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The relative contribution of nitrifiers to autotrophic nitrification across a pH-gradient in a vegetable cropped soil

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Abstract

Purpose Microbial nitrification plays an important role in nitrogen cycling in ecosystems. Nitrification is performed by ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) including complete ammonia oxidizers. However, the relative importance of nitrifiers in autotrophic nitrification in relation to soil pH is still unclear.

Materials and methods Combining DNA-based stable isotope probing (SIP) and molecular biological techniques, we investigated the abundance, structure, and activity of AOA, AOB, and NOB along a pH-gradient (3.97–7.04) in a vegetable cropped soil.

Results and discussion We found that AOA abundance outnumbered AOB abundance and had a significantly negative relationship with soil pH. The abundances of NOB *Nitrospira* 16S rRNA, *nxrB* gene, and *Nitrobacter nxrA* gene were affected by soil pH. Incubation of soil with $^{13}CO_2$ and DNA-SIP analysis demonstrated that significant $^{13}CO_2$ assimilation by AOA rather than by AOB occurred in the acidic soils, whereas the labeled ^{13}C level of AOA was much less in the neutral soil than in the acidic soils. There was no evidence of $^{13}CO_2$ assimilation by NOB except for *Nitrobacter* with *NxrB* gene at pH 3.97. Phylogenetic analysis of AOA *amoA* gene in the ^{13}C- and ^{12}C-labeled treatments showed that the active AOA mainly belonged to *Nitrososphaera* in the acidic soils.

Conclusions These results suggested that the main performer of nitrification was AOA in the acidic soils, but both AOA and AOB participated in nitrification in the neutral soil with low nitrification activity. NOB *Nitrospira* and *Nitrobacter* did not grow in the soils with pH 4.82–7.04 and other populations of NOB were probably involved in nitrite oxidation in the vegetable cropped soil.

Keywords Nitrifiers · Soil pH · Stable isotope probing · Vegetable soil

1 Introduction

The processes of nitrogen cycling driven by microorganisms include nitrogen fixation, ammoniation, nitrification, and denitrification (Gruber and Galloway 2008). Among these steps, nitrification is very important in biogeochemical cycles, leading to nitrate loss and pollution (Zhao et al. 2010). Aerobic autotrophic nitrification is a two-step process driven

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by ammonia oxidizers and nitrite oxidizers, respectively. However, recent studies suggested that some species of *Nitrospira* can conduct complete ammonia oxidation (comammox) (Hu and He 2017). Ammonia oxidation is the first step in which ammonia is oxidized to nitrite, also the rate-limiting step of nitrification. It is carried out by chemolithoautotrophic AOB and AOA by the key enzyme, ammonia mono-oxygenase (AMO). AOB was previously believed to be the exclusive performer in microbial ammonia oxidation until the discovery of AOA and archaeal *amoA* gene (de Boer and Kowalchuk 2001; Venter et al. 2004). Recent investigation into the *amoA* gene revealed that this gene encodes the first subunit of AMO enzyme, and that AOA, in addition to AOB, play a main role in ammonia oxidation in acidic soil or infertile soil (Leininger et al. 2006; He et al. 2007).

AOA and AOB are widely distributed in terrestrial systems. They are found in paddy soil, vegetable soil, seawater, sediment, wetland, rhizosphere soil, and hot spring (Francis et al. 2005; Chen et al. 2008; Santoro and Casciotti 2011; Zhang et al. 2011; Lee et al. 2014). The two groups of ammonia oxidizers are different in cellular biochemistry and physiology, which cause the differential of abundance and community structure of ammonia oxidizers in different agroecosystems (Shen et al. 2008; Yao et al. 2011; He et al. 2012). Many factors can affect ammonia oxidizers, such as soil pH, oxygen and substrate concentration, temperature, etc. (Santoro et al. 2008; Shen et al. 2008; Morimoto et al. 2011). Among them, soil pH is an important driver of ammonia oxidizer activity, abundance, and community structure (Stephen et al. 1998; Nicol et al. 2008). Hu et al. (2013) confirmed that the distribution of AOA and AOB was driven by soil pH, and the AOA/AOB ratio decreased with an increase in soil pH. They used 65 soil samples collected from different regions and found that AOA had a competitive advantage over AOB. Nicol et al. (2008) found that the AOA *amoA* gene abundance decreased with increasing soil pH, while AOB *amoA* gene abundance generally increased with increasing pH. Generally, the quantity of AOA is greater than the quantity of AOB in acidic soils, demonstrating that AOA has a stronger ability to adapt to low pH habitats (Leininger et al. 2006).

Relative contribution of AOA and AOB to ammonia oxidation depends on soil conditions and ecosystems (Prosser and Nicol 2008). Autotrophic AOB has been adjudged the foremost contributor to ammonia oxidation before (Purkhold et al. 2000; Bock et al. 2006). However, the contribution of AOA to autotrophic nitrification should not be underestimated in light of *amoA* gene and AOA research (Yao et al. 2011; Zhang et al. 2011). Yao et al. (2011) found that AOA was more abundant than AOB in Chinese tea orchard soils. In addition, soil nitrification potential and AOA abundance were positively correlated, but there was no significant relationship between nitrification potential and AOB abundance, indicating that AOA had a greater role in soil ammonia oxidation. Most studies showed that AOA accounts for about 10% of the total quantity of soil microorganisms, and that AOA *amoA* gene copies were up to 3000-fold more abundant than AOB *amoA* gene copies (Leininger et al. 2006). Nonetheless, these observations do not definitively mean that ammonia oxidation is exclusively or mainly governed by AOA as implied by the extremely high abundance (Jia and Conrad 2009; Souza 2009). A previous study on agricultural soil found close correlation between the activity of ammonia oxidation and AOB *amoA* gene abundance, and observed incorporation of ^{13}C-CO_2 into the AOB *amoA* gene, but not AOA (Jia and Conrad 2009). In addition, a study of long-term field experimental plots revealed that AOA had higher abundance compared with AOB in all the treatments. However, the two types of ammonia oxidizers had significant positive relationships with potential nitrification rates, demonstrating that both AOA and AOB drove the process of autotrophic ammonia oxidation (He et al. 2007).

