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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 N_2O 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 N_2O fluxes by combining functional responses (N_2O 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 N_2O 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 N_2O 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 (CH_4) and nitrous oxide (N_2O) 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.

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In addition to plant straw, a second by-product of the sugarcane ethanol industry is vinasse (Mutton *et al*.,

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2010). 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 (Bouwman *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 being 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 *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 *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 (Critchfield, 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 ($n = 4$; 8×9 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 (1.10^5 l ha^{-1}) and mineral fertilizer containing P; CV (concentrated vinasse) – concentrated vinasse as K source (1.10^4 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 N_2O (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 NO_3^- and Krom (1980) for NH_4^+.

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 (GTGCCAGCMGCCGCGG-TAA) and 806R (TAATCTWTGGGVHCATCAGG) (Caporaso *et al*., 2010) and the LIB-L kit for unidirectional sequencing. Duplicate 50 μl PCRs were performed as follows: 5 μl Roche 10 × PCR buffer with $MgCl_2$, 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 Qiagen 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, Brandford, 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 taxa abundance on N_2O 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 'MuMIn' R package version 1.10.5 (Barton, 2014) with the Akaike information criterion (AIC) for ranking. To verify the effect of OTUs on N_2O 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 N_2O 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 ($P < 0.001$), N fertilization ($P < 0.001$), straw ($P = 0.016$), and moisture ($P = 0.004$) increased the fluxes of N_2O from the soil. In contrast, NO_3^- reduced ($P = 0.065$) the fluxes of N_2O from the soil. Ammonium (Fig. S1) and soil temperature did not explain N_2O 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 N_2O 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 N_2O fluxes but also reduced soil 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 N_2O fluxes than soil inorganic N. The parameter estimation for fixed effects in global and best fitted models used to explain the N_2O fluxes from soil considering physicochemical parameters and management practices are presented in Table S3. Despite the high N_2O peaks verified in treatments without straw (Fig. 1), annual N_2O 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 N_2O fluxes compared to treatments without straw (illustrated in Fig. 1).

During the first 60 days of sampling, the cumulative N_2O 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 N_2O 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 N_2O 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_2O 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.

