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REGULAR ARTICLE

Linking soil N_2O emissions with soil microbial community abundance and structure related to nitrogen cycle in two acid forest soils

Hongling Qin · Xiaoyi Xing · Yafang Tang · Haijun Hou · Jie Yang · Rong Shen · Wenzhao Zhang · Yi Liu · Wenxue Wei

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Abstract
Aims Tree species and seasonal change influence N_2O flux and microbial communities, but the mechanisms are unclear. We studied N_2O flux in soils planted with slash pine and oil-seed camellia trees. We sampled on typical days of the four seasons. We tested whether N-cycling communities respond more to tree species or seasonal change. We assessed how tree species affect N_2O flux.
Methods We used qPCR and RFLP to determine abundance and community composition of *amoA*-containing bacteria (AOB) and archaea (AOA), and denitrifiers that contain the *narG*, *nirK*, *nirS*, and *nosZ* genes.
Results N_2O flux rate and soil characteristics varied significantly between forest soils and sampling seasons. Abundance of all detected genes, but not of the *nirS* gene, was significantly affected by tree species. Differences in gene abundance between days in different seasons were found only for *narG*, *nirK,* and *nosZ*. Functional microbial community composition in the soil varied between the tree species for most of the genes studied, but varied, not significantly, slightly among sampling days. Differences in the abundance and community composition of nitrifiers and denitrifiers between tree species depended on soil concentration of NH_4^+, NO_3^-, and dissolved organic carbon (DOC). N_2O flux rate was affected by community composition, but not abundance of nitrifiers and denitrifiers. Temperature, NO_3^-, and DOC concentrations significantly affected N_2O flux.
Conclusions Tree species influenced N_2O flux more than seasonal change, by altering community composition and environmental factors rather than nitrifier/denitrifier abundance.

Keywords N_2O emission · Nitrification · Denitrification · Acid forest soil · Tree species · Seasonal change

Hongling Qin and Xiaoyi Xing contributed equally to this research.

Highlights
- Tree species sharply affected *in situ* N_2O flux and functional microbe community
- Abundance and structure of nitrifiers and denitrifiers varied between tree species depending on soil substrate availability
- Community structure of nitrifiers and denitrifiers was more affected by tree species than by seasonal change
- Microbial community composition rather than abundance determined N_2O flux in acid forest soils

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H. Qin · X. Xing · H. Hou · J. Yang · R. Shen · W. Zhang · Y. Liu · W. Wei (✉)
Key Laboratory of Agro-ecological Processes in Subtropical Regions, Taoyuan Agro-ecosystem Research Station, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China
e-mail: wenxuewei@isa.ac.cn

X. Xing · Y. Tang
University of Chinese Academy of Sciences, Beijing 100000, China

Y. Tang
Hubei Key Laboratory of Quality Control of Characteristic Fruits and Vegatbables, College of Life Science and Technology, Hubei Engineering University, Xiaogan 432000, China

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Introduction

Nitrous oxide (N_2O) is a powerful and enduring greenhouse gas whose warming effect in the troposphere is 265-times stronger than that of carbon dioxide, on a 100-year time horizon (IPCC 2014). Further, N_2O reacts with ozone in the stratosphere and is a dominant compound that threatens to deplete ozone in the twenty-first century (Ravishankara et al. 2009). N_2O emissions from temperate forest soils amount to 1.0 (0.2–2.0) Tg N y^{-1} (Morishita et al. 2011). Thus, strategies to mitigate N_2O emissions should take forest soils into account.

There have been some artificial afforestation experiments to study how tree species affect N_2O flux, but the results are contradictory. A study on artificial afforestation in Siberia predicted that deciduous species produce more N_2O than conifers, because of a large discrepancy between N_2O production and reduction rates under deciduous species (Menyailo et al. 2002). In contrast, Oura et al. (2001) reported that coniferous stands emit more N_2O than adjacent deciduous stands that were affected by nitrogen deposition. A study of a wide range of European forest soils revealed that N_2O emissions were highest in deciduous forests, but these forests were also situated on more fertile soils (Ambus et al. 2006). Further, Liu and Greaver (2009) found that soil N status rather than forest type could be the main factor determining the magnitude of N_2O emissions. The role of tree species in modulating N_2O flux, therefore, remains unclear.

In general, microbiological processes of nitrification and denitrification regulate the production and consumption of N_2O, while both processes are influenced by soil temperature, moisture content, pH, and carbon and nitrogen content (Brown et al. 2012). Microbial ammonia oxidation is the first and also the rate-limiting step in nitrification. The functional gene *amoA*, which codes for ammonia monooxygenase, is used as a biomarker for ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA). Several genes have been used as molecular markers of the denitrification steps: *narG* (which codes for membrane-bound nitrate reductase)*; nirK* (for copper-containing nitrite reductase); *nirS* (for Cyt cd1-containing nitrite reductase); and *nosZ* (for nitrous oxide reductase) (Zhang et al. 2016). The contribution of nitrification and denitrification to N_2O emissions depends on climate and soil conditions (Wolf and Brumme 2002). Seasonal changes in soil temperature and moisture may cause shifts in N_2O emissions and in the microbial communities involved in nitrification and denitrification. The N_2O flux increases with increasing soil moisture when denitrifying microorganisms are promoted (Menyailo and Hungate 2015). N_2O emissions increase with increasing soil temperature, because the rates of enzymatic processes increase with temperature, if other factors are not limiting (Schindlbacher et al. 2004). In forest ecosystems, soil temperature and moisture do not change simultaneously. The influence of seasonal change on N_2O emissions and on related functional microbial communities is therefore a complex phenomenon.

In the subtropical red soil hilly areas in China, the planting area of oil-seed camellia has increased annually because of its economic importance (Luo et al. 2016). Slash pine is an important tree species for afforestation because of drought resistance and high survival rate (Xu et al. 2018). Previously, we documented that tree species affects soil biological fertility (Zheng et al. 2006): natural secondary forest and tea-oil plantations had higher soil biological fertility than Chinese fir and slash pine plantations. In that study, we ascribed this difference to the higher litter production and quality, higher root biomass, richer plant species, better soil ecological condition and faster plant growth of the secondary forests and tea-oil plantations. Soil fertility drives the effect of vegetative cover on N_2O emissions (Guardia et al. 2016). Thus, we expect different tree species to affect soil N_2O production differently.

