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IMPACTO DA ADIÇÃO DE RESÍDUOS ORGÂNICOS NA COMUNIDADE MICROBIANA DO SOLO E NA EMISSÃO DE N_2O

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciência do Solo, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Ciência do Solo**

Orientador: Prof. Dr[a]. Zaida Inês Antoniolli

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RESUMO

IMPACTO DA ADIÇÃO DE RESÍDUOS ORGÂNICOS NA COMUNIDADE MICROBIANA DO SOLO E NA EMISSÃO DE N_2O

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Terras agrícolas tem recebido resíduos de culturas e de animais como opção de fornecimento de nutrientes adicionais em substituição aos fertilizantes inorgânicos. Apesar da ideia do retorno de resíduos produzidos na agricultura ao solo ser sustentável, sua aplicação continua contribuindo para produção de gases de efeito estufa como o óxido nitroso (N_2O). Entretanto, o impacto dos distúrbios provocados pelo uso de resíduos agrícolas sobre a comunidade microbiana do solo ainda não são claros. O objetivo deste trabalho foi estudar o impacto do retorno de resíduos produzidos na agricultura como dejetos, vinhaça e palha sobre a comunidade bacteriana do solo acessada por sequenciamento de nova geração de ácidos nucleicos. Foram realizados dois experimentos de campo a curto prazo foram realizados com o primeiro experimento apresentando os tratamentos: controle, fertilizante mineral, dejeto e dejeto com inibidor de nitrificação dicianodiamida (DCD), enquanto o segundo experimento apresentou os seguintes tratamentos: controle, palha de cana-de-açúcar, vinhaça e vinhaça em conjunto com palha de cana-de-açúcar. O efeito das emissões de óxido nitroso também foram analisadas. A adição de resíduos orgânicos apresentou o maior impacto na mudança da comunidade bacteriana do solo e afetaram a presença de microrganismos conforme o resíduo aplicado quando em comparaçao com solos com solos com ou sem fertilizantes, mas sem a aplicaçao de resíduos. A aplicação de dejetos animais afetou a comunidade ao terceiro dia do experimento principalmente devido a um aumento na abundância de *Bacteroidetes*, *Proteobacteria* e *Firmicutes*, mas a comunidade voltou ao seu estado inicial após 50 dias de experimento. O impacto da aplicação de resíduos vegetais vinhaça e palha foi visualizada com a aplicação da palha modificando a abundância de *Bacteroidetes* e *Beta-Proteobacteria*. A adição de vinhaça provocou um aumento nas proporções de *Verrucomicrobia* enquanto *Firmicutes* foram mais abundantes no tratamento com adição de vinhaça em conjunto com a palha nos 46 dias do experimento. Entre as funções afetadas pela adição de resíduos de origem animal e vegetal, a aplicação de dejetos com e sem DCD, assim como a aplicação de palha e vinhaça, atuaram sobre grupos específicos de microrganismos do ciclo do nirogênio. Além disso, ciclos de fósforo, ferro e nitrogênio foram observados em diferentes tratamentos de resíduos vegetais. Diferentes microrganismos foram responsáveis pelas mesmas funções nos ciclos biogeoquímicos em distintos tratamentos com resíduos vegetais indicando possível redundância funcional. A aplicação de todos os resíduos também contribuiu para o aumento da emissão de N_2O, com exceção do tratamento com adição de DCD em que mostrou que o inibidor foi efetivo em retardar o processo de nitrificação. Os resultados são importantes para entender o gerenciamento dos resíduos das culturas nas comunidades e funções microbianas do solo sob o esforço atual de práticas agrícolas sustentáveis.

Palavras-chave: Dejetos. Vinhaça. Palha. Gases de efeito estufa. Ecologia microbiana.

ABSTRACT

IMPACT OF ORGANIC RESIDUES ADDITION IN SOIL MICROBIAL COMMUNITY AND N_2O EMISSIONS

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Agricultural lands receive crop and animal residues as option for additional nutrients to replace inorganic fertilizers. Although the idea of discard residues is sustainable, its implementation contributes to the production of greenhouse gases such as nitrous oxide (N_2O). However, the impact of disturbances caused by crop wastes on soil microbial community is still not clear. The aim was to study the impact of agricultural residues on the bacterial community accessed by next generation sequencing of nucleics acids. Two field experiments were carried out with the first experiment with the treatments control, mineral fertilizer, slurry and slurry with nitrification inhibitor dicyandiamide (DCD) while the second experiment presented the following treatments: control, sugarcane straw, vinasse and vinasse with sugarcane straw. Nitrous oxide emissions were also analysed. The organic fertilizers were the main drivers on changes in microbial community structure and they affected the microorganisms differently conformable to the applied residue compared with soils with or without fertilizer, but without residues. Slurry application changed the community in the third day of experiment temporarily due to increases in the abundance of *Bacteroidetes, Proteobacteria* and *Firmicutes* but the metabolically active microbial community was resilient returning to the original state after 50 days of experiment. The impact of plant residues were visualized in the treatment than microbial dynamics with only straw application modifying the abundance of *Bacteroidetes* and *Beta-Proteobacteria*. High proportions of *Verrucomicrobia* were found in vinasse treatment, whereas *Firmicutes* were overrepresented in vinasse plus straw treatment. Plants and animal origin residues as slurry with and without DCD, straw and vinasse affected specific groups of microorganisms that participate in nitrogen cycle. Furthermore, phosphorus, iron and nitrogen cycles were altered in plants residues treatments. Different microorganisms were responsible for the same functions in biogeochemical cycles in different treatments with plant residues indicating possible functional redundancy. All agricultural residues amendments also contributed to increase N_2O emissions, except for the treatment with DCD which was effective against the nitrification process. In conclusion, the results are important to understand the appropriate crop residues managements in microbial compositions and functions under the current effort of sustainable agricultural practices.

Keywords: Manure. Vinasse. Straw. Green house gases. Microbial Ecology.

SUMÁRIO

1 INTRODUÇÃO GERAL

A agricultura moderna é dependente das entradas regulares de fertilizantes minerais e essa tendência provavelmente continuará nas próximas décadas. Esse distúrbio de pequena escala para o solo provocado pelo adição de nutrientes é uma prática agrícola comum, que visa melhorar a nutrição das plantas a alcançar alto rendimento, contudo pode alterar as propriedades físicas, químicas e biológicas do solo (Nemergut et al., 2008). Com o objetivo de aumentar a sustentabilidade na agricultura, a estratégia de utilização de resíduos orgânicos e de culturas vem se expandindo a fim de proporcionar um fornecimento de nutrientes mais equilibrado, e a liberação de nutrientes de forma reaproveitável, quando comparado com fertilizantes sintéticos (Dittmar et al., 2000, Chen, 2006).

No passado, como os resíduos eram retirados no campo, a aplicação de fertilizantes com fonte de N era elevada para aumentar o rendimento das culturas. Nas últimas décadas, a prática de incorporação dos resíduos de colheitas nos solos como o retorno da palha como cobertura dos campos favorece a conservação do solo aumentando assim a sua resistência ao vento, evitando a erosão pela água (Glab e Kulig, 2008) além de ser uma opção vantajosa e favorável economicamente para descartar e reciclar resíduos nas propriedade rurais. No entanto, a pallha contém de 30-50% de celulose com alta polimerização e alta relação C:N fazendo com que se decomponha lentamente no campo (Wang e Bakken, 1997). Por isso, resíduos orgânicos como dejetos ou vinhaça, por exemplo, são geralmente aplicados, dependendo da região do Brasil ou do mundo, em conjunto com a palha para acelerar o seu processo de decomposição e como forma de descarte.

O cultivo da cana-de açúcar para a produção de álcool resulta na geração de vinhaça. Este resíduo líquido resultante como subproduto oriundo da indústria do açúcar e etanol é composto por água, orgânicos sólidos e minerais sendo que para cada litro de álcool produzido, são gerados de dez a dezoito litros de vinhaça (Silva et al., 2007, Laime et al., 2011). Considerando a expansão da produção de etanol no Brasil, necessitam-se métodos alternativos para tratar ou re-utilizar a vinhaça como sua aplicação no solo em campos agrícolas, um processo chamado de fertirrigação. A utilização da vinhaça como fertilizante natural resolveu os problemas do descarte da substância indevidamente e ainda se reaproveitam minerais e resíduos orgânicos, evitando a necessidade de utilização de adubos para reposição de tais elementos. Outra forma de acelerar a decomposição da palha é através da adição de dejetos animais (compostos principalmente pela mistura de fezes, urina e água) como fertilizante, porque este contém uma série de elementos químicos prontamente

disponíveis, ou após o processo de mineralização estarão disponíveis e poderão ser absorvidos pelas plantas (Bhandral et al., 2009). Com a valorização da pecuária, a população mundial de animais aumentou, consequentemente, elevando a produção de dejetos (Oenema et al., 2005, Steinfeld et al., 2006). O Brasil, por exemplo, produz mais do que 38 milhões de suínos anualmente, o quarto maior produtor mundial com 300 milhões de litros de dejeto suíno por dia (Balota et al., 2014). No entanto, a eficiência do uso de N desse resíduo orgânico produzido pelos animais é pobre já que apenas 20-50% do N aplicado é recuperado por culturas (Van der Hoek et al., 1998, Oenema et al., 2005, Ma et al., 2010).

Apesar da aplicação desses resíduos ser considerada mais plausível ambientalmente, a aplicação desses resíduos agrícolas assim como a adição de fertilizantes sintéticos continua contribuindo para a poluição ambiental devido ao excesso de nutrientes introduzidos no solo. A aplicação de resíduos orgânicos pode aumentar substancialmente a produção de gases de efeito estufa a partir de solo como o óxido nitroso (N_2O), com potencial de aquecimento 298 vezes maior do que o CO_2 em um período de 100 anos (IPCC, 2007). Esse gás ainda está envolvido na depleção da camada estratoférica de ozônio (IPCC, 2013) e estimativas apontam que fontes agrícolas respondem por 84% das emissões globais antropogênicas de N_2O (Scheehle e Krueger, 2006).

Com essas práticas, o solo está constantemente sob pressão ambiental que altera sua capacidade de executar serviços essencias do ecossistema. Isso porque a decomposição e a reciclagem da matéria orgânica é, em grande parte realizada por microrganismos do solo capazes de converter os componentes orgânicos em nutrientes disponíveis para as plantas (Steinberger e Shore, 2009). Para manter essas atividades essenciais, é importante conhecer como os microrganismos respondem aos distúrbios ou mudanças ambientais. A comunidade sujeita a um distúrbio pode reagir de diferentes formas podendo permanecer intacta e resistir ao distúrbio, ser modificada e após esse fato retornar ao seu estado inicial ou atingir um outro estado estável com comunidade microbiana diferente (Allison e Martiny, 2008).

Em estudos anteriores, verificou-se que as práticas agrícolas afetam a estrutura da comunidade bacteriana pelo fornecimento adicional de carbono derivado de resíduos (Navarro-Noya et al., 2013; Calderon et al., 2016). O efeito da palha na comunidade microbiana geralmente é acessado através da comparação de práticas conservasionistas com convencionais. Ambas podem alterar as comunidades bacterianas favorecendo grupos microbianos específicos, sendo que a primeira prática favorece organismos oligotróficos (Ramírez-Villanueva et al., 2015). Embora seja claro que as práticas agronômicas afetam a comunidade bacteriana, o impacto da palha recém-adicionada no solo em conjunto com

resíduo orgânico não é percebido, já que não são avaliados sozinhos, e questões ambientais mais aplicadas e específicas em conjunto com outros resíduos são colocadas em foco. Um dos resíduos orgânicos que é aplicado em conjunto com a palha é a vinhaça. Apesar das pesquisas conduzidas desde 1950, os estudos sobre os seus efeitos na comunidade microbiana é ainda recente (Navarrete et al., 2015; Pitombo et al., 2015). Por ser fonte de nutrientes, matéria orgânica e água, os grupos taxonômicos microbianos são modificados em resposta a aplicação de vinhaça e palha de cobertura em solos cultivados com cana-de-açúcar (Navarrete et al., 2015). Além disso, desnitrificadoras e táxons relacionados ao consumo de N_2O foram identificados (Pitombo et al., 2015), já que a aplicação desses resíduos tem grande impacto na emissão de N_2O quando comparadas com outros fertilizantes como a ureia (Paredes et al., 2014).

Resultados contraditórios são encontrados na literatura em relação aos impactos da fertilização orgânica derivada de dejetos animais e inorgânico na comunidade microbiana. Alguns estudos indicam impactos significativos aumentando a atividade dos microrganismos do solo (Ge et al., 2008, Mandal et al., 2007) enquanto outros demonstraram pouco ou nenhum efeito sobre a diversidade e a atividade microbiana do solo (Okano et al, 2004, Treseder, 2008). As pesquisas são muitas vezes ambíguas, particularmente porque os sistemas experimentais e as definições de manejo variam amplamente. Especificamente, a adição de dejetos aumenta a acumulação de carbono orgânico no solo, que por sua vez estimula a biomassa e atividade microbiana entre 16 a 20% induzindo mudanças na estrutura da comunidade microbiana quando comparado aos fertilizantes inorgânicos (Dinesh et al. 2010, González et al., 2010, Chaudhry et al., 2012). Devido a problemática das perdas de N por emissão de N_2O, resultante da adição de dejetos nos processos de nitrificação e desnitrificação realizadas por grupos específicos participantes do ciclo do nitrogênio, uma estratégia seria a utilização de inibidores de nitrificação como a diacianodiamida (DCD). A DCD é um composto químico que pode retardar a oxidação de NH_4^+ a NO_2^- e NO_3^- diminuindo as atividades dos oxidantes de amônia no processo de nitrificação (Amberger, 1989) reduzindo eficientemente as emissões de N_2O, quando aplicada no campo com somente fertilizante mineral, urina ou dejetos (Weiske et al., 2001, Di e Cameron, 2002, Di et al., 2007, Smith et al., 2008, Zaman et al., 2009). O seu efeito já é conhecido para nitrificantes e os estudos estão em progresso para a comunidade desnitrificante (O'Callaghan et al., 2010, Di et al., 2014), no entanto, não se tem estudos sobre os efeitos da DCD na comunidade microbiana geral com a aplicação dos dejetos animais.

Uma complexa interação microbiana metaboliza os resíduos de cultura, mas ainda pouco se sabe sobre quem é impactado e quem está envolvido na sua degradação (McGuire e Treseder, 2010). Na última década, uma variedade de ferramentas moleculares tem melhorado o conhecimento sobre a diversidade e composição microbiana que não pode ser identificada utilizando abordagens tradicionais taxonômicos entre os vários sistemas orgânicos e convencionais (Dong et al., 2014). Entretanto, mesmo com o advento dessas técnicas, parece difícil formar uma conclusão sólida sobre diferentes práticas agrícolas e as comunidades microbianas. Diferentes métricas, técnicas e concepções experimentais têm frequentemente afetado o poder de resolução e confiabilidade de acessar as diferenças microbianas (Hartmann e Widmer, 2006, Hartmann et al., 2015). Além de diversos estudos serem geralmente baseados em uma única amostragem temporal em experimentos de curto ou longo prazo sem um acesso da dinâmica na comunidade microbiana com amostragens periódicas (Lazcano et al., 2013, Pan et al., 2014). Com a era do sequenciamento de alto rendimento, esses métodos promissores estão recentemente sendo aplicados para investigar diferentes genes microbianos, inclusive em sistemas agrícolas (Delmont et al., 2012, Souza et al., 2015).

Neste contexto, o objetivo geral desta tese foi avaliar o impacto de resíduos orgânicos na comunidade bacteriana em experimentos de campo a curto prazo baseando-se em técnicas moleculares como sequenciamento de nova geração. A apresentação dos resultados obtidos foi dividida em dois capítulos. No **primeiro capítulo** intitulado de "***Temporal variability of soil microbial communities after application of dicyandiamide-treated swine slurry and mineral fertilizers***" objetivou-se avaliar o impacto da dinâmica da comunidade bacteriana ativa devido a aplicação de dejetos suínos com DCD e fertilizante mineral. O **segundo capítulo** intitulado de "***Recycling crop residues in agriculture impacts soil-borne microbial structure and function and N_2O emissions***" teve como objetivo estudar o efeito da adição de diferentes resíduos sobre a dinâmica dos táxons e funções microbianas.

2 HIPÓTESES E OBJETIVOS

2.1 HIPÓTESES

a) Os fertilizantes orgânicos mudam a estrutura e funções das comunidades microbianas do solo.

b) Os fertilizantes orgânicos aumentam as emissões do N_2O.

2.2 OBJETIVO GERAL

Avaliar o impacto de resíduos orgânicos na comunidade bacteriana em experimentos de campo a curto prazo baseando-se em técnicas moleculares como sequenciamento de nova geração

2.3 OBJETIVOS ESPECÍFICOS

a) Avaliar o impacto da dinâmica da comunidade bacteriana ativa devido a aplicação de dejetos suínos com DCD e fertilizante mineral

b) Estudar o efeito da adição de diferentes resíduos sobre a dinâmica dos táxons e funções microbianas.

3 ARTIGO I

Temporal variability of soil microbial communities after application of dicyandiamide-treated swine slurry and mineral fertilizers*

* Artigo elaborado de acordo com as normas da Soil Biology and Biochemistry (publicado em 16 de março de 2016).

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3.1 Abstract

In modern agriculture, mineral and organic fertilization account for most of the global anthropogenic N_2O emissions. A strategy to prevent or to reduce emissions of greenhouse gases such as N_2O is the use of nitrification inhibitors, which temporarily inhibit the microbial conversion of soil ammonium to nitrate. However, information about the magnitude and duration of disturbance caused by organic fertilization with nitrification inhibitor on the microbial community is lacking. Here we examined N dynamics and how potentially active soil microbial communities changed through time by the addition of dicyandiamide-treated swine slurry and mineral fertilizers. A field experiment (corn/cereal succession under no-tillage system) was carried out using the following treatments: (I) unfertilized control, (II) surface application of mineral nutrients, (III) surface application of swine slurry, and (IV) surface application of swine slurry with dicyandiamide. Soil samples were collected at 0, 3, 6, 11, 25 and 50 days after start of experiment. Total RNA was extracted, synthesized to cDNA and used as template to amplify and sequence the 16S rRNA. Nitrous oxide emissions were also quantified. The organic fertilizers were the main drivers on changes in microbial community structure. Slurry application decreased microbial diversity and changed the microbial structure temporarily but the metabolically active microbial community was resilient, recovering to the original status 50 days post-fertilization. DCD had no effect on metabolically active microbial community and was pathway-specific, having impact only on nitrifiers during a short-term period, which in turn reduced the N_2O emissions.

Keywords
Nitrous oxide emission; Nitrification inhibitor; Mineral fertilizer; Organic fertilizer; Next generation sequencing; 16S rRNA

3.2 Introduction

Nutrient enrichment through fertilizers input is a source of small-scale anthropogenic disturbance for soil habitats. Interest in the use of organic fertilizers, such as animal manure, has increased with aims to reduce the use of mineral fertilizers for crop production and to develop sustainable global agriculture (De Vries et al., 2015). Animal manure is cost-effective and provides nutrients to plant growth, primarily nitrogen (Gale et al., 2006).

In many regions of Brazil, like in other countries of the globe, the field application of manure is a common practice. According to Aita et al. (2015), doses greater than 100 m^3 ha^{-1} $year^{-1}$ of animal manure (e.g. swine slurry) are commonly used in Southern Brazil. Long and short-term fertilization practices might result in positive, neutral or negative effects in soil microbial community structure (Biederbeck et al., 1996, Hu et al., 2011, Lazcano et al., 2013, Williams et al., 2013 and Pan et al., 2014). Hu et al. (2011) documented that fertilizers affected microbial functional diversity, metabolic activity and metabolic quotient. Long-term swine slurry application increased soil microbial quality indicators of biomass and enzyme activity (Balota et al., 2014) while short-term fertilizer regimes stimulated microbial growth, altered the structure of the soil microbial community and increased enzyme activity (Lazcano et al., 2013). However, there is so far very little evidence of a connection between alterations in activity and effects on microbial community composition over time series post disturbances.

Although organic fertilizers are effective in increasing nutrient availability, improving grain yield and are more environmental friendly than mineral fertilizers, the disturbance caused by animal manure application might also impose some risks. An important aspect of organic fertilization is the introduction of high loads of exogenous microbes in soil (Durso et al., 2011) and chemical components that might represent a perturbation for soil microbial communities through stimulating specific populations and suppressing others. On the other hand, the soil microbial communities are highly resilient and tend to exclude exotic populations attempting to occupy niches already occupied by the indigenous population (Levine and D'Antonio, 1999).

Furthermore, organic fertilizer additions result in environmental pollution (e.g. nitrate contamination of ground water, eutrophication of water bodies and increase of ammonia and greenhouse gas emissions) when manure is applied beyond the soil retention capacity or above the plant nutrient requirements (Luo et al., 2010). Agricultural sources, including mineral and organic fertilizers, are estimated to account for 60% of the global anthropogenic N_2O emissions (Aguilera et al., 2013) and animal manure is considered the major source of N_2O global emissions (Davidson, 2009). In the 21st century this greenhouse gas (GHG) has become the greatest threat to the ozone layer (Ravishankara et al., 2009) mainly because it has higher global warming potential compared to CO_2 and CH_4. Strategies to avoid adverse impacts on the environment are under development aiming to prevent and/or reduce emissions of N_2O.

The use of nitrification inhibitors such as dicyandiamide (DCD, $C_2H_4N_4$) is a potential alternative to reduce N_2O emissions caused by agricultural use of N sources such as urine and animal manure. DCD temporarily inhibits the microbial conversion of soil ammonium to nitrate by binding to the active site of the enzyme ammonia monooxygenase (AMO), that is responsible for the first step of the nitrification pathway (Amberger, 1989). Dicyandiamide is a white crystalline powder, highly soluble in water, low volatility, biodegradable and totally decomposed in soil to ammonium (NH_4^+) and CO_2 (Amberger, 1989). DCD biodegradation rate is a function of temperature. The half-life of DCD in soils at 25 °C is around 6–20 days depending of DCD rates and experiment conditions (e.g. laboratory or field conditions) (Singh et al., 2008, Kelliher et al., 2008 and Kelliher et al., 2014). On the other hand, high rates of DCD (20 mg kg^{-1} or higher) might cause phytotoxic effects including leaf tip and margin chlorosis and necrosis, and reduction in the biomass yield (Prasad and Power, 1995 and Macadam et al., 2003). DCD applied to the soil mixed with manure and urine, presents variable effectiveness on mitigating of N_2O emissions ranging from 11 to 97% depending on the system (Kumar et al., 2000, Luo et al., 2013, Aita et al., 2014 and Huang et al., 2014). The effects of DCD on nitrifier and denitrifier communities are relatively well known (O'Callaghan et al., 2010, Wakelin et al., 2013, Di et al., 2014 and Morales et al., 2015), but there are no studies on the general effect of DCD-treated swine slurry on the active soil microbial community.

Although organic fertilization is a practice disseminated around the world, information about the N dynamics, magnitude and duration of disturbance caused by organic fertilization with nitrification inhibitor as well as the resistance and resilience of microbial communities in a time series still need to be better understood. Microbial communities can change abruptly in

response to perturbations and recover quickly to its original state. A time series study allows us to analyze the stability and dynamics of microbial communities while single sampling points might only capture a specific status of soil microbes that might not represent the true microbial response to perturbations. The aim of this study was to examine N dynamics and how the potentially active soil microbial communities changed through time by the addition of DCD-treated swine slurry and mineral fertilizers using 'post-light'-based sequencing technology. Special attention was devoted to nitrous oxide emissions because of the link to the nitrogen cycle and the importance in global warming potential.

