Research Paper

Manure fertilization alters the population of ammonia-oxidizing bacteria rather than ammonia-oxidizing archaea in a paddy soil

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Manure fertilizers are widely used in agriculture and highly impacted the soil microbial communities such as ammonia oxidizers. However, the knowledge on the communities of archaeal versus bacterial ammonia oxidizers in paddy soil affected by manure fertilization remains largely unknown, especially for a long-term influence. In present work, the impact of manure fertilization on the population of ammonia oxidizers, related potential nitrification rates (PNRs) and the key factors manipulating the impact were investigated through studying two composite soil cores (long-term fed with manure fertilization versus undisturbed). Moreover, soil incubated with NH_4^+ for 5 weeks was designed to verify the field research. The results showed that the copy numbers of bacterial *amoA* gene in the manure fed soil were significant higher than those in the unfed soil ($p < 0.05$), suggesting a clear stimulating effect of long-term manure fertilization on the population of ammonia-oxidizing bacteria (AOB). The detected PNRs in the manure fed soil core ($14\text{--}218 \text{ nmol L}^{-1} \text{ N g}^{-1} \text{ h}^{-1}$) were significant higher than those in the unfed soil core ($5\text{--}72 \text{ nmol L}^{-1} \text{ N g}^{-1} \text{ h}^{-1}$; $p < 0.05$). Highly correlations between the PNRs and the bacterial *amoA* gene copies rather than archaeal *amoA* gene were observed, indicating strong nitrification capacity related to bacterial ammonia oxidizers. The $\text{NH}_4^+\text{-N}$ significantly correlated to the abundance of AOB ($p < 0.01$) and explained 96.1% of the environmental variation, showing the $\text{NH}_4^+\text{-N}$ was the main factor impacting the population of AOB. The incubation experiment demonstrated a clear increase of the bacterial *amoA* gene abundance (2.0×10^6 to $8.4 \times 10^6 \text{ g}^{-1} \text{ d.w.s.}$ and 1.6×10^4 to $4.8 \times 10^5 \text{ g}^{-1} \text{ d.w.s.}$) in both soil but not for the archaeal *amoA* gene, in agreement with the field observation. Overall, our results suggested that manure fertilization promoted the population size of bacterial ammonia oxidizers rather than their archaeal counterparts whether in long-term or short-term usage and the $\text{NH}_4^+\text{-N}$ was the key impact factor.

Abbreviations: PNR – potential nitrification rate; AOA – ammonia-oxidizing archaea; AOB – ammonia-oxidizing bacteria; AMO – ammonia monooxygenase; qPCR – quantitative polymerase chain reaction; PBS – phosphate buffer solution; TN – total nitrogen; TP – total phosphate; TOM – total organic matter; COD – chemical oxygen demand; PCA – principal component analysis

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Introduction

Application of ammonium-based N fertilizers has substantially raised the rice yield over the past decades. However, only 27–33% of the applied N fertilizer could be recovered in the plant material [1]. In agricultural ecosystem, nitrification as the first step in coupled

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nitrification–denitrification is a key step in the losses of N fertilizers. The nitrification, the conversion of NH_3 to hydroxylamine, is carried out by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) with the key ammonia monooxygenase (AMO) enzyme [2, 3]. Distinct physiology [4] and metabolism [5] of AOA and AOB indicated that environmental factors such as pH, organic carbon, NH_4^+ availability and salinity may determine the distribution and functional importance of both guilds in natural ecosystems [6–11]. The cultivation-based studies and molecular surveys targeting the *amoA* gene which encodes catalytic subunit A of AMO have been widely used to investigate the effect of fertilization on the ammonia oxidizers [6, 8, 12, 13]. AOA, affiliated into a new division within the *Archaea* of *Thaumarchaeota* [14, 15] were numerically dominated over their bacterial counterparts in various soils [16]. However, limited knowledge is known about the communities of archaeal versus bacterial ammonia oxidizers in paddy soil affected by manure fertilization, especially for a long-term influence.

