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

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Funding information
FAPESP, Grant/Award Number: 2014/24141-5, 2013/12716-0; NWO, Grant/Award Number: 729.004.003, 2013/50365-5; CNPq, Grant/Award Number: 311.197/2013-2

Abstract
Organic compounds and mineral nitrogen (N) usually increase nitrous oxide (N2O) 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 N2O emissions when applied with N in sugarcane plantations, a common practice. However, the biological processes involved in N2O emissions under this management practice are unknown. This study investigated the roles of nitrification and denitrification in N2O emissions from straw-covered soils amended with different vinasses (CV: concentrated and V: nonconcentrated) before or at the same time as mineral fertilizers at different time points in the sugarcane cycle in two seasons. N2O emissions were evaluated for 90 days, the period that occurs most of the N2O emission from fertilizers; the microbial genes encoding enzymes involved in N2O 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 N2O emissions than the application of N fertilizer alone. The strategy of vinasse application 30 days before mineral N reduced N2O 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 N2O emissions, which indicates that nitrification by AOB is the main microbial-driven process linked to N2O emissions in tropical soil. Interestingly, fungal nirK was also significantly correlated with N2O emissions, suggesting that denitrification by fungi contributes to N2O emission in soils receiving straw and vinasse application.

KEYWORDS
denitrification, dry-wet season, greenhouse gas, microbial ecology, nitrification, quantitative real-time PCR, residue, straw, sugarcane, vinasse
1 INTRODUCTION

Vinasse is the major residue generated during ethanol production from sugarcane. For each liter of ethanol produced, approximately 10–15 L of vinasse are generated (Christofoletti, Escher, Correia, Marinho, & Fontanetti, 2013). Brazil grows 9 Mt ha of sugarcane, and approximately half of it is used for ethanol production (CONAB, 2017). A dark-brown wastewater with high organic and nutrient contents (Christofoletti et al., 2013; Elia-Neto & Nakahodo, 1995; Fuess & Garcia, 2014; Macedo, Seabra, & Silva, 2008), vinasse is widely applied on sugarcane fields as fertilizer, mainly in the ratatou 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 contents (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, Rossetto, & Mutton, 2014; Parnauede, Condon, Oliver, Cazeveille, & Recous, 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 N₂O (Carmo et al., 2013; Pitombo et al., 2015). N₂O is a nitrogen (N) cycle product with major environmental and ecological impacts. N₂O is both an ozone-depleting substance (Ravishankara, Daniel, & Portmann, 2009) and a greenhouse gas with global warming potential 298 times greater than that of carbon dioxide (CO₂) (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, N₂O emissions were lower than when vinasse and N fertilizer were applied simultaneously (Paredes et al., 2014, 2015). However, there is little information about N₂O 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 N₂O when concentrated vinasse was used.

N₂O is produced and consumed by biotic and abiotic soil processes. The abiotic process, chemo denitrification, occurs through chemical decomposition of hydroxylamine (NH₂OH), nitroxyl hydride (HNO), or NO₂⁻ in the presence of organic and inorganic compounds at low pH (<4.5). By contrast, the biotic process requires autotrophic and heterotrophic microorganisms, that is, bacteria, archaea, and fungi (Hayatsu, Tago, & Saito, 2008; Higgins et al., 2016; Hink, Nicol, & Prosser, 2016). N₂O is produced in soil via nitrification and denitrification processes (Martins, Nazaries, Macdonald, Anderson, & Singh, 2015; Németh, Wagner-Riddle, & Dunfield, 2014; Soares et al., 2016; Stevens & Laughlin, 1998; Xu et al., 2017). In the oxic, well-drained soils typical of most agricultural soils, N₂O is mainly produced by ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Baggs, Smales, & Bateman, 2010; Bateman & Baggs, 2005; Bollmann & Conrad, 1998; Hink et al., 2016). However, under suboxic or anoxic conditions, facultative denitrifiers (Di, Cameron, Podolyan, & Robinson, 2014; Tiedje, Saxstone, Myrold, & Robinson, 1983) dominate N₂O production. According to Soares et al. (2016), AOB are the main contributors to N₂O 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 N₂O emission, the control of N₂O 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 N₂O losses in sugarcane-planted soils receiving different fertilization regimes with vinasse during different seasons (spring-rainy/winter-dry). Concentrated (CV) and nonconcentrated (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 N₂O emissions from vinasse-fertilized sugarcane-planted soils. We hypothesized that (I) application of vinasse residue before N fertilizer application drastically reduces N₂O emissions; (II) nitrification is the major pathway contributing to N₂O emissions from sugarcane-planted soils; and (III) N₂O 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 N₂O emissions from sugarcane-planted soil as well as the key functional genes related to N₂O emissions during different seasons, that is, archaeal
and bacterial amoA, fungal and bacterial nirK, and bacterial nirS and nosZ. Additionally, we determined the total bacterial and fungal abundances.

