1 **Stimulatory effect of exogenous nitrate on soil denitrifiers and**
2 **denitrifying activities in submerged paddy soil**

3 Ling Wang^{a,b}, Rong Sheng^a, Huicui Yang^{a,b}, Qing Wang^{a,b}, Wenzhao Zhang^a, Haijun
4 Hou^a, Jinshui Wu^a, Wenxue Wei^{a,*}

5 ^a*Key Laboratory of Agro-ecological Processes in Subtropical Regions and Taoyuan Station of*
6 *Agro-Ecology Research, Institute of Subtropical Agriculture, Chinese Academy of Sciences,*
7 *Changsha 410125, China*

8 ^b*University of Chinese Academy of Sciences, Beijing 100049, China*

9
10 * *Corresponding author:*

11 *Wenxue Wei*

12 *Tel: +86-731-84615210*

13 *Fax: +86-731-84612686*

14 *E-mail: wenxuewei@isa.ac.cn*

Abstract Paddy soil is submerged under water for most of the rice growing season, but our understanding of the driving mechanisms of nitrogen cycling, N₂O production, and N₂O consumption under submerged conditions is limited. In this study, intact paddy soil cores were sampled and an incubation experiment was conducted with nitrate amendments under flooding. N₂O concentrations in the soil profile and N₂O flux rates were measured by gas chromatography. The community compositions and abundances of *narG*- and *nosZ*-containing denitrifiers were analyzed by terminal restriction fragment length polymorphism (T-RFLP) and real-time quantitative polymerase chain reactions (qPCR), respectively. The results showed that N₂O emissions and N₂O concentrations in the submerged soil profile were significantly affected by the nitrate inputs. Higher NO₃-N additions stimulated a sharp increase in N₂O flux rate, which suggested an obvious stimulation caused by the increasing nitrate input. This effect was closely related to the significant increases in the population size of *narG*-containing denitrifiers and obvious alterations in its community composition. Therefore, high nitrate concentrations in submerged paddy soil can stimulate much higher N₂O production and emissions, and in this process, *narG*- rather than *nosZ*-containing denitrifiers are the important drivers. We also observed that N₂O concentrations in the 0-5 cm soil layer were clearly lower than those in the 5-10 cm layer, but the nitrate contents in these layers were reversed, indicating large N₂O losses occur from the 0-5 cm layer. The emitted N₂O is derived mainly from the uppermost soil layer (0-5 cm).

Key words Paddy Soil, Submergence, Nitrate, Nitrous oxide, Soil Profile, Denitrifier

1. Introduction

Nitrous oxide (N₂O), an important greenhouse gas, greatly contributes to global warming and stratospheric ozone depletion (Ravishankara *et al.*, 2009). Agricultural land emits a large amount of N₂O to the atmosphere and accounts for about 84% of global anthropogenic N₂O emissions (Smith *et al.*, 2008). In agricultural ecosystems, rice paddy fields are a major land use, with > 135 million ha utilized worldwide for rice cultivation (Li *et al.*, 2014). Unlike upland soils, paddy soils are submerged during most of the rice growing season and they maintain a high denitrifying potential (Ferre *et al.*, 2012; Wang *et al.*, 2015). Although previous studies have demonstrated that N₂O fluxes in paddy fields occur during flooding-drying cycles (Chen *et al.*, 1997; Hua *et al.*, 1997; Ruser *et al.*, 2006), under flooded conditions, this greenhouse gas is clearly emitted after nitrogen (N) fertilizer applications (Weitz *et al.*, 2001; Yao *et al.*, 2012; Das and Adhya, 2014), and low emission rates are also maintained consistently (Cai *et al.*, 1997; Zheng *et al.*, 2000; Hansen *et al.*, 2014). The cumulative amount of emitted N₂O during paddy soil submergence is considerable, however, the mechanisms of N transformation and N₂O emission of paddy soils under submerged conditions have barely been investigated.

Long term submergence induces a strict anaerobic environment in paddy soils, and under such a situation O₂ availability is very limited, thus, denitrification is the

60 dominant N₂O-producing process (Mathieu *et al.*, 2006). It has been reported that
61 paddy soil clearly harbors lower copy numbers of denitrifiers under constant flooded
62 conditions than under drying conditions (Liu *et al.*, 2012; Yang *et al.*, 2016), which
63 implies that submerged soils might have low denitrifying activities. On the contrary,
64 other studies have reported that water saturated soils possess much higher
65 denitrification potentials than unsaturated soils (Bettez and Groffman, 2012; Song *et al.*,
66 2014; Brauer *et al.*, 2015). Since the determination of denitrification potential is
67 conducted using exogenous nitrate as a substrate, it is obvious that nitrate addition can
68 stimulate the denitrifying reactions, however, the microbial mechanisms by which
69 nitrate amendments influence denitrification potentials remains unknown. It has also
70 been reported that submerged paddy soils normally contain very low NO₃⁻
71 concentrations (Wang *et al.*, 2011; Liu *et al.*, 2012; Pittelkow *et al.*, 2013). Therefore,
72 the fact that anaerobic paddy soils host low denitrifying populations might be strongly
73 linked to the low nitrate concentrations. Previous studies have demonstrated that
74 nitrate amendments induce increases in N₂O emissions from sediment slurries and
75 low marsh soils (Royer *et al.*, 2004; Hernandez and Mitsch, 2007; Hu *et al.*, 2015),
76 and different amounts of added NO₃⁻ induce different patterns of N₂O flux
77 (Lalissegrundmann *et al.*, 1988; Gillam *et al.*, 2008; Wang *et al.*, 2013). However,
78 other studies have indicated that high nitrate concentrations in soil solution can
79 significantly inhibit denitrification (Senbayram *et al.*, 2012). Thus, it is imperative to
80 explore the mechanisms by which denitrifiers respond to variations in soil nitrate
81 contents.

82 An additional factor influencing microbes is soil heterogeneity, which is a common
83 feature in both cultivated and natural soils (Franzluebbers, 2002). This phenomenon
84 also exists within the 0-5 cm topsoil layer, and it has been demonstrated that the
85 distributions of soil nutrients and denitrifying microorganisms in the uppermost soil
86 exhibits strong variation (Uchida *et al.*, 2014; Yang *et al.*, 2016). The surface soil
87 normally retains higher amounts of soil nutrients and obviously harbors a higher
88 density of microorganisms compared to the subsoil (Fierer *et al.*, 2003; Minamikawa
89 *et al.*, 2013; Gao *et al.*, 2014). It has also been shown that this heterogeneity includes
90 the denitrifying microbial communities and denitrification enzyme activities existing
91 within the topsoil (plough layer) (Mergel *et al.*, 2001; Dhondt *et al.*, 2004). Although
92 N₂O emissions are measured directly at the soil surface, the correlations between N₂O
93 fluxes and N₂O production and consumption within the soil profile are still not well
94 understood.

