Differences in N$_2$O Fluxes and Denitrification Gene Abundance in the Wet and Dry Seasons Through Soil and Plant Residue Characteristics of Tropical Tree Crops

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The conversion of forest to agricultural soils is a widespread activity in tropical systems, and its link to nitrous oxide (N$_2$O) fluxes and nitrogen cycling gene abundance is relevant to understand environmental drivers that may interact with climate change. A current challenge to estimating N$_2$O emissions from land use conversion is an incomplete understanding of crop-specific impacts on denitrifier communities and the N$_2$O fluxes driven by differences in the above- and below-ground inputs with crop type. To address this knowledge gap in tree crops, we evaluated N$_2$O fluxes and denitrification gene abundance and their relationships with soil and plant residue characteristics in citrus and eucalyptus plantations in the field and in soil incubations. We found that the accumulated N$_2$O fluxes from soil were lower for the two agricultural field sites than those for their adjacent forest sites in dry and wet seasons. The N$_2$O fluxes were higher in the wet season, and this seasonal difference persisted even when the soils collected from both seasons were incubated under the same moisture and temperature conditions in the lab for 30 days. Increased N$_2$O fluxes in the wet season were accompanied by an increase in soil *nir*K and *nos*Z gene abundance, the dissolved organic carbon (DOC) concentration, and the total soil carbon (C) and nitrogen (N) content. In turn, the abundance of denitrifiers, as indicated by *nir*K, *nir*S, and *nos*Z gene copy numbers, showed a low but significant positive correlation with soil bulk density. Our results suggest that soil moisture, leaf litter, and crop residues influence the seasonal differences in both N$_2$O fluxes and abundance of denitrifiers in citrus- and eucalyptus-cultivated soils, likely through effects on soil physicochemical characteristics. These findings highlight the overwhelming role of environmental drivers that can make investigating microbial drivers difficult in the field and open the possibility for a better understanding of N cycling processes in tropical soils based on paired field- and incubation-based experimentation.

Keywords: tropical soil, denitrifiers, nitrous oxide, citrus, eucalyptus

INTRODUCTION

Many gaseous nitrogen (N) emissions from soils are a consequence of soil microbial processes (Hallin et al., 2018). In soils, nitrous oxide (N_2O) is produced by N cycling nitrification and denitrification reactions, which are mediated by enzymes encoded by *nir*K, *nir*S, and *nos*Z genes (Levy-Booth et al., 2014). Denitrification in agricultural soils is a leading source of anthropogenic N_2O, especially in humid climates (Lammel et al., 2015; Kanter et al., 2016). Atmospheric N deposition is chronically elevated and predicted to increase in tropical regions (Reay et al., 2008; IPCC, 2014) and may stimulate additional N cycling pathways in these regions. A high demand for wood material and orange exports causes eucalyptus and citrus crops to play important roles in the agricultural sector in Brazil (Neves et al., 2010; Cavararo, 2014). However, the impacts of N additions on N_2O emissions have been studied most often in natural forests and rarely in tropical plantations (Martinson et al., 2013; Mori et al., 2013; Zhang et al., 2014). The effects of agricultural management of tree crops (e.g., citrus orchards and eucalyptus plantations) on the N_2O balance remain unclear.

It is critical to understand the sensitivity of the microbial communities that regulate ecosystem N cycling to the soil, and the changes in both environmental and crop-specific conditions that accompany natural forest loss via conversion to agriculture. Microbe-mediated N_2O production begins with the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), followed by the reduction of NO_2^- to nitric oxide (NO), which is catalyzed by enzymes encoded by *nir*K and *nir*S genes (Pajares and Bohannan, 2016). The conversion of one-third of NO to N_2O is followed by its reduction to N_2 by N_2O reductases encoded by *nos*Z genes, thereby partially mitigating N_2O release to the atmosphere (Jones et al., 2008; Hallin et al., 2018). The presence of crop residues and other leaf litter affects the rate of denitrification primarily by increasing organic C availability, which is one of the most important factors influencing denitrification (Handerson et al., 2010). While different studies have investigated the links between N_2O fluxes and key marker genes (Lammel et al., 2015; Liu et al., 2016), few have investigated the interplay between tree crop species, their residues, and denitrifiers that lead to different N_2O emission profiles from various types of agricultural management.

In this study, we evaluated seasonal patterns in N_2O fluxes and denitrification gene abundance and their relationships with soil and plant residue characteristics in citrus and eucalyptus plantations. We evaluated the following hypotheses: (1) that citrus- and eucalyptus-cultivated soils emit higher amounts of N_2O to the atmosphere than their adjacent natural forest soil counterparts and (2) that the abundance of denitrifier genes, as indicated by *nir*K, *nir*S, and *nos*Z gene copy numbers, would be increased in citrus- and eucalyptus-cultivated soils in comparison to that in natural forest soils. We predicted that these observed differences would be driven in part by seasonal environmental variables, the chemical characteristics of plant residues and soil physicochemical characteristics. To evaluate these expectations, we determined N_2O soil atmosphere trace gas fluxes, *nir*K, *nir*S, and *nos*Z gene copy numbers, and soil physicochemical and plant residue chemical characteristics in samples taken in the dry and wet seasons in the field and using soil and plant residue incubations. The results from our study provide the first comprehensive picture of the complex determinants of N_2O cycling in the tree crops that are widespread in the tropics.

MATERIALS AND METHODS

Site Descriptions

Four different field sites were chosen in Southeastern Brazil; two sites (Citrus and Forest 1) are located in the Sorocaba municipality (23° 34′ 30.76″ S; 47° 31′ 58.95″ W and 23° 34′ 14.21″ S; 47° 30′ 50.23″ W, respectively) and two others (Eucalyptus and Forest 2) are located in the Salto de Pirapora municipality (23° 36′ 07.92″ S; 47° 33′ 42.72″ W and 23° 36′ 54.13″ S; 47° 33′ 58.56″ W, respectively) in the state of São Paulo, Brazil (**Figure 1**).