2 MATERIALS AND METHODS

2.1 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 (2015) or Oxisol by soil taxonomic (USDA, 2014), and the physicochemical properties (Camargo, Moniz, Jorge, & Valadares, 1986; Van Raij, Andrade, Cantarella, & Quaggio, 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 12 November 2013. The dry season (DS) experiment was conducted during the 2014/2015 sugarcane cycle and began on 15 July 2014. Both experiments lasted 90 days, period that most of GHG emissions occur. High N2O 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 regrow up to five times after the first harvest (raton 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 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, N2O 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 in Table 2.

The N (ammonium nitrate) fertilizer application rate was 100 kg/ha 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, Cantarella, Quaggio, & Furlani, 1996). In both sites, in treatment with vinasse, a volume of 100 m³/ha 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 approximately 100 m³/ha which corresponds approximately to 100 m³/ha 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

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Physicochemical properties of soil layer (0 to 20 cm) in rainy (RS) and dry (DS) seasons (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>pH (CaCl₂; 0.0125 mol/L)</td>
</tr>
<tr>
<td>RS</td>
<td>5.3</td>
</tr>
<tr>
<td>DS</td>
<td>5.0</td>
</tr>
</tbody>
</table>

¹Organic matter.
²Available phosphorus, K, Ca, and Mg were extracted with ion exchange resin.
³Buffer solution (Calcium acetate 0.5 M, pH 7.0).
⁰Cation exchange capacity.
⁴Soil texture determined by the densimeter method.
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 in Table S1.

### 2.2 | CO2 and N2O measurements, soil sampling, and chemical analysis

Fluxes of CO2 and N2O 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 CO2 determination (Hutchinson & Mosier, 1981) and an electron capture detector for N2O determination (Hutchinson & Mosier, 1981). The overall CO2 and N2O fluxes were calculated by linear interpolation of the three sampling times.

CO2 and N2O 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 were CV and mineral N fertilizer were applied and most GHG 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 N2O-N emission factors (EF).

The emission factors for N2O were calculated based on the amounts of N applied as vinasse and mineral N fertilizer inside the chamber according to the formula:

\[
EF = \frac{N_{2O} \text{-} N_{\text{treat}} - N_{2O} \text{-} N_{\text{control}}}{N_{\text{applied}} \text{ (fert + Vinasse)}} \times 100
\]

EF is the N2O-N emission factor (%), N2O-N_{treat} and N2O-N_{control} are the cumulative emissions in the fertilized and unfertilized chambers, respectively, and N_{applied} 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 RS.
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\(_3^–\)) and ammonium (NH\(_4^+\)). 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 hr. Soil mineral N (NH\(_4^+\)-N, NO\(_3^–\)-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\(^3\) in RS and DS) and porosity determined at the beginning of the experiment.

2.3 | 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.

