Microbial communities in Pampa soils; *impact of land-use changes, soil type and climatic conditions*

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Content

Chapter 1
General introduction

Soils are heterogeneous environments where abiotic factors such as pH, nutrient availability, soil porosity, moisture and biotic factors such as interactions with other organisms are key to the creation of niches available for microorganisms to survive and proliferate (Dumbrell et al. 2009). Because soils provide a huge range of different habitats, they support an enormous biomass and diversity of microbes, with an estimated abundance of 10^7 - 10^{10} cells/cm^3 and up to 10^4 species/cm^3 (Gans et al. 2005). This makes soils to be the most diverse habitats on Earth (Torsvik et al. 2002).

The biodiversity of soil microbial communities has important implications for the stability and functioning of natural and disturbed ecosystems, but also their interactions with other microbes and higher organisms, in particular plants, are of prime importance for the role of microbes in terrestrial ecosystems (Bell et al. 2008). Any modification of the interaction between plants and microorganisms might affect the composition and diversity of the soil microbiome, which, in turn, will influence the functionality of ecological processes (Chapin III et al. 2000). Also, seasonal environmental forces and anthropogenic disturbances of a soil ecosystem will lead to modifications in the composition and functioning of the microbial community and as a result to substantial impacts on soil functioning (Rudolf & Rasmussen 2013).

There are significant gaps in our understanding of the influence of land-use changes by the removal of natural vegetation and of seasonal climatic variations on soil microbiome dynamics. Based on the importance of microbial community dynamics for soil ecosystem functioning, the studies described in

my thesis were carried out to achieve **three main goals**: I - *To investigate how land use systems and soil type affect the composition and activity of the soil microbial community,* II - *To assess the responses of the soil microbiome to seasonal climatic variations, and* III - *To understand microbe-microbe associations and detect keystone species in different land use systems by the use of network approaches.* The studies described here are the first attempts to identify key drivers of the microbiome in the Pampa ecosystem. It may help to develop and implement policies and regulations to minimize the consequences of changes in biodiversity of the Pampa ecosystems and to find future solutions for anthropogenic impacts.

1.1 The Pampa biome

Biomes are climatically and geographically contiguous areas. They are classified according to the dominant vegetation and distinguished by particular adaptations of the inhabiting organisms to the prevailing climatic conditions (Woodward et al. 2004). Brazil is a megadiverse country, with six terrestrial biomes - Amazon, Atlantic Forest, Caatinga, Cerrado, Pantanal and Pampa. The Brazilian Pampa biome is located in the south of Brazil, between latitudes 28° 00' S and 34° 00' S and longitudes 49° 30' W and 58° 00' W, occupying an area of 63% of the Rio Grande do Sul State (Fig. 1.1).

Due to its geographic position, the Pampa biome lies between the subtropical climate zone in the north and extending southward into the temperate region, with four defined seasons, including hot summers and cold winters. Climate is the most important factor determining the presence of a mosaic of grassland ecosystem associated with gallery and small areas of forests in this region (Fig. 1.1 A) (Overbeck et al. 2007). Subtropical and temperate grasslands are ecosystems, dominated by grass species, as opposed to other biomes (*e.g.*, tropical forest) where trees or large shrubs occupy the largest portion of the territory surface. Among them, Pampa grasslands

represent one of the major biomes. Pampa grasslands appear to be homogeneous regarding to floristic composition but a larger macro- and micro-organisms diversity has developed in response to regional climate and soil conditions (Overbeck et al. 2006).

Large areas of the Pampa grassland have sandy type soils, which are highly sensitive to seasonal shifts (water and wind erosion) and to anthropogenic disturbances (land-use changes) (Hevia et al. 2003; Roesch et al. 2009); only relatively small spots of loamy soil types can be found (Streck et al. 2002; Pillar et al. 2009). This fragility implies that inappropriate human activities bear great risks and might lead to intense soil degradation and decrease of soil fertility.

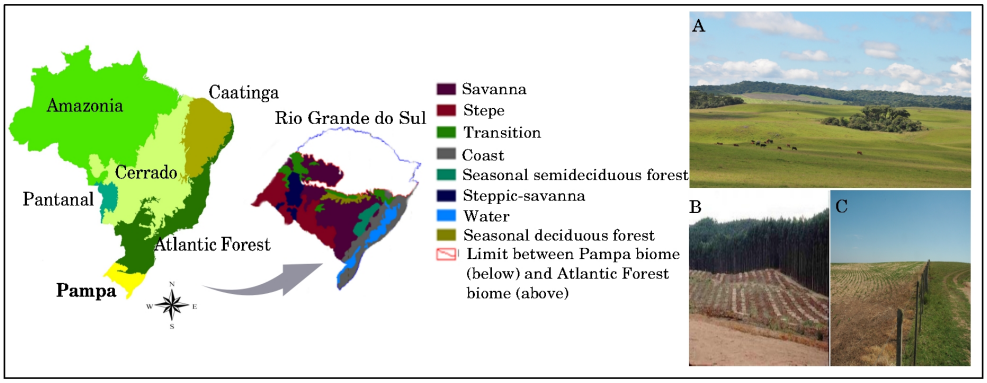

Fig. 1.1 Biomes in Brazil, indicating the geographic localization and physiographic regions of the Pampa biome, based on the distribution of trees and grassland. The landscapes images show (A) the most predominant vegetation (grassland in front and gallery forest behind) and the land-use changes here depicted as the introduction of (B) tree plantation and (C) soybean cultivation in the landscape of Pampa biome.

