**Responses of nitrifying and denitrifying bacteria to flooding-drying
cycles in flooded rice soil**

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Abstract The flooding-drying cycle can cause obvious increases of nitrous oxide (N₂O) emissions from paddy soil. However the relationships between N₂O flux and N₂O concentrations in soil and the microbial driving mechanisms during the flooding-drying process are unclear. In this study, a flooding-drying incubation experiment was carried out with a paddy soil. The topsoil (0-6 cm) was divided into 6 micro-sublayers each of 1 cm depth which were sampled independently. Terminal restriction fragment length polymorphism (T-RFLP) and real-time quantitative polymerase chain reaction (qPCR) were employed to determine the community composition and abundance of nitrifiers and denitrifiers, respectively. Results showed that the dynamics of N₂O flux were more closely related to the N₂O concentrations at 2-3 cm in comparison with that at 4-5 cm depth in the soil profile. During the peak period of N₂O flux, the top three micro-sublayers (0-3 cm) simultaneously harbored significantly higher ammonia oxidizing bacteria (AOB) population sizes, and contained higher nitrate and lower ammonia concentrations. Therefore, the top soil (0-3 cm) possesses a strong ability to produce nitrate substrate for denitrification during the flooding-drying process, and the drying surface soil, with O₂ penetration, favoured N₂O generation. In contrast, although the bottom soil (4-6 cm) contained abundant nitrate reductase gene (*narG*) copy numbers, it maintained low levels of AOB abundance, which could suggest that low nitrifying activity would be the major restriction limiting N₂O production in this layer. In conclusion, the flooding-drying process induced significant N₂O emissions from the paddy soil, which were closely related to the increasing nitrifying capability in the topsoil within 0-3 cm and the dynamics of N₂O concentrations at 2-3 cm depth.

Key words Paddy Soil · Flooding-Drying · Micro-sublayer · Nitrifier · Denitrifier

1. Introduction

Nitrous oxide (N₂O) is a potent greenhouse gas contributing to global warming with 298 times higher global warming potential than carbon dioxide (CO₂) on a 100-year

time scale (Pachauri and Reisinger, 2007). N₂O is also involved in the destruction of stratosphere ozone (Ravishankara et al., 2009). Agricultural systems are the main emission source of anthropogenic N₂O, and accounted for 58% of the global anthropogenic N₂O emissions in 2005 (Metz et al., 2007). Paddy rice fields comprise about 10% of the world's cultivated land, and the properties of paddy soils are greatly affected by human activities. Among various management practices, flooding-drying is a common manipulation for rice cultivation, and large amounts of N₂O are emitted during this process (Xu et al., 1997; Peng et al., 2011). However, the microbial driving mechanisms of N₂O emissions during this process are unclear.

Soil N₂O emissions are influenced by various environmental factors, such as temperature, moisture, substrate availability and pH and etc. (Skiba et al., 1998; Hou et al., 2000; Smith et al., 2003). Among them, soil moisture is considered as a key factor (Weitz et al., 2001; Jauhiainen et al., 2012). Soil moisture influences soil redox status (Xing et al., 2002) and also affects the activities of nitrifiers and denitrifiers (Jha et al., 1996; Amha et al., 2011; Ruser et al., 2006; Scheer et al., 2008). It has been shown that substantial N₂O fluxes occur when water filled porosity (WFPS) is between 70 to 90% (Dobbie et al., 1999; Hou et al., 2012; Xu et al., 2013). In paddy fields, N₂O fluxes mainly occur during the flooding-drying process while they are at minimum levels under flooding (Minami et al., 1987; Chen et al., 1997; Tsuruta et al., 1997).

N₂O fluxes from agricultural soils have been intensively studied in recent years (Freney, 1997; Bateman and Baggs, 2005; Lin et al., 2012), but the relationships between the N₂O flux from soil and the N₂O concentration produced in soil profile and the mechanisms involved have rarely been investigated. Previous research has demonstrated that soil N₂O concentrations are largely related to the water table or water level, and N₂O accumulates in the narrow depth just above water table (van Groenigen et al., 2005; Jørgensen et al., 2012; Minamikawa et al., 2013). However, N₂O emissions may not always be directly related to the N₂O concentration in the soil profile (Hosen et al., 2000, 2002; Gao et al., 2014), but are dependent on its location. Generally, N₂O in the subsoil is barely emitted through the topsoil due to the

microbial consumption of N₂O during long distance diffusion (Arah et al., 1991; Hopkins et al., 1997). It is predicted that the N₂O produced in surface soil might be the important source for N₂O emissions (Gao et al., 2014; Denmead et al., 1979). Gradients of soil texture, chemical and biochemical properties within topsoil still exist, which could largely influence N₂O production, consumption and emission (Weitz et al., 2001), especially during the flooding-drying process, but the mechanisms involved remain unknown.