95 In the present study, we collected intact soil cores (0-20 cm depth) from a paddy
96 field and conducted laboratory incubation experiments with applications of nitrate
97 under flooding conditions. Intact soil cores maintain in situ soil properties and
98 structure, and show more sensitive responses to waterlogging events and higher N₂O
99 emission than sieved soil (Li *et al.*, 2014; Uchida *et al.*, 2014). We hypothesised that
100 the heterogeneities of the biogeochemical properties in the top layers of the paddy soil
101 profiles would result in differential denitrifying activities for coping with nitrate
102 inputs under submerged conditions. The objectives of this study were to explore (1)
103 the responses of N₂O production and emissions to nitrate additions in submerged

paddy soil, (2) the links between the dynamics of N_2O concentrations and the shifts
indenitrifying bacterial communities as influenced by NO_3^- amendments, and (3) the
relationships between N_2O emissions and N_2O concentrations at various soil depths.

2. Materials and methods

The sampling field is located in Changsha, China ($28^\circ14'08''\text{N}$; $113^\circ13'05''\text{E}$), and
has grown double rice crops for more than 100 years. The paddy soil, derived from
quaternary red clay and classified as loamy clay (Hydragric Anthrosols) (Soil Survey
Staff, 2010), was sampled in February 2014 during the winter fallow period. Soil
properties were as follows: Gravimetric water content, 26.14 g H_2O per 100 g dry soil;
soil pH (H_2O) 5.11; $\text{NH}_4^+\text{-N}$, 2.57 mg kg^{-1} ; $\text{NO}_3^-\text{-N}$, 2.73 mg kg^{-1} ; organic matter
content, 30.50 g kg^{-1} ; organic carbon content, 1.77 g kg^{-1} . The soil bulk densities of
the 0-5, 5-10, 10-15 and 15-20 cm layers were 1.00, 1.04, 1.07 and 1.30 g cm^{-3}
respectively.

2.1. Intact soil column collection

PVC cylinders (15 cm diameter, 25 cm high) and bottom circular PVC plates (18
cm diameter) were prepared before soil sampling, two adjacent holes (1.6 cm
diameter) were drilled in the cylinder wall at positions of 2.5, 7.5, 12.5 and 17.5 cm
from the bottom of cylinder, respectively. In the field, plant residues were manually
removed from soil surface and soil columns were dug. PVC cylinders were fitted to
the soil columns by trimming off extra soil with a spade. Afterwards, the intact soil

column (0-20 cm) was removed from the field, the bottom was covered with a plate and the cylinder was wrapped with film to prevent water loss. In total, 40 intact soil columns were collected and transported to the laboratory. For each column, after the bottom plate was sealed to the cylinder with glue, two horizontal tunnels at each level were made across the soil column with a stainless tube (1.6 cm diameter) through the holes in the PVC cylinder. Each tunnel was filled with a gas sampler, which was constructed as follows. Silicon tubes (each 14 cm long, 1.2 cm internal diameter, 0.2 cm wall thickness) were closed with silicone septa at both ends, and twin parallel tubes connected with a U type stainless steel tube at one end were inserted into a pair of tunnels. The stainless steel U tubes (2mm) were inserted into the silicon tubes through the septa and three-way stopcocks were fixed to the steel tubes outside the cylinders (Fig.1). The holes in PVC wall were then sealed with glue and the space between the soil columns and PVC cylinders was filled with soil slurry.

2.2. Soil incubation

Three treatments were used with nitrate (KNO_3) applications of 0 mg N per pot (N0), 100 mg N per pot (N100) and 350 mg N per pot (N350), which amounts to the N-fertilizer applications of 0, 60 and 200 kg N per hectare in the field practice. Each treatment consisted of 12 pots, 3 for gas sampling and 9 for soil sampling at 3 different times. In effect there were 3 experimental replicates for each treatment. All the pots were randomly arranged. The nitrate solutions of 0, 1.50, 5.04 g KNO_3 L⁻¹ were added to the corresponding columns from the soil surface, the volume was 500

ml for each pot and 2 cm of free surface water maintained throughout the experiment. Immediately after application of the treatments, the silicon tubes in the soil columns were flushed with high-purity helium gas three times. The pots were incubated at 28°C.

2.3. Gas and soil sampling

Gas samples for determining N₂O flux were taken between 9 and 10 am on days 1, 3, 5, 7, 9, 13, 17, 21, 25, 30, 34 and 38 after nitrate application using a static chamber method (Liu *et al.*, 2012). Gas samples were taken at 0 min and 60 min each time, the N₂O concentrations were measured by GC (C0 and C60 respectively) and the N₂O emission during this hour was calculated by the following formula:

$$\text{N}_2\text{O flux (mg N/m}^2\text{/h)} = \frac{(C_{60} - C_0) \cdot 44}{22.4} \cdot h \cdot \frac{273}{273 + 28}$$

For detecting soil N₂O concentrations in the soil profile, gas samples (2 mL) from the top to the bottom layer 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 immediately transferred to evacuated 12 mL vials (Labco limited high Eycombe, UK), and after 28 mL N₂ was injected into the vials to increase the pressure, which was needed during N₂O determination by a gas chromatograph equipped with an electron capture detector (Agilent 7890A, USA).

Soil samples were taken on days 1, 7 and 13 after nitrate application, representing the increasing, peak and decreasing phases of N₂O emission based on the dynamics of N₂O fluxes determined in a preliminary experiment. The soil sampler consisted of two

half stainless steel tubes (25 cm length, 4 cm inner diameter) held together with a rubber belt. After the surface water was removed from the soil column, the sampler was inserted vertically into the soil column, on removal from the column, the sampler was separated, and the soil sample was quickly divided into 0-5cm (A layer), 5-10cm (B layer), 10-15cm (C layer) and 15-20cm (D layer). This process was carried out three times in each pot. 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, the other was stored at 4°C for soil chemical analysis.

2.4. Measurements of soil basic physio-chemical properties

Soil gravimetric water content was determined by drying the soil at 105°C for 24 h, and all results were expressed on an oven-dry base. Soil NH_4^+ and NO_3^- 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 determined by a pH meter (FE-20, METTLER TOLEDO, China) in the suspension of 1 M KCL (dry soil: solution = 1:5, W/V). Three redox electrodes (FJA-3, Nanjing, China) were vertically inserted into each layer at 2.5, 7.5, 12.5 and 17.5 cm depth, and Eh values were recorded before gas sampling.

2.5. DNA extraction

Total soil DNA was extracted from 0.3 g fresh soil using the method of Chen et al. (2010) with minor modifications. At the first step, the MP Fast Prep1-24 (MP Biomedicals, USA) was used instead of a 1 h water bath treatment. The extracted DNA was quantified using Nanodrop ND-1000 (Wilmington, DE, USA).

2.6. Quantitative PCR

Quantitative PCR (qPCR) was performed to quantify the nitrate reductase gene (*narG*) and nitrous oxide reductase gene (*nosZ*). Primer sets of *narG*-571F/*narG*-773R and *nosZ*-1126F/*nosZ*-1381R were used (Table 1). 10 μ L reaction mixtures contained 5 μ L SYBR green mixII, 0.2 μ L Rox (Takara, Dalian, China), 0.3 μ L (0.4 μ mol L⁻¹) of both forward and reverse primers, 1 μ L (5 ng μ L⁻¹) of sample template DNA, and made up to volume with deionized water. Thermal programs were run with an ABI prism 7900 system (Applied Biosystem, USA) in triplicate samples using the following thermal conditions: (1) for *narG*: 95 °C, 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 10 s; (2) for *nosZ*: 95 °C, 30 s; 40 repeats of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 10 s. Standard curves used to quantify the *narG* and *nosZ* genes were created using a 10-fold dilution series of plasmids containing the target gene.