3.3 Material and Methods

3.3.1 Experimental field description and soil sampling

The experiment was carried out in the Federal University of Santa Maria (UFSM), Rio Grande do Sul State, Brazil (29°43′ S, 53°43' W, altitude 105 m). The soil in the experimental area was characterized as Typic Paleudult (USDA classification). Soil samples were collected from the 0.00–0.10 m soil layer and contained: 19.2% of clay and 44.3% of sand (pipette method, Embrapa, 1997); pH 5.9 pH (H_2O) 5.9, determined in a soil:water suspension (1:1, m/v). The exchangeable concentrations of Ca, Mg and Al were 9.8, 3.1 and 0.0 cmolc kg^{-1}, respectively (extractor KCl 1 mol L^{-1}) (Tedesco et al., 1995). The determination of the concentrations of Ca and Mg in the extracts was performed in an atomic absorption spectrophotometer (AAS) and Al by titration (Tedesco et al., 1995). The concentration of available P was 6.7 mg kg^{-1} and exchangeable K was 39.0 mg kg^{-1} (extractor Mehlich 1 solution – HCl 0.05 mol L^{-1} + H_2SO_4 0.0125 mol L^{-1}) (Tedesco et al., 1995). The P was measured using a spectrophotometer and the K was measured using a flame photometer. Cation exchange capacity (CEC) was 12.9 cmolc kg^{-1}. The CEC was calculated following Summer and Miller (1996). Total C (20.5 g kg^{-1}) and N (1.6 g kg^{-1}) contents were analyzed by dry combustion with a graphite furnace (FlashEA 1112, Thermo Finnigan, Milan, Italy). For more details about the soil and experimental area see Aita et al. (2014).

The experimental field was divided into plots of 5.25 m × 6.00 m and each treatment was replicated three times in a complete randomized block design. Four treatments were applied: (I) control (unfertilized), (II) surface application of urea (NPK), (III) surface application of swine slurry (slurry) at 50 m^3 ha^{-1}, and (IV) surface application of swine slurry with dicyandiamide (slurry with DCD) at 50 m3 ha^{-1}. Rates of swine slurry were determined

to provide a target total N supply of 130–140 kg total N ha^{-1}, equivalent to the application of urea in the treatment II.

The field was cultivated during 2 years before soil sampling (2011 and 2012) with a corn/cereal succession (corn/oat/corn/wheat) under a no-tillage system. The treatments were applied twice a year during two years (before the summer - corn; and the winter crops - oat or wheat) on soil surface with residues of the preceding crop (a few days before sowing the next crop). The average daily temperature was obtained from the University meteorological station, located approximately 500 m from the experiment. The amount of rainfall and irrigation water at each site was measured using rain gauges. The average annual rainfall was 1700 mm, and the mean annual temperature was 19.9 °C (Supplementary Figure 1).

The swine slurry was obtained from fattening pigs and stocked in an anaerobic tank at the experimental field. The main characteristics of the swine slurry at each growing no-tillage succession season are presented in the Supplementary Table 1. The swine slurry applied in the experiment presented pH 7.6, 23 kg m^{-3} of dry matter, 8.2 kg m^{-3} of total carbon, 3.29 kg m^{-3} of total N and 2.6 kg m^{-3} of total ammoniacal N (NH3 + NH_4^+). In treatment IV, DCD was applied at a rate of 10 kg ha^{-1}, and mixed with the slurry swine immediately prior to soil application. The surface application was performed using watering cans. During application, samples of slurry and slurry with DCD were collected for microbial molecular analysis. Urea fertilizer was applied on the soil surface of treatment II at a target rate of 130 kg N ha^{-1}, one third pre-plant and two thirds at the maize sixth-leaf stage (34 days after sown). The treatment with urea was also fertilized with P and K in a rate of 125 kg ha^{-1} and 90 kg ha^{-1}, respectively.

For microbial and chemical analysis, a composite soil sample (mixture of 8 random points in each plot to form a single composite sample) was taken of the top soil (0–5 cm) from the triplicate plots covered with 6.1 t ha^{-1} of wheat residues (*Triticum aestivum* L.; cv. Quartz). Four days after treatment application, maize (*Zea mays* L.; hibrid 32R22 Herculex) was sown manually, with an interrow distance of 0.7 m. Pre-experiment soil samples were collected one day before and after 3, 6, 11, 25 and 50 days of start of experiment for molecular analyses. The samples were immediately placed in liquid nitrogen and stored at −80 °C until total RNA extraction.

3.3.2 RNA extraction and reverse transcription

The PowerSoil Total RNA Isolation Kit (MO-BIO Laboratories, Inc., CA) was used to extract the total RNA from two grams of soil per sample (n = 72) by following the

manufacturer's instructions. The microbial RNA was also extracted from the swine slurry used for soil fertilization with and without DCD addition in three replicates (n = 6). The extracted RNA was treated with TURBO DNA-free Kit (ABI, USA) to remove the remaining DNA. Superscript III reverse transcriptase kit (Invitrogen, Paisley, UK) and random hexamer primers were used for cDNA synthesis. The resulting cDNA was used for subsequent PCR reactions.

3.3.3 16S library preparation and sequencing with PGM Ion Torrent

The V4 region of the 16S rRNA gene was amplified and sequenced using the PGM Ion Torrent (Life Technologies) using archaeal/bacterial primers 515F and 806R in accordance with the protocol described in Caporaso et al. (2012). To minimize the biases that may occur in PCR reactions, three independent reactions were performed for each sample and the products were pooled before sequencing. Multiple samples were PCR-amplified using barcoded primers linked with the Ion adapter "A" sequence (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) and Ion adapter "P1" sequence (5′-CCTCTCTATGGGCAGTCGGTGAT-3′) to obtain a sequence of primer composed for A-barcode-806R and P1-515F adapter and primers. Each of the 25 μL of PCR mixture consisted of 12.5 μL 5X KAPA HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, Inc., Boston, MA, USA), 0.1 μM of each primer, and 100 ng of cDNA template. The PCR conditions used were 94 °C for 2 min, 25 cycles of 94 °C per 45 s denaturation; 55 °C per 45 s annealing and 72 °C per 1 min extension; followed by 72 °C per 6 min. The resulting PCR products were purified with the Agencourt® AMPure® XP Reagent (Beckman Coulter, USA). The final concentration of the PCR product was quantified by using the Qubit Fluorometer kit (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Finally, the reactions were combined in equimolar concentrations to create a mixture composed by 16S gene amplified fragments of each sample. This composite sample was used for library preparation with Ion OneTouch™ 2 System with the Ion PGM™ Template OT2 400 Kit Template. The sequencing was performed using Ion PGM™ Sequencing 400 on Ion PGM™ System using Ion 314™ Chip v2 (Thermo Fisher Scientific, Waltham, MA, USA). A total of five runs were performed to obtain high sequence coverage. After sequencing, the reads were pre-filtered within the PGM software to remove low quality and polyclonal sequences.

3.3.4 Sequence processing and statistical analysis

The 16S rRNA raw sequences were analyzed following the recommendations of the Brazilian Microbiome Project (Pylro et al., 2014). Briefly, the OTU (Operational Taxonomic Unit) table was built using the UPARSE pipeline (Edgar, 2013) in which the reads were truncated at 200 bp and quality filtered using a maximum expected error of 0.5. Filtered reads were dereplicated and singletons were removed. The sequences were clustered into OTUs at 97% similarity cutoff and chimeras checked to obtain representative sequences for each phylotype. Taxonomic classification was carried-out in QIIME (Caporaso et al., 2012) based on the UCLUST method against the Greengenes 13.5 database (McDonald et al., 2012) with a confidence threshold of 80%. Sampling effort was estimated using Good's coverage (Good, 1953). Ordination of the 16S rRNA sequencing was performed based on UniFrac matrix calculated by QIIME software and presented in a weighted and unweighted PCoA (Principal Coordinate Analysis) to visualize differences in bacterial/archaeal community composition among species and treatments during each sampling point of the experiment. Permutational multivariate analysis of variance (PERMANOVA) was used to assess statistical differences in community composition among treatments (Anderson, 2001). Factors in the PERMANOVA were treatments, time and their interaction. Taxonomic alpha diversity metrics, including Chao1 (Chao, 1984) and phylogenetic diversity (Faith, 1992) were calculated using QIIME and Shannon diversity index was calculated using PAST on all samples rarified to the same sequencing depth (1836 sequences per sample). Finally, Statistical Analysis of Metagenomic Profiles (STAMP) was used to determine differences in the relative abundances of categories (i.e. taxa) between treatments (Parks et al., 2014). The statistical hypothesis tests were performed using the Welch's t test while confidence intervals were calculated using the Welch's inverted method and Bonferroni multiple test for p-value correction.

Raw sequences were submitted to the NCBI Sequence Read Archive under the experiment number SRX1294673, run number SRR2533758.

3.3.5 Soil pH, inorganic N and N_2O *emissions*

Soil pH (1:2.5 soil:water) was measured in distilled water using a pH probe (Ohaus, Starter 2100, USA). Soil inorganic N concentrations (NH_4^+ and NO_3^-) were quantified using the composited soil samples collected from each plot. Specifically, soils were extracted by shaking 20 g of field-moist fresh soil in 80 mL of KCl 1M solution for 30 min (1:5 soil:

solution). After decanting, the supernatant was filtered and nitrate and ammonium concentrations were determined using spectrophotometry San++ Automated Wet Chemistry Analyzer (Skalar, Breda, Netherlands).

Nitrous oxide (N_2O) emissions were quantified on days 1, 3, 6, 11, 15, 25 and 50 after start of experiment using insulated, fan-mixed, non–flow-through, non–steady-state chambers (40 cm length, 35 cm width, and 20 cm height) manufactured at Federal University of Santa Maria. After treatment application, a galvanized steel base adjacent to a maize row was pressed into the soil (5 cm depth). Each metallic base had a water-filled trough that the chamber lid was placed in during sampling to prevent gas exchange to the external atmosphere. For the measurements (between 9:00 am and 11:00 am), gas samples (20 mL) were collected using propylene syringes at 0, 15, 30, and 45 min and were taken using valve fitted in the top of the chamber. N_2O was analyzed using a gas chromatograph (GC-2014, Shimadzu Corp., Kyoto, Japan) equipped with an electron capture detector. The N_2O flux was calculated according to Rochette and Bertrand (2008). Air temperature and moisture inside the chamber was recorded using a thermo-sensor model 7664.01.0.00(Incoterm, Porto Alegre, Brazil) and soil temperature at 5 cm depth next to the chamber was recorded using a thermo-sensor model 6032.08.1.00 (Incoterm, Porto Alegre, Brazil). The results were compared between treatments and time of sampling with two-way repeated measures ANOVA that compares the mean differences between independent variables.

3.4 Results

3.4.1 Sequencing coverage and overall microbial abundance

Most of the studies on impact of fertilization in soil microbial community target microbial genomic DNA, however in this study we assessed bacterial and archaeal communities by sequencing 16S cDNA gene fragments. Microbial RNA is considered an index of potential microbial activity and acclimation. After quality filtering, a total of 727,968 high quality sequences remained for soil community analysis and a total of 11,837 and 20,856 high quality sequences were obtained from the swine slurry with and without DCD, respectively. All treatments presented comprehensive sampling of the bacterial community with high sequence coverage (76–98%) as shown in Supplementary Table 2.

There was consistently higher abundance of bacterial (99.5%) than archaeal (0.48%) 16S rRNA fragment sequences across treatments. Taking into account the average of all time

points, 39 bacterial phyla were identified with the majority affiliated to seven major phyla. Overall, *Proteobacteria* (59.3% ± 5.2), *Bacteroidetes* (12.4% ± 1.9), *Acidobacteria* (7.8% ± 2.2) and to a lesser extent *Verrucomicrobia* (4.6% ± 0.9), *Actinobacteria* (3.8% ± 1.5), *Chloroflexi* (3.5% ± 1.02) and Planctomycetes (3.1% ± 0.96) were the dominant bacterial phyla. The 16S rRNA from the microbial communities from swine slurry and swine slurry with DCD used as fertilizer was also sequenced. The swine slurry had *Proteobacteria* as the most abundant phylum (32% ± 1.3) followed by *Bacteroidetes* (28.9% ± 5.6), *Firmicutes* (25.9% ± 3.1) and WWE1 (6.4% ± 0.2), Synergistetes (2.3% ± 1.28) and Spirochaetes (2.02% ± 0.5). The swine slurry with DCD presented similar phyla with similar abundances as observed in the swine slurry without DCD. They were: *Proteobacteria* (34.5% ± 3.3), *Bacteroidetes* (27.1% ± 3.5), *Firmicutes* (26.1% ± 3.8), WWE1 (6.04% ± 1.6), Spirochaetes (2.4% ± 1.4) and Synergistetes (1.6% ± 0.8). The abundances of other bacterial phyla were <1%.

The three dominant Archaea phyla in soils were Crenarchaeota (0.47% ± 0.18), Euryarchaeota (0.01% ± 0.01) and Parvarchaeota (0.01% ± 0.01); however, no archaeal sequences were detected in the swine slurry (with or without DCD).

3.4.2 Soil active microbial community variation among treatments

The Principal Coordinates Analysis (PCoA) was applied to detect soil microbial beta diversity variations during the experiment by using weighted and unweighted UniFrac distances (Fig. 1 and Fig. 2) and PERMANOVA (Table 1) at 97% cutoff similarity level for OTU grouping. Days, treatments and their interaction were the forces structuring the microbial community with pseudo-F values of 4.18, 13.13 and 2.37 ($p < 0.01$), respectively (Table 1).

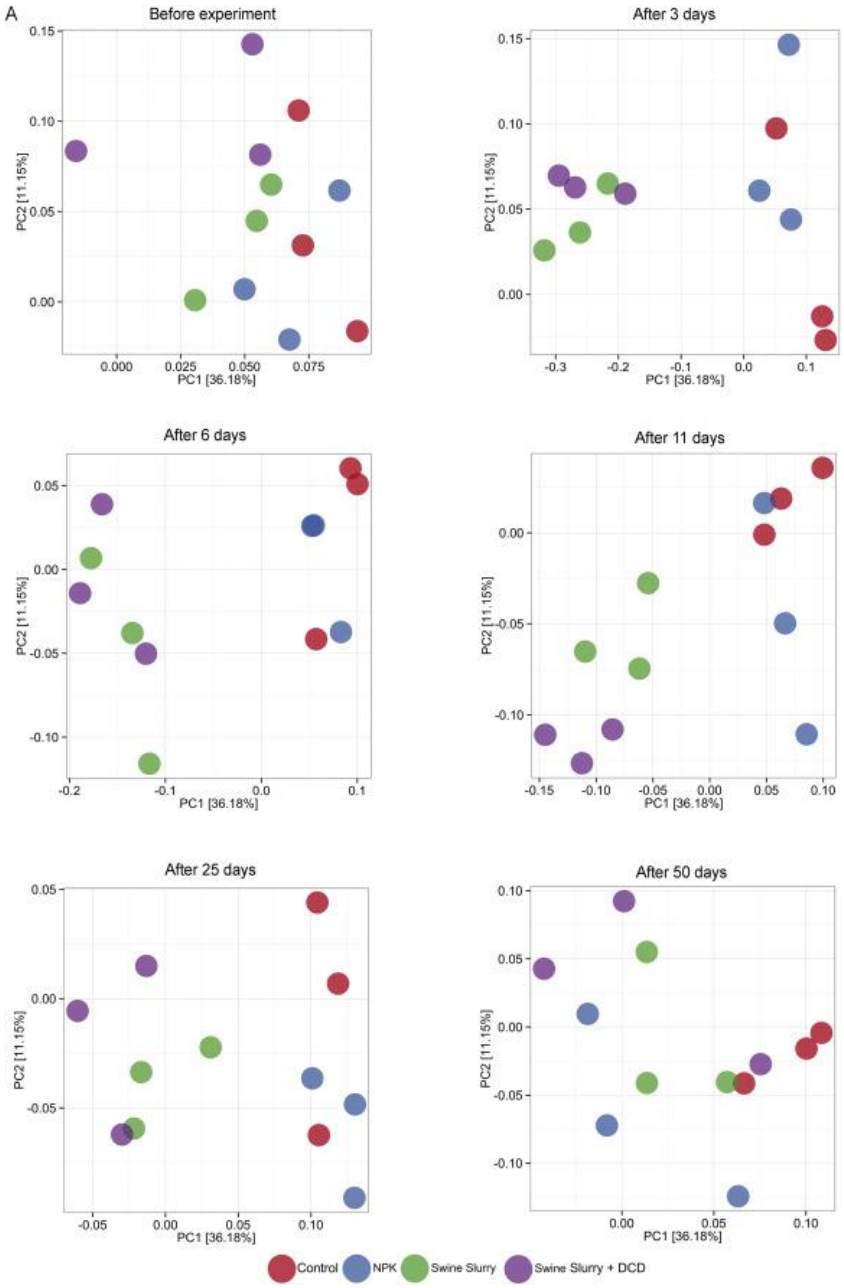

Fig. 1.

Temporal changes in the active soil microbial community depicted by weighted (which accounts for changes in the relative abundance of taxons) Principal Coordinates Analysis (PCoA) in soils cultivated with corn during six periods following applications of NPK, swine slurry and swine slurry with dicyandiamide. Each point represents an individual sample, with colors indicating treatments.

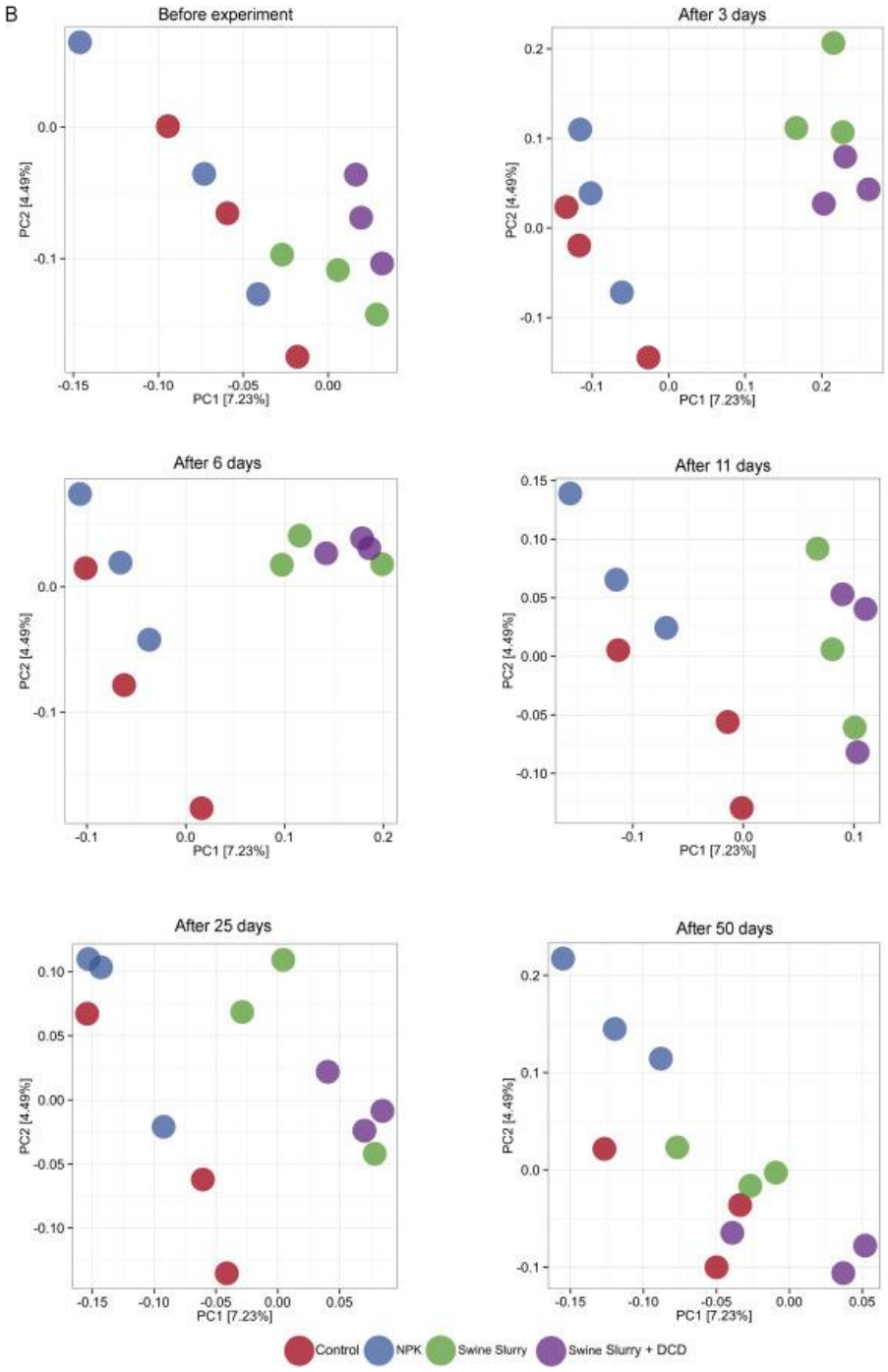

Fig. 2.

Temporal changes in the active soil microbial community depicted by unweighted Principal Coordinates Analysis (PCoA) (which accounts for presence/absence of taxons) in soils cultivated with corn during six periods following applications of NPK, swine slurry and swine slurry with dicyandiamide. Each point represents an individual sample, with colors indicating treatments.

Table 1.
Permutational Analysis of Variance (PERMANOVA) showing the differences among soil microbial communities.

Main test[a]	Pseudo-F (F)	p value[*]
Days	**4.18**	**0.005**
Treatments	**13.13**	**0.001**
Days x Treatments	**2.37**	**0.015**
Pairwise test[b]	Univariate t-statistic (t)	p value[*]
Treatments		
Control 0 x Slurry 0	0.95	0.5
Control 3 x Slurry 3	**3.66**	**0.001**
Control 6 x Slurry 6	**2.42**	**0.001**
Control 11 x Slurry 11	1.5	0.11
Control 25 x Slurry 25	**1.57**	**0.001**
Control 50 x Slurry 50	1.14	0.32

[a] Main factors represent days (0, 3, 6, 11, 25 and 50) and treatments (Control, NPK, Slurry and Slurry with DCD).
[b] Pairwise comparisons between control and slurry treatments with weighted UniFrac distance.
[*] Values at P<0.05 are shown in bold.

According to the weighted UniFrac distances (Fig. 1) that took into account the abundance of OTUs, all microbial communities pre-experiment were similar. After 3 days the microbial communities from soils fertilized with swine slurry with and without DCD differed from those that received NPK or were unfertilized (controls). The effect of swine slurry application at three, six and 25 days was confirmed by PERMANOVA with t-statistic of 3.66, 2.42 and 1.57 respectively ($p < 0.001$). After 6 days, the dissimilarity between microbial communities from soils with addition of swine slurry and controls decreased. This dissimilarity remained decreasing at each time of sampling and finally the microbial community recovered to original status after 50 days following fertilization. This trend was also supported by PERMANOVA results (Table 1).

The PCoA calculated by unweighted UniFrac distances (Fig. 2) demonstrated similar trends observed by the weighted PCoA, but the axes explained smaller variance than those of the weighted PCoA. The unweighted UniFrac distance account for changes in the presence/absence of microbial phylotypes. Thus, the composition of the bacterial community differed whether or not the abundance of taxa was considered however, the low variation explained by the first two PCoA axes indicated that the major factor contributing to the microbial community variance was the changes in abundance of OTUs. The use of nitrification inhibitor DCD did not affect the overall soil microbial communities as observed in Fig. 1 and Fig. 2. In addition, no differences between microbial communities from the

control and NPK were detected in any sampling time. Furthermore, the soil samples from all treatments presented very different communities from the swine slurry input (with and without DCD) as observed by the well-separated groups in the microbial community clustering analysis (Supplementary Fig. 2).

The effect of swine slurry fertilization on alpha diversity was in agreement with beta diversity analysis. Calculations of species richness (Chao1), phylogenetic diversity (Faith's PD) and Shannon diversity indices were initially similar before fertilization but diverged after swine slurry application. All indices showed decrease in alpha diversity in treatments with addition of swine slurry (with and without DCD) at days 3, 6 and 25 of the experiment but after the initial days, the diversity returned to the original status (Table 2).

Table 2.
Changes in active soil microbial alpha-diversity measured by three different metrics during six periods following applications of NPK, swine slurry and swine slurry with dicyandiamide over wheat residues. The average for each treatment on each sampling date is showed with standard deviation of the mean.

