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Nitrogen loss by anaerobic oxidation of ammonium in rice rhizosphere

San'an Nie[1], Hu Li[1], Xiaoru Yang[1], Zhaoji Zhang[1], Bosen Weng[1], Fuyi Huang[1], Gui-Bing Zhu[2] and Yong-Guan Zhu[1]

[1]Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China and [2]Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China

Anaerobic oxidation of ammonium (anammox) is recognized as an important process for nitrogen (N) cycling, yet its role in agricultural ecosystems, which are intensively fertilized, remains unclear. In this study, we investigated the presence, activity, functional gene abundance and role of anammox bacteria in rhizosphere and non-rhizosphere paddy soils using catalyzed reporter deposition–fluorescence *in situ* hybridization, isotope-tracing technique, quantitative PCR assay and 16S rRNA gene clone libraries. Results showed that rhizosphere anammox contributed to 31–41% N_2 production with activities of 0.33–0.64 nmol N_2 g^{-1} soil h^{-1}, whereas the non-rhizosphere anammox bacteria contributed to only 2–3% N_2 production with lower activities of 0.08–0.26 nmol N_2 g^{-1} soil h^{-1}. Higher anammox bacterial cells were observed (0.75–1.4 × 10^7 copies g^{-1} soil) in the rhizosphere, which were twofold higher compared with the non-rhizosphere soil (3.7–5.9 × 10^6 copies g^{-1} soil). Phylogenetic analysis of the anammox bacterial 16S rRNA genes indicated that two genera of 'Candidatus Kuenenia' and 'Candidatus Brocadia' and the family of *Planctomycetaceae* were identified. We suggest the rhizosphere provides a favorable niche for anammox bacteria, which are important to N cycling, but were previously largely overlooked.
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Introduction

With the rapid increasing use of chemical fertilizers in agriculture, high nitrogen (N) loss in many densely populated countries is becoming an important issue from both environmental and agronomic perspectives. China is one of the largest rice producing countries and consumers of chemical N fertilizer in the world (FAO 2013, available at www.fao.org/publications/sofa.), which results in large amounts of N loss through NH_3 volatilization, NO_3^- runoff and leaching and N_2O emissions (Xing and Zhu, 2000). However, >10% of the total N fertilizers applied on arable soils remain uncharacterized (Zhu, 2008).

The discovery of anaerobic oxidation of ammonium (anammox) in natural ecosystems provides new insights into the mechanisms responsible for N loss (Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Kuypers et al., 2003). Recently, anammox bacteria were found to

be widely distributed in agricultural fields (Humbert et al., 2010; Wang et al., 2012b; Shen et al., 2013; Wang and Gu., 2013). However, these few published articles mainly focused on the distribution and phylogenetic diversity of anammox bacteria. The quantification of anammox activity and its contribution to the N cycle are not well known. To date, there are no studies that address anammox processes in the rhizosphere, which is ubiquitous (Jones and Hinsinger, 2008) and has a key role in N cycling (Richardson et al., 2009; Jackson et al., 2012; Liu et al., 2014). The role of anammox bacteria in rhizospheric N cycling is unknown.

Hence, the objectives of the present study were to investigate the occurrence, activity, contributions and role of anammox to N loss in the rhizosphere and non-rhizosphere zones of a fertilized paddy soil from Southern China.

Materials and methods

Soil sampling
Paddy soil was collected from the Red Soil Ecological Experiment Station, Chinese Academy of Sciences, located in subtropical southern China, in Changde city, Hunan province (N: 28°57'; E: 110°30'). Soil samples (0–20 cm) were collected in the field on 22 November 2010 and composited. Prior to the start of the experiment, the soil was air-dried, then homogenized and sieved (< 2 mm).

Experimental setup
Rhizo-bags (30-µm nylon mesh, 7.5 cm diameter, 12 cm height) filled with 475 g sieved soil were placed in the center of polyvinyl chloride pots (15 cm diameter, 23 cm height), which were then filled with 3 kg soil. The rhizo-bag was separated into two compartments, which allowed smaller molecular substrates to penetrate but prohibited penetration by roots (Supplementary Figure S1). Two treatments (control and N fertilization) were evaluated, and for each treatment, we sampled rhizosphere and non-rhizosphere soils. In the control treatment, no fertilizers were applied. In treated soil, N was applied as urea (100 mg kg^{-1} dry soil). The pot experiment was carried out using a randomized design with three replicates for each treatment.