To date, the community composition and abundance of nitrifiers and denitrifiers in upper soil layers (0-15 cm or 0-20 cm) in relation to N₂O emissions have been well investigated. It has been demonstrated that the variations in ammonia oxidizing archaea (AOA) are not correlated to N₂O fluxes in cultivated soils (Di et al., 2010a; Andert et al., 2011) except for acidic soils where AOA are the major functional nitrifiers (Lehtovirta-Morley et al., 2011; Yao et al., 2011; Jung et al., 2014). In contrast, ammonia oxidizing bacteria (AOB) are the important nitrifying community in most soils (Di et al., 2009; Xia et al., 2011) and have been linked to N₂O emissions (Di et al., 2010b; Xia et al., 2013; Di et al., 2014). Among the denitrifiers, the role of the nitrate reductase gene (*narG*), nitrite reductase genes (*nirK*, *nirS*) and nitrous oxide reductase gene (*nosZ*) have commonly been studied. It has been reported that shifts in *narG*-containing communities, including cell numbers and community composition, in the top layer (0-5 cm) of paddy soils were closely related to N₂O emissions during flooding-drying cycles while *nosZ*-containing communities, although sensitive to water content changes, were not linked to N₂O fluxes (Liu et al., 2012). Recently, Uchida et al. (2014) also found that the abundance of denitrifying gene transcripts (*nirK*, *nirS* and *nosZ*) in the top 1 cm layer of soil was more significantly related to N₂O flux than those in the 1-3 cm layer. These results suggest that sublayers within the top soil might differentially produce and emit N₂O. Therefore, it is important to understand the differential microbial driving mechanisms of N₂O production and emission of defined micro-sublayers within the top soil, which would be crucial for efficient nitrogen fertilization and manipulation of N₂O emissions in paddy fields. We hypothesise that the differential responses of nitrifiers

and denitrifiers to the flooding-drying process within the upper soil profile could trigger the variations of N₂O production, consumption and emission.

In the present study, micro-sublayers of one centimeter thickness were sequentially sampled from soil cores. The objectives were to investigate the relationships between N₂O concentrations at various soil depths and N₂O emissions during the flooding-drying process, and the microbial driving mechanisms of N₂O production and consumption.

2. Materials and Methods

The soil sampling site is located in Changsha, China (28°14'08" N and 113°13'05" E), which had been under double rice cropping for over 100 years. The paddy soil (0-20 cm) was collected after late rice harvest in December 2012. After air-drying the soil was sieved through a 2 mm sieve while visible plant residues were removed manually. This soil was derived from quaternary red clay and classified as loamy clay (Hydragric Anthrosols) (Soil Survey Staff, 2010), its basic properties were as follows: Soil pH=5.3, total C=16 g C kg⁻¹, total N=1.7 g N kg⁻¹, NH₄⁺-N=36 mg kg⁻¹, NO₃⁻-N=5.2 mg kg⁻¹, organic matter=27 g kg⁻¹.

2.1 Soil incubation

The soil incubation experiment consisted of two groups, one for gas collection with 6 pots continuously flooded (CF) and flooding-drying (FD) treatments. The other was for soil sampling at four time points during the flooding-drying process with 12 pots. All the treatments had three replicates. Each pot (19 cm diameter, 20 cm high) contained 5 kg air-dried soil which was well mixed with 1.09 g urea (100 mg N kg⁻¹ dry soil). The pots were filled with distilled water leaving about 2 cm of free water on the soil surface and incubated at 28 °C. After 17 days under flooding, the pots for the flooding-drying treatment were drained (Liu et al., 2012) while the rests remained flooded.

For soil gas collection, twin silicon tubes (total 30 cm long, internal diameter 10

mm, wall thickness 2 mm) connected with a U type stainless steel tube were horizontally positioned at 2-3 cm and 4-5 cm from the soil surface, separately (Fig. 1). Both ends of silicon tube were closed with silicone septa. At one end, a right-angled 2-mm stainless steel tube was inserted to connect the silicone tube in the soil with the soil surface. The end of the steel tube was fitted with a three-way stopcock that enabled the soil air to be sampled with 5-ml syringes.

2.2 Gas and soil sampling

Gas samples were taken between 9 and 10 am on days 1, 6, 11, 16, 22, 26, 31, 36 after drainage using a static chamber for the determination of N₂O flux. For detecting soil N₂O concentrations, gas samples (3 mL) were collected from the silicon tubes using a syringe on the above dates, and an equal volume of helium was injected back into the tubes afterwards. The gas samples were transferred to evacuated 12 mL vials immediately (Labco limited high Eycombe, UK), and after 27 mL N₂ was injected into the vial the N₂O was analyzed using a gas chromatograph equipped with an electron capture detector (Agilent 7890A, USA). The procedures for taking gas samples to determine N₂O flux were carried out as described by Liu et al. (2012).

