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Title page

Comammox *Nitrospira* play an active role in nitrification of agricultural soils amended with nitrogen fertilizers

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Abstract

The recent discovery of complete ammonia oxidizers (comammox *Nitrospira*) challenged the paradigm of the two-step nitrification mediated by two distinct groups of nitrifiers, and raised fundamental questions regarding their niche specialization and relative contribution to nitrification in agricultural soils. Previous studies suggest that comammox *Nitrospira* have an oligotrophic lifestyle and would outcompete canonical ammonia oxidizers (ammonia-oxidizing bacteria and ammonia-oxidizing archaea) under ammonia-limited conditions. Here, we demonstrated that comammox *Nitrospira* clade A were significantly more abundant than canonical ammonia oxidizers and $^{13}CO_2$-DNA-stable isotope probing revealed that comammox *Nitrospira* clade A incorporated $^{13}CO_2$ into their genomes in fertilized agricultural soils during the microcosm incubation. Phylogenetic analysis of the *amoA* gene revealed that $^{13}CO_2$-labeled comammox *Nitrospira* clade A belonged to the *Nitrospira inopinata*-related cluster and a new cluster that was distinct from the known comammox isolates. These results demonstrated the potential important role of comammox *Nitrospira* in autotrophic ammonia oxidation in agricultural soils amended with nitrogen fertilizers and their lifestyle may be not strictly restricted to oligotrophic habitats. There is a potential contribution of comammox *Nitrospira* to soil nitrification, which calls re-evaluation of the microbial nitrogen cycling processes and the subsequent impacts on agriculture and the environment.

Key words: Comammox *Nitrospira*; ammonia-oxidizing archaea; ammonia-oxidizing bacteria; complete nitrification; nitrification inhibitors; agricultural soils

1 Introduction

Nitrification, the sequential microbial oxidation of ammonia (NH_3) via nitrite (NO_2^-) to nitrate (NO_3^-), is a central process of the global nitrogen (N) cycling. The classic two-step nitrification process includes conversion from NH_3 to NO_2^- by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), and conversion from NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB) (Costa et al., 2006). Since the identification of archaea being capable of oxidizing ammonia to nitrite (Könneke et al., 2005), substantial efforts have been devoted to exploring the ecological significance and niche specialization of AOA and AOB in terrestrial ecosystems during the past decade (He et al., 2007; Hu et al., 2014; Prosser and Nicol, 2012). Although the relative contribution of AOA and AOB to nitrification is still debated, numerous physiological and genomic studies have supported their potential niche separation: AOA are more adapted to oligotrophic conditions characterized by acidic soils and nitrogen-depleted environments (He et al., 2007; Lehtovirta-Morley et al., 2011; Zhang et al., 2012); AOB have a eutrophic lifestyle and are more dominant in nitrogen-rich environments (Di et al., 2009).

In 2015, the discovery of complete ammonia oxidizers (termed “comammox”) within the *Nitrospira* genus that are capable of oxidizing NH_3 to NO_3^- in a single organism radically challenged our long-held perspective of classic nitrification and the relative contribution of canonical ammonia oxidizers (Daims et al., 2015; van Kessel et al., 2015). Notably, in earlier theoretical studies, comammox organisms were predicted to play a crucial role under substrate-limiting conditions, such as in ammonia-depleted biofilms (Costa et al., 2006). Indeed, the enrichment of comammox organisms was successfully obtained from a deep oil exploration well (Daims et al., 2015) and an aquaculture system (van Kessel et al., 2015) containing low ammonium substrate influx. In 2017, a pure culture of comammox bacterium, *Nitrospira inopinata* was isolated from a microbial biofilm (Kits et al., 2017), and kinetic characterization revealed its high affinity for ammonia which facilitates the competitive advantage of comammox in oligotrophic habitats. Comparison of the ammonia oxidation kinetics for *N. inopinata*, AOA and AOB cultures suggested that, in contrast to earlier perceptions (He *et al*., 2012; Hu et al., 2014; Prosser and Nicol, 2012), comammox *Nitrospira* may out-compete AOA and AOB in ecological niches characterized by low ammonia availability (Kits et al., 2017).

Metagenomic profiling in published databases and PCR-based molecular investigations revealed the broad distribution of comammox *Nitrospira* in various aquatic and terrestrial habitats and engineered environments, including forest soils, freshwater sediments, wastewater treatment plants, and drinking water treatment systems (Bartelme et al., 2017; Beach and Noguera, 2019; Daims et al., 2015; Fowler et al., 2018; Hu and He, 2017; Palomo et al., 2016; Pinto et al., 2015; Pjevac et al., 2017; van Kessel et al., 2015; Wang et al., 2017; Yu et al., 2018). The majority of these habitats are characterized by low ammonium concentrations. These findings raised fundamental questions regarding the functional importance of comammox *Nitrospira* and the niche separation between comammox *Nitrospira* and canonical nitrifiers in terrestrial ecosystems. However, we have a very incomplete understanding of the relative contribution of comammox *Nitrospira* and canonical nitrifiers to the global nitrogen cycle, especially under copiotrophic conditions. As a novel group of ammonia oxidizers, it is imperative to bridge our knowledge gap regarding the potential contribution of comammox *Nitrospira* in nitrification of agricultural soils, which usually receive a large amount of N fertilizers to sustain agricultural production and might be unfavourable for the growth of comammox *Nitrospira*.

The aim of this study was to examine the potential contribution of comammox *Nitrospira* to nitrification and the niche differentiation between comammox *Nitrospira* and canonical nitrifiers in fertilized agricultural soils. We established a laboratory microcosm incubation to: (1) determine the responses of net nitrification rates and comammox *Nitrospira*, AOA and AOB populations to addition of N fertilizer and two nitrification inhibitors (NIs) (C_2H_2 and C_8H_{14}) in the pasture and arable soils; (2) assess the incorporation of $^{13}CO_2$ into the genomes of comammox *Nitrospira*, AOA and AOB using $^{13}CO_2$-DNA-stable isotope probing (SIP) technique; and (3) characterize the community composition of $^{13}CO_2$-incorporated comammox *Nitrospira* through cloning library, sequencing and phylogenetic analysis.

2 Materials and methods

2.1 Site description and soil sampling

Soil samples were collected in May 2018 from two long-term research farms: a dairy farm site (pasture soil) at Dookie (36° 33’ S, 145° 69’ E) and a vegetable farm site (arable soil) at Clyde (38° 13’ S, 145° 33’ E), Victoria, Australia. The Dookie site has been used as a dairy farm for more than 30 years, including around 43 hectares of irrigated pastures with a feeding capacity for 180 cows. The pastures at the Dookie site are a mixture of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). The arable field at Clyde is planted with celery, and has been treated with chicken manure and inorganic fertilizers and conventionally managed in the previous years. The mean annual maximum and minimum temperatures at Dookie are 21°C and 9°C, respectively, and the mean annual rainfall is 554 mm. The mean annual maximum and minimum temperatures at Clyde are 20°C and 10°C, respectively, and the mean annual rainfall is 816 mm. The soil texture of the Dookie pasture is silty loam, while the soil of Clyde arable plot is loamy sand. The pH values of the two soils are neutral (6.90 at Dookie and 7.13 at Clyde). Approximately 5 kg soils consisted of around 40 soil cores (5 cm in diameter) were randomly collected from the upper 10 cm of soils across the whole field of each site, mixed well into a composite sample, placed into plastic buckets, and shipped on ice to the laboratory. The soil samples were sieved through a 2 mm sieve to remove roots and stones, mixed thoroughly and stored at 4°C for 3~5 days prior to construction of soil microcosms.

