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**Dominance of bacterial ammonium-oxidizers and fungal denitrifiers in the complex nitrogen cycle pathways related to nitrous oxide emission**

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## ABSTRACT

Organic compounds and mineral nitrogen (N) usually increase nitrous oxide (N<sub>2</sub>O) emissions. Vinasse, a by-product of bio-ethanol production that is rich in carbon, nitrogen and potassium, is recycled in sugarcane fields as a bio-fertilizer. Vinasse can contribute significantly to N<sub>2</sub>O emissions when applied with N in sugarcane plantations, a common practice. However, the biological processes involved in N<sub>2</sub>O emissions under this management practice are unknown. The present study investigated the roles of nitrification and denitrification in N<sub>2</sub>O emissions from straw-covered soils amended with different vinasses (CV: concentrated and V: non-concentrated) before or at the same time as mineral fertilizers at different time points of the sugarcane cycle in two seasons. N<sub>2</sub>O emissions were evaluated for 90 days, the period that occur most of the N<sub>2</sub>O emission from fertilizers; the microbial genes encoding enzymes involved in N<sub>2</sub>O production (archaeal and bacterial *amoA*, fungal and bacterial *nirK*, and bacterial *nirS* and *nosZ*), total bacteria and total fungi were quantified by real-time PCR. The application of CV and V in conjunction with mineral N resulted in higher N<sub>2</sub>O emissions than the application of N fertilizer alone. The strategy of vinasse application 30 days before mineral N reduced N<sub>2</sub>O emissions by 65% for CV, but not for V. Independent of rainy or dry season, the microbial processes were nitrification by ammonia-oxidizing bacteria (AOB) and archaea and denitrification by bacteria and fungi. The contributions of each process differed and depended on soil moisture, soil pH, and N sources. We concluded that, *amoA*-AOB was the most important gene related to N<sub>2</sub>O emissions, which indicates that nitrification by AOB is the main microbial-driven process linked to N<sub>2</sub>O emissions in tropical soil. Interestingly, fungal *nirK* was also significantly correlated with N<sub>2</sub>O emissions, suggesting that denitrification by fungi contributes to N<sub>2</sub>O emission in soils receiving straw and vinasse application.

## INTRODUCTION

Vinasse is the major residue generated during ethanol production from sugarcane. For each liter of ethanol produced, approximately 10 to 15 liters of vinasse are generated (Christofoletti *et al.*, 2013). Brazil grows 9 M ha of sugarcane and approximately half of it is used for ethanol production (CONAB, 2017). A dark-brown wastewater with high organic and nutrient content (Christofoletti *et al.*, 2013, Elia-Neto & Nakahodo, 1995, Fuess & Garcia, 2014, Macedo *et al.*, 2008), vinasse is widely applied on sugarcane fields as fertilizer, mainly in the ratoon cycle, complementing mineral fertilization. In 2016, the annual production of vinasse was 360 billion liters in Brazil (CONAB, 2017). However, this immense volume of vinasse is difficult to manage for utilization as fertilizer. Concentration of vinasse by evaporation reduces the water content and consequently the volume, providing an alternative residue with high nutrient and carbon content (Christofoletti *et al.*, 2013). Following evaporation, concentrated vinasse can be applied in the field, often in bands close to the plant row in a manner similar to that of mineral fertilizers, which reduces application cost and facilitates nutrient absorption by crops (Mutton *et al.*, 2014, Parnaudeau *et al.*, 2008).

Mineral nitrogen (N) is often applied simultaneously with vinasse in sugarcane fields to ensure sufficient availability of N for sugarcane plant uptake. This combination may stimulate biological activity in the soil and subsequent N transformations, including the production of N<sub>2</sub>O (Carmo *et al.*, 2013, Pitombo *et al.*, 2015). N<sub>2</sub>O is a nitrogen (N) cycle product with major environmental and ecological impacts. N<sub>2</sub>O is both an ozone-depleting substance (Ravishankara *et al.*, 2009) and a greenhouse gas with global warming potential 298 times greater than that of carbon dioxide (CO<sub>2</sub>) (IPCC, 2006). Carmo *et al.* (2013) and Pitombo *et al.* (2015) reported that the proportion of N emitted was three and two times

higher, respectively, when mineral N was applied together with vinasse compared to mineral N alone in sugarcane field. When vinasse was added to the soil a few days before or after N fertilizer, N<sub>2</sub>O emissions were lower than when vinasse and N fertilizer were applied simultaneously (Paredes *et al.*, 2015, Paredes *et al.*, 2014). However, there is little information about N<sub>2</sub>O emissions for the application of concentrated vinasse as a fertilizer; the only published result, of Pitombo *et al.* (2015), reported that 1.6% of total N applied as vinasse was lost as N<sub>2</sub>O when concentrated vinasse was used.

N<sub>2</sub>O is produced and consumed by biotic and abiotic soil processes. The abiotic process, chemodenitrification, occurs through chemical decomposition of hydroxylamine (NH<sub>2</sub>OH), nitroxyl hydride (HNO) or NO<sub>2</sub><sup>-</sup> in the presence of organic and inorganic compounds at low pH (< 4.5). By contrast, the biotic process requires autotrophic and heterotrophic microorganisms, *i.e.*, bacteria, archaea and fungi (Hayatsu *et al.*, 2008, Higgins *et al.*, 2016, Hink *et al.*, 2016). N<sub>2</sub>O is produced in soil via nitrification and denitrification processes (Martins *et al.*, 2015, Németh *et al.*, 2014, Soares *et al.*, 2016, Stevens & Laughlin, 1998, Xu *et al.*, 2017). In the oxic, well-drained soils typical of most agricultural soils, N<sub>2</sub>O is mainly produced by ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Baggs *et al.*, 2010, Bateman & Baggs, 2005, Bollmann & Conrad, 1998, Hink *et al.*, 2016). However, under suboxic or anoxic conditions, facultative denitrifiers (Di *et al.*, 2014, Tiedje *et al.*, 1983) dominate N<sub>2</sub>O production. According to Soares *et al.* (2016), AOB are the main contributors to N<sub>2</sub>O emissions via the nitrification pathway in soils planted with sugarcane. However, the actual management practices of sugarcane production in Brazil involve a combination of vinasse recycling, inorganic fertilization and cultivation of sugarcane in fields covered with sugarcane straw from the previously cultivated sugarcane crop.

Despite considerable knowledge of the processes involved in N<sub>2</sub>O emission, the control of N<sub>2</sub>O emissions from tropical soils planted with sugarcane has only recently been addressed. The most important region for sugarcane production in Brazil is the Central-Southern region, which has two defined seasons: rainy summers with high temperatures and dry winters with mild temperatures. Sugarcane fertilization usually occurs between April and December, encompassing fall, winter and the end of spring, which have completely different climatic conditions. Therefore, the aim of this study was to evaluate the N<sub>2</sub>O losses in sugarcane planted soils receiving different fertilization regimes with vinasse during different seasons (spring-rainy/winter-dry). Concentrated (CV) and non-concentrated (V) vinasse were applied before or at the same time as mineral fertilizers. Furthermore, we investigated the potential role of nitrification and denitrification processes in N<sub>2</sub>O emissions from vinasse-fertilized sugarcane-planted soils. We hypothesized that (I) application of vinasse residue before N fertilizer application drastically reduces N<sub>2</sub>O emissions; (II) nitrification is the major pathway contributing to N<sub>2</sub>O emissions from sugarcane-planted soils; and (III) N<sub>2</sub>O emissions are lower in winter (dry) than in spring (rainy) due to differences in climatic conditions at the time of mineral N and vinasse application to soil. To test these hypotheses, we quantified N<sub>2</sub>O emissions from sugarcane-planted soil as well as the expression of key functional genes related to N<sub>2</sub>O emissions during different seasons, *i.e.*, archaeal and bacterial *amoA*, fungal and bacterial *nirK*, and bacterial *nirS* and *nosZ*. Additionally, we determined the total bacterial and fungal abundances.

## **MATERIALS AND METHODS**

*Experimental setup and soil sampling* - The study comprised two experiments conducted in two experimental fields planted with sugarcane variety RB86-7515. The experimental fields were located at the Paulista Agency for Agribusiness Technology (APTA), Piracicaba, Brazil.

