

## ORIGINAL ARTICLE

# Autotrophic growth of nitrifying community in an agricultural soil

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**The two-step nitrification process is an integral part of the global nitrogen cycle, and it is accomplished by distinctly different nitrifiers. By combining DNA-based stable isotope probing (SIP) and high-throughput pyrosequencing, we present the molecular evidence for autotrophic growth of ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) and nitrite-oxidizing bacteria (NOB) in agricultural soil upon ammonium fertilization. Time-course incubation of SIP microcosms indicated that the *amoA* genes of AOB was increasingly labeled by <sup>13</sup>CO<sub>2</sub> after incubation for 3, 7 and 28 days during active nitrification, whereas labeling of the AOA *amoA* gene was detected to a much lesser extent only after a 28-day incubation. Phylogenetic analysis of the <sup>13</sup>C-labeled *amoA* and 16S rRNA genes revealed that the *Nitrosospira* cluster 3-like sequences dominate the active AOB community and that active AOA is affiliated with the moderately thermophilic *Nitrososphaera gargensis* from a hot spring. The higher relative frequency of *Nitrosospira*-like NOB in the <sup>13</sup>C-labeled DNA suggests that it may be more actively involved in nitrite oxidation than *Nitrobacter*-like NOB. Furthermore, the acetylene inhibition technique showed that <sup>13</sup>CO<sub>2</sub> assimilation by AOB, AOA and NOB occurs only when ammonia oxidation is not blocked, which provides strong hints for the chemolithoautotrophy of nitrifying community in complex soil environments. These results show that the microbial community of AOB and NOB dominates the nitrification process in the agricultural soil tested.**

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## Introduction

Nitrification is the only oxidative process that links the reduced and oxidized pools of inorganic nitrogen to sustain the global nitrogen cycle (Gruber and Galloway, 2008). The rate-limiting step in microbial nitrification is the oxidation of ammonia to nitrite, which is then rapidly oxidized further to nitrate (Koops *et al.*, 2006). Soil ammonia oxidation has long been considered to be performed by only a few phylogenetically restricted microorganisms belonging to *Proteobacteria* (Kowalchuk and Stephen, 2001). However, recent studies have expanded the known ammonia oxidizers from the domain *Bacteria* to

*Archaea* in marine habitats (Könneke *et al.*, 2005) and soil (Treichel *et al.*, 2005). The widespread presence of archaeal *amoA* gene (Francis *et al.*, 2005; Leininger *et al.*, 2006; Erguder *et al.*, 2009) and autotrophic lifestyle of cultured ammonia-oxidizing archaea (AOA) (Könneke *et al.*, 2005; Hatzenpichler *et al.*, 2008) have suggested its important role in the global cycling of nitrogen and carbon. However, the relative contribution of AOA and ammonia-oxidizing bacteria (AOB) to ammonia oxidation in agricultural soil remains unclear. For instance, AOB has been shown to dominate the microbial ammonia oxidation in German agricultural soil (Jia and Conrad, 2009), whereas archaeal ammonia oxidation prevails in Scottish agricultural soil (Offre *et al.*, 2009; Zhang *et al.*, 2010).

Unlike AOB, the ecological study of nitrite-oxidizing bacteria (NOB) has been hampered because there is no universal 16S rRNA gene probe that can specifically target the highly diverse NOB community in complex soil environments (Freitag

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*et al.*, 2005). In addition to the newly discovered *Nitrotoga* of *Betaproteobacteria* (Alawi *et al.*, 2007), NOB is composed of four genera, namely *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira*, which are phylogenetically affiliated to *Alpha*-, *Gamma*-, provisionally *Deltaproteobacteria* and the deep-branching bacterial phylum *Nitrospirae*, respectively (Bock and Wagner, 2006). For a long time, *Nitrobacter* has been studied most intensively and is believed to play a dominant role in nitrification, but an increasing line of evidence suggests the functional importance of *Nitrospira*. A recent study has shown that *Nitrobacter*-like NOB responds positively to high N availability and high nitrite oxidation potential, whereas *Nitrospira*-like NOB seems favored when soil nitrite oxidation activity is low (Attard *et al.*, 2010). The autotrophic lifestyle of known NOB in culture makes it suitable for DNA-stable isotope probing (SIP) investigation to directly link NOB identity with nitrification activity in a way similar to that of AOB in agricultural soil (Jia and Conrad, 2009). Instead of focusing on the NOB-specific 16S rRNA gene, barcoded pyrosequencing of the total 16S rRNA in  $^{13}\text{C}$ -labeled DNA could provide useful insights into the relative role of *Nitrospira* and *Nitrobacter* in soil nitrification in agricultural ecosystems.

Agricultural ecosystems annually receive approximately 25% of global nitrogen input, mostly in the form of ammonium (Gruber and Galloway, 2008). The ammonium must be oxidized at least once to nitrate by a nitrifying community. A comprehensive understanding of microbial nitrification requires simultaneous characterization of AOB, AOA and NOB. However, no study appears to have been able to clearly link nitrite oxidation activity to the phylogenetic identity of *Nitrobacter* and/or *Nitrospira* in agricultural soil. In fact, soil nitrite concentration is generally below the detection limit, indirectly suggesting that some NOB may have high substrate affinity and are, therefore, extremely efficient for nitrite oxidation. As for ammonia oxidizers, direct evidence is still missing for ammonia oxidation by group I.1b AOA, which is commonly observed in soil (Schleper, 2010). Notably, active AOA in Scottish soil is phylogenetically most closely associated with group I.1a, which is typically dominant in marine habitats (Offre *et al.*, 2009). Taking into account the recent conflicting observations (Gubry-Rangin *et al.*, 2010), an investigation with regard to microbial nitrification in geographically broader regions is needed to assess accurately the global relevance of AOA and AOB in nitrogen cycling in agricultural ecosystem.

Therefore, by combining  $^{13}\text{C}$ -based DNA-SIP and high-throughput 454 pyrosequencing techniques, we aim to investigate the autotrophic growth of active nitrifying community upon ammonium fertilization in agricultural soil from a region typical of agricultural areas in the North China Plains. In addition,  $\text{C}_2\text{H}_2$  inhibition technique was also

exploited in an attempt to determine whether  $^{13}\text{C}$ - $\text{CO}_2$  assimilation would occur in the absence of soil nitrification, which is thought to provide the sole energy source in support of the lithotrophic growth of the nitrifying community.

