Exploring soil microbial 16S rRNA sequence data to increase carbon yield and nitrogen efficiency of a bioenergy crop

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Abstract
Crop residues returned to the soil are important for the preservation of soil quality, health, and biodiversity, and they increase agriculture sustainability by recycling nutrients. Sugarcane is a bioenergy crop that produces huge amounts of straw (also known as trash) every year. In addition to straw, the ethanol industry also generates large volumes of vinasse, a liquid residue of ethanol production, which is recycled in sugarcane fields as fertilizer. However, both straw and vinasse have an impact on N₂O fluxes from the soil. Nitrous oxide is a greenhouse gas that is a primary concern in biofuel sustainability. Because bacteria and archaea are the main drivers of N redox processes in soil, in this study we propose the identification of taxa related with N₂O fluxes by combining functional responses (N₂O release) and the abundance of these microorganisms in soil. Using a large-scale in situ experiment with ten treatments, an intensive gas monitoring approach, high-throughput sequencing of soil microbial 16S rRNA gene and powerful statistical methods, we identified microbes related to N₂O fluxes in soil with sugarcane crops. In addition to the classical denitrifiers, we identified taxa within the phylum Firmicutes and mostly uncharacterized taxa recently described as important drivers of N₂O consumption. Treatments with straw and vinasse also allowed the identification of taxa with potential biotechnological properties that might improve the sustainability of bioethanol by increasing C yields and improving N efficiency in sugarcane fields.

Keywords: Anaeromyxobacter, nitrous oxide, straw, sugarcane, sustainability, vinasse

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Introduction
Bioethanol from sugarcane is becoming an increasingly important alternative energy source worldwide. The sugarcane planted area in Brazil is around 9 million-ha (CONAB, 2013) and 26 million-ha worldwide (Kao et al., 2014). The environmental benefits of replacing fossil fuels by ethanol from sugarcane can be only achieved if management practices are applied which lead to a minimum of greenhouse gases (GHG) losses to the atmosphere. Approximately 45% of the total amount of methane (CH₄) and nitrous oxide (N₂O) emitted by the cultivation of sugarcane are derived from straw burning (Macedo et al., 2008); this practice also releases charcoal particles, causing health problems for humans and animals. Recently, legislation was passed that restricts burning, which is being phased out. The unburned cane (green cane) leaves a thick mulch of plant material after harvest (trash or straw) ranging from 10 to 20 t ha⁻¹ (dry matter) consisting of leaves and tops. The conversion from burning to green management of sugarcane will impact the biogeochemical cycling of carbon and nitrogen in the plant–soil system. Straw preservation affects the entire production process of sugarcane, influencing yields, fertilizer management and application, soil erosion, soil organic matter dynamics, and GHG emissions.

In addition to plant straw, a second by-product of the sugarcane ethanol industry is vinasse (Mutton et al.,...
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Each liter of ethanol generates 10–15 l of vinasse, which is a liquid organic residue that has a high potential for polluting water streams. For this reason, there is legislation regulating the destination of vinasse. Currently, Brazil produces approximately 337 billion liters of vinasse that is almost entirely recycled as fertilizer in sugarcane fields (Mutton et al., 2010). Vinasse has a high dissolved organic carbon (DOC) content resulting in high biochemical oxygen demand (BOD) and contains several nutrients; it is particularly rich in potassium and sulfur and contains considerable amounts of phosphorus, nitrogen, calcium, magnesium, and micronutrients. Although vinasse use is of relevance for nutrient cycling in sugarcane fields its application contributes to GHG emissions especially when applied with chemical fertilizers and on the straw (Carmo et al., 2013).

The major sources of atmospheric \( N_2O \) are the nitrification and denitrification processes (Bourgman et al., 2013). Globally, approximately 10.9 Tg N-\( N_2O \) is estimated to be lost from land surfaces every year (Galloway et al., 2004). Although the metabolic pathways that result in \( N_2O \) production during nitrification are not entirely clear, some authors suggest that this process might represent an even larger source of \( N_2O \) from agricultural fields than denitrification (Khalil et al., 2004; Toyoda et al., 2011). Moreover, nitrification supplies oxidized N compounds that can be used as electron acceptors, subsequently resulting in \( N_2O \) release. Denitrification can also be a detoxification pathway as a result of the accumulation of \( NO_2 \) in the system (Zhu et al., 2013). Nitrogen oxidation regulates nutrient availability for plant and the element mobility on the system, making the nitrification process of agronomical and environmental interest. Nitrification is a microbial-restricted niche (Hayatsu et al., 2008) while denitrification can be performed by a broad diversity of microorganisms, especially prokaryotes (Philippot et al., 2007). Denitrification has been described as a modular pathway in which different cells may drive different steps to the complete reduction of nitrate to \( N_2 \) being \( N_2O \) an intermediate product (Zumft, 1997). Complementarily, the diversity of potential \( N_2O \) reducers goes beyond \textit{sensu stricto} denitrifiers (Jones et al., 2013), which makes it difficult to identify the main players in \( N_2O \) production and consumption. Identifying these players will improve our understanding of these processes in the field and promote more sustainable fertilizer use.