2.2 Soil microcosm incubation

The soil incubation was performed in 250-ml plastic vials containing 20 g soil samples (oven dry weight equivalent). Acetylene (C_2H_2) has been widely used as an effective and unspecific inhibitor of AOA and AOB ammonia monooxygenase (AMO) (Offre et al., 2009), and 1-octyne (C_8H_{14}) is a selective inhibitor that specifically impedes AOB AMO (Hink et al., 2017). However, the response of comammox *Nitrospira* to C_2H_2 and C_8H_{14} remains unknown, and thus the effects of both NIs were examined in the soil microcosm experiments in this study. Four treatments were established with three replicates: (i) control; (ii) $(NH_4)_2SO_4$; (iii) $(NH_4)_2SO_4$ + C_2H_2 (0.1% v/v) in the headspace; and (iv) $(NH_4)_2SO_4$ + C_8H_{14} (0.03 % v/v) in the headspace. The $(NH_4)_2SO_4$ fertilizer was added to soils to reach a final concentration of 100 mg NH_4^+-N kg^{-1} soil. The concentrations of C_2H_2 and C_8H_{14} in the headspace of incubation vials were based on previous studies, which revealed high inhibitory effects on canonical ammonia oxidizers in different soils (Offre et al., 2009; Taylor et al., 2013; Hink et al., 2017). All microcosms were incubated in a temperature-controlled incubator at 25°C in the dark for 28 days. Soil moisture content was maintained at 55% water filled pore space (WFPS) by weekly adding water to the incubation vials based on the weight changes. Aerobic conditions, C_2H_2 and C_8H_{14} contents were maintained every three days by opening the vials and replenishing. Soil microcosms were destructively collected on days 0, 3, 7, 14, 21 and 28, and stored at -80°C before molecular analysis and determination of mineral N at the same time. The flow chart of the main steps in this study is shown in Fig. 1.

2.3 Soil physicochemical analysis

Soil pH was analysed with a soil to water ratio of 1: 5 using an Orion Star A211 pH meter (Thermo Fisher Scientific Inc., Melbourne, Australia). Total carbon (C) and N were determined via the Dumas combustion method on an isotope ratio mass spectrometer (Sercon Hydra, Crewe, UK). Soil NH_4^+-N and NO_3^--N were extracted by 1 M KCl solution with a soil to solution ratio of 1:5, filtered through a Whatman Grade 42 filter paper (Sigma-Aldrich Co., St. Louis, MO, USA), and analysed with a segmented flow analyzer (SAN++; Skalar, Breda, Holland). Available Phosphorus (P) were measured via the Colwell P test on a segmented flow analyzer (SAN++; Skalar, Breda, Holland). Available sulphur (S) and potassium (K) were extracted with a soil to Mehlich 3 ratio of 1:10 and estimated by Optima 8300 ICP-OES spectrometer (PerkinElmer Inc., Waltham, WA, USA). The basic properties of soils are shown in Table 1.

2.4 DNA-stable isotope probing (DNA-SIP) microcosm

We constructed a laboratory DNA-SIP microcosm incubation to identify the metabolically active comammox *Nitrospira* and canonical ammonia oxidizers which can incorporate ^{13}C into their genomes in the pasture and arable soils. Ten grams of sieved fresh soils were sealed in 160-ml serum bottles with butyl rubber stoppers and aluminium caps. Four treatments with three replicates were set up: (i) $(NH_4)_2SO_4$ (100 mg NH_4^+-N soil kg^{-1} soil) plus 5% (vol/vol) $^{12}CO_2$; (ii) $(NH_4)_2SO_4$ plus 5% $^{13}CO_2$ (99 atom%; Sigma-Aldrich Co., St. Louis, MO, USA); (iii) $(NH_4)_2SO_4$ plus 5% $^{13}CO_2$ plus C_2H_2 (0.1% v/v); and (iv) $(NH_4)_2SO_4$ plus 5% $^{13}CO_2$ plus C_8H_{14} (0.03% v/v). The microcosms were incubated at 25°C in the dark for 28 days and maintained at 55% WFPS during the incubation. The concentrations of CO_2, C_2H_2 and C_8H_{14} in the headspace were maintained every three days following aeration. Soil samples were destructively collected at days 0 and 28 and immediately frozen at -80°C.

2.5 DNA extraction and SIP fractionation

Nucleic acids were extracted from 0.25 g of soil using MoBio PowerSoil DNA isolation kits (MoBio Laboratories, Carlsbad, CA, USA). The extracted DNA concentrations were determined using the NanoDrop ND2000c spectrophotometer (NanoDrop Technologies).

Density gradient centrifugation was performed in 4.9-ml OptiSeal polyallomer tubes (Beckman Coulter, Palo Alto, CA, USA) in a VTi 90 vertical rotor (Beckman Coulter), subject to isopycnic centrifugation at 56,200 rpm (228,166 × gav) at 20°C for 24 h (Hu et al., 2015; Zhang et al., 2012). The extracted DNA (around 2 µg) was added into the CsCl gradient buffer with an initial density of 1.696 g ml^{-1} in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Centrifuged gradients were fractionated into 25 equal volumes (around 200 µl) by displacing the CsCl solution with sterile water at the top of the tube, using a fraction recovery system (Beckman Coulter) and a syringe pump (New Era Pump System, USA). The buoyant density of each collected fraction was calculated by measuring the refractive index of 30 µl aliquots using a hand-held refractometer (Uni-It, Bellingham, WA, USA). DNA was subsequently precipitated from the CsCl gradients through the addition of two volumes of 30% (wt/vol) polyethylene glycol 6000 in 1.6 M NaCl, washed twice with ice-cold 70% ethanol, and dissolved in 30 µl of sterilized water. The cleaned DNA samples were used to determine the *amoA* gene abundances of AOA, AOB and comammox *Nitrospira* in each fraction using quantitative PCR (qPCR).

2.6 Real-time qPCR assay of *amoA* gene

All qPCR assays were performed on a Bio-Rad CFX384 optical real-time PCR detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The primer sets and amplification conditions for quantification of AOA, AOB and comammox *Nitrospira amoA* gene abundances are shown in Table S1. The 10-µl reaction mixture contained 5 µl of SensiMix SYBR No-ROX reagent (Bioline, Sydney, Australia), 0.5 µl of each primer (10 µM), and 2 µl of template DNA. Standard curves were generated using 10-fold serial dilutions of plasmids containing correct inserts of the target genes. Melting curve analysis was performed at the end of each qPCR run to check the specificity of PCR products, before identification by agarose gel electrophoresis. Based on the gel electrophoresis results, we failed to obtain a high-quality single band for comammox *Nitrospira* clade B in either pasture or arable soil, and therefore comammox *Nitrospira* clade B were excluded from the downstream analysis. The amplification efficiencies of all *amoA* gene were 85-99% and the r^2 values were > 0.99.