2.7. Terminal restriction fragment length polymorphism assay

The primer pairs of *narG*-145F/*narG*-773R and *nosZ*-F/*nosZ*-R were used to amplify targeted gene fragments of *narG* (629 bp) and *nosZ* (706 bp), respectively

(Table 1). The forward primers were labeled with 6-carboxyfluorescein at the 5'ends. The PCR reactions were performed in a 50 μ L volume including 2 μ l (0.2 μ mol L⁻¹) of both forward and reverse primers, 25 μ L PCRMix (Tiangen, China), 4 μ l (20 ng μ l⁻¹) of sample template DNA, and made up to volume with deionized water. Thermal cycling was carried out using a Thermal Cycler (Eppendorf AG 2233, Hamburg, Germany) in duplicate according to the following thermal conditions: (1) for *narG*: 95 °C, 5 min; 2 cycles of 95 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min; 5 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min; 30 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 1 min; 72 °C, 8 min; (2) for *nosZ*: 95 °C, 3 min; 10 cycles of 95 °C for 30 s, 65 °C (-1 °C/cycle) for 40 s, 72 °C for 40 s; 30 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 40 s; 72 °C, 7 min.

The PCR products were purified using a TIAN gel Midi Purification Kit DP209 (Tiangen biotech co., Ltd., China). The endonucleases enzymes *HhaI* and *CfoI* (Thermo Scientific FastDigest) were used to digest *narG* and *nosZ* genes, respectively. The Terminal restriction fragment length polymorphism (T-RFLP) analysis was conducted with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA).

2.8.Data treatment and statistical analysis

Consumption rate (*CRi*) of soil NO₃-N in each soil layer (mg NO₃-N day⁻¹) was calculated by Eq. (1):

$$CRi = \frac{(Ct1-Ct2) \cdot (BDi \cdot Vi) / 1000}{t2-t1} (1)$$

where i is a specific soil layer ($i=A, B, C$ and D); $t1$ and $t2$ are the start and end time of incubation (d) respectively; BD_i and V_i are bulk density (g cm^{-3}) and volume (cm^3) of the specific soil layer respectively; C_{t1} and C_{t2} are $\text{NO}_3\text{-N}$ concentrations (mg kg^{-1} dry soil) in each soil layer at $t1$ and $t2$ respectively. The total $\text{NO}_3\text{-N}$ consumption rate (CR) of the soil profile ($\text{mg NO}_3\text{-N day}^{-1}$ per pot) was calculated by Eq. (2):

$$TR = \sum CR_i(2)$$

The total consumption rate of soil $\text{NO}_3\text{-N}$ in soil profile on day 1 was estimated by Eq. (3), as Eq. (2) is not suitable since the initial $\text{NO}_3\text{-N}$ concentrations in the soil profile could not be determined.

$$TR1 = \left(A + 2.73 \times \sum \frac{BD_i \times V_i}{1000} \right) - \sum \frac{C_i \times BD_i \times V_i}{1000}(3)$$

Where A is the amount of $\text{NO}_3\text{-N}$ addition (mg); 2.73 was soil background $\text{NO}_3\text{-N}$ concentration before experiment (mg kg^{-1} dry soil); C_i is $\text{NO}_3\text{-N}$ concentration (mg kg^{-1} dry soil) in a specific soil layer on day 1.

All statistical analyses were performed using SPSS 13.0 (SPSS Inc., USA). Due to the nonnormal distribution of data, nonparametric test was employed to analyze the variance of N_2O fluxes and N_2O concentrations in the soil profile over time and among treatments. Also the significant differences of $\text{NO}_3\text{-N}$ concentrations and denitrifying gene abundance in relation to soil depth and the nitrate amendments were tested by nonparametric methods. All nonparametric tests were carried out using the Kruskal-Wallis test. Linear regression analysis was conducted to match NO_3^- content to N_2O concentrations in the soil profile. The correlations between N_2O flux and soil N_2O concentration, and the relationships of N_2O emissions and soil variables in soil

profile were assessed by Spearman's test. Significance was accepted at a level of probability of $P < 0.05$. T-RFLP data was processed as described by Liu et al. (2012), and Principal Component Analysis (PCA) (CANOCO version 4.53) was used for community analysis.

3. Results

3.1. Dynamics of NO_3^- concentrations in the soil profile after nitrate amendments

Nitrate concentrations decreased significantly with increasing depth (0-20cm) in all three nitrate treatments over the incubation ($P < 0.001$) (Fig.2). However, the N100 and N350 treatments had larger differences in NO_3^- concentrations in their corresponding soil profiles than the N0 treatment, such that the ratios of NO_3^- concentrations in the A:D layers on day 1 were 2.68, 19.81 and 25.04 in the N0, N100 and N350 treatments, respectively, showing that more NO_3^- was located in the top soil as nitrate addition increased. The $\text{NO}_3\text{-N}$ concentrations in the whole soil profile were increased proportionally with the increase in nitrate application, indicating the added nitrate had permeated into the entire soil column. In relation to the nitrate consumption rates of different treatments (Table 2), when the original soil nitrate content plus the amendment is treated as the nitrate source, after 24 h incubation, the total consumption rates of the N100 and N350 treatments were 50.59 and 59.96 $\text{mg NO}_3\text{-N pot}^{-1} \text{ day}^{-1}$, respectively, which were very similar. However, during the following period the difference in rates between the two treatments widened greatly, and during days 1 to 7, the average rates were 9.31 and 32.38 $\text{mg NO}_3\text{-N pot}^{-1} \text{ day}^{-1}$, respectively,

the rate for the N350 treatment being about 3.5 times higher than for the N100 treatment. Soil $\text{NO}_3\text{-N}$ in the N100 treatment was nearly exhausted to the level of the N0 treatment by day 13 and that in the N350 treatment still remained at a relative high level (about $14.43 \text{ mg NO}_3\text{-N kg}^{-1}$ dry soil). Furthermore, the nitrate consumption rates in different soil layers also varied significantly, with the A layer having the highest rates during the incubation followed by the B, C and D layers. During days 1 to 7 in both the N100 and N350 treatments, the consumption rates in the A, B and C layers were about 16.06, 5.04 and 2.61 times higher than in the D layer respectively. This result implies that the upper soil in the profile possesses a higher nitrate transformation capacity than soil lower down the profile.

