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Nitrosospira cluster 3-like bacterial ammonia oxidizers and *Nitrospira*-like nitrite oxidizers dominate nitrification activity in acidic terrace paddy soils

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Abstract

The isolation of acid-adapted ammonia-oxidizing bacteria (AOB) has suggested the functional importance of AOB in acidic soils. However, there is, currently, no convincing evidence that links AOB activity to nitrification in acidic paddy soils. Here we demonstrated the incorporation of $^{13}CO_2$ into the genomes of ammonia-oxidizing archaea (AOA), AOB and nitrite-oxidizing bacteria (NOB) following urea application by using stable isotope probing (SIP) in three acidic terrace paddy soils with altitudes of 200 m (E200), 600 m (E600) and 1100 m (E1100), respectively. Nitrification activity increased from E200 to E600 and then to E1100, accompanied with significant growth of AOB over the 56-day incubation, while the abundance of archaeal *amoA* gene declined significantly in all soils after incubation. DNA-SIP demonstrated that active AOB outnumbered AOA and were much more heavily labeled than AOA and NOB, implying their more significant contributions to nitrification in these soils. Phylogenetic analysis indicated that *Nitrosospira* cluster 3-like AOB predominantly catalyzed bacterial ammonia oxidation. ^{13}C-labeled NOB was dominated by *Nitrospira moscoviensis* in E1100, while in E200 and E600, *Nitrospira marina* and *Nitrospira japonica* were as prevalent as *Nitrospira moscoviensis*, respectively. Canonical correlation analysis and the Mantel test indicated the importance of soil physiochemical properties (e.g., pH, available phosphorus (AP) and soil oxidation capacity (OXC)) in determining the composition of the active nitrifying populations. These results suggest a greater functional importance of AOB in ammonia oxidation in the tested acidic paddy soils and the

existence of a broader ecological niche for AOB than previously considered.

Keywords: Bacterial ammonia oxidation; *Nitrosospira* cluster 3-like AOB; *Nitrospira*-like nitrite oxidizers; Acidic paddy soil; DNA stable isotope probing

1. Introduction

Acidic soils (pH<5.5) are widely distributed around the world, occupying approximately 30% of the land area (Von Uexküll and Mutert, 1995). Because of low nitrate concentrations, it was previously believed for a long time that biologically mediated ammonia oxidation did not occur in acidic soils, and pH-raising measures (e.g. liming) are required for nitrate production (Noyes and Conner, 1919; Heubült, 1929). However, increasing evidence in recent decades has demonstrated nitrification in many acidic soils, even in those below pH 3 (Weber and Gainey, 1962; De Boer et al., 1992; Norton and Stark, 2011; Liu et al., 2018). Nitrification rates in acidic soils were found to equal or even exceed those in neutral soils (Lehtovirta-Morley et al., 2011). Exploring the nitrification in acidic soils is of great significance to provide a basis for the management of the global nitrification process and the mitigation of nitrate leaching and nitrous oxide emissions. Although encouraging progress has been made, the role of active nitrifiers in low pH soils remains unclear.

Most cultured isolates of the widespread ammonia-oxidizing bacteria (AOB) are found to be inactive in nitrification in batch culture with pH lower than 5.5 and the tolerance of AOB to low pH stress has been questioned (Jiang and Bakken, 1999; Zhang et al., 2012). The mechanism of nitrification in acidic soils remained a mystery until the discovery of ammonia-oxidizing archaea (AOA), which were found to be dominant in ammonia oxidation in acidic soils, benefitting from their high substrate affinity (Leininger et al., 2006; Lu and Jia, 2013; Wang et al., 2013; Jiang et al., 2015; Li et al., 2017; Yu et al., 2018).

Following the development of molecular biological technology, the growth of AOB was also observed under acidic conditions (Burton and Prosser, 2001; Xu et al., 2017) and AOB have even been identified as dominant ammonia oxidizers both quantitatively and functionally in acidic soils (Petersen et al., 2012; Huang et al., 2018). Recently, an AOB strain isolated from an acidic agricultural soil was proved to be able to grow between pH 5-7.5 and survive under highly acidic condition (pH 2) (Hayatsu et al., 2017). More recently, autotrophic ammonia oxidation in an acidic forest soil was reported to be conducted by the *Nitrosospira* cluster 3a.2 AOB (Huang et al., 2018). All the above work indicates that the contribution of AOB to ammonia oxidation in acidic soils cannot be excluded and more research is required to understand their functional role.

Nitrite-oxidizing bacteria (NOB) typically exist in conjunction with ammonia oxidizers to facilitate rapid oxidation of nitrite to nitrate. Due to their close relationship, even small changes in the community of ammonia-oxidizing microorganisms (AOM) could have a great influence on the abundance and composition of NOB communities (Gieseke et al., 2003; Knapp and Graham, 2007).

Recent research has also demonstrated the presence of “NOB-AOM consortia”, where *Nitrospira* converts urea to ammonia and CO_2 and supplies urease deficient AOM with ammonia, which is then oxidized to nitrite, thereby providing the NOB with energy (Koch et al., 2015). Therefore, the role of NOB in nitrification is more complicated than previously believed. In addition, the great competition between AOA, AOB and NOB for oxygen in flooded paddy soils may also influence their performance in nitrification. In contrast to the ammonia oxidizers, current understanding of the ecology of NOB is scarce and their functional activity in nitrification, as determined by stable isotope technologies like DNA-stable isotope probing (SIP), remains poorly understand.

Terraces are leveled surfaces built on hill slopes for agricultural use. Rice production on terraces requires large amounts of N fertilizer and comprises a substantial amount of China’s rice harvest. Paddy soils are characterized by constantly alternation of wetting and drying, which will lead to great changes in soil properties and oxygen content (Atere et al., 2018), and influence the activity of nitrifiers compared with upland soils (Hu et al., 2013; Wei et al., 2017). DNA-SIP, as a powerful technique, can directly link the active lineages with defined activities in the complex soil environment. For example, $^{13}CO_2$-DNA-SIP has been previously applied to determine active nitrifiers which are responsible for CO_2 incorporation (Jia and Conrad, 2009; Zhang et al., 2012; Huang et al., 2018).

Here, $^{13}CO_2$-DNA-SIP combined with high-throughput sequencing was used to identify the active AOA, AOB and NOB groups assimilating $^{13}CO_2$ for growth during functional ammonia oxidation activity in three acidic paddy soils of different altitudes in the Yunhe terrace, China. As CO_2-C is considered to be the sole C source supporting the autotrophic growth of nitrifiers, the acetylene inhibition technique was applied to determine whether the assimilation of $^{13}CO_2$ occurred only in the presence of soil nitrification (Xia, et al., 2011). In addition, the correlations between active nitrifiers and edaphic factors were determined to explore the principal driving factors of the active nitrifying community in the tested acidic terrace paddy soils.

2. Materials and methods

2.1 Site description and soil sampling

Soil samples for the microcosm incubations were collected from the largest terraced fields of the Eastern China, the Yunhe terrace in Zhejiang province. The terraced fields are located in a subtropical monsoon climate area, with rice cultivation for more than 100 years. Three soils (Stagnic Anthrosols) at altitudes of 200 m (E200), 600 m (E600) and 1100 m (E1100) were chosen. The mean annual temperatures ranged from 12.1 ^{o}C in E1100 to 17.5 ^{o}C in E200 (Table 1). Approximately 300 kg urea-N ha^{-1} is applied during the rice growing season each year. Soil sampling at each elevation was carried out in three separate fields. In each field, three random soil cores (>50 m apart, approximately 20 cm×20 cm in area, and 15 cm depth) were collected and pooled to provide a single sample. The samples were stored on ice in an incubator and transported to the laboratory immediately after sampling. Plant residues and other materials, such as obvious macro-fauna and stones, were removed before the samples were sieved moist $<$ 2 mm. The sieved soil from each replicate plot was subdivided into two subsamples, one was stored at 4 ^{o}C prior to construction of microcosms and the other was air dried for physiochemical analysis. All results are means of triplicate measurements and expressed on an oven dry soil basis (24 h, 105 ^{o}C) (Table 1).