In paddy field, the intensified application of chemical and organic fertilizers supplies ample substrate for the growth of AOA and AOB under *in situ* conditions. Furthermore, the vertical ammonia flux/concentration and redox gradient formed in paddy soil along depth highly impact the structure and function of bacteria, fungi, and archaea including AOA in microhabitats [6, 12, 17]. These variations in vertical profiles provide a convenient system for investigating the influence of changing environment on the ammonia oxidizing communities in paddy soil.

In this study, we aimed to investigate the vertical profiles of AOA and AOB population and their response to the manure fertilization in paddy soil. To achieve these goals, core samples were collected from a long-term manure fertilized paddy field and an adjacent site without fertilization and rice planting. And then a 5 weeks incubation experiment was performed to verify the field study and investigate the dynamic of AOA and AOB in response to the addition of ammonium substrate. The abundance of AOA and AOB were determined by the

quantitative polymerase chain reaction (qPCR) targeting the *amoA* genes. The soil properties and PNRs were also measured. The relationship between soil chemistry and the abundance of AOA and AOB was correlated to find out the key factors manipulating their population size and the putative contribution of AOA and AOB to the nitrification in paddy soil.

Materials and methods

Sampling sites and sampling

Samples were taken in a paddy field at a typical rice planting region in Jiaxing city (Zhejiang Province, south China). Manure fertilization (two times per month from April to October of the growth season) has been applied for more than 20 years in a rice–wheat rotation system. The manure was mainly constituted of livestock waste including urine, dung, and flushing water which were primarily fermented from the neighbor farm and fed into the paddy field through two channels. The manure contains high ammonium ($\text{NH}_4^+\text{-N}$) and chemical oxygen demand (COD; Table 1). Within them, ammonium accounts for 49–60% of the total nitrogen (TN) content. An adjacent plot without manure influence and rice planting was chosen as a comparison. The soil samples were collected after the harvest without water flooding from the depths of 0–100 cm in November 2009. Three soil cores (approximately 5 cm diameter) were taken from each plot and samples in the same depth (every 10 cm interval) were mixed to form one composite sample. Sample was sealed in a sterile plastic bag on ice when transported to the laboratory. One part was stored at 4 °C for the incubation experiments and another part was sieved through a 2.0-mm sieve for the analyses of soil characteristics. The rest were store at –80 °C for the DNA extraction and downstream analysis.

Chemical properties and potential nitrification rates measurement

The ammonium, nitrite, and nitrate in soil were extracted with 2 M KCl extraction buffer and measured by a Continuous Flow Analyzer (Skalar + Analytical, The

Table 1. The profiles^a of the manure wastewater fed into the paddy field.

	pH	COD ^b	Salinity (‰)	$\text{NH}_4^+\text{-N}^c$	$\text{NO}_x^-\text{-N}^c$	$\text{PO}_4^{3-}\text{-P}^c$	TN ^c	TP ^c
Channel 1	7.6	2245	0.17	55.86	3.50	0.21	91.57	10.71
Channel 2	7.4	2318	0.22	53.48	3.93	0.21	108.50	11.77

TP, total phosphorus.

^aMeasurements were carried out according to the standard method for waste water measurement [36].

^bUnits are mg L^{-1} .

^cUnits are mM.

Netherlands). The soil pH was determined with a soil to water ratio of 1:2.5 and measured by PB-10 pH meter (Sartorius, Germany). Soil organic matter (TOM) and TN was determined according to the standard method [18]. Three replicates were carried out for each measurement. The oxygen concentration in fresh soil was measured *in situ* using OXY Meter S/N 4164 stainless electrode sensor (Unisense, Denmark). The measurements of potential nitrification rates were carried out using a chlorate inhibition method according to previous reports [19, 20]. Briefly, 5.0 g soil was added into a 50-ml centrifuge tube containing 20 ml phosphate buffer solution (PBS; g/L: NaCl, 8.0; KCl, 0.2; Na₂HPO₄, 0.2; NaH₂PO₄, 0.2; pH 7.4) with 1 mM (NH₄)₂SO₄. Potassium chlorate with a final concentration of 10 mM was added to inhibit the nitrite oxidation. The suspension was incubated in a dark incubator at 25 °C. The nitrite was extracted with 5 ml of 2 M KCl solution and determined by a spectrophotometer at a wavelength of 540 nm with *N*-(1-naphthyl) ethylenediamine dihydrochloride. Apparent potential nitrification rates were calculated from the linear increase in the concentration of NO₂⁻-N during the first 6 h.