In this work, we assessed in situ N_2O flux, soil environmental factors, abundance and microbial (genetic) community composition involved in nitrification and denitrification in two soils of oil-seed camellia forests and slash pine forests, from 2012 to 2014, and over the four seasons. Our objectives were 1) to investigate the seasonal change in N_2O flux in soils planted with different tree species; 2) to test whether N-cycling communities varied with tree species and seasonal change; and 3) to explain how tree species affect N_2O flux. We hypothesized that tree species would induce a shift in in situ N_2O flux; that soils from oil-seed camellia forests would have higher N_2O flux rates than soils from slash pine forests; and that tree species would induce specific soil characteristics that affect the abundance and community composition of nitrification and denitrification microbes, thereby influencing the in situ N_2O flux.

Materials and methods

Experimental site

The sampling site is located at the Taoyuan Agroecosystem Research Station (111°26′ E, 28°55′ N and altitude 92.2–125.3 m) and represents a typical hilly area of the Hunan Province, China. The soil is developed from quaternary red clay, and the region is characterized by a subtropical humid monsoon climate, with annual average air temperature of 16.5 °C, annual average precipitation of 1448 mm, daily average sunshine duration of 15 h 13 min, and an annual frost-free period of 283 days (Qin et al. 2013).

Soil samples were collected from slash pine (*Pinus elliottii*) and oil-seed camellia (*Camellia oleifera* Abel.) forests in a long-term land use experiment at the Taoyuan Agroecosystem Research Station. The experiment, with eight land use plots of 62 m × 20 m each, was established in 1995. The field has a 15° slope, a length of 62 m, and an area of 10,000 m^2. Each land use plot extended from the top to bottom of the sloped field. The field was too small to allow replicated plots. At the start of the experiment, the slash pine and oil-seed camellia forest plots had the same soil properties, were free of human disturbance, and the trees were of the same age. Soil properties at the start of the experiment in 1995 were pH (H_2O), 4.45; soil organic matter (SOM), 28.9 g kg^{-1}; and total N, 1.93 g kg^{-1}. The similarity of initial site conditions provided an adequate background for comparison of the tree-mediated effects on soils (Binkley and Giardina 1998). Thus, we assumed that any soil differences between two corresponding treatments revealed by the study at the same site would be an effect of tree species. Soil properties in the two forests are summarized in Table 1. As can be seen, in 2012, higher SOM and total N was registered in soils of the oil-seed camellia forest.

Soil samples were collected on typical days of the four seasons: winter, spring, summer, and autumn. We chose typical days based on temperature (Supplementary Fig. 1). In 2012 we sampled on 20 October. In 2013, we sampled on 17 January, 12 April, 9 July, and 22 October. In 2014 we sampled on 19 January, 3 April, and 19 July. The days in October were typical days of autumn, those in January of winter, those in April of spring, and those in July of summer, respectively. We defined Oct 2012–July 2013 as year 1 and Oct 2013–July 2014 as year 2. For each plot in the slash pine and oil-seed camellia forests, three replicate sampling sites (50 m × 5 m) were selected at the same altitude across the land use plot. At each sampling site, ten soil cores along a "S" shaped line were collected (0–20 cm) and mixed thoroughly. In all, 48 soil samples were collected (2 species × 4 seasons × 2 years × 3 replicates). From each sample, a sub-sample (approximately 100 g) was packed into a sterile bag, which was immediately stored in liquid nitrogen in the field. Each sub-sample was then freeze dried (Neocool, Yamato Scientific Co., Ltd., Tokyo, Japan) in the lab under sterile conditions, ground to a fine powder after removal of root residues, and stored at −80 °C for subsequent molecular analysis. The remaining soil from each sample (approximately 400 g) was stored at 4 °C for chemical analysis.

For all samples, we analysed soil moisture, soil temperature and soil substrate availability of nitrate-N (NO_3^--N), ammonia-N (NH_4^+-N), and dissolved organic carbon (DOC). Soil moisture was determined by the oven drying method. Soil temperature at 5 cm soil depth was measured using a geothermometer. Soil NO_3^--N and NH_4^+-N were extracted with 1 M KCl and analysed with a continuous-flow injection analyser (FIAstar 5000, Foss Corporation, Hillerod, Denmark). DOC was extracted using 0.5 M K_2SO_4 and analysed for total oxidizable carbon (TOC-VMP, Shimadzu Corporation, Kyoto, Japan).

N_2O emission measurement

In situ N_2O gas fluxes were monitored by the static opaque chamber gas-chromatography (GC) method according to Zheng et al. (2008). The chamber (50 cm long × 50 cm wide × 20 cm high) was composed of insulated stainless-steel sheet metal and was equipped with a fan to ensure thorough gas mixing. The base was inserted 10 cm into the soil 2 months before the commencement of gas sampling to minimize the effects of disturbance. The static opaque chamber was equipped in the middle of each soil sampling site. On the same day of soil sampling, gases were sampled in the same time from each land use plot of slash pine and oil-seed camellia forests. Each sampling site, a 30-mL samples were collected using gas-tight syringes. We sampled 5 times per site on each day, at 15 min intervals from 9:00 to 10:00, when the air temperature is as close as possible to the mean daily temperature (Zhang et al. 2012). Concentrations were determined using a gas chromatograph (Agilent 7890A, Agilent Technologies, Santa

Table 1 Basic properties of the two forest soils used in this study of soil N_2O emissions

Tree species	pH (H_2O)	SOM (g kg^{-1})	Alkaline N (mg kg^{-1})	Olsen P (mg kg^{-1})	available K (mg kg^{-1})	Total-N (g kg^{-1})	Total P (g kg^{-1})	Total K (g kg^{-1})
Oil-seed camellia	4.33	32.93	139.45	3.30	46.82	1.61	0.31	9.83
Slash pine	4.31	27.22	125.83	2.53	62.13	1.33	0.32	12.68

Clara, CA, USA) fitted with an electron capture detector (ECD) for N_2O analyses at 350 °C. The in situ N_2O fluxes were determined by linear regression and expressed in μg N m^{-2} h^{-1}.

DNA extraction

Soil microbial DNA was extracted from 0.5 g soil stored at −80 °C, as described by Chen et al. (2010). DNA quality and concentration were measured using a NanoDrop NA-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The extractions were replicated three times, pooled, and stored at −80 °C for further analysis.

