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The study described in this thesis was performed at the Netherlands Institute of Ecology, NIOO-KNAW; the practical work was performed at the Paulista Agency for Agribusiness Technology (APTA), Agronomic Institute of Campinas (IAC) and Netherlands Institute of Ecology (NIOO-KNAW).

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"It always seems impossible until it's done."

Nelson Mandela

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Chapter 1

General introduction

Modern agriculture is dependent of mineral fertilizers and it is expected that this will increase in the next decades. World fertilizer nutrient ($N+P_2O_5+K_2O$) consumption was estimated to be around 187 million tons in 2016 (FAO, 2017). In order to reduce the abundant use of mineral fertilizers the recycling of organic residues and the optimization of the use of nutrients in agriculture are widely used strategies. Organic residues are produced in huge amounts and in some extent have been considered contaminants. However, the application of organic residues as fertilizer is one of the best options to decrease this problem. Organic residues can be an important source of nutrients for crops, especially in regions nearby the production site, and these residues can replace a significant portion of the inorganic fertilizers input (Ussiri et al., 2009; Christofoletti et al., 2013; Trivelin et al., 2013). Furthermore, application of organic residues has been proposed as a useful option to improve soil structure and protection by reducing erosion and runoff (Rossetto et al., 2010; Boulal et al., 2011; Bhattacharyya et al., 2013; Jemai et al., 2013; Brouder and Gomez-Macpherson, 2014; Carvalho et al., 2017; Menandro et al., 2017). However, the inadequate and indiscriminate discharge of residues in the environment may cause an unwanted disturbance of the soil system. If residues are applied beyond the soil retention capacity or above the plant nutrient requirements soil, water and atmosphere contamination may occur (Carmo et al., 2013; Di et al., 2014; Navarrete et al., 2015a; Pitombo et al., 2015; Tao et al., 2015; Castro et al., 2017). Besides, the application of organic residues in the soil may also affect seriously the soil microbial community and consequently the process carried out by the soil biota including the production of greenhouse gases (GHG), *i.e.* CO_2, CH_4 and N_2O as it has been observed after vinasse and sewage sludge applications in soil as fertilizer (Carmo et al., 2013; Pitombo et al., 2015; Tao et al., 2015; Soares et al., 2016; Suleiman et al., 2016).

Microbial communities can change abruptly in response to perturbations and may recover quickly to its original state. Understanding of how organic residues in combination with mineral fertilizer and seasonal climatic variations affect the diversity, composition and dynamics of the resident soil microbes is required to reduce negative side effects of its application in agriculture. Only by using time series approaches the stability and dynamics of microbial communities' response to perturbations can be assessed properly. Thus, the main objectives of the study described in this thesis are to assess the impact of bioenergy organic residue amendments , *i.e.* vinasse and sugarcane straw, on the structure and functioning of the soil microbial community and to determine the link with the nitrous oxide (N_2O) production and emission, which is the most important GHG emitted from sugarcane soils (Cerri et al., 2009), after the application of these residues. The information from this study may help to develop and implement sustainable agricultural cropping systems in which recycling of residues is linked with adequate nutrient management without side effects of GHG's emissions and/or nutrient runoff and leaching.

1. Vinasse

Brazil is the world's largest producer of sugarcane, and the second largest producer of ethanol, with about 685 million tons of sugarcane produced in 2016/2017 on an area of 9 million hectares (CONAB, 2017). São Paulo state has the largest area of sugarcane, approximately 52% of the total area of sugarcane in Brazil. Moreover, 53% of the total Brazilian sugarcane production is destined for the production of ethanol (CONAB, 2017). Up to date, ethanol from sugarcane is considered one of the most economical and sustainable biofuels in the world so far (Goldemberg et al., 2008) and one of the best options to replace fossil fuels (Lisboa et al., 2011). Studies conducted by Macedo et al. (2008) and Seabra et al. (2011) indicated that ethanol emits about 80% less GHG's than gasoline. However, some management practices may counter this benefit, for example, the recycling of the residues generated during the ethanol production in the sugarcane fields as organic fertilizer and the application of inorganic nitrogen (N) fertilizer (Galdos et al., 2010; De Figueiredo and La Scala Jr, 2011; Carmo et al., 2013; Pitombo et al., 2015; Siqueira Neto et al., 2016). Depending on the management practices, the N_2O emitted from organic and inorganic fertilization during the sugarcane crop season can increase the total amount of GHG emitted to the atmosphere from the production and use of ethanol to a level similar to that of the use of fossil fuel (Crutzen et al., 2008; Lisboa et al., 2011; Carmo et al., 2013).

Vinasse is a major residue generated during sugarcane fermentation to ethanol (Figure 1). For each liter of ethanol produced, 10 to 15 liters of vinasse are generated. It was estimated in 2016/2017, that Brazil produced up to 360 billion liters of vinasse per year (27.5 billion liters of ethanol) (CONAB, 2017). Vinasse is a dark-brown wastewater with high organic content (biochemical oxygen demand of 2-20.8 mg L^{-1} and chemical oxygen demand of 2-49.5 mg L^{-1}), rich in potassium (2056 mg L^{-1}), and nitrogen (357 mg N L^{-1}) (Elia-Neto and Nakahodo, 1995; Macedo et al., 2008; Christofoletti et al., 2013; Fuess and Garcia, 2014). The chemical composition of sugarcane vinasse is quite variable and varies with sugarcane variety, stage of plant development, soil type and distillation process (Christofoletti et al., 2013; Mutton et al., 2014) (Figure 1). Thus, effluents (vinasse) from the distillation of molasses, sugarcane juice or the combination of both are different, depending on whether the industry is producing ethanol or sugar in a certain period of the year (Christofoletti et al., 2013; Fuess and Garcia, 2014). Higher sugar production rates increase the volumes of molasses, a residue that is obtained after evaporation and crystallization and subsequently directed to the production of ethanol (Figure 1), providing vinasse with high levels of organic and inorganic compounds. In contrast, the direct use of sugarcane juice to fermenters provides a more diluted vinasse in terms of organic and inorganic compounds.

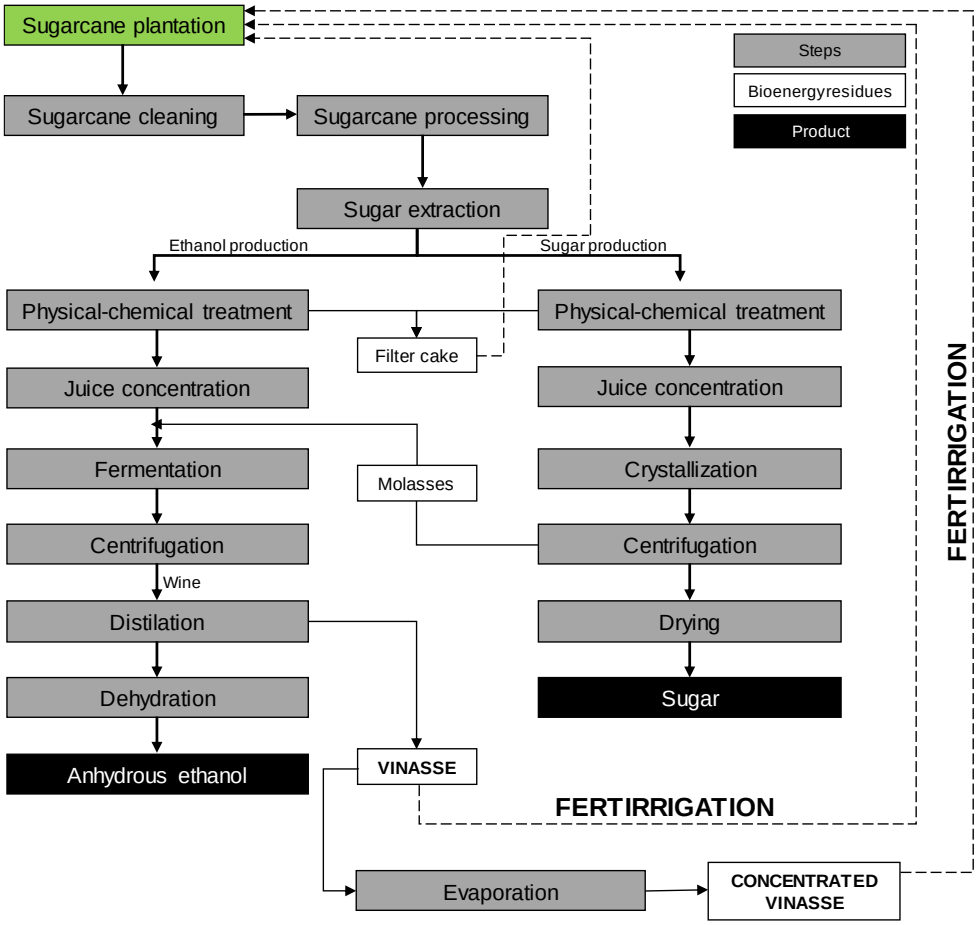

Figure 1 | Simplified flowchart of Brazilian sugarcane-based biorefineries for the production of ethanol and sugar and the associated production of residues; adapted from Fuess et al. (2017).

Because of its chemical characteristics, especially the potassium concentration, vinasse is often directly applied on sugarcane fields as liquid organic fertilizer, which process is called fertirrigation (i.e., the utilization as a liquid fertilizer for plants) (Silva et al., 2014). Although there are different methods to recycle vinasse, including the use as fodder (Christofoletti et al., 2013), incineration for production of energy (Akram et al., 2015) and fermentation (Moraes et al., 2015), fertirrigation is the number one management method of vinasse recycling in Brazil. Due to the high amount of potassium as mentioned before, its effectiveness in terms of potassium fertilization is equivalent to that of an inorganic fertilizer. By law the concentration of potassium in both soil and vinasse must be taken into account for proper application of vinasse in sugarcane fields (Uyeda et al., 2013; CETESB, 2014), as high levels of vinasse may cause soil and groundwater contamination. However, vinasse cannot always be used in fertirrigation due to the

huge volume and high costs of transport to the field. Concentration of vinasse by evaporation, therefore, is an option to reduce the volume without loss of nutrients and so to reduce the transportation costs (Christofoletti et al., 2013). This procedure increased largely in recent years. Concentrated vinasse is applied in the plant row similarly to the application of inorganic fertilizer allowing higher amounts of nutrients close to the plants. However, there is little information about the efficiency of concentrated vinasse as fertilizer and information on its environmental impacts is scarce.

Despite its benefits, ethanol from sugarcane has been highly criticized for its negative environmental effects (Fuess et al., 2017; Rodrigues Reis and Hu, 2017). One of the main points of criticism concerned the use of vinasse. Vinasse has been shown to have negative effects on soil, groundwater and crops on the long term (Christofoletti et al., 2013). Vinasse can cause soil salinization, as the continuous application of this residue leads to the accumulation of salts in the soils. The acid characteristic of the vinasse (pH 3.0–4.7) can also cause acidification of water resources (Fuess et al., 2017; Rodrigues Reis and Hu, 2017). The input of organic carbon and organic N from vinasse, may lead to the reduction of the oxygen present in soil and groundwater directly effecting the microbial activity, and consequently changing soil processes, for example favoring denitrification and so N_2O production (Carmo et al., 2013). While for many agriculture and industrial residues (e.g., municipal wastewater, swine manure), a vast literature about the impact of residues on soil physical, chemical and biological constitution, including the resident soil microbial community, is available, for vinasse this information is limited. In addition, the process of ethanol production from sugarcane does not occur under sterile conditions and, so, the contamination of soil and water by microbes inhabiting the vinasse complex may also occur (Costa et al., 2015a; Brexó and Sant'Ana, 2017). In general, the main contaminants during ethanol production include *Acetobacter*, *Bacillus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Staphylococcus*, *Streptococcus* and *Weissella* (Costa et al., 2015a; Brexó and Sant'Ana, 2017). Costa et al. (2015a) found that after fermentation of the wine stage where vinasse is produced, *Lactobacillus* dominated the microbial community of contaminants. To our knowledge, up to date, there is no study on the fate of microbial contaminantes in the vinasse residue, consequently no study has been published on the potential invasion of these microbes in soils receiving the vinasse.

2. Microbial community responses to disturbances

The microbial community composition of soils is influenced by physical, chemical, and biological factors, and by management and environmental disturbances. These disturbances include tillage (Sengupta and Dick, 2015), cover cropping (Navarrete et al., 2015a), crop rotation (Soman et al., 2016), fertilization (Su et al., 2015; Cassman et al., 2016), and organic amendments (Navarrete et al.,

2015a; Suleiman et al., 2016; Lupatini et al., 2017). Furthermore, also soil type, (Ulrich and Becker, 2006; Wakelin et al., 2008; Lupatini et al., 2013a; Mendes et al., 2015a; Mendes et al., 2015b), pH and other chemical factors (Lauber et al., 2008; Kuramae et al., 2011; Kuramae et al., 2012; Navarrete et al., 2015b; Ying et al., 2017), moisture (Stark and Firestone, 1995; Valverde et al., 2014), and temperature (Lipson, 2007; Prevost-Boure et al., 2011), as well as shifts in seasonality (Bardgett et al., 1999; Steenwerth et al., 2006; Buckeridge et al., 2013) can alter the microbial community functions and composition. Many of these factors interact with each other and have both direct and indirect effects on the soil microbial community. For example, straw left on top of the soil would add organic carbon to the soil through decomposition, and it would reduce water evaporation (Carvalho et al., 2017). Moreover, the application of organic residues as fertilizer introduce not only organic carbon to the soil, but also mineral nutrients and, depending on the type of the residue, it may change substantially soil pH (Silva et al., 2014), which may counter the stimulatory effect of extra carbon input (Canellas et al., 2003).

Soil microbes are primary mediators of organic matter decomposition (Kuramae et al., 2013) and nutrient cycling (Rousk and Bengtson, 2014). Organic and inorganic fertilizer amendments are used to increase nutrient availability to plants, but they can also affect the soil microbial community and its functionality by directly or indirectly affecting the physical and chemical properties of soil. The application of organic and inorganic fertilizers may disturb microbial communities such that community members die or change their abundances (Rykiel, 1985; Suleiman et al., 2016). Disturbances are often classified as pulses or presses depending on their duration (Bender et al., 1984; Shade et al., 2012). In general, organic and inorganic fertilizer additions are pulse disturbances, they are relatively discrete, short-term events, whereas presses are long-term or continuous, such as liming, that change the soil pH. The soil microbial community may show to be resistant or resilient to the disturbances or if the community appears to be sensitive, it may perform differently (Figure 2) or appears to be functionally redundant. Resistance is defined as the degree to which a community is insensitive to a disturbance (Allison and Martiny, 2008) and resilience is the phenomenon that a community returns to its original composition after being disturbed (Allison and Martiny, 2008); commonly referred to as community recovery (Shade et al., 2012; Griffiths and Philippot, 2013). Finally, functional redundancy refers to the property that even when the community composition is sensitive and not resilient or resistant, its functions remain similarly to the original community (Allison and Martiny, 2008). The functionally redundant microbial community is related to the presence of functionally redundant species in the community. However, the concept of functional redundancy remains controversial (Shade et al., 2012). Thus, depending on the disturbance, duration and microbial community stability, the community's response can differ substantially.

The stability of microbial communities can be investigated in terms of functional or compositional parameters. If functions are carried out by many taxa (Schimel, 1995) changes in community composition may not lead to functional changes (Allison and Martiny, 2008). On the contrary, if functions are performed by few microbes, changes in the community composition may change these functions. Shade et al. (2012) analysed 378 studies of microbial responses to biotic and abiotic disturbances, in 82% of the cases the community appeared to be sensitive to the disturbance, 31% were changes in composition, 26% in functionality and 43% showed changes in both composition and function. Only a few studies measured resilience (Shade et al., 2012) and a small fraction, 23%, the community returned to the pre-disturbance condition, of which 56% in composition, 35% in function, and 9% to both. The authors also reported that microbial communities may be more resilient after short-term than after long-term disturbances. Besides recovery from short-term disturbances was reported by Shade et al. (2012) more often for the microbial community functionality than for the composition, while recovery from long-term disturbances was approximately the same for both function and composition.

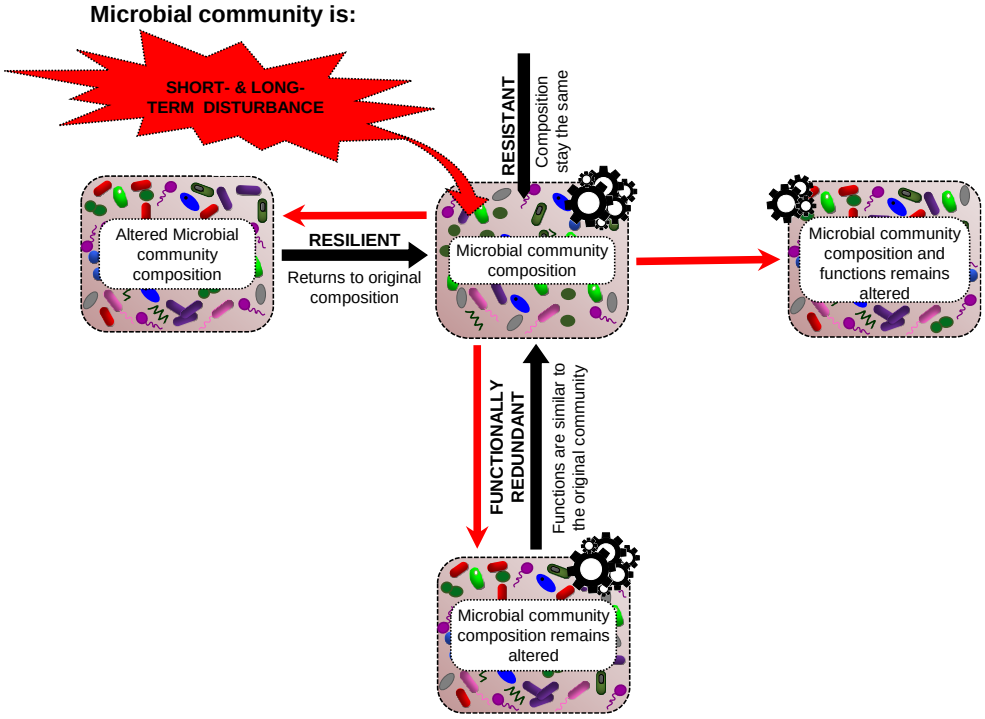

Figure 2 | Scheme of how disturbances can change microbial community composition and functions. Adapted from Allison and Martiny (2008).

Organic residues may differ in organic matter composition, for example C/N ratio, which affects the decomposition rate and the microbial community structure and function. For instance, the presence of labile organic components in

the organic residue promotes the growth of microorganisms with copiotrophic lifestyle that grow rapidly in nutrient-rich environments compared to organisms adapted to nutrient-poor conditions (oligotrophic lifestyle) (Navarrete et al., 2015a), while straw additions enhance cellulolytic microorganisms (Kuramae et al., 2013; Kielak et al., 2016b). Thus, application of inorganic or organic compounds on a short or long-term basis might result in positive, neutral or negative effects in soil microbial community structure (Biederbeck et al., 1996; Hu et al., 2011; Williams et al., 2013; Balota et al., 2014; Cassman et al., 2016; Suleiman et al., 2016). In general soil microbial communities are resilient to biotic disturbances and usually exclude successfully exotic organisms (Levine and D'Antonio, 1999). Suleiman et al. (2016) documented that pig manure used as fertilizers affected microbial functional diversity, and changed the microbial structure temporarily. The metabolically active microbial community was resilient recovering to its original status. Nevertheless, there is so far very little evidence of a connection between alterations on microbial community composition and function over time series after input of bioenergy organic residues.

Microbial community responses to pulse- and press-type disturbances are important to consider in the context of the sustainability of bioethanol production and global climate change. The organic residues produced during sugar and ethanol production, i.e. straw and vinasse do affect the microbial community structure (Navarrete et al., 2015a; Pitombo et al., 2015). Results of field studies have shown that different management strategies with straw (Huang et al., 2012) and vinasse (Navarrete et al., 2015a), alter the soil bacterial community composition. In general, straw application increases the microbial community metabolic activity (Navarro-Noya et al., 2013) and vinasse amendment causes positive or negative effects on specific microbial groups (Pitombo et al., 2015). Thus, understanding of how microbial communities and functions change over time after vinasse and straw applications is important to understand processes such as succession after or recovery from perturbations and so to assess the consequences of the use of these residues in tropical agricultural systems.

Also changes in climatic conditions through changes in water content and temperature are important factors regulating the composition and activity of microbial communities in soils (Bell et al., 2008). Thus, the responses of the soil microbial community to organic and inorganic fertilizers will be season dependent. For example in a rainy season the labile organic carbon input from organic fertilizers may be less important than in a dry season, due the larger decomposition of native soil organic matter under rainy conditions. Previous studies showed that water content plays an important role in the composition and diversity of microbial communities over seasons in environments such as sediments (Valverde et al., 2014), forest soils (Bouskill et al., 2013) and agriculture soil (Phillips et al., 2015). Low water content inhibits microbial activity by restricting substrate supply and selecting for only species adapted to survive under these conditions (Stark and Firestone, 1995; Valverde et al., 2014). The maximum aerobic microbial activity

occurs at moisture levels of around 70% of water holding capacity. Changes in temperature may also influence the structure of bacterial communities and temperature is positively correlates with microbial activity (Lipson, 2007). Seasonal variations in water content and temperature have considerable impact on important processes such as organic matter decomposition (Stark and Firestone, 1995; Karhu et al., 2014). However, it is only poorly understood how microbial communities respond to seasonal variations in moisture and temperature after application of mineral and organic residues. Few studies show that seasonality may affect the structure of microbial communities and functional properties, suggesting that microbial dynamics is influenced by seasonal variability (Smith et al., 2015). On the other hand, others studies showed that bacterial communities are not strongly tied to seasonal variations (Landesman and Dighton, 2010). The central-Southern region of Brazil, i.e. the most important region for sugarcane production, has two defined seasons, rainy summers with high temperature and dry winters with mild temperatures. Therefore, understanding the impact of seasonal variability in combination with fertilization on the soil microbial community will help to develop better strategies to optimize the use of mineral and organic fertilizers.

3. Greenhouse gas emissions

The increase in the concentration of greenhouse gases (GHG) in the atmosphere after the industrial revolution is one of the main problems causing global warming. Nitrous oxide (N_2O), carbon dioxide, (CO_2) and methane (CH_4) are the main GHG emitted due to anthropogenic activities. The global warming potentials of N_2O and CH_4 are 298 and 34 times greater than CO_2 (IPCC, 2013). In addition, N_2O is one of the main molecules that are responsible for the destruction of ozone layer (Ravishankara et al., 2009).

In Brazil, N_2O is the most important GHG emitted from sugarcane soils (Cerri et al., 2009). Recent studies showed that N_2O emissions from inorganic fertilizer are lower than reported by Crutzen et al. (2008). They claimed that 3 to 5% of the total N applied, and Lisboa et al. (2011) claimed 3.9% of N applied being emitted as N_2O from sugarcane fields (Vargas et al., 2014; Soares et al., 2015; Siqueira Neto et al., 2016). Such high N_2O emissions almost denied the use of sugarcane biofuel as an option to decrease GHG emission. However, other studies showed that the N_2O emission from sugarcane fields in Brazil ranged from 0.2 to 1% of applied N (Filoso et al., 2015) which is even lower than the default value of 1% of the N applied in the field (IPCC, 2013). These data suggest that sugarcane might be a sustainable alternative bioenergy source in terms of the reduction of GHG emissions as compared to fossil fuel (Boddey et al., 2008; Crutzen et al., 2008; Galdos et al., 2010). However, when vinasse was applied with N fertilizer, the emissions increased up to 3% of applied N (Carmo et al., 2013). Similar results were obtained by Pitombo et al. (2015), who found that the proportion of N emitted

as N_2O was 2.4% when vinasse and N were applied combined in the soil. Paredes et al. (2014) also examined the effect of vinasse and fertilizer application in a field experiment. The N_2O emission after application of inorganic N was 0.2%, but reached 0.6 and 0.7% when N was applied with vinasse with a difference of application timing over two days in the same area. The authors found similar results when vinasse was applied with a delay of 3 or 15 days related to the moment of inorganic fertilizer application; 0.77% and 0.78% of applied N was lost as N_2O (Paredes et al., 2015) against 0.58% of N applied when only inorganic N was applied. The results of N_2O emissions in literature are quite variable, but in most cases application of vinasse with mineral N in the same area increased N_2O emissions.

The high N_2O emissions observed in studies when vinasse is applied were assigned to the increase in soil microbial respiration (Carmo et al., 2013; Paredes et al., 2014; Paredes et al., 2015) and high water content (Barton and Schipper, 2001; Carmo et al., 2013). Barton and Schipper (2001) observed similar results on the increase of emissions of N_2O and CO_2 in soils that received inorganic N plus dairy farm effluent when compared to inorganic fertilizer applied with water. The authors impute these increased emissions to the larger organic C availability, higher soil water content and lower aeration resulting in depletion of O_2 in the soil, which stimulate the production of N_2O by denitrification.

Furthermore, the soil reactions that result in GHG emissions are affected by climatic conditions. The sugarcane harvest period in São Paulo State and in Central-Southern region of Brazil is between April and November, which covers three seasons, starting in the fall (April to June) and ending in the spring (October-December). In the early and mid-season (fall and winter) temperatures are moderate with long dry periods. However, at the end of the season (spring) the temperatures are higher with occurrence of rain, i.e. ideal conditions for high N_2O production by denitrification. Therefore, changes in temperature and moisture due seasonality and nutrient availability by application of vinasse and inorganic N may affect the structure and functionality of microbial communities including those involved in N-cycling. Thus, in order to assess the GHG emission factors it is necessary to take into the account the timing of the mineral fertilizer and vinasse application.

N_2O is produced in soil via biotic as well as abiotic process. The abiotic process, chemodenitrification, is based on chemical decomposition of hydroxylamine (NH_2OH), nitroxyl hydride (HNO) or nitrite (NO_2^-) with organic and inorganic compounds at low pH (<4.5). The potential to biotic N_2O production has been observed in more than 60 bacterial and archaeal genera and more recently also in fungi N_2O production has been demonstrated (Hayatsu et al., 2008; Higgins et al., 2016; Hink et al., 2016). Production of N_2O in soils occur mainly due to the processes of nitrification and denitrification (Figure 3) (Stevens and Laughlin, 1998; Németh et al., 2014; Martins et al., 2015; Soares et al., 2016; Xu et al., 2017). In oxic soils, well-drained soils, typical for agricultural soils (<60% water-filled pore

space - WFPS), N_2O is mainly produced by organisms involved in the first step of nitrification, i.e., ammonium oxidation (bacteria and archaea) (Bollmann and Conrad, 1998; Bateman and Baggs, 2005; Baggs et al., 2010; Hink et al., 2016). However, under suboxic or anoxic conditions (60-90% WFPS), facultative heterotrophic denitrifiers (Tiedje et al., 1983; Di et al., 2014) dominate N_2O production.

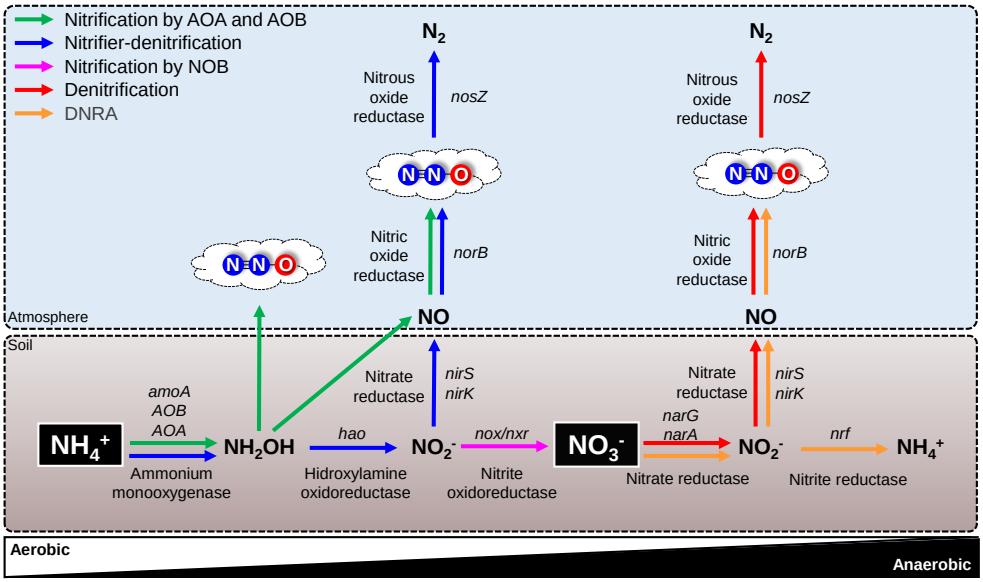

Figure 3 | Schematic diagram of the major microbial pathways of N_2O production in soils. The multiple pathways include nitrification (ammonia oxidation performed by AOA and AOB and nitrite oxidation by NOB), nitrifier denitrification (performed by AOA and AOB), denitrification (heterotrophic denitrification by heterotrophic bacteria), DNRA (dissimilatory nitrate reduction to ammonium, by unknown microorganisms) and anammox (anaerobic ammonium oxidation, by anaerobic ammonia oxidizers). Enzymes: *amoA* (ammonia monooxygenase); *hao* (hydroxylamine oxidoreductase); *narG* (membrane-bound nitrate reductase); *napA* (periplasmic nitrate reductase); *nirK* (copper-containing nitrite reductase); *nirS* (cytochrome cd1 nitrite reductase); *nxr* (nitrite oxidoreductase); *norB* (nitric oxide reductase) *nosZ* (nitrous oxide reductase) and *nrf* (Nitrite reductase). Different microbial groups and pathways are indicated clearly by different colors. Adapted from Hu et al. (2015).

Nitrification is the aerobic oxidation of ammonia (NH_3) to nitrate (NO_3^-) and it occurs in two phases mediated by autotrophic microorganisms (Figure 3). In the first phase ammonia-oxidizing bacteria (AOB) or archaea (AOA) oxidize NH_3 to nitrite (NO_2^-), and subsequently NO_2^- is oxidized to NO_3^- by nitrite-oxidizing bacteria (NOB) ($NO_2^- \rightarrow NO_3^-$). The first phase ($NH_3 \rightarrow NH_2OH/HNO \rightarrow NO_2^-$), i.e., ammonia oxidation, is catalyzed by the *amo*A gene encoding ammonia

monooxygenase. It is known to be present in β- *or γ*-proteobacteria (AOB) and the newly described *Thaumarchaeota* phylum (AOA). The *nxr*B gene encodes the nitrite oxidoreductase and regulates the second phase of nitrification. The first main N_2O-yielding pathway during nitrification occurs under aerobic conditions, N_2O emission from AOB results from the incomplete oxidation of NH_2OH to either nitroxyl (HNO) or NO (nitric oxide) (Smith and Hein, 1960; Hu et al., 2015) and subsequently N_2O is produced. Recently Caranto et al. (2016) demonstrated that another, direct enzymatic pathway from NH_2OH to N_2O at anaerobic conditions exists, and this pathway is mediated by cytochrome P460. The second N_2O-yielding route is named nitrifier denitrification and occurs at both high and low oxygen concentration. AOB possess machinery that reduces NO_2^- to N_2O via a nitric oxide (NO) intermediate (Ritchie and Nicholas, 1972; Shaw et al., 2006; Stein, 2011). Recently it has been found that nitrification can occur during a single step performed by bacteria of the *Nitrospira* genus (Daims et al., 2015; van Kessel et al., 2015); however, it is not yet known whether N_2O emission occurs in this one-step process.

Denitrification is a multistep reaction performed by a variety of bacteria and fungi. During denitrification oxidized mineral forms of N (NO_3^- and NO_2^-) are reduced to the gaseous products NO, N_2O and N_2 under oxygen-limited condition ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Figure 3). The sequential processes of bacterial denitrification are regulated by divergent reductases encoded by distinct functional genes; *nar*G or *nap*A genes encode nitrate reductase, *nirK* or *nirS* genes encode two entirely different types of nitrite reductase; *cnor*B or *qnor*B genes encode nitric oxide reductase and *nos*Z gene encodes nitrous oxide reductase (Philippot et al., 2007; Jones et al., 2013).

Despite considerable knowledge of the processes involved in N_2O production, most of the work was conducted under controlled conditions, thus in studies in which the impact of climatic conditions and variations during the year was not taken into account. The prevalence of the processes that control N_2O production in tropical soils during the growth of sugarcane has only begun to be addressed.

4. *Research aims and thesis outline*

The major goal of the research described in this thesis was to understand to what extent organic vinasse applications and sugarcane straw in combination with inorganic fertilizers affect the composition, functions and dynamics of the soil microbiome at seasonal climatic variations (Figure 4). Modern molecular techniques such as new generation sequencing were used to analyze microbial communities in field samples. N_2O production over time was also measured in the field and linked to data on microbial community structure and functioning.

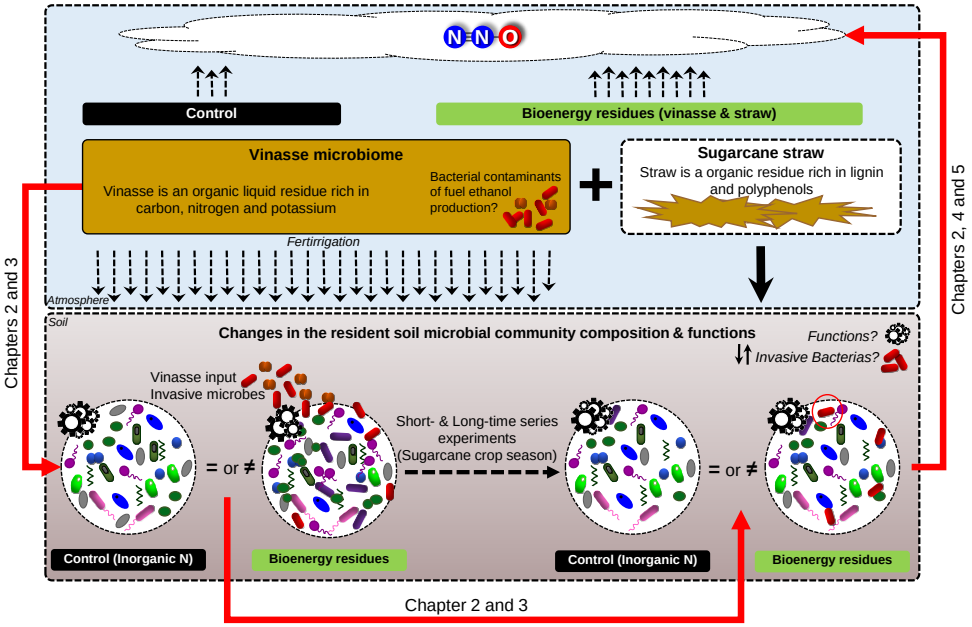

Figure 4 | Schematic overview of the chapters presented in this thesis.

The research questions addressed are:

(i) To what extent are the composition and functionality of the resident microbial community in a sugarcane field affected by organic residue and inorganic fertilizer amendments (sugarcane straw, organic vinasse and inorganic nitrogen)?

(ii) How do the single and combined applications of vinasse, straw and inorganic fertilizers influence N_2O emissions from soil?

(iii) Is the microbial community resistant or resilient to a pulse disturbance brought about by the application of organic residues and inorganic fertilizers?

(iv) How do climatic conditions affect the responses of the microbial community involved in N_2O production to disturbances?

(v) Which microbial process, i.e. nitrification or denitrification, contributes most to the N_2O production?

(vi) Do fungal denitrifiers contribute to N_2O production in tropical soils amended with straw?

This thesis starts with an assessment of how the soil microbial community's composition and functions are affected by bioenergy residues (organic vinasse and sugarcane straw) and inorganic fertilization and how these residues are linked with N_2O emissions. In *Chapter 2* a short-term sugarcane field experiment (crop season 2012/2013) is described that was designed to assess the changes in the soil microbial community composition and functions through time by analyzing shotgun metagenomics data and N_2O emissions.

In *Chapter 3*, the effect of organic vinasse and inorganic N fertilizer application on the resident soil microbial community was monitored during an

entire sugarcane crop season (season of 2014/2015) as well as CO_2 emission. This allowed for evaluating the stability and dynamics of the microbial community in response to perturbations. The microbial community was analyzed by PCR-amplified 16S ribosomal DNA. In addition, the microbes present in vinasse were tracked back into the soil and the potential invasiveness of those microbes was evaluated.

In *Chapter 4 and 5* investigations on the N_2O losses from sugarcane planted soils receiving different fertilization regimes (organic vinasse and inorganic N fertilizer) and the potential role of nitrification and denitrification processes in N_2O productions are described. In *Chapter 4* I studied how different seasons (spring-rainy/winter-dry, crop season 2013/2014 and 2014/2015, respectively) affected the N_2O losses from sugarcane planted soils receiving concentrated and non-concentrated vinasse. Furthermore, in this chapter I described the assessment of the abundance of microbial genes encoding proteins involved in the N cycle and N_2O production, such as archaeal and bacterial *amoA*, fungal and bacterial *nirK*, and bacterial *nir*S and *nosZ*. In *Chapter 5* I describe a study on the main microorganisms responsible for the N_2O production in soil after amendments of bioenergy crop residues.

Finally, in *Chapter 6* I combine the main obsrevations described this thesis and further discuss the role of bioenergy residues in the N_2O emissions from sugarcane production fields and the changes in the soil microbial community composition and functions. Here, I present a future outlook on the potential strategies to optimize the sustainable use of organic vinasse and inorganic N fertilizers in the sugarcane and ethanol production leading to low N_2O emissions.

Chapter 2

Recycling bioenergy residues as fertilizer impacts microbial community composition and function and increases N_2O emissions

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Abstract

Recycling residues is a sustainable alternative to improve soil structure and increase the stock of nutrients. However, information about the magnitude and duration of disturbances caused by crop and industrial wastes on soil microbial community structure and function is still scarce. The objective of this study was to investigate how added residues from industry and crops together with nitrogen (N) fertiliser affect the microbial community structure and function, and nitrous oxide (N_2O) emissions. The experimental sugarcane field had the following treatments: (I) control with nitrogen, phosphorus, and potassium (NPK), (II) sugarcane straw with NPK, (III) vinasse (by-product of ethanol industry) with NP, and (IV) vinasse plus sugarcane straw with NP. Soil samples were collected on days 1, 3, 6, 11, 24 and 46 of the experiment for DNA extraction and metagenome sequencing. N_2O emissions were also measured. Treatments with straw and vinasse residues induced changes in soil microbial composition and potential functions. The change in the microbial community was highest in the treatments with straw addition with functions related to decomposition of different ranges of C-compounds overrepresented while in vinasse treatment, the functions related to spore-producing microorganisms were overrepresented. Furthermore, all additional residues increased microorganisms related to the nitrogen metabolism and vinasse with straw had a synergetic effect on the highest N_2O emissions. The results highlight the importance of residues and fertiliser management in sustainable agriculture.

1. INTRODUCTION

Anthropogenic activities impact soil properties and consequently soil functioning. Agricultural practices such as crop residue retention from the previous or different crops have been proposed as alternatives to improve soil structure and soil protection by reducing erosion (Boulal et al., 2011; Brouder and Gomez-Macpherson, 2014), and increasing the stock of plant nutrients and soil organic matter content, thus enhancing soil fertility (Bhattacharyya et al., 2013; Jemai et al., 2013) and crop yields (Ussiri et al., 2009). In sustainable agriculture, it is common practice to add crop residues in different forms such manure and compost (Ge et al., 2009), and other agricultural waste products like straw, wood chips, sewage sludge, or sawdust to increase soil quality (Scotti et al., 2015).

The return of straw to the soil is an effective management regime providing available carbon (C) and N (Li et al., 2013). However, the inadequate and indiscriminate discharge of other agricultural wastes in the environment may have a specific and negative impact on the soil. Examples include the amendments of manure (Suleiman et al., 2016) and, more recently, vinasse residue generated as a by-product mainly of the sugar-ethanol industry from sugar crops (beet, sugarcane), starch crops (corn, wheat, rice, cassava), and/or cellulosic material (sugarcane bagasse and wood residues) (Christofoletti et al., 2013). The large sugarcane ethanol production in Brazil generates about 8–15 litters of vinasse for every litre of alcohol produced (Freire and Cortez, 2000). Researchers have been suggesting alternative usages of vinasse in order to avoid discharge it in rivers. One alternative is the application of vinasse as fertiliser on sugarcane plantations (Fuess et al., 2017). Vinasse is a source of organic matter and potassium, nitrogen and phosphorus. However, the combination of vinasse and inorganic fertiliser applications contributes significantly to the increase of greenhouse gas (GHG) emissions, especially N_2O. Moreover, if this combination of vinasse and fertiliser is added to soil containing straw, the N_2O emissions are much higher (Carmo et al., 2013). Therefore, adequate soil management practices for sugarcane cultivation with recycling residues are urgently needed. These practices not only affect environmental issues but also soil quality and health.

Fertilisation practices, tillage, and crop residue management effect the soil microbial community structure (Kuramae et al., 2013; Lupatini et al., 2013b; Carbonetto et al., 2014; Cassman et al., 2016; Suleiman et al., 2016), which soil microbes are the primary mediators of organic matter decomposition (Kuramae et al., 2013; Kielak et al., 2016b), and nutrient cycling (Rousk and Bengtson, 2014). Results of field studies have shown that different management strategies with straw (Huang et al., 2012) and vinasse (Navarrete et al., 2015a) alter soil bacterial community composition. Furthermore, straw application increases the microbial metabolic activity (Navarro-Noya et al., 2013) and vinasse amendment causes positive or negative effects on different microbial groups (Pitombo et al., 2015). However, most of the studies about the effects of agricultural management on soil

microorganisms focus on the changes in the soil living biomass and their community composition (Navarro-Noya et al., 2013; Sengupta and Dick, 2015). Quantifying how microbial communities and functions change through time is important to understanding processes such as succession or recovery from perturbations. However, the understanding of the direct and indirect effect of residues generated from agricultural practices on the structure and functioning of microbial communities and the consequences for the functioning of agroecosystems is limited. This study aimed to determine the effect of industrial and crop residue amendments on the dynamics of microbial community composition and function, and the N_2O production in a short-term field experiment. We hypothesise that different residues have distinct effects on microbial communities, with straw having no or less impact on microbial community and traits than vinasse, while treatments with vinasse having temporary impacts favouring copiotrophic (i.e., fast-growing, low C use efficiency) taxa. Furthermore, we postulate that residues added to soil increase N_2O emission. The results are of primary importance for a proper management of residues in agriculture.

2. MATERIAL AND METHODS

2.1. Experimental setup and soil sampling

The field experiment was situated in the Piracicaba municipality, São Paulo state, Brazil (22°41019.34″S; 47°38041.97″W; 575 m above sea level). The mean air temperature and precipitation were 25.9 °C and 234 mm, respectively over the 46 days of the study (Figure A.1). The soil is classified as Haplic Ferralsol with a pH of 5.1, organic matter of 23 g dm^{-3}, P of 16 mg dm^{-3}, K^+ of 0.7 $mmol_c$ dm^{-3}, Ca^{+2} (calcium) of 19 $mmol_c$ dm^{-3}, Mg^{+2} (magnesium) of 11 $mmol_c$ dm^{-3}, $H^+ + Al^{+3}$ (hydrogen and aluminium) of 34 $mmol_c$ dm^{-3}, and cation-exchange capacity (CEC) of 64.7 $mmol_c$ dm^{-3}.

The experimental field was cultivated with sugarcane and consisted of four treatments with three replicates. Each treatment consisted of a 4.8 x 9 m plot separated from each other by 2 m in a complete randomised block design as follows: (i) control (amended with NPK), (ii) sugarcane straw (with NPK), (iii) vinasse (with N and P), (iv) vinasse plus sugarcane straw (with N and P). Vinasse was used as a K source and its composition is presented in Supplementary Table A.1. The composition of straw was 364.8 g C kg^{-1} , 4.5 g N kg^{-1} , 0.5 g P kg^{-1} , 9.5 g K kg^{-1} , 6.6 g Ca kg^{-1} , 2.2 g Mg kg^{-1} , 1.3 g S kg^{-1} , and 80:1 of C:N ratio. After harvesting, the straw (10 t ha^{-1}) was left from a previous sugarcane crop season in the treatments with straw and vinasse plus straw and removed for the remaining treatments. For all treatments, soil sampling was carried out at 8 time points after 1, 3, 8, 14, 20, 24, 30, and 46 days of residues addition and collected (top 10 cm) from three soil cores at the fertiliser line position. As usually performed in commercial areas, vinasse (1.10^5 l ha^{-1}) was applied to the total area of the plots with the relevant treatments, and mineral fertiliser with N as ammonium nitrate (100

kg N ha^{-1}), P as superphosphate (17 kg ha^{-1}), and K as potassium chloride (100 kg ha^{-1}) were applied in lines parallel to the crop line.

2.2. DNA extraction and library preparation

Total soil DNA was extracted from 0.25 g of each soil sample using the MoBio PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions. DNA concentration and quality were determined by spectrophotometry (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA), and by agarose gel electrophoresis.

Shotgun metagenome libraries were constructed following the Illumina Paired-End Prep kit protocol and sequenced at Macrogen Inc. Company, South Korea using 2 × 300 bp sequencing run on Illumina MiSeq2000 (Illumina, San Diego, CA) technology.

2.3. Annotation of metagenome sequences and data analysis

Generated reads were uploaded and annotated with MG-RAST (Rapid Annotation using Subsystems Technology for Metagenomes) server (Meyer et al., 2008) using associated metadata files for taxonomic affiliations and functional annotations into different metabolic subsystems. Raw, unassembled reads were annotated using best hit classification against the Refseq and subsystem databases with a maximum e-value cut-off of 10^{-5}, a minimum percent identity cut-off of 60% and a minimum alignment length cut-off of 15 and Hierarchical Classification subsystems with a maximum e-value cut-off of 10^{-5}, a minimum percent identity cut-off of 60% and a minimum alignment length cut-off of 15. All compared distributions were normalised as a function of the number of annotated sequences for each metagenome library.

The microbial sequences were normalised via random sub-sampling at 14,065 and 5,529 reads per sample to determine the taxonomy and function, respectively, for downstream analyses. We used four additional indices to assess differences in bacterial and archaeal community diversities, including Shannon (Ludwig and Reynolds, 1988), observed taxonomical units (OTUs), Chao 1 (Chao, 1984), and Simpson (Simpson, 1949). To test whether sample categories harboured significantly different metagenomes or microbial communities, we used PERMANOVA analysis implemented in R software. The multivariate regression tree analyses (De'ath, 2002; De'ath, 2007) with time scales of days after vinasse application was used to identify the days that best explain the variation in microbial community composition. Discriminant analysis of the principal components (DAPC) was used to examine the dissimilarity between the different treatments based on the taxonomical and functional datasets. DAPC was performed using a square root-transformed data table with the dapc function of the R Adegenet v2.0.0 package (Jombart et al., 2010) in R. This method is based on the assumption of defined prior groups to construct the plot based on treatment groups. The canonical loading plots were used to identify microbial orders and functions

capable of differentiating the microbial communities according to the defined clustering groups using the user-defined threshold (1/4 of the highest value) (Pajarillo et al., 2014). To assess the link between the microbial community composition and function, the Procrustes approach expressed in terms of m^2 (Gower, 1975) was tested with 9,999 permutations with the Monte-Carlo test (Peres-Neto and Jackson, 2001). The m^2 value is a closeness of fit between to the two sets and is based on the sum of the squared deviations (Gower, 1971). Data corresponding to both taxonomic and functional distributions were also statistically analysed with STAMP software (Parks and Beiko, 2013). Relative abundances of individual taxa or functions of samples were compared using pairwise t tests followed by the Welch's t test ($p < 0.05$). Reads assigned by MG-RAST v3.0 to Refseq databases related to N metabolisms were filtered and taxonomically classified using BLASTX against the subsystem database in the MG-RAST v3.0.