3.2. Dynamics of N_2O flux and N_2O concentrations in the soil profile

Nitrate additions affected ($P < 0.05$) N_2O emissions from day 1 to day 30 (Fig.3). The N_2O flux rates of the N100 and N350 treatments were immediately increased on day 1 to 2.11 and $2.99 \text{ mg N}_2\text{O-N m}^{-2} \text{ h}^{-1}$, respectively, although the increases were not significantly different from each other. However, the dynamics of N_2O emissions from these two treatments behaved differently thereafter. The N100 treatment maintained a relatively steady emission rate for about 4 days and then, the rate quickly decreased to the level of the N0 treatment. In contrast, the N_2O emission rate from the N350 treatment increased steeply from $2.99 \text{ mg N}_2\text{O-N m}^{-2} \text{ h}^{-1}$ on day 1 to a peak of $15.49 \text{ mg N}_2\text{O-N m}^{-2} \text{ h}^{-1}$ on day 7, indicating the existence of different mechanisms for driving N_2O flux. Compared to the N100 treatment, the dynamics of

the N₂O emission rates from the N350 treatment after the peak was also different, the rate dropped 80% to about 3 mg N₂O-N m⁻² h⁻¹ within 6 days of the peak, and then it took another 17 days to reach the level observed in the N0 treatment.

The N₂O concentrations in the soil were largely dependent on the position in the soil profile and nitrate input (Fig.4). Both the N100 and N350 treatments showed that the dynamics of N₂O concentrations versus depth shared similar trends with time, which were closely correlated to the variations in N₂O emission patterns ($P<0.01$)(Table 3), and were significantly influenced by nitrate inputs ($P<0.001$). The higher input in the N350 treatment resulted in significantly higher N₂O concentrations in the whole soil profile than for the N100 treatment. The N₂O concentrations of the N100 and N350 treatments significantly decreased with soil depth with one exception, that the A layers clearly contained lower N₂O concentrations than the B layers, about 0.26-37.84 % less for the N100 treatment and 8.10-28.85 % less for the N350 treatment.

This difference in N₂O concentrations between the A and B layers, is a mismatch to the significantly higher NO₃⁻ contents in the A layer compared with the B layer. Linear equations were derived for the relationships between N₂O concentrations and NO₃⁻ contents in the B, C, D layers of the N100 and N350 treatments. The equations were all significant at the $P<0.01$ level except for N100 on day 13. On the basis of these equations, the theoretical N₂O concentrations in A layers were calculated (Table 4). All the theoretical values in the A layers were higher than the measured N₂O concentrations in the B layers, demonstrating the upper soil could possess a higher

N₂O production ability than the lower soil.

Moreover the N₂O deficit between the theoretical and measured values in the A layers of both N100 and N350 treatments were much higher than the N₂O emissions over the same periods. The N₂O emissions as percentages of the N₂O deficits were highest on day 1, about 10.53% and 14.70% for the N100 and N350 treatments, respectively. During the rest of the incubation, these percentages decreased, and over 90% of the N₂O produced in the A layers was appeared to be lost by other pathways.

3.3. Influence of nitrate amendments on population size of functional genes

The population sizes of *narG*- and *nosZ*-containing denitrifiers were determined with *qPCR* (Fig.5). It is obvious that the abundance of both denitrifiers had similar distributions with soil depth, that is the A layer clearly hosting the highest copy numbers followed by the B, C and D layers ($P<0.01$). This distribution features were also common among all nitrate treatments.

On day 1, only the abundances of the *narG*-containing community in the A layers of the N100 and N350 treatments were increased ($P<0.05$) by about 34.92% and 36.35%, respectively compared to the N0 treatment. The copy numbers of *narG* genes in the remaining layers did not show clear responses to the nitrate amendments during the first 24 h. However, the copy numbers of the *narG* gene in the A, B, C and D layers were all positively related to the nitrate inputs on day 7 ($P<0.01$), especially in the N350 treatment, and the highest *narG* gene copies compared to other treatments occurred in all soil layers of the N350 treatment when N₂O emissions peaked. By day

13, the *narG*-containing population sizes of the N100 treatment were similar to those for the N0 treatment, while only the A layer of the N350 treatment still maintained significantly ($P<0.01$) higher copy numbers (about 45.09% more than for the N0 treatment). The population sizes of *narG*-containing communities in the D layers were obviously lower than for the other soil layers and clearly did not respond to nitrate amendments.

On day 1, there were no significant differences between treatments in *nosZ*-containing community abundances in any soil layer. In comparison with the N0 treatment the *nosZ* gene copy numbers of the N100 and N350 treatments in the A layers increased by about 19.55% and 30.22% on day 7, respectively, but the differences were not significant. At the same time, the *nosZ*-containing population size of the B layer of the N100 treatment was significantly ($P<0.01$) higher than for the N0 and N350 treatments, but there were no significant differences among the three treatments in C and D layers. However the copy numbers of *nosZ* in A layer was increased with the nitrate inputs increasing on day 13 ($P<0.01$), and no significant difference was in the rest layers. In general, nitrate amendments had a very different and limited effect on the abundances of *nosZ*-containing community; the highest abundance occurring for the N100 treatment was of 1.69×10^8 copies g^{-1} dry soil on average on day 7 while for the N350 treatment was of 1.89×10^8 copies g^{-1} dry soil on average on day 13.

3.4.Impact of nitrate addition on denitrifying community compositions

The T-RFLP data on days 1, 7 and 13, representing the increasing, peak and decreasing phases of N₂O fluxes from the treated soil cores, was employed to show the effects of exogenous NO₃⁻ additions to flooded paddy soil on denitrifying community compositions. The results of the PCA analysis indicated that both NO₃-amendments caused some shifts in the community composition of *narG*-containing denitrifiers on day 1(Fig.6a), but the shifts were not significant. Then, by day 7, a further and obvious shift in the community structures of *narG*-containing denitrifiers had resulted from the 350 mg NO₃-N addition, whereas the 100 mg NO₃-N addition had not induced any significant change (Fig.6b). With extended flooding (day 13) the community structures of *narG*-containing denitrifiers for both NO₃- amendments were different from that of the N0 treatment (Fig.6c). Unlike for the *narG*-containing denitrifiers, the *nosZ*-containing communities for the N0, N100, and N350 treatments indicated no significant effect of nitrate addition on *nosZ* gene community compositions. Another feature of the data is that the differences in both denitrifying community compositions between soil layers were consistent for all treatments from day 1 to day 13.

3.5. Correlation analysis

After nitrate addition, the influences of changed soil parameters with soil profiles on the N₂O emissions were analyzed by spearman's correlation coefficients (Table 5). The data shows that the N₂O flux was significantly correlated to NO₃-N contents and soil Eh in all 4 soil layers ($P<0.01$), while no obvious relations were found to soil DOC and PH. Besides, N₂O flux was only correlated to the *narG* gene abundance in A layer significantly ($P<0.01$), no clear relationships of N₂O gas emissions to the

abundances of *narG* in B, C, D layers and *nosZ* in all 4 soil layers.

4. Discussion

4.1. Responses of the denitrifying process to nitrate amendments

Generally, a well developed soil possesses relatively stable physio-chemical properties, such as soil porosity, aggregation and fertility etc (Franzluebbers, 2002).