While experiments on micro and mesocosm scales have been performed to identify microbial drivers of the N cycle and specifically linked to \( N_2O \) fluxes from soils (e.g. Ishii et al., 2011; Jung et al., 2014), field conditions cannot be reproduced. Surely such micro and mesocosm studies give important insights into the microbes involved in N cycle and specific processes. However, the dynamic system of soil/plants in addition to weather instability results in a complex microbial community that goes beyond cultivable microorganisms. Thus, in this study by combining intensive \textit{in situ} \( N_2O \) monitoring, high-throughput sequencing of soil microbial 16S rRNA gene and powerful statistical methods we have identified potential microbial taxa that might be explored to improve the sustainability of sugarcane as a bioenergy crop as a result of their biotechnological potential and/or their role in nutrient cycling.

Materials and methods

Site description and experimental design

The experimental site is located in Piracicaba, São Paulo state, Brazil (22°41’19.34”S; 47°38’41.97”W). More than 50% of the sugarcane of Brazil is grown in this state (Conab, 2013). The site’s altitude is 575 m, the mean annual temperature is 21°C, and the mean annual precipitation is 1390 mm. The climate is defined as humid tropical with a rainy summer and a dry winter and is considered to be a Cwa type according to the Köppen classification system (Crittfield, 1960). The soil is classified as Haplic Ferralsol (FAO, 2006); fertility parameters are listed in Table S1. Prior to the experiment, the sugarcane crop of the plant cycle was harvested without burning; thus, the field was in the first ratoon stage. After harvesting, the site was subdivided into two subsites. The straw was left onto soil of subsite 1 (10 t ha\(^{-1}\)) and removed mechanically of subsite 2. The removal of straw simulated the scenario that occurs when the sugarcane straw is used as raw material for biofuel production.

Ten different treatments based on combination of organic, mineral fertilizers and straw were applied to the experimental site. The plots (\( n = 4; 8 \times 9 \) m) were randomly distributed and separated from each other by 2 m borders. All treatments received single superphosphate as the phosphorus source (17 kg ha\(^{-1}\) of P). The source of potassium was potassium chloride (100 kg ha\(^{-1}\) of K) or vinasse (normal or concentrated). The nitrogen source was ammonium nitrate applied at a rate of 100 kg N ha\(^{-1}\). The treatments without straw are described as the following: Ctrl (without N) – mineral fertilizer containing P and K; MN (mineral N) – mineral fertilizer containing N, P, and K; MN_V (mineral N plus vinasse) – vinasse as K source (1.10^5 l ha\(^{-1}\)) and mineral fertilizer containing N and P; V (vinasse) – vinasse as K source (1.10^4 l ha\(^{-1}\)) and mineral fertilizer containing P; CV (concentrated vinasse) – concentrated vinasse as K source (1.10^5 l ha\(^{-1}\)) and mineral fertilizer containing P. The same treatments were used with straw (Ctrl_S; MN_S; MN_V_S; V_S; and CV_S). Fertilization was carried out on 19 November, 2012. Vinasse was applied to the total area, while the mineral fertilizers and concentrated vinasse were applied in bands parallel to the crop line as is usually performed in commercial areas. There was no treatment with CV and N because they could be applied to different sides of the plant; thus, the combination of C and N that results in high \( N_2O \)
emissions was avoided. The composition of the vinasse and concentrated vinasse is presented in Table S2.

Greenhouse gas flux measurement and emission estimation

Soil GHG fluxes were measured using the chamber-based method (Varner et al., 2003). In each plot, a PVC chamber (30 cm in diameter) was placed on the soil in which it was partially inserted (3 cm deep). After closing the chambers, 60 ml samples were collected at time points 1, 10, 20, and 30 min using syringes and stored under pressure in 20-ml-evacuated penicillin flasks sealed with gas-impermeable butyl-rubber septa (Bellco Glass 2048). All samplings were performed between 8:00 and 12:00 a.m. The first 31 samplings were collected within 60 days after fertilization, followed by 4 weekly samplings and finally fortnightly intervals until the crop was harvested. Each gas chamber flux was calculated from slope regression between the gas concentration and collection time according to Carmo et al. (2013). Measurements of atmospheric pressure, chamber height, and air temperature were taken during gas sampling to determine the air chamber volume and to calculate GHGs emissions.

The gas samples were analyzed in a GC-2014 model gas chromatograph with electron capture for N2O (Shimadzu, Kyoto, Japan). Soil temperature was measured at a depth of 10 cm to assist in the interpretation of the results. All of the chambers were installed at the fertilized sugarcane line position. GHG emissions were calculated by linear interpolation of fluxes between sampling events (Allen et al., 2010). We estimated that the fertilized bands accounted for 20% of the total experimental area; the remaining 80% of the area consisted of the spaces between the rows. Fertilizer emission factors (EF) were calculated according to Allen et al. (2010) and Carmo et al. (2013).

Soil sampling and soil chemical analysis

Soil sampling and gas collection initiated 1 day after fertilization. After collecting the gases, a composite soil sample 0–10 cm deep from three points was collected from each plot at the fertilizer band position. The samples were stored at −20°C. Soil moisture was determined by gravimetry, and mineral N content was determined by colorimetry of the soil extracts (KCl 2 mol l−1) using flow injection analysis (FIAlab 2500) based on the methods proposed by Kamphake et al. (1967) for NO3 and Krom (1980) for NH4.