Archaeal 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-

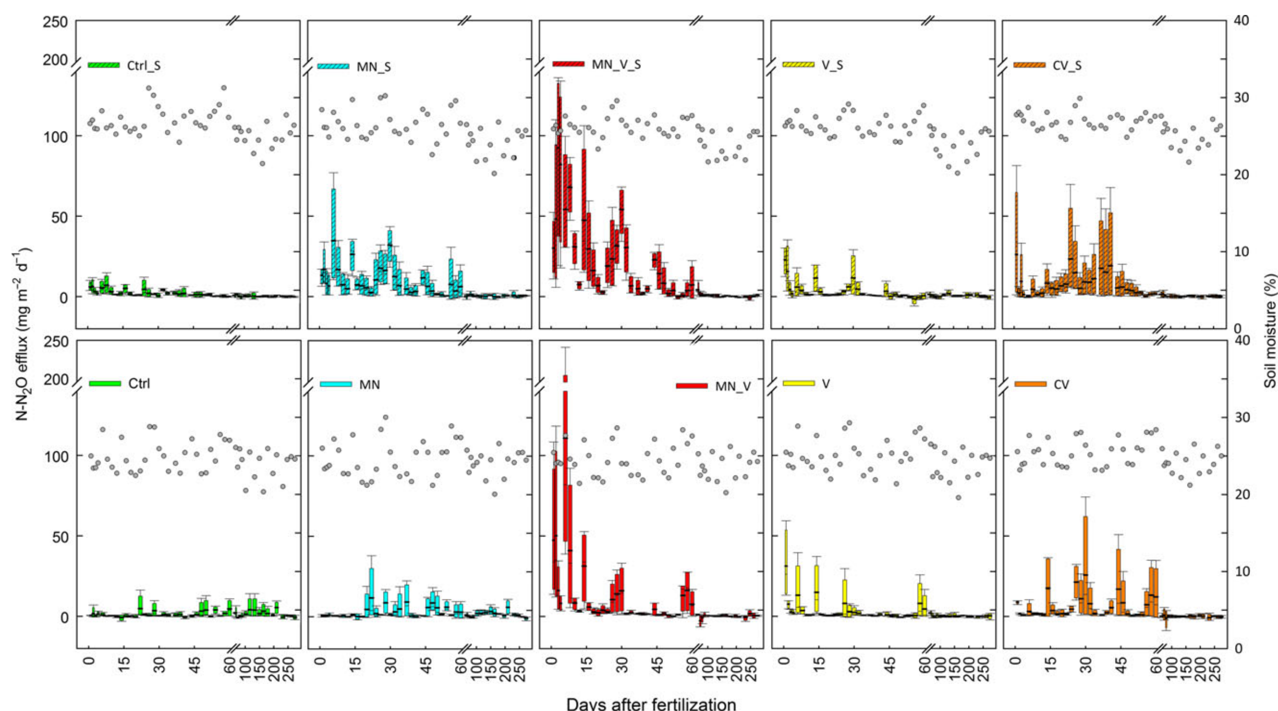

Fig. 1 Box-plot representation of nitrous oxide fluxes from soil with sugarcane crop after soil fertilization. Ctrl: no nitrogen addition; MN: mineral nitrogen fertilization; V: vinasse; CV: concentrated vinasse; S: straw left on the soil. *Daily measurements for samplings 1–4; 3–4 times/week measurements for samplings 5–31; once a week measurements for samplings 32–35; fortnightly measurements for samplings 36–47 (just before the crop harvesting). The first sampling was 1 day after fertilization.

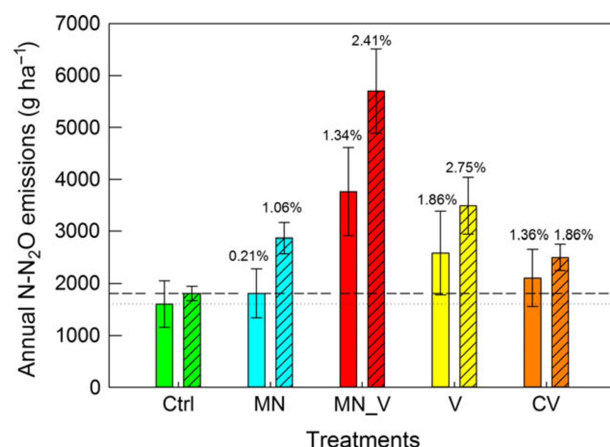

Fig. 2 Annual nitrous oxide emissions from soils with sugarcane and respective emission factors (%). Bars are the standard errors. Reference lines are the calculated emissions from the control (Ctrl) treatments without straw (dotted line) and with straw (dashed line). Ctrl: no nitrogen addition; MN: mineral nitrogen fertilization; MN_V: mineral nitrogen plus vinasse fertilization; V: vinasse; CV: concentrated vinasse. Transversal lines: treatments with straw left on soil.

