The phenological stage of rice growth determines anaerobic ammonium oxidation activity in rhizosphere soil

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ABSTRACT

Anaerobic oxidation of ammonium (anammox) plays an important role in nitrogen (N) loss from agricultural systems. Recently, the rice rhizosphere was demonstrated to be a hotspot for anammox, yet the dynamics of anammox activity and the distribution of anammox bacteria in rhizosphere soil at different phenological stages of rice growth are still unknown. In this study, the activity, diversity and abundance of anammox bacteria in both rhizosphere and bulk soils were investigated over the entire rice growth season. From tillering to ripening stage, significantly higher anammox bacterial abundance was detected in rhizosphere soils compared to bulk soils. The rhizosphere soils also had significantly higher anammox rates at tillering and booting stages (0.71 and 0.32 nmol N g⁻¹ dry soil h⁻¹, respectively) compared to bulk soils. The anammox rate in rhizosphere soil was positively correlated to the concentrations of NO₃⁻ (total of nitrate and nitrite) and acetate. The abundance of anammox bacteria was significantly correlated with the concentration of succinate in rhizosphere soils. A total of five anammox genera of *Brocadia*, *Kuenenia*, *Anammoxoglobus*, *Jettenia* and *Scalindua* were detected, with *Brocadia* predominating in all examined samples. The distribution of anammox bacteria in rhizosphere and bulk soils varied with phenological stages. Statistical analysis indicated that C/N ratio, formate, citrate and ammonium were key factors influencing the composition of anammox bacteria. Variations in activity, abundance and distribution of anammox bacteria in rhizosphere were observed over the phenological progression, demonstrating that the root exudates might be influential for the anammox process. This study implies that future efforts in estimating the rate of anammox should consider the temporal variation during plant life cycles.

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1. Introduction

Anammox, the oxidation of ammonium coupled to the reduction of nitrite with N₂ as the end-product under anaerobic conditions, greatly contributes to the loss of fixed nitrogen to the atmosphere in terrestrial ecosystems (Humbert et al., 2010; Shen et al., 2013; Zhu et al., 2011a). Agricultural fields with intensive

application of mineral fertilizers have been reported as ideal habitats for anammox bacteria (Shen et al., 2013; Yang et al., 2015). Broad distribution of diverse anammox bacteria has been detected in agricultural soils (Zhu et al., 2011a; Shen et al., 2013; Wang and Gu, 2013), and a considerable proportion of N₂ production in paddy soils in Southern China could be attributed to the anammox process based on incubation studies (Yang et al., 2015).

Rhizosphere soils are important in N cycling, which has been largely overlooked in the past. Our previous work demonstrated the presence of anammox bacteria in rhizosphere soils, contributing higher N₂ production in comparison to bulk soils at the tillering stage of rice growth (Nie et al., 2015). However, the dynamics of the activity and diversity of anammox bacteria in

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rhizosphere soils over the entire growth period of rice remain unknown. Root exudates have been suggested to play a critical role in shaping the microbial community in soils (Lugtenberg et al., 2002; Hartmann et al., 2008; Shi et al., 2013). Also shifts in diversity and abundance of microorganisms (e.g. methane oxidizing bacteria) during rice growth have been observed (Eller and Frenzel, 2001; Dianou et al., 2012), which could be partially attributed to the shift in the quantity and quality of root exudates during the growth of rice (Badri and Vivanco, 2009; Chaparro et al., 2013).

We hypothesize that (i) the diversity and activity of anammox bacteria are different between the rhizosphere and non-rhizosphere zones in a fertilized paddy soil, and shift over the growth period of rice plants; and (ii) the quantity and composition of root exudates, especially the low molecular weight organic acids (LMWOAs), influence the rate and distribution of the anammox during various phenological stages. To address these hypotheses, we set up a pot experiment where rhizosphere and bulk soils were collected at different phenological stages of rice growth. The activity, abundance and composition of anammox bacteria were investigated using a ^{15}N -tracing experiment, qPCR and clone library analysis. The secretion activity of the rice root, and the composition of root exudates and soil chemical properties were measured to determine the factors influencing the anammox process in rhizosphere and bulk soils.

