Soil Biology & Biochemistry 107 (2017) 218–225

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

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

Ammonia-oxidizing bacteria rather than archaea respond to short-term urea amendment in an alpine grassland

Xingjia Xiang [a, d], Dan He [a], Jin-Sheng He [b], David D. Myrold [c], Haiyan Chu [a, *]

[a] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, East Beijing Road 71, Nanjing 210008, China
[b] Department of Ecology, College of Urban and Environmental Sciences, and Key Laboratory for Earth Surface Processes of the Ministry of Education, Peking University, Beijing 100871, China
[c] The Department of Crop and Soil Science, Oregon State University, Corvallis OR 97331, Oregon, USA
[d] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 5 May 2016
Received in revised form
29 December 2016
Accepted 17 January 2017

Keywords:
Grassland
Fertilization
Ammonia-oxidizers
Pyrosequencing

ABSTRACT

Chemical fertilizers, especially nitrogen (N) and phosphorus (P), are used in grasslands to maximize plant biomass production for livestock. Despite a substantial body of work on how fertilization affects aboveground plant and belowground microbial communities, the short-term response of soil ammonia-oxidizing microbial communities, which play the central role in nitrification, to fertilization is not well understood. The responses of ammonia-oxidizing microbial communities to short-term (3 years) N and/or P additions were investigated in an alpine grassland of the Qinghai-Tibetan Plateau. Quantitative polymerase chain reaction (qPCR) analysis of the *amoA* genes showed that ammonia-oxidizing archaea (AOA) dominated over ammonia-oxidizing bacteria (AOB) in non-amended soil. Short-term urea addition significantly increased the abundance of AOB, whereas AOA abundance remained unchanged, resulting in a shift from AOA to AOB dominance, which suggests that AOB are likely to be more important in the first step of the nitrification following urea amendment. Pyrosequencing demonstrated a significant shift in AOB but not AOA community composition within short-term N fertilizer plots, indicating that AOB community composition was more sensitive than AOA in response to urea amendment. This study demonstrated that the abundance and composition of AOA and AOB communities responded differently to 3 years of urea addition, suggesting that N fertilizer may be an important controller of ammonia-oxidizing microbial communities in managed alpine grasslands, such as those of the Qinghai-Tibet Plateau.

1. Introduction

The Qinghai-Tibetan Plateau is the youngest (~2.4 × 10^8 years), largest (~2.27 × 10^6 km^2), and highest (~4000 m on average) plateau in the world. Most (85%) of the plateau is covered by grassland, accounting for 30% of all the grassland in China (Wen et al., 2013). Due to long-term livestock overgrazing and climate change, the entire grassland is facing extensive degradation, which has created serious social, environmental, and economic issues (Wen et al., 2013). Shang and Long (2007) reported that almost 30% of the alpine grassland of the Qinghai-Tibet Plateau was severely degraded, resulting in lower grass productivity, which reduces livestock production, leads to desertification, and triggers dust storms (Akiyama and Kawamura, 2007).

Chemical fertilizers are used in grasslands to maximize aboveground production for livestock (Beauchamp et al., 1989; Reijs et al., 2005; Hacker et al., 2011), as nitrogen (N) and/or phosphorus (P) are commonly the limiting nutrients in terrestrial ecosystems (Vitousek et al., 2010; Harpole et al., 2011). In addition to increasing plant biomass, N and P additions can significantly affect plant species diversity (Elser et al., 2007; Clark and Tilman, 2008; Bai et al., 2010) and influence soil carbon (C) fractions (Li et al., 2014) in grassland ecosystems. Furthermore, soil microbial biomass decreased significantly in response to N fertilization in grasslands of England (Lovell et al., 1995) and America (Allison et al., 2013). Previous studies have demonstrated that fertilization significantly altered soil bacterial (Fierer et al., 2012; Leff et al., 2015; Zeng et al.,

* Corresponding author.
E-mail address: hychu@issas.ac.cn (H. Chu).

http://dx.doi.org/10.1016/j.soilbio.2017.01.012
0038-0717/

2016) and fungal (Antoninka et al., 2011; Liu et al., 2012; Klabi et al., 2015; He et al., 2016; Xiang et al., 2016) community composition in grasslands. Fertilization may influence the community structure of soil ammonia-oxidizers, which play an important role in nitrification and maintaining grassland sustainability (van der Heijden et al., 2008). The impact of fertilization on plant and microbial communities has been studied extensively; however, the effects of short-term fertilization on ammonia-oxidizing microbial communities remain largely unexamined in alpine grasslands.

Ammonia-oxidizers convert ammonia to nitrite, which is the first and rate limiting step in nitrification (Pester et al., 2012). Previous studies have shown that ammonia-oxidizing bacteria (AOB) can be more abundant in high ammonia soils, whereas ammonia-oxidizing archaea (AOA) dominate under low substrate conditions (Jia and Conrad, 2009; Lu et al., 2012; Hatzenpichler et al., 2008). Nitrogen fertilization may change ammonia availability (Wu et al., 2011) and thereby affect ammonia-oxidizing microbial communities and regulate nitrification in soil (van der Heijden et al., 2008). A recent study showed that AOB community composition was sensitive to inorganic N addition while AOA community composition was significantly altered by organic N amendment following long-term (44 years) fertilization of a temperate grassland (Zhou et al., 2015). In this study, we designed a 3-year fertilization experiment to evaluate the response of ammonia-oxidizing microbial communities to fertilization in an alpine grassland of the Qinghai-Tibet Plateau. We aimed to know how short-term (i.e., 3 years) N and/or P amendment affects soil ammonia-oxidizing microbial communities in an alpine grassland.

2. Materials and methods

2.1. Site selection and soil sampling

The study area is at the Haibei Alpine Grassland Ecosystem Experimental Station (37°29′-37°45′N, 101°12′-101°23′E; 3220 m), which is located in the northeast of the Qinghai-Tibetan Plateau. The area has a long and cold winter because of its continental climate. Based on weather records of the Haibei Alpine Grassland Ecosystem Experimental Station (http://www.nwipb.ac.cn/gystxyjzx/hbghcdstxtyjz/hbzjj/200906/t20090623_1771380.html), the average annual temperature is −1.7 °C, with an extreme maximum temperature of 27.6 °C and extreme minimum temperature of −37.1 °C.