The soil sampling dates were decided based on the dynamics of N₂O emissions, where day 0 represents the flooded status just before drainage, days 6, 22 and 30 were in the middle of the initial, increasing and decreasing phases of N₂O emission, respectively. Soil samples from the 0-1, 1-2, 2-3, 3-4, 4-5 and 5-6 cm depths (named micro-sublayers 1, 2, 3, 4, 5 and 6) were collected independently on the above dates. The sampling was destructive and the sampled pots were discarded. For the continuously flooded treatment, the soil samples were taken immediately after the surface water was removed. The samples were divided into two portions, one was packed in foil and flash frozen in liquid nitrogen, and stored at -80 °C for molecular analysis, the other was temporary stored at 4 °C and used for chemical measurements.

2.3 Measurements of soil NH₄⁺-N and NO₃⁻-N concentrations

Soil NH_4^+ -N and NO_3^- -N in the CF and FD treatments were extracted with 50 mL 2 mol L^{-1} KCl from 10 g fresh soil, and determined using an Automatic Flow Injection Analyzer (FIA star 5000, FOSS, Sweden).

2.4 DNA extraction

Soil total DNA was extracted according to Chen et al. (2010) with minor modifications. At the first step, the MP FastPrep[®]-24 (MP Biomedicals, USA) was used instead of a 1 h water bath treatment. Total DNA was quantified using Nanodrop ND-1000 (Wilmington, DE, USA).

2.5 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was performed to quantify the archaeal and bacterial ammonia oxidase genes (*amoA*), nitrate reductase genes (*narG*) and nitrous oxide reductase genes (*nosZ*). The primer pairs employed are listed in Table 1. The reaction mixture (10 μL) contained 5 μL SYBR green mix (Takara, Dalian China), 0.4 $\mu\text{mol L}^{-1}$ of forward and reverse primer, and 5 ng of template DNA. Thermal cycling conditions for the archaea *amoA* and the bacterial *amoA* gene were as follows: 95 °C for 2 min; 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Thermal cycling conditions for *narG* and *nosZ* were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 10 s. The standard curves used to quantify the archaea *amoA*, bacterial *amoA*, *narG* and *nosZ* genes were created using a 10-fold dilution series of plasmids containing the target gene.

2.6 PCR amplification and T-RFLP assay

The primers of Arch-*amoA* 23F/616R, *narG* 145F/773R and *nosZ*-F/*nosZ*-R (Table 1)

were used for PCR amplification of archaea *amoA*, *narG* and *nosZ* genes, respectively. The forward primers were labeled with 6-carboxyfluorescein at the 5' ends. The reaction was performed in a volume of 50 μ L with 25 μ L PCR mix (TransGen, China), 0.2 μ mol L⁻¹ forward and reverse primer, about 80 ng of template DNA for archaea *amoA*, *narG* gene; 0.1 μ mol L⁻¹ forward and reverse primer and about 100 ng of template DNA for the *nosZ* genes. Touchdown PCR for the archaea *amoA* gene was carried out according to Qin et al. (2013), and *narG* and *nosZ* as described by Liu et al. (2012).

Amplification of bacterial *amoA* gene sequences was done using a nested polymerase chain reaction (PCR) protocol. First-round PCR primers used were *amoA*-2F/5R (Table 1) in a 25 μ L solution containing 12 μ L PCR mix (TransGen, China) 0.2 μ mol L⁻¹ forward and reverse primer, and about 25 ng of soil DNA. PCR amplification was carried out using the touchdown method (Webster et al., 2002). Second-round PCR primers *amoA*-1F/2R (Table 1) were used to amplify a 491-bp product using 4 μ L of the first PCR mixture as the template; the reaction solution was the same as described of archaea *amoA* above. Touchdown PCR for the bacterial *amoA* gene was carried out according to Qin et al. (2013). To generate bacterial *amoA* amplicons for T-RFLP analysis, a 6-carboxyfluorescein-labeled *amoA*-1F primer was used in the second-round PCR.

The PCR products were purified using a TIAN gel Midi Purification Kit DP209 (Tiangen biotech co., Ltd., China). T-RFLP profiles for archaeal *amoA*, bacterial *amoA*, *narG* and *nosZ* genes were generated with the endonucleases *MspI*, *TaqI*, *RsaI* and *CfoI*, respectively. The digested products were analyzed with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). The size and relative abundance of terminal restriction fragments (T-RFs) were quantified using Peak Scanner software (version 1.0, Applied Biosystems, Inc.). Fragments with a signal above 1% of the sum of all peak heights were included in the analysis. Peak positions that differed in size by less than 1 bp in an individual profile were binned and considered as one fragment (Dunbar et al., 2001; Sait et al., 2003). Principal

Component Analysis (PCA) (CANOCO version 4.53) was used for community analysis.

2.8 Statistical analysis

Statistical analysis was carried out using SPSS 17.0 (SPSS Inc., USA). One-way ANOVA was employed to test the effects of the drying process on the copy numbers of bacterial *amoA*, *narG* and *nosZ* genes, ammonium and nitrate contents in soil. Pearson's correlation analysis was carried out to test the the relationships between N_2O emission and the abundances of bacterial *amoA*, *narG* and *nosZ* genes and between N_2O concentrations and the population sizes of AOB, *narG*- and *nosZ*-containing denitrifiers. Significance was accepted at a level of probability of $P < 0.05$.