## Materials and methods

### DNA-SIP microcosm

Soil samples were collected in triplicate from the State Key Experimental Station for Ecological Agriculture (35°00' N, 114°24' E) of the Chinese Academy of Sciences; the field information is detailed in Supplementary Materials. DNA-SIP microcosm was constructed in triplicate to investigate the active soil-nitrifying community as previously described in detail (Jia and Conrad, 2009). Briefly, 6.58 g of fresh soil (equivalent to 6.0 g dry weight gram soil) was incubated at 60% maximum water-holding capacity in 100 ml serum bottles, which were tightly capped with black butyl stoppers at 27 °C in the dark. Incubation of the soil microcosm was performed with 5% of  $^{13}\text{C}$ - $\text{CO}_2$  or  $^{12}\text{C}$ - $\text{CO}_2$  with or without 100 Pa  $\text{C}_2\text{H}_2$ , a suicide substrate inhibitor of microbial ammonia oxidation. The treatment was renewed once a week, including the addition of 100  $\mu\text{g}$   $(\text{NH}_4)_2\text{SO}_4\text{-N}$  g dry weight gram soil and of 5%  $^{13}\text{C}$ - $\text{CO}_2$  or  $^{12}\text{C}$ - $\text{CO}_2$ . Thus, the soil microcosms received a total of 400  $\mu\text{g}$   $(\text{NH}_4)_2\text{SO}_4\text{-N}$  g dry weight gram soil over an incubation period of 4 weeks. The headspace of the bottle was flushed weekly with pressurized synthetic air (20%  $\text{O}_2$ , 80%  $\text{N}_2$ ) for 1 min to maintain oxic conditions, and, if stated, the addition of 100 Pa  $\text{C}_2\text{H}_2$  was performed immediately after the headspace air was fully exchanged. The  $^{13}\text{C}$ - $\text{CO}_2$  (99 atom% carbon) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA) and  $^{12}\text{C}$ - $\text{CO}_2$  was made by acidifying sodium carbonate. The headspace  $\text{CO}_2$  concentration was measured as described previously (Meng *et al.*, 2005). Before incubation of the DNA-SIP microcosm, a 12-day pre-incubation of soil at 40% maximum water-holding capacity was carried out to reduce the amount of soil-respired  $\text{CO}_2$  so that the target concentration of 5%  $\text{CO}_2$  could be maintained at a constant level over the entire incubation period. The pre-incubation also increased the labeling efficacy of targeted microorganisms because the dilution of  $^{13}\text{C}$ - $\text{CO}_2$  by soil-respired  $^{12}\text{C}$ - $\text{CO}_2$  could be decreased significantly as reported previously (Jia and Conrad, 2009). In addition, a 3-day pre-incubation with 100 Pa  $\text{C}_2\text{H}_2$  was executed to fully inactivate the AOB community for all the  $\text{C}_2\text{H}_2$  treatments immediately before SIP incubation was started.

The destructive sampling was performed in triplicate at days 3, 7 and 28. About 2.0 g of fresh soil was removed from each replicate and immediately frozen at -20 °C for molecular analysis. The rest of the soil replicate was homogenized with 10 ml of 2 M KCl by shaking at 200 r.p.m. for 30 min,

and then passed through filter paper for the determination of  $\text{NH}_4^+$ -N,  $\text{NO}_2^-$ -N and  $\text{NO}_3^-$ -N using a Skalar SAN Plus segmented flow analyzer (Skalar Inc., Breda, The Netherlands). The nitrite concentration was below the detection limit and nitrification was assessed by the changes in  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N content in the soil. These were then calculated based on oven-dried soil weight.

#### *Nucleic acid extraction and SIP gradient fractionation*

Soil DNA was extracted using a FastDNA spin kit for soil (MP Biomedicals, Cleveland, OH, USA), according to the manufacturer's instruction. Soil DNA quantity and purity were determined by a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). SIP fractionation was performed as described previously (Neufeld *et al.*, 2007). For each treatment, 2.0  $\mu\text{g}$  of soil DNA extract was mixed well with CsCl stock solution to achieve an initial CsCl buoyant density of 1.725  $\text{g ml}^{-1}$  before ultracentrifugation at 177 000  $g$  for 44 h at 20 °C. DNA fractionation was carried out by displacing the gradient medium with sterile water from the top of the ultracentrifuge tube using an NE-1000 single syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA) with a precisely controlled flow rate of 0.38  $\text{ml min}^{-1}$ . Up to 14 or 15 DNA gradient fractions were generated with equal volumes of about 380  $\mu\text{l}$ , and a 65  $\mu\text{l}$  aliquot of each fraction was used for refractive index measurement using an AR200 digital hand-held refractometer (Reichert, Inc., Buffalo, NY, USA). The fractionated DNA was purified and dissolved in 30  $\mu\text{l}$  TE buffer as discussed previously (Jia and Conrad, 2009).

#### *Real-time quantitative PCR*

Real-time quantitative PCR was performed in triplicate to verify the efficacy of  $^{13}\text{C}$  incorporation into the genomes of the autotrophic ammonia-oxidizing prokaryotes by analyzing the copy number of bacterial and archaeal *amoA* genes in the fractionated DNA across the entire buoyant density gradients from DNA-SIP microcosms as detailed previously (Jia and Conrad, 2009) on a CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc., Hercules, CA, USA). Bacterial and archaeal 16S rRNA genes were also quantified using domain-specific primers. The primers and PCR conditions are detailed in Supplementary Table S6. The real-time PCR standard was generated using plasmid DNA from one representative clone containing *amoA* gene or 16S rRNA genes of *Bacteria* and *Archaea*, and a dilution series of standard template over six to eight orders of magnitude per assay was used to optimize the real-time PCR conditions. Blanks were always run with water as the template instead of soil DNA extract. Specific amplification was verified by melting curve

analysis, which always results in a single peak, and by agarose gel electrophoresis.