2.4 | 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 μl containing 6 μl of SYBR Green Master Mix and 4 μl of DNA (1.25 ng/μl), except fungal nirK, which was amplified in a total volume of 10 μl containing 1 μl of undiluted DNA. The primer combinations, reaction descriptions, and thermal cycler conditions for each gene amplification are listed in Table S2. 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. 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\(^{–8}\). Samples were analyzed with two technical replicates. The reaction efficiency varied from 80% to 105%, and the \(R^2\) values ranged from .94 to .99.

2.5 | Statistical analysis

The cumulative emissions of N\(_2\)O and CO\(_2\) 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\(_2\)O were compared per orthogonal contrasts using sissvar statistical software (Ferreira, 2011). Soil pH was transformed to H\(^+\): 10\(^–pH\) 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\(_2\)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, Hastie, & Tibshirani, 2010). The lasso penalty is a regression method that performs both shrinkage and variable selection (Osborne, Presnell, & Turlach, 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, Field, Mills Flemming, & Ronchetti, 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 log10 transformation for both N\(_2\)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).

3 | RESULTS

3.1 | Weather conditions and soil analysis

The mean air temperature varied between 13 and 28°C (Figure S1). 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 (NH₄⁺-N and NO₃⁻-N) approximately 40 days after mineral N fertilizer application. In DS, the mineral N (NH₄⁺-N + NO₃⁻-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/kg of dry soil in RS and DS, respectively (Figure S2).

### 3.2 Carbon dioxide emissions

The emissions of CO₂-C from sugarcane were similar in the two seasons, with high emissions immediately after vinasse application (Figure S3). The treatments with CV had higher CO₂ emission fluxes in the fertilized bands than the treatments with V in both seasons with peaks of 33 and 17 g m⁻² day⁻¹ of C for CV and V, respectively. The cumulative CO₂-C emissions were 97 and 126 g/m² 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 CO₂-C emissions; however, in the rainy season, the treatments with CV application emitted more CO₂-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 CO₂-C emissions by 89 g/m² (on average) (Table 3).

### 3.3 Nitrous oxide emissions

In both, rainy and dry seasons (RS and DS), the N₂O emission fluxes of the control treatment were similar, approx. 0.06 mg m⁻² day⁻¹ of N (Figure 1c,d). In RS, the N₂O emission fluxes were similar in all treatments (0.61 mg m⁻² day⁻¹ of N), except the CV+N treatment, in which N₂O fluxes were much higher (46.49 mg m⁻² day⁻¹ of N) (Figure 1a,c). In DS, the highest N₂O emissions were observed in treatments with mineral N. The application of vinasse 30 days before mineral N (Vₜ+N and CVₜ+N) decreased the N₂O emission fluxes compared to the application of vinasse together with mineral N (Figures 1d, 2d, and 3d). In RS, only V was applied prior to N. In both experiments, the maximum N₂O emission peaks occurred soon after application of mineral N and vinasses (CV and V) and immediately after rain events (Figures 1 and S1).

In the treatments with mineral N, the cumulative N₂O-N emissions in the fertilized bands were higher in DS than in RS; the total N emitted was 49 and 89 mg N₂O-N/m² greater than in the control treatment (12 and 6 mg/m² of N₂O-N), respectively, corresponding to 0.08% and 0.14% of total N applied (Figure 2). The application of vinasse (CV and V), mineral N, or the combination of both resulted in higher (+184 mg N/m²) cumulative N₂O emissions than in the control in DS (Table 3). In addition, the application of vinasse plus mineral N (CVₜ+N, CVₜ+N Vₜ+N, and V+N) resulted in an increase in emissions of nearly 328 and 221 mg N/m² compared to treatment with either vinasse alone (CV and V). Comparing the effect of both vinasses plus mineral N, CV increased N₂O emissions over V by 875 and 233 mg N/m² in RS and DS, respectively (Table 3). However, the application of CV 30 days before N reduced N₂O-N emissions by 65% compared with those of CV and mineral N applied at the same time. The N₂O-N emissions represented 0.26 and 0.65% of the total N applied in the CVₜ+N and CV+N treatments, respectively (Table 3). On the other hand, the application of V 30 days before mineral N did not reduce N₂O-N emissions (Figure 2).