DNA extraction, 16S rRNA gene amplification and sequencing

DNA was extracted from 250 mg of soil using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer’s protocol. We performed DNA extraction in samples from eight time points of each treatment corresponding to 240 samples (10 treatments × 8 time points × 3 replicates). Archaeal and bacterial community structures were assessed by sequencing the V4 region of the 16S rRNA gene using primers 515F (GTgccagcmgcccgccg-TAA) and 806R (TAATCTWGGVHCATCAGG) (Caporaso et al., 2010) and the LJB-L kit for unidirectional sequencing. Duplicate 50 μl PCRs were performed as follows: 5 μl Roche 10 × PCR buffer with MgCl2, 2 U of Roche Taq DNA polymerase, 2 μl of dNTP (10 mM), 1 μl each primer (5 pmol μl−1), and 1 μl of DNA template. Thermocycling conditions consisted of an initial denaturation at 94 °C for 5 min, 30 cycles at 95 °C for 30 s, 53 °C for 1 min, 72 °C for 1 min, and an final extension at 72 °C for 10 min. Technical replicates were pooled and purified using the Qagen PCR purification kit. The 240 samples were equimolar mixed and then sequenced (Macrogen Inc. Company, South Korea) on a Roche 454 automated sequencer using the GS-FLX Titanium system (454 Life Sciences, Branford, CT, USA).

Sequence data analysis

Sequence data were processed using the workflow of the NG6 system (Mariette et al., 2012), installed on a local server, which system depends on Mothur version 1.31.1 (Schloss et al., 2009). The flowgrams were demultiplexed and sizes between 350 and 750 flows were selected. The flowgrams were corrected using the shhh.flows command, which is the Mothur implementation of the original PyroNoise algorithm (Quince et al., 2011). Next, the results of the different sff files were combined for further analysis. The merged sequences were aligned to the bacterial and archaeal reference alignment provided on the Mothur website (http://www.mothur.org/wiki/Silva_reference_alignment) based on the SILVA 102 release of the SSURef database (Quast et al., 2013). Only reads that fell into region 13 862–22 580 of the reference alignment were kept. The pre.cluster command was used to reduce typical sequence errors due to sequencing PCR products from high-diversity DNA samples. This command assigns sequences that are within two mismatches to the most abundant sequence (Huse et al., 2010). Chimeric sequences were identified and removed using the chimera.uchime command (Edgar et al., 2011). Operational taxonomic units (OTUs) were formed at a maximum distance of 0.03 using the dist.seqs command and average neighbor clustering. All sequences were taxonomically classified using the Mothur implementation of the RDP classifier (Wang et al., 2007) using the training set (version 9) provided on the Mothur website (http://www.mothur.org/wiki/RDP_reference_files) and a bootstrap cutoff of 80%. OTUs were defined at an identity cutoff of 97%. We are aware that the Mothur developers state this training set as of poor quality and it is small in size. However, RDP taxonomy is one of the only sets that take subgroups of Acidobacteria into account. For each OTU, consensus taxonomy was determined using the classify.otu command. Representative sequences for each OTU were re-aligned to the Silva reference alignment and a neighbor joining tree was created using the clear-cut program (Sheneman et al., 2006). Taxonomic classification and OTU clustering data were combined into the BIOM format (McDonald et al., 2012) for further downstream statistical analysis with the Phyloseq (McMurdie & Holmes, 2013) R package.
The raw 454 pyrosequencing data of the 16S rRNA are available at the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/) under the study Accession Number PRJEB8973.

Statistical analyses

Differential analyses of the count data (Anders & Huber, 2010) were applied to verify the effect of different management practices on the abundance of operational taxonomic units (OTU). Time points were not considered for these analyses. The control (Ctrl) treatment was used as the reference, and the negative binomial generalized linear model was applied using the DESeq2 package (Love et al., 2014) in R. OTU fold changes were considered statically significant using \( P < 0.01 \) as the criterion.

Generalized linear mixed models (Bolker et al., 2009) were used to test the effect of different management practices, soil parameters, and soil tax abundance on \( \text{N}_2\text{O} \) fluxes. The sampling event was used as the random factor. First, we fitted a global model using the ‘lme4’ R package version 1.1–7 (Bates et al., 2014). Model selection was performed using the ‘MuMln’ R package version 1.10.5 (Barton, 2014) with the Akaike information criterion (AIC) for ranking. To verify the effect of OTUs on \( \text{N}_2\text{O} \) fluxes, we performed screening steps to reduce the number of parameters before applying the ‘dredge’ function, which imposes limited number of parameters. From each global model, we consecutively fitted nested models selecting the OTUs with \( P \) values <0.05, <0.1, and <0.2, until we obtained approximately 15 OTUs. Thus, the ‘dredge’ function was applied to select the best fitted model based on all parameter combinations. The AIC was used as a criterion for model selection. In general, \( P \) values in generalized linear mixed models should be only used as guides to test nested models (Bolker et al., 2009). Thus, once the best model for each management practice was selected, and all OTUs were considered relevant for explaining \( \text{N}_2\text{O} \) fluxes from soil even at \( P > 0.05 \).