The second step of nitrification is nitrite oxidation. In this step, nitrite is oxidized to nitrate by enzyme nitrite oxidoreductase (NXR), which is mediated by nitrite-oxidizing bacteria (NOB). Because ammonia oxidation is the rate-limiting step, the second step is often given little attention. NOB are classified into four genera, namely *Nitrobacter*, *Nitrospira*, *Nitrococcus*, and *Nitrospina.* Among them, research into *Nitrobacter* and *Nitrospira* has dominated (Bartosch et al. 2002; Freitag et al. 2005). Unlike ammonia oxidizers, target-specific 16S rRNA primers to NOB are not available for complicated environment systems (Freitag et al. 2005, Li et al. 2018). In contrast, 16S rRNA primers and nxrB primers can be successfully used to target *Nitrospira* strains (Pester et al. 2014), and *nxrA* (Poly et al. 2008) or *nxrB* (Pester et al. 2014) is widely used for studying *Nitrobacter* groups in soil. The NXR active site in *Nitrobacter* is cytoplasmic-orientated, whereas the NXR active site in *Nitrospira* faces the periplasm (Spieck and Bock 2005; Sorokin et al. 2012). The difference of adaptation and niche differentiation among NOB species could be due to the different membrane orientations of the active site of NXR (Spieck and Bock 2005). Previously, most of the researches focused on *Nitrobacter* and suggested that *Nitrobacter* played a main role in nitrite oxidation, but recent studies suggest *Nitrospira* may be of significant importance as well.

DNA-SIP technique has been successfully used to link microorganisms to specific functions within different ecosystems (Radajewski et al. 2000; Uhlík et al. 2009). Lately, $^{13}CO_2$-DNA-SIP experiments persuasively linked autotrophic nitrification activity and ammonia oxidizers together in an acidic soil (Lehtovirta-Morley et al. 2011), whereas previously, this was only shown in the neutral sediment of lakes (Whitby et al. 2001), estuaries (Freitag et al. 2006), and neutral agricultural soil (Jia and Conrad 2009). However, few studies have been able to clearly demonstrate the dominating nitrifiers along a pH-gradient of arable soil. Thus, our work investigated the

relative contributions of AOA, AOB, and NOB to autotrophic nitrification and the active microbial groups at four pH levels (3.97–7.04) of vegetable soil using molecular tools. Our hypothesis is that the abundance, structure, and activity of AOA, AOB, and NOB are altered by soil pH.

2 Materials and methods

2.1 Study site and soil sampling

Soil samples at a depth of 0 to 20 cm were collected in June 2015 from a vegetable field, located in Ningbo (121° 51′ N, 29° 54′ E), China. The soil was derived from quaternary red earth and classified as red soil (equivalent to Ultisols in US soil taxonomy), with a texture of sandy loam and pH of 3.97. The detailed information about the sampling site has been previously described (Xi et al. 2017). The soil samples were sieved through a 2-mm mesh after air-drying. Soil pH was determined using a pH meter (soil/water, 1:2.5).

2.2 Experimental design

Two-kilogram soil was homogeneously mixed with lime at the rates of 0, 2.0, 8.0, and 40.0 g $CaCO_3$ kg^{-1} dry soil, and soil pH was adjusted to 3.97, 4.82, 6.07, and 7.04, respectively. Soil samples were pre-incubated at 25 °C and 50% WHC (water-holding capacity) for 1 week to allow the effects of lime on soil pH to stabilize. Potential nitrification rate (PNR) was measured by incubating soil (added with 200 mg NH_4^+-N kg^{-1}) at 25 °C and 50% WHC for 24 h and determining the changes in nitrate concentrations using a continuous flow analyzer, according to a procedure described by Yao et al. (2011). Two treatments for the four soil pH levels employed in the incubation were 5% (*v*/*v*) ^{12}C-CO_2 and 5% ^{13}C-CO_2 (99 atom%; Sigma-Aldrich, USA) with three replicates. Stable isotope probing incubations were conducted in 120-ml serum bottles containing 10 g of fresh soil samples covered with rubber stoppers and aluminum caps. The head-space air in the bottles was refreshed every 2 days to provide oxygen for microcosms. Then, CO_2 concentration was reestablished by injection. The soil samples were incubated at 25 °C in the dark for 28 days. Destructive sampling was conducted at day 28, and then stored at − 80 °C for molecular analysis.

2.3 DNA extraction and SIP fractionation

DNA was extracted from 500-mg freeze-dried soil using the FastDNA® SPIN Soil Kit following the user manual. The extracted DNA was dissolved in 100 μl of RNase-free sterile water. DNA concentration was checked using a NanoDrop ND-1000 spectrophotometer (USA), then DNA samples were put into a − 20 °C freezer for further analysis.

SIP fractionation was performed in centrifuge tubes (Beckman Coulter, USA), and subject to centrifugation at 190000×*g* for 44 h at 20 °C. The purpose of CsCl solution and gradient buffer was to regulate the refractive index to 1.3999 by hand-held refractometer (Reichert, NY). After centrifugation, samples in tubes were divided into 15 samples evenly by a single-channel syringe pump (LSP01-1A, China). The method for DNA purification using PEG 6000 and 70% ethanol washing was described by Griffiths et al. (2000). The final product was dissolved in 30 μl of sterile water.

2.4 Real-time quantitative polymerase chain reaction

We used quantitative polymerase chain reaction (Q-PCR) to quantify targeted genes on a Light Cycler Roche 480 instrument (Roche Molecular Systems). The primers for AOA, AOB, and NOB were listed in Table 1. Amplification was performed with 20-μl of solution consisting of 0.5 μM each primer, 10 μl of SYBR® Premix, and 1 μl of 10-fold dilution DNA template. Amplification conditions of AOA and AOB were as follows: 95 °C for 5 min, 40 cycles of 5 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C. Annealing temperatures of *Nitrobacter* and *Nitrospira* were 58 and 52 °C, respectively. Other amplification conditions were same as for AOA and AOB. The amplification efficiencies were higher than 90%, with r^2 values between 0.95 and 0.99 for all qPCR assays.

