Effects of land-use change and fertilization on N₂O and NO fluxes, the abundance of nitrifying and denitrifying microbial communities in a hilly red soil region of southern China

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ABSTRACT

Nitrous oxide (N₂O) and nitric oxide (NO) are generally interrelated in soil nitrogen biogeochemical cycles. However, the effects of land-use change and fertilization on N₂O and NO fluxes and the underlying mechanisms are not well understood, especially in fields converted from rice paddies (RPs) to upland cultivation. This study investigated the effects of land-use change from RPs to a citrus orchard (OR) as well as fertilization on N₂O and NO fluxes, and the gene abundance of nitrifying and denitrifying microbial communities by using static chamber-gas chromatography and quantitative real-time PCR (qPCR). Structural equation modeling was performed to determine whether soil properties and the abundances of nitrifiers and denitrifiers influenced *in situ* N₂O and NO fluxes. Land-use change from RPs to an OR and fertilization significantly increased N₂O and NO fluxes. Land-use change increased AOA abundance, did not affect AOB abundance, and decreased *nirK*, *nirS*, and *nosZ* abundances. The growth of AOA and AOB abundance in response to fertilization was generally stimulated by providing more NH₄⁺, but sometimes was inhibited by lower pH. Fertilization did not significantly affect the abundance of denitrifiers. Soil properties and functional microbial groups were explanatory variables, while land-use change altered their role in predicting *in situ* N₂O and NO fluxes. For N₂O fluxes, dissolved organic carbon was the strongest predictor in the RPs, whereas the primary explanatory factor was *nirK* abundance in the OR. For NO fluxes, soil temperature was the most important explanatory variable. Our results demonstrate the need for comprehensive approaches incorporating nitrifying and denitrifying processes to improve our understanding of biogeochemical cycles.

1. Introduction

It is of great concern worldwide that nitrogenous gases contribute to regional and global-scale environmental issues (IPCC, 2013). Nitrous oxide (N₂O) is one of the main potent long-living greenhouse gases that contributes to global warming and participates in the destruction of stratospheric ozone (Ravishankara et al., 2009). Nitric oxide (NO) is not only a precursor for tropospheric ozone but is also involved in the regulation of the oxidation balance of the atmosphere (Bouwman et al., 2002). In most agricultural soils, N₂O and NO emissions strongly depend on the activity of the microbiological nitrogen processes of

nitrification and denitrification, in which both gases are produced as byproducts or intermediate products (Bouwman et al., 2002).

Land-use change, which is regarded as the second largest anthropogenic source of greenhouse gas emissions, can significantly alter the dynamics of the global nitrogen cycle (IPCC, 2013; van Lent et al., 2015). Although the effects of land-use change on greenhouse gas emissions have been widely reported, few *in situ* studies have been conducted after land-use change from conventional rice paddies (RPs) to upland cultivation, especially including year-round continuous measurements of both N₂O and NO fluxes (Yao et al., 2015; Zhang et al., 2016). Several previous studies have demonstrated relatively

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lower N_2O emissions from RPs than from upland soils because anaerobic conditions limit nitrate availability and strict anaerobiosis favors complete denitrification to N_2 (Zou et al., 2005; Liu et al., 2016). However, by conducting two years of field measurements of N_2O flux from four different types of land uses in subtropical red soil, Lin et al. (2012) reported that N_2O emissions from rice paddies were approximately 54.4% higher than those from orchard soil. This discrepancy might be partly attributed to the differences in the land-use legacies and fertilization managements (Scheer et al., 2008; Shao et al., 2017). Fertilization is the management practice most frequently used for agricultural soils and has long been identified as an important factor in regulating N_2O and NO fluxes (Bouwman et al., 2002; Dai et al., 2013; Liu et al., 2015). Fertilization is widely accepted to cause a significant increase in N_2O and NO emissions by providing more inorganic N contents (Bouwman et al., 2002; Rowlings et al., 2013). However, few year-round field studies have investigated the response of N_2O and NO emissions to fertilization simultaneously from the red soil region of China, resulting in uncertainties about the emission inventory of nitrogenous gases (Bouwman et al., 2002; Akiyama et al., 2005).

Nitrification is the biological oxidation of ammonium (NH_4^+) to nitrite (NO_2^-), which is performed by ammonia-oxidizing archaea (AOA) and bacteria (AOB), followed by the oxidation of the NO_2^- to nitrate (NO_3^-) (Rotthauwe et al., 1997). Denitrification is the stepwise reduction of NO_3^- to N_2 via NO_2^- , NO and N_2O by denitrifiers, in particular the NO_2^- reductase encoded by either *nirK* or *nirS* gene and the N_2O reductase encoded by *nosZ* gene (Throbäck et al., 2004). Land-use change from paddies to uplands involves niche specialization with environmental factors (e.g., oxygen, ammonia and pH) that are key factors influencing nitrifiers and denitrifiers (Alam et al., 2013; Yang et al., 2016). However, detailed measurements of the abundances of nitrifying and denitrifying microbial communities with simultaneous N_2O and NO flux determination are still limited, especially for the red soil regions in China. Moreover, though a large number of studies have documented nitrifiers and denitrifiers in response to fertilization (Szukics et al., 2009; Alam et al., 2013), some discrepancy remains, resulting from different land-use types (He et al., 2007; Di et al., 2014; Long et al., 2016). Furthermore, because microbial communities play an essential role in the production and consumption of N_2O and NO (Bergaust et al., 2010; Medinets et al., 2015), conceptual models have been used to predict the dynamics of trace gas fluxes (Petersen et al., 2012; Lammel et al., 2015; Martins et al., 2015). Therefore, to improve our understanding of the dynamics of N_2O and NO fluxes and the underlying mechanisms, detailed studies of the abundances of soil nitrifiers and denitrifiers following land-use change and fertilization are needed.

