Soil Biology and Biochemistry 208 (2025) 109847

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Molecular evidence for microbial methane oxidation associated with complete ammonia oxidizers in paddy soils

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ARTICLE INFO

Keywords:
Comammox *Nitrospira*
Ammonia monooxygenase
Methane oxidation
Paddy soil

ABSTRACT

The evolutionary relatedness of ammonia monooxygenase (AMO) of ammonia oxidizers and methane monooxygenase (MMO) of methane oxidizers has long been recognized, and both aerobic AMO and MMO-carrying microorganisms can mutually catalyze oxidation of methane and ammonia. However, this process remains unclear for complete ammonia oxidizer (comammox) *Nitrospira*. The absence of pure cultures of comammox *Nitrospira* from soil, combined with the lack of effective selective chemical inhibitors to distinguish between ammonia or methane oxidation, poses a technical challenge in directly assessing their contribution to methane oxidation. Ammonium oxidation almost stops below 4 °C, but methane oxidation and comammox *Nitrospira* remained active in paddy soils. To address this gap, we conducted $^{13}CO_2$ or $^{13}CH_4$-DNA-based stable isotope probing (SIP) incubation experiments using purple and black paddy soils employing 0 °C to selectively inhibit ammonia oxidation in soil microcosms to assess the involvement of comammox *Nitrospira* in methane oxidation. Results showed that at 25 °C, *amoA* genes from comammox clade A, ammonia oxidizing archaea (AOA) and bacteria (AOB) were labelled in $^{13}CO_2$ microcosms, and *amoA* genes from comammox clade B and *pmoA* genes from methanotrophs were labelled in both $^{13}CO_2$ and $^{13}CH_4$ microcosms, indicating both ammonia oxidation and methane oxidation. However, at 0 °C, only *amoA* genes from comammox clade B and *pmoA* genes from methanotrophs were labelled in $^{13}CO_2$ or $^{13}CH_4$ microcosms, with detectable methane oxidation and no evidence for ammonia oxidation. These findings suggest that comammox clade B retains activity at near-freezing temperatures, potentially contributing to methane oxidation under cold conditions. This study underscores a previously unrecognized potential methane-oxidizing function of comammox clade B, offering new insights into their metabolic versatility and broader ecological role in the carbon cycle.

1. Introduction

Ammonia oxidizing bacteria (AOB) and archaea (AOA) play critical roles in the global nitrogen cycle. The discovery of complete ammonia oxidation (i.e., comammox) has fundamentally reshaped our understanding of nitrogen transformations. Nitrite-oxidizing bacteria within the *Nitrospira* II lineage have acquired the ammonia monooxygenase (AMO) gene from AOB through genetic drift (Palomo et al., 2018), enabling them to oxidize ammonia to nitrate (van Kessel et al., 2015; Daims et al., 2015). Comammox *Nitrospira* are further categorized

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https://doi.org/10.1016/j.soilbio.2025.109847
Received 25 December 2024; Received in revised form 7 May 2025; Accepted 9 May 2025
Available online 12 May 2025

into clade A and clade B based on the phylogenetic of *amoA* genes (encoding the AMO subunit A) (Daims et al., 2015). However, comammox *Nitrospira* differ from canonical ammonia oxidation microorganisms. For example, *Nitrospira inopinata* (Kits et al., 2017) and *Candidatus* Nitrospira kreftii (Sakoula et al., 2021) have been shown to outcompete most AOB and AOA under oligotrophic conditions due to their higher affinity for ammonia. Nonetheless, at high ammonia concentrations, ammonia oxidation rates and growth of *N. inopinata* are lower than those of most AOB and AOA (Kits et al., 2017).

Aerobic ammonia oxidation in soil is known to be highly temperature-sensitive (Ouyang et al., 2017), with ammonium oxidizing activity of AOB and AOA has been observed spanning 4–37 °C (Taylor et al., 2017), but nearly ceasing below 4 °C (Avrahami and Conrad, 2003). However, our previous study revealed that comammox *Nitrospira* maintains significant *amoA* transcriptional activity even during winter, suggesting a potential for metabolic activity under cold conditions (Wang et al., 2022). The possibility that comammox *Nitrospira* may contribute to methane oxidation in soils is an intriguing hypothesis, given that methane can serve as a substrate for AMO (Ward, 1987; Stein et al., 2012). This hypothesis is supported by several lines of evidence. First, *Nitrospira* exhibits remarkable physiological versatility (Daims et al., 2016) and their genomes encode pathways for utilizing acetate, pyruvate, formate, and glycerol as substrates (Daims et al., 2001; Freitag et al., 2005; Lücker et al., 2010) and can derive energy from hydrogen oxidation (Palomo et al., 2018), cyanate (Yang et al., 2020) and guanidine (Palatinszky et al., 2024). These metabolic capabilities have not been documented in AOA or AOB. Second, comammox *Nitrospira* remain active and retain stable community composition in cold environments, where both AOA and AOB populations decline. For example, in wastewater treatment systems (Zhou et al., 2021) and agricultural soils (Wang et al., 2022), the abundance of AOA and AOB decreases significantly during winter, while comammox *Nitrospira* maintain high activity. Notably, recent studies have demonstrated the resilience of comammox clade B to freeze-thaw cycles (Tang et al., 2023). Third, methane oxidation shows greater resistance to cold stress than ammonia oxidation and has been observed in alpine regions such as Siberia (Liebner et al., 2008), the Arctic tundra (Voigt et al., 2023) and the Tibetan Plateau (Zhang et al., 2020).