Results

GHG fluxes and emissions

Taking into account management practices and soil physicochemical factors, we found by the best fitted model that organic C addition (\( P < 0.001 \)) and straw (\( P = 0.016 \)) increased the \( \text{N}_2\text{O} \) fluxes from soil. In contrast, \( \text{NO}_3^- \) reduced (\( P = 0.065 \)) the \( \text{N}_2\text{O} \) fluxes from the soil. Ammonium (Fig. S1) and soil temperature did not explain \( \text{N}_2\text{O} \) fluxes according to the best fitted model. The intercept of the model highly correlated with soil moisture (\( r^2 = 0.949 \)). The relationship of nitrate with \( \text{N}_2\text{O} \) production is well known. In our study, the models showed an association but not a cause-effect relationship because straw and organic fertilization increased the \( \text{N}_2\text{O} \) fluxes but also reduced soil \( \text{NO}_3^- \) (e.g., by immobilization; Fig. S2). Thus, N fertilization by itself (i.e., available N and not the total N amount) better explained the \( \text{N}_2\text{O} \) fluxes than soil inorganic N. The parameter estimation for fixed effects in global and best fitted models used to explain the \( \text{N}_2\text{O} \) fluxes from soil considering physicochemical parameters and management practices are presented in Table S3. Despite the high \( \text{N}_2\text{O} \) peaks verified in treatments without straw (Fig. 1), annual \( \text{N}_2\text{O} \) emissions were greater in the plots receiving treatment with straw (Fig. 2). We assumed that the effect of high moisture in the treatments with straw resulted in longer slopes with high \( \text{N}_2\text{O} \) fluxes compared to treatments without straw (illustrated in Fig. 1).

During the first 60 days of sampling, the cumulative \( \text{N}_2\text{O} \) emissions reached more than 70% of the total emissions observed during the sugarcane cycle for all treatments (Fig. S3). At the 89th day, the cumulative emissions reached at least 85%. After 60 days, the soil inorganic N content reached a similar content to that before fertilization (Figs S1 and S2), which was expected as sugarcane plants were actively taking up nutrients from the soil. Therefore, we selected soil samples from the first 60 days to assess the microbial community related to N redox in soil.

In accordance with the modeling showing that organic fertilization, mineral fertilization, and straw addition increased \( \text{N}_2\text{O} \) fluxes (Fig. 1), the calculated annual emissions were also affected by these management practices (Fig. 2). The emission factors (EF) showed that the amount of N from fertilizer released as \( \text{N}_2\text{O} \) was equivalent to the following: (i) 210 g ha\(^{-1}\) when only mineral nitrogen was applied to the soil without straw; (ii) 2157 g ha\(^{-1}\) when mineral nitrogen was combined with vinasse; (iii) 1141 g ha\(^{-1}\) only with vinasse; and (iv) 503 g ha\(^{-1}\) when concentrated vinasse was used as fertilizer. In the treatments with straw, the N-N\(_2\text{O}\) released from the fertilizers increased to 1060 g ha\(^{-1}\) in the treatment containing mineral nitrogen; 3380 g ha\(^{-1}\) in the treatment with mineral nitrogen combined with vinasse; 1677 g ha\(^{-1}\) in the treatment with vinasse; and 688 g ha\(^{-1}\) when concentrated vinasse was applied.

Archaenal and Bacterial microbial community

Overall sequencing of 16S rRNA gene marker yielded >1.3 million reads with an average length of 283 bp. After quality trimming, a total of 836 750 sequences were obtained from 240 samples. These sequences covered 21 657 OTUs; a total of 7037 of these OTUs were represented by more than five reads. Differential analyses showed the effects of the management practices on 423 OTUs. Time points were not discriminate for this purpose. From these, 32 belonged to Acidobacteria (Fig. 3c), 46 to Actinobacteria (Fig. 3f), 29 to Bacterioide-
tes (Fig. 3b), 24 to Chloroflexi (Fig. 3a), 58 to Firmicutes (Fig. 3d), 13 to Gemmatimonadetes (Fig. 3b), 3 to Nitrospirae (Fig. 3b), 8 to Planctomycetes (Fig. 3b), 124 to Proteobacteria (Fig. 3e), 2 to Thaumarcheota (Fig. 3b), 3 to Thermotogae (Fig. 3b), 5 to Verrucomicrobia (Fig. 3b), and 76 are unclassified bacteria or belong to other phyla.

Interestingly, taxa classified as Chloroflexales were increased in abundance only in the treatments in which the straw was left on the soil. All OTUs in this order belonged to the genus *Roseiflexus*. In contrast, the differential analysis showed that the members of *Ktedobacterales* were underrepresented in soils with straw. There was a significant effect of straw in reducing the abundance of 12 OTUs from the *Ktedobacterales* order, while in the plots without straw, only 4 OTUs were underrepresented. These OTUs were underrepresented 23 times with straw and only five times without straw (Fig. 3a), suggesting an inhibitory effect of straw on *Ktedobacterales* members.

The taxa recognized as ammonia oxidizers and affected by the treatments with straw and mineral N were from Thaumarcheota genus *Nitrososphaera* (OTU-123, OTU-333, Fig. 3b) and bacterial genus *Nitrosospira* (OTU-487, Fig. 3e, Fig. S4) (*Nitrosomonadales* order). *Nitrososphaera* OTUs were overrepresented only in treatments with straw, while *Nitrosospira* sp. OTU increased abundance in treatments with the addition of mineral N. The thousand most archaeal OTUs belong to the
Within the entire 16S rRNA dataset, there was a clear dominance of the OTU 487 classified as ammonia oxidizer bacteria, which representative sequence presents 99% of identity with *N. multiformis*. The other member of the *Nitrosomonadales* order (OTU 2796, *Gallionellaceae*) was low abundant. Among the nitrite oxidizers, only *Nitrospira* spp. (OTU-82, OTU-190, OTU-481, Fig. 3b) showed overrepresentation in treatments with straw and vinasse. The OTU-136 (Fig. 3d) classified as *Ammoniphilus* sp. also related to nitrogen cycle increased abundance in treatment with the addition of vinasse. *Ammoniphilus* is obligatory oxalotrophic, haloalkalitolerant bacteria and requires high ammonium concentration for growth (Zaitsev *et al.*, 1998).