The soils were first incubated for 1 month at 105% of water-holding capacity at 25 °C, then added to pots as described above. Rice seeds (cv. Xiangzaoshan 45) were sterilized in 30% H_2O_2 for 10 min and then thoroughly washed with de-ionized water. Three days after germination, uniform seedlings were transplanted into the rhizo-bags. After 37 days of rice growth in a greenhouse (illumination 1500 E m^{-2} s^{-1}, ambient temperature 35 °C ± 2, 30 °C night; humidity, ambient 90%), rhizosphere and bulk soils were sampled. The rhizosphere and bulk soils were each divided into three parts. One part was used for *in situ* cell fixation for catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) as described below, another part was immediately frozen in liquid N_2 and archived at −80 °C for molecular analysis, and the other part was incubated to determine anammox and denitrification activity.

Chemical analytical procedures
Soil pH was determined in a 1:2.5 soil/water suspension. Soil organic matter, total organic C and total N were determined using a total carbon analyzer (TOC-V CPH, SHIMADZU, Japan). Ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) were extracted from the soil with 2 M KCl and diluted prior to determination by a flow injection analyzer (FIA QC8500, Lachat, Loveland, CO, USA). Soil grain size was analyzed using a laser scattering particle analyzer (MS2000, Malvern Instruments, Malvern, UK) after sieving (2 mm) to remove the gravel and plant roots. All analyses were performed in triplicate.

Fluorescence in situ hybridization and catalyzed reporter deposition
CARD-FISH was applied to capture photographic documentation of anammox bacterial cells in the rhizosphere and bulk soils. The horseradish peroxidase (HRP)-labeled oligonucleotides probe Amx820 (5′-AAAACCCCTCTACTTAGTGCCC-3′) (Schmid et al., 2000) was used for the detection of anammox bacteria, including 'Candidatus Brocadia' and 'Candidatus Kuenenia', in the soil samples. To check for unspecific staining and autofluorescence, the HRP-

labeled probe Non338 (5′-ACTCCTACGGGAGG CAGC-3′) (Wallner et al., 1993) was used. Total cells were determined on the basis of staining with DAPI (4,6-diamidino-2 phenylindole) and were recorded with an LSM 710 confocal laser scanning microscopy (Carl Zeiss, Inc., Oberkochen, Germany). Sampling and processing for CARD-FISH followed standard protocols (see Supplementary Table S1).

Measuring anammox and denitrification rate with ^{15}N labeled ammonium and nitrate
The activity and potential role of anammox and denitrification were measured at *in situ* soil temperatures with a ^{15}N-tracing technique (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen et al., 2004). Approximately 3.5 g soil (fresh weight, three replicates) were transferred to 12.0 ml glass vials (Exetainer, Labco, High Wycombe, Buckinghamshire, UK) together with N_2-purged media water from *in situ* irrigation water (total C: 0.89 mg l^{-1}; total N: 0.40 mg l^{-1}; NH_4^+-N: 0.08 mg l^{-1}; NO_3^--N: 0.29 mg l^{-1}). The resulting paddy soils were then preincubated to remove residual NO_x^- and oxygen (Supplementary Figure S2). Subsequently, 100 µl of N_2-purged stock solution of each isotopic mixture, namely, (1) $^{15}NH_4^+$ (^{15}N-$(NH_4)_2SO_4$ at 99.14%, 12 mM N), (2) $^{15}NH_4^+ + ^{14}NO_3^-$ (KNO$_3$, 12 mM N) and (3) $^{15}NO_3^-$ (^{15}N-KNO$_3$ at 98.15%, 12 mM N), was injected through the septa of each vial, resulting in a final concentration of about 100 µM N. All isotope solutions were flushed with He prior to addition. The incubations were performed at temperature 35 ± 1 °C. At five intervals over 24 h (0, 3, 6, 12, 24 h, respectively), reactions in three replicate vials from each treatment were inhibited by injecting 200 µl of a 7 M ZnCl$_2$ solution. The rate and potential contribution to N_2 formation by anammox or denitrification were calculated from the excess production of $^{29}N_2$ and $^{30}N_2$ in the $^{15}NO_3^-$ treatment, measured by continuous flow isotope ratio mass spectrometry (MAT253 with Gasbench II and autosampler (GC-PAL), Bremen, Thermo Electron Corporation, Finnigan, Germany), and the fraction of ^{15}N in NO_3^-. The equations used and their explanations as described by Thamdrup and Dalsgaard (2002) are summarized in Supplementary Information (Supplementary Table S2).

