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RESEARCH ARTICLE

Elevated N_2O emission by the rice roots: based on the abundances of *narG* and bacterial *amoA* genes

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Abstract Rice fields are an important source of nitrous oxide (N_2O), where rice plants could act as a key factor controlling N_2O fluxes during the flooding-drying process; however, the microbial driving mechanisms are unclear. In this study, specially designed equipment was used to grow rice plants and collect emitted N_2O from the root-growing zone (zone A), root-free zones (zones B, C, and D) independently, at tillering and booting stages under flooding and drying conditions. Soil samples from the four zones were also taken separately. Nitrifying and denitrifying community abundances were detected using quantitative polymerase chain reaction (qPCR). The N_2O emission increased significantly along with drying, but the N_2O emission capabilities varied among the four zones under drying, while zone B possessed the highest N_2O fluxes that were 2.7~4.5 times higher than those from zones C and D. However, zone A showed N_2O consumption potential. Notably, zone B also harbored the highest numbers of *narG*-containing denitrifiers and *amoA*-containing nitrifiers under drying at both tillering and booting stages. This study demonstrates that drying caused significant increase in N_2O emission from rhizosphere soil, in which the higher abundance of AOB would help to produce more nitrate and significantly higher *narG*-containing microbes would drive more N_2O production and emission.

Keywords N_2O emission · Flooding-drying · *amoA* · *narG* · *nosZ* · Rice rhizosphere

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Introduction

Nitrous oxide (N_2O), the third most important greenhouse gas after carbon dioxide and methane, contributes significantly to global warming and destruction of stratospheric ozone (Ravishankara et al. 2009). Agricultural soil is considered to be a major source of atmospheric N_2O due to the continuous increase of nitrogen fertilization (Conrad et al. 1983; Bouwman et al. 2002) and accounts for about 81% of anthropogenic N_2O emission (Isermann 1994). Paddy rice fields occupy approximately 22% of the world's grain-producing cropland (Frolking et al. 2002), and the flooding-drying management in rice production can stimulate N_2O emission (Akiyama et al. 2005; Liu et al. 2010); thus, paddy fields are an important source of N_2O emission (Minami 1987; Cai et al. 1997; Mosier et al. 1998; Yang et al. 2003; Xing et al. 2009).

Previous studies suggest that drainage can cause substantially more N_2O emission than the continuous flooded period during the rice growing season (Zheng et al. 2000; Li et al. 2005; Jiao et al. 2006) and that the growing rice roots could play an important role in this process (Reddy 1982; Jackson et al. 2008; Ishii et al. 2011). Rice roots can release oxygen (O_2) to the rhizosphere to change the redox status and promote nitrate (NO_3^-) formation by nitrification (Savant and DeDatta 1982; Keeney and Sahrawat 1986). NO_3^- can then diffuse into adjacent anaerobic zones where it is mainly denitrified into N_2O by the denitrifiers taking the *narG* genes (Liu et al. 2012),

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and N_2O is further reduced to N_2 by denitrifiers with the *nosZ* genes (Jensen et al. 1993; Liu et al. 2014). It has been indicated that the potential denitrifying activity decreases rapidly within the first few millimeters away from maize roots (Smith and Tiedje 1979). Similarly, Li et al. (1999) suggested that potential nitrification also rapidly decreases with increasing distance from the rice rhizoplane. Furthermore, Arth and Frenzel (2000) using multichannel microelectrodes to locate nitrification and denitrification in a fertilized rice paddy showed that nitrification occurred at a distance of 0–2 mm from the root surface and denitrification occurred at 1.5–5.0 mm.

Following the introduction of molecular techniques into soil science, microbial communities in the plant rhizosphere have been widely investigated. Bacterial abundance may be dynamically modified in the rhizosphere during plant growth (Philippot et al. 2013). For the functional microorganisms involved in nitrogen cycling, Hamonts et al. (2013) found that the rhizosphere of wheat hosted a bigger population of denitrifying communities than that in bulk soil. Many studies indicated that ammonia-oxidizing bacteria (AOB) are predominant nitrifiers in the paddy soils, especially in the root environment of rice (Briones et al. 2002; Nicolaisen et al. 2004; Bowatte et al. 2006, 2007; Chu et al. 2010). Nevertheless, it is unclear how the rice rhizosphere affects N_2O emission during flooding-drying cycles and what the potential microbial driving mechanisms are.