Vinasse organic fertilization resulted in an increase in 37 Firmicutes taxa; however, the *Lactobacillales* order was the most responsive in terms of fold change (Fig. 3d). The *Lactobacillales* OTUs were also highly abundant based on the model intercept analysis (Fig. S5). Many OTUs (24) within *Clostridiales* were stimulated by the different treatments. However, 18 of these OTUs had low-positive fold changes with treatments including concentrated vinasse and straw left on the soil (Fig. 3d). Shifts in members of family *Leuconostocaceae*
only occurred with concentrated vinasse treatment, while shifts in members of family *Alicyclobacillaceae* were observed with normal vinasse treatment. OTU-215 and OTU-818 belonging to genera *Megasphaera* and *Dialister*, respectively, were shifted in all treatments with normal vinasse (Fig. 3d).

Regarding the phylum Actinobacteria, members of class *Thermoleophilia* were clearly overrepresented in treatments including the presence of straw. Order *Microccales* from the class *Actinobacteria* was overrepresented in soils with the absence of straw, while the OTU belonging to the order *Micromonosporales* was overrepresented in soils with straw combined with mineral nitrogen fertilization (Fig. 3f).

In general, straw on the soil and the input of nutrients (organic or mineral) reduced the abundance of OTUs within phylum Acidobacteria. However, no pattern among specific Acidobacteria OTUs and fertilization practices was observed. The presence of straw resulted in 36 negative fold changes within 19 different OTUs; in treatment without straw, there were only 12 negative fold changes within 10 OTUs. Only 9 OTUs belonging to phylum Acidobacteria slightly increased their abundance in soil with straw (Fig. 3c).

Overall, Proteobacteria was favored by organic fertilization and inhibited by the absence of straw (Fig. 3e). The treatments without straw resulted in 38 negative fold changes within 25 OTUs, while treatment with straw resulted in 19 negative fold changes within 14 OTUs (Fig. 3e). In treatments with organic fertilization, *Alphaproteobacteria* OTU-52, OTU-71, and OTU-317 that were classified as *Novosphingobium*, *Gluconacetobacter* and *Acetobacter*, respectively, were highly overrepresented (Fig. 3e). In addition to organic fertilization with concentrated vinasse, members of order *Rhodocyclales* (Betaproteobacteria) needed soil covered with straw to be overrepresented. The ability of members of order *Rhodocyclales* to use complex organic sources and perform important steps of the nitrogen cycle, such as denitrification during waste treatment, has been previously described (Hesselsoe et al., 2009). The class *Deltaproteobacteria* is another group that was overrepresented in soil with straw. Additionally, the members of this group were underrepresented in soil without straw (Fig. 3e), indicating a condition of dependency on straw. Interestingly, there were different effects at the OTU level within the *Anaeromyxobacter* spp. (Deltaproteobacteria) in the different treatments. From this taxon, OTU-50 and OTU-434 were overrepresented in soil with straw, OTU-100 and OTU-248 were inhibited with the input of mineral nitrogen, and OTU-289 had a positive fold change with the addition of concentrated vinasse. Among the members of *Gammaproteobacteria*, OTU-76 and OTU-120 belonging to genera *Acetinobacter* and *Dyella*, respectively, were highly abundant in the treatments without straw when compared with the treatments with straw. Moreover, OTU-120, assigned to *Dyella* sp., was increased in soil with mineral nitrogen treatment without straw.

OTU-121 and OTU-224 of the phylum Gemmatimonadetes were overrepresented in treatments with straw (Fig. 3b). Within the phylum Verrucomicrobia, OTUs belonging to the order *Chthoniodibacteres* presented negative fold changes in all treatments (Fig. 3b). One of these OTUs (OTU-398, classified as *Candidatus Xiphinemaotobacter*) was underrepresented only in the treatments with straw. Members of this genus are obligate endosymbionts of nematodes of commercial importance (Vandekerckhove et al., 2000), suggesting that straw might reduce the amount of this parasite in soil. Therefore, this taxon might be considered as a bioindicator of soil quality.