DNA extraction and PCR
DNA was extracted from approximately 0.25 g soil using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. DNA concentration and quality were measured by a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA). Thermal cycling and data analysis were carried out with a Real-time PCR Detection System (Roche480, Roche, Indianapolis, IN, USA) to assess the abundance of anammox *hzsA* gene with the primers of *hzs*A_1594F and *hzs*A_1857R (Kartal et al., 2011;

Harhangi *et al.*, 2012). The reaction mixture consisted of 4 µl DNA as template, 0.6 µl of each primer, 10 µl of SYBR 2 Premix Ex Taq, 1.0 µl BSA (20 mg ml^{-1}) and 3.8 µl of dd H$_2$O. Thermal cycling conditions were as follows: 3 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 63 °C, 10 s at 72 °C, and 10 s at 82 °C. Three independent quantitative PCR assays were performed for each sample. Negative controls without DNA template were included in each amplification reaction. Standard curves were obtained using 10-fold dilutions of standard plasmid containing *hzs*A gene, which were amplified with the primers mentioned above. Every sample was quantified in three parallel quantitative PCR reactions to ensure the correct amplification. Only the reactions with efficiencies between 90% and 110% were accepted.

A nested PCR approach was conducted to detect the anammox bacterial 16S rRNA genes. The initial amplification was fulfilled using the PLA46f-630r primer combination with a thermal profile of 96 °C for 10 min, followed by 35 cycles of 60 s at 96 °C, 1 min at 56 °C, and 1 min at 72 °C (Juretschko *et al.*, 1998; Neef *et al.*, 1998). Afterward, a 500 times diluted PCR product was used as template for the second amplification with Amx368f - Amx820r primers using a thermal profile of 96 °C for 10 min, followed by 25 cycles of 30 s at 96 °C, 1 min at 58 °C, and 1 min at 72 °C. The PCR mixture and thermal cycling programs were conducted as described by Hefting *et al.* (2006). The amplified products were examined by electrophoresis using a 1.0% agarose gel.

Cloning and sequencing
The PCR amplified anammox bacterial 16S rRNA gene fragments were cloned using the pMD19-T vector cloning kit (TaKaRa, Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Plasmid DNA was isolated with the GeneJET Plasmid Miniprep Kit (Fermentas, Lithuania). At least 30 positive clones from each sample were randomly selected for sequencing (Invitrogen, Shanghai, China). The quality of the recovered sequences was checked using the Chromas Lite (version 2.01, Technelysium Pty, QLD, Australia) program. The occurrence of chimeric sequences was further examined using UCHIME (31). The Phylogenetic analysis of the 16S rRNA gene was performed with the MEGA 6.0 software (http://www.megasoftware.net) by maximum likelihood method. A bootstrap analysis with 1000 replicates was applied to estimate the confidence values of the tree nodes. The sequences obtained in this study for

anammox bacteria are available from Genbank under accession numbers KJ523975–KJ524101.

Statistical analysis
Results were given on a soil dry weight basis (oven dry, 24 h, 105 °C). Data were expressed as the mean of replicates ± s.e. except where otherwise noted. One-way analysis of variance (Duncan, $P < 0.05$) and Student's *t*-test ($P < 0.05$) were used to determine differences between groups. All analyses were assessed by SPSS for Windows version 14.0 software (SPSS Inc., Chicago, IL, USA).

Results

Soil properties and inorganic N pools after planting
Paddy soil was selected for this study with high ammonia concentration, which was also used in many anammox bioreactor studies to stimulate anammox bacteria (Strous *et al.*, 1998; Sliekers *et al.*, 2002; Kartal *et al.*, 2008). The chemical characteristics of the soil are shown in Table 1. Because of long-term application of chemical fertilizers, the content of paddy soil organic matter, C_{mic}, N compounds (total N, N_{mic}) and available P and K were high. The pools of NH_4^+ and NO_x^- from planted soils are shown in Figure 1a. Overall, the NH_4^+ concentrations were almost 1–2 orders of magnitude higher than those of NO_x^-. The NH_4^+ concentrations in the bulk soil were significantly higher than in the rhizosphere. In the rhizosphere, the NO_3^- concentrations were significantly higher than in the bulk soil. The NO_2^- concentration in the non-rhizosphere soil ranged from 1.7 mg kg^{-1} to 2.8 mg kg^{-1} but was undetected in the rhizosphere soil. The NH_4^+ and NO_3^- concentrations in the rhizosphere did not change significantly between fertilized and non-fertilized soil.