The climate, according to the Köppen classification system, is considered to be a Cwa type defined as humid tropical with a dry winter and a rainy summer (Critchfield, 1960). The mean annual air temperature and precipitation of the region are 21 °C and 1,390 mm, respectively. Temperature and precipitation measurements during the experiment were obtained from a meteorological station located nearby the experimental fields (Figure S1). The soil is classified as a Ferralsol by FAO (FAO, 2015) or Oxisol by soil taxonomic (USDA, 2014), and the physicochemical properties (Camargo *et al.*, 1986, Van Raij *et al.*, 2001) of the 0- to 20-cm soil layer are shown in Table 1. The main difference between the two experiments was the season in which they were conducted (spring-rainy vs. winter-dry). The rainy season (RS) experiment was conducted during the 2013/2014 sugarcane cycle and began on November 12, 2013. The dry season (DS) experiment was conducted during the 2014/2015 sugarcane cycle and began on July 15, 2014. Both experiments lasted 90 days, period that most of GHG emissions occur. High N<sub>2</sub>O emission occurs soon after vinasse and mineral N fertilizer application with little variation in the emission after 90 days (Carmo *et al.*, 2013, Paredes *et al.*, 2015, Pitombo *et al.*, 2015, Soares *et al.*, 2016).

Prior to both experiments, the sugarcane already planted in the experimental field was mechanically harvested, and the straw was left on top of the soil, a common practice in Brazilian sugarcane fields. Sugarcane can re-grow up to five times after the first harvest (ratoon cycle); in the experiments, the plants were grown for the third (RS) and fourth (DS) time, and the amount of straw left on top of the soil was 12 and 16 t ha<sup>-1</sup> on a dry matter basis in the RS and DS experiments. Experiments were conducted in a randomized block design with three replicates. The treatments included different application times of concentrated (CV) and non-concentrated (V) vinasse in relation to the time of mineral N fertilization. Vinasse was applied either 30 days before or at the same time as N fertilizer. In both

experiments, N<sub>2</sub>O was also monitored in control treatments without N fertilization. The rainy season experiment comprised eight treatments (24 plots) fertilization because of two missed plots, whereas the dry season experiment had two additional CV treatments, resulting in a total of ten treatments (30 plots) (Table 2). The treatments are described on Table 2.

The N (ammonium nitrate) fertilizer application rate was 100 kg ha<sup>-1</sup> for both experiments. The amount of mineral N applied to the experimental followed commercial sugarcane plantation guidelines in the state of São Paulo, Brazil (Van Raij *et al.*, 1996). In both sites, in treatment with vinasse, a volume of 100 m<sup>3</sup> ha<sup>-1</sup> of V was sprayed over the entire experimental plot using a motorized pump fitted with a flow regulator; this volume represents the average application rate of vinasse in sugarcane plantations in the State of São Paulo. CV is produced by removing water of vinasse (5.8 times less volume) by evaporation. CV was applied in bands at a rate of 17.2 m<sup>3</sup> ha<sup>-1</sup> which corresponds approximately to 100 m<sup>3</sup> ha<sup>-1</sup> of V. The ammonium nitrate and CV were surface-applied in 0.2-m-wide bands 0.1 m from the plant rows, which is common practice in commercial sugarcane production. The rates of N applied as vinasse could not be standardized for all treatments and times of application (Table 2) because the vinasse composition varies according to the sugarcane feedstock variety being processed, stage of plant development and soil type (Mutton *et al.*, 2014). As vinasse undergoes fast microbial reactions and we employed large volumes in the experiments, vinasse could not be stored. Therefore, new vinasses (CV and V) were used in each vinasse application. The chemical properties of vinasses (CV and V) applied in the experiments are described on Table S1 in the Supporting information.

*CO<sub>2</sub> and N<sub>2</sub>O measurements, soil sampling and chemical analysis* - Fluxes of CO<sub>2</sub> and N<sub>2</sub>O were measured using PVC static chambers with a height of 20 cm and a diameter of 30 cm according to the method described by Varner *et al.* (2003). The chambers were inserted 5 cm into the soil and 10 cm from the sugarcane rows. The chamber cap had two openings that were each fit to a valve, one for gas sampling and the other for pressure equilibrium. The chambers remained open until gas sampling. Gases were sampled with plastic syringes (60 mL of gas) at three time intervals (1, 15, and 30 min) after the chambers were closed. The samples were transferred to pre-evacuated glass vials (12 mL) for storage and analyzed in a gas chromatograph (model GC-2014, Shimadzu Co.) with a flame ionization detector (FID) (250 °C) for CO<sub>2</sub> determination (Hutchinson & Mosier, 1981) and an electron capture detector for N<sub>2</sub>O determination (Hutchinson & Mosier, 1981). The overall CO<sub>2</sub> and N<sub>2</sub>O fluxes were calculated by linear interpolation of the three sampling times.

CO<sub>2</sub> and N<sub>2</sub>O measurements were conducted for 90 days during both experiments. Throughout the experiments, gas samples were collected in the morning, beginning at least five days before fertilizer and vinasse application. Once the treatments were established, the gases were sampled every day during the first week and three times per week thereafter.

Cumulative fluxes were calculated for each treatment using the emission values measured in the fertilized bands near the crop rows where CV and mineral N fertilizer were applied and most GHGs emissions occur. Cumulative emissions were calculated by linear interpolation between adjacent sampling dates (Soares *et al.*, 2016). For treatments with V, which were applied over the whole field, cumulative emissions on a hectare basis were also calculated (Table S3). The fertilized bands accounted for 16% of the total experimental area and the space between fertilized bands (inter-row) for 84%. Proper controls (plots with no mineral

fertilizer and vinasse, or plots with vinasse without fertilizer were used to calculate the inputs of N<sub>2</sub>O-N emission factors (EF).

The emission factors for N<sub>2</sub>O were calculated based on the amounts of N applied as vinasse and mineral N fertilizer inside the chamber according to the formula:

$$EF = \frac{N_2O-N_{treat} - N_2O-N_{control}}{N_{applied} (fert+vinasse)} \times 100$$

*EF* is the N<sub>2</sub>O-N emission factor (%), *N<sub>2</sub>O-N<sub>treat</sub>* and *N<sub>2</sub>O-N<sub>control</sub>* are the cumulative emissions in the fertilized and unfertilized chambers, respectively, and *N<sub>applied</sub>* is the mass of N fertilizer added to the chamber with ammonium nitrate and/or N from vinasse (V and CV).

Air and soil temperatures were measured at the same time as gas sampling. Six soil samplings per plot were performed throughout the experiments. Soil sampling was performed 1, 3, 7, 22, 24, and 54 days after mineral N application in RS and -30, 1, 11, 19, 45 and 52 days after mineral N application in DS. For all treatments, soil samples were collected from the 0- to 10-cm layer near the gas chambers. The soil samples were used to measure moisture content, pH, and concentrations of nitrate (NO<sub>3</sub><sup>-</sup>-N) and ammonium (NH<sub>4</sub><sup>+</sup>-N). Soil subsamples (30 g) were stored at -80 °C for molecular analyses. Soil moisture was determined gravimetrically by drying the soil at 105 °C for 24 h. Soil mineral N (NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N) was measured with a continuous flow analytical system (FIALab-2500 System) after extraction with 1 M KCl, and the results were expressed per gram of dry soil. The water-filled pore space (WFPS) was calculated based on the soil bulk density (1.45 and 1.49 g cm<sup>-3</sup> in RS and DS) and porosity determined at the beginning of the experiment.

*DNA extraction* - Total soil DNA was extracted from 0.25 g of soil using the MoBio PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions. DNA quantity and quality were determined using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). The extracted DNA was visualized on 1% (w/v) agarose gels under UV light.

*Quantitative real-time PCR* - The abundances of the functional genes *amoA*, *nirS*, *nirK*, and *nosZ*, which encode proteins involved in nitrification and denitrification processes, and ribosomal RNA genes indicating total bacteria (16S rRNA) and total fungi (18S rRNA) were quantified by quantitative real-time PCR (qPCR). qPCR was performed in a 96-well plate (Bio-Rad) using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). qPCR was performed in a total volume of 12  $\mu$ L containing 6  $\mu$ L of SYBR Green Master Mix and 4  $\mu$ L of DNA (1.25 ng/ $\mu$ L), except fungal *nirK*, which was amplified in a total volume of 10  $\mu$ L containing 1  $\mu$ L of undiluted DNA. The primer combinations, reaction descriptions and thermal cycler conditions for each gene amplification are listed in Table S2 in the Supporting information. Data were acquired at 72 °C, and melting curve analysis was performed to confirm specificity. Amplicon sizes were confirmed on 1% (w/v) agarose gels under UV light. Plasmid DNA from microorganisms containing the gene of interest or from environmental samples was used to construct standard curves and then cloned into vectors. Standard curves were performed 10 times using serial dilutions from 10 to 10<sup>-8</sup>. Samples were analyzed with two technical replicates. The reaction efficiency varied from 80 to 105%, and the R<sup>2</sup> values ranged from 0.94 to 0.99.