Red soil, one of the typical agricultural soils in southern China, covers approximately 11.8% of the country's land surface, produces 80% of the rice, and supports 22.5% of the population of China (Liu et al., 2016). In recent decades, the red soil regions have been undergoing remarkable land-use change due to increasing socio-economic developments and demands for livestock products. In particular, changing rice paddies to upland cultivation for growing vegetables, fruits and economic forests have been locally recommended to meet the increasing market demands and obtain higher economic returns in these regions (Lin et al., 2012). This process has led to a diverse range of land uses with different cultivation, irrigation and fertilizer rates, which could result in significant differences in soil nitrifiers and denitrifiers and subsequent N_2O and NO fluxes. Therefore, understanding the effects of land-use change from paddies to orchards on N_2O and NO fluxes as well as the abundances of nitrifying and denitrifying microbial communities is critical for elucidating the mechanisms and processes of nitrogenous gas emissions. However, few studies have simultaneously paid attention to the changes in N_2O /NO fluxes and the abundances of nitrifying and denitrifying microbial communities in these regions. The present study thus aimed to (1) assess the effects of land-use change and fertilization on *in situ* N_2O and NO fluxes; (2) examine how land-use

change and fertilization affect the abundances of nitrifying and denitrifying microbial communities; and (3) evaluate the existing relationships among N_2O /NO fluxes, soil properties, and functional gene abundance.

2. Materials and methods

2.1. Field site and experimental setup

The study site is located at the Qianyanzhou Experimental Station (26°44'N, 115°04'E), Chinese Academy of Science (CAS), in Jiangxi Province, southern China. This region experiences a subtropical warm and humid monsoon climate. During the period 1989–2010, the mean annual air temperature and precipitation were 18.0 °C and 1509.0 mm, respectively (Liu et al., 2016). The soil is typical red soil found in middle-subtropical China and classified as Cambisol according to the Ultisol classification. The soil texture is sandy loam, consisting of 58% sand, 31% silt, and 11% clay. Double-cropping rice is the main cropping pattern in this region.

The two most common land-use types in the hilly red soil regions of southern China were selected for the present study, namely, rice paddy (*Oryza sativa* L.) and citrus orchard (*Citrus reticulata* L.). Conventional rice paddies had been continuously cultivated for approximately 10 years following the regime of local field managements, and a portion of the fields was converted to orchard in June 2012. Under each land-use type, two fertilizer treatments (i.e., conventional fertilization and no fertilization) were established. The fertilization treatment followed the local cropping regimes and farmer fertilization practices. The fertilizers used were compound fertilizer (15% N) and urea (46% N). The other was treated as a control without fertilization, with additional management practices being the same as in the fertilization treatment. Therefore, four treatments—rice paddies with fertilization (RP-F) and without fertilization (RP-NF) and citrus orchards with fertilization (OR-F) and without fertilization (OR-NF)—were arranged in a completely randomized block design with four replicates (12 × 14 m). Detailed descriptions of the experimental site, management practices and experimental design can be found in previous publications (Liu et al., 2016, 2017). To ensure survival and yield, a floodwater layer of 5–7 cm was maintained in the RPs until the mid-season drainage, from July 29 to Sep 15, 2013 for the late rice and from April 18 to Jun 10, 2014 for the early rice. The cultivations and fertilization practices in the RP and OR are shown in Table 1.

2.2. Measurement of N_2O and NO fluxes

In-situ fluxes of N_2O and NO were simultaneously measured from July 2013 to August 2014 using a static chamber-based method as described by Mei et al. (2009) and Yao et al. (2015). A stainless steel collar (diameter = 40 cm) was pre-installed in the centre of each plot before rice transplanting or orchard planting. The top edge of the collar contains a groove (5 cm in depth) filled with water to seal the rim of a chamber during gas collection. Cylindrical sampling chambers with a diameter of 40 cm and height of 0.39 or 0.69 m (according to the plant height) were covered with a layer of thermal insulation to minimize air temperature changes inside the chamber and equipped with a circulating fan to ensure complete gas mixing during the gas sampling period. The base frames were kept in the same location throughout the entire measurement period in the orchard plots, whereas those in the paddy fields were removed before tillage and placed (24 h before the measurement) in the location marked for subsequent measurements.

Generally, the flux measurements were carried out daily within duration of 5–7 days after each fertilization event, and performed once or twice per week for the remaining period. On each sampling day, gas samples were collected between 09:00 and 11:00 am. Five samples for the N_2O analysis were taken from the headspace of chamber using 20 mL polypropylene syringes at an interval of 10 min after chamber

Table 1
Management practices for the experimental fields.

Management	Date
Rice paddies (RPs)	
Flooding period	Jul 29, 2013–Sep 15, 2013
Rice transplanting	Jul 30, 2013
Compound fertilizer (72 kg N ha ⁻¹) applied to RP-F	Jul 30, 2013
Urea (106 kg N ha ⁻¹) applied to RP-F	Aug 10, 2013
Rice harvest	Nov 14, 2013
Drainage period	Sep 15, 2013–Apr 18, 2014
Flooding period	Apr 18, 2014–Jun 10, 2014
Rice transplanting	Apr 19, 2014
Compound fertilizer (72 kg N ha ⁻¹) applied to RP-F	Apr 19, 2014
Urea (106 kg N ha ⁻¹) applied to RP-F	Apr 29, 2014
Rice harvest	Jul 23, 2014
Drainage periods	Jun 10, 2014–Jul 23, 2014
Citrus orchards (ORs)	
Compound fertilizer (67.2 kg N ha ⁻¹) and urea (20.8 kg N ha ⁻¹) applied to OR-F	Jul 17, 2013
Compound fertilizer (67.2 kg N ha ⁻¹) and urea (20.8 kg N ha ⁻¹) applied to OR-F	Apr 17, 2014

closure. Each sample was immediately injected into 20 mL vacuum Exetainer® borosilicate vials (Labco, High Wycombe, UK). Within 24 h, each gas sample was evacuated with a sampling pump via stainless steel tubes, dried in a condenser and automatically injected into the gas chromatography (Agilent 7890A, California, USA) equipped with an electron capture detector (ECD). For the NO analysis, two gas samples from the headspace of each chamber were taken at the beginning and end of the chamber enclosure period at an interval of 7–10 min (Mei et al., 2009). Within 4 h after sampling, the NO concentrations were analyzed using a model 42i chemiluminescence NO–NO₂–NO_x analyzer (Thermo Electron Corporation, Waltham, USA). The N₂O and NO fluxes were determined from the linear or nonlinear changes in gas concentrations in the enclosed chamber headspace with time (Mei et al., 2009; Yao et al., 2015), and corrected for the chamber air temperature and ambient air pressure influences. Cumulative N₂O and NO emissions were sequentially computed from the fluxes between every two adjacent intervals of measurements and were estimated by linear interpolation (Wu et al., 2010; Yao et al., 2015). The direct emission factor (EF) of applied N for N₂O and NO is defined as the emissions from fertilized plots minus the emissions from unfertilized plots, indicated as the percentage of N₂O and NO emitted as a proportion of the N applied (IPCC, 2006). The equation is as follows:

$$EF = \left(\frac{E_N - E_0}{N} \right) \times 100\%$$

where E_N and E₀ are the cumulative N₂O or NO emissions (kg N ha⁻¹) from the fertilization treatment and the non-fertilization treatment,

respectively. N is the total N (kg N ha⁻¹) input.