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

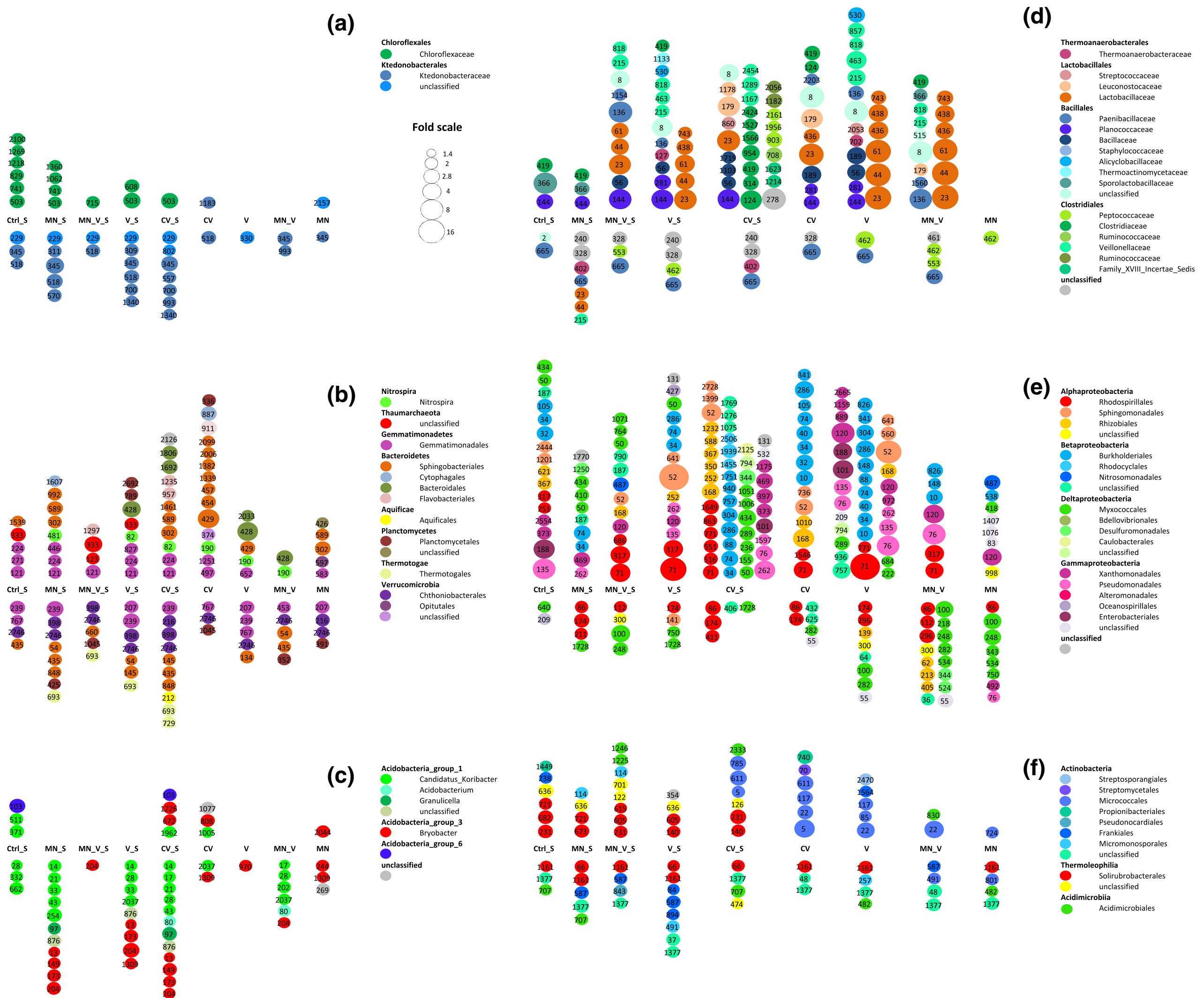

Fig. 3 Effect of different treatments on OTUs belonging to Chloroflexi (a), Acidobacteria (c), Firmicutes (d), Proteobacteria (e), Actinobateria (f), or other phyla (b). Numbers represent the OTU identification. Color groups at Family (a, d), Order (b, e, f), or Genus (c) level. Circle size indicates the fold change of the respective OTU when compared to the control treatment (Ctrl). Overrepresented OTUs are in the upper part of the plot and underrepresented OTU are in the below part of the plot. Ctrl_S: no N with straw; MN_S: mineral nitrogen with straw; MN_V_S: mineral nitrogen plus vinasse with straw; V_S: vinasse with straw; CV_S: concentrated vinasse with straw; CV: concentrated vinasse; V: vinasse; MN_V: mineral nitrogen plus vinasse; MN: mineral nitrogen.

genus *Nitrososphaera*. 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 *Micrococales* from the class *Actinobacteria* was overrepresented in soils with the absence of straw, while the OTU belonging to the order *Micronomosporales* 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 *Chthoniobacterales* presented negative fold changes in all treatments (Fig. 3b). One of these OTUs (OTU-398, classified as *Candidatus Xiphinematobacter*) 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_2O fluxes