*Statistical analysis* - The cumulative emissions of N<sub>2</sub>O and CO<sub>2</sub> were checked for normal distribution of residues by the Shapiro-Wilk test, and the data were subsequently transformed using the Box-Cox transformation method (Statistica, version 10). Total cumulative emissions of N<sub>2</sub>O were compared per orthogonal contrasts using SISVAR statistical software (Ferreira, 2011). Soil pH was transformed to H<sup>+</sup>: 10<sup>-pH</sup> before statistical analysis.

Gene abundance values were checked for normal distribution of residues by the Shapiro-Wilk test, and the data were subsequently transformed by log(x) transformation and rechecked to obtain a normal distribution of residues and variance stability (Statistica, version 10). The correlations between N<sub>2</sub>O flux and microbial gene abundance were calculated by Spearman correlation analysis (SystatSoftware, 2014). Additionally, to evaluate the influence of variables (genes/soil factors plus genes), we fit a general linear model with the lasso penalty using cyclical coordinate descent, computed along a regularization path (Friedman *et al.*, 2010). The lasso penalty is a regression method that performs both shrinkage and variable selection (Osborne *et al.*, 2000). To select the most appropriate model, we adopted cross-validation criteria with the “one-standard error” rule by checking the lambda value that minimized the mean square error and choosing the largest value of lambda within one standard error of the minimum (Cantoni *et al.*, 2007). This criterion facilitates the selection of a model that minimizes both the square error and selected variables. We included the treatments as dummy variables. We applied log<sub>10</sub> transformation for both N<sub>2</sub>O emissions and microbial genes (archaeal and bacterial *amoA* genes, fungal and bacterial *nirK*, bacterial *nirS* and *nosZ*, 16S rRNA and 18S rRNA) and standardized soil factor variables. Our analysis was performed in the R environment with the package ‘glmnet’ (Friedman *et al.*, 2010).

## RESULTS

*Weather conditions and soil analysis* - The mean air temperature varied between 13 and 28 °C (Fig. S1 in the Supporting information). The minimum mean air temperature was 19 and 12 °C, and the maximum mean temperature was 32 and 29 °C in RS and DS, respectively. During the 90 days of the experiment, the cumulative rain was approximately 276 mm and 103 mm, whereas the average WFPS on soil sampling days was 77% and 66% in RS and DS, respectively. Both cumulative rain values are lower than the historical values recorded for the region (RS = 561 mm, DS = 121 mm, average of 100 years) (ESALQ, 2016). In DS, plant development was highly affected by the lack of water during the first months after fertilization.

In RS, part of the mineral N applied in the field area was still detectable in mineral form ( $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N) approximately 40 days after mineral N fertilizer application. In DS, the mineral N ( $\text{NH}_4^+$ -N +  $\text{NO}_3^-$ -N) concentration was stable throughout the entire experimental period. The mineral N concentrations in the treatments with ammonium nitrate were approximately 140 and 80 mg N  $\text{kg}^{-1}$  of dry soil in RS and DS, respectively (Fig. S2 in the Supporting information).

*Carbon dioxide emissions* - The emissions of  $\text{CO}_2$ -C from sugarcane were similar in the two seasons, with high emissions immediately after vinasse application (Fig. S3 in the Supporting information). The treatments with CV had higher  $\text{CO}_2$  emission fluxes in the fertilized bands than the treatments with V in both seasons with peaks of 33 and 17 g  $\text{m}^{-2}$   $\text{d}^{-1}$  of C for CV and V, respectively. The cumulative  $\text{CO}_2$ -C emissions were 97 and 126 g  $\text{m}^{-2}$  higher in the treatments with vinasse (CV and V) and mineral N than in the control in RS and DS, respectively (Table 3). The combined application of vinasse (CV or V) plus mineral N did not

further increase the cumulative CO<sub>2</sub>-C emissions; however, in the rainy season, the treatments with CV application emitted more CO<sub>2</sub>-C than the treatments with V, regardless of the timing of the application of mineral N (Table 3). In both seasons, the application of vinasse (CV and V) prior to mineral N reduced the cumulative CO<sub>2</sub>-C emissions by 89 g m<sup>-2</sup> (on average) (Table 3).

*Nitrous oxide emissions* - In both, rainy and dry seasons (RS and DS), the N<sub>2</sub>O emission fluxes of the control treatment were similar, approx. 0.06 mg m<sup>-2</sup> d<sup>-1</sup> of N (Fig. 1C, 1D). In RS, the N<sub>2</sub>O emission fluxes were similar in all treatments (0.61 mg m<sup>-2</sup> d<sup>-1</sup> of N), except the CV+N treatment, in which N<sub>2</sub>O fluxes were much higher (46.49 mg m<sup>-2</sup>d<sup>-1</sup> of N) (Fig. 1A, 1C). In DS, the highest N<sub>2</sub>O emissions were observed in treatments with mineral N. The application of vinasse 30 days before mineral N (V<sub>b</sub>+N and CV<sub>b</sub>+N) decreased the N<sub>2</sub>O emission fluxes compared to the application of vinasse together with mineral N (Fig. 1D, 2D, 3D). In RS, only V was applied prior to N. In both experiments, the maximum N<sub>2</sub>O emission peaks occurred soon after application of mineral N and vinasses (CV and V) and immediately after rain events (Fig. 1 and Fig. S1 in the Supporting information).

In the treatments with mineral N, the cumulative N<sub>2</sub>O-N emissions in the fertilized bands were higher in DS than in RS; the total N emitted was 49 and 89 mg N<sub>2</sub>O-N m<sup>-2</sup> greater than in the control treatment (12 and 6 mg m<sup>-2</sup> of N<sub>2</sub>O-N), respectively, corresponding to 0.08% and 0.14% of total N applied (Fig. 2). The application of vinasse (CV and V), mineral N or the combination of both resulted in higher (+184 mg N m<sup>-2</sup>) cumulative N<sub>2</sub>O emissions than in the control in DS (Table 3). In addition, the application of vinasse plus mineral N (CV<sub>b</sub>+N, CV+N V<sub>b</sub>+N and V+N) resulted in an increase in emissions of nearly 328 and 221 mg N m<sup>-2</sup> compared to treatment with either vinasse alone (CV and V). Comparing the effect of both

vinasses plus mineral N, CV increased N<sub>2</sub>O emissions over V by 875 and 233 mg N m<sup>-2</sup> in RS and DS, respectively (Table 3). However, the application of CV 30 days before N reduced N<sub>2</sub>O-N emissions by 65% compared with those of CV and mineral N applied at the same time. The N<sub>2</sub>O-N emissions represented 0.26 and 0.65% of the total N applied in the CV<sub>b</sub>+N and CV+N treatments, respectively (Table 3, Fig. 2). On the other hand, the application of V 30 days before mineral N did not reduce N<sub>2</sub>O-N emissions in both seasons (Table 3). The cumulative N<sub>2</sub>O emissions in the treatments with V were 54 and 79 mg N m<sup>-2</sup> in V<sub>b</sub>+N and V+N in RS, respectively, and 137 and 241 mg N m<sup>-2</sup> d<sup>-1</sup> in V<sub>b</sub>+N and V+N in DS, respectively (Fig. 2). The N emitted as N<sub>2</sub>O was approximately 0.10 and 0.26% (V<sub>b</sub>+N and V+N in average) of the total N applied in RS and DS, respectively (Fig. 2).

Because the amount of N of CV and mineral N fertilizer were higher in the application bands, the N<sub>2</sub>O emissions of bands area were higher than those of the whole field. However, when the N<sub>2</sub>O emissions were reported in hectare basis, the relative values of N<sub>2</sub>O emissions of the treatments with V tend to be higher than those of CV and mineral N. N<sub>2</sub>O emissions from CV and mineral N occurred only in 16% of the field whereas emissions from V occurred in the whole field (Table S3). However, the interpretation of the treatment effect was similar regardless of emissions expressed per band or per hectare. For instance, the N<sub>2</sub>O-N emission factors, (EF, % of N applied) calculated with original N<sub>2</sub>O emission values expressed per chamber area (mg N m<sup>-2</sup>) or field basis (g N ha<sup>-1</sup>) were similar (Figure 2 and Table S3), the background emissions (control in the inter-row) and the V emissions were relatively small (Table S3). The exception was the EF values of V+N in the dry season DS, which were 0.33% (calculated per chamber area) and 0.71% (calculated on a hectare basis) (Figure 2 and Table S3). This was caused by the N<sub>2</sub>O emissions of V applied without mineral N in August 2015, which were much higher than any other single vinasses (Table S3)

probably caused by the high N concentration of that vinasse lot, equivalent to 89 kg N ha<sup>-1</sup> and the weather conditions. Nevertheless, both EF values were lower than the IPCC default values (1% of the applied N).

