ORIGINAL ARTICLE

Nitrogen loss by anaerobic oxidation of ammonium in rice rhizosphere

San’an Nie¹, Hu Li¹, Xiaoru Yang¹, Zhaoji Zhang¹, Bosen Weng¹, Fuyi Huang¹, Gui-Bing Zhu² and Yong-Guan Zhu¹

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

An aerobic 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 rice 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% N₂ production with activities of 0.33–0.64 nmol N₂ g⁻¹ soil h⁻¹, whereas the non-rhizosphere anammox bacteria contributed to only 2–3% N₂ production with lower activities of 0.08–0.26 nmol N₂ g⁻¹ soil h⁻¹. Higher anammox bacterial cells were observed (0.75–1.4 × 10⁶ copies g⁻¹ soil) in the rhizosphere, which were twofold higher compared with the non-rhizosphere soil (3.7–5.9 × 10⁵ 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.

The ISME Journal (2015) 9, 2059–2067; doi:10.1038/ismej.2015.25; published online 17 February 2015

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 NH₃ volatilization, NO₃⁻ runoff and leaching and N₂O 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% H₂O₂ 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 nitrogen 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 (NH₄⁺), nitrate (NO₃⁻) and nitrite (NO₂⁻) were extracted from the soil with 2 M KCl and diluted prior to determination by a flow injection analyzer (FIA LC8500, 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′-AAAAACCCCTCTACTTAGTGGCC-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-phenyindole) 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 ¹⁵N labeled ammonium and nitrate

The activity and potential role of anammox and denitrification were measured at in situ soil temperatures with a ¹⁵N-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 N₂-purged media water from in situ irrigation water (total C: 0.89 mg l⁻¹; total N: 0.40 mg l⁻¹; NH₄⁺-N: 0.08 mg l⁻¹; NO₃⁻-N: 0.29 mg l⁻¹). The resulting paddy soils were then preincubated to remove residual NO₃⁻ and oxygen (Supplementary Figure S2). Subsequently, 100 μl of N₂-purged stock solution of each isotopic mixture, namely, (1) ¹⁵NH₄Cl (¹⁵N-(NH₄)₂SO₄ at 99.14%, 12 mM N), (2) ¹⁵NH₄Cl+¹⁴NO₃⁻ (KNO₃, 12 mM N) and (3) ¹⁵NO₃⁻ (¹⁵N-KNO₃ 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 mM ZnCl₂ solution. The rate and potential contribution to N₂ formation by anammox or denitrification were calculated from the excess production of ²⁰N₂ and ¹⁸N₂ in the ¹⁵NO₃⁻ 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 ¹⁵N in NO₃⁻. 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 hzsA_1594F and hzsA_1857R (Kartal et al., 2011;
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 H₂O. 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 hzsA 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 cloning (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, Cmic, N compounds (total N, Nmic) and available P and K were high. The pools of NH4⁺ and NO3⁻ from planted soils are shown in Figure 1a. Overall, the NH4⁺ concentrations were almost 1–2 orders of magnitude higher than those of NO3⁻. The NH4⁺ concentrations in the bulk soil were significantly higher than in the rhizosphere. In the rhizosphere, the NO3⁻ concentrations were significantly higher than in the bulk soil. The NO3⁻ 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 NH4⁺ and NO3⁻ 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      | SOM, g kg⁻¹ | Total N, g kg⁻¹ | Cmic, mg kg⁻¹ | Nmic, 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: Cmic, microbial biomass carbon; Nmic, microbial biomass nitrogen; SOM, soil organic matter. Parent material: quaternary red clay. Mean ± s.d. (n = 3).
Nitrogen loss by anammox in rice rhizosphere
S Ne et al