2. Materials and methods

2.1. Soil and pot experiment

The paddy soil (quaternary red clay, 0–20 cm) was collected from Taoyuan (28°55'15" N, 111°27'35" E), Hunan province of China on November 22, 2010. The characteristics of soil were listed as follows: pH (1: 2.5 soil/MilliQ water), 5.17; soil organic carbon (SOC), 47.7 g kg⁻¹; total nitrogen (TN), 2.68 g kg⁻¹; available phosphorus (AP), 5.36 mg kg⁻¹ and available potassium (AK), 56.8 mg kg⁻¹. The soil sample was air-dried and sieved through a 2 mm mesh before pot experiment. Basic fertilizers (urea-N, 250 mg kg⁻¹; P as calcium superphosphate, 60 mg kg⁻¹; K₂O as KCl, 100 mg kg⁻¹) were well mixed with soil at the beginning of the experiment. No more fertilizers were applied during the growth periods. Each pot and rice seeds (cv. Xiangzaoshan 45) were pre-incubated as previously described (Nie et al., 2015). Rhizo-bag, allowing smaller molecular substrates to penetrate but prohibiting penetration by roots, was used to separate rhizosphere and bulk soils (Nie et al., 2015). The rice plants were incubated in greenhouse with natural illumination and humidity over the growth period in flooded condition with the temperature at 30 ± 2 °C during the day and 25 ± 2 °C in the night. The rhizosphere soils, bulk soils, rhizosphere pore water, bulk pore water and plants were sampled at each phenological stage (seedling, 12 d; tillering, 37 d; booting, 62 d; flowering, 82 d and ripening stage, 122 d) of rice growth. The pore water of rhizosphere and bulk soils were collected using one porous plastic soil moisture sampler (Rhizon SMS, Rhizosphere Research Products, Wageningen, Netherlands). The soils were divided into two parts. One part was used for analysis of anammox activity and chemical properties such as pH, moisture, NH_4^+ and NO_3^- , the other part was freeze-dried for genomic DNA extraction, SOC, TC and TN measurement. Analysis was carried out on soil samples from three separate pots at the same phenological stage.

2.2. Chemical analytical procedures

Soil pH was determined at a soil/MilliQ water ratio of 1:2.5 with a pH analyzer. The NH_4^+ and NO_3^- of soils were extracted with 2 M KCl at a soil/KCl ratio of 1:10 and filtered through a 0.22 μm

hydrophilic PEPT needle filter (ANPEL, Shanghai, China). Subsequently, the filtrates were analyzed by a flow injection analyzer (FIA QC8500, Lachat, America). For the determination of TC, TN and SOC, the soils were sieved through a 0.15 mm mesh. The TC, TN and SOC were analyzed using a C/N analyzer (Vario MAX C/N, Germany) and a total carbon analyzer (TOC-V CPH, SHIMADZU, Japan), respectively. AP and AK were determined as previously described (Bao, 2000a, 2000b). Moisture of soil was assessed gravimetrically by drying the soil at 105 °C for 16 h. All samples were analyzed in triplicate.

2.3. Measurement of organic acids

The rice roots were sampled for LMWOA determination in the pot experiment. The soils around the rice roots were gently washed off with tap water, and the roots were washed finally using MilliQ water. As every care was taken, this procedure facilitated the removal of soil without injuring the roots. A 500 mL brown glass bottle with 300 mL MilliQ H₂O was used to collect root exudates from individual plant. After about 6 h extraction (Lu et al., 2007), the liquid was collected and filtered through a 0.22 μm hydrophilic PEPT needle filter (ANPEL, Shanghai, China). The collected filtrates and the pore water of rhizosphere and bulk soils sampled above were used for analysis of LMWOAs by ion chromatograph (ICS-3000; Dionex).