2.3. Soil sampling and chemical analysis

Daily precipitation and air temperature were measured at the Qianyanzhou meteorological station. Soil temperature and moisture (0–10 cm) for each treatment were measured using a portable digital thermometer (JM624, Tianjin, China) and a moisture probe meter (TDR100, Spectrum, USA), respectively. Soil samples from the top layer (0–10 cm) were taken once per month or once every 2 months from July 2013 to August 2014. From each plot, five soil cores (diameter = 3 cm) were acquired, pooled, and sieved at 2 mm. Subsequently, subsamples of each soil sample were stored at 4 °C for analyses of soil characteristics or at –80 °C for DNA extraction. Soil pH was measured at a soil:water ratio of 1:2.5 using a pH meter. Soil organic carbon (SOC), total nitrogen (TN), and C:N ratio were measured using an element analyzer (Elementar, Hanau, Germany). Soil ammonium (NH₄⁺-N) and nitrate (NO₃⁻-N) were extracted from 20 g of fresh soil with 1 M KCl (soil:water = 1:5 w/v) and quantified using a flow injection analyzer (Seal AA3, Norderstedt, Germany). Soil dissolved organic carbon (DOC) was analyzed using a TOC-TN analyzer (Elementar, Laurel, Germany) after extraction with distilled water (soil:water = 1:5 w/v).

2.4. Soil DNA extraction and quantification of nitrifier and denitrifier abundances

According to the manufacturer's instructions, soil DNA was extracted from 0.3 g of freeze-dried soil using Mobio Powersoil™ DNA isolation kit (MoBio Laboratories, Carlsbad, USA). The quality and concentrations of the extracted DNA were estimated using agarose gel electrophoresis and a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). DNA extraction yields fell in the range of 19.7–68.3 ng/μL with an average of 38.32 ± 10.5 ng/μL and an A260/280 ratio of 1.80 ± 0.15 ng/μL. All soil DNA extracts were diluted with nuclease-free DI water to a ratio of 1:5 to reduce potential PCR inhibition and were stored at –80 °C until further use.

Quantification real-time PCR (qPCR) was used to quantify AOA, AOB, *nirK*, *nirS* and *nosZ* abundances. The qPCR assays were performed on an iCycler IQ2 Thermocycler (Bio-Rad Laboratories, Hercules, USA). Amplification was performed in a 25 μL reaction mixture containing 12.5 μL of SYBR® Premix Ex Taq™ (TaKaRa Biotechnology, Dalian, China), 0.5 μL bovine serum albumin (20 mg mL⁻¹), 0.5 μL each primer (10 μM) and 2 μL 5-fold-diluted DNA as a template. The standard curves for all the detected genes were created using 10-fold dilution series (10¹ ~ 10⁷ copies) of the respective plasmids containing the targeted gene fragments. The presence of PCR inhibitors in DNA was examined by a 10-fold dilution series before qPCR, and inhibition was not detected after more than a 5-fold dilution when qPCR was performed. Details of the primer sequence and amplification conditions for AOA *amoA*, AOB *amoA*, *nirK*, *nirS* and *nosZ* genes are shown in Table 2. For each primer pair, the no-template control reactions in each run had an

Table 2
Primer pairs and PCR conditions used for qPCR amplification.

Primers	Fragment length	Primer pair	Sequence(5'-3')	Annealing temperature and time	References
AOA <i>amoA</i>	635 bp	CrenamoA23-F CrenamoA616-R	ATGGTCTGGCTWAGACG GCCATCCATCTGTATGTCCA	53 °C for 45 s	Tourna et al. (2008)
AOB <i>amoA</i>	491 bp	<i>amoA</i> -1F <i>amoA</i> -2R	GGGGTTTCTACTGGTGGT CCGCTCKGSAAGCCTTCTTC	55 °C for 45 s	Rothauwe et al. (1997)
<i>nirK</i>	472 bp	F1aCu R3Cu	ATCATGGTSGTCCGCG GCCTGATCAGRTTGTGGTT	63 °C for 30 s	Hallin and Lindgren (1999)
<i>nirS</i>	425 bp	Cd3aF R3 cd	GTSACGTSAAAGGARACSGG GASTTCGGRTGSGTCTCTTGA	57 °C for 30 s	Throbäck et al. (2004)
<i>nosZ</i>	453 bp	<i>nosZ</i> -F <i>nosZ</i> 1662R	CGYTGTTCMTGACAGCCAG CGSACCTTSTTGCCSTYGGC	57 °C for 30 s	Throbäck et al. (2004)

undetectable amplification. Each reaction was performed in triplicate. Standards used in the qPCR analysis were prepared by cloning AOA *amoA*, AOB *amoA*, *nirK*, *nirS*, and *nosZ* genes from genomic DNA from *Nitrosomonas europaea* spp., *Nitrospira multiformis* spp., *Alcaligenes xylooxidans* spp., *Pseudomonas aeruginosa* spp., and *Pseudomonas fluorescens* spp., respectively. PCR efficiencies were 90% for AOA, 91% for AOB, 90% for *nirK*, 85% for *nirS* and 95% for *nosZ*, respectively, and the R^2 values were between 0.990 and 0.998. The final copies of genes were obtained by calibrating against the extracted plasmid DNA copies based on the slope of linear regression between copies and cycle threshold.