*Abundances of nitrogen cycle genes* - The abundances of N cycle genes related to N<sub>2</sub>O emissions are shown in Figure S4, S5 and S6 in the Supporting information for all treatments and sampling time points. The abundance of *amoA* (AOB) followed the pattern of N<sub>2</sub>O emissions (Fig. S4A, S4B, S4C, S4D in the Supporting information). The abundance of *amoA* bacteria (AOB) was higher in the treatments with CV plus mineral N (CV+N) than in the other treatments, regardless of season. During the entire experiment (combination of all time points), the abundance of AOB was correlated significantly with N<sub>2</sub>O emissions in both RS ( $R^2 = 0.17, p \leq 0.05$ ) and DS ( $0.24; p \leq 0.05$ ) (Fig. 3). However, the correlations were not positive at all sampling time points. In RS, on day 22, N<sub>2</sub>O emissions were positively correlated with *amoA*-AOB, with a coefficient of correlation ( $R^2$ ) of 0.46 ( $p \leq 0.01$ ) (Table 4). By contrast, in DS, N<sub>2</sub>O emissions were positively correlated with *amoA*-AOB on days 45 ( $R^2 = 0.50; p \leq 0.01$ ) and 52 ( $R^2 = 0.47; p \leq 0.01$ ) (Table 3). Overall, for RS and DS, a significant correlation between the abundance of AOA *amoA* and N<sub>2</sub>O emissions was detected (RS:  $R^2 = 0.15, p \leq 0.10$ ; DS:  $R^2 = 0.13, p \leq 0.10$ ) (Fig. 3). However, the abundance of AOA *amoA* was higher in RS than in DS, although no significant correlation between AOA *amoA* abundance and N<sub>2</sub>O emission was observed on specific days (Table 4).

The correlations between the abundances of bacterial denitrification genes (*nirK*, *nirS* and *nosZ*) and N<sub>2</sub>O emissions differed between seasons (Fig. 3). For RS overall, N<sub>2</sub>O emissions were correlated significantly with *nirS* ( $R^2 = 0.22, p \leq 0.01$ ) and *nosZ* ( $R^2 = 0.19; p \leq 0.05$ ), whereas for DS overall, *nirK* ( $R^2 = 0.16, p \leq 0.05$ ) and *nirS* ( $R^2 = 0.24, p \leq 0.01$ ) were

positive correlated with N<sub>2</sub>O emissions (Fig. 3). The abundances of the *nirK*, *nirS* and AOA-*amoA* genes increased linearly with time with the increase in water availability (Fig. S1, S4 S5, S6 in the Supporting information).

The abundance of total bacteria (16S rRNA gene) had no defined pattern in either RS or DS, whereas the abundance of total fungi (18S rRNA gene) was significantly and positively correlated with N<sub>2</sub>O emissions in both seasons (RS:  $R^2 = 0.30$ ,  $p \leq 0.01$ ; DS:  $R^2 = 0.37$ ,  $p \leq 0.01$ ) (Fig. 3). Total fungi were most abundant in the treatments with vinasse application (with or without nitrogen) (Fig. S6 in the Supporting information). In RS, the abundance of the fungal *nirK* gene was significantly correlated with N<sub>2</sub>O emissions on day 22 ( $R^2 = 0.36$ ,  $p \leq 0.10$ ), whereas in DS, the abundance of the fungal *nirK* gene was significantly correlated with N<sub>2</sub>O emissions on days 11 ( $R^2 = 0.35$ ,  $p \leq 0.15$ ) and 19 ( $R^2 = 0.47$ ,  $p \leq 0.01$ ) (Table 4).

The positive correlation between N<sub>2</sub>O emissions and N cycle genes indicates that nitrification and denitrification likely occurred during the entire experimental period in both seasons. To assess the main microbially driven processes related to N<sub>2</sub>O emissions, the ratios between gene abundances and their correlation with N<sub>2</sub>O emissions were calculated (Table 4). In both seasons, nitrification by *amoA*-AOB appeared to be the dominant process related to N<sub>2</sub>O emissions due to the negative correlation between N<sub>2</sub>O emissions and the ratio of denitrifier to nitrifier genes (RS:  $(nirK+nirS)/(AOB+AOA)$ ,  $R^2 = -0.26$ ,  $p \leq 0.01$ ;  $(nirK+nirS)/amoA$ -AOB,  $R^2 = -0.22$ ,  $p \leq 0.01$ ; and *nirK*-Fungi/*amoA*-AOB,  $R^2 = -0.17$ ,  $p \leq 0.05$ ; similar results were obtained for DS) (Table 5). The general linear model also provided evidence of the predominance of nitrification (Table 6A); N<sub>2</sub>O emissions were dependent on the abundance of *amoA*-AOB in both seasons when N cycle genes, 16S rRNA and 18S rRNA were taken into account (Table 6).

To evaluate the relative influences of functional genes, treatments, and climatic factors on N<sub>2</sub>O emissions, we fit the general linear model to both seasons. The models were consistent with the Spearman's correlation results. Both analyses identified relationships of N<sub>2</sub>O emissions with the abundance of nitrogen-cycle genes and environmental variables, as shown in Tables 4 and 6. However, in both seasons, WFPS was the most important factor controlling N<sub>2</sub>O emissions. In RS, N<sub>2</sub>O emissions increased with soil moisture, soil temperature, mineral N (NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N), *nosZ* and total bacteria, whereas in DS, N<sub>2</sub>O emissions increased with soil moisture, air temperature, mineral N (NO<sub>3</sub><sup>-</sup>-N), *amoA* (AOB) and *nosZ*. Application of vinasse (CV and V) plus mineral N increased N<sub>2</sub>O emissions in both seasons (Table 6B).

## DISCUSSION

The application of vinasse (CV and V) 30 days prior to mineral N fertilizer reduced the cumulative N<sub>2</sub>O emissions from straw-covered sugarcane fields by 65% and 37% compared to the application of vinasse and mineral N simultaneously in the first three months after vinasse application. The interval of 30 days between the application of vinasse and N fertilizer appears to be sufficient to ameliorate the anaerobic conditions induced by vinasse application and thereby decrease heterotrophic denitrification by nitrifiers and denitrifiers. In addition, since vinasse is a source of carbon and N, this 30-day period permits vinasse-carbon decomposition and vinasse-N mineralization and/or N uptake by plants (Parnaudeau *et al.*, 2008, Silva *et al.*, 2013), which may lead to a low N<sub>2</sub>O emission rate. The N<sub>2</sub>O emissions from the treatments with vinasse (CV and V) plus N were similar to or higher than those of the single mineral N treatment, regardless of the timing of application. Surprisingly, N<sub>2</sub>O emissions were higher in the dry season than in the rainy season. Denitrification conditions are expected to occur for a longer period in the rainy season than in the dry season, leading to high N<sub>2</sub>O emissions. However, the phenology of the sugarcane plant may provide insights on

the lower N<sub>2</sub>O emissions in all treatments in the rainy season. Sugarcane is a fast-growing plant, with high N demand during the initial stages of ratoon growth (Franco *et al.*, 2011, Mariano *et al.*, 2016), and can accumulate 30 to 60 t ha<sup>-1</sup> of dry matter in a single season (Cantarella *et al.*, 2012, CONAB, 2017). If N is applied during the growing stage of the plant, the rapid uptake of nutrients, including N, will reduce the available N for microbial-related processes of N<sub>2</sub>O emission. In the dry season, N<sub>2</sub>O emissions were nearly 2-fold higher compared to the rainy season. In the rainy season, fertilizers were applied at the beginning of summer, when the plants were 1.5 m high; by contrast, in the dry season, N was applied at the beginning of winter, when the plants were starting to sprout. Therefore, at the beginning of the dry season, plants were not able to take up as much N, which allowed the applied N to remain longer in the soil to support microbial reactions leading to N<sub>2</sub>O emission.

The variation of N<sub>2</sub>O emissions in the treatments with either type of vinasse (CV or V) and mineral N can be explained by the complex combination of available C and N present in the vinasse and environmental factors (pH, organic matter, porosity, temperature, moisture) (Halvorson *et al.*, 2014, Liang *et al.*, 2015, Subbarao *et al.*, 2006, Vargas *et al.*, 2014). The large variation of environmental conditions in the present study likely caused rapid changes in the nitrifier and denitrifier community abundances.