<table>
<thead>
<tr>
<th>Selected contrasts</th>
<th>Contrast calculation</th>
<th>Mean of the parameters measured</th>
<th>CO₂ (g C m⁻²)</th>
<th>N₂O (mg N/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rainy season</td>
<td>Dry season</td>
</tr>
<tr>
<td>1</td>
<td>N effect (vinasse-N or N)</td>
<td>(All treatments) – control</td>
<td>97**</td>
<td>126***</td>
</tr>
<tr>
<td>2</td>
<td>N plus vinasse effect</td>
<td>(All vinasses +N) – (all vinasses)</td>
<td>29ns</td>
<td>11ns</td>
</tr>
<tr>
<td>3</td>
<td>Type of vinasse</td>
<td>CV - V</td>
<td>142***</td>
<td>12ns</td>
</tr>
<tr>
<td>4</td>
<td>V: Anticipating vs. ST</td>
<td>Vₜ - V</td>
<td>-102**</td>
<td>-89*</td>
</tr>
<tr>
<td>5</td>
<td>CV: Anticipating vs. ST</td>
<td>CVₜ - CV</td>
<td>-104**</td>
<td>-104**</td>
</tr>
<tr>
<td>6</td>
<td>Type of vinasse + N</td>
<td>(CV+N) - (V+N)</td>
<td>216***</td>
<td>23**</td>
</tr>
<tr>
<td>7</td>
<td>V+N: Anticipating vs. ST</td>
<td>(Vₜ+N) - (V+N)</td>
<td>-34ns</td>
<td>-143**</td>
</tr>
<tr>
<td>8</td>
<td>CV+N: Anticipating vs. ST</td>
<td>(CVₜ+N) - (CV+N)</td>
<td>-89*</td>
<td>-89*</td>
</tr>
</tbody>
</table>

*Contrasts 1 and 2 compare the overall effect of N on N₂O emissions; contrasts 3 through 8 compare the effects of type of vinasse with and without N fertilizer; contrasts within each group are orthogonal.

*N: mineral N fertilizer, ammonium nitrate; CV: concentrated vinasse; V: nonconcentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: nonconcentrated vinasse plus mineral N; Vₜ: Vinasse application 30 days before N fertilization; ST: application at the same time.

*Net effect on emissions for the indicated contrast. Significant difference: *p ≤ .10; **p ≤ .05; ***p ≤ .01; ns: nonsignificant.
emissions in both seasons (Table 3). The cumulative N₂O emissions in the treatments with V were 54 and 79 mg N/m² in V_b+N and V+N in RS, respectively, and 137 and 241 mg N m⁻² day⁻¹ in V_b+N and V+N in DS, respectively (Figure 2). The N emitted as N₂O was approximately 0.10 and 0.26% (V_b+N and V+N in average) of the total N applied in RS and DS, respectively (Figure 2).

Because the amount of N of CV and mineral N fertilizer were higher in the application bands, the N₂O emissions of bands area were higher than those of the whole field. However, when the N₂O emissions were reported in hectare basis, the relative values of N₂O emissions of the treatments with V tend to be higher than those of CV and mineral N. N₂O 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 N₂O-N emission factors (EF, % of N applied) calculated with original N₂O emission values expressed per chamber area (mg N/m²) or field basis (g N/ha) were similar (Figure 2 and Table S3), and 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 N₂O 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 and the weather conditions. Nevertheless, both EF values were lower than the IPCC default values (1% of the applied N).

