Time-dependent shifts in populations and activity of bacterial and archaeal ammonia oxidizers in response to liming in acidic soils

Miao-Miao Zhang^a, Ricardo J.E. Alves^b, Dan-Dan Zhang^{a, c}, Li-Li Han^a, Ji-Zheng He^a, Li-Mei Zhang^{a, *}

^a State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

^b Department of Ecogenomics and Systems Biology, Division of Archaea Biology and Ecogenomics, Faculty of Life Sciences, University of Vienna, Althanstrasse 14 UZA I, A-1090 Vienna, Austria

^c University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:

Received 17 November 2016

Received in revised form

30 April 2017

Accepted 1 May 2017

Available online 10 May 2017

Keywords:

Ammonia-oxidizing bacteria (AOB)

Ammonia-oxidizing archaea (AOA)

Nitrification

N fertilizers

Liming

Acidic soils

ABSTRACT

During the past decades, extensive nitrogen fertilization and acid deposition have greatly contributed to soil acidification in agroecosystems. Liming, the addition of calcium- and magnesium-rich material to soil, is an effective management strategy used to improve fertility and productivity of agricultural soils degraded by acidification. Nitrification plays a central role in nitrogen (N) availability in agroecosystems and contributes to soil acidification. However, little is known regarding the effects of liming on this process and microbial populations that drive it. Here, we investigated population dynamics and activity of ammonia oxidizers in response to a 2-year liming field trial in acidic soils received long-term fertilization with chemical N fertilizers and short-term lime amendment in microcosm incubations. Our results showed that activity, abundance and population structure of both ammonia-oxidizing archaea (AOA) and bacteria (AOB) were sensitive to liming in fertilized acidic soils. AOB abundance and potential nitrification rates increased in field plots subjected to liming, whereas the opposite was observed for AOA. In microcosm incubations, AOA abundance increased progressively over 60 days, namely under low CaO levels, whereas AOB abundance was greatly stimulated only during the first week under high CaO levels. ¹³CO₂-SIP-DNA experiments further supported that AOA were the most active ammonia oxidizers in fertilized field soils. However, in N-fertilized soils freshly amended with CaO, only autotrophic growth of AOB was observed after seven days, but not after 30 days when growth of AOA was observed. Taken together, our results indicated that both AOA and AOB play a role in nitrification following liming in fertilized acidic soils, likely through selection of better adapted clades of organisms. Although AOA were likely the main drivers of nitrification in these soils in the long-term, liming stimulated AOB activity in the short-term.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Acidic soils, defined as having pH below 5.5, occupy a vast extension of the terrestrial surface, comprising about 30% of the world's total ice-free areas (Uexküll and Mutert, 1995). During the past decades, high levels of nitrogen fertilizer application and acid deposition have led to soil acidification and consequent lower crop yields and disturbances of the N cycle (Guo et al., 2010; Wallace, 1994). Liming, the application of calcium- and magnesium-rich

material to soils, such as limestone (Álvarez et al., 2009), calcium carbonate (Fornara et al., 2011) and calcium oxide (CaO) (Kerley, 2000; Zaniewicz-Bajkowska et al., 2007), is commonly employed to improve quality and productivity of acidic soils (Caires et al., 2008; Lollato et al., 2013). It has been reported that liming can decrease water repellence in sandy soils and increase microbial biomass and respiration associated with acid-intolerant soil microorganisms (Avrahami and Bohannan, 2007; Park et al., 2006; Xiao et al., 2014). The influence of lime amendment on N turnover in acidic soils have also been reported, including N mineralization, N fixation, nitrification and N₂O emissions (Abell et al., 2012; Avrahami and Bohannan, 2007; Barton et al., 2013; Shanmugam et al., 2014).

* Corresponding author.

E-mail address: zhanglm@rcees.ac.cn (L.-M. Zhang).

Nitrification, the conversion of ammonia to nitrate via nitrite, is a central process in global nitrogen (N) cycling that greatly contributes to lower N fertilizer efficiency, and groundwater and atmospheric pollution through nitrate leaching and emission of reactive N species, respectively (Farmaha, 2014; Wrage et al., 2001). Furthermore, nitrification is also directly and indirectly involved in soil acidification through generation of nitrous acid and proton release, and loss of basic cations through nitrate leaching (Bernhard, 1986; Bolan and Hedley, 2005; Helyar and Porter, 1989). Ammonia oxidation, the first and rate-limiting step of nitrification, was thought to be catalyzed primarily by autotrophic ammonia-oxidizing bacteria (AOB) belonging to β - or γ -proteobacteria, with very limited contributions from heterotrophic bacteria and fungi in some soils (De Boer and Kowalchuk, 2001; Prosser, 1990). However, metagenomic and cultivation studies revealed that ammonia-oxidizing archaea (AOA) are also able to grow chemolithoautotrophically by aerobically oxidizing ammonia to nitrite (Konneke et al., 2005; Leininger et al., 2006; Treusch et al., 2005; Venter et al., 2004). AOA and AOB generally occupy distinct environmental niches, with AOA being numerically and functionally dominant in most acidic soils (Prosser and Nicol, 2012). Nitrification activity in some acidic soils has been positively correlated with AOA abundance (Yao et al., 2011; Zhang et al., 2012), and changes in nitrification activity have also been associated to changes in AOA community composition (Gubry-Rangin et al., 2010; Lu et al., 2012; Zhang et al., 2012). Further evidence was provided by $^{13}\text{CO}_2$ -DNA-SIP experiments showing autotrophic growth of AOA, but not of AOB, in nitrifying acidic tea orchard soil (Zhang et al., 2012). By contrast, AOB have been shown to generally dominate ammonia oxidizer populations and nitrification activity in neutral and alkaline soils, as shown by $^{13}\text{CO}_2$ -DNA-SIP experiments (Jia and Conrad, 2009; Xia et al., 2011), as well as in soils subjected to high nitrogen inputs, based on inhibition experiments (Di et al., 2009). Nevertheless, AOB are often present in acidic soils and in some cases their abundance is comparable, or higher, than that of AOA (Andert et al., 2011; Long et al., 2012; Pereira et al., 2012). Moreover, potential nitrification rates in some acidic soils have been shown to be positively correlated with abundance and community structure dynamics of AOB (Fan et al., 2011; Gleeson et al., 2010; Ying et al., 2010). DNA-SIP experiments also indicated that recovery of nitrification and subsequent zinc tolerance after long-term zinc exposure in an acidic soil was initially driven by AOB rather than AOA (Ruyters et al., 2013). Such evidence indicates that, despite AOA dominance, acidic soils also harbour active and dynamic AOB populations, although their role in nitrification and response to disturbances in such soils remain unclear. In fact, increases in AOB abundance have been observed following lime application in acidic soils (Barton et al., 2013; Che et al., 2015; Rotthauwe et al., 1997), although long-term and short-term effects of lime application on ammonia oxidizer community structure and nitrification activity have not been evaluated.

In our previous work with upland red soils (pH 3.7–6.0) from the Qiyang Red Soil Experimental Station in Hunan Province, China, which was continuously fertilized over a 16-year period, we found that inorganic N fertilization significantly reduced soil pH and AOB abundance, and markedly changed AOA community composition (He et al., 2007). In 2010, field plots under inorganic N fertilization were subjected to liming with ~1000 ppm CaO, as an attempt to alleviate soil acidification. The current study aims to assess the long- and short-term responses of AOA and AOB populations to liming in acidic soils under continuous fertilization and their effect on nitrification. Additionally, we aim to further elucidate the particular role of AOB in acidic soils, as it might be currently underestimated. We addressed these questions by analyzing NH_4^+ and NO_3^- pool dynamics, potential nitrification rates (PNRs) and

ammonia oxidizer abundance and community structure, through quantitative PCR (qPCR) and *amoA* gene sequencing, respectively, in soils subject to different fertilizers after a 2-year field liming trial and 2-month laboratory soil incubations with and without lime amendment. Furthermore, $^{13}\text{CO}_2$ -DNA-SIP experiments were performed following similar laboratory soil incubations to determine the active ammonia populations after short-term lime amendment.

