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Ammonia- and Methane-Oxidizing Bacteria: The Abundance, Niches and Compositional Differences for Diverse Soil Layers in Three Flooded Paddy Fields

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Abstract: Ammonia oxidizing bacteria (AOB), Ammonia oxidizing archaea (AOA) and methane oxidizing bacteria (MOB) play cogent roles in oxidation and nitrification processes, and hence have important ecological functions in several ecosystems. However, their distribution and compositional differences in different long-term flooded paddy fields (FPFs) management at different soil depths remains under-investigated. Using qPCR and phylogenetic analysis, this study investigated the abundance, niches, and compositional differences of AOA, AOB, and MOB along with their potential nitrification and oxidation rate in three soil layers from three FPFs (ShaPingBa (SPB), HeChuan (HC), and JiDi (JD)) in Chongqing, China. In all the FPFs, CH₄ oxidation occurred mainly in the surface (0–3 cm) and subsurface layers (3–5 cm). A significant difference in potential methane oxidation and nitrification rates was observed among the three FPFs, in which SPB had the highest. The higher *amoA* genes are the marker for abundance of AOA compared to AOB while *pmoA* genes, which is the marker for MOB abundance and diversity, indicated their significant role in the nitrification process across the three FPFs. The phylogenetic analysis revealed that AOA were mainly composed of Nitrososphaera, Nitrosospumilus, and Nitrosotalea, while the genus Nitrosomonas accounted for the greatest proportion of AOB in the three soil layers. MOB were mainly composed of Methylocaldum and Methylocystis genera. Overall, this finding pointed to niche differences as well as suitability of the surface and subsurface soil environments for the co-occurrence of ammonia oxidation and methane oxidation in FPFs.

Keywords: AOA; AOB; MOB; q-PCR; Paddy field

1. Introduction

Nitrification, through which ammonia (NH₃) is converted into nitrite (NO₂[−]) or nitrate (NO₃[−]), is a key process in global nitrogen (N) cycling and is strongly linked with the release of greenhouse gases to the atmosphere, NO₂[−] leaching into ground water and N availability for plant use [1]. The biological oxidation of NH₃ into NO₃[−] is currently considered to be catalyzed by Ammonia oxidizing Bacteria (AOB) within two Proteobacteria subclasses (β and γ subclasses Proteobacteria) and Ammonia oxidizing Archaea (AOA) [2]. Conversely, the oxidation of the second most abundant greenhouse

gas, methane (CH_4), is conducted by methane-oxidizing bacteria (MOB) [3]. In paddy fields, MOB minimize the amounts of CH_4 emitted into the atmosphere [4]. MOB are Gram-negative bacteria that utilize CH_4 as the exclusive carbon and energy source. They oxidize CH_4 in the aerobic environments such as the rhizosphere and the soil-water interface before it is transported into the atmosphere [5]. Studies have shown that the rate of oxidation is approximately 50% to 90% of the amount of CH_4 produced [6]. Therefore, elucidating on MOB abundance and activity in the aerobic region, particularly in paddy fields, is critical for enhancement of the understanding of CH_4 oxidation and the reduction of greenhouse gases from rice fields.

With more than half of the world's population consuming rice, several hectares of lands are continuously being converted for rice production [7]. In China, paddy field management is widely distributed and remains the main source of N_2O and CH_4 [3]. Because of the fundamental roles of AOA, AOB, and MOB in the N biogeochemical cycle, the oxidation of CH_4 , ecological issues and soil health, particularly in paddy field, have attracted considerable attention.

According to a previous study, nitrous oxide, which is a major greenhouse gas, is a by-product of both AOB and AOA activity [8]. In addition, AOB produces nitrogen monoxide, which decreases ozone levels in the stratosphere [9]. It has also been reported that AOA could possess a genetic capacity to exploit organic carbon and small organic molecules [10,11]. However, several environmental factors, including soil moisture, temperature, NH_4^+ -N concentrations, among others, could influence the abundance and community structure of soil AOA, AOB and MOB [12]. Studies have reported archaeal *amoA* to be more abundant than bacterial *amoA* in most soils [13,14]; however, the differences in the abundance of different types of *amoA* may be due to their distinct ecological niches. For example, in an agricultural soil with N amendments, bacterial *amoA* are predicted to be more abundant [15], while in soil where mineralization of organic N influences the nitrification process, archaeal *amoA* are expected to be more abundant. Soil type, pH, vegetation types, and locations influence both nitrifiers and the nitrification process in soil ecosystems [16]. According to Lehtovirta-Morley et al. (2011) [17], in terrestrial ecosystems, although a specific AOA cluster is likely to occur in neutral environments, *Nitrosotalea devanatterra*, which is another cluster with an AOA origin, exists in acidic soils, suggesting that different AOA species respond differently to soil alkalinity or acidity. Similarly, in paddy soils, the coupling of nitrification and denitrification has been reported, with AOA exhibiting higher activity in acidic paddy soils, while AOB are primarily dominant in neutral and alkaline paddy soils, particularly in intensively N-fertilized soil [18,19]. However, the distribution and compositional differences of AOA, AOB, and MOB in long-term nitrogen saturated flooded paddy fields (FPFs) management at different soil depths remains rarely examined.