After identifying the management practices (i.e., straw, mineral nitrogen fertilization, and organic fertilization) that increased N_2O 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_2O 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_2O 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 *Rummeliibacillus* OTUs, significantly explaining the N_2O 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

Table 1 OTUs that explain N_2O 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

	Global model§			Best model			Taxonomy			
OTU	S	MN	V	S	MN	V	Phylum	Order	Family	Genus
103				†			Acidobacteria			*Acidobacteria Gp6*
5	***			*			Actinobacteria	*Actinomycetales*	*Micrococcaceae*	*Arthrobacter*
231	*						Actinobacteria	*Solirubrobacterales*	*Unclassified*	
429	*			**			Bacteroidetes	*Sphingobacteriales*	*Sphingobacteriaceae*	*Sphingobacterium*
503						**	Chloroflexi	*Chloroflexales*	*Chloroflexaceae*	*Roseiflexus*
23		*	***		*	***	Firmicures	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
61		**	*		**	***	Firmicures	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
436	*						Firmicures	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
179			*			*	Firmicutes	*Lactobacillales*	*Leuconostocaceae*	*Leuconostoc*
56	*						Firmicutes	*Bacillales*	*Bacilaceae*	*Bacillus*
136	***	**	*	***	**	**	Firmicutes	*Bacillales*	*Paenibacillaceae*	*Ammoniphilus*
144	*						Firmicutes	*Bacillales*	*Paenibacillaceae*	*Rummeliibacillus*
189			*			***	Firmicutes	*Bacillales*	*Bacilaceae*	Unclassified
366	***			***			Firmicutes	*Bacillales*	*Sporolactobacillaceae*	*Sporolactobacillus*
124			*			*	Firmicutes	*Clostridiales*	*Clostridiaceae*	*Clostridium*
215					**		Firmicutes	*Selenomonadales*	*Veillonellaceae*	*Megasphaera*
121			***			***	Gemmatimonadetes	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*
224	**						Gemmatimonadetes	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*
34	**		**				Proteobacteria	*Burkholderiales*	*Comamonadaceae*	Unclassified
40			*				Proteobacteria	*Burkholderiales*	*Burkholderiaceae*	*Burkholderia*
74			*			†	Proteobacteria	*Burkholderiales*	*Oxalobacteraceae*	Unclassified
88	*		*	†			Proteobacteria	*Burkholderiales*	*Burkholderiaceae*	*Burkholderia*
341	*						Proteobacteria	*Burkholderiales*	*Burkholderiaceae*	*Ralstonia*
826						***	Proteobacteria	*Burkholderiales*	Unclassified	
101						*	Proteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	Unclassified
50		*			**		Proteobacteria	*Myxococcales*	*Cystobacteraceae*	*Anaeromyxobacter*
289	***			*			Proteobacteria	*Myxococcales*	*Cystobacteraceae*	*Anaeromyxobacter*
434				*			Proteobacteria	*Myxococcales*	*Cystobacteraceae*	*Anaeromyxobacter*
317	*						Proteobacteria	*Rhodospirillales*	*Acetobacteraceae*	*Acetobacter*
641			***			***	Proteobacteria	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*
131	*			**			Proteobacteria	Unclassified		
120			*			**	Proteobacteria	*Xanthomonadales*	*Xanthomonadaceae*	*Dyella*
373	**			*			Proteobacteria	*Xanthomonadales*	*Xanthomonadaceae*	*Arenimonas*
469	*						Proteobacteria	*Xanthomonadales*	*Sinobacteraceae*	*JTB255*
333	*						Thaumarchaeota	*Nitrososphaerales*	*Nitrososphaeraceae*	*Nitrososphaera*
172					‡		Unclassified			
206		*			**		Unclassified			
242	**	*	*		†	‡	Unclassified			

§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.

that explain N_2O 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 N_2O 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-