2.4. Anammox and denitrification rate measuring

The rates of anammox and denitrification in rhizosphere and bulk soils were measured by ^{15}N stable isotope tracing technique as previously described (Yang et al., 2015) with some modifications. About ~3.5 g soil was transferred into a 12 ml gastight vial (Exetainer, Labco, High Wycombe, Buckinghamshire, UK), which were then filled totally with N₂-purged overlaying water (Wang et al., 2012a) and sealed with a gastight lid. The resulting slurries were pre-incubated for 1 day to remove residual NO_x^- and oxygen according to our preliminary experiment when both NO_2^- and NO_3^- were under the detection limit of ion chromatograph (0.05–0.1 ppm and 0.075–0.1 ppm for NO_2^- and NO_3^- , respectively). Subsequently, 100 μL of N₂-purged stock solution of each isotopic mixture: (1) $^{15}\text{NH}_4^+$ (^{15}N -(NH_4)₂SO₄, ^{15}N at.‰: 99.14), (2) $^{15}\text{NH}_4^+ + ^{14}\text{NO}_3^-$, and (3) $^{15}\text{NO}_3^-$ (^{15}N -KNO₃, ^{15}N at.‰: 98.15), was injected through the stopper of each vial, resulting in a final concentration of about 100 μM N, which was added in access based on the maximum TN concentration (ca. 70 μM N) detected in all collected samples. The incubations were performed at a temperature of 25 ± 1 °C. Time course incubations were carried out in duplicate (time points 0, 3, 6, 12, and 24 h). ZnCl₂ solution (200 μL, 7 M) was added at each time point during the incubation in order to stop microbial activity (Zhu et al., 2011b). The N₂ in slurries was determined within 24 h after incubation. For the measurement of N₂ production, 2 mL headspace water sample of gastight vial was transferred into a new gastight vial filled with analytical grade (99.99%) helium using a 5 ml syringe in a condition without N₂. The vials were shaken violently for about 1 min and stored upright at room temperature to allow N₂ to equilibrate. The content of $^{28}\text{N}_2$, $^{29}\text{N}_2$ and $^{30}\text{N}_2$ in headspace was then analyzed by Isotope Ratio Mass Spectrometers (MAT253 with Gasbench II and autosampler (GC-PAL), Bremen, Thermo Electron Corporation, Finnigan, Germany) using air with constant $^{15}\text{N}/^{14}\text{N}$ as standards. The equations and their explanations were described by Thamdrup and Dalsgaard (2002).

2.5. Genomic DNA extraction and clone library construction

Approximately 0.5 g soil was used for genomic DNA extraction using a FastDNA™ SPIN Kit for Soil (MP Biomedicals, USA) according to the protocols provided by manufacturer's instructions. Anammox 16S rRNA genes were amplified using a nested PCR approach. In the first round of this PCR, Planctomycetales 16S rRNA genes were amplified using the primer set Pla46f-630r (Juretschko et al., 1998; Schmida et al., 2005). In the next round, the primer set Amx368f-Amx820r (Schmid et al., 2000, 2003) was used to amplify anammox 16S rRNA genes using amplicons of Planctomycetales 16S rRNA genes as templates. The PCR reactions and thermal cycles were performed as previous study (Zhu et al., 2011b). The amplified products were checked by electrophoresis in a 1.0% agarose gel, and then purified using a Universal DNA Purification Kit (Tiangen, Beijing China). The purified products were cloned into pMD19-T vector, in accordance with the instruction. At least 40 positive clones of each clone library were randomly selected for sequencing. The quality of sequence was examined using the Chromas LITE (version 2.01, Technelysium Pty, QLD, Australia) program and the existence of chimeric sequence was further checked using QIIME (version 1.8). After that, the sequences were aligned with the MEGA (version 6.0) software and manually checked and trimmed.