Days	Treatments	Chao	Phylogenetic Diversity	Shannon
0	Control	1888.4±155.4	52.2±1.6	6.3±0.1
	NPK	2101.5±171.0	53.1±0.5	6.4±0.1
	Slurry	1818.3±57.1	52.7±0.7	6.3±0.1
	Slurry with DCD	1959.4±85.1	51.4±3.7	6.3±0.1
3	Control	1969.5±236.1	53.9±2.8	6.4±0.1
	NPK	2077.4±35.8	52.3±1.9	6.3±0.1
	Slurry	1402.2±137.8	39.8±2.7	5.2±0.3
	Slurry with DCD	1758.8±137.9	47.7±1.3	5.5±0.2
6	Control	2063.4±83.0	53.8±1.1	6.3±0.1
	NPK	2047.8±144.5	53.0±1.8	6.4±0.1
	Slurry	1770.5±143.9	49.4±3.8	5.9±0.1
	Slurry with DCD	1704.3±118.0	50.0±2.0	5.9±0.1
11	Control	1843.4±148.4	52.2±1.3	6.3±0.1
	NPK	1914.42±89.1	51.5±1.3	6.3±0.0
	Slurry	1795.0±194.6	49.0±0.4	6.1±0.1
	Slurry with DCD	1883.8±59.9	51.3±2.7	6.0±0.2
25	Control	2055.7±71.5	51.7±1.1	6.3±0.0
	NPK	1953.1±171.8	53.8±2.6	6.4±0.1
	Slurry	1746.6±139.3	49.2±3.0	6.2±0.1
	Slurry with DCD	1848.3±292.8	50.3±2.9	6.2±0.1
50	Control	2010.4±207.1	54.0±2.0	6.3±0.1
	NPK	2007.3±272.4	52.2±2.0	6.3±0.1
	Slurry	2130.4±129.1	54.0±2.0	6.3±0.1
	Slurry with DCD	1951.2±200.7	51.1±2.0	6.2±0.1

Having found statistically significant differences between microbial communities after three days of swine slurry application, the next step was to identify microbial taxa responsible for the observed differences. At phylum level, *Bacteroidetes*, *Proteobacteria* and *Firmicutes*

increased significantly with swine slurry fertilization either with or without DCD while *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, Planctomycetes and *Verrucomicrobia* decreased (Fig. 3). The analysis of specific microbial phyla also revealed the negative influence of DCD on Nitrospirae, an important microbial group related to the nitrification process (Ehrich et al., 1995 and Daims et al., 2001). Most microbial groups showed no differences with addition of swine slurry ($p \leq 0.05$) but shifts in specific microbial groups were observed. At day 3, out of 302 microbial OTUs grouped at order taxonomical level, only 36 (approximately 12%) and 43 (approximately 14%) were found to be responsible for the differences between microbial communities from unfertilized soil (control) compared to soils fertilized with swine slurry with and without DCD, respectively (Fig. 3).

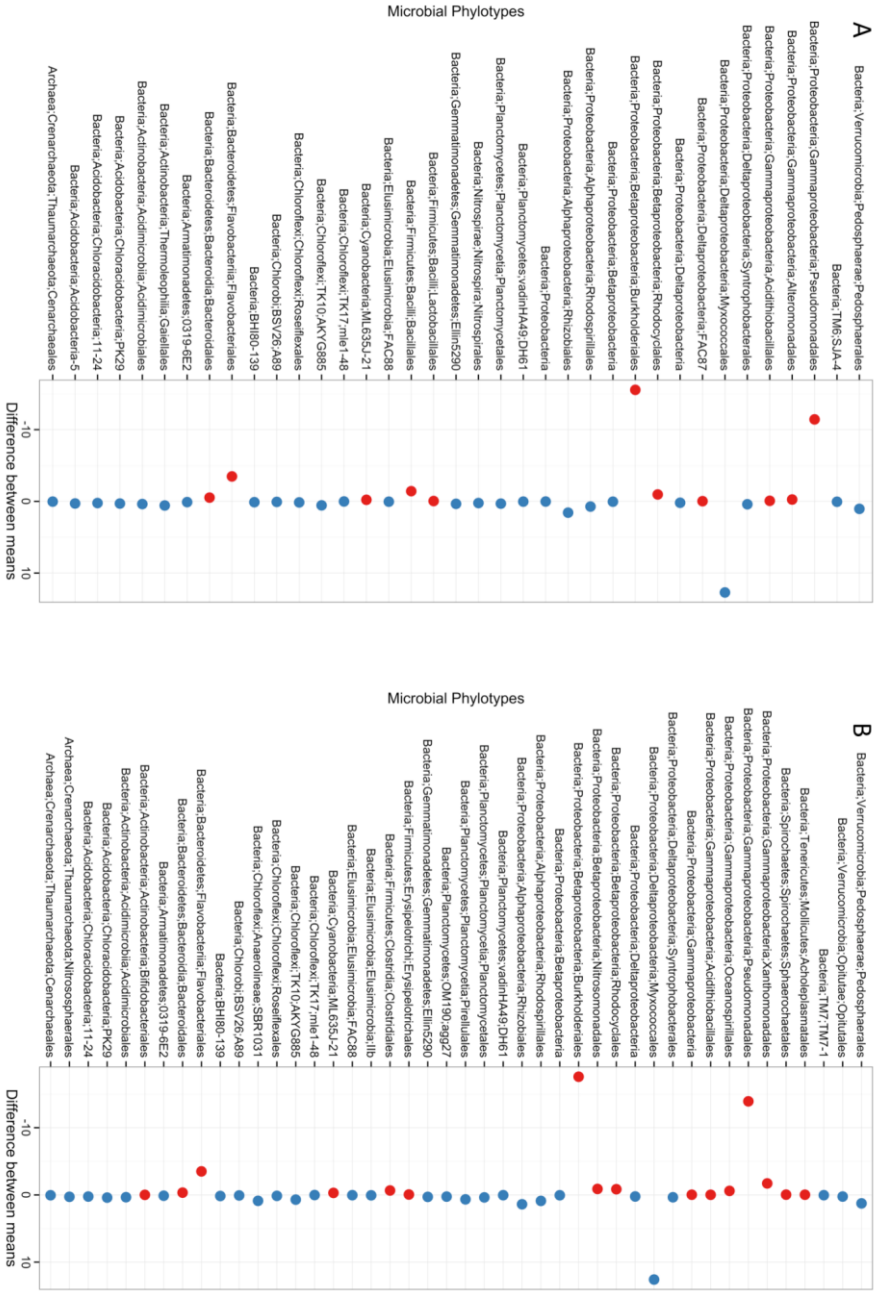

Fig. 3.

Differences in the relative abundance of microbial orders between soils with no fertilization (control) versus soils fertilized with swine slurry with dicyandiamide (A) and between soils with no fertilization versus soils fertilized with swine slurry without dicyandiamide (B) in the third day of the experiment. Red circles represent microbial orders that were in greater abundance in the slurry treatments while blue circles represent microbial orders in greater abundance in treatments without fertilization. The differences between groups were calculated using the Welch's inverted method. Corrected p-values were calculated with Bonferroni Multiple test correction. Only significant differences at $p \leq 0.05$ are presented.

The relative abundance of taxa in the active microbial communities from soils fertilized with swine slurry with and without DCD at the third day after fertilization was analyzed (Fig. 4). Twenty-four OTUs presented significant differences between treatments. Among these, 21 phylotypes were significantly more abundant in the presence of DCD ($p \leq 0.05$). Specifically, the uncultured iii1-8, *Acidobacteria*-6, Chlor*Acidobacteria*, Planctomycetia, Phycisphaerae, C6, OM190, Opitutae, Caulobacteriales, *Chromatiales*, Anaeroliae, Ellin6529 increased with addition of DCD, whereas Thermoleophilia, Elusomicrobia and Nitrosomonadales increased with the application of swine slurry only. Notably, the Nitrosomonas genus of this order is the main player responsible for the first step of nitrification pathway (Di et al., 2009 and Taylor et al., 2010); Nitrosomonadales increased with application of swine slurry but reduced their relative abundance ($p \leq 0.05$) with the application of the nitrification inhibitor DCD.

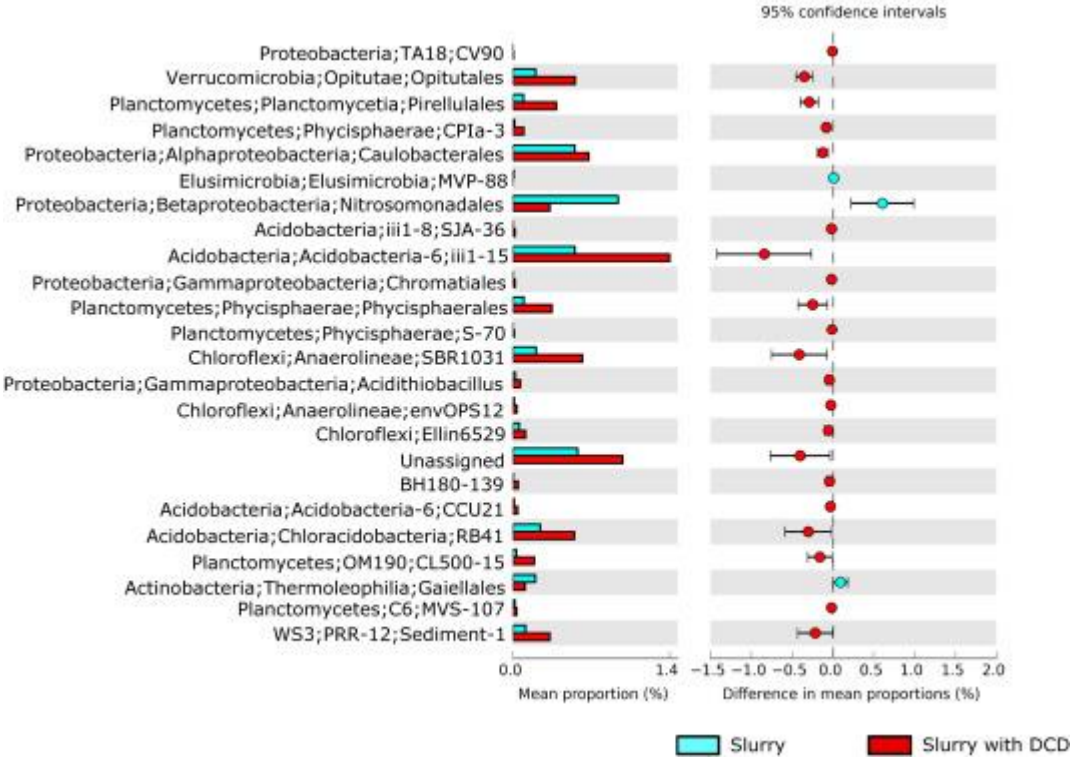

Fig. 4.

Relative abundance of active soil microbial orders whose abundances differed statistically (p-value ≤ 0.05) between treatments in the third day of experiment. The differences between groups was calculated using the Welch's inverted method. Corrected p-values were calculated using Bonferroni Multiple test correction. The error bars show calculated standard deviation of triplicate samples. The colored circles represent the 95% confidence intervals.

3.4.3 Effect of DCD on soil pH, N_2O emissions, soil mineral N concentrations and their relationship with beta diversity

Application of swine slurry with or without DCD did not affect the soil pH ($p \leq 0.05$) (Fig. 5A). Overall, soil pH declined significantly by approximately 0.63 units compared with the other treatments when NPK was applied ($p \leq 0.05$).

To obtain a comprehensive view about DCD effects on soil processes, N_2O emissions and N mineral concentrations were quantified. The mixture of DCD with swine slurry aims to slow down the conversion of NH_4^+ to NO_3^- resulting in treatments with DCD having lower N_2O emissions, higher NH_4^+ and lower NO_3^- concentrations. Fluxes of N_2O over the seven sampling dates were mainly detected during the first three days of the experiment, after slurry

application with lower levels thereafter (Fig. 5B) ($p \leq 0.01$, treatments comparisons). The emissions of N_2O were higher in treatments with addition of slurry. In the largest peak observed in N_2O emissions at the third day of experiment, the DCD application significantly reduced soil N_2O fluxes by 70% comparing to slurry application ($p \leq 0.01$, treatments comparisons). After 3 days post fertilization, the N_2O emission in soils fertilized with swine slurry declined sharply and was not different from slurry with DCD until the end of the experiment ($p \leq 0.01$, treatments and days comparisons). The N_2O emissions from the control and NPK treatments remained very low throughout the experimental period (Fig. 5B) and the total amount of $N–N_2O$ emitted in each treatment decreased in the seventh evaluation in the following order: Control (2.9 g ha^{-1}); NPK (4.6 g ha^{-1}), Slurry (28.2 g ha^{-1}) and slurry with DCD (22.3 g ha^{-1}).

The soil NH_4^+ content presented significant differences in slurry and slurry with DCD treatments compared to the control in the third day ($p \leq 0.01$) (Fig. 5C). Compared to the control, the addition of slurry with and without DCD resulted in elevated contents of NH_4^+ in soil representing an increment of 6 and 8 times, respectively ($p \leq 0.05$). Additionally, at the 3rd day after start of experiment, the NPK treatment increased 2 times the soil NH_4^+ content relative to control, reaching 29.3 mg kg^{-1} dry soil ($p \leq 0.05$). Also for the sixth day, the DCD was efficient to increase the NH_4^+ contents because the presence of the nitrification inhibitor increased 8 times comparing with control while in the absence of DCD increased just 1.7 times. The decrease of soil NH_4^+ contents over time was evident, reaching background levels along the 50 days of experiment. Furthermore, the increase of NO_3^- content (Fig. 5D) by 44% at day six showed the rapid nitrification of ammonium-swine slurry and the addition of DCD delayed this process in the following days of experiment ($p \leq 0.01$). The levels of NO_3^- were also significantly higher for NPK treatment during the first six days (14.7 mg N kg^{-1} dry soil) ($p \leq 0.01$) compared with the control (2.9 mg N kg^{-1} dry soil) but this levels also declined along the experiment. At day 11 the soil NO_3^- content in slurry treated with DCD plots was higher than slurry without DCD evidencing that DCD quickly lost its inhibitor effect.

Last, relationships between microbial beta diversity and soil properties within each time of sampling in each treatment were analyzed using Mantel test. No significant relationships between measured soil gas fluxes, inorganic soil N concentrations and pH within any microbial community were found (Table 3).

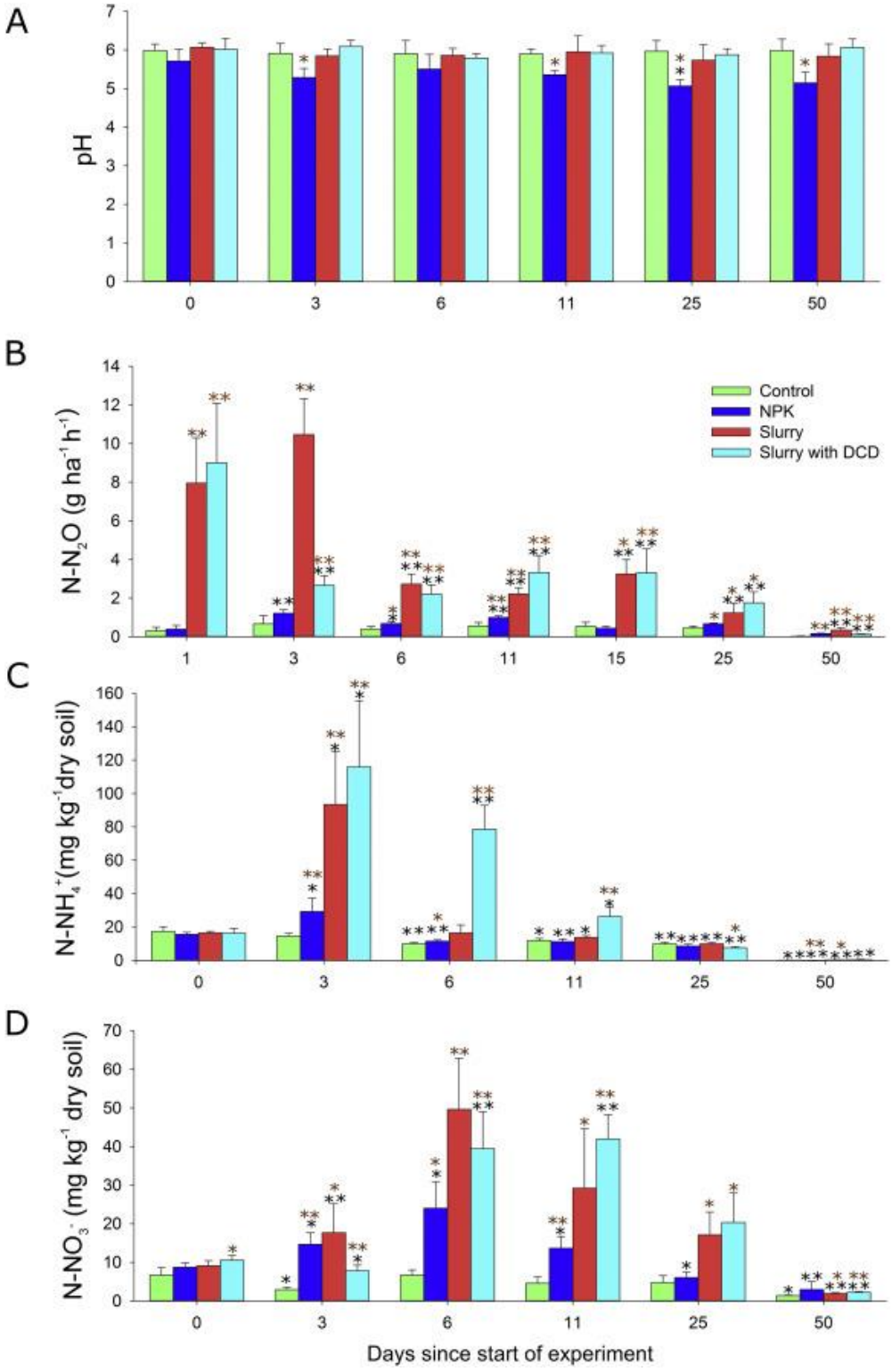

Fig. 5.

Soil pH, nitrous oxide (N_2O) emissions, concentrations of soil ammonium (NH_4^+) and soil nitrate (NO_3^-) following applications of NPK, swine slurry and swine slurry with dicyandiamide over wheat residues. Error bars indicate the standard error of mean (n = 3). Brown asterisks depict differences between treatments and the control. Black asterisks depict differences between days of experiment relative to the initial day. *$p \leq 0.05$, **$p \leq 0.01$.

Table 3.
Mantel correlation coefficients between distance matrixes derived from environmental parameters (N_2O emissions, concentrations of soil NH_4^+, concentrations of soil NO_3^- and soil pH) and UniFrac distance matrixes derived from microbial communities found in soil samples under no treatment (control), NPK, swine slurry and swine slurry with dicyandiamide.

Treatment*	**N-N_2O**	**N-NH_4^+**	**N-NO_3^-**	**pH**
		Before experiment		
Control	1	0,5	0,5	0,5
NPK	-1	-1	-0,5	-0,5
Slurry	0,5	-0,5	-1	1
Slury with DCD	0,5	-0,5	1	1
		3rd Day		
Control	-1	0,5	-1	-1
NPK	-0,5	1	0,5	-0,5
Slurry	-0,5	1	0,5	0,5
Slury with DCD	0,5	0,5	-0,5	0,5
		50thDay		
Control	0	0,5	-0,5	-1
NPK	0	-0,5	-0,5	-1
Slurry	0,5	0,5	1	-0,5
Slury with DCD	0,87	-0,5	0,5	-1

Normalized soil data were used to calculate pairwise Euclidean distances before performing Mantel correlations. *All results were non-significant with $p>0.05$.

3.5 Discussion

Here we tested the effect of mineral and organic fertilizers as liquid swine manure (mixture of mainly feces, urine and water but can also contain feed leftovers and dust) and a nitrification inhibitor (DCD) on the potentially active soil microbial community through high-throughput sequencing using total microbial RNA instead of DNA. To our knowledge, this is the first work evaluating the influence of nutrient enrichment and management strategies using organic and mineral fertilizers and a nitrification inhibitor (dicyandiamide) under field conditions including periodic soil sampling in no-till soil conditions. Ribosomal RNA abundance data is considered an index of potential microbial activity and acclimation (Blazewicz et al., 2013). The approach proposed here was able to characterize the microbial ribosomal RNA turnover under repeated patterns of environmental changes caused by organic (swine slurry) and mineral fertilization, providing robust understanding on how soil microbial communities adapt and respond to disturbance caused by slurry treated or not with the nitrification inhibitor DCD. Another substantial contribution of this study is the analysis of the microbial community through time. Our results showed that organic fertilizers have a

significant short-term impact on the structure of the active soil microbial community. In previous studies (Lazcano et al., 2013 and Su et al.,2015) the microbial communities were analyzed only in a single sampling date. As consequence, such studies indicate only short-term or long-term effects on microbial community and therefore, not microbial community dynamics.

Fertilization with mineral and organic nutrient sources is one of the typical disturbances that soil ecosystems experience under human-driven global change (Allison and Martiny, 2008). Prior and large numbers of reports have been initiated worldwide to evaluate the effects of organic and mineral fertilizers on microbial diversity in different systems. However, reports on the effects of organic farming on microbial diversity are often ambiguous, in particular because the experimental systems and management definitions vary widely. Manure application can have a stronger influence on soil microbial communities than either unfertilized or mineral fertilizer applications (Williams et al., 2013 and Dong et al., 2014). In the present study, the treatments with slurry and slurry with DCD showed substantially negative effects in the third day on soil bacterial diversity comparing with the control and NPK (Table 2). In general, the literature shows opposite results with microbial diversity being significantly decreased with application of mineral fertilizers and increased in treatments with manure (Dong et al., 2014 and Hartmann et al., 2015). However, we have to point out here that these studies were DNA-based microbial community analyses (including dead, dormant microbes and extracellular DNA) whereas our study was on the RNA-based microbial community (metabolically active microbes). Other studies found neutral effects of manure addition with increased amount of cultivable microorganisms and microbial biomass but stable microbial diversity (Zhen et al., 2014). Hence, it seems difficult to conclude on the effect of mineral and organic fertilizers on soil microbial diversity based on the available literature. This issue might be caused by the different methods and measurements used in each experiment and by the differences in the experimental design. As we assessed temporal changes on soil active microbes, our data provided a better view of the entire microbial shifts during the decomposition of the organic matter added by swine slurry application, not only representing a snapshot of the microbial community in a single moment.

The microbial community changed immediately after addition of swine slurry and recovered gradually to its original structure and to similar levels as the control treatment in the fiftieth day. Shade et al. (2012) have drawn attention on microbial community responses to press (long-term) and pulse (short-term) disturbances in a variety of habitats. In response to a disturbance, a microbial community may be resistant, resilient (returning to original condition

after disturbance) or move to a different but stable status following perturbation. Our results indicated that the soil microbial communities rapidly responded to a small-scale stress (nutrient amendment). Organic amendments are proposed to improve soil resilience to disturbance (Griffiths and Philippot, 2013) as organic fertilizers are not only nutrient-rich, but also longer lasting than chemical fertilizers providing required carbon, nitrogen and energy for microbial growth (Zeng et al., 2007).

3.5.1 Effects of DCD on the active soil microbial community and nitrogen cycle

Overall, swine slurry fertilization, with and without DCD, appeared to be the major driver of changes in active microbial community structure causing transient changes in microbial community throughout the experiment. Soil microbial communities in treatments with addition of swine slurry solely and swine slurry with DCD were significantly different from control and NPK treatments after 3 days, mainly due to increases in the abundance of *Bacteroidetes*, *Proteobacteria* and *Firmicutes* and decrease in the abundance of *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, Planctomycetes and *Verrucomicrobia*. These results can be explained partly by the dynamics of copiotrophic and oligotrophic bacteria. Evidence suggest that *Bacteroidetes* and a range of *Proteobacteria* have typically copiotrophic attributes and are most abundant in soils that have relatively large amounts of labile organic carbon (Fierer et al., 2007). Thus, the higher abundance of those phyla may be associated with their ability to utilize a wide variety of carbon substrates.