2. Materials and methods

2.1. Soil collection and sample characterization

Soils were collected from a long-term fertilization trial at the Qiyang Red Soil Experimental Station (26°45'N, 111°52'E), Hunan Province, China. The site has a subtropical monsoon climate with an annual rainfall of 1300 mm and annual average temperature of 18 °C, and the soil developed from quaternary red clay earth and classified as agri-udic ferrosols. The long-term fertilization trial was established in 1990 with a wheat-maize rotation system, including three replicate plots under eight treatments of fallow, control without fertilizers (Control) and different combinations of nitrogen (N), potassium (K), phosphate (P) and organic manure fertilizers (He et al., 2007). Due to high soil acidification in plots subject to annual inputs of N, NK, NP and NPK fertilizers since 1990, at rates of 300 kg ha⁻¹ N, 120 kg ha⁻¹ P₂O₅, 120 kg ha⁻¹ K₂O, in 2010, half of the plots were amended with 2250 kg ha⁻¹ CaO (equivalent to 1000 ppm) to alleviate soil acidification. CaO powder was broadcasted in fallow season only once in 2010 and mixed with soil by subsequent manual plough. For the current study, we selected soils under chemical N fertilizer treatments (N, NK, NP, NPK), the corresponding liming treatments (N + CaO, NK + CaO, NP + CaO and NPK + CaO) and untreated Control soil. Soil samples were collected from a depth of 0–20 cm in July 2012. Five soil cores were collected from each plot and homogenized into one composite sample. All samples were passed through a 2.0 mm sieve and stored at 4 °C for soil physico-chemical analyses and microcosm incubations, whereas subsamples for molecular analyses were immediately stored at –80 °C.

Soil pH_(KCl) was determined using a Delta 320 pH-meter (Mettler-Toledo Instruments Co., Shanghai, China) after suspended with 1 M KCl. Soil ammonium and nitrate pools were extracted with 1 M KCl and measured with a Continuous Flow Analyzer (SAN++, Skalar, Breda, Holland). Potential nitrification rates (PNR) were determined using the chlorate inhibition method as described by Kurola and colleagues (Kurola et al., 2005). Briefly, for each assay, 5.0 g of fresh soil was suspended in 20 mL phosphate buffer solution (PBS) (g L⁻¹: NaCl 8.0, KCl 0.2, Na₂HPO₄ 0.2, NaH₂PO₄ 0.2, pH 7.4) and 1 mM (NH₄)₂SO₄ as substrate. Potassium chlorate was added to a final concentration of 10 mM to inhibit nitrite oxidation. The soil suspension was incubated in the dark for 24 h at 25 °C, while shaking at 180 rpm. Soil nitrite was extracted with 5 mL 2 M KCl and measured at 540 nm based on the Griess method using sulphonamide and naphthylethelene diamide. Soil organic carbon (SOC) was extracted with 70 °C hot water and measured with a Total Organic Carbon Analyzer (Fusion/Torch, Tekmar Dohrmann, USA), as described by Wang and Wang, 2011.

2.2. Soil microcosm incubations

Microcosm experiment 1 (CaO amendment): Short-term soil incubations were performed with soil from the long-term field N treatment amended prior to incubation with 500, 1000 and 2000 ppm CaO powder, in addition to the control without CaO, and designated N + 500, N + 1000 and N + 2000 and N (control), respectively. Incubations were performed in 120 ml serum bottles

containing 10 g fresh samples and covered with aluminium foil. Briefly, CaO powder was separately added into each bottle with a final concentration of 500, 1000 and 2000 ppm, and thoroughly mixed with the soils prior to adjust soil moisture to 60% field moisture capacity. Microcosms were incubated at 28 °C in the dark. The water content of soils was maintained by resupplying the lost water every 3 days throughout the incubation. Three replicates from each treatment were destructively sampled at 0, 7, 15, 30 and 60 days, immediately subsampled for ammonium and nitrate extractions and the remaining samples were frozen at –80 °C for molecular analysis.

Microcosm experiment 2 (Stable Isotope Probing incubation): Short-term soil incubations for SIP analysis were performed with soils from the long-term field N and N + CaO treatments. An additional incubation was performed with soil from the field N treatment amended with 1000 ppm CaO prior to incubation (N + 1000). Microcosms were performed in 120 ml serum bottles containing 10 g of the fresh samples and sealed with rubber stoppers and aluminium caps. The headspace of the bottles was adjusted to 5% (v/v) of either ^{12}C -CO₂ or ^{13}C -CO₂ (99 atom%, Sigma-Aldrich Co., St Louis, MO, USA) by injection through the rubber septum. Air in the headspace was renewed every 3 days by opening the bottles and then re-establishing the CO₂ concentration. Microcosms were incubated at 28 °C in the dark, and three replicates from each treatment were destructively sampled at 0, 7 and 30 days, as described above.

2.3. DNA extraction and quantitative PCR of *amoA* genes

Soil DNA was extracted from 0.25 g samples with the MoBio PowerSoil™ DNA Kit (MO BIO laboratories, CA, US), according to the manufacturers' protocol. Extracted DNA was verified on 1% agarose gel and concentrations were determined photometrically using a Nanodrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Archaeal and bacterial *amoA* genes were quantified by quantitative PCR (qPCR) using the primers Arch-*amoA*F/Arch-*amoA*R (Francis et al., 2005) and *amoA*-1F/*amoA*-2R (Rotthauwe et al., 1997), respectively. PCR reactions were performed in 25 µl volumes containing 12.5 µl SYBR® Premix Ex Taq™ (TaKaRa Biotechnology, Otsu, Shiga, Japan), 200 nM of each primer and 2 µl of 5-fold diluted template DNA (1–10 ng). qPCR was performed in an iCycler iQ 5 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following thermal conditions: 2 min at 95 °C, 35 cycles of 10 s at 95 °C, 30 s at 53 °C for AOA and 55 °C for AOB, and 1 min at 72 °C followed by a plate reading step at 81 °C. Standard curves for the qPCR assays were generated as described previously (He et al., 2007), and quantification efficiencies varied between 80%–120% with R² values > 0.99. All qPCR reactions were performed in triplicate.

2.4. Cloning, sequencing and phylogenetic analysis

A preliminary screening of AOA and AOB community compositions with Denaturing Gradient Gel Electrophoresis (DGGE) of *amoA* genes indicated that ammonia oxidizer population structure was similar among N fertilizer treatments (N, NK, NP, NPK) and among liming treatments (N + CaO, NK + CaO, NP + CaO, NPK + CaO) (data not shown), but differed mostly between non-liming and liming treatments. Therefore, five field samples, Control, N, NPK, N + CaO and NPK + CaO, and samples collected at days 0, 7 and 30 from the N, N + 1000 and N + CaO treatments of the ^{13}C -SIP-DNA incubations (experiment 2) were selected for *amoA* gene cloning and sequencing. Archaeal and bacterial *amoA* genes were amplified with primers CrenamoA-23f/CrenamoA-616r

(Tournai et al., 2008) and *amoA*-1F/*amoA*-2R (Rotthauwe et al., 1997). PCR was performed in a 25 µl reaction volume containing 12.5 µl Premix Ex Taq™ (TaKaRa Biotechnology, Otsu, Shiga, Japan), 200 nM primer for AOA or 160 nM primer for AOB and 2 µl DNA template, using the following thermal condition: 5 min 95 °C, 10 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, then 25 cycles of 30 s at 92 °C, 30 s at 55 °C, and 1 min at 72 °C followed by 72 °C at 10 min. All PCR reactions were performed in triplicate.

A total of 1047 archaeal (20–48 clones per sample) and 862 bacterial (20–34 clones per sample) *amoA* gene sequences were obtained from soil samples of five field plots under different treatments and from three time-points of SIP microcosm incubations under three different treatments. Triplicate clone libraries were performed from all samples/time-points, except from day 0 of the incubation experiment (one or two replicates per day 0 sample, for AOA and AOB, respectively). No bacterial *amoA* genes were obtained from day 30 samples of the incubation, due to unsuccessful PCR amplification. Individual archaeal and bacterial *amoA* gene datasets were aligned with MAFFT (FFT-NS-i method) (Katoh and Standley, 2013), primers were trimmed and sequences containing stop codons or indels causing frame-shifts were excluded. Datasets were filtered for chimeras and clustered into operational taxonomic units (OTUs) at 97% sequence identity with UPARSE (Edgar, 2013). The quality-checked datasets comprised 1013 sequences with 592 bp clustered into 33 OTUs for AOA, and 818 sequences with 447 bp clustered into 44 OTUs for AOB. Selection of the best-fit model of DNA evolution, tree inference and calculation of Ultrafast Bootstrap (UFBoot) (Minh et al., 2013) and SH-aLRT (Guindon et al., 2010) support values (1000 replicates each) were performed by maximum-likelihood with IQ-TREE (Nguyen et al., 2015). Phylogenetic tree calculations were based on the TVM and K3Pu models of DNA evolution for archaeal and bacterial *amoA* gene sequences, respectively, both with a FreeRate model of substitution rate heterogeneity with four categories (+R4) (Soubrier et al., 2012). Nucleotide sequences obtained in this study were deposited in GenBank under accession numbers KX666100 to KX666917 and KX666918 to KX667930 for bacterial and archaeal *amoA* genes sequences, respectively.