The intensive application of N fertilizers has increased in recent decades, particularly in paddy fields, which has aggravated soil acidification and accelerated the rate at which most paddy fields become acidic [1]. Generally, N fertilizer is critical for increasing grain yield in paddy fields. However, intensive N fertilizer application could alter the abundance and composition of AOB in the soil [20]. For example, Shen et al. (2011) [21] observed long-term N stocking considerably influenced the abundance and composition of AOB but not AOA in a semi-arid grassland. In addition, as soil becomes flooded in the course of rice growth, oxygen (O_2) is depleted at a more rapid rate due to an interface a few millimeters beneath the soil-water [5], suggesting that the abundance of AOA and AOB, which are widespread in paddy fields, varies across soil layers. However, it is unclear how factors associated with soil depth influence AOA, AOB and MOB distribution in FPFs.

Therefore, investigating the community responses of AOA, AOB and MOB in different soil layers could enhance our understating of the soil N cycle. In this study, soil samples were collected from three soil layers from three FPFs in Chongqing, China. The aim was to investigate the key microbial mechanisms of NH_3 and CH_4 oxidation in the FPFs and evaluate changes in AOA, AOB and MOB abundance and composition along the paddy field soil profiles. The study aimed to also evaluate the contributions of AOA, AOB and MOB in different soil layers nitrification and oxidation processes.

We applied qPCR techniques to identify AOA, AOB and MOB and compared their abundance and occurrence among three FPFs.

2. Materials and Methods

2.1. Soil Sampling and Site Characteristics

The soil samples used in the present study were selected from three different soil layers, including the surface (0–3 cm), the subsurface (3–5 cm) and the bottom (5–20 cm) layers at three FPFs following crop harvest. The three experimental fields are at the Purple Soil Ecology Experimental Station of Southwest University (JD) (30°26'N, 106°26'E), Hechuan (HC) (36°1'12''N, 118°59'31.2''E), and Shapingba (SPB) (29°43'39''N, 106°19'57''E), Chongqing, China. The average annual rainfall in the FPFs were 1105.4 mm, 1100–1300 mm and 1100 mm in JD, HC and SPB, respectively, and the average temperatures were 18.3 °C, 18.1 °C and 18.2 °C, respectively. The management practices in the three FPFs included conventional fertilization treatments. At the JD sites, the application of 190 kg hm^{−2} human and animal feces or urine containing urea, 500 kg hm^{−2} calcium superphosphate, 75 kg hm^{−2} potassium chloride, 80 kg hm^{−2} urea topdressing of rice at the tillering stage and 75 kg hm^{−2} potassium chloride were common practices. At the HC and SPB sites, ammonium sulfate combined with human waste was applied as a fertilizer.

In October 2016, three replicate soil samples were randomly collected at three layers in each of the paddy fields using a ZYA-QY Earth drill soil sampler (70 mm × 300 mm, Hangzhou Billion McNair Tech., Zhejiang, China). The soils were homogenized by using manually mixing and removing visible plant debris, rocks and soil micro-fauna, and were then stored at 4 °C. Soil samples for molecular analysis were stored at −20 °C. Other soil samples were air-dried and used for the determination of basic physical and chemical properties.

2.2. Determination of Soil Properties

Soil pH was measured in a 1:2.5 (soil:water) suspension using a pH meter (320-S, Mettler-Toledo Instruments Co., Ltd., Shanghai, China). Soil organic matter (SOM) concentration was measured using the dichromate oxidation method [22]. Total nitrogen (TN) was measured using the kjeldahl method [23] and bulk density using the ring knife method. Redox potential was calculated using the depolarization method (FJA-4 redox depolarization automatic analyzer) [24]. The results for the soil chemical property tests are presented in Table 1.