2.7 Cloning library, sequencing and phylogenetic analysis

The PCR products of AOA, AOB and comammox *Nitrospira* clade A *amoA* genes from the heavy fractions (considered as the active ammonia oxidizers incorporating ^{13}C into their genomes during the DNA-SIP incubation) in the $^{13}CO_2$ microcosms at day 28 were purified using the Wizard® SV gel and PCR clean-up system (Promega, Sydney, Australia) and quantified using the NanoDrop ND2000c spectrophotometer (Thermo Fisher Scientific Inc., Melbourne, Australia). The resultant PCR products were cloned into the pGEM®-T Easy vector and transformed into *Escherichia coli* JM109 competent cells based on the manufacturer’s instructions. Positive clones were sent to Macrogen Sequencing Department, South Korea to analyse the sequences containing the correct inserts of the targeted gene sequences. The obtained sequences were checked for chimeras and aligned with reference sequences using MUSCLE in MEGA 7.0 (Kumar et al., 2016). Phylogenetic analysis was performed through constructing a neighbour-joining tree with the translated amino acid sequences using the Kimura 2-parameter distance with 1,000 replicates to produce bootstrap values. The obtained nucleotide sequences were deposited at the Genbank database under the accession numbers MK581036 to MK581050 for comammox *Nitrospira* clade A, MK573226 to MK573236 for AOA, and MK570514 to MK570519 for AOB.

2.8 Statistical analysis

One-way analysis of variance (ANOVA) based on the Duncan test was conducted to test the difference in ammonium and nitrate concentrations, the abundances of comammox *Nitrospira* clade A, AOA and AOB across different treatments and sampling times in SPSS Statistics 25 (IBM, USA). Differences at a P value of < 0.05 were statistically significant.

3. Results

3.1 Changes in soil properties during the microcosm incubation

The changes of NH_4^+-N and NO_3^--N concentrations were measured after the application of $(NH_4)_2SO_4$ fertilizer and two NIs, C_2H_2 and C_8H_{14} in the pasture and arable soils in a short-term microcosm incubation. In the control treatments, the NH_4^+-N concentrations remained largely unchanged in both soils (Fig. 2A and 2B), while the NO_3^--N concentrations continuously increased over time, indicating a sustaining nitrification activity in both soils (Fig. 2C and 2D). In the $(NH_4)_2SO_4$ treatment, the NH_4^+-N concentrations rapidly reduced to the same levels as that in the control treatment and the NO_3^--N concentrations sharply increased, indicating a high rate of ammonia oxidation. The $(NH_4)_2SO_4$ treatment had significantly ($P < 0.05$) higher net nitrification rates at ~9.36 (±0.26) and ~4.64 (±0.13) mg NO_3^- -N kg^{-1} soil per day than the control treatment (~5.86 (±0.02) and ~1.44 (±0.04) mg NO_3^--N kg^{-1} soil per day) in the pasture and arable soils, respectively. Addition of C_2H_2 significantly increased the NH_4^+-N concentrations in the pasture soil (Fig. 2A), probably due to the NH_4^+-N accumulation from the mineralization of organic N, but C_2H_2 addition had no significant effect in the arable soil (Fig. 2B). The NO_3^--N concentrations in the treatments with NIs had significantly ($P < 0.05$) lower values than that in the $(NH_4)_2SO_4$ treatment. Addition of C_2H_2 resulted in almost complete inhibition of nitrification, and addition of C_8H_{14} partially inhibited nitrification in both soils (Fig. 2C and 2D).

3.2 Changes of the *amoA* gene abundance during the microcosm incubation

Comammox *Nitrospira* clade A, AOB and AOA communities were quantified using the qPCR analysis. In the control treatments, the *amoA* gene abundances of comammox *Nitrospira* clade A ranged from 4.32×10^7 ($\pm 9.71 \times 10^5$) to 9.26×10^7 ($\pm 1.10 \times 10^7$) copies g^{-1} soil in the pasture soil and 1.77×10^7 ($\pm 3.33 \times 10^6$) to 3.28×10^7 ($\pm 3.22 \times 10^6$) copies g^{-1} soil in the arable soil (Fig. 3), with the highest values observed at day 3. Compared to control, addition of $(NH_4)_2SO_4$ significantly ($P < 0.05$) increased the comammox *Nitrospira* clade A abundance in the pasture soil at days 7 and 14, but no significant effect was observed in the arable soil (Fig. 3). The comammox *Nitrospira* clade A abundances showed opposite responses to the two NIs. Compared with the $(NH_4)_2SO_4$ treatment, addition of C_2H_2 significantly ($P < 0.05$) decreased comammox *Nitrospira* clade A abundance from 7.98×10^7 ($\pm 3.40 \times 10^6$) at day 0 to 2.62×10^7 ($\pm 9.79 \times 10^5$) copies g^{-1} soil at day 28 in the pasture soil, and from 1.63×10^7 ($\pm 1.32 \times 10^6$) at day 0 to 8.67×10^6 ($\pm 9.41 \times 10^5$) copies g^{-1} soil at day 28 in the arable soil (Fig. 3). By contrast, the C_8H_{14} addition showed no significant inhibition on the comammox *Nitrospira* clade A abundance after day 3 in either soil (Fig. 3).

The responses of AOA abundance to $(NH_4)_2SO_4$ and NIs were similar with comammox *Nitrospira* clade A in both soils. The AOA abundance was significantly increased ($P < 0.05$) after adding $(NH_4)_2SO_4$ in both soils (Fig. 3). Compared with the $(NH_4)_2SO_4$ treatment, C_2H_2 significantly ($P < 0.05$) inhibited the growth of AOA whereas C_8H_{14} had no obvious inhibitory effects on the AOA abundance in either soil (Fig. 3). In the $(NH_4)_2SO_4 + C_8H_{14}$ treatment of arable soil, the AOA abundance had a significant ($P < 0.05$) increase over time (Fig. 3). The AOB abundance tended to decrease over time in the control treatments of both soils (Fig. 3). Compared with control, $(NH_4)_2SO_4$ application significantly ($P < 0.05$) increased the AOB abundances in both soils. Addition of C_2H_2 and C_8H_{14}

significantly ($P < 0.05$) inhibited the growth of AOB in both soils but to varying degrees during the incubation (Fig. 3).