#### *Pyrosequencing, cloning and phylogenetic analysis*

Pyrosequencing was carried out on a Roche 454 GS FLX Titanium sequencer (Roche Diagnostics Corporation, Branford, CT, USA) by analyzing the V4 regions of 16S rRNA genes in the fractionated DNA across the entire buoyant density gradients from the  $^{13}\text{CO}_2$ -treated SIP microcosms with or without  $\text{C}_2\text{H}_2$ . Taq sequences were used to barcode the PCR amplicons in each DNA gradient fraction from labeled and control treatments (Supplementary Tables S1 and S2). Triplicate amplicons were pooled, purified and visualized on 1.2% agarose gels. The concentration of purified PCR amplicons was determined, and the purified PCR amplicons were then combined in equimolar ratios into a single tube in preparation for pyrosequencing analysis. In addition, pyrosequencing of the archaeal 16S rRNA genes was performed using the archaea-specific primer shown in Supplementary Table S6.

In total, 196 000 high-quality sequence reads were obtained after removing about 20% of the original reads by RDP online pyrosequencing analysis (Cole *et al.*, 2009). Taxonomy of the high-quality sequence reads was assigned using the RDP classifier with a minimum support threshold of 60% and the RDP taxonomic nomenclature (Cole *et al.*, 2009), and only sequence reads highly similar to the putative nitrifying microorganisms were further analyzed. From each sample of the 28 DNA gradient fractions (Supplementary Table S1) and 13 DNA gradient fractions (Supplementary Table S2), all sequences affiliated with AOB, NOB and crenarchaeota or unclassified archaea by the RDP classifier were extracted. The targeted sequence reads from the  $^{13}\text{C}$ -labeled 'heavy' DNA fractions were clustered into operational taxonomic unit (OTU) at 97% cutoff using the mothur software (Schloss *et al.*, 2009), and the OTUs containing less than 10 reads were removed. A representative sequence was then used from each OTU for phylogenetic analysis.

Clone libraries of 16S rRNA and *amoA* genes were also constructed from the SIP microcosm after incubation for 4 weeks. Bacterial and archaeal 16S rRNA genes were amplified in the  $^{13}\text{C}$ -labeled 'heavy' DNA fractions for clone library construction using the universal primers 27f-1492r (Lane, 1991) and Arch21f-Arch958R (DeLong, 1992) as detailed in Supplementary Table S6. The bacterial *amoA* genes were amplified in the  $^{13}\text{C}$ -labeled 'heavy' DNA fractions and the 'light' DNA fractions from the  $^{13}\text{CO}_2$ -labeled microcosm for clone library construction (each containing 25 clones) using *amoA*-1F/*amoA*-2R assay (Rotthauwe *et al.*, 1997). PCR amplicons of archaeal *amoA* genes were obtained in both the  $^{13}\text{C}$ -labeled 'heavy' DNA fractions and the total DNA from the  $^{13}\text{CO}_2$ -labeled microcosm for

clone library construction (each containing 20 clones). Triplicate PCR products were pooled and purified before cloning. The *Escherichia coli* JM109-competent cells were used for transformation. Sequencing of the clones containing the correct insert was performed by the Invitrogen Sequencing Department (Invitrogen, Shanghai, China) and analyzed by DNASTAR software package (DNASTAR, Inc., Madison, WI, USA).

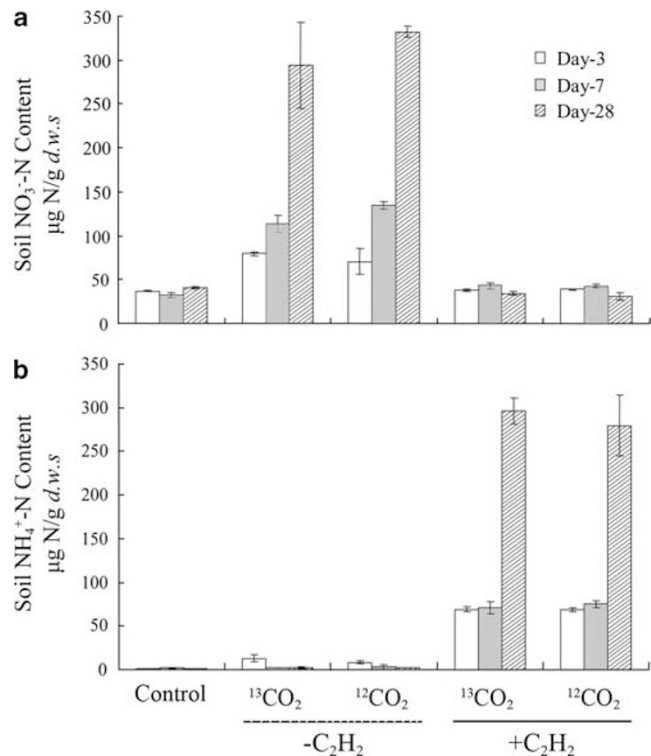
Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA 4.0) software package (Kumar *et al.*, 2004). For the bacterial 16S rRNA sequence highly affiliated with AOB, the basic tree of sequences larger than 1.4 kb was constructed through a neighbor-joining algorithm. The closest relatives to the  $^{13}\text{C}$ -labeled AOB sequences in this study and those from literature were then selected to reconstruct a phylogenetic tree inferred from the distance matrix using the Fitch algorithm with global rearrangement and a randomized input order of sequences. The tree topology was checked by the neighbor-joining algorithm and the minimum evolution method (Kumar *et al.*, 2004). Phylogenetic analysis of the bacterial and archaeal *amoA* genes were performed as described previously (Jia and Conrad, 2009).

#### Accession numbers of nucleotide sequences

The nucleotide sequences have been deposited at GenBank with accession numbers HQ678241–HQ678248 and HQ678202–HQ678208 for 16S rRNA genes of AOA and AOB, respectively, as well as HQ678187–HQ678201 and HQ678209–HQ678240 for the *amoA* genes of AOA and AOB, respectively, from the DNA-SIP experiment. The pyrosequencing reads have been deposited at GenBank with accession number DRA000318.