2.2 Physicochemical properties of the soil

Soil pH was measured at a soil-to-water ratio of 1:2.5 after vortexing the soil suspension for 30 min and permitting it to settle for 1 h using a pH electrode (Metter-Toledo SevenMulti™, Switzerland). Particle sizes were measured using a rapid sieving procedure by Kettler et al. (2001). Inorganic N (NO_3^--N, NO_2^--N and NH_4^+-N) was extracted with 1 M KCl (1:10) and analyzed by flow injection analysis (SAN++, Skalar, Holland). Total N (TN) and organic C (SOC) were determined following Kjeldahl digestion (Yuen and Pollard, 1953) and the dichromate oxidation method (Mebius, 1960), respectively. Available phosphorus (AP) was extracted using 0.025 mol l^{-1} HCl + 0.03 mol l^{-1} NH_4F and measured by a visible spectrophotometer (Bray and Kurtz, 1945). Available K (AK) was determined by flame photometry using the ammonium acetate extracts (Carson, 1980). Soil oxidation capacity (OXC), representing the soil potential capacity to accept electrons, was calculated from: soil

$OXC=5\times[NO_3^-]+2\times[Mn(IV)]+[Fe(III)]+8\times[SO_4^{2-}]$ (Chadwick and Chorover, 2001; Zhang et al., 2009). The millimolar concentrations (mmol kg^{-1}) of NO_3^-, Mn(IV), Fe(III) and SO_4^{2-} were used in the OXC equation.

2.3 Construction of DNA-SIP microcosms

For each of the three soils, one labeled treatment ($^{13}CO_2$) and two control treatments ($^{12}CO_2$ and $^{13}CO_2 + C_2H_2$) were prepared. The soils were incubated at 60% of the maximum water-holding capacity at 25 °C in the dark for 56 days with three replicates of each treatment. The microcosms in the 120 ml serum bottles contained 10 g sieved moist soil and were sealed with rubber stoppers and aluminum caps. 6 ml of ^{12}C-CO_2 or ^{13}C-CO_2 (99 at% carbon) was injected into the bottles through the rubber septum. 100 Pa C_2H_2 was also added in the $^{13}CO_2 + C_2H_2$ treatments. All microcosms received 100 μg urea-N g^{-1} soil, equivalent to the annual fertilizer N input in the field. ^{13}C-urea was applied in the $^{13}CO_2$ and $^{13}CO_2+C_2H_2$ microcosms to reduce the dilution of $^{13}CO_2$ by CO_2 released from the catalysis of urea and to ensure that the active nitrifiers were isotopically labeled. The $^{12}CO_2$ treatments received ^{12}C-urea. The ^{13}C-urea and ^{12}C-urea (100 μg urea-N g^{-1} soil) were both applied weekly during the 8-week incubation period by dropwise addition of freshly prepared urea solution. A 14-day pre-incubation was conducted at 40% of the maximum water-holding capacity before the application of urea and CO_2 to decrease the dilution of $^{13}CO_2$ by soil-respired $^{12}CO_2$, as previously reported (Jia and Conrad, 2009). The headspace

CO_2 concentration was measured every three days by gas chromatography (Shimadzu) (Meng et al., 2005). Pre-incubation caused significant declines in the basal respiration of the soil in microcosms of E200 (from 1.21 to 0.20 μmol CO_2 g^{-1} soil day^{-1}), E600 (from 1.16 to 0.30 μmol CO_2 g^{-1} soil day^{-1}) and E1100 (from 1.76 to 0.54 μmol CO_2 g^{-1} soil d^{-1}). Therefore, subsequent treatment with 5% $^{13}CO_2$ in the headspace maintained a constant concentration of about 4.8% $^{13}CO_2$ during the incubation.

2.4 Analysis of the DNA-SIP microcosms and gradient fractionation

Sampling was performed on separate replicate microcosms for each treatment at days 0, 7, 14, 28 and 56, and immediately transferred to a -80 °C freezer prior to subsequent molecular analysis. The three bottles in each treatment were used as individual replicates in the measurement of NH_4-N, NO_2-N and NO_3-N concentrations and DNA extraction. The extraction of soil DNA was performed with a FastDNA Spin Kit for Soil (MP Biomedicals, Cleveland, OH, USA), and the obtained DNA samples from each treatment were used for qPCR, 16s rRNA sequencing and *amoA* gene sequencing (Table S1).

SIP fractionation of the three replicates was performed according to Xia et al. (2011). Approximately 3.0 μg DNA extract with an initial CsCl buoyant density of 1.725 g ml^{-1} was placed in a 5.1 ml Quick-Seal polyallomer ultracentrifugation tube and centrifuged in a Vti65.2 vertical rotor at 177, 000 g for 44 h at 20 °C (Beckman Coulter Inc., Palo Alto, CA, USA). Fifteen DNA gradient fractions (~380 μl) were generated and their refractive index was measured with an AR200 digital hand-held refractometer (Reichert Inc., Buffalo, NY, USA) using a 65 μl aliquot of each fraction. The purified fractionated DNA was dissolved in 30 μl of TE buffer (Freitag et al., 2010).

2.5 Quantitative PCR (qPCR) analysis of amoA genes

The abundance of *amoA* genes in the total DNA and the fractionated DNA over the 56-day incubation were quantified with LightCycler 480 II (Roche Applied Science). The qPCR primers and thermal-cycling conditions are described in Table S2. Each 20 μl PCR contained 1 μl of DNA template, 10 μl SYBR Premix Ex Taq (TaKaRa, Dalian, China), 0.4 mM of each primer, and 8.68 μl Milli-Q water. The qPCR standards with 1.15×10^1 to 1.15×10^7 and 2.33×10^1 to 2.33×10^7 copies per assay were used for the AOA and AOB *amoA* genes, respectively.

All total DNA extracts were diluted 1:10 with nuclease-free water to reduce potential PCR inhibition, but no dilution was applied to the fractionated DNA. Three technical replicates were performed for each of the triplicate biological replicates. Amplification efficiencies were 96-102%, with R^2 values of 0.99. The specificity of the amplification products was determined by standard agarose gel electrophoresis and melting-curve analysis.

The cell-specific rate of archaeal or bacterial ammonia oxidation was calculated from: [(soil nitrate production rate)/(the active AOA or AOB cell numbers in the

^{13}C-DNA heavy fractions from the $^{13}CO_2$ microcosms spanning 8 week incubation)], assuming that soil nitrate production was solely from AOA or AOB and each cell has the same activity.

2.6 Sequencing and processing of sequences

Miseq sequencing of 16S rRNA genes in the V4-V5 regions was conducted with primers 515F-907R (Table S2). Total DNA extracts at days 0 and 56 and the fractionated heavy DNA (fractions 3-8) at day 56 in $^{13}CO_2$ and $^{13}CO_2$+C_2H_2 microcosms were measured using an Illumina MiSeq platform. The raw reads were submitted to the NCBI Sequence Read Archive database with assigned Study SRP139012.

Pyrosequencing of AOA and AOB *amoA* genes in the total DNA extracted from the $^{13}CO_2$ microcosms at days 0 and 56 was also performed. Primers Arch-*amoA*F/Arch-*amoA*R and *amoA*-1F/*amoA*-2R were used for AOA and AOB (Rotthauwe et al., 1997; Francis et al., 2005), respectively on a Roche 454 GS FLX+ Titanium platform (Roche 454 Life Sciences, Branford, CT, U.S.) (Table S2). The sequence data were deposited in the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP139021 for AOA and SRP139024 for AOB).