3.4 | Abundances of nitrogen cycle genes

The abundances of N cycle genes related to N₂O emissions are shown in Figures S4–S6 for all treatments and sampling.
The correlations between the abundances of bacterial denitrification genes (nirK, nirS, and nosZ) and N2O emissions differed between seasons (Figure 3). For RS overall, N2O emissions were correlated significantly with nirS ($R^2 = .22, p \leq .01$) and nosZ ($R^2 = .19; p \leq .05$), whereas for DS overall, nirK ($R^2 = .16, p \leq .05$) and nirS ($R^2 = .24, p \leq .01$) were positively correlated with N2O emissions (Figure 3). The abundances of the nirK, nirS, and AOA-amoA genes increased linearly with time with the increase in water availability (Figures S1, S4–S6).

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 N2O emissions in both seasons (RS: $R^2 = .30, p \leq .01$; DS: $R^2 = .37, p \leq .01$) (Figure 3). Total fungi were most abundant in the treatments with vinasse application (with or without nitrogen) (Figure S6). In RS, the abundance of the fungal nirK gene was significantly correlated with N2O emissions on day 22 ($R^2 = .36, p \leq .10$), whereas in DS, the abundance of the fungal nirK gene was significantly correlated with N2O emissions on days 11 ($R^2 = .35, p \leq .15$) and 19 ($R^2 = .47, p \leq .01$) (Table 4).

The positive correlation between N2O 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 N2O emissions, the ratios between gene abundances and their correlation with N2O emissions were calculated (Table 4). In both seasons, nitrification by amoA-AOB appeared to be the dominant process related to N2O emissions due to the negative correlation between N2O emissions and the ratio of denitrifier to nitrifier genes (RS: $(nirK+nirS)/(AOB+AOA)$, $R^2 = -.26, p \leq .01$; $(nirK+nirS)/amoA-AOB$, $R^2 = -.22, p \leq .01$; and nirK-Fungi/amoA-AOB, $R^2 = -.17, p \leq .05$; similar results were obtained for DS) (Table 5). The general linear model also provided evidence of the predominance of nitrification (Table 6A); N2O emissions were dependent on the abundance of amoA-AOB in both seasons when N cycle genes, 16S rRNA and 18S rRNA genes, were taken into account (Table 6).

To evaluate the relative influences of functional genes, treatments, and climatic factors on N2O 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 N2O 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 N2O emissions. In RS, N2O emissions increased with soil moisture, soil temperature, mineral N (NH$_4^+$-N and NO$_3^-$-N), nosZ and total bacteria, whereas in DS, N2O emissions...
increased with soil moisture, air temperature, mineral N (NO$_3^-$-N), amoA (AOB), and nosZ. Application of vinasse (CV and V) plus mineral N increased N$_2$O emissions in both seasons (Table 6B).

4 | DISCUSSION

The application of vinasse (CV and V) 30 days prior to mineral N fertilizer reduced the cumulative N$_2$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, as 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, Rossetto, Bonnecene, Piemonte, & Muraoka, 2013), which may lead to a low N$_2$O emission rate. The N$_2$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$_2$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$_2$O emissions. However, the phenology of the sugarcane plant may provide insights on the lower N$_2$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 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$_2$O emission. In the dry season, N$_2$O emissions were nearly twofold 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 N₂O emission.

The variation in N₂O 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, Snyder, Blaylock, & Del Grosso, 2014; Liang, Eberwein, Allsman, Grantz, & Jenerette, 2015; Subbarao et al., 2006; Vargas et al., 2014). The large variation in 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 nonmineral 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 \textit{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 N₂O is emitted to the atmosphere (Soares et al., 2016). Many studies have shown

### Table 4

Spearman’s correlation coefficients between N₂O emission flux (mg m⁻² day⁻¹) and abundance of archael and bacterial \textit{amoA}, fungal and bacterial \textit{nirK}, bacterial \textit{nirS} and \textit{nosZ}, and total bacterial 16S rRNA, and total fungal 18S rRNA (gene copy/g dry soil) in the rainy and dry seasons