Among the four sampling sites, Forest 1 and Citrus are located on São Pedro Farm, with a total area of 431.5 ha with 333.7 ha of conventional citrus orchards. According to the records of 18 August 1965 and 29 July 1970, Forest 1 can be considered to be in an advanced regeneration stage without recorded anthropogenic disturbances within the last 52 years. The conversion from forest to citrus orchard occurred 48 years before our study. The orchards have been planted every 10 years since. On an annual basis, the citrus orchard received 856.8 kg ha^{-1} of NPK (15-10-15). This dose was split into three applications as follows: the first application was 55.1 kg ha^{-1} of N as NH_4NO_3, 36.7 kg ha^{-1} of P_2O_5 as triple superphosphate and 55.1 kg ha^{-1} of K_2O as KCl, and the second and third applications were 36.7 kg ha^{-1} of N as NH_4NO_3, 24.5 kg ha^{-1} of P_2O_5 as triple superphosphate and 36.7 kg ha^{-1} of K_2O as KCl. Although fertilizer recommendations were expressed in kg ha^{-1}, all the fertilizers were mechanically spread under the canopy, which occupied half of the field area. In contrast, the Eucalyptus site is located in a 38 ha plantation. Fertilization was performed in the furrow once during eucalyptus planting and consisted of 50 kg of N in the form of urea, 250 kg of P_2O_5 (triple superphosphate), and 50 kg of K_2O (potassium oxide) per hectare. Broadcast fertilization was performed 9 and 18 months after planting with 55 kg of N, 20 kg of P_2O_5, and 75 kg of K_2O per hectare. The Eucalyptus site was planted 4 years prior to our study. The native 10 ha vegetation area adjacent to the eucalyptus (Forest 2) is in an advanced natural regeneration stage without any recorded anthropogenic disturbance in the last 50 years.

The regional climate is subtropical warm (humid temperatures with a warm summer according to Köppen's classification) with annual precipitation of 1,285 mm and 16.8°C and 30.1°C as the minimum and maximum average temperatures, respectively. The air and soil temperature varied <7 and 5°C, respectively, among the different sampling sites during the gas and soil sample collection. The only soil in the study sites is an Udox in a hyperthermal soil temperature regime according to the classification of Soil Survey Staff (1999).

N_2O Fluxes From the Soil

The N_2O flux from the soil was measured using the chamber-based method (Davidson et al., 2002) in the regional dry

FIGURE 1 | Sampling site locations for Citrus, Forest 1, Eucalyptus and Forest 2. The chambers for gas sample collection (red and yellow points in the diagram) were installed at each sampling site in dry and wet seasons. Soil and plant residue samples (also the yellow points in the diagram—soil and plant residue sampling cores were randomly chosen next to the yellow point chambers) were taken at each sampling site (for a total of five samples per sampling site at each season).

(July 2016) and wet (January 2017) seasons. Gas sampling was performed at all four sampling sites (Citrus, Eucalyptus, Forest 1, and Forest 2). At each site, eight cylindrical PVC bases (30 cm diameter, 22 cm height) were installed in soils under the tree canopies. All gas sampling was performed between 7:00 and 12:00 a.m. After closing the chambers, 60 mL samples were collected at 1, 10, 20, and 30 min using syringes and were transferred and stored under pressure in 20 mL evacuated penicillin flasks sealed with gas-impermeable butyl rubber septa (Bellco Glass 2048). The gas sampling was performed over 5 days at each site in each season for a total of 1,280 gas samples (i.e., 32 gas samples for each sampling site per day) in order to fit to the soil sampling and it may not represent behavior over the entire seasons. The N_2O mole fractions were determined by gas chromatography with an electron capture detector (GC 2014 Shimadzu, Columbia, MD, USA). The system was equipped with a HayeSep[TM] N packed column (1.5 m, 80–100 mesh) for N_2O separation. Helium was used as the carrier gas. Gas samples were stored at $20°C$ and analyzed 1–2 weeks after collection. Measurements of atmospheric pressure, chamber height and air temperature were taken during the gas sampling to determine the total moles of air in the chamber volume. The N_2O fluxes were calculated for each sampling event in each site and season according to equation (Jantalia et al., 2008):

$$f = (\Delta G/\Delta t) \times (C/A) \times (m/Vm)$$

Where: f = gas flux, N_2O (mg $N\text{-}N_2O$ m^{-2} day^{-1});

ΔG = concentration of N_2O during sampling;

Δt = time period that chamber remained closed;

V and A = volume and soil surface area covered by chamber, respectively;

m = molecular weight of the N_2O.

Weekly accumulated N_2O fluxes were estimated by plotting daily fluxes through time, interpolating linearly between them and integrating the area under the curve.

Soil, Leaf Litter, and Crop Residue Sampling

Soil samples were collected from five different points at each sampling site from the upper 10 cm (topsoil) layer on the 3rd day of gas sampling in both the dry and wet seasons. At each of the 5 sampling points at the Citrus and Eucalyptus sites, crop residues (i.e., leaves, stalk, and husk) (**Supplementary Figures 1A,C**) were collected from a 30 cm diameter area and stored in plastic bags, and soil samples were collected using a 5 cm diameter aseptic cylindrical corer. The same sampling approach was taken at 5 sampling points in the Forest 1 and Forest 2 sites for soil and plant residues, where litter layer samples (**Supplementary Figures 1B,D**) were collected in the same

manner as crop residues in the agricultural sites. A total of 40 soil samples were collected from the field (4 sampling sites × 5 sampling points × 2 sampling periods). The samples were transported to the laboratory on ice, stored at −20°C, and processed within 72 h of sampling.

Soil Physicochemical and Plant Residue Chemical Analysis

The soil physicochemical analysis was performed on each of the 40 soil samples. The granulometric analysis was performed as described in Camargo et al. (2009) using the soil texture triangle from IAC (Agronomic Institute of Campinas). Soil moisture was determined by the gravimetric method as described in EMBRAPA (2009). Bulk density was measured by the volumetric flask method (EMBRAPA, 2009). For chemical analysis, a subsample of each sample was air dried, passed through a 2 mm mesh sieve, and shaken in pure water. Of this solution, 100 μL was acidified with 1.5% of 2 M HCl, followed by a purge with synthetic air for 5 min to remove the inorganic carbon. Then, the dissolved organic carbon (DOC) content was determined on a Shimadzu TOC-5000 A analyzer (Shimadzu, Columbia, MD, USA). Another soil subsample was air dried and passed through a 0.149 mm mesh for total C and N determination by dry combustion on a LECO® CN-2000 elemental analyzer (PerkinElmer, Waltham, MA, USA). Soil NH_4^+-N and NO_3^--N were extracted by 2 M potassium chloride (KCl) and quantified using spectrophotometry as described by Norman et al. (1985) and Krom (1980), respectively. The soil pH in $CaCl_2$, soil moisture, total soil porosity, and particle density were analyzed according to EMBRAPA (2009). The determination of particle density was based on the relationship between soil mass and the volume occupied by the soil particles. All analyses were performed in triplicate for each soil sample. Total C and N, the only analyses performed on plant residues (leaf litter and crop residues), was performed by dry combustion on a LECO® CN-2000 elemental analyzer (PerkinElmer, Waltham, MA, USA) after the samples were completely dried at 65°C and ground.