Table 1. Soil properties at the experimental sites.

	Bei Bei (JD)			He Chuan (HC)			Sha Pingba (SPB)		
	0–3 cm	3–5 cm	5–20 cm	0–3 cm	3–5 cm	5–20 cm	0–3 cm	3–5 cm	5–20 cm
Average rainfall (mm)		1105.4			1100–1300			1100	
Average temperature (°C)		18.3			18.1			18.2	
Average sunshine (h)		1276.7			1300			1150	
Frostless period (d)		334			331			325	
pH	6.6	6.8	6.5	6.7	6.6	6.8	6.7	6.8	6.6
Eh (mV)	361 ^b	296 ^d	252 ^f	375 ^a	301 ^d	288 ^e	344 ^c	296 ^d	254 ^f
Bulk density (g/cm ³)	1.44 ± 0.02 ^b	1.46 ± 0.01 ^b	1.49 ± 0.01 ^a	1.12 ± 0.01 ^d	1.13 ± 0.02 ^d	1.33 ± 0.01 ^c	1.31 ± 0.03 ^c	1.33 ± 0.01 ^c	1.43 ± 0.02 ^b
Organic matter (g/kg)	23.8 ± 0.2 ^d	25.0 ± 0.3 ^b	22.9 ± 0.1 ^e	18.0 ± 0.1 ^h	19.5 ± 0.2 ^f	18.5 ± 0.1 ^g	25.1 ± 0.1 ^b	25.7 ± 0.2 ^a	24.3 ± 0.1 ^c
Total N (g/kg)	1.31 ± 0.02 ^c	1.43 ± 0.03 ^b	1.34 ± 0.01 ^c	1.17 ± 0.01 ^e	1.23 ± 0.01 ^d	1.11 ± 0.02 ^f	1.44 ± 0.02 ^b	1.49 ± 0.01 ^a	1.46 ± 0.01 ^b

Notes: 0–3 cm: surface layer, 3–5 cm: subsurface layer, 5–20 cm: bottom layer. Different lower-case letters (a–f) indicate significant differences $p < 0.05$. The differences at a 0.05 significance level were separately checked among soil samples.

2.3. Potential Nitrification Rates (PNRs) and Methane Oxidation Potentials (MOP)

Potential nitrification rates (PNRs) were tested using the Chlorate (sodium chlorate) inhibition method [13]. Dry soil samples (5 g) were added into a 50 mL centrifuge tube that contained 20 mL of 1 mM (NH₄)₂SO₄ and 1 mL of sodium chlorate solution. The tube was maintained at 25 °C for 24 h in the dark and later retrieved for further analysis. The oxidation rate was calculated as follows:

$$\frac{(S - C) \cdot 2,510,000}{5.5 \cdot \%dm} = n(Ng^{-1} \cdot dm \cdot 5h^{-1}). \quad (1)$$

S—Soil sample value (mg N); C—Control (mg N)

The determination of soil CH₄ oxidation potential was carried out according to Lü et al. (2005) [25] with some modifications. Briefly, the CH₄ gas initially added to the pre-culture samples was released following the opening of the culture flask. As 1% CH₄ gas was added to the samples, 1% difluoromethane was also added to the control treatment as a CH₄ oxidation inhibitor. The culture suspensions were carefully shaken after sealing the pinhole. Each treatment had three replicates and was incubated at 25 °C in the dark. Sampling and soil CH₄ oxidation potential determination were carried out on the first, fourth, seventh and tenth days after the beginning of the culture. The CH₄ oxidation rate was calculated based on the slope when the concentration of CH₄ in the bottle decreased linearly.

The ratio of the difference of the CH₄ concentration to the sampling time interval measured based on two consecutive samples during the culture period represented the CH₄ oxidation rate during the period, and the oxidation rate was calculated as follows:

$$Q = \frac{d_c}{d_t} \times \frac{V}{W} \times \frac{MW}{MV} \quad (2)$$

where: *Q*: the oxidation rate of CH₄ (μg g⁻¹h⁻¹); *dc/dt*: change of CH₄ concentration per unit time (ppm mol⁻¹ h⁻¹); *V*: the volume of gas in the culture flask (L); *W*: the dry soil weight (g); *MW*: the molecular weight (g) of CH₄; *MV*: the volume (L) of 1 mol of gas in a standard state.