Nitrification by AOB during vinasse application occurred in both non-mineral N-fertilized and mineral N-fertilized sugarcane fields in both seasons. These results show that the application of ammonium nitrate-based fertilizer and/or different vinasses induced and enhanced the number of copies of the bacterial *amoA* gene, which is related to a strong nitrification process. In tropical soils with high drainage capacity, such as the soil in our experiment, nitrification is the main process by which N<sub>2</sub>O is emitted to the atmosphere

(Soares *et al.*, 2016). Many studies have shown that N<sub>2</sub>O emissions are significantly and positively correlated with ammonia oxidation by AOB under controlled conditions (Law *et al.*, 2012, Regina *et al.*, 1996). AOA also played a role in N<sub>2</sub>O emissions from soil amended with vinasse (CV and V) and mineral N. In both the rainy and dry seasons, the abundance of AOA was related to N<sub>2</sub>O emissions. The soil conditions at our sites were acidic (pH 5.1), and *amoA*-AOA gene abundance usually increases with decreasing pH (Nicol *et al.*, 2008, Zhang *et al.*, 2012). Although ammonia oxidation by AOA was also responsible for the N<sub>2</sub>O emissions, the *amoA*-AOB/*amoA*-AOA ratio and regression analysis of our results showed that *amoA*-AOB was the most important gene related to N<sub>2</sub>O emissions, thus indicating that nitrification by AOB dominates the nitrification process and N<sub>2</sub>O emissions in sugarcane fields. It has been reported that AOAs, although present in soils, do not respond to NH<sub>4</sub><sup>+</sup>-N fertilization or N<sub>2</sub>O production in intensively managed agricultural soils, in contrast to AOB (Di *et al.*, 2009, Hink *et al.*, 2016, Yang *et al.*, 2017). Independent of soil pH, the concentration of NH<sub>4</sub><sup>+</sup>-N is a key factor determining the niche separation of AOA and AOB (Zhang *et al.*, 2012). In the same region as our study, Soares *et al.* (2016) observed that nitrification by AOB, rather than AOA or denitrification, was the main process responsible for N<sub>2</sub>O emissions, but neither vinasse nor sugarcane straw was applied in that study

In addition to the considerable importance of N<sub>2</sub>O emissions during ammonia oxidation by AOB, the consumption of O<sub>2</sub> by heterotrophic microorganisms triggers denitrification, as indicated by the increases in the abundance of *nirS* and *nirK*. These results suggest that AOB will actively grow under high NH<sub>4</sub><sup>+</sup>-N concentrations, leading to microoxic or anoxic conditions, which in turn will induce denitrification by heterotrophic denitrifiers or by nitrifiers, resulting in high N<sub>2</sub>O emission fluxes. The significant correlation between *nosZ* and N<sub>2</sub>O indicates that complete denitrification is also occurring in the soil, *nosZ* is the key

enzyme involved in the N<sub>2</sub>O reduction to N<sub>2</sub> (Orellana *et al.*, 2014, Samad *et al.*, 2016). This cascade is further reinforced by N fertilization, especially when N is applied with a rich carbon source, such as vinasse (Di *et al.*, 2014). Previous studies of sugarcane fields have shown that high N<sub>2</sub>O fluxes occur immediately after N fertilization (Carmo *et al.*, 2013, Navarrete *et al.*, 2015, Pitombo *et al.*, 2015, Soares *et al.*, 2015, Soares *et al.*, 2016). However, denitrification appears to be less important than AOB for N<sub>2</sub>O emissions under our experimental field conditions.

N<sub>2</sub>O emissions and the total fungal abundance showed significant positive correlations over time, suggesting a contribution of fungal denitrifiers to N<sub>2</sub>O emissions. This relationship was further confirmed by the significant positive correlation between fungal *nirK* and N<sub>2</sub>O emissions in both seasons on different days. A role of fungi in N<sub>2</sub>O emissions has recently been reported (Mothapo *et al.*, 2015, Shoun *et al.*, 2012, Wu *et al.*, 2017), albeit in crop and management systems other than sugarcane. In contrast to bacteria, fungi do not have genes encoding nitrous oxide reductase (*nosZ*), which reduces N<sub>2</sub>O to N<sub>2</sub>, and thus fungal denitrification terminates at N<sub>2</sub>O (Phillips *et al.*, 2016, Shoun *et al.*, 2012). Therefore, an increase in fungal denitrification might increase N<sub>2</sub>O emissions. The high amount of sugarcane straw, which has a high C:N ratio (77:1), present in our experiments might trigger an increase in fungal biomass (Allison & Killham, 1988). Consistent with this expectation, Wu *et al.* (2017) determined that N<sub>2</sub>O emissions in soil with wheat straw were initially predominantly derived from bacterial denitrification but later mainly resulted from fungal denitrification. Similar to 18S rRNA, the abundance of the fungal nitrite reductase gene (*nirK*) increased significantly with N<sub>2</sub>O emissions after swine manure application (Xu *et al.*, 2017).

No study has investigated the abundance of nitrifier and denitrifier genes and their links to N<sub>2</sub>O emissions in soil amended with vinasse and mineral N for the cultivation of sugarcane crops with straw, a common agricultural practice for sugarcane cultivation in Brazil in the past ten years. Our results suggest that nitrification and denitrification by nitrifiers and denitrifiers occur simultaneously in the soil. The mineral N source, ammonium nitrate, resulted in N<sub>2</sub>O emissions by NH<sub>4</sub><sup>+</sup>-N oxidation or nitrification-denitrification as well as by NO<sub>3</sub><sup>-</sup>-N reduction by heterotrophic denitrification. The significant positive correlations between N<sub>2</sub>O emissions and the abundances of the bacterial *nirK*, *nirS* and *nosZ* genes show that the production of N<sub>2</sub>O is due to favorable conditions for denitrification. Rain events and vinasse fertirrigation induce low oxygen concentrations in soil microsites (Di *et al.*, 2014), consistent with the significant correlation between N<sub>2</sub>O emissions and WFPS. In addition, vinasse is an organic residue rich in carbon with high biological oxygen demand (Fuess & Garcia, 2014). The input of labile organic compounds from vinasse in soils might have two effects: (1) greatly increased soil microbial activities, resulting in intense oxygen consumption (Renault *et al.*, 2009); (2) the creation of microoxic or anoxic conditions, resulting in anaerobic microsites (Torbert & Wood, 1992). Therefore, after vinasse application, anaerobic conditions may prevail for a short time due to the large organic C load and soil moisture, promoting reducing conditions in the soil. In this way, anaerobic processes may cause N<sub>2</sub>O emissions. However, this situation may be transient since drying of the soil within a few hours or days will favor N<sub>2</sub>O emissions by nitrification. By contrast, fungal denitrifiers can release N<sub>2</sub>O under both aerobic and anaerobic conditions (Shoun *et al.*, 2012, Zhou *et al.*, 2002). Other, less-characterized processes may also be involved in N<sub>2</sub>O emissions, such as nitrifier denitrification in suboxic conditions and NO<sub>2</sub><sup>-</sup> accumulation (Kool *et al.*, 2011), aerobic denitrification, and co-denitrification (Joo *et al.*, 2005, Spott *et al.*, 2011, Zhao *et al.*, 2012). However, nitrification by AOB and denitrification by fungi were the

prevalent processes leading to high N<sub>2</sub>O emissions in both experiments and therefore could be useful indicators for mineral N management strategies to mitigate N<sub>2</sub>O emissions in tropical soils with organic residue application.

Understanding the prevalent microbial processes related to N<sub>2</sub>O in sugarcane fields is a considerable challenge, given the myriad of conditions that may occur simultaneously. In this study, we investigated the microbial processes involved in N<sub>2</sub>O emissions in a complex field soil ecosystem where different bioenergy residues, i.e., types of vinasse and straw, were applied to soil in combination with N fertilizer in two different seasons. Independent of season, different contributions of nitrification by bacteria, nitrification by archaea and denitrification by bacteria and fungi were observed, dependent on soil moisture, soil pH and nitrogen source. A practical finding is that the strategy of prior vinasse application 30 days before mineral N reduced N<sub>2</sub>O emissions by 65% and 37% for concentrated and non-concentrated vinasse, respectively.

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## AUTHOR CONTRIBUTIONS

K.S.L., J.B.C., E.E.K. and H.C. designed the research; K.S.L. and J.R.S. conducted the experiments; K.S.L., M.R.D. and A.P. conducted the qPCR analyses; K.S.L. performed the statistical analyses; K.S.L. wrote the paper. E.E.K., H.C. and J.A.V critically reviewed the manuscript. All authors reviewed the manuscript.

## ADDITIONAL INFORMATION

The authors declare no conflict of interest.

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## Figures

**Figure 1.** Daily mean fluxes of N<sub>2</sub>O with (a, b) or without (c, d) nitrogen in sugarcane ratoon in different treatments in the rainy (a, c) and dry (b, d) seasons. The treatments are as follows: Control; N: mineral N fertilizer, ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N;  $\nu$ : vinasse application (30 days before N fertilization). Vertical bars indicate the standard error of the mean (n = 3).