Detection of anammox bacterial cells
For the analysis of anammox bacterial cells in flooded rice soils, CARD-FISH analysis was used, which allowed for the localization of native microbial cells in the rhizosphere and bulk soils *in situ*. Probe Amx820 hybridized with their 16S rRNA was constructed to specifically detect anammox organisms, including '*Ca*. Brocadia' or '*Ca*. Kuenenia'. The acquisition of CARD-FISH signals and autofluorescence were performed by laser scanning confocal microscopy for an improved visualization of microbial cells. Images showing discrete fluorescent signals of high intensity were observed, which

Table 1 Characteristics of the paddy soil used in this study

pH 1:2.5	SOM, g kg^{-1}	Total N, g kg^{-1}	C_{mic}, mg kg^{-1}	N_{mic}, mg kg^{-1}	Available P, mg kg^{-1}	Available K, mg kg^{-1}	Clay content %
5.9 ± 0.1	41.7 ± 2.3	2.3 ± 0.2	1092.6 ± 21.4	128.2 ± 3.2	9.5 ± 0.8	62.8 ± 5.6	13.2

Abbreviations: C_{mic}, microbial biomass carbon; N_{mic}, microbial biomass nitrogen; SOM, soil organic matter. Parent material: quaternary red clay. Mean ± s.d. ($n = 3$).
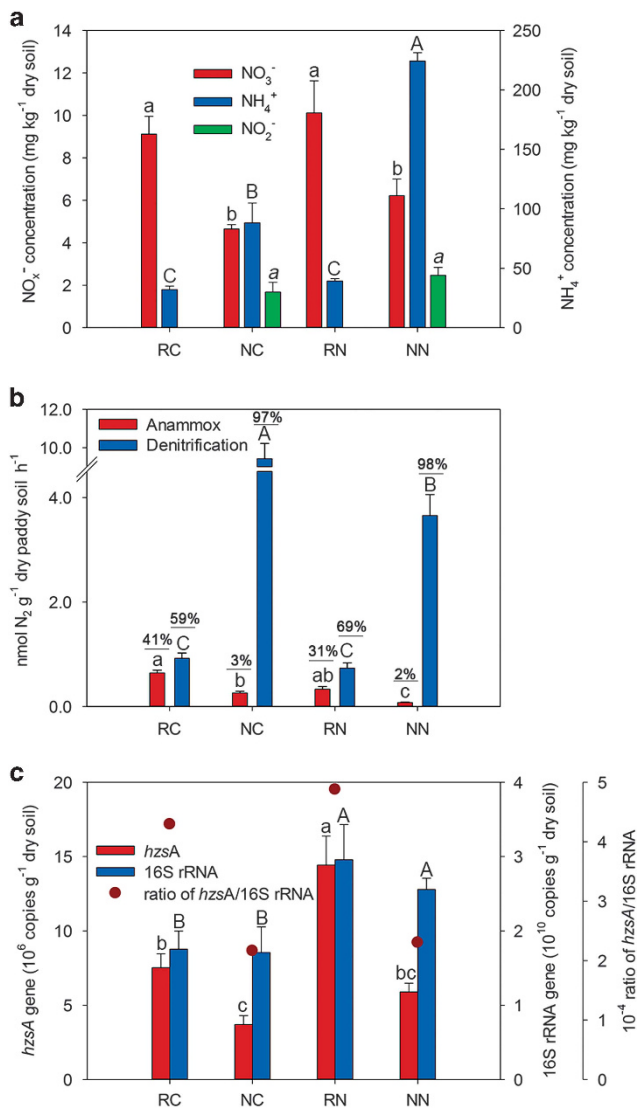
Figure 1 The concentration of NO_3^-, NO_2^- and NH_4^+ (**a**), anammox, denitrification activity and their contributions to total N_2 production (**b**) and abundance of anammox bacteria targeting the *hzs*A gene and total bacteria targeting the 16S rRNA gene (**c**) both in the rhizosphere and non-rhizosphere soils. The soil samples evaluated were (1) rhizosphere in control (RC); (2) non-rhizosphere in control (NC); (3) rhizosphere in N fertilization (RN); and (4) non-rhizosphere in N fertilization (NN). $n = 3$, Duncan test or *t*-test at $P < 0.05$ level, letters with different labels indicate significant differences.

represented individual microbial cells in the soils (Figure 2). Anammox bacteria, including genera affiliated with 'Candidatus Brocadia' and/or 'Candidatus Kuenenia,' were detected in samples from both the oxic and anoxic zones and verified by CARD-FISH and sequencing of their 16S rRNA genes.