2.4. N_2O measurements and soil chemical analysis

The fluxes of N_2O were measured using closed chambers using the chamber-based method (Soares et al., 2016) at the fertilised sugarcane line position. The chambers were inserted to a soil depth of 3 cm. On each sampling day, gas samples (60 mL) were collected between 8:00 am and 12:00 pm at 1, 10, 20, and 30 min after chamber closure using syringes, with 20-ml-evacuated penicillin flasks sealed with gas-impermeable butyl-rubber septa (Bellco Glass 2048) and analysed by gas chromatography (GC-2014 model) with electron capture for N_2O (Shimadzu, Kyoto, Japan). The flux rates of N_2O were calculated by linear interpolation of fluxes between sampling events (Soares et al., 2016). Each gas chamber flux was calculated from slope regression between the gas concentration and collection time according to Carmo et al. (2013). During the sampling period, we also monitored environmental temperature and precipitation as well as ambient N_2O concentration to check the order of magnitude of the N_2O concentration in the chambers. The concentrations of NH_4^+ (Krom, 1980) and NO_3^- (Kamphake et al., 1967) in the filtered extract were determined colourimetrically by a using flow injection analysis (FIAlab 2500).

3. RESULTS

3.1. General overview of the soil microbial community data analysis

From a total of 96 samples, 90 samples could be annotated and recovered from each of the eight sampling time points, with three replicates per time point. The quality of the samples and the excluded samples are shown in Supplementary Table A.2. On average, 98.35% of the shotgun metagenome reads were assigned to prokaryotes with the majority assigned to bacteria (97.26%) and a small fraction (1.09%) to archaea (Figure A.2a). The remaining reads were assigned to Eukaryota (1.63%) and to viruses (0.03%). We proceeded with the analysis with bacteria and archaea domains due to their highest representation in the shotgun

metagenome data. The bacterial community was composed of 28 phyla, dominated by Proteobacteria (40.2%) followed by Actinobacteria (24.7%), Acidobacteria (9.2%), Firmicutes (6.4%), Chloroflexi (4.6%), Bacteroidetes (3.4%), Deferribacteres (2.2%), Verrucomicrobia (2.1%) Planctomycetes (2.0%), and Gemmatimonadetes (0.9%), while the archaeal community was composed of the 3 main phyla Euryarchaeota (0.8%), Crenarchaeota (0.2%), and Thaumarchaeota (0.1%) (Figure A.2b). Functional analysis classified the sequences in 28 subsystems (Figure A.3). The top five categories belonged to carbohydrates (15%), clustering-based subsystems (functional coupling evidence but unknown function) (13%), amino acids and derivatives (10%), protein metabolism (9%), and miscellaneous (6%).

3.2. Taxonomic and function structure pattern in distinct residues amendments

In order to assess the temporal effect of the residues amendment on the microbial community structure, the taxonomic and functional profiles were compared at different time points with a dissimilarity test. PERMANOVA analysis showed no interaction between treatment and time of determining taxonomy and function (Pseudo-F values = 1.07 and 0.92, respectively; $P > 0.05$, Table 1). Considering that the factor treatment had a significant effect on the microbial community structure and function (Pseudo-F values = 3.68 and 1.55, respectively; $P < 0.05$, Table 1), further analyses were done, neglecting time as a factor. The discriminant analysis of the principal components (DAPC) revealed that the microbial community structure was markedly different among treatments (Figure 1a). In contrast, microbial functions were similar in different residue treatments (Figure 1b). However, the control treatment slightly differed from treatments with the addition of vinasse and/or straw. Taxonomic (Pseudo-F values = 4.36, 2.27, and 2.37 for straw, vinasse and vinasse + straw, respectively; $P < 0.01$, Table 1) and function (Pseudo-F values = 1.43, 1.53, and 1.92 for straw, vinasse, and vinasse + straw, respectively; $P < 0.10$, Table 1) pairwise comparison analyses showed significant differences for residue type compared with the control. Straw seems to be more determinant for changes in taxonomy while both residues, straw and vinasse, seem to alter soil functions similarly. Despite no interaction between time and treatment, it is worth mentioning that treatments with vinasse changed microbial community in the first week after application of vinasse with higher sample dispersion when compared with the addition of straw alone (Figure A.4).

The alpha diversity of microbial communities measured by the Shannon and Simpson indices was significantly ($P < 0.05$) higher in the straw and straw with vinasse treatments than in the treatment with vinasse alone (Figure A.5). Though the richness of OTUs tended to increase with the addition of vinasse, the results were not statistically significant. For functions, both treatments with straw (straw and vinasse+straw) were significantly higher for Shannon and Simpson indices diversity (Figure A.6). To assess the degree of concordance between community

composition and their potential function, we compared the microbial community composition through Procrustes analyses. A significant concordance with high m^2 value between ordinations was found (m^2 = 0.824, P = 0.000, based on 9999 permutations), suggesting that distinct communities were associated with distinct functions.

Table 1 | Effects of crops residues amendments and Permanova pairwise comparisons on taxonomy and functions of the soil microbial community.

	Taxonomy	**Functions**		
Main test*	Order	Level1	Level2	Level3
Treatment	**3.68*****	**1.55*****	**1.14****	**1.11*****
Time	**2.07*****	1.13	**1.13*****	**1.06*****
Interaction	1.07	0.92	0.95	1.01
C x S	**4.36*****	**1.43***	**1.31****	**1.22*****
C x V	**2.27*****	**1.53****	1.17	1.00
C x V+S	**2.37*****	**1.92*****	1.18	**1.14*****
S x V	**6.29*****	**2.01*****	**1.31****	**1.17*****
S x V+S	**2.25*****	0.94	0.88	1.03
V x V+S	**2.69*****	**1.52****	1.04	1.08

Abbreviations: (C) Control; (S) Straw; (V) Vinasse; (V+S) Vinasse plus straw; Values represent the univariate t-statistic (t). Significance : '***' p ≤ 0.01, '**', p ≤ 0.05 and '*' p ≤0.10.

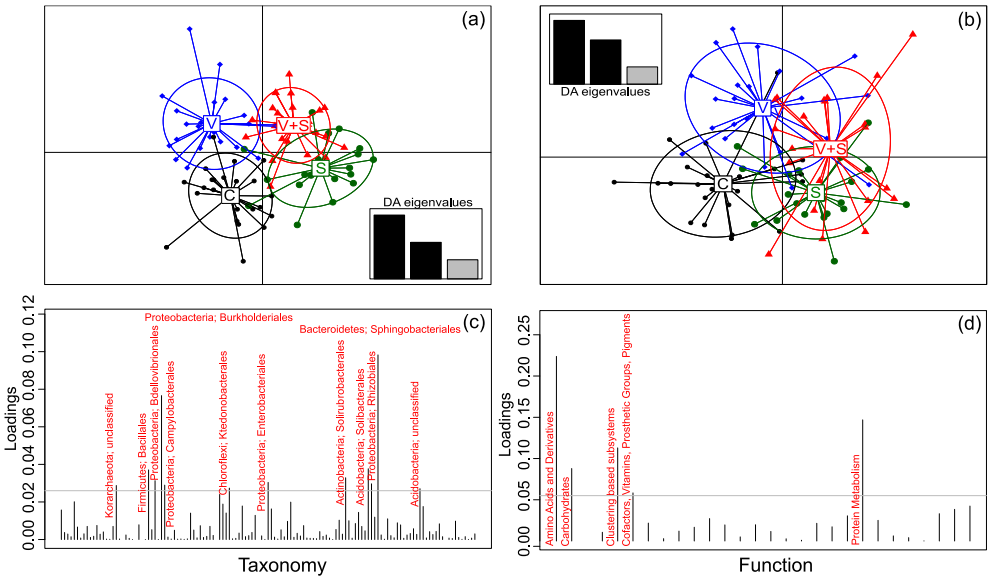
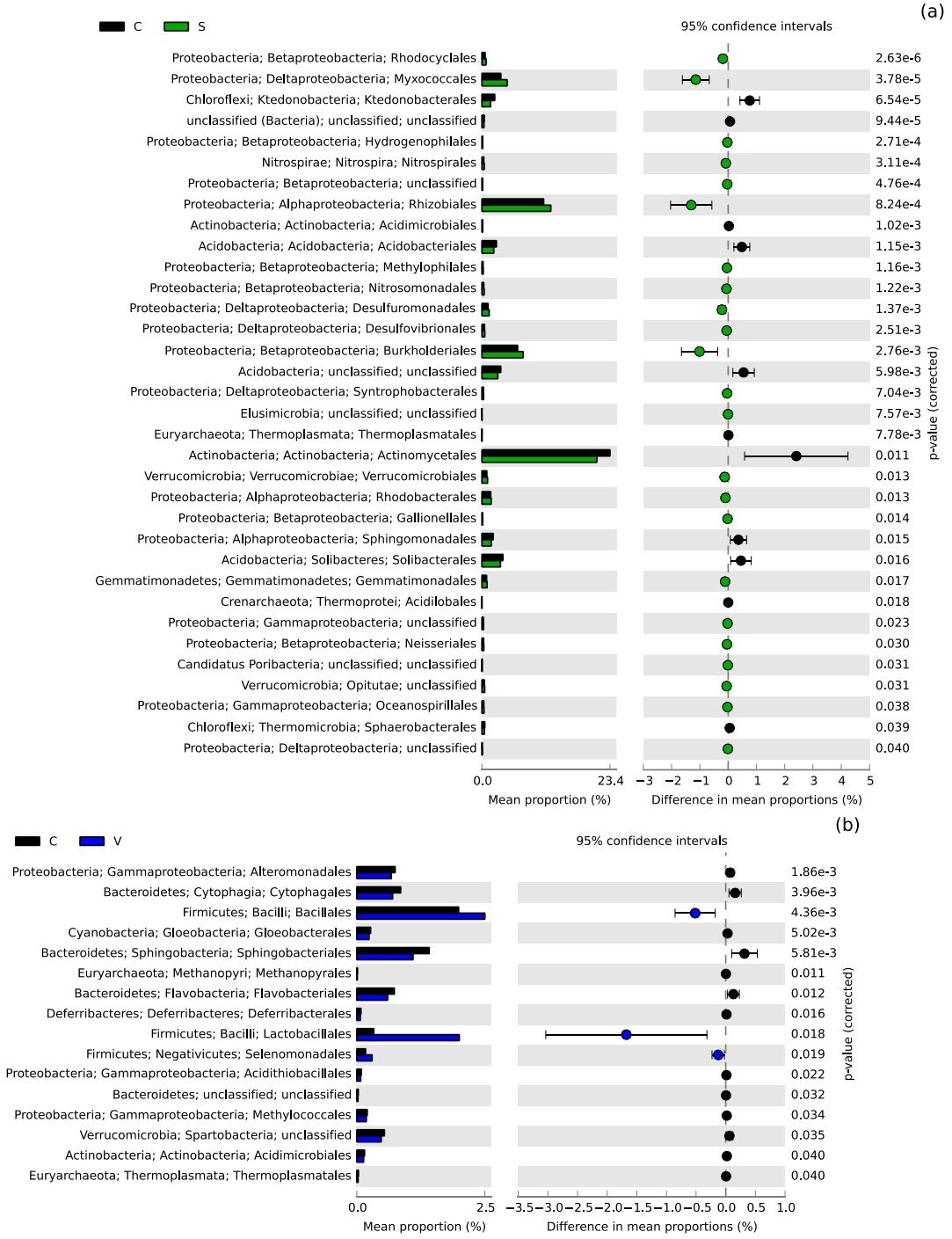


Figure 1 | Discriminant analysis of principal components (DAPC) plot of the effect of crop residues on soil microbial (a) taxonomy and (b) functions. Canonical loading plot of the main contributor (c) orders and (d) functions of the DAPC analysis of the different treatments (C) control, (S) straw, (V) vinasse and (V+S) vinasse + straw soil metagenomes. Only contributors above ¼ of the highest grey horizontal line are indicated for the sake of clarity.

3.3. Differences between taxa and functions for each residue

The main taxonomic orders responsible for the differences among treatments in DAPC analysis belonged to Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, and Korarchaeota. The relative abundance of Alphaproteobacteria (Rhizobiales, Rhodobacterales), Betaproteobacteria (Burkholderiales, Gallionellalis, Hydrogenophilales, Methylophilales, Neisseriales, Nitrosomonadales, Rhodocyclales), Deltaproteobacteria (Desulphuromonadales, Desulfovibrionales, Myxococcales, Syntrophobacterales), Gammaproteobacteria (Oceanospirillales), Gemmatimonadetes (Gemmatimonadales), Nitrospirae (Nitrospirales), and Verrucomicrobia (Verrucomicrobiales) increased significantly in straw treatment. High proportions of Firmicutes (Bacillales, Lactobacillales, and Selenomonadales) was found in vinasse treatment, whereas Alphaproteobacteria (Rhizobiales, Rhodobacterales, Rhodospirillales), Betaproteobacteria (Burkholderiales, Rhodocyclales), and Deltaproteobacteria (Myxococcales) were overrepresented in vinasse plus straw treatment (Figure 2). In the control treatment, higher proportions of Acidobacteria (Acidobacterales, Solibacterales), Actinobacteria (Actinomycetales), Alphaproteobacteria (Sphingomonadales), and Cloroflexi (Ktedonobacterales) were found when compared with straw residue, whereas Bacteroidetes (Cytophagales, Sphingobacteriales, Flavobacteriales) had higher abundance in the control than in vinasse treatment.

For functions, taking into account all the treatments, carbohydrates, amino acids, clustering-based subsystems, ‘cofactors, vitamins and pigments’, ‘virulence, disease and defence’, stress response and protein, sulphur and potassium metabolisms were the nine categories that contributed the most to discriminant functions created by DAPC (Figure 1). Pairwise comparisons showed dominance of core metabolic functions (e.g., carbohydrates, membrane transport, motility and chemotaxis, and amino acids) in all treatments. However, the functions of virulence, disease, and defence; and dormancy and sporulation were higher in residues treatments than in control (Figure 3). While vinasse treatment had core metabolic functions in the highest abundance, the nitrogen metabolism subsystem appeared to be specific to straw residue addition.


(a)
C
S
95% confidence intervals
Proteobacteria; Betaproteobacteria; Rhodocyclales
Proteobacteria; Deltaproteobacteria; Myxococcales
Chloroflexi; Ktedonobacteria; Ktedonobacterales
unclassified (Bacteria); unclassified; unclassified
Proteobacteria; Betaproteobacteria; Hydrogenophilales
Nitrospirae; Nitrospira; Nitrospirales
Proteobacteria; Betaproteobacteria; unclassified
Proteobacteria; Alphaproteobacteria; Rhizobiales
Actinobacteria; Actinobacteria; Acidimicrobiales
Acidobacteria; Acidobacteria; Acidobacteriales
Proteobacteria; Betaproteobacteria; Methylophilales
Proteobacteria; Betaproteobacteria; Nitrosomonadales
Proteobacteria; Deltaproteobacteria; Desulfuromonadales
Proteobacteria; Deltaproteobacteria; Desulfovibrionales
Proteobacteria; Betaproteobacteria; Burkholderiales
Acidobacteria; unclassified; unclassified
Proteobacteria; Deltaproteobacteria; Syntrophobacterales
Elusimicrobia; unclassified; unclassified
Euryarchaeota; Thermoplasmata; Thermoplasmatales
Actinobacteria; Actinobacteria; Actinomycetales
Verrucomicrobia; Verrucomicrobiae; Verrucomicrobiales
Proteobacteria; Alphaproteobacteria; Rhodobacterales
Proteobacteria; Betaproteobacteria; Gallionellales
Proteobacteria; Alphaproteobacteria; Sphingomonadales
Acidobacteria; Solibacteres; Solibacterales
Gemmatimonadetes; Gemmatimonadetes; Gemmatimonadales
Crenarchaeota; Thermoprotei; Acidilobales
Proteobacteria; Gammaproteobacteria; unclassified
Proteobacteria; Betaproteobacteria; Neisseriales
Candidatus Poribacteria; unclassified; unclassified
Verrucomicrobia; Opitutae; unclassified
Proteobacteria; Gammaproteobacteria; Oceanospirillales
Chloroflexi; Thermomicrobia; Sphaerobacterales
Proteobacteria; Deltaproteobacteria; unclassified
2.63e-6
3.78e-5
6.54e-5
9.44e-5
2.71e-4
3.11e-4
4.76e-4
8.24e-4
1.02e-3
1.15e-3
1.16e-3
1.22e-3
1.37e-3
2.51e-3
2.76e-3
5.98e-3
7.04e-3
7.57e-3
7.78e-3
0.011
0.013
0.013
0.014
0.015
0.016
0.017
0.018
0.023
0.030
0.031
0.031
0.038
0.039
0.040
p-value (corrected)
0.0
23.4
Mean proportion (%)
−3 −2 −1 0 1 2 3 4 5
Difference in mean proportions (%)
(b)
C
V
95% confidence intervals
Proteobacteria; Gammaproteobacteria; Alteromonadales
Bacteroidetes; Cytophagia; Cytophagales
Firmicutes; Bacilli; Bacillales
Cyanobacteria; Gloeobacteria; Gloeobacterales
Bacteroidetes; Sphingobacteria; Sphingobacteriales
Euryarchaeota; Methanopyri; Methanopyrales
Bacteroidetes; Flavobacteria; Flavobacteriales
Deferribacteres; Deferribacteres; Deferribacterales
Firmicutes; Bacilli; Lactobacillales
Firmicutes; Negativicutes; Selenomonadales
Proteobacteria; Gammaproteobacteria; Acidithiobacillales
Bacteroidetes; unclassified; unclassified
Proteobacteria; Gammaproteobacteria; Methylococcales
Verrucomicrobia; Spartobacteria; unclassified
Actinobacteria; Actinobacteria; Acidimicrobiales
Euryarchaeota; Thermoplasmata; Thermoplasmatales
1.86e-3
3.96e-3
4.36e-3
5.02e-3
5.81e-3
0.011
0.012
0.016
0.018
0.019
0.022
0.032
0.034
0.035
0.040
0.040
p-value (corrected)
0.0
2.5
Mean proportion (%)
−3.5 −3.0 −2.5 −2.0 −1.5 −1.0 −0.5 0.0 0.5 1.0
Difference in mean proportions (%)

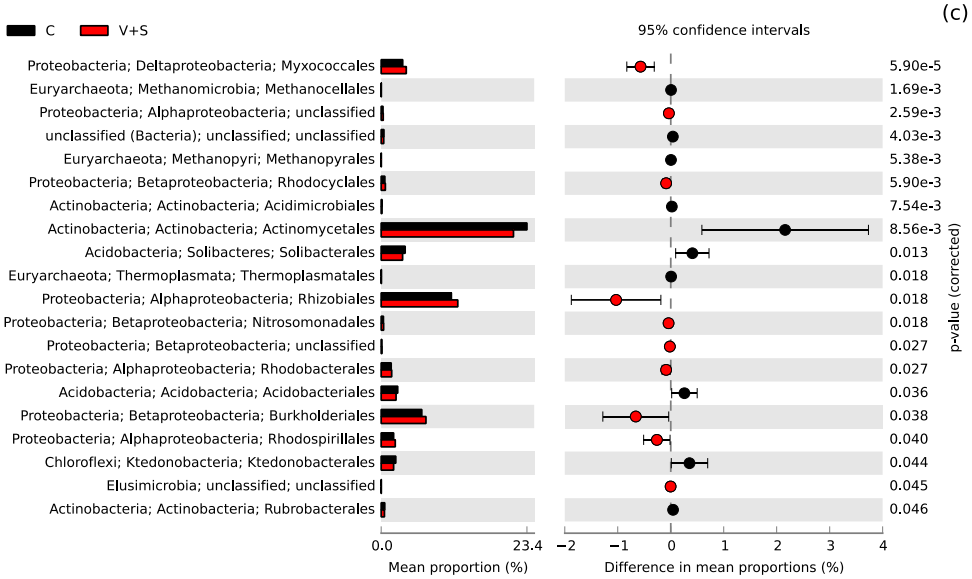


Figure 2 | Differences in the relative abundance of microbial orders between soils without crop residues (control) and soils with different crop residues (a) straw, (b) vinasse and (c) vinasse plus straw. The differences between groups were calculated using Welch's inverted method. Only significant differences at $p \leq 0.05$ are presented.

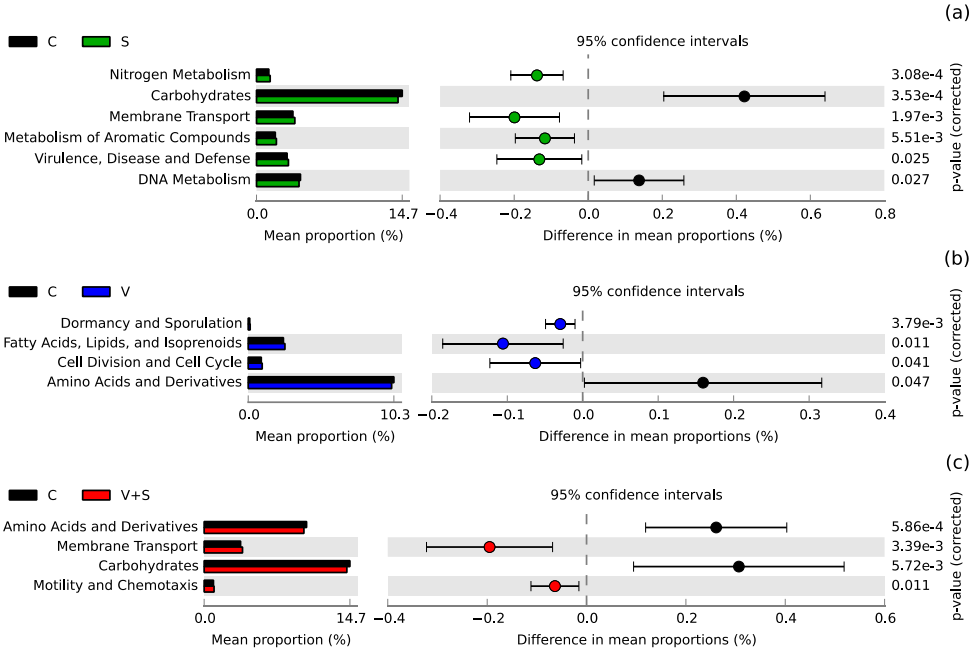


Figure 3 | Differences in the relative abundance of functions between soils without crop residues (control) and soils with different crop residues (a) straw, (b) vinasse and (c) vinasse plus straw. The differences between groups were calculated using Welch's inverted method. Only significant differences at $p \leq 0.05$ are presented.

3.4. N_2O emissions and mineral N

The application of the residues affected the temporal dynamics of nitrous oxide emissions. During the 46 days of sampling, the presence of straw increased N_2O emissions (Figure 4a). Both treatments with vinasse (vinasse and vinasse+straw) had higher emissions of N_2O than the control treatment, although the emissions from soil with the vinasse plus straw treatment were generally higher than those with vinasse alone. N_2O production rates from soil where vinasse was applied together with straw were high during the first four sampling days followed by the treatments solely vinasse and solely straw. From day 20, the treatments with all residues increased N_2O emissions until day 30. After that, the fluxes of N_2O emissions were reduced. Compared to the control, the total average of N_2O emission rates from the soils were 2.8, 3.2, and 8.9 times higher for straw, vinasse, and vinasse plus straw treatments, respectively.

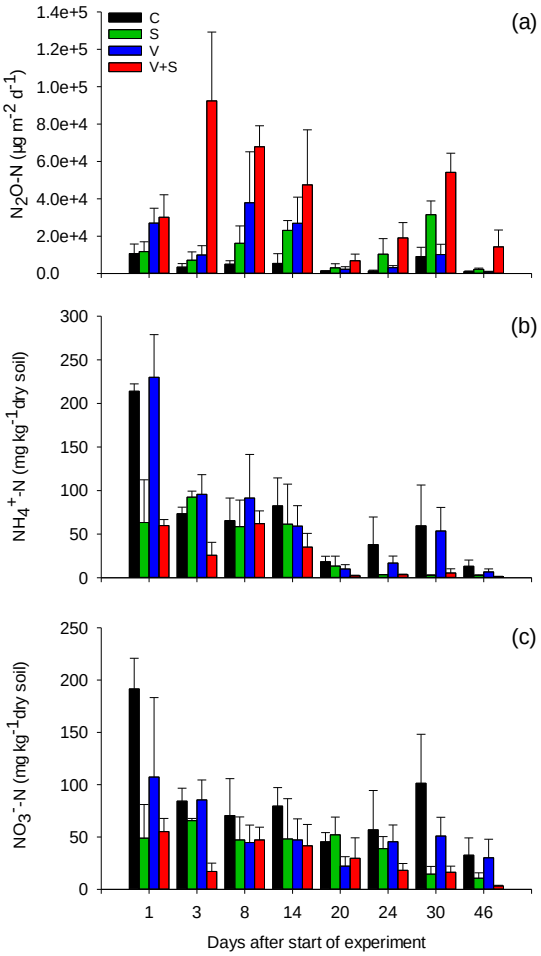


Figure 4| Nitrous oxide (N_2O) emissions, concentrations of soil ammonium (NH_4^+-N) and soil nitrate (NO_3^--N) in different treatments (C) control, (S) straw, (V) vinasse and (V+S) vinasse + straw. Error bars indicate the standard error of mean (n = 4).

The same pattern of N_2O emissions was shown for the NH_4^+-N content (Figure 4b). In general, NH_4^+-N content from vinasse plus straw was always less than other treatments during the entire experiment period. At day 1, the application of straw decreased 3 times as much NH_4^+-N content when compared to the control treatment. At day 3, in all treatments there was a decrease of soil NH_4^+-N content relatively similar to the control. After day 20 only vinasse had a similar amount of NH_4^+-N to the control while straw and vinasse plus straw treatments showed lower NH_4^+-N contents. The NH_4^+-N content of straw and vinasse plus straw treatments decreased twice as much as the control treatment.

The dynamics of NO_3^--N content showed a different pattern to that of N_2O. NO_3^--N content in the control and vinasse treatments was always higher than in the other treatments. NO_3^--N content in soil treated with vinasse plus straw was on average 28 mg kg^{-1} dry soil as compared to 82 mg kg^{-1} dry soil in the control (Figure 4c). During the 46 days of the experiment, all treatments with organic residues application decreased the NO_3^--N content compared to the control and the levels of NO_3^--N were declining for those treatments till the end of the experiment.

3.5. Taxa associated with nitrogen cycle

We analysed the phylogenetic bins, at order taxonomic level, of nitrogen metabolism traits for a better understanding of which microbes were linked to this function since the addition of residues increased N_2O emissions. The abundances of the taxa presumed to contribute to N metabolism and pathways associated with N in the soil are shown in Figure 5. *Betaproteobacteria* (*Nitrosomonadales*) was the common taxa related to nitrogen metabolism that increased with the two types of residue amendments. Shared taxa related to nitrogen metabolism were also found for both straw and vinasse plus straw treatments (Figure 5) with the highest proportions of *Deltaproteobacteria* (*Myxococcales*) and *Gammaproteobacteria* (*Pseudomonadales*). Specific residue type treatments had unique taxa related to N metabolism (Figure 5) as compared to the control. For the straw treatment, microbes with the highest relative abundances related to N metabolism belonged to *Gammaproteobacteria* (*Alteromonadales*), and for the vinasse treatment, to *Betaproteobacteria* (*Neisseriales*). In the combined vinasse and straw treatment, these same organisms were found again to have the highest abundance.

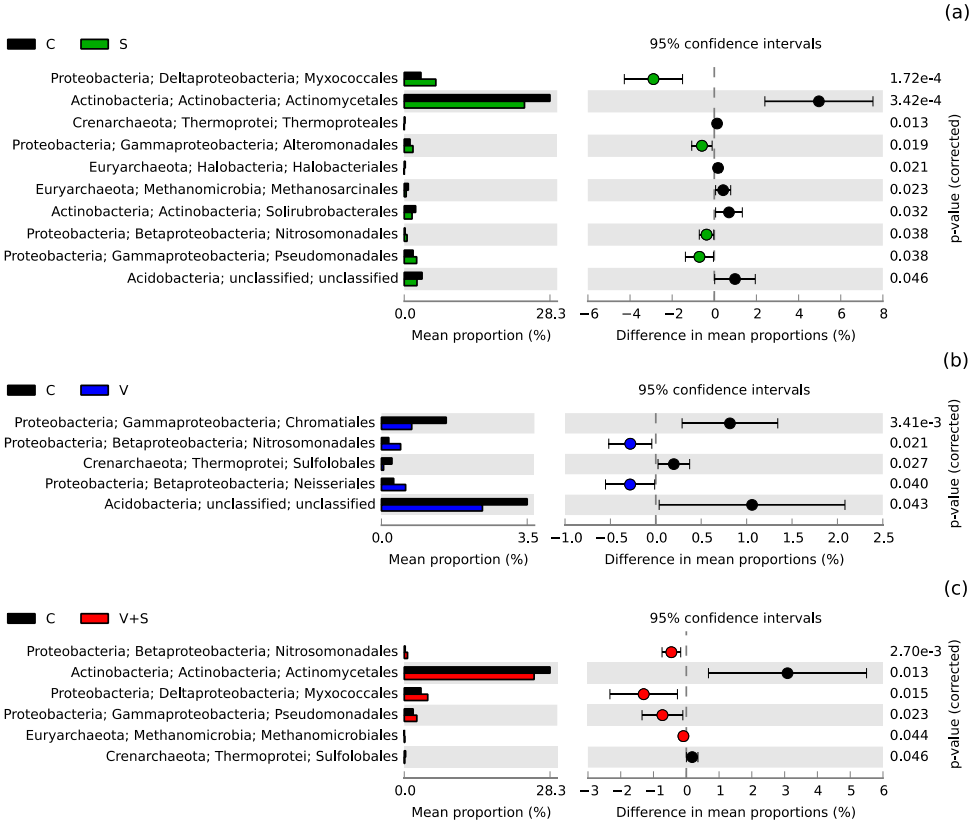


Figure 5| Microbial order contributing to nitrogen metabolism correlated between soils without crop residues (control) and soils with different crop residues, (a) straw, (b) vinasse and (c) vinasse plus straw (Welch's two-sided test; $P < 0.05$). The bars indicate the percentage of contribution of microbial order to each the selected functional category.

4. DISCUSSION

The addition of residues as by-products of crop production is a common practice in agriculture. Since crop residues are sometimes considered a problem, a set of different management practices, including reduced crop residue retention, has been proposed as a promising management option to support farm productivity, reduce soil degradation, and improve nutrient cycling in the agroecosystem. It has also been reported that straw (Liang et al., 2007; Zhang et al., 2013) and residues considered organic fertilisers, such as manure (Chadwick et al., 2011; Aita et al., 2015) and vinasse (Paredes et al., 2015), contribute to extra emissions of greenhouse gases (GHG), thereby accelerating greenhouse effects. Therefore, in this study we monitored the dynamics of the taxonomic and functional structure of the soil microbial community and the emission of nitrous oxide (N_2O) in soils amended with different agricultural and industrial residues. The 16S rRNA gene sequence based analyses has been previously shown to be a

valuable taxonomic genetic marker for analysing microbial communities, including those associated with residues like straw and vinasse (Navarrete et al., 2015a; Pitombo et al., 2015). Here, however, we used a shotgun metagenome approach to provide insight into both the taxonomic and the potential functional profiles of soil microorganisms. The short-term effect of residues addition revealed treatment-impact rather than temporal effect on soil microbial community. Some consistent patterns were found for specific organic residues amendments. For example, there were no shared taxa or core metabolic functions for all fertilised treatments with and without residues. Members of Firmicutes phyla and the dormancy and sporulation function were predominant mainly in the presence of vinasse, while orders related with decomposition; the nitrogen cycle; and the virulence, disease, and defence function prevail in straw. Furthermore, shared taxonomic orders in straw treatments suggest that straw is the determinant to drive microbial changes while residues alter soil functions.

The first factor we wanted to examine was the temporal dynamics of soil microbial communities as they may change as an immediate response to the disturbance caused by the organic matter addition and return later to their original stable state (Allison and Martiny, 2008). In soil, there are considerable time-scale studies in literature focused on microbial driven biogeochemical processes and specific functions as an indirect answer for their activity (Strickland et al., 2009). However, there are a limited number of studies examining through time how general microbial composition and function respond to agricultural disturbances. The different treatments did not present temporal variability in microbial community structure during the short-term experiment. Yet, the vinasse application caused the largest change in the microbial community in the first week of the experiment. Our findings are in disagreement with other studies on disturbances due to organic additions to soil. Suleiman et al. (2016) found that microbial diversity changed temporarily after slurry fertilisation, but the community recovered later to the original status. Despite the insignificant time-depending variation, our study revealed consistent residue addition effects. In most cases long-term studies are used to assess the effects of fertilisation (Pan et al., 2014; Cassman et al., 2016) and crop residues retention on the soil (Sradnick et al., 2013; Sun et al., 2015). Yet, we believe that short-term experiments are also relevant for a better understanding of these effects, particularly related to soil microbiota which could change rapidly, in the time frame of this study (Allison and Martiny, 2008; Suleiman et al., 2016).

Our study shows that treatments with agricultural and industrial residues induced changes in soil microbial composition and functions. In straw systems, for instance, the crop residue is left on the soil surface to be subject to decomposition, however, this residue is recalcitrant organic matter with high concentrations of lignin and polyphenols (Abiven et al., 2005) and needs to be degraded by specific microorganisms. Usually, the annual decomposition rates of sugarcane straw, range from 60% to 98% throughout the crop season (Oliveira et al., 1999; Fortes et

al., 2012; Carvalho et al., 2017). Our results on the performance of the microbial community in soil where straw was added are in disagreement with those from Rachid et al. (2016), who suggested that different levels of straw on sugarcane (0%, 50%, and 100% of the original straw deposition) have no effect on the bacterial community.

The combination of straw and vinasse had no drastic effect on the microbial community structure and functions, except on the functions of the nitrogen cycle. In addition, this combination had an effect on the N_2O emissions. The high temperature and precipitation during the experiment may have favoured the rapid decomposition of straw on the soil surface (10 t ha^{-1}) and probably the vinasse carbon input was not as much as required to boost changes in the bacterial community expected with the addition of both residues (straw and vinasse) (Devêvre and Horwáth, 2000).

Since our interest was in the impact of each organic residues amendments on microbial community composition and function, we compared the different treatments with the addition of NPK only (control) in pairs because major differences could be masked if analysing all the treatments together. Relatively few groups of bacteria responded to different residues application compared to the control. Some of the groups with higher abundances in the straw treatments are known to have traits related to functions associated with C-compounds degradation and methylotrophic metabolism as well as with functions related to nitrogen metabolism including nitrogen fixation, denitrification and nitrification. For example, some species of *Burkolderiales*, *Rhizobiales, Myxococcales*, and *Rhodospirillales* are nitrogen fixing, denitrifier bacteria and characterised as having strong catabolic versatility, which property enables them to degrade a wide range of C-compounds including cellulose or lignin (DeAngelis et al., 2011; Orlando et al., 2012; Jones, 2015; Saarenheimo et al., 2015; Sacco et al., 2016). Moreover, these bacterial groups could be endophytic of sugarcane plants as representative species belonging to these groups have been isolated from sugarcane roots, stems, and leaves (Muangthong et al., 2015). Although the previously mentioned bacterial groups have been studied substantially, less is known about other groups, such as the Gemmatimonadales and Verrucomicrobiales. Members of Gemmatimonadetes have been found to be more active in soil with the addition of biochar made from rice straw (Xu et al., 2014; Whitman et al., 2016), while Verrucomicrobiales are generally oligotrophic with a slow-growing life strategy and found in high abundance in soil with straw blanket coverage (Ramirez et al., 2012; Navarrete et al., 2015a; Navarrete et al., 2015b). *Rhodobacterales* and *Rhodocyclales* are also decomposers with diverse physiological capabilities allowing the anaerobic reduction of nitrate with the degradation of aromatic hydrocarbons or halogenated compounds (Hesselsoe et al., 2009; Dong et al., 2014).

Furthermore, other anaerobic-like organisms such as Methylophilales, have been identified as methanol-consuming denitrifiers (Fan et al., 2014; Phan et al., 2016), while Desulphuromonadales and Desulphovibrionales are

sulphate/sulphur-reducers and capable of oxidising saturated fatty acids via sulphur reduction (Gittel et al., 2014; Islam et al., 2015; Ihara et al., 2017). However, aerobic organisms were also found in higher abundances in straw treatments, such as Nitrosomonadales and Nitrospirales which are involved in the bottleneck of nitrification (Prosser et al., 2014). Previously, Pitombo et al. (2015) demonstrated that straw amendments in sugarcane crop increased the orders involved with nitrification. Similarly, Navarro-Noya et al. (2013) and Navarrete et al. (2015a) found that sugarcane straw retained on the soil surface had a significant positive effect on the relative abundance of members of *Betaproteobacteria, Gammaproteobacteria*, *and Verrucomicrobia*. These results are evidence that straw selected specialised microbes, mainly decomposers, that degrade a high molecular weight of organic compounds which are favoured by straw surface application (Fierer et al., 2007; Kielak et al., 2016b). Besides that, this crop residue may have functioned as a barrier to water loss providing anaerobic microsites, ideal for anaerobic microbes related to N_2O emission. The microbial decomposers utilise different organic and inorganic C in the added residues as substrate for metabolism by retaining some C in their biomass and releasing the others as metabolites or CO_2. From the results, we suggest that the decomposition is not only related to C but also to N, as microbes could be closely coupled with other essential microbial metabolisms.

Interestingly, orders of Actinobacteria and Bacteroidetes decreased in treatments with straw and vinasse residues, respectively. This could be related to the copiotrophic lifestyle as members of Actinobacteria thrive in conditions of elevated labile organic substrates exhibiting relatively rapid growth rates (Eilers et al., 2010; Goldfarb et al., 2011). In addition, the increment of Actinobacteria and Bacteroidetes is relatively common in soils with inorganic N fertilisation, similar to our control treatment (Fierer et al., 2011; Ramirez et al., 2012; Pan et al., 2014; Huang et al., 2017). Navarrete et al. (2015a) also find decreased Actinobacteria abundance with sugarcane straw addition in a mesocosm experiment. Contrastingly, Acidobacteria decreased with residues addition despite being oligotrophic, however, ammonium nitrate fertilisation through nitrogen could decrease soil pH (Pierre, 1928; Fierer et al., 2007), which is favourable for Acidobacteria growth (Sait et al., 2006; Kielak et al., 2016a).

The vinasse amendment in soil might stimulate r-strategist bacteria with faster growth rates. This was predicted mainly in vinasse application treatments since vinasse is rich in labile carbon; *Firmicutes* (Bacillales, Lactobacillales and Selenomonadales) were highly abundant in vinasse treatments and members of this phylum are known to be fast-growing when stimulated in a C-rich environment, capable of fermenting various organic substrates and forming spores, which increase their ability to survive stressful climatic conditions (Hayden et al., 2012; Sharmin et al., 2013). *Firmicutes* have been reported to be present in vinasse (Costa et al., 2015b), and they survive the stressful conditions of the thermophilic treatment of vinasse production. Therefore, vinasse application might be a great

chance to add members of *Firmicutes* to soil. Moreover, Pitombo et al. (2015) pointed out that general fermenters such as *Lactobacillus* (*Firmicutes*) are present in vinasse, and when vinasse is applied to soil those microorganisms might contribute to N_2O emissions. Thermophilic microorganisms both have a tolerance to high temperatures and also change the pH in the fermenters. These microorganisms, acidophiles belonging to *Firmicutes*, thrive in vinasse to pH 4.0. Considering the substantial amount of vinasse that is applied to the soil, vinasse may affect the microbial activity and relative abundance of specific taxonomic groups in sugarcane-cultivated soils by introducing exogenous acidophilic microbes (Cassman et al., 2018). Apparently, these bacteria could persist for a short time in the soil. Pitombo et al. (2015) observed an increase in the abundance of *Lactobacillaceae* in treatments with vinasse, but after 14 days, the relative abundance decreased showing that vinasse-exogenous microbes are unable to survive in the soil conditions after certain period.

The overall potential microbial function in soil inorganic fertiliser (control) is found in genes associated with a higher abundance of general metabolic functions such as carbohydrates and amino acids. This may indicate an abundance of reads-related functions for the maintenance of basic cellular machinery, enabling the growth and metabolism of microbes (Moran, 2009). As straw is characterised as having relatively large amounts of highly lignified structural carbohydrates (cellulose, hemicellulose, and lignin) and a small amount of structural proteins, microorganisms involved in the metabolism of aromatic compounds were overrepresented in straw treatments when compared with control suggesting that these microbes could compete with other decomposers that are able to access lower recalcitrance polymers. This possible competition is evidenced by the decrease of carbohydrate metabolism in both treatments with straw addition. Furthermore, the treatment with only straw showed a relatively high abundance of the 'virulence, disease, and defence' category. Mendes et al. (2014) also found this function in soil, but it is difficult to draw solid conclusions on this observation because to date, no studies have focused on these categories in the metagenome data of soils under agricultural practices. However, as mentioned previously, endophytic microorganisms were found in higher abundances in straw treatments and many of the endophytes produce secondary metabolites which have antifungal and antibacterial properties and could inhibit the growth of other microorganisms. The addition of only vinasse, incremented the proportions of genes associated with dormancy and sporulation. This fact was to some extent expected since the phylum of *Firmicutes* increased, including the orders of *Bacillales* and *Selenomonadales*, both of which are well known spore-forming microorganisms (Hayden et al., 2012; Sharmin et al., 2013). However, more studies will be required to capture a more comprehensive understanding to tease apart the effect of N fertilisation from residues amendments.

In our work, the organic residues contributed to increased N_2O emissions. The largest emission of N_2O was observed for vinasse mixed with straw, for which

treatment the N_2O emission increased 8.9 times than the control. The vinasse and straw alone showed increases of 3.2 and 2.8 times compared to the control, respectively. Carmo et al. (2013) also observed that the application of vinasse with crop residue in the soil surface of sugarcane fields resulted in significant increase in the emissions of GHGs, especially N_2O. In a recent study, Pitombo et al. (2015), using 16S gene amplicon sequences, found similar orders, including *Burkholderiales*, *Myxococcales*, and *Lactobacillales*, which can explain the N_2O fluxes from soil. Looking into nitrogen metabolism, we found microorganisms related to nitrification, denitrification, and nitrogen fixing pathways in the treatments with residues. As the three different treatments with residues showed higher abundances of Nitrosmonadales when compared with control, this could be evidence that nitrification is one of the main pathways responsible for N_2O emissions in sugarcane fields. Ammonia-oxidising bacteria (AOB) were previously shown to be the main drivers of N_2O emissions via the nitrification pathway in sugarcane plantations (Soares et al., 2016).

Our results indicate that the addition of residues cause changes in the structure and functions of microbial communities, in particular in the presence of straw. The addition of straw resulted in the increase of functions related to carbon metabolism and vinasse increased genes associated with sporulation. The different organic residues added into soil resulted in increases of microorganisms related to the nitrogen metabolism contributing to increased N2O emissions.

5. Author contributions

A.K.A.S., K.S.L., L.M.P., H.C. and E.E.K designed research; L.M.P. and J.B.C. conducted the experiment; L.M.P., A.P. and E.E.K. obtained the data; A.K.A.S, K.S.L., L.W.M. and L.F.W.R. performed the statistical analyses; A.K.A.S., K.S.L. and E.E.K wrote the paper. All authors reviewed the manuscript.

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Supplementary Data

Supplementary Tables

Table S1| Chemical composition of the vinasse applied as crop residue into soil.

Parameter	Vinasse
[a]COD (g O_2 L^{-1})	18.60
[b]BOD - Δt = 5 days (g O_2 L^{-1})	6.00
[c]Organic C (g L^{-1})	6.97
pH	4.20
Conductivity (dS m^{-1})	4.00
Hardness as $CaCO_3$ (g L^{-1})	3.20
Total N (g L^{-1})	0.61
N-NH_4^+ (mg L^{-1})	51.10
N-NO_3^- (mg L^{-1})	5.00
N-NO_2^- (mg L^{-1})	<1.00
Na^+ (mg L^{-1})	73.60
K^+ (g L^{-1})	1.87
Ca^{++} (g L^{-1})	0.67
Mg^{++} (g L^{-1})	0.37
SO_4^- (g L^{-1})	2.60
PO_4^- (g L^{-1})	0.19

[a] Chemical Oxygen Demand;
[b] Biological Oxygen Demand;
[c] Organic Carbon determined according to COD values.

Table S2| Number of sequencing reads, base pairs, reads assigned to SEED Subsystems and percentages of predict proteins before and after quality control given by the MG-RAST pipeline from the treatments control, straw, vinasse and vinasse plus straw in sugarcane experiment.