2.5. Statistical analyses

Repeated-measures ANOVA with Duncan's *post hoc* test was used to examine the effect of land-use change and fertilization on soil N_2O and NO fluxes, AOA, AOB, *nirK*, *nirS*, and *nosZ* abundance, soil temperature, soil moisture, pH, SOC, TN, C:N ratio, NH_4^+-N , NO_3^--N , and DOC from Aug 2013 and Jul 2014. The experimental treatment was set as the between-subjects factor, and the date was selected as the within-subjects factor. The general linear model (GLM) for univariate analysis was used to examine the effects of land-use change and fertilization on cumulative N_2O and NO emissions. The T test was used to calculate the differences in the EF for N_2O-N and $NO-N$ between the RP and OR. All above statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, USA). Structural equation modeling (SEM) was used to investigate the causal-effects linkages among soil properties, microbial communities, and *in situ* N_2O and NO fluxes in the ORs and RPs. Path analyses were performed as described by Petersen et al. (2012) on the basis of a reduction of the full models and selection of the best models in which all paths were statistically significant ($P < 0.05$). The coefficients, calculated as standardized coefficients for each path, were determined by the analysis of the correlation matrix and are presented in the figures. Path coefficients (values on the arrows) indicate by how many standard deviations (SDs) the effect variable would change if the causal variable were changed by SD. The general fit of the model is shown, with non-significant χ^2 test ($P > 0.05$) and fitness index. The SEM was carried out using Amos 22.0 (Amos Development Co, Maine, USA).

3. Results

3.1. Environmental conditions and soil properties

Air temperature at the experimental site ranged from 0.02 °C (Feb 2014) to 31.9 °C (Aug 2013), with an average value of 19.3 °C (Fig. 1a). The annual precipitation was 1424.8 mm, lower than the long-term average (1509.0 mm), and 57% of the rainfall occurred between March and June (Fig. 1a).

The temporal variations of soil properties were pronounced and mainly driven by management practices and environmental conditions (Fig. S1). As expected, land-use change and fertilization significantly changed the soil properties (Fig. 2 and Table 3). Specifically, land-use change from paddies to orchards significantly increased soil temperature (Fig. 2a and Table 3), but soil moisture in the RPs was higher than that in the ORs (Fig. 2b). Both land-use change and fertilization significantly reduced soil pH ($P < 0.001$), and the lowest pH was observed in the OR-F (Fig. 2c and Table 3). Land-use change significantly increased the SOC, TN, and C:N ratio ($P < 0.05$) (Fig. 2d–f and Table 3). However, fertilization did not affect soil temperature, soil moisture, SOC or C:N ratio ($P > 0.05$) (Table 3). Fertilization was shown to significantly influence soil TN contents ($P < 0.05$) (Table 3). Land-use change significantly increased soil NO_3^--N contents ($P < 0.001$), but did not affect soil NH_4^+-N contents ($P = 0.055$) (Fig. 2g,h and Table 3). The NH_4^+-N and NO_3^--N contents in the fertilization treatment were significantly higher than those in the non-

Fig. 1. Seasonal variations of the daily air temperature and precipitation (a), N_2O fluxes (b, c) and NO fluxes (d, e) in the RPs and ORs during the observation period. The data ($n = 83$) are shown as the means with standard errors ($n = 4$). Gray arrows represent the application of fertilizer to RP-F. Black arrows represent the application of fertilizer to OR-F. Horizontal bars indicate the period of flooding in the paddy plots.

fertilization treatment in the RPs and ORs (Fig. 2g,h). Moreover, soil DOC contents were significantly lower in the ORs than that in the RPs (Fig. 2i). Fertilization significantly increased soil DOC contents in the RPs ($P < 0.05$) (Fig. 2i and Table 3).

3.2. N_2O and NO fluxes

As shown in Fig. 1b–e, all treatments showed seasonal variations of N_2O and NO fluxes. In the RPs, N_2O fluxes varied from 3.30 to 689.61 $\mu g N m^{-2} h^{-1}$ and from 1.47 to 192.06 $\mu g N m^{-2} h^{-1}$ for the RP-F and RP-NF, respectively (Fig. 1b). In the flooding period, unperceivable N_2O fluxes were observed, whereas fertilization triggered the N_2O pulses in the mid-season drainage (Fig. 1b). In the ORs, N_2O fluxes ranged from 4.93 to 955.06 $\mu g N m^{-2} h^{-1}$ and from 4.54 to 952.73 $\mu g N m^{-2} h^{-1}$ in the OR-F and OR-NF, respectively (Fig. 1c). Land-use change from paddies to orchards significantly increased N_2O emissions ($P < 0.001$) (Fig. 1b,c and Table 4). Compared to the control, the application of fertilizer in both land-use types significantly increased N_2O emissions ($P < 0.001$) (Fig. 1b,c and Table 4). Meanwhile, the annual cumulative N_2O emissions ranged from 9.03 to 12.24 kg N ha $^{-1}$ in the ORs, significantly higher than those in the RPs (Table 5). Accordingly, direct EFs for N_2O were estimated to be 1.11% and 1.82% in the RP and OR, respectively (Table 5).

In the RPs, the NO fluxes ranged from 2.07 to 31.96 $\mu g N m^{-2} h^{-1}$ for the RP-F and from 0.055 to 11.88 $\mu g N m^{-2} h^{-1}$ for the RP-NF, respectively (Fig. 1d). Maximum NO fluxes in the later rice season occurred after the transplanting, whereas those in the early rice season were observed in the mid-season drainage. In the ORs, the NO fluxes from the OR-NF were relatively stable (ranging from 9.97 to 58.43 $\mu g N m^{-2} h^{-1}$) (Fig. 1e). In the OR-F, the NO fluxes were characterized by pulse emission event, with the maximum being observed in

Fig. 2. Soil temperature (a), soil moisture (b), pH (c), SOC (d), TN (e), C:N (f), NH_4^+ -N (g), NO_3^- -N (h), and DOC (i) under the four treatments. The bottom, middle and top lines of the box denote the 25%, 50%, and 75% percentiles, respectively. The upper and lower whiskers extending from each quartile denote the highest and lowest value, respectively. The squares in the box denote mean values ($n = 36$). Different lowercase letters indicate significant differences ($P < 0.05$).

the mid-Apr 2014 (Fig. 1e). During the entire observation period, both land-use change from paddies to orchards and fertilization significantly increased NO fluxes ($P < 0.001$) (Fig. 1 and Table 4). The annual cumulative NO emissions were 2.28 and 3.19 kg N ha⁻¹ in the OR-NF and OR-F, respectively, which were significantly higher than those in the RPs (Table 5). As a result, land-use change from paddies to orchards increased the EF for NO from 0.12% to 0.52% (Table 5).