2.5 Cloning and sequence analyses

We constructed AOA and AOB clone libraries from the treatments at four pH levels with the primers listed in Table 1. The sequences displaying more than 97% nucleotide similarities with each other were considered to belong to the same operational taxonomic unit (OTU) by the DNAMAN software, then selected representative sequences were blasted and phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis (MEGA 6.0) software using the neighbor-joining method (Tamura et al. 2013).

2.6 Statistical analysis

Quantitative PCR data were log-transformed before further analysis. To compare the abundance of AOA, AOB, and NOB among all treatments, data were analyzed using ANOVA with the SPSS 19.0 software (IBM, USA). Pearson correlation analysis ($P < 0.05$) was also performed to test the correlation between soil pH and microbial abundances. In order to predict the variations among the ammonia oxidizer communities, the clone libraries data were analyzed in the CANOCO version 4.5. The phylogenetic trees were constructed using the MEGA version 6.0 software (Tamura et al. 2013).

Table 1 Primers of nitrifiers used in this study

Target gene	Primer	Sequence (5′-3′)	Length of amplicon (bp)	Reference
AOA *amoA*	CrenamoA23f CrenamoA616r	ATGGTCTGGCTWAGACG GCCATCCATCTGTATGTCCA	635	(Nicol et al. 2008)
AOB *amoA*	amoA-1F amoA -2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	(Rotthauwe et al. 1997)
Nitrobacter nxrA	F1nxrA R2nxrA	CAGACCGACGTGTGCGAAAG TCCACAAGGAACGGAAGGTC	322	(Franck et al. 2008) (Wertz et al. 2008)
Nitrobacter nxrB	nxrB1F nxrB1R	ACGTGGAGACCAAGCCGGG CCGTGCTGTTGAYCTCGTTGA	411	(Vanparys et al. 2007)
Nitrobacter nxrB	nxrB179F nxrB403R	ACACGTTGGGARGACCAGAC/ACCCGTTGGGAAGA CCAGAC GCCTCRATSGTGTCCATGTA	263	(Meng 2016)
Nitrospira 16S rRNA	Nspra675f Nspra746r	GCGGTGAAATGCGTAGAKATC G TCAGCGTCAGRWAYGTTCCAGAG	72	(Graham et al. 2007)
Nitrospira nxrB	nxrB169f nxrB638r	TACATGTGGTGGAACA CGGTTCTGGTCRATCA	485	(Pester et al. 2014)

A bootstrap analysis with 500 replicates was performed to evaluate the cluster stability.

2.7 Accession numbers of nucleotide sequences

Sequence data were deposited at GenBank with accession numbers MF323888-MF324007, MF324008-MF324127, MF324128-MF324247, and MF324248-MF324367 for the *amoA* gene of AOA-labeled treatments at pH 3.97, 4.82, 6.07, and 7.04, respectively; MF324368-MF324487, MF324488-MF324607, MF324608-MF324727, and MF324728-MF324847 for the *amoA* gene of AOA-unlabeled treatments at pH 3.97, 4.82, 6.07 and 7.04, respectively; MF322928-MF323047, MF323048-MF323167, MF323168-MF323287, and MF323288-MF323407 for the *amoA* gene of AOB at pH 3.97, 4.82, 6.07, and 7.04, respectively.

3 Results

3.1 The abundance of AOA, AOB, and NOB

The population sizes of AOA, AOB, and NOB in both labeled and unlabeled treatments at different pH levels on day 28 were shown in Figs. 1 and 2. The AOA abundances ranged from 3.08×10^6 to 1.01×10^9 copies g^{-1} dry soil and remained at higher levels compared to AOB abundances. The highest and lowest AOA populations were observed at soil pH 3.97 and 7.04, respectively. A negative correlation was found between the AOA *amoA* gene abundance and soil pH ($r = -0.953$, $P < 0.01$). The mean AOB abundance was 2.31×10^4 copies g^{-1} dry soil, with no significant differences among the four pH levels. Mineral N (NH_4^+-N and NO_3^--N) contents during the incubation have been reported by Xi et al. (2017). Briefly, soil NH_4^+-N and NO_3^--N concentrations ranged from 60 to 100, and 100 to 175 mg kg^{-1} after the one-week pre-incubation, respectively. After 28 days of incubation, the soil NO_3^--N concentrations increased by 80–100 mg kg^{-1} at pH 3.97–6.07. However, the NO_3^--N concentration remained relatively stable at pH 7.04 and was much lower than those at the other pH levels. Soil PNRs were 5.9 ± 0.5, 7.7 ± 0.4, 4.9 ± 0.5, and 0.9 ± 0.1 mg kg^{-1} day^{-1} at pH 3.97, 4.82, 6.07, and 7.04, respectively. The PNRs were positively correlated with the AOA abundances ($r = 0.889$, $P < 0.01$), but there was no significant relationship between PNRs and AOB abundances.

For NOB, *Nitrobacter* and *Nitrospira* abundances changed with the pH gradient (Fig. 2). However, the five functional genes showed different trends. The abundance of *Nitrobacter nxrA*, ranging from 2.34×10^4 to 1.32×10^6 copies g^{-1} dry soil, decreased with the increase in soil pH, but the abundance of *Nitrobacter nxrB* with two different primers showed no significant differences among the four pH levels. There were significant differences in the abundance of *Nitrospira* 16S rRNA and *nxrB* genes among the four pH levels. Lower *Nitrospira* 16S rRNA gene abundances at pH 3.97 and pH 4.82 were found compared to the other two pH levels. The abundance of *Nitrospira nxrB* gene increased with the increase in soil pH.