3. Results

3.1 N_2O flux and N_2O concentration

Based on the variations in N_2O emissions (Fig. 2), the drying process can be divided into three phases: an initial phase, an increasing phase and a decreasing phase. The initial phase was characterised by low levels of N_2O emission maintained from the initial drainage up to 16 days of drying. The increasing phase was from 16 to 26 days of drying during which period the N_2O emissions sharply increased by more than 8 times. The decreasing phase was from 26 to 36 days of drying during which time the N_2O flux rapidly reduced. The peak period of N_2O emission was between the 22nd and 30th days after drainage. The N_2O concentrations at both 2-3 and 4-5 cm depths in the FD treatment showed the same initial phase as for N_2O emissions and the N_2O concentrations remained as low as in the CF treatment during this period. However, the N_2O concentrations at the two soil depths responded differentially to further drying. Firstly, the N_2O concentrations at 2-3 cm increased much quicker than those at 4-5 cm, for example, the concentration at 2-3 cm on day 26 was 160 mg (N_2O) L⁻¹

which was 63.5% higher than that at 4-5 cm. Secondly, between days 31 and 36, the variations in N_2O concentrations at the two depths showed different trends. During this period, the N_2O concentration at 2-3 cm depth decreased quickly whereas at 4-5 cm they remained steadily increasing.

3.2 Variations in soil NH_4^+ -N and NO_3^- -N concentrations

Under flooded conditions (day 0) it was observed that ammonium concentrations differed between the micro-sublayers, with the lowest NH_4^+ -N concentration occurring in micro-sublayer 1 and concentrations increasing with soil depth (Fig. 3a). The NH_4^+ -N concentrations in the micro-sublayers clearly increased during 6 days of drying with one exception, the NH_4^+ -N content in micro-sublayer 1 decreased about 55% compared to day 0 (flooded conditions). At this stage, micro-sublayer 1 contained significantly lower NH_4^+ -N than micro-sublayer 2 while the other sublayers had higher NH_4^+ -N concentrations than in micro-sublayer 2 but similar concentrations to each other. Further drying up to day 22 (increasing phase of N_2O emissions) induced obvious decreases in NH_4^+ -N concentrations in all the micro-sublayers. In comparison with day 6, the greatest reductions occurred in micro-sublayers 2 and 3, which decreased by about 145 and 157 mg NH_4^+ -N kg^{-1} , respectively. The smallest decrease (35 mg NH_4^+ -N kg^{-1}) appeared in micro-sublayer 1. It was observed that the NH_4^+ -N concentrations showed a clear sequential order with depth and were significantly different from each other ($P<0.05$), with the lowest concentration occurring in the top micro-sublayer. This NH_4^+ -N concentration order was maintained

during further drying until day 30 by which time the $\text{NH}_4^+\text{-N}$ concentrations in the micro-sublayers had tended to become relatively stable.

The distribution of nitrate concentrations between the micro-sublayers showed a different picture to that for $\text{NH}_4^+\text{-N}$ (Fig. 3b). Under flooding (day 0), the $\text{NO}_3^-\text{-N}$ concentrations in all micro-sublayers were uniformly low at about 0.5 mg kg^{-1} . It was observed that the drying process induced significant increases in $\text{NO}_3^-\text{-N}$ concentration in micro-sublayer 1 alone, reaching 7.59 mg kg^{-1} on day 22 before decreasing back to 3.46 mg kg^{-1} at day 30. Similarly, drying also caused significant increases of NO_3^- contents in the remaining micro-sublayers on day 22 and 30 compared to the corresponding flooded controls although they were far less than that in micro-sublayer 1 (supplementary Fig. 1).

3.3 Influence of flooding-drying on the abundance of the amoA gene

The abundance of the bacterial *amoA* gene (AOB) in the micro-sublayers was differentially affected by drainage (Fig. 4) while the copy numbers of archaea *amoA* was not clearly influenced (data not shown). When soil was under flooding (day 0), micro-sublayer 1 harbored the highest AOB copy numbers with 2.8×10^7 copies g^{-1} dry soil while the other sublayers possessed similar AOB population sizes ranging from 3.7×10^6 to 7.1×10^6 copies g^{-1} dry soil. After 6 days drying, AOB population sizes in the micro-sublayers increased slightly compared to their respective flooded controls (day 0). However, when the drying process reached the increasing phase of N_2O emission on day 22, the most significant increases in AOB abundances occurred in micro-sublayers 1 and 2, being 3 and 15 fold greater than at day 6, respectively. However, the remaining micro-sublayers maintained similar low AOB population

sizes to those observed on day 6. Further drying up to day 30 resulted in a significant and substantial elevation of AOB copy numbers in micro-sublayer 3, being about 11 times higher than on day 6. The AOB population sizes in micro-sublayers 1 and 2 clearly reduced between days 22 and 30 but were still higher than those on day 6. The AOB abundances in the bottom three micro-sublayers remained at relatively low levels from day 6 onwards.