Quantitative PCR

Primer pairs AOB-*amoA*-1F/AOB-*amoA*-2R, AOA-*amoA*-23F/AOA-*amoA*-616R, *narG*-571F/*narG*-773R, *nirK*-876F/*nirK*-1055R, *nirS*-cd3aF/*nirS*-R3cd, and *nosZ*-1126F/*nosZ*-1381R were used for quantifying gene abundance (Supplementary Table 1). The qPCR assay was performed with an ABI 7900HT (Applied Biosystems, Foster City, CA, USA). The 10 μL reaction mixture consisted of 5 μL 2× SYBR green mix II (TaKaRa Biotechnology Co. Ltd., Dalian, China), 0.2 μL 50× Rox Reference Dye (TaKaRa Biotechnology Co. Ltd., Dalian, China), 0.4 μL each 10 μM forward and reverse primers, and 5 ng DNA template. A standard curve was obtained from serial 10× dilutions of linearized plasmids containing the target gene. Three parallel PCR replicates of all samples were performed on each plate. A melting curve analysis was conducted following the assay to verify the specificity of the amplification product. The PCR efficiency was in the range of 90–110%.

T-RFLP

The terminal-restriction fragment length polymorphism (T-RFLP) method was used for community composition analysis. Amplification of AOB *amoA* gene sequences from soil microbial DNA was done using a nested polymerase chain reaction (PCR) protocol. First-round PCR primers used were *amoA*-2F/5R and second-round PCR primers *amoA*-1F/2R. The primer pairs *amoA*-23F/616R, *narG*-145F/773R, *nirK*-517F/1055R, *nirS*-cd3aF/R3cd and *nosZ*-2002F/2002R were used for PCR amplification of AOA *amoA*, *narG*, *nirK*, *nirS* and *nosZ* genes (Supplementary Table 1). Forward primers were labelled with 6-carboxyfluorescein at the 5′ ends, except for *amoA*-2F. Approximately 200 ng of each amplicon was digested with 5 U restriction enzymes; TaqI were used for AOA *amoA*, AOB *amoA*, *narG*, *nirK* fragments, HaeIII and MspI for *nirS* and *nosZ* fragments, respectively. There are 11 T-RFs for AOA *amoA*, 28 T-RFs for AOB *amoA*, 26 T-RFs for *narG*, 29 T-RFs for *nirK*, 37 T-RFs for *nirS* and 13 T-RFs for *nosZ* genes.

Statistical analyses

Two-way analysis of variance (ANOVA) with repeated measures of 2 years was used to determine significant differences in the interaction of tree species and sampling seasons. The copy numbers of all functional genes were log-transformed, and the normality of all data was checked before running the ANOVA test. The data were found to be normally distributed. One-way ANOVA with repeated measures was used to evaluate the effect of tree species and seasons on the soil properties and abundance of genes, since two-way ANOVA did not reveal any significant interaction between tree species and sampling seasons for most parameters measured. Significant differences between treatments were identified by the least significant difference (LSD) test at the 5% level. Pearson correlation and regression analysis were conducted to determine the relationships among N_2O flux, abundance of N-associated functional genes, and soil parameters using all data from the 48 plots. All statistical analyses were performed using SPSS

10. Principal component analysis (PCA) based on the T-RFLP data was used to assess the similarities between the nitrifying and denitrifying microbial community structures in the slash pine and oil-tea camellia soils) among days from four seasons. In addition, redundancy analysis (RDA) was used to evaluate the effects of soil properties on microbial community composition. A Monte Carlo test with 999 random permutations was used to test for significant associations between environmental factors (soil temperature, moisture, NO_3^--N, NH_4^+-N, DOC and N_2O flux) and species (nitrifying and denitrifying microbial community composition). PCA and RDA were performed using Canoco v.4.5 for Windows.

Results

In situ N_2O flux rate and soil properties

Significant differences were detected for in situ N_2O flux rate and for all measured soil characteristics, except soil temperature, between the oil-seed camellia and slash pine soils. In addition, sampling days from different seasons also had significant effects on all soil properties and in situ N_2O flux rate (Table 2). In situ N_2O fluxes ranged from 3.78 to 596.16 μg N m^{-2} h^{-1}, with average values of 128.89 and 30.87 μg N m^{-2} h^{-1} in oil-seed camellia and slash pine soils, respectively. In situ N_2O flux rate, soil moisture, and soil NH_4^+-N and DOC content were higher in the soil of the oil-seed camellia forest than that of the slash pine forest. However, NO_3^--N content revealed an opposite trend (Table 3). In situ N_2O flux rate varied among days from different seasons, with the highest rate observed in summer days and lowest in autumn days. Soil temperature ranged from 6.62 to 24.64 °C and varied strongly among sampling days from different seasons. Soil moisture, soil NO_3^--N, soil NH_4^+-N and soil DOC were higher in summer days and in autumn days, but lower in spring (Table 4).

Abundance of nitrification and denitrification functional genes

There was significant difference in functional gene abundance between the oil-seed camellia and slash pine soils, except for the *nirK* gene. However,

Table 2 Two-way analysis of variance of N_2O flux, soil environmental factors, and gene abundances

Variable		df	N_2O flux	Temperature	Moisture	NO_3^--N	NH_4^+-N	DOC	AOA *amoA*	AOB *amoA*	*narG*	*nirK*	*nirS*	*nosZ*
Tree species	F ratio	1	1975.49	0.42	9.08	220.09	108.89	51.57	20.88	9.11	4.74	0.21	15.86	15.23
	p		**0.00**	0.52	**0.01**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.04**	0.65	**0.00**	**0.00**
Season	F ratio	3	1610.40	1742.32	3.27	32.67	4.90	10.67	2.30	0.90	6.47	4.49	2.18	3.25
	p		**0.00**	**0.00**	**0.03**	**0.00**	**0.01**	**0.00**	0.10	0.45	**0.00**	**0.01**	0.11	**0.03**
Tree species × season	F ratio	3	706.24	2.76	1.50	26.80	8.52	1.85	1.71	0.13	0.19	0.78	1.20	1.24
	p		**0.00**	0.06	0.23	**0.00**	**0.00**	0.16	0.18	0.94	0.90	0.51	0.32	0.31

AOA *amoA*, AOB *amoA*, *narG*, *nirK*, *nirS*, and *nosZ* are the copy numbers of these genes. And the copy numbers of genes were log10 transformed; Bold typing indicates significance at 5%

Table 3 One-way table of means of soil properties and abundance of genes between two tree species. Abundances of genes were log10 transformed