The resulting sequences were processed using the Quantitative Insights into Microbial Ecology (QIIME) software (Caporaso et al., 2010). Sequences with mismatched primers, ambiguous characters, < 20 average quality score, or shorter than 200 bp were removed and only sequences that overlapped more than 10 bp were assembled. Operational Units (OTUs) were clustered with 97% similarity cutoff using UPARS (version 7.1). The taxonomy of 16S rRNA gene sequences and *amoA* gene sequences were analyzed by RDP Classifier against the Silva 16S rRNA database (Release 123) and FGR (Release 7.3) respectively, with a confidence threshold of 70%. Reads classified as AOA (*Thaumarchaeota* at phylum level), AOB (*Nitrosomonas*, *Nitrosococcus* and *Nitrosospira*) and NOB (*Nitrobacter*, *Nitrospira*, *Nitrotoga*, *Nitrolancetus*, *Nitrococcus* and *Nitrospina*) were screened out, and their Neighbour-joining phylogenetic trees were constructed using representative sequences of the 16S rRNA and *amoA* genes with the Kimura 2-parameter distance in MEGA (version 4.0) using 1000 bootstrap replicates (Tamura et al., 2007). The relative abundance of specific lineages was calculated from: (Reads attributed to this lineage)/(the total 16S rRNA reads).

2.7 Statistical analysis

A one-way analysis of variance was used to assess the differences in physicochemical properties between soils, and the differences in ammonia concentration, nitrate concentration and *amoA* gene copy numbers between different treatments. Multiple comparisons were performed by Tukey's *post hoc* tests. All analyses were conducted using the SPSS 17.0 (IBM, Armonk, NY, USA). Values at *P*<0.05 were considered statistically significant. The Mantel test, Pearson correlation and canonical correspondence analysis (CCA) were used to explore the relationship between abiotic factors and the nitrifying populations in the three terrace paddy soils with the vegan package in R.

3. Results

3.1 Soil nitrification activity

Physiochemical properties of the three soils are shown in Table 1. Nitrate production occurred in $^{13}CO_2$ and $^{12}CO_2$ microcosms during the 56-day incubation, with no significant difference between the labeled and control treatments (Fig. 1a and S1). The presence of C_2H_2 totally inhibited the production of nitrate in all soils (Fig. S1c, f and i). The nitrite concentration in this study was below the detection limit (0.05 μg g^{-1}), so the nitrification activity could be largely represented by the rate of increase in soil nitrate concentrations since little nitrate would be consumed by denitrifiers under oxic conditions. Net nitrification activity was calculated from the mean values of triplicate replicates, assuming linear kinetics. The values increased from E200 to E600 and finally to E1100, with 6.84, 7.14 and 9.15 μg NO_3-N g^{-1} soil d^{-1}, respectively. This was in accordance with a decreasing trend in soil accumulated ammonium concentrations, with 426.3, 398.6 and 275.4 μg NH_4^+-N g^{-1} soil at day 56 in the E200, E600 and E1100 soils, respectively (Fig. S1e). Soil pH increased slightly during the first seven days, and then decreased gradually to be even lower than the initial value during incubation without C_2H_2 (Fig. S1g and h). In contrast, in the C_2H_2 treatment, soil pH increased with increasing incubation time (Fig. S1i).

3.2 Abundance and composition of the soil nitrifying communities

Quantitative PCR (qPCR) of the *amoA* genes was applied to assess the population sizes of putative ammonia oxidizing archaea and bacteria before and after the 56-day incubation. The AOA *amoA* gene abundance declined to different extents in all treatments (Fig. 1b). While the copy number of AOB *amoA* genes increased during the incubation, from 6.60×10^5 to 2.41×10^7, 4.90×10^5 to 1.04×10^8 and 3.64×10^6 to 3.56×10^8 g^{-1} soil in E200, E600, E1100, representing 35.5-, 211- and 96.8-fold increases, respectively (Fig. 1c). The increase in bacterial *amoA* gene copies was eliminated by the addition of C_2H_2 in all three soils (Fig. 1 c).

Miseq sequencing of the 16S rRNA genes of total DNA at days 0 and 56 was carried out and the rarefaction curves were prepared to assess the range of soil microbial diversity. In total, 1,721,299 high-quality reads were obtained (Table S1) and the cumulative number of species reached an asymptote at 20,000 reads in most samples (Fig. S2). The nitrifying community comprised a tiny fraction of the total microbial community in the soil (Fig. 1d and S3). The relative abundance of AOA declined in all soils during the incubation, while the AOB populations were stimulated and their relative abundance was greater than AOA after the 56-day incubation (Fig. 1d and S3a), in agreement with the changes in the number of *amoA* gene copies (Fig.

1b and c). Specifically, the AOB relative abundances increased dramatically from lower than 0.1% at day 0 in all soils to 0.7%, 0.6% and 1.1% at day 56 in E200, E600 and E1100, representing 82.4-, 150- and 79.8-fold increases, respectively (Fig. 1d). The relative abundance of NOB decreased after incubation (Fig. S3b).

The *amoA* gene pyrosequencing of the total DNA extracted from $^{13}CO_2$ treatments before and after incubation was also performed. The cumulative number of species reached an asymptote at 2,000 reads in all samples (Fig. S4). Approximately 129,253 and 128,194 high quality *amoA* reads were obtained for archaea and bacteria, respectively (Table S1). The phylogeny of the *amoA* genes for both AOA and AOB was largely similar to that of the 16S rRNA genes (Fig. S5 and S6). Most AOA reads fell within the marine group 1.1a-associated lineage (Fig. S5) and AOB reads were classified into the *Nitrosospira* cluster 3 lineage (Fig. S6). With NOB, all reads had high sequence similarities to members within the genera *Nitrospira* and *Nitrobacter*, while *Nitrospira* had the highest relative abundance and dominated the nitrite-oxidizing populations in all soils (Fig. S7).

3.3 SIP analysis of active ammonia- and nitrite-oxidizing populations

To separate the ^{13}C-labeled DNA from the ^{12}C native DNA, isopycnic centrifugation of the total DNA extracts at day 56 was conducted. Heavy labeling of both AOA and AOB was demonstrated by the quantitative analysis of the *amoA* genes in the DNA fractions of the three soils (Fig. 2). In control treatments ($^{12}CO_2$ and $^{13}CO_2$

+ C_2H_2), the archaeal *amoA* gene copy numbers peaked in the "light" fractions, with a buoyant density of 1.710 g ml^{-1} (Fig. 2a, c and e). However, in the $^{13}CO_2$ microcosms, with all three soils, the highest copy number of the AOA *amoA* genes occurred in the fractions with a buoyant density ranging from 1.732 to 1.739 g ml^{-1}, a typical range for "heavy" DNA fractions. A second small peak appeared at 1.720 g ml^{-1} with E600 and E1100 soils, suggesting the partial labeling of some AOA.

Similarly, most bacterial *amoA* genes occurred in the "heavy" DNA fractions with buoyant densities of 1.735 to 1.742 g ml $^{-1}$ in the three soils of the $^{13}CO_2$-labeled microcosms (Fig. 2b, d and f). In the $^{13}CO_2$+C_2H_2 treatment, there was no labeling of *amoA* genes, implying that the assimilation of $^{13}CO_2$ by AOA and AOB depends on ammonia oxidation (Fig. 2). The buoyant density of AOB was higher than AOA, which is consistent with a higher DNA G+C content. The percentages (%) of (archaeal *amoA* gene copy numbers in heavy DNA)/(archaeal *amoA* gene copy numbers in all DNA fractions) increased from E200 to E600 and then to E1100, with 54.7%, 63.6% and 86.1%, respectively (Table S3). With AOB, the percentages increased to 98.2%, 98.9% and 97.9%, respectively in the three soils (Table S3). It could be concluded that the labeling of AOB cells was much greater than that of AOA during the nitrification process (Fig. 2 and Table S3).

High-throughput sequencing of the total 16S rRNA genes revealed a significant enrichment of the AOA, AOB and NOB populations in the labeled DNA from the $^{13}CO_2$ microcosms. For example, the percentages (%) of (AOA reads)/(total 16S rRNA gene reads) in the 'heavy' DNA fractions were 2-, 16- and 117-fold higher than those in the control microcosms in E200, E600 and E1100 soils, respectively (Table 2). With AOB, the enrichment was larger. The percentages (%) (AOB 16S rRNA genes)/(total gene sequence reads) in the labeled microcosms reached 29.1%, 59.8% and 65.5% in E200, E600 and E1100 soils, respectively, compared to less than 0.4% in their respective controls. Similarly, the percentages (%) (NOB reads)/(total 16S rRNA gene reads) in the labeled microcosms were also much higher than those in the control microcosms (Table 2). Thus, $^{13}CO_2$ assimilation by nitrifiers occurred only when ammonia oxidation was not inhibited, suggesting chemolitho-autotrophic growth of the nitrifying community in the paddy soils.