The present characteristics of vegetation, climate and soil types, make the Pampa a unique ecosystem, with a high plant, animal and microbial

diversity (Overbeck et al. 2006; Wilson et al. 2012; Carbonetto et al. 2014). Although considered to be one of the priority areas for flora and fauna conservation (MMA 2002) this biome is the most unknown of the Brazilian biomes regarding its biodiversity, although the agricultural practices and the associated cultivation of monocultures, and the introduction of exotic species, among others factors, have contributed to the degradation of this unique ecosystem (Roesch et al. 2009; Overbeck et al. 2007).

1.2 Anthropogenic disturbances in the Pampa biome: land-use changes by the expansion of agri- and silvicultural production

As other grasslands ecosystems all over the world, the Pampa biome has experienced for a long time anthropogenic disturbances which have altered the natural communities with significant consequences at temporal and spatial scales (Faggi et al. 2008; Carbonetto et al. 2014). Anthropogenic disturbances have changed the natural structure of plant and animal communities, through the conversion of native areas (grassland and gallery forest) to agricultural lands or grazing areas and more recently through the establishment of forest plantations (exotic tree species). It is estimated that a large portion of the original vegetation has been removed and 60% of the vegetation is under human management (Santos & Silva 2012). Up to now, land-use changes in the Pampa have been documented poorly as compared to other regions of Brazil (*e.g.*, Amazonia, Cerrado) (Lapola et al. 2013), and the socio-economic causes and ecological consequences of these changes have scarcely been investigated (Overbeck et al. 2007).

The Pampa biome has a long agricultural history (Viglizzo et al. 2001). Before the 16th century, the local population had relatively low impact on the natural ecosystem. However, with the arrival of Europeans immigrants and the

introduction of cattle production (Pillar et al. 2009), grazing and fire became important regulators of the structure and composition of the native grasslands (Soriano 1992). At the 20th century, the Pampa grassland was confronted with new challenges. The increase of cropping practices and the intense use of agrochemical inputs promoted further intensification of agricultural production, accelerating the expansion of the agricultural impact on biotic and abiotic properties of this region (Caride et al. 2012). The decrease in the total area of natural vegetation has accelerated in the past 30 years due to unrestricted expansion of agricultural activities for crop production, mainly soybean, rice and wheat crops (Pillar et al. 2009) (Fig 1.1 B and C).

Furthermore, the cultivation of exotic trees for pulp production has increased substantially on the Pampa biome. The present area of tree cultivation in southern Brazil is about 450.000 ha and new projects have been planned for the near future (ABRAF 2011). Natural grasslands areas have been transformed into plantations of *Acacia* sp., *Eucalyptus* sp. and *Pinus* sp. over large areas (Overbeck et al. 2007) (Fig. 1.1 B). Plantations are usually systems where part of the original species composition remains but the dense tree monocultures do not allow for much natural understory plants to grow, leading to loss of native grassland species (Pillar et al. 2009). Further, surrounding grassland areas are often being invaded by these exotic species, because of their effective seed dispersal and germination capacity (Bustamante & Simonetti 2005). Reliable, recent quantitative data on the spatial expansion of tree plantations and thus up-to-date data on the impact of these plantations on flora and fauna are, however, not yet available for southern Brazil.

Anthropogenic disturbances and land-use changes are causing unprecedented shifts in biodiversity in grasslands ecosystems, as native animal and plant species are driven to extinction locally and globally (Pimm et al. 1995; Sala et al. 2000). Not only grasslands, but also gallery forest degradation causes substantial environmental problems such as threat or extinction of local

species, soil erosion and impoverishment of soil chemical properties (Roesch et al. 2009; Costella et al. 2013). Although apparent changing patterns are emerging for macro-organisms in Pampa grassland (del Pilar Clavijo et al. 2005; Azpiroz et al. 2012), we have limited understanding on how these anthropogenic shifts by biotic (plant cover, agricultural crops, invasive species) and abiotic (chemical and physical soil properties, chemical inputs, soil management) disturbances affect the soil microbiome in the Pampa ecosystem.

Of particular importance for the composition and functioning of the soil microbiome are the effects by the changes in the vegetation and so, in the release of different carbon compounds through rhizodeposition (Berthrong et al. 2009), and the quantity and quality of litter inputs (Zhang et al. 2014) as well as allelopathic effects (Lorenzo et al. 2010). Shifts in the structure of microbial communities are also associated with changes in a number of soil properties including pH, organic matter, and nitrogen availability (Nacke et al. 2011; Bissett et al. 2013). While pH changes may lead to an altered composition of the microbial community able to survive under the prevailing conditions (Bååth & Anderson 2003), changes in the availability of organic substrate and nutrients in soil will also be reflected in shifts in the metabolic capabilities of the microbial community, for instance related to decomposition (Ramirez et al. 2012) and nutrient mineralization rates (Hartman & Richardson 2013). Thus, land-use changes can directly affect community composition, resulting in changes in biodiversity as well as in alteration of microbial functions (Philippot et al. 2013) *e.g.,* related to carbon and nitrogen cycling and so influence ecosystem functioning (Paula et al. 2014).