3.3. Abundances of AOA, AOB, *nirS*, *nirK* and *nosZ* genes

The abundances of *amoA* genes for AOA and AOB were calculated based on the qPCR results and ranged, respectively, from 6.96×10^6 to 4.66×10^8 copies g⁻¹ dry soil, and from 1.29×10^6 to 9.03×10^6 copies g⁻¹ dry soils (Fig. S2a,b and Fig. 3a,b), suggesting that the AOA abundance was consistently higher than the AOB abundance. The AOA abundance was significantly altered by land-use change from paddies to orchards ($P < 0.001$), fertilization ($P < 0.05$), and their interactions ($P < 0.001$) (Table 4). The AOA abundance was significantly higher in the ORs than in the RPs (Fig. 3a). Fertilization significantly increased AOA abundance in the ORs but reduced AOA abundance in the RPs (Fig. 3a). The conversion of RPs to ORs did not distinctly affect the AOB abundance ($P = 0.070$) (Table 5). However, the AOB abundance in the fertilization treatments was significantly higher than that with the non-fertilization treatments in the OR and RP ($P < 0.05$) (Fig. 3b).

The numbers of *nirK*, *nirS* and *nosZ* gene copies ranged, respectively, from 4.64×10^6 to 1.05×10^9 copies g⁻¹ dry soil, from 1.28×10^7 to 5.34×10^8 copies g⁻¹ dry soil, and from 1.50×10^8 to 1.55×10^9 copies g⁻¹ dry soil (Fig. S2c–e and Fig. 3c–e). The abundances of *nirK*, *nirS* and *nosZ* significantly decreased after land-use change ($P < 0.001$), but were not affected by fertilization ($P > 0.05$)

Table 3
Results of repeated-measures ANOVA on the effects of land-use change, fertilization, and their interactions on soil properties.

Source	Soil temperature		Soil moisture		pH		SOC		TN		C:N		NH_4^+ -N		NO_3^- -N		DOC	
	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F
Land use change	0.021	58.694	0.000	107.25	0.000	8.483	0.013	135.984	0.000	8.483	0.005	25.065	0.055	4.607	0.000	539.686	0.000	101.587
Fertilization	0.235	0.123	0.435	10.789	0.000	2.485	0.141	28.173	0.033	2.485	0.416	5.762	0.000	87.116	0.000	87.390	0.026	7.022
Land-use change × Fertilization	0.465	10.465	0.475	11.235	0.995	0.176	0.682	0.000	0.165	0.176	0.045	2.186	0.019	7.612	0.004	13.042	0.821	0.054

Significant effects are highlighted in bold ($P < 0.05$).

Table 4Results of repeated-measures ANOVA on the effects of land-use change, fertilization, and their interactions on N₂O and NO fluxes, and abundances of AOA, AOB, *nirK*, *nirS* and *nosZ* genes.

Source	N ₂ O fluxes		NO fluxes		AOA		AOB		<i>nirK</i>		<i>nirS</i>		<i>nosZ</i>	
	P	F	P	F	P	F	P	F	P	F	P	F	P	F
Land-use change	0.000	49.469	0.000	585.863	0.000	814.462	0.070	3.967	0.001	18.429	0.000	64.256	0.000	55.384
Fertilization	0.000	15.287	0.000	53.061	0.004	12.910	0.000	44.996	0.279	1.286	0.171	2.123	0.991	0.000
Land-use change × Fertilization	0.603	0.286	0.111	2.969	0.000	130.709	0.060	4.308	0.250	1.462	0.950	0.007	0.405	0.750

(Fig. 3c–e and Table 5).

3.4. Relationships between nitrifiers and denitrifiers gene abundances, soil properties and *in situ* N₂O and NO fluxes

Due to the large differences of the soil properties, soil microorganisms, and *in situ* N₂O and NO fluxes, we conducted SEM analyses separately for the RPs and ORs. After the removal of non-significant paths and fitting to the test model to the observed data, the final models fitted well to N₂O data in the RPs and ORs (Fig. 4). In the RPs, the best model explained 39% of the variation of *in situ* N₂O fluxes containing soil temperature, soil moisture, NH₄⁺-N, NO₃⁻-N, DOC, AOA and *nirS* abundances, whereas DOC (with highest total effects) was the primary explanatory factor (Table S3). In the RPs, N₂O fluxes were positively related to *nirS* abundance, and negatively correlated with AOA abundance (Fig. 4a). In the ORs, the final model explained 58% of the variation of N₂O fluxes containing soil temperature, soil moisture, pH, NO₃⁻-N, AOA, and *nirK* abundances (Fig. 4b). In the ORs, N₂O fluxes were negatively correlated with AOA and *nirK* abundances (Fig. 4b). Soil *nirK* abundance was the most important factor explaining *in situ* N₂O from the ORs (Table S3). Fertilization could indirectly affect the N₂O fluxes through mediating the soil properties and microbial gene abundance (Fig. 4). In addition, some of the soil properties directly affected *in situ* N₂O and NO fluxes via impacts on gene abundance (Fig. 4 and Table S3).

For NO fluxes, path analysis suggested that the conceptual models fitted the observed data in the RPs and ORs (Fig. 5), which explained 68% and 40% of the *in situ* NO fluxes, respectively (Fig. 5). In the RPs, the final model included soil temperature, soil moisture, NH₄⁺-N, NO₃⁻-N, DOC, AOA, and AOB abundances (Fig. 5a). In the RPs, NO fluxes were negatively and positively correlated to AOA and AOB abundances, respectively (Fig. 5). In the ORs, the best model contained soil temperature, C:N, NO₃⁻-N, AOB, *nirK* and *nirS* (Fig. 5b). Moreover, NO fluxes were positively correlated with AOB and *nirS*, but negatively correlated with *nirK* abundances (Fig. 5b). Soil temperature was the most important factor explaining *in situ* NO fluxes from the RPs and ORs (Table S4). In addition, fertilization could affect the N₂O fluxes directly and indirectly (Fig. 4). Similarly, some of the soil properties could indirectly affect *in situ* N₂O and NO fluxes from the ORs (Fig. 5 and Table S4).