			Before QC					After QC								
Sample ID	Metagenome ID	treatment_day	bp Count	Sequences Count	Mean Sequence Length	Mean GC percent	Artificial Duplicate Reads: Sequence Count	bp Count	Sequences Count	Mean Sequence Length	Mean GC percent	Predicted Protein Features	Predicted rRNA Features	Identified Protein Features	Identified rRNA Features	Identified Functional Categories
32	4617696	C_day1	19756738	190462	104 ± 49	62 ± 9	99	18691950	164777	113 ± 46	62 ± 9	124228	3164	34611	113	26656
38	4617702	C_day1	18408192	186313	98 ± 47	62 ± 9	126	17164039	156327	109 ± 44	62 ± 9	114956	3174	32654	101	25465
55	4617720	C_day1	20258634	201515	100 ± 48	61 ± 9	91	18953867	169752	111 ± 44	61 ± 9	126522	3336	34983	111	26861
63	4617728	C_day3	23798947	245059	97 ± 48	61 ± 10	156	22022974	201516	109 ± 44	61 ± 9	146795	4064	37831	140	28694
69	4617734	C_day3	9283626	91185	102 ± 50	60 ± 10	33	8713074	77203	113 ± 46	60 ± 10	57485	1423	14839	54	11231
85	4617751	C_day3	13125940	122086	108 ± 52	61 ± 9	53	12438714	105377	118 ± 48	61 ± 9	81053	1932	21944	59	16405
183	4617622	C_day8	18011656	176125	102 ± 49	60 ± 10	91	16905288	149204	113 ± 45	60 ± 10	111508	2947	30135	115	22838
189	4617628	C_day8	12098485	119820	101 ± 49	61 ± 10	36	11336692	101241	112 ± 45	61 ± 10	75790	2063	20791	95	16116
205	4617647	C_day8	19580787	210761	93 ± 46	61 ± 11	138	17887010	169260	106 ± 42	61 ± 10	120159	3830	36095	204	28395
99	4617766	C_day14	21589295	207308	104 ± 51	61 ± 9	93	20299204	175962	115 ± 47	61 ± 9	132994	3508	37980	148	29408
115	4617549	C_day14	19743224	185705	106 ± 51	61 ± 8	71	18648891	158945	117 ± 47	61 ± 8	122196	2931	34962	93	27039
123	4617558	C_day20	22993630	210264	109 ± 52	61 ± 10	83	21861556	183140	119 ± 48	61 ± 10	142119	3298	42634	168	32613
129	4617564	C_day20	15666164	147528	106 ± 50	61 ± 10	40	14868021	128128	116 ± 46	61 ± 10	99034	2486	27285	110	20910
145	4617582	C_day20	10521797	101100	104 ± 50	61 ± 9	65	9904722	86190	115 ± 46	61 ± 9	65828	1675	17987	58	13858
153	4617590	C_day24	13770586	133190	103 ± 49	62 ± 9	54	12980205	113862	114 ± 45	62 ± 9	86728	2307	25519	107	19890
159	4617596	C_day24	17013123	169461	100 ± 48	62 ± 9	108	15880349	141863	112 ± 44	62 ± 9	106345	2821	28937	106	22229
175	4617613	C_day24	16690570	161521	103 ± 49	61 ± 10	86	15717633	137909	114 ± 45	61 ± 10	104439	2787	29631	120	22710
213	4617656	C_day30	23064431	246240	94 ± 46	62 ± 10	181	21134741	198686	106 ± 43	62 ± 9	141682	4393	37435	104	28679
219	4617662	C_day30	15482606	148941	104 ± 49	62 ± 9	114	14578471	127050	115 ± 45	62 ± 9	97219	2416	27471	91	21317
235	4617680	C_day30	20050723	191029	105 ± 49	61 ± 10	144	18956800	164440	115 ± 45	61 ± 9	126461	3080	35319	96	27113
2	4617640	C_day46	8587733	75273	114 ± 50	60 ± 9	20	8282206	67878	122 ± 46	60 ± 9	54471	1087	15560	55	11902
8	4617745	C_day46	15201268	144882	105 ± 49	61 ± 10	70	14400615	125570	115 ± 46	61 ± 10	94980	2480	26331	133	20019
24	4617685	C_day46	20511300	203755	100 ± 48	60 ± 9	64	19267441	173591	110 ± 44	60 ± 9	128456	3448	36261	120	28112
34	4617698	S_day1	16994110	166469	102 ± 49	62 ± 10	112	15962657	141259	113 ± 45	62 ± 10	106056	2791	29780	135	23068
65	4617730	S_day3	15048383	153233	98 ± 48	61 ± 10	90	14008545	127640	110 ± 44	61 ± 10	94259	2711	26682	122	21164
90	4617757	S_day3	18553875	182287	102 ± 51	62 ± 9	67	17355743	152883	114 ± 47	63 ± 9	114337	3172	32145	121	24989
185	4617624	S_day8	15230014	153324	99 ± 47	61 ± 9	76	14223406	128632	110 ± 44	61 ± 9	95740	2670	26178	107	20418
190	4617630	S_day8	15891435	164262	97 ± 47	62 ± 10	113	14698834	134890	109 ± 43	62 ± 9	99045	2822	27850	100	21720

210	4617653	S_day8	15363603	156261	98 ± 48	62 ± 10	113	14267141	129396	110 ± 44	62 ± 10	95329	2736	26683	109	20792
95	4617762	S_day14	19489188	192190	101 ± 50	63 ± 9	83	18234362	161430	113 ± 46	63 ± 9	120667	3274	33899	112	26395
120	4617555	S_day14	24941381	234328	106 ± 51	61 ± 9	118	23617648	202154	116 ± 47	61 ± 9	154635	3852	45673	168	35745
125	4617560	S_day20	20833996	195170	107 ± 50	62 ± 10	108	19780145	169620	117 ± 47	62 ± 9	130982	3141	38091	113	29798
130	4617566	S_day20	19182340	185241	104 ± 51	61 ± 10	78	18041159	157548	115 ± 47	61 ± 10	118078	3093	31999	157	24239
150	4617588	S_day20	14154055	135596	104 ± 51	61 ± 10	71	13317864	115313	115 ± 47	61 ± 9	87565	2078	24270	90	18642
155	4617592	S_day24	12988657	129719	100 ± 49	62 ± 9	58	12107118	108148	112 ± 45	62 ± 9	80932	2194	22493	73	17557
180	4617619	S_day24	10255202	102196	100 ± 47	61 ± 9	68	9583445	85835	111 ± 43	61 ± 9	64561	1695	17474	72	13366
181	4617620.3	S_day24	17,118,971	171,967	100 ± 48	62 ± 10	100	15,992,578	144,333	111 ± 44	62 ± 10	106,983	2,853	28,974	123	22,648
215	4617658	S_day30	20185294	201754	100 ± 48	62 ± 10	186	18852138	169259	111 ± 44	62 ± 10	127035	3397	34609	139	26808
220	4617664	S_day30	22383208	229170	98 ± 47	62 ± 10	177	20784057	190141	109 ± 43	62 ± 9	140231	3818	37307	127	28780
240	4617686	S_day30	10574978	101826	104 ± 48	61 ± 9	66	9991103	87594	114 ± 44	61 ± 9	67400	1681	18340	43	14152
4	4617703	S_day46	12329201	112795	109 ± 49	61 ± 9	47	11796501	99848	118 ± 45	61 ± 9	78606	1791	21893	94	16985
9	4617756	S_day46	12597023	117551	107 ± 49	60 ± 9	49	12024926	103932	116 ± 45	60 ± 9	80487	1870	21323	72	16383
29	4617692	S_day46	13032158	123854	105 ± 48	61 ± 9	35	12408545	108820	114 ± 45	61 ± 9	83450	2103	23234	77	18119
42	4617706	V_day1	14101683	139604	101 ± 49	61 ± 9	59	13195514	117457	112 ± 45	61 ± 9	87349	2472	24926	107	18968
46	4617710	V_day1	20751005	207032	100 ± 49	61 ± 10	122	19398661	174096	111 ± 45	61 ± 10	128983	3455	34550	135	25846
51	4617716	V_day1	17526702	174904	100 ± 49	61 ± 10	114	16387783	147184	111 ± 45	61 ± 10	108956	3056	31654	157	23848
73	4617738	V_day3	9802446	96155	102 ± 50	61 ± 10	33	9199545	81363	113 ± 46	61 ± 10	60649	1658	16974	100	13089
77	4617742	V_day3	16628499	162490	102 ± 50	61 ± 9	70	15574334	136714	113 ± 46	61 ± 9	102280	2650	26646	73	20417
82	4617748	V_day3	20510105	204269	100 ± 50	60 ± 10	129	19133546	170621	112 ± 46	60 ± 10	125746	3540	36252	205	27395
193	4617633	V_day8	15587816	154345	100 ± 47	61 ± 9	49	14644895	131304	111 ± 43	61 ± 9	98919	2671	28490	153	22128
197	4617637	V_day8	20081677	208954	96 ± 47	61 ± 9	147	18541309	171019	108 ± 43	61 ± 9	124607	3550	34240	147	26350
202	4617644	V_day8	19415831	202242	96 ± 47	61 ± 10	192	17881753	165186	108 ± 44	61 ± 10	119582	3573	36117	249	28129
103	4617536	V_day14	20568455	201664	101 ± 50	62 ± 9	93	19266952	169994	113 ± 46	62 ± 9	127711	3460	36874	142	28833
107	4617540	V_day14	23347515	229894	101 ± 50	61 ± 9	92	21822894	192645	113 ± 47	61 ± 9	143422	3801	40072	143	30764
112	4617546	V_day14	18142574	172518	105 ± 50	61 ± 9	64	17127715	147801	115 ± 46	62 ± 9	113288	2852	35783	194	27674
137	4617573	V_day20	14689727	141985	103 ± 50	60 ± 9	43	13835656	121233	114 ± 46	60 ± 9	91531	2327	24646	80	18670
142	4617579	V_day20	15228488	148154	103 ± 51	63 ± 9	72	14279519	125066	114 ± 47	63 ± 9	94022	2578	27967	120	21725
163	4617600	V_day24	15042758	145719	103 ± 49	62 ± 9	71	14144138	123839	114 ± 45	62 ± 9	94506	2445	27614	113	21434
167	4617604	V_day24	19359687	198691	97 ± 47	62 ± 9	103	17973997	164547	109 ± 43	62 ± 9	120539	3320	32998	95	25285
172	4617610	V_day24	18478557	194337	95 ± 47	62 ± 9	104	17016713	158276	108 ± 43	62 ± 9	114466	3200	32490	125	25103

223	4617667	V_day30	5289468	57531	92 ± 40	60 ± 10	24	4925268	48660	101 ± 36	60 ± 9	35052	1057	8869	51	6836
227	4617671	V_day30	19583347	186888	104 ± 49	60 ± 9	118	18485277	160364	115 ± 45	61 ± 9	122581	2929	34631	99	26088
232	4617677	V_day30	12035251	121141	99 ± 48	61 ± 9	57	11218277	101179	110 ± 44	61 ± 9	74900	2032	22349	92	17393
12	4617554	V_day46	14591382	138923	105 ± 49	61 ± 9	65	13813541	120071	115 ± 45	61 ± 9	91759	2221	25041	78	19182
16	4617597	V_day46	15013781	139575	108 ± 50	61 ± 9	53	14315628	122731	117 ± 46	61 ± 9	94876	2249	26580	86	20273
21	4617652	V_day46	16932146	175326	96 ± 44	60 ± 9	82	15879763	149906	105 ± 41	60 ± 9	108460	3181	27308	148	21003
44	4617708	V+S_day1	13792023	137984	100 ± 49	61 ± 9	87	12868395	115401	112 ± 45	61 ± 9	86103	2201	23268	76	18001
48	4617712	V+S_day1	21766038	219321	99 ± 49	60 ± 11	166	20261353	182651	111 ± 45	60 ± 10	134458	3798	38821	239	29320
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75	4617740	V+S_day3	9208498	88222	104 ± 52	60 ± 11	26	8664820	74810	116 ± 48	60 ± 11	56371	1567	17036	137	13009
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135	4617571	V+S_day20	18362468	173941	105 ± 50	61 ± 9	66	17352371	149495	116 ± 47	61 ± 9	114265	2807	32615	132	25288
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169	4617606	V+S_day24	12393359	117551	105 ± 48	61 ± 10	53	11762543	102197	115 ± 44	61 ± 9	78821	1960	22112	79	17039
173	4617611	V+S_day24	10370504	102097	102 ± 48	60 ± 10	50	9726502	86334	113 ± 44	60 ± 10	65368	1645	17612	70	13647
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229	4617673	V+S_day30	17612825	174106	101 ± 47	61 ± 9	117	16541476	147876	111 ± 43	61 ± 9	111715	2906	31189	95	24074
233	4617678	V+S_day30	13149100	122972	107 ± 48	61 ± 10	63	12527162	107905	116 ± 44	61 ± 10	84038	2082	23454	98	18166
14	4617576	V+S_day46	17348928	170286	102 ± 48	61 ± 9	63	16342296	145838	112 ± 45	61 ± 9	109214	2846	30195	112	23367
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22	4617663	V+S_day46	18667092	184765	101 ± 48	62 ± 9	59	17587038	158519	111 ± 45	62 ± 9	117365	3204	32795	121	25417

Supplementary Figures

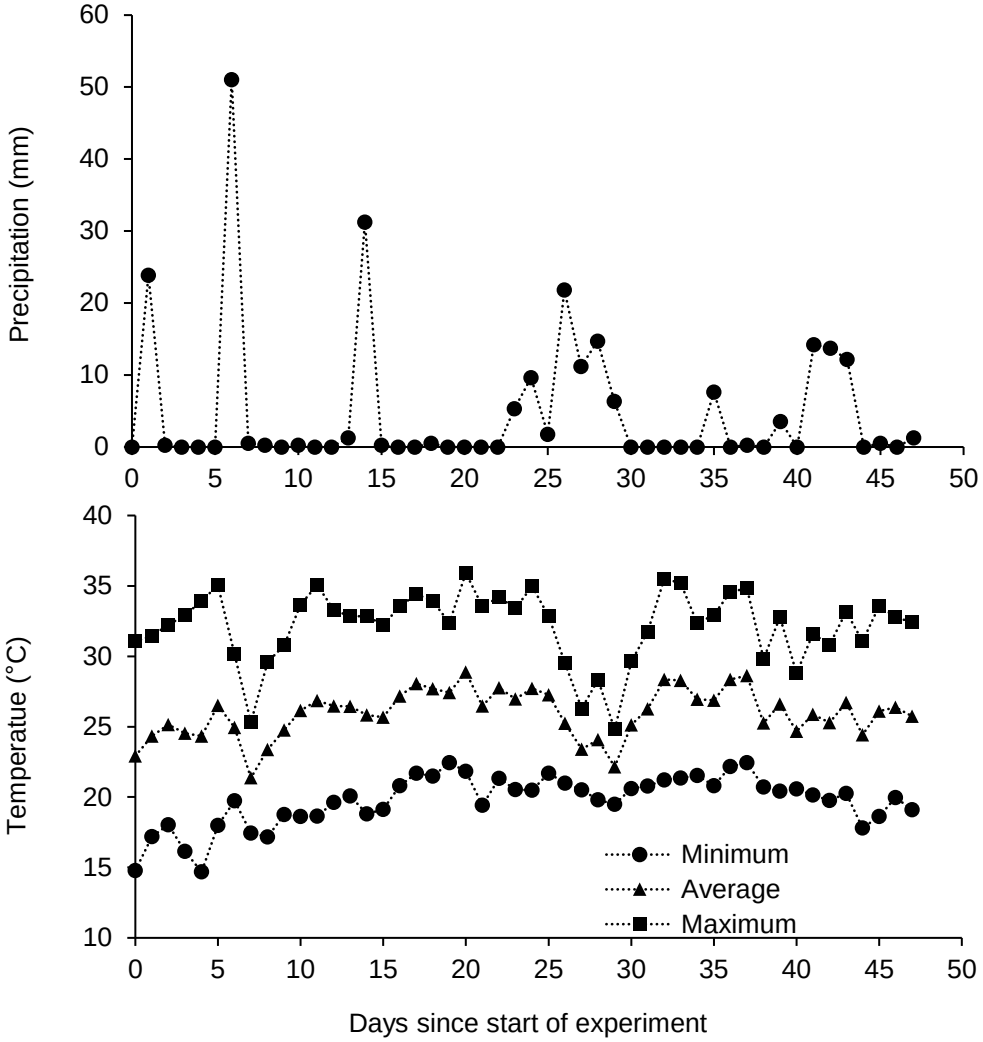

Figure S1| Averages of daily precipitation and air temperature during the experimental period.

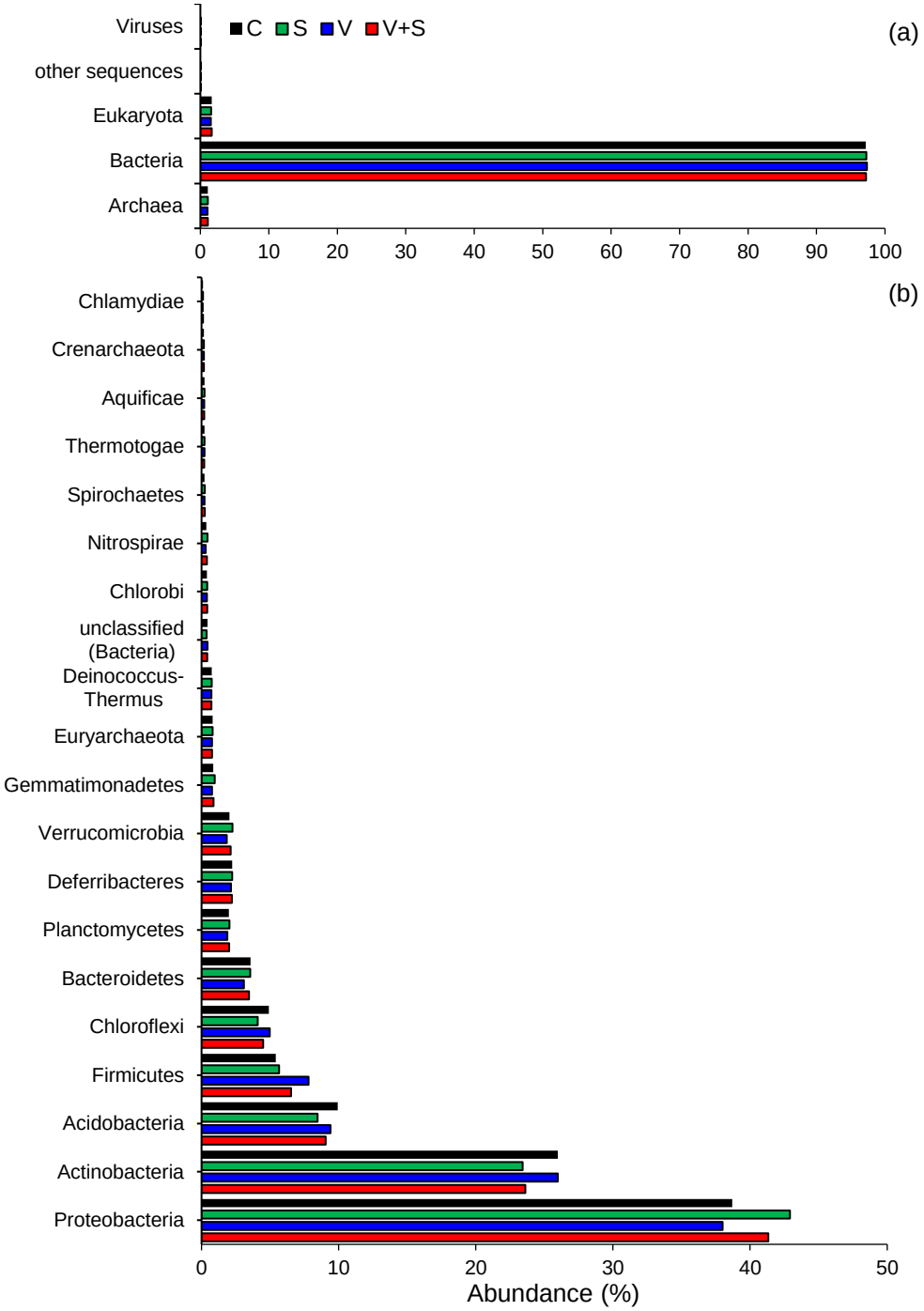

Figure S2| Sequence abundance (a) domain and (b) phylum of Bacteria and Archaea Domains based on Refseq database using normalized values between 0 and 100 (%) for (C) control, (S) straw, (V) vinasse and (V+S) vinasse + straw soil metagenomes (99.3% of the total microbial community).

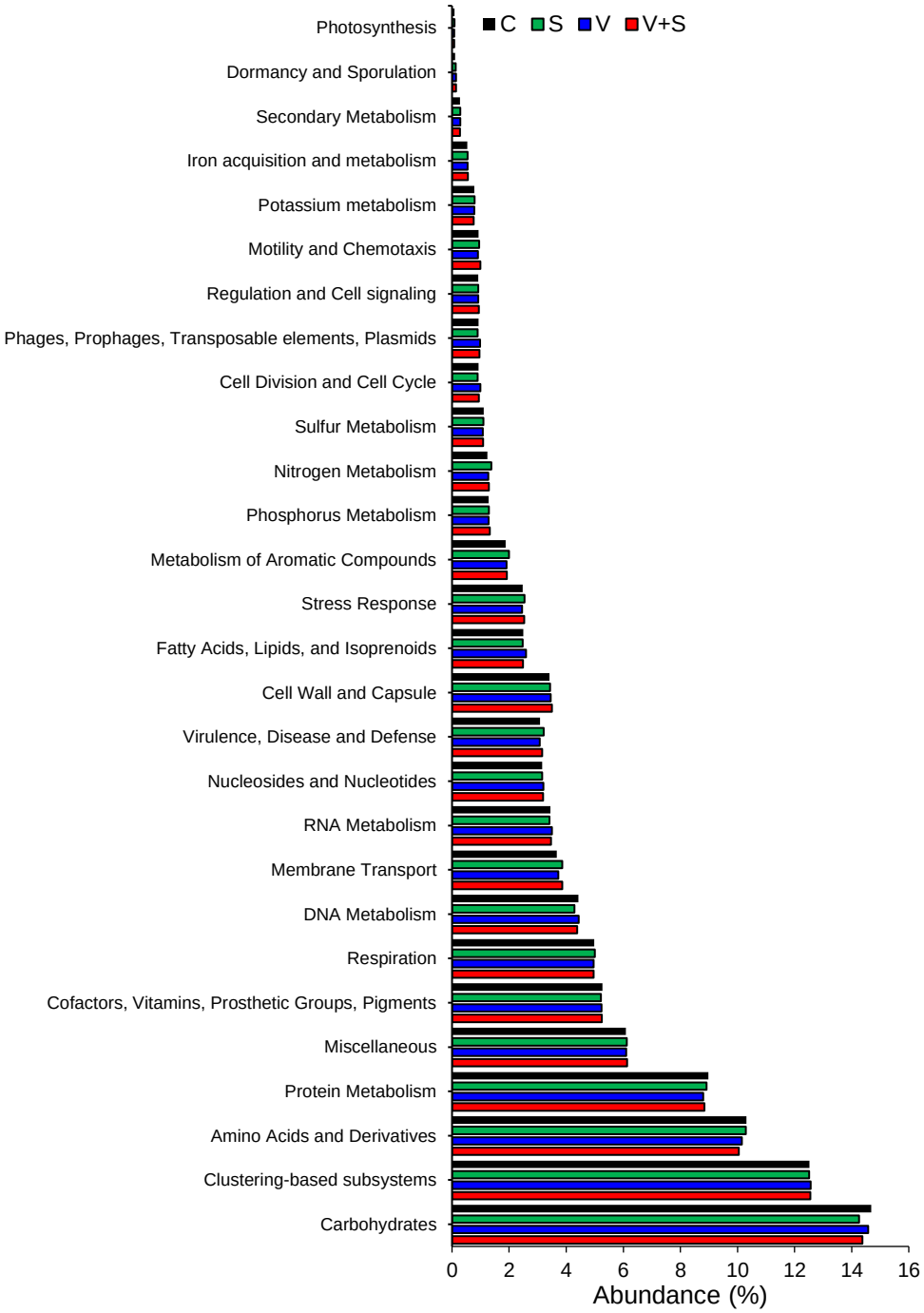

Figure S3 | Functional analysis generated by MG-RAST classified the sequences in 28 subsystems. Abundance of functional classification in subsystems categories using normalized values between 0 and 100 % for (C) control, (S) straw, (V) vinasse and (V+S) vinasse + straw soil metagenomes.

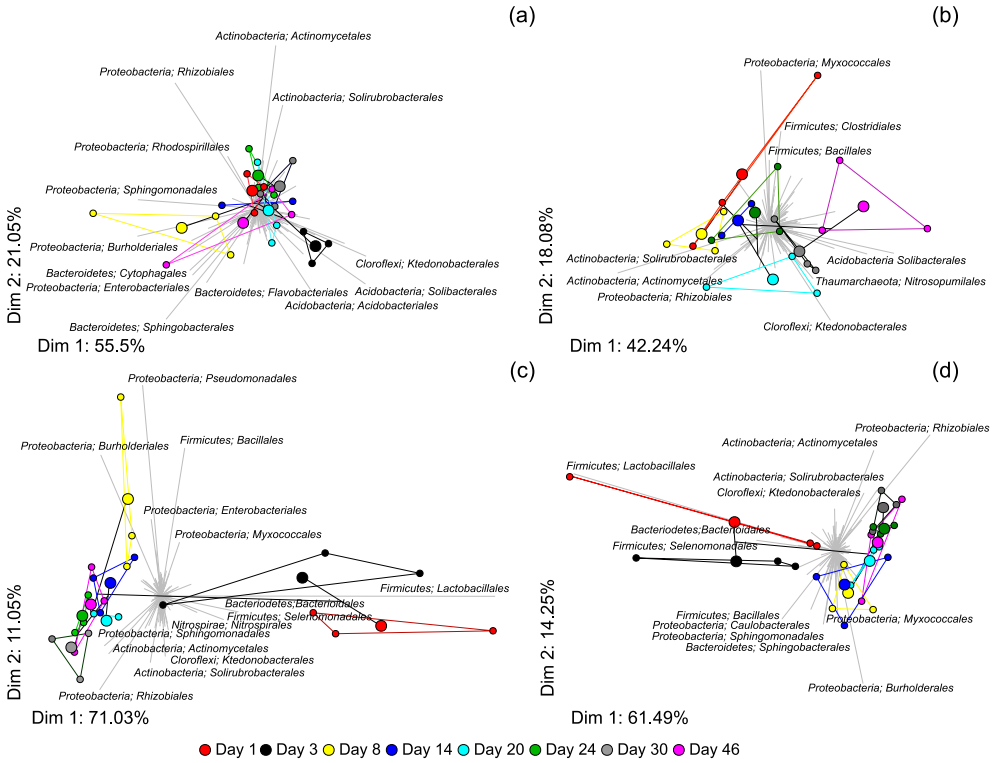

Figure S4 | Multivariate regression tree (MRT) analysis of community composition at different time points in different treatments (a) control, (b) straw, (c) vinasse, and (d) vinasse + straw. Eight (a, c, d) and seven (b) different leaves (large colored circles) were defined based on microbial abundance and composition. The community composition within leaves is represented in a principal component analysis (PCA) plot, where small points represent individual samples and large points represent the group mean (within the leaf). The grey barplot in the background indicates families whose differential abundance explains the variation in the PCA plot.

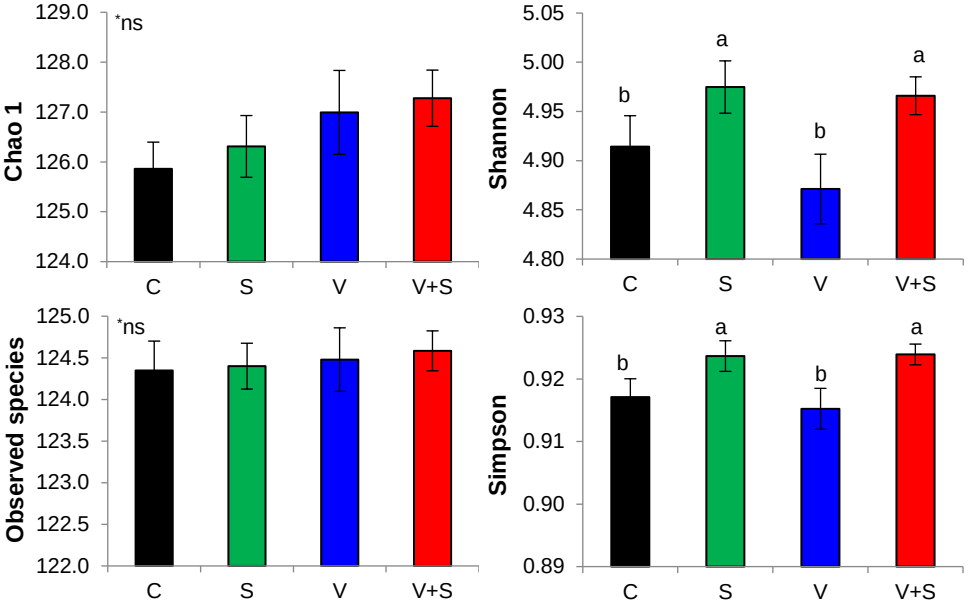

Figure S5 | Soil microbial alpha-diversity measured for different treatments (C) control; (S) straw; (V) vinasse; and (V+S) vinasse + straw. Means followed by the same lowercase letter at each treatment do not differ significantly by the Scott-Knott's test ($p < 0.05$) and *ns means non-significant difference.

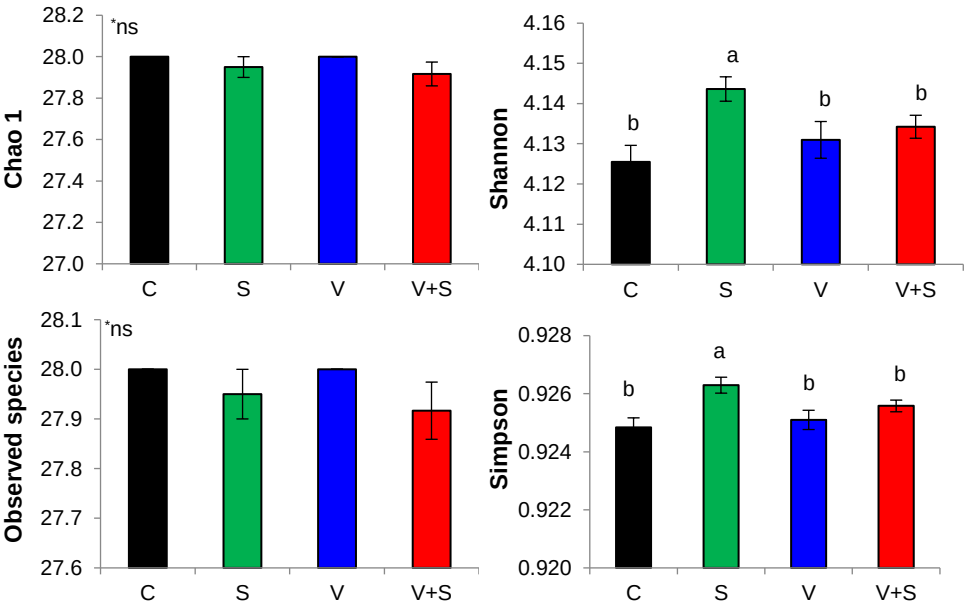

Figure S6 | Soil microbial function diversity measured for different treatments (C) control; (S) straw; (V) vinasse; and (V+S) vinasse + straw. Means followed by the same lowercase letter at each treatment do not differ significantly by the Scott-Knott's test ($p < 0.05$) and *ns means non-significant difference.

Chapter 3

Resilience of the resident soil microbial community to organic and inorganic amendment disturbances and to temporary bacterial invasion

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Abstract

Vinasse, a by-product of sugarcane ethanol production, is recycled in sugarcane plantations as a fertilizer due to its rich nutrient content. However, the impact of the chemical and microbial composition of vinasse on the soil microbiome dynamics are unknown. Here, we employed a 16S rRNA sequencing approach to evaluate the recovery of the native soil microbiome after multiple disturbances caused by the application of organic vinasse, inorganic nitrogen (N) or a combination of both during the sugarcane crop-growing season (389 days). Additionally, we evaluated the resistance of the resident soil microbial community to the invasion of bacteria inhabiting the vinasse. Vinasse is a source of microbes, nutrients and organic matter, and the combination of these factors drove the changes in the resident soil microbial community rather than seasonal fluctuations. However, these changes were restricted to a short period due to the capacity of the resident microbial community to recover. The invasive bacteria present in the vinasse were unable to survive in the soil conditions and disappeared after 31 days, except of members of the *Lactobacillaceae* family. Our analysis showed that the resident soil microbial community was not resistant to vinasse and inorganic N application but was highly resilient.

1. INTRODUCTION

Bioethanol production uses feedstocks (e.g., beet, sugarbeet, corn) and produces large amounts of organic residues that can be recycled as organic fertilizers. Brazil is currently the largest sugarcane ethanol producer (659.1 million tons of sugarcane annually) and generates approximately 10–15 litters of vinasse for every liter of alcohol produced (~360 billion liters of vinasse annually) (Freire and Cortez, 2000; CONAB, 2017). Vinasse is a by-product of ethanol production from sugarcane and is usually acidic (pH 3.5–5) with a high organic matter content (chemical oxygen demand: 50–150 g L^{-1}). To avoid discharge in rivers, alternative uses of vinasse have been explored, including fertilization to sugarcane plantations (Freire and Cortez, 2000) as a source mainly of potassium (K) but also organic matter, nitrogen (N), and phosphorus. Due to the high content of K, the regulations to the application rate of vinasse as organic fertilizer are based on the capacity of the soil to hold on cations (cation exchange capacity - CEC). Leaching of cations can occur if the amount of K applied in the soil is higher than soil CEC, with potential for groundwater contamination. So, the total amount of N appied as vinasse is not sufficient to supply the N required by the plants. Consequently, vinasse is commonly applied in combination with mineral N fertilizers in sugarcane fields. The combined application of inorganic and organic fertilizers contributes to increased greenhouse gas emissions, especially nitrous oxide (N_2O) and carbon dioxide (CO_2), due to the high water and organic matter content of vinasse (Carmo et al., 2013; Pitombo et al., 2015).

Organic fertilizers are considered more environmentally friendly than inorganic fertilizers because the former allow the nutrients produced in agricultural systems to be recycled and improve soil quality. However, the application of organic residues might disturb the resident soil microbial community. Short- and long-term impacts of inorganic fertilization practices on microbial community structure have been reported (Hu et al., 2011; Williams et al., 2013; Balota et al., 2014; Cassman et al., 2016). However, few studies have evaluated the impact of organic fertilizer on the resident microbial community, particularly immediately after application and throughout the plant-growing season (Suleiman et al., 2016; Leite et al., 2017). Organic fertilizers cause small-scale disturbances of soil due to their water content, chemical and organic components, and introduction of exogenous microbes (depending on the feedstock source) (Suleiman et al., 2016). The soil microbial community is usually resistant and/or resilient to exogenous microbes and returns to the original state (Levine and D'Antonio, 1999; Suleiman et al., 2016). Previous studies of sugarcane have shown that the combined application of vinasse and mineral N fertilizer can alter specific bacterial groups and favors high emissions of CO_2-C and N_2O-N (Navarrete et al., 2015a; Pitombo et al., 2015). When vinasse is added a few days before or after N fertilizer as an option to decrease GHG emissions, N_2O and CO_2 emissions may decrease compared with combined application (Paredes et al., 2015), but the impact on the microbial

community is unknown. In addition, no studies have considered the dynamics of the soil microbial community after vinasse application during an entire year, the soil microbiome capacity to recovery from the impact of vinasse, or the potential invasion of the resident soil microbial community by microorganisms from vinasse. Given the crucial importance of maintaining soil functions, the response of soil ecosystems to disturbances (organic and inorganic fertilizers) or environmental changes (seasonality) must be elucidated.

In this study, we evaluated the recovery of the native soil microbiome after (i) multiple pulse disturbances caused by the application of organic vinasse residue, inorganic nitrogen or both throughout the sugarcane crop-growing season and (ii) the introduction of the residue-inhabiting microbiome to the soil. The study was conducted under field conditions for 389 days using the management practices of sugarcane farmers in Brazil. This study is the first to reveal the changes in the resident soil microbial community of a sugarcane plantation over time after disturbances caused by the application of vinasse, N fertilizer and the vinasse microbiome in association with seasonal effects.

2. MATERIAL AND METHODS

2.1. Experimental setup and soil sampling

The study was conducted in an experimental field planted with sugarcane variety RB86-7515 located at Paulista Agency for Agribusiness Technology (APTA), Piracicaba, Brazil. The soil is classified as an Oxisol soil (soil taxonomy), and the physicochemical properties (Camargo et al., 1986; Van Raij et al., 2001) are shown in Table S1. The experiment began on July 15, 2014, and the last sampling was performed on August 8, 2015, one day before harvest. The sugarcane was mechanically harvested, and the straw (16 t ha^{-1}) was left on the soil.

The experiment was conducted in a randomized block design with three replicate blocks and a total of 12 plots (4 treatments x 3 blocks). In each plot, four 8-m-long rows spaced at 1.5 m were planted with sugarcane. In each treatment, the application time of vinasse in relation to the time of mineral N fertilization differed. Vinasse was applied either 30 days before or at the same time as N fertilization. We used two vinasses from different batches from the same sugar mill and ethanol production process. The first vinasse (V_f) application was performed on day zero (July 15, 2014). Nitrogen fertilizer and the second vinasse (V_s) application were performed on day 30. The treatments were as follows: 1) V_f: vinasse applied at day 0; 2) N: inorganic fertilizer ammonium nitrate, applied at day 30; 3) V_f|N: vinasse applied at day 0 and ammonium nitrate applied at day 30; 4) V_s+N: vinasse plus ammonium nitrate applied only at day 30. The treatments were chosen based on previous results for sugarcane management practices described in Chapter 2 and Pitombo et al. (2015).

The N fertilizer rate was 100 kg ha^{-1} of ammonium nitrate. A volume of 100 m^3 ha^{-1} of vinasse (V_f and V_s) was sprayed over the entire experimental plot using a motorized pump fit with a flow regulator. This volume of vinasse corresponds to the average application rate in sugarcane plantations. The mineral fertilizer was surface-applied on a 0.2-m-wide row 0.1 m from the plant, a common practice in commercial sugarcane production. The treatments with vinasse had a higher input of N than the mineral N treatment because vinasse contains mineral and organic N. The chemical characteristics of the vinasses applied in the experiments are shown in Table S2.

Soil samples (6 per plot, three samples from the two central sugarcane rows of each plot) were obtained at eleven time points 1, 3, 8, 31, 36, 42, 50, 76, 113, 183 and 389 days after the first vinasse (V_f) application. For all treatments, soil samples (0-10 cm) were collected for determination of moisture content, NO_3^--N and NH_4^+-N concentrations, pH, and DNA extraction. Soil subsamples (30 g) were stored at -80 °C for molecular analysis. Soil moisture was determined gravimetrically by drying the soil at 105 °C for 24 h. Soil mineral N (NH_4^+-N, NO_3^--N) was measured with a continuous flow analytical system (FIAlab-2500 System) after extraction with 1 M KCl, and all results are expressed per gram of dry soil. The water-filled pore space (WFPS) was calculated based on the soil bulk density (1.49 g cm^{-3}) and the porosity determined at the beginning of the experiment. Climatic data were obtained from a meteorological station located approximately 500 m from the experiment.

2.2. *Respiration measurement*

Fluxes of CO_2 were measured according to the method described by Soares et al. (2016) using PVC static chambers with a height of 20 cm and a diameter of 30 cm. The chambers were inserted 5 cm into the soil and 10 cm from the sugarcane rows. The two openings of the chamber cap were each fit with a valve: one for gas sampling and the other for pressure equilibration. Gases were sampled with plastic syringes (60 mL of gas) at three time intervals (1, 15, and 30 min) after the chambers were closed. The samples were transferred to pre-evacuated glass vials (12 mL) and analyzed in a gas chromatograph (model GC-2014, Shimadzu Co.) with an flame ionization detector (FID; 250 °C) (Hutchinson and Mosier, 1981). Before FID detection, CO_2 was reduced to CH_4 by a methanizer accessory coupled to the GC. The CO_2 flux was calculated by linear interpolation of the data from the three sampling times. CO_2 measurements were conducted for 389 days during the experiment. Throughout the experiment, gas samples were collected in the mornings. The gases were sampled every day during the first week, three times per week for the first 4 months, and weekly or biweekly thereafter in all treatments. Cumulative fluxes were calculated for each treatment using the emission values measured in the crop rows (Soares et al., 2016).

2.3. *DNA extraction and library preparation*

Total soil DNA was extracted from 0.25 g of soil using the MoBio PowerSoil DNA Isolation Kit (MO BIO, Solana Beach, CA, USA) according to the manufacturer's instructions. Three replicates of each vinasse batch were also used for DNA extraction. These replicates were treated as individual samples of the same vinasses applied in the field; we considered these samples independent in the subsequent statistical analysis. Two 50-mL aliquots of each vinasse sample were centrifuged at 10,621 g for 10 min on a benchtop centrifuge (Sigma 2-16P) to separate the cells from the liquid, and the pellets were combined. Total DNA was extracted from the pellets with the MoBio PowerSoil kit according to the manufacturer's instructions. Soil and vinasse DNA quantities and qualities were determined using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). The extracted DNA was also visualized on a 1% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer.

The extracted DNA was used for amplification and sequencing of the 16S rRNA. Targeting the variable V4 regions (forward primer, 515F-5′-GTGCCAGCMGCCGCGGTAA-3′; reverse primer 806R - 5′-GGACTACHVGGGTWTCTAAT-3′) resulted in amplicons of ~300-350 bp. Dual-index and Illumina sequencing adapters were attached to the V4 amplicons. After library quantification, normalization and pooling, MiSeq V3 reagent kits were used to load the samples for MiSeq sequencing. The samples were sequenced on the Illumina MiSeq System (BGI, China)

PANDASeq (Masella et al., 2012) was used to merge paired-end reads with a minimum overlap of 50 bp and a Phred score of at least 25. Sequences were converted to FASTA format and concatenated into a single file for downstream analyses. Briefly, the OTU (operational taxonomic unit) table was built using the UPARSE pipeline (Edgar, 2013); reads were truncated at 200 bp and quality-filtered using a maximum expected error of 0.5. After discarding replicates and singletons, the remaining reads were assigned to OTUs with a threshold of 97% identity. The chimera removal processes were then performed. Finally, bacterial and archaeal representative sequences were searched against the Greengenes 13.5 database (McDonald et al., 2012) with a confidence threshold of 80%.

2.4. Microbial community composition and data analysis

Sampling efficiency was estimated by Good's coverage (Good, 1953). Alpha diversity analyses of rarefied OTUs were calculated using QIIME software (Caporaso et al., 2012). The samples were rarefied to 3,267, 2,864 and 2,741 reads to compare the effects of vinasse on the soil microbial community, to compare the differences between treatments, and to compare vinasses, respectively. The diversity indices measured were Shannon, Simpson, and Chao1 (Chao, 1984).

To calculate the beta diversity between groups of samples (treatments or days), a non-rarefied OTU table was used to calculate non-metric Bray-Curtis dissimilarity. The Bray-Curtis dissimilarity between treatments was calculated using QIIME software and presented in a principal coordinate analysis (PCoA) to visualize the differences in bacterial community composition (Caporaso et al., 2012). Differences in community structure between treatments, time and their interaction were tested using permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) and analysis of similarity (ANOSIM) (Clarke, 1993). PERMANOVA and ANOSIM were performed using the 'vegan' package (Oksanen et al., 2017) in R package version 2.4-4 with 10,000 permutations and the 'adonis' and 'anosim' functions, respectively. The PERMANOVA and ANOSIM tests are both sensitive to dispersion, and thus we first tested for dispersion in the data by performing an analysis of multivariate homogeneity (PERMDISP) (Anderson, 2006) in PRIMER v7 software.

We used multivariate regression tree (MTR) analyses (De'ath, 2002) in the R 'mvpart' package (Therneau and Atkinson, 1997; De'ath, 2007) with the goal of identifying the temporal variation (time) that best explained the difference in microbial community composition in each treatment. MTR analysis is particularly useful to investigate both linear and non-linear relationships between community composition and a set of explanatory variables without requiring residual normality (Ouellette et al., 2012). For the analysis, the OTU table was log-transformed, and the tree was plotted after 500 cross-validations (Breiman et al., 1984), avoiding overfitting. Subsequently, the function 'rpart.pca' from the 'mvpart' package was used to plot a PCoA of the MTR.

The relative abundances of taxa in each treatment, environmental factors and daily CO_2 fluxes were checked for normal distribution of residues by the Kolmogorov-Smirnov (KS) test, and the data were subsequently log10-transformed. The normalized data set was used for further analyses. Soil pH was transformed to H^+ content:10^{-pH} before statistical analysis. Boxplots and statistical analyses were performed in R version 3.4.0.

To explore the biological factors involved in the differences between days and treatments, we identified taxonomic biomarkers at the family level. We used linear discriminant analysis effect size (LEfSe) in Microbiome Analyst (Dhariwal et al., 2017), a web-based tool, to identify the families that were most enriched in the soil (Segata et al., 2011). Based on the normalized relative abundance matrix, the LEfSe method uses the Kruskal-Wallis rank-sum test to detect features with significantly different abundances between the assigned taxa and performs linear discriminant analysis (LDA) to estimate the effect size of each feature. A significance level of $\alpha \leq 0.05$ was used for all biomarkers evaluated in this study. The relative abundances present in vinasse and in soil (vinasse-exogenous microbes) at the taxonomic level of family were compared by Tukey's test at $P \leq 0.05$.

To investigate the taxa–environment relationship, we performed a redundancy analysis (RDA) (Rao, 1964) with the log10-transformed OTU table. The matrices of explanatory environmental parameters (soil and air temperatures, pH, soil moisture, NH_4^+-N and NO_3^--N) were also log-transformed due to differences in units. RDA of microorganisms that differed significantly between days or treatments was performed to determine if interactions between environmental variables better explained the changes in the bacterial community. RDA was performed using CANOCO software for Windows 5 (Biometris, Wageningen, The Netherlands).

3. RESULTS

3.1. *Soil microbial diversity and composition*

After quality filtering, a total of 1 911 455 16S rRNA sequences with an average of 15 170 reads per sample clustered into 8 178 OTUs. Comprehensive sampling was obtained for all treatments, with an average sequence coverage of 99%. The Simpson index revealed that microbial diversity was highest in the days immediately after vinasse application and lowest on days 36, 42 and 76 (Table S3). The treatments had no effect on the Chao1 index, with similar values between treatments and days (Table S3). At days 1 and 31, application of V_f had no effect on the alpha-diversity. However, at days 36 and 42 (5 and 11 days after mineral N fertilization), the treatments with combined application of vinasse and mineral N (V_f | N and V_s+N) had higher soil microbial alpha-diversity than the treatments with mineral N or V_f (high Simpson and Shannon index). These changes explain the difference in the PCoA based on Bray-Curtis dissimilarity between the treatments with combined application of vinasse and N and the treatments with mineral N or V_f alone. However, after 113 days, neither treatment nor seasonal climatic variation showed a significant effect on the soil microbial alpha-diversity.

There was a consistently higher abundance of bacterial (97.35%) than archaeal (2.65%) sequences across treatments and days. In general, 29 bacterial phyla were identified, including eight major phyla: Proteobacteria (28.0%), Acidobacteria (19.0%), Actinobacteria (15.9%), Chloroflexi (12.5%), Planctomycetes (6.2%), Verrucomicrobia (4.9%), Gemmatimonadetes (3.0%), and Bacteroidetes (2.9%). The abundances of the other bacterial phyla were <7.6%. The two dominant Archaea phyla were Crenarchaeota (2.6%) and Euryarchaeota (0.03%) (Figure S1).

3.2. *Impact of multiple pulse disturbances on the soil microbial community over time*

PCoAs based on Bray-Curtis dissimilarity (Figure 1) showed that the soil community changed during the experiment. On day 36 (5 days after mineral N application), the microbial communities of the V_s+N and V_f | N treatments differed from those that received either only V_f at day 0 or only N at day 30. The effect of

fertilization explained the variation in community structure until day 50. This dissimilarity between treatments continued to decrease at each sampling time, and the microbial communities ultimately became similar after 113 days, suggesting long-term stability of the microbial community on the time scale of one year.