2.4. Molecular Analyses

A FastDNA SPin Kit for Soil (MP Biomedicals, LLC, United States) was used to extract soil DNA. Amplification of the qPCR products for AOA, AOB and MOB was conducted on a CFX-96 Optical Real-Time PCR System (Bio-Rad Inc. Hercules, CA, US). The primer pairs amoA-1F/amoA-2R [26], Arch-amoAF/Arch-amoAR [27], and A189f/682r [28], A189f/mb661 [29] (Table 2) were adopted for the quantitative PCR (q-PCR) assays of AOA, AOB and MOB abundance, respectively. The total volume of the PCR reaction system was 20 μL, which was 10 μL of SYBR® Premix EX Taq™ (TaKaRa), 0.3 μL of the upstream and downstream primers (20 pmol μL⁻¹), 1.0 μL of the sample and 8.4 μL of sterilized double distilled water (ddH₂O). The reaction conditions are listed in Table 3. The standard curve for quantitative PCR was used to clone an AOA and AOB *amoA* or an MOB *pmoA* functional gene into a vector plasmid (from Shanghai Meiji Sequencing Co., Ltd.).

Table 2. Specific primers for functional genes used in the present study.

Target	Primer Sets	Primer Sequences(5′-3′)	References
AOB <i>amoA</i>	amoA-1F	GGGGTTTCTACTGGTGGT	Rotthauwe(1997)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	
	Arch-amoAF	STAATGGTCTGGCTTAGACG	
	Arch-amoAR	GCGGCCATCCATCTGTATGT	
AOA <i>amoA</i>	A189gc	GGNGACTGGGACTTCTGG	Beman (2006)
MOB <i>pmoA</i>	682r	GAASGCNAGAGAAGAASGC	Liebner (2009)
	A189gc	GGNGACTGGGACTTCTGG	Zhou (2008)
	mb661	CCGGMGCAACGTCYTACC	

Table 3. PCR reaction conditions for microorganisms in the present study.

Target	General PCR Reaction Conditions	Quantitative PCR Reaction Conditions
AOB <i>amoA</i>	94 °C 5 min; 32 × (94 °C 45 s, 55 °C 45 s, 72 °C, 1 min); 72 °C, 10 min, step at 4 °C.	95 °C, 5.0 min; 35 × (95 °C, 30 s; 56 °C, 45 s; 72 °C, 1 min.); Melt curve 65.0 °C to 95.0 °C, increment 0.5 °C, 0:05+ plate read.
AOA <i>amoA</i> MOB <i>pmoA</i>	94 °C 5 min, 32 × (94 °C 45 s, 53 °C 45 s, 72 °C 90 s); 72 °C, 10 min, step at 4 °C. Round 1: 94.0 °C, 3 min; 30 × (94.0 °C, 30 s; 56.0 °C, 1 min; 72.0 °C, 1 min); 72.0 °C, 7 min, step at 4 °C. The product obtained by the first round of PCR amplification is used as a template. Round 2: 95.0 °C, 3 min, 35 × (94.0 °C, 30 s; 56.0 °C, 1 min; 72.0 °C, 1 min); 72.0 °C, 7 min, step at 4 °C.	95.0 °C, 90 s; 40 × (95.0 °C, 10 s; 55.0 °C, 30 s; 72.0 °C, 30 s; 80.0 °C, 5 s with plate read; Melt curve 65.0 °C to 95.0 °C, increment 0.5 °C, 0:05 + plate read.

2.5. Cloning, Sequencing, and Phylogenetic Analysis

The target gene and *Escherichia coli* were recombined in vitro and then introduced into competent cells for expression and amplification. The positive clones were sent to Shanghai Meiji Company for further sequencing using a pEASY-T3 Cloning Kit (TransGen Biotech, Beijing, China). The result obtained for the cloned sequences was subjected to sequence quality control using Chromas (Technelysium, Helensvale, Queensland, Australia), and the vector sequences were deleted from the text to obtain the target gene sequences. A Blast comparison was conducted by searching the National Center for Biotechnology Information (NCBI) Gen Bank database to obtain highly similar homologous gene sequences. MEGA v4.0 [30] was used for the analysis and the sequences of all gene clones were classified based on Operating Taxonomic Units (OTUs). The neighbor joining method was used to establish phylogenetic trees to determine the phylogenetic status of sequencing clones based on their positions and genetic distances in phylogenetic trees.