## Results

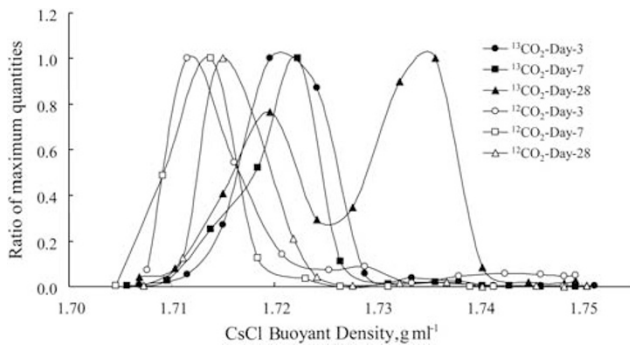
SIP incubation was established by feeding the soil microcosm with 5%  $^{13}\text{CO}_2$  or 5%  $^{12}\text{CO}_2$  with or without 100 Pa  $\text{C}_2\text{H}_2$ . Ammonium fertilization with  $100\ \mu\text{g}\ \text{NH}_4^+\text{-N g dry weight gram soil}$  was performed once a week at days 0, 7, 14 and 21 as described previously (Jia and Conrad, 2009). In the absence of  $\text{C}_2\text{H}_2$ , active nitrification activity was observed through significant nitrate production, which was completely abolished in the  $\text{C}_2\text{H}_2$ -treated soil microcosms at days 3, 7 and 28 (Figure 1). At day 28, the recovered ammonium concentration in the  $\text{C}_2\text{H}_2$ -treated soil was largely consistent with the nitrate produced in the soil in the absence of  $\text{C}_2\text{H}_2$ , suggesting the effective inhibition of  $\text{C}_2\text{H}_2$  on soil nitrification activity. The total DNA was extracted and subjected to isopycnic centrifugation for each of the four different treatments ( $^{13}\text{CO}_2$ ,  $^{12}\text{CO}_2$ ,  $^{13}\text{CO}_2 + \text{C}_2\text{H}_2$  and  $^{12}\text{CO}_2 + \text{C}_2\text{H}_2$ ) at days 3, 7 and 28 in triplicates for molecular analysis.



**Figure 1** Change in concentrations of soil nitrate (a) and ammonium (b) in surface agricultural soil incubated with either  $^{12}\text{CO}_2$  or  $^{13}\text{CO}_2$  in the presence or absence of  $\text{C}_2\text{H}_2$  after incubation for 3, 7 and 28 days. The error bars of soil nitrate and ammonium contents indicate standard deviations of triplicate microcosm incubations.

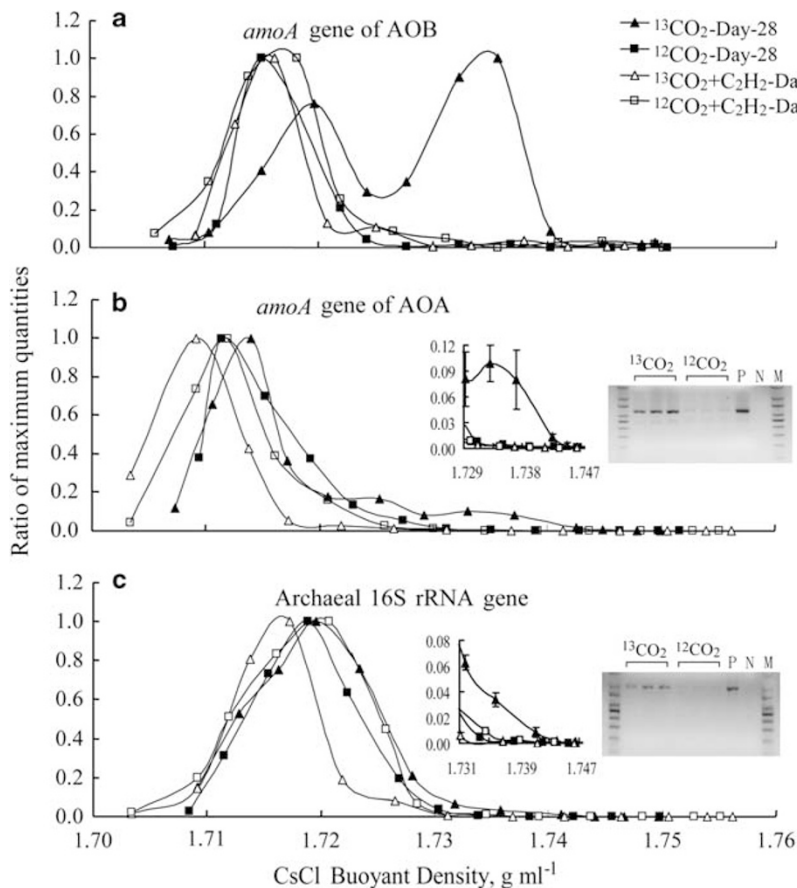
Real-time quantitative PCR indicated that the copy number of bacterial *amoA* genes peaked in the DNA fractions with buoyant densities with the  $^{13}\text{CO}_2$ -labeled treatment having a slightly higher value than those from the  $^{12}\text{CO}_2$  control treatment. This implies that the genomes of AOB cells were partially labeled in the  $^{13}\text{CO}_2$ -treated soils after incubation for 3 and 7 days (Figure 2). At day 28, the majority of bacterial *amoA* gene was observed in the heavier DNA fractions, indicating a much greater degree of labeling of the AOB cells during active nitrification (Figures 2 and 3a). Similarly, real-time quantitative PCR revealed that the abundance of archaeal *amoA* (Figure 3b) and 16S rRNA genes (Figure 3c) were significantly higher in the 'heavy' DNA fractions from the  $^{13}\text{CO}_2$ -labeled treatment than those from the control treatments (that is,  $^{12}\text{CO}_2$ ,  $^{12}\text{CO}_2 + \text{C}_2\text{H}_2$  and  $^{13}\text{CO}_2 + \text{C}_2\text{H}_2$ ) at day 28. However, the labeling of archaeal *amoA* and 16S rRNA genes was not detected at days 3 and 7. In addition, the PCR signals of archaeal *amoA* and 16S rRNA genes in the 'heavy' DNA fractions were clearly visualized on agarose gel from the  $^{13}\text{CO}_2$ -labeled treatment, but not from the  $^{12}\text{CO}_2$  control treatment (Figures 3b and c).

