Activity, abundance and community structure of anammox bacteria along depth profiles in three different paddy soils

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Abstract

Anaerobic ammonium oxidation (anammox) is a globally important nitrogen-cycling process mediated by specialized microbes, and has been demonstrated to be ubiquitous in anoxic natural settings and bioreactors. However, our knowledge of its prevalence in different paddy soil types and along the depth profiles remains largely undocumented. Here, mesocosm incubations were constructed to investigate the activity and community compositions of anammox bacteria across different depth layers of three different paddy soils. ^{15}N tracer experiments showed that anammox rates were prevalent in almost all depth layers of an inceptisol (Binhai, BH) and an oxisol (Leizhou, LZ), but was absent in an ultisol (Taoyuan, TY). The functional gene *hzsB* of anammox bacteria was only detected in BH and LZ soil profiles with detected anammox activity. Anammox accounted for 0.4% to 12.2% of the total N_2 production, and anammox rates were 0.02 to 0.77 nmol N g^{-1} dry soil h^{-1} and significantly correlated with soil pH and electric conductivity. Anammox activity and abundance of *hzsB* genes were significantly lower in the surface soil layers (0-5 cm) than in the sub-surface soil layers (20-60 cm). *Candidatus* Brocadia, *Candidatus* Kuenenia and *Candidatus* Jettenia were detected by cloning and sequencing, with *Candidatus* Brocadia being dominant in all the three soils and *Candidatus* Jettenia being more frequently detected in the LZ soil. The operational taxonomic unit number and diversity indices of anammox bacterial 16S rRNA gene increased with soil depth. Our results demonstrate that anammox is more common and active in alkaline soils than in acidic soils, in deep paddy soil layers than in surface soil layers, and that anammox activities are likely to be regulated by soil chemical properties such as pH, salinity and redox potential.

Key words: anammox, *hzs* gene, rice paddy soil, soil profile, ^{15}N tracer, denitrification

1. Introduction

Nitrogen (N) loss from soil through nitrate leaching and gas emissions as N₂ and nitrous oxide (N₂O) is of global concern in agricultural ecosystems. Denitrification has been long considered the sole pathway for N₂ generation until the discovery of the anaerobic ammonium oxidation process (anammox, $\text{NH}_4^+ + \text{NO}_3^- \rightarrow \text{H}_2\text{O} + \text{N}_2$) mediated by anammox bacteria under anoxic conditions, which was firstly reported in a wastewater treatment plant (Mulder et al., 1995). Subsequent detections of anammox in various natural settings have significantly revised our traditional perceptions of the N cycling since anammox can also form N₂ gas as an end product aside from denitrification (Dalsgaard et al., 2003; Lam et al., 2009; Thamdrup and Dalsgaard, 2002). Anoxic environments are crucial for the occurrence of anammox due to the inhibition effect of oxygen (O₂) on anammox bacteria (Jetten et al., 2009), therefore most of previous studies focused on anoxic environments such as oceanic sediments and oxygen minimum zones (OMZs). It was found that in environments where anammox occurs, it can account for more than 50% of the N₂ production (Engström et al., 2005; Rich et al., 2008; Thamdrup and Dalsgaard, 2002). Prevalence of anammox in terrestrial ecosystem such as freshwater wetlands was later reported (He et al., 2012; Humbert et al., 2010; Zhu et al., 2011a). As anammox bacteria are responsible for N loss from farmlands which receive large amount of N fertilizers, studies that focus on anammox in agricultural soils are highly desirable (Long et al., 2013; Shen et al., 2013). Paddy soils are frequently flooded, making these habitats quite similar to freshwater wetlands. Thus, we suggest that anammox may occur and be one of the critical N transformation processes in paddy soils.

The recent progress in anammox activity was paralleled by the molecular studies on the 16S rRNA gene and hydrazine synthase gene *hzs* of anammox bacteria, which have unravelled the wide-spread distribution of anammox bacteria in various habitats. To date, phylogenetic analysis based on 16S rRNA genes suggested that anammox bacteria are affiliated within the phylum *Planctomycetes*, including five genera of *Candidatus* Anammoxoglobus, *Candidatus* Brocadia, *Candidatus* Jettenia,

Candidatus Kuenenia and *Candidatus* Scalindua (Schmid et al., 2005). Recently, functional *hzs* genes were identified as a specific molecular marker for quantifying and characterizing anammox bacteria (Harhangi et al., 2012), and the *hzsA* and *hzsB* genes have been found in a few wetlands, agricultural and marine ecosystems (Humbert et al., 2012; Lipsewers et al., 2014; Shen et al., 2013). Furthermore, anammox bacteria are considered to be habitat-specific with certain groups observed to be dominant in distinct environments (Humbert et al., 2010). For instance, *Candidatus* Scalindua was mainly discovered in marine ecosystems (Li et al., 2010; Penton et al., 2006; Rich et al., 2008), while *Candidatus* Brocadia and Kuenenia were more predominant in agricultural soils (Bai et al., 2015; Shen et al., 2013). However, a recent study reported the dominance of *Candidatus* Scalindua in paddy soils in Northeast China, which raised the question of a heterogeneous distribution of anammox bacteria in different soil types (Wang and Gu, 2013).