3.2 DNA-SIP of nitrifiers in the vegetable soil

Enrichments of ^{13}C for nitrifiers were identified by adding different inorganic carbon sources and determining the abundances of targeted genes after incubation. Fifteen fractions were obtained from the ultracentrifugation. The *amoA* gene

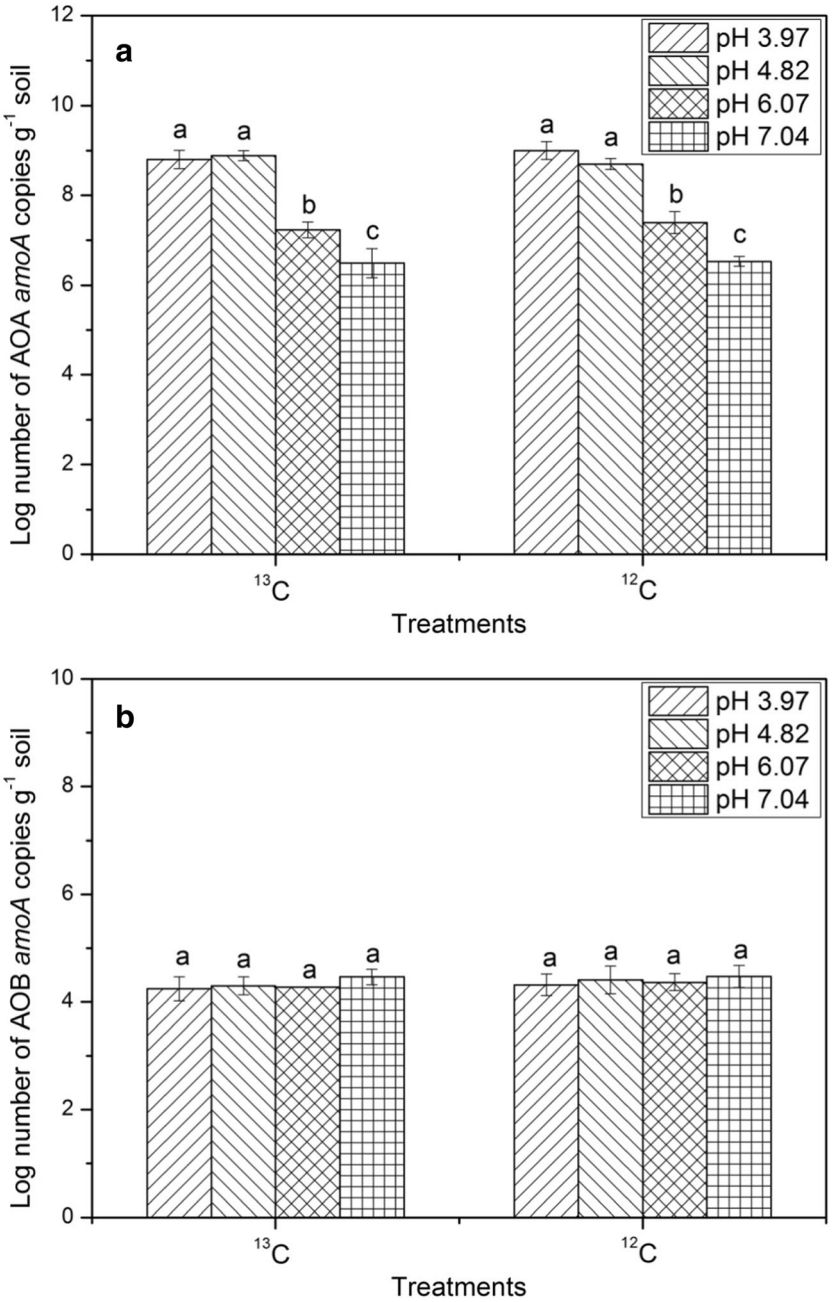

Fig. 1 Archaeal (a) and bacterial (b) *amoA* gene copies at the end of DNA-SIP incubation with ^{13}C- and ^{12}C-labeled CO_2 at soil pH 3.97–7.04. Error bars indicate standard errors of three replicates. Different small letters indicate a significant difference ($P < 0.05$)

of AOA in the ^{12}C-CO_2 microcosms was predominantly in the fractions with buoyant densities of 1.681–1.691 g ml^{-1} (Fig. 3). For the ^{13}C-CO_2 treatments, except at pH 7.04, the *amoA* gene of AOA was mostly found in a heavier fraction with a buoyant density of approximately 1.708 g ml^{-1}. There were clear discrepancies in DNA buoyant density between the ^{12}C- and ^{13}C-labeled treatments at pH 3.97–6.07 (Fig. 3a–c). For the ^{13}C-CO_2 treatment at pH 7.04, the AOA *amoA* genes were mostly contained in the fraction with a buoyant density at around 1.694 g ml^{-1}, with a slight shift toward a lower DNA buoyant density than at other pH levels (Fig. 3d). This implied that AOA assimilated more ^{13}C-CO_2 in the acidic soils.

For AOB, the abundance of *amoA* gene copies reached the maximum value in a buoyant density fraction at around 1.701 g ml^{-1} for both ^{13}C-CO_2 and ^{12}C-CO_2 treatments at pH 3.97–7.04 (Fig. 4). There was no significant variation in the distribution of AOB abundance between the ^{13}C-CO_2 and ^{12}C-CO_2 treatments, which implied that no ^{13}C-CO_2 was assimilated into bacterial DNA during the microcosm incubation.