Pyrosequencing analysis of the total microbial community was performed in DNA gradient fractions

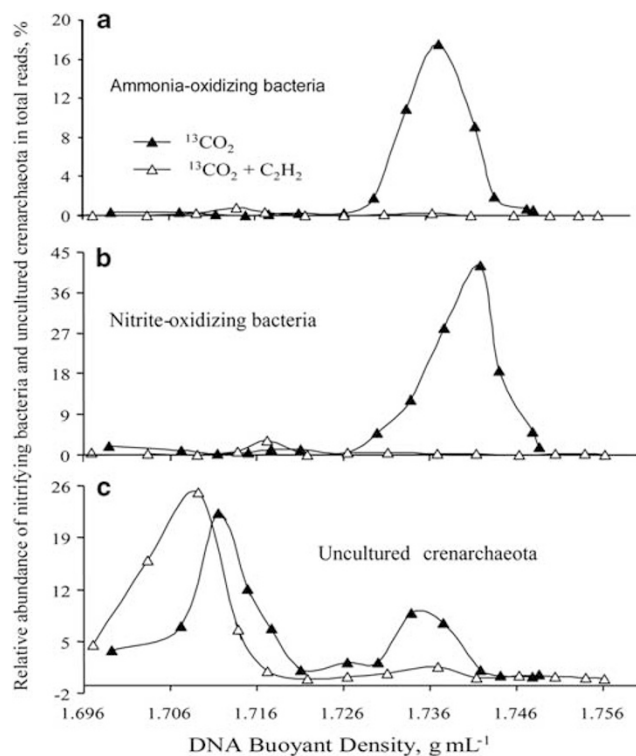


**Figure 2** Quantitative distribution of bacterial *amoA* gene copy numbers across the entire buoyant density gradient of the fractionated DNA from the soil incubated with either  $^{13}\text{CO}_2$  or  $^{12}\text{CO}_2$  without  $\text{C}_2\text{H}_2$  after incubation for 3, 7 and 28 days. The normalized data are the ratio of the gene copy number in each DNA gradient fraction to the maximum quantities from each treatment, as described previously (Lueders *et al.*, 2004). For clarity, the standard error bar from the triplicate microcosm incubations was removed.

from the labeled microcosm ( $^{13}\text{CO}_2$ ) and the control treatment ( $^{13}\text{CO}_2 + \text{C}_2\text{H}_2$ ) at day 28 (Supplementary Tables S1 and S2). About 196 000 high-quality sequence reads were obtained with an average length of about 370 bp in the V4 region of the 16S rRNA gene. Across the entire DNA gradient, the highly enriched 16S rRNA gene sequence of AOB (Figure 4a) and of NOB (Figure 4b) were only observed in the labeled microcosm in which ammonia oxidation was not blocked by  $\text{C}_2\text{H}_2$  (Figure 1). Up to 13.2% of bacterial 16S rRNA gene sequences in the  $^{13}\text{C}$ -labeled DNA were closely related to the *Nitrosospira* cluster 3 (Figure 5a), whereas sequences affiliated with the *Nitrosomonas* sp. comprised 4.5% of the total bacterial community in the  $^{13}\text{C}$ -labeled DNA fraction (Figure 5b). Similarly, NOB consisted of *Nitrospira*- and *Nitrobacter*-like 16S rRNA reads, accounting for 27% and 15% of total 16S rRNA sequences in the  $^{13}\text{C}$ -labeled DNA, respectively (Figures 5c and d). Phylogenetic analysis indicated that the majority of *Nitrospira*-



**Figure 3** Quantitative distribution of the *amoA* gene copy numbers of *Bacteria* (a), *Archaea* (b) and of the archaeal 16S rRNA gene copy number (c) across the entire buoyant density gradient of the fractionated DNA from the soil incubated with either  $^{12}\text{CO}_2$  or  $^{13}\text{CO}_2$  in the presence or absence of  $\text{C}_2\text{H}_2$  after incubation for 28 days. For clarity, the standard error bar from the triplicate microcosm incubations was removed, whereas it is shown in the insets. Strong signals of the PCR-amplified archaeal *amoA* (b) and 16S rRNA genes (c) were visualized by agarose gel electrophoresis in the  $^{13}\text{C}$ -labeled DNA fraction-6 from three biological replicates of the  $^{13}\text{CO}_2$ -treated soil, but not from the  $^{12}\text{CO}_2$ -treated soil. P, N and M denote positive control, negative control and DNA marker, respectively.



**Figure 4** Relative frequency of the 16S rRNA gene sequence reads affiliated with ammonia-oxidizing bacteria (a), nitrite-oxidizing bacteria (b) and uncultured crenarchaeota (c) across the entire buoyant density gradient of the fractionated DNA from the <sup>13</sup>CO<sub>2</sub>-treated soil after incubation for 28 days with or without 100 Pa acetylene. The abundance is expressed as the percentage of the targeted sequence reads to the total 16S rRNA gene reads in each DNA gradient fraction.

like sequences could be grouped into five OTUs, accounting for 95% of the total NOB sequences at 97% sequence similarity (Supplementary Table S3). On the other hand, more than 98% of the *Nitrobacter*-like sequences were assigned to a single OTU group (Supplementary Table S4), which was most closely affiliated with the NOB enriched from wastewater treatment plant (Alawi *et al.*, 2009). A similar result was observed for the uncultured crenarchaeal 16S rRNA reads, of which the relative frequency was apparently higher in the 'heavy' DNA fractions from the labeled microcosm than those in the control microcosms (Figure 4c). In addition, the crenarchaeal 16S rRNA reads in the labeled microcosm appeared to have a slightly higher buoyant density compared with those in the control microcosm (Figure 4c). This may imply an incremental labeling of uncultured crenarchaeota during active nitrification, despite the fact that the <sup>13</sup>C label seems to be assimilated to a much lesser extent compared with the bacterial *amoA* gene (Figure 2).