Anammox and denitrification rate and contribution to N_2 production
To determine anammox rate and the potential role of anammox as a N_2 producer, incubations were performed with rhizosphere soil and bulk soil under

in situ temperature using a ^{15}N isotope-tracing technique. The results showed that, in the soil samples amended with $^{15}NH_4^+$, no significant accumulation of $^{15}N_2$-labeled gas could be detected in the rhizosphere or bulk soils (Supplementary Figure S3A), indicating that all ambient $^{14}NO_x^-$ was consumed during preincubation. When both $^{15}NH_4^+$ and $^{14}NO_3^-$ were added, $^{29}N_2$ was accumulated but not $^{30}N_2$ (Supplementary Figure S3B). Significant rates of both anammox and denitrification were observed in the incubations amended with $^{15}NO_3^-$ only (Supplementary Figure S3C). It is possible that potential rates may overestimate the actual *in situ* activity because NH_4^+ and NO_3^- availability may limit the process *in situ*. However, the *in situ* concentration of NH_4^+ and NO_3^- were quite high (32.0–224.3 mg kg^{-1} and 4.6–10.1 mg kg^{-1}, respectively). Our samples were incubated with NH_4^+ or NO_3^- to a final concentration corresponding to a maximum of $< 10\%$ of the *in situ* concentration, therefore the potential rates of anammox and denitrification may not be seriously overestimated.

Anammox and denitrification rates calculated using $^{29}N_2$ and $^{30}N_2$ production values from the $^{15}NO_3^-$ incubations are shown in Figure 1b. High potential rates of anammox were observed (0.33–0.64 nmol N_2 g^{-1} soil h^{-1}) in the rice rhizosphere for both treatments, contributing 31% and 41%, respectively, to their total N_2 loss. In the non-rhizosphere zone, however, the rates of denitrification (3.66–9.42 nmol N_2 g^{-1} soil h^{-1}) were much higher than that of anammox (0.08–0.26 nmol N_2 g^{-1} soil h^{-1}). Approximately 2–3% was produced by anammox, while the remainder was denitrified to N_2. Though differences were observed in denitrification activity between rhizosphere (0.74–0.92 nmol N_2 g^{-1} h^{-1}) and non-rhizosphere (3.66–9.42 nmol N_2 g^{-1} h^{-1}), denitrification was still the main contributor of N_2 production in paddy soil (59–69% in rhizosphere and 97–98% in non-rhizosphere). The isotope tracing technique revealed that the ratio of contribution to N_2 production by anammox in rhizosphere (31–41%) was mostly 15 times higher than that in non-rhizosphere (2–3%), suggesting high variability in N loss between the two zones in the paddy soil.

Abundance of anammox bacteria
To obtain more detailed information on the anammox bacteria, the primer pair of *hzs*A_1594F and *hzs*A_1857R was applied for the quantification of anammox bacterial *hzs*A gene abundance in the soil using quantitative PCR assay (Harhangi *et al.*, 2012; Wang *et al.*, 2012b). The *hzs*A gene abundance was up to $0.75–1.4 \times 10^7$ copies g^{-1} dry soil in the rhizosphere, whereas the number of anammox bacterial genes decreased to $3.7–5.9 \times 10^6$ copies g^{-1} dry soil in the non-rhizosphere (Figure 1c). The quantitative PCR assays on 16S rRNA gene was also performed and showed the proportion of anammox cell numbers to total bacteria were maintained at a high level
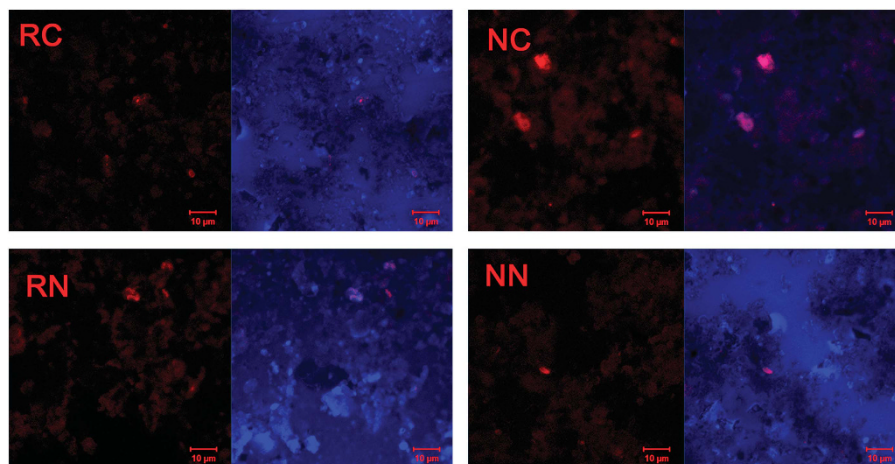
Figure 2 *In situ* mapping of anammox bacteria from rhizosphere and bulk soils by confocal laser scanning confocal microscopy. Anammox bacteria stained by CARD-FISH probe Amx820 specific for genera 'Candidatus Brocadia' and 'Candidatus Kuenenia' (left) and combination of DAPI-stained cells and cells stained with probes specific for anammox bacteria (right) are shown. NC, non-rhizosphere in control; NN, non-rhizosphere in N fertilization; RC, rhizosphere in control; RN, rhizosphere in N fertilization.