For the three functional genes (*Nitrobacter nxrA*, *Nitrobacter nxrB*, and *Nitrospira nxrB*) of NOB, the maximum relative abundance overlapped at approximately 1.700–1.720 g ml^{-1} for the ^{13}C-CO_2 and ^{12}C-CO_2 treatments

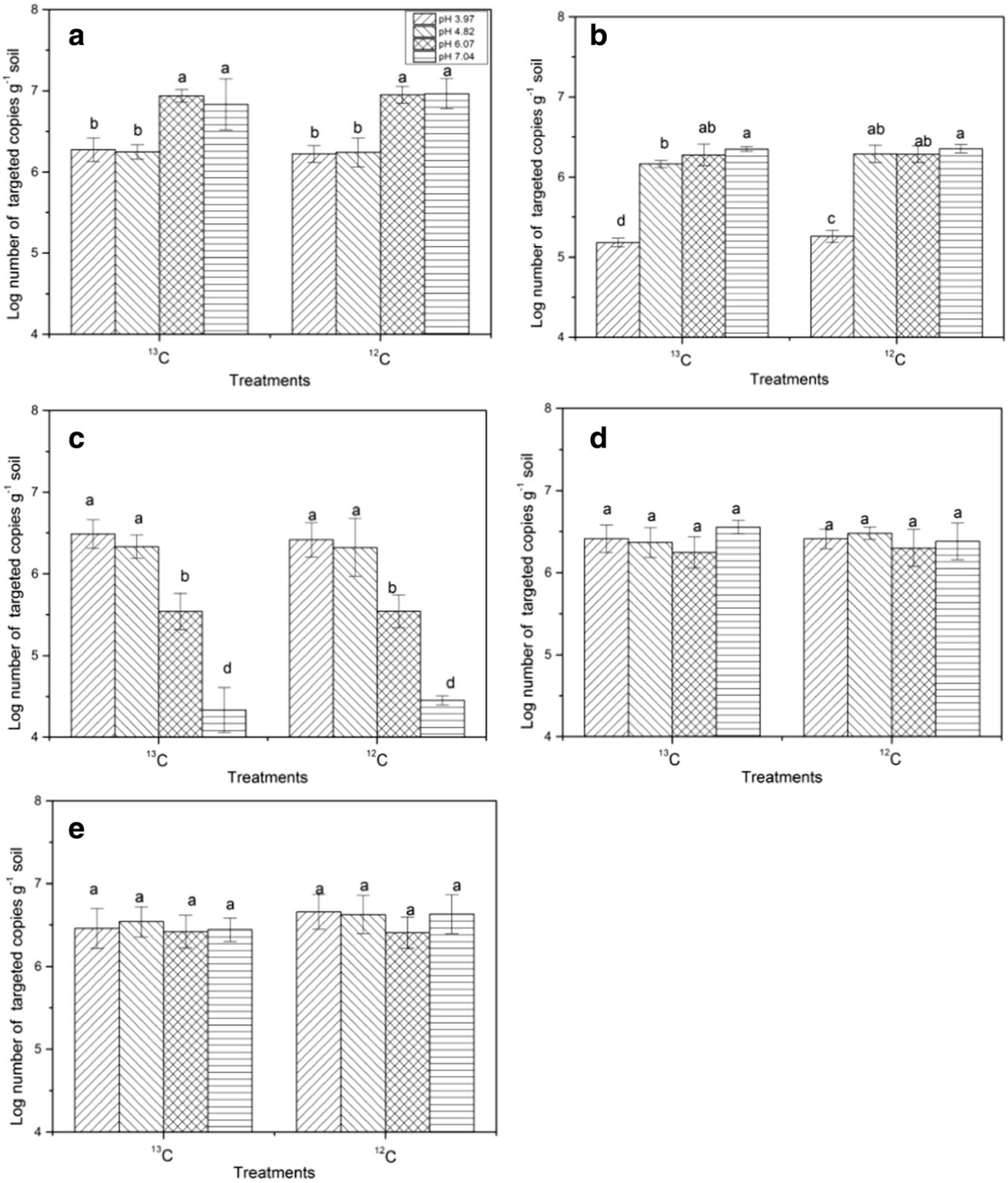

Fig. 2 Targeted gene copies of nitrite-oxidizing bacteria at the end of DNA-SIP incubation with ^{13}C- and ^{12}C-labeled CO_2 at soil pH 3.97–7.04. Error bars indicate standard errors of three replicates; different small letters indicate a significant difference ($P < 0.05$). (a, *Nitrospira* 16S rRNA gene; b, *Nitrospira nxrB*; c, *Nitrobacter nxrA*; d, *Nitrobacter nxrB* genes with primers *nxrB1F/nxrB1R*; e, *Nitrobacter nxrB* genes with primers *nxrB179F/403R*)

at pH 4.82–7.04 (Fig. S1, Electronic Supplementary Material), demonstrating a lack of incorporation of ^{13}C-CO_2 into genomic DNA of either *Nitrospira* or *Nitrobacter*. Only a slight shift of the peak value in *Nitrobacter nxrB* gene was found at pH 3.97. The *Nitrobacter nxrB* gene copies reached the maximum value in a buoyant density fraction at around 1.716 and 1.705 g ml^{-1} for the ^{13}C-CO_2 and ^{12}C-CO_2 treatments, respectively.

3.3 Phylogenetic affiliation of *amoA* sequences

Clone libraries of AOA *amoA* genes from the fraction with maximum relative abundance in the $^{12}CO_2$ and $^{13}CO_2$ microcosms at four pH levels were constructed, and 40 clones from each replicate were randomly selected for sequencing. Since there was no significant variation in the distribution of AOB abundance in density fractions between the ^{13}C-CO_2 and ^{12}C-