Studies on wastewater treatment reactors have recognized pH and salinity as important factors influencing activity of anammox bacteria (Jin et al., 2012). Neutral to alkaline conditions were found to be more suitable for the growth of anammox bacteria in bioreactors, but the anammox activity can be inhibited by large amounts of saline wastewater (Strous et al., 1999). The pH value was also identified as an essential driver shaping the anammox bacterial community and abundance in natural habitats like mangrove sediments (Han and Gu, 2013). Anammox bacteria were also detected in environments with a salinity gradient from freshwater to marine ecosystems (Wang and Gu, 2014). Several studies have shown the existence of anammox in paddy soils (Wang and Gu, 2013; Zhu et al., 2011b), and environmental factors such as pH and NH_4^+ were found to be significantly correlated with the community structure of anammox bacteria (Bai et al., 2015; Yang et al., 2015). However, most of these studies concentrated more on the molecular ecology characteristics of anammox bacteria, relatively little is known about the potential linkage between anammox activity and bacterial community and the determining environmental factors.

Concentrations of soil O₂ decrease with depth in the soil profile, and could significantly impact the dynamics of soil microbial communities (Noll et al., 2005). Variation in activity and community composition of anammox bacteria were observed along the vertical depth profiles in marine sediments (Bale et al., 2014; Lipsewiers et al., 2014). Similarly, O₂ and redox conditions in paddy soils gradually shift with soil depth profiles (Ratering and Schnell, 2000). The characteristics of the anammox process and anammox bacteria across vertical horizons in paddy soils and their potential correlations are also of great interest.

Paddy ecosystems are essential components of the agricultural systems, and occupy a large area in China with diverse parent material types of soil which represent various soil conditions and N cycle performance. Therefore, in this study, three types of paddy soil characterized with distinct pH, electric conductivity (EC), organic content and redox potential (*Eh*) were chosen to answer the following hypothesis: (1) Does anammox vary with the range of soil conditions that characterize paddy soils in China? (2) Does anammox activity increase with soil depth due to changes in soil O₂ conditions? (3) Does the performance of anammox correlate with the molecular ecology characteristics of anammox bacteria? The chosen soils included parent material types of inceptisol, oxisol and ultisol, which represented three typical paddy soil types in China. To address the above hypothesis, samples from 4 horizon layers (0-5 cm, 5-20 cm, 20-40 cm and 40-60 cm) of the flooded mesocosm columns of the three types of soil were subjected to anammox rates measurement using ¹⁵N isotope tracer and molecular analysis on the community structure and abundance of anammox bacteria.

2. Materials and Methods

2.1 Soil sampling and mesocosm incubation

The paddy soil samples used for mesocosm were originally collected from Binhai (BH, 119.84° E, 34.01° N), Taoyuan (TY, 111.48° E, 28.90° N) and Leizhou (LZ,

110.04° E, 20.54° N) in three rice production areas in Southeast China developed from three distinct parent material types, including inceptisol (BH), oxisol (LZ) and ultisol (TY), respectively. The mesocosms columns (50 cm in diameter and 70 cm in height) have been receiving similar water and rice plantation management as in the field since the establishment of mesocosm in greenhouse in 2012. For each soil type, two replicate columns were setup, each column was further vertically divided into two compartments, and one profile were sampled from each compartment during the waterlogged period and thus resulting in four replicates for each soil type.

In addition, two fresh paddy soils (BH-F and TY-F, 0-20 cm) were taken from the fields where the column soils (BH and TY) were originally collected, and one fresh black soil (HLJ-F, 0-20 cm) was collected from Heilongjiang in the summer of 2014. These fresh samples were used for the ¹⁵N isotope tracer experiment as well.

2.2 Physicochemical determinations and DNA extraction

Soil pH and EC were measured with a pH meter and a conductivity meter, respectively. NO₃⁻ and NH₄⁺ were extracted with 1 M KCl and determined by using a flow analyzer (AA3, SEAL analytical, Germany). *Eh* values of the soil samples in mesocosm columns were recorded immediately after sampling using a PRN-41 soil *Eh* meter (DKK, TOA, Tokyo, Japan). Concentrations of dissolved O₂ along the soil depth profiles were also measured with an O₂ microsensor electrode (Unisense, Denmark) precisely positioned by a micromanipulator (Unisense). The total genomic DNA was extracted from 0.25 g of soil using the Power Soil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) as per the manufacturer's instructions. All DNA samples were stored at -40°C before downstream analysis.