Soil, Leaf Litter, and Crop Residue Incubation Experiment

To evaluate the N_2O fluxes and denitrification gene abundance across our sites while normalizing the influence of environmental parameters (e.g., moisture and temperature variability) on the soil, a set of microcosms were prepared with the soils and plant residues collected from the field sites in the dry and wet seasons. The soil characteristics such as the water holding capacity (WHC) of the soil, soil moisture, and soil density were analyzed prior to soil incubation as mentioned above for these soil samples. The WHC was determined by transferring the soil into a metal ring with a paper filter until it was fully covered, followed by a water soaking step until water completely diffused in the ring. The weights of the soil, ring, and wet set (ring + wet soil) were previously noted for further calculations. After all of these analyses, the samples were incubated at 105°C for 24 h and then weighed. The WHC was calculated from the ratio between the water and soil weight multiplied by 100 (results

in %). For each sampling site and season, five microcosms were prepared by placing ~300 g of soil into 500 mL sterilized Schott bottles (63.58 cm^2 basal area) covered by plant residues. The plant residues from the corresponding site were added on top of the soils to achieve the same coverage as observed in the field (visual evaluation); the forest sites had thicker litter layers than the citrus and eucalyptus site crop residue layers. The microcosms were incubated at room temperature (between 20° and 22°C) for 1 day at a moisture level of 45% of WHC, after which the soil moisture was adjusted to 70% of WHC by adding water and weighing. The microcosm soil moisture was maintained at this level for the remaining 30 days by weighing daily to check and adjust for moisture loss. The N_2O fluxes were measured after the first 24 h (at 45% of WHC) and then at 1, 7, 15, and 30 days following wetting (at 70% of WHC). For each flux measurement, five gas samples were taken from the microcosm headspace using syringes at 1, 30, 60, and 90 min after closure of the microcosm, followed by the respirometric assay proposed by Pitombo et al. (2018). The N_2O concentrations were determined by gas chromatography, and the fluxes were calculated as described above. On the last day of gas sampling, soil samples were taken from each of the 40 microcosms (4 sampling sites × 5 microcosms × 2 sampling periods) and stored at −20°C until the DNA extraction process.

Isolation of DNA From the Soil and Quantitative Real-Time PCR Assays for the Denitrification Genes

Genomic DNA was extracted from 250 mg of soil from the field and soil incubation experiments using the Power Lyzer™ Power Soil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA purity and concentration were assessed spectrophotometrically (Nanodrop ND-1000, NanoDrop Technologies, Inc., Wilmington, DE, USA) to determine absorbance at the following wavelengths: 230, 260, 280, and 320 nm. All 80 DNA samples were stored at −20°C until use. Quantitative real-time PCR (qPCR) using the *nir*K, *nir*S, and *nos*Z genes as biomarkers was performed to assess the abundance of the denitrifying bacterial community in each of the 40 soil samples from the sampling sites and 40 incubation experiment samples. Amplicons of *Sinorhizobium meliloti* (DSM 30135), *Pseudomonas fluorescens* (DSM 50090), and *Bradyrhizobium japonicum* (DSM 1755) were used as standards for the *nir*K, *nir*S, and *nos*Z genes, respectively. DNA standard curves were generated by dilution series of 10^2-10^7 copies μL^{-1} using duplicate 10-fold dilutions of *S. meliloti*, *P. fluorescens,* and *B. japonicum* standard DNA.

The following primer pairs were used for qPCR of *nir*K, *nir*S, and *nos*Z gene fragments (**Table 1**).

Each 10 μL reaction contained 5 μL of SYBR green master mix (Applied Biosystem, Foster City, CA, USA), 0.7 μL of each primer (0.3 μM for *nir*K and *nir*S and 0.5 μM for *nos*Z), 0.5 μL of bovine serum albumin (BSA; 0.12 mg mL^{-1}) and 1–2 ng μL^{-1} (final concentration) of template DNA. The following conditions were chosen for *nir*K amplification after optimization: initial denaturation for 10 min at 95°C and 40 cycles of 30 s at

95°C, 30 s at 58°C, and 40 s at 72°C. The PCR conditions for nirS amplification were as follows: initial denaturation for 2 min at 94°C and 50 cycles of 15 s at 95°C, 1 min at 57°C, and 1 min at 72°C. For nosZ amplification, the following PCR conditions were used: initial denaturation for 10 min at 95°C and 45 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. A negative (no template) control reaction in which soil DNA was substituted for nuclease-free water was used in qPCR assays. PCR amplification and product quantification were performed using the QuantStudio™ 3 Real Time PCR System (Applied Biosystems, Foster, CA, USA). A melting curve analysis of amplicons was conducted to confirm that the fluorescent signals originated from specific amplicons and not from primer dimers or other artifacts. Automated analyses of PCR amplicon quality (e.g., PCR baseline subtraction, Ct threshold set to the linear amplicon phase) and quantity were performed using QuantStudio® Design and Analysis Software (Applied Biosystems).

Statistical Analysis

A Tukey's HSD test was used to determine the significance of the differences in N_2O fluxes between dry and wet seasons within the same sampling site and agricultural and adjacent forest site in the same season based on data obtained in field and incubation experiment. The statistical comparison of N_2O fluxes was performed using Statistica v. 10.0 software (Statsoft Inc., Tulsa, OK, USA).

Pearson's correlation analysis was performed to explore the relationships among the abundances of denitrification genes (nirK, nirS, and nosZ), N_2O fluxes, and soil physicochemical characteristics by using the function "prcomp" in the R packages "factoextra" and "FactoMineR" (version 3.3.3; R Core Team, 2017). The explicit relationships among these variables were examined by constrained ordination generated by principal component analysis (PCA). The PCA and Pearson's correlation analyses were performed based on a data frame in which all variables had the same row length. The field N_2O flux data in these analyses were based on averages by "day" across "chamber," resulting in eight unique flux measurements per sampling site per season. The soil incubation N_2O flux measurements were averaged by "day" across microcosm bottles only.

Linear mixed effects models (R version 3.3.3; R Core Team, 2017) with "chamber" nested in "day" were used to test N_2O fluxes across sites and seasons; P-values were obtained using Satterthwaite's approximation of degrees of freedom (lmerTest).

Mean N_2O fluxes were compared with Tukey HSD and were considered significantly different at $P \leq 0.05$ (lsmeans).

Data for Citrus and its adjacent forest (Forest 1), and Eucalyptus and its adjacent forest (Forest 2) were analyzed independently; four mixed effects models were tested (**Table 2**). Model 1 used field data from Citrus and Forest 1; Model 2 used field data from Eucalyptus and Forest 2; Model 3 used lab data from Citrus and Forest 1; Model 4 used lab data from Eucalyptus and Forest 2. All models contained data from both wet and dry seasons. Analyses of Models 2–4 were done on the natural log scale; constants were added in these models to make all N_2O flux values >0 so that data could be log-transformed. Constants' units were in mg m^{-2} day^{-1} in the field data and mg kg soil^{-1} day^{-1} in lab data. LSmeans were back transformed and the respective constant was subtracted for presentation and interpretation.