Fig. 3. Abundances of ammonia-oxidizing archaea (AOA) (a) and bacteria (AOB) (b), *nirK* (c), *nirS* (d) and *nosZ* (e) genes under the four treatments. The bottom, middle and top lines of the box denote the 25%, 50%, and 75% percentiles, respectively. The upper and lower whiskers extending from each quartile denote the highest and lowest value, respectively. The squares in the box denote mean values. Different lowercase letters indicate significant differences ($P < 0.05$).

4. Discussion

4.1. Effects of land-use change and fertilization on N₂O and NO fluxes

Land-use change was found to have a pronounced influence on N₂O and NO fluxes (van Lent et al., 2015; Liu et al., 2016). In our study, N₂O and NO fluxes were significantly higher in the ORs than in the RPs, which might be mainly caused by the anaerobic conditions prevailing in the RPs (Bouwman et al., 2002; Scheer et al., 2008). The RPs showed relatively lower N₂O fluxes in the flooding period (Fig. 1b). This finding is consistent with earlier studies by Zou et al. (2005) and Hou et al. (2012), who observed that N₂O emissions released from flooded rice paddies were negligible. However, the aerobic conditions in the ORs formed in fine-textured soils suitable for N₂O and NO emissions (Bouwman et al., 2002). The relatively greater *nosZ* abundance might also account for the lower N₂O fluxes in the RPs (Fig. 3e), since the *nosZ*-containing denitrifiers mainly reduced N₂O to N₂. The lower NO fluxes from the RPs might reflect the greater abundances of *nirK* and

Table 5Cumulative N₂O and NO emissions (means ± SE, n = 4) from the four treatments and EF (means ± SE, n = 4) for N₂O and NO in the RPs and ORs.

Treatments	Total N application (kg N ha ⁻¹ Y ⁻¹)	N ₂ O (kg N ha ⁻¹ Y ⁻¹)	NO (kg N ha ⁻¹ Y ⁻¹)	EF for N ₂ O (%)	EF for NO (%)
RP-F	356	6.02 ± 1.65c	0.67 ± 0.03c	1.11 ± 0.04b	0.12 ± 0.01b
RP-NF		2.07 ± 0.19d	0.25 ± 0.008d	–	–
OR-F	176	12.24 ± 1.49a	3.19 ± 0.11a	1.82 ± 0.05a	0.52 ± 0.01a
OR-NF		9.03 ± 0.61b	2.28 ± 0.15b	–	–

Different letters in the same column indicate significant differences ($P < 0.05$) between corresponding treatments.

Fig. 4. Structural equation model for the effects of soil properties and AOA, AOB, *nirK*, *nirS* and *nosZ* gene abundances on *in situ* N₂O fluxes in the RPs and ORs. Path coefficients (values on arrows) correspond to the standard coefficients computed based on the analysis of correlation matrices. Tests of path significance are given in Table S1. Coefficients differing significantly from 0 are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 5. Structural equation model for the effects of soil properties and AOA, AOB, *nirK*, *nirS* and *nosZ* gene abundances on *in situ* NO fluxes in the RPs (a) and ORs (b). Path coefficients (values on arrows) correspond to the standard coefficients computed based on the analysis of correlation matrices. Tests of path significance are given in Table S2. Coefficients differing significantly from 0 are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

nirS (Fig. 3c,d) because they are capable of consuming NO as an alternative electron acceptor in the anaerobic conditions (Wrage et al., 2001). The increases in N₂O emissions accompanying land-use change were also related to the changes in the ammonia oxidizers, which perform the rate-limiting steps of nitrification (Leininger et al., 2006; He et al., 2007; Di et al., 2009). Although ammonia oxidizers were not consistently correlated with *in situ* N₂O and NO fluxes in the ORs and RPs (Figs. 4 and 5), both were tightly related to ammonia-oxidation

rates (Liu et al., 2017). In addition, the higher NO₃⁻-N could be another reason for higher N₂O and NO fluxes in the ORs since higher soil NO₃⁻-N concentrations have been known to promote N₂O and NO emissions (Rowlings et al., 2013; Liu et al., 2015). Moreover, higher SOC contents appear to accelerate nitrogen transformation, such as the nitrification and denitrification rates in soil, as reported by Li et al. (2005). We measured a relatively higher SOC content after land-use change from paddies to orchards (Fig. 2d). Thus, the involved mechanisms of the increases in N₂O and NO fluxes induced by land-use change from paddies to orchards could be summarized as inducing N₂O/NO production and depressing N₂O/NO-consumption.

In this study, the seasonal variations of N₂O fluxes were similar to NO, characterized by emissions pulses following drainage and fertilization in the RPs and following fertilization and rainfall in the ORs. These results are consistent with numerous studies conducted in paddy fields (Zou et al., 2005; Hou et al., 2012) and orchard plots (Rowlings et al., 2013; Liu et al., 2016), showing that fertilization has a pronounced influence on N₂O and NO fluxes. It is likely that fertilization, the vast majority of which is supplied as inorganic N in the form of NH₄⁺-N and NO₃⁻-N, promotes N₂O and NO production (Liu et al., 2015; Yao et al., 2015). This viewpoint was further reinforced by our observation of higher NH₄⁺-N and NO₃⁻-N concentrations in the fertilization treatments of both land uses. Generally, soil carbon availability (e.g., SOC and DOC) promotes labile energy for nitrifiers and denitrifiers (Bouwman et al., 2002; Zou et al., 2005). Indeed, in our analysis, the DOC contents from the fertilization treatment were higher than in the non-fertilization treatment, albeit with no significant difference in the ORs (Fig. 2i). Another relevant factor was the lower pH induced by the fertilization because lower pH could be beneficial for the survival of some fungi and enhances heterotrophic nitrification (Marusenko et al., 2013). Furthermore, the responses of N₂O and NO fluxes to fertilization are microbiologically dependent (Cui et al., 2016; Zhong et al., 2016). In this study, fertilization significantly affected the abundance of nitrifiers but did not affect the abundance of denitrifiers. These findings provide support for the argument that nitrifiers, rather than denitrifiers, were the main contributors of the differences in N₂O and NO fluxes induced by fertilization (Martins et al., 2015). Accordingly, fertilization increased N₂O and NO flux in this region mainly via impacts on the production-mediated processes.