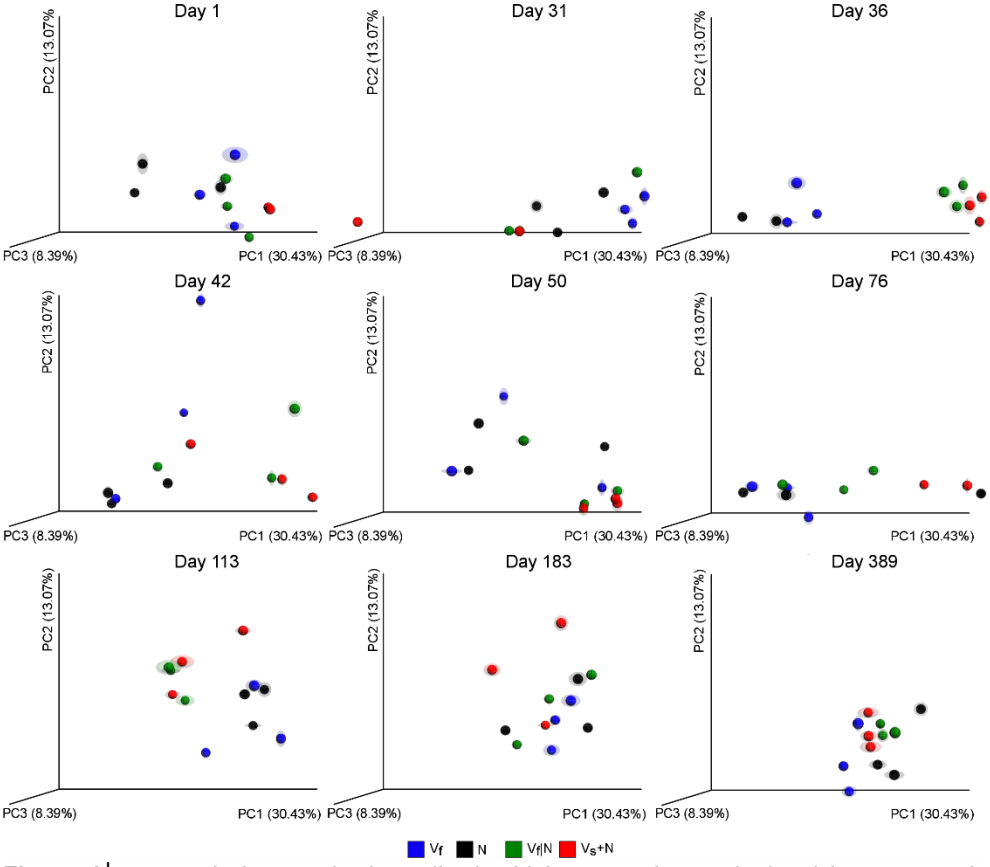

Figure 1 | Temporal changes in the soil microbial community as depicted by Bray-Curtis dissimilarity. Principal coordinate analysis (PCoA) of soils cultivated with sugarcane was performed at nine time points. The treatments were as follows: Vf, vinasse applied at day 0; N, inorganic fertilizer ammonium nitrate applied at day 30; Vf | N, vinasse applied at day 0 and ammonium nitrate applied at day 30; and Vs+N, vinasse plus ammonium nitrate applied together at day 30. Each point represents an individual sample, with colors indicating treatments.

To more clearly track the changes in community composition we assessed the difference in community composition using PERMANOVA ($p \leq 0.04$) and ANOSIM ($p \leq 0.00$) due to the homogeneity of multivariate dispersions within the groups (PERMDISP $p=0.10$ and $p=0.11$). Treatment, day and their interaction were the forces structuring the microbial community, pseudo-F values of 2.21, 1.95 and 1.61, respectively. To further explore temporal signals in the data for different treatments, we used an MRT approach. The PCA given by MRT analysis showed that the microbial community dynamics appeared to be cyclical (Figure 2), with a

return to approximately the same composition after disturbance in all treatments except Vf|N (Figure 2A and Figure 2C, respectively).

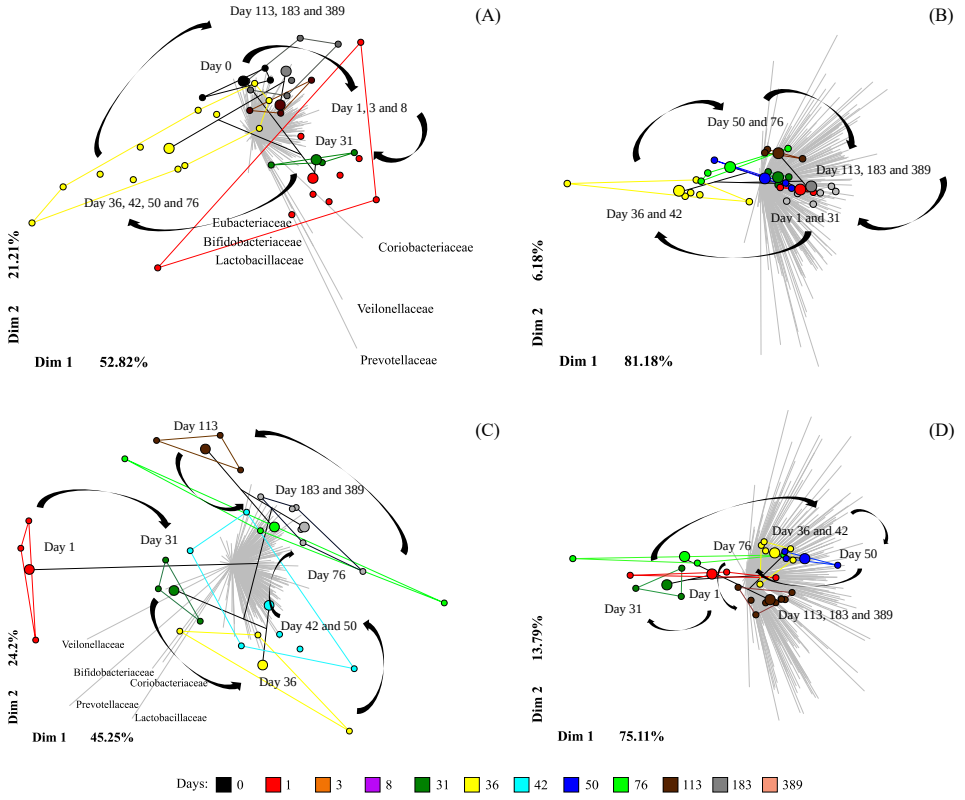

Figure 2|Multivariate regression tree (MRT) analysis showing the cyclical community composition dynamics for each treatment, (A) Vf, vinasse applied at day 0; (B) N, inorganic fertilizer ammonium nitrate applied at day 30; (C) Vf|N, vinasse applied at day 0 and ammonium nitrate applied at day 30; and (D) Vs+N, vinasse plus ammonium nitrate applied only at day 30. Six (A, D) and seven (B, C) different leaves (large colored circles) were defined based on microbial abundance and composition. The community composition within leaves is represented in a principal component analysis (PCA) plot, where small points represent individual samples and large points represent the group mean (within the leaf). The gray barplot in the background indicates families of which differential abundance explains the variation in the PCA plot.

To explore the biological factors involved in the differences in microbial communities between treatments, we identified taxonomic biomarkers at the family level on the days that had the highest microbial diversity and dissimilarity (days 36 and 42). Based on LEfSe analysis, the most enriched families in the soil were in Vf|N and Vs+N (Figure S2). The top five biomarkers were *Acetobacteraceae*, *Lactobacillaceae*, *Gaiellaceae*, FFCH4570 and *Micrococcaceae* on day 36 and *Dolo_23*, *Micrococcaceae*, *Burkholderiaceae*, *Lactobacillaceae* and *Oxalobacteraceae* on day 42.

3.3. *Weather conditions, soil analysis and CO_2 emissions*

The climatic conditions during the experimental period are shown in Supplementary Figure S3A. The mean air temperature was 21.96 °C, with minimum and maximum air temperatures of 3.4 and 39.1 °C, respectively. Over the 389 days of the study, the cumulative rain was approximately 1 064 mm (July 14 to August 15). The average WFPS was 66% on the sampling days (range of 60% to 94% WFPS). Part of the mineral N applied in the field was available in mineral form (NH_4^+-N and NO_3^--N) for approximately 80 days and the pH was similar for all treatments through time (Figure S4).

CO_2 emissions were highest in the V_s+N treatment, nearly 18 g C $m^{-2}d^{-1}$. The N fertilizer treatment had the lowest CO_2 emissions (Figure S3B). However, CO_2-C emissions increased through time with rain events and increasing temperature. Microbial activity was lower in the dry period (days 0 and 389) than in the rainy period (days 113 and 183).

Among all environmental factors, weather conditions, soil characteristics and nutrient availability, soil moisture was the explanatory factor that most explained the microbial community changes in soil with vinasse, N and combined N and vinasse application with 18.70% (Figure 3; pseudo-F=4.7, p=0.002). The others environmental variables explained less variation and acted in the opposite direction of soil moisture. Together, these environmental variables explained ~21.7% of the variation, suggesting that unmeasured biotic or abiotic factors explain the majority of the variation.

3.4. *Effect of the vinasse microbiome on the soil microbial community*

Because the two vinasses were from different batches from the same sugar mill, we assessed the microbial community composition of the V_f and V_s vinasses and determined the impact of the vinasse microbiome on the dynamics of the soil resident microbial community after vinasse application. We then tracked back the vinasse-exogenous microorganisms using the V_f treatment.

The two vinasses (V_f and V_s) had similar Chao1 indices. However, the Simpson and Shannon indices were higher in V_f than V_s (Table S4). The main families found in the vinasses were *Veillonellaceae*, *Lactobacillaceae* and *Eubacteriaceae* from the phylum Firmicutes (93.5%), *Bifidobacteriaceae* and *Coriobacteriaceae* from Actinobacteria (3.8%), *Prevotellaceae* from Bacteroidetes (2.1%), and *Acetobacteraceae* from Proteobacteria (0.4%). V_s was dominated by a single bacterial family (Figure S5). The greatest difference between the vinasses was the dominance of *Megasphaera* (79.3%) from the family *Veillonellaceae* in V_f and *Lactobacillus* (96.5%) from *Lactobacillaceae* in V_s; both of these families belong to the phylum Firmicutes. No archaeal sequences were detected in the vinasse samples (Figure S5). To assess the changes, dynamics and resilience of the soil microbial community after vinasse-microbiome application, samples were obtained at eleven time points plus samples without fertilizer collected at day 1 (day 0 in the analysis).

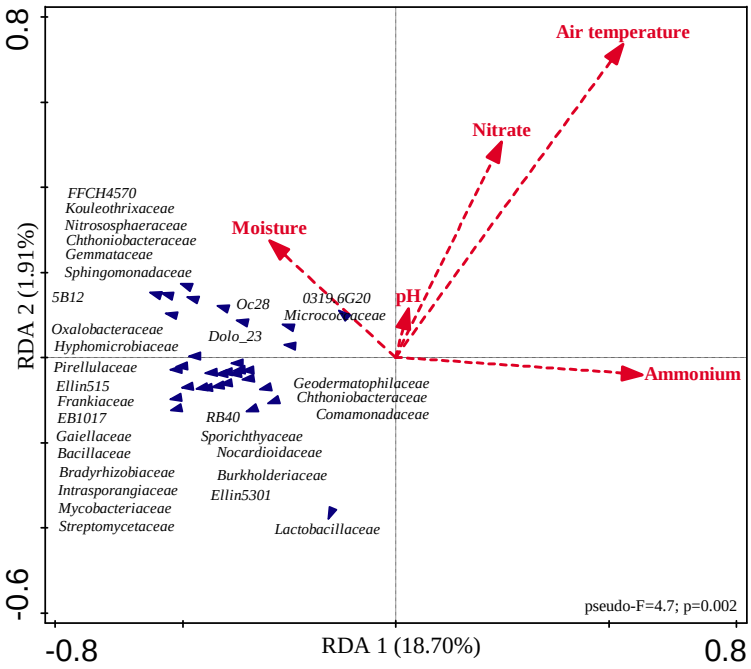

Figure 3| Redundancy analysis of environmental factors and the microbial community in all treatments. The treatments were as follows: Vf, vinasse applied at day 0; N, inorganic fertilizer ammonium nitrate applied at day 30; Vf|N, vinasse applied at day 0 and ammonium nitrate applied at day 30; and Vs+N, vinasse plus ammonium nitrate applied only at day 30.

The application of vinasse to the soil altered the resident soil microbial community (Figure 4). However, the difference in community composition could not be assessed by PERMANOVA because the invasive bacteria found in the vinasse caused high dispersion (PERMDISP p=0.04). This was solved by removing the vinasse input counts (PERMDISP p=0.20). The effect of vinasse application on the resident soil microbial community was confirmed by PERMANOVA and ANOSIM with a pseudo-F value of 1.48 ($p<0.04$) and an R value of 0.20 ($p<0.00$), respectively. To better visualize the effects of vinasse and environment (seasonality) on the resident soil microbial community, the PCoA was split into two figures, Figures 4A and 4B. According to the Bray-Curtis dissimilarity after 1 day, the microbial community in soil fertilized with vinasse differed from that of unfertilized soil (day zero) (Figure 4A). The dissimilarity continued to increase at each sampling time until day 8 and differed from day zero until day 31 (Figure 4A). Finally, after 36 days, the microbial community recovered to the original state and remained stable until day 76 (Figure 4B). The soil microbial community subsequently changed to an another stable state probably due to increases in temperature and soil moisture, with frequent rainy events (Figure 4B).

To more clearly track the changes in microbial community composition over time scales of days throughout the year, we used an MRT approach (Figure 2A). Consistent with the Bray-Curtis dissimilarity (Figure 4), the microbial community changes through time revealed resilience. The PCA ordination based on MRT (R^2 = 0.303) (Figure 2A) showed that the microbial community dynamics appeared to be cyclical, with a return to approximately the same compositional stage as day zero after 36 days. To determine if the variation observed during the year was driven by the vinasse-exogenous microorganisms, the MRT analyses were performed again after removing all microbial sequences also found in vinasse. A similar MRT result was obtained (R^2=0.34).

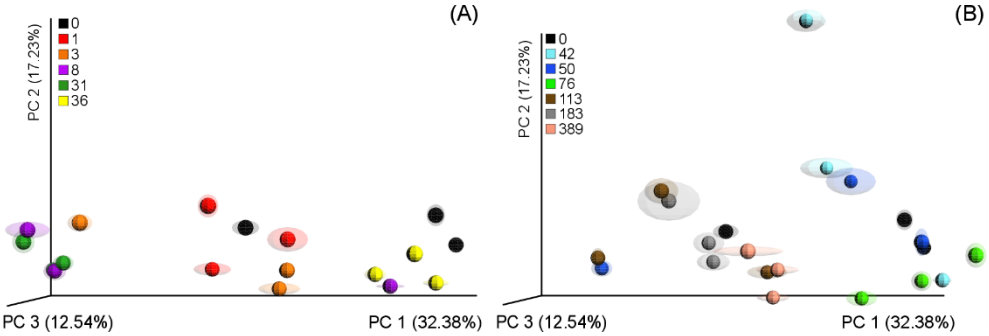

Figure 4 | (A) Temporal changes in the soil microbial community until 36 days and (B) from 42 until 389 days after the first vinasse (Vf) application, as depicted by Bray-Curtis dissimilarity. Each point represents an individual sample, with colors indicating treatments.

The LEfSe analyses showed that the relative abundances of the *Lactobacillaceae*, *Prevotellaceae*, *Veillonellaceae*, *Micrococcaceae*, *Hyphomicrobiaceae*, *Bacillaceae* and *Nitrospiraceae* families changed significantly after vinasse application in the soil (Table S5). The exogenous microorganisms found in vinasse were subsequently tracked in the soil samples. The main exogenous families disappeared or returned to the original state after 31 days (Figure 5). The highest abundances of all bacteria found in vinasse were observed on day 3. The most abundant families were *Lactobacillaceae*, *Veillonellaceae* and *Prevotellaceae*; surprisingly, the relative abundance of the *Lactobacillaceae* family increased after 183 days (Figure S6).

For the vinasse-only treatment (V_s), RDA showed that nitrate concentration (NO_3^--N) was the best explanatory environmental variable for soil microbial community change (Figure S7; pseudo-F=2.8, p=0.002). Nitrate concentration explained ~36.6% of the microbial community variation (axis 1: 31.7%; axis 2: 3.80%).

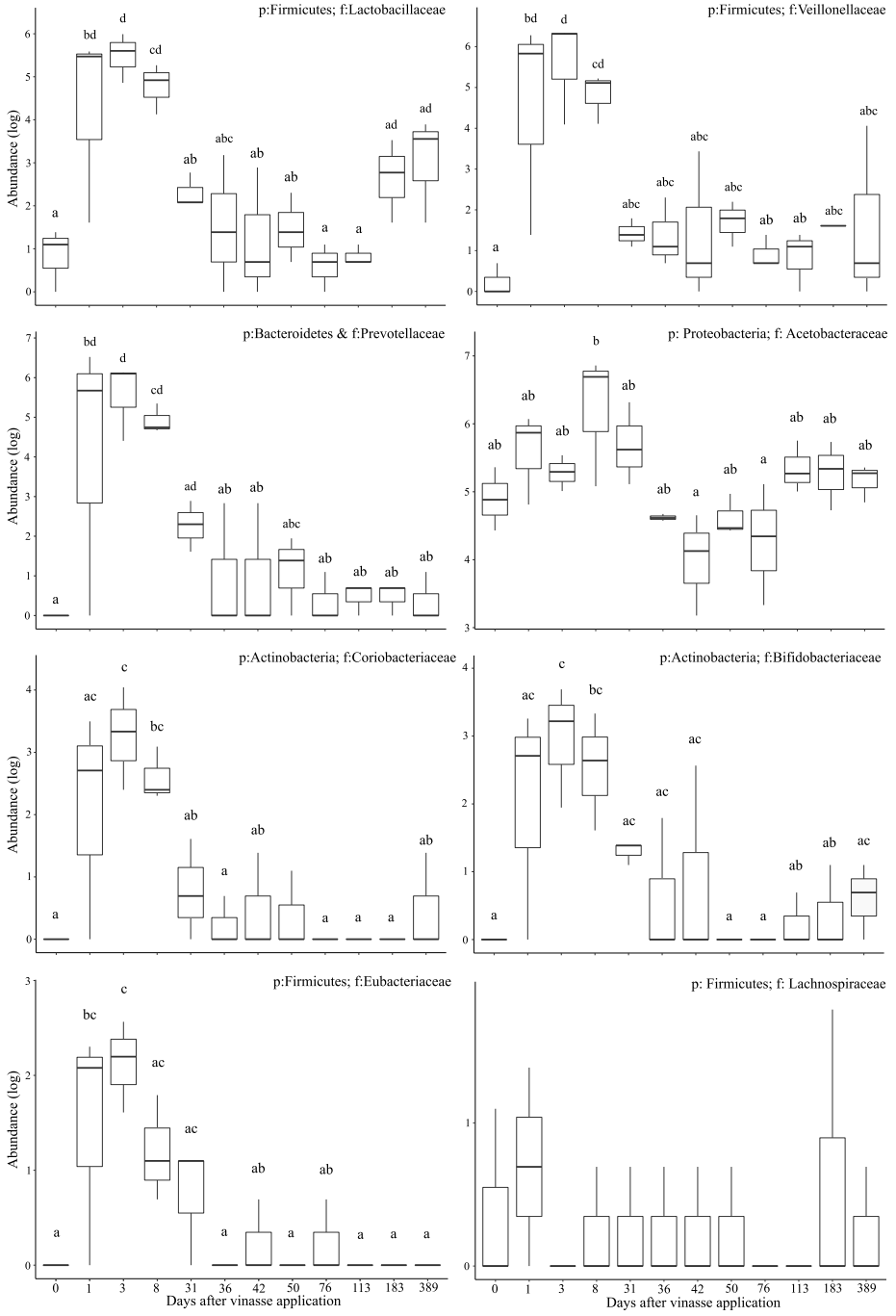

Figure 5| Relative abundance of bacterial families (families found in pure vinasse) in the soil after the first vinasse application. The abundances (log of relative abundance) of the phyla (p:) and families (f:) in three replicates per day were used. Different letters indicate significant differences between days by Tukey's HSD test (Tukey, P≤0.05).

4. DISCUSSION

In this study, the resident soil microbial community was highly resilient but not resistant to disturbances caused by the application of vinasse alone or in combination with N fertilizer. Vinasse is an organic residue rich in organic-C, N and potassium. When applied to soil, vinasse increases pH, cation exchange capacity, nutrient availability and water retention and improves soil structure (Mutton et al., 2014). In response, the abundances and activities of some members of the microbial community in the soil, particularly bacteria with a copiotrophic lifestyle, increase (Navarrete et al., 2015a; Suleiman et al., 2016). The high nutrient availability due to only vinasse application resulted in increased abundances of *Bacillaceae, Micrococcaceae* (Actinobacteria), *Hyphomicrobiaceae* and *Nitrospiraceae* families. These findings are similar to those observed in the field (Pitombo et al., 2015) and at mesocosm conditions (Navarrete et al., 2015a). Members of *Bacillaceae* are mostly aerobic or facultatively anaerobic heterotrophs that grow rapidly in response to available organic-C, such as that found in vinasse, (Pitombo et al., 2015; Mandic-Mulec et al., 2016). Members of Actinobacteria are also considered to have developed adaptations to nutrient-rich soils (Navarrete et al., 2015a). Surprisingly, *Hyphomicrobiaceae* from Alphaproteobacteria and *Nitrospiraceae* from Nitrospirae were the families that increased the most in soil after vinasse application. Many species of *Hyphomicrobiaceae* are oligocarbophilic and chemoheterotrophs that thrive only in low concentrations of carbon sources and are unable to grow in rich media. However, these organisms are capable of using NO_3^- as a source of N. By contrast, *Nitrospiraceae* includes chemolithoautotrophic aerobic nitrite-oxidizing bacteria that can use N from vinasse and straw mineralization (Daims, 2014; Navarrete et al., 2015a). Therefore, the nitrogen input from vinasse and sugarcane straw mineralization probably explains the increase in the abundances of *Hyphomicrobiaceae* (Oren and Xu, 2014) and *Nitrospiraceae*.

The application of vinasse and N fertilization alone or in combination had different effects on the soil microbial community. However, application of vinasse on the same day or 30 days before N application resulted in similar changes in the soil microbial community. The differences between the microbial communities in the treatments with combined application of vinasse plus mineral N and with sole application of mineral N or V_f were obvious until eleven days after mineral N application (day 42). The responses of the resident microbial community to the first pulse disturbance, i.e., application of vinasse, and the second pulse disturbance 30 days later, i.e., application of mineral N, were similar to the response to the single pulse disturbance caused by combined application of vinasse plus mineral N. Apparently, the time between the V_f and N applications was not sufficient to allow significant C decomposition and N mineralization from vinasse and/or N fertilizer uptake by plants. Parnaudeau et al. (2008) and Silva et al. (2013) evaluated the net and potential N mineralization of vinasses and found that vinasse released N

and C at a slow rate (Parnaudeau et al., 2008; Silva et al., 2013). It is likely that organic-C was still present in the soil at the time of mineral N application. The presence of organic-C could stimulate the resident soil microbiota, and subsequent decreases in the C:N ratio would favor fast-growing microbes with a copiotrophic lifestyle, resulting in an increase in their relative abundance (Navarrete et al., 2015a; Suleiman et al., 2016). However, the microbial communities appeared to be resilient, and after 76 days, the dissimilarity between the communities decreased. After four months, the communities were similar in all treatments.

Vinasse may affect the microbial activity and relative abundance of specific taxonomic groups in sugarcane-cultivated soils by altering soil chemical factors and introducing exogenous microbes. The vinasse-exogenous microbes were unable to survive in the soil conditions and disappeared after 31 days, with the exception of *Acetobacteraceae* (natural from soil) and *Lactobacillaceae*. Pitombo et al. (2015) observed an increase in the abundance of *Lactobacillaceae* in treatments with vinasse, but after 14 days, the relative abundance decreased and was similar to the treatments without vinasse. However, *Pitombo et al. (2015)* evaluated the microbial community for only a short period (46 days). Although the resident community in the present study was resilient and returned to the original state 36 days after vinasse application, an increase in the relative abundance of *Lactobacillaceae* was observed in all treatments with vinasse during the rainy period (days 113 and 183) that persisted in the soil even after one year. Notably, no vinasse was applied in the experimental area previously. *Lactobacillus* are generally aero-tolerant or anaerobic (Salvetti et al., 2012; Costa et al., 2015b) and are found in rich habitats with carbohydrate-containing substrates (Salvetti et al., 2012). The straw on top of the soil likely enabled *Lactobacillus* survival due to the availability of labile organic-C (straw mineralization) and higher moisture content (straw retention) (Leal et al., 2013; Carvalho et al., 2017). Based on the literature and our findings, the main contaminants of bioethanol production from sugarcane are lactic acid bacteria such as *Lactobacillus* (Costa et al., 2015b; Brexó and Sant'Ana, 2017). This study is the first to show the persistence of invasive vinasse-exogenous bacteria in soil, and further studies elucidating persistence and ecological functions in soils are needed.

The soil microbial community variation was cyclical in all treatments, with small variations over time after recovery from the disturbance caused by vinasse and mineral N. Seasonal variations may result in a microbial community that is adapted to fluctuations in temperature and precipitation (Cregger et al., 2012; Evans and Wallenstein, 2012), thus resulting in a diminished response of the resident soil microbial community to changes in temperature and rainfall during the year. We found that the soil microbial community were more responsive to organic and inorganic fertilizers than fluctuations in seasonal temperature and rainfall. Other studies have demonstrated that when microbial communities are adapted to multiple dry-wet episodes, their response is diminished with each repeated event (Steenwerth et al., 2005; Evans and Wallenstein, 2012). In additional, the high

amount of sugarcane straw (16 t ha^{-1}) on soil surface in the beginning of the experiment may have functioned as a barrier to water loss and soil temperature variation (Carvalho et al., 2017). This barrier effect may be responsible for the small difference in the community between the dry and rainy seasons.

The interpretation of our results for the impacts of vinasse and vinasse-exogenous microbes on the soil resident community is subject to methodological limitations. First, the exogenous microbes present in vinasse and later found in the soil were considered invasive bacteria in our study. By definition, a microbial invader is a microbe that was not part of the resident community prior to the time point of observation (Kinnunen et al., 2016). In our study the microbes from vinasse were not found in the soil before vinasse application or in the N treatment, with the exception of the Acetobacteraceae and *Lactobacillaceae*. The average observed number of OTUs for these two families was 142 and 2, respectively, and an observation of 2 OTUs could represent a mistake during sequencing. Besides, we did not use specific primers or label the vinasse-exogenous microbes to track them back in the soil; instead, we used the number of OTU counts found in the 16S rRNA datasets for vinasse and for the soil samples. In our case, this approach was sufficient to answer the question regarding soil microbial invasion. Second, the OTU data were compositional (Gloor and Reid, 2016). Removing reads does not remove their influence on other OTUs because of the dependent structure of compositional data (Gloor and Reid, 2016; Morton et al., 2017). This dependence could explain why there were no apparent differences in soil community diversity after removing bacterial families found in the vinasse community. However, the removal of reads is analogous to the common practice of removing eukaryotic or archaeal reads from 16S rRNA data. Removing reads creates a bias in the remaining data; however, the same bias is likely introduced for all days of sampling, and thus sample comparisons should remain valid. A similar approach was used by Tromas et al. (2017) to predict cyanobacterial blooms in lakes.

This study reveals soil microbial community dynamics in response to the application of organic and/or inorganic fertilizers along the sugarcane cycle. Vinasse was the main driver of changes in microbial community structure, and the soil resident communities were not resistant to vinasse application but appeared to be resilient. The invasive bacteria in vinasse microbiome were unable to survive in the soil and disappeared after 31 days, except of *Lactobacillaceae*. Further studies are needed to determine the consequences of the invasive *Lactobacillus* and consecutive vinasse application to the resident soil microbial community.

5. Author contributions

K.S.L., A.K.A.S, E.E.K, and. H.C. designed research; K.S.L. conducted the experiment; K.S.L. and A.P. conducted the PCR analyses; K.S.L. and A.K.A.S performed the statistical analyses; K.S.L., A.K.A.S, J.A.V., and E.E.K wrote the paper. All authors reviewed the manuscript.

6. Acknowledgments

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Supplementary Data

Supplementary Tables

Table S1| Physicochemical properties parameters of soil (0- to 20-cm) (mean ± standard deviation).

pH [a]	OM [b]	P [c]	K	Ca	Mg	H+Al [d]	CEC [e]	Soil texture [f]		
								Clay	Silt	Sand
	g dm^{-3}	mg dm^{-3}	----------		mmol$_c$$dm^{-3}$	----------		---------	g kg^{-1}	---------
5.0 ±0.1	21.1±1.3	14.6±1.1	0.7±0.1	17.4±3.2	11.9±2.7	34.9±3.0	65.1±5.5	631±11	151±8	218±2

Abbreviations are as follows:
[a] ($CaCl_2$; 0.0125 mol L^{-1})
[b] Organic matter.
[c] Available phosphorus, K, Ca, and Mg were extracted with ion exchange resin.
[d] Buffer solution (pH 7.0).
[e] CEC (Cation exchange capacity).
[f] Soil texture determined by the densimeter method.

Table S2| Chemical characteristics of the different batches of vinasses from the first (V_f) and the second (V_s) vinasse application to the soil.

Vinasse [a]	Application time	pH	C org [b]	N tot [c]	NH_4^+-N [d]	NO_3^--N [e]	P	K	C/N
			g L^{-1}	g L^{-1}	mg L^{-1}	mg L^{-1}	g kg^{-1}	g kg^{-1}	
V_f	Jul. 15, 2014	4.8	28.8	0.51	45.7	8.8	0.11	3.5	57/1
V_s	Aug. 15, 2014	3.9	31.4	0.89	41.6	4.1	0.23	4.7	35/1

Abbreviations are as follows:
[a] V_f: Vinasse applied at day zero (15 July, 2014) and V_f: Vinasse applied at day 30 (Aug. 15, 2014).
[b] C org: Total organic carbon.
[c] N tot: Total organic nitrogen.
[d] NH_4^+-N: ammonium.
[e] NO_3^--N: nitrate.

Table S3| Soil microbial alpha-diversity measured at nine time points. The treatments are: V_f: vinasse applied at day 0; N: inorganic fertilizer ammonium nitrate, applied at day 30; V_f|N: vinasse applied at day 0 and ammonium nitrate applied at day 30; and V_s+N: vinasse plus ammonium nitrate applied only at day 30.

ANOVA test [a]	Chao1	Simpson	Shannon
Treatment	ns	***	**
Day	**	***	**
Treatment x Day	ns	***	***

Tukey's test [b]	DAYS AFTER VINASSE APPLICATION								
	1	31	36	42	50	76	113	183	389
	Chao 1								
	ab	ab	ab	a	a	ab	b	a	ab
V_f	201.22	218.13	219.91	212.54	230.82	210.89	185.53	214.14	212.52
N	232.48	216.78	204.68	234.14	206.96	197.23	201.37	222.83	216.59
V_f|N	206.85	209.47	209.96	216.01	206.51	218.05	200.74	228.71	205.68
V_s+N	204.39	212.88	212.28	212.83	226.00	214.54	191.00	202.92	224.94
	Simpson								
V_f	0.98 aA	0.98 aA	0.96 abAB	0.95 bB	0.97 abA	0.95 bB	0.97 abA	0.97 abA	0.97 abA
N	0.97 aA	0.97 aA	0.95 aB	0.96 aBC	0.97 aA	0.95 aBC	0.97 aA	0.97 aA	0.97 aA
V_f|N	0.98 aA	0.97 aAB	0.98 aA	0.97 aAB	0.98 aA	0.97 aAB	0.96 aA	0.97 aA	0.97 aA
V_s+N	0.98 aA	0.95 bB	0.98 aA	0.98 aA	0.98 aA	0.98 aA	0.96 abA	0.97 abA	0.97 abA
	Shannon								
V_f	6.16 aA	6.21 aA	5.89 abAB	5.64 bB	5.96 abA	5.75 abB	5.99 abA	6.09 abA	6.07 abA
N	6.04 aA	6.15 aA	5.67 aB	5.83 aAB	5.93 aA	5.80 aAB	6.01 aA	5.98 aA	6.01 aA
V_f|N	6.14 aA	6.09 aAB	6.11 aA	6.12 aA	6.17 aA	6.07 aAB	5.74 aA	5.99 aA	6.03 aA
V_s+N	6.13 aA	5.72 aB	6.12 aA	6.23 aA	6.22 aA	6.22 aA	5.78 aA	5.97 aA	6.12 aA

[a] Symbols in the caption refer to overall ANOVA results for the given experiment; Significant difference: * p≤ 0.05; ** p≤ 0.01 and ns: Non-Significant.
[b] Means followed by the same capital letter in the column at each treatment and lowercase letter at each day of sampling do not differ significantly by the Tukey's test ($p < 0.05$).

Table S4| Soil (12 time points) and vinasse (V_f and V_s) microbial alpha-diversities.

Treatment [a]	Chao1	Simpson	Shannon
Day	*Vinasse Effect*		
	ns	***	**
0	225.27	0.97 ab	6.01 a
1	207.52	0.98 ab	6.14 a
3	225.45	0.98 a	6.17 a
8	214.68	0.97 ab	6.13 a
31	221.33	0.98 a	6.20 a
36	207.19	0.96 bc	5.86 a
42	231.88	0.95 c	5.65 a
50	216.62	0.97 ab	5.95 a
76	211.39	0.95 bc	5.73 a
113	205.23	0.97 ab	5.98 a
183	222.39	0.97 ab	6.09 a
389	228.26	0.97 ab	6.02 a
Vinasses	*Comparison Between Vinasses Input* [b]		
	ns	***	***
V_f	41.83	0.26 a	0.93 a
V_s	39.00	0.07 b	0.32 b

[a] Symbols in the caption refer to overall ANOVA results for the given experiment. Difference between vinasses or days. Significant difference: ** p≤ 0.05; *** p≤ 0.01 and ns: Non-Significant.
[b] Means followed by the same letter in the column at each vinasse or day of sampling do not differ significantly by the Tukey's test ($p \leq 0.05$).

Table S5| Microbial community at the family level of which the abundances differed statistically by linear discriminant analysis effect size (p-value ≤ 0.01) between days after first vinasse (V_f) application in the soil.

Significative difference between days – Vinasse Effect [a]	"Pvalues"	LDAscore
p__Actinobacteria_c__Actinobacteria_o__Actinomycetales_f__Micrococcaceae	0.004	1.73
p__Firmicutes_c__Bacilli_o__Lactobacillales_f__Lactobacillaceae	0.012	1.88
p__Proteobacteria_c__Alphaproteobacteria_o__Rhizobiales_f__Hyphomicrobiaceae	0.012	2.23
p__Firmicutes_c__Bacilli_o__Bacillales_f__Bacillaceae	0.013	1.54
P__Nitrospirae_c__Nitrospira_o__Nitrospirales_f__Nitrospiraceae	0.020	0.94
p__Verrucomicrobia_c__.Pedosphaerae._o__.Pedosphaerales._f__Ellin517	0.021	1.2
p__Gemmatimonadetes_c__Gemmatimonadetes_o__Gemmatimonadales_f__Ellin5301	0.021	1.45
p__Actinobacteria_c__Actinobacteria_o__Actinomycetales_f__Mycobacteriaceae	0.022	1.38
p__Proteobacteria_c__Deltaproteobacteria_o__Myxococcales_f__Myxococcaceae	0.027	1.43
p__Actinobacteria_c__Acidimicrobiia_o__Acidimicrobiales_f__EB1017	0.029	1.20
p__Proteobacteria_c__Alphaproteobacteria_o__Rhizobiales_f__Bradyrhizobiaceae	0.032	2.04
p__Planctomycetes_c__Planctomycetia_o__Pirellulales_f__Pirellulaceae	0.034	1.23
p__Verrucomicrobia_c__.Pedosphaerae._o__.Pedosphaerales._f__Ellin515	0.034	1.41
p__Proteobacteria_c__Betaproteobacteria_o__Burkholderiales_f__Comamonadaceae	0.034	1.72
p__Proteobacteria_c__Alphaproteobacteria_o__Sphingomonadales_f__Sphingomonadaceae	0.036	1.97
p__Actinobacteria_c__Actinobacteria_o__Actinomycetales_f__Streptomycetaceae	0.040	1.34
p__Firmicutes_c__Clostridia_o__Clostridiales_f__Clostridiaceae	0.041	1.12
p__Bacteroidetes_c__Bacteroidia_o__Bacteroidales_f__Prevotellaceae	0.043	1.98
p__Chloroflexi_c__Anaerolineae_o__SBR1031_f__oc28	0.043	1.58
p__Proteobacteria_c__Alphaproteobacteria_o__Rhodospirillales_f__Rhodospirillaceae	0.043	1.9
p__Firmicutes_c__Clostridia_o__Clostridiales_f__Veillonellaceae	0.044	1.87
p__Chloroflexi_c__TK10_o__AKYG885_f__Dolo_23	0.048	1.91
p__Actinobacteria_c__Actinobacteria_o__Actinomycetales_f__Nocardioidaceae	0.052	0.93
p__Actinobacteria_c__Thermoleophilia_o__Gaiellales_f__Gaiellaceae	0.053	2.01

[a] p: and f: means Phylum and Family level.

Supplementary Figures

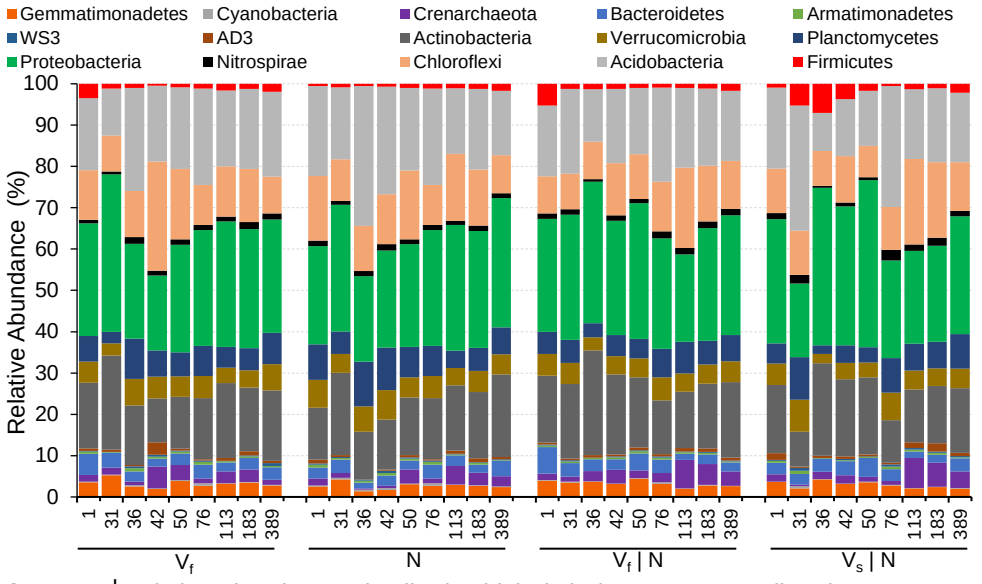


Figure S1| Relative abundance of soil microbial phyla in sugarcane soils. The treatments are: V_f: vinasse applied at day 0; N: inorganic fertilizer ammonium nitrate, applied at day 30; V_f | N: vinasse applied at day 0 and ammonium nitrate applied at day 30; and V_s+N: vinasse plus ammonium nitrate applied only at day 30. The value of each bacterial group percentage is the mean of soil samples collected from three different replicates.

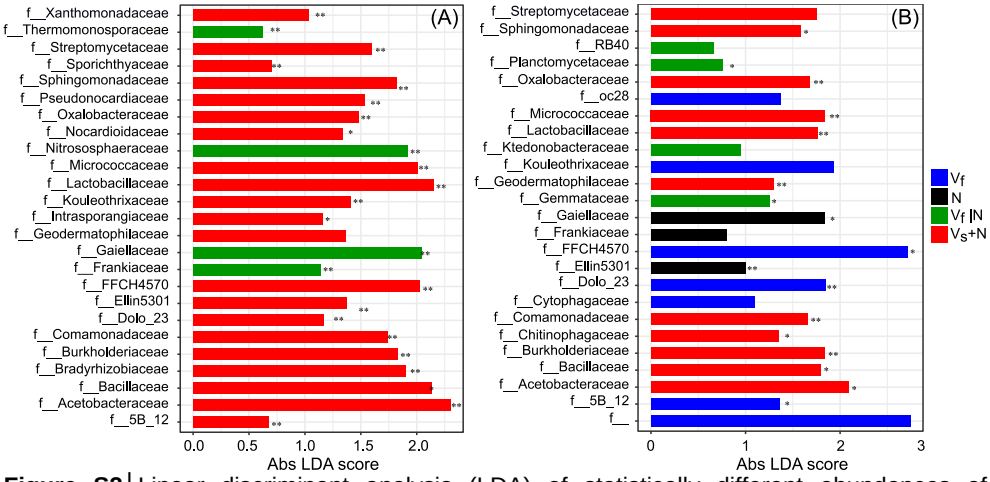


Figure S2| Linear discriminant analysis (LDA) of statistically different abundances of bacterial families between treatments at (A) day 36 and (B) day 42. The treatments are: V_f: vinasse applied at day 0; N: inorganic fertilizer ammonium nitrate, applied at day 30; V_f | N: vinasse applied at day 0 and ammonium nitrate applied at day 30; and V_s+N: vinasse plus ammonium nitrate applied only at day 30. Significant difference: *p≤0.10; ** p≤ 0.05; and *** p≤ 0.01. f: means Family level.

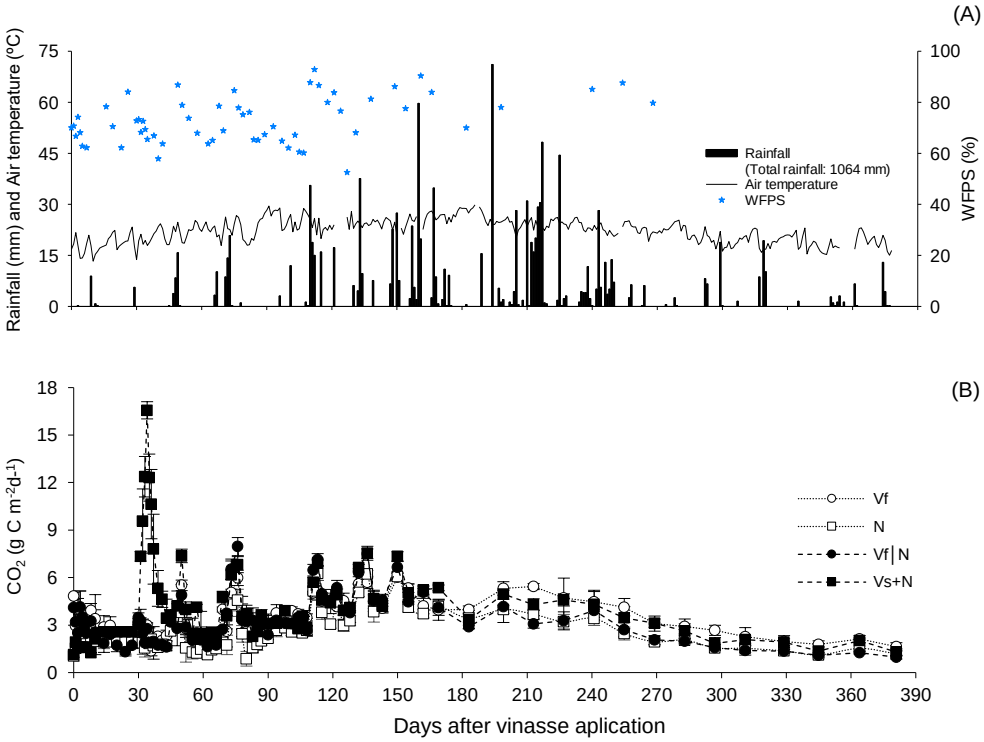


Figure S3| (A) Rainfall, air temperature and water-filled pore space - WFPS and (B) total daily mean fluxes of CO_2-C from soils with sugarcane for different treatments. The treatments are: V_f: vinasse applied at day 0; N: inorganic fertilizer ammonium nitrate, applied at day 30; V_f|N: vinasse applied at day 0 and ammonium nitrate applied at day 30; and V_s+N: vinasse plus ammonium nitrate applied only at day 30. Vertical bars indicate the standard error of the mean (n = 3).

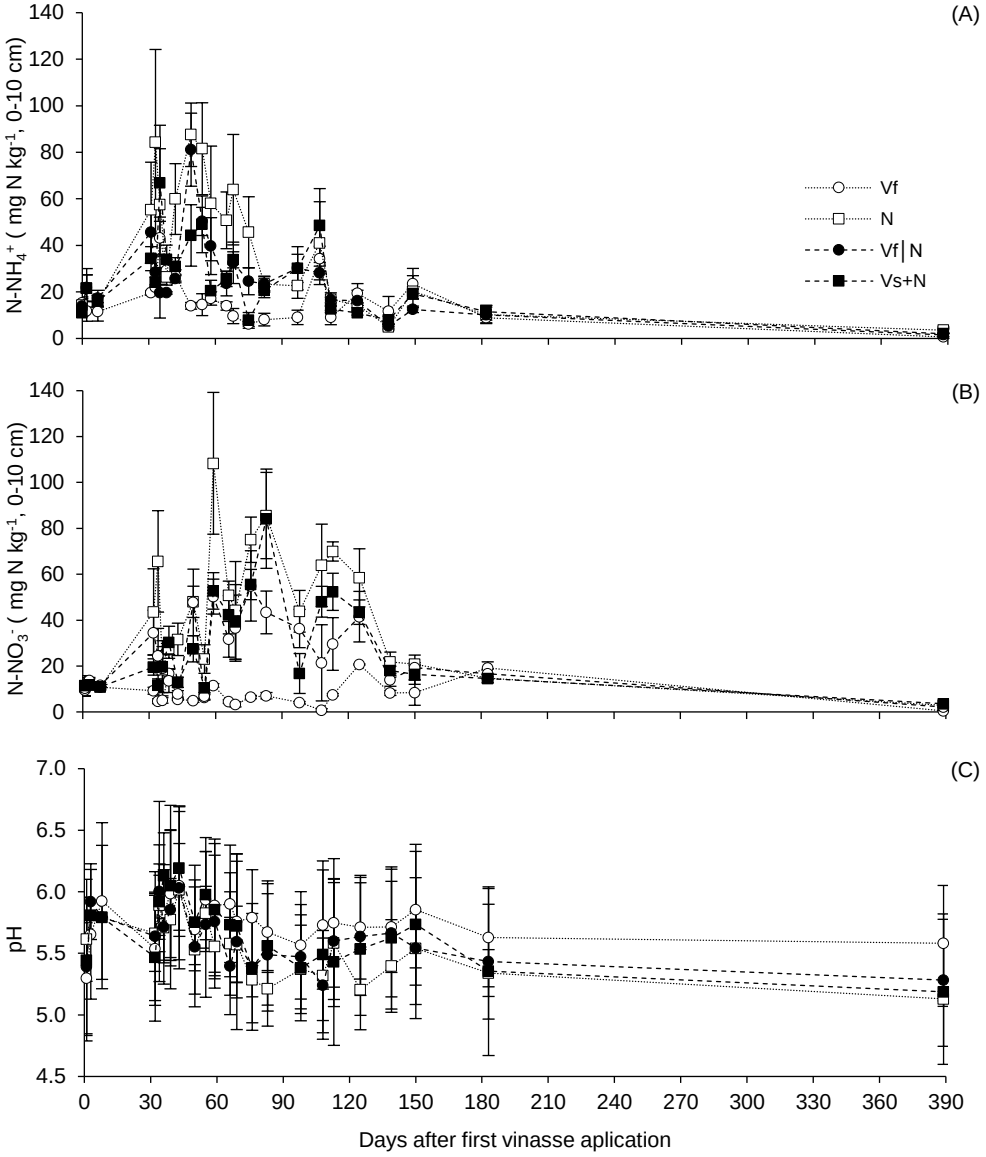


Figure S4| (A, B) Soil mineral N (NH_4^+-N + NO_3^--N) content (mg N kg^{-1} of dry soil) and (C) pH. The treatments are: V_f: vinasse applied at day 0; N: inorganic fertilizer ammonium nitrate, applied at day 30; V_f|N: vinasse applied at day 0 and ammonium nitrate applied at day 30; and V_s+N: vinasse plus ammonium nitrate applied only at day 30.