Items		Slash pine	Oil-seed camelli
The abundance of genes (copies per g dried soil)	AOA *amoA*	7.33 (0.42) a	6.75 (0.76) b
	AOB *amoA*	6.27 (0.41) b	6.83 (0.83) a
	narG	8.93 (0.44) a	8.67 (0.55) b
	nirK	7.31 (0.42) a	7.36 (0.54) a
	nirS	6.35 (0.59) a	5.78 (0.51) b
	nosZ	7.55 (0.35) b	7.87 (0.50) a
Soil moisture (% by weight)		21.53 (4.47) b	23.62 (5.17) a
Soil NH_4^+-N (mg kg^{-1})		7.32 (2.66) b	14.86 (6.58) a
Soil NO_3^--N (mg kg^{-1})		6.40 (3.69) a	1.66 (0.63) b
Soil DOC (mg kg^{-1})		305.23 (50.68) b	375.81 (74.69) a
Soil temperature (°C)		15.88 (6.47) a	15.77 (6.93) a
N_2O flux rate (μg N m^{-2} h^{-1})		30.87 (30.52) b	128.89 (183.06) a

Numbers within parenthesis are standard deviations, $n = 24$; Different letters within rows indicate significant difference between tree species ($p < 0.05$)

significant differences between sampling days from different seasons were detected only for denitrification functional genes of *narG*, *nirK,* and *nosZ* (Table 2). The abundance of AOA *amoA*, *narG,* and *nirS* genes was higher in soils of the slash pine forest than in those of the oil-seed camellia forest, while the opposite was true for AOB *amoA* and *nosZ* genes. (Table 3). The abundance of the denitrifying genes was higher in the days of winter than in any other season, with average abundance values of 1.10×10^9, 5.42×10^7 and 7.39×10^7 copies g^{-1} dry soil, for *narG*, *nirK* and *nosZ*, respectively, in the soil of the oil-seed camellia forest. In contrast, no difference was found in soils of the slash pine forest (Fig. 1). We also detected a positive relationship between the abundance of genes *narG* and *nirK* and soil moisture content (Table 5).

Community composition of nitrification and denitrification functional genes

The average relative abundance of T-RFs for AOA *amoA*, AOB *amoA*, *narG*, *nirK*, *nirS,* and *nosZ* genes is shown in Fig. 2. We detected a difference in community composition between the soils of the two forest types. However, soil microbial community composition of all detected genes showed no significant difference among days from different seasons. In most instances, T-RFs were greater in the oil-seed forest than in the slash pine forest soils: these included dominant T-RFs of 54 bp for AOA *amoA* and 282 bp for AOB *amoA* (by 30.56% and 25.94%, respectively); four dominant T-RFs, of 206, 222, 246, and 260 bp for *narG* (by 12.58%, 18.64%, 8.11%, and 7.62%, respectively);

Table 4 One-way table of means of soil properties for four seasons

Season	N_2O flux (μg N m^{-2} h^{-1})	Soil temperature (°C)	Soil moisture (% by weight)	Soil NO_3^--N (mg kg^{-1})	Soil NH_4^+-N (mg kg^{-1})	Soil DOC (mg kg^{-1})
Autumn	22.40 (28.17) d	17.85 (0.84) b	23.66 (8.27) a	5.61 (4.77) a	11.38 (7.14) a	302.02 (39.52) bc
Winter	53.44 (43.08) b	6.62 (1.08) d	22.02 (2.63) bc	2.59 (1.61) b	12.75 (8.62) a	353.18 (53.17) a
Spring	32.48 (10.36) c	14.21 (0.32) c	21.08 (2.13) c	2.32 (0.93) b	8.90 (3.78) b	329.82 (108.67) b
Summer	211.21 (232.60) a	24.64 (1.22) a	23.55 (4.20) ab	5.61 (4.09) a	11.31 (4.40) a	377.06 (53.85) a

Numbers within parenthesis are standard deviations, $n = 12$; Different letters within columns indicate significant difference among four seasons ($p < 0.05$)

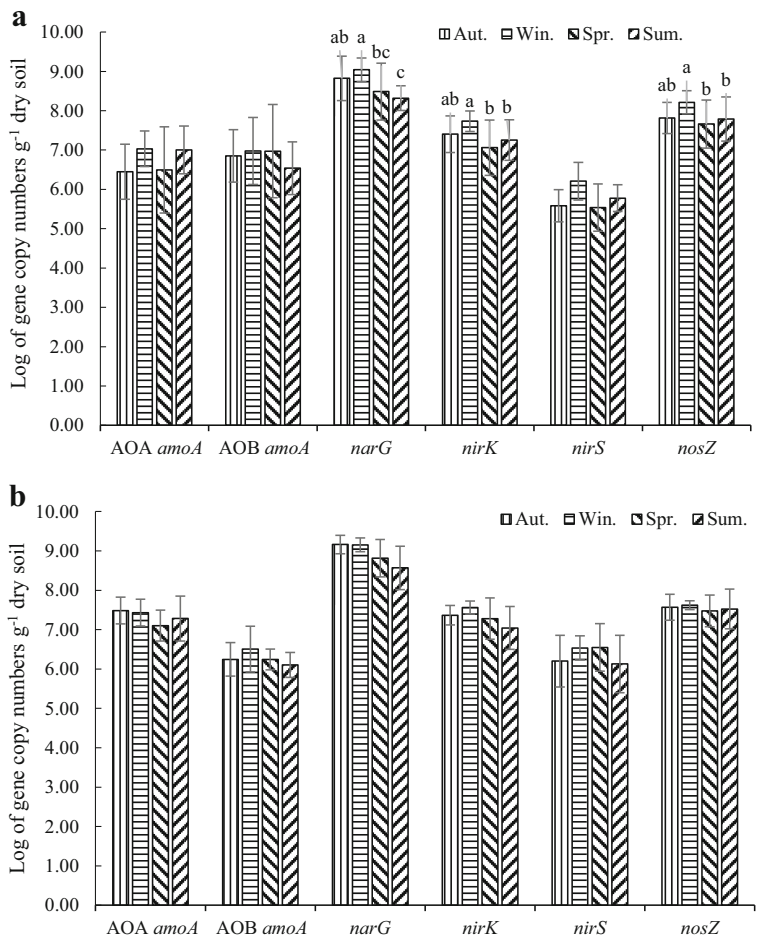

Fig. 1 Abundance of AOA *amoA*, AOB *amoA*, *narG*, *nirK*, *nirS*, and *nosZ* genes in soils under different forest vegetation of oil-seed camellia (**a**) and slash pine (**b**). Spr., spring; Sum., summer; Aut., Autumn; Win., winter, the same below. Error bars are standard deviations for seasons. Different letters above bars indicate significant differences among seasons ($P < 0.05$)