2.6. Quantitative PCR

The abundance of *hzsB* gene was determined in triplicate using a Light Cycler 480 with the primer set HSBeta396F-HSBeta742R (Wang et al., 2012b). The 20 μ L qPCR reaction contained 10 μ L 2 $\times$ TransStart® Top Green qPCR SuperMix (AQ131, Transgen biotech, Beijing, China), 0.25 μ M each primer, 0.8 μ L bovine serum albumin (BSA, 20 mg mL⁻¹) and 2 μ L of 5-fold diluted DNA as a template. The standard curve was obtained using 10-fold serial dilutions of plasmid DNA with target-gene of *hzsB*. Three non-template controls were carried out for each quantitative assay. The thermal profile was performed as follows: 95 °C 3 min, followed by 48 cycles of 95 °C for 15 s and 62 °C for 34 s. A melting curve for each reaction showed that only one special peak at temperature of 86 °C was detected. Only the reactions with efficiencies between 90% and 110% (Malte et al., 2015), and standard curves with correlation coefficient above 0.99 were employed in this study.

2.7. Phylogenetic and statistical analysis

The sequences from each clone library were identified by blasting in NCBI GenBank database. Operational taxonomic units (OTUs) were defined using 97% similarity in the nucleotide sequences by Mothur (version 1.34.0). The related reference sequences and our representative sequences were aligned, and the neighbor-joining phylogenetic tree was constructed by MEGA (version 6.0). A bootstrap analysis based on 1000 replicates was applied to assess the cluster stabilities. The diversity, community composition of anammox and relationship between anammox community composition and soil chemical properties showed by redundancy analysis (RDA) were performed using R (version 2.14.0) software. ANOVAS-test analyzing the differences/similarities was performed by R. Pearson correlation and *t*-test was performed using SPSS when necessary. The plots in this study were created using Origin program (version 9.0).

2.8. Nucleotide sequence accession numbers

The sequences obtained in this study have been deposited in NCBI GenBank under accession numbers KR064787 to KR065414.

3. Results

3.1. Soil properties at different phenological stages

The soil properties at different phenological stages of rice growth are shown in Table 1. Soil pH varied from 6.5 to 5.0 and 6.7 to 5.4 in rhizosphere and bulk soils, respectively. Soil pH (except at seedling and ripening stages) and NH₄⁺ concentration (184–12.8 mg kg⁻¹) in rhizosphere soil was significantly lower than those in bulk soils at the stages of tillering, flowering and ripening. The NO₃⁻ concentrations in rhizosphere soils (8.4–48.9 mg kg⁻¹) were significantly higher than those in bulk soils (1.64–7.57 mg kg⁻¹) at the stages of seedling, tillering and booting. SOC in rhizosphere soils increased from 21.2 g kg⁻¹ to 23.4 g kg⁻¹, but decreased in bulk soils. The concentration of SOC in rhizosphere was significantly higher than that in bulk soils at the booting, flowering and ripening stages. The C/N ratio increased in both rhizosphere and in bulk soils, and C/N ratio in the rhizosphere was significantly higher than that in bulk soils at the last three phenological stages.

3.2. Organic acids in root exudates

The quantity of organic acids secreted by rice roots ranged from 2.06 to 265 μ g h⁻¹, with formate (2.0–14.3%), citrate acid (3.1–42.8%) and lactate (16.7–71.8%) being the dominant components. The composition of LMWOAs varied with phenological stages. The amounts of LMWOAs were lowest at seedling stage, and it gradually increased and reached the peak at ripening (Fig. 1a). The total quantity of LMWOAs in rhizosphere pore water was ~1.1–8.9 fold of that in bulk soils (Fig. 1b).