Biological processes of nitrification and denitrification are mainly performed by nitrifiers and denitrifiers (Romain et al. 2005). Ammonia oxidation is the primary and rate-limiting step in nitrification, which in turn is the rate-limiting step for denitrification and coupled nitrification-denitrification in rice paddy soils (Rao et al. 1984). The *amoA* gene encodes the key subunit of the enzyme ammonia monooxygenase catalyzing ammonia oxidation (Braker and Conrad 2011). Recent studies with different agricultural soils revealed that AOB were functionally more important in ammonia oxidation (Di et al. 2009; Jia and Conrad, 2009). Therefore, in the present experiment, we targeted the *amoA* gene to analyze the AOB community. For the denitrification, nitrate reductase and nitrite reductase are encoded by the *narG* and *nosZ* genes, respectively. In previous studies of denitrification in natural and incubated soil samples, *narG* and *nosZ* genes were preferred molecular indicators to reveal the dynamic variations of denitrifying bacteria (Palmer et al. 2010; Liu et al. 2012).

In this study, we tried to compare N_2O emission between rhizosphere and non-rhizosphere soils under flooding and drying conditions, as well as the change of N-related microbial groups (bacterial *amoA*, *narG*, and *nosZ*) at tillering and booting stages of rice growing. We aim to understand the microbial driving mechanisms underlying N_2O fluxes influenced by the growing rice roots.

Materials and methods

Paddy soil for experiment

A paddy soil (0–20 cm), derived from quaternary red clay, was collected from a rice field (28°14′08″N, 113°13′05″E, Changsha, China) in March 2013. The paddy soil had a pH 5.78 (soil/1 M KCl = 1:5), 13.28 mg C g^{-1} soil (total carbon), and 1.58 mg N g^{-1} soil (total nitrogen). The soil was air-dried, ground, sieved through 2 mm, and then mixed well for subsequent use.

Experiment design and rice cultivation

The experiment consisted of two treatments with three replications, including flooding-drying (FD) and continuous flooding (CF). Each treatment contained 24 pots, 12 for gas and soil sampling at tillering and the other 12 for sampling at the booting stage. At each sampling time, three pots were used for the following determinations: pot N_2O emission, soil redox potential (Eh), N_2O fluxes from different zones, and soil sampling. The pots for rice cultivation were specially designed (Fig. 1) and were assembled as follows: a polyvinyl chloride (PVC) cylinder (20 cm height and 15 cm diameter) was fixed on a circular PVC plate (16 cm diameter and 4 mm thickness), and a small cylinder (15 cm height and 4 cm diameter) framed with stainless wire and covered with nylon mesh (50 μm) was then installed in the pot center. Each pot including the cylinder was filled with 3.5 kg soil amended with 1.5 g urea, 0.9 g KH_2PO_4, and 0.6 g KCl. Deionized water was added to establish a 3-cm depth of free surface water, then three healthy 16-day-old rice seedlings (*Oryza sativa* L.) were transplanted into the central cylinder. The plants were grown in a greenhouse with an average day temperature of 32 °C and night temperature of 25 °C. The 3-cm depth of free surface water was maintained during the plant growth period.

The FD treatment was established as follows: on days 20 (tillering stage) and 44 (booting stage) after transplanting, the surface water was removed at 8 a.m. with a syringe and the pots were allowed to dry naturally in the greenhouse.

Gas sampling and measurement

Gas samples were collected with a static chamber (Supplementary Fig. A1) between 9:00 and 11:00 a.m. during the five consecutive days after water was drained (FD) at both tillering and booting stages; at the same time, the gas samples from CF treatment were also taken.

For determining N_2O emission from different zones, the soil core was sampled in four zones (Fig. 1): zone A (the root growing zone, 4 cm diameter) was located in the pot center enclosed by the nylon mesh (50 μm),

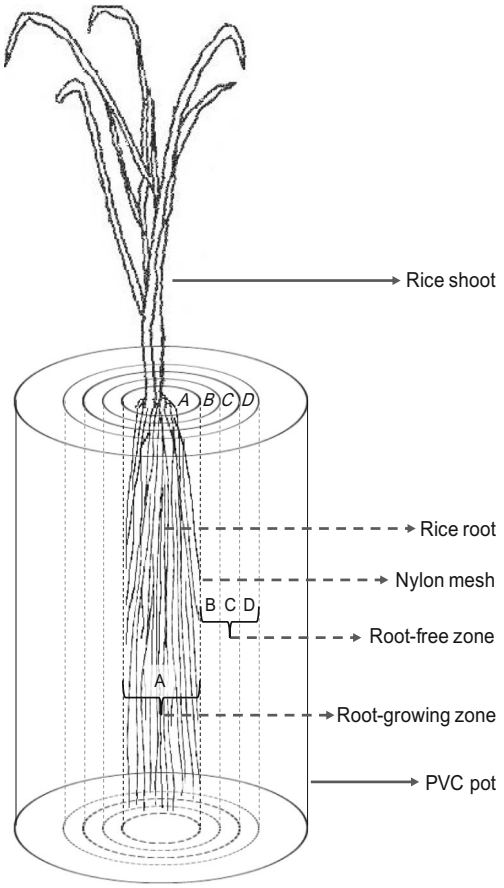

Fig. 1 Schematic diagram of the pot for rice planting. Based on the distance from rice roots, the root-free zone is divided into three zones, including zone B, zone C, and zone D; each zone has a 1-cm thickness and surrounds zone A one by one. *A*, *B*, *C*, and *D* represent zone A, zone B, zone C, and zone D, respectively. Zone A is the root-growing zone confined within the nylon mesh (50 μm) column with a 4-cm diameter. Zone B is 0–1 cm away from the nylon surface, zone C is 1–2 cm away from the nylon surface, and zone D is 2–3 cm away from the nylon surface