The current understanding of comammox *Nitrospira* is primarily based on five enriched cultures, including *Candidatus* Nitrospira nitrosa, *Candidatus* Nitrospira nitrificans, etc., as well as a pure culture of the strain *N. inopinata* from engineered systems (Daims et al., 2015; van Kessel et al., 2015; Sakoula et al., 2021). However, no pure cultures of comammox *Nitrospira* have been isolated from soil samples (Li et al., 2019; Wang et al., 2019; Hsu et al., 2022; Tan et al., 2022; Zhang et al., 2024). Additionally, there are no effective selective chemical inhibitors for distinguishing between methanotrophs and ammonia oxidizers, making it impossible to test the methane oxidation potential of comammox *Nitrospira* using standard culturing or inhibition techniques. To overcome these challenges, we employed DNA-based stable isotope probing (SIP) with $^{13}CO_2$ or $^{13}CH_4$ to investigate the activity of AMO in methane oxidation by comammox *Nitrospira*. We applied low temperature (0 °C) to suppress ammonia oxidation selectively in soil microcosms. We hypothesized that if comammox *Nitrospira* participates in methane oxidation, methane oxidation activity should be detectable without corresponding ammonia oxidation at 0 °C.

2. Materials and methods

2.1. Description of the paddy field sites

Two paddy soils were used in this study, both classified as hydragric anthrosols (FAO, 1988). The purple paddy soil, developed from purple mudstone, was collected in November 2022 from the Purple Soil Ecology Experimental Station of Southwest University (30° 26′ N, 106° 26′ E) in Chongqing, China. Rice (*Oryza sativa*) was cultivated in both summer and winter under flooded conditions since 1989. The site is characterized by a subtropical monsoon climate with a mean annual temperature of 18.2 °C and total rainfall of 1080 mm. The black paddy soil was collected in December 2022 from Shuangjingzi town (42° 28′ N, 123° 42′ E) in the Tieling, Liaoning Province, China, where rice was also grown in summer under flooded conditions since 2016. This site has a temperate monsoon climate, with a mean annual temperature of 8.5 °C and an annual precipitation of 600 mm. Soil samples were taken from three replicate 4 × 5 m^2 plots at each site. From each plot, three field replicates were collected from the 0–20 cm soil depth, with each replicate comprising five individual soil cores (5.5 cm diameter). The collected soils were stored on ice and immediately transported to the laboratory. Composite samples from each site were air-dried to a 15% moisture content (equal to 24% of maximum water-holding capacity), passed through a 2 mm-sieve to homogenize, and stored at −4 °C until use.

The physicochemical properties of the soils were as follows: Purple soils were pH 6.9, 1.3 mg kg^{-1} total N, 24.7 g kg^{-1} organic matter, and a maximum water-holding capacity 40%; black soils were pH 6.5, 1.1 mg kg^{-1} total N, 32.3 g kg^{-1} organic matter, and a maximum water-holding capacity 49%. Soil pH was determined using a Mettler Toledo 320-S pH meter (Mettler-Toledo Instruments Co. Ltd., Shanghai, China) with a water-to-soil ratio of 2.5. NH_4^+-N and NO_3^--N were extracted using 2 M KCl, and their concentrations were measured using the salicylate method and the single reagent method, respectively (Bao, 2000; Song et al., 2007), on a Unico UV-2102 PC spectrophotometer (Unico Co., Shanghai, P.R. China).

2.2. Soil incubation

Air-dried paddy soils (equivalent to 9.5 g dry-weight) were pre-incubated at 70% water-holding capacity in 200-ml serum bottles for 14 days at 25 °C in darkness before the start of the experiment, with the aim of depleting the organic carbon and minimizing CO_2 emissions within the microcosms (Daebeler et al., 2014). To maintain oxic conditions, microcosms were flushed with synthetic air (20% O_2, 80% N_2) for 1 min prior to the addition of CH_4, CO_2, or C_2H_2. The bottles were then sealed with rubber stoppers. Half of the microcosms were supplemented with C_2H_2 reach the target concentration of C_2H_2 in the headspace at 1.5% (vol/vol) and sealed with aluminum caps, while the remaining microcosms were not supplemented with C_2H_2. The microcosms were pre-incubated at either 0 °C or 25 °C for 3 days. CH_4 (0.5%, v/v) and CO_2 (5%, v/v), or C_2H_2 (1.5%, v/v) were added to the headspace of the vials in six different combinations (each in triplicate): (i) $^{12}CO_2$+$^{12}CH_4$, (ii) $^{13}CO_2$+$^{12}CH_4$, (iii) $^{12}CO_2$+$^{12}CH_4$+C_2H_2 (iv) $^{13}CO_2$+$^{12}CH_4$+C_2H_2, (v) $^{12}CO_2$+$^{13}CH_4$, (vi) $^{12}CO_2$+$^{13}CH_4$+C_2H_2. Both purple and black paddy soils underwent the same treatments (see details in Table S1). Microcosms supplemented with $^{13}CH_4$ or $^{13}CO_2$ were used to track the activity of methanotrophs and ammonia oxidizer. Acetylene (C_2H_2) was applied to inhibit both methane and ammonia oxidation. The labelled and unlabelled CO_2 and CH_4 (99 atom% ^{13}C) were purchased from Wuhan Isotope Technology Co., Ltd (Wuhan, China).

For the microcosms without C_2H_2 at 25 °C, the concentrations of CH_4 were measured weekly. In microcosms incubated at 25 °C with combinations (i) $^{12}CO_2$+$^{12}CH_4$, (ii) $^{13}CO_2$+$^{12}CH_4$, and (v) $^{12}CO_2$+$^{13}CH_4$, CO_2 and CH_4 were renewed weekly. Destructive sampling of the microcosms was performed after 28 and 56 days of incubation. The 56 days incubation was selected to compensate for suppressed microbial metabolic activity at low temperatures, thereby significantly enhancing the probability of successful microbial assimilation and isotopic labeling with the ^{13}C-substrate. A 2.0 g soil sample was stored at −20 °C for molecular analysis, while the remaining soil was used to measure pH, soil moisture content, NH_4^+-N and NO_3^--N concentrations. Gas in headspace of the vials were harvest at weekly, and CH_4 concentrations in gas samples were analyzed using a gas chromatograph (Agilent 7890A, China).