3.3. Anammox and denitrification rates and contribution to N₂ production

Anammox and denitrification rates were quantified using ¹⁵N-tracing. No significant accumulation of ¹⁵N-labeled gas was detected in soil samples amended with ¹⁵NH₄⁺ after anoxic incubation (Supplementary material, Fig. S1a), while considerable amount of ²⁹N₂ (¹⁵N¹⁴N) and/or ³⁰N₂ (¹⁵N¹⁵N) were detected in treatments amended with both ¹⁵NH₄⁺ and ¹⁴NO₃⁻ (Supplementary material, Fig. S1b), and treatments amended with only ¹⁵NO₃⁻ (Supplementary material, Fig. S1c), demonstrating the occurrence of anammox in both rhizosphere and bulk soils, and all ambient NO_x⁻ was depleted after pre-incubation, since both NO₂⁻ and NO₃⁻ were under the detection limit of ion chromatography (0.05–0.1 ppm and 0.075–0.1 ppm for NO₂⁻ and NO₃⁻, respectively). The anammox and denitrification rates were calculated using the quantity of N₂ (²⁸N₂, ²⁹N₂, ³⁰N₂) in treatments amended with Na¹⁵NO₃ only (Fig. 2). In rhizosphere soils, anammox rate increased to 0.71 $\pm$ 0.04 nmol N g⁻¹ dry soil h⁻¹ at tillering stage, and thereafter decreased to 0.27 $\pm$ 0.03 nmol N g⁻¹ dry soil h⁻¹ at flowering stage. The rate of anammox in rhizosphere at tillering was significantly higher than at other phenological stages; nevertheless, in bulk soil there was no significant difference in anammox rates among phenological stages. Significantly lower anammox rates were observed in bulk soils at tillering and booting stages in comparison with those in rhizosphere soils. In rhizosphere, the anammox rate was significantly positive correlated to NO_x⁻ and acetate (Supplementary Material Table S1).

3.4. Abundance of anammox bacteria

The *hzsB* gene abundance ranged from 5.29 $\times$ 10⁵ to 3.04 $\times$ 10⁶ copies g⁻¹ dry soil in the rhizosphere soil, which was significantly

Table 1
Changes in chemical properties of soils at different phenological stages of rice growth.

Source	Phenological stage ^a	pH	Moisture (%)	NH ₄ ⁺ (mg kg ⁻¹)	NO _x ⁻ (mg kg ⁻¹)	SOC (g kg ⁻¹)	C/N ratio
Rhizosphere	Seedling	6.45 ± 0.04 ^{a/A}	49.28 ± 0.38 ^{a/A}	176.44 ± 21.64 ^{a/A}	8.38 ± 0.81 ^{a/C}	21.15 ± 0.18 ^{a/B}	8.66 ± 0.08 ^{a/C}
	Tillering	6.09 ± 0.08 ^{b/B}	47.84 ± 0.96 ^{a/AB}	183.88 ± 11.88 ^{b/A}	48.92 ± 0.41 ^{a/A}	21.46 ± 0.40 ^{a/B}	9.24 ± 0.42 ^{a/B}
	Booting	5.04 ± 0.12 ^{b/D}	45.77 ± 2.69 ^{a/BC}	77.72 ± 35.06 ^{a/B}	22.35 ± 1.73 ^{a/B}	22.75 ± 0.31 ^{a/A}	10.60 ± 0.15 ^{a/A}
	Flowering	5.57 ± 0.13 ^{b/C}	44.71 ± 1.24 ^{b/C}	49.58 ± 5.42 ^{b/BC}	1.59 ± 0.04 ^{a/D}	22.83 ± 0.26 ^{a/A}	10.94 ± 0.04 ^{a/A}
	Ripening	5.19 ± 0.10 ^{a/D}	44.28 ± 0.40 ^{b/C}	12.75 ± 2.72 ^{b/C}	1.73 ± 0.40 ^{a/D}	23.14 ± 0.26 ^{a/A}	10.82 ± 0.22 ^{a/A}
Bulk soil	Seedling	6.43 ± 0.05 ^{a/B}	52.65 ± 9.70 ^{a/A}	199.56 ± 14.75 ^{a/B}	6.63 ± 0.26 ^{b/A}	21.02 ± 0.13 ^{a/A}	8.67 ± 0.15 ^{a/C}
	Tillering	6.62 ± 0.03 ^{a/A}	50.75 ± 1.66 ^{a/A}	213.54 ± 5.01 ^{a/A}	1.64 ± 0.20 ^{b/B}	21.15 ± 0.28 ^{a/A}	8.58 ± 0.19 ^{a/C}
	Booting	6.37 ± 0.08 ^{a/B}	49.83 ± 0.71 ^{a/A}	125.03 ± 1.54 ^{a/C}	7.57 ± 1.80 ^{b/A}	21.00 ± 0.25 ^{b/A}	9.14 ± 0.28 ^{b/B}
	Flowering	6.69 ± 0.04 ^{a/A}	50.31 ± 1.98 ^{a/A}	64.30 ± 1.49 ^{a/D}	1.58 ± 0.03 ^{a/B}	20.76 ± 0.22 ^{b/AB}	9.65 ± 0.25 ^{b/A}
	Ripening	5.41 ± 0.15 ^{a/C}	52.39 ± 1.52 ^{a/A}	28.67 ± 4.49 ^{a/E}	1.68 ± 0.10 ^{a/B}	20.37 ± 0.24 ^{b/B}	9.83 ± 0.23 ^{b/A}