3.3 DNA-SIP microcosm to track active ammonia oxidizers

We constructed a 28-day DNA-SIP microcosm incubation to identify the functionally active ammonia oxidizers labelled by $^{13}CO_2$ during the active nitrification. The qPCR analysis of the *amoA* gene in the fractioned DNA revealed that comammox *Nitrospira* clade A and AOA were labelled in both soils and AOB were only labelled in the arable soil. The relative abundances of comammox *Nitrospira* clade A in the $^{12}CO_2$ treatments reached the maximal value around a buoyant density of ~1.70 - 1.71 g ml^{-1} in both soils (Fig. 4). After the 28-day incubation, the peak of comammox *Nitrospira* clade A communities shifted to heavy fractions of ~1.72 g ml^{-1} in both soils (Fig. 4), indicating that the majority of comammox *Nitrospira* clade A assimilated $^{13}CO_2$ into their DNA during the active nitrification. The peak of comammox *Nitrospira* clade A in the $^{13}CO_2$ + C_2H_2 treatment remained in the light fractions in both soils at day 28 (Fig. 4), indicating that their activity was effectively inhibited by C_2H_2. However, the inhibitory effect of C_8H_{14} on the activity of comammox *Nitrospira* clade A was not obvious in either soil (Fig. 4).

The AOA abundance clearly shifted after the $^{13}CO_2$ addition from the light fractions (~1.69 g ml^{-1}) to the heavy fractions (~1.72 g ml^{-1}) at day 28 in both soils (Fig. 4). The peak of the AOA abundance in the $^{13}CO_2$ + C_2H_2 treatment overlapped with that in the $^{12}CO_2$ treatment at day 28 in both soils, indicating that C_2H_2 significantly inhibited the assimilation of $^{13}CO_2$ into AOA (Fig. 4). As expected, no obvious effect of C_8H_{14} addition on the activity of AOA was observed in either soil (Fig. 4). The majority of AOB populations were labelled by $^{13}CO_2$ and distributed in the heavy fractions of ~1.72 g ml^{-1} at day 28 in the arable soil (Fig. 4). Compared with the $^{13}CO_2$ treatment, C_2H_2 and C_8H_{14} effectively inhibited the growth of AOB in the arable soil (Fig. 4). By contrast, no apparent change in the AOB abundance between the $^{12}CO_2$ and $^{13}CO_2$ treatments was detected in the pasture soil, suggesting that $^{13}CO_2$ was not incorporated into AOB (Fig. 4).

3.4 Phylogenetic analysis of ^{13}C-labelled nitrifiers

The phylogenetic trees of the *amoA* gene were constructed using the translated amino acid sequences obtained from the heavy regions in the $^{13}CO_2$ microcosms of both soils at day 28, except the metabolically inactive AOB community in the pasture soil. The results revealed that 96% of the ^{13}C-labelled comammox *Nitrospira* clade A community in the arable soil (based on 54 positive clones) fell into Cluster 1 that was highly related to many published comammox *amoA* gene sequences (Fig. 5). Intriguingly, majority (82%) of ^{13}C-labelled comammox *Nitrospira* clade A community in the pasture soil (based on 45 positive clones) belonged to a separate cluster, temporarily named Cluster 2 (Fig. 5). The ^{13}C-labeled AOA community in the arable soil (based on 39 positive clones) was classified into *Nitrosopumilus* and *Nitrososphaera*, accounting for 67% and 31% of the obtained AOA *amoA* gene sequences, respectively (Fig. S1). The ^{13}C-labeled AOA community in the pasture soil (based on 41 positive clones) was mainly affiliated with *Nitrosotalea*, representing 95% of the obtained AOA *amoA* gene sequences (Fig. S1). The ^{13}C-labeled AOB community in the arable soil (based on 18 positive clones) was affiliated with the *Nitrosopira* Cluster 3, and 78% of them belonged to Cluster 3a (Fig. S2).

4. Discussion

Previous studies have recognized ammonia availability as a major factor shaping the niche differentiation between AOA and AOB (Hu et al., 2014; Prosser and Nicol, 2012). Agricultural soils usually receive a large amount of N-based fertilizers to promote plant growth, and pasture soils receive a majority of N from animal excreta return (Di et al., 2009; Di et al., 2010). The theoretical NH_3 concentrations in soil aqueous solution calculated based on the ionization equilibrium ($NH_4^+ \leftrightarrow NH_3 + H^+$; $pK_a = 9.25$ at 25℃) ranged from 159~893 nM in the pasture soil and from 408~781 nM in the arable soil (Table S2), which are remarkably higher than the ammonia affinity ($K_{m\ (NH3)}$) of the pure comammox *Nitrospira inopinata* around 63 nM (Kits et al., 2017). Therefore, comammox *Nitrospira*, which are assumed to have an oligotrophic lifestyle (Kits et al., 2017), are hypothesized be less competitive than canonical ammonia oxidizers in agricultural systems. However, contrasting to the hypothesis, our study demonstrated that comammox *Nitrospira* clade A remained one order of magnitude more abundant than AOA and AOB in both agricultural soils, the application of N fertilizer significantly increased the comammox *Nitrospira* clade A abundance in the pasture soil, and comammox *Nitrospira* clade A can incorporate ^{13}C-CO_2 into their genomes during the incubation. These results provide evidence that comammox *Nitrospira* clade A may play an active role in nitrification of agricultural soils amended with N fertilizers.

The majority of ^{13}C-labelled comammox *Nitrospira* in both soils belonged to Clusters 1 and 2 of clade A. To our knowledge, this is the first characterization of the ^{13}C-labelled comammox *Nitrospira* clade A community in agricultural soils, and this study provides valuable insights into Cluster 2 of comammox *Nitrospira*, which is separated from Cluster 1 containing comammox *Nitrospira* mostly from ecosystems characterized by low ammonia concentrations (Fig. 5). Cluster 2 may represent assemblages of comammox *Nitrospira* clade A that can play a potential role in nitrification of agricultural soils. A recent study reported that comammox *Nitrospira* clade B grew autotrophically in the forest and paddy soils and may contribute to nitrification in the absence of ammonium amendment (Wang et al., 2019). Therefore, we assume that there is a possible niche separation between comammox *Nitrospira* clade A and clade B in the soil environment probably driven by the ammonia availability, and it requires future investigation of a range of soils with differing N mineralization potentials or that are exposed to different rates of ammonium application that drive different rates of nitrification to verify the potential niche separation. Phylogenetic analyses of the AOA *amoA* genes retrieved from $^{13}CO_2$-labeled heavy fractions in DNA-SIP microcosms showed that the active AOA belonged to *Nitrosopumilus*, *Nitrosotalea* and *Nitrososphaera* clusters, which have been widely reported to be associated with autotrophic ammonia oxidation in various soil environments (Gubry-Rangin et al., 2010; Offre et al., 2009; Shi et al., 2016; Zhang et al., 2012).