Linear mixed effects models were also conducted using total gene abundances as the response variable. These models had the same data groupings as above; replicate was used as the random effect.

RESULTS

Field and Soil Incubation Fluxes of N_2O

The field N_2O fluxes increased by 10–77% from the dry to wet season. At all sites, the field average N_2O fluxes were lower in the dry season than in the wet season (**Figure 2A**). The N_2O fluxes were lower for the two agricultural sites than those from their adjacent forest sites in both the dry and wet seasons. These differences in average N_2O fluxes were significant (Tukey's HSD, $P < 0.05$) between the dry and wet seasons and between agricultural sites and their respective adjacent forest sites (**Figure 2A**), except for the Eucalyptus and Forest 2 sites that showed no difference between the dry and wet seasons (**Figure 2A**). The accumulated flux for dry season was between

TABLE 1 | Primer pair sequences used to quantify nirK, nirS, and nosZ genes.

Gene	*Primers* sequences	References
nirK	nirK876 (ATYGGCGGVAYGGCGA) nirK1040 (GCCTCGATCAGRTRTGG)	Henry et al., 2004 Braker et al., 1998
nirS	nirScd3f (GTNAAYGTNAARGARACNGG) nirSR3cd (GASTTCGGRTGSGTCTTGA)	Throbäck et al., 2004
nosZ	nosZ2F (CGCRACGGCAASAAGGTSMSSGT) nosZ2R (CAKRTGCAKSGCRTGGCAGAA)	Henry et al., 2006

qPCR efficiency (%): nirK = 103.2, nirS = 82.1, nosZ = 98.8.

TABLE 2 | The effect of site and season on N_2O flux in field and lab experiments.

Effect	Model 1	Model 2	Model 3	Model 4
P-VALUE●				
Site	<0.001	<0.001	0.47	<0.001
Season	<0.001	0.08	<0.001	0.04
Site vs. Season	0.004	0.85	0.009	0.21
LSmeans*				
Cultivated	–	81451 B	–	4843.6 B
Control	–	1261502.2 A	–	13172.3 A
Dry Season	–	–	–	1685.7 B
Wet Season	–	–	–	24604.7 A
Cultivated Dry	78089.6 C	–	7925.3 AB	–
Control Dry	284768.5 B	–	11613.3 A	–
Cultivated Wet	345826.8 B	–	685.1 B	–
Control Wet	859560 A	–	26131.7 A	–

*N_2O flux was measured as mg^{-1} m^{-2} day^{-1} in the field and mg kg soil^{-1} day^{-1} in the lab.
● P-values were assigned by Satterthwaite approximation in models fit with restricted maximum likelihood (REML) (package "lmerTest" package; R version 3.3.3).
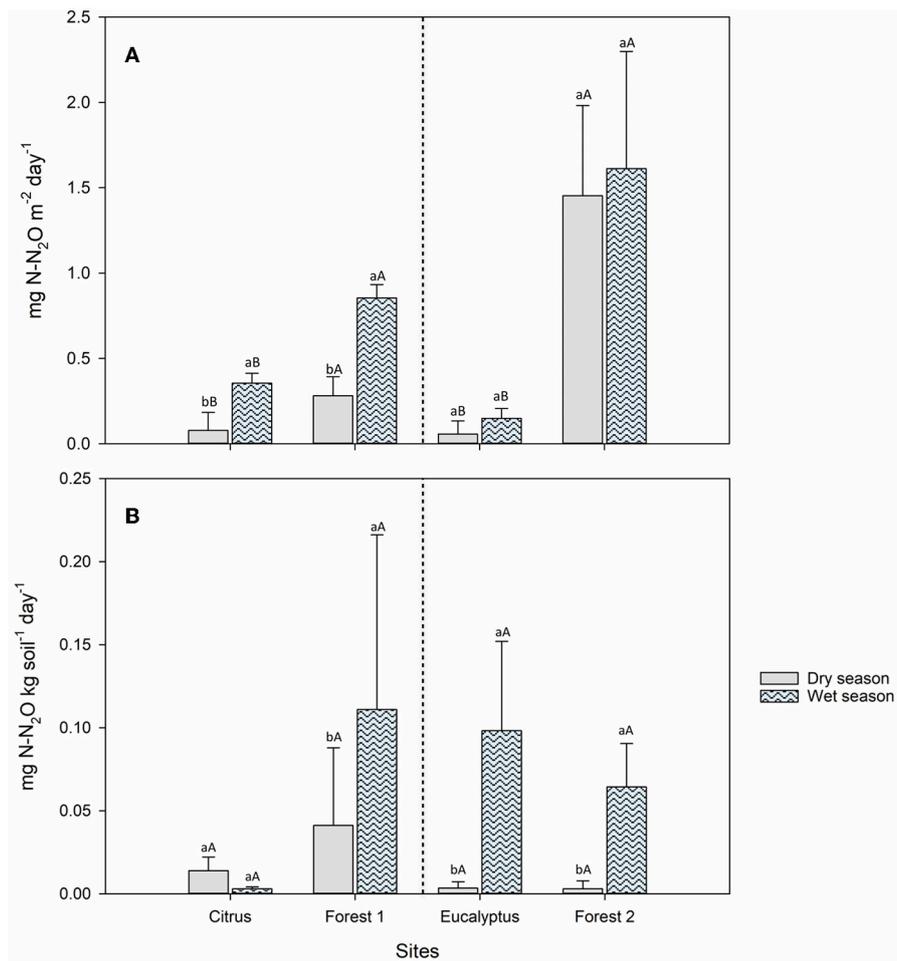
FIGURE 2 | Average N_2O fluxes ($n = 5$) from Citrus, Forest 1, Eucalyptus and Forest 2 sites for measurements made in the dry (solid) and wet (pattern) seasons. **(A)** Field measurements **(B)** Soil incubation measurements, moisture at 70% of water holding capacity. Tukey's HSD test ($P < 0.05$) was performed to compare the dry and wet seasons within the same sampling site (lower-case letters) and the agricultural vs. adjacent forest site (Citrus vs. Forest 1 and Eucalyptus vs. Forest 2) in the same season (upper-case letters).

0.3 and 6.9 mg N-N_2O m^{-2} week^{-1}, and for wet season was between 0.7 and 8.15 mg N-N_2O m^{-2} week^{-1}.