2.5. SIP fractionation

DNA extracted from ^{12}C - and ^{13}C -CO₂ incubations was subjected to isopycnic density gradient centrifugation, as described by Zhang et al. (2012). Briefly, a CsCl solution with a buoyant density of 1.696 g ml^{–1} was prepared in TE buffer (pH 8.0) and adjusted to a refractive index of 1.3999 with an ATAGO-R-5000 hand-held refractometer (UNI-IT, Tokyo, Japan). DNA (~2 µg) was added to the CsCl solution in 4.9 ml OptiSeal polyallomer tubes (Beckman Coulter, Palo Alto, CA, USA), and then subjected to centrifuge at 227,962 g (56,200 rpm) for 24 h at 20 °C in a VTi 90 vertical rotor (Beckman Coulter). Centrifuged CsCl gradients were fractionated into 24 equal volumes (~200 µl) by injecting sterile water on the top to replace CsCl gradient solution using a fraction recovery system (Beckman Coulter). Buoyant densities of each fraction were measured by determining their refractive index in 20 µl aliquots. Nucleic acids were precipitated from CsCl overnight with two volumes of PEG 6000 in 1.6 M NaCl, washed with 70% ethanol and then eluted in 30 µl of sterile water.

2.6. Statistical analysis

Analysis of variance in soil pH, SOC, NH₄⁺ and NO₃[–] concentrations, PNR and log-transformed *amoA* gene copy numbers were performed with one-way ANOVA followed by S-N-K (n ≥ 3) and paired T (n = 2) tests using SPSS version 19.0 (IBM Co., Armonk, NY,

USA), respectively. *P* values below 0.05 were considered statistically significant.

3. Results

3.1. Field soil properties, potential nitrification rates and ammonia oxidizer populations

Significant differences ($P < 0.05$) in soil pH (Table 1) were observed between mineral N fertilizer treatments (N, NK, NP, NPK) and the corresponding liming treatments (N + CaO, NK + CaO, NP + CaO and NPK + CaO). Soil pH_(KCl) in plots treated with mineral N fertilizers (N, NK, NP and NPK) was significantly lower than in untreated control plots, ranging between 3.35 and 3.47, whereas liming changed soil acidity, resulting in significantly higher soil pH in all limed plots (4.10–4.46; $P < 0.05$) (Table 1). Liming also induced significantly higher PNRs, which ranged between 0.02 and 0.14 mg NO₂-N kg⁻¹ h⁻¹ in the N fertilized soils and between 0.22 and 0.34 mg NO₂-N kg⁻¹ h⁻¹ in the corresponding limed soils ($P < 0.05$) (Table 1). Similar to our previous observations in the same experimental plots 6 years earlier (He et al., 2007), PNRs in unfertilized control soils were significantly higher than in N fertilized soils and showed a significant positive correlation with soil pH ($r = 0.78$, $n = 27$, $P < 0.01$) (Table 1, Fig. S1). Ammonium concentrations did not differ significantly between treatments, whereas three of the N-fertilized soils (i.e., N, NK, NP) showed significantly higher NO₃⁻ concentrations than soils under any other treatment ($P < 0.05$) (Table 1). SOC concentrations in three of the P-fertilized soils (i.e., NP, NPK and NPK + CaO) were significantly higher than those in soils under other treatments ($P < 0.05$) (Table 1).

The largest AOA and AOB populations were detected in the Control and NPK + CaO treatment, and the smallest under N + CaO and N treatments, respectively (Table 1). AOA abundance were significantly more abundant than AOB in all treatments without CaO ($P < 0.05$), with AOA:AOB ratios between 10.9 and 148.7. Paired T-test analysis showed that AOB abundance increased, while AOA decreased significantly ($P < 0.05$) in limed soils (N + CaO, NK + CaO, NP + CaO, NPK + CaO) than in the corresponding treatments without lime amendment (N, NK, NP, NPK) (Table 1), resulting in lower AOA:AOB ratios between 0.83 and 1.94. Soil PNR showed no significant correlation with AOA nor AOB abundance but was rather positively correlated with total ammonia oxidizer population size, i.e., the sum of AOA and AOB abundances ($r = 0.40$, $n = 9$, $P < 0.05$).

Five field treatments (Control, N, N + CaO, NPK and NPK + CaO) were selected for in-depth community analysis by *amoA* gene cloning and sequencing. Archaeal *amoA* gene sequences were affiliated with nine distinct phylogenetic clades associated with the order *Nitrososphaerales*, following the classification by Alves et al.

(2013) (clades A, B, C and *Nitrososphaera*), extended here to accommodate diversity not detected in that study (clades E and F), as well as an additional clade, clade *Nitrosotalea*, after the genus *Ca. Nitrosotalea* representing the order *Ca. Nitrosotaleales* (Kerou et al., 2016) (Fig. 1a and S2). As previously observed by He et al. (2007), long-term application of chemical N fertilizers greatly affected AOA community composition in these soils (Fig. 1a). Liming, however, had a pronounced effect on AOA community composition in soils fertilized with only N but not with NPK. Clade B dominated most AOA populations, comprising 68–79% of AOA under all treatments, except in soils fertilized only with N (Fig. 1a). Clade B represents a phylogenetically broad lineage encompassing distinct sub-lineages, which responded differently to soil treatments and therefore were designated here as sub-clades B1 to B4 (Fig. 1a). Nearly all AOA in clade B were affiliated with subclades B1 or B3, with the later dominating in most soils. Clade C and *Nitrosotalea* were detected in fertilized soils but not in untreated control soil, with relative abundances ranging from 12 ± 4% to 53 ± 8% and from 2 ± 1% to 15 ± 4%, respectively (Fig. 1a). The majority of bacterial *amoA* gene sequences detected were affiliated with six clades represented by cultivated *Nitrosospira* strains, mostly corresponding to environmental clades defined by (Zhang et al., 2009) (Fig. 1b and S3). AOB affiliated with the *Nitrosomonas* genus, related to *Nitrosomonas* sp. Is79A3, were only detected in untreated control soils. AOB populations were dominated by *Nitrosospira* sp. Nsp65/L115 clade under N, NPK and NPK + CaO treatments, and by *Nitrosospira* sp. Nsp2/Nsp17 clade in the control and N + CaO-treated soils (Fig. 1b). Both fertilization and liming had a profound effect on the relative abundance of the dominant AOB clades: the relative abundance of *Nitrosospira* sp. Nsp65/L115 clade was, in average, higher in both non-limed fertilized soils (N: 51 ± 12% and NPK: 90 ± 10%) than in the Control (37 ± 17%), whereas the opposite was observed for *Nitrosospira* sp. Nsp2/Nsp17 clade, which was less abundant under N treatment (26 ± 5%) than in the Control (48 ± 18%) and not detected under NPK treatment (Fig. 1b). Moreover, the relative abundance of *Nitrosospira* sp. Nsp2/Nsp17 clade was consistently higher in limed soils (N + CaO: 84 ± 3% and NPK + CaO: 20 ± 6%) than in the corresponding non-limed soils (N: 26 ± 5% and NPK: undetected).

3.2. Microcosm incubation of soils amended with CaO

In order to assess short-term responses of ammonia oxidizers to liming and associated changes in pH, microcosms with soil subjected to N treatment were incubated for two months without CaO addition or with addition of 500, 1000 or 2000 ppm CaO (N, N + 500, N + 1000 and N+2000 treatments, respectively). Liming generally increased soil pH significantly which remained stable in every treatment throughout the incubation period. The highest pH

Table 1
Soil properties, potential nitrification rates (PNR) and ammonia oxidizer abundances in soils from the 2-year field trials.

Treatment	H ₂ O (%)	pH (KCl)	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	SOC (mg kg ⁻¹)	PNR (mg NO ₂ -N kg ⁻¹ d ⁻¹)	AOB (× 10 ⁷ copies g ⁻¹)	AOA (× 10 ⁸ copies g ⁻¹)
Ctrol ^a	10.64 ± 0.10 ^b	4.43 ± 0.09c	10.88 ± 0.57a	0.69 ± 0.19a	54.63 ± 10.79ab	10.46 ± 0.39h	0.94 ± 0.14	13.9 ± 4.68
N	12.29 ± 0.24	3.35 ± 0.01a	19.86 ± 3.08a	12.93 ± 0.34d	60.46 ± 4.54ab	0.56 ± 0.10a	0.41 ± 0.08	0.85 ± 0.22
NK	13.48 ± 0.40	3.41 ± 0.01a	14.44 ± 2.01a	12.46 ± 0.56cd	69.22 ± 9.32ab	2.17 ± 0.10c	1.13 ± 0.44	2.11 ± 0.53
NP	12.92 ± 0.99	3.42 ± 0.03a	13.30 ± 0.43a	11.66 ± 0.49c	125.84 ± 12.01c	1.42 ± 0.06b	5.13 ± 2.73	5.59 ± 1.87
NPK	13.53 ± 0.68	3.47 ± 0.02a	6.75 ± 0.48a	1.94 ± 0.40ab	158.40 ± 14.09d	3.33 ± 0.04d	4.59 ± 1.80	8.15 ± 2.17
N + CaO	11.15 ± 0.21	4.10 ± 0.01b	8.81 ± 0.24a	1.08 ± 0.18ab	69.98 ± 3.64ab	6.54 ± 0.12f	6.38 ± 3.63	0.53 ± 0.08
NK + CaO	11.61 ± 0.09	4.46 ± 0.08c	6.87 ± 0.56a	2.27 ± 0.26b	42.35 ± 3.08a	5.27 ± 0.31e	8.44 ± 1.99	1.64 ± 0.23
NP + CaO	11.70 ± 0.18	4.15 ± 0.04b	12.05 ± 1.20a	1.21 ± 0.19ab	92.14 ± 9.84b	8.13 ± 0.13g	9.66 ± 4.68	1.87 ± 0.49
NPK + CaO	10.97 ± 0.08	4.31 ± 0.01c	13.37 ± 1.36a	0.63 ± 0.12ab	123.48 ± 6.94c	6.93 ± 0.09f	24.2 ± 5.42	4.58 ± 0.03

^a Treatment: control without fertilizers (Ctrol), and with fertilizers N, NK, NP, NPK, and their counterpart with CaO, N + CaO, NK + CaO, NP + CaO, NPK + CaO.