3.4 Impact of flooding-drying on the abundance of denitrifying genes

Flooding-drying also influenced the abundance of denitrifying communities, but the responses of the nitrate reductase gene (*narG*) and the nitrous oxide reductase gene (*nosZ*) to the drying process were different. It was observed that the average *narG* copy numbers of the six micro-sublayers during the flooding-drying process were 3.53×10^9 , 4.15×10^9 , 1.39×10^{10} and 1.38×10^{10} copies g⁻¹ dry soil on day 0, 6, 22 and 30, respectively (Fig. 5a), indicating that during the initial phase of N₂O emission the *narG*-containing population sizes were similar to those under flooding, but during the increasing and decreasing phases of N₂O fluxes the copy numbers significantly increased and remained at high levels. With regard to the individual micro-sublayers, during the first 6 days drying the *narG* abundances in all the micro-sublayers showed no obvious changes in comparison with the flooded samples (day 0). By day 22 the *narG*-containing population sizes in all micro-sublayers had obviously increased, but there were no significant differences among the micro-sublayers. Further drying (day

30) caused no additional clear changes in *narG* copy numbers, apart from micro-sublayer 1, and all micro-sublayers possessed similar *narG*-containing populations sizes.

Unlike *narG*-containing communities, *nosZ*-containing population sizes responded to drainage much quicker (Fig. 5b). After 6 days of drying, except for micro-sublayers 4 and 5, the *nosZ* copy numbers in the micro-sublayers obviously increased compared to flooded conditions (day 0), especially for the top three micro-sublayers, whose *nosZ* population sizes increased by 46%, 104% and 183%, with the highest numbers appearing in micro-sublayer 1. Thereafter, the *nosZ* abundances were generally maintained at similar high levels during further drying at both the increasing and decreasing phases of N₂O emission, with some fluctuations among the micro-sublayers.

3.5 Impact of flooding-drying on nitrifying and denitrifying community compositions

The T-RFLP data on day 22 of drying representing the increasing phase of N₂O flux, compared with flooded conditions (day 0) was employed to show the effects of flooding-drying on nitrifying and denitrifying community compositions. The plots indicated clearly that flooding-drying caused obvious shifts in the community compositions of AOB, *narG*- and *nosZ*-containing denitrifiers in comparison with the corresponding micro-sublayers under flooding, especially for the *narG*-bearing community (Fig. 6).

3.6 Correlation analysis

The data shows that the N₂O flux was significantly correlated with the N₂O concentrations at 2-3 cm ($R^2=0.817$, $P<0.01$) and 4-5 cm ($R^2=0.673$, $P<0.01$). Furthermore, it was significantly related to AOB abundances in micro-sublayers 0-1, 1-2 and 2-3 cm, but there were no clear relations between N₂O fluxes and the AOB population sizes in micro-sublayers 3-4, 4-5 and 5-6 cm (Table 2). Besides, N₂O fluxes were significantly correlated to the abundances of *narG* and *nosZ* in all 6 micro-sublayers except for the *nosZ* copy numbers in micro-sublayer 0-1 cm. The N₂O concentration at 2-3 cm was significantly correlated with the abundances of *narG* in the top three micro-sublayers and the population sizes of *nosZ*-containing microbes in micro-sublayers 2 and 3, but was only significantly related to the AOB population size in micro-sublayer 3. In addition, the N₂O concentration at 4-5 cm was significantly correlated with the copy numbers of *narG* and *nosZ* in micro-sublayers 4, 5 and 6 ($P<0.01$), but was not related to AOB abundance.

4. Discussion

Prior studies, based on stable isotope techniques, have demonstrated that the surface soil might be the important contributor to N₂O fluxes (Yano et al., 2014; Maeda et al., 2013), and that soil physical and biochemical characteristic can influence nitrogen cycling and N₂O emissions (Uchida et al., 2014; Weitz et al., 2001). In this study, we discovered that the N₂O concentrations at 2-3 cm were markedly higher than those at 4-5 cm during the increasing phase of N₂O emissions following the drying of flooded

paddy soil. Another interesting feature was that the N₂O concentration at 2-3 cm dropped significantly after N₂O emissions decreased, but continued to steadily increase at 4-5 cm at the same stage. Furthermore, statistical analysis showed that the correlation between N₂O flux and N₂O concentration at 2-3 cm ($R^2=0.817$, $P < 0.001$) was clearly higher than that between N₂O flux and N₂O concentration at 4-5 cm ($R^2=0.673$, $P < 0.001$). These findings strongly suggest that N₂O emission is more closely related to N₂O production in the top micro-sublayer 2-3 cm than the bottom micro-sublayer 4-5 cm during the drying process. These results might be linked to the fact that the drying process results in water loss from the soil surface creating soil conditions suitable for N₂O emissions, such as water content and Eh (Liu et al., 2012; Rabot et al., 2015). Continuous water evaporation could bring nitrate towards the surface from lower parts of the soil profile causing greater denitrification in uppermost soil layer (Acosta et al., 2011; Kim et al., 2011; McLaughlin, 2008). Furthermore, to be emitted, the N₂O produced at 4-5 cm would need to be transported over a longer distance and during this process more N₂O could be consumed by the denitrifiers (Clough et al., 2005, 2006). More importantly, the responses of soil microorganisms to drying down the soil profile would directly influence N₂O production, consumption and emission (supplementary Fig. 2).