2.3 PCR, cloning libraries and sequencing of anammox bacterial 16S rRNA genes

A nested PCR approach was employed to examine the diversity and community structure of anammox bacteria by targeting the 16S rRNA gene. The initial PCR amplification was performed in a 25-μl reaction with primer-pair Pla46F/1037R

(Schmid et al., 2011). The 25- μ l PCR reaction mixture contained 12.5 μ l of 2 $\times$ TaKaRa Premix (TaKaRa Bio Inc., Shiga, Japan), 0.25 μ l of each primer (10 μ M), and 1 μ l of DNA template (1-20 ng). The thermo-cycling conditions were as follows: 96°C for 10 min, followed by 25 cycles of 60 s at 96°C, 1 min at 56°C, and 1 min at 72°C. Following the first step, PCR amplification was further performed with the primer set Amx368F/Amx820R (Schmid et al., 2000) with a thermal-cycling profile of 96°C for 10 min, followed by 35 cycles of 30 s at 96°C, 1 min at 58°C, and 1 min at 72°C. The 50 μ l reaction system contained 12.5 μ l of 2 $\times$ TaKaRa Premix, 1 μ l of each primer (10 μ M) and 2 μ l of templates from the initial amplification step.

The resultant PCR products were verified by running 1% agarose gels, and purified using Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). Equal amounts of the purified PCR products from the four replicates were pooled into a composite sample, and ligated into a pMD 18-T vector (TaKaRa Bio Inc., Shiga, Japan) at 4°C overnight according to the manufacturer's instructions. The ligation was then transformed into *E. coli* competent cells (JM109, TaKaRa Bio Inc., Shiga, Japan). Approximately 30 positive colonies were randomly selected and sequenced by an automatic sequencer ABI3730 (Applied Biosystems, USA).

2.4 Quantitative PCR of *hzsB* genes

Quantitative PCR (Q-PCR) was performed to target the functional gene *hzsB* of the anammox bacteria with the primer set *hzsB*_396F (5'-ARGGHTGGGGHAGYTGGAAG-3') and *hzsB*_742R (5'-GTYCCHACRTCATGVTCTG-3') (Wang et al., 2012b). The Q-PCR reactions were conducted in 25- μ l volume containing 12.5 μ l of SYBR Green Premix (TaKaRa Bio Inc.), 0.6 μ l of each primer (10 μ M), and 1 μ l of DNA template (1-20 ng). Non-template controls were also included for each Q-PCR run. A touchdown PCR protocol was carried out with an initial denaturation at 95°C for 5 min, followed by 5 cycles of 95°C for 1 min, 64°C (decreased by 1°C each cycle) for 1 min, and 72°C for 45 s, then continued with 35 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 45 s

and a final 72°C for 5 min. To obtain the standards for Q-PCR, *hzsB* genes amplified with the same primer pair as described above were cloned into a pMD 18-T vector (TaKaRa Bio Inc.), and positive clones were sequenced and the plasmids carrying correct inserts of the *hzsB* gene were extracted and purified. Copy numbers of *hzsB* gene were calculated based on concentrations of the plasmids. Ten-fold serial dilutions of the plasmid were then utilized as standards for Q-PCR reactions.

2.5 Slurry incubation experiments with $^{15}\text{NO}_3^-$ substrates

To measure the rates of anammox and denitrification in paddy soils, slurry incubation experiments with $^{15}\text{NO}_3^-$ were carried out using the protocol as previously described (Long et al., 2012). Briefly, 2 g of soil was added into 12-mL Exetainer tubes (Labco, High Wycombe, UK) and saturated with sterilized distilled water, and the tubes were vacuumed for 1 min and flushed with ultra-high pure N_2 for 2 min to create anoxic conditions. Tubes added with only distilled water were established as controls. The samples were pre-incubated in darkness for 24 h and the concentration of remaining NO_3^- was measured using a flow analyzer. After the initial incubation, the tubes were further flushed with N_2 for 1 min, and $\text{Na}^{15}\text{NO}_3$ (^{15}N at 98%) with a final concentration of 100 μM was injected into each tube and incubated for 48 h. At each time point, 200 μl of 7 M ZnCl_2 was added to eliminate microbial activity.

2.6 Gas isotope analysis and rate calculation

The tubes were shaken gently to equilibrate the gas between dissolved and gaseous phases before gas sampling. Gas was sampled from the headspace and measured for the ^{15}N abundance using a new gas chromatography isotope ratio mass spectrometer (GC-IRMS) approach (Yang et al., 2014). The ratios of $^{29}\text{N}_2/^{28}\text{N}_2$ (R_{29}) and $^{30}\text{N}_2/^{28}\text{N}_2$ (R_{30}) of sampled N_2 were obtained from the IRMS report. The molar fractions of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ in the sampled N_2 were calculated using equations (1) and (2):

$$f^{29} = \frac{{}^{29}\text{N}_2}{{}^{28}\text{N}_2 + {}^{29}\text{N}_2 + {}^{30}\text{N}_2} = \frac{\frac{{}^{29}\text{N}_2}{{}^{28}\text{N}_2}}{1 + \frac{{}^{29}\text{N}_2}{{}^{28}\text{N}_2} + \frac{{}^{30}\text{N}_2}{{}^{28}\text{N}_2}} = \frac{R_{29}}{1 + R_{29} + R_{30}} \quad (1)$$

