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.

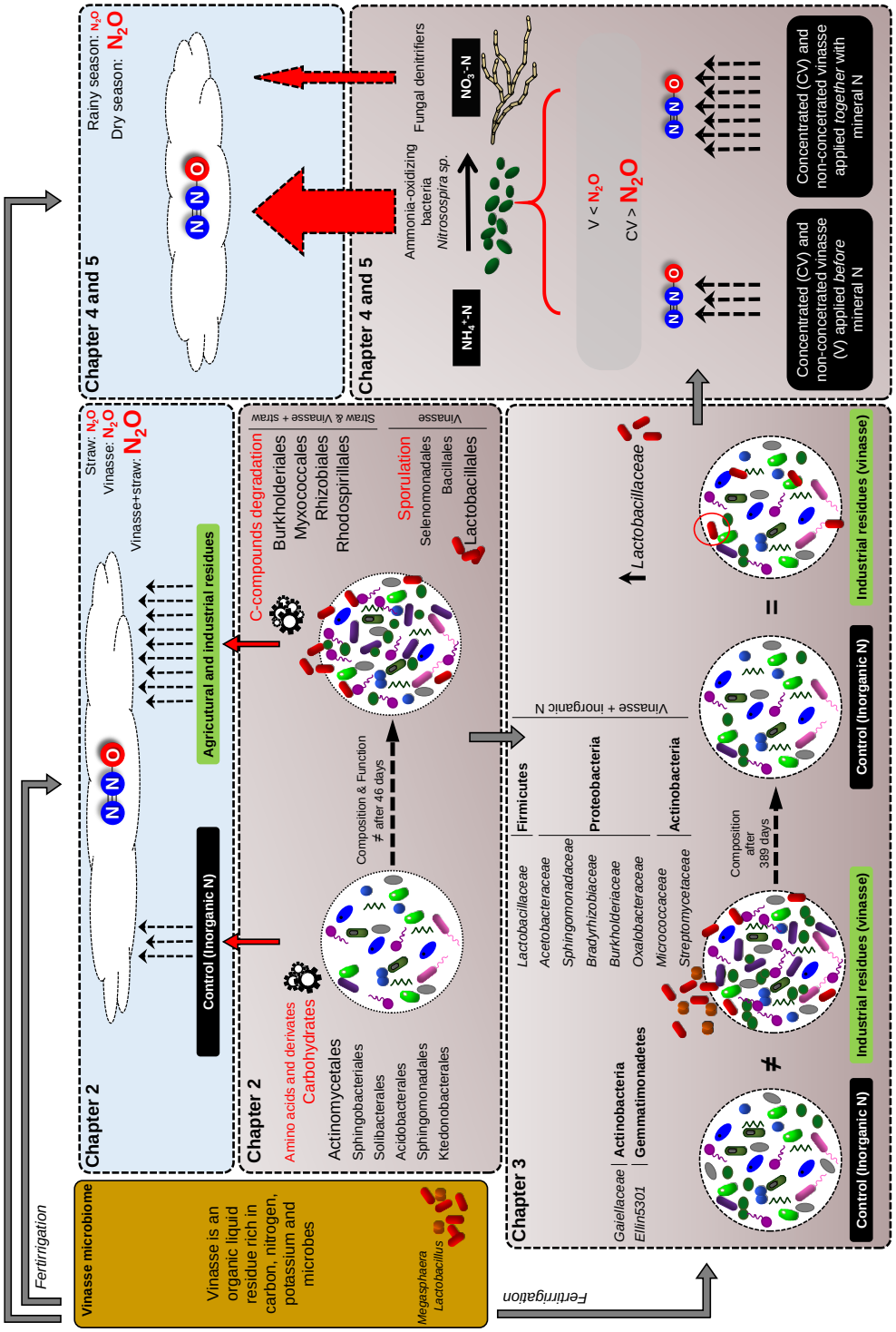

Figure 1 | Abstract of the thesis. Assessing the effect of organic and inorganic fertilizers on soil microbial community and N_2O emission.

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.

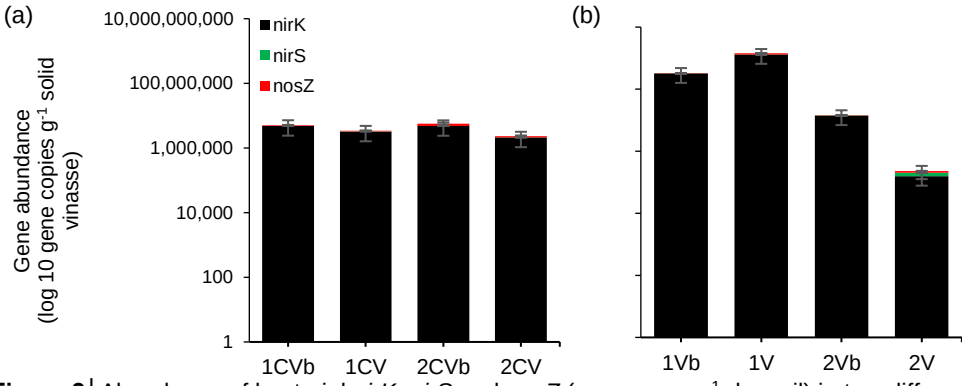

Figure 2 | Abundance of bacterial *nirK*, *nirS* and *nosZ* (gene copy g^{-1} dry soil) in two different vinasses, concentrated (a) and non-concentrated vinasse (b) in the rainy (1) and dry seasons (2).