^aDifferent letters following the value indicate significant difference between samples at $P < 0.05$, small letters represent difference between rhizosphere and bulk soil at the same phenological stage, and capital letters represent difference among phenological stages of rice growth.

Fig. 1. Low-molecular-weight organic acids produced by roots (a), and the concentrations of low-molecular-weight organic acids detected in the rhizosphere and bulk pore water (b). Letters in each plot indicate significant differences between samples at $P < 0.05$, the lowercase letters represent difference between rhizosphere and bulk soil at the same phenological stage, the capital letters (A, B, C and D in plot (a) for organic acids produced by roots, E, F and G in plot (a) for total organic acids; A, B, C and D in plot (b) for rhizosphere soil, E, F, G and H for bulk soil) represent differences between phenological stages of rice growth.

Fig. 2. The rates of anammox and denitrification in rhizosphere and bulk soils at phenological stages of rice growth. Letters in each plot indicate significant differences between samples at $P < 0.05$, the lowercase letters represent difference between rhizosphere and bulk soil at the same phenological stage, the capital letters were used for rhizosphere anammox (A and B), bulk soil anammox (C), rhizosphere denitrification (D, E and F), and bulk soil denitrification (G and H).

higher than that in bulk soil ($4.85\text{--}6.76 \times 10^5$ copies g⁻¹ dry soil) during the entire growth period (Fig. 3). The highest *hzsB* gene abundance in rhizosphere and bulk soil occurred at tillering stage and flowering stage, respectively. Succinate concentration significantly correlated with *hzsB* gene abundance in rhizosphere soils (Supplementary Material Table S1). The abundance of bacterial 16S

Fig. 3. The abundance of *hzsB* gene in rhizosphere and bulk soils at phenological stages of rice growth. Letters denote significant differences at $P < 0.05$, the lowercase letters represent differences between rhizosphere and bulk soil at the same phenological stage, and the capital letters (A, B and C for rhizosphere soil, D and E for bulk soil) represent differences between phenological stages of rice growth.

rRNA genes was also determined by qPCR assays (Supplementary Material Fig. S2). The proportion of anammox bacteria to total bacteria (*hzsB*/16S rRNA) in rhizosphere soils was maintained at a significantly greater level ($P < 0.05$) of $0.38\text{--}1.28 \times 10^{-4}$ compared to bulk soils ($2.62\text{--}8.25 \times 10^{-5}$). The relative abundance of

anammox bacteria compared to total bacteria in rhizosphere was about 1.5-fold greater than that observed in the adjacent bulk soils.