224

$$f^{30} = \frac{{}^{30}\text{N}_2}{{}^{28}\text{N}_2 + {}^{29}\text{N}_2 + {}^{30}\text{N}_2} = \frac{\frac{{}^{30}\text{N}_2}{{}^{28}\text{N}_2}}{1 + \frac{{}^{29}\text{N}_2}{{}^{28}\text{N}_2} + \frac{{}^{30}\text{N}_2}{{}^{28}\text{N}_2}} = \frac{R_{30}}{1 + R_{29} + R_{30}} \quad (2)$$

226

227 Based on the total N concentration in the headspace of the tubes during the incubation,
 228 the mass of headspace N₂ (m_{total}) was calculated using the equation (3) (Yang et al.,
 229 2014):

230

$$m_{\text{total}} = \text{Density of N}_2 \times \text{volume of headspace} \quad (3)$$

232

233 The production rates of ²⁹N₂ (p₂₉) and ³⁰N₂ (p₃₀) in the tubes could be calculated based
 234 on the molecular fractions of f^{29} and f^{30} using equations (4) and (5):

235

$$p_{29} = (m_{\text{total}} \times f^{29}) / (t \times m_{\text{soil}}) \quad (4)$$

237

$$p_{30} = (m_{\text{total}} \times f^{30}) / (t \times m_{\text{soil}}) \quad (5)$$

239

240 Where t represents the incubation time and m_{soil} represents the dry soil mass in tubes.

241 The calculation for the N₂ production from anammox (A_{total}) and denitrification (D_{total})
 242 was based on the method developed by Thamdrup and Dalsgaard (2002). This
 243 approach assumes that ²⁹N₂ (P₂₉) was derived from both anammox (A₂₉) and
 244 denitrification (D₂₉), and the production of ³⁰N₂ (P₃₀) was solely from denitrification
 245 (D₃₀).

246

247 2.7 Data analysis

248 SPSS was used for the Spearman's correlation and one-way analysis of variance
 249 (ANOVA) test. The calculation of operational taxonomic units (OTUs) at the 97%

identity and diversity indices of anammox bacteria 16S rRNA genes was carried out using QIIME software (Kuczynski et al., 2011). The phylogenetic tree was constructed by using the neighbor-joining method in MEGA 5 software with 1000 bootstraps (Kumar et al., 2008).

3. Results

3.1 Soil properties and redox potential

All the soil properties characterized in this study are given in Table 1. Soil pH ranged from 8.00-8.64 and 6.82-7.23 in the BH soil and LZ soil, respectively, to be slightly acidic around 5.90-6.01 in the TY soil. EC in the BH and LZ soils was an order of magnitude higher than that in TY soil, indicating a higher level of salinity in BH and LZ soils. Among soil profiles, BH soils had the lowest Eh , ranging from 134.0 to 234.0 mV, and TY soils had the highest Eh , ranging from 410.5 to 484.6 mV, giving their reductive and oxidative conditions, respectively. Dissolved O_2 concentration decreased with soil depth and reached zero at around 4 mm in all the soil types (Fig. 1).

3.2 Rates of anammox and denitrification

The stable isotope tracer experiment with $^{15}NO_3$ showed that $^{29}N_2$ accumulation in tubes was below the detection limit in all layers from the TY column profiles (Fig. 2), suggesting that all generated N_2 came from denitrification. Similarly, anammox-derived N_2 was not detected in the fresh field samples from the TY site (TY-F) (Data not shown). Anammox activity was detected in all soil layers of BH and LZ columns with the exception of the LZ surface soil, with the rates ranging between 0.02-0.77 $nmol\ N\ g^{-1}\ dry\ soil\ h^{-1}$ (Fig. 2). In the BH column soils, anammox activity peaked in the 5-20 cm layer with a rate of 0.77 $nmol\ N\ g^{-1}\ dry\ soil\ h^{-1}$, which was significantly higher in the deeper layer compared with the surface layer ($P < 0.05$). Anammox rates in LZ increased steadily within the range of 0.04-0.72 $nmol\ N\ g^{-1}\ dry\ soil\ h^{-1}$ along the depth gradient. The rates of anammox in fresh samples of BH-F and HLJ-F were 1.93 and 0.49 $nmol\ N\ g^{-1}\ dry\ soil\ h^{-1}$, respectively.

Denitrification was detected in all layers from the three soil types. Potential denitrification rates remained largely stable within the range of 5.33-7.56 nmol N g⁻¹ dry soil h⁻¹ (Fig. 2). No significant changes in denitrification rates could be observed with soil depth within the BH and TY soils, while the denitrification rates of the LZ soil were significantly lower in the 0-5 and 5-20 cm layers than in the bottom layer ($P < 0.05$).