A short-term (i.e., 3 years) fertilization experiment was initiated in 2011 and soils were collect on 12 August 2014. The experimental plots were constructed in randomized block design, which had four treatments with six replications of each: control (without fertilization); N (urea, 100 kg N $ha^{-1}yr^{-1}$); P (superphosphate, 50 kg P $ha^{-1}yr^{-1}$); and N + P (urea, 100 kg N $ha^{-1}yr^{-1}$; superphosphate, 50 kg P $ha^{-1}yr^{-1}$). The size of each plot was 36 m^2 (6 m × 6 m). Fertilizers were applied in three split applications at the beginning of June, July, and August, which is the main plant growing season. In each plot, soils at a depth of 0–10 cm were collected from four corners of a 4 m × 4 m square located 1 m from the plot edge, and then mixed together as one sample. After sampling, the soils were kept in a cooler and shipped refrigerated to the lab within 48 h. The samples were thoroughly mixed within each bag, sieved (2 mm) to remove roots and stones, and then divided into two parts: one part was stored at 4 °C for biogeochemical analysis within 2 weeks; the other was stored at −40 °C for DNA analysis.

2.2. Vegetation and soil analyses

Aboveground net primary productivity was measured in four, 0.5 × 0.5 m areas at the corners of each soil sampling square. Plant species were identified visually and aboveground plant portions were collected by clipping. Plant roots from three replicate soil cores collected using a 7-cm diameter soil auger were used to estimate belowground root biomass. Dry biomass for aboveground plant tissues and belowground roots (after washing) was calculated after drying for 48 h at 65 °C. Soil pH was measured in a soil water suspension (1:5 wt/vol), and soil moisture (SM) was measured gravimetrically. Standard methods were used for measuring soil available P (AP, Stahlberg, 1980), total P (TP, Bowman, 1988), total C (TC), and total N (TN) (Walkley and Black, 1934). Soil dissolved organic C (DOC), total dissolved N (TDN), and mineral N were measured after extraction by adding 50 ml of 0.5 M K_2SO_4 to 10 g fresh soil. Ammonium (NH_4^+) and nitrate (NO_3^-) contents in the extracts were determined colorimetrically by automated segmented flow analysis (Bran + Luebbe AAIII, Germany). A TOC-TN analyzer (Shimadzu, Kyoto, Japan) was used to measure DOC and TDN. Dissolved organic N (DON) was calculated as follows: $DON = TDN - NH_4^+ - NO_3^-$.

2.3. Soil DNA extraction and purification

DNA extractions were carried out on 0.5 g fresh soil according to the manufacturer's instructions (FastDNA® SPIN Kit for soil, MP Biomedicals, Santa Ana, CA). DNA was purified using a PowerClean® DNA Clean-Up Kit (MO BIO Laboratories, Carlsbad, CA, USA) to remove PCR inhibitors. The extracted DNA was dissolved in 50 μl of elution buffer, quantified by NanoDrop ND-1000 (Thermo Scientific, USA), and stored at −20 °C.

2.4. Quantitative real-time PCR for amoA genes

Primer sets amoA-1F/amoA-2R (Rotthauwe et al., 1997) for AOB and Arch-amo AF/Arch-amo AR (Francis et al., 2005) for AOA were used to determine the abundances of bacterial *amoA* and archaeal *amoA* by quantitative real-time PCR (qPCR) using a CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The 25-μl qPCR reaction mixture contained 12.5 μl SYBR®*Premix Ex Taq* (TliRNaseH Plus, 2 ×, Takara Bio, Japan), 0.5 μl PCR forward and reverse primer (both 10 μM), 2 μl DNA template, and 9.5 μl double distilled water (ddH2O). Quantitative real-time PCR parameters were: hold at 95 °C for 10 min, then 40 cycles with 95 °C for 15 s, and 60 °C for 1 min for both AOB and AOA. Plasmid DNA containing fragments of bacterial and archaeal *amoA* genes were used as standards. The potential for PCR inhibition in the extracts was tested by qPCR of dilutions; no inhibition was found. The qPCR showed excellent specificity, which was determined by melting curve analysis and agarose gel electrophoresis (Ririe et al., 1997). The efficiency of qPCR was 98.2% ($r^2 = 0.9999$) for AOA and 96.1% ($r^2 = 0.9881$) for AOB.

2.5. Pyrosequencing of amoA genes for ammonia-oxidizers

Primer sets amoA-1F/amoA-2R (Rotthauwe et al., 1997) for AOB and Arch-amo AF/Arch-amo AR (Francis et al., 2005) for AOA were used to amplify the *amoA* gene fragment for sequencing with the 454 GS-FLX pyrosequencing platform. PCR was carried out in 50-μl reaction mixtures containing each deoxynucleoside triphosphate at a concentration of 1.25 mM, 1 μl of forward and reverse primers, and 2 U of Taq DNA polymerase (TaKaRa, Japan). Each reaction mix received 2 μl of genomic DNA (50 ng) as a template. PCR conditions were: preheating at 94 °C for 5 min, then 30 cycles (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min for AOB; 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min for AOA), with a final extension at 72 °C for 7 min. Triplicate reaction mixtures per sample were pooled together, purified with the Agarose Gel DNA purification kit

(TaKaRa), and quantified using NanoDrop ND-1000 (Thermo Scientific, USA). The bar-coded PCR products from all samples were normalized in equimolar amounts before pyrosequencing. Data were processed by the QIIME pipeline (Caporaso et al., 2010). After denoising (Reeder and Knight, 2010), the poor-quality (below an average quality score of 25, homopolymer errors) and short (<200 bp) sequences were removed and those sequences which exactly matched with their barcode and primer were kept. The barcode and primer sequences were then removed. High quality sequences were assigned to operational taxonomic units (OTUs) using uclust (Edgar, 2010) at 88% similarity for AOB (Norton et al., 2002) and 85% similarity for AOA (Pester et al., 2012), because pure culture results showed that 88% *amoA* identity of AOB and 85% *amoA* identity of AOA have about 97% identity of their 16S rRNA genes. All singleton OTUs were deleted and chimeras were filtered to remove erroneous OTUs due to sequence errors using the USEARCH tool (version 1.8.0) in QIIME. The most abundant sequence within each OTU was selected as the representative sequence for that OTU. The representative sequences were checked against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and any non-*amoA* gene sequences (no hit in the database) were deleted. A maximum likelihood tree (MEGA 6; Tamura et al., 2013) was built for phylogenetic assignment by aligning dominant OTU sequences (relative abundance >1%) with known reference sequences. The reference sequences were downloaded from NCBI by their accession number (Avrahami and Conrad, 2003, Avrahami et al., 2003 for AOB and Pester et al., 2012; Stempfhuber et al., 2014 for AOA). To have similar sequencing effort and homogenize among samples, we used a randomly selected subset of 6000 sequences per sample for both AOB and AOA to compare relative differences among samples. Sequences (raw data) were submitted to the Sequence Read Archive (SRA) of NCBI under the accession number SRP071032 for AOB and SRP070789 for AOA.