Soil nitrifiers and denitrifiers are the important drivers of N₂O production and emission in soil ecosystems (Casella et al., 1986; Firestone and Davidson, 1989; Wrage et al., 2004; Poth and Focht, 1985; Webster and Hopkins, 1996). The drying process did not induce obvious shifts of bacterial *amoA*, *narG* and *nosZ* compositions between the micro-sublayers compared to the flooded treatment, however, population sizes of the above functional bacteria were altered differentially between micro-sublayers. Thus, the variations in the abundances could be more important in relation to N₂O emissions than the composition shifts. We observed that the flooding-drying process caused significant increases of AOB population sizes in the micro-sublayer 0-1 cm, 1-2 cm and 2-3 cm during the peak period of N₂O emissions (between 22 and 30 days), which might be linked to O₂ penetration as drying continued (Nicolaisen et al., 2004; Wang et al., 2009) and the increasing ammonium

supply from the decomposition of soluble organic matter (Smith and Jacinthe, 2014; Harrison-Kirk et al., 2013). High abundance of AOB normally means strong nitrification potential (Di et al., 2010a). It was also shown that the $\text{NH}_4^+\text{-N}$ decreased from the top to bottom micro-sublayers with the uppermost micro-sublayer containing by far the lowest $\text{NH}_4^+\text{-N}$ concentration during the drying process, further indicating that the top micro-sublayers possesses strong nitrifying ability (Di et al., 2010a).

Consequently, the denitrifying process would be stimulated by increasing $\text{NO}_3^-\text{-N}$ production. It was also observed that the abundance of *narG*-containing bacteria reached markedly high levels when the N_2O emission rate was high, which is in accordance with previous work (Liu et al., 2012). Since the *narG* gene encodes for nitrate reductase reducing nitrate to nitrite that can be further transformed into N_2O , *narG*-containing microorganisms are indicative for N_2O producers. We observed that the abundances of *narG* were similarly high within all the six micro-sublayers during the peak period of N_2O emission, which implies that the 6 micro-sublayers would possess similar N_2O generation potentials. Hence, the difference in N_2O concentrations in the top soil profile would mainly depend on the variations in nitrifying communities and nitrate supply. The significantly higher amounts of AOB in micro-sublayer 0-1 cm, 1-2 cm and 2-3 cm than that in the bottom micro-sublayers would suggest more nitrate could be produced in addition to nitrate accumulation by water evaporation, which is evidenced by the higher nitrate contents in these micro-sublayers (Fig 3b). As a result, the high denitrifier abundance plus adequate nitrate supply would mean more N_2O could be produced in micro-sublayer 0-1 cm, 1-2 cm and 2-3 cm. This can be further supported by the finding that the N_2O flux was significantly correlated to the copy numbers of both AOB and *narG* in the top three soil micro-sublayers. Therefore, the drying process resulted in the 0-3 cm soil possessing significantly higher AOB abundance and higher nitrate concentrations which were the important factors for the development of higher N_2O concentrations. However, AOB abundances remained low and were not clearly influenced by the drying process in micro-sublayer 3-4 cm, 4-5 cm and 5-6 cm, therefore, the nitrifying activities might be much lower even though the ammonium concentrations were

significantly higher there, which could be one of the major reasons for the lower N₂O concentrations.

nosZ-containing denitrifiers mainly reduce N₂O to N₂. Prior studies have demonstrated that *nosZ*-containing communities are sensitive to water drainage but are not closely related to N₂O flux in paddy soils during flooding-drying cycles (Liu et al., 2012; Uchida et al., 2014). It was observed that sharp increases in *nosZ* copy numbers during the initial drying phase only occurred in the top three micro-sublayers. This scenario might be the quick response of *nosZ*-containing microorganisms to the initial increase of N₂O concentration when the soil was drained (Liu et al., 2012; Liu et al., 2014). However, when further drying induced obvious N₂O emissions, all the micro-sublayers maintained similar high levels of *nosZ*-containing microbes. This suggests that there might be a threshold of N₂O concentration in the surface soil, below which the *nosZ*-containing population size quickly responds to increases of N₂O concentration, but above which the populations remain stable. In agreement with our results, a prior study has also reported that there is a saturation N₂O concentration for soil N₂O-reductase even under anaerobic conditions (Holtan-Hartwig et al., 2000; Qin et al., 2014). Under such a circumstance, N₂O emission would mainly depend on the increase in N₂O production. It can be seen that the *narG*-containing population sizes increased greatly during the increasing phase of N₂O emission, while on a relative basis, the increases in *nosZ* abundance were much smaller. In addition, a previous study revealed that the *nosZ* encoded enzyme is sensitive to O₂ (Knowles, 1982). When soil is drying, the surface micro-sublayers will primarily lose water and O₂ will penetrate into soil pores causing inhibition of nitrous oxide reductase (N₂OR) activity, thus favouring N₂O accumulation and emission.