Generally, anammox contributed 0.4-12% to N₂ production in the column samples (Table 1). Anammox activity was also detected in the fresh samples BH-F and HLJ-F, and accounted for 23% and 1.3% of N₂ formation, respectively (Table 1). Anammox rates were significantly correlated with soil pH ($P < 0.01$, $r = 0.336$, $n = 12$) and EC ($P < 0.05$, $r = 0.3$, $n = 12$).

3.3 Diversity, structure and abundance of anammox bacterial community

In total, 360 sequences were obtained from 12 clone libraries (30 sequences in each library), which could be classified into 77 OTUs at the 97% cutoff using QIIME software. BLAST searching in GenBank and phylogenetic analysis of the retrieved sequences in this study suggested that 22 OTUs (representing 273 sequences) were affiliated into the known anammox clusters (Fig. 3). The remaining 55 OTUs (representing 87 sequences) were grouped into non-anammox *Planctomycetes*, *Acidobacteria* and unclassified bacteria (Fig. S1).

Among the 22 taxonomy-known OTUs (Table 2), nine OTUs (representing 56% of the anammox bacterial sequences) were affiliated within the *Candidatus* Brocadia cluster and were frequently detected in the majority of the BH and LZ layers and in the 20-40 cm layer of the TY soil. Four OTUs representing 8.8% of the anammox bacterial sequences were classified into the *Candidatus* Kuenenia cluster, and three OTUs representing 6.2% of the anammox sequences shared 96% sequence identity to the *Candidatus* Kuenenia clones in GenBank (Fig.3). Six OTUs (79 sequences)

affiliated within the *Jettenia* cluster were detected in the deep layers of the BH and LZ profiles, and one OTU (OTU15) occupied 86% of the *Jettenia*-affiliated sequences.

All the retrieved sequences from BH and LZ soils fell into the known anammox bacteria clusters. The anammox bacteria communities in the BH column samples were mainly dominated by *Candidatus* Brocadia and Kuenenia with the relative abundance in individual layers in the range of 23-100% and 0-63%, respectively. The *Jettenia*-affiliated clones were only found in the bottom layer with a relative abundance of 3% in the BH samples. Conversely, *Candidatus* Kuenenia was absent in the LZ column samples while *Candidatus* Brocadia and *Jettenia* were abundant, with their relative abundance in individual layers within the range of 37-100% and 0-63%, respectively (Fig. 4). The lowest diversity of anammox bacteria was observed in the surface layers of both BH and LZ columns in which only one OTU was detected, while 8-14 and 3-5 OTUs were detected in the 5-60 cm layers of BH and LZ, respectively (Table 2).

Most sequences from the TY columns were grouped into non-anammox bacteria clusters, except for one OTU (representing 30 sequences) from the 20-40 cm layer and three OTUs (representing three sequences) from the bottom layer that were affiliated into *Candidatus* Brocadia and Kuenenia, respectively. The non-anammox bacteria in the TY columns consisted of 55 OTUs, in which 11 OTUs were affiliated into *Planctomycetes*, seven into *Acidobacteria*, and 37 into unclassified bacteria (Fig. S1).

Q-PCR assays showed that the *hzsB* genes were detected in all layers of the BH and LZ profiles, with their abundances varying between 8.4×10^5 and 2.7×10^6 copies g^{-1} soil in BH and between 2.7×10^3 and 2.6×10^4 copies g^{-1} soil in LZ (Table 2), but were not detected in all TY samples. Along the depth profiles, the *hzsB* gene copies were highest in the 20-40 cm layer and lowest in the 0-5 cm layer of BH, but the highest and lowest *hzsB* gene copies were recorded in the 40-60 cm and 0-5 cm layers for LZ,

respectively. The abundance of *hzsB* genes was significantly correlated with anammox rates ($P < 0.01$, $r = 0.352$, $n = 12$), and negatively correlated with soil *Eh* ($P < 0.05$, $r = 0.352$, $n = 12$).