Clone library construction of the bacterial *amoA* and 16S rRNA genes was performed in the <sup>13</sup>C-labeled and native DNA fractions, as described previously (Jia and Conrad, 2009). Out of the 62 clones containing the near full-length bacterial 16S

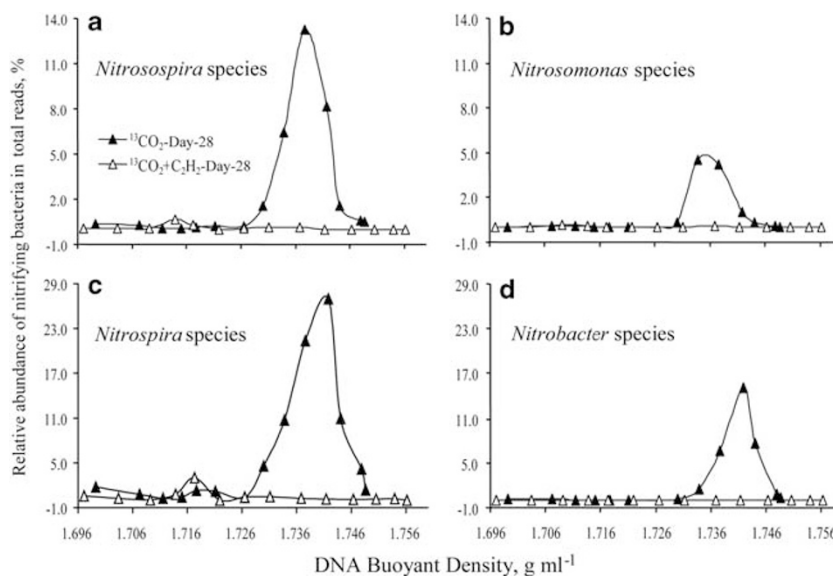
rRNA gene fragments, seven were identified as AOB sequences in the <sup>13</sup>C-labeled DNA (Supplementary Table S5). Phylogenetic analysis revealed that the active AOB were exclusively related to the *Nitrosospira* cluster 3 (Supplementary Figure S1). However, a molecular survey of the *amoA* gene indicated that two out of the 25 cloned *amoA* sequences were affiliated with the *Nitrosomonas* sp. Nm 58, whereas the other 23 sequences were placed within the *Nitrosospira* cluster 3 (Supplementary Figure S2). This observation was largely consistent with the pyrosequencing results (Supplementary Figure S1).

Clone libraries of archaeal 16S rRNA and *amoA* genes were also constructed from the <sup>13</sup>C-labeled DNA (Supplementary Figure S3). Phylogenetic analysis indicated that sequences affiliated with a moderately thermophilic AOA of *N. gargensis* accounted for the majority of archaeal *amoA* (33 out of 34 clones) and 16S rRNA genes (33 out of 64 clones). Pyrosequencing analysis of the archaeal 16S rRNA genes in the <sup>13</sup>C-labeled DNA revealed that 2918 out of 5504 reads were closely related to *N. gargensis* (Supplementary Figure S4A and Supplementary Table S2). This was supported by the specific enrichment (517 out of 1080) of sequences that were highly similar with *N. gargensis* when the total 16S rRNA gene was analyzed (Supplementary Figure S4B and Supplementary Table S1).

## Discussion

Microbial nitrification can serve as an ideal model for investigating community metabolism in complex soil environments by directly linking nitrification activity to the phylogenetic identity of physiologically distinct microorganisms. Using a SIP-targeted pyrosequencing approach, AOB and NOB were labeled to a much greater extent than AOA during active nitrification. Furthermore, the results in this study indicate that <sup>13</sup>CO<sub>2</sub> assimilation by AOB, AOA and NOB occurred only when nitrification was not inhibited, which suggests the chemolithoautotrophy of nitrifying community in the agricultural soil tested. These results imply that *Bacteria* dominated the nitrification activity in the agricultural soil tested, which is consistent with previous findings in German agricultural soil (Jia and Conrad, 2009).

The time-course incubation of SIP microcosms revealed that both AOB and AOA assimilated <sup>13</sup>CO<sub>2</sub> during active nitrification. The essential prerequisite for autotrophic ammonia oxidation is thought to be the *amoA* gene (Hyman and Arp, 1992). As shown in Supplementary Table S5, the *amoA* gene copy numbers of AOA and AOB in the 'heavy' DNA fractions from the labeled microcosm were generally one order of magnitude higher than those from the control microcosm. The <sup>13</sup>C-labeled AOB *amoA* genes accounted for 56.4% of the total AOB *amoA*



**Figure 5** Changes in the relative abundance of the 16S rRNA gene sequence reads affiliated with *Nitrosospira* (a) and *Nitrosomonas* (b) of the AOB community, and *Nitrospira* (c) and *Nitrobacter* (d) of the NOB community across the entire buoyant density gradient of the fractionated DNA from the  $^{13}\text{C}$ -treated soil after incubation for 28 days with or without 100 Pa acetylene. The relative abundance is expressed as the percentage of the targeted sequence reads to the total 16S rRNA gene sequence reads in each DNA gradient fraction.

gene abundance, whereas a constant background of about 2.62% was observed in the control treatments. With respect to AOA, 9.41% of the total archaeal *amoA* genes were retrieved in the  $^{13}\text{C}$ -labeled DNA (Supplementary Table S5) and 0.40–0.55% was found in the heavy fractions as background from the control microcosms. Similar observations were obtained for the 16S rRNA gene quantification of *Bacteria* and *Archaea* (Supplementary Table S5). Pyrosequencing has further suggested the exceptionally high abundance of AOB and NOB, up to 51.2% of the total microbial community in the  $^{13}\text{C}$ -labeled DNA (Figure 4). These findings provide strong hints that nitrification was primarily catalyzed by AOB and NOB in the agricultural soil tested. Assuming the ecological significance of AOA and AOB could be better revealed by the relative abundance of bacterial versus archaeal *amoA* genes in the  $^{13}\text{C}$ -labeled DNA, the archaeal contribution to nitrification activity was then estimated by multiplying the number of  $^{13}\text{C}$ -labeled *amoA*-containing AOA cells in the soil with the maximum ammonia oxidation rates estimated from known AOA cultures in marine habitats (Könneke *et al.*, 2005) and hot springs (de la Torre *et al.*, 2008). Theoretical calculation suggested that AOA could contribute 1.51% to 23.4% of the net nitrification activity from three biological replicates of SIP microcosm (Supplementary Table S5).