Incubation

To verify the field study and investigate the dynamics of AOA and AOB in response to the addition of ammonium substrate, the manure fed soil samples of the 40–50 cm layer where the AOA started to dominant over the AOB observed in the field study were incubated with 0.16 mM NH₄Cl solution to simulate the N loading rate. For the purpose of comparison, the sample of unfed soil was also introduced from the layers of 40–50 cm. The incubation was performed anoxically in dark for 5 weeks. The concentrations of NH₄Cl in the solution were measured every week. The copy numbers of archaeal and bacterial *amoA* genes in the sediment were quantified in the first, the third and the fifth week.

DNA isolation

DNA was extracted from 0.25 g soil using MoBio Ultra-Clean soil DNA isolation kits (MoBio, USA) according to the manufacturer's instructions with minor modifications: the bead tubes were held on the MoBio Vortex Adapter tube holder (MoBio Catalog no. 13000-V1) and vortexed at maximum speed for 5 min instead of 10 min. Afterwards, the tubes were bathed at 70 °C for another 5 min to facilitate the cell breaking. DNA was eluted with 50 µl sterilized MilliQ-filtered water and stored at –20 °C.

Quantitative PCR

Quantitative PCR were carried out with an ABI Prism 7300 Sequence Detection System (Applied Biosystems,

USA) as follows: 50 °C for 2 min, 95 °C for 30 s, followed by 40 cycles of 10 s at 95 °C, 30 s at 53 °C for AOA or 55 °C for AOB, 1 min at 72 °C. The 25 µl reaction volume contained 12.5 µl SYBRs Premix Ex Taq (TAKARA, Dalian, China), 200 nmol L⁻¹ of primers and 2 µl of tenfold diluted DNA as template [2, 21]. Three replicates were analyzed for each sample.

Plasmid DNA isolated from positive clones containing the bacterial or archaeal *amoA* gene was used as the standard curve for the quantitative PCR. The concentration of plasmid DNA was determined on a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA) and the copy numbers of *amoA* gene were calculated directly from the concentration. Tenfold serial dilutions of the plasmid DNA with known copy numbers were subjected to quantitative PCR in triplicate to generate an external standard curve. The results with amplification efficiency and correlation coefficient around 95% and 0.95 were employed. The specific amplification of *amoA* gene was checked by melting curve analysis, which resulted in a single peak.

Statistical analysis

Paired-samples *t*-test was used to test the significance of difference between archaeal and bacterial *amoA* gene copy numbers using SPSS version 13.0. To find the key environmental parameters impacting the abundance of *amoA* genes, correlation analysis and principal component analysis (PCA) based on Bray–Curtis distance matrix were used to extract the most important information of the environmental parameters. PCA was conducted at program R with the packages *vegan* and the correlation between the PCA extract and the abundance of AOA and AOB was conducted at PAST [22, 23]. The copy numbers of archaeal and bacterial *amoA* genes were log₁₀-transformed to normalize their distribution before analysis.

Results

Soil properties

The vertical profiles of ammonium (NH₄⁺-N), nitrite plus nitrate (NO_x⁻-N), TN, total organic matter (TOM), pH, and oxygen (DO) at every 10 cm were measured (Fig. 1).

In the manure fed soil, the NH₄⁺-N was 137.1 mg kg⁻¹ in the surface layer (0–10 cm) and peaked at 10–20 cm depth of 178.8 mg kg⁻¹. The highest concentrations of NO_x⁻-N, TN and TOM were observed in surface layer at 60.8 mg kg⁻¹, 3.9 g kg⁻¹, and 44.5 g kg⁻¹, respectively. The pH ranged from 6.69 (0–10 cm) to 7.05 (90–100 cm). The oxygen decreased rapidly to the limit of detection below 10 cm and therefore was turned to a limiting

Figure 1. Vertical distribution of soil profiles in manure fed soil (left) and unfed soil (right).

factor for the aerobic ammonia oxidation. Additionally, the ratio of $\text{NH}_4^+\text{-N}$, $\text{NO}_x^-\text{-N}$, TN, and TOM indicated a large proportion of organic nitrogen in the soil.