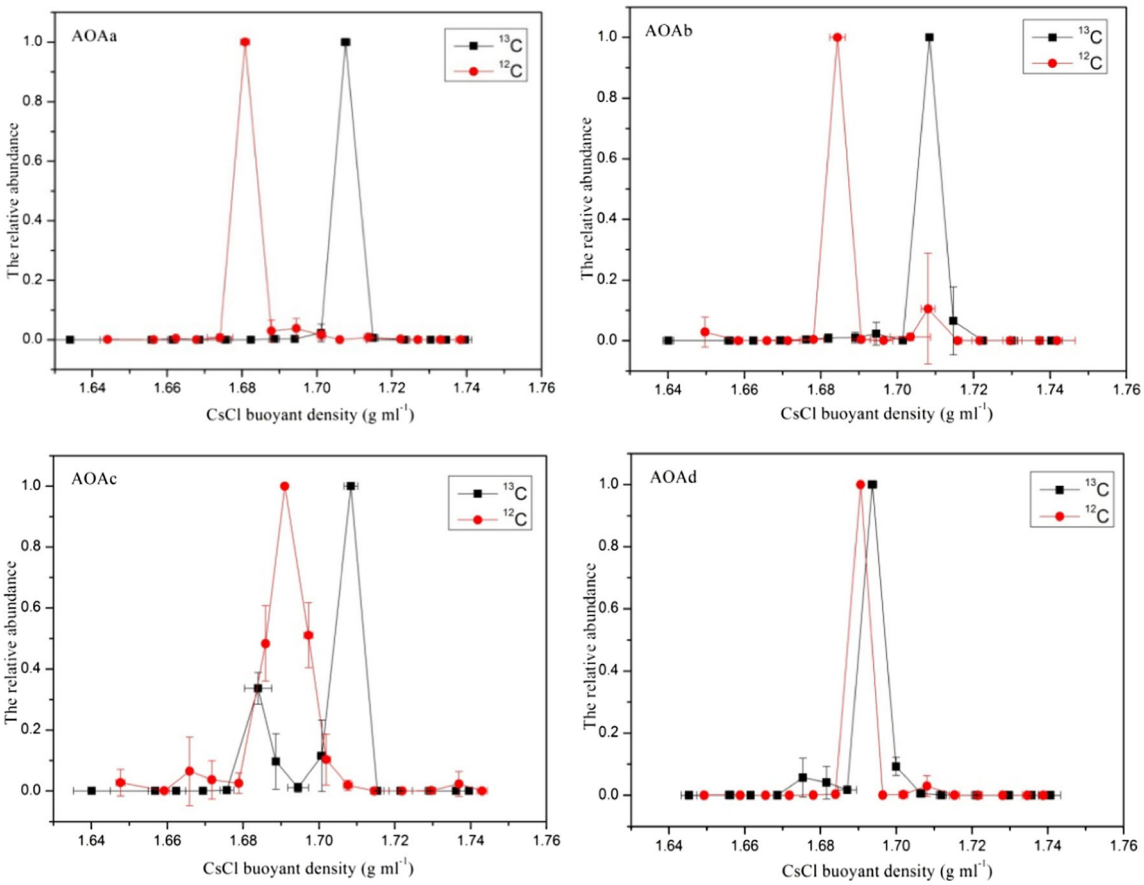

Fig. 3 Quantitative distribution of AOA *amoA* gene copy numbers along a buoyant density gradient for the ^{13}C-CO_2 and ^{12}C-CO_2 treatments. The relative abundance was the ratio of the *amoA* gene copy number in each DNA fraction to the maximum quantity in each treatment. *Y*-axis error bars indicate standard errors of the mean relative gene abundance, and *X*-axis error bars indicate the standard errors of the mean buoyant density (a, pH 3.97; b, pH 4.82; c, pH 6.07; d, pH 7.04)

CO_2 treatments, we constructed AOB clone library from the DNA before ultracentrifugation, and 120 clones from each pH level were randomly selected for sequencing.

For the AOA clone library, *amoA* gene sequences from the labeled and unlabeled treatments mainly fell into the same cluster (Fig. 5). In the ^{13}C-labeled treatments, OTU5 was the most abundant OTU which contained 49.2 and 53.3% sequences in the heavier fractions at pH 4.82 and 7.04, respectively. OTU2 was the most abundant OTU which contained 36.7% sequences in the heavier fractions at pH 3.97. OTU1 and OTU2 were the most abundant OTUs which contained 33.3 and 32.5% sequences in the heavier fractions at pH 6.07. OTU4 was the most abundant OTU which contained 79.2 and 57.5% sequences in the lighter fractions at pH 3.97 and 7.04 in the ^{12}C-labeled treatments, respectively. OTU5 was the most abundant OTU which contained 81.7 and 52.5% sequences in the lighter fractions at pH 4.82 and 6.07 in the ^{12}C-labeled treatments, respectively. OTU1, OTU2, OTU4, and OTU5 all belonged to *Nitrososphaera*.

For the AOB clone library, OTU9 was the most abundant OTU which contained 66.7, 55.0, 56.7, and 30.0% sequences at pH 3.97, 4.82, 6.07, and 7.04, respectively. OTU12 was the second most abundant OTU which contained 10.8, 15.8, 17.5, and 18.3% sequences at pH 3.97, 4.82, 6.07, and 7.04, respectively. Both OTU9 and OTU12 belonged to *Nitrosospira* (Fig. S2, Electronic Supplementary Material).

3.4 Discussion

The abundance of archaeal *amoA* gene was 100 to10,000 times higher than bacterial *amoA* gene, and increased significantly during the incubation. This result was consistent with the previous study, which reported that AOA were numerically dominant over AOB in many different habitats (Leininger et al. 2006; Morimoto et al. 2011; Liu et al. 2015). The negative correlation between soil pH and AOA *amoA* gene abundance demonstrated that some, if not all, AOA had a preference for acidic soils. The AOA *amoA* gene abundance at pH 7.04 was significantly lower than that at pH 3.97–6.07. This might be due to the death of a large proportion of AOA when the soil pH increased. In addition, a positive correlation between AOA *amoA* gene copies and PNR was found in this vegetable cropped soil. These results were in agreement with