Phylogenetic analysis of AOA, AOB and NOB in the heavy DNA fractions from the $^{13}CO_2$ treatments was performed. Members within 1.1a-associated cluster dominated the AOA communities, accounting for 70.9%, 98.8% and 99.4% of the ^{13}C-labeled archaeal 16S rRNA genes in the E200, E600 and E1100 soils, respectively (Table 3 and Fig. S8). Approximately 12.0% and 17.1% of the labeled AOA genes in soil E200 fell within group 1.1a cluster and 1.1b-29i4 cluster, respectively (Table 3 and Fig. S8). With active AOB, 99.9%, 99.8%, and 99.9% of the ^{13}C-labeled 16S rRNA gene reads showed high similarity with *Nitrosospira* cluster 3 in the E200, E600 and E1100 soils, respectively (Table 3 and Fig. S9). The ^{13}C-labeled NOB 16S rRNA genes were dominated by *Nitrospira*-like lineages, and approximately 37.5%, 36.8%, and 92.9% of them were assigned to *Nitrospira moscoviensis* in E200, E600 and E1100, respectively (Table 3 and Fig. 3). In addition, the *Nitrospira japonica* cluster comprised 18.3%, 45.9% and 2.4% of the ^{13}C-labeled NOB in E200, E600 and

E1100 respectively, whereas 42.0% of active NOB grouped into the *Nitrospira marina* cluster in the E200 soil (Table 3).

3.4 Correlation of soil properties and nitrifiers

Several soil chemical parameters were inter-related in the three soils, making it difficult to discern their individual contributions to the selection of nitrifiers. Nevertheless, the potential relationship between nitrifiers and environmental factors at day 0 can be inferred through canonical correlation analysis (CCA) and the Mantel test, which showed a close correlation between environmental factors and nitrifiers (Table S4). These variables explained most of the variance in the community composition of the active nitrifiers (Fig. 4). The first CCA axis was represented by pH, AP and OXC, and explained 58.1% of the variance in the phylotypes of nitrifiers. The second CCA axis explained 14.2% of the variance and was most strongly influenced by TN. The ^{13}C-labeled OTUs B1, B2, N2, N5 and N6 populations were closely related to high soil pH and low OXC. The ^{13}C-labeled A8 preferred high soil TN. Conversely, active nitrifiers A6, B3 and B4 were associated with low TN.

Correlations between the actual samples and physiochemical characteristics were also shown in Fig. 4. The communities of nitrifying population were very different between the three soils, due to different soil properties.

4. Discussion

4.1 Relative contributions of AOA and AOB to ammonia oxidation

Previous studies suggested the potential of bacterial ammonia oxidation in acidic soils (De Boer and Laanbroek, 1989; Petersen et al., 2012), which was confirmed recently in an acidic temperate forest soil (Huang et al., 2018). Our study, using stable isotope probing (SIP), is the first evidence of AOB activity in acidic paddy soil (Hu et al., 2014).

In the DNA-SIP based nitrification experiment, the relative importance of archaeal and bacterial ammonia oxidation could be largely reflected by the AOA/AOB ratio in ^{13}C-DNA (Wang et al., 2015; Pan et al., 2018). Both the *amoA* gene and 16S rRNA genes of AOB were much more heavily labeled than AOA (Table 2 and S3). Also, assuming that ammonia oxidation resulted solely from archaea, the cell-specific rate of AOA should be 2.01 to 32.4 fmol N per cell h^{-1} to reach the nitrate production in the three soils (Table S3), which was 2.4 to 53.9 times larger than the highest ammonia oxidation rate identified in group 1.1a strain *Nitrosopumilus maritimus* SCM1 (Könneke et al., 2005). These results indicated the great importance of bacterial ammonia oxidation in our acidic soils.

Labeled cells of AOB were much more numerous than those of AOA in the heavy fractions (Table S3) and, according to previous studies, the cell-specific rate of [(AOB/oxidized NH_3] is generally higher than that of AOA (Belser and Schmidt, 1980;

Ward et al., 1989; Jia and Conrad, 2009), implying a greater contribution of AOB to ammonia oxidation than AOA in the tested acidic soils. These results were contradictory to previous findings which showed that AOA dominated ammonia oxidation in acidic soils (Zhang et al., 2012; Jiang et al., 2015), indicating that soil pH may not always be the decisive factor of AOA and AOB differentiation in soils (Pan et al., 2016).

Nonetheless, we cannot discount the role of AOA in ammonia oxidation, especially in E1100, as AOA was significantly labeled (Fig. 2) and the AOA reads in heavy DNA occupied up to 11.8% of the total 16S rRNA genes detected (Table 2). Acetylene completely inhibited the enrichment of the ammonia oxidizers and nitrite oxidizers in the 'heavy' fractions, despite the application of $^{13}CO_2$, indicating that NOB obtains nitrite from ammonia oxidation, which is totally inhibited by acetylene (Fig. 2 and Table 2).

4.2 Active ammonia oxidizing phylotypes

The active AOA population was dominated by members within the marine group 1.1a and 1.1a-associated lineages in all soils. Approximately 17.1% of active AOA in soil E200 were classified as group 1.1b fosmid 29i4, a newly proposed lineage (Fig. S8 and Table 3) (Alves et al., 2013). Physiological investigations have demonstrated the large presence of group 1.1b in soils with relatively high pH (Tourna and Schleper, 2011). The 29i4 lineage was found to be highly stimulated by fresh organic substrates

(Xu et al., 2012) and to prefer microaerophilic environments (Wang et al., 2015). Interestingly, our results were exactly the opposite: the 29i4 lineage occurred only in E200, which had higher OXC and lower organic C (Table 1) than the other two soils, suggesting that the microaerophilic environment, high soil organic C and pH were not the only determining factors for the niche occupation of the 29i4 lineage.

Nitrosospira cluster 3 AOB dominated ammonia oxidation in soils with relatively high ammonium concentrations (Kowalchuk et al., 2000). The *Nitrosospira* cluster 3-like AOB had the highest relative abundance in our soils (Fig. S9 and Table 3), suggesting that they perform most of the bacterial ammonia oxidation. This result is consistent with another study which demonstrated that the autotrophic ammonia oxidation in an acidic forest soil was conducted by *Nitrosospira* cluster 3a.2 AOB (Huang et al., 2018). It was previously considered that urea hydrolysis could consume protons and increase pH, which might neutralize the microenvironment around AOB. However, our result showed the opposite and soil pH did not increase during incubation (Fig. S1), indicating that some *Nitrosospira* cluster 3-like phylotypes might be acid-tolerant.

The accumulation of ammonium in all microcosms indicated a rapid hydrolysis of urea (Fig. S1). The accumulation was immediate and the rate was greatest during 0-7 days, indicating possible extracellular urease activity. There was sufficient ammonium to supply all ammonia oxidizers and urease positive organisms would have no competitive advantage for ammonium. The common belief is that availability of ammonia substrate is a key factor leading to the metabolic divergence of ammonia oxidizers (Di et al., 2009; Verhamme et al., 2011; Prosser and Nicol, 2012), and the high ammonium concentrations may favor the activity of AOB in our acidic soils.