Similar variation was observed in the unfed soil but the concentration of each parameter was much lower. The $\text{NH}_4^+\text{-N}$ in the surface soil (0–10 cm) was lower than that in the subsurface (10–20 cm) and peaked at 10–20 cm depth at 20.1 mg kg^{-1} . The concentrations of $\text{NO}_x^-\text{-N}$, TN, and TOM peaked in surface at 9.1 mg kg^{-1} , 0.9 g kg^{-1} , and 9.8 g kg^{-1} , respectively. The pH increased slightly in deep soil and ranged from 6.65 (0–10 cm) to 8.19 (90–100 cm).

Abundance of *amoA* genes affected by long-term manure fertilization

The archaeal *amoA* gene was present in every layer of both manure fed and unfed soil cores. The copy numbers ranged from 1.2×10^6 to $1.3 \times 10^8 \text{ g}^{-1} \text{ d.w.s.}$ (per gram dry weight of soil) and they were much higher in upper layers (0–30 cm) than those of deep layers (70–100 cm) for average (Fig. 2). The highest copy number in the manure fed soil was observed in the layer of 20–30 cm depth at $4.8 \times 10^7 \text{ g}^{-1} \text{ d.w.s.}$ Nonetheless, it was still lower than that in the unfed soil of the same depth of $1.2 \times 10^8 \text{ g}^{-1} \text{ d.w.s.}$ Same trend was observed in other layers that the copy numbers of archaeal *amoA* gene in the unfed soil was slightly higher but it was not significant ($p > 0.05$). Therefore, no significant stimulating effect of manure fertilization to the population of AOA was concluded.

The stimulation of manure fertilization appeared to be obvious to the population of AOB. The copy numbers of bacterial *amoA* gene in the manure fed soil ranged from 8.8×10^5 to $3.2 \times 10^8 \text{ g}^{-1} \text{ d.w.s.}$ which were significant

higher than those in the unfed soil ranging from 1.6×10^4 to $6.3 \times 10^5 \text{ g}^{-1} \text{ d.w.s.}$ ($p < 0.05$). The bacterial *amoA* gene could only be detected in the layers above 50 cm at the unfed soil but was deeper at 70 cm in the manure fed soil. Additionally, the AOB in the layers around cultivated horizon and plough pan (0–30 cm) was more dominant than their archaeal counterparts in the manure fed soil. The AOA was more dominant in the deep layers (40–100 cm) due to the rapid decrease of the bacterial *amoA* gene copies. By comparison, the ammonia oxidizers were dominated by AOA in the samples of all depths in the soil without manure fertilization.

The effect of manure fertilization to the soil nitrification was also reflected by the variation of PNRs. The PNRs detected in the manure fed soil ($14\text{--}218 \text{ nmol L}^{-1} \text{ N g}^{-1} \text{ h}^{-1}$) were significant higher ($p < 0.05$) than those of unfed soil ($5\text{--}72 \text{ nmol L}^{-1} \text{ N g}^{-1} \text{ h}^{-1}$) with the highest value at the surface layer (0–10 cm). The PNRs decreased rapidly until the plough pan (20–30 cm) and maintained between $14\text{--}23$ and $5\text{--}14 \text{ nmol L}^{-1} \text{ N g}^{-1} \text{ h}^{-1}$ with minor variations at deep layers (60–100 cm) in the manure fed and unfed soil cores, respectively. Moreover, it was noted that the variation of PNRs was coincident with the variation of bacterial *amoA* gene copies.