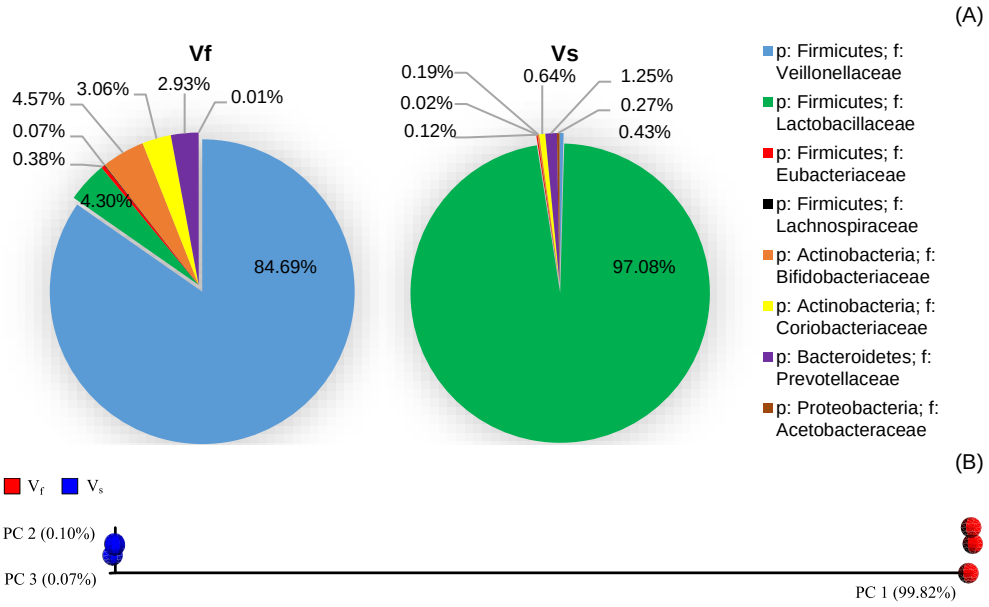
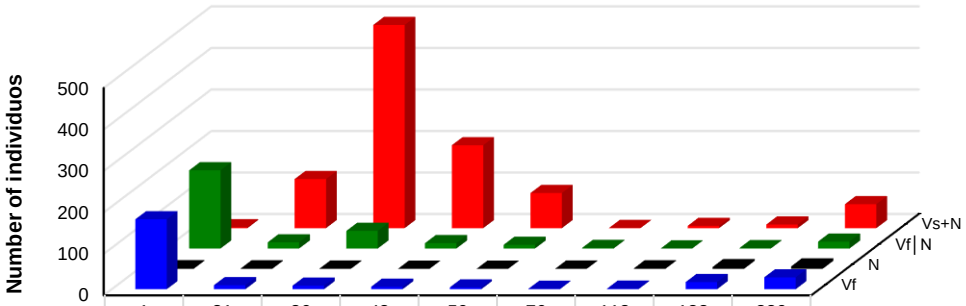


Figure S5| The bacterial community composition of the first (V_f) and second (V_s) vinasse batch, top 8 at family level (A) and differences between vinasse bacterial community depicted by Bray-Curts (B) (which accounts for changes in the relative abundance of Family). Principal Coordinates Analysis (PCoA) from two different vinasses. Each point represents an individual sample, with colors indicating V_f and V_s applied in the soil. p: and f: means Phylum and Family level.



	1	31	36	42	50	76	113	183	389
Vf	169	10	9	6	4	1	1	17	29
N	2	1	1	0	0	1	1	4	6
Vf|N	189	15	43	13	9	2	0	1	17
Vs+N	2	119	490	200	85	1	6	9	58

Figure S6| Relative abundance of *Lactobacillaceae* family in the soil after vinasse application. The abundance of three replicate per day was used. The treatments are: V_f: vinasse applied at day 0; N: inorganic fertilizer ammonium nitrate, applied at day 30; V_f|N: vinasse applied at day 0 and ammonium nitrate applied at day 30; and V_s+N: vinasse plus ammonium nitrate applied only at day 30.

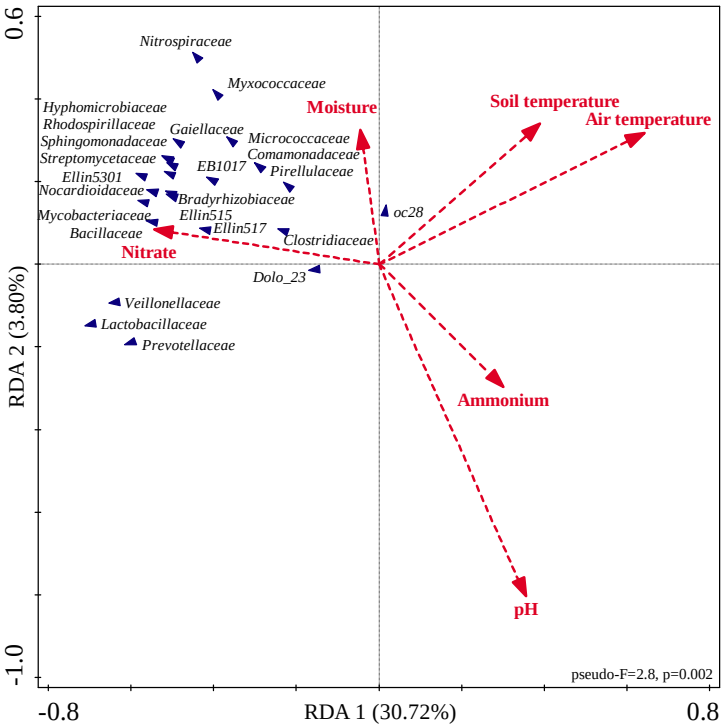


Figure S7| Redundancy analysis of environmental factors and microbial community in soils after the first vinasse (V_f) application.

Chapter 4

Dominance of bacterial ammonium-oxidizers and fungal denitrifiers in the production of nitrous oxide after vinasse applications

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Abstract

Organic compounds and mineral nitrogen (N) added to soil usually increase nitrous oxide (N_2O) emissions. Vinasse, a by-product of the bio-ethanol production that is rich in carbon, nitrogen and potassium, is recycled in sugarcane cultivation as a bio-fertilizer. Vinasse can contribute significantly to N_2O emissions when applied with N in sugarcane plantations in which the soil is covered with straw, a common practice. However, the biological processes involved in N_2O emissions under this management practice are not known. The present study investigated the roles of nitrification and denitrification in N_2O production in straw-covered soils amended with different vinasses (CV: concentrated and V: non-concentrated) before or at the same time as mineral fertilizers at different time points of the sugarcane cycle in two seasons. N_2O emissions were evaluated for 90 days, and the microbial genes encoding enzymes involved in N_2O production (archaeal and bacterial *amo*A, fungal and bacterial *nirK*, and bacterial *nirS* and *nosZ*), total bacteria and total fungi were quantified by real-time PCR. The application of CV and V in combination with mineral N resulted in higher N_2O emissions than the application of N fertilizer alone. The strategy of vinasse application 30 days before mineral N reduced N_2O emissions by 65% and 37% for CV and V, respectively. Independent of rainy or dry season, the microbial processes involved were nitrification by ammonia-oxidizing bacteria (AOB) and archaea and denitrification by bacteria and fungi. The contribution of each process differed and depended on soil moisture, soil pH, and N sources. However, amoA-AOB was the most important gene related to N_2O emissions overall, which indicates that nitrification by AOB is the main microbial-driven process linked to N_2O production in tropical soil. Interestingly, fungal *nirK* was also significantly correlated with N_2O emissions, suggesting that denitrification by fungi contributes to N_2O production in soils receiving straw and vinasse applications.

1. INTRODUCTION

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

Mineral nitrogen (N) is often applied simultaneously with vinasse to ensure sufficient availability of N for plant uptake. This combination may stimulate biological activity in the soil and subsequent N transformations, including the production of N_2O (Carmo et al., 2013; Pitombo et al., 2015). N_2O is a nitrogen (N) cycle product with major environmental and ecological impacts. N_2O is both an ozone-depleting substance (Ravishankara et al., 2009) and a greenhouse gas with global warming potential 298 times greater than that of carbon dioxide (CO_2) (IPCC, 2013). Carmo et al. (2013) and Pitombo et al. (2015) reported that the proportion of N emitted was three and two times higher, respectively, when mineral N was applied together with vinasse compared to mineral N alone. When vinasse was added to the soil a few days before or after N fertilizer, N_2O emissions were lower than when vinasse and N fertilizer were applied simultaneously (Paredes et al., 2014; Paredes et al., 2015). However, there is little information about N_2O emissions from the application of concentrated vinasse as a fertilizer; only Pitombo et al. (2015) reported that 1.6% of total N applied was lost as N_2O when concentrated vinasse was applied.

N_2O is produced and consumed by biotic and abiotic soil processes. The abiotic process, of chemodenitrification, occurs through chemical decomposition of hydroxylamine (NH_2OH), nitroxyl hydride (HNO) or NO_2^- in the presence of organic and inorganic compounds at low pH (< 4.5). By contrast, the biotic process requires autotrophic and heterotrophic microorganisms, *i.e.*, bacteria, archaea and fungi (Hayatsu et al., 2008; Higgins et al., 2016; Hink et al., 2016). N_2O is produced in soil via nitrification and denitrification processes (Stevens and Laughlin, 1998; Németh et al., 2014; Martins et al., 2015; Soares et al., 2016; Xu et al., 2017). In the oxic, well-drained soils typical of most agricultural soils, N_2O is mainly produced by ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Bollmann and Conrad, 1998; Bateman and Baggs, 2005; Baggs et al., 2010; Hink et al., 2016). However,

under suboxic or anoxic conditions, facultative denitrifiers (Tiedje et al., 1983; Di et al., 2014) dominate N_2O production. According to Soares et al. (2016), AOB are the main contributors to N_2O emissions via the nitrification pathway in soils planted with sugarcane.

Despite considerable knowledge of the processes involved in N_2O emission, the control of N_2O emissions from tropical soils planted with sugarcane has only recently been addressed. The most important region for sugarcane production in Brazil is the Central-Southern region, which has two defined seasons: rainy summers with high temperatures and dry winters with mild temperatures. Sugarcane fertilization usually occurs between April and December, encompassing fall, winter and the end of spring, which have completely different climatic conditions. Therefore, the aim of this study was to evaluate the N_2O losses in sugarcane planted soils receiving different fertilization regimes with vinasse during different seasons (spring-rainy/winter-dry). Concentrated (CV) and non-concentrated (V) vinasse were applied before or at the same time as mineral fertilizers. Furthermore, we investigated the potential role of nitrification and denitrification processes in N_2O production from vinasse-fertilized sugarcane-planted soils. We hypothesized that (I) application of vinasse residue before N fertilizer application drastically reduces N_2O production; (II) nitrification is the major pathway contributing to N_2O production in sugarcane-planted soils; and (III) N_2O emissions are lower in winter (dry) than in spring (rainy) due to differences in climatic conditions at the time of mineral N and vinasse application to soil. To test these hypotheses, we quantified N_2O emissions from sugarcane-planted soil as well as the expression of key functional genes related to N_2O emissions during different seasons, *i.e.*, archaeal and bacterial *amoA*, fungal and bacterial *nirK*, and bacterial *nirS* and *nosZ*. Additionally, we determined the total bacterial and fungal abundances.

2. MATERIAL AND METHODS

2.1. Experimental setup and soil sampling

The study comprised two experiments conducted in two experimental fields planted with sugarcane variety RB86-7515. The experimental fields were located at the Paulista Agency for Agribusiness Technology (APTA), Piracicaba, Brazil. The soil is classified as a Ferralsol (FAO, 2015), and the physicochemical properties (Camargo et al., 1986; Van Raij et al., 2001) of the 0- to 20-cm soil layer are shown in Table S1 in the Supporting information. The main difference between the two experiments was the season in which they were conducted (spring-rainy vs. winter-dry). The rainy season (RS) experiment was conducted during the 2013/2014 sugarcane cycle and began on November 12, 2013. The dry season (DS) experiment was conducted during the 2014/2015 sugarcane cycle and began on July 15, 2014. Both experiments lasted 90 days.

The treatments included different application times of concentrated (CV) and non-concentrated (V) in relation to the time of mineral N fertilization. Vinasse was applied either 30 days before or at the same time as N fertilizer. However, during the rainy season, CV was only applied together with mineral N fertilization (Table 1). In both experiments, N_2O was monitored in control treatments without N fertilization.

Table 1 | Time of application and corresponding nitrogen rate of mineral fertilizer (N: ammonium nitrate) and vinasse (V: non-concentrated vinasse and CV: concentrated vinasse) to sugarcane ratoon. The numbers in parentheses indicate the amount of N in kg ha^{-1} contained in vinasse. N was always applied at 100 kg ha^{-1} N.

	Rainy season (2013/2014 cycle)		Dry season (2014/2015 cycle)	
Treatments[a]	November 2013	December 2013	July 2015	August 2015
Control	-	-	-	-
N	-	N (100)	-	N (100)
V_b	V_b (53)	-	V_b (51)	-
CV_b	-	-	CV_b (30)	-
V_b +N	V_b (53)	N (100 kg N)	V_b (51)	N (100)
CV_b +N	-	-	CV_b (30)	N (100)
V	-	V (53)	-	V (89)
CV	-	CV (48)	-	CV+N (52)
V+N	-	V+N (53+100)	-	V+N (89+100)
CV+N	-	CV+N (48+100)	-	CV+N (52+100)

[a] $_b$: Vinasse application (V and CV) 30 days before N fertilization.

Prior to both experiments, the sugarcane already planted in the experimental field was mechanically harvested, and the straw was left on top of the soil. Sugarcane can re-grow up to five times after the first harvest; in the experiments, the plants were grown for the third (RS) and fourth (DS) time, and the amount of straw left on top of the soil was approximately 14 t ha^{-1} on a dry matter basis. Experiments were conducted in a randomized block design with three replicated blocks. The rainy season experiment comprised eight treatments (24 plots), whereas the dry season experiment had two additional CV treatments, resulting in a total of ten treatments (30 plots) (Table 1).

The N (ammonium nitrate) fertilizer application rate was 100 kg ha^{-1} for both experiments. The amount of mineral N applied to the experimental fields followed commercial sugarcane plantation guidelines in the state of São Paulo, Brazil (Van Raij et al., 1996). In both experiments, a volume of 100 m^3 ha^{-1} of V was sprayed over the entire experimental plot using a motorized pump fitted with a flow regulator; this volume represents the average application rate of vinasse in sugarcane plantations in the State of São Paulo. CV was applied in rows at a rate of 17.2 m^3 ha^{-1} for all experiments (Table S2 in the Supporting information) because the K content of CV was approximately 5.8 times that of the non-

concentrated vinasse. Vinasse application rates are restricted by K input rates. The ammonium nitrate and CV were surface-applied in a 0.2-m-wide band 0.1 m from the plant rows, which is common practice in commercial sugarcane production.

2.2. CO_2 and N_2O measurements, soil sampling and chemical analysis

Fluxes of CO_2 and N_2O were measured using PVC static chambers with a height of 20 cm and a diameter of 30 cm according to the method described by Varner et al. (2003). The chambers were inserted 5 cm into the soil and 10 cm from the sugarcane rows. The chamber cap had two openings that were each fit to a valve, one for gas sampling and the other for pressure equilibrium. The chambers remained open until gas sampling. Gases were sampled with plastic syringes (60 mL of gas) at three time intervals (1, 15, and 30 min) after the chambers were closed. The samples were transferred to pre-evacuated glass vials (12 mL) for storage and analyzed in a gas chromatograph (model GC-2014, Shimadzu Co.) with a flame ionization detector (FID) (250 °C) for CO_2 determination (Hutchinson and Mosier, 1981) and an electron capture detector for N_2O determination (Hutchinson and Mosier, 1981). The overall CO_2 and N_2O fluxes were calculated by linear interpolation of the three sampling times.

CO_2 and N_2O measurements were conducted for 90 days during both experiments. Throughout the experiments, gas samples were collected in the morning, beginning five days before fertilizer and vinasse application. Once the treatments were established, the gases were sampled every day during the first week and three times per week thereafter.

Cumulative fluxes were calculated for each treatment using the emission values measured near crop rows. Cumulative emissions were calculated by linear interpolation between adjacent sampling dates (Soares et al., 2016). The emission factors (EF) for N_2O were calculated based on the amounts of N applied with vinasse and mineral N fertilizer according to the formula:

$$EF = \frac{N_2O\text{-}N_{treat} - N_2O\text{-}N_{control}}{N_{applied}\ (fert + Vinasse)}\ x\ 100$$

EF is the N_2O-N emission factor (%), $N_2O\text{–}N_{treat}$ and $N_2O\text{–}N_{control}$ are the cumulative emissions in the fertilized and unfertilized chambers, respectively, and $N_{applied}$ is the mass of N fertilizer added to the chamber with ammonium nitrate and/or N from vinasse (V and CV).

Air and soil temperatures were measured in parallel at each gas sampling. Six soil samplings per plot were performed throughout the experiments. Soil sampling was performed 1, 3, 7, 22, 24, and 54 days after mineral N application in RS and -30, 1, 11, 19, 45 and 52 days after mineral N application in DS. For all treatments, soil samples were collected from the 0- to 10-cm layer near the gas chambers. The soil samples were used to measure moisture content, pH, and concentrations of nitrate (NO_3^-N) and ammonium (NH_4^+-N). Soil subsamples (30

g) were stored at -80 °C for molecular analyses. Soil moisture was determined gravimetrically by drying the soil at 105 °C for 24 h. Soil mineral N (NH_4^+-N, NO_3^--N) was measured with a continuous flow analytical system (FIAlab-2500 System) after extraction with 1 M KCl, and the results were expressed per gram of dry soil. The water-filled pore space (WFPS) was calculated based on the soil bulk density (1.45 and 1.49 g cm^{-3} in RS and DS) and porosity determined at the beginning of the experiment. Climatic data were obtained from a meteorological station located approximately 500 m from the experimental field.

2.3. *DNA extraction*

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

2.4. *Quantitative real-time PCR*

The abundances of the functional genes *amoA*, *nirS*, *nirK*, and *nosZ*, which encode proteins involved in nitrification and denitrification processes, and ribosomal RNA genes indicating total bacteria (16S rRNA) and total fungi (18S rRNA) were quantified by quantitative real-time PCR (qPCR). qPCR was performed in a 96-well plate (Bio-Rad) using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). qPCR was performed in a total volume of 12 µL containing 6 µL of SYBR Green Master Mix and 4 µL of DNA (1.25 ng/µL), except fungal *nirK*, which was amplified in a total volume of 10 µL containing 1 µL of undiluted DNA. The primer combinations, reaction descriptions and thermal cycler conditions for each gene amplification are listed in Table S3 in the Supporting information. Data were acquired at 72 °C, and melting curve analysis was performed to confirm specificity. Amplicon sizes were confirmed on 1% (w/v) agarose gels under UV light. Plasmid DNA from microorganisms containing the gene of interest or from environmental samples was used to construct standard curves and then cloned into vectors. Standard curves were performed 10 times using serial dilutions from 10 to 10^{-8}. Samples were analyzed with two technical replicates. The reaction efficiency varied from 80 to 105%, and the R^2 values ranged from 0.94 to 0.99.

2.5. Statistical analysis

The cumulative emissions of N_2O and CO_2 were checked for normal distribution of residues by the Shapiro-Wilk test, and the data were subsequently transformed using the Box-Cox transformation method (Statistica, version 10). Total cumulative emissions of N_2O were compared per orthogonal contrasts (Tukey $p \leq 0.05$) using SISVAR statistical software (Ferreira, 2011). Soil pH was transformed to H^+: 10^{-pH} before statistical analysis.

Gene abundance values were checked for normal distribution of residues by the Shapiro-Wilk test, and the data were subsequently transformed by log(x) transformation and rechecked to obtain a normal distribution of residues and variance stability (Statistica, version 10). The correlations between N_2O flux and microbial gene abundance were calculated by Spearman correlation analysis (SystatSoftware, 2014). Additionally, to evaluate the influence of variables (genes/soil factors plus genes), we fit a general linear model with the lasso penalty using cyclical coordinate descent, computed along a regularization path (Friedman et al., 2010). The lasso penalty is a regression method that performs both shrinkage and variable selection (Osborne et al., 2000). To select the most appropriate model, we adopted cross-validation criteria with the "one-standard error" rule by checking the lambda value that minimized the mean square error and choosing the largest value of lambda within one standard error of the minimum (Cantoni et al., 2007). This criterion facilitates the selection of a model that minimizes both the square error and selected variables. We included the treatments as dummy variables. We applied log10 transformation for both N_2O emissions and microbial genes (archaeal and bacterial *amoA* genes, fungal and bacterial *nirK*, bacterial *nirS* and *nosZ*, 16S rRNA and 18S rRNA) and standardized soil factor variables. Our analysis was performed in the R environment with the package 'glmnet' (Friedman et al., 2010).

3. RESULTS

3.1. *Weather conditions and soil analysis*

The mean air temperature varied between 13 and 28 °C (Figure S1 in the Supporting information). The minimum mean air temperature was 19 and 12 °C, and the maximum mean temperature was 32 and 29 °C in RS and DS, respectively. During the 90 days of the experiment, the cumulative rain was approximately 276 mm and 103 mm, whereas the average WFPS on soil sampling days was 77% and 66% in RS and DS, respectively. Both cumulative rain values were lower than the average historical values recorded for the region (RS = 561 mm, DS = 121 mm, average of 100 years) (ESALQ, 2016). In DS, plant development was highly affected by the lack of water during the first months after fertilization (Figure S2).

In RS, part of the mineral N applied in the field area was still detectable in mineral form (NH_4^+-N and NO_3^--N) approximately 40 days after mineral N fertilizer application. In DS, the mineral N (NH_4^+-N + NO_3^-- N) concentration was stable throughout the entire experimental period. The mineral N concentrations in the treatments with ammonium nitrate were approximately 140 and 80 mg N kg^{-1} of dry soil in RS and DS, respectively (Figure S3).

3.2. Carbon dioxide emissions

The emissions of CO_2-C from sugarcane were similar in the two seasons, with high emissions immediately following vinasse application (Figure S4). The treatments with CV had higher CO_2 emission fluxes than the treatments with V in both seasons, with peaks of 33 and 17 g m^{-2} d^{-1} of C for CV and V, respectively. The cumulative CO_2-C emissions were 97 and 126 g m^{-2} higher in the treatments with vinasse (CV and V) than in the control in RS and DS, respectively (Table 2). The combined application of vinasse (CV or V) plus mineral N did not further increase the cumulative CO_2-C emissions; however, in the rainy season, the treatments with CV application emitted more CO_2-C than the treatments with V, regardless of the timing of the application of mineral N (Table 2). In both seasons, the application of vinasse (CV and V) prior to mineral N reduced the cumulative CO_2-C emissions by 89 g m^{-2} (on average) (Table 2).

Table 2 | Statistical analysis using orthogonal contrasts for selected treatments. The mean values represent the difference between the amounts of N_2O and CO_2 emissions defined by the orthogonal contrast parameters (emission per chamber).

Selected contrasts[a]	Contrast calculation[b]	Mean of the parameters measured	CO_2 (g C m^2)[c]		N_2O (mg N m^{-2})	
			Rainy season	Dry season	Rainy season	Dry season
1	N effect (vinasse-N or N)	(All treatments) – control	**97****	**126*****	173^{ns}	**184***
2	N plus vinasse effect	(All vinasses +N) – (all vinasses)	29^{ns}	11^{ns}	**328*****	**221*****
3	Type of vinasse	CV – V	**142*****	12^{ns}	59^{ns}	**36***
4	V: Anticipating	V_b - V	**-102****	**-89***	-13^{ns}	-91^{ns}
5	CV: Anticipating	CV_b - CV	-	**-104****	-	-57^{ns}
6	Type of vinasse + N	(CV+N) - (V+N)	**216*****	23^{ns}	**875*****	**233****
7	V+N: Anticipating	(V_b+N) - (V+N)	-34^{ns}	**-143*****	-25^{ns}	-103^{ns}
8	CV+N: Anticipating	(CV_b+N) - (CV+N)	-	**-89***	-	**-407*****

[a] Contrasts 1 and 2 compare the overall effect of N on N_2O emissions; contrasts 3 through 8 compare the effects of type of vinasse with and without N fertilizer; contrasts within each group are orthogonal.
[b] N: mineral N fertilizer, ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N; V_b: Vinasse application 30 days before N fertilization.
[c] Net effect on emissions for the indicated contrast. Significant difference: *$p \leq 0.10$; **$p \leq 0.05$; ***$p \leq 0.01$; ns: non-significant.

3.3. *Nitrous oxide emissions*

In both seasons (RS and DS), the N_2O emission fluxes of the control treatment were similar, approx. 0.06 mg m^{-2} d^{-1} of N (Figure 1C, 1D). In RS, the measured N_2O emission fluxes were similar in all treatments (0.61 mg m^{-2} d^{-1} of N), except the CV+N treatment, in which N_2O fluxes were much higher (46.49 mg $m^{-2}d^{-1}$ of N) (Figure 1A, 1C). In RS and DS, the highest N_2O emissions were observed in treatments of vinasse with mineral N. In the application of vinasse prior to mineral N (V_b+N and CV_b+N) the N_2O emission fluxes were lower than when vinasse was

applied together with mineral N (Figure 1D, 2D, 3D). In RS, only V was applied prior to N. The highest N_2O emission fluxes measured in the V+N and V_b+N treatments were 12.6 and 3.8 mg m^{-2} d^{-1} of N, respectively (Figure 1A). In DS, the highest N_2O emission fluxes were 40.6 and 30.8 mg m^{-2} d^{-1} in the CV+N and V+N treatments respectively, and the highest N_2O emission fluxes in the treatments with vinasse applied before mineral N were 20.5 and 17.7 mg m^{-2} d^{-1} of N in CV_b+N and V_b+N, respectively (Figure 1C). In both experiments, the maximum N_2O emission peaks occurred directly after application of mineral N and vinasses (CV and V) and immediately after rain events (Figure 1).

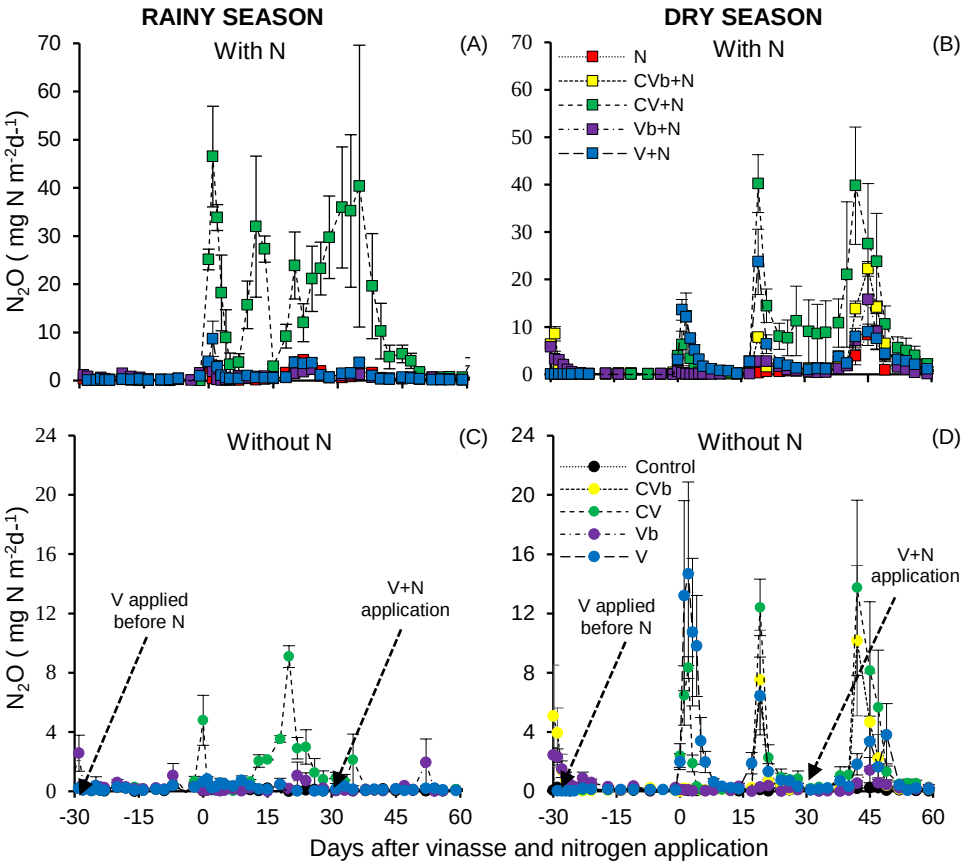

Figure 1 | Daily mean fluxes of N_2O with (A, B) or without (C, D) nitrogen in sugarcane ratoon in different treatments in the rainy (A, C) and dry (B, D) season. The treatments are as follows: Control; N: mineral N as ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N; V_b: Prior vinasse application (30 days before N fertilization). Vertical bars indicate the standard error of the mean (n = 3).

In the treatments with mineral N application, the cumulative N_2O-N emissions were higher in DS than in RS; the total N emitted was 89 and 49 mg m^{-2}

of N_2O^--N greater than in the control treatment, respectively, corresponding to 0.14% and 0.08% of total N applied (Figure 2, Table S4). The application of vinasse (CV and V), mineral N or the combined application of both fertilizers resulted in significantly higher cumulative N_2O emissions than in the control in DS (+184 mg N m^{-2}) (Table 2). In addition, the application of vinasse plus mineral N (CV_b+N, CV+N V_b+N and V+N) resulted in an increase in emissions of nearly 328 and 221 mg N m^{-2} as compared to treatment with either vinasse alone (CV and V). The application of CV plus mineral N increased N_2O emissions compared to the application of V by 875 and 233 mg N m^{-2} in RS and DS, respectively (Table 2). However, the application of CV 30 days before N reduced N_2O-N emissions by 65%. The N_2O-N emissions represented 0.26 and 0.65% of the total N applied as fertilizer in the CV_b+N and CV+N treatments, respectively (Table 2, Figure 2). The application of V, regardless of the application time or combined application with mineral N, resulted in similar N_2O-N emissions in RS and DS (Table 2). The cumulative N_2O emissions in the treatments with V were 54 and 79 mg N m^{-2} in V_b+N and V+N in RS, respectively, and 137 and 241 mg N m^{-2} d^{-1} in V_b+N and V+N in DS, respectively (Table S4). Although not significant, the application of V before mineral N reduced the total N emitted as N_2O by 37% (on average) in both seasons. The total N emitted as N_2O was approximately 0.10 and 0.26% on average for V_b+N and V+N of the total N applied in RS and DS, respectively (Figure 2).

3.4. *Abundances of nitrogen cycle genes*

The abundances of N cycle genes related to N_2O emissions are shown in Figure S5, S6 and S7 for all treatments and sampling time points. The abundance of *amoA* (AOB) followed the pattern of N_2O emissions (Figure S5A, S5B, S5C, S5D). The abundance of *amoA* bacteria (AOB) was higher in the treatments with CV plus mineral N (CV+N) than in the other treatments, regardless of season. During the entire experiment (combination of all time points), the abundance of AOB was correlated significantly with N_2O emissions in both RS (R^2 = 0.17, $p \leq$ 0.05) and DS (0.24; $p \leq 0.05$) (Figure 3). However, the correlations were not positive at all sampling time points. In RS, on day 22, N_2O emissions were positively correlated with *amoA*-AOB, with a coefficient of correlation (R^2) of 0.46 ($p \leq 0.01$) (Table 3). By contrast, in DS, N_2O emissions were positively correlated with *amoA*-AOB on days 45 (R^2 = 0.50; $p \leq 0.01$) and 52 (R^2 = 0.47; $p \leq 0.01$) (Table 3). Overall, for RS and DS, a significant correlation between the abundance of AOA *amoA* and N_2O emissions was detected (RS: R^2 = 0.15, $p \leq 0.10$; DS: R^2 = 0.13, $p \leq$ 0.10) (Figure 3). However, the abundance of AOA *amoA* was higher in RS than in DS, although no significant correlation between AOA *amoA* abundance and N_2O emission was observed on specific days (Table 3).

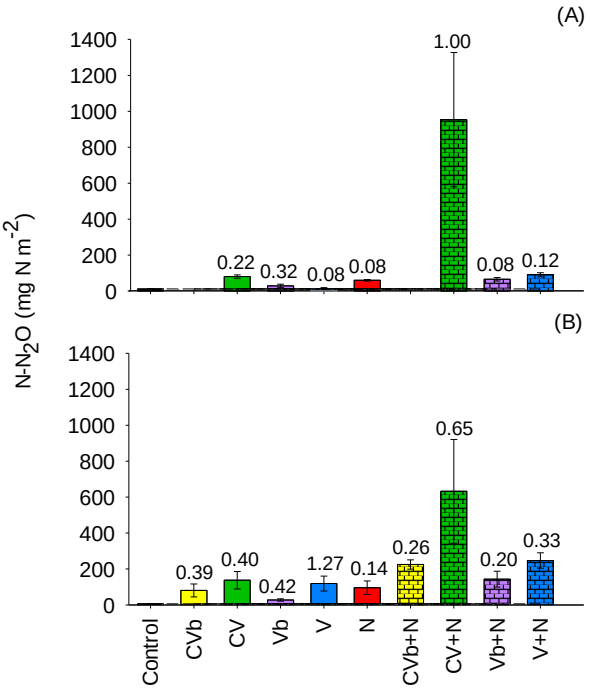

Figure 2| Cumulative fluxes of N_2O (mg N m^{-2}) and N fertilizer emission factor (%, values above bars) based on the rates of N fertilizer application during 90 days. Soil N_2O fluxes in (A) rainy and (B) dry seasons. The treatments are as follows: Control; N: mineral N fertilizer, ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N; V_b: Vinasse application 30 days before N fertilization. Vertical bars indicate the standard error of the mean (n = 3).

The correlations between the abundances of bacterial denitrification genes (*nirK*, *nirS* and *nosZ*) and N_2O emissions differed between seasons (Figure 3). For RS overall, N_2O emissions were correlated significantly with *nirS* (R^2 = 0.22, $p \leq$ 0.01) and *nosZ* (R^2 = 0.19; $p \leq$ 0.05), whereas for DS overall, *nirK* (R^2 = 0.16, $p \leq$ 0.05) and *nirS* (R^2 = 0.24, $p \leq$ 0.01) were positive correlated with N_2O emissions (Figure 3). The abundances of the *nirK*, *nirS* and AOA-*amoA* genes increased linearly with time with the increase in water availability (Figure S5, S6, S7 in the Supporting information). The abundance of total bacteria (16S rRNA gene) in RS and the abundance of total fungi (18S rRNA gene) was significantly and positively correlated with N_2O emissions in both seasons (RS: R^2 = 0.30, $p \leq$ 0.01; DS: R^2 = 0.37, $p \leq$ 0.01) (Figure 3). Total fungi were most abundant in the treatments with vinasse application (with or without nitrogen) (Figure S7).

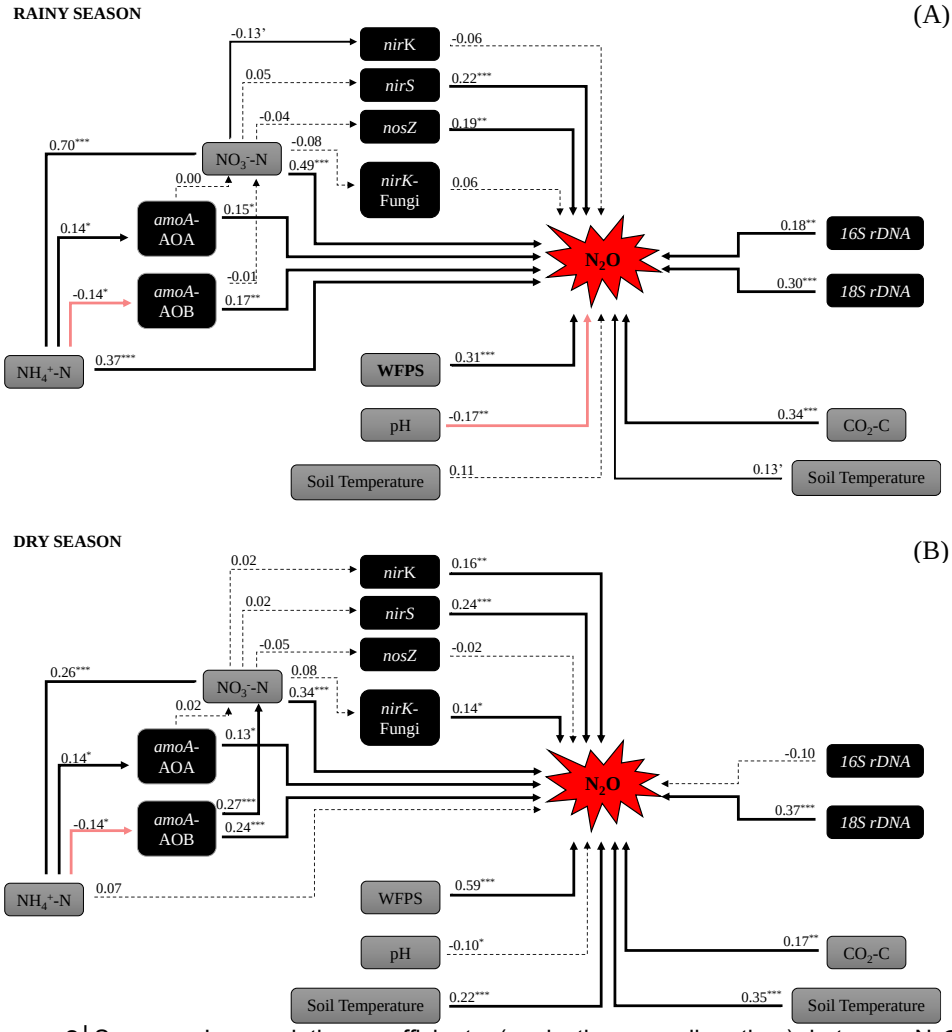

Figure 3| Spearman's correlation coefficients (neglecting sampling time) between N_2O emission fluxes (mg m^{-2} d^{-1}) and abundance of *amo*A (archaeal and bacterial), *nir*K (fungal and bacterial), *nir*S and *nos*Z (bacterial), total bacterial 16S rRNA and total fungal 18S rRNA (gene copies g^{-1} dry soil) and abiotic factors, mineral nitrogen, air and soil temperatures and CO_2-C emissions in the (A) rainy and (B) dry seasons. Abbreviations: WFPS: water-filled pore space; AOB: *amo*A belonging to ammonia-oxidizing bacteria; AOA: *amo*A belonging to ammonia-oxidizing archaea. Black bold lines indicate significant correlations; red bold lines indicate significant negative correlations; and dotted lines indicate no significant correlation between variables (n=144 and 180 for the rainy and dry seasons, respectively). Significant difference: '$p \leq 0.15$, $^{*}p \leq 0.10$, $^{**}p \leq 0.05$ and $^{***}p \leq 0.01$.

Table 3 | Spearman's correlation coefficients between N_2O emission flux (mg m^{-2} d^{-1}) and abundance of archaeal and bacterial *amoA*, fungal and bacterial *nirK*, bacterial *nirS* and *nosZ*, and total bacterial 16S rRNA and total fungal 18S rRNA (gene copy g^{-1} dry soil) in the rainy and dry seasons.

	Rainy season					
	Day 1 (n=24)	Day 3 (n=24)	Day 7 (n=24)	Day 22 (n=24)	Day 24 (n=24)	Day 54 (n=24)
WFPS	**0.60*****	0.07	-0.03	-0.31	-0.03	0.23
NH_4^+-N	**0.80*****	0.33	0.25	**0.83*****	**0.38****	-0.13
NO_3^--N	**0.68*****	**0.47****	0.22	**0.57*****	**0.59*****	0.39
pH	-0.31	-0.04	0.09	-0.02	-0.14	0.01
*amoA*_AOB	**-0.30'**	-0.13	-0.24	**0.46*****	0.28	0.16
*amoA*_AOA	0.08	-0.26	0.05	0.08	-0.15	0.08
nirK	0.01	-0.13	0.07	0.05	-0.08	-0.24
nirS	-0.05	0.00	0.06	0.17	-0.09	0.11
nosZ	-0.00	-0.10	-0.03	**0.43****	**0.42****	0.18
nirK-Fungi	-0.22	-0.24	0.10	**0.36***	0.25	0.22
16S rRNA	0.13	-0.05	0.01	0.14	0.31	0.15
18 rRNA	0.03	0.04	0.17	**0.53*****	**0.35*****	0.23
	Dry season					
	Day -30 (n=30)	Day 1 (n=30)	Day 11 (n=30)	Day 19 (n=30)	Day 45 (n=30)	Day 52 (n=30)
WFPS	0.04	**0.77*****	**0.45*****	-0.13	0.16	0.06
NH_4^+-N	-0.17	0.17	0.06	0.28	**0.42****	**0.45*****
NO_3^--N	-0.09	0.05	**0.41****	**0.33****	**0.66*****	**0.60*****
pH	-0.09	-0.22	-0.07	0.11	-0.06	-0.23
*amoA*_AOB	-0.07	0.09	0.26	0.10	**0.50*****	**0.47*****
*amoA*_AOA	0.17	-0.16	-0.25	0.00	-0.17	0.00
nirK	0.28	-0.36	0.02	0.10	0.09	0.26
nirS	0.29	-0.13	-0.14	0.19	-0.04	0.01
nosZ	0.15	-0.18	-0.12	0.30	0.10	0.33
nirK-Fungi	-0.15	0.09	**0.35***	**0.47*****	-0.12	-0.09
16S rRNA	**0.40****	-0.22	0.15	**0.17***	**0.06'**	-0.05
18 rRNA	0.16	-0.09	**0.47*****	**0.73*****	**0.32***	**0.48*****

Abbreviations: WFPS: water-filled pore space; AOB: *amoA* belonging to ammonia-oxidizing bacteria; AOA: *amoA* belonging to ammonia-oxidizing archaea. Significant difference: '$p \leq 0.15$, $^{*}p \leq 0.10$, $^{**}p \leq 0.05$ and $^{***}p \leq 0.01$.

The positive correlation between N_2O emissions and N cycle genes indicates that nitrification and denitrification likely occurred during the entire experimental period in both seasons. To assess the main microbial driven processes related to N_2O emissions, the ratios between gene abundances and their correlation with N_2O emissions were calculated (Table 4). In both seasons, nitrification by *amoA*-AOB appeared to be the dominant process related to N_2O emissions due to the negative correlation between N_2O emissions and the ratio of denitrifier to nitrifier genes (RS: (*nirK*+*nirS*)/(AOB+AOA), R^2 = -0.26, $p \leq 0.01$; (*nirK*+*nirS*)/*amoA*-AOB, R^2 = -0.22, $p \leq 0.01$; and *nirK*-Fungi/*amoA*-AOB, R^2 = -0.17, $p \leq 0.05$; similar results were obtained for DS) (Table 4). The general linear model also provided evidence of the predominance of nitrification (Table 5A); N_2O emissions were dependent on the abundance of *amoA*-AOB in both seasons when N cycle genes, 16S rRNA and 18S rRNA were taken into account (Table 5).

Table 4| Spearman's correlation coefficients between N_2O emission flux (mg m^{-2} d^{-1}) and the ratios of the abundances of nitrifier (archaeal and bacterial *amoA*) and denitrifier (fungal and bacterial *nirK*, bacterial *nirS* and *nosZ*, total bacterial 16S *rRNA* and total fungal 18S *rRNA*) genes in the rainy (RS) and dry seasons (DS).

	N_2O-N emission		
Spearman Correlation	**Rainy season (n=144)**	**Dry season (n=180)**	
(nirK+nirS)/(AOB +AOA)	**-0.26*** **	-0.08	RS: ↑(AOB +AOA) ↓Ratio↑N_2O (Nitrification) DS: ns
(nirK+nirS)/amoA-AOB	**-0.22*** **	**-0.18** **	RS: ↑AOB ↓Ratio↑N_2O (Nitrification) DS: ↑AOB ↓Ratio↑N_2O (Nitrification)
(nirK+nirS)/amoA-AOA	0.00	**-0.28*** **	RS: ns DS: ↑AOA ↓Ratio ↑N_2O (Nitrification)
amoA-AOB/amoA-AOA	0.08	**0.22*** **	RS: ns DS: ↑AOA ↓Ratio ↓N_2O (Nitrification by *amoA*-AOB)
nirK-Fungi*/amoA-AOB*	**-0.17** **	**-0.23*** **	RS: ↑ AOB ↓Ratio↑N_2O (Nitrification by *amoA*-AOB more important than denitrification by fungi) DS: ↑ AOB ↓Ratio↑N_2O (Nitrification by *amoA*-AOB more important than denitrification by fungi)
(nirK+nirS)/nosZ	**-0.26*** **	**0.19*** **	RS: ↑*nosZ* ↓Ratio↑N_2O (????_other process is occurring) DS: ↑*nosZ* ↓Ratio ↓N_2O (Complete denitrification as well)

AOB: *amoA* belonging to ammonia-oxidizing bacteria; AOA: *amoA* belonging to ammonia-oxidizing archaea. Significant difference: $^{*}p \leq 0.10$; $^{**}p \leq 0.05$; $^{***}p \leq 0.01$; ns: Non-significant.