Those phyla mentioned above negatively influenced by slurry application have been suggested to be slow-growing oligotrophs adapted to nutrient-limited environments. Isolation of *Acidobacteria* showed that they are slow-growing and seems to decline in numbers following substrate addition (Eichorst et al., 2007 and Goldfarb et al., 2011). *Actinobacteria* are well adapted to soil environments with reduced C and nutrient availability, and belong to groups responsible for the decomposition of recalcitrant organic matter (Ventura et al.,2007 and Sul et al.,2013). Though *Chloroflexi* remains relatively understudied bacterial lineage (Hug et al., 2013) this phylum is abundant in soils with low nutrient availability (Will et al., 2010). For Planctomycetes, most of the isolates that have been obtained are slow-growing and aerobic (Pearson et al., 2003 and Ward et al., 2006). *Verrucomicrobia* is oligotrophic and able to grow under conditions of low C availability (Eilers et al., 2012), which may explain their low abundance in this study.

Two hypotheses can explain the differences in microbial structure caused by slurry application. The first one is based on microorganism invasion from slurry, also called niche opportunity (Shea and Chesson, 2002). Slurry might serve as an inoculum for introducing novel taxa causing the community disturbance. Even seeming unlikely, this hypothesis is still possible because members of *Bacteroidetes* and *Firmicutes* were the most abundant in the slurry input used as fertilizer and these two phyla were enriched after the application of slurry in soil. The second hypothesis is that slurry served as substrate for indigenous taxa causing the increment of specific soil microbial groups adapted to degrade the new added organic sources. It is already well known that long-term applications of organic manure are beneficial for the accumulation of soil organic matter and thus improve different aspects of soil fertility (Liang et al., 2012). This organic amendment can either enhance or decrease the population of certain bacterial phyla as observed in this experiment.

An important aspect of this study was the attempt to better understanding the effect of DCD on active soil microbial communities. DCD temporarily blocks the active site of the enzyme ammonia monooxygenase, reducing the growth of microorganisms involved in the first step of nitrification process. Slowing down the nitrification pathway may also increase the availability of NH_4^+ in soil which might support the growth of specific microbial groups (Prosser and Nicol, 2012). Comparisons in the microbial relative abundances at the order level revealed significant differences between soils treated with slurry only and with slurry + DCD at the third day after fertilization. Inferring the ecological role of detected orders remains challenging because the information about their lifestyle is limited. Therefore, our explanation here is generalized.

The soil microbial structure was substantially similar in the presence or absence of DCD, indicating non-significant effects of this nitrification inhibitor in the overall active microbial community. DCD is highly soluble in water and easily leached after high intensity rainfalls. According to the data shown in Supplementary Figure 1, no high intensity rainfalls occurred during the experiment, suggesting that DCD was lost in deeper soil layers and that the lack of significant changes in microbial communities was not masked by DCD losses. A few recent studies have suggested that the use of DCD does not change the diversity of bacterial community, with predominant phyla remaining in similar proportions compared to unfertilized soils (O'Callaghan et al., 2010 and Morales et al., 2015). While these observations are largely in agreement with our study, these assessments tested the effect of DCD on microbial community with urine applied as nitrogen source whereas our study tested the effect of DCD on soil microbial community fertilized with swine slurry composed mainly of feces,

urine and water. Additionally, the previous studies were based on DNA-based microbial community while our study is RNA-based active microbial community.

The use of DCD does not change the overall microbial community structure but presented a significant effect on specific groups of microorganisms at genus level. Comparing microbial communities from soils fertilized with swine slurry (with and without DCD) with microbial communities from unfertilized soils, we found negative influence of DCD on the relative abundance of Nitrospirae, an important microbial group related to the nitrogen cycle. Furthermore, comparisons between the relative abundance of soil microbial communities fertilized with swine slurry with and without DCD at the third day of experiment revealed that DCD significantly reduced the relative abundance of Nitrosomonadales. This order includes the Nitrosomonas genus responsible for the first step of nitrification pathway. These results support the findings reported earlier that ammonia oxidizers are reduced when nitrification inhibitors are applied (Di et al., 2009, Di et al., 2014 and Zhang et al., 2012). In our work, the application of DCD decreased NO_3^- concentration by 44% and N_2O emissions by 70% compared to the treatment with slurry swine application without DCD during the 50 days of experiment. The greatest emission of N_2O was observed at day 3 coinciding with the most differences found in soil microbial community. Even so, the rainfall of 22 mm at day 3 (see Supplementary Figure 1) might facilitated the nitrous oxide production by contributing to the denitrification process. Although either rainfall or irrigation occurred during the days preceding the soil sampling at days 6, 25 and 50, the impact on N_2O emissions was smaller indicating that besides soil moisture, other factors, like the gradual reduction in available carbon, contributed to the decrease of this gas emission. The use of DCD clearly mitigated the N_2O emissions, but the differences in the effectiveness to reduce N losses reported in the literature probably occurred due to different experiments with variable factors such as soil type, moisture and temperature, nitrogen sources (urine or manure), different field cultivation systems and nitrogen application sources and rates (Kelliher et al., 2008, Sprosen et al., 2009, Dai et al., 2013, Luo et al., 2013, Aita et al., 2014 and Di et al., 2014). Differences in the indigenous soil microbial community might also contribute to such differences in effectiveness of DCD.

In conclusion, organic fertilizers were the main drivers of microbial community structure. Slurry application appeared to decrease diversity and changed the microbial structure temporarily. However, the metabolically active microbial community was resilient recovering to its original status after 50 days post fertilization. DCD had no effect on

metabolically active microbial community and was pathway-specific, having impact only on nitrifiers during a short-term period, which in turn reduced the N_2O emissions.

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SUPPLEMENTAL MATERIAL 1

Supplementary Table 1

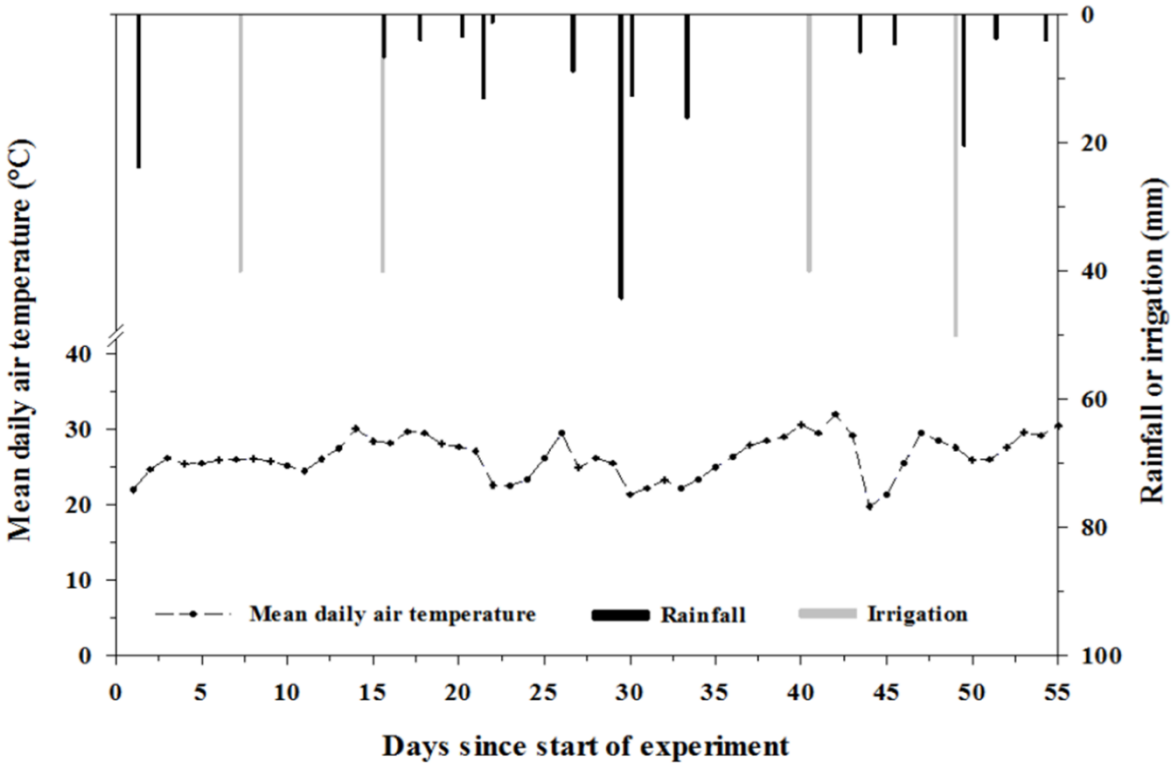
Application rate of swine slurry (SS), selected characteristics of the (SS), and quantities of total C, total N and total ammonia-N (TAN-N) present in soil samples at each growing season.

			Swine slurry composition				Soil characteristics[2]		
Crop/year of planting	SS rate/ m^3 ha^{-1}	pH	DM*	Total C	Total N	TAN-N	Total C	Total N	TAN-N**
			---------- kg m^{-3} ----------				---------- kg ha^{-1} ----------		
Corn/2011	50.0	8.2	27.0	7.0	2.99	2.35	351.0	149.5	117.5
Oat/2012	40.0	6.1	37.0	12.8	3.99	2.81	512.0	159.0	112.4
Corn/2012	49.5	7.2	23.0	7.3	3.28	2.42	362.8	162.3	119.8
Wheat/2013	40.0	6.9	41.1	10.8	3.76	2.75	431.6	150.2	110.0
Corn/2013[1]	50.0	7.6	23.0	8.2	3.29	2.60	410.0	164.5	130.0
Total amount	229.5	-	-	-	-	-	2067.4	785.5	589.7

[1] Soil sampling for the present work was made during the growing of Corn/2013).
[2] Evaluated after application of swine slurry
*DM (Dry Matter)
** TAN-N (total ammoniacal N) = $NH_3 + NH_4^+$
Total N and total ammoniacal N were determined in fresh SS by the Kjeldahl method and by distilling in the presence of MgO, respectively. Total C in manure solids was determined by dry combustion, and pH was measured directly in the slurry.

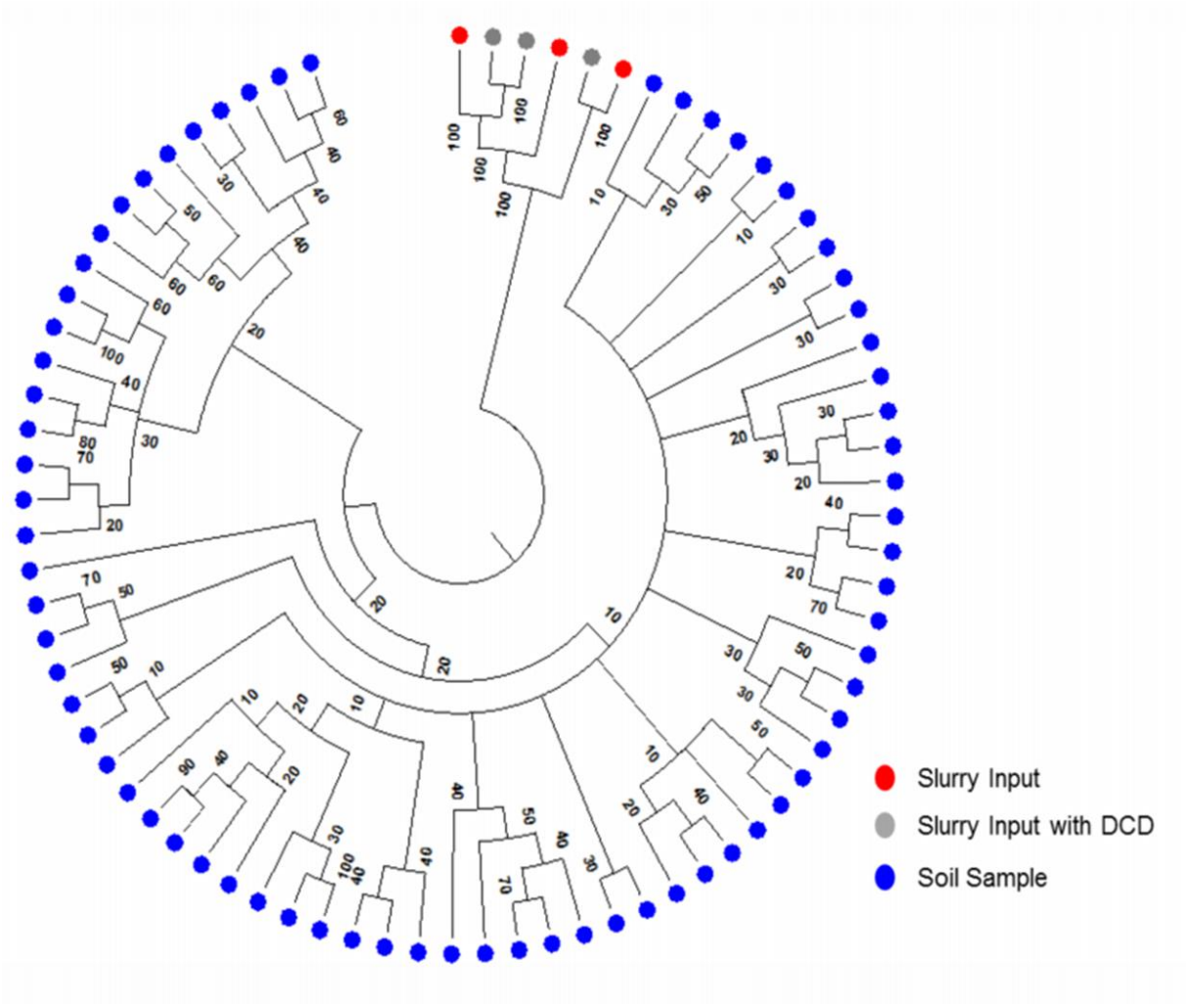
Supplementary Table 2. Per-library calculations of Good's coverage.

Days after fertilization	**Control**	**NPK**	**Slurry**	**Slurry with DCD**
Good's coverage				
0	0.92 ± 0.005	0.90 ± 0.032	0.89 ± 0.072	0.91 ± 0.020
3	0.90 ± 0.011	0.88 ± 0.056	0.92 ± 0.015	0.93 ± 0.000
6	0.89 ± 0.005	0.86 ± 0.015	0.86 ± 0.058	0.88 ± 0.045
11	0.88 ± 0.075	0.88 ± 0.034	0.89 ± 0.005	0.81 ± 0.062
25	0.85 ± 0.020	0.85 ± 0.010	0.87 ± 0.017	0.86 ± 0.010
50	0.90 ± 0.020	0.90 ± 0.011	0.90 ± 0.017	0.86 ± 0.060
Good's coverage				
slurry input	0.96 ± 0.011	-	-	-
slurry input + DCD	0.97 ± 0.005	-	-	-



Supplementary Figure 1.

Rainfall, irrigation and mean daily air temperature during N_2O emission analyses of experiment.



Supplementary Figure 2.

UPGMA dendrogram based on weighted UniFrac distances among microbial communities found in the swine slurry used for fertilization (slurry input and slurry input with dicyandiamide) and soil microbial communities across the 78 samples collected along different treatments and days after fertilization. Consistency of each cluster was measured by Jackknife support shown at each node.

4 ARTIGO II

Recycling crop residues in agriculture impacts soil-borne microbial structure and function and N_2O emissions*

* Artigo elaborado de acordo com as normas da Agriculture, Ecosystems & Environment

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4.1 Abstract

Conservational agriculture practices such as recycling and retention of crop residues from previous or others crops, have been proposed as sustainable alternative to improve soil structure and increase stock of nutrients. Returning straw to the field is a common management regime, however, the inadequate and indiscriminate discharge of agricultural wastes as vinasse in the environment may have a specific disturbance contributing significantly to nitrous oxide emissions (N_2O). Furthermore, information about the magnitude and duration of disturbances caused by crop wastes on soil microbial community is still scarce. The objective of this study was to investigate how tillage-crop residue management with N fertilizer application affected the bacterial community structure and function, and N_2O emissions. An experimental field was cultivated with sugarcane and carried out using the following treatments: (I) control with NPK, (II) sugarcane straw with NPK, (III) vinasse with NP, and (IV) vinasse plus sugarcane straw with NP. Soil samples were collected 1, 3, 6, 11, 25 and 46 days after start of experiment. Total DNA was extracted and used as template for shotgun Illumina sequencing. Nitrous oxide emissions were also quantified. Treatments with agricultural residues induce changes in soil microbial composition and microbial functions. The combination of straw and vinasse appeared to be more powerful in altering the bacterial community than the addition of those residues separately with different organisms responding differently. Phosphorus, iron and nitrogen metabolism subsystems were overrepresented in straw treatment. Phosphorus uptake and iron metabolism increased in vinasse plus straw treatment while in vinasse treatment did not present any function in higher abundance. Different microorganisms were related to same biogeochemical functions for distinct treatments indicating tendency for functional redundancy. All crop residues addition

contributed to increase N_2O emissions and the power combination of vinasse plus straw was not only perceptible in the taxonomic and functional view, but also contributed by 56.1% for N_2O emissions. The results are of great importance to reflect on the impacts of crop wastes and their crop residues managements under the ongoing issue of sustainable agricultural managments.
Key-words: straw, vinasse, metagenome, agricultural practices, sugarcane plantations, crop managments.

4.2 Introduction

Sustainability is a worldwide current concern in agriculture that brings important challenges for agroecosystem management. Agriculture anthropogenic activity is one of the most impacting practices that affect the soil properties and consequently soil functioning. Conservational agriculture practices, such as crop residues retention from the previous or others crops, have been proposed as alternative to improve soil structure and soil protection by reducing erosion and runoff (Boulal et al., 2011; Brouder and Gomez-Macpherson, 2014), increase stock of plant nutrients and soil organic matter content, enhance fertility and soil quality (Bhattacharyya et al., 2013; Jemai et al., 2013), and increase crop yields (Ussiri et al., 2009). Consequently, a major objective in any sustainable agricultural system is to add crop residues in different forms, e.g. types of manure and compost, straw and other agricultural wastes such as animal bedding (poultry litter), organic material from excess production or insufficient market (grass silage) and arable crop residues as husks.

In fact, the returning of straw to the field is a common management regime and results showed that crop straw application can provide a source of available C and N for soil (e.g., Li et al., 2013). However, the inadequate and indiscriminate discharge of agricultural wastes in the environment may have a specific disturbance and impact on the soil. One example is manure amendments and, more recently, vinasse residue generated as 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). Brazil, is currently one of the largest sugarcane ethanol producer generating great volumes about 8–15 litters of vinasse for every liter of alcohol produced (Freire and Cortez, 2000). Researchers have then focused on finding adequate uses and treatments for vinasse. As one alternative, this residue is applied mainly in sugarcane plantations as fertilizer that focuses on the rational use of natural resources. Preventing their discharge in rivers, vinasse is acknowledged to play a dominant role in soil fertility management through their short-term

effects in fertilizing agricultural land (fertirrigation) (Gianchini and Ferraz, 2009). Generally, the vinasse is applied directly to soil because of its organic matter and nutrient content (especially potassium, but also nitrogen and phosphorus). However, vinasse application together with inorganic fertilizers has a negative impact contributing significantly to the greenhouse gas (GHG) emissions to the atmosphere, especially of nitrous oxide (N_2O) and predominantly in the presence of the straw on the soil surface (Carmo et al., 2013). N_2O is an important component of the global biogeochemical nitrogen cycle, playing a significant role on global warming and the depletion of stratospheric ozone (IPCC, 2007; Ravishankara et al., 2009).

The effect of agricultural practices on soil microbial communities is important because their central role in ecological function and biological stability (Griffiths and Philippot 2013), which can be closely related to soil quality. Soil microbes are primary mediators of organic matter decomposition and nutrient cycling by playing important roles in several biogeochemical soil processes (Falkowski et al., 2008). Fertilization practices, tillage and crop residue management might also result in effects on soil microbial community structure (Carbonetto et al., 2014). Field studies have demonstrated that different management strategies with straw and vinasse can alter soil bacterial community composition (Huang et al., 2012; Navarrete et al., 2015). These studies revealed that straw application increased the microbial community metabolic activity and vinasse amendment can cause positive or negative effects on different microbial groups (Navarro-Noya et al., 2013; 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's living biomass and their community composition (Navarro-Noya et al., 2013; Sengupta and Dick, 2015) and no studies have applied metagenomics to investigate in temporal resolution the dynamics of microbial community responses to residues in agricultural management. Quantify how communities and their functions change through time are important to understand processes such as succession or recovery from perturbations. Then, ecologists and agronomists are still far from comprehend the effect of specific crop practices on microbial communities, on their ecological functions, and on their ultimate effects on agroecosystems.

To our knowledge, no metagenome study conducted in a sustainable agriculture perspective has yet investigated the impact of fertilizers with freshly agricultural residues in the dynamics of bacterial communities. Key issues that we aimed to address here are (i) how crop residue amendments affect the dynamics of microbial taxa and functions in short-term field experiment and (ii) how crop residue amendments influence the soil N_2O emissions

treatments. These results are of paramount importance to properly manage crop residues under the scenario of land use and land cover changes and increase the knowledge to predict the sustainability of a soil under a specific agronomic practice. Here, we hypothesized that different crop residues have distinct effects on microbial communities, with straw having no or less impact on bacterial communities and traits, while treatments with vinasse having temporary impacts favoring copiotrophic (i.e., fast-growing, low C use efficiency) bacterial taxa. The objective of this study was to investigate how tillage-crop residue management with N fertilizer application affected the bacterial community structure and function, and N_2O emissions.

4.3 Material and Methods

4.3.1 Experimental setup and soil sampling

The field experiment was established in Piracicaba municipality, São Paulo state, Brazil (22°41019.34″S; 47°38041.97″W; 575 m above sea level). The mean annual air temperature and precipitation were 21°C and 1390 mm, respectively. The soil was classified as Haplic Ferralsol with pH of 5.1, organic matter of 2.3 g dm^{-3}, P of 16 g dm^{-3}, K^{+} of 0.7 $mmol_c$ dm^{-3}, Ca^{+2} of 19 $mmol_c$ dm^{-3}, Mg^{+2} of 11 $mmol_c$ dm^{-3}, H^{+} + Al^{+3} of 34 $mmol_c$ dm^{-3} and 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 randomized block design as follow: (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 K source and its composition is presented in Supplementary Table 1. Before the experiment, all previous crop residues were removed from soil surface by harvesting and without burning. After harvesting, the straw was left in the treatment with straw and vinasse plus straw and removed for the others. For all treatments, soil sampling was carried out in 8 time points after 1, 3, 8, 14, 20, 24, 30, and 46 days of crops addition and were collected from three soil cores at the fertilizer line position and depth of the top 10 cm for each plot. As usually performed in commercial areas, vinasse (at rate of 1.10^5 l ha^{-1}) and straw was applied to the total area (at rate of 10 t ha^{-1}), mineral fertilizer with N as ammonium nitrate (at a rate of 100 kg N ha^{-1}), P as superphosphate (at rate of 17 kg ha^{-1}) and K as potassium chloride (at rate of 100 kg ha^{-1}) were applied in lines parallel to the crop line.

4.3.2 DNA extraction

Total community 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. All DNA samples were stored at -20 °C until used in downstream analyses. DNA concentration and quality were determined by spectrophotometry (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA), and by agarose gel electrophoresis.

4.3.3 Library preparation, annotation of metagenome sequences and data analysis

Shotgun metagenome libraries were constructed following the Illumina Paired-End Prep kit protocol and sequenced at Macrogen Inc. Company, South Korea using using 2 × 300 bp sequencing run on Illumina MiSeq2000 (Illumina, San Diego, CA) technology. Sequencing demultiplexing was performed on the Illumina MiSeq instrument using sample specific barcodes. 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 M5NR database with a maximum e-value cutoff of 10^{-5}, a minimum percent identity cutoff of 60% and a minimum alignment length cutoff of 15 and Hierarchical Classification subsystems with a maximum e-value cutoff of 10^{-5}, a minimum percent identity cutoff of 60% and a minimum alignment length cutoff of 15. All compared distributions were normalized as a function of the number of annotated sequences for each metagenome library.