<table>
<thead>
<tr>
<th></th>
<th>Rainy season</th>
<th>Dry season</th>
</tr>
</thead>
<tbody>
<tr>
<td>WFPS</td>
<td>0.60***</td>
<td>0.07</td>
</tr>
<tr>
<td>NH₄⁺-N</td>
<td>0.80***</td>
<td>0.33</td>
</tr>
<tr>
<td>NO₃⁻-N</td>
<td>0.68***</td>
<td>0.47**</td>
</tr>
<tr>
<td>pH</td>
<td>−0.31</td>
<td>−0.04</td>
</tr>
<tr>
<td>\textit{amoA}_{AOB}</td>
<td>−0.30⁺</td>
<td>−0.13</td>
</tr>
<tr>
<td>\textit{amoA}_{AOA}</td>
<td>0.08</td>
<td>−0.26</td>
</tr>
<tr>
<td>\textit{nirK}</td>
<td>0.01</td>
<td>−0.13</td>
</tr>
<tr>
<td>\textit{nirS}</td>
<td>−0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>\textit{nosZ}</td>
<td>−0.00</td>
<td>−0.10</td>
</tr>
<tr>
<td>\textit{nirK}-Fungi</td>
<td>−0.22</td>
<td>−0.24</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>0.13</td>
<td>−0.05</td>
</tr>
<tr>
<td>18 rRNA</td>
<td>0.03</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\*WFPS, water-filled pore space; AOB, \textit{amoA} belonging to ammonia-oxidizing bacteria; AOA, \textit{amoA} belonging to ammonia-oxidizing archaea. Bold value means significant difference: \* \(p \leq 0.15\), \*\* \(p \leq 0.10\), \*\*\* \(p \leq 0.05\) and \*\*\*\* \(p \leq 0.01\).
that N₂O emissions are significantly and positively correlated with ammonia oxidation by AOB under controlled conditions (Law, Ni, Lant, & Yuan, 2012; Regina, Nykönen, Silvola, & Martikainen, 1996). AOA also played a role in N₂O 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 N₂O emissions. The soil conditions at our sites were acidic (pH 5.1), and amoA-AOA gene abundance usually increases with decreasing pH (Nicol, Leininger, Schleper, & Prosser, 2008; Zhang, Hu, Shen, & He, 2012). Although ammonia oxidation by AOA was also responsible for the N₂O emissions, the amoA-AOB/amoA-AOA ratio and regression analysis of our results showed that amoA-AOB was the most important gene related to N₂O emissions, thus indicating that nitrification by AOB dominates the nitrification process and N₂O emissions in sugarcane fields. It has been reported that AOAs, although present in soils, do not respond to NH₄⁺-N fertilization or N₂O production in intensively managed agricultural soils, in contrast to AOB (Di et al., 2009; Hink et al., 2016; Yang, Zhang, & Ju, 2017). Independent of soil pH, the concentration of NH₄⁺-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 N₂O emissions, but neither vinasse nor sugarcane straw was applied in that study.

In addition to the considerable importance of N₂O emissions during ammonia oxidation by AOB, the consumption of O₂ 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 NH₄⁺-N concentrations, leading to microoxic or anoxic conditions, which in turn will induce denitrification by heterotrophic denitrifiers or by nitrifiers, resulting in high N₂O emission fluxes. The significant correlation between nosZ and N₂O indicates that complete denitrification is also occurring in the soil, nosZ is the key enzyme involved in the N₂O reduction to N₂ (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 N₂O fluxes occur immediately after N fertilization (Carmo et al., 2013; Navarrete et al., 2015; Pitombo et al., 2015; Soares et al., 2015, 2016). However, denitrification appears to be less important than AOB for N₂O emissions under our experimental field conditions.