A significant interaction between site and season was observed for N_2O flux in model 1 ($P = 0.004$, t-value = 2.90; lmerTest). Lsmeans determined Forest 1 Wet Season to have the greatest mean N_2O flux (859,560 mg N-N_2O m^{-2} day^{-1}) and Citrus Dry Season to have the smallest mean N_2O flux (78,089.6 mg N-N_2O m^{-2} day^{-1}); there was no mean flux difference between Citrus Wet Season (345,826.8 mg N-N_2O m^{-2} day^{-1}) and Forest 1 Dry Season (284,768.5 mg N-N_2O m^{-2} day^{-1}), which were between Forest 1 Wet Season and Citrus Dry Season ($P < 0.05$; **Table 2**). Model 2 showed site was the only significant predictor for N_2O flux ($P < 0.001$, t-value = 10.35); lsmeans determined Forest 2 had significantly greater flux (1,261,502.2 mg N-N_2O m^{-2} day^{-1}) than Eucalyptus (81,451 mg N-N_2O m^{-2} day^{-1}) ($P < 0.05$; **Table 2**).

The incubated soils demonstrated different trends in average N_2O fluxes with season and site than those observed in the

field (**Figure 2B**). The incubations of soil and plant residues collected in the dry and wet seasons showed higher average N_2O fluxes from the soils collected from the Eucalyptus, Forest 1, and Forest 2 sites in the wet season than those collected in the dry season even though soil moisture was fixed at 70% of WHC throughout all soil incubations (**Figure 2B**). The Citrus site showed the opposite pattern, with higher average N_2O flux in the dry season than in the wet season. In the Eucalyptus, Forest 1, and Forest 2 sites, the differences in average N_2O fluxes between the soils collected in the dry and wet seasons from a given sampling site were significant ($P < 0.05$), while the difference was not significant for the Citrus site (**Figure 2B**).

A significant interaction between site and season was observed for N_2O flux in model 3 ($P = 0.009$, t-value = 2.71; lmerTest). Lsmeans determined no difference in Forest 1 Wet Season and dry season fluxes, which had the greatest mean N_2O fluxes (11,613.3 and 26,131.7 mg N-N_2O kg soil^{-1} day^{-1}, respectively); Citrus Wet Season had the smallest mean N_2O flux (685.1 mg

N-N$_2$O kg soil^{-1} day^{-1}); there was no difference between Citrus Dry Season mean fluxes (7,925.3 mg N-N$_2$O kg soil^{-1} day^{-1}) and any of the other groups' ($P < 0.05$; **Table 2**). Model 4 showed site and season were both significant predictors for N$_2$O flux ($P < 0.001$, t-value = 4.29; $P = 0.04$, t-value = 2.10, respectively); lsmeans determined the wet season had a greater mean flux (24,604.7 mg N-N$_2$O kg soil^{-1} day^{-1}) than the dry season (2,685.7 mg N-N$_2$O kg soil^{-1} day^{-1}); and forests had a greater mean flux (13,172.3 mg N-N$_2$O kg soil^{-1} day^{-1}) than agricultural sites (4,843.6 mg N-N$_2$O kg soil^{-1} day^{-1}) ($P < 0.05$; **Table 2**).

Soil Physicochemical and Plant Residue Chemical Characteristics

The soil physical factors varied across the sites but did not change significantly with season. The range of soil texture fractions across all sites was 9–54% clay, 20–66% sand, and 12–35% silt. The soils were described as Forest 1 soil as clay, Citrus soil as clay-sandy, Forest 2 soil as sandy and Eucalyptus soil as clay. The soil pH ranged from 4.5 to 7.0 for the dry season and from 5.0 to 6.9 for the wet season. The pH was the lowest at the Eucalyptus site and the highest at the Forest 1 site in both seasons. These soil factors differed (Tukey's HSD, $P < 0.05$) between the Citrus and Forest 1 sites in both seasons and between the Eucalyptus and Forest 2 sites in the dry season only (**Table 3**) but not between dry and wet seasons at the same site (Tukey's HSD, $P > 0.05$).

Soil C and N varied with site and season in distinct ways for the tree crops and forests. Total soil C, N, and DOC were higher in Forest 1 than Citrus site in both seasons (**Table 3**). The same pattern was not observed for the Forest 2 and the Eucalyptus site, where differences were typically small. In general, soil C, N, and DOC increased by 121–162%, 117–181%, and 130–260%, respectively, in the wet season when the difference was significant (e.g., increase in DOC for the Eucalyptus and Forest 2 sites). The balance of the pools of C and N, expressed as the C/N ratio, was lower in the soil from the agricultural sites than from the forest sites (indicating more N) and tended to decrease further into the wet season.

The plant residue pool, which represents key matter inputs to the soil, was highly dynamic for the tree croplands but not for the forests. The plant residues (crop residues) in the Citrus and Eucalyptus sites were twice as high in C and N in the dry season compared to those in the wet seasons, while a corresponding change in the forest residues was not observed. The statistical analysis showed significant differences for total C and N of the plant residues between the Citrus and Forest 1 sites for both seasons and between Eucalyptus and Forest 2 in the dry season. The C/N of the plant residues was stable across the wet and dry seasons at all sites except for the Citrus and Eucalyptus sites, where the crop residue was higher in N in the dry season (C/N∼13.0 and ∼25, respectively) than in the wet season (C/N∼17.0 and ∼32, respectively).

The levels of the key denitrifying substrates nitrate and ammonium were the most variable in the forests, which drove the dynamics of the observed trends with site and season. Nitrate was higher in the forests than in the croplands, and the levels were significantly different (Tukey's HSD, $P < 0.05$) between the Citrus and Forest 1 sites in the dry and wet seasons and between the Eucalyptus and Forest 2 sites in the wet season (**Table 3**). The nitrate levels did not vary significantly with season, but the ammonium levels increased (between 4 and 197%) in the wet season for all sites except Forest 2. Ammonium increased significantly in Forest 1 and decreased in Forest 2 in the wet season, which drove their corresponding differences from the adjacent tree croplands that had lower significant increase on levels of ammonium across the seasons.

Denitrification Gene Abundance

The genetic markers of the denitrifiers (*nirK*, *nirS*, and *nosZ*) increased in abundance from the dry to wet season in all sites, except the Citrus site. The abundance of these three denitrification genes, as determined by qPCR (expressed in 10^5 gene copies per gram of soil), indicated that the *nirK* and *nosZ* gene copy numbers were generally highest in the wet season in both the field and incubation experiments (**Table 4**). These differences were for all sites and conditions (field vs. incubation), except for the Citrus site in the soil incubations, which demonstrated a higher *nosZ* abundance in the dry season soil incubations and little difference in *nirK*. Of all three gene targets, the *nirS* gene fragment had the lowest abundance at all sampling sites regardless of season or condition, and the differences with site and season were not significant. The gene abundance did not differ between the agricultural and adjacent forest sites in the same season. Finally, using linear mixed effects models, we found no significant effects ($P > 0.05$) of site and season on the total gene abundance (sum of *nosZ*, *nirK*, and *nirS*) in the field and lab experiments (**Table S1**).