Principal component analyses (PCAs) plots were calculated by using the affiliated taxonomic and functional abundance matrices in order to visualize the microbial taxonomic and functional structure among samples on normalized genus counts. To test whether sample categories harbored significantly different metagenomes or microbial communities, we used PERMANOVA analysis implemented in PRIMER 6+ (Clarke and Gorley, 2006). Data corresponding to both functional and taxonomic distributions also were statistically analyzed by STAMP software (Parks and Beiko 2010). Relative abundances of individual taxa or function of samples were compared using pairwise t tests followed by the Welch's t test ($p < 0.05$) with the Benjamini-Hochberg correction for multiple comparisons. Reads assigned by MG-RAST v3.0 to KEGG pathways related to nitrogen, potassium, phosphorus, sulphur and iron metabolism were extracted and classified taxonomically using BLASTX against the M5NR database on the MG-RAST v3.0.

4.3.4 N_2O *measurements and soil chemical analysis*

The fluxes of N_2O were measured using closed chambers using the chamber-based method (Varner et al., 2003), at the fertilized 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 and 12:00 a.m. at 1, 10, 20, 30 and 60 minutes after chamber closure using syringes, with 20-ml-evacuated penicillin flasks sealed with gas-impermeable butyl-rubber septa (Bellco Glass 2048) and analyzed 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 (Allen et al., 2010). 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 colorimetrically by a using flow injection analysis (FIAlab 2500).

4.4 Results

4.4.1 General overview of the soil communities

Using a shotgun metagenomics approach, 12,036,555 sequences were obtained after trimming (paired-end reads with average length of 113 bp). From the total of 96 samples, 84 samples could be annotated and recovered from each of the eight sampling time points, with three replicates per time point. The samples quality and excluded samples im MG-RAST are shown in Supplementary Table 2.

The taxonomic assignments elucidated in this investigation spanned prokaryote (bacteria and archaea). In average, 94.2% were assigned to prokaryotes with the majority of the shotgun metagenome reads assigned to bacteria (94.3%) and small fraction (5.7%) to archaea. The bacterial community was composed of 27 phyla, dominated by *Proteobacteria* (41.4%) followed by *Actinobacteria* (15.5%), *Bacteroidetes* (6.5%), Cyanobacteria (3.6%), *Firmicutes* (1.9%), *Chloroflexi* (1.17%) and Spirochaetes (1%), while archaeal community was composed by the 3 main phyla Euryarchaeota (56%), Crenarchaeota (21.5%) and Thaumarchaeota (1.7%).

4.4.2 Patterns of the dynamics of taxonomic and function structure among distinct crop residues amendments

In order to assess the temporal effect of the crop residues amendments on the microbial community structure, the taxonomic and functional profiles were compared at different time points using a combination of ordination and dissimilarity test. The Principal Component Analysis (PCA) plots revealed that treatment exhibited large within-sample variation (Figure 1 and 2). For bacteria and functions, the first and second principal components explained 55.8% and 39.6% of the variance, respectively. Comparative metagenomics analysis of the bacterial community structure revealed no clear separation among treatments for each time point for community composition and functions. PERMANOVA analysis corroborated with PCA analysis showing no differences between treatments in each day after crop residues addition in soil, i.e., considering the interaction between treatment and time (Table 1). However, a significant difference was found only for crop residue addition in soil (Pseudo-F values = 1.75, 1.69 and 1.65 for straw, vinasse and vinasse + straw, respectively; $P < 0.0001$, Table 1).

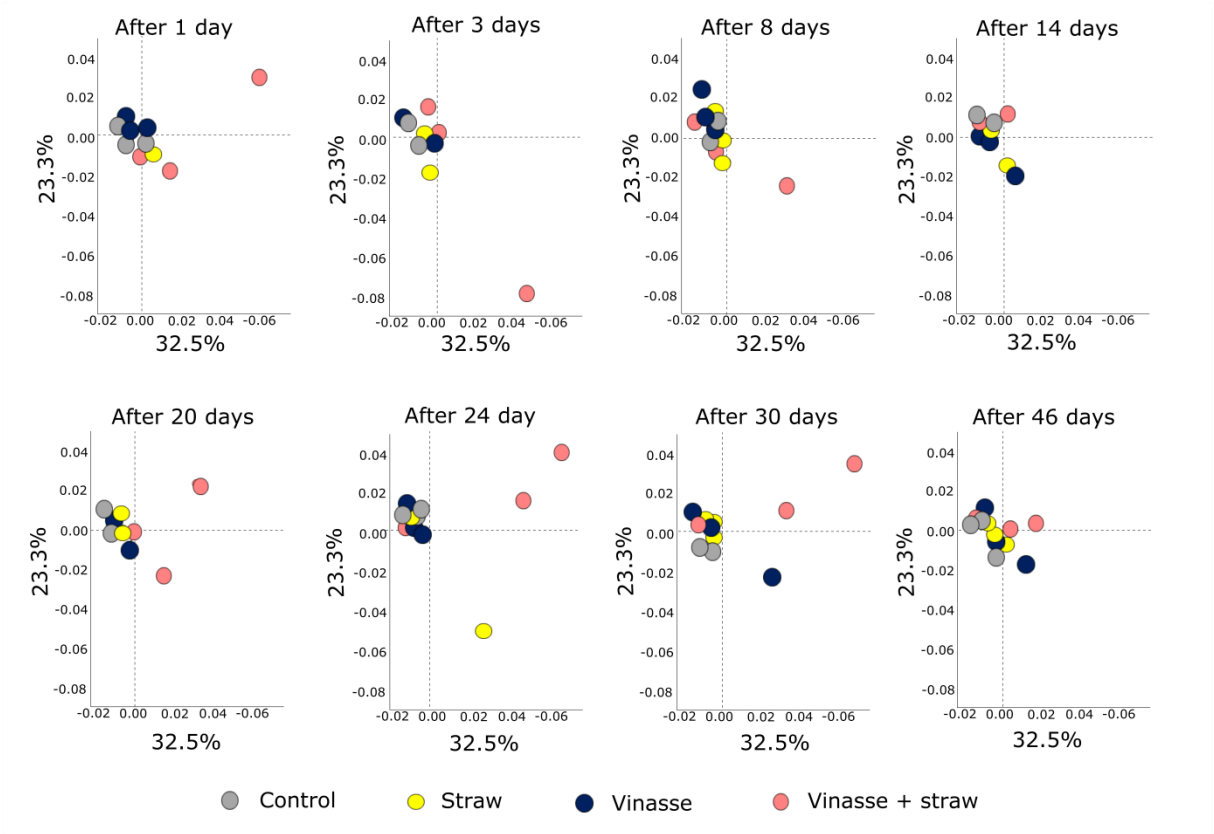

Fig.1.

Temporal changes in the soil microbial community depicted by Principal Component Analysis (PCA) in soils cultivated with sugarcane during eight periods following applications of different residues straw, vinasse and vinasse plus straw. Each point represents an individual sample, with colors indicating treatments.

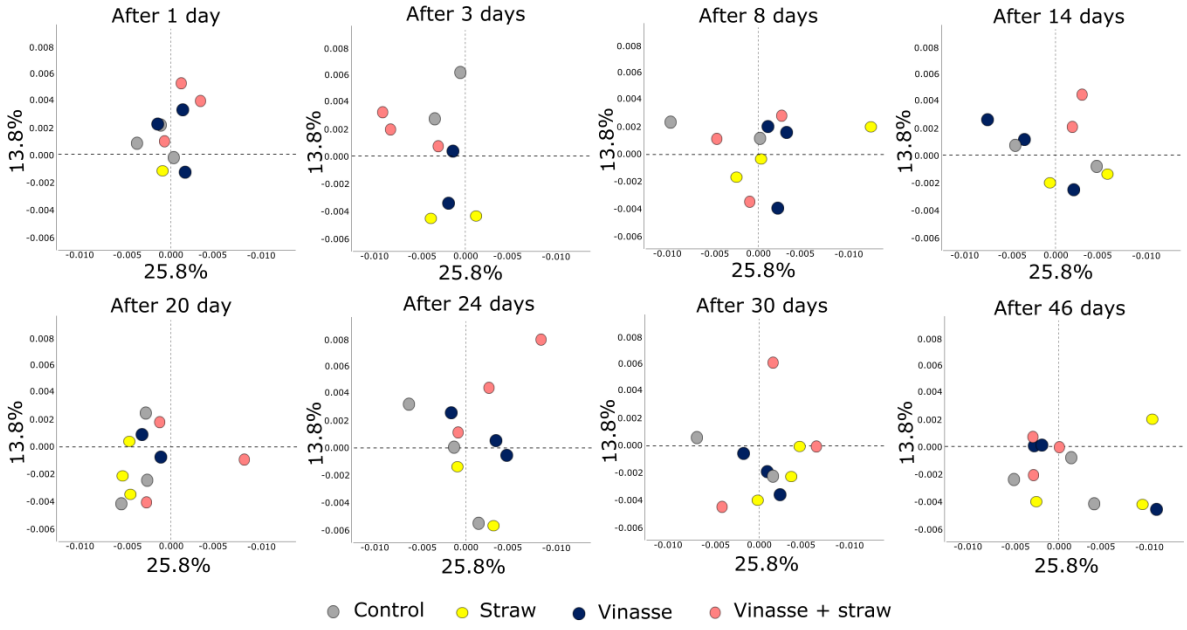

Fig. 2.

Temporal changes in the soil microbial community functions depicted by Principal Component Analysis (PCA) in soils cultivated with sugarcane during eight periods following applications of different residues straw, vinasse and vinasse plus straw. Each point represents an individual sample, with colors indicating treatments.

Table 1 - Effects of crops residues amendments and Permanova pairwise comparisons on taxa level and functions on soil microbial community.

	Taxonomy	**Functions**			
Main test*	Bacteria	Level1	Level2	Level3	Functions
Treatment	**3.06**	**2.26**	**1.26**	**1.2**	**1.15**
Time	0.89	1.13	1.04	1.02	1.01
Interaction	0.99	1.15	1.0	0.9	0.97
C x S	**1.75**	**1.33**	1.07	1.08	1.05
C x V	**1.69**	1.2	1.13	1.1	1.05
C x V+S	**1.65**	**1.61**	**1.27**	**1.17**	1.11
S x V	1	1.9	0.97	1.02	1.04
S x V+S	**1.47**	1.2	1.15	1.1	1.09
V x V+S	**1.5**	1.3	1.09	1.1	1.06

Abbreviations: C-Control; S-Straw; V-Vinasse; V+S-Vinasse plus straw; Values represent the univariate t-statistic (t). Values at P<0.05 are shown in bold.

Differences between taxa and functions for each crop residue

Considering that treatment factor has significant effect on the microbial structure, we investigated the pairwise comparisons between microbial community present in control and each treatment regardless the time. We found differences for pairwise comparisons considering the taxonomic affiliation (Table 1, PERMANOVA, $P<0.05$). In general, PCA clustered bacterial communities by treatment with each pair comparison displaying a greater spread between samples but with separation mainly for treatments with vinasse amendments (Figure 3) with the crop residues additions affected the microbial at order level differently. The relative abundance of *Bacteroidetes* (order *Bacteroidales*) and *Beta-Proteobacteria* (orders *Rodocyclales*, *Hydrogenophiales*, *Burkholderiales* and *Methynlophilales*) increased significantly in straw treatment. Treatments with the addition of vinasse also affected bacteria at phylum level. High proportions of *Verrucomicrobia* was found in vinasse treatment, whereas *Firmicutes* (order *Lactobacillales*) were overrepresented in vinasse plus straw treatment (Figure 4).

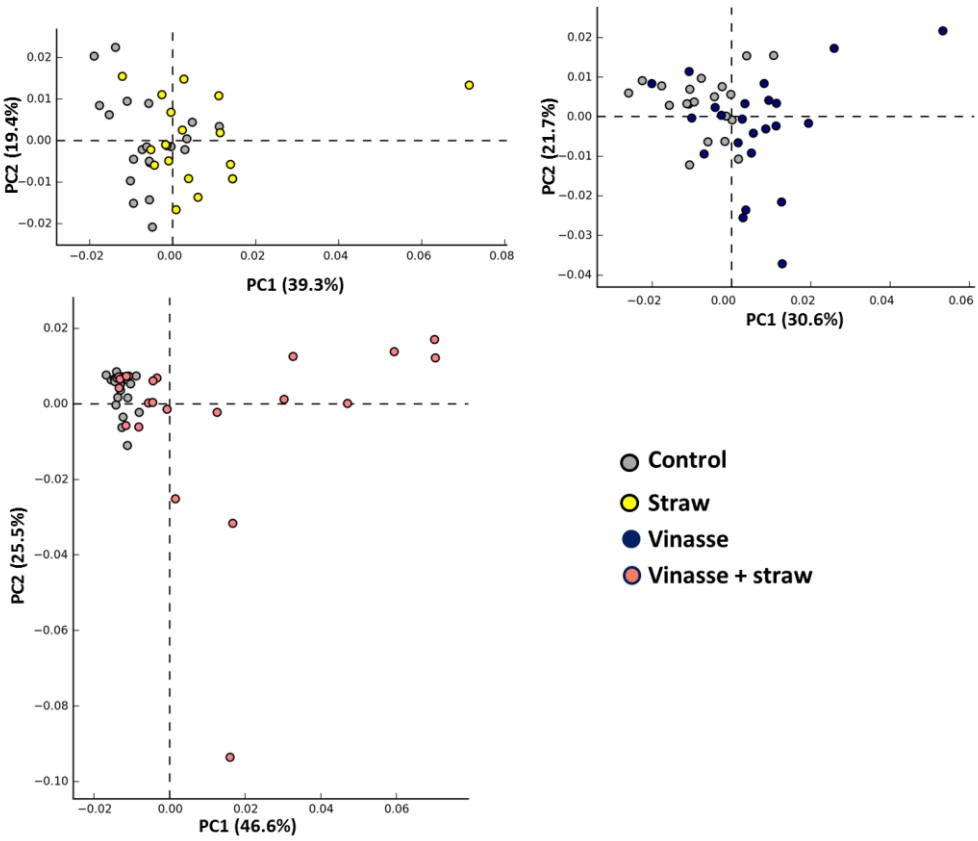

Fig. 3.

Soil microbial community structure depicted by Principal Component Analysis (PCA) in soils cultivated with sugarcane with comparisons in pairs between control and treatments with different applications of residues straw, vinasse and vinasse plus straw. Each point represents an individual sample, with colors indicating treatments.

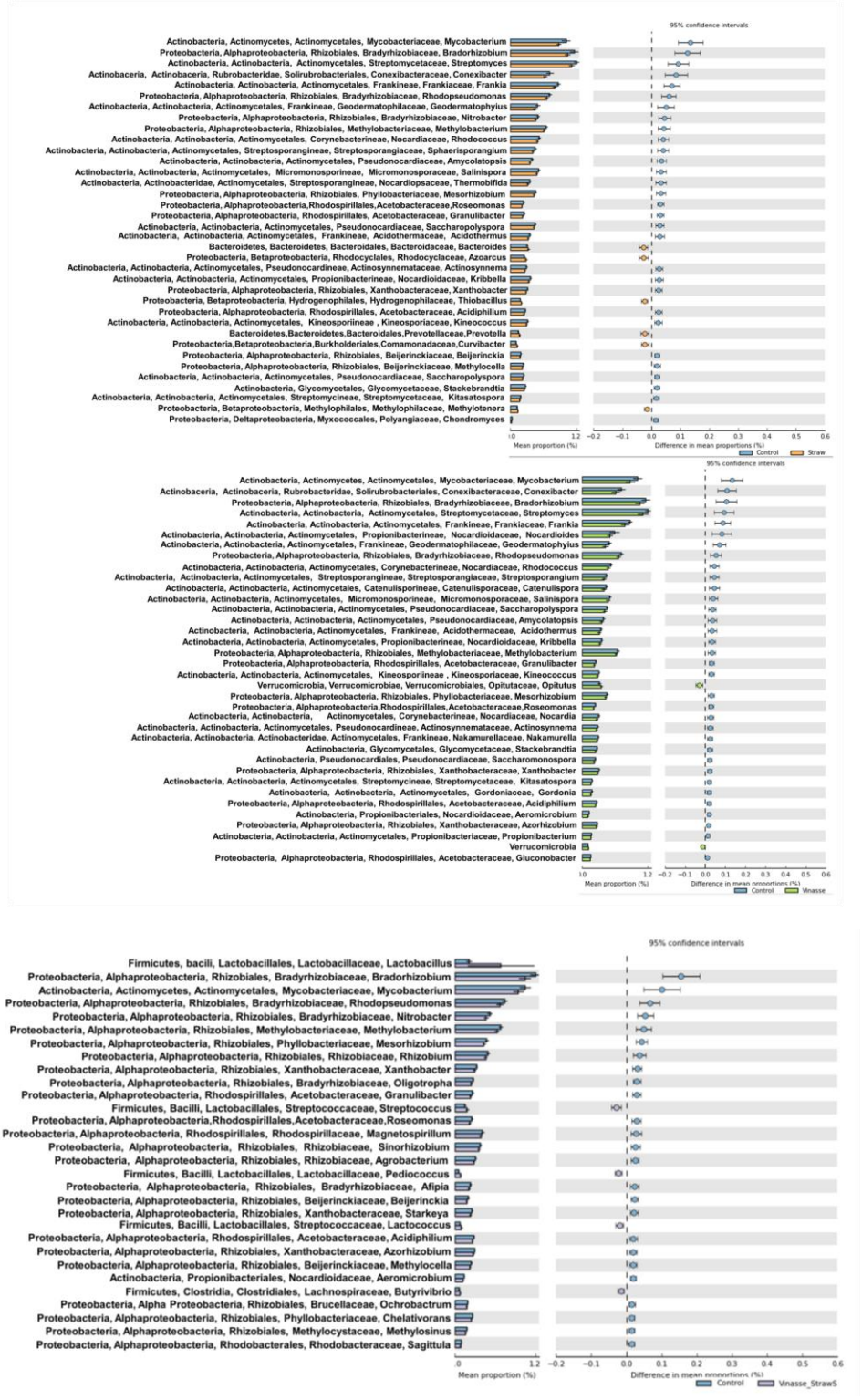

Fig. 4

Differences in the relative abundance of microbial genus between soils without crop residues (control) versus soils with different crop residues. The differences between groups were calculated using the Welch's inverted method. Corrected p-values were calculated with Benjamini -Hochberg test correction. Only significant differences at $p \leq 0.05$ are presented.

In the case of function, significant results were found for the effect of crop residues in microbial communities just for the treatments with only straw (SEED subsystem level 1) and vinasse plus straw (all SEED subsystem levels) comparing with control (Table 1, $P<0.05$). However, the pattern of separation of microbial community structure per treatment is still slightly observed in the PCA ordination analysis. Similarly to taxonomic affiliation, we found spread of microbial variation inter-samples with separation of crop residues addition when compared with control treatment for SEED subsystem level 1 (Figure 5). The PERMANOVA analysis based on SEED subsystems showed that the microbial community of vinasse plus straw compartmentalization comprises the largest source of functional variation compared to separated residues, only vinasse or only straw (Pseudo-$F = 1.61$; $P<0.05$). Different category functions (Figure 6) were selected for each treatment, however in general most of the categories related to core metabolic functions (e.g., respiration, carbohydrates and lipid metabolisms), virulence, aromatic, sulphur and potassium metabolisms showed relatively higher abundance in control than in crop residues treatments. Among the functional categories identified by MG-RAST, the shared categories were in treatments with straw and with vinasse plus straw as protein-, RNA-, DNA-, phosphorus-, secondary- and iron-metabolisms, motility and chemotaxis, vitamins, phages, signaling and photosynthesis. Moreover, interesting metabolic pathways appeared to be crop residue specific when compared with only NPK addition (control). Phosphorus, iron and nitrogen metabolism subsystems were overrepresented in straw treatment, phosphorus uptake and iron metabolism increased significantally in vinasse plus straw treatment while in vinasse treatment did not present any function in higher abundance.

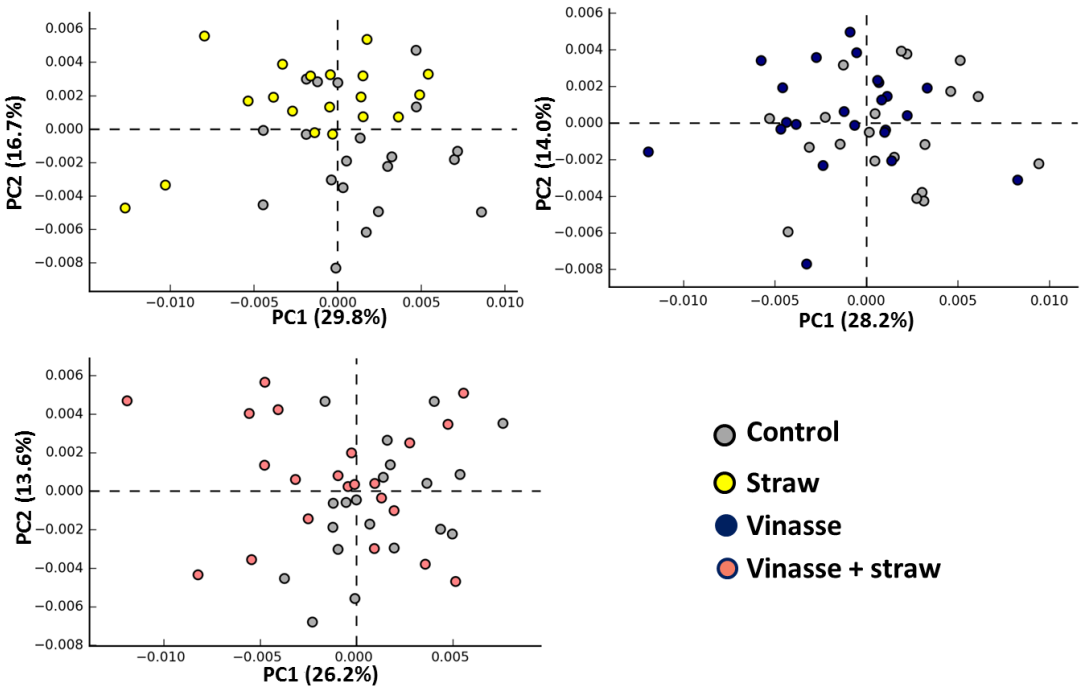

Fig. 5

Soil microbial community functions depicted by Principal Component Analysis (PCA) in soils cultivated with sugarcane with comparisons in pairs between control and treatments with different applications of residues straw, vinasse and vinasse plus straw. Each point represents an individual sample, with colors indicating treatments.

Fig. 6

Differences in the relative abundance of functions between soils without crop residues (control) versus soils with different crop residues. The differences between groups were calculated using the Welch's inverted method. Corrected p-values were calculated with Benjamini -Hochberg test correction. Only significant differences at $p \leq 0.05$ are presented.

4.4.3 Bacteria associated with nutrients and biogeochemical cycles

To get a more in-depth understanding of the functions traits selected in each treatment we analyzed at genus level, the phylogenetic bins of the sequences constructed from the community metagenomes. The abundances of taxa identified as the corresponding contributor of metabolism genes and pathways associated with important nutrients in soil and biogeochemical cycles as nitrogen, potassium, phosphorus, sulphur and iron are shown in Figures 7 to 11.

Mostly, using the functional classification as the starting point before identifying the responsible bacteria for that specific function we had almost the same picture about the modified microorganisms in the taxonomical results but with slight differences between control and crop residues treatments. Abundances of taxonomic-functional relations for the shotgun metagenome data for orders *Actinomycetales* and *Rhodospirillales* were not always supported in control. Moreover, the higher abundances of *Bacteroidetes* and *Beta-Proteobacteria* were not exclusively found in higher abundance in straw. Phylas *Verrucomicrobia* and *Firmicutes* were the only ones with higher abundances in vinasse treatments (Figures 7 to 11).