2.3. SIP gradient fractionation

The DNA was extracted from 0.5 g soil using a FastDNA spin kit for soil (MP Biomedicals, Cleveland, OH, USA), following to the manufacturer's instructions. Isopycnic density gradient centrifugation of the extracted DNA was performed to separate ^{13}C-DNA and ^{12}C-DNA in CsCl gradients (Jia and Conrad, 2009). For each sample, 3.0 μg DNA was added to a polyallomer tube containing CsCl solution with an initial buoyant density of 1.720 g ml^{-1}. The samples were centrifuged at 177, 000×*g* for 44 h at 20 °C in a Vti65.2 vertical rotor (Beckman Coulter, Palo Alto, CA, USA). After centrifugation, the CsCl gradients were fractionated into 15 fractions (~380 μl each) using a NE-1000 single syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA). A 60 μl aliquot from each fraction was used to determine the refractive index with an AR 200 digital hand-held refractometer (Reichert Inc., Buffalo, NY, USA). DNA from the fractionated CsCl solution was precipitated using 30% polyethylene glycol (PEG) 6000 solution in 1.6 mM NaCl, followed by a 70% ice-cold ethanol wash, as described previously (Freitag et al., 2005), and dissolved in 30 μl of TE buffer.

2.4. Quantitative PCR of amoA and pmoA genes

Quantitative PCR (qPCR) was used to quantify bacterial, archaeal, and comammox *Nitrospira amoA* genes in total DNA extracts and fractionated DNA-SIP samples using a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems of Life Technologies, Singapore). PCR primers used to amplify *amoA* genes were Arch-*amoA*F/Arch-*amoA*R for archaeal *amoA* (Francis et al., 2005) and *amoA*-1F/*amoA*-2R for bacterial *amoA* (Rotthauwe et al., 1997). For comammox clade A and B, primers CA377f/C576r and CB377f/C576r were used (Jiang et al., 2020), respectively. Bacterial *pmoA* genes were amplified using A189F/mb661r primers (Table S2) (Kolb et al., 2003). Template standards for each qPCR assay were prepared from individual clones of the target gene sequences in a plasmid and diluted from 10^1 to 10^8 copies. Each qPCR reaction (20 μl total) contained 10 μl SYBR Premix Ex Taq™ 2x mixture (Takara Biotech, Dalian, China), 0.5 μM of each primer, 0.4 μl ROX Reference Dye II (50 ×), 1 μl of DNA template (1–10 ng), and 7.6 μl of sterilized water. Each sample was analyzed in triplicate (biological and technical replicates). Thermocycling conditions are detailed in Table S1. All qPCR efficiencies ranged from 91 to 105%, with R^2 values between 0.995 and 0.999. Melt curve analysis confirmed specificity amplification of amplicons. A total of 15 DNA gradient fractions were obtained, the *amoA* and *pmoA* gene copy numbers in first and last fractions were below the detection limit, so we only showed the gene copy numbers in the other 13 fractions.

2.5. High-throughput sequencing and phylogenetic analysis

High-throughput sequencing of *amoA* and *pmoA* genes in fractionated DNA from $^{13}CO_2$-labelled SIP microcosms was performed to identify active ammonia oxidizers and methanotrophs. Amplicons were generated using primers specific for AOA, AOB, comammox clade A, comammox clade B *amoA* genes, and MOB *pmoA* genes (Table S2). PCR reaction mixtures and thermocycling profiles were the same as according to previously described (Wang et al., 2015). The PCR products were gel purified, pooled in equimolar concentrations, and sequenced using an illumina NovaSeq 6000 (PE250) platform. The raw fastq files were demultiplexed, quality-filtered using Trimomatic, and merged using FLASH (Magoč and Salzberg, 2011) under the following criteria: (i) Reads were truncated at any site with an average quality score <20 over a 10 bp sliding window; reads shorter than 50bp were discarded. (ii) Exact barcode matching with a 2 nucleotide mismatch in primer matching was allowed; reads with ambiguous characters were removed. (iii) Only sequences with overlaps longer than 10 bp were assembled. Non-assembled reads were discarded. Cleaned *amoA* and *pmoA* sequences of each sample were then classified to known *amoA* and *pmoA* groups or lineages using mothur as previously described (Dumont et al., 2014). For operational taxonomic unit (OTU) based analysis, *amoA* and *pmoA* sequences were clustered on their deduced amino-acid sequences by FunGene Pipeline with a distance cutoff of 0.07 (Degelmann et al., 2010). Representatives of these dominant OTUs and their closest relatives in GenBank were also used for phylogenetic analysis using MEGA 6.0 with 1000-fold bootstrap support. Tree topologies were checked using the neighbor-joining algorithm and minimum evolution method (Tamura et al., 2013).

2.6. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 18.0 (SPSS Inc., Cary, NC, USA). Methane concentration, ammonium nitrogen content, nitrate nitrogen content, and the abundance of *amoA* and *pmoA* genes in total DNA are expressed as the mean ± standard deviation of triplicate biological microcosms for each treatment. The relative abundance of *amoA* and *pmoA* genes in labelled DNA (fractions 6–7) is expressed as the mean value of triplicate technical replicates for each treatment. One-way ANOVA followed by Tukey's post hoc test was performed for multiple comparisons, and $p < 0.05$ between treatments considered statistically significant.