Another major finding of this study is that comammox *Nitrospira* clade A in soils were significantly inhibited by C_2H_2, which is the first report for the inhibitory of NIs on comammox *Nitrospira* in the soil environment. Before the discovery of comammox *Nitrospira*, many studies have attempted to identify the specific inhibitors exclusively targeting AOA or AOB in laboratory and field experiments. It was found that most NIs were not selective and they impeded the functionally dominant groups to a greater extent (Jia and Conrad, 2009; Offre et al., 2009). For example, AOB were effectively inhibited by C_2H_2 in agricultural soils functionally dominated by AOB (Jia and Conrad, 2009), while DCD significantly inhibited the growth and activity of AOA in acidic soils dominated by AOA (Zhang et al., 2012). The only selective NI that has been reported is C_8H_{14}, which is a recently discovered inhibitor specifically inhibiting AOB growth, activity and nitrous oxide production (Hink et al., 2017; Ouyang et al., 2016; Taylor et al., 2013). Our study confirmed that addition of C_2H_2 can inhibit AOA and AOB while C_8H_{14} exclusively inhibited AOB, and provided new evidence that the abundance of comammox *Nitrospira* clade A was significantly reduced after the application of C_2H_2. The results of DNA-SIP further revealed that incorporation of CO_2 into the genomes of comammox *Nitrospira* clade A was effectively impeded by C_2H_2, suggesting the autotrophic complete nitrification process in the soil can be manipulated by adding inhibitors. These results suggest that comammox *Nitrospira* AMO is catalytically active and can be inactivated by C_2H_2. The C_2H_2 treatment has significantly lower comammox *Nitrospira* abundance than the $(NH_4)_2SO_4$ treatment, probably because a sustained attempt by comammox bacteria to resynthesize AMO *de novo* over several days is impeded by C_2H_2, resulting in a ‘burn out’ of the comammox bacterial cells. Unlike AOB, there is no inhibition of C_8H_{14} on the abundances or activities of comammox *Nitropira* clade A in pasture and arable soils. Except for the specificity of C_8H_{14} for AOB, we assume that comammox *Nitrospira* AMO might be more similar to AOA AMO and less sensitive to longer-chain-length alkynes like C_8H_{14} (Taylor et al., 2015), or comammox *Nitrospira* prefer NO_2^- as the electron donor. Our results have significant implications for a better mechanistic understanding of the molecular mechanisms of NIs in soils, which is important for refining agricultural management of the microbial N cycle to promote N use efficiency and reduce N losses in agriculture.

5. Conclusions

Our understanding of the niche specification and metabolic versatility of comammox *Nitrospira* was restricted to investigations in engineered systems and aquatic and terrestrial ecosystems characterized by low ammonia concentrations. This study, by using $^{13}CO_2$-DNA-SIP and molecular approaches, demonstrated that, contrary to previous perceptions of the oligotrophic lifestyle of comammox *Nitrospira*, they are abundant and might play an active role in the two tested agricultural soils amended with N fertilizers. These findings provide new insights into the ecological niche of comammox *Nitrospira* in the terrestrial ecosystem, with implications for future management of the biogeochemical N cycle through manipulating the role of complete nitrification process.

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Table 1 Basic characteristics of the two soils in this study.

Property	**Pasture soil （Dookie）**	**Arable soil (Clyde)**
Soil pH (H_2O)	6.90	7.13
Total C (g kg^{-1})	35	25
Total N (g kg^{-1})	3.8	2.9
NH_4^+-N (mg kg^{-1})	0.82	0.17
NO_3^--N (mg kg^{-1})	38.60	21.83
Sulphur (mg kg^{-1})	22	45
Phosphorus (mg kg^{-1})	196	675
Potassium (mg kg^{-1})	348	129
Texture	Silty loam	Loamy sand

Figure Legends

Fig. 1 Flow chart of the main experimental steps in this study.

Fig. 2 Changes in the ammonium and nitrate concentrations during the microcosm incubation of the pasture soil (A and C) and the arable soil (B and D). Error bars indicate standard errors of triplicate samples.

Fig. 3 Changes in the *amoA* gene abundance of comammox *Nitrospira* clade A, AOA and AOB during the microcosm incubation of the pasture and arable soils. Error bars indicate standard errors of triplicate samples. * represents significant values $P < 0.05$ and ** represents $P < 0.001$.

Fig. 4 Distribution of the relative abundance of comammox *Nitrospira* clade A, AOA and AOB *amoA* gene retrieved from the treatments of $^{12}CO_2$, $^{13}CO_2$, $^{13}CO_2+C_2H_2$ and $^{13}CO_2+C_8H_{14}$ in the 28-day DNA-SIP microcosms of the pasture and arable soil. Error bars represent standard errors from three replicates.

Fig. 5 Phylogenetic analysis of the translated amino acid sequences of comammox *Nitrospira* clade A *amoA* gene retrieved from the ^{13}C-CO_2 DNA-SIP heavy fraction in the pasture and arable soils. The sequences for the pasture and arable soils were highlighted in blue and green, respectively. The red number represents the number of sequences with >98% sequence identity. Numbers at the nodes indicate the levels of bootstrap support based on the neighbour-joining analysis of 1,000 resampled data sets. The scale bar represents 5% sequence divergence, and bootstrap values (>50%) are indicated at branch points.