Land-use changes do not always lead to reduction of microbial diversity or to drastic changes in the composition and functioning of microbial communities (Jangid et al. 2008). A soil bacterial community well-adapted to a specific ecosystem condition, for instance soil type, which has been indicated as a dominant factor driving microbial community composition (Girvan et al.

2003), may be resistant or resilient to disturbances *e.g.*, by the introduction of new vegetation or modifications in edaphic properties effected by a change in the land usage (Upchurch et al. 2008). Finally, the soil microbiome might be more controlled by historical factors (*e.g.,* local history of land use, weather conditions, soil type) than by contemporary disturbances (*e.g.*, introduction of new vegetation) (Ge et al. 2008).

1.3 Seasonal variations and dynamics of the microbiome

The predominant climate in Pampa grasslands is subtropical with four well-defined seasons, with a large alteration in temperature between winter and summer. The annual mean temperature is 20°C, with a minimum of 0°C and maximum of 35°C. The rainfall is well divided during the year, with an annual rainfall around 1,200-1,600 mm, with rains concentrated in winter months (Overbeck et al. 2007). The climatic conditions in soil, *i.e.,* soil moisture and temperature conditions, are closely related to the seasonal patterns of rainfall and air temperature (Deng et al. 2012). The rainfall pattern is sufficient to maintain the subtropical soils in a moist condition through much of the year, with periods of drought during the summer and wet conditions in winter season (Veblen et al. 2007). The soil temperature, mainly in the surface layers, are closely associated with air temperature, but shifts in soil temperature are slowly than air temperature due to the dense characteristic of soil (Baumhardt et al. 2000).

Water content and temperature are well-known regulators of composition and functioning of microbial communities (Bell et al. 2008; Brockett et al. 2012). Seasonal variations in water content and temperature may have a decisive control on microbial growth and activity, which are determinants of key ecosystems processes such as organic matter decomposition and recycling of nutrients (Stark & Firestone 1995; Karhu et al.

2014). So, understanding of how bacterial communities become structured over seasons could provide clues to the underlying mechanisms and feedbacks that regulate community assembly and ecosystem functioning in a subtropical grassland. The few studies that have examined seasonal patterns in soil microbial communities showed a complex picture. In some cases, seasonality may affect the structure of microbial communities and functional properties, suggesting that microbial dynamics is strongly influenced by changing temperature, moisture and nutrient availability in tropical ecosystems (Smith et al. 2015). Contrary, others studies showed that bacterial communities are not strongly tied to seasonal variation such C allocation and soil temperatures in temperate ecosystems (Landesman & Dighton 2010). In temperate and subtropical soils, microbial communities experiencing regular episodic rainfalls and large variations in temperature may be more tolerant to seasonal variations than communities that do not experience regular events, such as those present in tropical or desert soils, suggesting that the response of microbial communities may be climate history-dependent (Frossard et al. 2015; Fierer et al. 2003; Bouskill et al. 2012).

1.4 Methodological developments in microbial ecological studies

During the last 35 years, microbial ecological research has experienced a transformation that has altered the view of microbiologists regarding microorganisms in complex ecosystems. It is now well recognized that microbiologists had grossly underestimated microbial diversity by relying on cultivation-based techniques, which capture only a selection of a few microbial taxa capable of growing rapidly at artificial laboratory conditions (Rappé & Giovannoni 2003). Studies in the last two decades have revealed that most (around 90 to more than 99%) of the microbes present in many environmental

samples cannot be cultivated and hence remain obscure regarding their behavior in the environment and their ecological functions (Skinner et al. 1952; Sørensen et al. 1997). The diversity of the unculturable microbial community was a relatively minor issue among microbiologists until 1985, when the idea arose that microbial diversity could be explored by applications of molecular biological approaches (Handelsman 2004). Culture-independent studies, based on direct isolation of DNA and RNA from environmental samples, recently in combination with high-throughput sequencing have gained new appreciation of the breadth and dynamics of the soil microbiome.

The most recent innovations in large-scale sequencing technologies, and evolution of bioinformatic tools allowed to advance from analysis of a few hundred sequences (when the microbes were accessed by cloning) per study to hundreds of millions of reads, and enabled us to dig deeper into microbial communities. As a consequence of the widespread use of PCR and amplicon sequencing (Caporaso et al. 2011), the 16S rRNA gene associated with barcode indexing approach (Hamady et al. 2008) has been so far the most frequently used molecular marker for community characterization. The reasons for the success of this approach is a combination of: (i) it has a slow rate of evolution (Isenbarger et al. 2008); (ii) it has a suitable length for bioinformatics purposes; (iii) the gene occurs at least in one copy per genome in bacteria (Acinas et al. 2004) and; (iv) there is an immense number of sequences available in databases for comparative purposes (Větrovský & Baldrian 2013).

After overcoming the difficulty in accessing the non-culturable microbes, the need arose to develop adequate computational tools that allow for analysis of the huge pile of information generated by machines. Multiple steps including sequence filtering, alignment, clustering (usually 16S rRNA sequences with 97% similarity represent the same microbial “species” - (Stackebrandt & Goebel 1994)) and comparison with reference information from databases such as SILVA and Greengenes are required to assign the 16S

rRNA sequences to microbial taxa present in databases. A wide number of tools such as QIIME, mothur, UPARSE and R packages that facilitate this task have been published in recent years (Gonzalez & Knight 2012).