3.5. Diversity and community composition of anammox in rhizosphere and bulk soils

A total of 628 unique sequences were obtained from the samples, which were clustered into 125 OTUs (3% cutoff), with 74 and 76 OTUs in the rhizosphere and bulk soil, respectively. Among these, 25 OTUs were shared in both rhizosphere and bulk soil. Five genera of *Brocadia*, *Kuenenia*, *Scalindua*, *Jettenia* and *Anammoxoglobus* were detected in the collected soils (Fig. 4a and Fig. 4b). *Brocadia* comprised 93.0% of amplified sequences, followed by *Scalindua* (2.75%), *Jettenia* (2.34%), *Kuenenia* (1.79%) and *Anammoxoglobus* (0.14%). *Anammoxoglobus* was only detected in bulk soils during the flowering stage and about 70% of *Kuenenia* were detected in rhizosphere soils. The anammox bacteria community was significantly different between rhizosphere/bulk soils and phenological stage (Supplementary Material Fig. S3 and Fig. S4). Redundancy analysis of the relationship between anammox bacteria community composition and physicochemical properties demonstrated that C/N ratio, NH_4^+ , citrate and formate concentration explained 53.0% of anammox bacterial variation (Fig. 5).

4. Discussion

To the best of our knowledge, this is the first study investigating the dynamics of anammox bacterial activity, abundance and diversity in rhizosphere over the entire growth period of rice. Our results showed that the rhizosphere was a hotspot for anammox in

Fig. 5. Redundancy analysis (RDA) to measure the relationship between the anammox community composition, soils properties, and low-molecular-weight organic acids. Only those factors that significantly describe variance in the composition of the anammox bacterial community determined by variation inflation factors (VIFs) calculated during RDA are shown here.

paddy fields, and the anammox activity depended on the phenological stage of rice growth, being highest at the tillering stage. Variation in the quality and quantity of LMWOAs across developmental stage was observed, with seedlings having the least root exudation, and the plant gradually increasing exudation

Fig. 4. Phylogenetic analysis (Neighbor-joining tree) based on anammox bacterial 16S rRNA gene sequences using p-distance with 1000 bootstraps in MEGA 6.0 (A), and the distribution of the five genera detected in this study (B). The sequences contained in this study are indicated in boldface. R = rhizosphere, and B = bulk soils. The value following the genus indicates the relative abundance.

throughout growth history, as shown previously (Aulakh et al., 2001; Eller and Frenzel, 2001; Bacilio-Jimenez et al., 2003).

At tillering and booting stages, anammox rates in rhizosphere were significantly higher than those in bulk soils, and the anammox rate in rhizosphere soil was significantly correlated with NO_3^- and acetate concentration, while no significant correlations were identified in bulk soils. As the rice rhizosphere is highly oxygenated compared to bulk soil, nitrite, which is a substrate for anammox, may be produced through both denitrification and nitrification (Lam et al., 2007; Zhu et al., 2011a; Wang et al., 2012a). In oxygen-limited bulk soil with low C/N ratio, where nitrification is inhibited, denitrifiers may reduce limited nitrate diffused from rhizosphere, to nitrite (Trimmer and Nicholls, 2009), then provide anammox with nitrite. Elevated anammox rates have been observed in surface paddy soils with higher organic matter, NO_3^- and oxygen, (Zhu et al., 2011a; Sato et al., 2012). The rhizosphere and surface layer of standing water are typical oxic-anoxic interfaces in wetland ecosystems. In this study, we separated bulk soil from rhizosphere using a nylon bag as described previously (Liu et al., 2006; Jia et al., 2013; Nie et al., 2015), however, in reality the micro-environment around the rhizosphere is a continual redox gradient that extends from the root surface to the bulk soil. In addition to NO_3^- in soils, acetate released in the rhizosphere due to both microbial and root respiration, would be an electron donor and/or carbon source for anammox bacteria (Kartal et al., 2008, 2011). Therefore, the redox gradient, higher exudation, and the oxygen concentration in rhizosphere soil could provide an ideal niche for anammox activity.