**Microbial taxa to explain N$_2$O fluxes**

After identifying the management practices (i.e., straw, mineral nitrogen fertilization, and organic fertilization) that increased N$_2$O fluxes, we statistically checked what OTUs explain gas fluxes under the respective conditions. As a threshold, only OTUs that were represented at least twice in treatments with the same management practice were used to compose the respective model. These included were 64 OTUs related to straw, 24 OTUs related to mineral nitrogen, and 64 OTUs related to organic fertilization. In contrast to the differential analysis, we used frequency-normalized data to troubleshoot convergence failures. The global models that explain N$_2$O fluxes for the different management practices using microbial taxa narrowed the field ($P < 0.05$) to 19 OTUs for straw, six for mineral nitrogen, and 14 for organic fertilization (Table 1). The best and global models fitted with microbial OTUs under different managements to explain N$_2$O are presented in Table 1. The most represented orders in these models were *Burkholderiales* (6 OTUs), *Bacillales* (5 OTUs), *Lactobacillales* (4 OTUs), *Myxococcales* (3 OTUs), and *Xanthomonadales* (3 OTUs). All nested models generated to obtain approximately 15 OTUs prior to application of the ‘dredge’ function (see Materials and methods for details) as well as the global and best models are presented in a supplemental file. The relative abundances of OTUs presented in Table 1 are illustrated for *Firmicutes* (Fig. S6), *Proteobacteria* (Fig. S7) and other phyla (Fig. S8). There was an effect of vinasse on *Lactobacillus*, concentrated vinasse on *Leuconostoc* and straw on *Rumelifibacillus* OTUs, significantly explaining the N$_2$O fluxes from soil (Fig. S6). Furthermore, the effect of straw and vinasse on relative OTU abundance within *Anaeromyxobacter* spp. was prominent (Fig. S7). The effects of straw on the OTUs

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that explain N₂O fluxes within the archaeal genus *Nitrososphaera* and bacterial genera *Roseiflexus* and *Gemmatimonas* are shown in Fig. S8.

### Discussion

Based on our field experiment, we expect to guide further efforts for mining specific members in the microbial community for sustainable biofuel production. A promising example for mining is our finding of the dominance of archaeal *Nitrososphaera* and bacterial *Nitrosospira* as ammonia oxidizers instead of *Nitrosomonas* commonly found in soils and used as model for ammonia oxidizing bacteria. One of the practices to reduce N₂O emissions in sugarcane is the use of nitrification inhibitors. However, the available inhibitors are expensive and some are applied in high amounts (up to 5% of the N is applied as nitrification inhibitor com-

### Table 1

OTUs that explain N₂O fluxes in soils under different sugarcane management practices (S = straw; MN = mineral nitrogen fertilization; V = vinasse fertilization) by the global models and by the best models

<table>
<thead>
<tr>
<th>OTU</th>
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<th>MN</th>
<th>V</th>
<th>S</th>
<th>MN</th>
<th>V</th>
<th>Phylum</th>
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§Only OTUs that presented $P < 0.05$ are shown. Complete list of OTUs are presented in a supplemental file.

$**P < 0.001; **P < 0.01; *P < 0.05; $P < 0.1; $not significant but is part of the best model selected by AIC.$
pounds) into soil (Soares et al., 2015). Furthermore, the available nitrification inhibitors were initially addressed to Nitrosomonas (Slangen & Kerkhoff, 1984) and some of them have different effect on Nitrospphaera and Nitrosospira (Shen et al., 2013). Therefore, based on our results, the use and development of specific products for these taxa might be addressed.

The genus Roseiflexus were present in all treatments, however, overrepresented in treatments with straw (Fig. 3a). Of the six Chloroflexi classes, Chloroflexia are able to perform photosynthesis (Gupta et al., 2013). Despite the fact that mixotrophy metabolism have been reported for Roseiflexus spp. (Klatt et al., 2013), it is difficult to suggest that photosynthesis occurred below the straw layer due to the absence/low incidence of light. Until recently, no other morphological, physiological, biochemical, or molecular traits were known to be shared by different groups from phylum Chloroflexi (Gupta et al., 2013). Nevertheless, Dellas et al. (2013) have described, using Roseiflexus sp. as reference organism, that the members of class Chloroflexia present a singular mevalonate pathway. In other words, they present a singular pathway to synthesize the precursor of all isoprenoids, which are compounds of biotechnological interest for a broad range of industrial areas (Maury et al., 2005). Additionally, some results have shown that members of phylum Chloroflexi are promising cellulose degraders (Stott et al., 2009) and the utilization of plant polymers may be widespread across the Chloroflexi phylum (Hug et al., 2013). Many efforts have being addressed to obtain cellulases by screening and sequencing new organisms as they have potential to become the largest group of industrially used enzymes worldwide (Wilson, 2009). These results highlight Roseiflexus as an important target for optimization of the C yield per harvested area from crops used for biofuel production.

Large amounts of N are exported from the sugarcane crop as compared to the N applied as fertilizer in field, what makes this crop one of the most efficient crops for bioenergy. One of the explanations is that sugarcane benefits from N2-fixing processes (Boddey et al., 2003; Urquiaga et al., 2012). Many bacterial isolates and bacteria consortia have been used to test this hypothesis without consistent results (Boddey et al., 2003; Martinez De Oliveira et al., 2006; Schultz et al., 2014). The reason might be that a great number of N2-fixing organisms exist in the field, both in soils and in plants, non-endophytic microorganisms contribute to N fixation (Fischer et al., 2012). Although nitrogenase activity has not yet been confirmed in Roseiflexus spp. strains, transcriptomic profiles similar to Oscillochloris trichoides have shown that Roseiflexus spp. fix atmospheric N and this activity is more intense during the night (Klatt et al., 2013).

Thus, deeper studies concerning soil living Roseiflexus spp. and their functional traits would contribute to better understanding of N cycle in soils.