**Figure 2.** Cumulative fluxes (90 days) of N<sub>2</sub>O (mg N m<sup>-2</sup>) and N fertilizer emission factor (EF, %, values above bars) based on rates of N fertilizer application. Soil N<sub>2</sub>O fluxes in (a) rainy and (b) dry seasons. The treatments are as follows: Control; N: mineral N fertilizer (ammonium nitrate); CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N;  $\nu$ : vinasse application 30 days before N fertilization. Vertical bars indicate the standard error of the mean (n = 3).

**Figure 3.** Spearman's correlation coefficients (neglecting sampling time) between N<sub>2</sub>O emission fluxes (mg m<sup>-2</sup> d<sup>-1</sup>) and abundance of *amoA* (archaeal and bacterial), *nirK* (fungal and bacterial), *nirS* and *nosZ* (bacterial), total bacterial 16S rRNA and total fungal 18S rRNA (gene copies g<sup>-1</sup> dry soil) and abiotic factors, mineral nitrogen, air and soil temperatures and CO<sub>2</sub>-C emissions in the (a) rainy and (b) dry seasons. Abbreviations: WFPS: water-filled pore space; AOB: *amoA* belonging to ammonia-oxidizing bacteria; AOA: *amoA* belonging to ammonia-oxidizing archaea. Black bold lines indicate significant correlations; red bold lines indicate significant negative correlations; and dotted lines indicate no correlation between variables (n=144 and 180 for the rainy and dry seasons, respectively). Significant difference:  $p \leq 0.15$ , \*  $p \leq 0.10$ , \*\*  $p \leq 0.05$  and \*\*\*  $p \leq 0.01$ .

**Table 1.** Physicochemical properties of soil layer (0- to 20-cm) in rainy (RS) and dry (DS) seasons (n=4).

Season	pH (CaCl <sub>2</sub> ; 0.0125 mol L <sup>-1</sup> )	Bulk density	OM <sup>†</sup>	P <sup>‡</sup>	K	Ca	Mg	H+Al <sup>§</sup>	CEC <sup>¶</sup>	Soil texture <sup>#</sup>		
										Clay	Silt	Sand
		g cm <sup>-3</sup>	g dm <sup>-3</sup>	mg dm <sup>-3</sup>	-----	-----	mmol <sub>c</sub> dm <sup>-3</sup>	-----	-----	g kg <sup>-1</sup>		
RS	5.3	1.45	23.5	10.5	0.55	45.5	20.5	31.5	98.5	619	145	236
DS	5.0	1.49	21.1	14.6	0.7	17.4	11.9	34.9	65.1	631	151	218

<sup>†</sup> Organic matter.

<sup>‡</sup> Available phosphorus, K, Ca, and Mg were extracted with ion exchange resin.

<sup>§</sup> Buffer solution (Calcium acetate 0,5 M, pH 7.0).

<sup>¶</sup> Cation exchange capacity.

<sup>#</sup> Soil texture determined by the densimeter method.

**Table 2.** Time of application and corresponding nitrogen rates of mineral fertilizer (N: ammonium nitrate) and vinasse (V: non-concentrated vinasse and CV: concentrated vinasse) to sugarcane ratoon. The numbers in parentheses indicate the amount of N in kg ha<sup>-1</sup> contained in vinasse. N fertilizer applied at 100 kg ha<sup>-1</sup> N.

Treatments <sup>†</sup>	Rainy season (2013/2014 cycle)		Dry season (2014/2015 cycle)	
	November 2013	December 2013	July 2015	August 2015
Control	-	-	-	-
N	-	N (100) <sup>‡</sup>	-	N (100)
V <sub>b</sub>	V <sub>b</sub> (53)	-	V <sub>b</sub> (51)	-
CV <sub>b</sub>	-	-	CV <sub>b</sub> (30)	-
V <sub>b</sub> +N	V <sub>b</sub> (53)	N (100 kg N)	V <sub>b</sub> (51)	N (100)
CV <sub>b</sub> +N	-	-	CV <sub>b</sub> (30)	N (100)
V	-	V (53)	-	V (89)
CV	-	CV (48)	-	CV+N (52)
V+N	-	V+N (53+100)	-	V+N (89+100)
CV+N	-	CV+N (48+100)	-	CV+N (52+100)

<sup>†</sup> b: Vinasse application (V and CV) 30 days *before* mineral N fertilization.

<sup>‡</sup> Rates of N for vinasses and mineral N fertilizer are represented in a hectare basis; however, when band-applied (N fertilizer and CV) the amount of N in the band (and in the GHG chambers) are much higher because the input are added in just 16% of the area.

**Table 3.** Statistical analysis using orthogonal contrasts for selected treatments. The mean values represent the difference between the amounts of N<sub>2</sub>O and CO<sub>2</sub> emissions defined by the orthogonal contrast parameters.

Selected contrasts <sup>†</sup>	Contrast calculation <sup>‡</sup>	Mean of the parameters measured	CO <sub>2</sub> (g C m <sup>-2</sup> ) <sup>§</sup>		N <sub>2</sub> O (mg N m <sup>-2</sup> )	
			Rainy season	Dry season	Rainy season	Dry season
1	N effect (vinasse-N or N)	(All treatments) – control	97**	126***	173 <sup>ns</sup>	184*
2	N plus vinasse effect	(All vinasses +N) – (all vinasses)	29 <sup>ns</sup>	11 <sup>ns</sup>	328***	221***
3	Type of vinasse	CV – V	142***	12 <sup>ns</sup>	59 <sup>ns</sup>	36*
4	V: Anticipating vs ST	V <sub>b</sub> - V	-102**	-89*	-13 <sup>ns</sup>	-91 <sup>ns</sup>
5	CV: Anticipating vs ST	CV <sub>b</sub> - CV	-	-104**	-	-57 <sup>ns</sup>
6	Type of vinasse + N	(CV+N) - (V+N)	216***	23 <sup>ns</sup>	875***	233**
7	V+N: Anticipating vs ST	(V <sub>b</sub> +N) - (V+N)	-34 <sup>ns</sup>	-143***	-25 <sup>ns</sup>	-103 <sup>ns</sup>
8	CV+N: Anticipating vs ST	(CV <sub>b</sub> +N) - (CV+N)	-	-89*	-	-407***

<sup>†</sup> Contrasts 1 and 2 compare the overall effect of N on N<sub>2</sub>O emissions; contrasts 3 through 8 compare the effects of type of vinasse with and without N fertilizer; contrasts within each group are orthogonal.

<sup>‡</sup> N: mineral N fertilizer, ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N; <sub>b</sub>: Vinasse application 30 days before N fertilization; ST: application at the same time.

<sup>§</sup> Net effect on emissions for the indicated contrast. Significant difference: \* $p \leq 0.10$ ; \*\* $p \leq 0.05$ ; \*\*\* $p \leq 0.01$ ; ns: non-significant.

**Table 4.** Spearman's correlation coefficients between N<sub>2</sub>O emission flux (mg m<sup>-2</sup> d<sup>-1</sup>) and abundance of archaeal and bacterial *amoA*, fungal and bacterial *nirK*, bacterial *nirS* and *nosZ*, and total bacterial 16S rRNA and total fungal 18S rRNA (gene copy g<sup>-1</sup> dry soil) in the rainy and dry seasons.