zone B (root-free zone 1) with a 1-cm thickness surrounding zone A, zone C (root-free zone 2) with a 1-cm thickness surrounding zone B, and zone D (root-free zone 3) with a 1-cm thickness surrounding zone-C. N_2O gas emitted from each zone was sampled after 48 h drying. All gas samples were taken 30 min after a static chamber had been placed on the columns (Supplementary Figs. A1-A2). The procedure was as follows: the first gas sample was taken for determining N_2O emission from the whole pot, the second was collected after the root column (zone A) was replaced with a top sealed cylinder, the third was taken after zones A and B were blocked with the same method as above, and the fourth was carried out after zones A, B, and C were blocked. The detailed information about the equipment and gas sampling methodology are provided in the Supplementary Material. N_2O was detected using a gas chromatograph with a uECD detector (Agilent 7890A, USA).

Soil sampling and analysis of physicochemical properties

The pots for soil sampling were assembled from two semicircular PVC pipes held together by two stainless steel hoops and sealed onto a PVC bottom plate with silicone gel (Supplementary Fig. A3). Rice plants were cultivated as stated above. Soil samples in the FD treatment were taken 48 h after the surface water was drained. While soil samples from the CF treatment were collected immediately after the surface water was removed. The sampling procedure started by taking the hoops away and cautiously removing one semicircular PVC pipe. Then, part of soil was quickly cut off to produce a vertical soil profile with a sterilized scalpel. Three sterilized soil samplers shaped as squared pipe with an open side (20 cm length and 1 cm width) were immediately and vertically inserted into the soil profile side by side just beside the root cylinder and then separated from the soil profile with a sterilized L-shaped knife. Soil samples were collected from each sampler by discarding the top and bottom parts (2 cm each). These processes were carried out repeatedly until the amounts of soil collected were enough for analysis. These soil samples were labeled as zones B, C, and D. Finally, soil samples inside the root growth cylinder (zone A) were collected by cutting the nylon mesh and taking the soil sample within a 1-cm distance of the nylon mesh. Root residues were removed from these samples. All the samples were well mixed and divided into two portions: one was quickly frozen in liquid nitrogen and stored at −80 °C for molecular analysis, and the other was stored at 4 °C for soil chemical analysis.

Soil moisture was determined gravimetrically by drying the soil at 105 °C for 24 h, and all results were expressed on an oven-dry basis. Soil ammonium and nitrate were extracted with 2 M KCl (1:5, *w*/*v*) and measured by an Automatic Flow Injection Analyzer (FIAstar 5000, FOSS, Sweden). Dissolved organic carbon (DOC) was extracted by 0.5 M K_2SO_4 (1:4, *w*/*v*) and measured by an organic carbon analyzer (TOC-VMP, Shimadzu, Japan). Soil pH was measured through the suspension of 1 M KCI (dry soil/solution = 1:5, *w*/*v*) with a pH meter (FE-20, Mettler Toledo, China). Four redox electrodes were vertically inserted into each zone at 5 cm depth, and in situ soil Eh was determined by an Eh meter (PRN-41, Fujiwara, Japan).

Soil DNA extraction

Total soil DNA was extracted from 0.3 g soil (dry weight) by the method according to Chen et al. (2010). At the end of extraction, DNA quality was determined using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA), then stored at −20 °C for analysis.

Quantitative PCR

Primer sets of amoA-1F/amoA-2R (Rotthauwe et al. 1997), narG-571F/narG-773R, and nosZ-1126F/nosZ-1381R (Chen et al. 2012) were used to amplify targeting gene fragments of bacterial *amoA* (491 bp), *narG* (203 bp), and *nosZ* (256 bp), respectively. The 10 μL reaction mixture contained 5 μL of SYBR Green PCR Master Mix (Takara SYBR® Premix Ex Taq™), 0.3 μL of each primer (10 μM), 0.2 μL of ROX Reference Dye II, and 5 ng of DNA or 1 μL plasmid DNA containing objective gene fragments. Thermal programs were run with an ABI PRISM 7900 system (Applied Biosystems, USA) in triplicate using the following thermal conditions: (1) for bacterial *amoA*, 95 °C, 2 min; 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s; (2) for *narG*, 95 °C, 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 10 s; and (3) for *nosZ*, 95 °C, 30 s; 40 repeats of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 10 s. Standard curves were established using 10-fold serial dilutions of plasmid DNA as described by Henry et al. (2004). The specific amplification of each gene was checked by dissociation curves or gel electrophoresis.