1.4.1 Network-based approaches

Microorganisms play key roles in soil food-webs and biogeochemical processes, but specific ecological relationships among microbial species are largely unknown yet. Microbes rarely live in isolation, but interact with neighboring species in complex assemblages (Marx 2009). Interactions can be positive, for instance when two species exchange complementary metabolites (Woyke et al. 2006) or negative, for instance in case of competition for a limiting resource (Lidicker 1979) or when producing antimicrobial metabolites (Whipps 1997). To infer these interactions, experimental systems of reduced complexity based on combinations of isolates (synthetic communities) have been used (Saleem et al. 2013; Großkopf & Soyer 2014). However, this approach is limited because the constructed assemblages fail in reflecting the real communities in soil taking into account that the largest fraction of microorganisms in soil may be so-far uncultured.

Traditionally, biodiversity studies in soil microbial ecology consider only species richness/diversity and composition and ignore the co-occurrence among different microorganisms (Barberán et al. 2011). However, microbe-microbe interactions may be more important to ecosystem processes and functions than species diversity especially in complex ecosystems such as the soil. Biotic and abiotic disturbances caused by land-use changes, management and plant cover may also lead to changes in microbial interaction patterns, such as in the control of plant pathogens and the associated suppression of plant diseases, which is depending of specific interactions between pathogens and certain groups of beneficial pathogen-controlling micro-organisms (Postma

et al. 2008).

Integrative methodology to study the interactions between (micro)organisms in an ecosystems (biology system concept, Kitano 2002), has developed rapidly in last decades. The approach of network development has been applied by mathematicians, computer and social scientists (Borgatti et al. 2009), and recently, it was used to examine complex interactions among microbes (Chaffron et al. 2010; Duran-Pinedo et al. 2011). It was shown that this statistical approach can be applied to large soil microbial datasets thereby offering new insights into the microbial community and the ecological rules guiding community structure and functioning under natural conditions or at anthropogenic shifts in ecosystems (Barberán et al. 2011; Zhou et al. 2011).

Network approaches include modeling of microbial communities using basic rules on growth and association patterns. After abundance data (based on relative recovery of sequences) have been accessed and OTUs or "species" have been identified, microbial relationships can be predicted by applying association metrics or correlation coefficients to multiple pair-wise combination (all possible connections within a set of OTUs). The outcome is a network comprised of nodes and edges; nodes are defined as OTUs, "species" or any relevant taxonomic level and edges represent a significant relationship between nodes (Faust & Raes 2012). Microbial associations can, subsequently, derived from the networks; when two nodes show similar abundance pattern over multiple samples, a positive association between them is assumed; when they show the inverse patterns, a negative relationship is predicted (Steele et al. 2011). Thereafter, properties potentially relevant for community roles and functioning, such as clustering betweenness, centrality, closeness centrality, can be determined by the application of mathematical methods derived from the graph theory (Albert & Barabasi 2002).

The network-based approach is essential to explain fundamental questions still unclear in microbial ecology, such as the identification and role

of keystone species. The keystone-species concept was borrowed from macro-organisms theories and was defined by Paine (1969) as species exerting an important influence on the patterns of species interactions, occurrence and distribution within a biological community. To date the identification of keystone species is critical given the complexity, high diversity, and uncultivated status of a large portion of microbial communities (Berry & Widder 2014). Keystone species may be the core players in trophic interactions, for instance when one microorganism is dependent on another one for the degradation of specific substrates (Kato et al. 2008) or in cellular communication between microorganisms (Ng & Bassler 2009). Thus, the loss of keystone species by anthropogenic disturbances associated with land-use changes may lead to community fragmentation affecting ecosystem functioning (Coleman & Whitman 2005; Nielsen et al. 2011).

1.5 Research aims and thesis outline

The major goal of the research described in this thesis was to understand to what extent land-use changes in conjunction with soil type and seasonal climatic variations affect the diversity, composition and dynamics of the soil microbiome in Pampa grasslands. Therefore, I performed an evaluation of the soil microbial communities across different soil types and land use systems in three landscapes (field studies). In addition, a controlled experiment mimicking natural variation in soil moisture and temperature was performed to gain insight into the impact of climatic conditions of temperature and moisture on the dynamics of the microbial communities and in particular the differentiation in active and dormant fractions.

The research questions addressed are:

(i) To what extent is the soil microbial community of Pampa ecosystems determined by soil type ?

(ii) Do land-use changes in the Pampa biome affect the soil microbiome ?

(iii) Do the active and dormant communities respond in the same way to seasonal variations in soil moisture and temperature in a subtropical grassland of the Pampa biome ?

(iv) Is the network approach useful to identify keystone species in the soil microbial community ?

1.6 Approaches

In order to assess how soil microbial communities are affected by land-use changes, soil properties and soil type, I analyzed microbial communities by analyzing a PCR-amplified region of 16S ribosomal DNA and ITS (Internal Transcribed Spacer), the most commonly used taxonomic indicators. For the detection of the impact of soil type, I sampled a typical sequence of soils (toposequence) of native grasslands. The effect of land-use change and soil properties on the structure, diversity and function of the microbial community was studied at three sites: (*i*) the first site was characterized by two natural vegetation types, *i.e.*, grassland and forest and two adjacent arable sites, *i.e.*, soybean crop and *Acacia* tree plantation introduced after the removal of the natural vegetation; (*ii*) the second site is similar to the first site (two natural vegetation and two anthropogenic uses), where crop production (watermelon and soybean fields) and *Eucalyptus* tree plantation were introduced after replacement of the natural grassland and; (*iii*) the third site consisted of a pristine forest and an eight-year-old grassland, which resulted from the clear-cutting of the forest.