Figure 1 The concentration of NO3−, NO2− and NH4+ (a), anammox, denitrification activity and their contributions to total N2 production (b) and abundance of anammox bacteria targeting the hzsA 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 N2 production
To determine anammox rate and the potential role of anammox as a N2 producer, incubations were performed with rhizosphere soil and bulk soil under in situ temperature using a 15N isotope-tracing technique. The results showed that, in the soil samples amended with 15NH4+ no significant accumulation of 15N2-labeled gas could be detected in the rhizosphere or bulk soils (Supplementary Figure S3A), indicating that all ambient 14NO2 was consumed during preincubation. When both 15NH4+ and 14NO2 were added, 29N2 was accumulated but not 30N2 (Supplementary Figure S3B). Significant rates of both anammox and denitrification were observed in the incubations amended with 15NO3 only (Supplementary Figure S3C). It is possible that potential rates may overestimate the actual in situ activity because NH4+ and NO3− availability may limit the process in situ. However, the in situ concentration of NH4+ and NO3− were quite high (32.0–224.3 mg kg−1 and 4.6–10.1 mg kg−1, respectively). Our samples were incubated with NH4+ or NO3− 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 29N2 and 30N2 production values from the 15NO3− incubations are shown in Figure 1b. High potential rates of anammox were observed (0.33–0.64 nmol N2 g−1 soil h−1) in the rice rhizosphere for both treatments, contributing 31% and 41%, respectively, to their total N2 loss. In the non-rhizosphere zone, however, the rates of denitrification (3.66–9.42 nmol N2 g−1 soil h−1) were much higher than that of anammox (0.08–0.26 nmol N2 g−1 soil h−1). Approximately 2–3% was produced by anammox, while the remainder was denitrified to N2. Though differences were observed in denitrification activity between rhizosphere (0.74–0.92 nmol N2 g−1 soil h−1) and non-rhizosphere (3.66–9.42 nmol N2 g−1 soil h−1), denitrification was still the main contributor of N2 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 N2 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 hzsA_1594F and hzsA_1857R was applied for the quantification of anammox bacterial hzsA gene abundance in the soil using quantitative PCR assay (Harhangi et al., 2012; Wang et al., 2012b). The hzsA gene abundance was up to 0.75–1.4 × 107 copies g−1 dry soil in the rhizosphere, whereas the number of anammox bacterial genes decreased to 3.7–5.9 × 106 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
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 $\mathrm{N}_2$ production with rates of $0.33-0.64 \ \mathrm{nmol} \ \mathrm{N}_2 \ \mathrm{g}^{-1} \ \mathrm{soil} \ \mathrm{h}^{-1}$ was contributed by anammox, whereas approximately 2–3% of N was produced through anammox in bulk soils $(0.08-0.26 \ \mathrm{nmol} \ \mathrm{N}_2 \ \mathrm{g}^{-1} \ \mathrm{soil} \ \mathrm{h}^{-1})$. These findings improve our understanding of N cycle in paddy fields.

The observed anammox rates $(0.33-0.64 \ \mathrm{nmol} \ \mathrm{N}_2 \ \mathrm{g}^{-1} \ \mathrm{h}^{-1})$ in the rhizosphere were significantly higher than that in the bulk soils $(0.08-0.26 \ \mathrm{nmol} \ \mathrm{N}_2 \ \mathrm{g}^{-1} \ \mathrm{h}^{-1})$, 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 $\mathrm{NO}_3^-$ production could be the key factors in determining anammox activity.

In the present study, great variation in denitrification rate was observed between rhizosphere $(0.74-0.92 \ \mathrm{nmol} \ \mathrm{g}^{-1} \ \mathrm{soil} \ \mathrm{h}^{-1})$ and bulk soils $(3.66-9.42 \ \mathrm{nmol} \ \mathrm{N}_2 \ \mathrm{g}^{-1} \ \mathrm{soil} \ \mathrm{h}^{-1})$ (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 × 10^6 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\(_3^−\) 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).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This study is financially supported by the Natural Science Foundation of China (41002022), the Strategic Priority Research Program of Chinese Academy of Sciences (XDB15020300, XDB15020400) and the International Science and Technology Cooperation Program of China (2011DFB91710). The author Gui-bing Zhu gratefully acknowledges the support of National Natural Science Foundation of China (41322012), Humboldt Research Fellowship (1152633) and Beijing Nova Program (2011095). We also thank Ms Bei Wei for her assistance. We thank Professor Sarah Rothenberg from the University of South Carolina, USA for proofreading the manuscript.

References

Armstrong W. (1971). Radial oxygen losses from intact rice roots as affected by distance from the apex, respiration and waterlogging. Physiol Plant 25: 192–197.

Brune A, Frenzel P, Cypionka H. (2000). Life at the oxic-anoxic interface: microbial activities and adaptations. FEMS Microbiol Rev 24: 691–710.

Caffrey JM, Kemp WM. (1991). Seasonal and spatial patterns of oxygen production, respiration and root rhizome release in Potamogeton perfoliatus L and Zostera marina L. Aquat Bot 40: 109–128.