^b Mean ± SD ($n = 3$). Values within the same column followed by the same letter do not differ at $P < 0.05$.

Fig. 1. Community composition of AOA (a) and AOB (b) based on *amoA* gene sequences in soils from field trials with different N fertilizers, with or without CaO. Soils were subjected to annual fertilization for 12 years and to CaO amendment for two years. See also phylogenetic tree in Fig. S2 and S3. Error bars represent the standard error of the mean ($n = 3$).

was measured in the N + 2000 treatment (pH 4.37), followed by N + 1000 and N + 500 treatments (3.82 and 3.62, respectively). The lowest pH value of 3.35 was measured in the N treatment without CaO addition. Nitrate concentrations generally increased throughout the incubation while ammonia concentration decreased sharply after 7 days in all treatments (Fig. 2), which

indicated active nitrification. Net nitrification rates, defined as net accumulation of NO_3^- per gram dry soil per day, were considerably higher during the first seven days of incubation, and significantly higher in the N + 2000 treatment ($0.34 \mu\text{g NO}_3^- \text{N g}^{-1} \text{d}^{-1}$) than in treatments with lower or no CaO addition ($0.21\text{--}0.25 \mu\text{g NO}_3^- \text{N g}^{-1} \text{d}^{-1}$).

Fig. 2. Ammonium (a) and nitrate (b) concentrations during incubation of N-fertilized soil amended with 0, 500, 1000 or 2000 ppm CaO. Error bars represent the standard error of the mean ($n = 3$).

AOA abundance, inferred from archaeal *amoA* gene abundance, increased steadily throughout the incubation period under all treatments, although this increase was only significant in soils amended with the lowest CaO concentration (N + 500) and without CaO addition (N) ($P < 0.05$ at day 60). AOA abundance increased significantly from 8.51×10^7 *amoA* gene copies g^{-1} dry soil at day 0 to 5.40×10^8 and 5.86×10^8 *amoA* gene copies g^{-1} dry soil at day 60 in the N and N + 500 treatments, respectively (Fig. 3a). Increases in AOA abundance were less pronounced under high CaO treatments (N + 1000 and N + 2000), resulting in significantly higher AOA abundance in lower (N + 500) or no CaO (N) than in high CaO treatments (N + 1000, N + 2000) at day 15, 30 and 60 ($P < 0.05$) (Fig. 3a). By contrast, AOB abundance did not change significantly in the N and N + 500 treatments, despite an apparent small decrease, particularly in the untreated control. However, AOB abundance increased sharply and significantly over the initial seven days of incubation under the highest CaO amendments, from 4.08×10^6 to 1.65×10^7 (N + 1000; $P < 0.05$) and 1.70×10^7 (N + 2000; $P < 0.05$) *amoA* gene copies g^{-1} dry soil at day 7, followed by a significant decrease to 5.10×10^6 (N + 1000) and 2.59×10^6 (N + 2000) *amoA* gene copies g^{-1} dry soil at day 60, respectively ($P < 0.05$) (Fig. 3b).

Fig. 3. AOA (a) and AOB (b) abundance, based on *amoA* gene quantification, during microcosm incubations of N-treated soil amended with 0, 500, 1000 or 2000 ppm CaO. Error bars represent the standard error of the mean ($n = 3$). * means significant difference ($P < 0.05$).

3.3. DNA-SIP incubation

In order to assess the active ammonia oxidizer populations in response to liming, soil from field N and N + CaO treatments, and from field N treatment soil freshly amended with 1000 ppm CaO (N + 1000), were incubated in microcosms with addition of $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$ to the headspace, followed by DNA-SIP analysis. Similar to what we observed in microcosm experiment 1, soil pH in the N + 1000 treatment increased by approximately 0.5 units in relation to the N-fertilized soil without CaO and remained stable over the incubation period. Likewise, pH in N + CaO soils remained at about 4.1 throughout the incubation. In general, $\text{NH}_4^+\text{-N}$ concentrations decreased over the incubation period, concomitantly with an increase in $\text{NO}_3^-\text{-N}$ concentrations, indicating active nitrification in all treatments (Table 2). AOA were consistently more abundant than AOB in most treatments throughout incubation, except at days 0 and 7 in the N + CaO soil incubation. Abundance of both AOA and AOB remained relatively constant over time in most treatments, except in N + CaO soil incubations, where abundance of both AOA and AOB increased during the first 7 days (AOA: $P < 0.05$, AOB: $P > 0.05$) but decreased between days 7 and 15 (AOA and AOB: $P < 0.05$), followed by another increase in abundance of AOA

Table 2
Soil properties and ammonia oxidizer abundances in DNA-SIP incubations under different treatments.

Treatment	N ^a				N+1000				N + CaO			
	0 d	7 d	15 d	30 d	0 d	7 d	15 d	30 d	0 d	7 d	15 d	30 d
pH _(KCl)	3.35 ± 0.02 ^b	3.54 ± 0.02	3.47 ± 0.01	3.47 ± 0.06	3.35 ± 0.02	4.02 ± 0.00	3.97 ± 0.03	3.99 ± 0.10	4.10 ± 0.02	4.12 ± 0.05	4.11 ± 0.02	4.11 ± 0.03
NH ₄ ⁺ -N (mg kg ⁻¹ soil)	17.51 ± 0.79	13.42 ± 1.92	6.30 ± 1.51	2.82 ± 0.60	17.51 ± 0.79	4.94 ± 0.41	0.00 ± 0.00	0.00 ± 0.00	8.53 ± 0.82	1.14 ± 0.54	2.94 ± 1.42	1.35 ± 0.65
NO ₃ ⁻ -N (mg kg ⁻¹ soil)	12.77 ± 0.41	12.72 ± 0.56	16.08 ± 0.61	16.94 ± 0.84	12.77 ± 0.41	14.73 ± 0.95	15.30 ± 1.08	17.15 ± 0.49	1.10 ± 0.02	1.39 ± 0.17	1.95 ± 0.18	2.80 ± 0.09
AOA (× 10 ⁷ copies g ⁻¹ soil)	0.65 ± 0.19	0.68 ± 0.14	0.59 ± 0.16	0.35 ± 0.11	0.65 ± 0.19	0.70 ± 0.08	0.58 ± 0.12	0.84 ± 0.20	2.07 ± 0.48	4.37 ± 0.49	1.66 ± 0.38	2.48 ± 0.35
AOB (× 10 ⁷ copies g ⁻¹ soil)	0.29 ± 0.14	0.12 ± 0.02	0.07 ± 0.02	0.02 ± 0.01	0.29 ± 0.14	0.17 ± 0.02	0.12 ± 0.03	0.11 ± 0.02	4.43 ± 1.56	6.33 ± 0.69	1.44 ± 0.52	1.58 ± 0.20

^a Treatment: N and N + CaO, field soil with fertilizer N and the counterpart with CaO, N + CaO; N+1000, Treatment N amended with 1000 ppm CaO before incubation.

^b Mean ± SD (n = 6).

between days 15 and 30 of incubation ($P > 0.05$), but not of AOB ($P > 0.05$) (Table 2).