Furthermore, to obtain better understanding of how seasonal variations are important to shape the coexisting active and dormant communities in soil in a subtropical grassland in Pampa biome, I followed the active and dormant communities dynamics in a well-controlled microcosm system containing soil

from a Pampa grassland site, where individual and combined effects of soil moisture and temperature mimicking winter and summer conditions were evaluated.

Finally, to assess the usefulness of the network approach to predict how land-use changes influence microbial ecological interactions and to identify keystone species associated with each land use system, I performed a network analysis approach using a data base from two biomes in Brazil, Pampa (using the data set from the sites described above) and Cerrado (two natural vegetations - grassland and forest - and two anthropogenic usages - coniferous forest and sugarcane field).

My specific research objectives are addressed in 5 chapters:

I- Investigate how soil type and land use systems affect the dynamics of the soil microbial community

In *Chapter 2* I analyze and discuss to what extent land-use changes and different soil types are key factors determining the microbiome structure and diversity in Pampa soils.

In *Chapter 3* which is a continuation of the work described in *Chapter* 2, with a focus on thorough, in-depth analysis of bacterial communities in the same land use systems and soil type.

In *Chapter 4* I aim to understand how modifications in plant cover and soil properties affect microbial community composition and diversity. I investigated bacterial communities in a natural forest soil and in an adjacent grassland, resulted from the replacement of the forest.

II- Assess the responses of soil microbiome related to modification in seasonal climatic variations

In *Chapter 5* I provide information on soil microbiome responses in a microcosm experiment with different combinations of moisture and temperatures mimicking the natural climate conditions over the year in Pampa biome.

III- Use of network approach to understand the microbe-microbe interactions and detect keystone species in different land use systems

In *Chapter 6* I evaluate how land-use changes influence microbial interactions and I identify the presence of keystone species associated with each land use system using a network-based approach.

Finally, in *Chapter 7* the main findings of this thesis are combined with conceptual approaches to discuss the implications of land-use changes in conjunction with seasonal climatic variations for microbial communities and ecological interactions in a subtropical grassland.

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Chapter 2

Land-use changes and soil type are drivers of fungal and archaeal communities in the Pampa biome

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Abstract

The current study aimed to test the hypothesis that both land-use changes and soil type are responsible for the major changes in the fungal and archaeal communities structure and functioning of the soil in the Brazilian Pampa biome. Soil samples were collected at sites with different land uses (native grassland, native forest, *Eucalyptus* and *Acacia* plantation, soybean and watermelon fields) and in a typical toposequence in Pampa biome formed by Paleudult, Albaqualf and alluvial soils. The structure of the soil microbial community (archaeal and fungal) was evaluated by Ribosomal Intergenic Spacer Analysis and soil functional capabilities were measured by microbial biomass carbon and metabolic quotient. We detected different patterns in microbial community structure driven by land-use changes and soil type, showing that both factors are significant drivers of fungal and archaeal community structure confirming the hypothesis that both land-use changes and soil type are drivers of archaeal and fungal community structure as well as of soil functional capabilities. Fungal community structure was more affected by land use and archaeal community was more affected by soil type. Irrespective of the land use or soil type, a large percentage of Operational Taxonomic Units (OTUs) were shared among the soils. Moreover, we also suggest the existence of a soil microbial core.

Keywords

Intergenic spacer analysis; Microbial community; Afforestation; Soil microbial core

2.1 Introduction

The Pampa biome covers an area shared by Brazil, Argentina and Uruguay in the southern part of South America and is characterized by typical vegetation of native grassland, with sparse shrub and tree formations (Overbeck et al. 2007). In Brazil, this biome occupies part of Rio Grande do Sul State and was officially recognized by the Brazilian Institute of Geography and Statistics in 2004 (IBGE 2007). It presents distinctive characteristics of vegetation, climate and soil types, making it a unique ecosystem on the planet, capable of holding a high plant and animal diversities. Although considered one of the priority areas for flora and fauna conservation (MMA 2002), this biome is the most unknown of the Brazilian's biomes in relation to its biodiversity.

Despite its ecological importance, the Pampa biome is not adequately protected under current conservation policies (Overbeck et al. 2007). In recent years, this biome has undergone severe modifications through land-use changes by the conversion of natural vegetation to agricultural land (rice and soybean) and to large areas with exotic tree plantation (*Acacia* sp., *Eucalyptus* sp. and *Pinus* sp.). It is estimated that half of its original vegetation has been removed and transformed into other types of vegetation (Pillar et al. 2009). Furthermore, another important characteristic is that this biome has a wide specific variety of soil types, formed mainly upon sandy material. These soils are fragile and very susceptible to natural or anthropogenic erosion, which may enhance the effects of land-use changes (for more information see Overbeck et al. (2007) and Roesch et al. (2009)).