4.3 Active nitrite oxidizing phylotypes

The highest nitrification rate occurred with E1100, which had the largest ratio of (NOB)/(total 16S rRNA genes) in the ^{13}C-labeled treatment (Table 2). This is consistent with NOB having a central role in nitrification activity. Phylogenetic analysis showed marked differences in *Nitrospira* composition between different soils (Fig. 3). Previous studies also found differences between the three *Nitrospira* sublineages in terms of cell morphology, cell size, optimum growth temperature, utilization of organic substrates and response to nitrite concentration (Watson et al., 1986; Daims et al., 2006; Ushiki et al., 2013). The growth of *Nitrospira marina* is optimal at atmospheric oxygen pressure, and inhibited at low oxygen partial pressure (Watson et al., 1986). Similarly, in our study, *Nitrospira marina* had the highest relative abundance in soil E200, and was almost absent in the other two soils, which might be attributed to the lowest moisture content and the highest OXC in soil E200. Pearson correlation analysis also revealed a positive correlation between the relative abundance of *Nitrospira marina* and soil OXC ($r=0.678$, $P<0.05$) (Table S5). There were also significant positive correlations between *Nitrospira moscoviensis* and C/N ratio ($r=0.849$, $P<0.01$) and pH ($r=0.895$, $P<0.01$), while *Nitrospira japonica* was negatively correlated with soil organic C ($r=-0.742$, $P<0.05$) (Table S5). Both

Nitrospira moscoviensis and *Nitrospira japonica* belonged to sublineage II, but their response to environmental factors were quite different, implying the ecological versatility of *Nitrospira*.

4.4 Correlating soil properties with nitrifiers

Hu et al. (2015) found that ammonia oxidizers in paddy soils were not randomly dispersed but showed distinct microbial biogeographical patterns. The nitrifying population in our study was closely related to soil pH (Fig. 4), indicating the important role of pH in the selection and adaptation of particular phylogenetic nitrifying populations by determining the chemical form, availability and concentration of nitrification substrates (Curtin et al., 1998; Kemmitt et al., 2006; Nicol et al., 2008; Jiang et al., 2015; Luo et al., 2016). Soil OXC, representing the redox potential, also played a vital role in shaping the structure of the active nitrifying community in the complex soil environment (Fig. 4), in accordance with a previous study (Wang et al., 2015).

A positive correlation between the abundance of group 1.1a (in terms of *amoA* genes) and the concentration of P was reported in waters of the North Sea (Herfort et al., 2007). In contrast, in our study, most active AOA OTUs occurred in soils with low AP content (Fig. 4). One interpretation for this discrepancy might because the presence of *amoA* genes do not equate to their functional activity (Jia and Conrad, 2009). Another explanation might be that the content of P in our soils was hundreds of times higher than the concentration in the North Sea, implying that the correlation between AOA and P might depend on its concentration. This was in good agreement with another study, in which a positive correlation between nitrification rates and AP was observed only in soils with less than 6 μg g^{-1} of AP (Purchase 1974). Nitrite-oxidizers were more sensitive to P deficiency compared with ammonia-oxidizers and P deficiency might cause a delayed or negligible response of NOB to applied ammonium (Purchase 1974). This also occurred in our study, which showed that the highest ratio of [(NOB reads)/(total 16S rRNA gene reads)] in ^{13}C-DNA appeared in E1100 which had the highest AP content.

5. Conclusions

The assimilation of $^{13}CO_2$ by AOA, AOB and NOB indicated that nitrification in our acidic soils was conducted by multi-tiered and complex autotrophic communities. DNA-SIP indicated that members within *Nitrosospira* cluster 3 predominantly catalyzed the ammonia oxidation. Nitrite oxidation was dominated by *Nitrospira*-like NOB, with distinct phylotypes in different soils, which was driven by soil properties. Our results provided the first unequivocal evidence for ^{13}C-labeling of AOB in acidic paddy soils and suggested that soil nitrification activity was influenced by the differences in the relative abundance and community structure of active nitrifiers.

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Table and Figure Legends

Table 1 Physiochemical properties of the three paddy soils.

Table 2 Sequencing summary of the 16S rRNA genes in the DNA of heavy fractions at day 56.

Table 3 Proportions of active nitrifying populations in the ^{13}C-DNA of the three paddy soils.

Fig. 1 Change in soil nitrate concentration (a) and abundance of ammonia oxidizers in SIP microcosms before and after the 56-day incubation. The archaeal (b) and bacterial (c) *amoA* gene copy numbers were determined using qPCR. Relative abundance of AOB (d) is calculated as the percentage: (the AOB 16S rRNA gene reads)/(the total microbial 16S rRNA gene reads) in each microcosm. The standard deviations of the triplicate microcosms were represented by the error bars. A significant difference ($P < 0.05$) based on the analysis of variance was indicated by the different letters above the columns.

Fig. 2 The relative frequency of the archaeal (a,c,e) and bacterial (b,d,f) *amoA* genes based on qPCR across the entire buoyant density gradient of the DNA fractions from the $^{12}CO_2$, $^{13}CO_2$ or $^{13}CO_2$ +C_2H_2 microcosms at day 56. The standard errors of the triplicate microcosms were represented by the error bars.

Fig. 3 Phylogenetic analysis of NOB 16S rRNA genes in ^{13}C-labeled DNA at day 56. The designation 'E600-NOB-16S rRNA-HF-OTU-1-17-(8.1%)' indicates that OTU-1 accounts for 8.1% of the total NOB 16S rRNA gene reads in the ^{13}C-DNA from the heavy fractions of soil E600, containing 17 reads with 97%

sequence similarity. Bootstrap values higher than 50% are indicated at the branch nodes. The scale bars represent 1% nucleic acid sequence divergence for the 16S rRNA genes.

Fig. 4 Canonical correspondence analysis (CCA) between nitrifying populations and physiochemical properties of the three paddy soils. The phylotypes of nitrifiers were grouped on the basis of the OTU taxa of 16S rRNA sequencing. A1-A9 denote the AOA phylotypes, B1-B5 refer to AOB phylotypes, and N1-N7 denote the NOB phylotypes as shown in Supporting Fig. S5, S6 and S7. The solid red, green and black triangles indicated active AOA, AOB and NOB in the ^{13}C-DNA, respectively. The abbreviations AP, SOC, TN and OXC represent the available phosphorus, soil organic C, total nitrogen and soil oxidation capacity, respectively. Bacterial communities of the three soils at day 56 were also shown (E200: blue circle; E600: grey circle; E1100: orange circle).

Table 1 Physiochemical properties of the three paddy soils

	E 200	E 600	E 1100
MAT(^{o}C)	17.5 a	15.1 b	12.1 c
pH	5.48 b	5.29 c	5.67 a
Moisture (%)	26.4 c	34.7 a	32.2 b
SOC (g/kg)	10.6 c	22.5 b	34.4 a
DOC (mg/kg)	52.7 c	69.1 b	81.5 a
TN (g/kg)	0.98 c	2.23 b	2.82 a
C/N	10.8 b	10.1 b	12.2 a
Available P (mg/kg)	4.96 c	31.0 b	89.0 a
Available K (mg/kg)	23.0 b	127 a	25.2 b
NO_3-N (mg/kg)	2.91 c	4.22 a	3.67 b
NH_4-N (mg/kg)	9.22 c	15. 6 a	13.3 b
CEC (cmol/kg)	6.42 b	10.6 a	10.7 a
SO_4^{2-} (mg/kg)	10.7 b	17.8 a	16.1 a
Fe(Ⅲ) (g/kg)	3.05 a	1.81 b	0.43 c
Mn(Ⅳ) (mg/kg)	27.6 a	20.4 b	10.4 c
OXC (mmol/kg)	248 a	161 b	55.6 c
Sand (%)	61.2 a	48.5 c	54.1 b
silt (%)	23.5 c	30.9 a	28.3 b
clay (%)	15.3 b	20.6 a	17.6 b

Abbreviations: MAT, mean annual temperature; SOC, soil organic carbon; TN, total nitrogen; CEC, cation exchange capacity; OXC, soil oxidation capacity. Data shown in this table were average values and a, b, and c indicated a significant difference ($P<0.05$) based on the analysis of variance.

Table 2 Sequencing summary of the 16S rRNA genes in the DNA of heavy fractionations at day 56.