of $4.3–4.9 \times 10^{-4}$ in the rhizosphere in comparison to non-rhizosphere ($2.2–2.3 \times 10^{-4}$). Anammox bacterial abundance and their proportion to total bacteria detected in rhizosphere were about twofold higher than those observed in the adjacent bulk soils.

Community structure of anammox bacteria
To investigate the community structure of anammox bacteria, the anammox 16S rRNA gene clone libraries were constructed from four representative soil samples (RC, NC, RN and NN). A total of 28 operational taxonomic units (OTUs) (97% cutoff) were obtained from the rhizosphere (11 OTUs from RC and 9 OTUs from RN) and the non-rhizosphere (11 OTUs from NC and 8 OTUs from NN) as shown in Figure 3. Phylogenetic analyses of anammox 16S rRNA bacterial sequences and related sequences deposited in GenBank showed 19 OTUs were assigned to *Planctomycetaceae* (rhizosphere, 62.1%; non-rhizosphere, 37.9%). The OTU 3# and OTU 7# in the non-rhizosphere were most closely affiliated to 'Candidatus Kuenenia'. The remaining OTUs were affiliated with 'Candidatus Brocadia' (rhizosphere, 13.8%; non-rhizosphere, 86.2%).

Discussion

To the best of our knowledge, this is the first report of the abundance and activity of anammox bacterial in rice rhizosphere. Up to 31–41% of rhizospheric soil N_2 production with rates of $0.33–0.64\,\mathrm{nmol\,N_2}$ $\mathrm{g^{-1}\,soil\,h^{-1}}$ was contributed by anammox, whereas approximately 2–3% of N was produced through anammox in bulk soils ($0.08–0.26\,\mathrm{nmol\,N_2\,g^{-1}\,soil}$-$\mathrm{h^{-1}}$). These findings improve our understanding of N cycle in paddy fields.

The observed anammox rates ($0.33–0.64\,\mathrm{nmol}$ $\mathrm{N_2\,g^{-1}\,h^{-1}}$) in the rhizosphere were significantly higher than that in the bulk soils ($0.08–0.26\,\mathrm{nmol\,N_2\,g^{-1}\,h^{-1}}$),

indicating that rhizospheric anammox process might be an overlooked pathway for N loss from paddy soils. In the rhizosphere, which is exposed to oxygen, both partial denitrification and nitrification may produce nitrite for anammox bacteria, which have been reported in many environments, including marine (Kuypers *et al.*, 2005; Lam *et al.*, 2007), freshwater (Zhu *et al.*, 2010, 2011a; Wang *et al.*, 2012a) and soil (Zhu *et al.*, 2011b). In oxygen-limited bulk soil, where nitrification is inhibited, denitrification may provide anammox with nitrite. Higher anammox rates were also detected in the surface layer of other paddy soils (Zhu *et al.*, 2011b; Sato *et al.*, 2012). The rhizosphere and surface layer of standing water are typical oxic–anoxic interfaces in wetland ecosystems; therefore, we propose that the redox gradient in the rhizosphere is a hotspot for anammox activity. In waterlogged ecosystems, oxygen exposure and NO_x^- production could be the key factors in determining anammox activity.

