N fertilization. For example, the OTU20, which tended to increase with N fertilization rates, are affiliated with cluster 2 that were mainly found at low soil pH (Stephen et al.,

Fig. 5. PCA analysis of bacterial *amoA* sequences based on proportions of different OTUs. Distributions of different treatments are shown in black graphs with numbers indicating the N fertilization rate ($\text{g m}^{-2} \text{yr}^{-1}$), NH_4^+ addition rate ($\text{mg N kg}^{-1} \text{soil}$) or soil pH. Loadings of variables are shown in grey graphs, and some variables that varied greatly between different experiments or treatments are indicated with arrows and their OTU numbers.

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