Sample	CsCl Buoyant Density, g ml^{-1}	High Quality Read Number		Percentage of AOA reads to total 16S rRNA gene reads, %		Percentage of AOB reads to total 16S rRNA gene reads, %		Percentage of NOB reads to total 16S rRNA gene reads, %	
		$^{13}CO_2$	$^{13}CO_2$+C_2H_2	$^{13}CO_2$	$^{13}CO_2$+C_2H_2	$^{13}CO_2$	$^{13}CO_2$+C_2H_2	$^{13}CO_2$	$^{13}CO_2$+C_2H_2
E200	1.7273	72621	73097	-	-	-	-	-	-
	1.7319	91456	67408	0.3	0.1	0.6	0.3	0.2	-
	1.7354	77647	59220	0.2	-	10.4	0.4	-	-
	1.7388	70968	51007	-	-	29.1	-	0.5	-
	1.7422	90070	58109	-	-	5.6	-	0.2	-
	1.7457	69931	68506	-	-	0.6	-	-	-
E600	1.7273	53991	82054	0.2	0.1	-	-	0.1	0.1
	1.7319	47789	70334	2.4	0.7	0.3	-	0.2	0.1
	1.7354	51269	74126	5.1	0.3	9.1	0.1	0.2	0.1
	1.7388	43596	63851	0.9	0.1	59.8	0.2	0.3	-
	1.7422	42922	55786	1.0	0.1	17.2	-	-	-
	1.7457	57732	51903	0.2	0.1	0.5	0.2	-	-
E1100	1.7273	62282	75917	0.2	-	-	-	-	-
	1.7319	60777	85531	8.6	0.2	1.5	-	0.1	0.1
	1.7354	52201	73185	11.8	0.1	38.8	0.1	1.5	0.2
	1.7388	43697	54983	3.1	0.3	65.5	0.2	1.2	0.1
	1.7422	50925	62434	2.1	0.2	41.7	-	0.4	0.1
	1.7457	56016	60022	2.2	-	8.3	-	0.4	-

Annotation: The grey boxes indicate the significantly higher percentages (%) of (AOA, AOB and NOB reads) /(total 16S rRNA gene reads) in the labeled treatments ($^{13}CO_2$) compared to their control treatments ($^{13}CO_2$+C_2H_2). The dashed line indicates that the percentage was lower than 0.05.

Table 3 Proportions of active nitrifying populations in the ^{13}C-DNA of the three paddy soils

	Nitrifier Phylotype[a]	^{13}C-16S rRNA genes		
		E200 (%)	E600 (%)	E1100 (%)
AOA	Group 1.1a	12.0	0.6	0.3
	Group 1.1a-associated	70.9	98.8	99.4
	Group 1.1b-Soil 29i4 fosmid	17.1	-	-
	Group 1.1c	-	0.6	0.2
AOB	*Nitrosospira* cluster 3	99.9	99.8	99.9
	Nitrosomonas oligotropha	0.1	0.2	0.1
NOB	*Nitrospira moscoviensis*	37.5	36.8	92.9
	Nitrospira japonica	18.3	45.9	2.4
	Nitrospira marina	42.0	-	1.6
	Nitrospira calida	-	4.3	-
	Unclassified Nitrospira	-	8.1	1.3
	Nitrobacter hamburgensis	2.2	4.8	1.8

Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; NOB, nitrite-oxidizing bacteria. The dashed line indicates that no sequences were detected.[a]Phylogenetic affiliations of active nitrifiers are delineated in Supplementary Figure S8 (AOA), Supplementary Figure S9 (AOB) and Figure 3 (NOB).

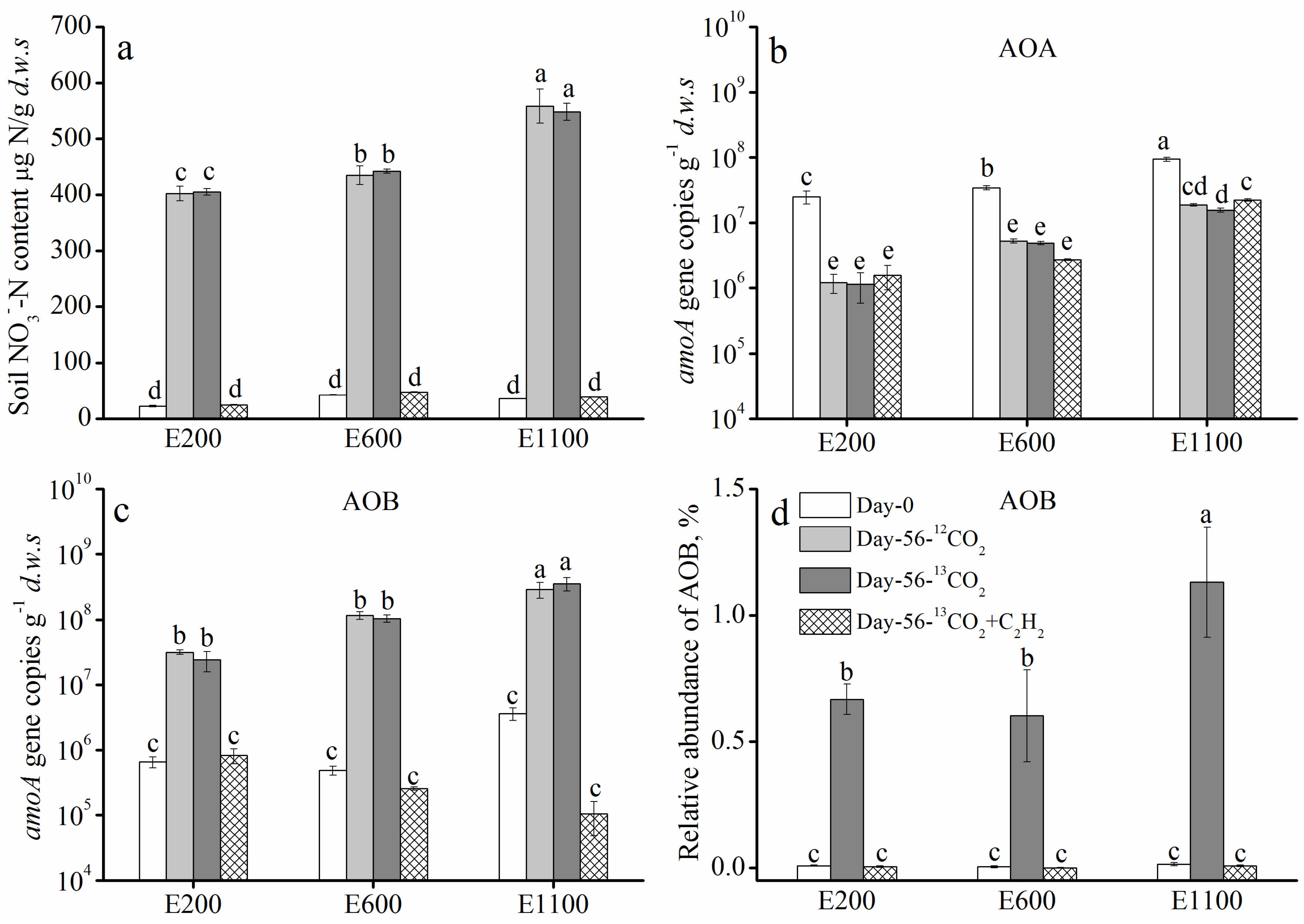
a
Soil NO_3^--N content μg N/g *d.w.s*
0
100
200
300
400
500
600
700
d c c d
d b b d
d a a d
E200
E600
E1100
b
AOA
amoA gene copies g^{-1} *d.w.s*
10^4
10^5
10^6
10^7
10^8
10^9
10^{10}
c e e e
b e e e
a cd d c
E200
E600
E1100
c
AOB
amoA gene copies g^{-1} *d.w.s*
10^4
10^5
10^6
10^7
10^8
10^9
10^{10}
c b b c
c b b c
c a a c
E200
E600
E1100
d
AOB
Relative abundance of AOB, %
0.0
0.5
1.0
1.5
Day-0
Day-56-$^{12}CO_2$
Day-56-$^{13}CO_2$
Day-56-$^{13}CO_2$+C_2H_2
c b c
c b c
c a c
E200
E600
E1100