Many studies have shown that both land use and the soil type determine the composition of soil bacterial communities with emphasis on the effects of edaphic parameters on this domain (da C Jesus et al. 2009; Wallenius et al. 2011; Osborne et al. 2011). However, there are few reports and little is known about the effect of land-use changes or soil type on soil fungal and archaeal communities (Wakelin et al. 2013; Carson et al. 2010; Taketani & Tsai 2010; Takada Hoshino et al. 2011). According to recent findings, land-use changes and soil type can affect the structure of both fungal and archaeal communities in different ways. While studies show that the fungal community is more affected by vegetation or soil management (Kasel et al. 2008), the modification of the archaeal community structure is more affected by soil types or chemical variables (Chen et al. 2010). In addition to modifying the structure of soil microbial communities, these factors can also influence the soil ecosystem processes and important functions mediated by fungal and archaeal communities (Bissett et al. 2011; Berthrong et al. 2009).

Microbial communities can have a profound influence on biochemical processes in soil. Studying how environmental changes affect these groups could help to predict how biogeochemical cycles will respond to different soil modifications. For example, ammonia-oxidizing archaea are considered to be major contributors for the first step in nitrification, the transformation of ammonium (NH_4^+) to nitrite (NO_2^-) (Leininger et al. 2006; Gubry-Rangin et al. 2010), a key process in the global nitrogen cycle. The soil type is a determinant factor that affects the population and structure of these communities in soil (Chen et al. 2010; Takada Hoshino et al. 2011) and different land uses also influence these communities, especially through changes in soil chemical properties (Taketani & Tsai 2010). Fungal communities are thought to be the main active players in the decomposition, a key process of the carbon cycle, because of their ability to degrade complex polymers, such as cellulose and hemicelluloses, and recalcitrant compounds such as lignin (Schneider et al.

2012). There are indications that soil fungal communities are less correlated with modifications or differences in soil chemical properties (Wakelin et al. 2008), but this group is mainly influenced by litter nutrient content (Schneider et al. 2012). Therefore, the new vegetation type introduced by land-use changes might be the main factor affecting soil fungal structure and function (Lauber et al. 2008). Although several works focus on finding differences in microbial communities in distinct environments, recently more attention has been given to microorganisms widespread across habitats, suggesting the existence of a soil microbial core which harbor microorganisms presents in all environment regardless of soil type or land use (Shade & Handelsman 2012).

Nevertheless, there are no studies in the Brazilian Pampa biome about the effects of land-use changes on soil microbial communities and there is no information about the differences between microbial communities in different soil types of this biome. Due to these factors, we attempted here to address a simple question: which factor, land use or soil type, is responsible for the structuring of the fungal and archaeal communities in the Brazilian Pampa biome? In this study, we hypothesized that both land-use changes (*e.g.*, introduction of agricultural cropland and exotic tree plantations) and soil types in the Pampa biome have a significant effect on the soil archaeal and fungal community structure and functioning. To study the effect of land-use changes on the microbial communities, we sampled two sites with the same soil type (Paleudult) and with different usages (natural grassland, natural forest, agricultural cropland and exotic trees plantation). To verify the effect of different soil types on microbial communities we sampled a typical sequence of soils (toposequence) of this biome under native grassland. Soil microbial community structure was characterized using the Ribosomal Intergenic Spacer Analysis (RISA) approach and the overall status of the microbial community was evaluated by measuring microbial biomass and metabolic activity.

2.2 Materials and methods

2.2.1 Study sites and soil sampling

To analyze the impact caused by land-use changes in fungal and archaeal communities, soil samples were collected at two sites in the state of Rio Grande do Sul, Brazil: site A, located in Rosário do Sul municipality, and site B, located in São Gabriel municipality, approximately 23 away far from site A (the sampling sites can be visualized in Fig. S2.1). To minimize the effect of climate and soil type on the microbial communities at each site, samples were collected at the same day (December 17th, 2010), in adjacent areas and under the same soil type (Paleudult, U.S. Soil Taxonomy; Alisols, WRB-FAO). To analyze the effect of different soil types of the Pampa biome on microbial communities, soil samples were collected in a common toposequence (sequence of related soils which differ from each other, especially due to the topography as a factor soil formation but having the same parent material) in the landscape of this region with uniform vegetation, formed by native grassland, currently used for grazing cattle. The toposequence was denominated site C, located in São Gabriel municipality, approximately 4.5 km far from site B is formed by Paleudult (U.S. Soil Taxonomy; Alisols, WRB-FAO), Albaqualf (U.S. Soil Taxonomy; Planosols, WRB-FAO) and typical alluvial soil.

At site A, soil samples were collected in areas with four different land uses: NGA - native grassland currently used for grazing of cattle (30° 00′ 38.2″ S and 54° 50′ 17.4″ W, altitude 121 m); NFA - native forest used only for preservation of wildlife (30° 00′ 39.7″ S and 54° 50′ 05.6″ W, altitude 120 m); SFA - soybean field cultivated under no-tillage system on oat straw, with plants in early stage of growth (30° 00′ 40.3″ S and 54° 50′ 13.2″ W, altitude 122 m); and APA - 9-year-old *Acacia* tree plantation (*Acacia mearnsii* Willd.) (30° 00′ 27.5″ S and 54° 50′ 10.2″ W, altitude 120 m).

At site B, soil samples were also collected in areas with four different land uses: NGB - native grassland currently used for grazing of cattle (30° 11′ 03.6″ S and 54° 42′ 48.3″ W, altitude 172 m); NFB - native forest used only for preservation of wildlife (30° 10′ 57.5″ S and 54° 42′ 54.8″ W, altitude 173 m); WFB - watermelon field cultivated in conventional tillage, with plants in early stage of growth (30° 10′ 57.5″ S and 54° 42′ 54.8″ W, altitude 173 m); and EPB - 5-year-old *Eucalyptus* tree plantation (*Eucalyptus urophylla*) (30° 10′ 57.3″ S and 54° 42′ 48.5″ W, altitude 168 m).