two dominant T-RFs of 190 and 224 bp for *nirK* (by 12.37% and 10.33%, respectively); three dominant T-RFs of 116, 136, and 210 bp for *nirS* (by 8.14%, 11.60%, and 16.32%, respectively); and one dominant T-RF of 350 bp for *nosZ*, by 9.75%. The dominant T-RFs of 130 bp for AOA *amoA* and 280 bp for AOB *amoA* were less, by 33.95% and 27.35%, respectively, in the oil-seed camellia than the slash pine forest soils. One dominant T-RFs of 248 bp for *narG* was less in the oil-seed camellia forest, by 13.71%; this T-RF, and some minor T-RFs for *narG* in soils of the slash pine forest (228 bp, 244 bp, 250 bp, and 266 bp), disappeared from soils of the oil-seed camellia forest. Two dominant T-RFs for *nirK* of 192 bp and 436 bp were less, by 11.89% and 5.07%, respectively, in the oil-seed camellia than in the slash pine forest soils. Four dominant T-RFs for *nirS* of 78 bp, 188 bp, 190 bp, and 406 bp were less, by 7.99%, 7.53%, 18.28%, and 9.60%, respectively, in the oil-seed camellia than in the slash pine forest soils. Two T-RFs for *nosZ* of 206 bp and 220 bp were less, by 8.24% and 9.34%, respectively, in the oil-seed camellia than in the slash pine forest soils.

PCA showed that variations in the nitrification and denitrification microbial community structure were affected more by tree species than by seasonal change (Fig. 3). The first two principal components (PCs) accounted for 98.8 and 1.0%, 98.8 and 1.0%, 76.2 and 16.1%, 47.5 and 28.9%, 70.4 and 17.0%, and 51.7 and 34.8%, respectively, of the total variability recorded in AOA *amoA*, AOB *amoA*, *narG*, *nirK*, *nirS*, and *nosZ* genes. For all the detected genes, except for *nosZ* gene, the main separation of slash pine and oil-seed camellia forests along PC1 reveals that differences between the two tree species caused most of the variation in the data, while the smaller separation of the seasons along PC2 was responsible for less of the variation. The Monte Carlo test based on

Table 5 Pearson correlation coefficients for gene abundance and soil properties in two forest soils

Items		AOB *amoA*	*narG*	*nirK*	*nirS*	*nosZ*	Soil moisture	Soil NH_4^+-N	Soil NO_3^--N	Soil DOC	Soil temperature	N_2O flux
Oil-seed camelli	AOA *amoA*	0.23	**0.47**	**0.63**	**0.48**	**0.54**	0.28	0.37	0.21	−0.25	−0.04	0.39
	AOB *amoA*		0.33	**0.45**	0.34	**0.49**	0.35	−0.06	−0.22	−0.04	−0.17	−0.07
	narG			0.79	**0.56**	**0.71**	**0.41**	0.17	−0.30	**−0.40**	**−0.40**	−0.26
	nirK				**0.72**	**0.86**	**0.45**	0.30	−0.16	−0.34	−0.27	0.03
	nirS					**0.64**	0.23	0.16	−0.17	−0.25	−0.30	0.07
	nosZ						**0.41**	0.29	−0.05	−0.18	−0.30	0.22
	Soil moisture							−0.06	−0.39	−0.11	0.11	0.14
	Soil NH_4^+-N								0.28	−0.07	−0.19	0.21
	Soil NO_3^--N									0.02	0.27	**0.54**
	Soil DOC										0.08	0.33
	Soil temperature											**0.42**
Slash pine	AOA amoA	**0.49**	**0.49**	**0.55**	0.20	**0.80**	**0.55**	0.20	0.30	−0.27	−0.07	−0.21
	AOB amoA		**0.44**	0.47	0.11	**0.48**	0.25	0.18	−0.11	−0.03	−0.34	−0.29
	narG			**0.88**	0.21	**0.46**	0.06	−0.07	−0.10	−0.15	**−0.40**	**−0.66**
	nirK				0.25	**0.62**	0.06	0.07	−0.12	−0.21	**−0.42**	**−0.53**
	nirS					0.04	−0.27	0.19	−0.36	−0.39	−0.29	−0.32
	nosZ						**0.50**	0.10	0.24	−0.24	−0.12	−0.12
	Soil moisture							0.05	**0.51**	−0.06	0.21	0.18
	Soil NH_4^+-N								−0.14	−0.38	0.19	0.11
	Soil NO_3^--N									0.10	**0.64**	0.28
	Soil DOC										0.10	**0.41**
	Soil temperature											**0.65**