4. Discussion

4.1 Anammox and denitrification activity in the three paddy soils

Numerous studies have reported the occurrence of anammox in various anoxic environments such as marine OMZs (Stewart et al., 2012), aquatic sediments (Li et al., 2010) and wetland marshes (Zhu et al., 2011a). In the present study, anammox contributed 0.4-12.2% of N_2 production (Table 1), consistent with a recent report that anammox contributed 0.6-15% of N_2 production across 12 surface paddy soils from southern China (Yang et al., 2015). Despite these findings suggesting the functional importance of anammox, the contributions to N_2 production from denitrification were approximately 10-150 times of that from anammox in the BH and LZ columns, and denitrification was responsible for all N_2 emission in the TY column and field-fresh soil. Similar trends are also observed in other paddy soils (Yang et al., 2015; Zhu et al., 2011b), freshwater sediments (Wang et al., 2012a; Zhu et al., 2011a; Zhu et al., 2013) and mangrove sediments (Fernandes et al., 2012), suggesting a more dominant contribution of denitrification than anammox to N_2 production in terrestrial ecosystems. In contrast, the contribution of anammox to N_2 production was reported to exceed denitrification in marine ecosystems (Engström et al., 2005; Thamdrup and Dalsgaard, 2002). Such a phenomenon could be attributed to the relatively higher organic matter content in paddy soils than in seawaters. Compared to autotrophic anammox bacteria, heterotrophic denitrification bacteria are apparently more active and responsive to organic-rich environments such as flooded soils and sediments (Dalsgaard et al., 2005). Furthermore, previous studies have found that a relatively low C/N ratio is favored to denitrification while the opposite is true for anammox (Kraft et al., 2014; Schmidt et al., 2011; Tiedje et al., 1982). Due to the frequent N fertilizer application in agriculture, the paddy soils system received much more N input than marine system and resulted in a low C/N value, which might explain the

reason of denitrification over anammox in paddy soils.

4.2 Detection of anammox bacteria in paddy soils

Anammox bacteria are generally considered as habitat-specific microbes. For instance, the genus *Candidatus Scalindua* was frequently discovered in marine ecosystems (Dang et al., 2013; Humbert et al., 2010; Lam et al., 2009), while *Candidatus Brocadia* and *Kuenenia* were mainly detected in soil and freshwater ecosystems (Sonthiphand et al., 2014). Therefore, it is not surprising that *Candidatus Scalindua* was absent but *Candidatus Brocadia* and *Kuenenia* were predominant in paddy soils in this study. Recent investigations have also demonstrated the wide prevalence of these two genera in paddy soils (Bai et al., 2015) and dry agricultural soils (Shen et al., 2013) across broad areas of China. Observations from laboratory-scale experiments indicated that *Candidatus Brocadia* had relatively higher tolerance to O₂ and a higher growth rate (Oshiki et al., 2011). A metagenomic study also showed that the versatile metabolism system of *Candidatus Brocadia* (Gori et al., 2011). All these facilitate their adaption to various environments. *Candidatus Kuenenia* have a higher affinity for ammonia and nitrite (van der Star et al., 2008), thus making this genus more abundant and active in the habitats with high substrates input such as paddy soils. Furthermore, a recent study on the biogeography of anammox bacteria based on data from both natural and engineered ecosystems indicated that *Candidatus Brocadia* and *Kuenenia* are the closest co-occurring groups among the five genera, which implies that these genera would share similar habitats (Sonthiphand et al., 2014). Comparing to other four genera, *Candidatus Scalindua* was observed to be favorable to high salinity environments such as oceans and marine sediments (Dale et al., 2009), which partially explained its absence in the tested soils in this study. Contrary to our observation, another study reported that *Candidatus Scalindua* was the most abundant and diverse group in paddy soils in Northeast China (Wang and Gu, 2013). These studies together suggested the possible variation of anammox bacterial distribution in an even larger scale.

In this study, six *Jettenia*-affiliated OTUs (79 sequences, representing 29% of the anammox sequences) were detected (Fig. 3; Fig. 4). Notably, the LZ soil harbored 94% of the detected *Candidatus Jettenia* sequences, and *Jettenia*-affiliated sequences accounted for 54% of anammox sequences detected in the LZ profiles (Fig. 4). In many previous studies, the detection frequency of *Candidatus Jettenia* was much lower in comparison to *Candidatus Brocadia* and *Kuenenia*, and was even undetectable in some paddy soils (Bai et al., 2015; Yang et al., 2015). However, it was also shown that *Candidatus Jettenia* occupied a large portion of anammox bacterial groups in a farmland field (Long et al., 2013). Similarly, there was variation in detection of *Candidatus Jettenia* in two recent studies in which *Candidatus Jettenia* was observed in river water columns but was absent in another study in a freshwater wetland (Lee et al., 2014; Sun et al., 2014). Unlike the other four genera which were largely observed in natural habitats, the *Candidatus Jettenia* group was typically detected in engineered environments such as bioreactors and wastewater treatment plants (Sonthiphand et al., 2014). Such pattern implied that this genus might favor conditions with high level of substrates load. In the present study, LZ soils have the highest nitrate and ammonium concentrations across the three paddy soils, which subsequently provided an ideal habitat for *Candidatus Jettenia*. Likewise, *Candidatus Jettenia* was also recovered from a manure pond soil with relatively high nitrogen input in a previous study (Sher et al., 2012). All these results suggested that diverse soil types or environmental conditions may be inhabited by diverse anammox bacterial groups, and a broader scale of investigation is highly desirable to understand the distribution of anammox bacteria and the anammox process in terrestrial ecosystems.