Figure

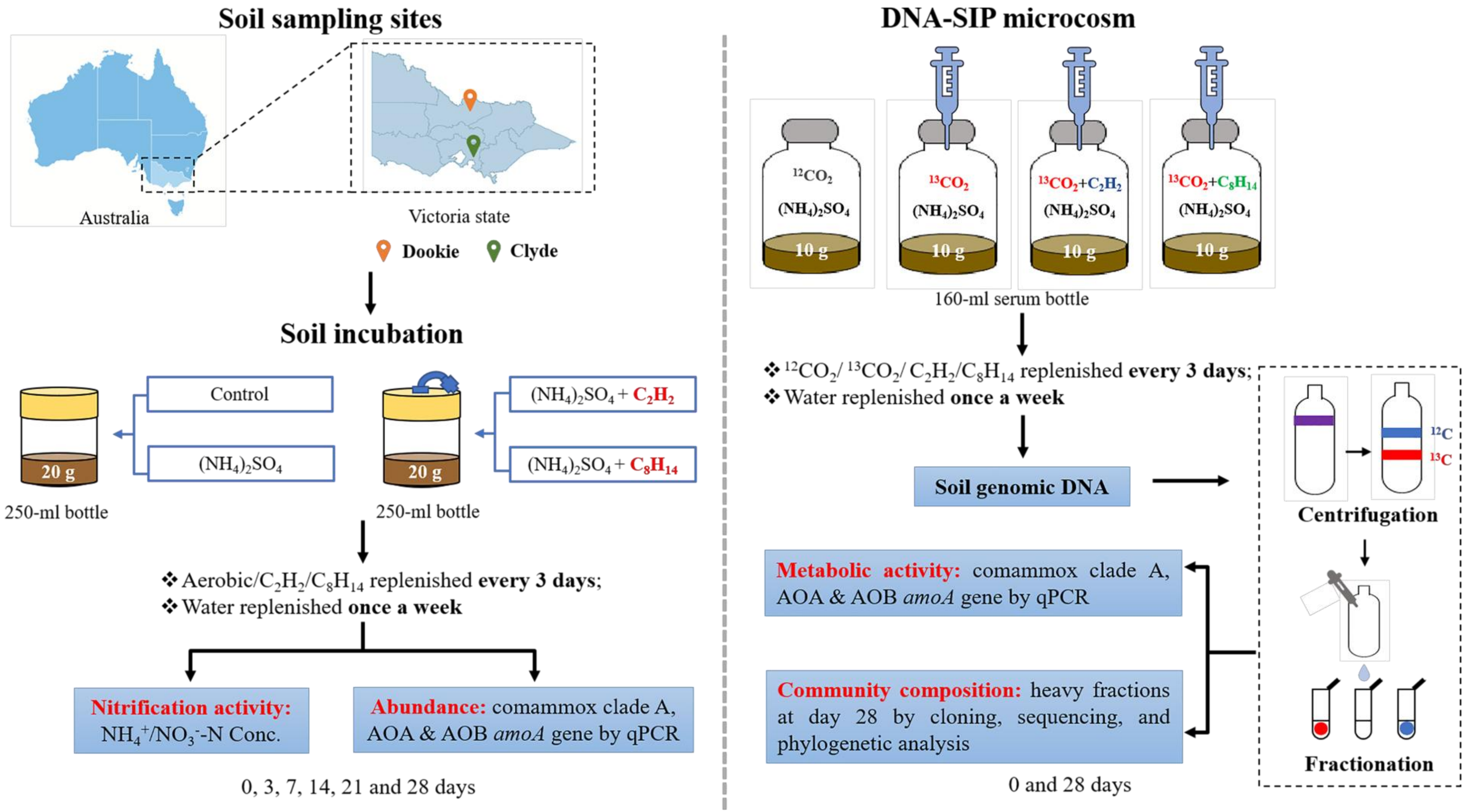

Figure 1

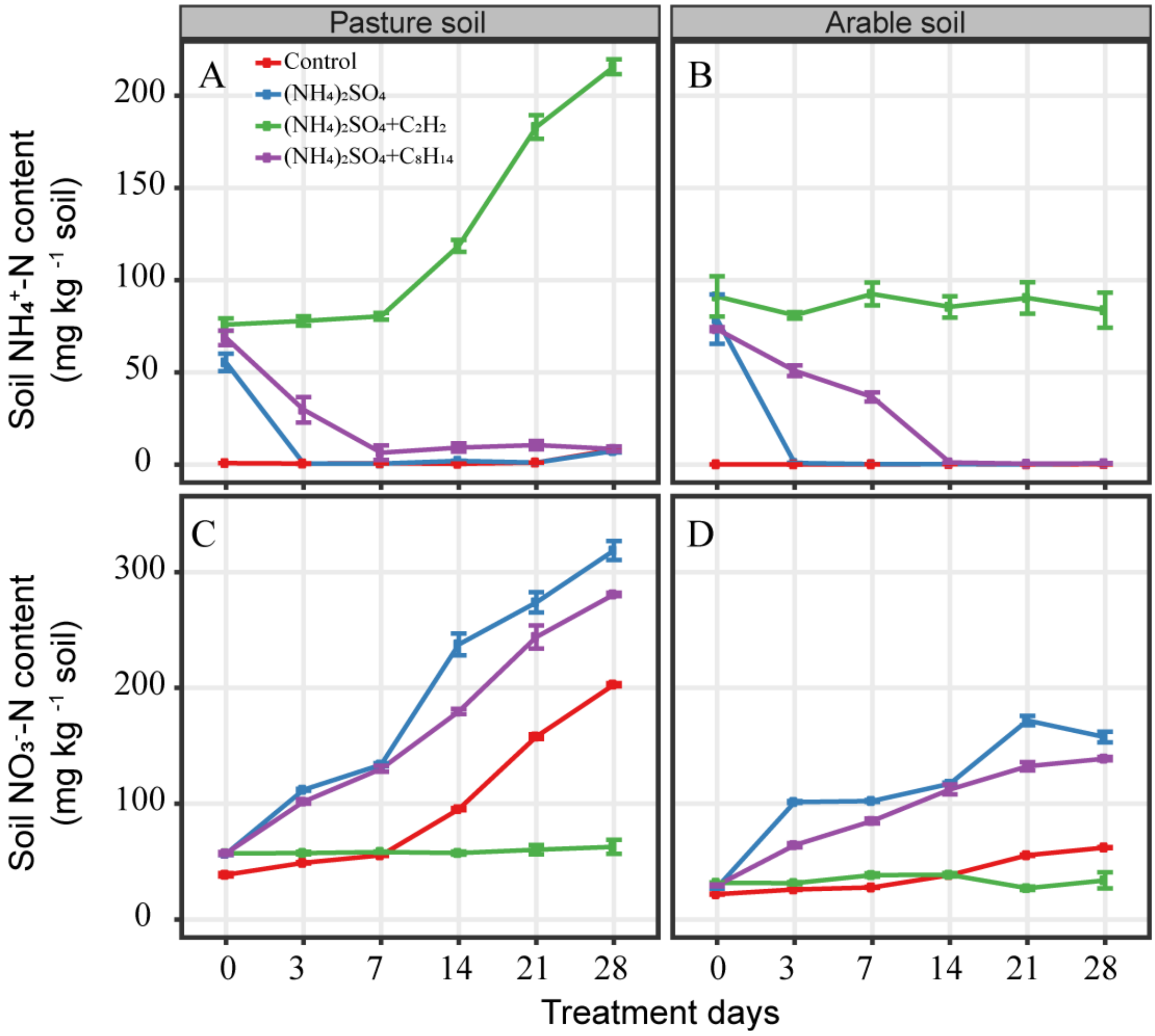
Pasture soil
Arable soil
A
B
C
D
Control
(NH4)2SO4
(NH4)2SO4+C2H2
(NH4)2SO4+C8H14
Soil NH4+-N content (mg kg -1 soil)
Soil NO3--N content (mg kg -1 soil)
0
50
100
150
200
0
100
200
300
0
3
7
14
21
28
Treatment days