3. Statistical Analysis

Statistical analyses were conducted using IBM SPSS Statistics 19.0 (IBM, Armonk, NY, US). Nitrification potential and microbial abundance were visualized using Origin 8.6 (OriginLab Corp., Northampton, MA, US). Gene sequencing was performed using Chromas, NCBI-Blast (<https://www.ncbi.nlm.nih.gov/>) and Mega v4.0 analysis and mapping.

4. Results

4.1. Potential Nitrification Rates

Potential nitrification rates were different among the FPFs and among the different soil layers. The nitrification activities ranged from 0.0150 $\mu\text{g N g}^{-1} \text{h}^{-1}$ in HC soil to 0.1680 $\mu\text{g N g}^{-1} \text{h}^{-1}$ in SPB soil (Figure 1). In all the FPFs, nitrification activity was significantly higher in the subsurface layers (3–5 cm), although at different rates. Compared to the surface soil layer (0–3 cm), the potential nitrification rate in HC soil was significantly lower in the bottom layer (5–20 cm), although it was higher in SPB and JD soil (Figure 1).

Figure 1. Nitrification activity in different soil layers in all the flooded paddy fields. Values are the averages of triplicate samples. Vertical bars indicate standard deviations. Different letters above the columns indicate significant differences $p < 0.05$.

4.2. CH_4 Oxidation Potentials

The CH_4 oxidation rates in different soil layers of the three FPFs are illustrated in Figure 2. In all the FPFs, the CH_4 oxidation rates were rapid initially and then decreased with an increase in incubation time (Figure 2). The average CH_4 oxidation capacity of SPB soil was the highest and that of HC soil was the lowest, irrespective of the number of soil layers (Figure 2). The net CH_4 oxidation rates in SPB soil were 0.46 ± 0.40 , 0.63 ± 0.46 and $0.43 \pm 0.26 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$ at 0–3 cm, 3–5 cm and 5–20 cm, respectively. In addition, the net CH_4 oxidation rates in HC soil were $0.43 \pm 0.33 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$, $0.26 \pm 0.15 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$ and $0.22 \pm 0.19 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$ at 0–3 cm, 3–5 cm and 5–20 cm, respectively. The net CH_4 oxidation rates in JD soil were 0.022 ± 0.018 , 0.020 ± 0.79 and $0.91 \pm 0.93 \mu\text{g CH}_4 \text{ g}^{-1} \text{ h}^{-1}$ at 0–3 cm, 3–5 cm and 5–20 cm.

4.3. AOA, AOB, and MOB Abundance

The results of the q-PCR analysis of *amoA* and *pmoA* were used to determine the AOA, AOB and MOB abundance in the SPB, HC and JD soils. AOA abundance ranged from 5.28 to 1.09×10^7 /g dry soil, 5.36 to 1.59×10^7 /g dry soil and 3.64 to 1.10×10^7 /g dry soil archaeal *amoA* gene copies in the SPB, HC and JD soils, respectively (Figure 3a). The highest AOA *amoA* abundance was observed in the bottom (5–20 cm) layer of the SPB and JD soils. AOB abundance ranged from 3.81 to 7.50×10^6 /g dry soil, 1.10 to 4.27×10^6 /g dry soil, and 1.43 to 3.14×10^6 /g dry soil bacterial *amoA* gene in the SPB, HC and JD soil samples, respectively (Figure 3b). The AOB *amoA* gene copies in the subsurface (3–5 cm) layer of the SPB, HC and JD soils were significantly higher than in the bottom (5–20 cm) layer. MOB abundance ranged from 2.56 to 1.05×10^6 /g dry soil, 7.46 to 1.02×10^6 /g dry soil, and 2.92 to 1.04×10^6 /g dry soil *pmoA* gene copies in the SPB, HC and JD soils, respectively (Figure 3c). The MOB *pmoA* gene copies at the SPB site decreased with an increase in soil layer depth; however, no consistent trend in MOB *pmoA* abundance was observed in the HC and JD sites among the soil layers. The AOA/AOB

ratio at 0–3 cm and 5–20 cm was greater than 10 across the three sites (Table 4) while the AOB/MOB ratio in all the three sites and at the different layers was much lower than 10.

Figure 2. Methane oxidation rate in different soil layers in all the flooded paddy fields. Values are the averages of triplicate samples. Vertical bars indicate standard deviations.