Here, we showed that organic fertilization was the management practice that most affected the abundance of Firmicutes in soils (Fig. 3d). As proposed by Sharmin et al. (2013), Firmicutes-type bacteria play a dominant role in the sugarcane industry due to their physiological ability to ferment sugars. Firmicutes, in particular lactic acids producers, are historically recognized as contaminants by the ethanol industry because their metabolites effectively inhibit yeast ethanol production (Muthaiyan et al., 2011). Thus, because most of the OTUs of Lactobacillus and Leuconostoc were not observed in treatments without vinasse application and Lactobacillus is the dominant taxa in vinasse (Assis Costa et al., 2015), vinasse residues might be the main source of these taxa to the soil (Fig. S6). As verified in this work and by Carmo et al. (2013), vinasse increases N2O emissions from sugarcane fields. The main reason might be because vinasse residues are rich in labile C and have high biological oxygen demand (BOD) values (Carmo et al., 2013; Christofoletti et al., 2013); thus, carbon and moisture are the key factors related with N2O release. General fermenters and Lactobacillus are present in vinasse and when vinasse is applied to soil those microorganisms might be additional important factor for the N2O balance. In accordance with Philippot et al. (2011), we also propose that the increase in the proportion of these organisms in soils here studied might result in increases in N2O release as truncated denitrification pathways are found in Lactobacillus spp. (Shapleigh, 2013). Four of the taxa that explained N2O fluxes from soil in our study were probably added to soil by vinasse organic fertilization. These taxa are from the genera Lactobacillus and Leuconostoc and were not detected in treatments without vinasse application (Fig. S6).

Another important observation is that nitric oxide (NO) is a natural product from amino acids reduction during fermentation processes as well from amino acids oxidation (Medinets et al., 2015). NO can also be used as an electron acceptor by microorganisms such as the non-denitrifier Anaeromyxobacter spp., which have the ability to reduce NO and N2O (Sanford et al., 2012). Thus, the question arises of whether the proportion of nitrous oxide reducers can improve the N2O balance. Nishizawa et al. (2014) showed that the inoculation into the soil of N2-generating denitrifiers (Azotococcus sp., Niastella sp. and Burkholderia sp.) reduced the release of N2O. Nevertheless, it is not adequate in fields with mineral fertilization because denitrifiers also tend to reduce the efficiency of the applied N. One promising approach is the overexpression of the
nosZ gene by denitrifiers to improve the N$_2$O balance, for instance, when N is not dependent on artificially fixed sources (Itakura et al., 2013). In theory, microorganisms lacking genes related to N$_x$O generation (NO and N$_2$O) or those with a greater affinity for N$_x$O than other N species could also be applied to fertilized fields (Miyahara et al., 2010). Sanford et al. (2012) proposed that the proportion of nitrous oxide reducers would improve the N$_2$O balance between the soil and the atmosphere, with special attention paid to the non-denitrifier Anaeromixobacter spp. This genus was among the most abundant taxa identified in this study (OTUs-50, -100, -133, -150, -236, -289, -434, -764, -1051, -1435, and -1593; Figs S5 and S7). Microorganisms that use trace gas as nutrient source or electron acceptors require efficient enzymatic systems because the concentration of these gases is very low (Conrad, 1996). By consensus, the more specialized the function is, the more diversity is required as the rare (micro) organisms exert these functions as has been shown for methane consumption (Pester et al., 2010). Thus, it is not clear whether trace gas consumption is a specialized niche or whether diversity is not required to maintain active specialized niches. Because Anaeromixobacter spp. can use sources other than N$_x$O as an electron acceptor (i.e., iron oxides; Hori et al., 2010), we assume that the reduction of trace gases is not a specialty of Anaeromixobacter. According to the thermodynamic theory, oxygen>nitrogen>manganese>iron>sulfur oxides are reduced sequentially (Ponnampuruma, 1972; Thauer et al., 1977). Therefore, in theory when N$_x$O are available in the environment, Anaeromixobacter spp. tend to use these gases rather than other oxides. Thus, N$_x$O reduction does not represent the niche of these microorganisms and the reduction of N$_x$O does not require specialized microorganisms. OTUs classified as Anaeromixobacter spp. were identified both by the global models and by the best fitted models explaining N$_2$O fluxes from soil with straw and soil with mineral N fertilization (Table 1). To date, there are four strains of Anaeromixobacter spp. with available genome sequences that are able to reduce NO and N$_2$O; however, they do not possess the capacity to produce NO from NO$_3^-$ (Sanford et al., 2012). Our findings linking this taxon to N$_2$O fluxes reinforce the hypothesis that Anaeromixobacter spp. are important contributors to N$_2$O consumption. However, in this present study, some OTUs of Anaeromixobacter spp. were overrepresented, while others were underrepresented under the management practices that increase N$_2$O emissions. These results indicate that the selection of groups related to N$_2$O balance at different taxonomical levels should be considered. For instance, our results suggest that different OTUs might share the same ability to perform a specific function, but they might respond differently under conditions of stress (i.e., fertilizers).

Most OTUs identified in this study seem to be related to processes involved in N$_2$O release. For example, one OTU of Nitrososphaera significantly explained the fluxes of N$_2$O on the global model in straw management (Table 1, Fig. 3b, Fig. S8). This archaeal genus is well recognized as an ammonia oxidizer (Spang et al. 2010) with the capacity for N$_2$O production from ammonia oxidation (Jung et al., 2014; Stiegler et al., 2014).