Figure 1

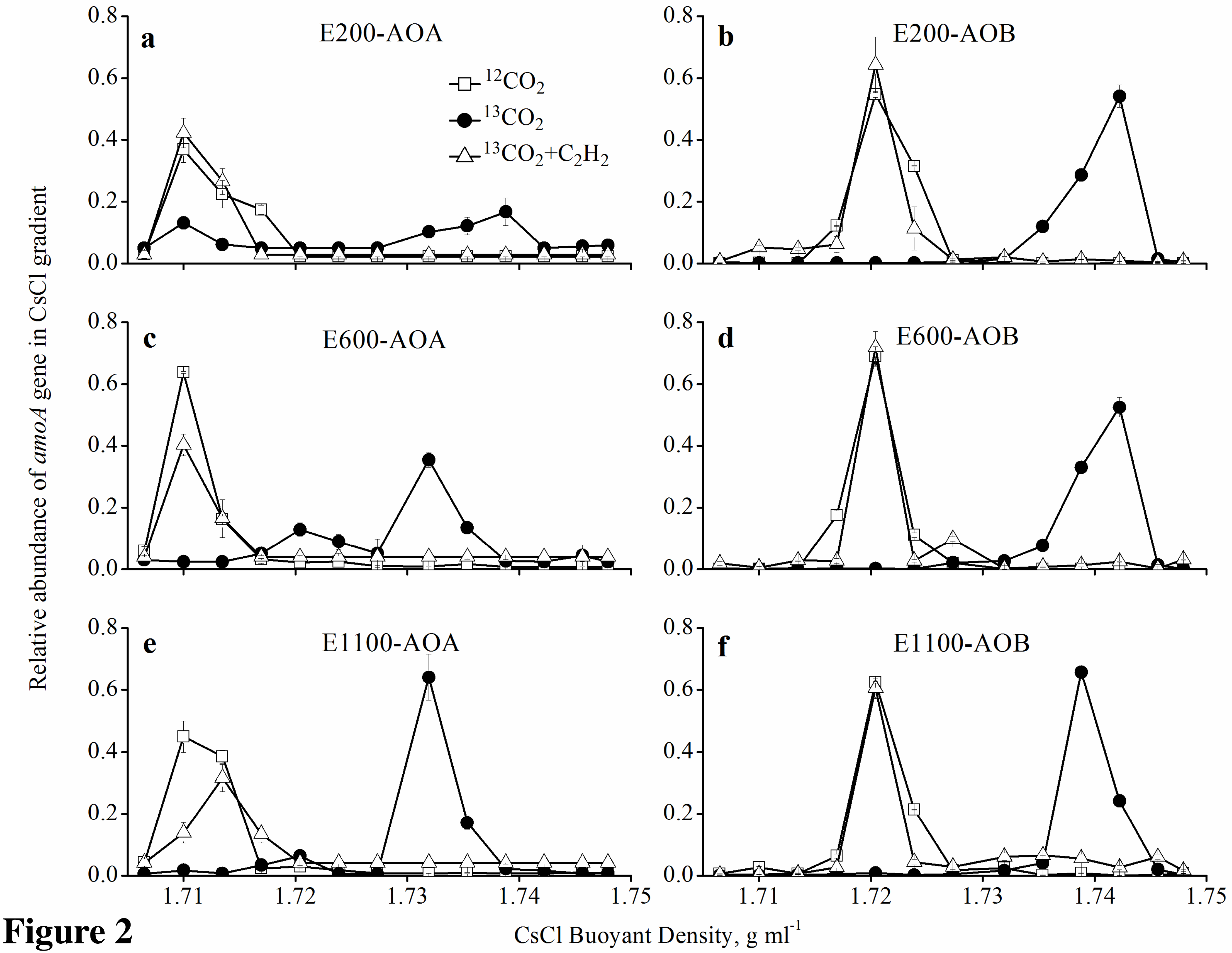
a
E200-AOA
b
E200-AOB
c
E600-AOA
d
E600-AOB
e
E1100-AOA
f
E1100-AOB
$^{12}CO_2$
$^{13}CO_2$
$^{13}CO_2+C_2H_2$
0.0
0.2
0.4
0.6
0.8
1.71
1.72
1.73
1.74
1.75
Relative abundance of *amoA* gene in CsCl gradient
CsCl Buoyant Density, g ml^{-1}

Figure 2

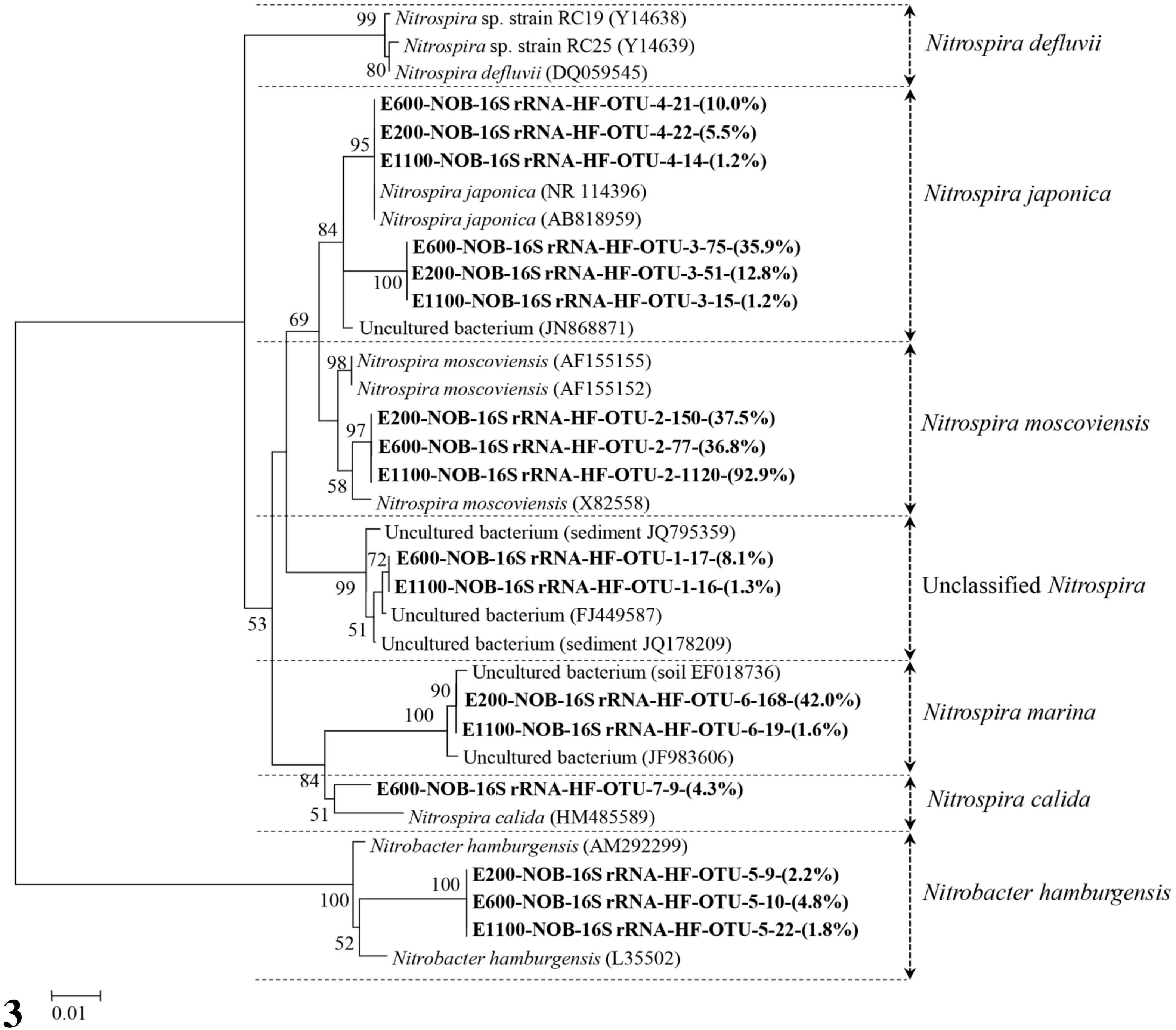
99
Nitrospira sp. strain RC19 (Y14638)
Nitrospira sp. strain RC25 (Y14639)
80
Nitrospira defluvii (DQ059545)
Nitrospira defluvii
E600-NOB-16S rRNA-HF-OTU-4-21-(10.0%)
E200-NOB-16S rRNA-HF-OTU-4-22-(5.5%)
95
E1100-NOB-16S rRNA-HF-OTU-4-14-(1.2%)
Nitrospira japonica (NR 114396)
Nitrospira japonica (AB818959)
84
Nitrospira japonica
E600-NOB-16S rRNA-HF-OTU-3-75-(35.9%)
E200-NOB-16S rRNA-HF-OTU-3-51-(12.8%)
100
E1100-NOB-16S rRNA-HF-OTU-3-15-(1.2%)
69
Uncultured bacterium (JN868871)
98
Nitrospira moscoviensis (AF155155)
Nitrospira moscoviensis (AF155152)
E200-NOB-16S rRNA-HF-OTU-2-150-(37.5%)
97
E600-NOB-16S rRNA-HF-OTU-2-77-(36.8%)
Nitrospira moscoviensis
E1100-NOB-16S rRNA-HF-OTU-2-1120-(92.9%)
58
Nitrospira moscoviensis (X82558)
Uncultured bacterium (sediment JQ795359)
72
E600-NOB-16S rRNA-HF-OTU-1-17-(8.1%)
99
E1100-NOB-16S rRNA-HF-OTU-1-16-(1.3%)
Unclassified *Nitrospira*
Uncultured bacterium (FJ449587)
53
51
Uncultured bacterium (sediment JQ178209)
Uncultured bacterium (soil EF018736)
90
E200-NOB-16S rRNA-HF-OTU-6-168-(42.0%)
100
Nitrospira marina
E1100-NOB-16S rRNA-HF-OTU-6-19-(1.6%)
Uncultured bacterium (JF983606)
84
E600-NOB-16S rRNA-HF-OTU-7-9-(4.3%)
Nitrospira calida
51
Nitrospira calida (HM485589)
Nitrobacter hamburgensis (AM292299)
E200-NOB-16S rRNA-HF-OTU-5-9-(2.2%)
100
100
E600-NOB-16S rRNA-HF-OTU-5-10-(4.8%)
Nitrobacter hamburgensis
E1100-NOB-16S rRNA-HF-OTU-5-22-(1.8%)
52
Nitrobacter hamburgensis (L35502)
0.01