4.3 Linkage between anammox activity, bacterial abundance and soil properties

In the present study, the examined paddy soils represented three parent material types with alkaline (BH), neutral (LZ) and acidic (TY) conditions respectively. The much lower diversity and detection frequency of anammox bacteria in TY soils than in the BH and LZ soils, and the absence of anammox performance and *hzsB* gene in all

depth layers of TY columns, consistently revealed the scarcity of anammox bacteria and activity in this acidic soil (Fig. 2, Fig. 4; Table 2). Previous studies have reported that soil pH was a key factor influencing the activity of anammox bacteria and shaping the anammox bacterial community structure (Bai et al., 2015; He et al., 2012; Yang et al., 2015). Studies on bioreactors have also suggested that the physiological pH for anammox bacteria was within the range of 6.5-9.0 and the optimum pH was around 8 (Egli et al., 2001; Jin et al., 2012; Strous et al., 1999). In this study, the BH (pH 8.00-8.64) and LZ (pH 6.78-7.23) soils performed much higher anammox activity than the TY (pH 5.97-6.01) soil, which further indicated that soil pH was crucial in determining anammox activity and that anammox bacteria are more adapted to neutral and relatively alkaline conditions than relatively acidic conditions. The preference of alkaline soils by anammox bacteria could be explained by the characteristic of their enzymes. For instance, the hydrazine oxidizing enzyme (HZO), which catalyzes the final step of anammox process, was reported to be stable between pH 6.0 and 9.0, and was more active between pH 7.5 and 8.5 (Shimamura et al., 2007). In addition to soil pH, our results also indicated that soil EC, a parameter reflecting soil salinity, had a positive relationship with anammox rates and the functional gene abundance. A similar observation was obtained in mangrove sediment incubation in which higher salinity treatments markedly promoted the growth and diversity of anammox bacteria (Wang and Gu, 2014). These observations imply that soil salinity is also an essential factor affecting the activity of anammox bacteria.

Redox potential is reported as a controlling parameter of anammox progress in bioreactors due to its role in regulating aerobic and anaerobic phases (Lackner and Horn, 2012; Lackner et al., 2012). In the present study, *Eh* was significantly correlated with the rates of N₂ production in denitrification ($P < 0.05$), and the *hzsB* gene copy numbers and soil *Eh* were negatively correlated, implying a possible inhibition effect of less negative *Eh* on anammox bacteria. This could be explained by the limitation of Fe²⁺ under less negative *Eh* conditions. Since the ions greatly affect the biosynthesis of Haem-C, an important electron carrier for energy metabolism of anammox bacteria,

and a low supply in Fe^{2+} substrate could reduce the growth of anammox bacterial cells (Huang et al., 2014).

Anammox activity in surface layers of the BH and LZ soils was lowest or undetectable even though the 16S rRNA and *hzsB* genes were detected by cloning-sequencing and Q-PCR in these surface layers (Fig. 2; Table 2). These results indicated that the surface paddy soils may provide a habitat for anammox bacteria but do not necessarily support the anammox activity, and deeper soil layers may be more suitable for them to survive and function. Coincidentally, our previous investigation on the molecular ecology of anammox bacteria in a large-scale paddy field also showed that anammox bacteria were absent in most of the surface paddy soils (Bai et al., 2015). Growth and metabolism of anammox bacterial cells can be greatly inhibited by O_2 (Jetten et al., 2009). Thus the low detection frequency of anammox bacteria in surface soil could be partially attributed to the relatively high O_2 concentration in the 0-5 cm soil layer due to its pervasion into the air-water interface. In the present study, dissolved O_2 was detected at approximately 0-4 mm depths of the surface soils, but was absent in deeper layers in all columns (Fig. 1), which corroborated this. Some previous studies also observed that O_2 concentrations gradually decreased to zero at a depth around 3 mm in water-logged paddy soil profile (Lüdemann et al., 2000; Ratering and Schnell, 2000). These results indicated that the anammox process in the surface paddy soils was weak due to the inhibition of dissolved O_2 , which distinctly differed from the observation that the hotspot of anammox reaction occurred in the water-sediment interface in the Baiyangdian Lake reported by Zhu et al. (2013). A possible reason is that the concentration of organic matter in the surface of lake sediment is higher than in surface paddy soils, and the degradation of organic matter resulted in the lack of oxygen.

5. Conclusions

In the present study, stable isotope tracer technique in combination with molecular approach provided a strong line evidence that anammox process is more active in

alkaline and neutral soil than in acidic soil, in the depth layers than in the surface layers, but denitrification contributed to N₂ production predominantly over anammox under all cases. Our study further indicated that the differentiated performance of anammox bacteria in different soil types was correlated with soil properties such as pH and salinity which are largely determined by parent materials of soils. Moreover, the concentration of dissolved O₂ along the soil profile depths is another key limiting factor to anammox activity. Our findings suggest that anammox is more important in alkaline saline soils than that in acidic soils, while anammox contributes little to total N₂ production in paddy soils in comparison with denitrification process. These results shed light on the understanding of anammox process in terrestrial ecosystems.

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Table captions and Figure captions

Table 1 Soil properties and contribution of anammox to N₂ production in along three soil profiles.