At site C, four soil samples were collected in a line about 120 m from the highest (top of a hill) to the lowest point (near small stream), with distance between sampling points of approximately 30 m: T1C - point with highest altitude, top of a hill, Paleudult soil (30° 13′ 34.3″ S and 54° 42′ 24.6″ W, altitude 178 m); T2C - point with upper middle altitude, Paleudult soil (30° 13' 34.1″ S and 54° 42' 25.9″ W, altitude 175 m); T3C - point with lower middle altitude, Albaqualf soil (30° 13′ 33.9″ S and 54° 42' 27.4″ W, altitude 172 m); and T4C - point with the lower altitude (30° 13' 34.1″ S and 54° 42′ 29.8″ W, altitude 167 m), alluvial soil constituted by fluvial sediments deposited by stream. This occurs because soils in the region are sandy and very susceptible to erosion.

For microbial community analysis, a total of 60 soil samples were collected following the experimental design proposed by Baker et al. (2009). The samples were taken by drawing five randomly distributed 1 m² plots per land use or point in the toposequence. A composite sample was collected by taking samples in every corner and in the center of the square. The soil was collected with a sterile spatula to a depth of 5 cm and stored in sterile bottles in ice, until transport to the laboratory in the same day. Each soil sample was used to extract the total DNA separately.

For physical, chemical and microbial metabolic activity analyses, soil samples from each land use or point in the landscape, were pooled to make a

single composite sample, resulting in 12 samples. The samples were collected in the same day for microbial community analysis, with a sterile spatula to a depth of 5 cm and stored in sterile bottles in ice, until transport to the laboratory.

2.2.2 Physical, chemical and microbial metabolic activity analyses

The physical and chemical analyses were performed according to the recommendations of the Brazilian Society of Soil Science (Silva 2009). Total carbon and nitrogen were determined by combustion using an automated C/N analyser (Flash EA 1112, Thermo Finnigan, Milan, Italy). Soil moisture content was determined gravimetric method by oven drying soils at 105°C for 24h. Clay was determined by densimeter method, soil pH was measured in a 1:1 soil/distilled water suspension. Potassium and phosphorus were extracted by Mehlich-1. Aluminum, Calcium and Magnesium were extracted by KCl 1 mol L^{-1}. Copper and Zinc were extracted by HCl 0.1 mol L^{-1}, sulfur was extracted with $Ca(H_2PO4)_2$ and boron was extracted with hot water. Physical and chemical data from the sites studied are show in Table S2.1. The estimation of microbial biomass carbon (MBC) was conducted by fumigation-extraction method (Vance et al. 1987) and metabolic quotient (qCO_2) was calculated by ratio between basal respiration (Stotzky 1965) and microbial biomass (three repetitions was used to estimate microbial biomass and qCO_2).

2.2.3 Soil DNA extraction and RISA

Soil DNA was extracted with Kit PowerSoil®DNA (MoBio Laboratories, Inc) according to the manufacturer's instruction with the exception that 1 g rather than 0.25 g of soil was used and the final DNA extracts were eluted into 50 µl of ultrapure H_2O rather than solution C6. DNA concentrations were

determined using NanoVue™ (GE Healthcare) and all DNA samples were stored at -20°C until needed. The genetic structure of the dominant portion of the soil fungal and archaeal communities was determined by RISA. The fungal internal transcribed spacer (ITS) was amplified according to Sequerra et al. (1997) and the archaeal intergenic spacer region was amplified according to Summit and Baross (2001). The gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Molecular Probes) following the manufacturer's instructions. An aliquot of 20 µl PCR products of fungal and archaeal was loaded into 8% non-denaturing polyacrylamide gel in TBE buffer and resolved by electrophoresis for 14 h at 60 V and 5 mA. The gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Molecular Probes) following the manufacturer's instructions.

2.2.4 Data analysis

The RISA profiles were used to generate a binary matrix (presence/absence) with Gel-Pro Analyser program (Media Cybernetics, USA) with 5% threshold. The fungal and archaeal data were analyzed using multivariate techniques. To compare the genetic structure between samples, a matrix of similarity (Jaccard index) was calculated from the binary matrix with PRIMER 6 (Clarke & Gorley 2006). Changes in relative similarities of each site were measured using ranks of similarity represented by non-metric multidimensional scaling (NMDS) with PRIMER 6, where ordination distance ranking was showed using Kruscal's stress value, which indicates the degree of inconsistency between the similarity matrix and the final configuration of the plots. This value should be smaller than 0.2 to give a good and accurate representation of the similarities between the samples Clarke (1993). Associated with NMDS, one-way analysis of similarity (ANOSIM) tests were used to assess significant differences in microbial composition of land uses and

soil type, which use a similarity matrix (the same of NMDS) to calculate the R value. R value represent a difference of average rank similarities between and within groups, which can varies between 0 and 1. R values near 0 indicate a true null hypothesis of no difference between groups, whereas those greater than 0 indicate discrimination between groups (reaching a maximum of 1, when similarities within groups are less than any similarities between groups) (Clarke & Gorley 2006). R values smaller than 0.5 indicate that microbial composition do not differ statistically between samples and R values greater or equal to 0.5 indicate that the microbial composition differ significantly between samples (Wertz et al. 2007).