Relationships Among Denitrification Gene Abundances, N$_2$O Fluxes, and Soil and Plant Residue Physicochemical Factors

The relationships between *nirK*, *nirS*, and *nosZ* gene abundance, N$_2$O fluxes, and soil physicochemical factors were examined by PCA ordination analysis separately for each agriculture-forest pair from both the field and incubation results. The field data (**Figure 3**) revealed distinct groups for the geographical locations of the adjacent sampling sites (Citrus vs. Forest 1 and Eucalyptus vs. Forest 2). For the Citrus and Forest 1 sites, there was no association between *nirK*, *nirS*, and *nosZ* gene abundance and soil and plant residue chemical factors such as DOC, total C, or total N, which were related to soil bulk density (**Figure 3A**). A significant positive association ($P < 0.05$) was found among DOC, soil and plant residue total C and N, nitrate, ammonium, pH, and N$_2$O fluxes at the Citrus and Forest 1 sites. Soil moisture was positively correlated with the soil and plant residue chemical factors, as well as with N$_2$O fluxes. The PCA ordination revealed a positive correlation among N$_2$O fluxes, ammonium and DOC for the Eucalyptus and Forest 2 sites (**Figure 3B**). The denitrification genes showed a positive correlation with bulk density and plant residue total C and N for these sampling sites.

A similar PCA ordination analysis performed on the soil incubation experiment dataset (**Figure 4**) showed the same

TABLE 3 | Leaf litter and crop residue chemical factors and soil physicochemical factors from Citrus, Forest 1, Eucalyptus and Forest 2 sites collected in the field in dry and wet seasons.

Factors	Dry season				Wet season			
	Citrus	**Forest 1**	**Eucalyptus**	**Forest 2**	**Citrus**	**Forest 1**	**Eucalyptus**	**Forest 2**
LEAF LITTER/CROP RESIDUES								
Total C (g kg^{-1})	15.6[¶]aB ± 0.8[§]	22.3aA ± 2.0	25.8aA ± 4.4	10.1aB ± 0.5	8.5bB ± 0.9	22.9aA ± 3.1	10.7bA ± 3.4	9.9aA ± 1.4
Total N (g kg^{-1})	1.2aB ± 0.1	1.4aA ± 0.1	0.9aA ± 0.2	0.6aA ± 0.2	0.5bB ± 0.1	1.5aA ± 0.1	0.4bB ± 0.1	0.6aA ± 0.1
C/N	13.1bB ± 0.7	16.2aA ± 1.7	25.8bA ± 1.9	20.4aB ± 2.2	17.5aA ± 1.9	14.7aA ± 0.9	32.1aA ± 1.8	18.5aB ± 1.0
SOIL								
DOC (ppm)	7.7aB ± 0.5	15.7aA ± 2.0	4.2bB ± 0.2	4.3bB ± 0.1	7.4aB ± 0.3	14.4aA ± 2.8	6.8aA ± 1.0	6.4aB ± 0.3
Total C (g kg^{-1})	19.0bB ± 1.2	45.6bA ± 5.1	20.8aB ± 2.8	16.0aB ± 1.6	24.4aB ± 2.3	69.1aA ± 6.7	21.2aA ± 2.1	27.1aA ± 6.0
Total N (g kg^{-1})	2.4bB ± 0.3	3.6bA ± 0.5	1.5bA ± 0.3	1.2bA ± 0.4	3.1aB ± 0.2	6.3aA ± 0.6	2.1aA ± 0.3	2.3aA ± 0.4
C/N	7.8aA ± 0.9	12.3aB ± 0.5	12.1aA ± 1.9	13.3aA ± 2.2	7.7aB ± 0.6	10.9aA ± 0.7	9.9aA ± 1.8	11.5bA ± 0.9
Nitrate (mg kg^{-1})	2.1aB ± 1.1	12.7aA ± 1.9	0.0aA	2.9aA ± 1.9	3.1aB ± 1.1	16.0aA ± 7.9	0.0aB	3.3aA ± 1.5
Ammonium (mg kg^{-1})	4.6bA ± 1.4	3.8bA ± 0.3	1.8bB ± 0.05	7.6aA ± 3.1	4.8bA ± 0.9	11.3aA ± 7.5	2.5aA ± 0.4	1.8bB ± 0.5
pH	5.8aB ± 1.0	7.0aA ± 0.1	5.4aA ± 0.3	4.5aB ± 0.5	5.7aB ± 0.7	6.9aA ± 0.2	5.4aA ± 0.4	5.0aA ± 0.4
Soil moisture (%)	18.3bB ± 2.1	32.4bA ± 1.6	15.9aA ± 1.6	17.7bA ± 3.2	27.7aB ± 2.4	46.8aA ± 3.5	23.3aA ± 3.2	24.3aA ± 2.6
Density (g cm^{-3})	0.89	0.65	0.96	0.96	0.89	0.65	0.96	0.96
Clay (%)	37.4	53.4	44.1	9.0	37.4	53.4	44.1	9.0
Sand (%)	50.5	34.1	20.9	65.8	50.5	34.1	20.9	65.8
Silt (%)	12.1	16.3	35.0	25.2	12.1	16.3	35.0	25.2

[¶]*Average of five soil replicates.*

[§]*Standard deviation of the average of five soil replicates.*

Tukey's HSD test was performed separately to compare the dry and wet seasons at the same sampling site (lower-case letters) and the agricultural vs. adjacent forest site (Citrus vs. Forest 1 and Eucalyptus vs. Forest 2) in the same season (upper-case letters).

Values with the same lower or upper-case letters were not significantly different (P < 0.05) based on Tukey's HSD test between contrasted samples.

grouping pattern by sampling site locations as revealed by the field dataset for the Citrus and Forest 1 soils (**Figures 3A**, **4A**). A positive correlation was revealed between N$_2$O fluxes and the soil chemical factors in the incubated soil samples (**Figure 4A**), where the abundance of the *nir*K, *nir*S, and *nos*Z genes was not strongly associated with the other variables. Both the Eucalyptus and Forest 2 sites revealed a correlation between N$_2$O fluxes and soil DOC, in which the *nir*K, *nir*S, and *nos*Z gene abundance was more positively correlated with soil pH, soil total C and N, and soil moisture than with N$_2$O fluxes (**Figure 4B**).

Pearson correlations were determined between all data from each of the four sampling sites in both the field and soil incubation experiments and season (**Supplementary Figure 2**). A greater number of negative correlations were observed in the agricultural Citrus and Eucalyptus sites (**Supplementary Figures 2A,C**) than in the Forest 1 and Forest 2 sites (**Supplementary Figures 2B,D**). For instance, *nir*K and *nir*S gene abundance revealed a negative correlation with DOC in the Citrus and Eucalyptus sites, while in both forest sites, the same correlation was positive for the field and soil incubation experiments. The correlations common to both the field and soil incubation experiment data included those between soil total C and N and the *nir*K, *nir*S, and *nos*Z genes. Furthermore, in all sampling sites, soil moisture was positively correlated with N$_2$O fluxes for the field measurements. In the soil incubation experiment, DOC was correlated with N$_2$O fluxes at all sampling sites (**Supplementary Figure 2**).