Factors influencing *amoA* gene abundance

Correlation analysis between the *amoA* gene copies and chemical properties showed that the bacterial *amoA* gene copies in the manure fed soil were significant positively correlated with all the parameters tested, such as $\text{NH}_4^+\text{-N}$, $\text{NO}_x^-\text{-N}$, TN, and TOM (Table 2). However, the archaeal *amoA* gene copies only significantly correlated to the pH. The correlation between soil chemistry and *amoA*

Figure 2. Vertical distribution of archaeal, bacterial *amoA* genes copies and potential nitrification rates in the manure fed site (F) and the unfed soil (U). The samples deeper than 70 cm for bacterial *amoA* gene in manure fed site and samples deeper than 50 cm in unfed soil was excluded from the quantitative analysis because the yield of environmental DNA was too low to obtain reliable qPCR results.

gene suggested that manure fertilization highly impacted the population size of bacterial ammonia oxidizer rather than the archaeal counterparts. It was also noted that significant positively correlation were observed between the PNRs and the bacterial *amoA* gene copies, suggesting high activity in nitrification related to the bacterial ammonia oxidizers. PCA based on Bray–Curtis distance matrix was used to extract the most important information of the environmental parameters. PCA explained 99.8% variation in the environmental data set including five factors of pH, $\text{NH}_4^+\text{-N}$, $\text{NO}_x^-\text{-N}$, TN, and TOM. Within PCA, PC1 was composed of $\text{NH}_4^+\text{-N}$ and PC2 was composed of other parameters explained 96.1 and 3.8% of the variation in the environmental data set, respectively. The extracted PC1 significantly correlated to the abundance of AOB ($p < 0.001$) but no significant correlation was observed to the AOA ($p = 0.303$), suggesting $\text{NH}_4^+\text{-N}$ was the key factor impacted the abundance of AOB.

Abundance of *amoA* genes affected by short-term fertilization

The NH_4^+ was the main content of manure and other fertilizers and it was also identified to be the key factor impacting the population of ammonia oxidizers in this study. Therefore, to simulate the response of AOA and AOB population to the fertilization, 5 weeks incubation with the addition of ammonium was carried out using the soil samples in the 40–50 cm layer where the AOA started to be dominant over AOB observed in field study. Clear increases of the bacterial *amoA* gene copies were observed in both incubations (2.0×10^6 to $8.4 \times 10^6 \text{ g}^{-1} \text{ d.w.s.}$ in the manure fed soil and 1.6×10^4 to $4.8 \times 10^5 \text{ g}^{-1} \text{ d.w.s.}$ in the unfed soil) and the $\text{NH}_4^+\text{-N}$ decreased simultaneously during the incubation (0.16 to 0.03 mM and 0.16 to 0.09 mM). The archaeal *amoA* gene copies varied from 8.9×10^6 to $1.8 \times 10^7 \text{ g}^{-1} \text{ d.w.s.}$ in the manure fed soil and 2.1×10^6 to $2.3 \times 10^7 \text{ g}^{-1} \text{ d.w.s.}$ in the unfed soil. The archaeal *amoA* gene copies decreased

Table 2. Correlation^a between AOA and AOB^b *amoA* gene copy numbers and the soil properties.

Parameters	Correlations with the chemical properties					Correlations with the PNRs
	$\text{NH}_4^+\text{-N}$	$\text{NO}_x^-\text{-N}$	TN	TOM	pH	
Log number of AOA <i>amoA</i> gene copies	0.234	0.215	0.367	0.337	−0.568 ^c	0.318
Log number of AOB <i>amoA</i> gene copies	0.757 ^c	0.564 ^c	0.833 ^c	0.828 ^c	−0.687 ^c	0.803 ^c

^aPearson correlation coefficient, $n = 20$.

^bCopy numbers of samples below the detection limitation were assumed to be the detection limitation of qPCR assay (10^2 copies).

^cCorrelation is significant at the 0.01 level (2-tailed).

Figure 3. Variations of $\text{NH}_4^+\text{-N}$ and relevant copy numbers of archaeal and bacterial *amoA* gene in 5 weeks incubation. The quantification of archaeal and bacterial *amoA* gene copies were performed in the first, third, fifth weeks. The sample was introduced from the layers of 40–50 cm where the AOA was more dominant than AOB in the manure fed site (A). For the purpose of comparison, the sample of control site was also introduced from the layers of 40–50 cm (B).

slightly but no clear pattern as their bacterial counterparts was observed (Fig. 3).