Statistical analysis

The one-way ANOVA method was used to test the significant differences of soil properties, gene abundance, and N_2O fluxes using SAS system for Windows 8.0. Figures were generated using OriginPro 9.0. Pearson's correlation coefficients were calculated using SPSS 13.0.

Results

Influence of growing roots on soil properties

The results indicate that there were similar trends in soil properties among the four soil zones at both the tillering and booting stages (Table 1). The root growing zone (zone A) showed major differences compared with the root-free zones; it possessed significantly lower water, NH_4^+-N and NO_3^-N contents, and significantly higher pH and DOC concentrations. Among the three root-free zones, zone B was obviously affected by the growing roots. The soil pH and DOC contents in zone B were higher than those in zone D, while the NH_4^+-N contents were significantly lower. Most soil properties were not significantly different between zone C and zone D except for the NH_4^+-N content at the booting stage. The results indicate that the soil properties of zone A and zone B were obviously influenced by the growing rice roots. It was observed that DOC, soil Eh, and moisture were changed dramatically after water was drained, compared with other three properties.

Dynamics of N_2O emission

The flooding-drying (FD) treatment caused continuously sharp increases in N_2O emission rates after drainage at the tillering stage, from 2 μmol m^{-2} h^{-1} at the beginning of drying to 57 μmol m^{-2} h^{-1} on day 4 (Fig. 2a). A similar increasing trend appeared at the booting stage, but the flux rates were much lower than the counterparts at tillering and declined at day 3 as the drying continued. In contrast, the N_2O fluxes in the continuous-flooding (CF) treatment remained at baseline levels ranging from 0.03 to 3.81 μmol m^{-2} h^{-1} at both growth stages.

Variations of the N_2O emission across soil zones

The N_2O emission rates from all the zones under continuous flooding remained at baseline levels ranging from −12.5 to 2.5 μmol m^{-2} h^{-1} (Fig. 2b). Clearly, the drying process resulted in elevated N_2O emission with different N_2O flux strength across the four zones. The averaged overall N_2O emission rates of zone A at tillering and booting were negative values (i.e., −118.6 and −93.5 μmol m^{-2} h^{-1}), suggesting that zone A contributed little to the N_2O emission of the pots. In contrast, zone B was the major contributor because the highest N_2O fluxes (i.e., 164.0 and 75.7 μmol m^{-2} h^{-1}) were consistently observed from this zone at both tillering and booting stages. Drying also caused a significant increase in N_2O emission rates from zones C and D in comparison with the corresponding zones of the CF treatment. The rates were similar for these two zones at both tillering and booting stages, but they were only about 22.6 and 33.8% of the emission from zone B at tillering and booting, respectively.

Variations in the abundances of nitrifiers and denitrifiers

The abundance of bacterial *amoA* (AOB) obviously varied across the soil zones, ranging from 8.7×10^5 to 2.3×10^6 copies per gram of dry soil (Fig. 3a). Compared to the CF treatment, there were significantly ($P < 0.05$) higher AOB abundances in the FD treatment in all soil zones at both tillering and booting stages. In addition, the distribution patterns of AOB among the soil zones differed between the two growth stages. At the tillering stage, the AOB population sizes in the FD treatment were all high with no significant differences between the zones. However, there were significant differences among the zones at the booting stage, with zone B hosting the highest amount of AOB and zone A containing the lowest copy numbers. Overall, AOB was enriched in zone B under both flooding and drying conditions.

In most cases, the FD treatment resulted in significant increases in the abundances of denitrifying *narG* gene in all the soil zones in comparison with the CF treatment, and the *narG* copy numbers of the zones of the FD treatment at tillering

Table 1 Soil properties from the four zones in flooding-drying (FD) and continuous flooding (CF) treatments at tillering and booting stages