DISCUSSION

Through complementary field- and incubation-based analyses, we characterized the fluxes of N$_2$O from the soil and their plant residue covers to evaluate our research hypotheses. In contrast to the first hypothesis that citrus- and eucalyptus-cultivated soils emit higher amounts of N$_2$O to the atmosphere than their adjacent natural forest soils counterparts, we observed higher average N$_2$O fluxes from the natural forest soils than from the adjacent crop-cultivated soils (**Figure 2**; **Table 2**). Season emerged as a key driver of N$_2$O fluxes and marker gene abundance for denitrifiers, which did not significantly differ with land use (**Table 4**; **Table S1**), in contrast to our second hypothesis. The factors such as soil moisture and plant residue characteristics that may drive the observed differences with season, land use, and their combinations are discussed below.

Our results showed a strong influence of season on N$_2$O fluxes, with the N$_2$O flux increasing in the wet season in both the field and soil incubation experiments. Field moisture was an important predictor of the N$_2$O fluxes in the field and incubations (**Figures 3**, **4**, and **Table 2**). Clough et al. (2017) also showed higher N$_2$O fluxes in soil at high moisture levels once a lower oxygen content and, consequently, an anaerobic environment, stimulate denitrification (Van Spanning et al., 2007). Leaf litter and crop residues can contribute to increased soil moisture retention and may consequently promote N$_2$O production (Kravchenko et al., 2017). Forest 1 and Forest 2 soils had thicker

TABLE 4 | Absolute abundance of *nir*K, *nir*S, and *nos*Z genes measured by quantitative real-time PCR from field and incubation experiment soil samples in dry and wet seasons.

Genes	Dry season				Wet season			
	Citrus	Forest 1	Eucalyptus	Forest 2	Citrus	Forest 1	Eucalyptus	Forest 2
FIELD								
*nir*K	34.1¶bA ± 2.0§	28.1bA ± 2.3	39.6bA ± 11.1	19.1bA ± 7.1	40.2aA ± 1.3	34.4aA ± 3.0	148.0aA ± 27.1	30.6aA ± 3.2
*nir*S	0.05aA ± 0.03	0.05aA ± 0.005	0.5aA ± 0.2	0.2aA ± 0.1	0.1aA ± 0.03	0.01aA ± 0.004	0.4 aA ± 0.2	0.2aA ± 0.1
*nos*Z	5.1bA ± 1.4	6.8bA ± 3.3	37.0bA ± 14.7	24.0bA ± 9.2	10.1aA ± 1.1	0.3bA ± 0.1	116.0aA ± 18.4	46.9aA ± 9.8
INCUBATION EXPERIMENT								
*nir*K	21.3aA ± 3.5	14bA ± 1.7	32.9bA ± 2.1	30.5bA ± 4.4	20.8aA ± 3.3	31.5aA ± 7.2	42.3aA ± 3.6	61.7aA ± 17.9
*nir*S	0.03aA ± 0.01	0.1aA ± 0	0.2 aA ± 0.05	0.2aA ± 0.1	0.1aA ± 0	0.04aA ± 0	0.2aA ± 0.05	0.4aA ± 0.2
*nos*Z	28.0aA ± 3.5	8.2aA ± 1.9	32.7bA ± 8.2	34.0bA ± 5.5	18.3bA ± 2.6	3.2bA ± 2.6	67.3aA ± 17.7	79.9aA ± 9.3

The values are expressed as 10^5 gene copies per gram of fresh soil.

¶Average of five soil replicates.

§Standard deviation of the average of five soil replicates.

Tukey's HSD test was performed to separately compare the dry and wet seasons at the same sampling site (lower-case letters) and the agricultural vs. adjacent forest site (Citrus vs. Forest 1 and Eucalyptus vs. Forest 2) in the same season (upper-case letters).

Values with the same lower or upper-case letters were not significantly different ($P < 0.05$) based on Tukey's HSD test between contrasted samples.

FIGURE 3 | Principal component analysis plot based on the nirK, nirS, and nosZ gene abundances, N$_2$O fluxes, and soil physicochemical and plant residue chemical factors as determined in the field samples. **(A)** Citrus and Forest 1 sites and **(B)** Eucalyptus and Forest 2 sites. The symbols refer to replicates of soil from the following sampling sites and seasons: agricultural sites in the dry season (ADS), agricultural sites in the wet season (AWS), forest sites in the dry season (FDS), and forest sites in the wet season (FWS). C/N, from soil total C and N; DOC, Dissolved organic carbon; N$_2$O, Nitrous oxide; RC, Residue total carbon; RN, Residue total nitrogen; SD, Soil density; SM, Soil moisture; STC, Soil total carbon; and STN, Soil total nitrogen.

FIGURE 4 | Principal component analysis plot based on the nirK, nirS, and nosZ gens abundances, N$_2$O fluxes, and soil physicochemical and plant residue chemical factors as determined in the soil incubation experiment samples. **(A)** Citrus and Forest 1 sites and **(B)** Eucalyptus and Forest 2 sites. The symbols are identical to those defined in **Figure 3**.

litter layers than the cultivated soils, and our results are therefore consistent with the higher N$_2$O fluxes from soils under thicker plant residue layers. In addition, Taketani et al. (2017) stated that soil moisture influences changes in soil microbial communities by increasing nutrient transportation during the high moisture period. Thus, plant residues can promote soil moisture retention, oxygen limitation, nutrient transport, and the corresponding microbial community composition in ways known to promote the denitrification process, a critical component of N$_2$O fluxes.

We discovered that the drivers of the differences in the soil N$_2$O fluxes in the wet and dry seasons were not limited to the soil moisture conditions. If that had been the case, we

would have seen the differences in the average N_2O fluxes observed in the field (**Figure 2A**) vanish upon incubation under identical moisture conditions in the lab (**Figure 2B**). Instead, we found that the soil collected from the two seasons demonstrated persistent differences in N_2O flux dynamics in lab incubations. Therefore, this persistent and surprising legacy behavior was used to evaluate the key drivers of N_2O fluxes based on the chemical characteristics of leaf litter and crop residues and soil physicochemical characteristics. The differences among soil texture as showed for the studied sites may be also an important factor on N_2O production, as previous state by Harrisson-Kirk et al. (2013) and Skiba and Ball (2002). However, considering a Udox soil under a hyperthermal soil temperature regime, which C and N turnover is faster, nutrient cycling may have a more significant effect on N_2O fluxes.