The relative proportion of *amoA* genes across CsCl gradients was similar between N and N + CaO treatments (Fig. 4). The maximum relative proportion of archaeal *amoA* genes was initially detected in the light fractions (around a buoyant density of 1.69 g ml⁻¹) under ¹²CO₂ headspace but shifted to the heavy fractions (around a buoyant density of 1.72 g ml⁻¹) under ¹³CO₂ headspace at both days 7 and 30 (Fig. 4a), indicating that AOA grew autotrophically by assimilating CO₂ during active nitrification. By contrast, no significant shift in the relative proportion of bacterial *amoA* genes was observed in ¹²CO₂ and ¹³CO₂ incubations throughout the N and N + CaO soil incubations (Fig. 4b), thus showing no evidence of autotrophic growth by AOB. However, very different population dynamics were observed in the N + 1000 treatment: the relative proportion of bacterial *amoA* genes was maximal in the heavy fractions from ¹³CO₂ incubations after 7 days (around a buoyant density of 1.72 g ml⁻¹) but shifted to the light fractions after 30 days, whereas the relative proportion of archaeal *amoA* genes shifted to the heavy fractions only after 30 days (Fig. 4). These results indicated that AOB growth is stimulated by CaO amendment only in the short-term, although within a short period AOA take over as main active autotrophic ammonia oxidizers.

Archaeal and bacterial *amoA* genes were amplified and sequenced from bulk DNA extracted from samples collected at days 0, 7 and 30 of all ¹³CO₂-SIP microcosms to assess changes in community composition throughout the incubation period. Due to unsuccessful PCR amplification, no bacterial *amoA* sequences were obtained from day 30 of the N and N + 1000 treatments. This was likely due to the much lower microbial biomass in these particular samples and general low AOB abundance. Sample storage at 4 °C for approximately four months before using for SIP incubation had considerable impact in the community composition of ammonia oxidizers in most soils, as seen in communities at the beginning of the microcosm incubations in relation to those detected soon after soil sampling (Figs. 1 and 5). The main changes in AOA communities consisted of a relative decrease in Clade C and increase in clade B1 in both N- and N + CaO-treated soils after storage, whereas for AOB, only an increase in relative abundance of *N. multiformis* clade was observed in N-treated soil. In most cases, however, both AOA and AOB communities greatly reverted towards the clade composition initially present in the samples (Figs. 1 and 5). Clade B dominated AOA communities in all microcosms, sub-clade B1 was the most abundant group in N and N + 1000 incubations, with little variability in relative abundance during incubation (Fig. 5a). In turn, the relative abundance of sub-clade B3 decreased from 20% to 1 ± 1% during the first 7 days in the N treatment microcosm and was undetectable at day 30 in the N + 1000 treatment. Clade C was more abundant in N and N + 1000 microcosms than in those with N + CaO soil, where it represented <5% of the community from day 7 onwards (Fig. 5a). The relative abundance of clade C remained relatively constant throughout incubation in the N + 1000 treatment (16%–20 ± 4%) but increased progressively in the N treatment, from 16% at day 0 to 30 ± 4% at day 30 (Fig. 5a). Clade *Nitrosotalea* was detected in all microcosms at day 0, making up 4% and 21% of the AOA communities in N/N + 1000 and N + CaO microcosms, respectively, although it was undetectable after 7 days of incubation (Fig. 5a). AOB communities were initially dominated by *N. multiformis* clade in the N treatment, although after 7 days AOB communities in both N and N + 1000 treatments were very similar to that present in the original soil, dominated by *Nitrosospira* sp. Nsp65/L115 and Nsp2/Nsp17 clades (Figs. 1b and 5b). The main differences between N

Fig. 4. Relative abundance of *amoA* genes of AOA (a) and AOB (b) along a CsCl buoyant density gradient from $^{13}\text{CO}_2$ and $^{12}\text{CO}_2$ treatments in DNA-SIP microcosm incubation of N-fertilized soil after short- (N+1000; 30 days) or long-term liming (N + CaO; 2 years). Seventeen fractions from each CsCl spin were analysed, representing a density range from 1.64 to 1.75 g ml^{-1} . Abundance of archaeal and bacterial *amoA* genes in each fraction was converted to proportion of total *amoA* gene abundance throughout the gradients. Vertical bars represent standard errors of the mean relative abundance ($n = 3$), and horizontal error bars represent the standard error of the buoyant density of the same order fractions ($n = 3$).

and N + 1000 microcosms at day 7 were a higher abundance of *N. multiformis* clade in the former ($26 \pm 17\%$) than in the later ($5 \pm 5\%$), and the detection of *N. briensis* clade only in the N + 1000 microcosm (Fig. 5b). AOB community composition in N + CaO microcosms was nearly identical to that in the original soil and remained relatively unchanged throughout incubation, despite a small increase in the relative abundance of *Nitrosospira* sp. Nsp65/L115 from $13 \pm 4\%$ to $26 \pm 9\%$ (Figs. 1b and 5b).

4. Discussion

Our results show that activity, abundance and population structure of both AOA and AOB are sensitive to liming in fertilized acidic soils. Both AOB abundance and PNRs increased significantly in field plots after a 2-year liming trial, whereas the opposite was observed for AOA abundance. In microcosm incubations amended with different CaO concentrations, the peak of AOB abundance occurred at day 7 in treatments with high CaO levels (1000 and 2000 ppm), whereas AOA abundance peaked at day 60 in treatments with the lowest CaO levels (Control and 500 ppm CaO). These results suggest that liming generally favours AOB growth. Both field samples and incubation microcosm experiments showed that N fertilizer application markedly changed the community composition of AOA and AOB, whereas liming appeared to selectively stimulate and maintain specific AOA and AOB groups, such as clade B and *Nitrosospira* sp. Nsp2/Nsp17 clade (3a.1), respectively. Furthermore, $^{13}\text{CO}_2$ -SIP-DNA experiments showed autotrophic growth of AOA concomitant with nitrification activity in soil from the N and N + CaO field treatments throughout 30-days incubations, suggesting that AOA drive nitrification in these acidic soils regardless of long-term liming, which is consistent with previous studies in other acidic soils (Zhang et al., 2012). However, in N-treated soils freshly amended with CaO, only autotrophic growth of AOB was observed based on $^{13}\text{CO}_2$ -SIP-DNA after seven days of incubation, but not after 30 days when $^{13}\text{CO}_2$ assimilation was only observed for AOA. This indicates that AOB activity is stimulated by CaO addition in acidic soils in the short-term, although within less than one month, nitrification activity was again mainly driven by AOA. Taken together, our results provide evidence for time-dependent shifts between autotrophic ammonia oxidation by AOB and AOA in response to liming in acidic soils.

4.1. Liming induced AOB functioning in acidic soils

Current evidence indicates that AOA are numerically and functionally dominant over AOB in most acidic soils, whereas the latter generally predominate in neutral to alkaline soils (Di et al., 2009; Jia and Conrad, 2009; Prosser and Nicol, 2012; Schleper, 2010; Zhang et al., 2012). Due to ionization of NH_3 to NH_4^+ at low pH, acidic soils are generally ammonia-limited (De Boer and Kowalchuk, 2001). Most cultivated AOA strains have much lower ammonia concentration tolerance ($0.89\text{--}356\text{ }\mu\text{M}$) than cultured AOB ($394\text{--}40,000\text{ }\mu\text{M}$) (He et al., 2012) and, at least some strains, have also lower half-saturation constants (K_m) and higher affinity for ammonia (Martens-Habbena et al., 2009; Park et al., 2010). These physiological features have thus been proposed to confer AOA a general adaptive advantage over AOB in acidic environments. Consistent with these assumptions, AOA outnumbered AOB in all acidic soils studied here and were the only ammonia oxidizers with detectable activity in short-term soil incubations under native conditions, regardless of long-term continuous fertilization over 16 years and lime amendment over 2 years. However, following the 2-year liming trial (1000 ppm CaO), soil pH increased significantly by $0.73\text{--}1.05$ units, with concomitant increases in PNRs by $2.1\text{--}11.7$ -fold and AOB abundance by $1.9\text{--}15.6$ -fold. These results

demonstrate that, despite general dominance of AOA in these acidic soils, AOB populations were favoured by liming in the long-term, leading to larger populations over time. Moreover, microcosm incubations and $^{13}\text{CO}_2$ -DNA-SIP experiments showed that AOB can become active and take over the dominant role in autotrophic ammonia oxidation in the short-term (i.e., one week), shortly after lime amendment. AOB typically dominate ammonia oxidizer communities in N-rich environments, such as neutral-alkaline fertilized soils, and are more stimulated than AOA by increased ammonia availability which can be enhanced by liming in different ways (Schauss et al., 2009; Verhamme et al., 2011). For instance, higher release of soil DOM (dissolved organic matter) and N-mineralization have been observed in response to increases in soil pH following lime application (Persson et al., 1990; Curtin et al., 1998), potentially leading to higher ammonia supply rates and concomitant faster turn-over rates, despite no changes in net NH_4^+ accumulation, as observed here. Liming has also been observed to stimulate NH_3 volatilization in acidic soils, with each increment of 0.1 pH unit driven by liming resulting in an increase of NH_3 emissions to 37.9 kg ha^{-1} compared with no lime treatment at 34.6 kg ha^{-1} (Mkhabela et al., 2006; Smith et al., 2009; Sommer and Ersboll, 1996). Considering NH_3 as the direct substrate for AOB, higher NH_3 volatilization due to liming could alleviate the substrate limitation imposed by greater ionization under acidic conditions. Liming may also increase ammonia availability in acidic soils by stimulating hydrolysis of natively-produced urea or that derived from fertilizers. For example, in pine forest soils subjected to lime amendment, $\text{NH}_4^+\text{-N}$ accounted for $15.5\text{--}18.3\%$ of total applied urea-N 144 h after fertilization, whereas it accounted for $<13\%$ without lime amendment (Niemiera et al., 2014).