	Rainy season					
	Day 1 (n=24)	Day 3 (n=24)	Day 7 (n=24)	Day 22 (n=24)	Day 24 (n=24)	Day 54 (n=24)
WFPS <sup>†</sup>	<b>0.60</b> <sup>***</sup>	0.07	-0.03	-0.31	-0.03	0.23
NH <sub>4</sub> <sup>+</sup> -N	<b>0.80</b> <sup>***</sup>	0.33	0.25	<b>0.83</b> <sup>***</sup>	<b>0.38</b> <sup>**</sup>	-0.13
NO <sub>3</sub> <sup>-</sup> -N	<b>0.68</b> <sup>***</sup>	<b>0.47</b> <sup>**</sup>	0.22	<b>0.57</b> <sup>***</sup>	<b>0.59</b> <sup>***</sup>	0.39
pH	-0.31	-0.04	0.09	-0.02	-0.14	0.01
<i>amoA</i> _AOB	<b>-0.30</b> <sup>†</sup>	-0.13	-0.24	<b>0.46</b> <sup>***</sup>	0.28	0.16
<i>amoA</i> _AOA	0.08	-0.26	0.05	0.08	-0.15	0.08
<i>nirK</i>	0.01	-0.13	0.07	0.05	-0.08	-0.24
<i>nirS</i>	-0.05	0.00	0.06	0.17	-0.09	0.11
<i>nosZ</i>	-0.00	-0.10	-0.03	<b>0.43</b> <sup>**</sup>	<b>0.42</b> <sup>**</sup>	0.18
<i>nirK-Fungi</i>	-0.22	-0.24	0.10	<b>0.36</b> <sup>*</sup>	0.25	0.22
16S rRNA	0.13	-0.05	0.01	0.14	0.31	0.15
18 rRNA	0.03	0.04	0.17	<b>0.53</b> <sup>***</sup>	<b>0.35</b> <sup>***</sup>	0.23
	Dry season					
	Day -30 (n=30)	Day 1 (n=30)	Day 11 (n=30)	Day 19 (n=30)	Day 45 (n=30)	Day 52 (n=30)
WFPS	0.04	<b>0.77</b> <sup>***</sup>	<b>0.45</b> <sup>***</sup>	-0.13	0.16	0.06
NH <sub>4</sub> <sup>+</sup> -N	-0.17	0.17	0.06	0.28	<b>0.42</b> <sup>**</sup>	<b>0.45</b> <sup>***</sup>
NO <sub>3</sub> <sup>-</sup> -N	-0.09	0.05	<b>0.41</b> <sup>**</sup>	<b>0.33</b> <sup>**</sup>	<b>0.66</b> <sup>***</sup>	<b>0.60</b> <sup>***</sup>
pH	-0.09	-0.22	-0.07	0.11	-0.06	-0.23
<i>amoA</i> _AOB	-0.07	0.09	0.26	0.10	<b>0.50</b> <sup>***</sup>	<b>0.47</b> <sup>***</sup>
<i>amoA</i> _AOA	0.17	-0.16	-0.25	0.00	-0.17	0.00
<i>nirK</i>	0.28	-0.36	0.02	0.10	0.09	0.26
<i>nirS</i>	0.29	-0.13	-0.14	0.19	-0.04	0.01
<i>nosZ</i>	0.15	-0.18	-0.12	0.30	0.10	0.33
<i>nirK-Fungi</i>	-0.15	0.09	<b>0.35</b> <sup>*</sup>	<b>0.47</b> <sup>***</sup>	-0.12	-0.09
16S rRNA	<b>0.40</b> <sup>**</sup>	-0.22	0.15	<b>0.17</b> <sup>*</sup>	<b>0.06</b> <sup>†</sup>	-0.05
18 rRNA	0.16	-0.09	<b>0.47</b> <sup>***</sup>	<b>0.73</b> <sup>***</sup>	<b>0.32</b> <sup>*</sup>	<b>0.48</b> <sup>***</sup>

<sup>†</sup> Abbreviations: WFPS: water-filled pore space; AOB: *amoA* belonging to ammonia-oxidizing bacteria; AOA: *amoA* belonging to ammonia-oxidizing archaea. Significant difference: <sup>†</sup> $p \leq 0.15$ , <sup>\*</sup> $p \leq 0.10$ , <sup>\*\*</sup> $p \leq 0.05$  and <sup>\*\*\*</sup> $p \leq 0.01$ .

**Table 5.** Spearman's correlation coefficients between N<sub>2</sub>O emission flux (mg m<sup>-2</sup> d<sup>-1</sup>) and the ratios of the abundances of nitrifier (archaeal and bacterial *amoA*) and denitrifier (fungal and bacterial *nirK*, bacterial *nirS* and *nosZ*, total bacterial *16S rRNA* and total fungal *18S rRNA*) genes in the rainy (RS) and dry seasons (DS).

	N <sub>2</sub> O-N emission		
	Rainy season (n=144)	Dry season (n=180)	
<b>Spearman Correlation<sup>†</sup></b>			
<b>(<i>nirK+nirS</i>)/(AOB +AOA)</b>	<b>-0.26***</b>	-0.08	RS: ↑(AOB +AOA) ↓Ratio↑N <sub>2</sub> O (Nitrification) DS: ns
<b>(<i>nirK+nirS</i>)/<i>amoA</i>-AOB</b>	<b>-0.22***</b>	<b>-0.18**</b>	RS: ↑AOB ↓Ratio↑N <sub>2</sub> O (Nitrification) DS: ↑AOB ↓Ratio↑N <sub>2</sub> O (Nitrification)
<b>(<i>nirK+nirS</i>)/<i>amoA</i>-AOA</b>	0.00	<b>-0.28***</b>	RS: ns DS: ↑AOA ↓Ratio ↑N <sub>2</sub> O (Nitrification)
<b><i>amoA</i>-AOB/<i>amoA</i>-AOA</b>	0.08	<b>0.22***</b>	RS: ns DS: ↑AOA ↓Ratio ↓N <sub>2</sub> O (Nitrification by <i>amoA</i> -AOB)
<b><i>nirK</i>-Fungi/<i>amoA</i>-AOB</b>	<b>-0.17**</b>	<b>-0.23***</b>	RS: ↑ AOB ↓Ratio↑N <sub>2</sub> O (Nitrification by <i>amoA</i> -AOB more important than denitrification by fungi) DS: ↑ AOB ↓Ratio↑N <sub>2</sub> O (Nitrification by <i>amoA</i> -AOB more important than denitrification by fungi)
<b>(<i>nirK+nirS</i>)/<i>nosZ</i></b>	<b>-0.26***</b>	<b>0.19***</b>	RS: ↑ <i>nosZ</i> ↓Ratio↑N <sub>2</sub> O (????_other process is occurring) DS: ↑ <i>nosZ</i> ↓Ratio ↓N <sub>2</sub> O (Complete denitrification as well)

<sup>†</sup> AOB: *amoA* belonging to ammonia-oxidizing bacteria; AOA: *amoA* belonging to ammonia-oxidizing archaea. Significant difference: \**p* ≤ 0.10; \*\**p* ≤ 0.05; \*\*\**p* ≤ 0.01; ns: Non-significant.

**Table 6.** (A) Standardized coefficients of regression analysis with the lasso penalty for the influence of gene abundance on N<sub>2</sub>O emissions. (B) Standardized coefficients of regression analysis with the lasso penalty for the influence of gene abundance on N<sub>2</sub>O emissions with soil factors, days and treatments included as dummy variables.

(A)	Dependent variable	Intercept	<i>amoA</i> - AOB <sup>†</sup>	<i>amoA</i> - AOA	<i>nirK</i>	<i>nirS</i>	<i>nosZ</i>	<i>nirK</i> - Fungi	16S rRNA	18S rRNA	r <sup>2</sup>
RS	N <sub>2</sub> O	-0.831	0.011	_	-0.319	0.142	0.051	_	0.126	0.228	0.230
DS	N <sub>2</sub> O	-0.005	0.158	_	_	0.097	-0.036	_	_	_	0.107

(B)	Dependent variable	Intercept	Treatments <sup>‡</sup>	Day	WFPS	air Tem.	Soil Tem.	NH <sub>4</sub>	NO <sub>3</sub>	pH	<i>amoA</i> AOB	<i>amoA</i> AOA	<i>nirK</i>	<i>nirS</i>	<i>nosZ</i>	<i>nirK</i> - Fungi	16S rRNA	18S rRNA	r <sup>2</sup>			
RS	N <sub>2</sub> O	-0.82	CV+N	0.59	_	0.09	_	0.01	0.02	0.03	-0.02	_	_	_	_	0.03	_	0.08	_	0.58		
			V	-0.04																		
			V+N	0.10																		
			Vb	-0.03																		
DS	N <sub>2</sub> O	-0.186	CV	0.289	-0.13	0.60	0.02	_	-0.04	0.12	-0.05	0.05	-0.05	_	-0.04	0.05	_	_	_	0.57		
			CV+N	0.576																		
			CVb+N	0.369																		
			V+N	0.382																		
			Vb	-0.052																		
			Vb+N	0.21																		

<sup>†</sup> Abbreviations: WFPS: Water-filled pore space; air Tem.: Air temperature; soil Tem.: soil temperature; AOB: *amoA* belonging to ammonia-oxidizing bacteria; AOA: *amoA* belonging to ammonia-oxidizing archaea; Fungi: *nirK* belonging to denitrifier fungi.

<sup>‡</sup> N: mineral N fertilizer, ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N. <sub>b</sub>: Vinasse application 30 days before N fertilization.