This status decides the reactions of the bulk soil to the addition of exogenous materials. Although previous studies have suggested that submerged paddy soils and sediments hardly emit much N₂O to the atmosphere (Cai *et al.*, 1997; Chen *et al.*, 1997; Hansen *et al.*, 2014), these soils have high denitrification potentials (Brauer *et al.*, 2015; McPhillips and Walter, 2015). There have been different hypotheses put forward for this phenomenon, including substrate limitation and complete denitrification to form N₂, but the mechanisms of denitrifying reactions under flooded conditions are not clear. In the present study, we applied three nitrate amendment levels to intact paddy soil cores under a constant flooding environment. It was clear that nitrate inputs induced N₂O emissions, and the more nitrate added, the greater the N₂O flux. This suggests that the low N₂O emissions from submerged paddy soils are probably due to nitrate substrate restriction. Previous studies have shown that increases in initial NO₃⁻ concentrations significantly enhance denitrification potentials and N₂O emissions (Casella *et al.*, 1986; Wang *et al.*, 2013), whereas negligible N₂O emissions from submerged paddy soils in the presence of low NO₃⁻ concentrations have been observed in laboratory incubations (Liu *et al.*, 2012) and in field plots (Wang *et al.*, 2011; Pittelkow *et al.*, 2013). In the present study, the nitrate

consumption rates between the N100 and N350 treatments during the first 24 h after nitrate addition showed no significant difference, neither did the N₂O fluxes for the two treatments, implying that both nitrate input levels had reached or exceeded the soils initial capacity for reducing nitrate. This capacity would be directly linked to the original soil properties and microbial habitats (D'Haene *et al.*, 2003; Khalil and Richards, 2011). From this point, if the soil maintains its initial nitrate reducing ability without further responding to the high nitrate amendment, it would be predicted that the different nitrate inputs would share similar maximum N₂O flux rates. However, as the incubation continued, we observed that the N350 treatment showed significantly higher nitrate consumption rates in the whole soil profile than for the N100 treatment, and the peak N₂O flux rate of the N350 treatment was about 5 times higher than for the N100 treatment. Since the difference in nitrate inputs was 3.5 times between the two treatments, it is obvious that the soils responded differently to the various rates of exogenous nitrate. The higher nitrate input caused significantly higher nitrate consumption and N₂O emission rates, indicating that a stimulatory effect induced by high nitrate input existed in the soil. If this is the case, some soil characteristics would change to deal with the high amount of substrate. In our study, the N₂O flux were significantly affected by the soil Eh and NO₃-N concentrations in soil profiles, not by the DOC and Ph changes after nitrate addition. Although paddy soil Eh would be decreased by continuous flooding, high NO₃⁻ concentrations in soil could delay the decreasing rates (Kralova *et al.*, 1992; Thind and Rowell, 1999). Then the high redox potential stimulated the N₂O flux through acceleration of electron transfer, resulting in

more N₂O being produced (Liu *et al.*, 2012). Besides, the bio-characteristics could shift in response to the various substrate concentrations (Cheneby *et al.*, 2010). Since nitrate is the direct substrate for denitrification, soil denitrifying communities can adapt to nitrate amendments by shifting the population size and community compositions (Bowen *et al.*, 2013; Huang *et al.*, 2015). We discovered that the population size of *narG*-containing bacteria significantly increased in the N350 treatment compared to the N100 treatment, and its community compositions were also obviously changed. Because nitrate reductase encoded by the *narG* gene catalyzes the first reaction of denitrification and is considered as the rate limiting step (Enwall *et al.*, 2005), the elevation of *narG* copy numbers and the shifts in *narG*-containing community composition suggest that high nitrate input stimulated a larger *narG*-containing population with more active denitrifiers. However, the exact nature of the active components of the altered community composition is not clear. Thus the variations in *narG*-containing denitrifiers in response to the increase in nitrate concentration would be the dominant generator for the stimulatory effect. Although *nosZ*-containing bacteria are also denitrifiers which reduce N₂O to N₂, the abundances and compositions in the soil profile did not show significant differences between the nitrate addition treatments, therefore, this denitrifying community was clearly not affected by the nitrate amendments and would not play a significant role in the priming effect of exogenous nitrate reduction.

4.2. Relationships between nitrous oxide emissions and concentrations in the top soil

profile

Top-dressing of nitrogen fertilizers results in nitrogen distribution patterns which gradually decrease from the top to the lower soil layers (D'Haene *et al.*, 2003; Jahangir *et al.*, 2012). It was determined that nitrate distributions followed this pattern within the 0-20 cm profile in both the N100 and N350 treatments. Soil nitrate is an important limiting factor for N₂O production and emissions and soil N₂O concentrations have been proportionally correlated to soil NO₃⁻ concentrations (Casella *et al.*, 1986; Lalissegundmann *et al.*, 1988; Senbayram *et al.*, 2012). We observed that N₂O concentrations were significantly and linearly correlated ($P < 0.01$) with NO₃⁻ concentrations within the 5-20 cm soil layer, but the 0-5 cm layer did not fit this relationship. This top layer retained the highest NO₃⁻ concentrations but significantly lower N₂O concentrations than in the 5-10 cm layer. The question is whether this top soil layer has a lower ability to produce N₂O or it possesses a strong capability to generate N₂O which is lost. The topsoil (0-5 cm) contained significantly higher NO₃⁻ concentrations than the other sublayers and it also possessed better conditions for denitrification, such as nutrient contents and distribution of denitrifying microorganism. Therefore, the soil should have a strong ability to produce N₂O (D'Haene *et al.*, 2003; Khalil and Richards, 2011). The generated N₂O could then be lost either through emission or complete reduction to N₂. Although the top soil harbored the highest amounts of *nosZ*-containing bacteria, the copy numbers did not change greatly during the incubation, especially during the peak period of N₂O emission, suggesting that the capacity for N₂ generation would not have increased

significantly. Furthermore, the distance for N₂O diffusion from this top layer to the atmosphere is the shortest compared to the lower layers (Castle *et al.*, 1998; Jahangir *et al.*, 2012). Therefore, the only possibility is that N₂O was lost either to the atmosphere or to water. Based on the linear equations relating N₂O and NO₃⁻ concentrations within the 5-20 cm layers, the differences between the estimated and measured N₂O concentrations in the 0-5 cm layer were calculated. If the differences are defined as N₂O loss, the amounts of daily N₂O emissions accounted for only about 10% of these differences in soil N₂O concentrations, which could strongly suggest that N₂O production in the 0-5 cm soil layer contributes most of the N₂O emissions. This soil layer is effectively regarded as a ‘hot spot’ for N₂O production and emissions (Uchida *et al.*, 2014; Yano *et al.*, 2014). The other portion of N₂O loss from the 0-5 cm soil layer would be related to N₂ production, and dissolution in the flooded water layer (Clough *et al.*, 2005). To better understand the N₂O fate in soil before emission, more specific studies should focus on the quantification of different pathways of N₂O consumption in the soil profile.

5. Conclusions

Nitrate application to a submerged paddy soil stimulated N₂O production and emission. Based on the variations in the community compositions and abundance of *narG*- and *nosZ*-containing denitrifiers, in response to nitrate inputs, *narG*-containing bacteria is the key player in the formation of the priming effect, whereas, *nosZ*-containing microorganisms did not play a significant role in the intensified

nitrate reduction. According to the linear correlations between nitrate contents and N₂O concentrations in the soil profile and significant lower N₂O concentrations in the 0-5 cm compared to 5-10 cm soil layer, it can be hypothesized that N₂O emissions would be mainly from N₂O production in the 0-5 cm soil layer. Further field investigations, such as the application of various nitrogen fertilizers with isotope labeling, would be valuable to solidify the relationships between N₂O emissions and soil N₂O concentrations.