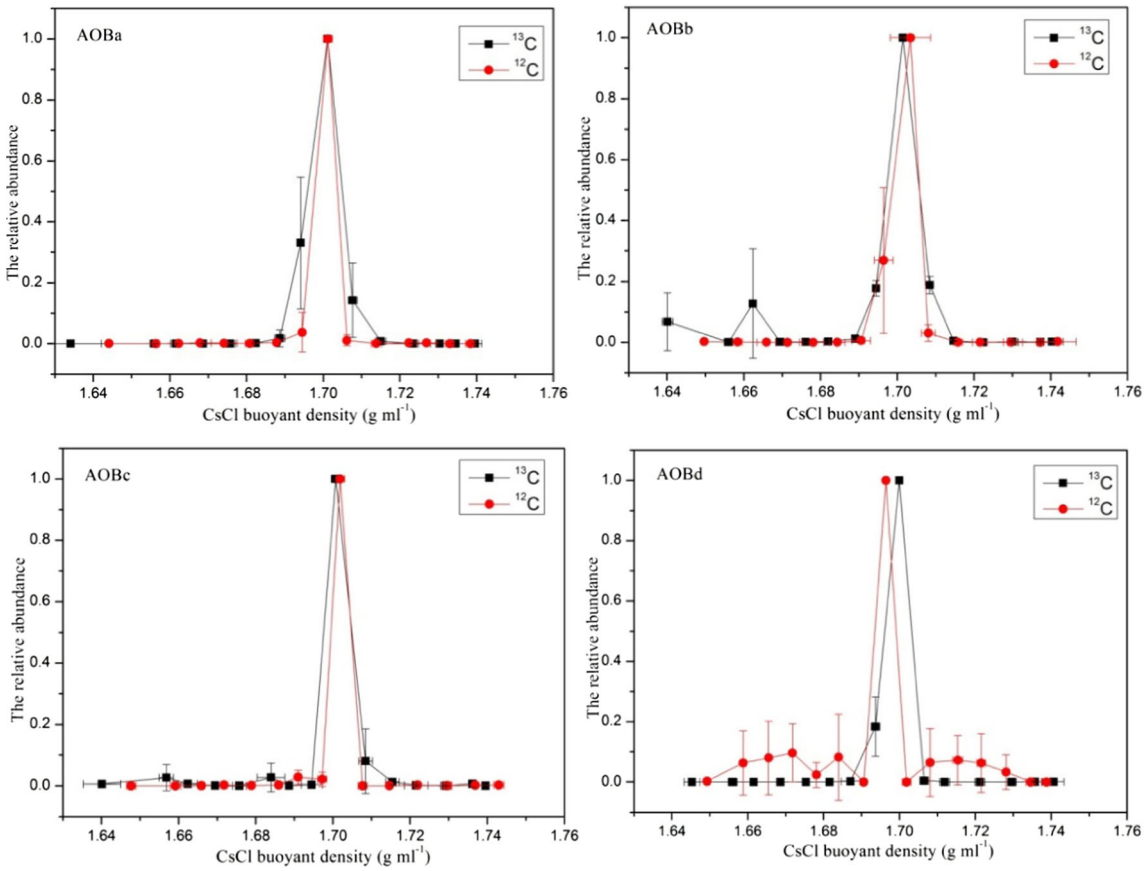

Fig. 4 Quantitative distribution of AOB *amoA* gene copy numbers along a buoyant density gradient for the ^{13}C-CO_2 and ^{12}C-CO_2 treatments. The relative abundance was the ratio of the *amoA* gene copy number in each DNA fraction to the maximum quantity in each treatment. *Y*-axis error bars indicate standard errors of the mean relative gene abundance, and *X*-axis error bars indicate the standard errors of the mean buoyant density (a, pH 3.97; b, pH 4.82; c, pH 6.07; d, pH 7.04)

previous findings that AOA primarily drove the process of ammonia oxidation in acidic soils (Stopnisek et al. 2010; Lehtovirta-Morley et al. 2011). Generally, AOB dominate nitrification in neutral pH soils that have had a history of receiving N fertilizer inputs (Di et al. 2009; Li et al. 2018; Long et al. 2018). However, AOB abundance and nitrification activity in the neutral soil were quite low and there was no significant difference in AOB abundance across the pH gradient. The results demonstrated that the short-term increase in soil pH could not promote the growth

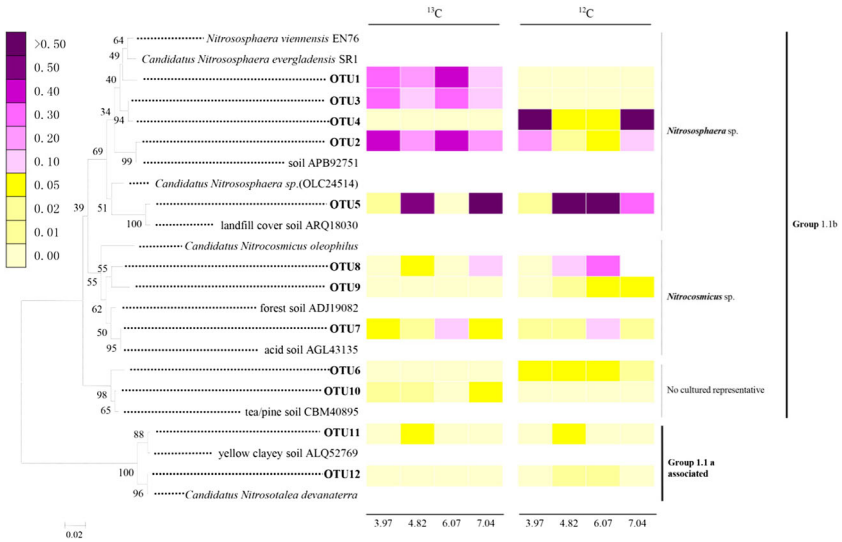

Fig. 5 Phylogenetic tree of AOA *amoA* gene sequences retrieved from selected density fractions of the ^{13}C- and ^{12}C-labeled treatments. Their relative abundances are shown as a heatmap distribution. The scale bar of 0.02 represents 2% estimated sequence divergence

and activity of the original AOB species, perhaps due to the niche discrimination of active nitrifiers adapted to different pH ranges.