			Parameters					
Growing period	Treatments	Zones	Eh (mv)	pH	Moisture (*w/w* %)	NH_4^+-N (mg N kg^{-1})	NO_3^--N (mg N kg^{-1})	DOC (mg C kg^{-1})
Tillering stage	FD	A	249 ± 36b	5.94 ± 0.02a	22.9 ± 2.0b	51 ± 29c	0.64 ± 0.02b	1036 ± 204a
		B	340 ± 21a	5.64 ± 0.10ab	35.4 ± 0.2a	286 ± 16b	0.90 ± 0.04a	271 ± 31b
		C	315 ± 12a	5.45 ± 0.14bc	34.5 ± 0.7a	328 ± 45ab	0.86 ± 0.06a	254 ± 36b
		D	347 ± 9a	5.33 ± 0.13c	35.4 ± 0.2a	367 ± 17a	0.88 ± 0.02a	233 ± 39b
	CF	A	–238 ± 2a	6.26 ± 0.09a	36.0 ± 1.1b	44 ± 33c	0.67 ± 0.01a	1523 ± 102a
		B	–246 ± 2b	5.42 ± 0.11b	45.7 ± 0.9a	256 ± 14b	0.71 ± 0.02a	430 ± 37b
		C	–248 ± 4b	5.37 ± 0.12b	45.2 ± 0.9a	348 ± 17a	0.69 ± 0.02a	398 ± 35b
		D	–247 ± 4b	5.44 ± 0.03b	45.2 ± 0.9a	371 ± 12a	0.70 ± 0.03a	402 ± 24b
Booting stage	FD	A	310 ± 9b	6.85 ± 0.19a	17.2 ± 0.9b	5.9 ± 1.0 d	0.68 ± 0.09b	2117 ± 402a
		B	402 ± 13a	5.04 ± 0.21b	31.8 ± 0.5a	171 ± 8c	0.84 ± 0.02a	212 ± 40b
		C	400 ± 23a	4.93 ± 0.10b	31.8 ± 0.5a	247 ± 8b	0.72 ± 0.01ab	183 ± 18b
		D	410 ± 18a	4.88 ± 0.15b	32.4 ± 0.1a	308 ± 15a	0.68 ± 0.01b	170 ± 14b
	CF	A	–264 ± 5b	6.80 ± 0.05a	31.3 ± 1.1b	4.4 ± 0.4 d	0.67 ± 0.06b	2246 ± 94a
		B	–234 ± 7a	5.41 ± 0.21b	45.1 ± 1.2a	137 ± 8c	0.81 ± 0.07a	296 ± 28b
		C	–241 ± 1a	5.23 ± 0.12b	44.4 ± 0.5a	246 ± 7b	0.73 ± 0.01ab	242 ± 14b
		D	–244 ± 2a	5.07 ± 0.10b	44.4 ± 0.5a	329 ± 14a	0.71 ± 0.03ab	251 ± 30b

The lowercase letters mean the significant differences among the four zones in each treatment within each growing stage ($P < 0.05$)

Eh soil oxidation-reduction potential, *pH* soil pH, *DOC* dissolved organic carbon, *TN* total nitrogen. Values are means with standard deviation from three replicates

were almost double those of the corresponding zones of the same treatment at the booting stage (Fig. 3b). In contrast, the CF treatment maintained similarly low *narG* abundances between the two stages and among the soil zones. It is obvious that the distribution patterns of *narG* abundance between soil zones in the CF treatment were quite similar between the tillering and booting stages. Zone B harbored the highest abundances of *narG* with 1.2×10^9 and 6.6×10^8 copies per gram of dry soil at tillering and booting, respectively, which were significantly higher than those of other zones ($P < 0.05$) at both stages. In addition, zones A and C had similar population sizes of *narG* and they were significantly higher than for zone D at the tillering stage. However, at the booting stage, zone C still harbored a significantly higher abundance of *narG* than zone D, whereas the *narG* copy number in zone A dropped to the lowest level.

The distributions of the denitrifying *nosZ* gene among the four soil zones were different from those observed for *narG*. The FD treatment also induced significant increases of *nosZ* copy numbers across the soil zones at both growth stages compared with the CF treatment, and the *nosZ* abundances at the tillering stage were about two times higher than at the booting stage (Fig. 3c). However, the population sizes of *nosZ*-containing denitrifiers between the zones were not significantly different either at the tillering stage or at the booting stage under drying, although zones C and D contained slightly higher *nosZ* copies than zones A and B. The *nosZ* copy numbers maintained similar low levels under flooding (CF) at both growth stages, with a downward trend from zone A to zone D, varying from 1.1×10^7 to 3.4×10^7 copies per gram of dry soil.

Pearson's correlation coefficients between N_2O fluxes and soil parameters

N_2O fluxes from the root-free zone were positively and significantly related to the abundance of *narG* ($r = 0.797$), *nosZ* ($r = 0.624$), bacterial *amoA* ($r = 0.470$), soil Eh ($r = 0.567$), and NO_3^- ($r = 0.619$) (Table 2). Soil NO_3^- was positively and significantly related to the numbers of bacterial *amoA* ($r = 0.756$) and soil Eh ($r = 0.468$). Meanwhile, the population of *narG*-containing denitrifiers was positively and significantly related to the population of nitrifiers ($r = 0.720$, bacterial *amoA*).