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References

- Bettez, N.D., Groffman, P.M., 2012. Denitrification Potential in Stormwater Control Structures and Natural Riparian Zones in an Urban Landscape. *Environmental Science & Technology* 46, 10909-10917.
- Bowen, J.L., Byrnes, J.E.K., Weisman, D., Colaneri, C., 2013. Functional gene pyrosequencing and network analysis: an approach to examine the response of denitrifying bacteria to increased nitrogen supply in salt marsh sediments. *Frontiers in Microbiology* 4.
- Brauer, N., Maynard, J.J., Dahlgren, R.A., O'Geen, A.T., 2015. Fate of nitrate in seepage from a restored wetland receiving agricultural tailwater. *Ecological Engineering* 81, 207-217.

527 Cai, Z.C., Xing, G.X., Yan, X.Y., Xu, H., Tsuruta, H., Yagi, K., Minami, K., 1997.
528 Methane and nitrous oxide emissions from rice paddy fields as affected by nitrogen
529 fertilisers and water management. *Plant Soil* 196, 7-14.

530 Casella, S., Leporini, C., Picci, G., 1986. Gaseous nitrogen losses from fertilized soil
531 during nitrification and denitrification using the acetylene blockage technique. *Biol.*
532 *Fertil. Soils* 2, 65-70.

533 Castle, K., Arah, J.R.M., Vinten, A.J.A., 1998. Denitrification in intact subsoil cores.
534 *Biol. Fertil. Soils* 28, 12-18.

535 Chen, G.X., Huang, G.H., Huang, B., Yu, K.W., Wu, J., Xu, H., 1997. Nitrous oxide
536 and methane emissions from soil-plant systems. *Nutr. Cycl. Agroecosyst.* 49, 41-45.

537 Chen, Z., Liu, J., Wu, M., Xie, X., Wu, J., Wei, W., 2012. Differentiated Response of
538 Denitrifying Communities to Fertilization Regime in Paddy Soil. *Microbial Ecology*
539 63, 446-459.

540 Cheneby, D., Bru, D., Pascault, N., Maron, P.A., Ranjard, L., Philippot, L., 2010. Role
541 of Plant Residues in Determining Temporal Patterns of the Activity, Size, and
542 Structure of Nitrate Reducer Communities in Soil. *Applied and Environmental*
543 *Microbiology* 76, 7136-7143.

544 Clough, T.J., Sherlock, R.R., Rolston, D.E., 2005. A review of the movement and fate
545 of N₂O in the subsoil. *Nutr. Cycl. Agroecosyst.* 72, 3-11.

546 D'Haene, K., Moreels, E., De Neve, S., Daguiar, B.C., Boeckx, P., Hofman, G., Van
547 Cleemput, O., 2003. Soil properties influencing the denitrification potential of
548 Flemish agricultural soils. *Biol. Fertil. Soils* 38, 358-366.

549 Das, S., Adhya, T.K., 2014. Effect of combine application of organic manure and
550 inorganic fertilizer on methane and nitrous oxide emissions from a tropical flooded
551 soil planted to rice. *Geoderma* 213, 185-192.

552 Dhondt, K., Boeckx, P., Hofman, G., Van Cleemput, O., 2004. Temporal and spatial
553 patterns of denitrification enzyme activity and nitrous oxide fluxes in three adjacent
554 vegetated riparian buffer zones. *Biol. Fertil. Soils* 40, 243-251.

555 Enwall, K., Philippot, L., Hallin, S., 2005. Activity and composition of the
556 denitrifying bacterial community respond differently to long-term fertilization.

557 Applied and Environmental Microbiology 71, 8335-8343.

558 Ferre, C., Zechmeister-Boltenstern, S., Comolli, R., Andersson, M., Seufert, G., 2012.

559 Soil microbial community structure in a rice paddy field and its relationships to CH₄

560 and N₂O fluxes. *Nutr. Cycl. Agroecosyst.* 93, 35-50.

561 Fierer, N., Schimel, J.P., Holden, P.A., 2003. Variations in microbial community

562 composition through two soil depth profiles. *Soil Biology & Biochemistry* 35,

563 167-176.

564 Franzluebbers, A.J., 2002. Water infiltration and soil structure related to organic

565 matter and its stratification with depth. *Soil Tillage Res.* 66, 197-205.

566 Gao, X., Rajendran, N., Tenuta, M., Dunmola, A., Burton, D.L., 2014. Greenhouse

567 Gas Accumulation in the Soil Profile is not Always Related to Surface Emissions in a

568 Prairie Pothole Agricultural Landscape. *Soil Science Society of America Journal* 78,

569 805-817.

570 Gillam, K.M., Zebarth, B.J., Burton, D.L., 2008. Nitrous oxide emissions from

571 denitrification and the partitioning of gaseous losses as affected by nitrate and carbon

572 addition and soil aeration. *Canadian Journal of Soil Science* 88, 133-143.

573 Hansen, M., Clough, T.J., Elberling, B., 2014. Flooding-induced N₂O emission bursts

574 controlled by pH and nitrate in agricultural soils. *Soil Biology & Biochemistry* 69,

575 17-24.

576 Hernandez, M.E., Mitsch, W.J., 2007. Denitrification in created riverine wetlands:

577 Influence of hydrology and season. *Ecological Engineering* 30, 78-88.

578 Hu, J., Inglett, K.S., Clark, M.W., Inglett, P.W., Reddy, K.R., 2015. Nitrous oxide

579 production and consumption by denitrification in a grassland: Effects of grazing and

580 hydrology. *Science of the Total Environment* 532, 702-710.

581 Hua, X., Guangxi, X., Cai, Z.C., Tsuruta, H., 1997. Nitrous oxide emissions from

582 three rice paddy fields in China. *Nutr. Cycl. Agroecosyst.* 49, 23-28.

583 Huang, C., Li, Z.L., Chen, F., Liu, Q., Zhao, Y.K., Zhou, J.Z., Wang, A.J., 2015.

584 Microbial community structure and function in response to the shift of sulfide/nitrate

585 loading ratio during the denitrifying sulfide removal process. *Bioresour. Technol.* 197,

586 227-234.

587 Jahangir, M.M.R., Khalil, M.I., Johnston, P., Cardenas, L.M., Hatch, D.J., Butler, M.,
588 Barrett, M., O'Flaherty, V., Richards, K.G., 2012. Denitrification potential in subsoils:
589 A mechanism to reduce nitrate leaching to groundwater. *Agriculture Ecosystems &*
590 *Environment* 147, 13-23.

591 Khalil, M.I., Richards, K.G., 2011. Denitrification enzyme activity and potential of
592 subsoils under grazed grasslands assayed by membrane inlet mass spectrometer. *Soil*
593 *Biology & Biochemistry* 43, 1787-1797.