We observed that the dynamics of nitrate concentrations in all the micro-sublayers shared the same trend during the drying process, which was that the highest nitrate contents occurred on day 22, and the nitrate concentrations during the increasing and decreasing phases of N₂O emission were significantly higher than in the initial phase. This phenomenon suggests that nitrate supply is essentially important for N₂O

emission. The distribution of nitrate in the soil profile during the drying process showed that micro-sublayer 0-1 cm contained much higher NO_3^- -N concentrations than all the other micro-sublayers. This would be linked to nitrate transportation and accumulation in the soil surface during the drying process, and the upper micro-sublayers possessed a stronger capacity for nitrate generation.

In conclusion, soil N_2O concentrations in the 2-3 cm soil layer responded to the flooding-drying process much quicker than that in the 4-5 cm layer, and the N_2O flux was more closely related to the N_2O concentrations in the uppermost layer in the soil profile. Drying did not cause obvious shifts of AOB, *narG* and *nosZ* compositions between the micro-sublayers, but induced significant increases of the abundances of AOB and *narG* in micro-sublayer 0-1 cm, 1-2 cm and 2-3 cm where the ammonium and nitrate contents were significantly lower and higher, respectively, compared to the bottom micro-sublayers. Thus the top soil would have a strong ability to produce nitrate that in turn stimulates the denitrifying process to generate N_2O . In contrast, the bottom soil (4-6 cm) maintained low levels of AOB abundance during the drying process, which suggests that low nitrifying activity would be the major restriction factor to limit N_2O production in the bottom soil layers.

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707 **Table 1** Primer sequence and references

Primer ^a	Primer sequence ^b (5'-3')	Reference
Arch- <i>amoA</i> 23F	ATGGTCTGGCTWAGACG	(Sahan et al., 2008)
Arch- <i>amoA</i> 616R	GCCATCCATCTGTATGTCCA	(Sahan et al., 2008)
<i>amoA</i> 2F	AARGCGGCSAAGATGCCGCC	(Webster et al., 2002)
<i>amoA</i> 5R	TTATTTGATCCCCTC	(Webster et al., 2002)
<i>amoA</i> 1F	GGGGTTTCTACTGGTGGT	(Rotthauwe et al., 1997)
<i>amoA</i> 2R	CCCCTCKGSAAAGCCTTCTTC	(Rotthauwe et al., 1997)
<i>narG</i> 145F	ACS CAY GGS GTD AAC TGY AC	(Chen et al., 2012)
<i>narG</i> 571F	CCG ATY CCG GCV ATG TCS AT	(Chen et al., 2012)
<i>narG</i> 773R	GGN ACG TTN GAD CCC CA	(Chen et al., 2012)
<i>nosZ</i> 1126F	GGG CTB GGG CCR TTG CA	(Chen et al., 2012)
<i>nosZ</i> 1381R	GAA GCG RTC CTT SGA RAA CTT G	(Chen et al., 2012)
<i>nosZ</i> -F	CGY TGT TCM TCG ACA GCC AG	(Rösch et al., 2002)
<i>nosZ</i> -R	CAT GTG CAG NGC RTG GCA GAA	(Rösch et al., 2002)

708 ^a The forward and reverse primers are indicated by the letters F and R, respectively.

709 ^b Y=C or T; R=A or G; D=A, G or T; V=A, C or G; B=C, G or T; N=A, C, T or G; M=A or C

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Table 2

Pearson's correlation coefficients for relationships: between N₂O concentrations (2-3 cm) and AOB, *narG* and *nosZ* copy numbers in micro-sublayers 0-1, 1-2 and 2-3 cm; between N₂O concentrations (4-5 cm) and AOB, *narG* and *nosZ* copy numbers in micro-sublayers 3-4, 4-5 and 5-6 cm; between N₂O flux and AOB, *narG* and *nosZ* copy number in all 6 micro-sublayers.

N ₂ O	Micor-sublayer	AOB	<i>narG</i>	<i>nosZ</i>
N ₂ O concentration in 2-3 cm	micro-sublayer 0-1 cm	0.495	0.758**	0.441
	micro-sublayer 1-2 cm	0.198	0.716**	0.750**
	micro-sublayer 2-3 cm	0.888**	0.679*	0.643*
N ₂ O concentration in 4-5 cm	micro-sublayer 3-4 cm	0.236	0.616*	0.665*
	micro-sublayer 4-5 cm	0.408	0.853**	0.812**
	micro-sublayer 5-6 cm	0.553	0.812**	0.632*
N ₂ O flux	micro-sublayer 0-1 cm	.657*	0.832**	0.298
	micro-sublayer 1-2 cm	0.531	0.879**	0.792**
	micro-sublayer 2-3 cm	0.742**	0.792**	0.580*
	micro-sublayer 3-4 cm	0.29	0.647*	0.785**
	micro-sublayer 4-5 cm	0.407	0.853**	0.832**
	micro-sublayer 5-6 cm	0.45	0.879**	0.703*

** Correlation is significant at the 0.01 level (two-tailed).

* Correlation is significant at the 0.05 level (two-tailed)

^a Correlation analysis is conducted by bivariate correlation analysis using the data from the soils drying 0, 6, 22 and 30 days (n = 3 for N₂O concentrations, flux and n = 9 for others). The correlations were presented as correlation coefficients within lines.