The putative archaeal contribution in this study has to be cautiously interpreted and re-assessed in the future because the cell-specific rate of  $^{13}\text{C}$ -labeled AOA cells in the soil tested may be highly different with that of *Nitrosopumilus maritimus* and of the *Candidatus Nitrosocaldus yellowstonii*. In fact, no ammonia oxidation activity

was observed after incubation of the soil tested for more than 5 months at 46 °C, which suggests that the labeled AOA was physiologically distinct from *N. gargensis*, despite the fact that they are the most phylogenetically related to one another. In addition, the carbon incorporation efficiency of AOA and AOB cannot adequately reflect nitrification activity because the relationship between  $\text{CO}_2$  fixation and ammonia oxidation is poorly understood. For instance, it remains unknown for AOA, whereas the mole ratio of  $\text{NH}_3$  oxidation to C incorporation is generally assumed to differ greatly among AOB species (Feliatra and Bianchi, 1993). Furthermore, archaeal ammonia oxidation activity may be underestimated by calculating AOA cells that are retrieved only in the ‘heavy’ DNA fractions containing the highly enriched  $^{13}\text{C}$  label (Supplementary Table S5). The overall distribution pattern of crenarchaeal 16S rRNA reads from the labeled microcosm showed a slightly drifting trend to the heavier DNA fractions when compared with that in the control microcosm (Figure 4c). Thus, the majority of AOA cells might have replicated at least once in the labeled soil microcosm even though only 9.41% of the total *amoA*-containing AOA cells actively replicated (Supplementary Table S5) and assimilated  $^{13}\text{C}$  to such an extent that they were sufficiently heavy to be revolved in the ‘heavy’ DNA fractions. Nevertheless, the results in this study show the chemolithoautotrophic growth of AOA within the soil crenarchaeota group I.1b lineage, which so far eludes cultivation in soil (Schleper, 2010).

The time-course experiment indicated that bacterial *amoA* genes were incrementally labeled during active nitrification upon ammonium fertilization (Figure 2), implying that AOB is actively involved

in soil nitrification. This was supported by the inferred cell-specific rate of ammonia oxidation. The AOB cell-specific rate fell well within the range reported in the literature (Okano *et al.*, 2004; Jia and Conrad, 2009), being sufficiently large to account for the ammonium consumption in the labeled microcosm (Supplementary Table S5). In contrast, the cell-specific rate of the labeled AOA was estimated to be 4.06 fmol per NH<sub>3</sub> oxidized cell per h, and it far exceeded the maximum capacity of the known AOA in cultures (0.59 fmol per NH<sub>3</sub> oxidized cell per h). This indicates that the observed nitrification activity could not have solely resulted from the AOA unless the growth of the labeled AOA in the soil is at least six-fold faster than that of the known AOA under optimal culture conditions. Thus, AOB seemed to dominate microbial ammonia oxidation by contributing >76.7% of the nitrification activity in the soil tested (Supplementary Table S5). This result is consistent with recent findings from studies involving German agricultural soil (Jia and Conrad, 2009), zinc-contaminated soils (Mertens *et al.*, 2009) and nitrogen-rich grassland soils (Di *et al.*, 2009).

A meta-analysis of the <sup>13</sup>C-labeled 16S rRNA genes in the literature was performed along with the active AOB retrieved in this study, showing physiologically distinct AOB ecotypes across various environments (Supplementary Figure S1). The *Nitrosospira* cluster 3 dominated active AOB population in this study, whereas the dominant sequence of active AOB in German agricultural soil (Jia and Conrad, 2009) was related to *Nitrosospira* sp. Nsp 65, of which the phylogenetic classification is still undefined (Koops *et al.*, 2006). In the estuarine sediments, the labeled 16S rRNA genes were related to the *Nitrosomonas* sp. Nm143 lineage and obligatorily halophilic *Nitrosomonas cryotolerans* lineage (Freitag *et al.*, 2006), whereas lake sediment AOB closely related to *Nitrosomonas europaea* or *Nitrosomonas eutropha* became metabolically active upon the addition of ammonium (Whitby *et al.*, 2001). In aquatic environments, AOB members within the *Nitrosospira* cluster 0 and *Nitrosomonas communis* lineage are exclusively found in ammonium-rich stream biofilms, whereas sequences within the *Nitrosomonas oligotroph* lineage are found in both ammonium-poor and ammonium-rich stream biofilms, as well as the mat of a Romanian Movile Cave without light (Chen *et al.*, 2009). The niche differentiation possibly selects the distinctly active nitrifiers in a wide variety of environments (Martens-Habbena *et al.*, 2009), which implies that AOB plays a major role in nitrification process in these environments. Niche separation may indeed explain the failure of detecting active archaeal nitrification in a previous study (Jia and Conrad, 2009). The community of AOA highly similar to *N. gargensis* (Supplementary Figure S3) was not observed in the German agricultural soil study. Thus, AOA in the previous study might have had lower growth rates than those affiliated with

*N. gargensis* in this study. The assimilation of <sup>13</sup>CO<sub>2</sub> might be too low to allow the detection of labeled AOA, as reported previously (Jia and Conrad, 2009). mRNA-SIP would be a powerful technique to elucidate the relative role of AOA and AOB in soil nitrification, with its much higher sensitivity than DNA-SIP (Huang *et al.*, 2009).

DNA-SIP clearly indicated that the active replication of *amoA*-containing AOA cells depended on ammonia oxidation activity as verified by the C<sub>2</sub>H<sub>2</sub> inhibition technique (Figures 3 and 4), providing strong evidence for the chemolithoautotrophic growth of AOA in complex soil environments. The widespread presence of archaeal *amoA* transcript suggests that AOA actively catalyzed ammonia oxidation under *in situ* conditions in soil (Treusch *et al.*, 2005; Nicol *et al.*, 2008) and marine habitats (Lam *et al.*, 2007; Santoro *et al.*, 2010). Archaeal ammonia oxidation is very likely a common feature that naturally occurs in a wide variety of environments on Earth, and its ecological importance varies greatly in accordance with the physicochemical characteristics of the habitats. For instance, recent studies showed that AOA within the marine crenarchaeota group I.1a lineage correlated well with nitrification activity in agricultural soil (Offre *et al.*, 2009), whereas archaeal ammonia oxidation in agricultural alkaline soil was performed by AOA members within the soil crenarchaeota group I.1b lineage in this study. The expression of archaeal *amoA* genes at high temperatures of up to 94 °C is observed in hot springs (Jiang *et al.*, 2010), implying that archaeal ammonia oxidation might have evolved under thermophilic conditions (de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008). In fact, comparative genomic analysis has provided strong support for the taxonomic assignment of the mesophilic *N. gargensis* and *N. maritimus* to the proposed Thaumarchaeota phylum (Spang *et al.*, 2010), a deeply branched group separate from, hyperthermophilic Crenarchaeota phylum. A considerable amount of crenarchaeota in moderate marine and terrestrial environments may not be ammonia oxidizers (Agogue *et al.*, 2008). Thus, the newly proposed phylum Thaumarchaeota appears to represent the best niche difference between mesophilic and hyperthermophilic habitats in which crenarchaeota live (Brochier-Armanet *et al.*, 2008), but not the putative primary physiology of archaeal ammonia oxidation (de la Torre *et al.*, 2008; Reigstad *et al.*, 2008).