3. Results and discussion

3.1. Comammox Nitrospira active in methane oxidation

Both methane and ammonia oxidation were detected in the paddy soils at 25 °C (Fig. 1 and Fig. S1). We employed a DNA-SIP incubation experiment to investigate the activity of ammonia oxidizers in methane oxidation across two paddy soils. The microcosms were amended with either $^{13}CO_2$ (5%, v/v) or $^{13}CH_4$ (0.5%, v/v) to trace the activity of comammox clade A and clade B, AOA, AOB, and methanotrophic bacteria (MOB). After 28 or 56 days of aerobic incubation at 25 °C, comammox clade A and clade B, AOA, AOB, and methanotrophs were labelled in $^{13}CO_2$ amended soil microcosms (Fig. 2 and Fig. S2), indicating the activity of these microorganisms in ammonia or methane oxidation. Given that AMO can catalyze methane oxidation (Ward, 1987; Stein et al., 2012; Fisher et al., 2018), it remains unclear whether these labeling ammonia oxidizers were involved in autotrophic ammonia oxidation, methane oxidation, or both.

Previous studies have shown that ammonia oxidizing microorganisms exhibit limited activity at low temperatures (Alves et al., 2013; Zhou et al., 2021), with ammonia oxidation nearly ceasing below 4 °C (Avrahami and Conrad, 2003). In contrast, methane oxidation demonstrates greater tolerance to low temperatures (Liebner et al., 2008; Voigt et al., 2023), with methane oxidation observed in the permafrost of Siberia at 0 °C (0.56 μg g^{-1} dry weight soil (*d.w.s*) day^{-1}) (Liebner and Wagner, 2007). To ensure complete inhibition of ammonia oxidation, we set the incubation temperature to 0 °C instead of 4 °C. Results showed that after 56 days of incubation at 0 °C, ammonium levels remained unchanged, and no increase in nitrate was observed, confirming the absence of net nitrification (Fig. S1). The DNA-SIP analyses revealed no detectable activity of comammox clade A, AOA, or AOB in both paddy soils at 0 °C, as evidenced by the lack of labeling in incubations with either $^{13}CO_2$+$^{12}CH_4$ or $^{12}CO_2$+$^{13}CH_4$ (Fig. 2 and Fig. S2). This result indicated that ammonia oxidation was completely inhibited at 0 °C.

Although methane oxidation rates at 0 °C (0.1–0.3 μg g^{-1} d.*w.s* day^{-1}) were lower than those at 25 °C, they were comparable to rates previously observed in permafrost soils (Liebner and Wagner, 2007). Notably, comammox clade B and MOB were labelled after 56 days of incubation with $^{13}CO_2$+$^{12}CH_4$ or $^{12}CO_2$+$^{13}CH_4$ at 0 °C (Fig. 2 and Fig. S5), suggesting that both comammox clade B and MOB are chemolithoautotrophically active and closely involved in the methane oxidation process. Furthermore, acetylene was used to inhibit both