In the present study, great variation in denitrification rate was observed between rhizosphere ($0.74–0.92\,\mathrm{nmol\,g^{-1}\,soil\,h^{-1}}$) and bulk soils ($3.66–9.42\,\mathrm{nmol\,N_2\,g^{-1}\,soil\,h^{-1}}$) (Figure 1b). As high concentrations of nitrate were detected in both zones, the possible reason for these changes may be the presence of oxygen, which was transported through the roots (Armstrong, 1971; Brune *et al.*, 2000). It is known that denitrification activity declines sharply in the presence of oxygen (Firestone *et al.*, 1979; Firestone and Tiedje., 1979). Hence, we propose that due to oxygen exposure denitrification activity in the rhizosphere declined significantly in comparison to the adjacent anoxic bulk soil. Although there are differences in denitrification rates between the rhizosphere and bulk soils, our results indicate that denitrification is still the main pathway for N loss in paddy soil (59–69% in the rhizosphere and 97–98% in the non-rhizosphere).
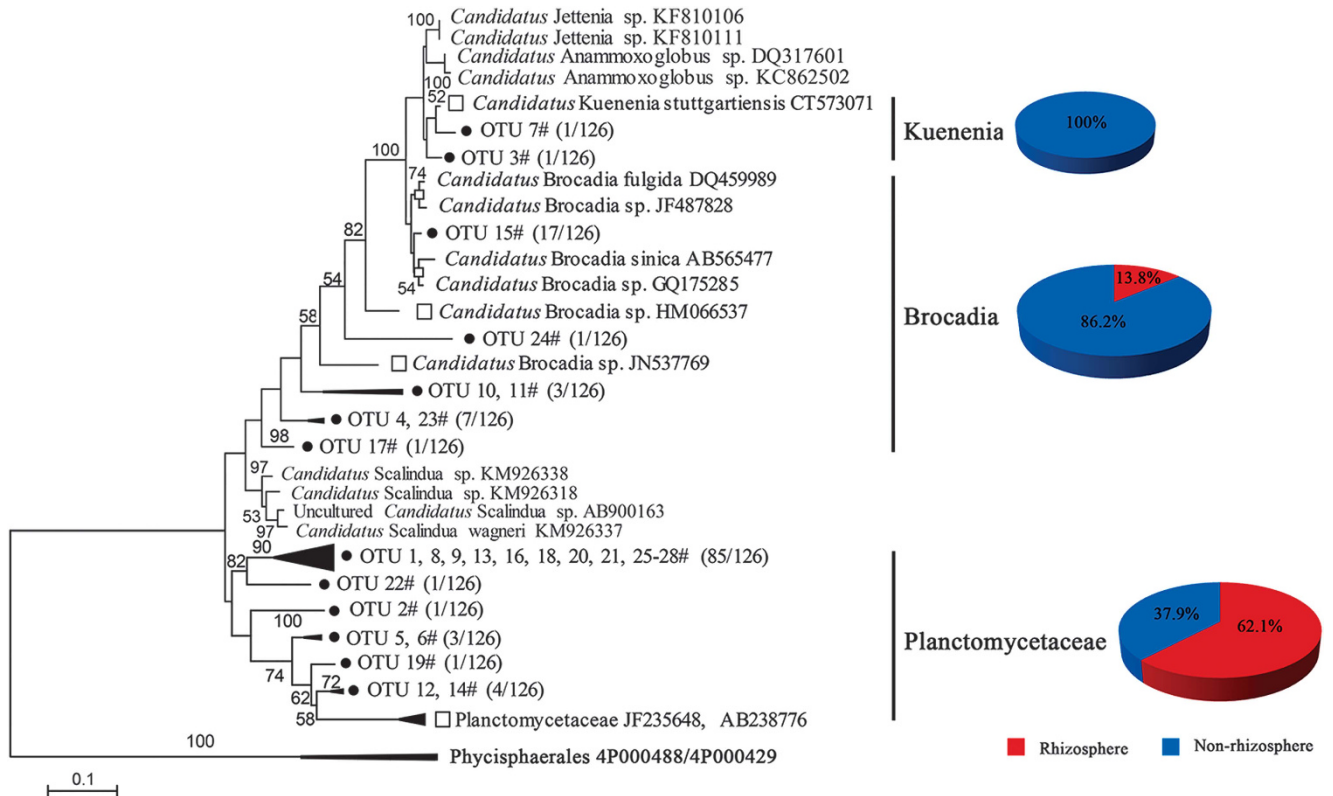
Figure 3 Phylogenetic tree of deduced anammox 16S rRNA gene sequences. Branches corresponding to partitions reproduced in < 50% bootstrap replicates were collapsed.
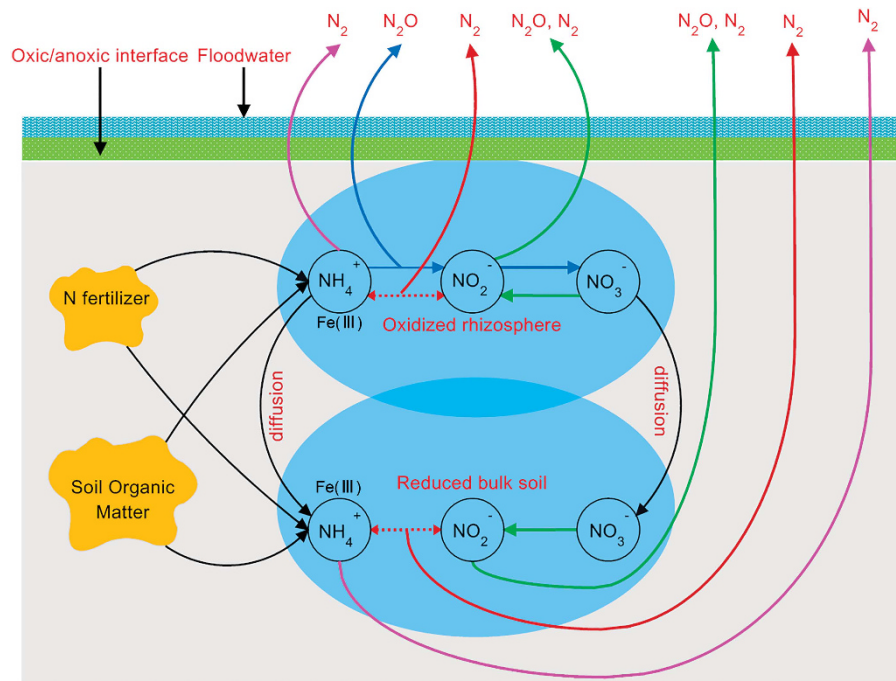