594 Kralova, M., Masscheleyn, P.H., Lindau, C.W., Patrick, W.H., 1992. PRODUCTION
595 OF DINITROGEN AND NITROUS-OXIDE IN SOIL SUSPENSIONS AS
596 AFFECTED BY REDOX POTENTIAL. *Water Air Soil Pollut.* 61, 37-45.

597 Lalissegundmann, G., Brunel, B., Chalamet, A., 1988. Denitrification in a cultivated
598 soil - optimal glucose and nitrate concentrations *Soil Biology & Biochemistry* 20,
599 839-844.

600 Li, X.B., Xia, L.L., Yan, X.Y., 2014. Application of membrane inlet mass
601 spectrometry to directly quantify denitrification in flooded rice paddy soil. *Biol. Fertil.*
602 *Soils* 50, 891-900.

603 Liu, J., Hou, H., Sheng, R., Chen, Z., Zhu, Y., Qin, H., Wei, W., 2012. Denitrifying
604 communities differentially respond to flooding drying cycles in paddy soils. *Applied*
605 *Soil Ecology* 62, 155-162.

606 Mathieu, O., Henault, C., Leveque, J., Baujard, E., Milloux, M.J., Andreux, F., 2006.
607 Quantifying the contribution of nitrification and denitrification to the nitrous oxide
608 flux using N-15 tracers. *Environ. Pollut.* 144, 933-940.

609 McPhillips, L., Walter, M.T., 2015. Hydrologic conditions drive denitrification and
610 greenhouse gas emissions in stormwater detention basins. *Ecological Engineering* 85,
611 67-75.

612 Mergel, A., Schmitz, O., Mallmann, T., Bothe, H., 2001. Relative abundance of
613 denitrifying and dinitrogen-fixing bacteria in layers of a forest soil. *Fems*
614 *Microbiology Ecology* 36, 33-42.

615 Minamikawa, K., Eguchi, S., Nishimura, S., Ihara, H., Maeda, M., Yagi, K., Komada,
616 M., 2013. Groundwater-induced emissions of nitrous oxide through the soil surface

617 and from subsurface drainage in an Andosol upland field: A monolith lysimeter study.
618 Soil Science and Plant Nutrition 59, 87-95.

619 Pittelkow, C.M., Adviento-Borbe, M.A., Hill, J.E., Six, J., van Kessel, C., Linquist,
620 B.A., 2013. Yield-scaled global warming potential of annual nitrous oxide and
621 methane emissions from continuously flooded rice in response to nitrogen input.
622 Agriculture Ecosystems & Environment 177, 10-20.

623 Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous Oxide (N₂O): The
624 Dominant Ozone-Depleting Substance Emitted in the 21st Century. Science 326,
625 123-125.

626 Rosch, C., Mergel, A., Bothe, H., 2002. Biodiversity of denitrifying and
627 dinitrogen-fixing bacteria in an acid forest soil. Applied and Environmental
628 Microbiology 68, 3818-3829.

629 Royer, T.V., Tank, J.L., David, M.B., 2004. Transport and fate of nitrate in headwater
630 agricultural streams in Illinois. Journal of Environmental Quality 33, 1296-1304.

631 Ruser, R., Flessa, H., Russow, R., Schmidt, G., Buegger, F., Munch, J.C., 2006.
632 Emission of N₂O, N₂ and CO₂ from soil fertilized with nitrate: Effect of compaction,
633 soil moisture and rewetting. Soil Biology & Biochemistry 38, 263-274.

634 Senbayram, M., Chen, R., Budai, A., Bakken, L., Dittert, K., 2012. N₂O emission and
635 the N₂O/(N₂O + N₂) product ratio of denitrification as controlled by available
636 carbon substrates and nitrate concentrations. Agriculture Ecosystems & Environment
637 147, 4-12.

638 Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B., Ogle, S.,
639 O'Mara, F., Rice, C., Scholes, B., Sirotenko, O., Howden, M., McAllister, T., Pan, G.,
640 Romanenkov, V., Schneider, U., Towprayoon, S., Wattenbach, M., Smith, J., 2008.
641 Greenhouse gas mitigation in agriculture. Philos. Trans. R. Soc. B-Biol. Sci. 363,
642 789-813.

643 Song, K., Hernandez, M.E., Batson, J.A., Mitsch, W.J., 2014. Long-term
644 denitrification rates in created riverine wetlands and their relationship with
645 environmental factors. Ecological Engineering 72, 40-46.

646 Thind, H.S., Rowell, D.L., 1999. Effects of algae and fertilizer-nitrogen on pH, Eh

647 and depth of aerobic soil in laboratory columns of a flooded sandy loam. *Biol. Fertil.*
648 *Soils* 28, 162-168.

649 Uchida, Y., Wang, Y., Akiyama, H., Nakajima, Y., Hayatsu, M., 2014. Expression of
650 denitrification genes in response to a waterlogging event in a Fluvisol and its
651 relationship with large nitrous oxide pulses. *Fems Microbiology Ecology* 88, 407-423.

652 Wang, J.Y., Jia, J.X., Xiong, Z.Q., Khalil, M.A.K., Xing, G.X., 2011. Water
653 regime-nitrogen fertilizer-straw incorporation interaction: Field study on nitrous oxide
654 emissions from a rice agroecosystem in Nanjing, China. *Agriculture Ecosystems &*
655 *Environment* 141, 437-446.

656 Wang, N., Ding, L.J., Xu, H.J., Li, H.B., Su, J.Q., Zhu, Y.G., 2015. Variability in
657 responses of bacterial communities and nitrogen oxide emission to urea fertilization
658 among various flooded paddy soils. *Fems Microbiology Ecology* 91, 11.

659 Wang, R., Feng, Q., Liao, T., Zheng, X., Butterbach-Bahl, K., Zhang, W., Jin, C.,
660 2013. Effects of nitrate concentration on the denitrification potential of a calcic
661 cambisol and its fractions of N₂, N₂O and NO. *Plant Soil* 363, 175-189.

662 Weitz, A.M., Linder, E., Frolking, S., Crill, P.M., Keller, M., 2001. N₂O emissions
663 from humid tropical agricultural soils: effects of soil moisture, texture and nitrogen
664 availability. *Soil Biology & Biochemistry* 33, 1077-1093.

665 Yang, H., Sheng, R., Zhang, Z., Wang, L., Wang, Q., Wei, W., 2016. Responses of
666 nitrifying and denitrifying bacteria to flooding-drying cycles in flooded rice soil.
667 *Applied Soil Ecology* 103, 101-109.

668 Yano, M., Toyoda, S., Tokida, T., Hayashi, K., Hasegawa, T., Makabe, A., Koba, K.,
669 Yoshida, N., 2014. Isotopomer analysis of production, consumption and
670 soil-to-atmosphere emission processes of N₂O at the beginning of paddy field
671 irrigation. *Soil Biology & Biochemistry* 70, 66-78.

672 Yao, Z., Zheng, X., Dong, H., Wang, R., Mei, B., Zhu, J., 2012. A 3-year record of
673 N₂O and CH₄ emissions from a sandy loam paddy during rice seasons as affected by
674 different nitrogen application rates. *Agriculture Ecosystems & Environment* 152, 1-9.