Figure 3. (a) AOA, (b) AOB, and (c) MOB community abundance in different soil layers in all the flooded paddy fields. Values are the averages of triplicate samples. Vertical bars indicate standard deviations. Different letters above the columns indicate significant differences $p < 0.05$.

Table 4. Relative abundances of functional gene copy numbers for different soil layers in flooded paddy fields.

	SPB			HC			JD		
	0–3 cm	3–5 cm	5–20 cm	0–3 cm	3–5 cm	5–20 cm	0–3 cm	3–5 cm	5–20 cm
AOA/AOB	20.7	7.04	28.6	57.2	12.6	68.4	43.5	11.6	76.9
AOA/MOB	7.72	13.1	42.6	17.2	5.25	10.1	7.14	12.5	20.5
AOB/MOB	0.373	1.87	1.49	0.301	0.419	0.147	0.164	1.07	0.647

Notes: 0–3 cm: surface layer, 3–5 cm: subsurface layer, 5–20 cm: bottom layer.

4.4. AOA, AOB, and MOB Community Composition

A total of 90 positive sequences were used for AOA, AOB *amoA* and MOB *pmoA* functional gene sequencing and the construction of a clone library (Figure 4). Phylogenetic analysis revealed that the AOA *amoA* in the surface layer in HC belonged to Group 1.1b, while 97% of AOA *amoA* in the subsurface layer belonged to the subordinates Group 1.1b and 3% to Group 1.1a associated. Sixty-four percent of AOA *amoA* in the bottom layer belonged to the subordinates Group 1.1b and 36% to Group 1.1a associated (Figure 4a). In addition, the phylogenetic analysis revealed that 92% of AOA *amoA* in the surface layer of SPB belonged to Group 1.1b, while 8% belonged to Group 1.1a. Fifty-six percent of AOA *amoA* in the subsurface layer of SPB belonged to subordinates Group 1.1b and 44% to Group 1.1a associated; while in the bottom layer, 12% of the AOA *amoA* belonged to Group 1.1b, and 88% of the subordinates belonged to Group 1.1a associated (Figure 4b).

At the JD site, 84% of AOA *amoA* belonged to Group 1.1b and 16% to Group 1.1a, while 36% belonged to Group 1.1b and 64% belonged to Group 1.1a in the subsurface and the bottom layers (Figure 4c). *Nitrosospora* clusters were observed in the surface and bottom layers, accounting for 17% and 14%, respectively. In addition, the community diversity of AOA exceeded that of AOB considerably, and AOB *amoA* was affiliated only with *Nitrosomonas* in the HC and SPB sites (Figure 5).

The phylogenetic analysis of MOB *pmoA* assigned 69% of the sequences to Type Ia and the residual 31% of the sequences to type II MOB in the HC surface layer. In the HC subsurface layer, MOB *pmoA* consisted of subordinate Type Ia (18%), Type Ib (37%) and Type II (45%). In the HC bottom layer, 24% of the sequences belonged to Type Ib and 76% belonged to Type II. In the surface layer at the SPB site, only 11% of the sequences were assigned to type II methanotrophs while the rest belonged to Type Ia (30%) and Type Ib (59%). In the SPB subsurface layer, 44% of the sequences were associated with Type Ib, and 56% were associated with Type I methanotrophs. Only 8% of MOB *pmoA* sequences were classified into Type Ia methanotrophs and the rest were associated with Type II. In the surface layer of the JD site, 17% of the sequences were assigned to Type Ia and the rest of the sequences were type Ib MOBs. The subsurface MOB *pmoA* belonged to Type Ib bacteria (38%) and Type II bacteria (62%). For the bottom layer soil at the JD site, the sequences belonged to Type Ia (25%), Type Ib (21%) and Type II (54%) (Figure 6).

(a)

Figure 4. Cont.

(b)

Figure 4. Cont.

(c)

Figure 4. Phylogenetic tree of AOA *amoA* in different soil layers in (a) HC, (b) SPB, and (c) JD soil samples. The number in brackets after the gene or clone name represents the sequence number of the OTU. The scale represents the genetic distance, and the number represents five differences in every 100 bases. H-1 represents a 0–3 cm soil layer, H-2 represents a 3–5 cm soil layer and H-3 represents a 5–20 cm soil layer in Figure 4a; Q-1 represents a 0–3 cm soil layer, Q-2 represents a 3–5 cm soil layer and Q-3 represents a 5–20 cm soil layer in Figure 4b; J-1 represents a 0–3 cm soil layer, J-2 represents a 3–5 cm soil layer, and J-3 represents a 5–20 cm soil layer in Figure 4c. The scale indicates the number of nucleotide substitutions per site.