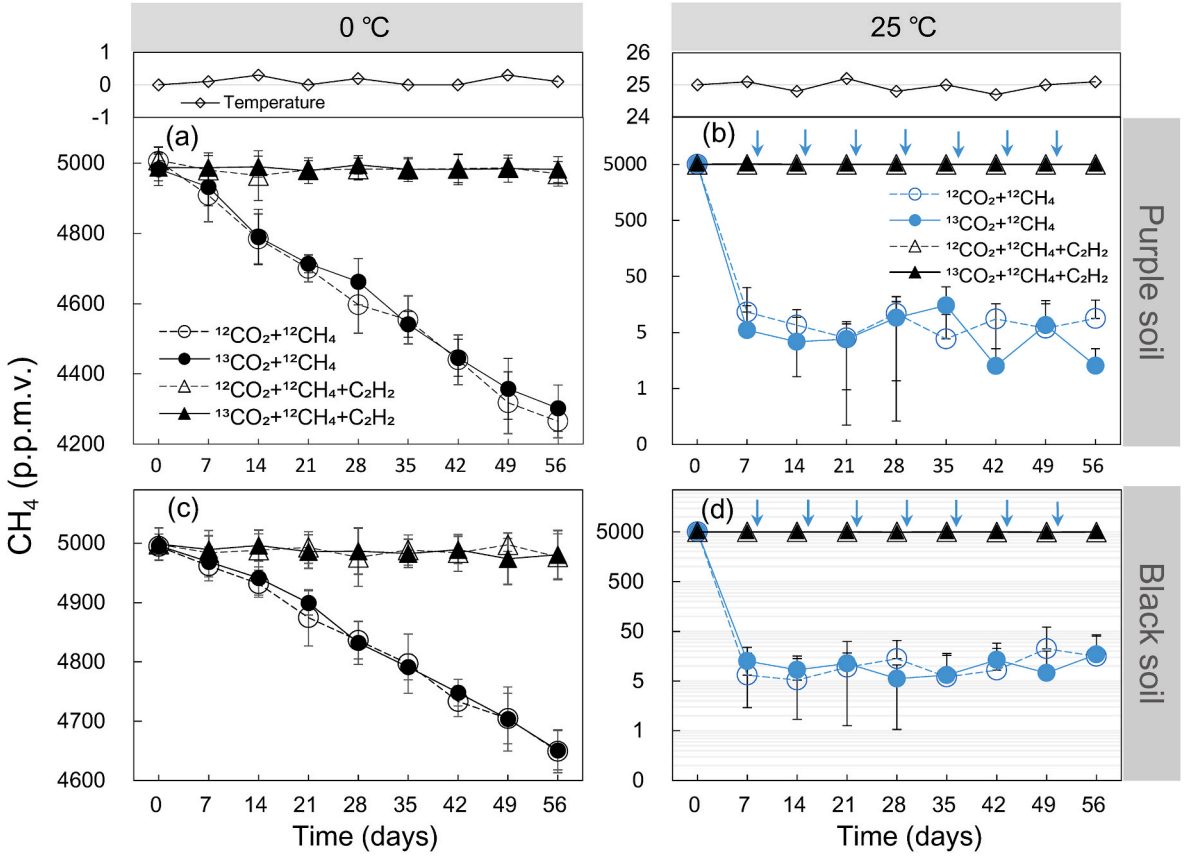

Fig. 1. Consumption dynamics of methane in paddy soil microcosms after 56-days incubation. Concentration of methane in purple paddy soil (a) and black paddy soil (c) microcosms at 0 °C and in the purple paddy soil (b) and black paddy soil (d) microcosms at 25 °C. $^{12}CO_2$+$^{12}CH_4$ represents a soil microcosm with $^{12}CO_2$ (5%, v/v) and $^{12}CH_4$ (0.5%, v/v); $^{13}CO_2$+$^{12}CH_4$ represents a soil microcosm with $^{13}CO_2$ (5%, v/v) and 12CH4 (0.5%, v/v); $^{12}CO_2$+$^{12}CH_4$+C_2H_2 represents a soil microcosm with $^{12}CO_2$ (5%, v/v), $^{12}CH_4$ (0.5%, v/v) and acetylene (1.5%, v/v); $^{13}CO_2$+$^{12}CH_4$+C_2H_2 represents a soil microcosm with $^{13}CO_2$ (5%, v/v), $^{12}CH_4$ (0.5%, v/v) and acetylene (1.5%, v/v). Circle and triangle represent paddy soils with and without the methane oxidation inhibitor acetylene (C_2H_2), respectively. Hollow and solid represent $^{13}CO_2$ treatments and $^{12}CO_2$ treatments, respectively. Blue arrow represents that $^{12}CO_2$ treatment and $^{13}CO_2$ treatment were weekly amended with $^{12}CO_2$ (5%, v/v)+$^{12}CH_4$ (0.5%, v/v) and $^{13}CO_2$ (5%, v/v)+$^{12}CH_4$ (0.5%, v/v), respectively. The error bars represent standard errors of three replicates for each treatment (N=3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ammonia and methane oxidation, and results showed that acetylene effectively inhibited both processes, regardless of temperature (25 °C or 0 °C) (Fig. 1 and Fig. S1). Neither comammox clade A and B, AOA, AOB, nor MOB were labelled in paddy soil microcosms amended with $^{13}CO_2$+$^{12}CH_4$+C_2H_2 or $^{12}CO_2$+$^{13}CH_4$+C_2H_2 amendment at 25 °C (Fig. 2, S2 and S5), confirming that acetylene effectively inhibited methanotroph and ammonia oxidizer activity. At 0 °C, acetylene similarly inhibited methane oxidation, as neither comammox clade B nor MOB were labelled with $^{13}CO_2$ or $^{13}CH_4$ (Fig. 2, S2 and S5), further suggesting that the metabolic activity of comammox clade B in paddy soils is closely linked to methane oxidation. Previous research identified poly (3-hydroxybutyrate) depolymerase (*phaZ*) genes in *Candidatus* Nitrospira CG24A, CG2C, and CG24E (Palomo et al., 2018; Yang et al., 2020). These genes are member of the polyhydroxybutyrate (PHB) depolymerase gene family (*pha*), which encompasses the three key functional genes (*phaCAB*) responsible for PHB synthesis via methane oxidation in *Methylocystis hirsuta* (Madison and Huisman, 1999; Pieja et al., 2011). Furthermore, formate dehydrogenase genes (*fdh*) were detected in five comammox clade B strains, with their expression significantly upregulated during active methane oxidation (Cai et al., 2016). These findings suggest that *phaZ* and *fdh* may play critical roles in methane oxidation by comammox clade B.

Comammox *Nitrospira* clade B was labelled during the 28-day incubation period at 0 °C with $^{13}CO_2$+$^{12}CH_4$ (Fig. S2a and c), whereas methanotroph labeling was not observed until 56 days of incubation (Fig. 2b–d and Fig. S2). This suggests that comammox clade B may exhibit higher activity than methanotrophs at low temperatures. However, the possibility of cross-feeding during incubation cannot be completely ruled out (Pan et al., 2021). Quantitative PCR targeting *amoA* and *pmoA* genes revealed that the *amoA* gene copy number of comammox clade B increased from 4.64 to 9.04 × 10^5 copies g^{-1} *d.w.s* after 56 days of incubation. In contrast, the abundance of methanotrophs, comammox clade A, AOB, and AOA significantly decreased (Fig. 4 and Fig. S4). These results imply that comammox clade B maybe more competitive than methanotrophs and other ammonia oxidizers at 0 °C, with its potential methane oxidation activity. Han et al. (2024) reported that comammox clade B is the primary driver of nitrification in coastal Antarctica, where the average annual temperature is approximately −10 °C. Comammox clade B may adapt to extreme cold by synthesizing trehalose (Elbein et al., 2003), competing for resources through high substrate affinity (Kits et al., 2017; Palomo et al., 2018), and utilizing supplementary metabolic pathways, such as the methionine salvage pathway (MSP), to survive in conditions ranging from −17 to 0 °C and in oligotrophic environments (Han et al., 2024).

3.2. Phylogenetic analysis of active ammonia oxidizers and methanotrophs

High-throughput amplicon sequencing was used to analyze *amoA* and *pmoA* genes in ^{13}C-DNA fractions from $^{13}CO_2$+$^{12}CH_4$ microcosms (Tables S3 and S4). OTUs similar to the *amoA* sequences of comammox clade B were detected in the ^{13}C-DNA fractions at both temperatures,