N₂O emissions and the total fungal abundance showed significant positive correlations over time, suggesting a contribution of fungal denitrifiers to N₂O emissions. This relationship was further confirmed by the significant positive correlation between fungal nirK and N₂O emissions in both seasons on different days. A role of fungi in N₂O emissions has recently been reported (Mothapo et al.,...
<table>
<thead>
<tr>
<th>(A)</th>
<th>Dependent variable</th>
<th>Intercept</th>
<th>amoA - AOB</th>
<th>amoA - AOA</th>
<th>nirK</th>
<th>nirS</th>
<th>nosZ</th>
<th>nirK - Fungi</th>
<th>16S rRNA</th>
<th>18S rRNA</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS</td>
<td>N₂O</td>
<td>-0.831</td>
<td>0.011</td>
<td>-0.319</td>
<td>0.142</td>
<td>0.051</td>
<td>-</td>
<td>0.126</td>
<td>0.228</td>
<td>.230</td>
<td></td>
</tr>
<tr>
<td>DS</td>
<td>N₂O</td>
<td>-0.005</td>
<td>0.158</td>
<td>-</td>
<td>0.097</td>
<td>-0.036</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>.107</td>
<td></td>
</tr>
</tbody>
</table>

| (B) | Dependent variable | Intercept | Treatmentsᵇ | Day | WFPS | Air Tem. | Soil Tem. | NH⁺ | NO⁻ | pH | amoA AOB | amoA AOA | nirK | nirS | nosZ | nirK - Fungi | 16S rRNA | 18S rRNA | r²  |
|-----|-------------------|-----------|--------------|-----|------|----------|-----------|-----|-----|----|----------|----------|------|------|-----|              |--------|--------|-----|
| RS  | N₂O               | -0.82     | CV + N      | 0.59| -0.04| -0.09    | 0.01      | 0.02| 0.03| -0.02| -        | -        | -    | -    | 0.03| -            | -      | -      | -    | .58 |
|     |                   |           | V            | -    | -    | -        | -         | -   | -   | -   | -        | -        | -    | -    | 0.03| -            | -      | -      | -    |     |
|     |                   |           | V + N        | 0.10| -    | -        | -         | -   | -   | -   | -        | -        | -    | -    | 0.03| -            | -      | -      | -    |     |
|     |                   |           | Vb           | -0.03| -    | -        | -         | -   | -   | -   | -        | -        | -    | -    | -   | -            | -      | -      | -    |     |
| DS  | N₂O               | -0.186    | CV + N      | 0.289| -0.13| 0.60    | 0.02      | -0.04| 0.12| -0.05| 0.05    | -0.05   | -0.04| 0.05 | -   | -            | -      | -      | -    | .57 |
|     |                   |           | CV           | 0.576| -    | -        | -         | -0.04| 0.12| -0.05| 0.05    | -0.05   | -0.04| 0.05 | -   | -            | -      | -      | -    |     |
|     |                   |           | CVb + N     | 0.369| -    | -        | -         | -0.05| 0.12| -0.05| 0.05    | -0.05   | -0.04| 0.05 | -   | -            | -      | -      | -    |     |
|     |                   |           | V + N       | 0.382| -    | -        | -         | -0.05| 0.12| -0.05| 0.05    | -0.05   | -0.04| 0.05 | -   | -            | -      | -      | -    |     |
|     |                   |           | Vb           | -0.052| -    | -        | -         | -0.05| 0.12| -0.05| 0.05    | -0.05   | -0.04| 0.05 | -   | -            | -      | -      | -    |     |
|     |                   |           | Vb + N      | 0.21 | -    | -        | -         | -0.05| 0.12| -0.05| 0.05    | -0.05   | -0.04| 0.05 | -   | -            | -      | -      | -    |     |

ᵇWFPS, water-filled pore space; air Tem., air temperature; soil Temp., soil temperature; AOB, amoA belonging to ammonia-oxidizing bacteria; AOA, amoA belonging to ammonia-oxidizing archaea; Fungi, nirK belonging to denitrifier fungi.