To generate total N₂O and NO emissions for inputting into the national inventory, fertilizer-induced N₂O and NO emissions were calculated as a product of the EF (IPCC, 2006). For the RPs, the EF for N₂O (1.11%) fell within the range 0.10%–1.78%, as observed by Zou et al. (2005) in China, but greatly higher than the suggested 0.3% used by the IPCC (IPCC, 2006) and 0.22% obtained in a review based on peer-reviewed journals (Akiyama et al., 2005). Higher EF values for N₂O of the paddies have been obtained in China because N fertilizer rates for rice systems are generally high (Ju et al., 2009; Linquist et al., 2012). The EF for NO for the RPs (0.12%) was lower than the value (0.20%) reported by Fang and Mu (2009). This result possibly occurred because the N fertilizer application rate (356 kg N ha⁻¹) in our study was higher than in the study (180 kg N ha⁻¹) by Fang and Mu. In the ORs, the EF for N₂O–N emissions (1.15%) was similar to the values in tropical and subtropical orchard fields listed by Rowlings et al. (2013). The conversion of paddies to orchard was shown to increase the EF for N₂O. Similarly, Linquist et al. (2012) found that the EF for N₂O was 1.21% and 1.06% for the upland cropping systems and 0.68% for the rice systems. However, no field observations have been made regarding the EF of NO–N for orchards; thus, *in situ* measurements of NO emissions from orchards are urgently needed. In summary, the EF of N₂O and NO displayed a wide variability across different land uses, which may also highly depend on the N fertilizer rate.

4.2. Effects of land-use change and fertilization on the abundance of nitrifying and denitrifying microbial communities

Our results showed that the AOA abundance was numerically more dominant than AOB abundance in acidic red soils, which was in good agreement with previous studies of agricultural soils (Leininger et al., 2006; He et al., 2007). Land-use change from paddies to orchards increased AOA abundance but did not affect AOB abundance, supporting the idea of niche differentiation between these two groups (He et al., 2007; Alam et al., 2013). Increases in the AOA rather than the AOB abundances might be ascribed to a higher affinity of AOA for oxygen (Szukics et al., 2009). However, other studies reported that AOA abundance was lower in uplands than in paddies, whereas AOB showed the opposite trend (He et al., 2007; Alam et al., 2013). It has been suggested that AOA abundance is likely to increase with decreasing pH (Nicol et al., 2008). Thus, the lower soil pH of the ORs than the RPs might have been more favorable for the AOA in the ORs. Meanwhile, pH has been identified as the main variable influencing AOB abundance, with greater abundance observed at a higher soil pH (Nicol et al., 2008). Moreover, Gleeson et al. (2010) reported that AOB abundance showed an increasing pattern with increasing soil moisture. The soil moisture and pH were lower in the ORs than the RPs, thus, the AOB abundance, in principle, should decrease after land-use change from paddies to orchards.

Fertilization decreased AOA abundance but increased AOB abundance in the RPs. In the ORs, both AOA and AOB abundances were increased by fertilization. A number of studies have demonstrated that the abundances of AOA and AOB remain unaffected or even decrease in response to fertilization, depending on the fertilizer rate (He et al., 2007; Di et al., 2014; Zhong et al., 2016). In contrast, other publications have indicated that the abundances of AOA and AOB were stimulated by fertilization in field studies (Alam et al., 2013; Dai et al., 2013) and laboratory incubations (Di et al., 2009). The different responses of AOA and AOB to the fertilization might be attributed to the ammonia (NH_4^+) availability. Generally, NH_4^+ is derived from either added inorganic NH_4^+ or mineralization of organic nitrogen (Tourna et al., 2008). However, the pH might affect the availability of NH_4^+ , as it would be ionized exponentially to NH_4^+ when pH decreased (He et al., 2007; Nicol et al., 2008). Therefore, in the RPs, lower pH caused by fertilization was a more important factor than the fertilizer itself as a substrate for AOA.

The abundances of *nirK*, *nirS* and *nosZ* genes decreased with land-use change from paddies to orchards, which might be explained by their different preferences with respect to oxygen supply (Wrage et al., 2001). When under the absolute anaerobic conditions, the denitrifier abundance was reduced (Uchida et al., 2014; Yang et al., 2016). Similarly, we also observed relatively lower denitrifier abundance during the flooding period of the RPs. However, the growth of denitrifiers is stimulated in the near-anaerobic conditions (Di et al., 2014; Uchida et al., 2014); thus, the flooding-drying patterns of the RPs would stimulate the growth of denitrifiers. Furthermore, significant increases in denitrifier abundance are associated with higher soil moisture (Uchida et al., 2014). Therefore, the lower soil moisture in the OR might also account for the lower *nirK*, *nirS*, and *nosZ* abundances.

The effect of fertilization on the abundances of the three denitrifying genes—*nirK*, *nirS* and *nosZ*—was not significant in either the RPs or the ORs, which was in agreement with the studies of Szukics et al. (2009) and Long et al. (2016). Conversely, Cui et al. (2016) observed distinct increases in the abundances of denitrifiers (i.e., *nirK*, *nirS* and *nosZ*) after long-term fertilization. Similarly, Hamonts et al. (2013) reported that the increases in NO_2^- , NO_3^- and DOC in the fertilizer-treated soils could lead to increases in the abundances of the denitrifiers. In addition, CO_2 emissions generated from the hydrolysis of fertilizer might allow the denitrifiers to proliferate (Hamonts et al., 2013). Moreover, low pH is known to slow down the turnover and assembly of N_2O reductase (Bergaust et al., 2010). In our study, NO_3^-

and DOC contents were increased by fertilization, whereas soil pH was decreased. Together, these factors might lead to the neutral effects of fertilization on the abundance of denitrifiers.