The N_2O fluxes were increased with the carbon content due its support of denitrifying organisms, and DOC may have been particularly important. Tiedje et al. (1983) suggested that organic C is more important than oxygen status in determining the denitrifying enzyme content of habitats. Higher concentrations of organic C usually support higher populations of heterotrophs, which include denitrifiers. Positive correlations among the DOC concentration, total soil C and N_2O fluxes were revealed in most soils in the field and soil incubation experiments. Higher DOC concentrations may drive more N_2O fluxes as labile C increases and plays a role as an electron acceptor for denitrifying microorganisms (Harter et al., 2014). Marques et al. (2012) showed a strong and positive relationship between the DOC concentration, total soil C and soil moisture when studying Amazonian soils under different land uses. In superficial soils (0–10 cm), as used in this study, the DOC concentration is mostly influenced by the type of plant residue on the soil surface (Marques et al., 2012). Harter et al. (2014) showed a positive correlation between the DOC concentration and N_2O fluxes in biochar-amended soil, possibly due to the high root quantity in forest fields, which supports DOC transportation throughout the soil in the dry season. This process makes the denitrification process efficient in the absence of water in soil under native forests when compared with that in agricultural fields (Marques et al., 2012).

As the substrate for denitrification, the availability and type of N in the soils and plant residues is an important factor in N_2O fluxes. In this study, the N_2O fluxes were positively correlated with soil total N mainly in the forest soils. This relationship has been described by previous studies, specifically for N in the nitrate form (NO_3^-) (Carmo et al., 2005; Cerri et al., 2006). However, the site with the highest ratios of N to C (lowest soil C/N) in the soil and in the crop residue inputs was the Citrus cultivation site. That site may have had greater N availability due to the annual fertilizer application, but, in fact, it had the lowest N_2O fluxes and a lower N_2O emission potential in the wet than the dry season, perhaps due to the increase in C/N in the wet season crop residue. The inorganic-based fertilizers used in citrus orchards are rapidly mineralized and have the possibility to supply NO_3^- in addition to NH_4^+, which is the preferred N species for uptake by most higher plants (Britto and Kronzucker, 2002). The NH_4^+ is constantly regenerated via

dissimilatory nitrate reduction to ammonium (DNRA) due to the redox fluctuation conditions in tropical soils (Silver et al., 2001; Templer et al., 2008), and with a subsequent influx of O_2, this NH_4^+ becomes re-available to ammonia oxidizing communities, disfavoring the denitrification process. Higher levels of nitrate and ammonia were observed in the forests compared to the cultivated sites that received historical or annual fertilization (see section Site Descriptions), and the concentrations increased in the wet season. Therefore, the site- and season-specific trends in N_2O flux are driven in part by changes in total N but perhaps most strongly by whether the N input is supplied through plant litter or fertilizer and how that impacts the relevant C/N ratios.

The soil pH may have also contributed to the site differences in N_2O fluxes, as denitrification rates are pH sensitive. Several studies have established that denitrification rates tend to decrease at low soil pH values (Simek et al., 2002; Saleh-Lakha et al., 2009). Parkin et al. (1985) previously demonstrated a 2-fold decrease in the denitrification rate and a 3-fold decrease in denitrification enzyme activity when soil pH decreased from approximately pH 6.0–4.1, suggesting that prolonged exposure to a low soil pH selected a denitrifier population that was more adapted to the low pH environment. Subsequently, the N_2O reduction by the acid-tolerant population was insignificant compared to the N_2O production rates. Although our results did not reveal a significant correlation between the N_2O fluxes and soil pH for the different samples, the relationship between N_2O fluxes and soil pH in the Citrus and Forest 1 sites corroborates these previous findings.

The denitrifying gene markers tracked changes in N_2O fluxes in some but not all cases, and their poor prediction of N_2O dynamics may indicate that other microbial N cycling processes were also important (Martins et al., 2015). Increased N_2O fluxes in the wet season in the field and incubation experiments were accompanied by increasing *nir*K gene abundances in the soil from all sampling sites. However, the gene-based measurements did not predict N_2O dynamics in all sites, experiments, and seasons (**Table S1**). Although, the higher levels of *nir*K than *nir*S indicate that the N_2O emission potential by microbial denitrification is consistent with the previous determinations for agricultural soils by Dandie et al. (2011) and Wang et al. (2017). In addition, the abundance of denitrifiers, as indicated by *nir*K, *nir*S, and *nos*Z gene copy numbers, neither differed between the agricultural and adjacent forest sites in the same season nor did they have a clear correlation with the chemical characteristics of the leaf litter and crop residues and soil physicochemical characteristics by site or condition (field vs. incubation). The exception was that the soil density has a low but significant positive correlation with denitrification gene abundance (**Supplementary Figure 2**); more dense soils may have lower porosity and higher moisture levels. Previous attempts to relate denitrifier abundance to environmental factors or N_2O emissions have been undertaken in a range of environments with varying results (Rich and Myrold, 2004; Philippot et al., 2009; Wertz et al., 2009; Enwall et al., 2010). Soil denitrifiers have been found not to respond to the environmental properties in grassland and agricultural fields, waterlogging, and changes between ecosystems, while the structure or composition of the denitrifying microbial communities indicated differences

(Philippot et al., 2009; Dandie et al., 2011; Wang et al., 2017). Therefore, we acknowledge that although surface soils have a more favorable environment for denitrifying microorganisms, the denitrification gene abundance does not necessarily relate to the real community composition. Despite this, it may be equally plausible that other N cycling pathways not represented by our three marker genes (*nir*K, *nir*S, and *nos*Z) are important factors in some or all of our sites. Additional pathways that produce N_2O or drive other N cycle conversions are known (Pajares and Bohannan, 2016) although their importance to N_2O cycling in tropical soils is unclear and variable. While beyond the scope of this study, the characterization of additional N cycling pathways may be necessary to explain the observed discrepancies between denitrification gene abundance and N_2O fluxes across seasons and sites.

CONCLUSION

Taken together, our findings through complementary field- and incubation-based analyses showed higher N_2O fluxes from natural forest soils than from the adjacent citrus- and eucalyptus-cultivated soils, with denitrification gene abundance not significantly different between the natural forest and cropland soils. Based on these findings, both hypotheses of this study were rejected. Additionally, we conclude that the differences observed in the N_2O fluxes and in some cases the *nir*K, *nir*S, and *nos*Z gene copy numbers are related to seasonal environmental variables and soil and plant residue characteristics, especially the DOC concentration, C and N content and density. Our results highlight the overwhelming role of environmental drivers (e.g., moisture dynamics) that can make investigating microbial drivers difficult in the field and open the possibility for a better comprehension of N cycling processes in tropical soils based on soil incubation experiments, particularly when both methods are comparatively employed as presented here.

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