4.2. Time-dependent effects of liming on AOA and AOB

Soil pH remained stable throughout incubation of microcosms amended with different CaO concentrations and net nitrification rates peaked between days 0 and 7 under all treatments, concomitant with the highest net NH_4^+ consumption rates (Fig. 2). An increase in AOB abundance was only observed in the first seven days of incubation under the highest CaO concentrations (N + 1000 and N + 2000). Moreover, incorporation of $^{13}\text{CO}_2$ into bacterial *amoA* genes was only observed in soils subjected to fresh CaO amendment (N + 1000) and only after 7 days, but not later, indicating that stimulation of AOB by liming is strongly time-dependent. In a previous study based on 91-day incubations of acidic soils amended with three CaO concentrations, the increase in respiratory quotient (RQ) with soil pH was strongly time-dependent, being greater at day 3 than at day 21, or day 91 (Bertrand et al., 2007). In a similar study following soil incubations for 57 days, the significant time-dependent effects of liming on N mineralization were observed in a soil with initial pH of 4.7 (Fuentes et al., 2006). These studies suggest that stimulation of N mineralization might occur immediately after lime application (i.e., at the beginning of incubation), likely providing sufficient ammonia to trigger activity and growth of AOB in the short-term but not enough to maintain the populations in the long-term. Similarly, in a study conducted in an acidic coniferous forest soil, Hermansson et al. (2004) compared the dynamics of AOB community between limed and non-limed forest soils in two sites and found that the increase in AOB abundance induced by lime was only observed in one of the two sites at the beginning of the growing season but not at the end. Our microcosm incubation experiment amended with medium and high CaO concentrations showed similar trends to those in the study by Hermansson et al. (2004). However, AOB abundance in our field plots were still significantly higher in all 2-year limed treatments than in their corresponding non-limed soils,

Fig. 5. Community composition of AOA (a) and AOB (b) based on *amoA* gene sequences during $^{12/13}\text{CO}_2$ -DNA-SIP incubations of N-fertilized soil under short- (N+1000) or long-term liming (N + CaO). See also phylogenetic tree in Fig. S2 and S3. Error bars represent the standard error of the mean ($n = 3$). n.a., not available.

in contrast to the less pronounced stimulation of AOB in our microcosm incubations and in the field trial by Hermansson et al. (2004). A possible explanation for this discrepancy is the fact that the field sites in our study are located in southern China, which is characterized by a subtropical monsoon climate and consequently great annual rainfall (1300 mm). CaO may be partially hygroscopic and incorporated into $\text{Ca}(\text{OH})_2$ or CaCO_3 aggregations after

application to soils, and frequent rainfall and agricultural practices may induce them to dissolve repetitively and occasionally, providing soil conditions similar to those following fresh lime application. Therefore, such events could possibly lead to repeated bursts in AOB growth and subsequent maintenance of larger populations even after 2 years. This hypothesis is further supported by observations that intact lime pellets were detected in soil more

than 220 days after lime application (Lollato et al., 2013).

4.3. Profiles of AOA and AOB community in response to liming

Analysis of ammonia oxidizer communities based on *amoA* genes showed prominent changes in relative abundance of phylogenetic clades within AOA and AOB in response to liming and fertilization, both in long and short terms. AOA communities were dominated by clade B, namely sub-clade B3, in all long-term fertilization field plots except under N treatment, where clade C predominated (Fig. 1a). Fertilization had a general selective effect on AOA communities, by comparison with unfertilized soil, as seen by the emergence of clades C, B1 and *Nitrosotalea*, and relative decrease of clades A, B2 and F, often to undetectable levels. In a study of distinct Arctic soils, clade B dominated ammonia oxidizer communities in an acidic and NH_4^+ -limited shrub tundra soil, where AOB were undetectable, and were likely responsible for threefold higher gross nitrification rates after ammonium addition (Alves et al., 2013). Moreover, most of AOA from clade B in that study (SO-S phylotype; Fig. S2) correspond to the sub-clade B1 defined here (Fig. S2), which were positively selected in all N-fertilized soils in our study (Fig. 1a). Our observations thus corroborate the dominant role in acidic soils and ammonium preferences of this (sub-)clade. The short-term relative decrease of AOA sub-clade B3 in SIP microcosms after fresh lime amendment is intriguing as it was not reflected on communities in field soils subjected to liming for two years. It is possible that such short-term dynamics were not reflected on AOA communities in the long-term, or resulted from artificial conditions imposed by laboratory storage and incubation. It is also possible that different sub-clades, such as B1 and B3, have different pH optima, despite their general adaptation to low pH, as previously observed across a great diversity of soil AOA (Gubry-Rangin et al., 2011). Clade C was only detected in fertilized soils and dominated AOA communities in soils fertilized only with N, where its relative abundance was drastically reduced after liming for two years (Fig. 1a). Similarly, the relative abundance of this clade increased in SIP microcosms with N-treated soil (N), whereas it remained unchanged after fresh lime amendment (N + 1000) and comprised $\leq 6\%$ of AOA communities in incubations of N + CaO-treated soil (Fig. 5a). Previous multivariate statistical analyses also suggested that AOA from clade C prefer wet organic soils with high N (particularly NO_3^-) and low DOC (dissolved organic carbon) to TDN (total dissolved nitrogen) ratios, such as N-rich peatlands (Alves et al., 2013). In agreement with these observations, the soils studied here were collected in a region with subtropical monsoon climate with high rainfall and N treatment soils contained the highest NH_4^+ and NO_3^- concentrations (Tables 1 and 2). Furthermore, a recent study also showed that AOA associated with this clade were enriched in soils following a 44-year N fertilization trial, by comparison with unfertilized soils (Zhou et al., 2015). These results indicate that clade C organisms are adapted to high N levels and appear to be inhibited by liming. Ammonia oxidation ability of AOA from clade C was first shown in enrichment cultures from Arctic soils (Alves et al., 2013), which also identified this *amoA* gene clade (also referred to as “*Nitrososphaera*-sister cluster”) (Pester et al., 2012) as being associated with the fosmid clone 2914 that represents an AOA group often found in soils. Remarkably, the recently isolated representative of clade C, *Ca. Nitrosocosmicus franklandus* C13, has the highest NH_4^+ tolerance and growth optimum among cultivated AOA, which are within the range of those of AOB (Lehtovirta-Morley et al., 2016).

AOB communities in field soils were dominated by *Nitrosospira* sp. clades Nsp65/L115 and Nsp2/Nsp17, whose relative abundance was greatly affected by both fertilization and liming: *Nitrosospira*

sp. Nsp65/L115 clade was generally favoured in the more acidic fertilized soils, whereas *Nitrosospira* sp. Nsp2/Nsp17 had higher relative abundance in the corresponding limed and control soils with higher pH (Fig. 1b and S3). These results are consistent with a meta-analysis of ammonia oxidizers in Chinese soils showing that *Nitrosospira* sp. Nsp2/Nsp17 clade (clade 3a.1) has been more frequently detected in neutral to alkaline soils and that *Nitrosospira* sp. Nsp65/L115 clade (clades 9–10) typically dominated strongly acidic soils (Shen et al., 2012). Additionally, at least one representative of *Nitrosospira* sp. Nsp65/L115 clade, *Nitrosospira* sp. strain AF, has been isolated from red acidic sandy soil (pH 4.0). In SIP microcosms with N-treated soils, the relative abundance of *Nitrosospira* sp. Nsp65/L115 clade recovered to levels similar to those in the original soil, although without noticeable difference after fresh lime amendment. This suggests that, similarly to what we observed for AOA communities, such short-term population dynamics did not necessarily reflect the net effect of liming in the field in the long-term, such as after the 2-year liming trial. Unfortunately, assessment of further AOB community composition dynamics in soil incubation was hindered by the inability to amplify bacterial *amoA* genes from day 30 samples. Fresh lime amendment might nevertheless stimulate soil ammonia oxidizers through transiently higher NH_3 availability derived from increased urea hydrolysis and DOM release and mineralization. For instance, the assimilation of $^{13}\text{CO}_2$ by AOB at day 7 after fresh lime amendment and concomitant peak in net nitrification without noticeable change in population size suggests that a flush of NH_3 could have stimulated activity by well-adapted and highly efficient nitrifiers. In fact, *Nitrosospira* sp. AF, a representative of *Nitrosospira* sp. Nsp65/L115 clade, is able to grow under highly acidic conditions at extremely slow rates (138 h) but with higher ammonia oxidation activity ($7.1\text{ fmol NO}_2^- \text{ cell}^{-1} \text{ h}^{-1}$) than at least some other *Nitrosospira* strains (e.g., strains 40K1, L115 and B6) (Jiang and Bakken, 1999). A previous study showed that AOA are also functionally heterogeneous, with different AOA clades harbouring distinct nitrification capabilities (Alves et al., 2013). Taken together, this suggests that the time-dependent selective effect of liming on populations of both AOA and AOB observed here is likely to affect gross nitrification activity and consequently also N losses, namely as reactive N species, depending on the interplay between fertilization and lime amendment regimes.