ᵇN, mineral N fertilizer, ammonium nitrate; CV, concentrated vinasse; V, nonconcentrated vinasse; CV + N, concentrated vinasse plus mineral N; V + N, nonconcentrated vinasse plus mineral N. b, Vinasse application 30 days before N fertilization.
2015; Shoun, Fushinobu, Jiang, Kim, & Wakagi, 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 N₂O to N₂, and thus fungal denitrification terminates at N₂O (Phillips et al., 2016; Shoun et al., 2012). Therefore, an increase in fungal denitrification might increase N₂O 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 N₂O emissions in soil with wheat straw were initially predominantly derived from bacterial denitrification but later mainly resulted from fungal denitrification. Similar to 18S rRNA gene, the abundance of the fungal nitrite reductase gene (nirK) increased significantly with N₂O emissions after swine manure application (Xu et al., 2017).

No study has investigated the abundance of nitrifier and denitrifier genes and their links to N₂O 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 N₂O emissions by NH₄⁺-N oxidation or nitrification–denitrification as well as by NO₃⁻-N reduction by heterotrophic denitrification. The significant positive correlations between N₂O emissions and the abundances of the bacterial nirK, nirS, and nosZ genes show that the production of N₂O is due to favorable conditions for denitrification. Rain events and vinasse fertilization induce low oxygen concentrations in soil microsites (Di et al., 2014), consistent with the significant correlation between N₂O 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 (Torbett & 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 N₂O emissions. However, this situation may be transient since drying of the soil within a few hours or days will favor N₂O emissions by nitrification. By contrast, fungal denitrifiers can release N₂O under both aerobic and anaerobic conditions (Shoun et al., 2012; Zhou et al., 2002). Other, less-characterized processes may also be involved in N₂O emissions, such as nitrifier denitrification in suboxic conditions and NO₂⁻ accumulation (Kool, Dolfing, Wrage, & Van Groenigen, 2011), aerobic denitrification, and codenitrification (Joo, Hirai, & Shoda, 2005; Spott, Russow, & Stange, 2011; Zhao, An, He, & Guo, 2012). However, nitrification by AOB and denitrification by fungi were the prevalent processes leading to high N₂O emissions in both experiments and therefore could be useful indicators for mineral N management strategies to mitigate N₂O emissions in tropical soils with organic residue application.

Understanding the prevalent microbial processes related to N₂O 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 N₂O emissions in a complex field soil ecosystem where different bioenergy residues, that is, 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 N₂O emissions by 65% and 37% for concentrated and nonconcentrated vinasse, respectively.

ACKNOWLEDGEMENTS

The authors thank Dr. André C. Vitti (APTA), Dr. Rafaeila Rossetto (APTA), MSc. Rafael M. Sousa, and Dr. Zaqueu F. Montezano (IAC) for technical assistance; Dr. Afnan K.A. Suleiman (NIOO-KNAW) for scientific discussions; and Dr. Leonardo Pitombo and MSc. Márcio F.A. Leite for the fitted general linear model applied to the data. This research was supported by FAPESP and The Netherlands Organization for Scientific Research (NWO) grant number 729.004.003, 2013/50365-5, FAPESP 2014/24141-5, FAPESP 2013/12716-0, and CNPq 311.197/2013-2. Publication 6512 of the Netherlands Institute of Ecology (NIOO-KNAW).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ 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 manuscript. E.E.K., H.C., and J.A.V critically reviewed the manuscript. All authors reviewed the manuscript.
REFERENCES


ESALQ (2016). Série de dados climatológicos do campus Luiz de Queiroz de Piracicaba, SP. pp Page, Escola Superior de Agricultura “Luiz de Queiroz”.


emission related to ammonia-oxidizing bacteria and mitigation options from N fertilization in a tropical soil. *Scientific Reports*, 6, 30349. https://doi.org/10.1038/srep30349


SystatSoftware (2014). SSI. Sigmaplot for Windows, version 13.0. San Jose, California, USA, Systat Software


**SUPPORTING INFORMATION**

Additional supplemental material may be found online in the Supporting Information section at the end of the article.