2.6. Statistical analysis

The differences of ammonia-oxidizing microbial *amoA* gene abundance, alpha-diversity, and relative abundances of AOB clusters or AOA subclusters among treatments were based on ANOVA with Tukey Honestly Significant Difference (HSD) Post-Hoc testing (SPSS 20.0 for Windows). Non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity of AOA or AOB OTUs, Analysis of Similarity (ANOSIM; permutations = 999), and Mantel tests (permutations = 999) were done with the *vegan* package (Version 2.0–2) of R v.2.8.1 project (R Development Core Team, 2006). The data followed a normal distribution except for available phosphorus (AP) and total phosphorus (TP). Pearson correlations were used for ammonia-oxidizing microbial data (*amoA* gene copy number and alpha-diversity) and biogeochemical factors with normal distribution, and Spearman correlations were used for ammonia-oxidizing microbial data (*amoA* gene copy number and alpha-diversity) and biogeochemical factors with non-normal distribution (SPSS 20.0 for Windows).

3. Results

3.1. Soil chemistry and vegetation

Compared to the control, soil NO_3^- and DON content significantly increased in N and N + P additions, and P and N + P addition significantly increased TP and AP content; there was no significant difference in other soil variables by N and/or P addition (Table 1). Combined N + P addition significantly increased graminoid, forb, and total aboveground biomass (TB), and significantly decreased sedge and legume biomass; root biomass was not significantly affected by fertilization (Table 1). Addition of N slightly increased graminoid biomass and decreased legume biomass; P addition slightly increased graminoid and legume biomass, and decreased sedge biomass (Table 1). Graminoid and sedge biomass were significantly correlated with AP ($r = 0.51$; $r = -0.62$, respectively; Table S1). Legume biomass showed significant negative correlation with NO_3^- ($r = -0.59$) and dissolved organic N (DON) ($r = -0.56$; Table S1). Forb biomass showed significant positive correlation with soil NO_3^- ($r = 0.72$) and DON ($r = 0.57$; Table S1). Total biomass showed significant positive correlation with soil NO_3^- ($r = 0.63$), DON ($r = 0.57$), and AP ($r = 0.54$; Table S1). In addition, N + P fertilization significantly reduced the number of plant species (Fig. S1), which showed significant negative correlation with soil NO_3^- content ($r = -0.68$; Table S1).

3.2. Soil ammonia-oxidizing microbial abundance and community composition

Soil AOA were more abundant than AOB in control and P addition soils, whereas N and N + P additions significantly increased AOB *amoA* gene copies by over 10-fold (Fig. 1). Fertilization did not significantly affect soil AOA *amoA* gene copies (Fig. 1). The AOB/AOA *amoA* gene ratio was 0.22 for control and 0.29 for P addition and significantly increased in N (3.64) and N + P (6.31) addition soils. Copies of AOB *amoA* genes showed significant positive correlation with NO_3^- ($r = 0.76$) and DON ($r = 0.65$), whereas AOA *amoA* gene copies positively correlated with pH ($r = 0.61$; Fig. S2).

Across all soil samples, we obtained a total of 356,962 high quality sequences (2407-23,439 sequences per sample) for AOB and 577,221 (4126-32,313 sequences per sample) for AOA. Sequences were rarified 6000 per sample, and those samples with less than 6000 sequences were excluded in further analyses, which resulted in the loss of two AOB and one AOA samples from the total of 24. The maximum likelihood tree showed that the dominant OTU sequences grouped into *Nitrosospira* clusters 1, 2, 3a, 3b, and 4 for AOB, and *Nitrososphaera* subclusters 1, 2, 4, 6, and 8 for AOA (Fig. S3). Compared to the control, addition of N significantly increased the relative abundance of AOB *Nitrosospira* cluster 3a; addition of N + P significantly increased AOB *Nitrosospira* cluster 1; both N and N + P additions significantly decreased the relative abundance of AOB *Nitrosospira* cluster 2 (Fig. 2). In contrast, the relative abundances of AOA subclusters were unaffected by fertilization (Fig. 2). Based on ANOSIM and NMDS, both N and N + P treatments resulted in significant shift of soil AOB community composition from the control, and the effect of N ($R = 0.637$) addition might be larger than N + P ($R = 0.293$) addition (Fig. 3a; Table 2); P addition did not have a significant effect on AOB community composition (Fig. 3a; Table 2). Fertilization did not significantly change AOA community composition (Fig. 3b; Table 2). Soil AOB and AOA community compositions were related to different biogeochemical variables (Table S2): AOB community composition was significantly positively correlated with soil NO_3^- ($r = 0.80$), forb biomass ($r = 0.46$), and TB ($r = 0.37$), whereas AOA community composition was significantly positively correlated with pH ($r = 0.42$), TN ($r = 0.25$), and TC ($r = 0.22$).

3.3. Soil ammonia-oxidizing microbial alpha-diversity

Alpha-diversity (Shannon index, evenness, OTU richness, and phylogenetic diversity) was surveyed at a depth of 6000 randomly selected sequences per sample for both AOB and AOA. The Shannon index and evenness of AOB significantly increased in N and N + P treatments, but fertilization did not have a significant effect on AOB OTU richness and phylogenetic diversity (Fig. 4a), or AOA alpha-diversity measures (Fig. 4b). Soil AOB Shannon index and

Table 1
Summary of the main soil and plant variables of sampling sites at Haibei Alpine Grassland Ecosystem Experimental Station, Qinghai-Tibetan Plateau. The values in brackets represent the standard deviation of the mean. Letters behind brackets represent significant differences from Tukey's HSD comparisons ($P < 0.05$; n = 6).