The targeted pyrosequencing of the total 16S rRNA gene allows the fingerprinting of active NOB communities with an unprecedented level of detail. Phylogenetic analysis indicated that nitrite oxidation was primarily catalyzed by *Nitrospira*- and *Nitrobacter*-like NOB communities (Figure 5). Nitrite oxidation has long been speculated to be predominantly performed by some organisms other than the best-investigated *Nitrobacter*-like NOB in freshwater and aquarium biofilters (Hovanec and



DeLong, 1996) and activated sludge (Wagner *et al.*, 1996). This hypothesis was confirmed when *Nitrospira*-like NOB was detected as dominant nitrite oxidizers in the aquatic environment (Hovanec *et al.*, 1998; Schramm *et al.*, 1998). The results in this study show that the labeled community of *Nitrospira*-like NOB comprised a much higher proportion of the total microbial community in the  $^{13}\text{C}$ -labeled DNA than that of the *Nitrobacter*-like NOB (Figures 5c and d), suggesting that the former is involved in nitrification more actively than the latter. This may be explained by the high substrate affinity of *Nitrospira* (Koops and Pommerening-Röser, 2001). For instance, the best growth of *Nitrospira moscoviensis* was observed with 0.35 mM nitrite concentration (Ehrich *et al.*, 1995), whereas 1.5 mM could result in complete growth inhibition of a novel marine *Nitrospira*-like bacterium (Off *et al.*, 2010). The soil nitrite concentration was under detection limit in this study, implying the rapid conversion of nitrite into nitrate by the NOB. In the meantime, owing to the highly heterogeneous nature of soil aggregate, considerably higher concentrations of nitrite might occur in certain microhabitats, in which the labeling of *Nitrobacter*-like NOB is enhanced. Thus, a large and highly diverse group of *Nitrospira*-like NOB may have been functionally active, whereas a low diversity of *Nitrobacter*-like NOB was labeled. This was confirmed by a single OTU comprising 98% of *Nitrobacter*-like sequence reads (Supplementary Table S4), whereas *Nitrospira*-like NOB community was composed of phylogenetically more diverse groups of 16S rRNA genes in the  $^{13}\text{C}$ -labeled DNA (Supplementary Table S3). The result presented here is consistent with those previously reported in grassland soil (Freitag *et al.*, 2005) and wastewater treatment plants (Daims *et al.*, 2001). The low diversity of the labeled *Nitrobacter*-like NOB may also be attributed to the fact that putatively dominant *Nitrobacter* in this study might grow as heterotrophs (Daims *et al.*, 2001) and could not be labeled with  $^{13}\text{CO}_2$ . A recent study elegantly showed the functional importance of *Nitrobacter*-like NOB rather than *Nitrospira*-like NOB in soils and suggested the *r*- and *K*-strategy for the former and the latter, respectively (Attard *et al.*, 2010). In combination with the functional gene assay (Poly *et al.*, 2008), DNA-SIP would enable better assessment of the relative contribution of *Nitrobacter* and *Nitrospira* on soil nitrification.

The results in this study provide strong evidence that AOB, AOA, NOB and uncultured crenarchaeota assimilated  $^{13}\text{CO}_2$  as a result of active nitrification in the soil microcosm (Figures 3 and 4). The SIP-targeted pyrosequencing can, in principle, allow detailed analysis of the active microorganisms involved in the metabolism of labeled substrate in the soil with unprecedented coverage. This is most important for the detection of active microbial populations that have a slow rate of substrate turnover and growth due to low energy yield, or that comprise only a tiny fraction of the total

microbial community. For instance, 20 out of 2744 AOB sequence reads were found to be closely related to the *Nitrosomonas oligotroph* lineage by pyrosequencing analysis (Supplementary Figure S1), and none of these sequences were detected by clone library analysis of 16S rRNA (Supplementary Figure S1) and bacterial *amoA* genes (Supplementary Figure S2). Similarly, AOB members affiliated with *Nitrosomonas* sp. accounted for a considerable proportion, up to 4.5%, of the total 16S rRNA genes in the  $^{13}\text{C}$ -labeled DNA (Figure 5), but these AOB sequences could not be retrieved by clone library analysis of the 16S rRNA genes (Supplementary Figure S1). Therefore, the active microorganisms with low abundance in the  $^{13}\text{C}$ -labeled DNA can be readily detected with tremendously improved resolution and sensitivity by pyrosequencing rather than by the conventional clone library technique. Interestingly, NOB was labeled to a much greater extent than AOB by pyrosequencing analysis (Figure 4). Sequences highly similar to *Nitrospira* and *Nitrobacter* sp. comprised 26.9% and 15.1% of the 16S rRNA genes in the  $^{13}\text{C}$ -labeled DNA fractions, respectively (Figure 5). The significantly simultaneous labeling of AOB and NOB has thus suggested the bacterial community dominate microbial nitrification in the soil studied. In addition, there might have been some other unknown organisms that provided trace amounts of nitrification activity and was overlooked in this study. However, the molecular survey showed that AOB highly similar to the *Nitrosospira* cluster 3 responded positively to *in situ* long-term fertilization and dominated the active nitrifying community under field conditions (Chu *et al.*, 2007). Thus, the results presented here might largely reflect the predominant nitrification process occurring under field conditions, although the SIP microcosm did not entirely reproduce the physiochemical and biological conditions in the field.

Taken together, the results of this study show that *Bacteria* dominated nitrification in the soil tested. The labeling of AOB, AOA and NOB by  $^{13}\text{CO}_2$  indicates that nitrification is driven by a complex and multi-tiered autotrophic community. The acetylene inhibition technique suggests the chemolithoautotrophic metabolism of nitrifying community in complex soil environments. With the rapid advancement of next-generation sequencing techniques, the SIP-targeted pyrosequencing approach described herein will be a powerful tool in microbial ecology for investigating ecologically important processes in a wide variety of ecosystems with labeled substrates.

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