Discussion

It was observed in this study that zone A showed negative N_2O flux rates, especially during the drying process. This phenomenon might be due to the following reasons. Firstly, all the rice roots were restricted to grow in zone A; consequently, available nutrients such as NH_4^+ could be exhausted by plant uptake, as evidenced by the significantly lower NH_4^+ concentration in this zone, which was consistent with other

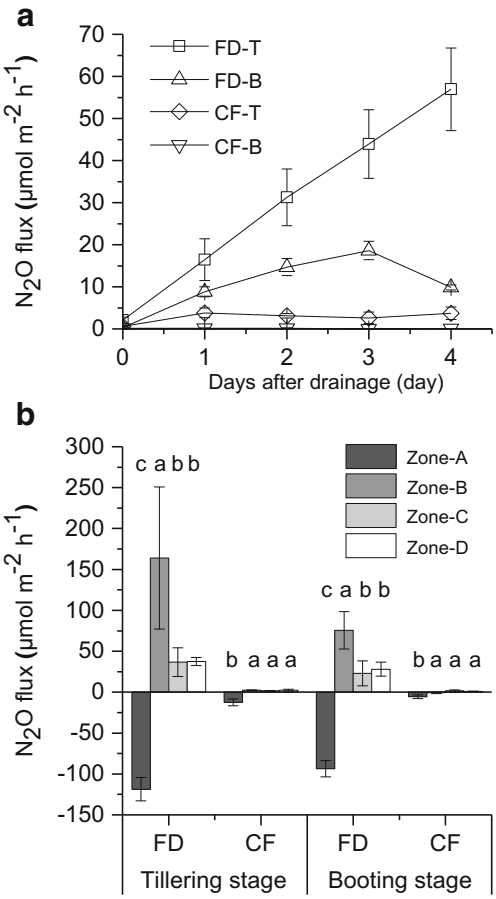

Fig. 2 Dynamics of total N_2O emission from the whole pot (**a**): *FD-T* flooding-drying at the tillering stage, *FD-B* flooding-drying at the booting stage, *CF-T* continuous-flooding at the tillering stage, *CF-B* continuous flooding at the booting stage. *Day 0* represents the day when water was drained. N_2O fluxes from the four different zones (**b**) in the two treatments at tillering and booting stages. Values are means with standard deviation from three replicates. *Means with the different letters* are significantly different between the zones within one treatment at each growing stage ($P < 0.05$)

researches (Li et al. 2008; Nie et al. 2015). Therefore, soil nitrification and denitrification would be restricted due to the limitation of substrates (Arth and Frenzel 2000). Secondly, as the plants grew, the volume occupied by the roots increased, which resulted in soil compression and changes in soil physicochemical characters such as the higher pH and the lower Eh compared with the root-free zones (Table 1), and similar results were observed due to intensive soil compaction (Stepniewski et al. 1994; Glab and Gondek 2013). Although we could not estimate such effects on N_2O emission in natural ecosystems, they might also exist in paddy soils. However, their influence might be very limited because natural fields are open systems and rice roots grow freely. The volume of a single rice root is very small and could not cause significant compression to its surrounding soil. Therefore, growing rice roots in the field might not result in an obvious N_2O consumption effect in the rhizosphere.

Zone B was adjacent to zone A in which most of the main roots were distributed along the nylon mesh (Supplementary Fig. A4). Accordingly, zone B could be intensively influenced by the growing roots and could also represent the rhizospheric effects. Although drying resulted in significant increases of N_2O fluxes from zones B, C, and D compared to their flooded controls, zone B emitted the highest amounts of N_2O that were several times higher than zones C and D at tillering and booting stages, suggesting that the N_2O emitting process might be unique and could be linked to the influence of the growing rice roots. Previous studies have demonstrated that growing rice roots can modify soil properties such as O_2, Eh, N, and other characteristics in the rhizospheric region (Kirk et al. 1993; Kirk 2001; Kögel-Knabner et al. 2010), but whether these influences favor N_2O emission is an open question. Since N_2O is closely related to soil Eh and water content, when soil Eh is between 300 and 400 mV (Hou et al. 2000; Jiao et al. 2006) and water