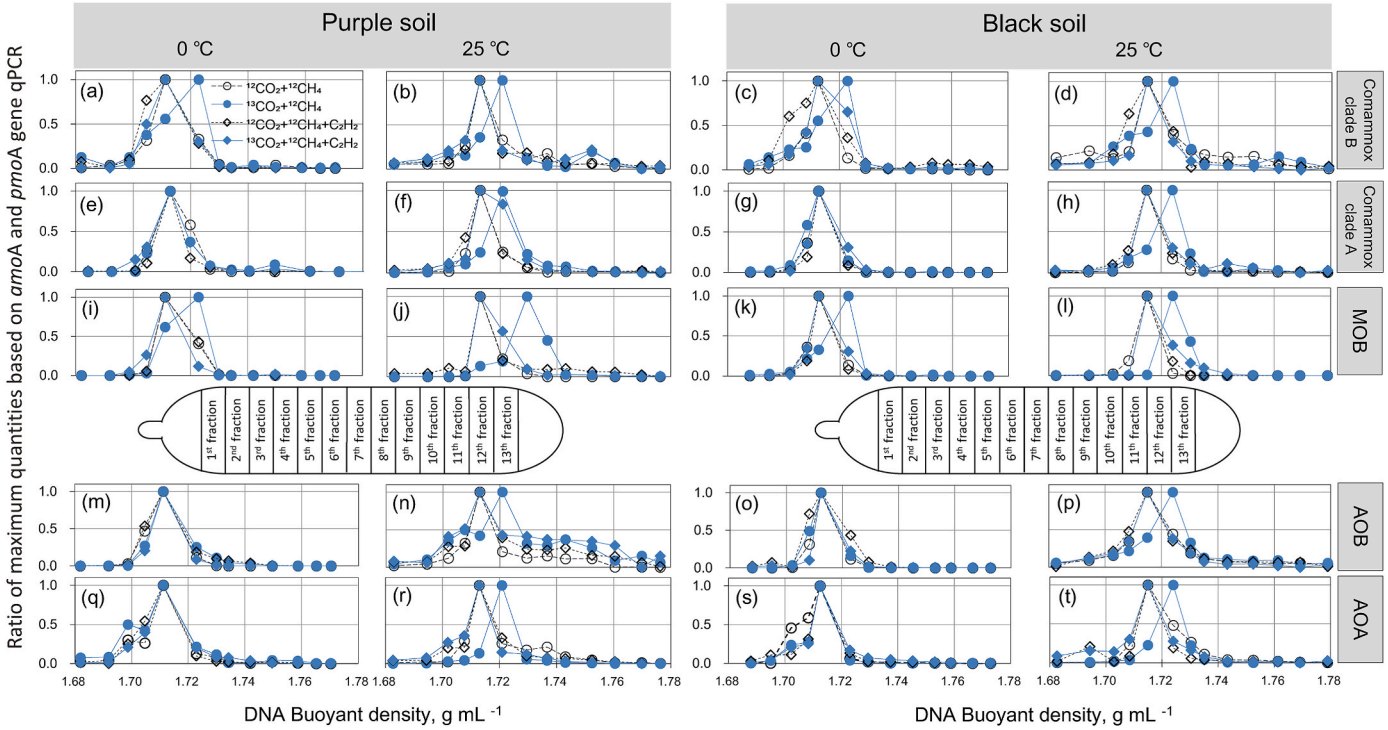

Fig. 2. Distribution of *pmoA* and *amoA* gene copy numbers in fractionated DNA across the buoyant density. Quantitative distribution of the comammox *Nitrospira* clade B (a–d), comammox *Nitrospira* clade A (e–h), AOB (m–p) and AOA (q–t) *amoA* genes and MOB (i–l) *pmoA* genes across the entire buoyant density gradient of the fractionated DNA from and black paddy soil microcosms incubated with and without addition of acetylene at 0 °C and 25 °C for 56 days. The normalized data are shown as the ratio of the gene copy number in each fraction to the maximum quantities in each treatment (N=3). Representation of treatments are as described in the legend for Fig. 1.

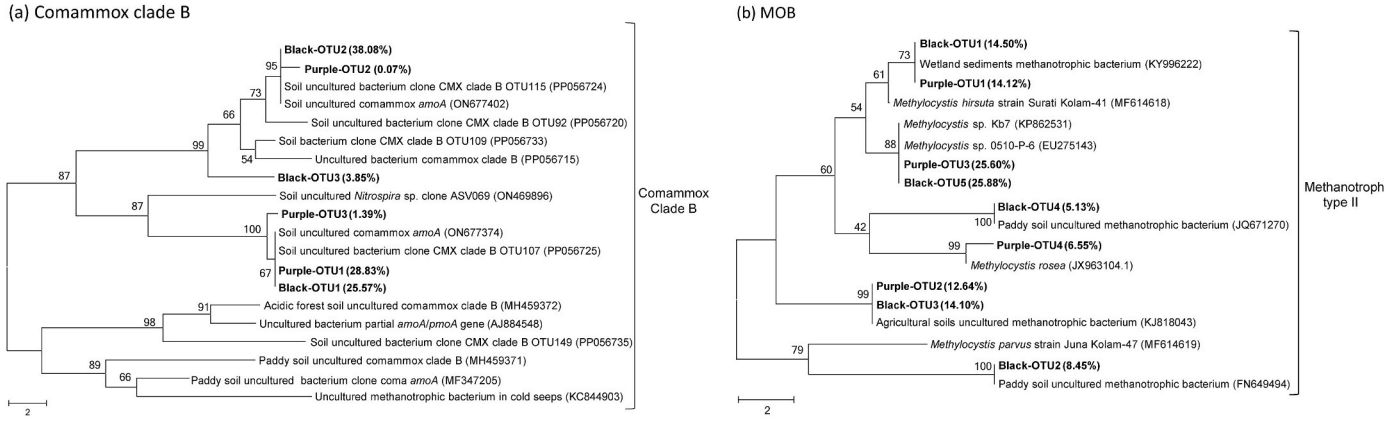

Fig. 3. Phylogenetic analysis *amoA* and *pmoA* genes amplified from ^{13}C-labelled heavy fraction DNA from $^{13}CO_2$-treated microcosms within 56-day at 0 °C. Phylogenetic analysis of *amoA* genes affiliated with the comammox *Nitrospira* clade B (a) and *pmoA* genes affiliated with MOB (b) in the ^{13}C-DNA from purple paddy soil and black paddy soil microcosms incubated without addition of acetylene at 0 °C for 56 days. Individual sequences are representative of an OTU containing sequences clustered at >97% sequence identity, with the % value and number in parentheses denoting the proportion and absolute number of sequence reads placed within that OTU, respectively. The scale bar represents 2% sequence divergence. Black-OTU indicates amoA and pmoA gene reads in ^{13}C-DNA from black paddy soils microcosms, Purple-OTU indicates *amoA* and *pmoA* gene reads in ^{13}C-DNA from purple paddy soils microcosms.