Table 2 The detected OTUs and diversity indice of anammox bacteria based on the clone library analysis of 16S rRNA genes by 3% cutoff, and the copy numbers of hzsB genes along three paddy soil profiles.

Fig.1. Depth profiles of oxygen concentrations measured with an O₂ microsensor electrode equipped with a micromanipulator.

Fig.2. Rates of anammox (left) and denitrification (right) in three paddy soil profiles. No anammox was detected in TY soils.

Fig.3. Phylogenetic tree of anammox bacteria 16S rRNA gene sequences retrieved with primer sets Pla46F/1037R and Amx368F/Amx820R from the three soil columns. The obtained sequences and their related sequences and standard species from GenBank were combined with neighbor-joining method. The OTUs identified in this study were labeled with black inverted triangle. The number after the OTUs denotes the number of sequences represented the OTU against the total number of sequences obtained in this study. The sequences obtained in this study are available in GenBank under accession numbers KR088215 to KR088262.

Fig.4. Relative abundance of anammox bacteria along three paddy soil profiles horizons 0-5 cm, 5-20 cm, 20-40 cm and 40-60 cm detected by primer sets Pla46F/1037R and Amx368F/Amx820R.

678 **Table 1 Soil properties and contribution of anammox to N₂ production in three soil profiles.**

Soil	Profile layer	NO ₃ ⁻ -N (mg kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)	pH	EC (dS M ⁻¹)	Eh (mV)	Contribution of
							anammox to N ₂ formation
BH ^a	0 - 5 cm	25.3	25.0	8.00	0.24	134.0	0.4%
	5 - 20 cm	34.3	23.9	8.25	0.36	163.0	11.3%
	20 - 40 cm	23.5	22.9	8.50	0.28	199.2	7.5%
	40 - 60 cm	25.0	23.3	8.64	0.24	234.0	6.6%
LZ ^a	0 - 5 cm	130.0	33.2	7.23	0.19	411.0	Not detected
	5 - 20 cm	60.0	29.6	6.82	0.26	431.9	0.5%
	20 - 40 cm	63.1	29.8	6.78	0.21	399.1	5.2%
	40 - 60 cm	51.1	33.9	6.88	0.21	383.6	12.2%
TY ^a	0 - 5 cm	28.2	25.6	5.97	0.030	484.6	Not detected
	5 - 20 cm	26.3	24.8	5.90	0.017	410.5	Not detected
	20 - 40 cm	25.7	23.9	6.01	0.016	422.6	Not detected
	40 - 60 cm	23.6	24.6	5.97	0.020	443.3	Not detected
BH-F ^b	0 - 20 cm	122.2	12.4	8.10	0.35	-	23.1%
HLJ-F ^b	0 - 20 cm	56.5	13.3	6.82	0.24	-	1.3%
TY-F ^b	0 - 20 cm	12.8	55.3	5.90	0.017	-	Not detected

700 ^a Soils sampled from flooded mesocosm columns (50 cm in diameter and 70 cm in height) which originally collected from Binhai (BH, 119.84° E, 34.01° N),
701 Taoyuan (TY, 111.48° E, 28.90° N) and Leizhou (LZ, 110.04° E, 20.54° N) in southeastern China.

702 ^b Soils freshly collected from the fields where BH and TY column soils originally located and one black soil (HLJ-F, 0-20 cm) collected from Heilongjiang in rice
703 growth season in 2014.

704

705 **Table 2 The detected OTUs and diversity indices of anammox bacteria based on the clone library analysis of 16S**
706 **rRNA genes by 3% cutoff, and the copy numbers of *hzsB* genes along the three paddy soil profiles.**

Soil column ^a	Soil layer	anammox OTUs	Non-anammox OTUs	Shannon index	Simpson index	Chao1	<i>hzsB</i> genes (copy g ⁻¹ dry soil)
BH	0 - 5 cm	1	0	0	0	1	1.85E+05 (±9.10E+04)
	5 - 20 cm	8	0	2.12	0.66	10	1.87E+06 (±1.20E+06)
	20 -40 cm	14	0	3.57	0.90	27	2.70E+06 (±7.73E+05)
	40 -60 cm	11	0	3.18	0.86	19.5	2.06E+06 (±6.88E+05)
LZ	0 - 5 cm	1	0	0	0	1	2.70E+03 (±1.10E+03)
	5 - 20 cm	5	0	1.80	0.64	5	6.35E+03 (±5.58E+03)
	20 -40 cm	3	0	1.56	0.65	3	9.57E+03 (±8.74E+03)
	40 -60 cm	4	0	1.47	0.54	4	2.58E+04 (±2.53E+04)
TY	0 - 5 cm	0	21	-	-	-	N/A
	5 - 20 cm	0	20	-	-	-	N/A
	20 -40 cm	1	0	0	0	1	N/A
	40 -60 cm	3	24	-	-	-	N/A

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