(a)

Figure 5. Cont.

(b)

Figure 5. Cont.

(c)

Figure 5. Phylogenetic tree of AOB amoA in different soil layers in (a) HC, (b) SPB and (c) JD soil samples. The number in brackets after the gene or clone name represents the sequence number of the OTU. The scale represents the genetic distance, and the number represents five differences in every 100 bases. H-1 represents a 0–3 cm soil layer, H-2 represents a 3–5 cm soil layer and H-3 represents a 5–20 cm soil layer in Figure 5a; Q-1 represents a 0–3 cm soil layer, Q-2 represents a 3–5 cm soil layer, and Q-3 represents a 5–20 cm soil layer in Figure 5b; J-1 represents a 0–3 cm soil layer, J-2 represents a 3–5 cm soil layer, and J-3 represents a 5–20 cm soil layer in Figure 5c. The scale indicates the number of nucleotide substitutions per site.

(a)

Figure 6. Cont.

Figure 6. Cont.

(c)

Figure 6. Phylogenetic tree of MOB *pmoA* in different soil layers in (a) HC, (b) SPB and (c) JD soil samples. The number in brackets after the gene or clone name represents the sequence number of the OTU. The scale represents the genetic distance, and the number represents five differences in every 100 bases. H-1 represents a 0–3 cm soil layer, H-2 represents a 3–5 cm soil layer and H-3 represents a 5–20 cm soil layer in Figure 6a; Q-1 represents a 0–3 cm soil layer, Q-2 represents a 3–5 cm soil layer and Q-3 represents a 5–20 cm soil layer in Figure 6b; J-1 represents a 0–3 cm soil layer, J-2 represents a 3–5 cm soil layer and J-3 represents a 5–20 cm soil layer in Figure 6c. The scale indicates the number of nucleotide substitutions per site. Numbers in parentheses against each abbreviation indicate the number of sequences recovered from each sample.

5. Discussion

5.1. Spatial Occurrence and Abundance of AOA, AOB and MOB among the FPFs

The present study explored the occurrence and niche distribution of AOA, AOB and MOB among three FPFs. Although some studies have suggested that the oxidation of NH_3 is mainly attributed to AOA [31,32], the presence of both AOA and AOB in all the three FPFs in the present study indicated that the two groups could jointly play a role in the transformation of NH_3 to NO_3^- . Consistent with the findings of previous studies across different ecosystems [16,33], we observed that AOA abundance has a more considerable role than AOB and MOB across the three FPFs, with the highest *amoA* observed in the bottom soil layers, particularly in the SPB and JD sites.

Although the results of previous studies have suggested that AOA numerically outnumber AOB in acidic soil, in the present study we observed that despite the soil pH values across the FPFs moving away from being acidic to alkaline (Table 1), the AOA abundance was still significantly higher than AOB abundance in all FPFs, and were consistent with the higher potential nitrification rates (PNRs), particularly in the SPB and JD sites. The results indicated a reduced affinity of soil pH and AOA for NH_3 , which enabled them to utilize low concentrations of NH_3 in the acidic soils and thus influenced their abundance in the present study. However, the higher AOA observed in the present study could be attributed to their capacity to adapt to low O_2 concentration environments, which is a common phenomenon in paddy fields [34], or the high organic matter concentrations that create NH_3 assimilation pathways in all the three sites (Table 1 in reference [35]). In addition, although the AOA/AOB ratio was higher than 10 in all the three sites, the AOB/MOB ratio was much lower than ten, indicating that the AOA *amoA* functional gene copy numbers in the three sites were significantly higher than the AOB *amoA* and MOB *pmoA* functional gene copy numbers, by at least one order of magnitude. Since potential nitrifying activity is linked strongly to soil NO_2^- content, the higher PNRs in the 3–5 cm soil layer indicated that the risk of NO_2^- leaching could be higher in the surface soil. Therefore, in all the studied flooded paddy fields, the surface soil requires more attention with regard to NO_2^- leaching.