further confirming the labeling and activity of comammox clade B in the $^{13}CO_2$+$^{12}CH_4$ microcosms across both paddy soils. Phylogenetic analysis of the *amoA* gene revealed that the labelled comammox clade B sequences aligned closely with previously reported sequences from comammox clade B in the water fluctuation zone of the Three Gorges Reservoir (ON677402, ON677374) (Ding et al., 2022) and agricultural soils (PP056724, PP056725) (Fig. 3a and Fig. S3c).

The community diversity of labelled comammox clade B and methanotrophs was higher at 25 °C compared to 0 °C (Fig. 3 and Fig. S3), suggesting that not all members of comammox clade B and methanotrophs tolerate cold environments. Previous studies have revealed the resilience of comammox clade B to freeze-thaw cycles, highlighting its endurance in low and subzero temperatures (Tang et al., 2023). Our data suggest that cold-adapted comammox clade B plays an active role in biological processes in temperate and subtropical paddy soils at 0 °C. At 25 °C, members of types I methanotrophs, including *Methylomicrobium*, *Methylosarcina*, and *Methylosarcina*, and type II methanotrophs, such as *Methylocystis,* were detected in the $^{13}CO_2$-labelled microcosms. These methanotrophs are widely distributed in black soil (Sultana et al., 2019) and purple soil (Cao et al., 2024). However, at

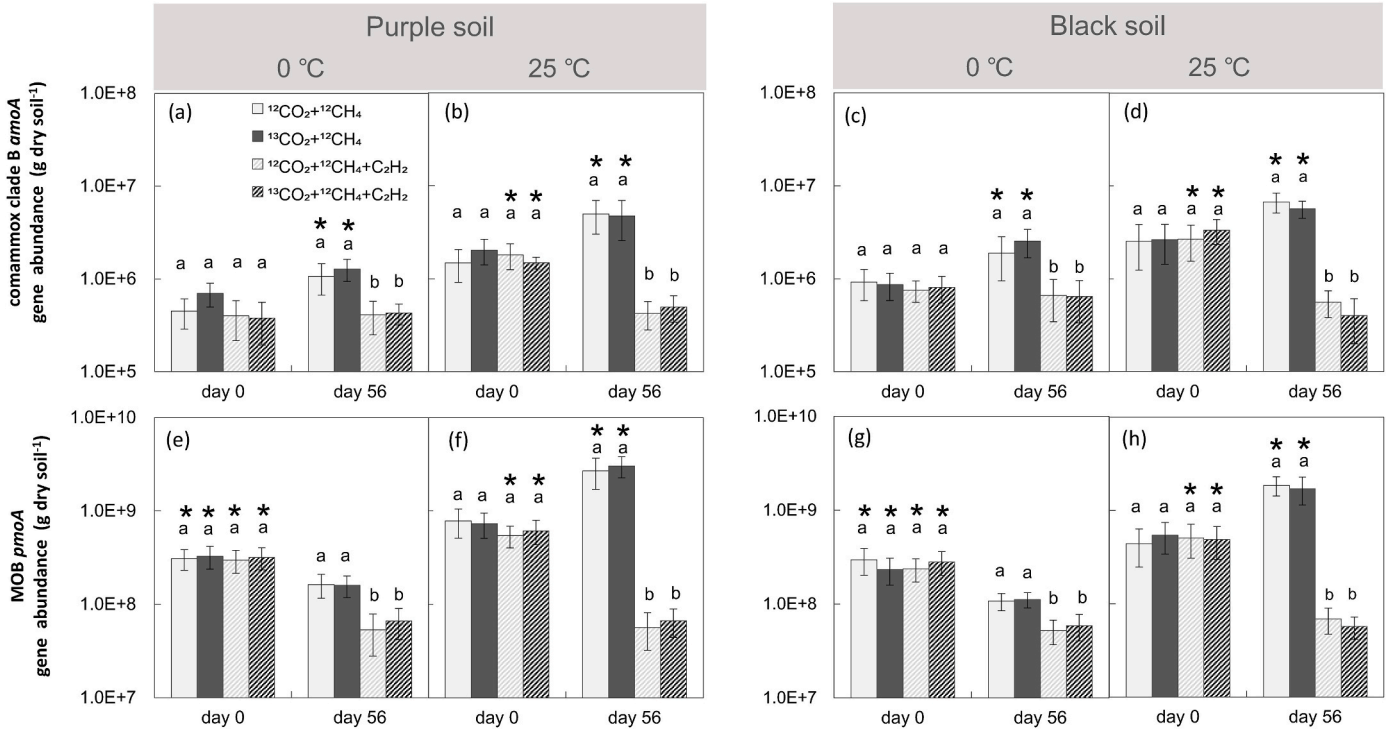

Fig. 4. Changes in abundance of the comammox clade B *amoA* and bacterial *pmoA* genes during incubation of paddy soil microcosms at 0 °C and 25 °C. The comammox clade B (a–d) *amoA* genes and MOB *pmoA* (e–h) genes copy numbers in purple and black paddy soil microcosms incubated with and without addition of acetylene at 0 °C and 25 °C on day 0 and day 56. Representation of treatments are as described in the legend for Fig. 1 The error bars represent standard errors of three replicates for each treatment (N=3). Different lower-case letters indicate significant differences among different treatments (Tukey method, $p < 0.05$).