Figure 3

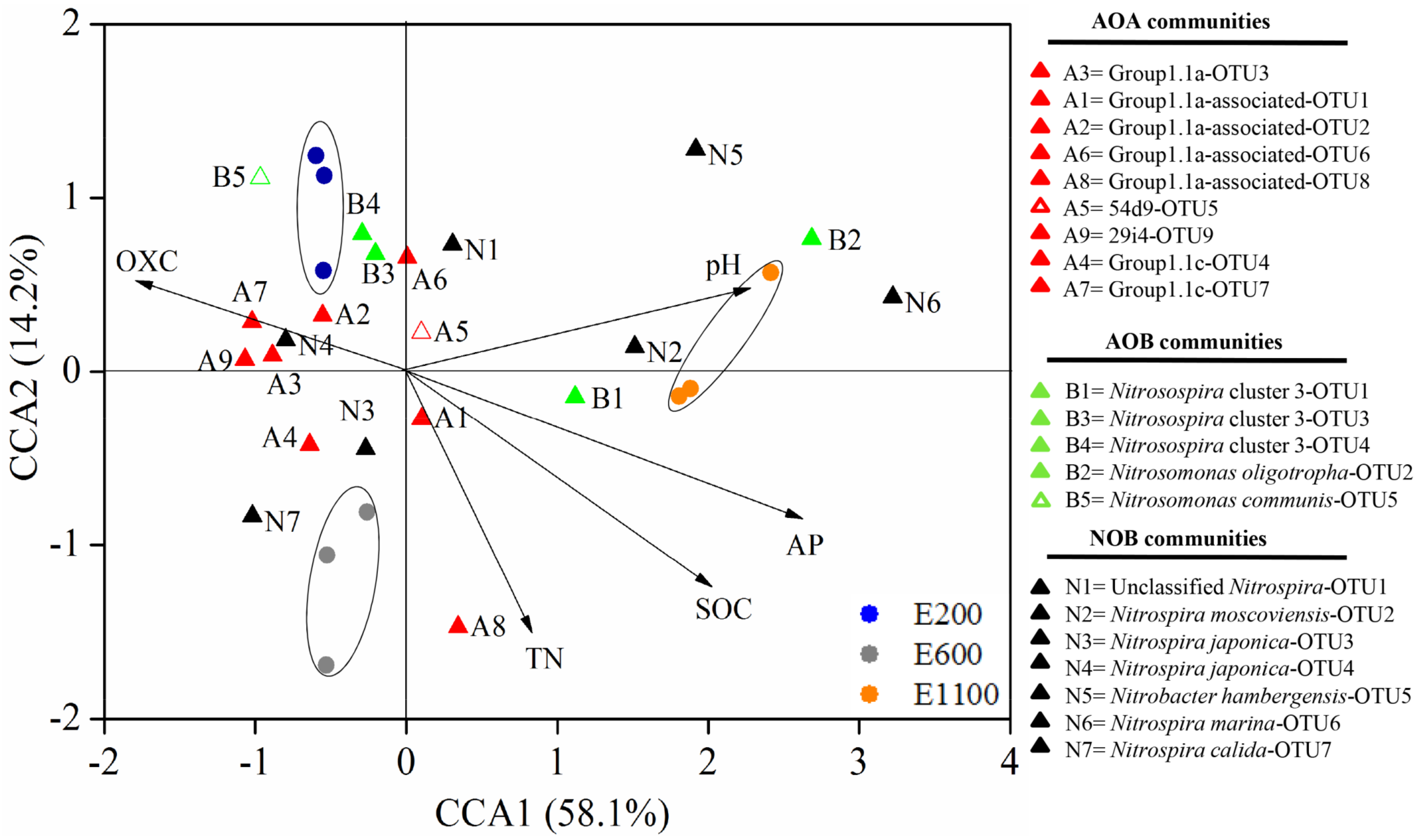
2
1
0
-1
-2
-2
-1
0
1
2
3
4
CCA2 (14.2%)
CCA1 (58.1%)
OXC
pH
AP
SOC
TN
B5
B4
B3
B2
B1
A7
A2
A6
A5
A9
A3
A4
A1
A8
N1
N2
N3
N4
N5
N6
N7
E200
E600
E1100
AOA communities
A3= Group1.1a-OTU3
A1= Group1.1a-associated-OTU1
A2= Group1.1a-associated-OTU2
A6= Group1.1a-associated-OTU6
A8= Group1.1a-associated-OTU8
A5= 54d9-OTU5
A9= 29i4-OTU9
A4= Group1.1c-OTU4
A7= Group1.1c-OTU7
AOB communities
B1= Nitrosospira cluster 3-OTU1
B3= Nitrosospira cluster 3-OTU3
B4= Nitrosospira cluster 3-OTU4
B2= Nitrosomonas oligotropha-OTU2
B5= Nitrosomonas communis-OTU5
NOB communities
N1= Unclassified Nitrospira-OTU1
N2= Nitrospira moscoviensis-OTU2
N3= Nitrospira japonica-OTU3
N4= Nitrospira japonica-OTU4
N5= Nitrobacter hambergensis-OTU5
N6= Nitrospira marina-OTU6
N7= Nitrospira calida-OTU7

Figure 4

Highlights:

Nitrification activity increased with the elevated altitudes

AOB rather than AOA dominated nitrification activity in the acidic paddy soils

Phylogenetically distinct NOB groups dominated nitrite oxidation in terrace soils

Soil properties played a vital role in shaping the composition of active nitrifiers