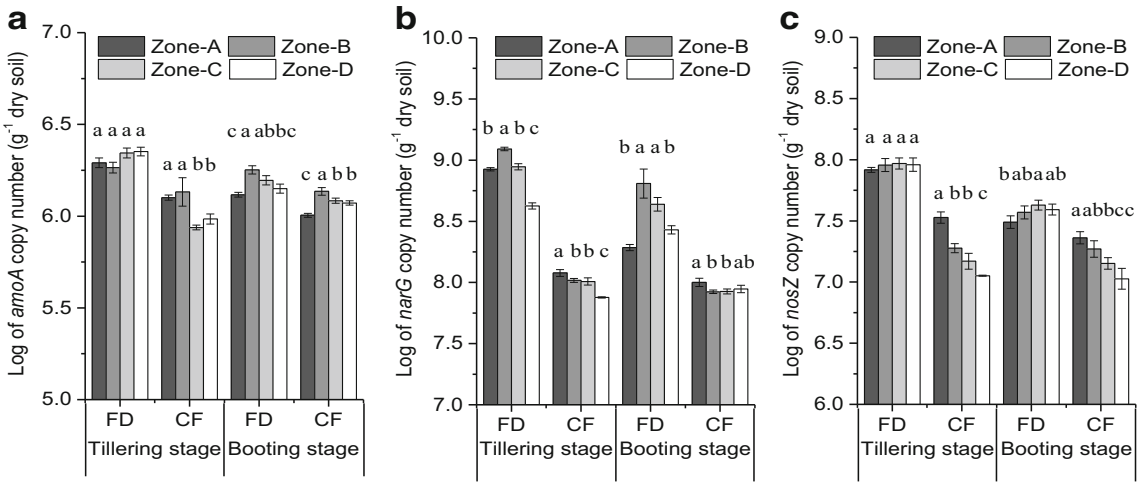

Fig. 3 Abundance of the bacterial *amoA* (**a**), *narG* (**b**), and *nosZ* (**c**) genes in the four different zones under flooding-drying (FD) and continuous flooding (CF) treatments at tillering and booting stages. Values are means with standard deviation ($n = 3$). *Means with the different letters* are significantly different between the zones within one treatment at each growing stage ($P < 0.05$)

Table 2 The relationships between parameters from soils in root-free zones in flooding-drying and continuous flooding treatments

Parameters	Pearson's correlation coefficients (n = 36)									
	N_2O	bacterial *amoA*	*narG*	*nosZ*	Eh	pH	Moisture	NH_4^+-N	NO_3^--N	DOC
Bacterial *amoA*	0.470**	1								
narG	0.797**	0.720**	1							
nosZ	0.624**	0.887**	0.819**	1						
Eh	0.567**	0.748**	0.726**	0.748**	1					
pH	0.257	0.067	0.25	0.27	–0.278	1				
Moisture	–0.510**	–0.691**	–0.673**	–0.660**	–0.986**	0.382*	1			
NH_4^+-N	–0.065	–0.032	–0.012	0.221	–0.018	0.174	0.071	1		
NO_3^--N	0.619**	0.756**	0.699**	0.737**	0.468**	0.383*	–0.384*	–0.169	1	
DOC	–0.29	–0.498**	–0.351*	–0.384*	–0.691**	0.588**	0.739**	0.24	–0.233	1

*Correlation is significant at the 0.05 level; **correlation is significant at the 0.01 level

content is between 60 and 90% WFPS or 30–40% mass water content (Skiba et al. 1997; Bateman and Baggs 2005; Ruser et al. 2006), a large amount of N_2O can be emitted to the atmosphere. We detected that soil Eh and water content were similarly high across the three root-free zones at both tillering and booting stages during drying and were favorable for N_2O emission; hence, these two factors should not be the causes for the differential N_2O emission between the zones. The availability of nitrogen substrates has been suggested as another important factor controlling N_2O emission (Delaune et al. 1998; Arth and Frenzel 2000). Although we observed that the NH_4^+-N content in zone B was significantly lower than in zones C and D at all cases, the minimum concentration was 137 mg kg^{-1}, which was still adequate for nitrifying and denitrifying reactions (Henderson et al. 2010; Jørgensen et al. 2012). Generally, NH_4^+ concentrations should be positively correlated to nitrate concentrations in a homogenous soil (Kleineidam et al. 2011; Wang et al. 2011). However, the variation in NO_3^--N concentrations in this study did not follow this rule. It was found that zone B possessed the highest NO_3^--N concentrations under flooding and drying conditions, indicating that nitrification in zone B might be stimulated by rice roots. Since NO_3^- is a main product of nitrification and an important substrate for denitrification, the measured NO_3^- concentrations were the balance of nitrification, denitrification, and plant uptake. The different distributions of ammonium and nitrate across the soil zones suggest that the nitrification and denitrification processes should be substantially different among the three root-free zones.

In relation to nitrifying microbes, it was found that zone B harbored the most abundant ammonia-oxidizing bacteria (AOB) among the zones under both flooding and drying conditions. Since ammonia oxidation is the first and rate-limiting step in the nitrification process (Chu et al. 2009), a high abundance of AOB has normally indicated a high nitrifying ability in various soils (Jia and Conrad 2009; Boyd et al. 2011; Wertz et al. 2012; Wang et al. 2015). Thus, the relatively higher AOB cell numbers in zone B would mean that more NH_4^+ could be converted into NO_3^- (Rao et al. 1984). Although nitrification can produce some N_2O (Braker and Conrad 2011), the water content in zone B was maintained at more than 31% under drying condition which is suitable for denitrification and generation of N_2O (Bateman and Baggs 2005). Therefore, the increased AOB population size in zone B would mainly help to produce nitrate for the further denitrification, which is supported by the distribution pattern of NO_3^--N concentrations in the root-free zones.