Discussion

The distribution and relative contributions of AOA and AOB were highly impacted by the soil properties [11, 24, 25]. Although manure fertilizer was frequently used in agriculture, little was known about its influence to the population of ammonia oxidizers in soil. By investigating on a long-term manure fertilized soil, we tested the populations of AOA and AOB in soil responding to the manure fertilization.

The abundance of AOB was significantly higher ($p < 0.05$) in the long-term manure fertilized soil (8.8×10^5 to 3.2×10^8 g⁻¹ d.w.s.) than those in the non-fertilized soil (1.6×10^4 to 6.3×10^5 g⁻¹ d.w.s.) but no significant difference for AOA were observed. Compared to other natural ecosystems like the soil of the Mount Everest [26] (5.4×10^4 to 3.1×10^6 copies g⁻¹ d.w.s.), an red soil [10] (5.8×10^5 to 9.3×10^7 copies g⁻¹ d.w.s.) or an alkaline sandy loam [19] (1.2×10^5 to 2.8×10^6 copies g⁻¹ d.w.s.), the abundance of AOB in manure fertilized soil was much higher. The results showed us a clear stimulating effect of manure fertilization to the population of AOB rather than AOA, which was in agree with previous observation in soil [6, 8].

Although manure was often a mixture of different compounds and rich in organic N, the effect of manure fertilizer to the population of AOA and AOB seemed to be similar to that of inorganic N fertilizers [6, 8, 27]. The PCA based on Bray–Curtis distance matrix showed that the $\text{NH}_4^+\text{-N}$, explaining 96.1% of the variation in the environmental data set, was the most important chemical parameter determining the abundance of AOB. Therefore, in spite of the manure fertilizer was characteristic of organic N, the inorganic N in manure was still the key factor impacting the abundance of AOB, which was further confirmed by the correlation analysis between $\text{NH}_4^+\text{-N}$ and bacterial *amoA* gene copies ($r^2 = 0.757$, $p < 0.01$). Adversely, it was hard to find any connection between the manure fertilization and the abundance of AOA. It was reported that the high rates of inorganic ammonium supply did not stimulate *thau-marchaeal* ammonia oxidation in different soils [8, 28–30] or even inhibited it [29, 31] whereas the population of AOA was stimulated by organic N [32]. Therefore, the unclear varieties of the abundance of AOA could be a comprehensive effect of both long-term inorganic and organic N supplying in manure. This speculation was confirmed by the 5 weeks incubation experiment. With the sole ammonium substrate, a clear increase of the bacterial *amoA* copies was observed, which was coincidence with previous studies that the abundance of AOB increased after 49 [27] and 28 days [8] incubations.

The PNRs in manure fertilized soil was significant higher ($p < 0.05$) than those of non-fertilized soil, which was agree with previous study that PNRs were enhanced by manure treatment but suppressed by mineral fertilizers [33] in soils. The simultaneous increase of PNRs and bacterial *amoA* copies indicated an increasing importance of AOB in nitrification. It was observed that the PNRs highly correlated to the abundance of AOB rather than that of AOA, showing that the AOB would be more important under the condition of higher substrate availability [9]. The *thaumarchaea* was reported to be the key driver of ammonia oxidation, but most of these conclusions were drawn under acidic soil condition which was unfavourable to AOB [25, 34]. Our results and previous studies showed that in a pH-neutral soil, the AOB would play a more important role in ammonia oxidation, especially with the addition of fertilizers [6, 8, 27]. However, there was also study which showed that the growth of *thaumarchaeal* was increased by the root exudation in a paddy rhizosphere soil, which revealed a significant role of root exudation to the communities of AOA and AOB [35]. In this study, the soil without manure fertilization was not planted with rice. Therefore, the results need to be interpreted with caution. Future studies focusing on the influence of different cultivation mode and plant to the communities of ammonia oxidizers in soil would be in priority of this field.

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Conflict of interest

The authors declare that no financial/commercial conflicts of interest in this manuscript.

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