0 °C, all *pmoA* genes detected in the two paddy soil microcosms were phylogenetically related to *Methylocystis* within type II methanotrophs (Fig. 2b). Notably, Purple-OTU2 and Black-OTU3 share 99% similarity with *Methylocystis* sp. from high-altitude agricultural soil in Yunnan (Yang et al., 2014), while Purple-OTU4 had 99% similarity with *Methylocystis rosea*, which was isolated from Arctic peat and has a minimum growth temperature of 5 °C (Wartiainen et al., 2006). The incubation temperature of 0 °C in this study likely restricted the growth of *Methylocystis* spp., resulting in the absence of methanotrophs labeling after 28 days of incubation at 0 °C. This finding suggests that cold-tolerant comammox clade B may be the dominant microbial group involved in methane oxidation in paddy soils under low and even subfreezing temperatures.

3.3. Ecological functional significance of comammox clade B

Previous studies have highlighted metabolic differences between comammox clade A and clade B, including variations in their metabolic pathways for substrates such as formate and hydrogen, as well as different ammonium uptake systems (Palomo et al., 2018). Additionally, the *amoA* genes of comammox clade A differs from that of comammox clade B, despite both clades sharing a common ancestor with the AMO of the betaproteobacterial AOB (Daims et al., 2015; van Kessel et al., 2015). In our study, comammox clade A was not labelled in ^{13}C–CH_4 microcosms at either temperature, in contrast to clade B (Fig. 2). The differential responses to CH_4 between the two clades may be attributed to structural differences in their AMO, reflecting their distinct evolutionary histories. The methane concentration gradients in terrestrial ecosystems, coupled with limited ammonia and urea availability, may create niches where clade B outcompetes clade A, a previously overlooked possibility. The relative abundance of comammox clade B accounted for 1.71–3.67% of total ammonia-oxidizers (Fig. 2, S2). Despite its low abundance, comammox clade B demonstrated significant activity (Wang et al., 2022; Han et al., 2024). However, its contribution to nitrification remains poorly quantified and lacks consensus in previous studies (Shi et al., 2018; Wang et al., 2019; He et al., 2019; Liu et al., 2021; Liu et al., 2023; Bai et al., 2024). Although its contribution to methane oxidation has not been precisely determined, the strong association between comammox clade B and methane oxidation processes suggests its potential role in the carbon cycle.

Soils act as a significant biological sinks for CH_4, a potent greenhouse gas. Methane oxidation activity in soils is sensitive to temperature decreases (Amodeo et al., 2018; Hu et al., 2018), with significant reductions in oxidation rates as soil temperature drop (De Visscher et al., 2001; Albanna and Fernandes, 2009). The activity of most methanotrophs at 0 °C is weak (Omelchenko et al., 1996; Bowman et al., 1997; Kalyuzhnaya et al., 1999; Dedysh et al., 2002; Dunfield, 2003; Dedysh, 2004; Yun et al., 2011), which is consistent with our findings (Fig. 2 and Fig. S2). Methane emissions are prevalent in low-temperature environments, including anthropogenic sources (Saunois et al., 2016) and natural sources (Chen et al., 2008; Høj et al., 2008; Juottonen et al., 2008). The metabolic activities of cold-adapted methane oxidizers in soils are crucial for maintaining its capacity to act as a methane sink in cold regions (~0 °C) such as Zoige Plateau (Chen et al., 2008), Arctic peatlands (Høj et al., 2008) and boreal fens in southern Finland (Juottonen et al., 2008). Our data suggest that active members of comammox clade B could potentially function as methane oxidizers, contributing to the reduction of methane emissions in soils around 0 °C. However, the absence of ammonium consumption and nitrate accumulation at 0 °C in our study does not conclusively rule out the occurrence of ammonia oxidation because of potential analytical errors, such as limitations in instrumental detection precision and thus the possibility of nitrification cannot be entirely dismissed. Therefore, while the methane-oxidizing capability of comammox clade B is plausible, it remains speculative and warrants further investigation.

4. Conclusions

This study highlights the potential involvement of comammox clade B in methane oxidation in paddy soils. The DNA-SIP experiment with two paddy soils revealed that comammox clade B was active in environments (i.e., 0 °C) where ammonia oxidation was suppressed, yet methane oxidation persisted. Results suggest that comammox *Nitrospira* clade B may function as a methane oxidizer in paddy soils, expanding our understanding of the metabolic versatility of comammox *Nitrospira*. Further investigation into the key functional genes of comammox clade B in the methane oxidation is essential to elucidate its role in carbon cycle, as it could provide critical insights into the ecological significance and biogeochemical roles of comammox *Nitrospira*.

CRediT authorship contribution statement

Zongjing Kang: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Shuling Wang:** Writing – review & editing, Methodology, Investigation. **Lu Lu:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation. **Xia Zhu-Barker:** Writing – review & editing, Writing – original draft, Supervision. **Junji Yuan:** Writing – review & editing, Writing – original draft, Data curation. **Yongxin Lin:** Writing – review & editing, Visualization. **Zhongjun Jia:** Writing – review & editing, Supervision. **Alan L. Wright:** Writing – review & editing, Writing – original draft. **Andreas Kappler:** Writing – review & editing, Writing – original draft. **Sara Kleindienst:** Writing – review & editing, Supervision. **Xianjun Jiang:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Data availability

The raw amplicon sequence datasets of AOA-, AOB-, and comammox *Nitrospira-amoA* and methanotrophs-*pmoA* genes have been deposited in the NCBI Sequence Read Archive (SRA) and are available under BioProject number PRJNA1128431.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (Nos. 42077035). We are grateful to the members of our lab for technical support and helpful discussion. We also thank Xueru Huang for sampling black paddy soils from Tieling, China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2025.109847.

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