Variables	Control	N	P	NP
Soil pH	7.32 (0.50)^{a}	7.18 (0.63)^{a}	7.67 (0.36)^{a}	7.01 (0.62)^{a}
Soil moisture (%)	26.5 (4.2)^{a}	22.0 (4.8)^{a}	23.3 (2.5)^{a}	22.8 (3.3)^{a}
NO_3^- (mg/kg)	19.6 (0.6)^{b}	26.3 (1.7)^{a}	18.9 (2.8)^{b}	27.3 (5.5)^{a}
NH_4^+ (mg/kg)	9.16 (2.11)^{a}	9.71 (1.42)^{a}	12.22 (4.28)^{a}	10.00 (5.17)^{a}
Total nitrogen (%)	0.65 (0.03)^{a}	0.61 (0.05)^{a}	0.63 (0.05)^{a}	0.64 (0.08)^{a}
Total carbon (%)	6.49 (0.34)^{a}	6.07 (0.55)^{a}	6.28 (0.74)^{a}	6.41 (1.02)^{a}
Total phosphorus (%)	0.79 (0.01)^{b}	0.82 (0.07)^{b}	0.94 (0.08)^{a}	0.90 (0.01)^{ab}
Dissolved organic N (mg/kg)	31.3 (4.1)^{b}	41.7 (5.1)^{a}	32.8 (7.4)^{b}	46.3 (10.1)^{a}
Dissolved organic C (mg/kg)	178.7 (18.6)^{a}	195.8 (37.3)^{a}	170.7 (14.4)^{a}	180.9 (29.9)^{a}
Available phosphorus (mg/kg)	6.20 (1.54)^{b}	4.86 (2.00)^{b}	36.3 (12.7)^{a}	46.2 (4.16)^{a}
Root biomass (kg/m^2)	2.06 (0.46)^{a}	2.06 (0.57)^{a}	2.25 (0.51)^{a}	2.33 (0.46)^{a}
Graminoid biomass (g/m^2)	233 (17)^{b}	265 (77)^{ab}	272 (67)^{ab}	414 (130)^{a}
Sedge biomass (g/m^2)	10.9 (5.0)^{a}	27.6 (15.4)^{a}	7.2 (4.5)^{ab}	1.7 (0.6)^{b}
Legume biomass (g/m^2)	21.2 (6.9)^{ab}	12.0 (4.3)^{b}	28.1 (9.1)^{a}	4.4 (3.6)^{c}
Forb biomass (g/m^2)	57 (17)^{b}	106 (21)^{b}	102 (61)^{b}	247 (98)^{a}
Total aboveground biomass (g/m^2)	322 (21)^{b}	411 (81)^{b}	409 (94)^{b}	667 (57)^{a}

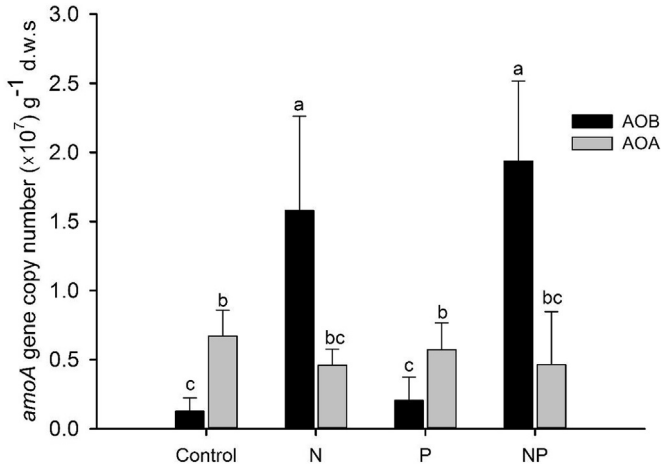

Fig. 1. Population sizes of ammonia-oxidizers estimated from copy numbers of *amoA* genes at Haibei Alpine Grassland Ecosystem Experimental Station, Qinghai-Tibetan Plateau. Bars represent mean; error bars denote standard deviation; letters above bars represent significant differences from Tukey's HSD comparisons ($P < 0.05$; n = 6 for both AOA and AOB).

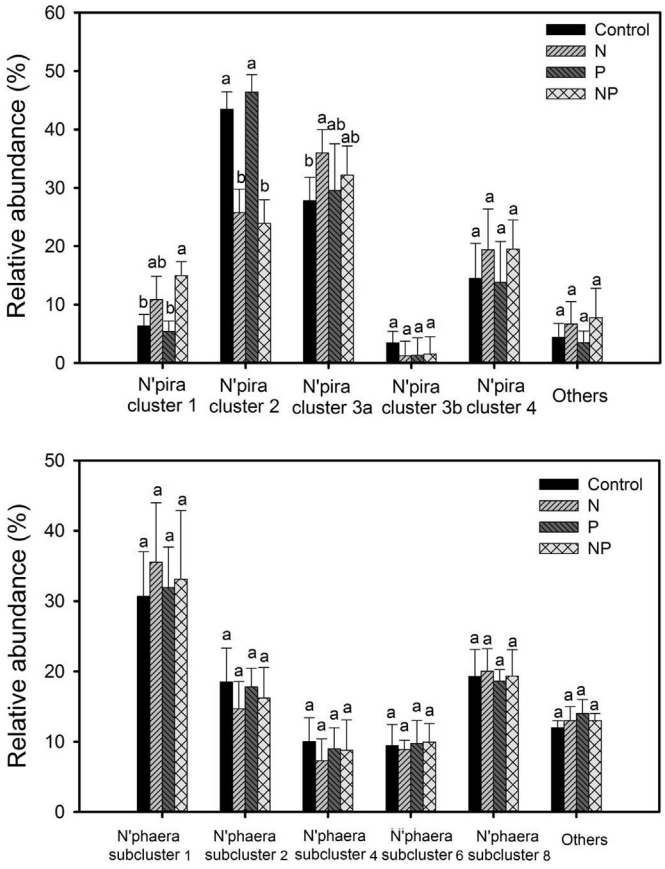

Fig. 2. The relative abundances of the dominant AOB clusters and AOA subclusters at the Haibei Alpine Grassland Ecosystem Experimental Station. Bars represent mean; error bars denote standard deviation; letters above bars represent significant differences from Tukey's HSD comparisons ($P < 0.05$; n = 5 for AOB Control and P treatments, and AOA P treatment; n = 6 for other AOB and AOA treatments). N'pira: *Nitrosospira*; N'phaera: *Nitrososphaera*.

evenness were positively correlated with soil NO_3^- ($r = 0.62$; $r = 0.57$, respectively) and DON ($r = 0.47$; $r = 0.45$, respectively). In contrast, the alpha-diversity of AOA did not correlate with any measured biogeochemical properties (Table S3).