Figure captions:

Fig. 1 Schematic diagram of the pot for gas sampling. **1**: silicon tubes, **2**: U type stainless steel tube, **3**: three-way stopcock, **4**: gas sampling cover, **5**: water.

Fig. 2 Dynamics of soil N₂O fluxes and N₂O concentrations in the soil profile during incubation. **(a)**: N₂O fluxes, **(b)**: N₂O concentrations in soil in 2-3 cm and 3-5 cm. CF: continuous flooding, FD: flooding drying. Data are mean ± standard error of the three replicates.

Fig. 3 Variations of soil ammonium **(a)** and nitrate **(b)** concentrations during soil flooding and drying incubation. Data are mean ± standard error of the three replicates.

Fig. 4 Dynamics of the AOB *amoA* gene copy numbers during the flooding-drying process. Data are mean ± standard error of the three replicates.

Fig. 5 Dynamics of the copy numbers of denitrifying genes during the flooding-drying process. **(a)**: *narG* gene; **(b)**: *nosZ* gene. Data are mean ± standard error of the three replicates.

Fig. 6 PCA analysis of nitrifying and denitrifying genes based on T-RFLP profiles of flooding control (F) and drying 22 days (D). **(a)**: the compositions of bacterial *amoA* (with *TaqI*); **(b)**: the community compositions of *narG* (with *RsaI*); **(c)**: the community compositions of *nosZ* (with *CfoI*).

Fig. 1

Fig. 2

Fig. 3a

Fig. 3b

Fig. 4

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Fig. 5a

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Fig. 5b

Fig. 6

Table 1 Primer sequence and references

Primer ^a	Primer sequence ^b (5'-3')	Reference
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<i>narG</i> 571F	CCG ATY CCG GCV ATG TCS AT	(Chen et al., 2012)
<i>narG</i> 773R	GGN ACG TTN GAD CCC CA	(Chen et al., 2012)
<i>nosZ</i> 1126F	GGG CTB GGG CCR TTG CA	(Chen et al., 2012)
<i>nosZ</i> 1381R	GAA GCG RTC CTT SGA RAA CTT G	(Chen et al., 2012)
<i>nosZ</i> -F	CGY TGT TCM TCG ACA GCC AG	(Rösch et al., 2002)
<i>nosZ</i> -R	CAT GTG CAG NGC RTG GCA GAA	(Rösch et al., 2002)

^a The forward and reverse primers are indicated by the letters F and R, respectively.

^b Y=C or T; R=A or G; D=A, G or T; V=A, C or G; B=C, G or T; N=A, C, T or G; M=A or C

Table 2

Pearson's correlation coefficients for relationships: between N₂O concentrations (2-3 cm) and AOB, *narG* and *nosZ* copy numbers in micro-sublayers 0-1, 1-2 and 2-3 cm; between N₂O concentrations (4-5 cm) and AOB, *narG* and *nosZ* copy numbers in micro-sublayers 3-4, 4-5 and 5-6 cm; between N₂O flux and AOB, *narG* and *nosZ* copy number in all 6 micro-sublayers.

N ₂ O	Micor-sublayer	AOB	<i>narG</i>	<i>nosZ</i>
N ₂ O concentration in 2-3 cm	micro-sublayer 0-1 cm	0.495	0.758**	0.441
	micro-sublayer 1-2 cm	0.198	0.716**	0.750**
	micro-sublayer 2-3 cm	0.888**	0.679*	0.643*
N ₂ O concentration in 4-5 cm	micro-sublayer 3-4 cm	0.236	0.616*	0.665*
	micro-sublayer 4-5 cm	0.408	0.853**	0.812**
	micro-sublayer 5-6 cm	0.553	0.812**	0.632*
N ₂ O flux	micro-sublayer 0-1 cm	.657*	0.832**	0.298
	micro-sublayer 1-2 cm	0.531	0.879**	0.792**
	micro-sublayer 2-3 cm	0.742**	0.792**	0.580*
	micro-sublayer 3-4 cm	0.29	0.647*	0.785**
	micro-sublayer 4-5 cm	0.407	0.853**	0.832**
	micro-sublayer 5-6 cm	0.45	0.879**	0.703*

** Correlation is significant at the 0.01 level (two-tailed).

* Correlation is significant at the 0.05 level (two-tailed)

^a Correlation analysis is conducted by bivariate correlation analysis using the data from the soils drying 0, 6, 22 and 30 days (n = 3 for N₂O concentrations, flux and n = 9 for others). The correlations were presented as correlation coefficients within lines.