Bold typing indicates significant difference ($P < 0.05$), $n = 24$

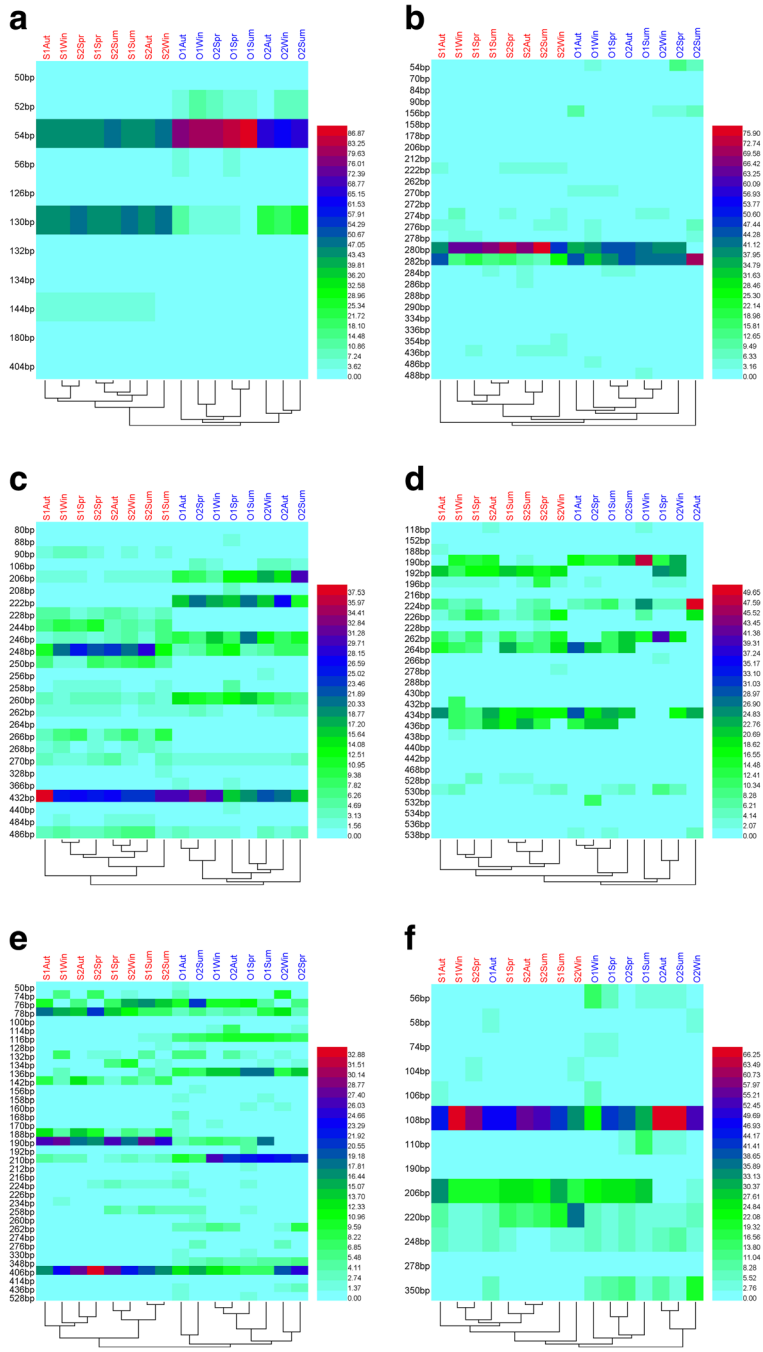

Fig. 2 Heat map of T-RFLP profiles showing the average relative abundances of AOA *amoA* (**a**), AOB *amoA* (**b**), *narG* (**c**), *nirK* (**d**) T-RFs with endonuclease TaqI, of *nirS* (**e**) and *nosZ* (**f**) T-RFs with endonuclease HaeIII and MspI, respectively. Relative abundances of T-RFs are given as percentages of total peak height. Fragments within the graph indicate the size (bp) of the experimental T-RFs as determined by T-RFLP. S, slash pine soil; O, oil-seed camellia soil. 1 and 2 mean the first and second sampling years

redundancy analysis (RDA) indicated that soil NO_3^--N, NH_4^+-N, and DOC significantly affected the nitrifying and denitrifying community compositions, except that DOC did not affect *nosZ*-containing denitrifier composition. Soil temperature and moisture significantly affected *nirK*-containing denitrifiers; and soil moisture also affected AOB *amoA*-containing nitrifiers (Table 6).

Relationships among N_2O flux, soil environmental factors, functional gene abundance and community composition

Pearson correlation coefficient analysis revealed that in situ N_2O flux rate was significantly and positively correlated with soil temperature and soil NO_3^--N in soils of the

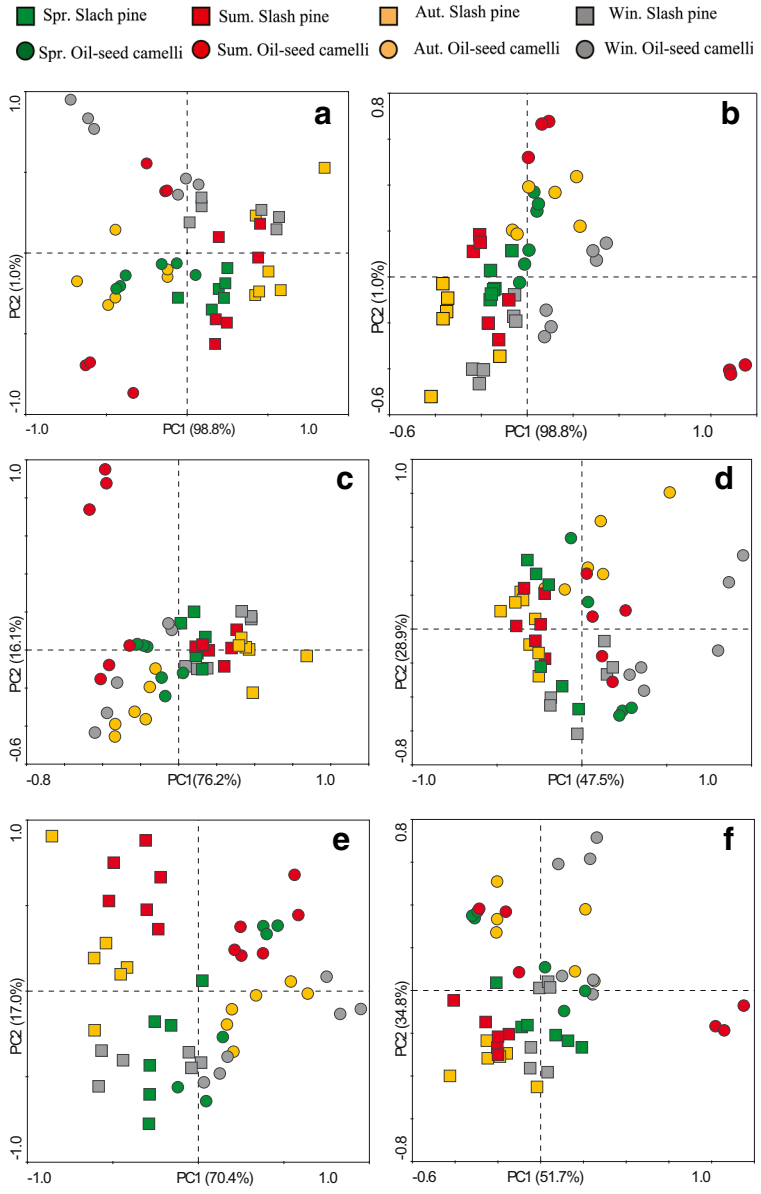

Fig. 3 Principal coordinate analysis based on the T-RFLP profile of AOA *amoA* (**a**), AOB *amoA* (**b**), *narG* (**c**), *nirK* (**d**), *nirS* (**e**) and *nosZ* (**f**) genes

Table 6 Monte Carlo test of redundancy analysis (RDA) based on the T-RFLP profile of nitrification and denitrification genes and soil properties