## Figures

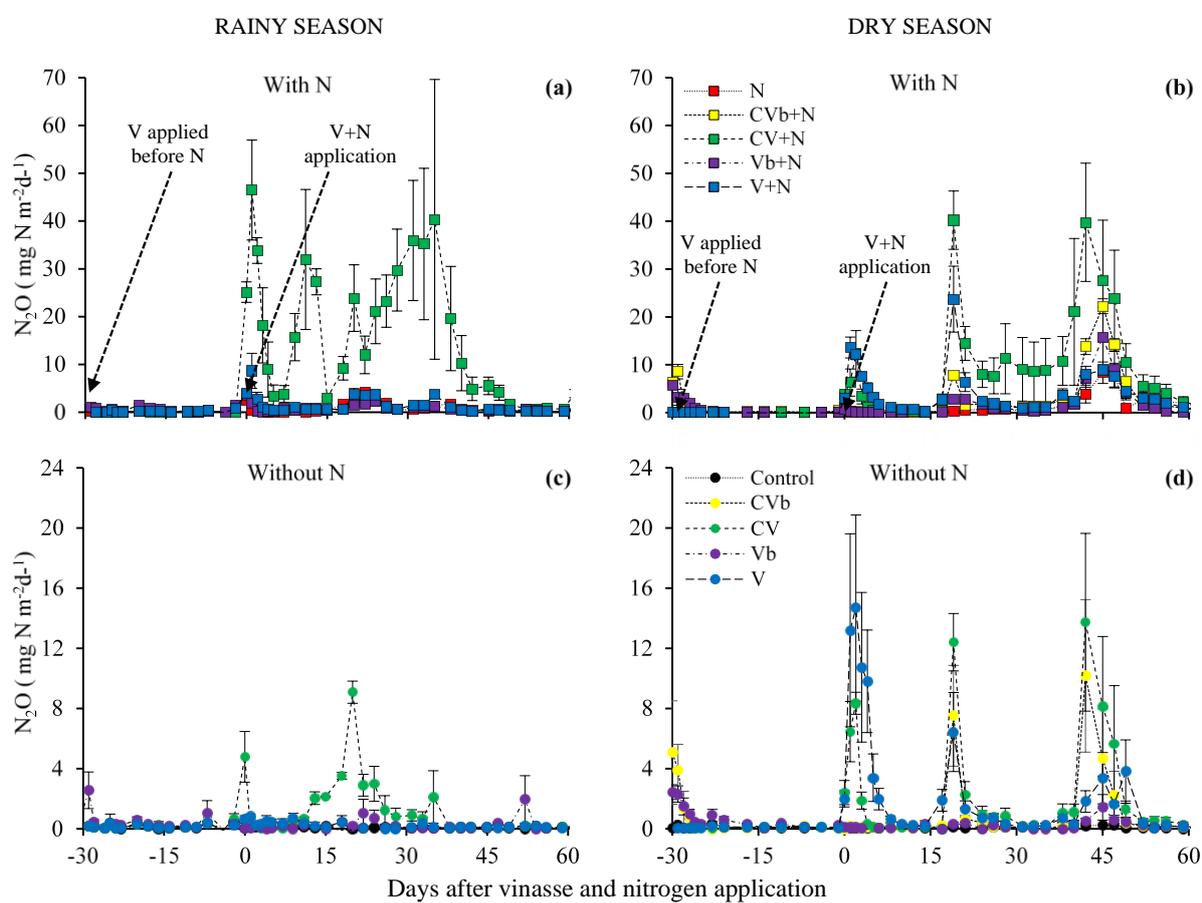


Figure 1

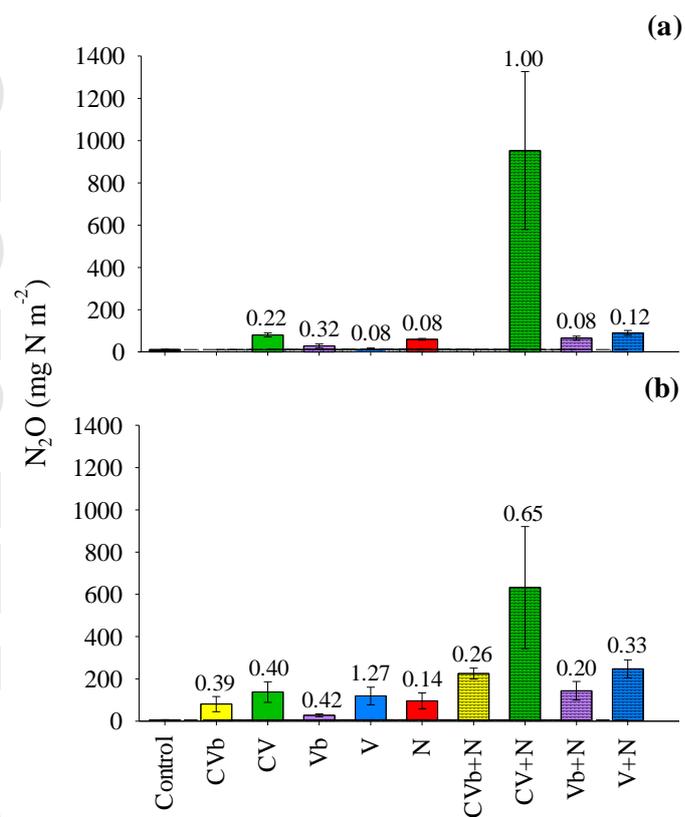
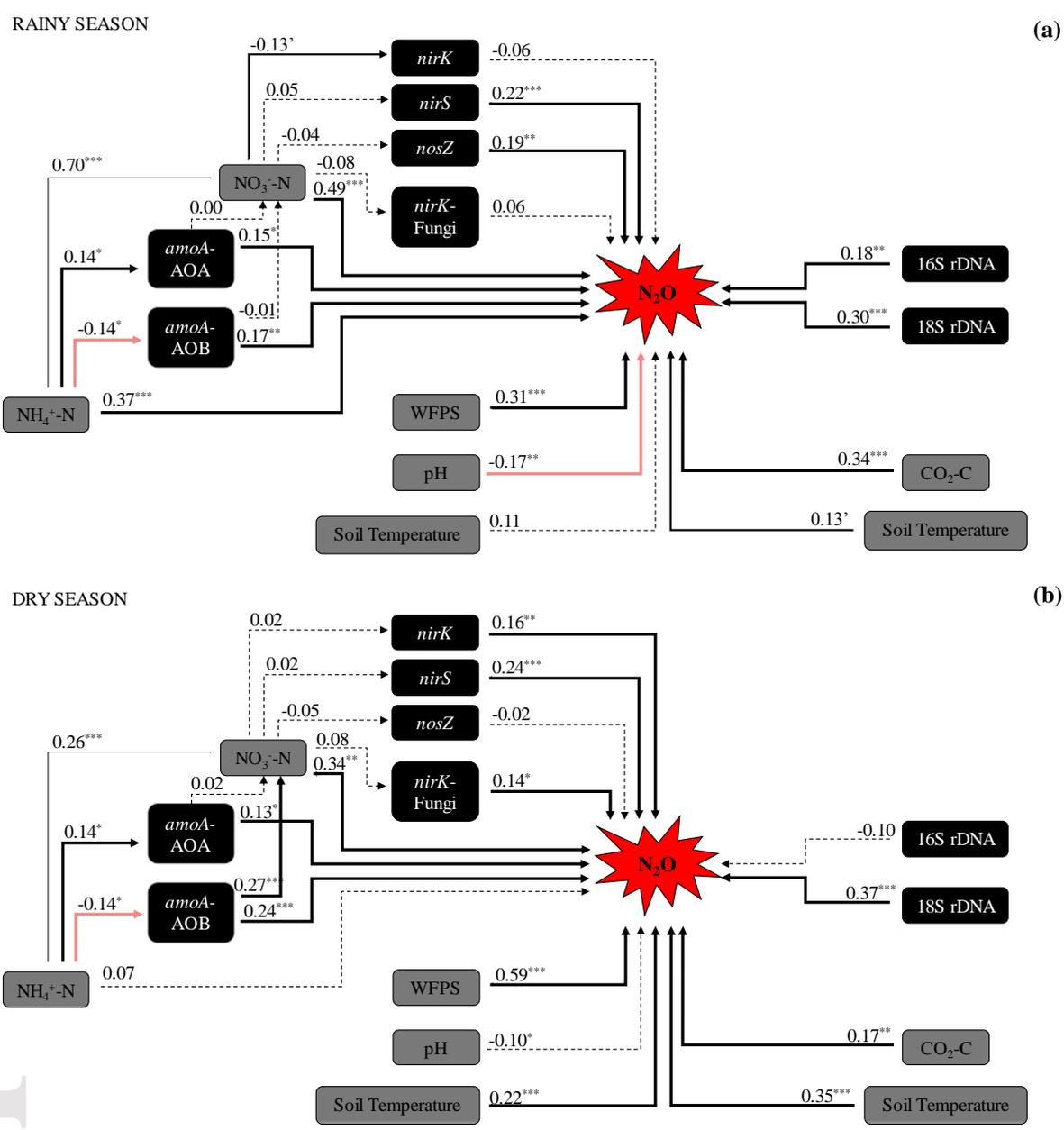


Figure 2



**Figure 3**