Dalsgaard T, Canfield DE, Petersen J, Thamdrup B, Acuña-González J. (2003). N\(_2\) production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. Nature 422: 606–608.

Fan G, Zhu G, Wang Y, Wang S, Wang C, Yin C. (2010). New functional microorganisms in nitrogen cycle restoration of river riparian ecosystems. Acta Scien Circum 30: 1558–1563.

Firestone MK, Smith MS, Firestone RB, Tiedje JM. (1979). Influence of nitrate, nitrite, and oxygen on the composition of the gaseous products of denitrification in soil. Soil Sci Soc Am J 43: 1140–1144.

Firestone MK, Tiedje JM. (1979). Temporal change in nitrous oxide and dinitrogen from denitrification following onset of anaerobiosis. Appl Environ Microbiol 38: 673–679.

Hammersley MR, Lavik G, Woebken D, Rattray JE, Lam P, Hopmans EC et al. (2007). Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. Limnol Oceanogr 52: 923–933.

Haug-Warburg HR, Le Roy M, van ALEN T, Hu B, Groen J, Kartal B et al. (2012). Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of anammox bacteria. Appl Environ Microbiol 78: 752–758.

Hefting M, Belmont B, Karlsson B, Rebel K, van Riessen M, Zopfi J. (2010). Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. ISME J 4: 450–454.
Humbert S, Zopfi J, Tarnawski SE. (2012). Abundance of anammox bacteria in different wetland soils. Environ Microbiol Rep 4: 484–490.

Jackson LE, Bowles TM, Hodson AK, Lazcano C. (2012). Soil microbial-root and microbial-rhizosphere processes to increase nitrogen availability and retention in agroecosystems. Curr Opin Environ Sci 5: 517–522.

Jetten MS, Strous M, Pas-Schoonen KT, Schalk J, Dongen UG, Graaf AA et al. (1998). The anaerobic oxidation of ammonium. FEMS Microbiol Rev 22: 421–437.

Jia Y, Huang H, Zhong M, Wang F-H, Zhang L-F, Zhu Y-G. (2013). Microbial arsenic methylation in soil and rice rhizosphere. Environ Sci Technol 47: 3141–3148.

Jones D, Hinsinger P. (2008). The rhizosphere: complex by design. Plant Soil 312: 1–6.

Juretschko S, Timmermann G, Schmid M, Schleifer KH, Pomerening-Roser A, Koops HP et al. (1998). Combined molecular and chemical analyses of nitrifying bacteria diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. Appl Environ Microbiol 64: 3042–3051.

Kartal B, Kelljens JT, Jetten M. (2008). The metabolism of anammox. Encyclopedia of Life Sciences (ELS). John Wiley & Sons Ltd: Chichester, UK, pp 1–9.

Kartal B, Maalcke WJ, de Almeida NM, Cîrpus I, Gloerich J, Geerts W et al. (2011). Molecular mechanism of anaerobic ammonium oxidation. Nature 479: 127–130.

Kuyppers MM, Slieters AO, Lavik G, Schmid M, Jørgensen BB, Kuenen JG et al. (2003). Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature 422: 608–611.

Kuyppers MM, Lavik G, Woebken D, Schmid M, Fuchs BM, Amann R et al. (2005). Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. Proc Natl Acad Sci USA 102: 6478–6483.

Lam P, Jensen MM, Lavik G, McGinnis DF, Muller B, Schubert CJ et al. (2007). Linking crenarchaeal and bacterial nitrification to anammox bacteria in the Black Sea. Proc Natl Acad Sci USA 104: 7104–7109.

Lee KS, Dunton KH. (2000). Diurnal changes in pore water sulfide concentrations in the seagrass Thalassia testudinum beds: the effects of seagrasses on sulfide dynamics. J Exp Mar Biol Ecol 255: 201–214.

Li M, Gu JD. (2011). Advances in methods for detection of anaerobic ammonium oxidizing (anammox) bacteria. Appl Microbiol Biotechnol 90: 1241–1252.

Liu D, Fang S, Tian Y, Chang SX. (2014). Nitrogen transformations in the rhizosphere of different tree types in a seasonally flooded soil. Plant Soil Environ 60: 249–254.

Liu WJ, Zhu YG, Hu Y, Williams PN, Gault AG, Meharg AA et al. (2006). Arsenic sequestration in iron plaque, its accumulation and speciation in mature rice plants (Oryza sativa L.). Environ Sci Technol 40: 5730–5736.