Figure 2

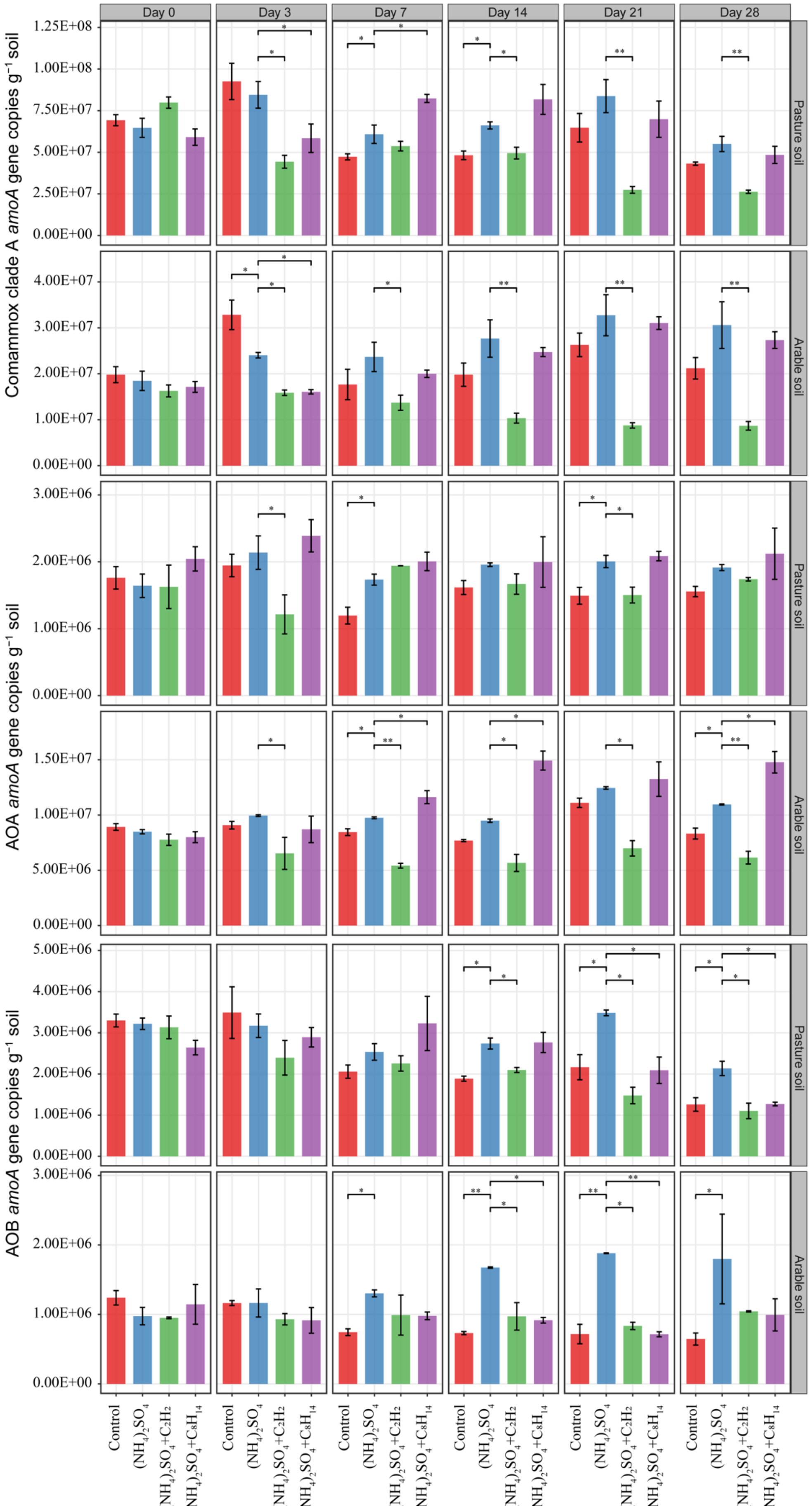
Day 0
Day 3
Day 7
Day 14
Day 21
Day 28
Comammox clade A amoA gene copies g⁻¹ soil
AOA amoA gene copies g⁻¹ soil
AOB amoA gene copies g⁻¹ soil
Pasture soil
Arable soil
Pasture soil
Arable soil
Pasture soil
Arable soil
1.25E+08
1.00E+08
7.50E+07
5.00E+07
2.50E+07
0.00E+00
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
3.00E+06
2.00E+06
1.00E+06
0.00E+00
1.50E+07
1.00E+07
5.00E+06
0.00E+00
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
3.00E+06
2.00E+06
1.00E+06
0.00E+00
Control
(NH4)2SO4
(NH4)2SO4+C2H2
(NH4)2SO4+C8H14
*
**

Figure 3

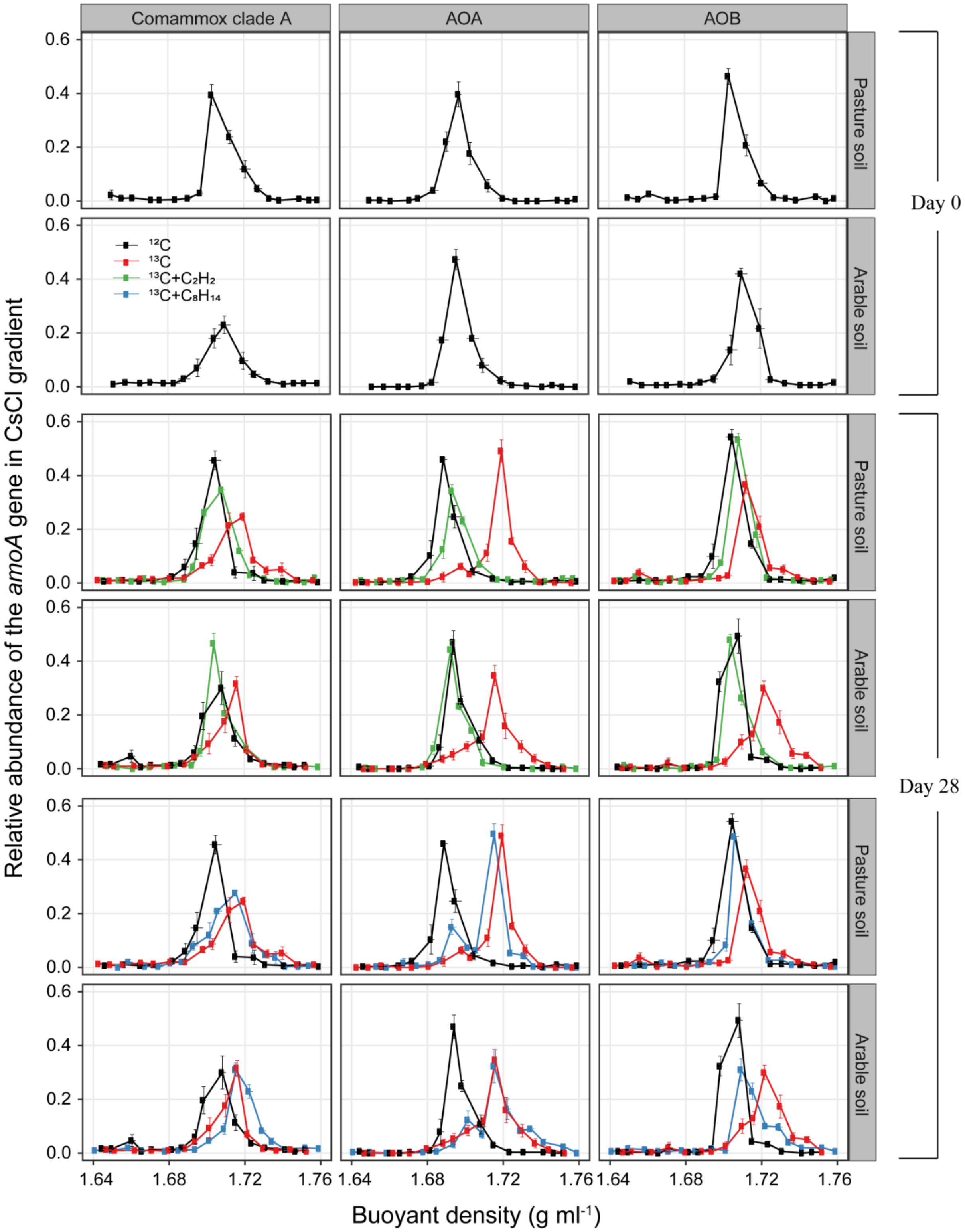
Comammox clade A
AOA
AOB
Pasture soil
Arable soil
Day 0
Day 28
^{12}C
^{13}C
$^{13}C+C_2H_2$
$^{13}C+C_8H_{14}$
Relative abundance of the *amoA* gene in CsCl gradient
0.0
0.2
0.4
0.6
1.64
1.68
1.72
1.76
Buoyant density (g ml^{-1})