To evaluate the relative influences of functional genes, treatments, and climatic factors on N_2O emissions, we fit the general linear model to both seasons. The models were consistent with the Spearman's correlation results. Both analyses identified relationships of N_2O emissions with the abundance of nitrogen-cycle genes and environmental variables, as shown in Tables 3 and 5. However, in both seasons, WFPS was the most important factor controlling N_2O emissions. In RS, N_2O emissions increased with soil moisture, soil temperature, mineral N (NH_4^+-N and NO_3^--N), *nosZ* and total bacteria, whereas in DS, N_2O emissions increased with soil moisture, air temperature, mineral N (NO_3^--N), *amoA* (AOB) and *nosZ*. Application of vinasse (CV and V) plus mineral N increased N_2O emissions in both seasons (Table 5B).

Table 5 | (A) Standardized coefficients of regression analysis with the lasso penalty for the influence of gene abundance on N_2O emissions. (B) Standardized coefficients of regression analysis with the lasso penalty for the influence of gene abundance on N_2O emissions with soil factors, days and treatments included as dummy variables.

(A)	Dependent variable	intercept	*amoA* - AOB	*amoA* - AOA	*nirK*	*nirS*	*nosZ*	*nirK* - Fungi	16S rRNA	18S rRNA	r^2
RS	N_2O	-0.831	0.011	_	-0.319	0.142	0.051	_	0.126	0.228	0.230
DS	N_2O	-0.005	0.158	_	_	0.097	-0.036	_	_	_	0.107

(B)	Dependent variable	Intercept	Treatments[a]		Day	WFPS	air Tem.	Soil Tem.	NH_4^+	NO_3^-	pH	*amoA* AOB	*amoA* AOA	*nirK*	*nirS*	*nosZ*	*nirK* Fungi	16S rRNA	18S rRNA	r^2
RS	N_2O	-0.82	CV+N	0.59	_	0.09	_	0.01	0.02	0.03	-0.02	_	_	_	_	0.03	_	0.08	_	0.58
			V	-0.04																
			V+N	0.1																
			Vb	-0.03																
DS	N_2O	-0.186	CV	0.289	-0.13	0.6	0.02	_	-0.04	0.12	-0.05	0.05	-0.05	_	-0.04	0.05	_	_	_	0.57
			CV+N	0.576																
			CVb+N	0.369																
			V+N	0.382																
			Vb	-0.052																
			Vb+N	0.21																

[a] N: mineral N fertilizer, ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N. [b]: Prior vinasse application 30 days before N fertilization.

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

4. DISCUSSION

The application of vinasse residue (CV and V) 30 days prior to mineral N fertilizer reduced the cumulative N_2O emissions from sugarcane planted with straw by 65% and 37% compared to the application of vinasse and mineral N simultaneously. The interval of 30 days between the application of vinasse and N fertilizer appears to be sufficient to ameliorate the anaerobic conditions induced by vinasse application and thereby decrease heterotrophic denitrification. In addition, since vinasse is a source of carbon and N, this 30-day period permits vinasse-carbon decomposition and vinasse-N mineralization and/or N uptake by plants (Parnaudeau et al., 2008; Silva et al., 2013), which may lead to a low N_2O production. The N_2O emissions from the treatments with vinasse (CV and V) plus N were similar to or higher than those of the single mineral N treatment, regardless of the timing of application. Surprisingly, N_2O emissions were higher in the dry season than in the rainy season. Denitrification conditions are expected to occur for a longer period in the rainy season than in the dry season, leading to high N_2O emissions. However, the phenology of the sugarcane plant may provide insights on the lower N_2O emissions in all treatments in the rainy season. Sugarcane is a fast-growing plant, with high N demand during the initial stages of ratoon growth (Franco et al., 2011; Mariano et al., 2016), and can accumulate 30 to 60 t ha^{-1} of dry matter in a single season (Cantarella et al., 2012; CONAB, 2017). If N is applied during the growing stage of the plant, the rapid uptake of nutrients, including N, will reduce the available N for microbial-related processes of N_2O production. In the dry season, N_2O emissions were nearly 2-fold higher compared to the rainy season. In the rainy season, fertilizers were applied at the beginning of summer, when the plants were 1.5 m high; by contrast, in the dry season, N was applied at the beginning of winter, when the plants were starting to sprout. Therefore, at the beginning of the dry season, plants were not able to take up as much N, which allowed the applied N to remain longer in the soil to support microbial reactions leading to N_2O production.

The variation of N_2O emissions in the treatments with either type of vinasse (CV or V) and mineral N can be explained by the complex combination of available C and N present in the vinasse and environmental factors such as pH, organic matter, porosity, temperature, moisture (Subbarao et al., 2006; Halvorson et al., 2014; Vargas et al., 2014; Liang et al., 2015). The large variation of conditions in the present study likely caused rapid changes in the microbial community.

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

indicated to be the main process by which N_2O is produced (Soares et al., 2016). Many studies have shown that N_2O emissions are significantly and positively correlated with ammonia oxidation by AOB under controlled conditions (Regina et al., 1996; Law et al., 2012). AOA also played a role in N_2O emissions from soil amended with vinasse (CV and V) and mineral N. In both the rainy and dry seasons, the abundance of AOA was related to N_2O emissions. The soil conditions at our sites were acidic, and *amoA*-AOA gene abundance usually increases with decreasing pH (Nicol et al., 2008; Zhang et al., 2012). Although ammonia oxidation by AOA was also responsible for the N_2O emissions, the *amo*A-AOB/*amo*A-AOA ratio and regression analysis of our results showed that *amo*A-AOB was the most important gene related to N_2O emissions, thus indicating that nitrification by AOB dominates the nitrification process and N_2O production in sugarcane fields. It has been reported that AOAs, although present in soils, do not respond to NH_4^+-N fertilization or N_2O production in intensively managed agricultural soils, in contrast to AOB (Di et al., 2009; Hink et al., 2016; Yang et al., 2017). Independent of soil pH (acidic soils and neutral or alkaline soils), the concentration of NH_4^+-N is a key factor determining the niche separation of AOA and AOB (Zhang et al., 2012). In the same region as our study, Soares et al. (2016) observed that nitrification by AOB, rather than AOA or denitrification, was the main process responsible for N_2O emissions, but neither vinasse nor sugarcane straw was applied in that study. In that study, the application of urea plus the inhibitor of nitrification 3,4-dimethylpyrazole phosphate decreased N_2O emissions by up to 95% compared to application of urea alone, with emissions comparable to those of the control treatment (no mineral N).

In addition to the considerable importance of N_2O production during ammonia oxidation by AOB, the consumption of O_2 by heterotrophc microorganisms may trigger denitrification, as indicated by the increases in the CO_2 production and abundance of *nir*S, *nir*K and *nosZ*. These results suggest that AOB will actively grow under high NH_4^+-N concentrations and the availability of labile vinasse-C for the fast-growing microorganisms may -lead to microoxic or anoxic conditions, which in turn will induce denitrification by heterotrophic denitrifiers or by nitrifiers, resulting in high N_2O emission fluxes but also N_2O consumption. The significant correlation between *nosZ* and N_2O indicates that complete denitrification is also occurring in the soil; *nosZ* is the key enzyme involved in the N_2O reduction to N_2 (Orellana et al., 2014; Samad et al., 2016). This cascade is further reinforced by N fertilization, especially when N is applied with a rich carbon source, such as vinasse (Di et al., 2014; Yang et al., 2017). Previous studies of sugarcane fields have shown that high N_2O fluxes occur immediately after N fertilization (Carmo et al., 2013; Navarrete et al., 2015a; Pitombo et al., 2015; Soares et al., 2015; Soares et al., 2016). However, denitrification appears to be less important than AOB for N_2O emissions under our experimental field conditions.

N_2O emissions and the total fungal abundance showed significant positive correlations over time, suggesting a contribution of fungal denitrifiers to N_2O

emissions. This relationship was further confirmed by the significant positive correlation between fungal *nirK* and N_2O emissions in both seasons on different days. A role of fungi in N_2O emissions has recently been reported (Shoun et al., 2012; Mothapo et al., 2015; Wu et al., 2017), albeit in crop and management systems other than sugarcane. In contrast to bacteria, fungi do not have genes encoding nitrous oxide reductase (*nosZ*), which reduces N_2O to N_2, and thus fungal denitrification terminates at N_2O (Shoun et al., 2012; Phillips et al., 2016). Therefore, an increase in fungal denitrification might increase N_2O emissions. The high amount of sugarcane straw, which has a high C:N ratio (77:1), present in our experiments might trigger an increase in fungal biomass (Allison and Killham, 1988). Consistent with this expectation, Wu et al. (2017) determined that N_2O emissions in soil with wheat straw were initially predominantly derived from bacterial denitrification but later mainly resulted from fungal denitrification. Similar to 18S rRNA, the abundance of the fungal nitrite reductase gene (*nirK*) increased significantly with N_2O emissions after swine manure application (Xu et al., 2017).

No study has investigated the abundance of nitrifier and denitrifier genes and their links to N_2O emissions in soil amended with vinasse and mineral N for the cultivation of sugarcane crops with straw, a common agricultural practice for sugarcane cultivation in Brazil in the past ten years. Our results suggest that nitrification and denitrification by nitrifiers and denitrifiers occur simultaneously in the soil. The mineral N source, ammonium nitrate, resulted in N_2O emissions by NH_4^+-N oxidation or nitrification-denitrification as well as by NO_3^--N reduction by heterotrophic denitrification. The significant positive correlations between N_2O emissions and the abundances of the bacterial *nirK, nirS* and *nosZ* genes show that the production of N_2O is due to favorable conditions for denitrification. Rain events and vinasse fertirrigation induce low oxygen concentrations in soil microsites (Di et al., 2014), consistent with the significant correlation between N_2O emissions and WFPS. In addition, vinasse is an organic residue rich in carbon with high biological oxygen demand (Fuess and Garcia, 2014). The input of labile organic compounds from vinasse in soils might have two effects: (1) greatly increased soil microbial activities, resulting in intense oxygen consumption (Renault et al., 2009); (2) the creation of microoxic or anoxic conditions, resulting in anaerobic microsites (Torbert and Wood, 1992). Therefore, after vinasse application, anaerobic conditions may prevail for a short time due to the large organic C load and soil moisture, promoting reducing conditions in the soil. In this way, anaerobic processes may cause N_2O emissions. However, this situation may be transient since drying of the soil within a few hours or days will favor N_2O emissions by nitrification. By contrast, fungal denitrifiers can release N_2O under both aerobic and anaerobic conditions (Zhou et al., 2002; Shoun et al., 2012). Other, less-characterized processes may also be involved in N_2O emissions, such as nitrifier denitrification, aerobic denitrification, and co-denitrification (Joo et al., 2005; Spott et al., 2011; Zhao et al., 2012). However, nitrification by AOB and denitrification by fungi were the prevalent processes leading to high N_2O emissions

in both experiments and therefore could be useful indicators for mineral N management strategies to mitigate N_2O emissions in tropical soils with organic residue application.

Understanding the prevalent microbial processes related to N_2O in sugarcane fields is a considerable challenge, given the myriad of conditions that may occur simultaneously. In this study, we investigated the microbial processes involved in N_2O emissions in a field soil ecosystem where different bioenergy residues, i.e., types of vinasse and straw, were applied to soil in combination with N fertilizer in two different seasons. Independent of season, different contributions of nitrification by bacteria, nitrification by archaea and denitrification by bacteria and fungi were observed, dependent on soil moisture, soil pH and nitrogen source. A practical finding is that the strategy of vinasse application 30 days before mineral N reduced N_2O emissions by 65% and 37% for concentrated and non-concentrated vinasse, respectively.

5. Author contributions

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

6. Acknowledgements

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

Supplementary Data

Supplementary Tables

Table S1| Physicochemical properties of soil layer (0- to 20-cm) in rainy (RS) and dry (DS) seasons (n=4).

Season	pH[a]	Bulk density	OM[b]	P[c]	K	Ca	Mg	H+Al[d]	CEC[e]	Soil texture[f]		
										Clay	Silt	Sand
		g cm^{-3}	g dm^{-3}	mg dm^{-3}	----------		$mmol_c dm^{-3}$		----------	---------	g kg^{-1}	---------
RS	5.3	1.45	23.5	10.5	0.55	45.5	20.5	31.5	98.5	619	145	236
DS	5.0	1.49	21.1	14.6	0.7	17.4	11.9	34.9	65.1	631	151	218

[a] (CaCl2; 0.0125 mol L-1)
[b] Organic matter.
[c] Available phosphorus, K, Ca, and Mg were extracted with ion exchange resin.
[d] Buffer solution (Calcium acetate 0,5 M, pH 7.0).
[e] Cation exchange capacity.
[f] Soil texture determined by the densimeter method.

Table S2| Chemical properties of vinasses applied in the experiments (n=4).

Exp.[a]	Vinasse[b]	Time application	pH	C org	N tot	NH_4^+-N	NO_3^--N	P	K	C/N
				g L^{-1}	g L^{-1}	mg L^{-1}	mg L^{-1}	g kg^{-1}	g kg^{-1}	
RS	V_b	Nov. 13	4.7	28.2	0.53	65.8	17.6	0.08	2.9	53/1
RS	V	Dec. 13	4.1	25.7	0.53	63.4	10.8	0.17	2.6	49/1
DS	V_b	Jul. 15	4.8	28.8	0.51	45.7	8.8	0.11	3.5	57/1
DS	V	Aug. 15	3.9	31.4	0.89	41.6	4.1	0.23	4.7	35/1
RS	CV	Dec. 13	4.0	69.7	2.80	119.8	21.2	1.00	17.3	25/1
DS	CV_b	Jul. 15	4.3	54.1	1.75	61.5	20.2	1.25	17.3	31/1
DS	CV	Aug. 15	4.2	65.3	3.00	100.9	23.7	0.53	21.0	22/1

[a]RS: Rainy season (2013/2014 cycle); DS: Dry season (2014/2015 cycle).
[b]CV: concentrated vinasse and V: no-concentrated vinasse and; $_b$: prior vinasse application (30 days before N fertilization).

Table S3 | Primers and thermocycler conditions used in gene abundance analysis by qPCR.

Target gene	Primers	Primer Sequence	Amplification size (bp)	Reaction	Cycling conditions	Reference
			12 µL of reaction			
AOA *amoA*	Arch-amoAF Arch-amoAR	5'-STAATGGTCT GGCTTAGACG-3' 5'-GCGGCCATC CATCTGTATGT-3'	635	6 µL of Sybrgreen Bioline SensiFAST SYBR non-rox mix, 0.125 µL of each primer (10 pmol), 1.75 µL of BSA and 4 µL of DNA (3 ng).	95°C-5 min.; 40x 95°C-10s, 64°C-10s, 72°C-20s	Francis et al. (2005)
AOB *amoA*	amoA1F amoA2R	5'-GGGGTTTCTA CTGGTGGT-3' 5'-CCCCTCKGSA AAGCCTTCTTC-3'	491	6 µL of Sybrgreen Bioline SensiFAST SYBR non-rox mix, 0.125 µL of each primer (10 pmol) and 4 µL of DNA (3 ng).	95°C-10min.; 40x 95°C-10s, 65°C-25s,	Rotthauwe et al. (1997)
nosZ	nosZ2F nosZ2R	5'-CGCRACGGCA ASAAGGTSMSSGT-3' 5'-CAKRTGCAKSG CRTGGCAGAA-3'	267	6 µL of Sybrgreen Bioline SensiFAST SYBR non-rox mix, 0.250 µL of each primer (10 pmol), 1.20 µL of BSA and 4 µL of DNA (3 ng).	95°C-5 min.; 40x 95°C-10s, 64°C-10s, 72°C-20s	Henry et al. (2006)
nirK	NirK876 NirK1040	5'-ATYGGCGGVAY GGCGA-3' 5'-GCCTCGATCAG RTTRTGGTT-3'	165	6 µL of Sybrgreen Bioline SensiFAST SYBR non-rox mix, 0.250 µL of each primer (10 pmol), 1.50 µL of BSA and 4 µL of DNA (3 ng).	95°C-5 min.; 40x 95°C-15s, 62°C-15s, 72°C-20s	Henry et al. (2004)
nirS	nirScd3aF nirSR3cd	5'-GTSAACGTSAA GGARACSGG-3' 5'-GASTTCGGRTG SGTCTTGA-3'	425	6 µL of Sybrgreen Bioline SensiFAST SYBR non-rox mix, 0.250 µL of each primer (10 pmol), 1.20 µL of BSA and 4 µL of DNA (3 ng).	95°C-5 min.; 40x 95°C-10s, 63°C-10s, 72°C-20s	Throbäck et al. (2004)
16S rRNA	Eub338 Eub518	5'-ACTCCTACGGG AGGCAGCAG-3' 5'-ATTACCGCGGC TGCTGG-3'	200	6 µL of Sybrgreen iQ™ SYBR® Green Supermix (Bio-Rad), 0.125 µL of each primer (10 pmol), 0.30 µL of BSA and 4 µL of DNA (3 ng).	95°C-3 min.; 40x 95°C-30s, 59°C-35s, 72°C-20s	Fierer et al. (2005)
18S rRNA	FF390 FFR1	5'-CGATAACGAAC GAGACCT-3' 5'-AICCATTCAATC GGTAIT-3'	390	6 µL of Sybrgreen iQ™ SYBR® Green Supermix (Bio-Rad), 0.250 µL of each primer (10 pmol), 0.30 µL of BSA and 4 µL of DNA (3 ng).	95°C-3 min.; 40x 95°C-30s, 52°C-45 s, 72°C-50 s	Vainio and Hantula (2000)
			10 µL of reaction			
nirK fungi	fnirK2F fnirK1R	5'-GTYCAYATYGCYA ACGGSATGTACGG-3' 5'-GCRTGRTCNAC MAGNGTRCGTCCC-3'	468	5 µL of Sybrgreen PowerUp™ SYBR® Green Master Mix (ThermoFisher), 0.250 µL of each primer (10 pmol) and 1 µL of DNA (undiluted).	50°C-2min; 95°C-2min; 45x 95°C-15s, 52°C-30s, 72°C-60 s	Long et al. (2015)

Table S4| Cumulative nitrous oxide emissions and N fertilizer emission relative to the rates of N fertilizer applications used in ratoon cane cycle experiment plus standard error. The treatments are: Control; (N) mineral N as ammonium nitrate; (CV) concentrated vinasse, (V) no-concentrated vinasse, (CV+N) concentrated vinasse plus mineral N, (V+N) non-concentrated vinasse plus mineral N. $_b$: Anticipated vinasse application (30 days before N fertilization).

	Rainy season, 2013/2014		Dry season, 2014/2015	
Treatments[a]	Cumulative N_2O–N emissions[b]		Cumulative N_2O–N emissions	
	mg N m^{-2}	% of N applied**	mg N m^{-2}	% of N applied
CV_b	-	-	74 ± 36	0.39 ± 0.19
CV	69 ± 9	0.22 ± 0.03	131 ± 49	0.40 ± 0.15
V_b	17 ± 10	0.32 ± 0.18	21 ± 7	0.42 ± 0.13
V	4 ± 3	0.08 ± 0.06	113 ± 42	1.27 ± 0.47
N	49 ± 4	0.08 ± 0.01	89 ± 38	0.14 ± 0.06
CV_b+N	-	-	219 ± 26	0.26 ± 0.03
CV+N	942 ± 373	1.00 ± 0.40	626 ± 289	0.65 ± 0.30
V_b+N	54 ± 10	0.08 ± 0.01	137 ± 44	0.20 ± 0.06
V+N	79 ± 11	0.12 ± 0.02	241 ± 43	0.33 ± 0.06

[a]Cumulative nitrous oxide emissions (mg N m^{-2}) and N fertilizer emission factor (EF) based on rates of N fertilizer applications used in the GHG chambers.
[b]Results from treatment without N subtracted for this calculation (Chambers); Background emission observed in the control treatments was 12 and 6 mg N m^{-2} to rainy and dry season.

Supplementary Figures

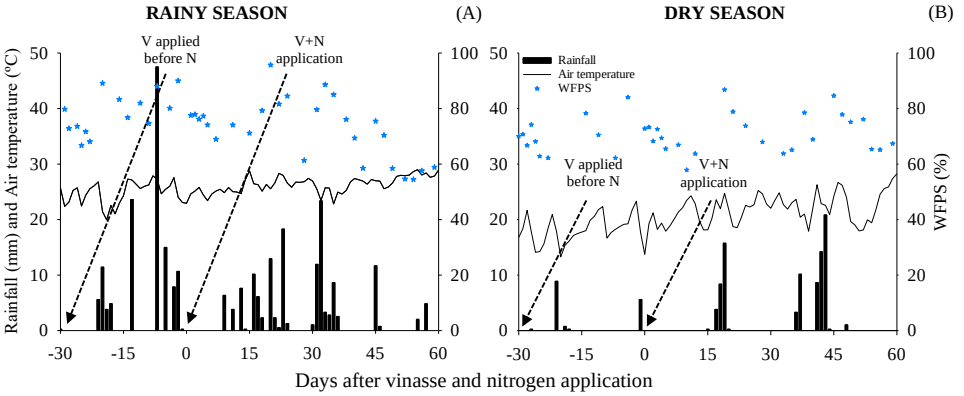

Figure S1| Rainfall (mm), air temperature (ºC) and water-filled pore space (WFPS, %) measured in the (A) rainy and (B) dry seasons.

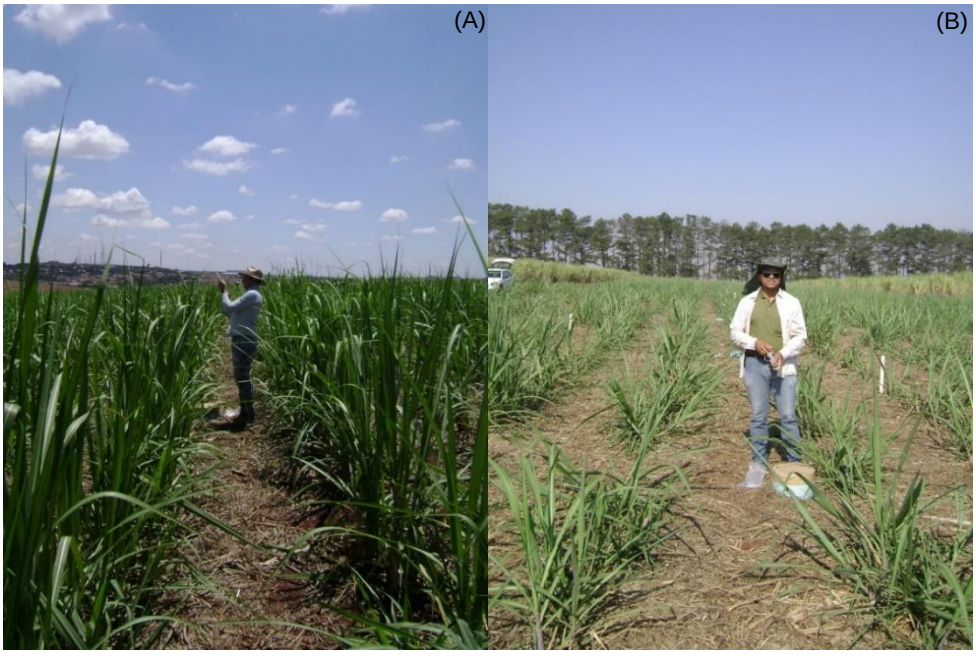

Figure S2| Picture of experiments in (A) rainy and in (B) dry seasons. The pictures were taken three and five months in the rainy and dry season, respectively.

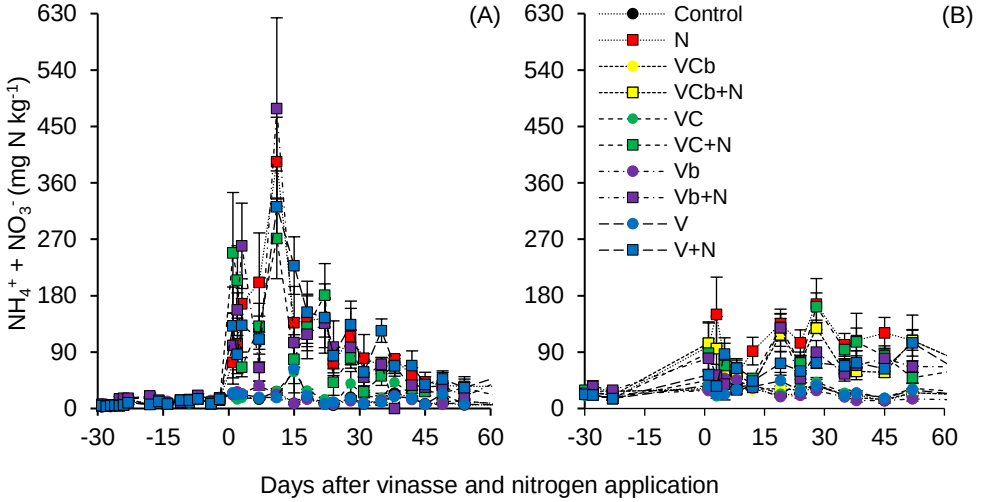

Figure S3 | Soil mineral N (NH_4^+-N + NO_3^--N) contents during rainy (A) and dry (B) seasons. The treatments are: Control; N: mineral N fertilizer, ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N. $_b$: Prior vinasse application (30 days before N fertilization). Vertical bars indicate the standard error of the mean (n = 3).

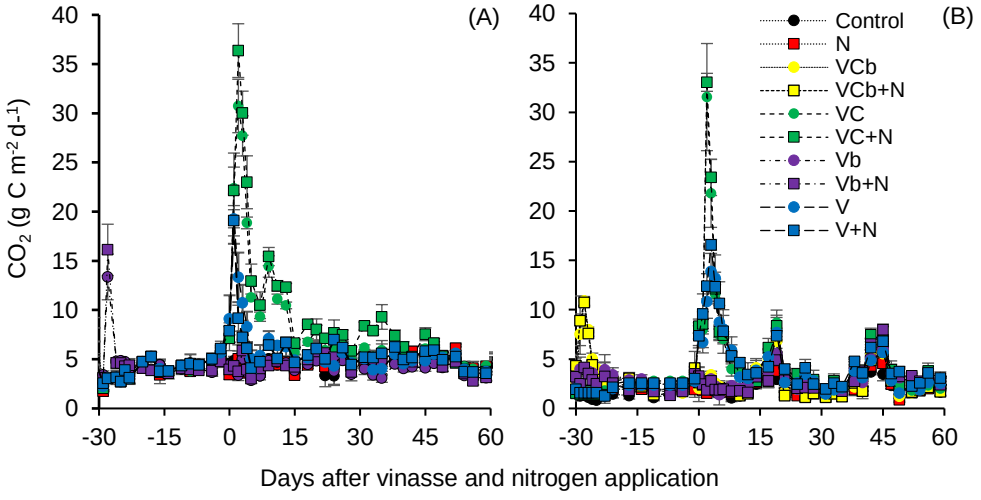

Figure S4 | Daily mean fluxes of CO_2-C measured in ratoon cane with concentrated vinasse and no-concentrated vinasse application. The mineral fertilizer and vinasse were applied in rainy (A) and dry (B) seasons. The treatments are: Control; N: mineral N as ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N. $_b$: Prior vinasse application (30 days before N fertilization). Vertical bars indicate the standard error of the mean (n = 3).

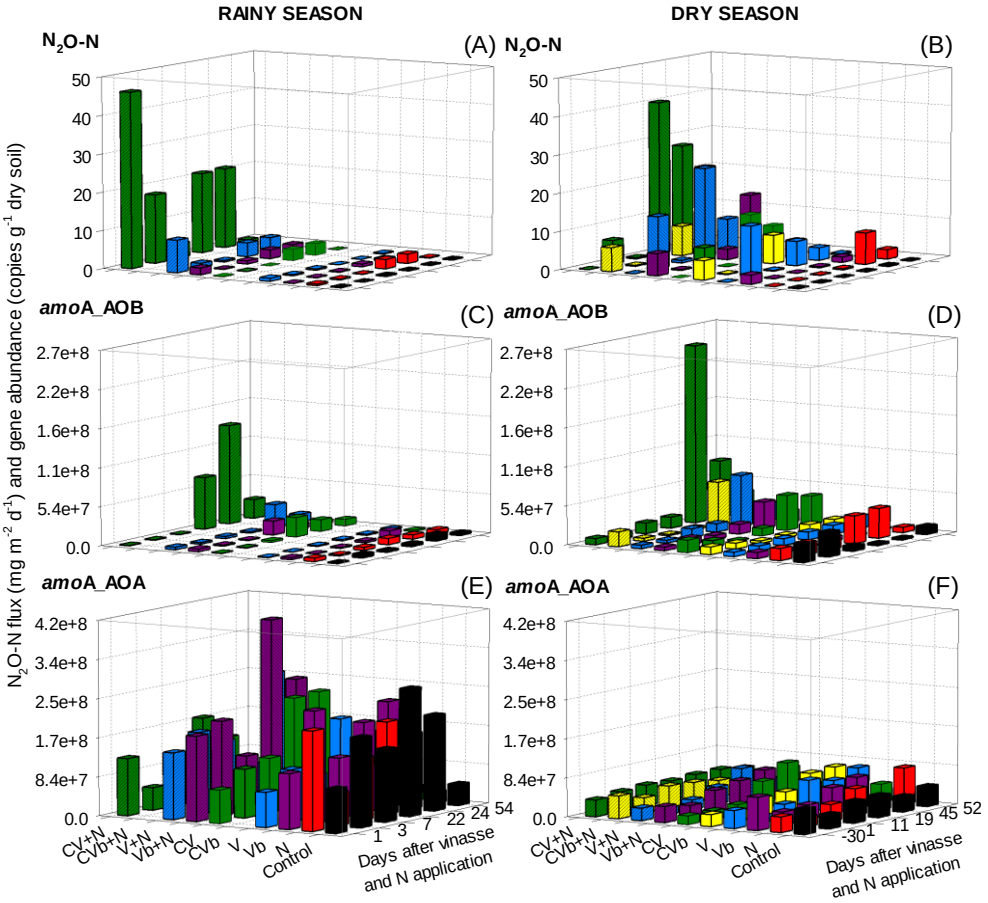

Figure S5| (A, B) Nitrous oxide fluxes (mg m^{-2} d^{-1}) and gene copy numbers of (C, D) ammonia-oxidizing bacterial (*amoA*-AOB) and (E, F) archaeal (*amoA*-AOA), obtained by qPCR. The mineral fertilizer and vinasses were applied in (A, C, E) and rainy (B, D, F) dry seasons. The treatments are: Control; N: mineral N as ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N (n = 3). $_b$: Prior vinasse application (30 days before N fertilization).

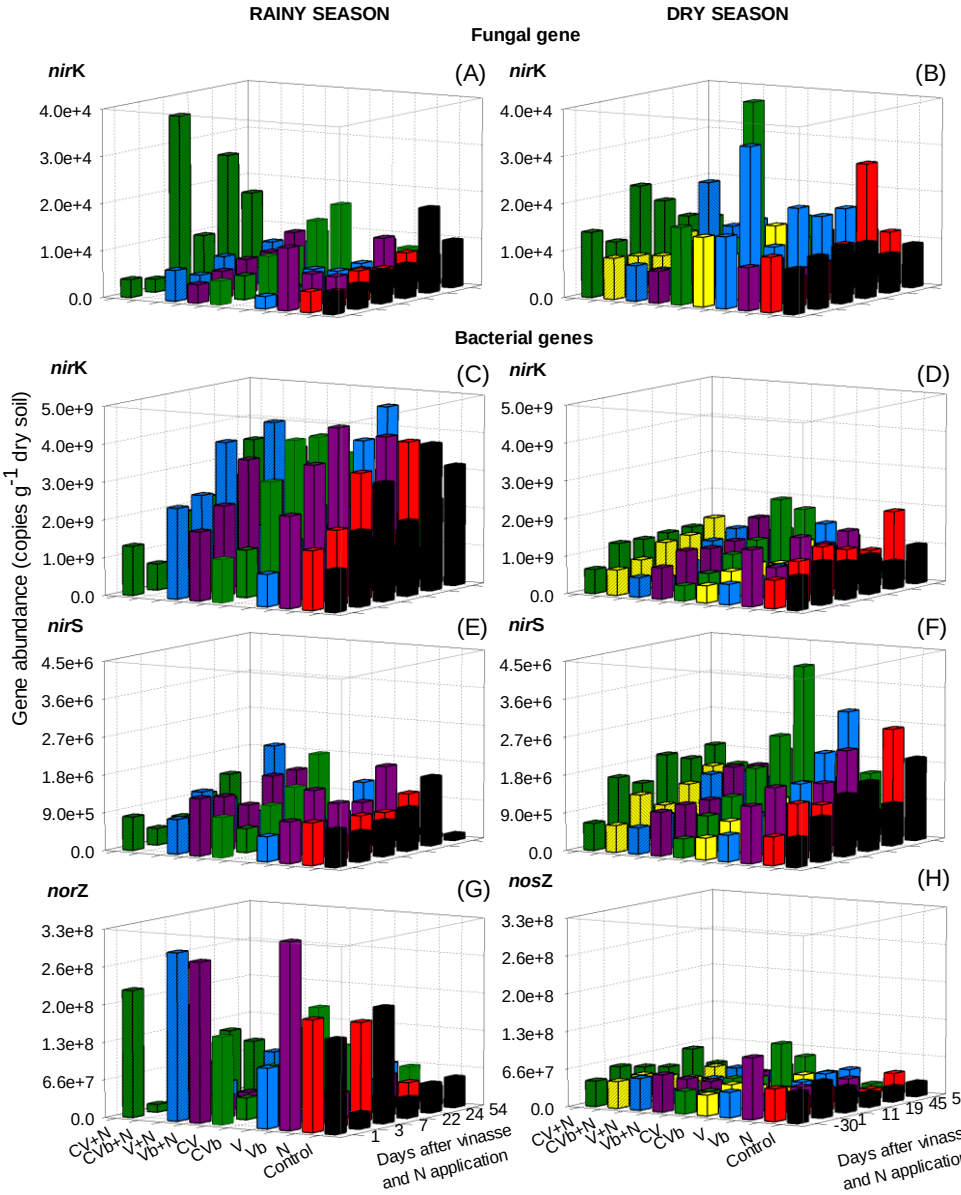

Figure S6| Gene copies numbers per gram of dry soil of fungal and bacterial *nirK* and bacterial *nirS* and *nosZ*, obtained by qPCR from soil with sugarcane in different treatments in (A, C, E) rainy and (B, D, F) dry seasons. The treatments are: Control; N: mineral N as ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N (n = 3). b: Prior vinasse application (30 days before N fertilization).

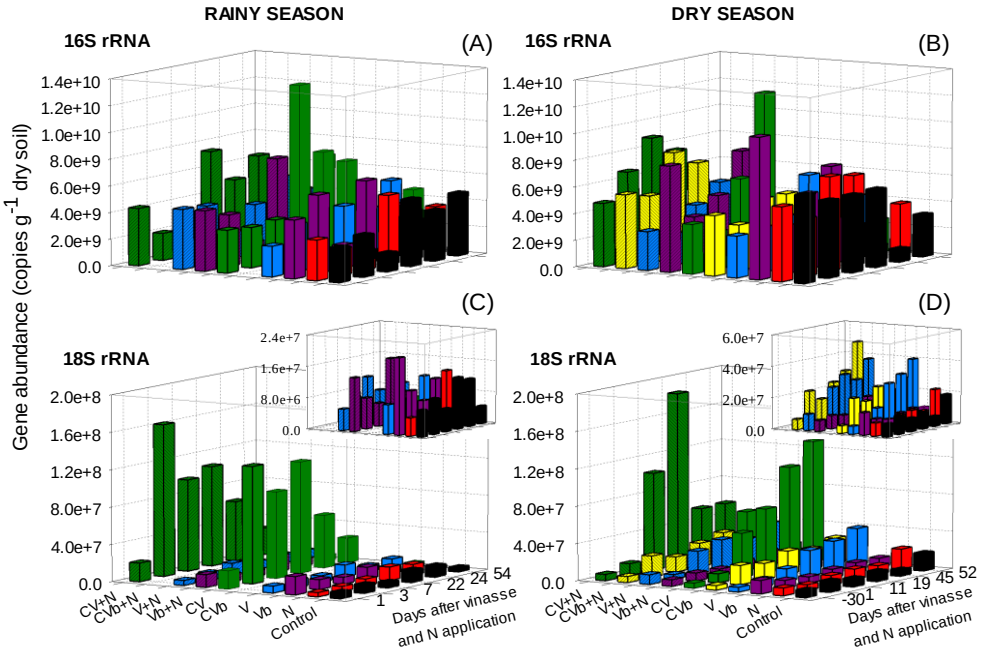

Figure S7 | Gene copies numbers per gram of dry soil of total bacteria (16S rRNA) and total fungi (18S rRNA) obtained by qPCR from soil with sugarcane in different treatments in (A, C) rainy and (B, D) dry seasons. The treatments are: Control; N: mineral N as ammonium nitrate; CV: concentrated vinasse; V: non-concentrated vinasse; CV+N: concentrated vinasse plus mineral N; V+N: non-concentrated vinasse plus mineral N (n = 3). $_b$: Prior vinasse application (30 days before N fertilization).

Chapter 5

Nitrosospira sp. govern nitrous oxide production in a tropical soil amended with residues of bioenergy crop

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(Submitted for publication)

Abstract

Vinasse, a residue produced during bioethanol production, increases nitrous oxide (N_2O) emissions when applied with inorganic nitrogen (N) fertilizer in soil. The present study investigated the role of the ammonia-oxidizing bacteria (AOB) community on the N_2O production in soils amended with vinasse (CV: concentrated and V: non-concentrated) plus inorganic N fertilizer. Soil samples and N_2O emissions were evaluated at day 11, 19 and 45 after fertilizer application, and the bacterial gene (amoA) encoding the ammonia monooxygenase enzyme and total bacteria were quantified by real time PCR. We also employed a deep amoA amplicon sequencing approach to evaluate the effect of treatment on the community structure and diversity of the soil AOB community. Both vinasse types plus inorganic N application increased the total N_2O emissions and the abundance of AOB. Nitrosospira sp. was the dominant AOB correlated with N_2O emissions. However, the diversity and the community structure of AOB were resistant to vinasse and inorganic N fertilizer amendment. The results highlight the importance of residues and fertilizer management in sustainable agriculture and can be used as a reference and an input tool to determine good management practices during organic fertilization.

1. INTRODUCTION

Brazil is the world's largest producer of sugarcane, with about 685 million tons of sugarcane produced from an area of 9 million hectares in 2016/2017 (CONAB, 2017). Roughly 53% of sugarcane production is directed toward bioethanol production, which is considered a sustainable biofuel (CONAB, 2017). Studies conducted by Macedo et al. (2008) and Seabra et al. (2011) indicated that ethanol production from sugarcane emits about 80% less greenhouse (GHG) gases than the production of fossil fuels. These benefits are reduced during the practice of recycling sugarcane straw and bioethanol production residues (Galdos et al., 2010; De Figueiredo and La Scala Jr, 2011; Carmo et al., 2013; Pitombo et al., 2015; Siqueira Neto et al., 2016; Soares et al., 2016). For each liter of ethanol produced, 10 to 15 liters of the liquid waste, called vinasse, are generated, which totals roughly 360 billion liters of vinasse per year. Vinasse is a major residue generated during the production of ethanol from sugarcane. Vinasse is rich in organic content, carbon (1-2 %), nitrogen (357 mg N L^{-1}) and especially potassium (2056 mg L^{-1}) (Elia-Neto and Nakahodo, 1995; Macedo et al., 2008; Christofoletti et al., 2013; Fuess and Garcia, 2014). To recycle these nutrients, vinasse is directly applied on sugarcane fields as a fertilizer. Recently the concentration of vinasse became popular due reduction of water volume and cost with transportation in the field. Thus, both types concentrated and non-concentrated vinasse are used as organic fertilizer (Rodrigues Reis and Hu, 2017). However, when vinasse is applied with N fertilizer in the soil, high nitrous oxide (N_2O) emissions were observed (Carmo et al., 2013; Paredes et al., 2014; Paredes et al., 2015; Pitombo et al., 2015).

Nitrous oxide (N_2O) is one of the molecules of the nitrogen (N) cycle with major environmental and ecological impacts. N_2O is both an ozone-depletion substance (Ravishankara et al., 2009) and a GHG with global warming potential 298 times higher than carbon dioxide (CO_2) (IPCC, 2013). Agricultural soils account for an estimated 65% of global N_2O emissions (IPCC, 2013). N_2O is produced in soil via biotic and abiotic processes. Biotic N_2O production processes are widely distributed over the soil microbiota and have been observed in more than 60 bacterial and archaeal genera (Philippot et al., 2007; Canfield et al., 2010; Nelson et al., 2016). Nitrous oxide is produced as a byproduct of nitrification or denitrification, which are the main biotic processes contributing to N_2O emissions in soil (Goreau et al., 1980; Wrage et al., 2001). Denitrification is widely responsible for soil N_2O productions at high water contents while nitrification has often been assumed to be the principal source of N_2O in soil under aerobic conditions (Mathieu et al., 2006; Soares et al., 2016).

Nitrification is the aerobic oxidation of ammonia (NH_3) to nitrate (NO_3^-), which occurs in two phases mediated mainly by autotrophic microorganisms. In the first phase, ammonia-oxidizing bacteria (AOB) or archaea (AOA) oxidize NH_3 to nitrite (NO_2^-); in the second phase, nitrite-oxidizing bacteria (NOB) oxidize NO_2^- to

NO_3^-. The ammonia oxidation phase ($NH_3 \rightarrow NH_2OH/HNO \rightarrow NO_2^-$) is catalyzed by the ammonia monooxygenase enzyme encoded by the *amo*A gene, which is carried by *β- or γ*-proteobacteria (AOB) and the newly described *Thaumarchaeota* phylum (AOA). The *nxr*B gene encodes the enzyme nitrite oxidoreductase and regulates the second phase of nitrification. The N_2O production by AOB is the result of incomplete oxidation of NH_2OH to either nitroxyl (HNO) or NO (Smith and Hein, 1960; Hu et al., 2015) which occurs under aerobic conditions. The second N_2O-yielding route related to nitrifiers is termed nitrifier denitrification and occurs under both high and low oxygen concentrations. AOB possess a machinery that reduces NO_2^- to N_2O via a nitric oxide (NO) intermediate (Ritchie and Nicholas, 1972; Shaw et al., 2006). Recently, Caranto et al. (2016) demonstrated another direct enzymatic pathway from NH_2OH to N_2O at anaerobic conditions, which is mediated by cytochrome P460.

In a recent study conducted in sugarcane fields in Brazil, AOB rather than AOA or denitrifier bacteria were associated with N_2O emissions (Soares et al., 2016), suggesting that nitrification is the dominant N_2O-producing process in these soils. While it is known that the application of vinasse plus inorganic N fertilizers increases N_2O emissions, there are no studies to date on the effects of these treatments on the AOB communities in these soils. Therefore, the aim of the current study was to evaluate the effects of vinasse plus inorganic nitrogen fertilization on the community abundance, structure, and diversity of the ammonia-oxidizing bacteria in a tropical soil planted with sugarcane. We hypothesized that the abundance and community structure of the AOB would respond to organic and inorganic inputs, i.e. vinasse and fertilization.

2. MATERIAL AND METHODS

2.1. Experimental setup and soil sampling

The field experiment was situated in Piracicaba, Brazil at APTA (Paulista Agency for Agribusiness Technology). The mean annual air temperature and precipitation of the region are 21 °C and 1,390 mm, respectively. Precipitation and daily temperature measurements during the experiment were obtained from a meteorological station located nearby the experimental field (Figure S1). The soil was classified as Ferrasol (FAO, 2015) pH of 5.0, organic matter of 21.1 g dm^{-3}, P of 14.6 mg dm^{-3}, K^+ of 0.7 $mmol_c$ dm^{-3}, Ca^{+2} of 17.4 $mmol_c$ dm^{-3}, Mg^{+2} of 11.9 $mmol_c$ dm^{-3}, $H^+ + Al^{+3}$ of 34.9 $mmol_c$ dm^{-3}, CEC of 65.1 $mmol_c$ dm^{-3} and soil bulk density of 1.49 g cm^{-3}. The experiment was carried out in a field planted with sugarcane variety RB86-7515. The sugarcane was mechanically harvested and the straw was left on top of the soil (16 Mg ha^{-1}). The experiment was conducted in a randomized block design with three replicate blocks. The treatments were: 1) Control: plot without inorganic N fertilization or vinasse; 2) N: inorganic N fertilizer only; 3) CV+N: concentrated vinasse plus inorganic N fertilizer; 4) V+N: non-concentrated vinasse plus inorganic N fertilizer.

The inorganic fertilizers and concentrated vinasse (CV) were surface-applied in a 0.2-m wide row, close to the plant (0.1 m) in agreement with common practices in commercial sugarcane production. The N fertilizer rate was 100 kg N ha^{-1} of ammonium nitrate (NH_4NO_3). Volumes of 1.0 $x10^5$ l ha^{-1} of non-concentrated vinasse (V) were sprayed over the experimental plots using a motorized pump fit with a flow regulator. This amount of V corresponded with recommended average application rates to sugarcane plantations in Sao Paulo. Concentrated vinasse (CV) was applied in fertilization rows at rate of 1.7 $x10^5$ l ha^{-1}. CV was produced by concentrating vinasse by a factor of 5.8, which is the average of sugar mill vinasse concentration processes. The chemical characteristics of the vinasses are shown in Table S1.

The experiment started on August 15, 2014 and 6 soil samplings per plot were carried out on three time points: 11, 19 and 45 days after inorganic N and vinasse applications. For each treatment, soil samples were collected from the 0-10 cm layer for measurements of moisture content, concentrations of NO_3^--N and NH_4^+-N, and pH. Soil subsamples (30g) were stored at -80 ºC for molecular analyses. In parallel, for each soil sample air and soil temperatures were measured. Soil temperatures were collected from the 0-10 cm layer with a digital thermometer. Soil moisture was determined gravimetrically by drying the soil at 105 °C for 24 h and the water-filled pore space (WFPS) was calculated considering soil moisture and bulk density. Soil mineral N (NH_4^+-N, NO_3^--N) was measured with a continuous flow analytical system (FIAlab-2500 System) (Kamphake et al., 1967; Krom, 1980).

2.2. N_2O measurements

Fluxes of N_2O were measured using PVC static chambers, 20 cm height and 30 cm diameter, according to the method described in Soares et al. (2016) and Pitombo et al. (2015). The gases were sampled with plastic syringes (60 mL) at three time intervals (1, 15, and 30 min) after the chambers were closed (Soares et al., 2016). The samples were transferred and stored in pre-evacuated 12 mL glass vials and analyzed in a gas chromatograph with an electron capture detector for N_2O determination (model GC-2014, Shimadzu Co.). Gas and soil samples were collected in the morning between 7:00 and 12:00 am. Overall N_2O flux was calculated by linear interpolation over the three sampling times.