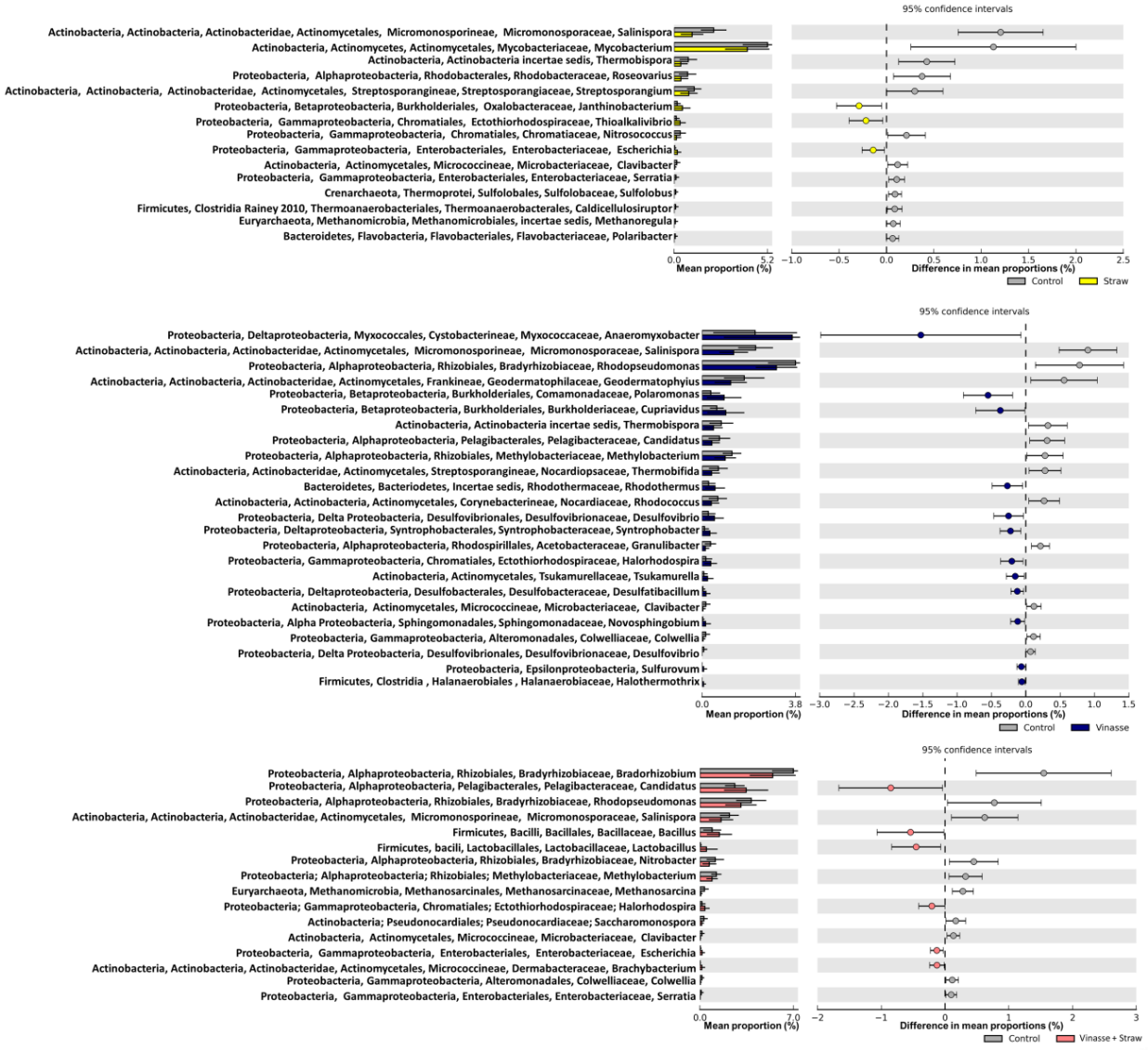

Fig.7.

Microbial genus contributing to nitrogen metabolism correlated between soils without crop residues (control) versus soils with different crop residues. The bars indicate the percentage of contribution of microbial genus to each the selected functional category. Corrected p-values were calculated with Benjamini -Hochberg test correction. Only significant differences at $p \leq 0.05$ are presented.

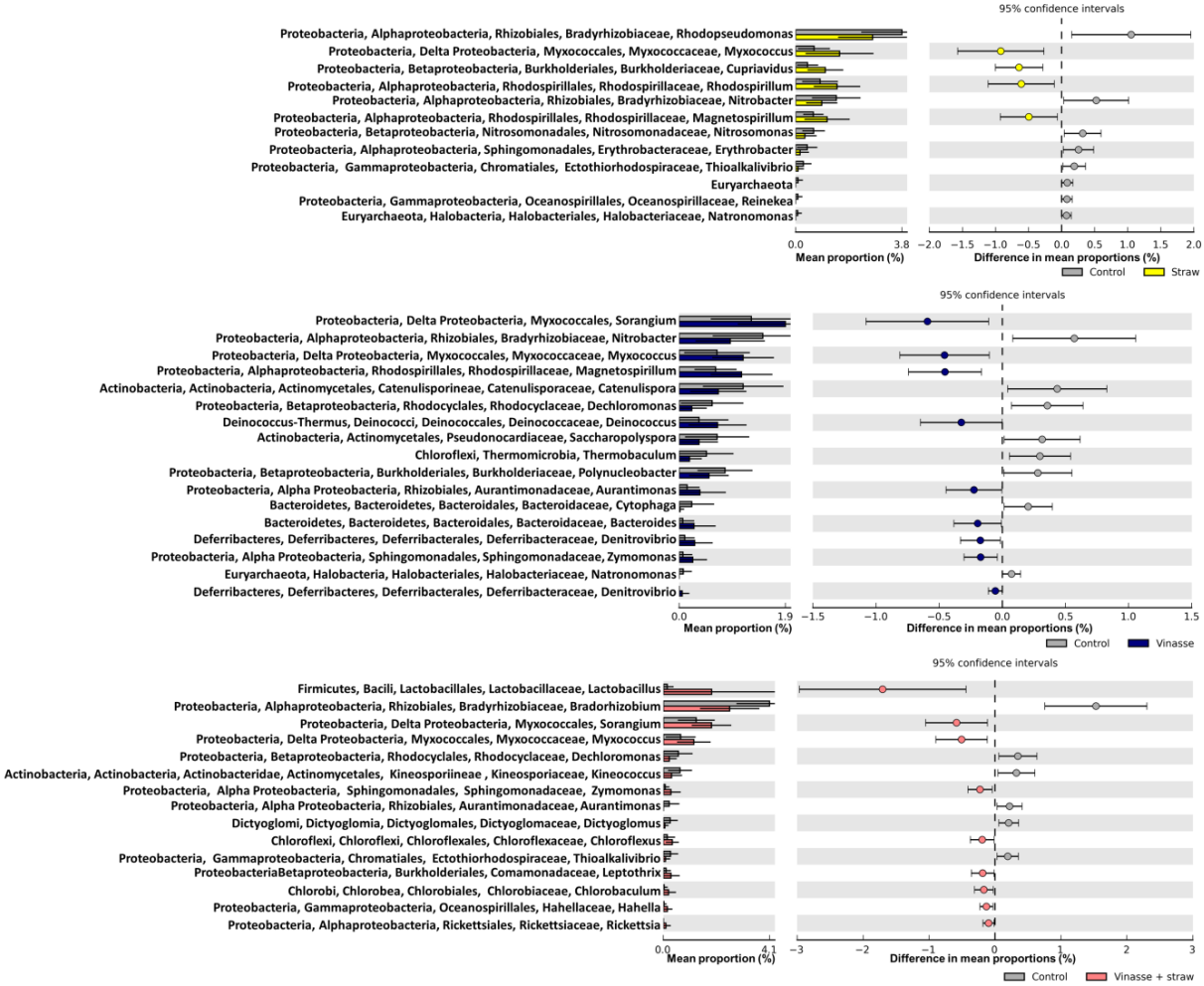

Fig. 8.

Microbial genus contributing to phosphorus metabolism correlated between soils without crop residues (control) versus soils with different crop residues. The bars indicate the percentage of contribution of microbial genus to each the selected functional category. Corrected p-values were calculated with Benjamini -Hochberg test correction. Only significant differences at $p \leq 0.05$ are presented.

Fig. 9.

Microbial genus contributing to sulphur metabolism correlated between soils without crop residues (control) versus soils with different crop residues. The bars indicate the percentage of contribution of microbial genus to each the selected functional category. Corrected p-values were calculated with Benjamini -Hochberg test correction. Only significant differences at $p \leq 0.05$ are presented.

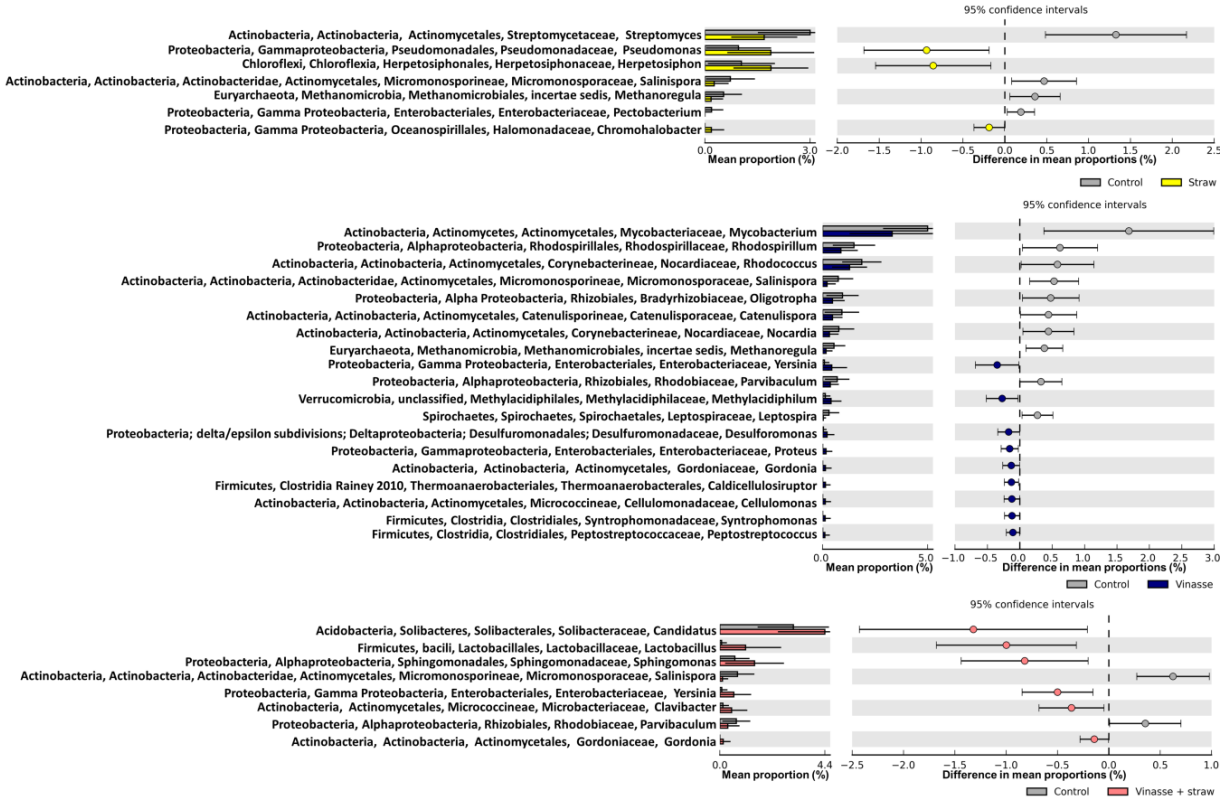

Fig. 10

Microbial genus contributing to potassium metabolism correlated between soils without crop residues (control) versus soils with different crop residues. The bars indicate the percentage of contribution of microbial genus to each the selected functional category. Corrected p-values were calculated with Benjamini -Hochberg test correction. Only significant differences at $p \leq 0.05$ are presented.

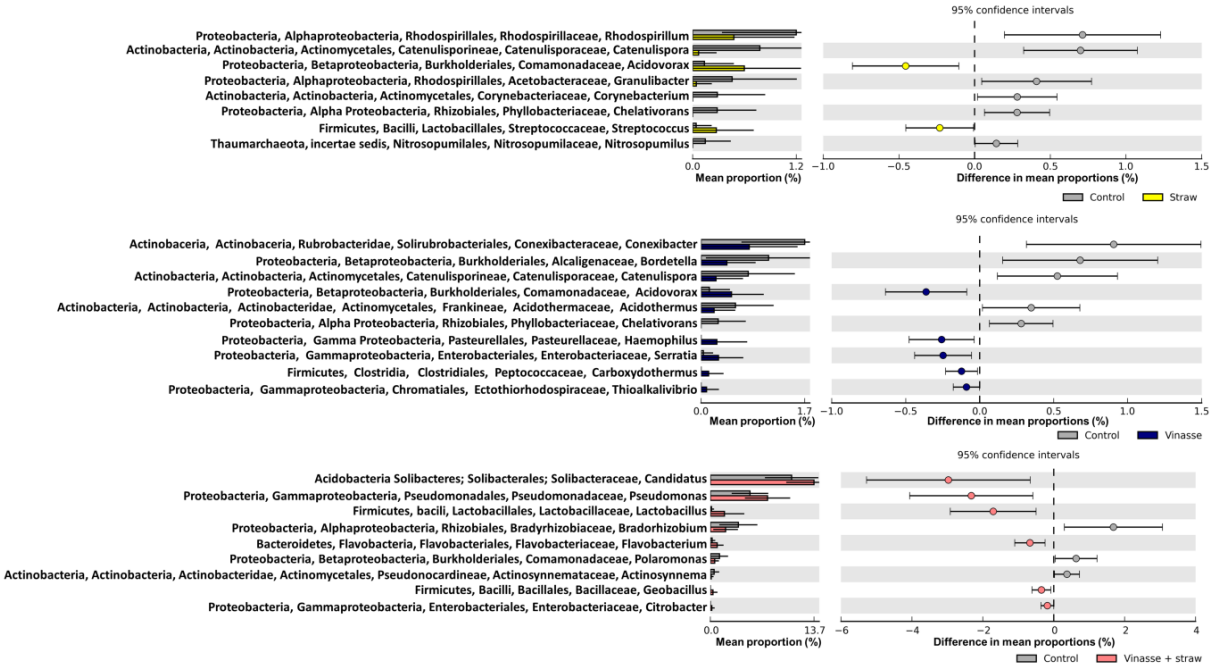

Fig 11.

Microbial genus contributing to iron metabolism correlated between soils without crop residues (control) versus soils with different crop residues. The bars indicate the percentage of contribution of microbial genus to each the selected functional category. Corrected p-values were calculated with Benjamini -Hochberg test correction. Only significant differences at $p \leq 0.05$ are presented.

In all the treatments with crop residues, the nitrogen metabolism category was represented in high abundance by *Gamma-Proteobacteria* (family *Ectothiorhodospiraceae*); the phosphorus metabolism category was represented by *Delta-Proteobacteria* (order *Myxococcales*); the sulphur category by *Beta-Proteobacteria* (*Burkholderiales*) and *Verrucomicrobia* (order *Verrucomicrobia*les). The others biogeochemical cycles did not present common taxa among different crop residues. Common taxa were also found for both vinasse treatments with phosphorus metabolism with higher proportions of *Alpha-Proteobacteria* (order *Shpingomonadales*); sulphur category overrepresented by phylums *Deinococcus* (order *Thermales*), *Firmicutes* (order *LactoBacillales*), *Gamma-Proteobacteria* (order *Alteromonadales*) and *Epsilon-Proteobacteria*; the potassium category was overrepresented by *Actinomycetales* (suborders *Micrococcineae* and family *Gordoniaceae*) while the iron metabolism had higher abundance for *Gamma-Proteobacteria* (order *Enterobacteriales*).

Still related to the selected functions of nutrients, we found some microorganisms more abundant in specific-treatment comparing with control. For straw treatment, potassium metabolism had the highest relative abundances of sequences assigned to *Gamma-Proteobacteria* (order *Oceanospirillales* and *Pseudomonadales*) and *Chloroflexi* (order Heretosiphonales); and sulphur category represented by *Alpha-Proteobacteria* (orders *Caulobacterales* and *Rhodobacterales*), *Gamma-Proteobacteria* (order *Vibrionales*), *Bacteroidetes* (order *Cytophagales*) and *Methanomicrobia* (order *Methanosarcinales*).

For vinasse treatment, the corresponding contributing bacterial taxa with higher proportions for nitrogen metabolism were *Alpha-Proteobacteria* (order *Sphingomonadales*), *Delta-Proteobacteria* (orders *Desulfobacterales*, *Syntrophobacterales*, *Myxococcales*), *Epsilon-Proteobacteria*, *Firmicutes* (order *Halanerobiales*) and *Bacteroidetes*; for phosphorus category contributing bacterial groups were affiliated to phyla *Deinococcus* (order *Deinococcales*), *Deferribacteres* (order *Deferribacterales*); for potassium category the bacterial contribuitors were from phyla *Verrucomicrobia* (order *Methylacidiphilales*), *Delta-Proteobacteria* (order *Desulfomonodales*) and *Firmicutes* (orders *Thermoanaerobacteriales* and *Clostridiales*); for sulphur category were *Bacteroidetes* (order *Flavobacteriales*) and; for iron metabolism were selected the taxa *Gamma-Proteobacteria* (orders *Pasteurallales*, *Chromatiales*) and *Firmicutes* (order *Clostridiales*).

When vinasse was applied with straw to the soil, the nitrogen metabolism were represented by reads affiliated to *Firmicutes* (orders *Bacillales* and *LactoBacillales*); the phosphorus metabolism represented by reads affiliated to phyla *Firmicutes* (order *LactoBacillales*), *Chlorobi* (order *Chlorobi*ales), *Alpha-Proteobacteria* (order *Rickettsiales*) and *Gamma-Proteobacteria* (order *Oceanospirillales*); the potassium metabolism by *Acidobacteria* (order *Solibacterales*), *Firmicutes* (order *LactoBacillales*) and *Alpha-Proteobacteria* (order *Sphingomonadales*); for sulphur metabolism reads assigned to *Gamma-Proteobacteria* (family *Idiomarinacea*), *Thermotogae* (order *Thermotogales*) and; for iron metabolism the reads were binned to *Acidobacteria* (order *Solibacterales*), *Gamma-Proteobacteria* (order *Pseudomonadales*), *Firmicutes* (orders *Bacillales*) and *Bacteroidetes* (order *Flavobacteriales*).

4.4.4 N_2O *emissions and mineral N*

The application of crop residues had effect on cumulative and dynamics of nitrous oxide emission during the experiment. During the 46 days of sampling period, the presence of straw on soil had an important role to raise N_2O emissions (Figure 12A). Both vinasse treatments picked higher emissions of N_2O, different from the control treatment but the emissions from soil with vinasse plus straw treatment were generally higher than those from solely vinasse. 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 which rapidly decreased the N_2O emissions later subsequently. After 20 days post experiment, the treatments with crop residues amendment started to increase the magnitude of N_2O emissions until the 30^{th} day. Afterwards, the fluxes of N_2O emissions were reduced. The total average of N_2O emission rates from the soils at 46 days of the experiment were 6.3%, 17.7%, 19.9% and 56.1% for control, straw, vinasse and vinasse plus straw, respectively.

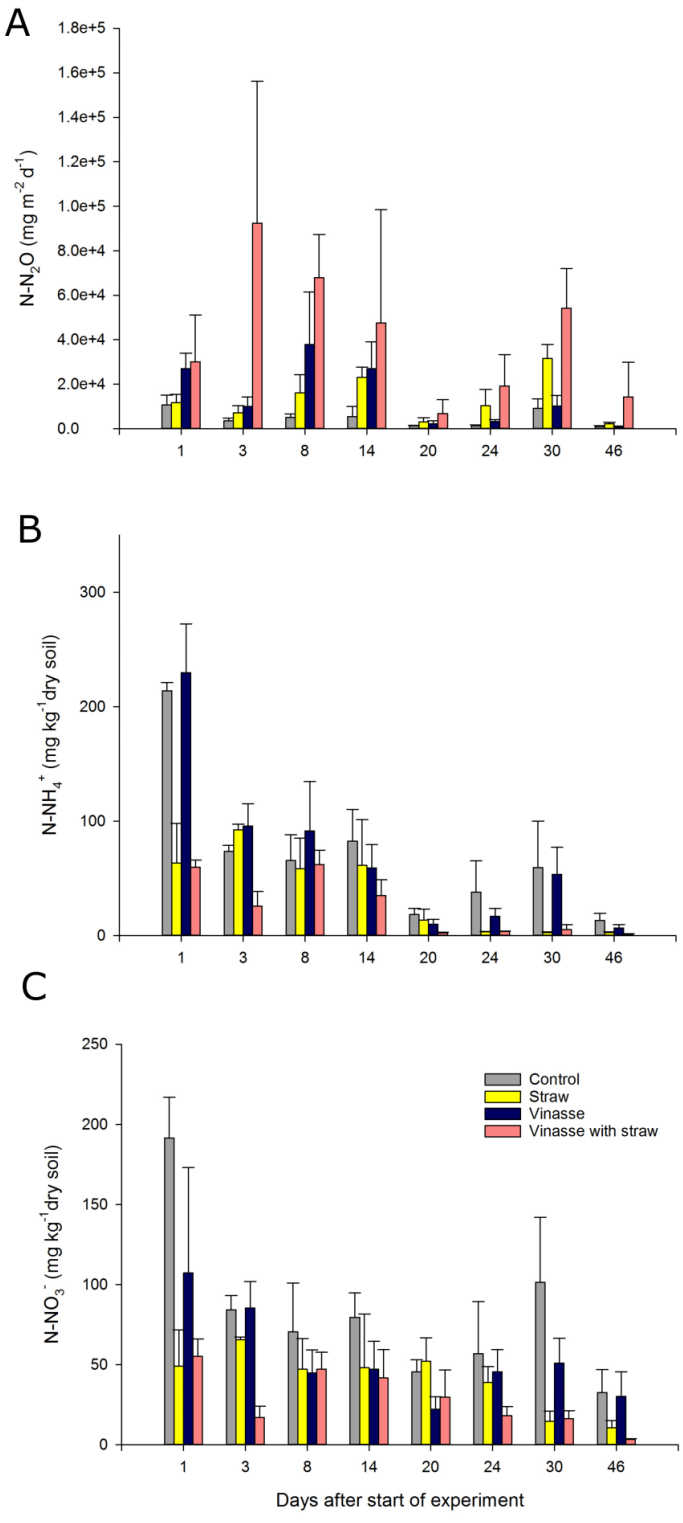

Fig. 12

Nitrous oxide (N_2O) emissions, concentrations of soil ammonium (NH_4^+) and soil nitrate (NO_3^-) following applications of crop residues. Error bars indicate the standard error of mean (n = 4).

The same pattern for N_2O emissions is shown for NH_4^+ content with differences before and after 2 weeks of experiment (Figure 12B). Compared to the control, the addition of crop residues resulted in different contents of NH_4^+ in soil. In general, NH_4^+ content from vinasse plus straw was always less than other treatments for each measured day. At the 1st day, the application of straw decreased 3 times the NH_4^+ content comparing to control treatment showing that the presence of straw altered NH_4^+ levels reaching 63.3 mg kg^{-1} dry soil ($p \leq 0.05$). At the 3rd day, in all treatments there was a decrease of soil NH_4^+ content relatively similar to the control, mainly the treatment of vinasse plus straw, which decreased by reaching 25.7 mg kg^{-1} dry soil ($p \leq 0.05$). After 20 days only vinasse had similar amounts of NH_4^+ to the control while straw and vinasse plus straw addition had less content. The NH_4^+ content of straw and vinasse plus straw treatments decreased twice when compared to control treatment. The NO_3^- content changed in a different pattern to that of NH_4^+ content and N_2O emissions. Soil NO_3^- content was always less in soil treated with vinasse plus straw with average of 28 mg kg^{-1} dry soil comparing to 82 mg kg^{-1} dry soil in control (Figure 12C). During the 46 days of experiment, all treatments with crop residues application decreased the NO_3^- content compared to control and the levels of NO_3^- were declining for those treatments till the end of experiment.