Neef A, Amann R, Schlesner H, Schleifer K-H. (1998). Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144: 3257–3266.

Pedersen O, Borum J, Duarte CM, Fortes MD. (1998). Oxygen dynamics in the rhizosphere of Cymodocea rotundata. Marine Ecol Progr Ser 169: 283–288.

Richardson AE, Barea J-M, McNeill AM, Prigent-Combaret C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321: 305–339.

Risgaard-Petersen N, Meyer RL, Schmid M, Jetten MSM, Enrich-Prast A, Rysgaard S et al. (2004). Anaerobic ammonium oxidation in an estuarine sediment. Aquat Microb Ecol 36: 293–304.

Sato Y, Ohta H, Yamagishi T, Guo Y, Nishizawa T, Rahman MH et al. (2012). Detection of anammox activity and 16S rRNA genes in ravine paddy field soil. Microbes Environ 27: 316–319.

Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M et al. (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst Appl Microbiol 23: 93–106.

Schmid MC, Maas B, Dapena A, de Pas-Schoonen KV, de Vossenberg IV, Kartal B et al. (2005). Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. Appl Environ Microbiol 71: 1677–1684.

Shen L, Liu S, Lou L, Liu W, Xu X, Zheng P et al. (2013). Broad distribution of diverse anaerobic ammonium-oxidizing bacteria in Chinese agricultural soils. Appl Environ Microbiol 79: 6167–6172.

Slieters AO, Derwert N, Campos-Gomez JL, Strous M, Kuenen JC, Jetten MSM. (2002). Completely autotrophic nitrogen removal over nitrite in one single reactor. Water Res 36: 2475–2482.

Steen E. (1984). Variation of root-growth in a grass ley studied with a mesh bag technique. Swed J Agric Res 14: 93–97.

Steen E, Atkinson D. (1991). Usefulness of the mesh bag method in quantitative root studies. Plant Root Growth: An Ecological Perspective. Blackwell scientific publications LTD: Oxford, England, UK, pp 75–86.

Strous M, Heijnen JJ, Kuenen JC, Jetten MSM. (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl Microbiol Biotechnol 50: 589–596.

Thamdrup B, Dalsgaard T. (2002). Production of N2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl Environ Microbiol 68: 1312–1318.

Wallner G, Amann R, Beisker W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14: 136–143.

Wang J, Gu J. (2013). Dominance of Candidatus Scalindua species in anammox community revealed in soils with different duration of rice paddy cultivation in Northeast China. Appl Microbiol Biotechnol 97: 1785–1798.

Wang S, Zhu G, Peng Y, Jetten MS, Yin C. (2012a). Anammox bacterial abundance, activity, and contribution in riparian sediments of the Pearl River estuary. Environ Sci Technol 46: 8834–8842.

Wang Y, Zhu G, Harhangi HR, Zhu B, Jetten MSM, Yin C et al. (2012b). Co-occurrence and distribution of nitrate-dependent anaerobic ammonium and methane-oxidizing bacteria in a paddy soil. FEMS Microbiol Lett 336: 79–88.

Xing GX, Zhu ZL. (2000). An assessment of N loss from agricultural fields to the environment in China. Nutr Cycl Agroecosys 57: 67–73.
Zhu G, Jetten MS, Kuschk P, Ettwig KF, Yin C (2010) Potential roles of anaerobic ammonium and methane oxidation in the nitrogen cycle of wetland ecosystems. *Appl Microbiol Biotechnol* **86**: 1043–1055.
Zhu G, Wang S, Feng X, Fan G, Jetten MS, Yin C. (2011a). Anammox bacterial abundance, biodiversity and activity in a constructed wetland. *Environ Sci Technol* **45**: 9951–9958.
Zhu G, Wang S, Wang Y, Wang C, Risgaard-Petersen N, Jetten MSM et al. (2011b). Anaerobic ammonia oxidation in a fertilized paddy soil. *ISME J* **5**: 1905–1912.
Zhu G, Wang S, Wang W, Wang Y, Zhou L, Jiang B et al. (2013). Hotspots of anaerobic ammonium oxidation at land-freshwater interfaces. *Nat Geosci* **6**: 103–107.
Zhu Z. (2008). Research on soil nitrogen in China. *Acta Pedologica Sin* **45**: 778–783.

Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)