Figure 4

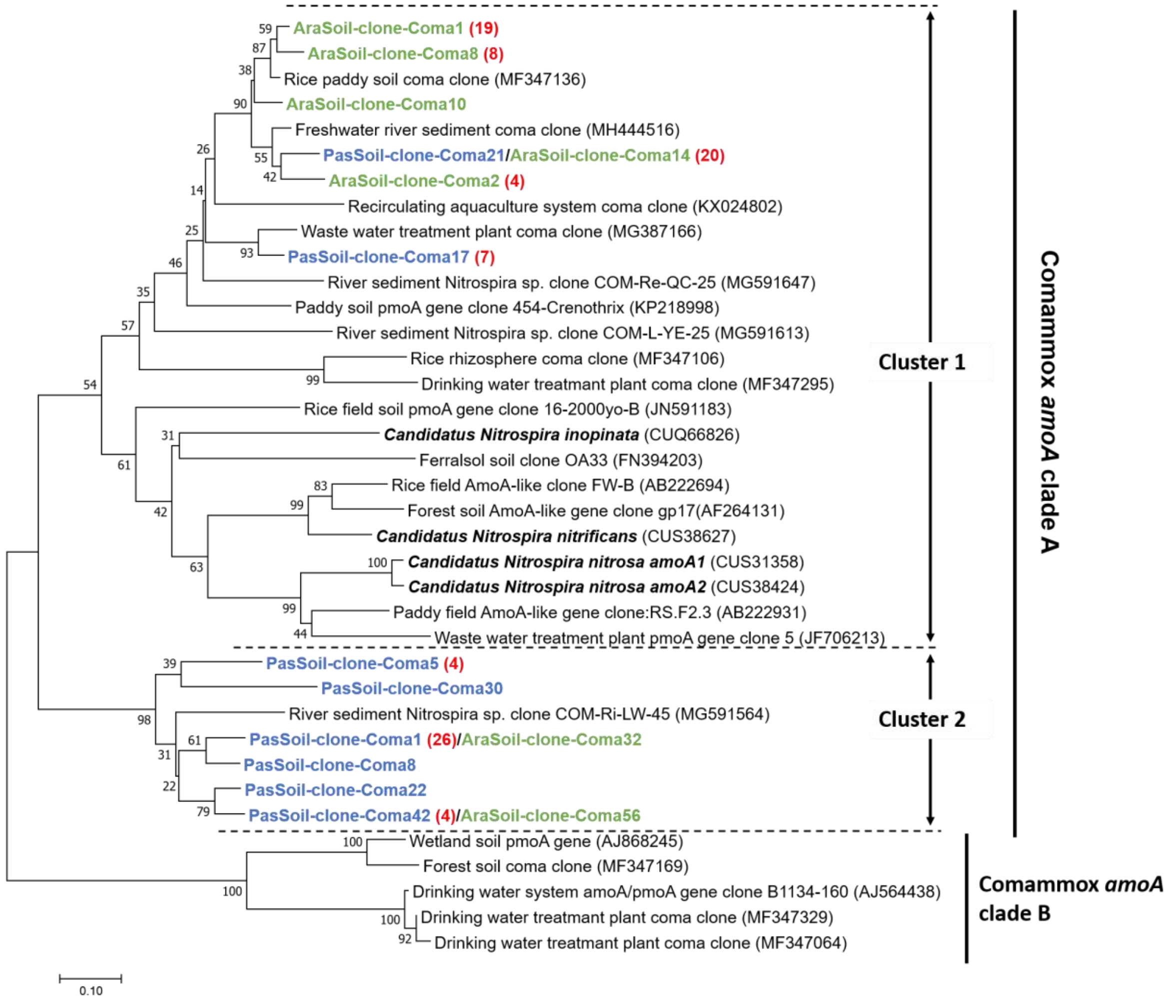
59
AraSoil-clone-Coma1 (19)
87
AraSoil-clone-Coma8 (8)
38
Rice paddy soil coma clone (MF347136)
90
AraSoil-clone-Coma10
Freshwater river sediment coma clone (MH444516)
26
55
PasSoil-clone-Coma21/AraSoil-clone-Coma14 (20)
42
AraSoil-clone-Coma2 (4)
14
Recirculating aquaculture system coma clone (KX024802)
25
Waste water treatment plant coma clone (MG387166)
93
PasSoil-clone-Coma17 (7)
46
River sediment Nitrospira sp. clone COM-Re-QC-25 (MG591647)
35
Paddy soil pmoA gene clone 454-Crenothrix (KP218998)
57
River sediment Nitrospira sp. clone COM-L-YE-25 (MG591613)
Rice rhizosphere coma clone (MF347106)
99
Drinking water treatmant plant coma clone (MF347295)
54
Rice field soil pmoA gene clone 16-2000yo-B (JN591183)
31
Candidatus Nitrospira inopinata (CUQ66826)
61
Ferralsol soil clone OA33 (FN394203)
83
Rice field AmoA-like clone FW-B (AB222694)
99
Forest soil AmoA-like gene clone gp17(AF264131)
42
Candidatus Nitrospira nitrificans (CUS38627)
63
100
Candidatus Nitrospira nitrosa amoA1 (CUS31358)
Candidatus Nitrospira nitrosa amoA2 (CUS38424)
99
Paddy field AmoA-like gene clone:RS.F2.3 (AB222931)
44
Waste water treatment plant pmoA gene clone 5 (JF706213)
39
PasSoil-clone-Coma5 (4)
PasSoil-clone-Coma30
98
River sediment Nitrospira sp. clone COM-Ri-LW-45 (MG591564)
61
PasSoil-clone-Coma1 (26)/AraSoil-clone-Coma32
31
PasSoil-clone-Coma8
22
PasSoil-clone-Coma22
79
PasSoil-clone-Coma42 (4)/AraSoil-clone-Coma56
100
Wetland soil pmoA gene (AJ868245)
Forest soil coma clone (MF347169)
100
Drinking water system amoA/pmoA gene clone B1134-160 (AJ564438)
100
Drinking water treatmant plant coma clone (MF347329)
92
Drinking water treatmant plant coma clone (MF347064)
Cluster 1
Cluster 2
Comammox *amoA* clade A
Comammox *amoA* clade B
0.10

Figure 5