Genes	Soil temperature		Soil moisture		Soil NO_3^--N		Soil NH_4^+-N		Soil DOC		N_2O flux	
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
AOA *amoA*	0.76	0.15	2.32	0.13	30.19	**0.00**	15.97	**0.00**	14.82	**0.00**	1.53	0.26
AOB *amoA*	0.53	0.52	5.27	**0.02**	7.87	**0.01**	8.78	**0.00**	7.78	**0.02**	26.09	**0.00**
narG	0.46	0.74	1.41	0.21	15.29	**0.00**	11.80	**0.00**	8.27	**0.00**	7.98	**0.00**
nirK	4.93	**0.00**	2.39	**0.03**	5.07	**0.01**	11.18	**0.00**	7.29	**0.00**	2.50	**0.04**
nirS	2.25	0.08	1.16	0.26	12.80	**0.00**	10.03	**0.00**	8.89	**0.00**	2.70	**0.03**
nosZ	1.49	0.18	0.64	0.62	4.33	**0.02**	5.72	**0.00**	2.33	0.07	6.08	**0.00**

Bold typing indicates significant difference ($p < 0.05$)

oil-seed camellia forest, and with soil temperature and DOC in soils of the slash pine forest (Table 5). Nevertheless, no significant positive relationship between in situ N_2O flux rate and abundance of nitrification and denitrification functional genes could be found in the soils of either forest type based on regression analysis (Fig. 4). However, the Monte Carlo test showed that in situ N_2O flux rate was significantly affected by all detected community compositions of all detected genes, except for AOA *amoA* gene (Table 6). Furthermore, correspondingly with N_2O flux in situ, the average relative abundance of some T-RFs of all detected genes in soils of the oil-seed camellia forest significantly exceeded that in soils of the slash pine forest, regardless of seasonal change (Fig. 2).

Discussion

Tree species influenced N_2O flux, with higher flux values in oil-seed camellia than slash pine forest soils, supporting our hypotheses. This confirmed that forests with deciduous species should produce more N_2O than forests with conifers. Slash pine is a conifer with needle-like leaves that contain large amounts of substances that are difficult to decompose, such as tannins, resins, cellulose, and lignin. In contrast, oil-seed camellia is a deciduous species, which produces leaf litter containing substances easily decomposed by bacteria, such as nitrogen and water (Yang et al. 2014). Zheng et al. (2006) found that oil-seed camellia forest soil had higher vegetation biomass and root biomass, and therefore, higher

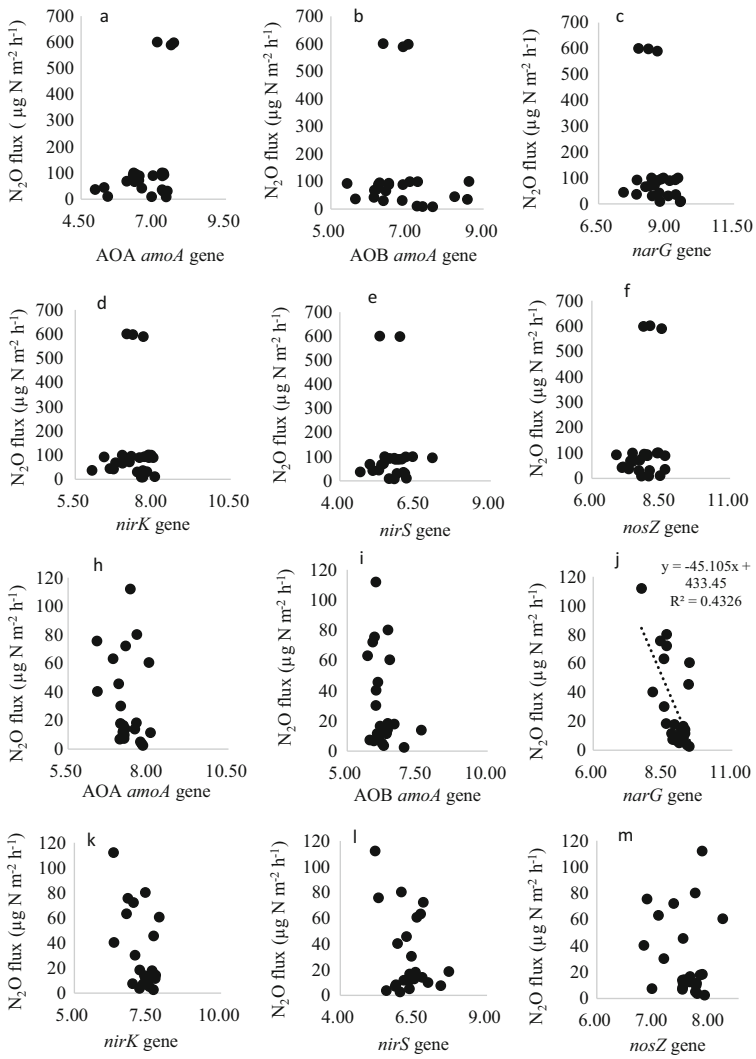

Fig. 4 Regression analysis between N_2O flux and abundance of AOA *amoA* (**a**), AOB *amoA* (**b**), *narG* (**c**), *nirK* (**d**), *nirS* (**e**), and *nosZ* (**f**) genes in oil-seed camellia soil; and AOA *amoA* (**h**), AOB *amoA* (**i**), *narG* (**g**), *nirK* (**k**), *nirS* (**l**), and *nosZ* (**m**) genes in slash pine soil. AOA *amoA* gene, AOB *amoA* gene, *narG* gene, *nirK* gene, *nirS* gene. and *nosZ* gene are the log10 transformed copy numbers per g dried soil of AOA *amoA*, AOB *amoA*, *narG*, *nirK*, *nirS* and *nosZ* genes

SOM content, than did slash pine forest soil. Likewise, we found that soils of the oil-seed camellia forest had higher soil moisture, NH_4^+-N, and DOC, but lower soil NO_3^--N, than soils of the slash pine forest. Soil environmental factors (temperature, concentration of NO_3^-, and DOC) were significantly related to N_2O fluxes. This indicates that tree species induced shifts in soil environmental factors, which were largely responsible for the variation in N_2O fluxes in acidic forest soils.