4.5 Discussion

The addition of crop residues as by-product of crop production is a common farming practice in conservation agriculture. Since crop residues are usually 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 agroecosystem. However, it has been reported that straw (Liang et al., 2007; Zhang et al., 2013) and wastes considered organic fertilizers such as manure (Chadwick et al., 2011; Aita et al., 2015) and vinasse (Paredes et al., 2014) contribute to extra gases emissions thereby accelerating greenhouse effects. Considering the fact that agricultural residues together with inorganic fertilizers accelerate gases emissions, in this study we monitored the dynamics of bacterial taxonomic and functional structure and the emission of nitrous oxide (N_2O) in soils amended with different agricultural residues. Studies with 16S rRNA gene have previously shown to be valuable taxonomic genetic marker for analyzing microbial communities, including those associated with residues as straw and vinasse (Navarrete et al., 2015, Pitombo et al., 2015). Here we used shotgun metagenome

approach to provide insight into the taxonomic and functional profiles of soil microorganisms. The short-term effect of crop residues addition revealed treatment-impact rather than temporal effect on soil bacterial community. In general, residue-sensitive taxa and functions were heterogeneously distributed across soils with different waste types. However, some consistent patterns, for example members of *Verrucomicrobia* and *Firmicutes* phyla in the presence of vinasse and metabolisms of phosphorus, iron and nitrogen were observed in different crop residues treatments. In each of biogeochemical cycles we found different organisms probably doing or supporting the same functions indicating functional redundancy. Furthermore, different soil-borne microbes were found mainly with the presence of vinasse which residue can increase N_2O emissions from soil.

4.5.1 Bacterial function and taxa associated to different crop residues amendments

The first factor that we wanted to examine is the temporal variability in soil microbial communities as they can change in response to disturbances and return rapidly to its original stable state (Alisson et al., 2008). In soil scenery, there is a quite considerable time-scale studies in literature focused on microbial driven biogeochemical processes and specific function as an indirect answer for their activity (Strickland et al., 2009) but there is a limited study examining through time how general microbial composition and function respond to agricultural disturbances. In our study, the different treatments did not present temporal variability in microbial community structure during short-term experiment. Our finding was in disagreement with other studies with different perturbation in soil. Gelsomino and Cacco (2006) found that bacterial diversity changed over time in heat disturbance experiment with functional resilience in soils. Furthermore, Suleiman et al. (2016) found that microbial diversity changed temporarily after slurry fertilization but the community was resilient, recovering to the original status. Despite the insignificant time-series variation for taxonomical and functional results, our approach revealed consistent residue addition effects, indicating high spatio variation. These results also draw attention for studies that takes conclusion about environment disturbances without time-scale sampling. Survey efforts lack the temporal resolution to capture rapid community changes, particularly in systems with actively growing microbial populations, in which generation times may be on few minutes (Shade et al., 2013). Although long-term studies are commonly used to assess the effects of fertilization (Pan et al. 2014; Cassman et al., 2016) and crop residues retention on the soil (Sradnick et al., 2013; Sun et al., 2015), short-term experiments are also important for understanding these effects, particularly on soil microbiota which could change rapidly

(Alisson et al., 2008; Suleiman et al., 2016). With that we would imagine different picture about the impact of any soil disturbance in microbial community. Therefore, different time frames have to be tested and adjusted to local environmental conditions in order to properly understand specific effects of disturbances in communities.

As we did not find any difference considering dynamics of microbial communities and functions we went further comparing in pairs the different residues treatments with the addition of NPK (control) since the differences seemed to be masked by analyzing all of the treatments together. Our study shows that treatments with agricultural residues induce changes in soil microbial composition and microbial function structure, in particular when vinasse is applied together with straw. The combination of straw and vinasse appeared to be more powerful in altering the bacterial community than the addition of those residues separately. The application of vinasse could be attributed for bacteria in soils that are already experienced to this regular disturbance and are less adapted to respond immediately to fresh organic material inputs comparing with straw. Additionally, in the vinasse systems, the microorganisms can get in direct contact with organic material as this organic fertilizer is applied in the liquid form with readily available source of nutrients. In solely straw system, however, the crop residue is left on the soil surface and although the total amount is much larger this residue have recalcitrant organic matter that needs to be degraded.

Relatively few genera responded to different residues application compared to mineral fertilizer application, in this case control. The classes *Actinomycetales* and *Alpha-Proteobacteria* (orders *Rhizobiales* and *Rhodospirillales*) were prevalent in control. In particular, soil bacterial groups that are generally considered to be more copiotrophic, including *Actinobacteria* and *Alpha-Proteobacteria*, increased in relative abundance with inorganic nutrient additions since control treatment had inorganic N, P and K fertilizers. As with time, carbon and nitrogen stocks decrease without crop residues (Babujia et al., 2010; Hungria et al., 2009; Wright et al., 2008), microorganisms that can utilize more effectively a variety of carbon sources are selected as we found with additions of crop residues. While *Bacteroidetes* (order Bacteriodales) and *Beta-Proteobacteria* (orders *Rodocyclalles*, *Hydrogenophiales*, *Burkholderiales* and *Methynlophilales*) increased significantly in only straw treatment, *Verrucomicrobia* increased only in soil with vinasse and *Firmicutes* (orders *LactoBacillales*) were more abundant in soil with vinasse plus straw. On the contrary that we were expecting, the relative abundance of bacterial groups that favors nutrient rich environments and is colonizers of soil as members of the phyla *Bacteroidetes* and *Beta-Proteobacteria* were higher in soil without crop residues additions than in solely mineral

fertilizer treatment (control). Similarly, Navarro-Noya et al. (2013) found that residue management retained on the soil surface had a significant positive effect on the relative abundance of *Bacteroidetes* and *Beta-Proteobacteria*. *Bacteroidetes* have been classified generally as high taxonomic rank bacteria and primary consumers capable to exploit easy C sources and due to their ability to degrade high molecular weight organic matter which favored by no tillage (Thomas et al., 2011; Fierer et al., 2007). Beta*Proteobacteria* grew faster with large amounts of available nutrients (Fierer et al., 2007) and when soil is amended with soluble carbon from plants residues, *Beta-Proteobacteria* was enriched at the end of residue decomposition when fresh organic matter is considered more complex and recalcitrant to degradation (Nicolardot et al., 2007). Bacteria in soil with vinasse amendment are expected to be r-selected with faster growth rates since vinasse is rich in labile carbon; but our results showed opposite trend toward as *Verrucomicrobia* has oligotrophic characteristics (Ramirez et al., 2012). However, a reduction in the abundance of *Verrucomicrobia* are already known to fluctuate with soil management practices (Buckley and Schmidt, 2001; Yin et al., 2010; Dorr de Quadros et al., 2012; Navarrete et al., 2015, Pan et al, 2015) and the role of these *Verrucomicrobia*l-microorganisms in terrestrial ecosystems is poorly understood. *Firmicutes* were high abundance in vinasse plus straw treatment and members of this phylum are known to be fast-growing stimulated in C-rich environment, capable of fermenting various organic substrates and forming spores, which increase their ability to survive stressful climatic conditions, such as warming and desiccation (Hayden et al., 2012; Sharmin et al., 2013). The *Firmicutes* members have been reported to be present in vinasse (Costa et al., 2015) since they survive to the stressful conditions of thermophilic treatment of vinasse residue. Therefore, there might be great chance to add members of *Firmicutes* through vinasse application in soil. Pitombo et al. (2015) pointed that general fermenters and *Lactobacillus* (*Firmicutes*) are present in vinasse and when vinasse is applied to soil those microorganisms might be contributors for N_2O emissions.

Our results on bacterial community in soil where straw was added are in disagreement with those from Rachid et al. (2016), who suggested that there are no effects of different levels of trash of sugarcane (0%, 50% and 100% of the original trash deposition) in bacterial community but with significant impact in fungal community. Navarrete et al. (2015) also indicated that different groups of bacteria, such as *Acidobacteria*, *Actinobacteria* and *Verrucomicrobia* are indicators of mineral fertilizer with vinasse amendment and straw retention in soils cultivated with sugarcane in a mesocosm conditions. Interestingly, Pitombo et al. (2015) assessed the soil microbial community by sequencing the 16S rRNA gene and

also specifically found the orders *LactoBacillales* and *Clostridiales* in soils with vinasse treatment, *Burkholderiales* in straw treatment and have identified taxa as important drivers of N_2O emissions and taxa of N_2O emissions consumption belonging to the same *Firmicutes* phylum.

The overall potential microbial function in soil inorganic fertilizer (NPK, control) are genes associated with higher abundance of general metabolic functions as respiration, carbohydrates and lipid metabolisms. This may indicate abundance of reads-related functions for the maintenance of basic cellular machinery, enabling growth and metabolism of microbes in any disturbed or undisturbed environments (Moran, 2009). Furthermore, the treatment with NPK inorganic fertilizers showed relatively high abundance of virulence and aromatic compounds categories, what functions are still difficult to draw solid conclusions since up to date, no studies have detected these categories in metagenome data of soils under agricultural practices. For crop residues treatments we found high proportions of genes associated with protein, RNA and DNA metabolisms, chemotaxis, vitamins, phages, secondary metabolism, signaling and photosynthesis which in some extend were expected since most of these categories could be overrepresented in copiotrophic than in oligotrophic bacteria (Fierer *et al.*, 2007; 2012). However, more shotgun sequencing will be required to capture a more compendious understanding of how N fertilization can affect the functional potential of the soil microbial communities to distinguish the effect of fertilizers from crop residues separately.

The functional reads involved in geochemical processes were investigated, although other functional genes involved in other functional catalogs were detected with significative differences in our analysis. Sulphur and potassium metabolisms were more abundant in the control than in the with crop residues treatments. For residues treatments, phosphorus, iron and nitrogen were highlighted in straw samples, vinasse plus straw samples shows significant representation of functions related to only phosphorus uptake and iron metabolism and only vinasse addition did not show any specific functions. Nitrogen (N), phosphorus (P), potassium (K), sulphur (S), and iron (Fe) metabolisms are important in sustaining ecosystem functioning, by providing nutrients for crop and soil microbiome growth. To infer the ecological role of detected taxa specifically those assigned to N, P, K, S and Fe metabolisms were not trivial since limited information is available. For each type of crop residue amendments, specific organisms were selected for the different biogeochemical cycles. Depending of the selected metabolism, basically straw treatments preferential exclusively the versatile *Proteobacteria* groups as *Alpha* and *Gamma-Proteobacteria*, *Bacteroidetes*,

Methanomirobia and *Chloroflexi*. For example, members of the selected orders are known to be associated with metabolism of cellulose as Caulobacterlales (Verastegui et al., 2014) and is involved in carbon cycle and with genus adapted to live in oligotrophic environments (Marks et al., 2010). Figueirola et al. (2012) showed also that bacterial populations affiliated to *Rhodobacterales* was also favored by no- till keeping the residues. Only vinasse application had higher abundance of the phylas *Alpha*, *Delta*, *Epsilon* and *Gamma-Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Deinococcus*, *Deferribacteres* and *Verrucomicrobia*. For vinasse plus straw, the phyla with higher abundance were *Acidobacteria*, *Chlorobi*, Alpha and *Gamma-Proteobacteria*, *Firmicutes*, *Thermotogae* and *Bacteroidetes*. Our results showed different phyla affected by vinasse application than Navarrete et al (2015), who found more abundance of only *Acidobacteria*, *Actinobacteria*, *Gamma-Proteobacteria* and *Verrucomicrobia* with regard to mineral and vinasse fertilization and straw retention. The presence of different phyla could be used as specific-taxa to characterize soil vinasse amendments. Furthermore, the microorganisms that were found as specific for each biogeochemical cycle could be isolated for fundamental understanding of their ecological roles as the metagenome provides a second tier of technical innovation that facilitates study of the physiology and ecology of environmental microorganisms.

Despite for each biogeochemical cycle we might await a relation of functional attributes specifically for the microorganisms who is doing the function, this could not always be the case as different taxa can share specific traits features and related taxa can have very divergent characteristics and status tolerances (Philippot et al., 2010). Furthermore, our results from shotgun metagenomic data indicated different microorganisms were related to same biogeochemical functions for different treatments. This tendency for the microbiomes of soils with crop residues towards adaptation to biogeochemical nutrient heterogeneity depending on which crop waste type is added to the soil. The different wastes in soils will select different bacterial guilds probably capable to do similar functions indicating functional redundancy. In previous studies (Bell et al., 2005, Nielsen et al., 2011) was found that functional redundancy in microbial communities is high, suggesting an initial change in microbial community was unlikely to considerably affect ecosystem functions. Souza et al. (2015) had similar conclusions with metagenomics analysis revealing microbial functional redundancies and specificities in a soil under different tillage and crop-management regimes. Since different species can have the same function in ecosystems, functional redundancy predicts that the change of species does not necessarily alter ecosystem functioning because of their replacement by other species for maintaining processes.

In our work, all crop residues addition contributed to increase N_2O emissions. The greatest emission of N_2O was observed for vinasse mixed with straw treatment that contributed by 56.1% followed by solely vinasse and solely straw treatments with 19.9 and 17.7%, respectively compared with 6.3% for the treatment without addition of agricultural wastes. In a recent study, Carmo et al. (2013) also observed that the application of vinasse with crop trash on soil surface to sugarcane fields in Brazil resulted in significant increases in the emissions of GHGs, especially N_2O. Pitombo et al (2015) using 16S gene amplicon sequences, a different approach used in this study - shotgun metagenomics, found similar results as the orders *Burkholderiales* and *Myxococcales* in straw treatment and *LactoBacillales* in vinasse plus straw which can explain the N_2O fluxes from soil. Looking deeper into nitrogen metabolism, we found also similar results of Pitombo et al (2015) with higher abundance reads binned to order *Burkholderiales* in treatments with any residue straw or vinasse and higher proportions of those binned to orders *Sphingomonadales*, *Bacillales*, *LactoBacillales* and *Clostridiales* in the presence of vinasse which could be related with N_2O emissions. Although shotgun metagenomics sequencing often fails to provide sufficient sequence depth, especially in complex microbial communities like those found in soils (Zhou et al., 2015), the comparison between two techniques, shotgun and 16S gene amplicon, do not necessarily have to give identical results. 16S rRNA gene regions recovered from the metagenomics data of this study can comprise the entire gene, whereas the amplicon amplification address only specific regions of the 16S rRNA which vary in the accuracy of their taxonomic affiliations (Liu et al., 2007; Fierer et al., 2012).

In conclusion, our results indicate that the addition of crop residues cause changes in taxonomic and functions of microbial communities mainly when straw and vinasse are mixed together. The short-term effects revealed a treatment-impact than temporal effects on soil bacterial community related with the pattern of copiotrophic organisms. Furthermore, residue-sensitive taxa and functions varied greatly across treatments suggesting possible functional redundancy among different biogeochemical metabolisms. The power of vinasse plus straw was not only perceptible in the taxonomic and functional view but also have impact on microorganisms related to N_2O emissions.

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SUPPLEMENTAL MATERIAL 1

Supplementary Table 1

Chemical composition of the vinasse applied as crop residue in soil

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

Parameter	**Vinasse**
[a]COD ($gO_2\ L^{-1}$)	18.60
[b]BOD - $\Delta t = 5$ days ($gO_2\ L^{-1}$)	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
$N\text{-}NH_4^+$ ($mg\ L^{-1}$)	51.10
$N\text{-}NO_3^-$ ($mg\ L^{-1}$)	5.00
$N\text{-}NO_2^-$ ($mg\ L^{-1}$)	<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

Supplementary Table 2 - Number of sequencing reads, base pairs, reads assigned to SEED Subsystems and percentages of predict proteins before and after quality control on MG-RAST pipeline from treatments control, straw, vinasse and vinasse plus straw in sugarcane experiment.

			Before QC					**After QC**								
Sample ID*	MG-RAST ID	Sample	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
116	4617550.3	C_day1	16,171,712	152,380	106 ± 50	61 ± 9 %	63	15,330,024	131,951	116 ± 46	61 ± 9	101,395	2,348	28,846	86	22,067
132	4617568.3	C_day1	19,269,158	192,667	100 ± 48	62 ± 9	71	18,005,428	161,716	111 ± 44	62 ± 9	120,505	3,258	34,539	125	26,986
225	4617669.3	C_day1	13,991,188	128,916	108 ± 49	60 ± 10	88	13,356,533	113,585	117 ± 45	60 ± 10	88,688	2,092	25,074	95	19,157
117	4617551.3	C_day3	15,272,748	140,402	108 ± 49	61 ± 9	45	14,599,880	124,059	117 ± 46	61 ± 9	97,012	2,226	28,629	114	22,168
226	4617670.3	C_day3	10,173,947	98,867	102 ± 49	61 ± 9	42	9,562,893	83,901	113 ± 44	61 ± 9	63,650	1,678	18,355	79	14,258
134	4617570.3	C_day8	18,256,904	175,724	103 ± 50	61 ± 9	65	17,193,984	149,817	114 ± 46	61 ± 9	113,632	2,856	31,487	124	24,429
227	4617671.3	C_day8	19,583,347	186,888	104 ± 49	60 ± 9	118	18,485,277	160,364	115 ± 45	61 ± 9	122,581	2,929	34,631	99	26,088
135	4617571.3	C_day14	18,362,468	173,941	105 ± 50	61 ± 9	66	17,352,371	149,495	116 ± 47	61 ± 9	114,265	2,807	32,615	132	25,288
228	4617672.3	C_day14	11,921,585	113,881	104 ± 48	61 ± 9	109	11,265,967	97,970	114 ± 44	61 ± 9	75,510	1,851	21,688	58	16,749
119	461753.35	C_day20	11,927,766	111,686	106 ± 47	61 ± 9	27	11,423,230	99,478	114 ± 43	61 ± 9	77,770	1,724	21,572	72	16,484
136	4617572.3	C_day20	18,668,007	186,603	100 ± 49	61 ± 9	56	17,415,825	156,003	111 ± 45	61 ± 9	116,055	3,050	31,368	119	24,249
229	4617673.3	C_day20	17,612,825	174,106	101 ± 47	61 ± 9	117	16,541,476	147,876	111 ± 43	61 ± 9	111,715	2,906	31,189	95	24,074
120	4617555.3	C_day24	24,941,381	234,328	106 ± 51	61 ± 9	118	23,617,648	202,154	116 ± 47	61 ± 9	154,635	3,852	45,673	168	35,745
137	4617573.3	C_day24	14,689,727	141,985	103 ± 50	60 ± 9	43	13,835,656	121,233	114 ± 46	60 ± 9	91,531	2,327	24,646	80	18,670
230	4617675.3	C_day24	17,964,897	175,610	102 ± 48	61 ± 9	143	16,872,108	148,920	113 ± 44	62 ± 9	113,184	2,919	31,688	93	24,472
121	4617556.3	C_day30	15,983,276	144,408	110 ± 51	60 ± 9	52	15,293,891	127,699	119 ± 47	61 ± 9	100,259	2,282	30,554	141	23,499
138	4617574.3	C_day30	20,275,230	193,438	104 ± 51	62 ± 9	92	19,065,873	163,939	116 ± 47	62 ± 8	125,058	3,065	37,402	140	29,274
122	4617557.3	C_day46	20,957,960	192,708	108 ± 52	62 ± 9	67	19,926,476	167,638	118 ± 48	62 ± 9	130,234	3,222	39,794	168	31,309
139	4617575.3	C_day46	13,925,209	144,490	96 ± 49	61 ± 9	62	12,827,062	117,513	109 ± 46	61 ± 9	84,597	2,411	22,601	78	17,599
232	4617677.3	C_day46	12,035,251	121,141	99 ± 48	61 ± 9	57	11,218,277	101,179	110 ± 44	61 ± 9	74,900	2,032	22,349	92	17,393
193	4617633.3	S_day1	15,587,816	154,345	100 ± 47	61 ± 9	49	14,644,895	131,304	111 ± 43	61 ± 9	98,919	2,671	28,490	153	22,128
180	4617619.3	S_day3	10,255,202	102,196	100 ± 47	61 ± 9	68	9,583,445	85,835	111 ± 43	61 ± 9	64,561	1,695	17,474	72	13,366
194	4617634.3	S_day3	17,419,265	172,444	101 ± 48	61 ± 10	135	16,310,610	145,395	112 ± 44	61 ± 9	109,012	2,928	32,339	142	25,394
1	4617532.3	S_day8	3,945,482	35,085	112 ± 50	61 ± 8	7	3,790,988	31,331	120 ± 46	61 ± 8	24,950	514	7,354	41	5,661
181	4617620.3	S_day8	17,118,971	171,967	99 ± 47	61 ± 9	100	15,992,578	144,333	110 ± 43	61 ± 9	106,983	2,853	28,974	123	22,648
195	4617635.3	S_day8	21,769,720	218,256	99 ± 48	61 ± 9	121	20,292,811	182,027	111 ± 44	61 ± 9	135,297	3,757	37,410	154	28,860
182	4617621.3	S_day14	11,182,868	102,615	108 ± 49	60 ± 9	42	10,694,441	90,756	117 ± 45	60 ± 9	71,470	1,564	20,794	75	16,011
196	4617636.3	S_day14	17,343,175	180,538	96 ± 46	61 ± 9	206	15,955,810	147,223	108 ± 42	61 ± 9	108,266	3,081	28,499	99	21,982
2	4617640.3	S_day20	8,587,733	75,273	114 ± 50	60 ± 9	20	8,282,206	67,878	122 ± 46	60 ± 9	54,471	1,087	15,560	55	11,902
183	4617622.3	S_day20	18,011,656	176,125	102 ± 49	60 ± 10	91	16,905,288	149,204	113 ± 45	60 ± 10	111,508	2,947	30,135	115	22,838