2.3. DNA extraction and real-time PCR

Total soil DNA was extracted using the MoBio PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA, USA). Of each soil sample, 0.30 g was used for DNA extraction according to the manufacturer's instructions. The quantity and quality of DNA were quantified and checked using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA), as well as visualized on 1% (w/v) agarose gel under UV light. The abundance of the *amo*A-AOB gene and total bacterial community was quantified by real-time PCR with a BIO-RAD CFX96 Touch™ Real-

Time PCR Detection System. Amplification of the *amo*A-AOB gene was performed in total volume of 12 µL, containing 6 µL Sybrgreen Bioline SensiFAST SYBR non-rox mix, 0.125 µL of each primer (10 pmol) and 4 µL of DNA (40 ng); the primer used was amoA1F (5'-GGGGTTTCTACTGGTGGT-3') and amoA2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') (Rotthauwe et al., 1997). The thermal cycler conditions were 95 °C-10 min; 40 times 95 °C-10 s, 65 °C-25 s; last, acquisition was done at 65 °C. The qPCR amplicon products (491bp) were checked by melting curve analysis and agarose gel electrophoresis. The efficiency of the *amo*A-AOB qPCR was 87% (R^2 = 0.99). For assessment of the abundance of the total bacterial community based on 16S rRNA gene qPCR was performed in total volume of 12 µL, containing 6 µL Sybrgreen iQ™ SYBR® Green Supermix (Bio-Rad), 0.125 µL of each primer (10 pmol), 0.30 µL of BSA and 4 µL of DNA (5 ng); the primer sets used was Eub338 (5'-ACTCCTACGGGAGGCAGCAG-3') and Eub518 (5'-ATTACCGCGGCTGCTGG-3') (Fierer et al., 2005). The thermal cycler conditions were 95 °C-3 min; 40 times 95 °C-30 s, 59 °C-35 s; 72 °C-20 s and acquisition was done at 59 °C. The qPCR amplicon products (200bp) were checked by melting curve analysis and agarose gel electrophoresis. The efficiency of the 16S rDNA qPCR was 96% (R^2 = 0.99). Plasmid DNA containing fragments of bacterial *amo*A and 16S rRNA genes were used as standards. Each run, in triplicate, included a DNA template, the standard positive control, and a negative control.

2.4. Sequencing of *amo*A genes for ammonia-oxidizing bacteria

Primer sets amoA-1F/amoA-2R (Rotthauwe et al., 1997) for AOB (same primers used in the qPCR) were used to amplify the *amo*A gene fragment for sequencing with Illumina MiSeq sequencing platform. The PCR was carried out in 20 µl reaction containing each 2 µl of deoxynucleoside triphosphate at a concentration of 2.0 mM, 0.25 µl of forward and reverse primers (10 pmol), 0.1 µl of FastStart *Taq* DNA Polymerase, 2µl of $MgCl_2$ buffer, and 0.5 µl of bovine serum albumin - BSA (4 mg ml^{-1}). Each reaction mix received 1 µl of genomic DNA as a template. The PCR conditions for the amplicons were: preheating at 95 ^oC for 5 min, then 35 cycles (95 ^oC for 30 s, 53 ^oC for 30 s, 72 ^oC for 30 s), with a final extension at 72 ^oC for 10 min. Triplicate reaction mixtures per sample were pooled together, purified with the Agarose Gel DNA purification kit (TaKaRa), and quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, USA). The bar-coded PCR products from all samples were normalized in equimolar amounts before sequencing. The amplicon library was prepared by adaptor ligation and PCR using the TruSeq nano DNA Library Prep Kit (Illumina, CatFC-121-4001) according to the TruSeq nano protocol (Illumina,FC-121-4003). Paired-end MiSeq sequencing was carried out by BGI Inc. (China).

2.5. Clustering and taxonomic classification of amoA OTUs

The raw data of *amo*A sequences were preprocessed using Mothur v 1.3.3 (Schloss et al., 2009). Raw sequences were merged (make.contigs command), then trimmed and sorted simultaneously (trim.seqs). Sequences were filtered out if average read quality was less than 25, there were more than two N's or if the read length was less than 150 bp; remaining sequences were filtered based on primer quality (≤ 2 errors), spacers (≤ 2 errors) and barcodes (≤ 1 error). Barcodes and primers were removed. Further, the sample reads were processed using the UCLUST pipeline implemented in a Snakemake workflow which is available upon request (Edgar, 2010). In summary, the *amo*A-AOB sequences were truncated to 480 bp, clustered into 90% OTUs and singletons and chimeras were removed (Norton et al., 2002). An OTU table was created at the 90% cutoff level. The OTUs were checked by comparison to the 2014-03-17 KEGG database using UProc version 1.2.0 with the uproc-dna command (Meinicke, 2015); those OTUs that did not match the *pmo*A-*amo*A (Particulate methane monooxygenase-ammonia monooxygenase) pathway K10944 were removed (5 of 236 OTUs). To further validate the OTUs, centroids were compared to the 2016-10-04 NCBI-nr database using diamond version 0.8.20 with the command blastx (Buchfink et al., 2015). The OTUs that were classified by the Last Common Ancestor algorithm from MEGAN version 6.5.8 as Eukaryote were removed (3 of 236 OTUs) (Huson and Weber, 2013). The centroid OTUs were finally classified using BLASTN (evalue cutoff of 0.02) against a custom *amo*A FunGene database described below which comprised 136 records (Fish et al., 2013). Last, the classification was added to the OTU table using a custom Perl script.

Due to poor classification results from classification against the NCBI nr database, a custom *amo*A database was created from FunGene *amo*A sequences as follows. High-quality *amo*A sequences with score above 350, size greater than 200 amino acids in length, HMM coverage of more than 85% and defined organism name were downloaded. The NCBI taxonomy of each unique record was obtained using a custom Perl script. Taxonomy information was refined as follows: 1) "environmental sample" was replaced with "unclassified", 2) "uncultured ammonia-oxidizing beta-proteobacterium" was annotated as an unclassified Beta-proteobacteria, and 3) "uncultured bacterium" or "uncultured soil bacterium" were annotated as unclassified Bacteria. The custom *amo*A sequences were aligned using ClustalW in MEGA7 (Kumar et al., 2016). From the first to the last conserved position of the aligned sequences (461 bp), a neighbor-joining tree was created to examine the phylogenetic relationships between the 138 records using as outgroup a *pmo*A cluster consisting of 25 Gamma-proteobacteria records (Saitou and Nei, 1987). Distances were computed using the Maximum Composite Likelihood method and a bootstrap test with 1000 replicates was conducted (Felsenstein, 1985). Because the *amo*A sequences clustered together at least at the Beta-proteobacteria level, the taxonomy of the records originally noted as unclassified Bacteria were updated as unclassified Beta-proteobacteria (see Figure S2). We

used the Interactive Tree of Life (iTOL) (Letunic and Bork, 2016) to plot the 30 most abundant sample amoA-AOB OTUs and their nearest neighbors in the custom FunGene amoA sequence database.

2.6. Statistical analyses of gas fluxes, gene abundances and amoA OTUs

All statistical analyses, except Spearman correlations, were carried out in RStudio version 1.0.136 running R version 3.3.1. Generalized linear models (Bolker et al., 2009) were used to test the effect of different treatments on N_2O fluxes and *amo*A gene copy number using the multcomp package (Hothorn et al., 2008) in R. The differences between treatments were analyzed for each sampling event. Treatments were considered statically significant using $P < 0.01$ as the criterion. To account for the increasing variation with the increase in the mean, we used Gamma (N_2O emission) and Poisson family (*amo*A and 16S gene copy number) distributions as criteria to the generalized linear models. Subsequently the glht function was used to evaluate the differences among treatments (Tukey $p \leq 0.01$). The correlation between N_2O flux and *amo*A-AOB gene abundances were calculated by Spearman correlation analysis in Sigma Plot, version 13.0 (SystatSoftware, 2014).

The phyloseq package was used to handle the *amo*A-AOB OTU abundance data (McMurdie and Holmes, 2013). The *amo*A data were rarefied to the size of the smallest sample (12,978 sequences) prior to alpha and beta diversity analyses. To determine whether AOB bacterial community diversity differed by treatment or day sampled, Renyi indexes were calculated using the BiodiversityR package and the values for average, normally distributed Shannon and Inverse Simpson indexes were compared between treatments (Tukey's HSD test with alpha of 0.05) using the multcomp package (Simpson, 1949; Kindt and Kindt, 2015). To test the effect of treatments on AOB bacterial community compositions, the rarefied AOB data was ordinated using PCoA using the Bray distance measure. The PERMANOVA test in the vegan package was used to ascertain group significance with 9999 permutations (Oksanen et al., 2015). In parallel, the data was ordinated using correspondence analysis and group significance was assessed with between-groups analysis applying a random permutation test (999 repetitions) from the ade4 package (Dray and Dufour, 2007). Last, a permutation test for homogeneity of multivariate dispersions was run on sample distances from the vegan package. Group tests were applied for treatment (Control, N, CV+N, V+N) and day (11, 19, 45) groups. We also used multivariate regression tree (MTR) analyses (De'ath, 2002) in the R 'mvpart' package (Therneau and Atkinson, 1997; De'ath, 2007) to identify the effect of the temporal variation (time) on AOB community composition (Ouellette et al., 2012). For the analysis, the rarefied AOB data was log-transformed, and the tree was plotted after 500 cross-validations (Breiman et al., 1984), avoiding overfitting. Subsequently, the function rpart.pca from the mvpart package was used to plot a PCoA of the MTR.

3. RESULTS

3.1. Weather conditions, greenhouse gas emission and soil analysis

The climatic conditions during the experimental period were shown in Supplementary Figure S1A. The lowest air temperature was 7 ^{o}C in the beginning of the experiment and the highest 35 ^{o}C. The mean temperature during the 45-day experiment was 22 ^{o}C (Figure S1A). A similar pattern was observed in soil temperature; the temperature increased through the experimental period from 17 to 22 ^{o}C in average.

Treatments with inorganic N plus vinasse application (CV or V) had higher N_2O emission than treatments with only inorganic N and control. At day 11 the emission was low due to lack of rain during the previous period, with even consumption of N_2O in the control treatment (Table 1). The CO_2 emissions were similar to N_2O emissions, with lower emission at day 11 than in day 19 and 45. The CO_2 emissions were higher for treatments with inorganic N plus vinasse in comparison to the control and only N (Figure S1B).

Table 1 | Ammonia-oxidizing bacteria (*amo*A-AOB) gene copy numbers (g dry soil^{-1}) and nitrous oxide fluxes (n = 3) for different treatments including Control; N: inorganic N fertilizer; CV+N: concentrated vinasse plus inorganic N fertilizer; V+N: non-concentrated vinasse plus inorganic N fertilizer.

	Day 11		Day 19		Day 45	
Treatment[a]	*amo*A[b]	N_2O-N[c]	*amo*A	N_2O-N	*amo*A	N_2O-N
Control	7.1 ±2.8a	-0.07 ±0.12a	2.8 ±1.1a	0.11 ±0.03a	2.4 ±0.5a	0.24 ±0.10a
N	12.8 ±6.5c	0.11 ±0.03a	38.6 ±12.4d	0.35 ±0.09a	41.4 ±22.1b	8.34 ±2.60b
CV+N	15.0 ±6.6d	0.33 ±0.05a	15.4 ±8.6c	40.22 ±7.04b	247.4 ±146.9d	27.54 ±14.65b
V+N	12.3 ±5.1b	0.70 ±0.09b	11.6 ±3.5b	23.71 ±7.95b	71.5 ±14.0c	8.93 ±1.09b

[a] Means followed by the same letter in the column at each treatment do not differ significantly by the Tukey's test ($p < 0.05$).
[b] x10^6 gene copies g^{-1} dry soil**;**
[c] mg N m^{-2} d^{-1}; Values followed by different letters are significantly different at $p \leq 0.05$ using the Tukey test.

For the treatments, the total NH_4^+-N content decreased through the time, while NO_3^--N content increased (Figure S3). The soil pH had overall low variation across treatments; in the CV+N treatment the pH slightly increased while in the inorganic N and V+N treatments the pH decreased over the time (Figure S3C). N_2O emissions were highly correlated with CO_2 emissions and other environmental parameters, including soil temperature, WFPS and NO_3-N (R^2 = 0.87; R^2=0.37; R^2 = 0.51; and R^2 = 0.63, respectively) (Figure 1).

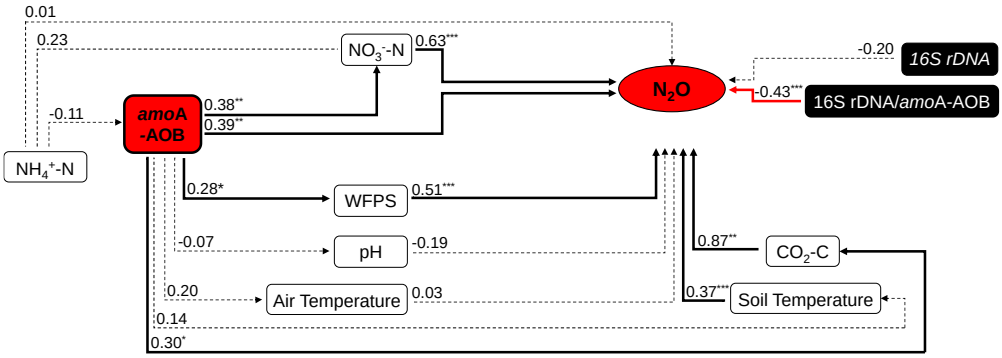

Figure 1 | Summary of Spearman's correlations between N_2O emission flux (mg N m^{-2} d^{-1}) and environmental variables, the abundance of bacterial *amo*A-AOB genes (gene copy g^{-1} dry soil) and the *16S rDNA/amoA* ratio (n=36). Black bold lines mean significant correlation, red bold lines significant negative correlation and dotted lines no correlation between variables (n=36). Significant difference: *p≤ 0.10; ** p≤ 0.05 and *** p≤ 0.01. Abbreviations: WFPS: Water-filled pore space; AOB: *amo*A belonging to ammonia-oxidizing bacteria.

3.2. Ammonia-oxidizing bacterial (AOB) abundance and community composition over time

Inorganic N plus organic vinasse (CV and V) significantly increased AOB *amo*A gene copies by more than 2, 8 and 51-fold at days 11, 19 and 45, respectively, compared to the control (Table 1). In contrast, the total bacteria (16S rDNA) abundance was similar for all treatments. The ratio between the abundance of the total bacteria and the *amo*A-AOB differed between treatments, with the lowest values for V and CV. This suggested that vinasse (CV and V) plus inorganic N treatment increased the *amo*A gene copies more than the total bacteria (Table 2). Furthermore, the abundance of *amo*A genes was significantly correlated with N_2O (R^2= 0.39) and CO_2 (R^2= 0.30) emissions; in addition, *amo*A gene abundances increased significantly with WFPS (R^2= 0.28) and soil NO_3^--N (R^2= 0.38) values (Figure 1).

A total of 1,661,482 high quality *amo*A-AOB sequences from 36 samples (4 treatments x 3 time points x 3 replicates) with an average of 46,152 reads (13,213 – 202,908 reads) per sample were clustered into 236 OTUs for *amo*A-AOB community analysis. Rarefaction curves indicated that the community diversity was well captured with our sequencing depth (Figure S4).

In order to assess the effects of the treatments or timepoints on the *amo*A-AOB community structure, the taxonomic profiles were compared at different time points using a combination of ordination and dissimilarity tests. Comparative analysis of the AOB community structure revealed no clear separation by treatment. The PERMANOVA and correspondence analysis-between class analysis revealed no differences between treatments; furthermore, the interaction between treatment and time was not significant (PERMANOVA: p=0.32) (Table S2; Figure S5). To further explore temporal effects we used a multivariate regression tree (MRT) approach and PCA ordination given by MRT analysis which further

showed that the microbial community composition did not change over time (Error = 0.92) (Figure 2). Moreover, the factors treatment or time did not affect alpha-diversity of the AOB communities (OTU richness, Chao1, Simpson and Shannon) (Table S3).

Table 2 | Ratios between the gene copy numbers (per gram of dry soil) of ammonia-oxidizing bacteria (*amo*A-AOB) and total bacteria 16S rDNA (n = 3). The treatments were: Control; N: inorganic fertilizer; CV+N: mineral fertilizer plus concentrated vinasse; V+N: mineral fertilizer plus no-concentrated vinasse.

	Ratio (16S rDNA /*amo*A-AOB)		
Treatments[a]	Day 11	Day 19	Day 45
Control	13891 ±13289c	4565 ±2029d	478 ±169d
N	18332 ±17408d	2974 ±2823c	180 ±123c
CV+N	1351 ±847b	2005 ±865b	89 ±43b
V+N	998 ±582a	1197 ±796a	26 ±3a

[a] Values followed by the same lowercase letter in the column are not significantly different at $p \leq 0.05$ using the Tukey test.

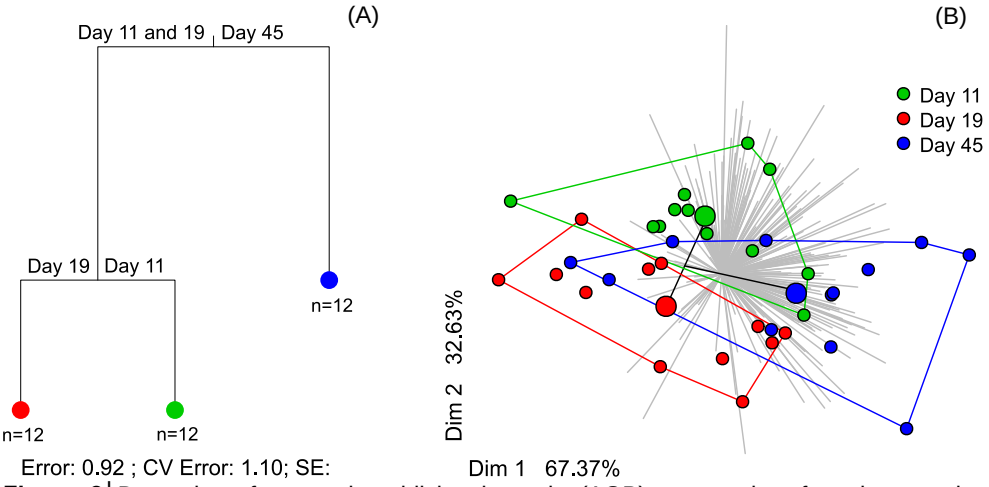

Figure 2 | Dynamics of ammonia-oxidizing bacteria (AOB) community after vinasse plus inorganic N application. Multivariate regression tree (MRT) analysis was used to estimate the impact of time on the AOB community structure, resulting in (A) the most parsimonious tree with three different leaves (large coloured circles) defined based on AOB abundance and composition and (B) the AOB community composition within leaves represented as a PCA plot, in which small points represent individual samples and big points the mean of the samples. The grey barplot in the background indicates the families whose differential abundance explains variation in the PCA plot.

The AOB community present in the soil was composed mainly of the β-Proteobacteria phylum and the *Nitrosomonadaceae* family, of which 20.8 % belonged to the genus *Nitrosospira* and 79.2% to unclassified β-proteobacteria (Figure 3). However, the phylogenetic tree showed that all of OTUs found in the soil used here clustered with *Nitrosospira* and *Nitrosovibrio* genus, except 2 OTUs (OTU 23 and OTU165) which clustered with the *Nitrosomonas* genus (Figure 3,

Figure 4). *Nitrosospira sp. PJA1* and *Nitrosovibrio sp. RY3C* had significant positive correlations (p≤ 0.10) with N_2O-N, NO_3-N and the number of *amo*A gene copies (Table 3). Surprisingly, *Nitrosospira multiformis* showed significant negative correlations with N_2O-N, NO_3-N and the *amo*A gene copy number (p≤ 0.10).

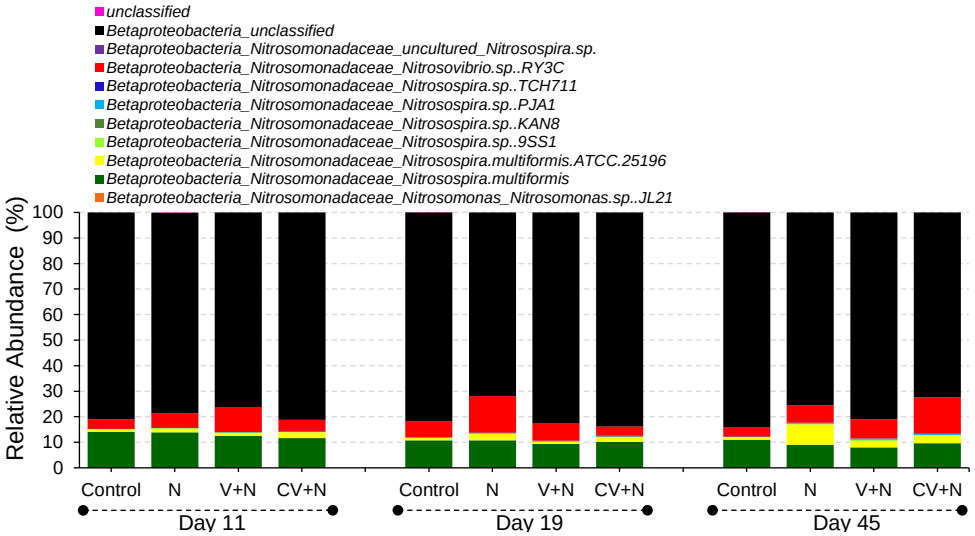

Figure 3 | Spearman's correlation coefficients between the amoA OTUs (classified at the species level) and *amo*A gene copy number (determined by qPCR), N_2O emission flux and mineral N (NH_4^+-N and NO_3^--N) values. The *amo*A-AOB OTUs were assigned to their taxonomic affiliations of ammonia-oxidizing bacteria by comparison to sequences in the *amo*A database from FunGene.

Table 3 | Spearman's correlation coefficients between the *amoA* OTUs (classified at the species level) and *amoA* gene abundances, N_2O emission flux and mineral N (NH_4^+-N and NO_3^-N) values. The *amoA*-AOB OTUs were assigned to their taxonomic affiliations of ammonia-oxidizing bacteria by comparison to sequences in the *amoA* database from FunGene.

Species-level classification[a]	N_2O-N[b]	NO_3^--N	NH_4^+-N	*amoA* AOB
Betaproteobacteria_Nitrosomonadaceae_Nitrosomonas_Nitrosomonas sp.JL21	-0.1	-0.02	**0.36****	-0.01
Betaproteobacteria_Nitrosomonadaceae_Nitrosospira multiformis	**-0.43*****	**-0.32***	0.20	**-0.47*****
Betaproteobacteria_Nitrosomonadaceae_Nitrosospira multiformis ATCC.25196	0.05	0.12	-0.00	**0.75*****
Betaproteobacteria_Nitrosomonadaceae_Nitrosospira sp. 9SS1	0.19	0.25	-0.14	**0.76*****
Betaproteobacteria_Nitrosomonadaceae_Nitrosospira sp. KAN8	0.08	0.11	-0.23	0.24
Betaproteobacteria_Nitrosomonadaceae_Nitrosospira sp. PJA1	**0.29***	**0.34****	-0.11	**0.84*****
Betaproteobacteria_Nitrosomonadaceae_Nitrosospira sp. TCH711	0.259	0.26	-0.25	**0.66*****
Betaproteobacteria_Nitrosomonadaceae_Nitrosovibrio sp. RY3C	**0.28***	**0.30***	-0.04	**0.81*****
Betaproteobacteria_Nitrosomonadaceae_uncultured.Nitrosospira sp.	-0.05	-0.22	0.06	-0.26
Betaproteobacteria_unclassified	-0.06	-0.19	-0.16	**-0.72*****
unclassified	-0.22	-0.26	-0.19	-0.16

[a] Significant difference: *p≤ 0.10; * * p≤ 0.05 and *** p≤ 0.01.
[b] (mg N m^{-2} d^{-1})

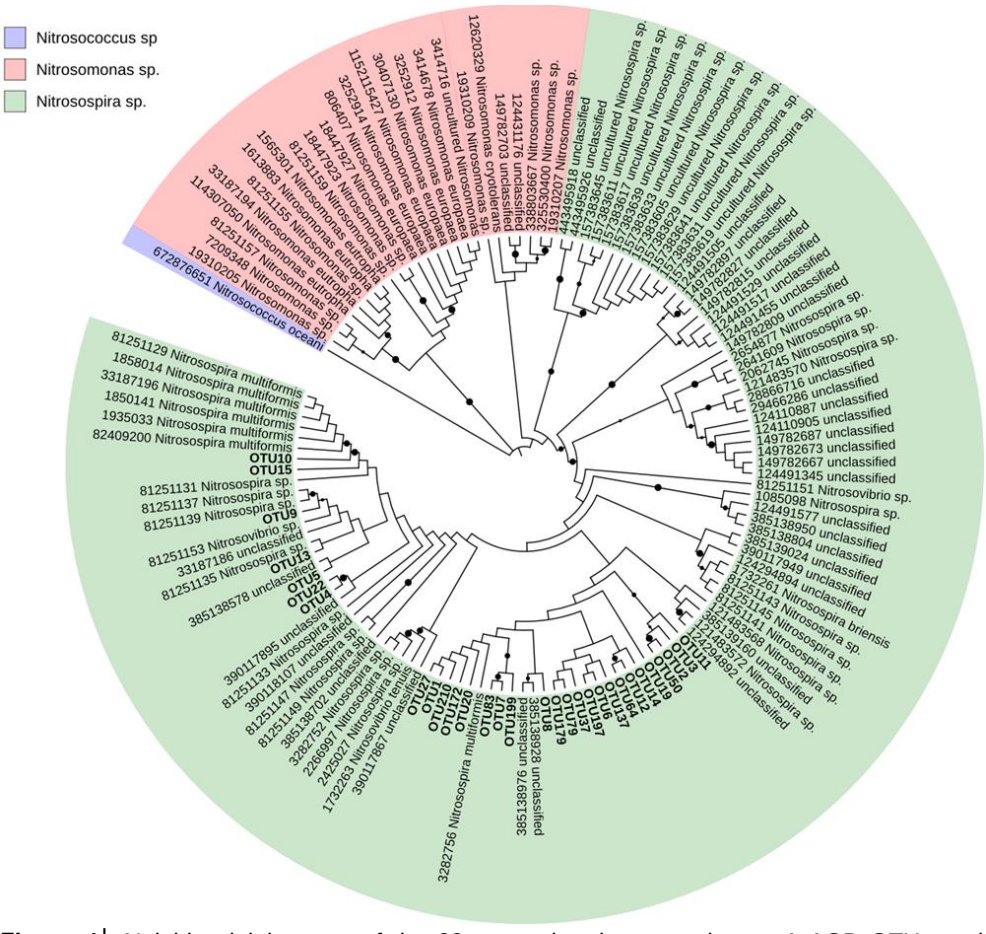

Figure 4| Neighbor-joining tree of the 30 most abundant sample amoA-AOB OTUs and their nearest neighbors in the custom FunGene amoA sequence database. The NCBI taxonomic classification of the database entries is included including the outgroup Nitrosococcus oceani. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (black points mean bootstrap value >75%). Evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 131 sequences and 305 positions and was conducted in MEGA7.

4. DISCUSSION

Based on previous results it is known that fertilization with N and concentrated and non-concentrated vinasse significantly increases N_2O emission, and the emissions are due to nitrification and denitrification processes. Specifically, nitrification by ammonia-oxidizing bacteria is the major process contributing to the higher N_2O emissions due to high drainage capacity of the tropical soil (Chapter 4). Similar results were found by Soares et al. (2016) in that nitrification by AOB was the main process responsible for N_2O emission in the same region with sugarcane

in Brazil, but in their study neither vinasse nor straw were applied in the soil. The main goal of our study was to complement the work done by Lourenço et al. (2018) to identify the main ammonia-oxidizing bacteria present in soil related with high N_2O emissions.

In our study the addition of inorganic N plus vinasse application (CV and V) boosted high N_2O emissions. Vinasse is an organic residue rich in organic compounds with high biological oxygen demand (Fuess and Garcia, 2014). The input of labelled carbon from vinasse in the soils increased soil microbial activities including intense oxygen consumption (Renault et al., 2009) and it creates microoxic or anoxic conditions, resulting in anaerobic microsites (Torbert and Wood, 1992). Both conditions favor denitrification; however, the anaerobic conditions may prevail only for a short time, since the soil drains well and will dry in a few hours. This then favors N_2O production by nitrification.

The AOB are aerobic microorganisms, which obtain energy by the oxidation of inorganic N compounds, allowing for N_2O production in the soil during aerobic conditions. While nitrification by AOB is usually associated with aerobic conditions, N_2O production by ammonia-oxidizing bacteria is also possible via nitrification under suboxic or anoxic conditions, although these situations are still relatively unstudied in soil field conditions (Caranto et al., 2016). Furthermore, nitrifier denitrification by AOB could also play a role in N_2O emissions in treatments with organic vinasse, under low oxygen and high concentration of nitrite (Joo et al., 2005; Spott et al., 2011; Zhao et al., 2012; Zhu et al., 2013). The positive correlation between *amo*A abundance and WFPS due to rain events and vinasse application suggested that nitrification and nitrifier-denitrification processes were occurring during anaerobic conditions (Di et al., 2014).

The application of different vinasses and inorganic N did not change the AOB community composition, but the applications increased the abundance of AOB in the soil. Thus, it is fair to conclude that, in the short time of the experiment, the community composition was resistant to the organic and inorganic fertilization. Studies have reported changes in AOB community composition in response to N fertilizers (Glaser et al., 2010; Ouyang et al., 2016; Xiang et al., 2017). Verhamme et al. (2011) found that the abundance and community structure of AOB changed only in the soil treatment with the highest ammonia concentration (200 mg g^{-1}). Other studies have also reported changes in AOB abundance without a corresponding change in composition with N additions (Phillips et al., 2000; He et al., 2007). In our experiment, we used the N rate recommended for sugarcane fields in Brazil, which is a relatively small input rate of 0.75 mg N g^{-1}. Therefore, we suggest that the community structure of AOB in soils with sugarcane was found to be unchanged after N fertilization due to the low application rate. Moreover, the AOB community in these fields may have already been adapted to the straw and annual application of inorganic fertilizer since sugarcane has been cultivated in this area for more the 20 years.

Interestingly, the AOB community is composed of only few species of bacteria in soils. The AOB found in soils generally belong to the β-Proteobacteria Phylum, *Nitrosomonas* and mainly *Nitrosospira genus* (Prosser et al., 2014). There is no reported evidence of γ- Proteobacteria ammonia oxidizers in soil. Here, the AOB phylogenetic tree revealed that *Nitrosospira* was the dominant genus (99.5 % of the total AOB community) in the soils with sugarcane. Recently, 16S rRNA and *amo*A gene sequencing studies have provided evidence that *Nitrosospira spp.* dominate most natural soil populations (Stephen et al., 1996; Pommerening-Röser and Koops, 2005). Surprisingly, we found only two OTUs with low abundance of *Nitrosomonas spp.* in this soil. Usually, they are prevalent in soils that have received high inputs of inorganic N (Hastings et al., 1997; Oved et al., 2001) and organic residues (Oved et al., 2001; Taylor and Bottomley, 2006; Habteselassie et al., 2013). Habteselassie et al. (2013) and Oved et al. (2001) showed that *Nitrosomonas* were not detected in soils that received inorganic fertilizer but were abundant in soils that received liquid dairy waste and wastewater effluent.

The dominance of *Nitrosospira sp*. could be explained by specific conditions such as soil pH which may have been consistent over the long period of over 20 years that this soil was used for sugarcane production. It has been postulated that pH may select for the presence of *Nitrosospira* group in acid soil (Pommerening-Röser and Koops, 2005) whereas strains of *Nitrosomonas* are not common in acidic environments (pH 4 - 5). The AOB isolated from acidic soils are generally *Nitrosospira* with ureolytic characteristics, for instance some of these AOB produced urease enzymes catalyzing the breakdown of urea to ammonia (De Boer and Kowalchuk, 2001). This advantage allows the ureolytic AOB to grow at relatively low pH with urea source (Pommerening-Röser and Koops, 2005; Ma et al., 2008). Our results showed that inorganic N application decrease soil pH over time. Therefore, the continual application of inorganic fertilizers could select the *Nitrosospira* population by lowering the soil pH.

Contrary to our hypothesis, the AOB community structure was resistant to vinasse and inorganic N fertilization. The long-time inorganic N fertilization may have resulted in an AOB community that is adapted to fluctuations in mineral N in the soil, thus resulting in a diminished response of the soil AOB community structure to changes in available mineral N, affecting only the growth of the whole AOB community. Furthermore, soils with sugarcane seem to select *Nitrosospira* over *Nitrosomonas*, and the first group was responsible for the N_2O emissions from soils with organic vinasse (CV and V) and inorganic N fertilizer. These results are of considerable importance for the sustainability of bioethanol production from sugarcane. The information about the microbes responsible for the N_2O emission may be helpful to define better strategies to mitigate the N_2O emissions due to inorganic N fertilizers and organic vinasse application.

5. Author contributions

K.S.L., H.C. and E.E.K. designed the research; K.S.L. conducted the experiments; K.S.L. and A.P. conducted the qPCR and PCR analyses; N.A.C. performed the bioinformatic steps; K.S.L. and N.A.C. performed the statistical analyses; K.S.L., J.A.V. and E.E.K. wrote the paper. All authors reviewed the manuscript.

6. Acknowledgments

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Supplementary Data

Supplementary Tables

Table S1| Chemical characteristics of the vinasse applied in the experiments.

		Concentrated vinasse - CV	No-concentrated vinasse - V
pH		4.2	3.9
C org	(g L^{-1})	65.3	31.4
N tot	(g L^{-1})	3.0	0.9
NH_4^+-N	(mg L^{-1})	100.9	41.6
NO_3^--N	(mg L^{-1})	23.7	4.1
P	(g kg^{-1})	0.53	0.23
K	(g kg^{-1})	21.0	4.7
C/N		22/1	35/1

Table S2| Result from permutational Analysis of Variance (PERMANOVA) testing the effect of treatment or day on the ammonia-oxidizing bacterial community structure based on Bray-Curtis distance.

	amoA community	
Main test	Pseudo-F (F)	p value
Treatments	0.91	0.54
Days	1.41	0.17
Treatments x Days	1.12	0.32

Table S3│Alpha diversity of the soil microbial communities and statistics comparing the factors treatment and timepoint. The treatments were: Control; N: inorganic N fertilizer; CV+N: concentrated vinasse plus inorganic N fertilizer; V+N: non-concentrated vinasse plus inorganic N fertilizer.

ANOVA test [a]	**Richness**	**Chao1**	**Simpson**	**Shannon**
Treatment	ns	ns	ns	ns
Day	ns	*	ns	ns
Treatment x Day	ns	ns	ns	ns
	Days after vinasse application			
	11	19	45	
	Richness			
Control	31.00±1.73	32.00±3.61	30.67±4.73	
N	35.33±2.08	27.67±2.08	29.67±5.86	
CV+N	31.67±2.31	31.00±0.58	27.67±5.03	
V+N	30.00±3.46	28.67±1.00	29.67±4.04	
	Chao 1			
Control	33.30±1.31	32.63±4.02	34.03±4.38	
N	37.50±3.50	30.43±3.50	31.17±6.71	
CV+N	38.73±6.09	38.10±8.63	28.03±5.62	
V+N	34.33±9.22	30.43±1.53	30.33±4.07	
	Simpson			
Control	0.21±0.02	0.21±0.06	0.21±0.08	
N	0.20±0.06	0.18±0.08	0.18±0.06	
CV+N	0.20±0.04	0.21±0.10	0.19±0.04	
V+N	0.16±0.02	0.17±0.01	0.15±0.03	
	Shannon			
Control	2.01±0.10	1.96±0.25	1.96±0.14	
N	2.06±0.23	2.05±0.33	2.10±0.22	
CV+N	2.06±0.14	2.01±0.29	2.10±0.19	
V+N	2.15±0.06	2.11±0.03	2.20±0.16	

[a] Symbols in the caption refer to overall ANOVA results for the given experiment.. Significant difference: * p≤ 0.10and ns: Non-Significant.

Supplementary Figures

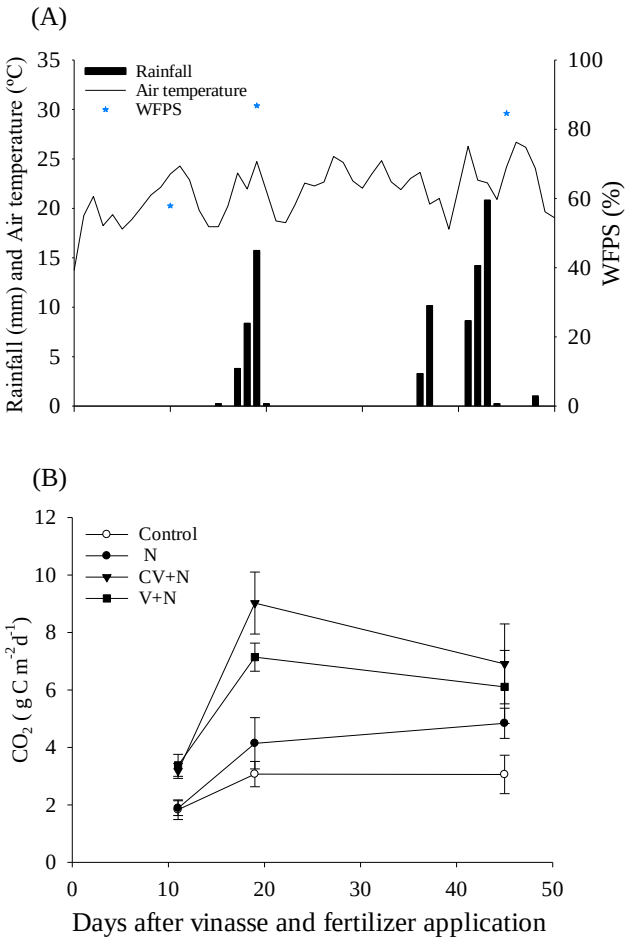

Figure S1| Plots depicting (A) rainfall, air temperature and water-filled pore space (WFPS) and (B) daily mean fluxes of CO2-C from soils for different treatments. The treatments were: Control; N: inorganic N fertilizer; CV+N: concentrated vinasse plus inorganic N fertilizer; V+N: non-concentrated vinasse plus inorganic N fertilizer. Vertical bars indicate the standard error of the mean (n = 3).

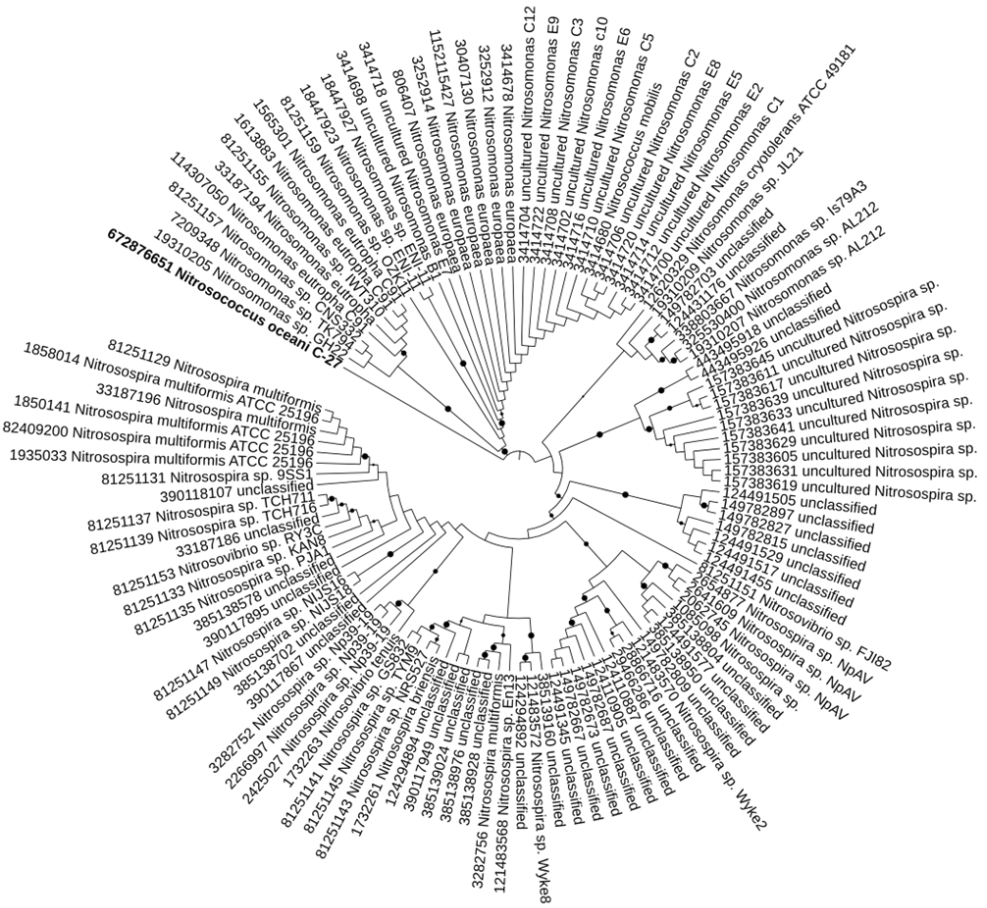

Figure S2| Neighbor-joining tree of the 138 amoA sequences taken from FunGene with NCBI taxonomy with a 25 member outgroup of Gammaproteobacterial amoA and the outgroup *Nitrosococcus oceani*. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (bootstrap values > 75%). Evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 138 sequences and 461 positions and was conducted in MEGA7.

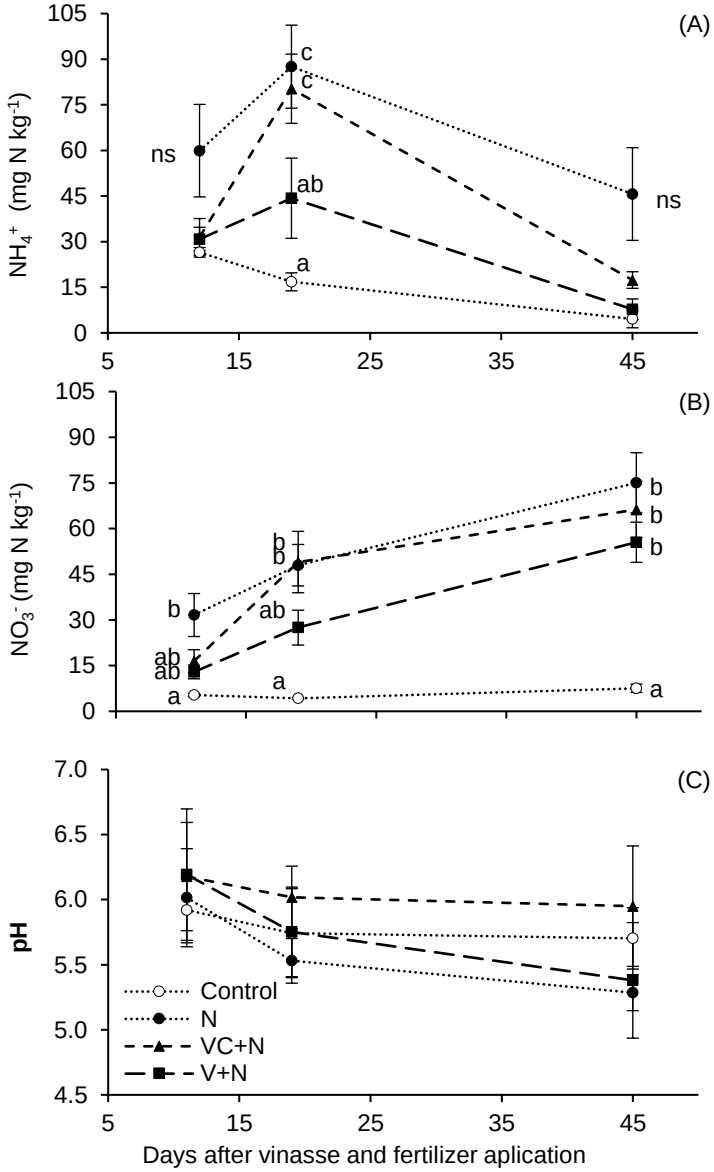

Figure S3│Plots of soil mineral N (NH_4^+-N + NO_3^--N) content (mg N kg^{-1} of dry soil) and soil pH. The treatments were: Control; N: inorganic N fertilizer; CV+N: concentrated vinasse plus inorganic N fertilizer; V+N: non-concentrated vinasse plus inorganic N fertilizer. Vertical bars indicate the standard error of the mean (n = 3). Values followed by the same lowercase letter in the column were not significantly different at $p \leq 0.05$ using the Tukey test.

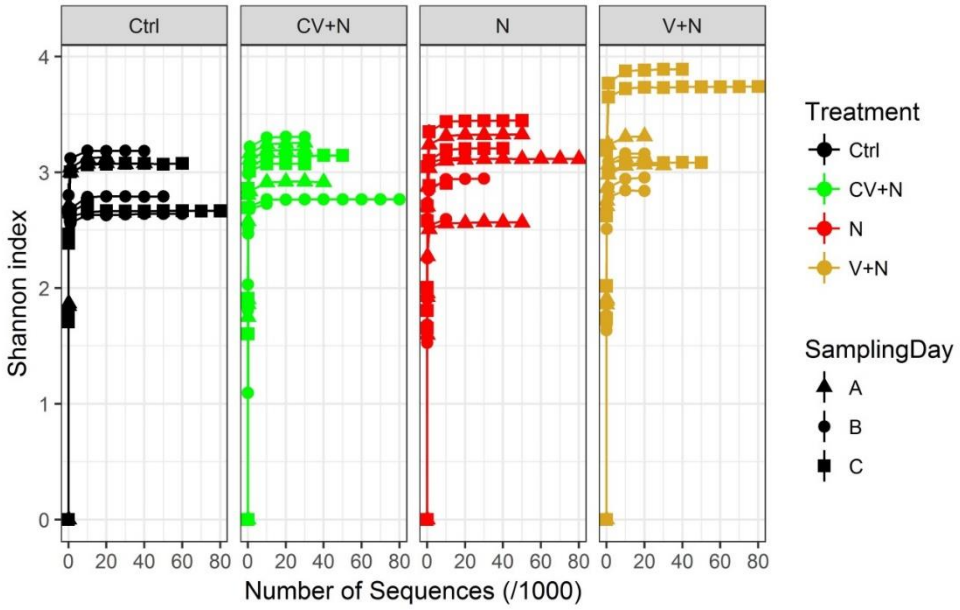

Figure S4| Rarefaction curves from the bacterial community in each treatment based on amoA sequence diversity. The treatments were: Control; N: inorganic N fertilizer; CV+N: concentrated vinasse plus inorganic N fertilizer; V+N: non-concentrated vinasse plus inorganic N fertilizer.