A significantly greater abundance of anammox bacteria was also observed in rhizosphere compared to bulk soils, possibly as a result of root exudates. Roots exudates, including sugars, amino acids, various LMWOAs such as succinate, malate and fumarate, may act as chemo-attractants for bacteria or simply stimulate bacterial growth (Haichar et al., 2014). Root exudates have a direct impact on carbon and nitrogen cycling through a priming effect, and are part of the rhizo-deposition process, which is the major source of soil organic carbon released by plant root, leading to a proliferation of microorganisms in the rhizosphere (Haichar et al., 2014). Plant roots excrete a compositionally diverse array of more than 100,000 different low-molecular mass natural products, which comprise a broad range of substrates and signaling molecules for rhizosphere-associated microorganisms (Bais et al., 2004; Haichar et al., 2014), possibly including anammox bacteria. The significant correlation between *hzsB* gene copies and succinate could indicate a potential role of succinate in anammox bacterial metabolism. Succinic acid is a chelator of poorly dissolved mineral nutrients (Haichar et al., 2014), which may be important for anammox bacterial growth.

Variation in soil denitrification rate was observed in the rhizosphere and bulk soils (Fig. 2). Oxygen excreted by rice roots (Brune et al., 2000) and nitrification (Chen et al., 2008) may explain the greater activity in the rhizosphere. The denitrification rates were significantly higher than previous measurements (Nie et al., 2015), possibly due to higher NO_3^- concentrations, which have been linked to denitrification rate (Bartoli et al., 2012; Song et al., 2014; Lan et al., 2015). Propionate was also significantly correlated with denitrification (Supplementary Material Table S1) as demonstrated previously (Paul and Beauchamp, 1989; Hallin and Pell, 1998; Chen et al., 2015). Our results demonstrated that denitrification contributed to 90% of the N loss in both rhizosphere and bulk soils, indicating that denitrification is the main pathway of N loss in paddy soils over the period of rice growth, even when the rhizosphere provides a more favorable habitat for anammox bacteria.

The five predominant genera of anammox bacteria identified in this study (see above) have been observed in many habitats, including soils (Humbert et al., 2010; Wang et al., 2012b; Hu et al., 2013; Wang and Gu, 2013) and sediments (Zhang et al., 2007; Van

de Vossenberg et al., 2008; Yoshinaga et al., 2011). Similar to previous reports, *Candidatus Brocadia* dominated in this paddy soils fields (Bai et al., 2015; Yang et al., 2015). Bacteria of this genus harbor diverse metabolism (Gori et al., 2011), with the ability to use short-chain organic acids as electron donors instead of NH_4^+ (Kartal et al., 2008), making it highly adaptable. The anammox community composition was significantly different between rhizosphere and bulk soils as observed previously (Nie et al., 2015). Changes in the concentration of NH_4^+ , C/N ratio, citrate and formate could explain 52.98% of the variation in anammox community composition. Ammonium is obviously a main substrate of anammox bacteria (Shen et al., 2013; Yang et al., 2015), but citrate and formate are not recognized as having a direct influence on anammox composition. However, the use of these LMWOAs as a nutrient source for anammox bacteria (Badri and Vivanco, 2009) or as a mechanism to reduce the toxicity of aluminum (Haichar et al., 2014), may explain this correlation.

5. Conclusions

In summary, our results demonstrated a shift in anammox activity in rice rhizosphere during the whole rice growth period, and NO_3^- and acetate were key factors influencing anammox rate. The anammox bacterial community composition, which was dominated by *Brocadia*, was significantly different between rhizosphere and bulk soils, and also varied between phenological stages. This variance could be partially attributed to the concentration and components of root exudates associated LMWOAs. Future studies are needed to investigate how the exudates influence anammox activity and the growth of anammox bacteria.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2016.05.015>.

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