At the same time, two denitrifying bacterial communities were investigated in the present study: (1) the *narG*-containing denitrifiers are linked to N_2O production, and (2) the *nosZ*-bearing communities are linked to N_2O consumption. It is obvious that drying resulted in significantly higher copy numbers of *narG* in zone B compared with the other zones at tillering and booting stages; however, there were no differences among the zones under flooding conditions. This scenario would imply that the growing roots did not influence the abundance of *narG*-containing microorganisms under flooding condition. In contrast, drying induced significant increases in *narG*-containing population sizes and obvious differentiations between the soil zones, although zone B was outstanding and possessed the highest *narG* copy numbers which were significantly higher than those in the other zones. These results mean that the increased *narG*-containing cell numbers, induced by drying in the rice rhizosphere, could be related to higher N_2O production.

Although rice roots can continuously secrete small molecules, such as DOC and O_2, to the rhizosphere which can influence soil microorganisms (Rovira 1965; Kraffczyk et al. 1984; Jones 1998; Baudoin et al. 2003; Bertin et al. 2003; Chaparro et al. 2014), such secretions would influence most rhizospheric bacteria rather than a specific group. If root

exudates can affect *narG*-containing community abundance, that could be reflected between the soil zones under flooding condition. However, we failed to detect such clear differences among the zones, which means that the growing rice roots might have little influence on *narG*-containing communities under continuous flooding. The question is why drying resulted in significant differentiations of *narG*-containing population size between the zones. From our results, drying caused significant reductions of water content in the three root-free zones to a suitable range for N_2O emission, which means that the soil conditions were suitable for the development of nitrifiers and denitrifiers (Bateman and Baggs 2005). The decreasing trend in NH_4^+-N concentrations from zone D to zone A under flooding would mainly reflect the transportation of NH_4^+ from high to low concentration pools driven by plant uptake (Nie et al. 2014, 2015). However, among the three root-free zones, drying only induced higher NH_4^+-N concentrations in zone B compared with the corresponding zone in the flooded treatment at both tillering and booting stages and also possessed higher NO_3-N contents. Since zone B obviously contained higher root exudates, e.g., DOC, than zones C and D, drying could stimulate the mineralization of organic nitrogen resulting in increases in NH_4^+ content (Devêvre and Horwáth 2000). Nitrification would then be enhanced increasing the NO_3^- supply; as a consequence, the cell numbers of the *narG*-containing denitrifier would be increased to convert the accumulated nitrate. If that is the case, the rice rhizosphere would have a strong capability to produce N_2O during flooding-drying cycles.

The function of *nosZ*-containing microorganisms is to consume N_2O in soil. We determined that drying induced sharp increases of *nosZ* copy numbers in all the soil zones, but there were no significant differences among the zones. This finding suggests that both the rhizosphere and bulk soil would possess similar N_2O consumption capabilities under drying conditions. In contrast, the *nosZ* copy numbers across the zones under flooding conditions remained at low levels but the highest *nosZ* abundance appeared in zone A, declining gradually from zone A to zone D. Although the reasons for the outstanding differential responses of *nosZ* copy numbers in relation to flooding and drying remain unclear (Liu et al. 2012), the amount of N_2O produced would be a key factor. Under the flooding environment, the N_2O production ability is very limited, but the rhizosphere possesses relatively higher N_2O generating ability than the bulk soil due to the influence of root exudates (Hamonts et al. 2013), therefore, rhizosphere soil contains higher *nosZ* copy numbers. However, drying stimulated significantly higher N_2O production and the copy number of *nosZ*-containing bacteria quickly increased to its maximum level as N_2O concentrations increased to a certain level; therefore, when rhizosphere and bulk soils reached or exceeded such a level, they harbored similar *nosZ*-containing population sizes without significant differences. The *nosZ* copy numbers would not respond to further increases in N_2O production; as a consequence, *nosZ* abundance under such a circumstance would not be closely related to N_2O fluxes during the flooding-drying process (Liu et al. 2012). The maximum *nosZ*-containing population size under drying would vary based on soil conditions and plant growth. The *nosZ* copy numbers at the tillering stage were much higher than those at the booting stage, which would be linked to the reduction of soil available nutrients during the longer plant growth period.

Conclusions

Rice growth can significantly increase N_2O emission during the flooding-drying process; this increase mainly occurred in the rhizosphere region. The major microbial mechanisms involved would be that drying caused significant elevation of the abundance of nitrifiers because of more accessible nitrogen sources in the rhizosphere; as a result, more nitrate could be produced in this region. Consequently, significantly higher numbers of denitrifiers (mainly N_2O producers) were generated. Therefore, stronger nitrifying and denitrifying activities coexisted in the rhizosphere during the drying process, thus driving N_2O production and emission.

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