4. Discussion

As in prior grassland studies, fertilization with N and P produced significant increases in available N and P, and resulted in higher total aboveground plant biomass for livestock (Ren et al., 2010; Li et al., 2014), indicating that N and P jointly limit plant productivity in this alpine grassland. However, increased forb biomass (Heil and Bruggink, 1987; Herron et al., 2001; Zhang et al., 2011) from N + P addition was associated with decreased numbers of plant species (Fridley, 2002; Clark and Tilman, 2008; Miller and Seastedt, 2009; Bai et al., 2010), suggesting that fertilization causes extinction of some rare species, which may be detrimental for sustainable animal husbandry and ecosystem biodiversity. Shifts in forb biomass and plant species numbers were highly correlated with soil NO_3^- content, which significantly increased after urea amendment, suggesting that nitrification following fertilization stimulates the expansion of nitrophilous (nitrate-loving) species and the competitive exclusion of others (Bobbink et al., 2010).

Aboveground community composition and diversity following fertilization have been studied extensively, but effects of short-term fertilization on soil ammonia-oxidizing microbial communities remain largely unexamined in alpine grassland ecosystems. As often found for non-fertilized soils, AOA were more numerous

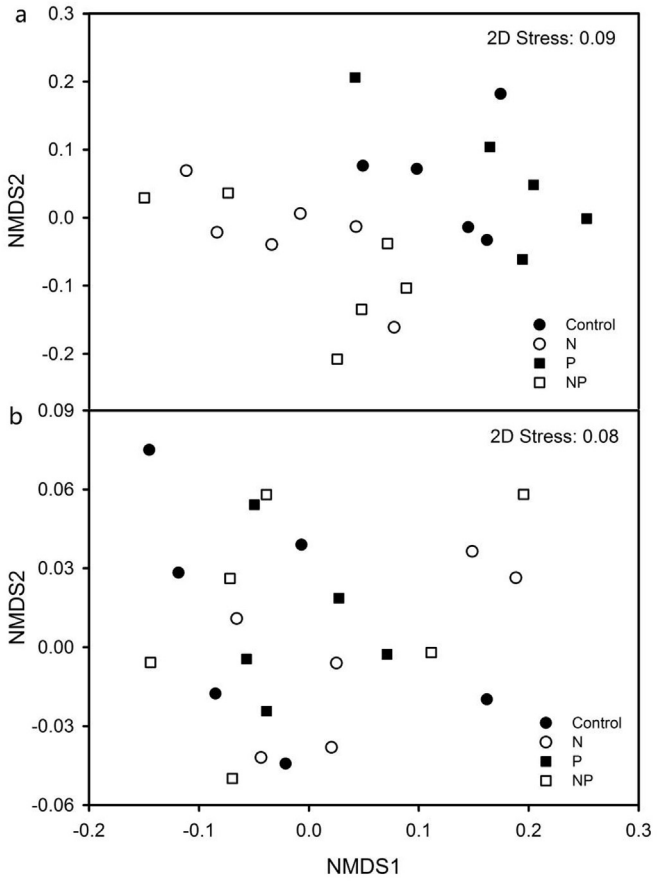

Fig. 3. Non-metric multidimensional scaling plot of (a) ammonia-oxidizing bacterial (AOB; n = 22) and (b) archaeal (AOA; n = 23) community composition in an alpine grassland across different nutrition additions.

Table 2
Differences of ammonia-oxidizing microbial community composition based on the similarity test of ANOSIM (n = 5 for AOB Control and P treatments, and AOA P treatment; n = 6 for other AOB and AOA treatments). Significant values shown in bold.

	AOB		AOA	
	R	*P*	*R*	*P*
Control vs N	**0.637**	**0.003**	0.020	0.316
Control vs P	0.164	0.144	−0.019	0.538
Control vs NP	**0.293**	**0.027**	−0.168	0.985
N vs P	**0.731**	**0.001**	0.017	0.380
N vs NP	0.057	0.221	−0.094	0.747
P vs NP	**0.341**	**0.010**	−0.087	0.866

than AOB (Leininger et al., 2006). Following 3 years of urea amendment, however, AOB became dominant over AOA (Fig. 1). Furthermore, short-term urea addition selectively altered AOB community composition and diversity but not that of AOA (Table 2; Figs. 3 and 4), indicating that AOB were more sensitive than AOA to urea amendment in this alpine grassland. A recent long-term (44 years) study of a temperate grassland found that AOB community composition responded more to inorganic N (ammonium nitrate) addition, whereas AOA community composition was more responsive to organic N (cattle slurry) inputs (Zhou et al., 2015). Sterngren et al. (2015) also found that soil bacterial *amoA* gene abundance was higher following addition of inorganic ammonium nitrate than organic N (amino acids) in grassland soil after 33 days incubation. In our study, AOB abundance and community composition rather than that of AOA rapidly respond to organic N (urea) amendment, possibly because urea behaves like inorganic N as it is quickly transformed into ammonia/ammonium or because some AOB can produce urease and thus respond directly to urea additions (Allison and Prosser, 1991; Burton and Prosser, 2001). The potential link between AOB and urease activity might be a worthy topic following urea amendment in grassland ecosystem. Collectively, these results implied that AOB are likely to be more important than AOA in regulating the first step of nitrification following short-term urea addition in alpine grasslands.

The dominant ammonia-oxidizers found in these alpine grassland soils were *Nitrosospira* (AOB) and *Nitrososphaera* (AOA), which is consistent with other studies that have shown the dominance of these two ammonia-oxidizing microbial groups in terrestrial ecosystems across the world (Bruns et al., 1999; Kowalchuk and Stephen, 2001; Carney et al., 2004; Pratscher et al., 2011). The result agreed with previous studies by showing that AOB *Nitrosospira* Cluster 2 might be less competitive than other AOB members in soils with high substrate concentration (Kowalchuk et al., 2000; Laverman et al., 2001). This research differs from other studies as we did not detect the enrichment of AOB within the *Nitrosomonas* lineage, which is usually favored by high N conditions (Koops and Pommerening-Roser, 2001; Zhou et al., 2015). As noted in other studies, fertilization did not have a significant effect on any of the AOA subclusters (Di et al., 2009; Jia and Conrad, 2009; Taylor et al., 2012), possibly because AOA have a high affinity for ammonia.

The composition and diversity of the AOB community showed primary correlation with elevated NO_3^- following urea amendment (Tables S2–3), suggesting that enriched AOB might quickly oxidize fertilizer N leading to NO_3^- accumulation in soil. The AOB community composition also showed significant correlation with forb and total aboveground biomass, which were significantly affected by NO_3^- derived from nitrification, suggesting that there might be tight link among AOB, NO_3^-, and aboveground biomass. The composition of the AOA community showed significant correlation with soil pH (Nicol et al., 2008; Hatzenpichler, 2012; Jiang et al., 2014), which was not affected by fertilization in this study; however, continuous fertilization might lead to acidification, which might trigger a competitive interaction between AOA and AOB.