Tree species also influenced the microbial community related to N_2O emission. The differences in the abundance and community composition of nitrifiers and denitrifiers between tree species depended on soil substrate availability. The higher abundance of the AOB *amoA* gene in the soil of the oil-seed camellia forest is consistent with the higher content of NH_4^+-N in this soil. However, the AOA *amoA* gene showed the opposite trend to AOB *amoA* gene. AOB and AOA have been found to co-exist in most soils, both mediating the first step of autotrophic nitrification (Schleper 2010). The relative roles of AOB and AOA have been debated since Leininger et al. (2006) first reported the dominance of AOA in 12 pristine agricultural soils from three climatic zones: they reported that AOB population was more sensitive to soil NH_4^+-N than AOA in acidic forest soils Those findings were supported by the findings of Yang et al. (2016), who observed that AOB were more responsive than AOA to nitrogen source in an agricultural soil. Conversely, in our study, higher abundance of the *narG* and *nirS* genes in the soils of the slash pine forest was consistent with the higher NO_3^--N content. Although both *nirK* and *nirS* encodes nitrite reductase, *nirK* abundance was not significantly different between tree species. Levy-Booth et al. (2014) reported that organisms that contain the *nirS* gene often do not contain the *nirK* gene. Our study suggested that the effect of tree species on populations of nitrite reducers was associated with the *nirS* gene, but not with the *nirK* gene. Finally, higher abundance of the *nosZ* gene in the soils of the oil-seed camellia forest was consistent with the higher N_2O flux rate in our study, consistent with the N_2O concentration in the surface soil (Wang et al. 2017). We found that the composition of all detected nitrifiers and denitrifiers varied between the two forest types, which were affected by soil NO_3^--N, NH_4^+-N, and DOC availability.

Production of N_2O is essentially mediated by microbial functional groups. Interestingly, data from this study revealed that community composition (not the abundance of nitrification and denitrification genes) was related to in situ N_2O flux in acidic forest soils. A few studies have found significant correlations between N_2O emissions and *narG*, *nirS*, and *nirK* gene numbers under favourable conditions for denitrification. For example, rainfall or irrigation induced a low oxygen concentration in soil microsites (Ju and Christie 2011; Zhang et al. 2017). However, in most cases the abundance of denitrification genes is probably not related to the N_2O flux (Cuhel et al. 2010; Liu et al. 2013). In the present study, the abundances of all detected nitrification and denitrification genes were not correlated with N_2O fluxes, indicating that the abundance of these genes might not be the limiting factor. Recent studies have shown that DNA-level analyses alone may not suffice to link soil microbial abundance with biogeochemical processes (Watanabe et al. 2009). Transcriptional analysis (mRNA) of functional genes might be applied to detect metabolically active organisms responsible for N_2O production in future studies. On the contrary, we show that the community compositions of nitrifiers and denitrifiers were more significantly related to N_2O fluxes than gene abundance, except for the AOA *amoA* gene. Why was the community composition of AOA not related to N_2O fluxes? It has been reported that AOAs are ubiquitous in soils but do not respond to NH_4^+ oxidization or N_2O production in intensively managed agricultural soils (Di et al. 2009; Jia and Conrad 2009). We found that *Nitrosospira*-like AOBs were dominant over AOAs in N_2O production. Previous studies showed that N_2O emissions could be reduced by up to 77% using nitrification inhibitors of 3,4-dimethylpyrazol phosphate (DMPP) in the field, or by up to 80–99% using DMPP or dicyandiamide (DCD) in laboratory experiments (Ju and Christie 2011; Ma et al. 2015). Our results provide molecular microbial evidence that the community composition of nitrification and denitrification genes was a key factor for generating N_2O in the studied soils. These results also explain why nitrification or denitrification inhibitors were effective for reducing N_2O emissions in these soils. However, based on T-RFLP analysis, we still do not know the exact species diversity or the identities of

individual species that affect N_2O production. High-throughput sequencing or cultivation-based approach might be used to identify the specific species and understand their roles in N_2O production.

Compared to tree species, seasonal change had a weak effect on soil-microbe related N_2O fluxes. For all detected nitrification and denitrification functional genes, the abundance of *narG*, *nirK*, and *nosZ* genes differed between seasons in soils of the oil-seed camellia forest, but not the slash pine forest. Community composition of the detected nitrifiers and denitrifiers varied slightly among sampling days. Although we selected seasonal sampling dates based on temperature, the variation in microbial community composition between soils was due to soil moisture, not to soil temperature. Soil moisture can enhance soil mineralization and denitrification rates: soil NH_4^+-N and DOC contents increase through mineralization, while soil NO_3^--N concentrations decrease through denitrification (Friedl et al. 2016; Fujita et al. 2013;). Therefore, alteration of soil NO_3^--N, NH_4^+-N, and DOC contents affects the microbial (gene) community composition of nitrifiers and denitrifiers, as we have shown. Szukics et al. (2010) found that both moisture and temperature affected nitrogen turnover rates by reversing the ratio of archaeal *amoA* to bacterial *amoA*. Phillips et al. (2014) found denitrification was more sensitive to moisture than to temperature, as moisture regulated the relative abundance of denitrifiers. A change in soil moisture implied that pH and oxygen concentrations in the soil might also be changed (DeAngelis et al. 2010). Therefore, soil conditions favouring denitrification would also rapidly alter the population of denitrifiers. Liu et al. (2012) reported that both abundance and composition of *narG*- and *nosZ*-containing denitrifiers responded to soil drying within only 1 day of surface water removal.

We found that in situ N_2O flux was higher in soils of the oil-seed camellia forest in all seasons, than in slash pine forest soils. We found that the relative abundance of some T-RFs of all detected genes in soils of the oil-seed camellia forest significantly exceeded those in the soils of the slash pine forest, irrespective of season. Further, there were significant differences in soil moisture, NH_4^+-N, and DOC contents between the two forest types. Therefore, we confirmed that tree species affected the soil environment in the study site, which affected the functional microbes, and ultimately induced difference in N_2O flux in the soil.

Conclusion

The difference in tree species, rather than between seasons, affected in situ N_2O flux, soil physic-chemical factors, nitrifier and denitrifier abundance, and microbial (gene) community structure. In situ N_2O flux was higher in soils of the oil-seed camellia forest than of the slash pine forest. The abundance and community composition of nitrifiers and denitrifiers was significantly affected by substrate availability, such as soil NO_3^--N and DOC contents. Community composition, but not abundance of nitrifier and denitrifier microorganisms, was related to N_2O flux in acidic forest soils. Soil temperature, NO_3^- and DOC concentrations, were significantly related to in situ N_2O fluxes. We found that differences between tree species caused variation in the soil environment and in the community structure of functional microbes, and that ultimately these variations induced the different in situ N_2O fluxes of the acidic forest soils. We suggest that in situ N_2O emission from these soils is regulated by the balance between nitrifying and denitrifying microbial communities and their different biological processes, as well as the abiotic processes mediated by soil temperature changes over the year.

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