197	46176 37.3	S_day 20	20,08 1,677	208,9 54	96 ± 47	61 ± 9	147	18,54 1,309	171,0 19	108 ± 43	61 ± 9	124,6 07	3,550	34,2 40	147	26,35 0
184	46176 23.3	S_day 24	11,18 0,955	105,0 92	106 ± 50	61 ± 9	40	10,59 4,162	90,67 7	116 ± 46	61 ± 9	70,62 7	1,651	19,6 40	70	15,25 1
198	46176 38.3	S_day 24	20,64 2,342	218,7 74	94 ± 46	61 ± 10	148	18,95 8,040	177,2 85	106 ± 43	61 ± 10	127,1 33	3,902	39,0 81	271	30,60 7
3	46176 93.3	S_day 30	12,49 5,712	115,5 12	108 ± 49	61 ± 9	52	11,90 2,421	101,1 72	117 ± 45	62 ± 8	79,28 5	1,886	22,2 88	79	17,22 1
185	46176 24.3	S_day 30	15,23 0,014	153,3 24	99 ± 47	61 ± 9	76	14,22 3,406	128,6 32	110 ± 44	61 ± 9	95,74 0	2,670	26,1 78	107	20,41 8
199	46176 39.3	S_day 30	20,65 9,808	211,3 56	97 ± 47	61 ± 9	173	19,19 5,595	175,5 32	109 ± 43	61 ± 9	128,5 60	3,595	34,3 07	143	26,44 0
4	46177 03.3	S_day 46	12,32 9,201	112,7 95	109 ± 49	61 ± 9	47	11,79 6,501	99,84 8	118 ± 45	61 ± 9	78,60 6	1,791	21,8 93	94	16,98 5
186	46176 25.3	S_day 46	9,515, 710	89,56 2	106 ± 48	61 ± 9	22	9,054, 518	78,36 5	115 ± 44	61 ± 9	60,93 7	1,404	17,0 98	49	13,39 1
200	46176 42.3	S_day 46	15,66 6,036	161,3 34	97 ± 46	61 ± 10	149	14,51 8,488	133,4 57	108 ± 42	61 ± 9	98,09 5	2,894	27,1 67	130	21,07 6
21	46176 52.3	V_da y1	16,93 2,146	175,3 26	96 ± 44	60 ± 9	82	15,87 9,763	149,9 06	105 ± 41	60 ± 9	108,4 60	3,181	27,3 08	148	21,00 3
52	46177 17.3	V_da y1	17,51 9,391	176,5 85	99 ± 47	61 ± 10	120	16,34 3,961	147,9 64	110 ± 44	61 ± 9	109,2 82	2,928	29,1 37	116	22,36 1
99	46177 66.3	V_da y1	21,58 9,295	207,3 08	104 ± 51	61 ± 9	93	20,29 9,204	175,9 62	115 ± 47	61 ± 9	132,9 94	3,508	37,9 80	148	29,40 8
22	46177 63.3	V_da y3	18,66 7,092	184,7 65	101 ± 48	61 ± 9	59	17,58 7,038	158,5 19	110 ± 44	61 ± 9	117,3 65	3,204	32,7 95	121	25,41 7
53	46177 18.3	V_da y3	39,11 9,210	394,6 80	99 ± 48	61 ± 9	493	36,45 2,673	330,0 46	110 ± 44	61 ± 9	243,6 92	6,787	64,5 62	231	49,78 9
23	46176 74.3	V_da y8	5,008, 030	51,22 9	97 ± 42	58 ± 9	13	4,712, 926	44,49 5	105 ± 39	58 ± 9	33,10 8	857	7,93 5	29	6,033
54	46177 19.3	V_da y8	20,56 0,205	208,5 24	98 ± 47	61 ± 9	95	19,13 6,143	173,6 41	110 ± 43	61 ± 9	128,6 02	3,417	37,6 76	160	29,40 4
101	46175 34.3	V_da y8	20,10 1,465	201,0 25	99 ± 49	61 ± 9	66	18,75 7,490	168,3 25	111 ± 45	61 ± 8	124,6 01	34,49 5	3,37 2	111	26,55 9
24	46176 85.3	V_da y14	20,51 1,300	203,7 55	100 ± 48	60 ± 9	64	19,26 7,441	173,5 91	110 ± 44	60 ± 9	128,4 56	3,448	36,2 61	120	28,11 2
55	46177 20.3	V_da y14	20,25 8,634	201,5 15	100 ± 48	61 ± 9	91	18,95 3,867	169,7 52	111 ± 44	61 ± 9	126,5 22	3,336	34,9 83	111	26,86 1
102	46175 35.3	V_da y14	18,68 9,719	182,3 00	102 ± 50	62 ± 9	87	17,52 9,616	154,0 68	113 ± 46	62 ± 9	115,4 26	3,009	34,7 48	180	27,04 8
56	46177 21.3	V_da y20	21,65 2,951	216,0 41	100 ± 48	61 ± 9	138	20,22 5,632	181,4 58	111 ± 44	61 ± 9	134,6 06	3,590	36,0 40	126	27,75 2
103	46175 36.3	V_da y20	20,56 8,455	201,6 64	101 ± 50	62 ± 9	93	19,26 6,952	169,9 94	113 ± 46	62 ± 9	127,7 11	3,460	36,8 74	142	28,83 3
25	46176 88.3	V_da y24	12,30 9,911	107,5 76	114 ± 50	60 ± 9	35	11,88 2,734	97,32 6	122 ± 46	60 ± 9	77,98 5	1,704	21,8 25	73	16,45 5
57	46177 22.3	V_da y24	17,28 8,271	167,2 81	103 ± 49	60 ± 10	97	16,28 2,981	142,8 72	113 ± 45	60 ± 10	107,7 59	2,713	29,7 57	122	23,09 9
104	46175 37.3	V_da y24	18,76 5,239	185,3 56	101 ± 50	62 ± 9	82	17,54 7,759	155,6 99	112 ± 46	62 ± 9	116,0 88	3,073	31,9 65	101	24,91 4
26	46176 89.3	V_da y30	20,82 2,724	197,5 23	105 ± 50	61 ± 9	113	19,72 1,585	170,7 59	115 ± 46	61 ± 9	130,1 67	3,290	36,5 51	127	28,54 8
58	46177 23.3	V_da y30	25,21 7,509	251,3 48	100 ± 49	61 ± 10	214	23,55 4,862	210,7 80	111 ± 45	61 ± 9	155,5 84	4,345	46,0 60	264	36,14 1
105	46175 38.3	V_da y30	18,19 8,284	179,5 89	101 ± 49	61 ± 9	60	17,06 5,014	151,9 44	112 ± 45	61 ± 9	113,4 91	3,108	31,5 32	152	24,18 3
27	46176 90.3	V_da y46	22,80 2,282	229,1 16	99 ± 49	59 ± 11	108	21,29 5,783	192,2 97	110 ± 45	59 ± 11	140,7 69	4,034	42,1 29	235	32,95 3
59	46177 24.3	V_da y46	16,38 4,948	158,4 79	103 ± 50	61 ± 9	68	15,40 9,202	134,6 26	114 ± 46	61 ± 9	101,2 36	2,636	28,4 21	116	21,93 5
36	46177 00.3	V+S_ day1	16,42 8,050	167,3 25	98 ± 47	61 ± 9	74	15,29 4,577	139,5 77	109 ± 43	61 ± 9	102,4 85	2,912	28,7 2	142	22,48 6
76	46177 41.3	V+S_ day1	13,91 8,904	144,2 75	96 ± 48	59 ± 11	78	12,71 7,101	116,5 29	109 ± 44	60 ± 11	84,05 8	2,671	23,7 99	196	18,15 6
108	46175 41.3	V+S_ day1	33,76 7,605	339,7 33	99 ± 48	61 ± 9	225	31,51 2,637	284,6 53	110 ± 44	61 ± 9	210,5 27	5,720	59,9 09	260	46,22 7
37	46177 01.3	V+S_ day3	16,55 7,501	165,0 61	100 ± 48	61 ± 10	82	15,51 9,527	139,8 72	110 ± 44	61 ± 9	103,1 31	2,886	30,2 78	174	23,45 6
77	46177 42.3	V+S_ day3	16,62 8,499	162,4 90	102 ± 50	61 ± 9	70	15,57 4,334	136,7 14	113 ± 46	61 ± 9	102,2 80	2,650	26,6 46	73	20,41 7
109	46175 42.3	V+S_ day3	22,53 8,075	225,1 00	100 ± 50	61 ± 9	99	20,97 8,858	187,0 07	112 ± 46	61 ± 9	138,0 50	3,826	37,8 65	148	29,07 1
38	46177 02.3	V+S_ day8	18,40 8,192	186,3 13	98 ± 47	62 ± 9	126	17,16 4,039	156,3 27	109 ± 44	62 ± 9	114,9 56	3,174	32,6 54	101	25,46 5
78	46177 43.3	V+S_ day8	14,15 2,673	139,4 61	101 ± 50	61 ± 10	64	13,23 2,192	116,8 43	113 ± 47	61 ± 9	86,82 7	2,426	25,1 76	131	19,43 6

110	4617544.3	V+S_day8	21,289,231	212,229	100 ± 49	62 ± 9	92	19,862,460	177,288	112 ± 46	62 ± 9	131,661	3,498	36,182	107	28,184
79	4617744.3	V+S_day14	14,038,074	140,584	99 ± 50	61 ± 9	53	13,039,180	115,876	112 ± 47	61 ± 9	85,330	2,347	22,609	94	17,357
111	4617545.3	V+S_day14	25,691,466	242,817	105 ± 50	61 ± 9	108	24,318,612	209,444	116 ± 46	61 ± 9	160,572	3,828	46,703	168	35,883
40	4617704.3	V+S_day20	14,348,055	143,319	100 ± 47	61 ± 9	59	13,507,379	123,069	109 ± 43	61 ± 9	90,631	2,501	24,421	95	18,286
80	4617746.3	V+S_day20	11,558,812	115,241	100 ± 50	61 ± 9	48	10,764,523	95,736	112 ± 46	61 ± 9	71,081	2,026	19,215	65	14,916
112	4617546.3	V+S_day20	18,142,574	172,518	105 ± 50	61 ± 9	64	17,127,715	147,801	115 ± 46	62 ± 9	113,288	2,852	35,783	194	27,674
41	4617705.3	V+S_day24	13,458,811	130,521	103 ± 48	60 ± 10	68	12,713,024	112,398	113 ± 45	60 ± 10	85,090	2,208	24,264	117	18,134
81	4617747.3	V+S_day24	8,759,100	83,018	105 ± 50	58 ± 10	26	8,312,019	72,104	115 ± 46	58 ± 10	54,262	1,456	14,966	149	11,159
113	4617547.3	V+S_day24	10,668,683	95,378	111 ± 50	61 ± 9	30	10,241,796	85,036	120 ± 46	61 ± 9	67,597	1,479	19,485	60	15,156
42	4617706.3	V+S_day30	14,101,683	139,604	101 ± 49	61 ± 9	59	13,195,514	117,457	112 ± 45	61 ± 9	87,349	2,472	24,926	107	18,968
82	4617748.3	V+S_day30	20,510,105	204,269	100 ± 50	60 ± 10	129	19,133,546	170,621	112 ± 46	60 ± 10	125,746	3,540	36,252	205	27,395
114	4617548.3	V+S_day30	21,246,550	204,604	103 ± 51	61 ± 9	66	19,969,678	173,259	115 ± 47	61 ± 9	131,192	3,439	37,228	146	28,692
43	4617707.3	V+S_day46	21,804,401	214,784	101 ± 49	60 ± 10	142	20,413,192	180,966	112 ± 46	60 ± 10	134,423	3,764	37,121	163	28,281
83	4617749.3	V+S_day46	14,049,709	135,689	103 ± 51	60 ± 9	89	13,188,368	114,601	115 ± 47	60 ± 9	86,602	2,255	23,811	119	18,080
115	4617549.3	V+S_day46	19,743,224	185,705	106 ± 51	61 ± 8	71	18,648,891	158,945	117 ± 47	61 ± 8	122,196	2,931	34,962	93	27,039
107	4617540.3	V_day46	23,347,515	229,894	101 ± 50	61 ± 9	92	21,822,894	192,645	113 ± 47	61 ± 9	143,422	3,801	40,072	143	30,764

Abbreviations: Treat-treatments; C-Control; S-Straw; V-Vinasse; V+S-Vinasse plus straw

* Samples could not be annoted on MG-RAST: C_day3; C_day8; C_day14; C_day30; S_day1 (2 samples); S_day3; S_day14; S_day24; V_day3; V_day20; V+S_day14.

5 DISCUSSÃO GERAL

O objetivo geral da tese foi examinar o impacto de resíduos de culturas e orgânicos na comunidade bacteriana em experimentos de campo a curto prazo. No primeiro capítulo tinha-se como objetivo acessar especificamente o efeito de fertilizantes minerais e dejetos líquidos suínos e o inibidor da nitrificação dicianodiamida (DCD) sobre a comunidade bacteriana potencialmente ativa do solo. Já no segundo capítulo tinha-se como objetivo acessar o impacto de diferentes resíduos como palha, vinhaça e a combinação de ambos na estrutura e funções microbianas do solo. Nos capítulos foram utilizados técnicas de sequencimento de nova geração para acessar a comunidade microbiana. O primeiro capítulo focou-se nas mudanças taxonômicas acessadas através da amplificação do gene 16S rRNA enquanto o segundo concentrou-se em análises metagenômicas para também examinar além dos microrganismos, as funções que foram modificadas no solo. Em geral, os resultados da tese mostram que os resíduos orgânicos modificam as comunidades microbianas no solo e contribuiram para o aumento das emissões de N_2O.

5.1 RESÍDUOS ORGÂNICOS PROVOCAM MUDANÇA NA ESTRUTURA DA COMUNIDADE BACTERIANA DO SOLO

A estrutura da comunidade microbiana é utilizada como indicador de mudança ambiental já que os microrganismos podem ser sensíveis a perturbações, e as alterações estruturais que ocorrem na comunidade decorridos aos distúrbios podem estar associadas a variações nos processos do ecossistema (Alisson e Martiny, 2008, Griffiths e Philippot, 2013). Seguindo nessa linha, foi demonstrado que a adição de resíduos orgânicos tem um forte papel na mudança da comunidade bacteriana do solo. Esta descoberta está de acordo com recentes estudos neste campo (Lazcano et al., 2013, Navarrete et al., 2015). Entretanto, o estudo traz uma visão mais detalhada devido a resolução temporal acessada através da captura da dinâmica microbiana após a aplicação de resíduos no solo. No caso dos dejetos suínos, sua aplicação com ou sem inibidor de nitrificação DCD, provocou alterações transitórias na comunidade microbiana durante todo o experimento. Essas mudanças foram mais significativas nos primeiros 3 dias do experimento quando comparadas ao tratamento controle e ao fertilizante mineral, mas a comunidade foi resiliente recuperando-se ao seu estado original em 50 dias após a fertilização orgânica. Quando da aplicação de vinhaça como resíduo orgânico, os diferentes tratamentos não apresentaram variabilidade temporal na

estrutura da comunidade microbiana, entretanto a adição de vinhaça em conjunto com a palha, em comparção ao controle, induziu a mudanças na composição e funções microbianas no solo. Os resíduos orgânicos, os quais derivam de excreções animais ou matéria vegetal fornecem nutrientes (Chen, 2006) e tem a capacidade de modificar as propriedades do solo (Bhattacharyya et al., 2007), provocando modificações afetando as comunidades microbianas do solo. Apenas poucos experimentos agrícolas visam comparar estratégias de manejo convencional e orgânico com diferentes fertilizações ao longo do tempo (Raupp et al., 2006). Especificamente para a aplicação de dejetos animais, enquanto inúmeros estudos investigaram os efeitos da adubação orgânica nas comunidades microbianas (Dinesh et al., 2010, Qiu et al., 2012) e que a diversidade bacteriana foi sempre maior em solos com a adição de dejetos independente do uso da terra ou estação do ano (Ge et al., 2008), o presente estudo mostrou que os tratamentos com a presença do dejetos afetaram negativamente a diversidade bacteriana ativa do solo. Com a aplicação da vinhaça, recentes e poucos estudos mostraram diferenças na relativa abundância de sequências metagenômicas relacionadas a fertilização do solo com vinhaça em conjunto com a retenção da palha no solo (Navarrete et al., 2015).

5.2 MICRORGANISMOS E FUNÇÕES MODIFICADOS COM A ADIÇÃO DE RESÍDUOS AGRÍCOLAS

A aplicação de diferentes resíduos agrícolas afetou os microrganimos diferentemente. Com a aplicação de resíduos animais o maior impacto na comunidade bacteriana geral foi observada ao terceiro dia do experimento, principalmente devido a um aumento na abundância de *Bacteroidetes*, *Proteobacteria* e *Firmicutes*. O impacto da aplicação de resíduos vegetais foi visualizada no tratamento como um todo com a aplicação da palha e de apenas vinhaça modificando siginificativamente a abundância de *Bacteroidetes* e *Beta-Proteobacteria* enquanto *Verrucomicrobia* e *Firmicutes* foram sobre-representados no tratamento com adição de apenas vinhaça e de vinhaça com palha, respectivamente. Os resíduos orgânicos selecionaram as comunidades distintamente, no entanto, as diferenças puderam ser explicadas pela dinâmica de bactérias copitróficas e oligotróficas, devido a adição de nutrientes prontamente disponíveis ou que se tornariam mais tarde disponíveis no solo. A explicação da diferença dos resultados podem ser atribuídas a distintas variações em comparação aos dois experimentos como a diferença na composição química dos resíduos como dejetos, palha e vinhaça e do solos dos experimentos. Por exemplo, além das diferenças geográficas das localidades experimentais, os resíduos orgânicos dejeto e vinhaça diferem em

relação ao pH, carbono e nitrogênio total enquanto que os solos diferem em relação a sua classificação, pH e concentração de nutrientes como P, K^{+}, Ca^{+2}, Mg^{+2} e CEC. Fatores ambientais e a biogeografia podem afetar a abundância e composição taxonômica das comunidade microbianas (Martiny et al., 2006, Allison e Martiny, 2008). Muitos estudos mostraram que fatores como o pH do solo, tipo de solo, atributos do solo, espécie da planta, tipo de manejo além de localização em escala regional ou continenetal influenciam a estrutura da comunidade microbiana (Rousk et al., 2010, Nüsslein and Tiedje, 1999, Wieland et al., 2001, Steenwerth et al., 2002, Hartman et al., 2008, Wakelin et al., 2008, Hollister et al., 2010, Fierer and Jackson, 2006, Lauber et al., 2009, Chu et al., 2010). Além dos fatores citados anteriormente, ambos os experimentos apresentaram métodos diferentes para o acesso da comunidade microbiana. A avaliação do impacto dos dejetos com ou sem inibidor de nitrificação na comunidade microbiana potencialmente ativa do solo através de sequenciamento de alto rendimento, usando RNA total ao invés de DNA, forneceu melhor entendimento sobre a resposta dos microrganismos em atividade frente a perturbação causada pelos dejetos no solo. O sequenciamento do DNA tem revelado a biodiversidade microbiana e clarificado as relações dos microrganismos com o ambiente, entretanto, as análises baseados em DNA podem não discriminar com eficiência entre a comunidade ativa funcionalmente e a população dormente. Como se sabe que o DNA persiste extracelularmente em ambientes após a morte deixando resíduos de DNA, o RNA parece ser mais lábil e representativo da comunidade ativa no solo (Mengoni et al., 2005). Apesar da avaliação do impacto de resíduos vegetais como a palha e vinhaça não ter sido feita avaliando-se a comunidade ativa do solo, o estudo usou da técnica de “shotgun” que é uma abordagem relativamente nova e poderosa de sequenciamento que fornece novas percepções sobre as funções e biodiversidade das comunidades. O sequenciamento por “shotgun” captura informação da comunidade como um todo e evita muitos dos vieses encontrados no sequenciamento de amplicons porque não requer amplificação por PCR antes do sequenciamento (Zhou et al., 2015).

Em relação às funções afetadas, observou-se que a aplicação de dejetos com e sem DCD apresentou um efeito significativo sobre grupos específicos de microrganismos do ciclo do nirogênio. Por exemplo, a aplicação de DCD afetou negativamente a abundância relativa de Nitrospirae, um importante filo relacionado ao ciclo do nitrogênio. Ao terceiro dia do experimento, a aplicação de DCD reduziu siginifiativamente a relativa abundância da ordem Nitrosomonadales que inclui o genero Nitrosomonas responsável pela primeira etapa do processo de nitrificação. Esses resultados estão de acordo com estudos anteriores que apresentaram de que microrganismos oxidantes de amônia foram significativamente afetados

pela DCD com redução na sua abundância e atividade (Di et al., 2010, Di et al., 2014, Zhang et al., 2012). Além disso, para os resíduos vegetais foram encontradas as ordens *Burkholderiales*, *Myxococcales,* para o tratamento com palha e *Sphingomonadales*, *Bacillales*, *LactoBacillales* e *Clostridiales* nos tratamentos com vinhaça em conjunto com palha os quais podem participar do fluxo de N_2O e, consequentemente, do ciclo de nitrogênio (Philippot et al., 2011, Rőszer et al., 2012, Nie et al., 2015, Pitombo et al., 2015). Resultados similares foram encontrados por Pitombo et al (2015) que mostraram amplificando o gene 16S as ordens *Burkholderiales* e *Myxococcales* nos tratamentos com palha, e *LactoBacillales* no tratamento com vinhaça e palha os quais são responsáveis pelo fluxo de N_2O do solo. Além das funções relacionadas ao ciclo do nitrogênio, a adição de resíduos vegetais no solo aumentou a proporção de genes associados ao metabolismo do fluxo de informaçao genética como DNA, RNA e proteínas, chemotaxas, vitaminas, fagos, metabolismo secundário, sinalização e fotossíntese. A maioria dessas categorias de genes citadas anteriormente poderiam ser mais comumente sobre-representadas em bactérias copiotróficas do que em oligotróficas (Fierer et al., 2007, 2012) ou essenciais para as atividades básicas da celula procariótica no solo (Xu et al., 2014). Além disso, metabolismos de fósforo, ferro e nitrogênio foram observados em diferentes tratamentos de resíduos de culturas mostrando que algumas atividades metabólicas são mais suscetíveis de ser associadas individualmente com resíduos vegetais. Tentando analisar os microrganismos associados aos ciclos biogeoquímicos, verificou-se que diferentes microrganismos são responsáveis pelas mesmas funções em distintos tratamentos indicando possível redundância funcional. Estudos em ecossistemas microbianos tem mostrado que a ligação entre a composição da comunidade e resposta metabólica não é direta, mas, em vez disso, é fortemente influenciada pela plasticidade metabólica e redundância funcional (Alisson et al., 2008, Comte et al., 2013). No entanto, mais estudos metagenomicos são necessários para a validação e diferenciação do efeito dos resíduos dos fertilizantes nas funções das comunidades do solo.

5.3 EMISSÕES DE N_2O RESULTANTES DO RETORNO DOS RESÍDUOS AO SOLO

Um aspecto importante deste estudo foi a tentativa de melhor compreender o efeito de resíduos animais e vegetais sobre as emissões do óxido nitroso já que a sua aplicação no solo afetou o ciclo do nitrogênio. A aplicação de todos os resíduos contribuíram para o aumento emissão de N_2O. Quando da aplicação de dejetos no solo, o maior pico de emissão foi no terceiro dia do experimento quando da adição da DCD, o inibidor de nitrificação foi eficiente

em reduzir as emissões em 70% em comparação a aplicação de dejeto sem DCD, mostrando claramente que a DCD foi eficiente na mitigação da emissão de óxido nitroso. Estudos já mostraram que a DCD pode substancialemnte reduzir as emissões de óxido nitroso após a aplicação de dejetos (Tao et al., 2008, Merino et al., 2002), embora outros estudos não observarem efeito algum (Pereira et al., 2010, Mkhabela 2006). Apesar do limitado número de estudos do efeito da DCD sobre as emissões de N_2O após a aplicação de dejeto animal, as diferenças relacionadas a efetividade da DCD em reduzir as perdas nitrogênio na literatura deve-se a experimentos sob ampla gama de condições de solo e climáticas assim como differentes fontes de nitrogênio que são aplicadas ao solo (Kelliher et al., 2008, Sprosen et al., 2009, Dai et al., 2013, Luo et al., 2013, Aita et al., 2014, Di et al., 2014). Em relação a adição dos outros resíduos, todos os resíduos vegetais contribuiram para um aumento nas emissões de N_2O. A maior emissão de N_2O foi observada para a vinhaça em combinação com palha que contribuiu por 56,1%, seguido por tratamento com apenas a aplicação de vinhaça e de somente palha com 19,9 e 17,7%, respectivamente, em comparação com 6,3% do tratamento sem adição de resíduos agrícolas. Apesar da retenção da palha ter sido adotada mundialmente por aumentar a produção agrícola, Lou et al. (2007) mostraram que as emissões acumulativas de N_2O aumentaram em solos com cultivo de arroz em que foram acrescentados de palha. Já para tratamentos com adição de vinhaça, Paredes et al. (2014) mostraram evidências de que a sequência de aplicação de fertilizantes com fonte de N e vinhaça aumentaram as emissões de N_2O. Carmo et al. (2013) também observaram que a aplicação de vinhaça com resíduos de culturas na superfície do solo aos campos de cana no Brasil resultou em aumentos significativos nas emissões de gases de efeito estufa, especialmente N_2O. Isso ocorre devido a adição de um fertilizante líquido em conjunto com o nitrogênio que favorece também o desenvolvimento de microrganismos que atuam na mineralização, imobilização do nitrogênio assim como nos processos de nitrificação, desnitrificação e fixação biológica no solo (Silva et al., 2007).

6 CONSIDERAÇÕES GERAIS

Em geral, fertilizantes orgânicos de origem animal ou vegetaI são os maiores responsáveis pelas mudanças na estrutura da comunidade microbiana do solo. Enquanto a aplicação de resíduos animais como dejetos afetaram a comunidade microbiana temporariamente com a comunidade sendo resiliente após 50 dias de fertilização, a aplicação de resíduos vegetais afetou o tratamento como um todo apresentando redundância funcional das funções relacionadas ao ciclos biogeoquímicos. Todos os resíduos mostraram efeito especificamente no ciclo do nitrogênio provocando consequentemente um aumento nas emissões de N_2O no curto prazo de tempo do experimentos. Os resultados encontrados para ambos os capítulos são de grande importância, pois servirão de base para encontrar soluções relacionadas a problemática da destinação e reaproveitamento de resíduos derivados de práticas agrícolas que aumentam emissões de N_2O decorrente de atividade microbiana.

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