5. Conclusion

In conclusion, liming of acidic soils greatly affects both the abundance and community structure of ammonia oxidizers, leading to changes in dominant clades and net nitrification activity that could not be individually explained by pH or NH_4^+ availability. As expected, AOA generally dominated these acidic soils, although AOB also played a role in response to liming, namely in the short-term, with AOA and AOB alternating as the main active ammonia oxidizers in a time-dependent manner. However, the possible repeated stimulation of highly adapted and efficient AOB due to recurrent effects of liming likely led to the greater AOB abundance and higher soil nitrification potential observed after two years of liming. Our results provide fundamental insights into nitrifier dynamics in response to liming in acidic soils and relevant knowledge for fertilization and liming strategies in agroecosystem management.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (41322007, 41171217) and Youth Innovation Promotion Association (2012031), Chinese Academy of Sciences. Ricardo J. E. Alves was funded by project P25369 of the Austrian Science Fund (FWF). We would like to thank Prof. Minggang Xu for access to the long-term experimental field plots, Mr Boren Wang and Mr Dongchu Li for assistance in soil sampling, and Dr. Hangwei Hu and Mr Tong Sun for technical support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2017.05.001>.

References

- Abell, G.C., Robert, S.S., Frampton, D.M., Volkman, J.K., Rizwi, F., Csontos, J., Bodrossy, L., 2012. High-throughput analysis of ammonia oxidiser community composition via a novel, *amoA*-based functional gene array. *PLoS One* 7, e51542.
- Álvarez, E., Viadé, A., Fernández-Marcos, M.L., 2009. Effect of liming with different sized limestone on the forms of aluminium in a Galician soil (NW Spain). *Geoderma* 152, 1–8.
- Alves, R.J., Wanek, W., Zappe, A., Richter, A., Svenning, M.M., Schleper, C., Urich, T., 2013. Nitrification rates in Arctic soils are associated with functionally distinct populations of ammonia-oxidizing archaea. *The ISME Journal* 7, 1620–1631.
- Andert, J., Wessén, E., Börjesson, G., Hallin, S., 2011. Temporal changes in abundance and composition of ammonia-oxidizing bacterial and archaeal communities in a drained peat soil in relation to N₂O emissions. *Journal of Soils and Sediments* 11, 1399–1407.
- Avrahami, S., Bohannan, B.J., 2007. Response of *Nitrosospora* sp. strain AF-like ammonia oxidizers to changes in temperature, soil moisture content, and fertilizer concentration. *Applied and Environmental Microbiology* 73, 1166–1173.
- Barton, L., Gleeson, D.B., Maccarone, L.D., Zúñiga, L.P., Murphy, D.V., 2013. Is liming soil a strategy for mitigating nitrous oxide emissions from semi-arid soils? *Soil Biology and Biochemistry* 62, 28–35.
- Bernhard, U., 1986. Natural and anthropogenic components of soil acidification. *Journal of Plant Nutrition and Soil Science* 149, 702–717.
- Bertrand, I., Delfosse, O., Mary, B., 2007. Carbon and nitrogen mineralization in acidic, limed and calcareous agricultural soils: apparent and actual effects. *Soil Biology and Biochemistry* 39, 276–288.
- Bolan, N.S., Hedley, M.J., 2005. Role of carbon, nitrogen, and sulfur cycles in soil acidification. In: Rengel, Z. (Ed.), *Handbook of Soil Acidity*. CRC press, New York, pp. 29–56.
- Caires, E.F., Garbuio, F.J., Churka, S., Barth, G., Corrêa, J.C.L., 2008. Effects of soil acidity amelioration by surface liming on no-till corn, soybean, and wheat root growth and yield. *European Journal of Agronomy* 28, 57–64.
- Che, J., Zhao, X.Q., Zhou, X., Jia, Z.J., Shen, R.F., 2015. High pH-enhanced soil nitrification was associated with ammonia-oxidizing bacteria rather than archaea in acidic soils. *Applied Soil Ecology* 85, 21–29.
- Curtin, D., Campbell, C., Jallil, A., 1998. Effects of acidity on mineralization: pH-dependence of organic matter mineralization in weakly acidic soils. *Soil Biology and Biochemistry* 30, 57–64.
- De Boer, W., Kowalchuk, G., 2001. Nitrification in acid soils: micro-organisms and mechanisms. *Soil Biology and Biochemistry* 33, 853–866.
- 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., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods* 10 (10), 996–998.
- Fan, F., Yang, Q., Li, Z., Wei, D., Cui, X., Liang, Y., 2011. Impacts of organic and inorganic fertilizers on nitrification in a cold climate soil are linked to the bacterial ammonia oxidizer community. *Microbial Ecology* 62, 982–990.
- Farmaha, B.S., 2014. Evaluating animo model for predicting nitrogen leaching in rice and wheat. *Arid Land Research and Management* 28, 25–35.
- Fornara, D.A., Steinbeiss, S., McNamara, N.P., Gleixner, G., Oakley, S., Poulton, P.R., Macdonald, A.J., Bardgett, R.D., 2011. Increases in soil organic carbon sequestration can reduce the global warming potential of long-term liming to permanent grassland. *Global Change Biology* 17, 1925–1934.
- 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 Science of the United States of America* 102, 14683–14688.
- Fuentes, J.P., Bezdicsek, D.F., Flury, M., Albrecht, S., Smith, J.L., 2006. Microbial activity affected by lime in a long-term no-till soil. *Soil and Tillage Research* 88, 123–131.
- Gleeson, D.B., Müller, C., Banerjee, S., Ma, W., Siciliano, S.D., Murphy, D.V., 2010. Response of ammonia oxidizing archaea and bacteria to changing water filled pore space. *Soil Biology and Biochemistry* 42, 1888–1891.
- Gubry-Rangin, C., Nicol, G.W., Prosser, J.I., 2010. Archaea rather than bacteria control nitrification in two agricultural acidic soils. *FEMS Microbiology Ecology* 74, 566–574.
- Gubry-Rangin, C., Hai, B., Quince, C., Engel, M., Thomson, B.C., James, P., Schlöter, M., Griffiths, R.I., Prosser, J.I., Nicol, G.W., 2011. Niche specialization of terrestrial archaeal ammonia oxidizers. *Proceedings of the National Academy of Science of the United States of America* 108, 21206–21211.
- Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* 59, 307–321.
- Guo, J.H., Liu, X.J., Zhang, Y., Shen, J.L., Han, W.X., Zhang, W.F., Christie, P., Goulding, K.W., Vitousek, P.M., Zhang, F.S., 2010. Significant acidification in major Chinese croplands. *Science* 327, 1008–1010.
- He, J.Z., Shen, J.P., Zhang, L.M., Zhu, Y.G., Zheng, Y.M., Xu, M.G., Di, H., 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. *Environmental Microbiology* 9, 2364–2374.
- He, J.Z., Hu, H.W., Zhang, L.M., 2012. Current insights into the autotrophic thaumarchaeal ammonia oxidation in acidic soils. *Soil Biology and Biochemistry* 55, 146–154.
- Helyar, K.R., Porter, W.M., 1989. Soil acidification, its measurement and the processes involved. In: Robson, A.D. (Ed.), *Soil Acidity and Plant Growth*. Academic press, Sydney, pp. 61–101.
- Hermansson, A., Bäckman, J.S.K., Svensson, B.H., Lindgren, P.E., 2004. Quantification of ammonia-oxidising bacteria in limed and non-limed acidic coniferous forest soil using real-time PCR. *Soil Biology and Biochemistry* 36, 1935–1941.
- Jia, Z., Conrad, R., 2009. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. *Environmental Microbiology* 11, 1658–1671.
- Jiang, Q., Bakken, L., 1999. Comparison of *Nitrosospora* strains isolated from terrestrial environments. *FEMS Microbiology Ecology* 30, 171–186.
- Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30, 772–780.
- Kerley, S.J., 2000. The effect of soil liming on shoot development, root growth, and cluster root activity of white lupin. *Biology and Fertility of Soils* 32, 94–101.
- Kerou, M., Alves, R.J.E., Schleper, C., 2016. *Nitrososphaeria*. *Bergey's Manual of Systematics of Archaea and Bacteria*, pp. 1–8.
- Konneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437, 543–546.
- Kurola, J., Salkinoja-Salonen, M., Aarnio, T., Hultman, J., Romantschuk, M., 2005. Activity, diversity and population size of ammonia-oxidising bacteria in oil-contaminated landfarming soil. *FEMS Microbiology Letters* 250, 33–38.
- Lehtovirta-Morley, L.E., Ross, J., Hink, L., Weber, E.B., Gubry-Rangin, C., Thion, C., Prosser, J.I., Nicol, G.W., 2016. Isolation of 'Candidatus Nitrosocosmicus franklandus', a novel ureolytic soil archaeal ammonia oxidiser with tolerance to high ammonia concentration. *FEMS Microbiology Ecology* 92, fiw057.
- Leininger, S., Urich, T., Schlöter, 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.
- Lollato, R.P., Edwards, J.T., Zhang, H., 2013. Effect of alternative soil acidity amelioration strategies on soil pH distribution and wheat agronomic response. *Soil Science Society of America Journal* 77, 1831–1841.
- Long, X., Chen, C., Xu, Z., Oren, R., He, J.Z., 2012. Abundance and community structure of ammonia-oxidizing bacteria and archaea in a temperate forest ecosystem under ten-years elevated CO₂. *Soil Biology and Biochemistry* 46, 163–171.
- Lu, L., Han, W., Zhang, J., Wu, Y., Wang, B., Lin, X., Zhu, J., Cai, Z., Jia, Z., 2012. Nitrification of archaeal ammonia oxidizers in acid soils is supported by hydrolysis of urea. *The ISME Journal* 6, 1978–1984.
- Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., Stahl, D.A., 2009. Ammonia oxidation kinetics determine niche separation of nitrifying archaea and bacteria. *Nature* 461, 976–979.
- Minh, B.Q., Nguyen, M.A., von Haeseler, A., 2013. Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution* 30, 1188–1195.
- Mkhabela, M.S., Gordon, R., Burton, D., Madani, A., Hart, W., 2006. Effect of lime, dicyandiamide and soil water content on ammonia and nitrous oxide emissions following application of liquid hog manure to a marshland soil. *Plant and Soil* 284, 351–361.
- Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution* 32, 268–274.
- Niemiera, A.X., Taylor, L.L., Shreckhise, J.H., 2014. Urea hydrolysis in pine tree substrate is affected by urea and lime rates. *HortScience* 49, 1437–1443.
- Park, H.D., Wells, G.F., Bae, H., Criddle, C.S., Francis, C.A., 2006. Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. *Applied and Environmental Microbiology* 72, 5643–5647.
- Park, B.J., Park, S.J., Yoon, D.N., Schouten, S., Sinninghe Damste, J.S., Rhee, S.K., 2010. Cultivation of autotrophic ammonia-oxidizing archaea from marine sediments in coculture with sulfur-oxidizing bacteria. *Applied and Environmental Microbiology* 76, 7575–7587.
- Pereira, E.S.M.C., Poly, F., Guillaumaud, N., van Elsas, J.D., Salles, J.F., 2012. Fluctuations in ammonia oxidizing communities across agricultural soils are driven by soil structure and pH. *Frontiers in Microbiology* 3, 77.