The 16S rRNA genes of *Nitrobacter* species are highly similar to those of some non-*Nitrobacter* genera within the *Bradyrhizobiaceae* (Vanparys et al. 2007), and thus, it is not suitable to use the 16S rRNA gene as a marker for *Nitrobacter*. Previous studies often used *nxrA* and *nxrB* genes to target *Nitrobacter* (Reeve et al. 2016; Stempfhuber et al. 2016). Hence, *Nitrospira* 16S rRNA, *nxrB*, *Nitrobacter nxrA*, and *nxrB* genes were used to characterize the dynamics of NOB in this study. Among the four target genes, the abundances of *Nitrospira* 16S rRNA, *nxrB* gene, and *Nitrobacter nxrA* gene were significantly affected by soil pH. This indicated that different groups/organisms are adapted to specific pH ranges. The different resistances to free nitrous acid (FNA) may potentially explain different pH preferences by *Nitrobacter* and *Nitrospira* (Vadivelu et al. 2007). Each group may have contrasting sensitivity to FNA. *Nitrospira* is more vulnerable to low concentration of FNA than *Nitrobacter* (Vadivelu et al. 2007). For *Nitrospira*, the increase in abundance at relatively high pH levels may be due to the high sensitivity to FNA. For *Nitrobacter*, the increase in abundance of *nxrA* gene with decreasing soil pH could be partially explained by the lower sensitivity to FNA. However, no evidence or proof has been given to explain the variation of *Nitrobacter nxrB* gene abundance with soil pH. It is possible that some other factors, such as H_2 oxidation and mixotrophic growth, could influence the abundance of the NOB group (Daims et al. 2016).

Berg et al. (2007) and Schramm et al. (1998) found that the pathways of AOA and AOB to fix CO_2 were 3-hydroxypropionate/4-hydroxybutyrate and Calvin-Bassham-Benson cycle, respectively. *Nitrobacter* and *Nitrospira* fix carbon with the Calvin-Bassham-Benson cycle and the reductive TCA (rTCA) cycle (Spieck and Bock 2005; Lücker et al. 2010). In our study, by determining the nitrifier abundances in the ^{13}C-labeled and ^{12}C-labeled treatments, the assimilation of ^{13}C into the genomes of nitrifiers could be demonstrated (Whitby et al. 2001; Jia and Conrad 2009; Lu and Jia 2013). There was clear incorporation of ^{13}C into AOA genomes except in the neutral soil. However, there was no evident ^{13}C incorporation into AOB genomes as shown by the similar density distribution for the ^{12}C-CO_2 and ^{13}C-CO_2 treatments. These results were consistent with the cloning and sequence analyses where AOA *amoA* gene sequences in the heavy DNA fractions of ^{13}C-labeled treatments were affiliated with OTU5 and OTU2, whereas the majority of the AOA sequences presented in the light DNA fractions of the ^{12}C-labeled treatments were affiliated with OTU5 and OTU4. The active AOA that contributed to ammonia oxidation in the acidic soils belonged to *Nitrososphaera*, demonstrating that some populations of *Nitrososphaera* were adapted to acidic environment. A large proportion of AOA *amoA* gene sequences in the ^{13}C-labeled and ^{12}C-labeled treatments fell within the same cluster at pH 3.97–6.07. A previous study using DNA-SIP analysis revealed that the majority of active AOA incorporated ^{13}C-CO_2 at pH 4.20 and fell into *Nitrosotalea* and *Nitrososphaera* groups (Zhang et al. 2011). Wang et al. (2014) also found that AOA were the main performers of nitrification activity, with greater incorporation of $^{13}CO_2$-derived carbon than AOB. No significant shift of AOA DNA buoyant density was found in the ^{13}C-labeled treatment at pH 7.04, suggesting that AOA could not assimilate CO_2 in neutral soils. Shen et al. (2008) reported that AOA was much more abundant than AOB, but AOB was the main driver for ammonia oxidation in an alkaline sandy loam soil. Our results further confirmed that the activity of AOA was very low in neutral and alkaline soils.

Niche differentiation of AOA and AOB can be strongly influenced by soil pH (Gubry-Rangin et al. 2011). The change in soil pH influences the equilibrium between ammonia and ammonium. In strongly acidic soils, ammonia concentration decreases because of the conversion of NH_3 to NH_4^+ ($NH_3 + H^+ \Leftrightarrow NH_4^+$). Furthermore, Martens-Habbena et al. (2009) reported that AOA had a higher substrate affinity than AOB in ammonia-limited conditions, based on an ammonia oxidation kinetic study with the assumption that the direct substrate for ammonia oxidizers was ammonia rather than ammonium (Suzuki et al. 1974). He et al. (2007) also demonstrated that AOA increased in relative abundance with decreasing soil pH. The ammonia concentrations increase with the increase of soil pH, which could stimulate nitrification by AOB and suppress the growth of AOA (Stempfhuber et al. 2015)

For NOB, we targeted three genes (*Nitrospira nxrB*, *Nitrobacter nxrA*, *Nitrobacter nxrB*) with DNA-SIP. Interestingly, there was no evidence of growth of either *Nitrospira* or *Nitrobacter* populations in the soils with pH 4.82–7.04. A lack of incorporation of ^{13}C-CO_2 into genomic DNA of either group indicated that these populations contributed to nitrite oxidation with no detectable growth. Alternatively, other populations of NOB, not targeted by the qPCR assays for *Nitrospira* and *Nitrobacter*, were probably involved in nitrite oxidation (Daims et al. 2015).

4 Conclusions

Our results provided strong evidence that AOA and *Nitrobacter* were the dominate nitrifiers in the acidic soils, whereas *Nitrospira* dominates in the near neutral soil. AOA dominated ammonia oxidation in the acidic soils. Labeling of AOA by ^{13}C-CO_2 indicated that nitrification was driven by *Nitrososphaera* in the acidic soils. The activities of both AOA and AOB were very low in the neutral soil. NOB was active and responsible for nitrite oxidation but active population growth of NOB was not identified.

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