Figure 4 Schematic representation of the N loss from paddy soil. The classical processes of nitrification (blue), denitrification (green) and recently discovered anammox (red) as well as anaerobic oxidation of ammonium coupled to Fe-reduction (magenta) are shown both in oxidized rhizosphere and reduced bulk soil.

In addition to their high activity, high cell numbers of anammox bacteria ($0.75–1.4 \times 10^7$ copies g^{-1} soil) in the rhizosphere in comparison to non-rhizosphere were also observed and were higher than those reported in other agricultural soils (Zhu et al., 2011b; Humbert et al., 2012; Shen et al., 2013). To the best of our knowledge, this is the highest anammox abundance recorded in agricultural soils. In the rhizosphere, the abundance was over twofold higher compared with the non-rhizosphere zone. The reason for the drastic increase in abundance was probably related to NO_x^- in the rhizosphere, which was in agreement with other studies (Hamersley et al., 2007; Shen et al., 2013; Zhu et al., 2013). Another possible reason may be attributed to the high background value of NH_4^+ ($32.0–224.3 \, mg \, kg^{-1}$) in comparison to NO_3^- ($4.6–10.1 \, mg \, kg^{-1}$). In the pot experiment, no differences in rhizospheric NH_4^+ concentrations were observed between fertilized and non-fertilized treatments, which was in agreement with literature reporting that 10 units of anammox reaction needed 10 units of ammonia and nitrate, respectively (Jetten et al., 1998; Zhu et al., 2013). This suggests that the rhizosphere provides a more favorable habitat for anammox bacteria.

In the present study, the anammox communities were closely related to two genera 'Candidatus Brocadia' and 'Candidatus Kuenenia', which have been observed to be dominant anammox community in soils and sediments (Fan et al., 2010; Humbert et al., 2010; Zhu et al., 2011a; 2011b; 2013; Shen et al., 2013). Moreover, results using a 16S rRNA-targeted oligonucleotide probe specific for these two genera demonstrated the actual presence of anammox bacteria (Figure 2) in rhizosphere and bulk soils. The Amx820 oligonucleotide probe was one of the most widely used probes for the CARD-FISH analysis (Schmid et al., 2005; Li and Gu, 2011). It was the first time that CARD-FISH was applied to detect anammox bacterial cells in flooded rice soils.

It should also be noted that the rhizosphere is an operational definition, which is dependent on experimental setup. In this study, we separated bulk soil from rhizosphere using a nylon bag, which was commonly adopted in other rhizosphere studies (Steen, 1984; Steen and Atkinson, 1991; Liu et al., 2006; Jia et al., 2013; Huang et al., 2014). Nonetheless, in reality the micro-environment around rhizosphere is a continual redox gradient that extends from the root surface to the bulk soil. This redox gradient is common for most wetland plants (Caffrey and Kemp, 1991; Pedersen et al., 1998; Lee and Dunton, 2000), therefore the findings from this study are important for both natural and constructed wetland ecosystems. This rhizosphere-driven anammox process was largely overlooked thus far. Taken together, we propose a conceptual model of N loss from paddy soil via different pathways along the redox gradient in paddy soils (Figure 4).

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