- Persson, T., Wiren, A., Andersson, S., 1990. Effects of liming on carbon and nitrogen mineralization in coniferous forests. *Water Air and Soil Pollution* 54, 351–364.
- 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.
- Prosser, J.I., 1990. Autotrophic nitrification in bacteria. *Advances in Microbial Physiology* 30, 125–181.
- Prosser, J.I., Nicol, G.W., 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends in Microbiology* 20, 523–531.
- Rothauwe, J., Witzel, K., 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.
- Ruyters, S., Nicol, G.W., Prosser, J.I., Lievens, B., Smolders, E., 2013. Activity of the ammonia oxidising bacteria is responsible for zinc tolerance development of the ammonia oxidising community in soil: a stable isotope probing study. *Soil Biology and Biochemistry* 58, 244–247.
- Schauss, K., Focks, A., Leininger, S., Kotzerke, A., Heuer, H., Thiele-Bruhn, S., Sharma, S., Wilke, B.M., Matthies, M., Smalla, K., Munch, J.C., Amelung, W., Kaupenjohann, M., Schlöter, M., Schleper, C., 2009. Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. *Environmental Microbiology* 11, 446–456.
- Schleper, C., 2010. Ammonia oxidation: different niches for bacteria and archaea? *The ISME Journal* 4, 1092–1094.
- Shanmugam, S., Abbott, L.K., Murphy, D.V., 2014. Clay addition to lime-amended biosolids overcomes water repellence and provides nitrogen supply in an acid sandy soil. *Biology and Fertility of Soils* 50, 1047–1059.
- Shen, J.P., Zhang, L.M., Di, H.J., He, J.Z., 2012. A review of ammonia-oxidizing bacteria and archaea in Chinese soils. *Frontiers in Microbiology* 3, 296.
- Smith, E., Gordon, R., Bourque, C., Campbell, A., Genermont, S., Rochette, P., Mkhabela, M., 2009. Simulated management effects on ammonia emissions from field applied manure. *Journal of Environmental Management* 90, 2531–2536.
- Sommer, S., Ersboll, A., 1996. Effect of air flow rate, lime amendments, and chemical soil properties on the volatilization of ammonia from fertilizers applied to sandy soils. *Biology and Fertility of Soils* 21, 53–60.
- Soubrier, J., Steel, M., Lee, M.S., Der Sarkissian, C., Guindon, S., Ho, S.Y., Cooper, A., 2012. The influence of rate heterogeneity among sites on the time dependence of molecular rates. *Molecular Biology and Evolution* 29, 3345–3358.
- Tourna, M., Freitag, T.E., Nicol, G.W., Prosser, J.I., 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. *Environmental Microbiology* 10, 1357–1364.
- Treusch, A.H., Leininger, S., Kletzin, A., Schuster, S.C., Klenk, H.P., Schleper, C., 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environmental Microbiology* 7, 1985–1995.
- Uexküll, H., Mutert, E., 1995. Global extent, development and economic impact of acid soils. *Plant and Soil* 171, 1–15.
- Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D., Paulsen, I., Nelson, K.E., Nelson, W., Fouts, D.E., Levy, S., Knap, A.H., Lomas, M.W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y.H., Smith, H.O., 2004. Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304, 66–74.
- Verhamme, D.T., Prosser, J.I., Nicol, G.W., 2011. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *The ISME Journal* 5, 1067–1071.
- Wallace, A., 1994. Soil acidification from use of too much fertilizer. *Communications in Soil Science and Plant Analysis* 25, 87–92.
- Wang, Q., Wang, S., 2011. Response of labile soil organic matter to changes in forest vegetation in subtropical regions. *Applied Soil Ecology* 47, 210–216.
- Wrage, N., Velthof, G., van Beusichem, M., Oenema, O., 2001. Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry* 33, 1723–1732.
- Xia, W., Zhang, C., Zeng, X., Feng, Y., Weng, J., Lin, X., Zhu, J., Xiong, Z., Xu, J., Cai, Z., Jia, Z., 2011. Autotrophic growth of nitrifying community in an agricultural soil. *The ISME Journal* 5, 1226–1236.
- Xiao, R., Chen, B., Liu, Y., Wang, C., Gu, J.D., Feng, H., Du, G., Ma, X., 2014. Higher abundance of ammonia oxidizing archaea than ammonia oxidizing bacteria and their communities in Tibetan alpine meadow soils under long-term nitrogen fertilization. *Geomicrobiology Journal* 31, 597–604.
- Yao, H., Gao, Y., Nicol, G.W., Campbell, C.D., Prosser, J.I., Zhang, L., Han, W., Singh, B.K., 2011. Links between ammonia oxidizer community structure, abundance, and nitrification potential in acidic soils. *Applied and Environmental Microbiology* 77, 4618–4625.
- Ying, J.Y., Zhang, L.M., He, J.Z., 2010. Putative ammonia-oxidizing bacteria and archaea in an acidic red soil with different land utilization patterns. *Environmental Microbiology Reports* 2, 304–312.
- Zaniewicz-Bajkowska, A., Rosa, R., Franczuk, J., Kosterna, E., 2007. Direct and secondary effect of liming and organic fertilization on cadmium content in soil and in vegetables. *Plant Soil and Environment* 53, 473–481.
- Zhang, L.M., Wang, M., Prosser, J.I., Zheng, Y.M., He, J.Z., 2009. Altitude ammonia-oxidizing bacteria and archaea in soils of Mount Everest. *FEMS Microbiology Ecology* 70, 52–61.
- Zhang, L.M., Hu, H.W., Shen, J.P., He, J.Z., 2012. Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. *The ISME Journal* 6, 1032–1045.
- 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.