Unlike AOA and AOB, approximately 50% to 90% of the CH_4 in the soil surface and the rhizosphere is oxidized by MOB prior to being emitted into the atmosphere, thus there is a need to pay further attention to their occurrence and distribution in FPFs [6]. Similar to AOA and AOB, MOB occurred in all the studied FPFs and there was no significant difference in their abundance among the sites. However, the potential CH_4 oxidation rates across the FPFs indicated that SPB paddy fields had the capacity to increase CH_4 oxidation capacity considerably compared to the other FPFs (Figure 2). MOB abundance across the FPFs was low compared to AOA and AOB abundance, which suggests that although MOB might contribute significantly to the nitrification process in all the three FPFs, their specific nitrification rates could be much lower than the AOA and AOB nitrification rates [5].

5.2. Community Structure of AOA, AOB, and MOB at Different Soil Depth among the FPFs

The results of the phylogenetic analysis in the present study revealed that the members of three archaea genera, including *Nitrososphaera*, *Nitrosopumilus*, and *Nitrosotalea* were enriched in the three FPFs. Members of bacterial genus *Nitrosomonas* were the most dominant NH_3 oxidizers in all the soils. In addition, genus *Nitrososphaera* was among the most abundant NH_3 oxidizers in the HC and the SPB surface soil. Members of genus *Nitrosotalea* were highly enriched in the JD subsurface and bottom soil layers, while members of *Nitrosopumilus* were only dominant in the JD subsurface and bottom soil layers, and *Nitrososphaera* were still dominant in the surface layer. The results indicated that different NH_3 oxidizers occupied different environmental niches in the FPFs. Several factors, including substrate concentration, pH, temperature and O_2 have been reported to influence the structural composition of NH_3 oxidizers in different ecosystems [36,37]. However, the distinction in the community structure across the FPFs in the present study could not be attributed solely to the environmental drivers because there were no significant differences in either temperature or pH among the FPFs.

In the present study, MOB *pmoA* distribution in the FPFs varied considerably; however, community distribution increased with an increase in the soil layer depth. Type I MOB were enriched in the surface and subsurface layers, although Type II MOB were enriched in the bottom layer. The results indicated that type I MOB abundance was higher than type II MOB, which was consistent with the findings of Steenbergh et al. (2010) [38], who proposed different life strategies for type I and type II methanotroph. It has been reported that Type I MOB preferentially grow at high O₂ and low CH₄ concentration environments, while Type II MOB preferentially grow at low O₂ and high CH₄ concentration environments [39]. Therefore, Type I and II MOB occupy different niches [40]. The results also demonstrated the uniqueness of each soil environment and the effects of ecological factors on MOB at the species level. The MOB communities in the FPFs were relatively stable. The Type I MOB belong to the *Methylococcaceae* type I methanotroph genera (Type I a) and the genus *Methylocaldum* (Type I b), while Type II MOB are the genus *Methylocystis*. The results are consistent with the findings of a previous study that suggested that the major contributor to CH₄ oxidation in the atmosphere is type II MOB, particularly *Methylocystis* sp [41]. Plant cultivation, moisture content and temperature also influence MOB diversity [42]. In addition, soil moisture could influence soil CH₄ absorption [43]. In the present study, the application of fertilizer in paddy fields enhanced CH₄ oxidation. The inorganic N concentration in the soil influenced MOB activity. The MOB CH₄ oxidation activity is near zero when N ions in the soil are exhausted, with three potential mechanisms: (1) NH₄⁺- or NO₂⁻-nitrogen increase the activity of CH₄ oxidizing microorganisms; (2) N participates in the synthesis of enzymes involved in CH₄ oxidation; and (3) an increase in nitrification bacteria diversity and activity could enhance CH₄ oxidation [44,45]. Nevertheless, the specific mechanism of action requires further study.

6. Conclusions

This study highlighted that AOA abundance was higher than AOB and MOB abundance in all the FPFs, which was consistent with the potential nitrification rates (PNRs), particularly in the SPB and JD sites. Three AOA genera (*Nitrososphaera*, *Nitrosopumilus*, *Nitrosotalea*) were observed in all the FPFs, and *Nitrososphaera* were dominant in the surface layers. In addition, *Nitrososphaera* were among the most abundant NH₃ oxidizers in the HC and in the SPB surface soil, while members of genus *Nitrosotalea* were considerably enriched in the JD subsurface and bottom layers. MOB community distribution increased with an increase in the depths of the soil layers, and Type I MOB were enriched in the surface and subsurface layers, while Type II MOB were enriched in the bottom layers. Overall, this study provides a basis for improving soil nitrogen use efficiency and mitigating soil greenhouse gas emissions in flooded paddy fields.

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