Jones et al. (2014) showed that 33% of the nosZ gene (encoding nitrous oxide reductase) sequences in diverse environmental samples were most closely related to Gemmatinoma spp., while 18% of all clones grouped with nosZ sequences were from phyla Bacteroidetes and Chloroflexi. These groups are members of the poorly characterized Clade II of the nitrous oxide-reducing microbial community (Jones et al., 2014), and they were represented in our fitted models explaining N$_2$O fluxes from sugarcane soil. However, due to their poor level of characterization and lack of experimental data tracing N$_2$O production or consumption such as for Anaeromixobacter spp., it is not possible to support a cause-effect relationship between these taxa and N$_2$O fluxes. It is also not possible to infer if they might contribute to N$_2$O production or consumption. In contrast, the well-characterized denitrifying Proteobacteria were identified within the OTUs explaining N$_2$O fluxes. For instance, strains of Burkholderia and Dyella have been tested to reduce the amount of N$_2$O released into the atmosphere after organic fertilization (Nishizawa et al., 2014). However, they might increase the N losses from the soil to atmosphere through denitrification and consequently reduce synthetic fertilizer efficiency. The OTU classified as Ammoniphilus sp. significantly explained N$_2$O fluxes in all fitted models (Table 1). Despite the fact that this genus is known to require high concentrations of ammonium and use oxalates exclusively as a C source, to date no metabolic pathways that could result in N$_2$O have been described for members of this genus. The available Ammoniphilus sp. type strains are not able to reduce NO$_3^-$ and are strictly aerobic (Zaitsev et al., 1998).

In an ideal context, a nitrogen fixer such as Azotococcus sp. that lacks the steps of denitrification prior to the production of N$_2$O (Krause et al., 2006) might be used to improve the N balance in bioenergy crops. Azotococcus strains have been tested for potential reduction of N$_2$O emissions from rice fields (Ishii et al., 2011; Nishizawa et al., 2014). For sugarcane, an analogous situation could arise with members of the family Acetobacteraceae. All of the known N-fixing organisms that are members of this family belong to the genera Acetobacter and Gluconacetobacter (Dutta & Gachhui, 2006). The association...
between these two genera and sugarcane was reported by James et al. (1994). These organisms are of special interest for sugarcane crop as they have the ability to fix N in aerobic conditions like well-drained soils. Additionally, *Acetobacter* and *Gluconacetobacter* can use a broad range of organic sources, including alcohols present in the vinasse (James et al., 1994; Parmaudeau et al., 2008). In our study, OTUs (OTU-71 and OTU-317, Fig. 3e) belonging to these two genera were strongly favored by vinasse organic fertilization and as showed by Assis Costa et al. (2015) they are also present in the ethanol production process. The same *Acetobacter* OTU favored by vinasse organic fertilization significantly explained N$_2$O fluxes from soil with straw by the global fitted model. Thus, *Acetobacter* and *Gluconacetobacter* should be highlighted as the closest representatives to an ideal situation for the improvement of N-cycling in sugarcane, both by acting as N-fixing organisms and potential drivers of processes related to N$_2$O release. Furthermore, *Acetobacter* and *Gluconacetobacter* could be added to vinasse used as organic fertilizer in sugarcane fields, and thus, contributing to redefine vinasse from a source to a sink of N$_2$ and N$_x$O. However, it must be acknowledged that the survival of microorganisms inoculated in the soil system is a challenge.

To our knowledge, this is the first study relating specific microbial taxa to the respective functional response (N$_2$O release) along the time and under different management practices in tropical soil. Here, we show that the limitation of linking microbial taxa with functional traits is particularly challenging because the microbial responses are at different taxonomical levels such as at OTU and genus levels, as observed for *Anaeromyzobacter* and *Roseiflexus*, respectively.

The effects of fertilization and straw on N$_2$O emissions have been previously reported (Carmo et al., 2013), however, not taken into account the microbial community. Besides the common Proteobacteria model organisms for denitrification, we have found taxa recently described as potential drivers of N$_2$O production and consumption (Jones et al., 2013). Additionally, we have identified taxa with potential biotechnological properties that might improve the sustainability of bioethanol by increasing C yields and improving N efficiency in sugarcane fields.

Acknowledgements

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Soil physicochemical characteristics of the experimental site.

**Table S2.** Chemical composition of the normal vinasse and concentrated vinasse applied as potassium source in soil.

**Table S3.** Parameter estimation for fixed effects on global and best models fitted to explain N₂O fluxes from soil considering phys-chemical parameters and management practices.

**Fig S1** Box-plot representation of ammonium concentration in soil after fertilization.

**Fig S2** Box-plot representation of nitrate concentration in soil after its fertilization.

**Fig S3** Cumulative (%) nitrous oxide emissions from soil under different treatments.

**Fig S4** Relative abundance of *Nitrosospira* sp.

**Fig S5** Intercept of the negative binomial generalized linear model fitted to verify the effect of treatments on abundance of OTUs.

**Fig S6** Relative abundance of Firmicutes OTUs that significantly explain N₂O fluxes.

**Fig S7** Relative abundance of Proteobacteria OTUs that significantly explain N₂O fluxes.

**Fig S8** Relative abundance of OTUs belonged to other phyla than Firmicutes and Proteobacteria that significantly explain N₂O fluxes.

**File S1.** Global, nested and best models fitted with microbial taxa to explain N₂O fluxes from soil.

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