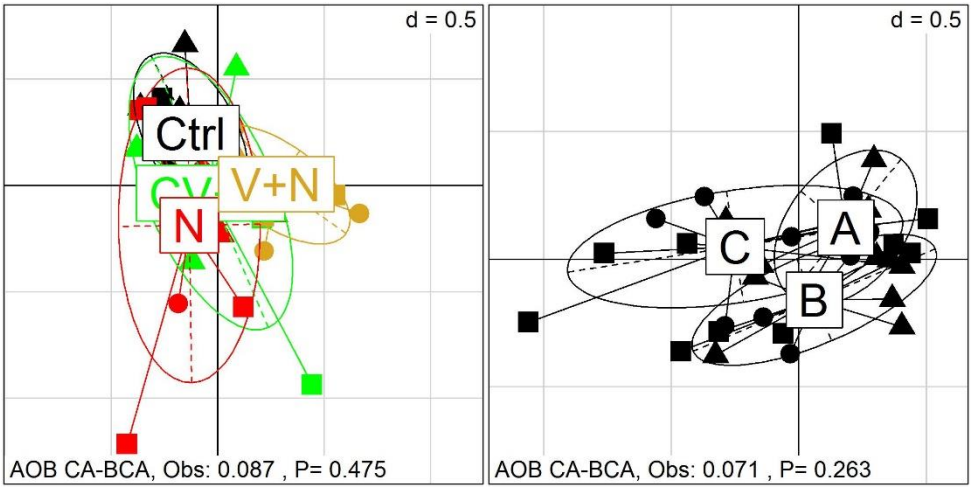

Figure S5| Between-Class Analysis (BCA) based on correspondence analysis of the abundance of OTUs from the ammonia-oxidizing bacteria (AOB) community (n=36) grouped by (A) treatment or (B) day. The treatments were: Control; N: inorganic N fertilizer; CV+N: concentrated vinasse plus inorganic N fertilizer; V+N: non-concentrated vinasse plus inorganic N fertilizer. The inertia between classes was 56.03% for treatment and 44.25%, for day and the Monte Carlo permutation level of significance was p=0.51 and p=0.27, respectively.

Chapter 6

General discussion, conclusion and future perspectives

The use of bioenergy residues, *i.e.* agricultural and industrial residues produced as by-products of the ethanol production from sugarcane, is a common farming practice in sugarcane production (Christofoletti et al., 2013; Mutton et al., 2014; Carvalho et al., 2017; Fuess et al., 2017). A set of different management practices with the use of crop residue additions has been proposed as promising management options to support sugarcane productivity, reduce soil degradation, and improve nutrient cycling in agroecosystems (Trivelin et al., 2013; Otto et al., 2016; Carvalho et al., 2017). However, it has been reported that straw (Liang et al., 2007; Zhang et al., 2013; Vargas et al., 2014) and other residues such as manure (Chadwick et al., 2011; Aita et al., 2015) and vinasse (industrial residue) (Carmo et al., 2013; Paredes et al., 2015) applied as organic fertilizers contribute to extra greenhouse gases emissions. In this thesis, we monitored the dynamics of the soil microbial community in relation to the emission of nitrous oxide (N_2O) in soils amended with different agricultural and industrial residues (sugarcane straw, concentrated vinasse - CV and non-concentrated vinasse - V). Furthermore, we determined the main N_2O producing processes in tropical sugarcane-planted soils and the microbes primarily responsible for these emissions.

The 16S rRNA gene has previously been shown to be a most valuable taxonomic marker for analysing the composition of microbial communities, including those associated with residues as straw and vinasse (Navarrete et al., 2015a; Pitombo et al., 2015). However, my thesis provides a more detailed view due to the temporal variation accessed through the capture of the microbial dynamics after the application of organic residues in the soil. Moreover, we used a shotgun metagenomics approach to obtain insight into the taxonomic and potential functional profiles of soil microorganisms (Chapter 2). We followed the changes in the soil microbial community after vinasse and inorganic fertilizer applications during the entire sugarcane crop season as well as the potential invasiveness of the vinasse-exogenous microbes (Chapter 3). The microbial genes encoding enzymes involved in N_2O production were quantified by quantitative PCR to assess the main processes responsible for these emissions (Chapter 4); and the main microbes related with N_2O production were targeted by specific-gene sequencing approach (Chapter 5). Figure 1 depicts the main research questions addressed in this thesis and summarizes the major findings.

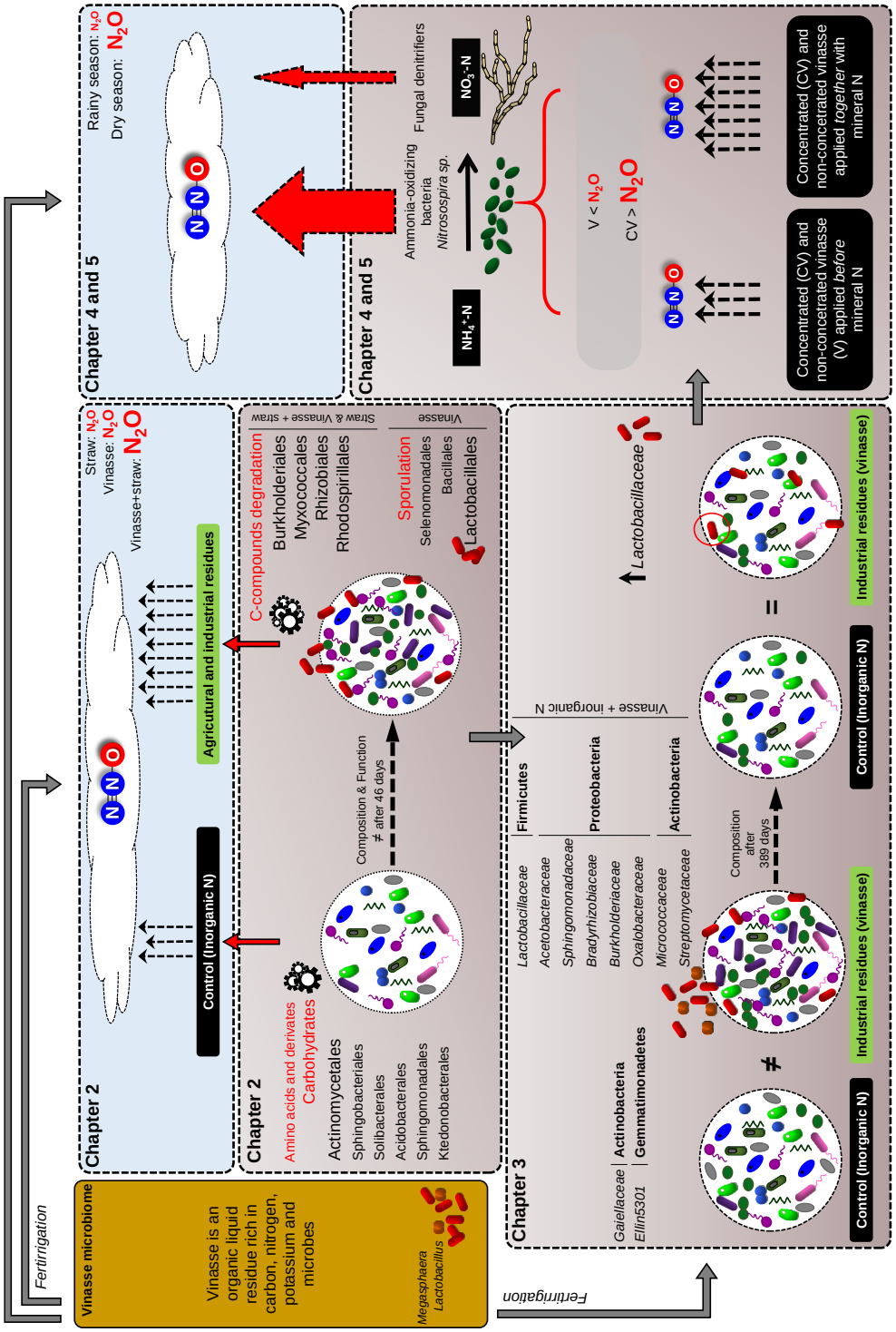

Figure 1 | Abstract of the thesis. Assessing the effect of organic and inorganic fertilizers on soil microbial community and N_2O emission.

1. *Structural and functional patterns in the soil microbiome after residues amendments*

My study showed that treatments with agricultural and industrial residues induced changes in soil microbial composition and functions compared with inorganic N fertilizer (Chapter 2). The difference in composition are related with the characteristics of each organic residue. In straw systems, for instance, the crop residue is left on the soil surface to be subject to decomposition; however, this residue is recalcitrant organic matter with high concentrations of lignin and polyphenols (Abiven et al., 2005; Barros et al., 2013; Landell et al., 2013) and it selects for specific microorganisms capable to degrade these compounds (Kumar et al., 2010; Mello et al., 2016). On the contrary, vinasse is an organic residue rich in labile organic-C, N and potassium (Rodrigues Reis and Hu, 2017). When applied to soil, vinasse increases cation exchange capacity, nutrient availability and water retention and improves soil structure (Mutton et al., 2014). In response, the abundances and activities of some members of the microbial community in the soil, particularly bacteria with a copiotrophic lifestyle increase, especially from the phylum Actinobacteria, Firmicutes and Proteobacteria. Despite the higher organic matter and nutrients input, the combined application of straw and vinasse had no drastic effect on the microbial community structure and functioning. The changes were similar to straw treatment, except for the functions related to the nitrogen cycle. This combination strongly boosted the N_2O emission. The high temperature and precipitation during the experiment may have favoured the rapid decomposition of straw on the soil surface and probably the vinasse carbon input was not as much as required to boost large extra changes in the bacterial community as one would expect about the combined addition of both residues (Devêvre and Horwáth, 2000).

No shared taxa and core metabolic functions were found for all fertilized treatments with and without organic residues amendments. Members of the phylum of Firmicutes and the functions related to ‘dormancy and sporulation’ were predominant mainly in the presence of vinasse (Chapter 2). This fact was to some extent expected since the phylum of Firmicutes increased, including the orders of *Bacillales* and *Selenomonadales* that are well known spore-forming microorganisms (Hayden et al., 2012; Sharmin et al., 2013). While orders related to decomposition and the cycling of nitrogen such as *Burkolderiales*, *Rhizobiales, Myxococcales* and *Rhodospirillales* and the functions related to ‘virulence, disease and defense’ prevailed in straw treatments, (DeAngelis et al., 2011; Orlando et al., 2012; Jones, 2015; Saarenheimo et al., 2015; Sacco et al., 2016). Furthermore, the shared taxonomic orders in the straw treatments suggest that straw is determinant for the structuring and functioning of microbial communities. As straw is characterized of having relatively large amounts of highly lignified and structural carbohydrates (cellulose, hemicellulose, and lignin) and a small amount of structural proteins (Szczerbowski et al., 2014), microorganisms containing genes

related to the metabolism of aromatic compounds were overrepresented in straw treatments as compared to the control treatment suggesting that these microbes successfully competed with other decomposers that are able to access lower recalcitrance polymers (Kielak et al., 2016b). This was confirmed by the observation of a decrease in genes related to carbohydrate metabolism in the straws treatment. Sidhu et al. (2017) evaluated the microbial interactions and metabolic potentials in pre- and post-treated sludge from a wastewater treatment plant and also found a decrease in the carbohydrate metabolism in treatments with high recalcitrance polymers.

2. Impact of multiple disturbances on the soil microbial community

Despite the absence of temporal effects in the short-term experiment (Chapter 2), the soil microbial community is not resistant to the disturbances caused by the application of vinasse, inorganic N or a combination of both but was highly resilient a shown in the long-time series experiment. In chapter 3 straw and inorganic N were applied on top of the soil in all treatments and the changes in the microbial community were followed until the end of the crop season (389 days). In addition vinasse was used as fertilizer for the first time in the experimental area. The disturbances caused by the vinasse and inorganic N applications had different effects on the soil microbial community. Application of vinasse on the same day or 30 days before N application resulted in similar effects on the soil microbial community. Apparently, application of vinasse prior to N application did not lead to substantial changes in C and/or N transformations. Parnaudeau et al. (2008) and Silva et al. (2013) found that C and N were released at a rather slow rate from vinasse. It is likely that part of the organic-C from vinasse was still present in the soil at the time of inorganic N application favouring fast-growing microbes that respond to C and inorganic N fertilizer, resulting in an increase in their relative abundance (Navarrete et al., 2015a; Suleiman et al., 2016). Furthermore, the application of vinasse changed the soil microbial community right after application. The microbial community was already different from the control at the time of inorganic N application, 30 days after vinasse. Probably the slow vinasse-C and organic N degradation plus the changes in the microbial community due to the vinasse application 30 days before inorganic N application boosted similar changes in the soil microbial community in treatments with vinasse plus inorganic N, regardless of the time of application. The variation in the composition of the soil microbial community was cyclical in all treatments. The composition of the soil microbial community was significantly diferent depending on treatments at 1.5 months after inorganic fertilizer application, but after 2.8 months the dissimilarity in composition of the communities was much smaller. The dynamics in the soil microbial community in the short-term experiment (Chapter 2) were, to some extent, similar to the dynamics in the long-time series experiment (Chapter 3), as we found largest differences among treatments in both experiments at 1.5 months

after inorganic N application. However, in the short-term experiment the sampling time was not enough to determine the capacity of the soil microbial community recovery.Thus, long-time series experiments give a better understanding of microbial communities' response to different disturbances. Therefore, it is fair to conclude that the evaluation of the impact of organic residue applications on soil microbial communities on the basis of one single time point or short-term studies may fail to show the real effect of such disturbances (Allison and Martiny, 2008; Shade et al., 2012).

Based on my results the soil microbial community is more responsive to organic and inorganic fertilizers applications than to fluctuations in seasonal temperature and rainfall (Chapter 3). The continuous seasonal variations may have resulted in a microbial community that is adapted to fluctuations in temperature and precipitation (Cregger et al., 2012; Evans and Wallenstein, 2012), thus resulting in a diminished response of the resident soil microbial community to changes in temperature and rainfall during the year. Other studies have demonstrated that when microbial communities are adapted to multiple dry-wet episodes, their response is diminished with each repeated event (Steenwerth et al., 2005; Evans and Wallenstein, 2012). In addition, the high amount of sugarcane straw (16 t ha^{-1}) on soil surface in the beginning of the experiment may have functioned as a barrier to water loss and soil temperature variation (Carvalho et al., 2017). This barrier effect may also be responsible for the small difference in the community between the dry and rainy seasons.

3. Impact of vinasse on the soil microbial community

Solely vinasse with straw, without inorganic N, affects the microbial activity and relative abundance of specific taxonomic groups in sugarcane-cultivated soils by altering soil chemical factors and introducing exogenous microbes. These effects occurred mainly up until 36 days after application to soil. Vinasse increased the abundances of *Bacillaceae, Micrococcaceae*, *Hyphomicrobiaceae* and *Nitrospiraceae* families (Chapter 3). These observations agree with other observations in field experiments (Pitombo et al., 2015) and in mesocosms (Navarrete et al., 2015a), but these studies did not show the dynamics and resilience of the soil microbial communities or the potential invasiveness of the vinasse-exogenous microbes. Members of *Bacillaceae* and Actinobacteria grow rapidly in response to available organic-C, such as found in vinasse (Pitombo et al., 2015; Mandic-Mulec et al., 2016), mainly in the first month after vinasse application. The nitrogen input from vinasse and sugarcane straw mineralization probably explains the increase in the abundances of *Hyphomicrobiaceae* and *Nitrospiraceae* (Daims, 2014; Navarrete et al., 2015a), as these organisms are depending on the availability of mineral N (Oren and Xu, 2014) and nitrite (Daims, 2014).

The microbes introduced into soil with the vinasse complex were unable to survive in the soil and disappeared after 31 days, with the exception of *Acetobacteraceae* and *Lactobacillaceae* (Chapter 3) that remained detectable in the soil. Pitombo et al. (2015) also observed an increase in the abundance of *Lactobacillaceae* in treatments with vinasse, but in their study after 14 days the relative abundance decreased and was similar to the treatments without vinasse. However, the authors could not prove that the *Lactobacillaceae* came with vinasse. So, up to now my study is the first that describes the vinasse microbiome. In the present study the resident community was resilient and returned to the original state 1 month after single vinasse application, which was earlier than in treatments with mineral N plus vinasse application. An increase in the relative abundance of *Lactobacillaceae* was observed in all treatments with vinasse during the rainy period (at days 113 and 183) that persisted in the soil even after one year. Notably, no vinasse was applied in the experimental area previously. *Lactobacillus* are generally aero-tolerant or anaerobic (Salvetti et al., 2012; Costa et al., 2015b) and are found in rich habitats with carbohydrate-containing substrates (Salvetti et al., 2012). The straw on top of the soil likely enabled *Lactobacillus* survival due to the availability of labile organic-C (straw mineralization) and higher moisture content (Leal et al., 2013; Carvalho et al., 2017).

4. Climatic conditions and N_2O emission

Surprisingly, N_2O emissions were higher in the dry season than in the rainy season (Chapter 4). As denitrification conditions are expected to occur for a longer period in the rainy season than in the dry season, leading to higher N_2O emissions. The phenology of the sugarcane plant may explain the lower N_2O emissions in all treatments in the rainy season. Sugarcane is a fast-growing plant, with high N demand during the initial stages of ratoon growth (Franco et al., 2011; Cantarella et al., 2012; Mariano et al., 2016; CONAB, 2017). If N is applied in the growing stage of the plant, plants will rapidly take up nutrients, including N, consequently reducing the available N for microbial-related processes including N_2O production. In the rainy season, fertilizers were applied at the beginning of summer, when the plants were 1.5 m high; by contrast, in the dry season, N was applied at the beginning of winter, when the plants were starting to sprout. Therefore, at the beginning of the dry season, the younger and smaller plants were not able to take up as much N, which allowed the applied N to remain longer in the soil and to be subject to microbial N_2O production processes.

5. Contribution of bioenergy residues to N_2O emissions and strategies for reduction

Bioenergy residues, *i.e.*, vinasse and straw, contributed to increase N_2O emissions. The largest emission of N_2O was observed for vinasse mixed with

straw, the N_2O emission increased to 9 times the production of N_2O (Chapter 2). Carmo et al. (2013) and Paredes et al. (2015) also observed that the application of vinasse with sugarcane straw onto the soil surface resulted in a significant increase in the emissions of N_2O. Furthermore, concentrated vinasse had 4.6 times higher N_2O emission than treatments with non-concentrated vinasse (Chapter 4). Concentrated vinasse is applied nearby the sugarcane plants, 20% of the total sugarcane field area; so, the total amount of vinasse-C in the area with inorganic N was around 2.2 times higher than treatments with non-concentrated vinasse. The higher amount of C in the fertilizered area plus inorganic N increased the N_2O production. Liang et al. (2015) found that N_2O emissions increased minimally with N additions, while without additional N, total N_2O emissions increased linearly with C additions. When both C and N were added together the largest increases in N_2O emissions occurred. So, in chapter 4 temporal strategies were used trying to control such high emissions.

The application of vinasse residue (concentrated and non-concentrated vinasse) 30 days prior to inorganic N fertilizer reduced the cumulative N_2O emissions from sugarcane fields with straw by 65% and 37% compared to the application of vinasse and inorganic N simultaneously (Chapter 4). The interval of 30 days between the application of vinasse and N fertilizer appears to be sufficient to minimize the anaerobic conditions induced by vinasse application and thereby decreasing denitrification. In addition, since vinasse is a source of N and carbon, this 30-day period permits that at least part of vinasse-carbon decomposed and vinasse-N mineralized and/or N taken up by plants (Parnaudeau et al., 2008; Silva et al., 2013), which may lead to a low N_2O emission rate as well.

In our study, I was not able to use standard vinasse with the same composition in all experiments. Although both concentrated and non-concentrated vinasse came from the same sugar mill, there was a 2.5-yr time span between the first and the last vinasse application. Vinasse cannot be stored because it rapidly deteriorates and high volumes were needed in field experiments. Vinasse composition may widely vary along the year due to its source (Elia-Neto and Nakahodo, 1995; Mutton et al., 2014). Thus, the composition of the nine vinasses used in the five application events was variable for both concentrated and non-concentrated vinasse. Although the vinasses composition could have had effects on greenhouse gases (GHG) emissions associated with the interaction of vinasse, N fertilizer, and time of application, I find it legitimate to compare the N_2O emissions and microbial community dynamics in the different experiments based on the relative effects compared to the control.

6. Microbes in control of N_2O production

My results suggest (Chapter 2 and 4) that nitrification by ammonium-oxidizers (bacteria and archaea) and denitrification by denitrifiers occur simultaneously in the soil, both resulting in the production of N_2O (Di et al., 2014;

Yang et al., 2017). The significant positive correlations between N_2O emissions and the abundances of the bacterial *nirK* and*, nirS* genes showed that the production of N_2O is due to favorable conditions for denitrification. Rain events and vinasse fertirrigation induce low oxygen concentrations in soil microsites (Di et al., 2014), consistent with the significant correlation with N_2O emissions, CO_2 emissions and water-filled pore space. In addition, vinasse is an organic residue rich in carbon with high biological oxygen demand (Fuess and Garcia, 2014). The input of labile organic compounds from vinasse in soils might greatly increase soil microbial activities, resulting in intense oxygen consumption (Renault et al., 2009); and the creation of microoxic or anoxic conditions due the high water content, resulting in anaerobic microsites (Torbert and Wood, 1992). Therefore, after vinasse application, anaerobic conditions may prevail for a short time and may cause N_2O production. However, this situation may differ fundamentally when drying of the soil within a few hours or days after the application of the vinasse may favor N_2O production by aerobic processes, *i.e.* nitrification (Soares et al., 2016). In spite of the occurrence of denitrification as indicated by the increase in denitrification related genes, nitrification by ammonia-oxidizing bacteria (AOB) and denitrification by fungi were in this study the prevalent N_2O production processes, and therefore could be useful targets for inorganic N management strategies to mitigate N_2O emissions in tropical soils (Jantalia et al., 2008; Soares et al., 2015). The amount of available organic C and the positive correlation with moisture give some indication that nitrifier denitrification by the ammonium-oxidizer bacteria could be an important pathways for the N_2O production, perhaps, even more important than denitrification by denitrifiers (Joo et al., 2005; Spott et al., 2011; Zhao et al., 2012).

In a recent study, Pitombo et al. (2015) using 16S gene amplicon sequences, found that orders as *Burkholderiales*, *Myxococcales and Lactobacillales* were mainly responsible for the N_2O production in soil*,* similar to our results with shotgun metagenomics approach (Chapter 2). Looking at the overall nitrogen metabolism, we found microorganisms related to nitrification, denitrification and nitrogen fixation to be abundantly present in the treatments with residues applications (Orlando et al., 2012; Prosser et al., 2014; Jones, 2015; Saarenheimo et al., 2015; Sacco et al., 2016), including bacteria such as *Deltaproteobacteria* (*Myxococcales*) and *Gammaproteobacteria* (*Pseudomonadales*). As the three different treatments with organic residues applications showed increased abundances of *Nitrosomonadales*, this could point to nitrification as one of the main pathways responsible for the N_2O production in sugarcane fields also in the short-term experiment (Chapter 2) (Stephen et al., 1996; Phillips et al., 2000; Prosser et al., 2014).

The application of different bioenergy residues and inorganic N increased the abundance of ammonium oxidizing bacteria (AOB) in the soil but the application did not change the AOB community composition. Mixed results having been reported in literature; some studies showed changes in AOB community

composition in response to N fertilizers (Glaser et al., 2010; Verhamme et al., 2011; Ouyang et al., 2016; Xiang et al., 2017) and other ones reported changes in AOB abundance only without a corresponding change in composition (Phillips et al., 2000; He et al., 2007). My results suggest that the application rate of N used in sugarcane fields do not lead to changes in community composition (Verhamme et al., 2011). The AOB community in these fields may have already been adapted to the straw and annual application of inorganic fertilizer since sugarcane has been cultivated in this area for more the 20 years, it is worth to remember that vinasse was never applied before in the soil (Francioli et al., 2016; Zhang et al., 2017). Remarkably, the AOB phylogenetic tree revealed that 99.5 % of the total AOB community consisted of species belonging to the *Nitrosospira* genus. The dominance of *Nitrosospira sp*. could be explained by specific conditions such as soil pH, which may have been consistent over the long period that this soil was used for sugarcane production. It has been postulated that pH may select for the presence of *Nitrosospira* group in acid soil (De Boer and Kowalchuk, 2001; Pommerening-Röser and Koops, 2005; Ma et al., 2008). Our results showed that inorganic N application decrease soil pH over time. Therefore, the continual application of inorganic fertilizers could select the *Nitrosospira* population by lowering the soil pH (Pierre, 1928; Fierer et al., 2007; Francioli et al., 2016; Zhang et al., 2017). Such a narrow range of organisms responsible for the majority of the N_2O production under these conditions provide an excellent opportunity for the development of strategies to limit the N_2O production when understanding the specific physiological and ecological characteristics of these *Nitrosospira*.

The positive correlation between total fungi and *nirK* fungi with N_2O emission in my experiments shows the importance of fungi to the N_2O emission at field condition. The role of fungi in the N_2O production is more common in soils than previously thought (Chen et al., 2014; Maeda et al., 2015). Maeda et al. (2015) investigated the N_2O-producing ability of a collection of 207 fungal isolates and concluded that N_2O production is a common and widespread trait in fungi (Shoun et al., 1992; Shoun et al., 2012; Maeda et al., 2015; Higgins et al., 2016). Many decomposer fungi, among them *Fusarium sp.*, *Trichoderma sp., Aspergillus sp*. and *Penicillium sp*., have the potential for N_2O emissions (Maeda et al., 2015; Mothapo et al., 2015; Higgins et al., 2016). By using fungal or bacterial inhibitors to distinguish the microbial origin of N_2O, previous studies have reported that fungi could contribute up to 18% of potential denitrification (Herold et al., 2012). The high amount of sugarcane straw, such as used in my experiments, with high C:N ratio (77:1), might have triggered fungal activity and associated fungal N_2O production (Allison and Killham, 1988). Wu et al. (2017) observed that N_2O production in soil with wheat straw were initially dominated by bacterial processes, in particular denitrification but later mainly resulted from fungal denitrification. Despite the importance of fungi in several soil functions, the production of N_2O by fungi has only been evaluated in a limited number of studies (Long et al., 2015; Maeda et al., 2015; Higgins et al., 2016). The lack of appropriated tools to determine the fungi

contribution to N_2O production is one of the main problems (Shoun et al., 2012; Long et al., 2015; Mothapo et al., 2015; Wei et al., 2015; Higgins et al., 2016).

7. *Outlook and future perspectives*

The research described here may be of importance for the development of sustainable ethanol production strategies from sugarcane by providing tools to reduce the GHG's emission contributing to global warming. Brazil is the biggest producer of sugarcane in the world and has the highest ethanol production after United States of America (Walter et al., 2011). On December 2015 during United Nations Climate Change Conference in Paris (COP 21) 196 countries, including Brazil, agreed by consensus to reduce their carbon output and to do their best to keep global warming below 2º C (Brazil, 2015). The government of Brazil committed to decrease the total amount of GHG emitted by 43% in 2030. There are different public policies to achieve this goal and the most important one is related with the increment of ethanol production from sugarcane; the initial plan is to almost double the production of ethanol, from 26 billion to 50 billion liters per year. In 2016, a Federal government program was built, RenovaBio (Brazil, 2016), with the objective to expand the production of biofuels and to increase the contribution of bioethanol from sugarcane in the Brazilian energy matrix from 6% to 18%. However, this implies that the sugar mills must adjust their production and residue management processes in order to provide a more sustainable biofuel production process. The plan is to create a decarbonisation credit. Therefore, the sugar mills need to reduce the GHG's emission across the ethanol production process, including the management of sugarcane production. In fact, N fertilization is the bottleneck in the overall ethanol production process; high N_2O emission during the sugar cane growing phase may deny the benefits of ethanol production (Crutzen et al., 2008; Lisboa et al., 2011). So, the assessment of the impact of organic and inorganic fertilization during sugarcane crop production on the N_2O production process in soil is of key importance.

In the present study, smaller N_2O emissions from the conventional fertilizer were found than in most results reported in literature for sugarcane (Lisboa et al., 2011; Carmo et al., 2013; Pitombo et al., 2015; Soares et al., 2016), and lower than the values used by the Intergovernmental Panel on Climate Change (1 %) (Jantalia et al., 2008; IPCC, 2013; Morais et al., 2013). Despite the low N_2O emissions from vinasses plus inorganic N treatments in most of the seasons, I demonstrated that N_2O emissions increased with N fertilizer and vinasses application, especially with concentrated vinasse. The cumulative emissions from concentrated vinasse plus inorganic N were 19 and 7 times (rainy and dry season respectively) higher than from inorganic N fertilizer only. The strategy to reduce N_2O emissions using a time gap between vinasse and N application of around 30 days may have a positive effect on the N_2O production. Another option to reduce the N_2O emission which was not tested here is the application of concentrated

vinasse and N fertilizer in opposite bands of the sugarcane line. However, sugarcane mills need to reduce operational costs as well, including the reduction of traffic of machines especially during vinasse and inorganic fertilizer application (Christofoletti et al., 2013; Fuess and Garcia, 2014). Therefore, there is a tendency to concentrate vinasse in the mills and more recently, the sugarcane industry proposed the use of a mixture of concentrated vinasse and different sources of inorganic fertilizers. Vinasses contain sufficient amounts of K to meet the demand of sugarcane (Carvalho et al., 2014; Dametie et al., 2014). With the addition of N and perhaps phosphorus and micronutrients to the concentrated vinasse, a complete and sufficient nutrient supply for the full growth of sugarcane may be formed. However, the low N_2O emission found when N fertilizers only are applied in sugarcane field (Paredes et al., 2014; Paredes et al., 2015; Soares et al., 2015; Siqueira Neto et al., 2016) would probably be reverted because of the increment in the N_2O emissions which I showed to be expected when both vinasse and fertilizer were applied together.

The results found in the chapter 4 showed that nitrification by AOB and denitrification by fungi are the main processes responsible for the N_2O production in soil after vinasse and inorganic N application. Therefore, there is the possibility to reduce the N_2O emission with the use of nitrification inhibitors (Soares et al., 2015; Soares et al., 2016). A strong reduction of up to 94 % in N_2O emissions by the addition of nitrification inhibitors (DMPP and DCD) to inorganic N fertilizers, however without vinasse application, were found by Soares et al. (2015 and 2016) in three consecutive seasons. Another option to reduce the N_2O emission would be the removal of part of straw from the sugarcane field (Vargas et al., 2014) which can be used as a valuable feedstock for second-generation ethanol production and bioelectricity cogeneration (Carvalho et al., 2017; Menandro et al., 2017).

Based on my results, *Lactobacillus* and *Megasphaera* from the *Lactobacillaceae* and *Veillonellaceae* families, respectively, are the main contaminants present in vinasse. *Lactobacillaceae* appears to have the ability to survive in the soil and are detectable even one year after application and, surprisingly, they increased their abundance at the end of the cropping season. Notably, no vinasse was applied in the experimental area previously. The survivability of the *Lactobacillaceae* was rather unexpected, as *Lactobacillus* is found in rich habitats with carbohydrate-containing substrates (Salvetti et al., 2012). Based on the functionality analyses the microbes present in vinasse encode genes for denitrification, mainly *nirK* (Figure 2). The question is if they contribute significantly to the N_2O production? Thus, it is advisable to investigate the persistence of the vinasse microbiome in soil after vinasse applications and the contribution to the overall N_2O emissions of the denitrification potential of the vinasse-inhabiting microbial community.

The results described in this thesis can be used as a reference and input tool to define and develop sustainable management practices for the ethanol production from sugarcane. This thesis provides important information to improve

our understanding of the negative sides of the recycling of bioenergy residues (vinasse and straw) as fertilizers. In addition, we also investigated strategies to minimize these problems, such as the application of vinasse prior to inorganic fertilization. The aforementioned results also emphasize the need for further long-term studies, i.e., over one sugarcane crop season, to better identify and quantify the environmental impacts associated with the reuse of organic fertilizers. Simultaneously, the development of several other strategies to reduce the N_2O load of vinasse is required in an effort to combine the environmental adequacy of the recycling process with the recovery of nutrients by plants (Fuess et al., 2017). One last item should be mentioned in terms of the calculation of the acceptable rates of vinasse application to soils. In Brazil, only the contents of potassium in vinasse and the soil are the parameters considered (CETESB, 2014). The amounts of other compounds, such as organic matter, nitrogen and vinasse-exogenous bacteria are not considered. The criteria for the disposal of sugarcane vinasse via fertirrigation should be defined at a more holistic perspective, considering at least the content of organic matter, which may trigger the most negative effects, as discussed in detail in this thesis.

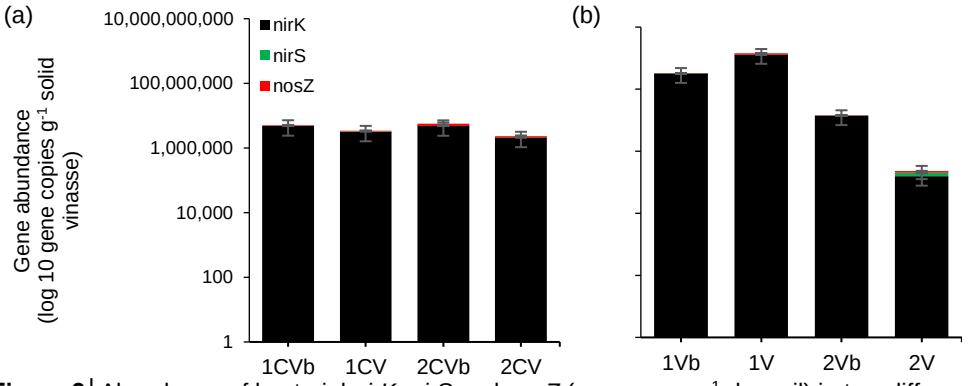

Figure 2 | Abundance of bacterial *nirK*, *nirS* and *nosZ* (gene copy g^{-1} dry soil) in two different vinasses, concentrated (a) and non-concentrated vinasse (b) in the rainy (1) and dry seasons (2).

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Summary

Recycling crop and industrial residues is a sustainable agricultural management practice, which also helps to improve soil structure and to increase the stock of nutrients. However, the addition of residues to agricultural fields causes disturbances to the soil ecosystem and to the soil microbial community in general. Until now, information about the magnitude and duration of these disturbances is scarce. Vinasse is a major by-product generated by the sugarcane biofuel industry. It is a source of microbes, nutrients and organic matter and often it is recycled as fertilizer. There is evidence that the application of vinasse together with mineral nitrogen (N) fertilizers in sugarcane fields affect the composition, functions and dynamics of the soil microbiome, thereby enhancing the emission of nitrous oxide (N_2O). However, it is still poorly understood how vinasse (and straw) affect the dynamics of the soil microbiome and the mechanisms that control the high N_2O emissions.

The research described in this thesis firstly addressed how organic residues - vinasse and sugarcane straw - added together with N fertilizer affect the soil microbial community structure and function and N_2O emission in a short-term experiment (Chapter 2). Vinasse and straw, both induced changes in the soil microbial community composition and potential functions, but straw additions triggered the stronger changes in particular related to functions involved in decomposition of different C-compounds. Functions related to spore-producing microorganisms were overrepresented in the vinasse treatment, which could be related with the presence of vinasse-inhabiting microorganisms and their survival in the soil. All additional residues increased the abundance of microorganisms related to nitrogen metabolism and N_2O emissions. However, treatments with vinasse plus straw applications showed highest N_2O emissions.

To investigate the capacity of the soil microbiome to recover from the impact of vinasse, or the potential invasion of the vinasse-inhabiting microorganisms, total microbial community analyses were performed during an entire sugarcane season (389 days) (Chapter 3). Vinasse, N fertilizer or a combination of both were applied in sugarcane plantations in which the soil was covered with straw, a common practice. Vinasse caused significant changes in the resident soil microbial community. However, these changes were restricted to a short period as the resident microbial community was able to recover. The invasive bacteria present in the vinasse were unable to survive in the soil and disappeared after one month, except of members of the Lactobacillaceae family that persisted in the soil even after one year. This study is the first to show the persistence of vinasse-inhabiting bacteria in soil, and further studies elucidating the ecological functions of these invaders in soil are urgently needed. Despite this, the resident soil microbial community was highly resilient to vinasse and N fertilizer application.

The higher abundance of bacteria of the order of Nitrosomodales in the treatments with organic residues was evidence that nitrification was one of the main pathways responsible for the N_2O production (Chapter 2). Therefore, I investigated the role of nitrification and denitrification in the N_2O production in straw-covered soils amended with concentrated and non-concentrated vinasses before or at the same time as N fertilizer at different time points of the sugarcane cycle in two seasons (Chapter 4). Independent of the (rainy or dry) season, the microbial processes involved in N_2O production were nitrification by ammonia-oxidizing bacteria (AOB) and archaea and denitrification by bacteria and fungi. The contribution of each process differed and depended on soil moisture, soil pH, and N sources. However, *amo*A-AOB and fungal *nir*K were the most important genes

related to N_2O emissions overall, which indicates that nitrification by AOB and denitrification by fungi are likely to be the main microbial-driven processes linked to N_2O production in tropical soil receiving straw and vinasse applications. Despite the increment in the AOB abundance in the soils receiving vinasses and N fertilizer, the diversity and the community structure of AOB did not change and was dominated by *Nitrosospira sp.* (Chapter 5). In addition, the application of vinasse 30 days prior to N fertilizer reduced N_2O emissions by 37-65%.

In conclusion, the research presented in this thesis showed for the first time the successional changes in the soil microbial community composition and functions after vinasse, straw and N fertilizer applications as well as the links of the dynamics of the soil microbiome with N_2O emissions. Also, it was the first time that the invasion potential of vinasse-inhabiting microbes was determined. A practical result of this research is that vinasse application 30 days before N fertilizer applications reduced the N_2O emissions. These results highlight the importance and limitations of recycling crop residues and fertilizer management and can be used as a reference and a practical tool to develop good management practices during organic fertilization as part of sustainable sugarcane production systems.

Samenvatting

Het recyclen van gewasresten en industriële afval is een duurzame landbouw praktijk, die ook helpt om de bodemstructuur en de nutriënten voorraad te verbeteren. Echter het opbrengen van deze resten in landbouwgrond veroorzaakt ook verstoringen van het bodemecosysteem en de microbiële gemeenschap in het bijzonder. Tot nu toe is de informatie over de mate en de duur van deze verstoringen beperkt. Vinasse is een belangrijk bijproduct van de suikerriet-biobrandstof industrie. Het is een bron van micro-organismen, nutriënten en organische stof en wordt vaak gebruikt als meststof. Er zijn aanwijzingen dat de toepassing van vinasse samen met minerale stikstof (N) in de teelt van suikerriet de samenstelling, functionaliteit en dynamiek van het bodemmicrobioom beïnvloedt, waardoor de emissie van di-stikstof oxide(N_2O) wordt versterkt. Het is echter niet duidelijk hoe vinasse (en stro) de dynamiek van het bodemmicrobioom en de mechanismen die ten grondslag liggen aan de hoge N_2O emissies beïnvloedt.

Het onderzoek dat in dit proefschrift wordt beschreven is in de eerste plaats gericht op de vraag hoe organische resten- vinasse en suikerriet stro- tezamen met minerale N de structuur en functionaliteit van de microbiële gemeenschap in de bodem en de emissie van N_2O beinvloeden in een korte termijn experiment (Hoofdstuk 2) Vinasse en stro induceren beide veranderingen in de samenstelling en de functionaliteit van de microbiele gemeenschap in de bodem , waarbij stro toevoegingen leiden tot grotere veranderingen, in het bijzonder in functies die te maken hebben met de decompositie van verschillende C-componenten. Functies die te maken hebben met sporevormende organismen kwamen meer voor in de vinasse behandeling, wat mogelijk het gevolg is van de aanwezigheid van micro-organismen, die in vinasse voorkomen en hun overleving in de bodem. Alle toegevoegde residuen verhoogden de hoeveelheid micro-organismen die betrokken zijn bij de N-cyclus en N_2O emissies. De hoogste N_2O emissies werden gevonden in de behandelingen waarin vinassee en stro tezamen werden toegevoegd.

Om de capaciteit van het bodem microbioom om te herstellen van het effect van vinasse en de mogelijke invasie van micro-organismen die in vinasse voorkomen te onderzoeken, heb ik gedurende een volledig suikerriet groeiseizoen (389 dagen) een totale microbiële gemeenschapsanalyse uitgevoerd in een bodem die bedekt was met stro, wat een normale praktijk is (Hoofdstuk 3). Vinasse veroorzaakte significante veranderingen in de microbiële gemeenschap van de bodem. Echter, deze veranderingen duurden slechts kort omdat de microbiële gemeenschap in staat bleek te zijn om zich ervan te herstellen. De invasieve bacteriën die aanwezig waren in de vinasse waren niet in staat om in de bodem te overleven en verdwenen binnen een maand, behalve bacteriën van de familie van de *Lactobacillaceae* die zelfs na een jaar nog aanwezig waren. Dit onderzoek is het eerste dat de persistentie van bacteriën die met vinasse in de bodem terecht komen, en het is van het uiterste belang om de ecologische karakteristieken van deze bacteriën in de bodem nader te onderzoeken. Ondanks de eerdergenoemde resultaten bleek het van nature aanwezige bodem microbioom in sterke mate resistent te zijn tegen de toediening van vinasse en N-kunstmest.

De grote hoeveelheid aan bacteriën van de order van de *Nitrosomodales* in de behandeling en met organische resten was een bewijs voor de veronderstelling dat nitrificatie één van de belangrijkste mechanismen voor de N_2O productie was (Hoofdstuk 2). Daarom onderzocht ik op verschillende momenten tijdens de groei van suikerriet in twee seizoenen de rol van nitrificatie en

denitrificatie in de N_2O productie in met stro bedekte bodems waaraan geconcentreerde en niet-geconcentreerde vinasse was toegevoegd voor of tegelijkertijd met N-kunstmest (Hoofdstuk 4). Onafhankelijk van het seizoen (regen- of droog), waren de microbiële processen betrokken bij de N_2O productie nitrificatie door ammonia-oxiderende bacteriën (AOB) en archaea en denitrificatie door bacteriën en schimmels. De bijdrage van elk van de processen verschilde en was afhankelijk van bodem vochtgehalte, pH, en N-bronnen. Echter, *amoA*-AOB en schimmel *nirK* waren de meest belangrijke genen gerelateerd aan de overall N_2O emissie, wat aangeeft dat nitrificatie door AOB en denitrificatie door schimmels waarschijnlijk de meest belangrijke microbiële processen zijn die betrokken zijn bij de N_2O productie in tropische bodems waaraan stro en vinasse zijn toegevoegd. Ondanks de toename in de AOB hoeveelheid in bodems waaraan vinasse en N-kunstmest zijn toegevoegd, veranderden de diversiteit en gemeenschapsstructuur van AOB niet en werd gedomineerd door *Nitrosospira* soorten (Hoofdstuk 5). Bovendien, de toevoeging van vinasse 30 dagen voor de toediening van N-kunstmest reduceerde de N_2O emissie met 37-65%.

Concluderend laat het onderzoek dat in dit proefschrift beschreven is voor de eerste keer de successievelijke veranderingen in de samenstelling en functionaliteit van de microbiële bodemgemeenschap zien na toevoeging van vinasse, stro en N-kunstmest alsmede de link tussen de dynamiek van het bodemmicrobioom en de emissie van N_2O emissies. Ook is voor de eerste keer het invasieve potentieel van in vinasse voorkomende micro-organismen bepaald. Een praktisch resultaat van dit onderzoek is dat de toevoeging van vinasse 30 dagen voor de toevoeging van N-kunstmest de N_2O emissie reduceerde. Deze resultaten benadrukken het belang en de beperkingen van het recyclen van gewasresten en adequaat mestmanagement en kunnen gebruikt worden als referentie en hulpmiddel voor het ontwikkelen van ‘good management practices’ voor organische bemesting als onderdeel van duurzame suikerriet productiesystemen.

Curriculum Vitae

Publications

Késia Silva Lourenço was born on 23th March 1988 in Ponte Alta (Santa Catarina), Brazil. In 2010, she completed her BSc degree in Agronomic Engineering at the University of Santa Catarina (UDESC), Brazil. During her bachelor, she worked on soil fertility and plant nutrition. After that, she continued her education. In 2011, she started her MSc studies in soil management at the University of Santa Catarina, Brazil. During her MSc thesis at the group of Chemical and Soil Fertility, she studied the reactions of N in the soil after application of organic and inorganic fertilizers in the presence or absence of urease inhibitors under the supervision of Prof. dr. Paulo Roberto Ernani. For this work, she received a grant from "Coordination for the Improvement of Higher Education Personnel (CAPES). In 2013, she started her PhD project in collaboration with Agronomic Institute of Campinas (IAC) and Netherlands Institute of Ecology (NIOO/KNAW) under the supervision of Prof. dr. J.A. van Veen, Dr. E. Kuramae and Dr. Heitor Cantarella. In 2013, she moved to Campinas, Brazil and started her PhD research under the supervision of Dr Heitor Cantarella (IAC), where she did the experimental work. In 2015 she moved to the Netherlands and continued her PhD under supervision of Dr. Eiko E. Kuramae (NIOO-KNAW) and Prof. dr. J.A. van Veen (NIOO-KNAW and Leiden University). The findings of her PhD research are described in this thesis.

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List of publications

Publications unrelated to this thesis:

Lourenço, K.S., Corrêa, J.C., Ernani, P.R., Lopes, L.D.S., and Nicoloso, R.D.S. (2013). Nutrient uptake and yield of common bean fertilized with poultry litters and mineral nutrients. *Brazilian Journal of Soil Science* 37, 462-471. doi:10.1590/s0100-06832013000200017

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Lourenço, K.S., Soares, J.R., Menegale, P.L.C., Gonzaga, L.C. and Cantarella, H. Nitrification inhibitor mitigates N_2O emission from organic residue combined with N fertilizer. (to be submitted).

Publications related to this thesis:

Lourenço, K.S.[*], Suleiman, A.K.A.[*], Pitombo, L.M., Mendes, L.W., Roesch, L.F.W., Pijl, A., Carmo, J.B., Cantarella, H., and Kuramae, E.E. (2018). Recycling organic residues in agriculture impacts soil-borne microbial community structure, function and N_2O emissions. *Science of The Total Environment* 631-632**,** 1089-1099. doi:10.1016/j.scitotenv.2018.03.116

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Lourenço, K.S., Cassman, N.A., Pijl, A., van Veen, J.A., Cantarella, H., Kuramae, E.E. (2018). *Nitrosospira sp.* govern nitrous oxide production in a tropical soil amended with residues of bioenergy crop. (Chapter 5, submitted).

The reaserch described in this thesis was performed in the Paulista Agency for Agribusiness Technology, Polo Regional Centro Sul (APTA), Laboratory of chemical and soil fertility of Agronomic Institute of Campinas (IAC), and the Department of Microbial Ecology at the Netherlands Institute of Ecology (NIOO/KNAW). This research was financially supported by the São Paulo Research Foundation (FAPESP) and The Netherlands Organization for Scientific Research (NWO).

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Cover: Picture of experiment field area with sugarcane in Brazil.
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