The effect of short-term P fertilizer on ammonia-oxidizing microbial communities was also investigated in this study. Compared with control soils, AOB *Nitrosospira* cluster 1 showed significant response to the combined N + P addition but not N alone; conversely, AOB *Nitrosospira* cluster 3a showed significant response to N addition rather than N + P addition (Fig. 2). Also, the effect of N (R = 0.637) addition on AOB community composition might be larger than N + P (R = 0.293) addition. These results suggested a potential effect of P addition on soil AOB when applied with N. This effect of P addition on AOB might depend on altered aboveground biomass (Liang et al., 2013) and/or other soil microbial groups (Chen et al., 2013), as they appear to be affected following P addition. In addition, P addition might affect N availability (Lü et al., 2013), which might trigger a potential effect of P on AOB. Overall, compared to urea addition, AOB abundance, community composition, and diversity showed less response following P addition (Table 2; Figs. 1, 3 and 4), indicating that urea amendment was more important than P addition in influencing the AOB communities in alpine grasslands of the Qinghai-Tibetan Plateau.

5. Conclusion

This study demonstrated the differential responses of AOB and AOA to short-term urea addition, which selectively affected AOB abundance, community composition, and diversity, whereas AOA

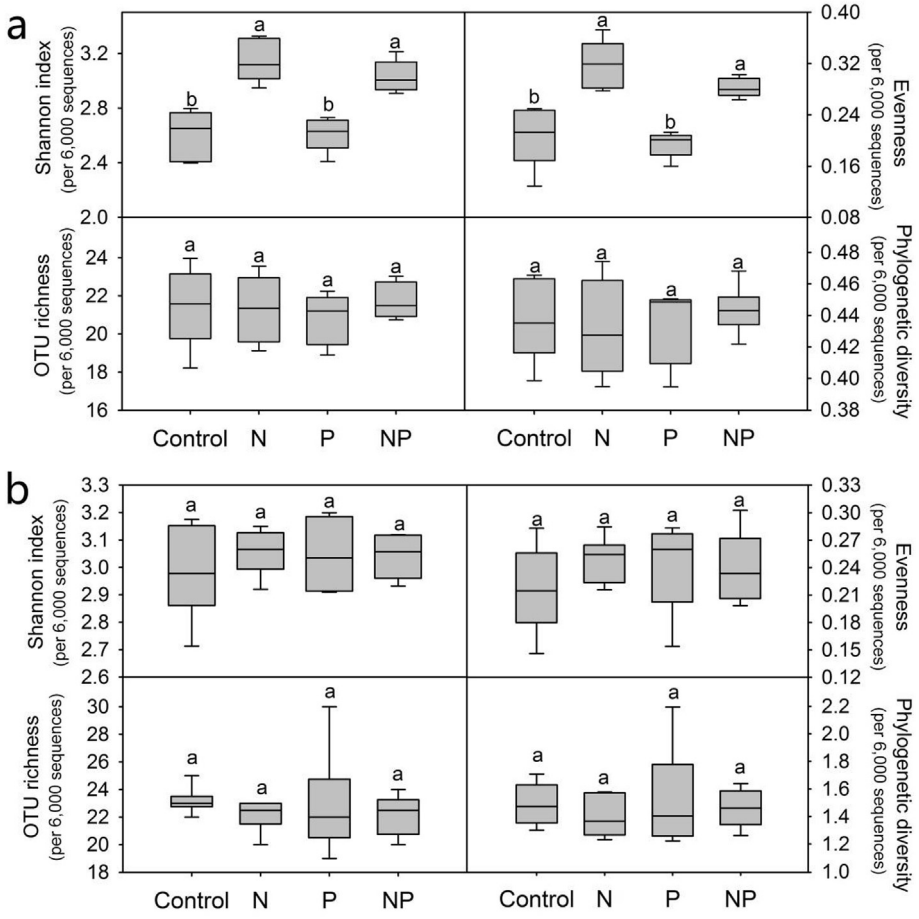

Fig. 4. Alpha-diversity of (a) ammonia-oxidizing bacteria (AOB) and (b) archaea (AOA) calculated at a rarefaction depth of 6000 randomly selected sequences per sample across different nutrient additions. The bottom and top of the box denote the first and third quartiles; the band inside the box denotes median; error bars denote standard deviation; different letters above bars represent significant differences from Tukey's HSD comparisons ($P < 0.05$; n = 5 for AOB Control and P treatments, and AOA P treatment; n = 6 for other AOB and AOA treatments).

remained stable. The results suggest that AOB might play a more important role in nitrification following urea amendment in grasslands, leading to the accumulation of NO_3^- which was associated with a decrease of plant species and increase of forb biomass. This work helps to build a more complete picture of how short-term fertilization affects aboveground biomass and belowground ammonia-oxidizing microbial communities in alpine grassland ecosystems. Future work should focus on detailed plant data, enzymatic activity (e.g. urease), and soil N cycling pathways mediated by microorganisms on different timescale following fertilization to develop a predictive framework for how fertilization affects grassland ecosystem functions.

Acknowledgements

We thank Ms. Yingying Ni and Mr. Yuntao Li from Institute of Soil Science, Chinese Academy of Sciences, for assistance in soil sampling. We also thank Dr. Sean M. Gibbons from Department of Biological Engineering, Massachusetts Institute of Technology, for useful discussion. This work was supported by the National Program on Key Basic Research Project (973 Program, Grant #2014CB954002), Strategic Priority Research Program (Grant #XDB15010101) of Chinese Academy of Sciences, and National Natural Science Foundation of China (41071121). The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.01.012.