4.3. Relationships between nitrifiers and denitrifiers gene abundances, soil properties and associated N_2O and NO fluxes

Generally, positive relationships exist between N_2O /NO fluxes and the abundances of ammonia oxidizers because of their possession of enzymatic mechanisms for N_2O and NO production (Martins et al., 2015; Medinets et al., 2015). However, direct links between the abundance of nitrifying microorganisms and N_2O fluxes were complicated (Cantarel et al., 2012; Zhong et al., 2014). Negative correlations between AOA abundance and N_2O fluxes in the ORs and RPs were measured in this study (Fig. 4). The complex soil conditions, sampling points, heterogeneity of the soil microbe distribution and methodological limitations could account for such relationships (Ma et al., 2011). Denitrifiers played significant roles in N_2O /NO production and consumption in the different soils (Medinets et al., 2015). In our study, the *nirK* and *nirS* abundances were significantly correlated with N_2O and NO fluxes, which are in accordance with previous studies (Miller et al., 2009; Yoshida et al., 2012). The *nosZ* gene encodes N_2O reductase activity responsible for the reduction of N_2O to N_2 under anaerobic conditions (Ma et al., 2011; Di et al., 2014). However, our study did not detect a relationship between *nosZ* abundances and N_2O or NO emissions, which has been also reported for various ecosystems (Hamonts et al., 2013; Zhong et al., 2014). This is not surprising because *nosZ* gene as a measure of gross N_2O consumption and gross N_2 production may not necessarily be related to net N_2O emissions. Moreover, Chen et al. (2015) reported that *nosZ* gene expression was closely related to N_2 production rather than N_2O emission and suggested that the change of gene abundances at DNA level may not be sensitive enough to demonstrate the dynamics of N gas fluxes. Therefore, further studies on the gene transcripts at mRNA level and the key end-products ($\text{N}_2 + \text{N}_2\text{O}$) of denitrification are highly required to elucidate the role of nitrifiers and denitrifiers genes in the associated N gas production and emission.

In the RPs and ORs, some of the soil parameters were indirectly correlated with N_2O and NO fluxes via impacts on functional gene abundance, indicating that N_2O and NO fluxes also depended on those indirect parameters. Therefore, these results highlighted the value of using path analysis in soil nitrogen cycling, which would more effectively reveal the subtler mechanisms driving *in situ* N_2O and NO emissions (Cantarel et al., 2012; Lammel et al., 2015). Soil DOC was the most important predicting factor of *in situ* N_2O fluxes from the RPs. The negative correlation between soil DOC contents and N_2O fluxes in RPs was mainly due to that higher DOC contents were observed in the flooding period (Fig. S1), during which less N_2O was released. In the ORs, the *nirK* abundance was the strongest explanatory factor of *in situ* N_2O fluxes, which is in good agreement with Cantarel et al. (2012), who found the *nirK* abundance to be a significant explanatory factor for N_2O emissions. Moreover, our results showed that the *nirK* community might be more sensitive to the changes of soil temperature than *nirS*, which is in agreement with previous studies conducted in other ecosystems (Jung et al., 2011; Wertz et al., 2013). However, the *nirS* abundances were significantly correlated with soil DOC contents. Saleh-Lakha et al. (2009) also found that the *nirS* community might adapt more easily to changes in some soil conditions, such as soil nitrate content. For the NO fluxes, soil temperature was the best predictive variable in the RPs and ORs. This hierarchy of measuring factors might introduce the ways in which land-use change distinctly alters the role of soil properties and microbial functional groups in predicting *in situ* N_2O and NO fluxes. In addition, fertilization could directly and indirectly affect NO fluxes, whereas it only indirectly affected N_2O fluxes via impacts on soil properties and microbial gene abundance.

Environmental conditions can cause rapid changes in the N_2O and

NO fluxes (hours-days), and soil properties affect long-term predictions (seasons-years), whereas gene abundance is a significant indicator for mid-term predictions (week-months) (Lammel et al., 2015). Petersen et al. (2012) reported that gene abundance might not be related to real-time process rates (i.e., traces gases fluxes) but could be associated with potential process rates, such as potential nitrifying and denitrifying rates. Moreover, they also suggested that qPCR technology could be applied to track large-scale and long-term changes of biogeochemical cycling, which may be difficult to assess using short-term measurements (Petersen et al., 2012; Lammel et al., 2015). Thus, despite of some counterintuitive relationships, our conceptual model would be useful for predicting N₂O and NO fluxes and generating informative data for future study, since our study corroborated the gene abundances as bio-indicators of nitrification and denitrification with actual gas fluxes measured *in situ* based on year-round measurements. Furthermore, improved primer designs and additional gene markers have expanded data for nitrogen processes (e.g., nitrification and denitrification rates), and long-term measurements could improve models for understanding the microbial regulation of nitrogen processes in soils.

5. Conclusion

Overall, our study demonstrated that the N₂O and NO fluxes were substantially increased after land-use change from rice paddies to citrus orchards and fertilization. Moreover, the conversion of rice paddies to citrus orchards significantly increased AOA abundance, had no impact on the AOB abundance, and decreased *nirK*, *nirS*, and *nosZ* abundances. The growth of AOA and AOB abundances in response to fertilization was generally stimulated by providing more NH₄⁺ but sometimes inhibited by lower pH, whereas fertilization had no significant impact on the abundance of denitrifiers. Furthermore, our results indicated that land-use change from rice paddies to citrus orchards increased N₂O and NO *via* impacts on inducing N₂O/NO production and depressing N₂O/NO consumption, whereas fertilization increased N₂O and NO fluxes mainly *via* impacts on the production-mediated processes. Meanwhile, fertilization could directly and indirectly affect NO fluxes, but it could only indirectly affect N₂O fluxes *via* impacts on soil properties and microbial gene abundance. Soil properties and microbial groups were significant explanatory variables, while land-use change would alter their roles in predicting *in situ* N₂O and NO fluxes. Therefore, our results provide supporting evidence of the abundances of nitrifiers and denitrifiers in relation to N₂O/NO production and N₂O/NO reduction, which could help elucidate the variations in N₂O and NO emissions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.apsoil.2017.08.004>.

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