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 *Nitrososphaera* 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 N_2-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 N_2-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 N_2O 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 N_2O 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 N_2O 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 N_2O release as truncated denitrification pathways are found in *Lactobacillus* spp. (Shapleigh, 2013). Four of the taxa that explained N_2O 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 nondenitrifier *Anaeromyxobacter* spp., which have the ability to reduce NO and N_2O (Sanford *et al*., 2012). Thus, the question arises of whether the proportion of nitrous oxide reducers can improve the N_2O balance. Nishizawa *et al*. (2014) showed that the inoculation into the soil of N_2-generating denitrifiers (*Azoarcus* sp., *Niastella* sp. and *Burkholderia* sp.) reduced the release of N_2O. 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

*nos*Z gene by denitrifiers to improve the N_2O balance, for instance, when N is not dependent on artificially fixed sources (Itakura *et al.*, 2013). In theory, microorganisms lacking genes related to NxO generation (NO and N_2O) or those with a greater affinity for NxO 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_2O 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 *Anaeromyxobacter* spp. can use sources other than NxO 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 *Anaeromyxobacter*. According to the thermodynamic theory, oxygen>nitrogen>manganese>iron>sulfur oxides are reduced sequentially (Ponnamperuma, 1972; Thauer *et al.*, 1977). Therefore, in theory when NxO are available in the environment, *Anaeromyxobacter* spp. tend to use these gases rather than other oxides. Thus, NxO reduction does not represent the niche of these microorganisms and the reduction of NxO does not require specialized microorganisms. OTUs classified as *Anaeromyxobacter* spp. were identified both by the global models and by the best fitted models explaining N_2O fluxes from soil with straw and soil with mineral N fertilization (Table 1). To date, there are four strains of *Anaeromyxobacter* spp. with available genome sequences that are able to reduce NO and N_2O; however, they do not possess the capacity to produce NO from NO_2^- (Sanford *et al.*, 2012). Our findings linking this taxon to N_2O fluxes reinforce the hypothesis that *Anaeromyxobacter* spp. are important contributors to N_2O consumption. However, in this present study, some OTUs of *Anaeromyxobacter* spp. were overrepresented, while others were underrepresented under the management practices that increase N_2O emissions. These results indicate that the selection of groups related to N_2O 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_2O release. For example, one OTU of *Nitrososphaera* significantly explained the fluxes of N_2O 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_2O production from ammonia oxidation (Jung *et al.*, 2014; Stieglmeier *et al.*, 2014).

Jones *et al.* (2014) showed that 33% of the *nos*Z gene (encoding nitrous oxide reductase) sequences in diverse environmental samples were most closely related to *Gemmatinoma* ssp., while 18% of all clones grouped with *nos*Z 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_2O fluxes from sugarcane soil. However, due to their poor level of characterization and lack of experimental data tracing N_2O production or consumption such as for *Anaeromyxobacter* spp., it is not possible to support a cause–effect relationship between these taxa and N_2O fluxes. It is also not possible to infer if they might contribute to N_2O production or consumption. In contrast, the well-characterized denitrifying Proteobacteria were identified within the OTUs explaining N_2O fluxes. For instance, strains of *Burkholderia* and *Dyella* have been tested to reduce the amount of N_2O 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_2O 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_2O 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 *Azoarcus* sp. that lacks the steps of denitrification prior to the production of N_2O (Krause *et al.*, 2006) might be used to improve the N balance in bioenergy crops. *Azoarcus* strains have been tested for potential reduction of N_2O 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; Parnaudeau *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_2O 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_2O 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 NxO. However, it must be acknowledged that the survival of microrganisms 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_2O 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 *Anaeromyxobacter* and *Roseiflexus*, respectively.

The effects of fertilization and straw on N_2O 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_2O 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.

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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 N_2O fluxes from soil considering physic-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 N_2O fluxes.
Fig S7 Relative abundance of Proteobacteria OTUs that significantly explain N_2O fluxes.
Fig S8 Relative abundance of OTUs belonged to other phyla than Firmicutes and Proteobacteria that significantly explain N_2O fluxes.
File S1. Global, nested and best models fitted with microbial taxa to explain N_2O fluxes from soil.