References

Akiyama, T., Kawamura, K., 2007. Grassland degradation in China: methods of monitoring, management and restoration. Grassland Science 53, 1–17.
Allison, S.M., Prosser, J.I., 1991. Urease activity in neutrophilic autotrophic ammonia-oxidizing bacteria isolated from acid soils. Soil Biology and Biochemistry 23, 45–51.
Allison, S.D., Lu, Y., Weihe, C., Goulden, M.L., Martiny, A.C., Treseder, K.K., Martiny, J.B.H., 2013. Microbial abundance and composition influence litter decomposition response to environmental change. Ecology 94, 714–725.
Antoninka, A., Reich, P.B., Johnson, N.C., 2011. Seven years of carbon dioxide enrichment, nitrogen fertilization and plant diversity influence arbuscular mycorrhizal fungi in a grassland ecosystem. New Phytologist 192, 200–214.
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.
Avrahami, S., Liesack, W., Conrad, R., 2003. Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. Environmental Microbiology 5, 691–705.
Bai, Y.F., Wu, J.G., Clark, C.M., Naeem, S., Pan, Q.M., Huang, J.H., Zhang, L.X., Han, X.G., 2010. Tradeoffs and thresholds in the effects of nitrogen addition on biodiversity and ecosystem functioning: evidence from inner Mongolia Grasslands. Global Change Biology 16, 358–372.
Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., Bustamante, M., Cinderby, S., Davidson, E., Dentener, F., Emmett, B., Erisman, J.W., Fenn, M., Gilliam, F., Nordin, A., Pardo, L., Vrie, W.D., 2010. Global assessment of nitrogen deposition effects on terrestrial plant diversity: a

synthesis. Ecological Application 20, 30–59.
Beauchamp, E.G., Trevors, J.T., Paul, J.W., 1989. Carbon sources for bacterial denitrification. Advances in Soil Science 10, 113–142.
Bowman, R.A., 1988. A rapid method to determine total phosphorus in soils. Soil Science Society of America Journal 52, 1301–1304.
Bruns, M.A., Stephen, J.R., Kowalchuk, G.A., Prosser, J.I., Paul, E.A., 1999. Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Applied and Environmental Microbiology 65, 2994–3000.
Burton, S.A.Q., Prosser, J.I., 2001. Autotrophic ammonia oxidation at low pH through urea hydrolysis. Applied and Environmental Microbiology 67, 2952–2957.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335–336.
Carney, K.M., Matson, P.A., Bohannan, B.J.M., 2004. Diversity and composition of tropical soil nitrifiers across a plant diversity gradient and among land-use types. Ecology Letters 7, 684–694.
Chen, Y.L., Chen, B.D., Hu, Y.J., Li, T., Zhang, X., Hao, Z.P., Wang, Y.S., 2013. Direct and indirect influence of arbuscular mycorrhizal fungi on abundance and community structure of ammonia oxidizing bacteria and archaea in soil microcosms. Pedobiologia 56, 205–212.
Clark, C.M., Tilman, D., 2008. Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. Nature 451, 712–715.
Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.Z., 2009. Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. Nature Geoscience 2, 621–624.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.
Elser, J.J., Bracken, M.E.S., Cleland, E.E., Gruner, D.S., Harpole, W.S., Hillebrand, H., Ngai, J.T., Seabloom, E.W., Shurin, J.B., Smith, J.E., 2007. Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. Ecology Letters 10, 1135–1142.
Fierer, N., Lauber, C.L., Ramirez, K.S., Zaneveld, J., Bradford, M.A., Knight, R., 2012. Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. The ISME Journal 6, 1007–1017.
Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., Oakley, B.B., 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proceedings of the National Academy of Sciences of the United States of America 102, 14683–14688.
Fridley, J.D., 2002. Resource availability dominates and alters the relationship between species diversity and ecosystem productivity in experimental plant communities. Oecologia 132, 271–277.
Hacker, R.B., Toole, I.D., Melville, G.J., 2011. Effects of nitrogen and phosphorus on vegetation dynamics of a degraded native grassland in semi-arid south-eastern Australia. The Rangeland Journal 33, 87–97.
Harpole, W.S., Ngai, J.T., Cleland, E.E., Seabloom, E.W., Borer, E.T., Bracken, M.E.S., Elser, J.J., Gruner, D.S., Hillebrand, H., Shurin, J.B., Smith, J.E., 2011. Nutrient co-limitation of primary producer communities. Ecology Letters 14, 852–862.
Hatzenpichler, R., Lebedeva, E.V., Spieck, E., Stoecker, K., Richter, A., Daims, H., Wagner, M., 2008. A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. Proceedings of the National Academy of Sciences of the United States of America 105, 2134–2139.
Hatzenpichler, R., 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Applied and Environmental Microbiology 78, 7501–7510.
He, D., Xiang, X.J., He, J.S., Wang, C., Cao, G.M., Adams, J.M., Chu, H.Y., 2016. Composition of the soil fungal community is more sensitive to phosphorus than nitrogen addition in alpine meadow on the Qinghai-Tibetan Plateau. Biology and Fertility of Soils 52, 1059–1072.
Heil, G.W., Bruggink, M., 1987. Competition for nutrients between *Calluna vulgaris* (L.) Hull and *Molina caerulea* (L.) Moench. Oecologia 73, 105–108.
Herron, G.J., Sheley, R.L., Maxwell, B.D., Jacobsen, J.S., 2001. Influence of nutrient availability on the interaction between spotted knapweed and bluebunch wheatgrass. Restoration Ecology 9, 326–331.
Jia, Z.J., Conrad, R., 2009. Bacteria rather than archaea dominate microbial ammonia oxidation in an agricultural soil. Environmental Microbiology 11, 1658–1671.
Jiang, H.C., Huang, L.Q., Deng, Y., Wang, S., Zhou, Y., Liu, L., Dong, H.L., 2014. Latitudinal distribution of ammonia-oxidizing bacteria and archaea in the agricultural soils of eastern China. Applied and Environmental Microbiology 80, 5593–5602.
Klabi, R., Bell, T.H., Hamel, C., Iwaasa, A., Schellenberg, M., Raies, A., St-Arnaud, M., 2015. Plant assemblage composition and soil P concentration differentially affect communities of AM and total fungi in a semi-arid grassland. FEMS Microbiology Ecology 91, 1–13.
Koops, H.P., Pommerening-Roser, A., 2001. Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiology Ecology 37, 1–9.
Kowalchuk, G.A., Stienstra, A.W., Heilig, G.H., 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.
Kowalchuk, G.A., Stephen, J.R., 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annual Review of Microbiology 55, 485–529.
Laverman, A.M., Speksnijder, A.G.C.L., Braster, M., Kowal-chuk, G.A., Verhoef, H.A., van Verseveld, H.W., 2001. Spatiotemporal stability on an ammonia-oxidizing community in a nitrogen-saturated forest soil. Microbial Ecology 42, 35–45.
Leff, J.W., Jones, S.E., Prober, S.M., Barberán, A., Borer, E.T., Firn, J.L., et al., 2015. Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe. Proceedings of the National Academy of Sciences 112, 10967–10972.
Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809.
Li, J.H., Yang, Y.J., Li, B.W., Li, W.J., Wang, G., Knops, J.M.H., 2014. Effects of nitrogen and phosphorus fertilization on soil carbon fractions in alpine meadows on the Qinghai-Tibetan Plateau. Plos One 9, e103266.
Liu, Y., Shi, G., Mao, L., Cheng, G., Jiang, S., Ma, X., An, L., Du, G., Johnson, N.C., Feng, H., 2012. Direct and indirect influences of 8 yr of nitrogen and phosphorus fertilization on Glomeromycota in an alpine meadow ecosystem. New Phytologist 194, 523–535.
Liang, Y., He, X., Liang, S., Zhang, W., Chen, X., Feng, S., Su, Y., 2013. Community structure analysis of soil ammonia oxidizers during vegetation restoration in southwest China. Journal of Basic Microbiology 54, 180–189.
Lovell, R.D., Jarvis, S.C., Bardgett, R.D., 1995. Soil microbial biomass and activity in long-term grassland - effects of management changes. Soil Biology and Biochemistry 27, 969–975.
Lu, L., Han, W.Y., Zhang, J.B., Wu, Y.C., Wang, B.Z., Lin, X.G., Zhu, J.G., Cai, Z.C., Jia, Z.J., 2012. Nitrification of archaeal ammonia oxidizers in acid soils is supported by hydrolysis of urea. The ISME Journal 6, 1978–1984.
Lü, X.T., Reed, S., Yu, Q., He, N.P., Wang, Z.W., Han, X.G., 2013. Convergent responses of nitrogen and phosphorus resorption to nitrogen inputs in a semiarid grassland. Global Change Biology 19, 2775–2784.
Miller, E.M., Seastedt, T.R., 2009. Impacts of woodchip amendments and soil nutrient availability on understory vegetation establishment following thinning of a ponderosa pine forest. Forest Ecology and Management 258, 263–272.
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.
Norton, J.M., Alzerreca, J.J., Suwa, Y., Klotz, M.G., 2002. Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. Archives of Microbiology 177, 139–149.
Pester, M., Rattei, T., Flechl, S., Grongroft, A., Richter, A., Overmann, J., Reinhold-Hurek, B., Loy, A., Wagner, M., 2012. *amoA*-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of *amoA* genes from soils of four different geographic regions. Environmental Microbiology 14, 525–539.
Pratscher, J., Dumont, M.G., Conrad, R., 2011. Ammonia oxidation coupled to CO_2 fixation by archaea and bacteria in an agricultural soil. Proceedings of the National Academy of Sciences of the United States of America 108, 4170–4175.
R Development Core Team, 2006. R: A language and Environment for Statistical Computing. R 21 Foundation for Statistical Computing, Vienna, Austria.
Reeder, J., Knight, R., 2010. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. Nature Methods 7, 668–669.
Reijs, J.W., Sonneveld, M.P.W., Sorensen, P., Schils, R.L.M., Groot, J.C.J., Lantinga, E.A., 2005. Utilization of nitrogen from cattle slurry applied to grassland as affected by diet composition. Agriculture Ecosystems and Environment 118, 65–78.
Ren, Z.W., Li, Q., Chu, C.J., Zhao, L.Q., Zhang, J.Q., Ai, D., Yang, Y.B., Wang, G., 2010. Effects of resource additions on species richness and ANPP in an alpine meadow community. Journal of Plant Ecology 3, 25–31.
Ririe, K.M., Rasmussen, R.P., Wittwer, C.T., 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical Biochemistry 245, 154–160.
Rotthauwe, J.H., Witzel, K.P., Liesack, W., 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied and Environmental Microbiology 63, 4704–4712.
Shang, Z.H., Long, R.J., 2007. Formation causes and recovery of the "Black soil type" degraded alpine grassland in Qinghai-tibetan plateau. Frontiers of Agriculture in China 1, 197–202.
Stahlberg, S., 1980. A new extraction method for estimation of plant-available P, K and Mg. Acta Agriculturae Scandinavica 30, 93–107.
Stempfhuber, B., Engel, M., Fischer, D., Neskovic-Prit, G., Wubet, T., Schöning, I., et al., 2014. pH as a driver for ammonia-oxidizing archaea in forest soils. Microbial Ecology 69, 879–883.
Sterngren, A.E., Hallin, S., Bengtson, P., 2015. Archaeal ammonia oxidizers dominate in numbers, but bacteria drive gross nitrification in N-amended grassland soil. Frontiers in Microbiology 6, 1350.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30, 2725–2729.
Taylor, A.E., Zeglin, L.H., Wanzek, T.A., Myrold, D.D., Bottomley, P.J., 2012. Dynamics of ammonia-oxidizing archaea and bacteria populations and contributions to soil nitrification potentials. The ISME Journal 6, 2024–2032.
van der Heijden, M.G.A., Bardgett, R.D., van Straalen, N.M., 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11, 296–310.
Vitousek, P.M., Porder, S., Houlton, B.Z., Chadwick, O.A., 2010. Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen-phosphorus interactions. Ecological Applications 20, 5–15.
Walkley, A., Black, I.A., 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid

titration method. Soil Science 37, 29–38.
Wen, L., Wen, L., Dong, S.K., Li, Y.Y., Li, X.Y., Shi, J.J., Wang, Y.L., Liu, D.M., Ma, Y.S., 2013. Effect of degradation intensity on grassland ecosystem services in the alpine region of Qinghai-Tibetan Plateau, China. Plos One 8, e58432.
Wu, Y.C., Lu, L., Wang, B.Z., Lin, X.G., Zhu, J.G., Cai, Z.C., Yan, X.Y., Jia, Z.J., 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, 1431–1439.
Xiang, X.J., Gibbons, S.M., He, J.S., Wang, C., He, D., Li, Q., Ni, Y.Y., Chu, H.Y., 2016. Rapid response of arbuscular mycorrhizal fungal communities to short-term fertilization in an alpine grassland on the Qinghai-Tibet Plateau. PeerJ 4, e2226.
Zeng, J., Liu, X.J., Song, L., Lin, X.G., Zhang, H.Y., Shen, C.C., Chu, H.Y., 2016. Nitrogen fertilization directly affects soil bacterial diversity and indirectly affects bacterial community composition. Soil Biology and Biochemistry 92, 41–49.
Zhang, R.Y., Gou, X., Bai, Y., Zhao, J., Chen, L.Y., Song, X., Wang, G., 2011. Biomass fraction of graminoids and forbs in N-limited alpine grassland N: P stoichiometry. Polish Journal of Ecology 59, 105–114.
Zhou, X., Fornara, D., Wasson, E.A., Wang, D., Ren, G., Christie, P., Jia, Z., 2015. Effects of 44 years of chronic nitrogen fertilization on the soil nitrifying community of permanent grassland. Soil Biology and Biochemistry 91, 76–83.