675 Zheng, X., Wang, M., Wang, Y., Shen, R., Gou, J., Li, J., Jin, J., Li, L., 2000. Impacts
676 of soil moisture on nitrous oxide emission from croplands: a case study on the

rice-based agro-ecosystem in Southeast China. *Chemosphere - Global Change Science* 2, 207-224.

Table 1 Primer sequence and quotations

Primer ^a	Primer sequence ^b (5'-3')	Reference
<i>narG</i> 145F	ACS CAY GGS GTD AAC TGY AC	(Chen <i>et al.</i> , 2012)
<i>narG</i> 571F	CCG ATY CCG GCV ATG TCS AT	(Chen <i>et al.</i> , 2012)
<i>narG</i> 773R	GGN ACG TTN GAD CCC CA	(Chen <i>et al.</i> , 2012)
<i>nosZ</i> 1126F	GGG CTB GGG CCR TTG CA	(Chen <i>et al.</i> , 2012)
<i>nosZ</i> 1381R	GAA GCG RTC CTT SGA RAA CTT G	(Chen <i>et al.</i> , 2012)
<i>nosZ</i> -F	CGY TGT TCM TCG ACA GCC AG	(Rosch <i>et al.</i> , 2002)
<i>nosZ</i> -R	CAT GTG CAG NGC RTG GCA GAA	(Rosch <i>et al.</i> , 2002)

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

^bY=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

Consumption rates of NO₃-N content in soil profile of three nitrate treatments during the submerged incubation of paddy soil cores.

	Soil layer	Day 0-1 ^a	Day 1-7 ^b	Day 7-13 ^b
N0	0-5cm	3.28(0.46)	0.02(0.02)	0.27(0.02)
	5-10cm		0.03(0.04)	0.23(0.01)
	10-15cm		-0.03(0.02)	0.25(0.01)
	15-20cm		-0.08(0.03)	0.22(0.02)
N100	0-5cm	50.59(10.17)	6.33(1.80)	0.73(0.34)
	5-10cm		1.70(0.48)	-0.11(0.04)
	10-15cm		0.86(0.34)	-0.17(0.03)
	15-20cm		0.42(0.08)	-0.21(0.04)
N350	0-5cm	59.96(10.71)	20.29(6.39)	5.65(2.17)
	5-10cm		7.14(2.13)	2.24(0.33)
	10-15cm		3.76(0.28)	1.09(0.31)
	15-20cm		1.19(0.53)	0.02(0.15)

^aDay 0-1 indicated the NO₃-N consumption rates of the whole soil core in first 24 h incubation caculated by Eq.(3). Values are means (standard error), n=3.

^bDay 1-7 indicated the NO₃-N consumption rates of the specific soil layers from incubation day 1 to day 7, and Day 7-13 was from incubation day 7 to day 13. Both were calculated by Eq. (1). Values are means (standard error), n=3.

Table 3

Nonparametric correlations by Spearman's test of N_2O flux and N_2O concentrations in soil profile with various nitrate amendments

Soil layer	N0	N100	N350
0-5 cm	-0.245	0.762**	0.958**
5-10 cm	-0.203	0.769**	0.895**
10-15 cm	-0.4168	0.902**	0.895**
15-20 cm	-0.224	0.867**	0.923**

**, Correlation is significant at the 0.01 level (2-tailed).

Table 4

Theoretical N₂O concentrations and N₂O losses in A soil layers based on the linear correlations between NO₃-N and N₂O concentrations in B, C and D layers of soil profile.

	Time	Linear equation	R ²	Sig.	Theoretical value ^a	Measured value	D-value ^b	Daily N ₂ O emission ^c	loss
					g N ₂ O-Nm ⁻³		mgN ₂ O-N		
N100	1	Y=0.308X+1.733	0.63	0.01	14.94	5.37	8.45	0.89	7.56
	7	Y=1.215X-0.518	0.79	0.00	8.33	0.98	6.49	0.37	6.12
	13	Y=0.001X-0.0008	0.42	0.06	0.002	0.001	0.001	0.004	-0.003
	Sum						14.94	1.204	13.74
N350	1	Y=0.062X+1.778	0.85	0.00	15.51	5.72	8.64	1.27	7.37
	7	Y=1.589X+24.53	0.87	0.00	148.54	51.55	85.64	6.57	79.07
	13	Y=0.824X+1.464	0.67	0.01	34.26	7.84	23.33	1.44	21.89
	Sum						117.61	9.28	108.33

^aThe theoretical N₂O concentrations in A soil layers (Y) were calculated by putting the NO₃-N contents of A layer (X) into the linear formulas respectively.

^bD-value was the deficits between the theoretical and measured N₂O concentrations in A layers multiply by soil layer volume.

^cDaily N₂O emission was estimated by the N₂O flux in 24h.

Table 5

Spearman's correlation coefficients for relationships between N₂O gas emissions and environmental variables in all 4 soil layers after addition of nitrate.

N ₂ O	Soil layer	NO ₃ -N	DOC	Ph	Eh	narG abundance	nosZ abundance
N ₂ O flux	A layer	0.888**	0.379	-0.282	0.766**	0.762**	0.341
	B layer	0.745**	0.220	-0.351	0.826**	0.359	0.053
	C layer	0.662**	0.142	-0.192	0.836**	0.124	-0.326
	D layer	0.643**	0.599**	-0.045	0.752**	0.043	0.045

**. Correlation is significant at the 0.01 level (2-tailed).

Figure captions:

Fig.1. Schematic diagram of the pot for gas sampling. **1:** silicon tubes, **2:** stainless steel tube, **3:** three-way stopcock, **4:** gas sampling static chamber, **5:** water. **A layer** represented 0-5 cm soil depth; **B layer** represented 5-10 cm soil depth; **C layer** represented 10-15 cm soil depth; and **D layer** represented 15-20 cm soil depth.

Fig.2. Dynamics of NO₃-N concentrations in various soil layers of (a) N350, (b) N100, (c) N0 treatments during flooded incubation. Data are mean ± standard error of the three replicates.

Fig.3. Dynamics of N₂O flux of N0, N100 and N350 treatments during flooded incubation. Data are mean ± standard error of the three replicates.

Fig.4. Dynamics of N₂O concentrations in different soil layers by (a) 350 mg NO₃-N addition, (b) 100 mg NO₃-N addition and (c) 0 mg NO₃-N addition. Data are mean ± standard error of the three replicates.

Fig.5. Dynamics of the copy numbers of denitrifying genes along soil profiles during the flooded incubation after three nitrate amendments. (a): *narG* gene; (b): *nosZ* gene. Data are mean ± standard error of the three replicates.

Fig.6. PCA analysis of *narG* composition (with *HhaI*) in four soil layers by three nitrate amendments on flooding (a) day 1, (b) day 7 and (c) day 13; *nosZ* composition (with *CfoI*) on flooding (d) day 1, (e) day 7 and (f) day 13 based on T-RFLP data. n=3.

Fig. 1

Fig.2

Fig.3

Fig.4

Fig.5

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757

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Fig.6a

759

760

Fig.6b

Fig.6c

Fig.6d

Fig.6e

Fig.6f