To display and analyze how the operational taxonomic unities (OTUs) were partitioned between samples we applied network-based analysis. The network was calculated with the same matrix of presence/absence from previous analysis (NMDS and ANOSIM), generated by using QIIME (Caporaso et al. 2010) and visualized with Cytoscape (Shannon 2003). In this type of analysis there are two types of nodes representing the treatments (in this case, the land uses or soil types) and the OTUs found in all treatments. OTUs found in only one node are connected by only one line (named edge) and OTUs found in more than one node are connected by more than one line. To compare the microbial biomass carbon and the metabolic quotient among different land uses and soil types we used Tukey's test at $p < 0.05$. Correlations were assessed by Pearson's coefficient.

2.3 Results

The average number of OTUs obtained by RISA from fungal and archaeal communities are presented in Fig. 2.1. The number of OTUs from fungal communities differed between land uses in both sites A and B, while the number of OTUs from archaea in site B did not present statistical differences.

Contrasting soil types (site C) also reflect differences in the number of OTUs for both fungal and archaeal communities. In both sites A and B, the smallest number of bands from fungal community was found in the cultivated forests followed by the agricultural sites.

To better judge the (di)similarities between microbial communities from different land usages and soil type, the RISA profiles were used to calculate non-metric multidimensional scaling (NMDS) plots associated with analysis of similarities (ANOSIM) of fungal and archaeal communities. These analyzes provide a way to test statistically whether there is a significant difference between two or more environments. According to the tests the fungal and archaeal communities were ordered according to the land uses and soil types (Fig. 2.2).

Changes in land use affected both fungal and archaeal communities at the two sites evaluated (Table 2.1). The global R, which is the average pairwise R for each group and indicates the general similarity of the microbial community analyzed was 0.83 for the fungal community and 0.51 for the archaeal community at site A. At site B, the global R was 0.48 for fungal and 0.80 for archaeal communities. Furthermore the NMDS and ANOSIM tests indicated that the native vegetation (grassland and forest) formed clusters apart from tree plantation and croplands (Fig. 2.2).

The highest fungal community similarities at site A were found between the native vegetation (pairwise R = 0.33) and the highest dissimilarities were found between *Acacia* plantation and the two native vegetation (Pairwise R = 1.0) (Table 2.1). The highest archaeal communities' dissimilarity at site A was found between the native grassland and the soybean field (Pairwise R = 1.0) (Table 2.1). At site B, the archaeal communities presented high dissimilarity between all the different land uses with the exception of *Eucalyptus* plantation and native grassland that presented an R value equal to 0.34. Those soil samples were collected from fields that were

about 20 m close to each other and far from the other sampling points.

In addition to the differences in fungal and archaeal communities from distinctive land uses, we also found that soil types under the same plant cover (site C) were able to support different fungal and archaeal communities. The archaeal communities were more influenced by the soil type than the fungal communities (archaeal global R equal to 0.81 and fungal global R equal to 0.48) (Table 2.1).

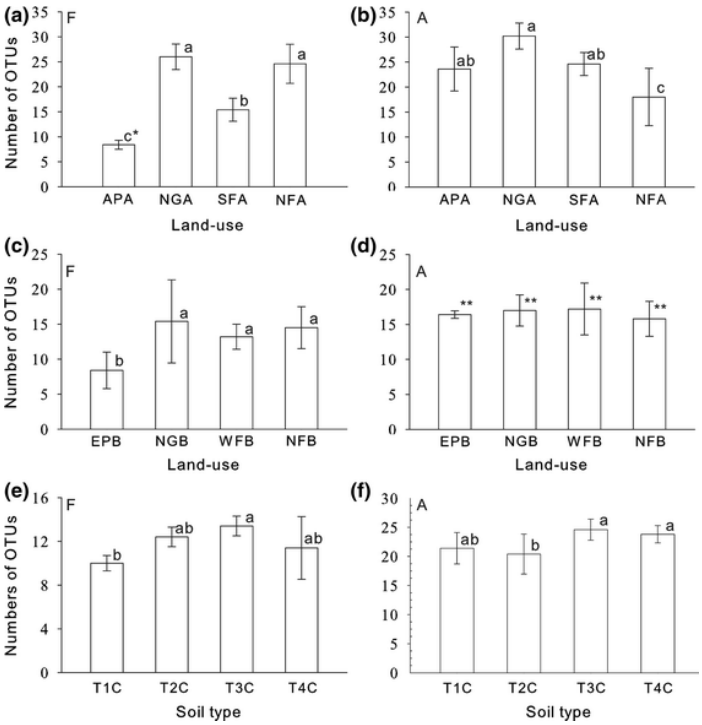

Fig. 2.1 Average number of OTUs obtained by RISA from fungal and archaeal communities from site A (a and b), site B (c and d) and site C (e and f), from Brazilian Pampa biome. APA, *Acacia* plantation; NGA, NGB, Native grassland; SFA, Soybean field; NFA, NFB, Native forest; EPB, *Eucalyptus* plantation; WFB, Watermelon field; T(1:4)C, Toposequence. Bars are mean ± SE, $n = 5$. *Different letters in each columns denote a significant mean difference in OTU number ($p < 0.05$). ** Indicate no difference between numbers of OTUs.

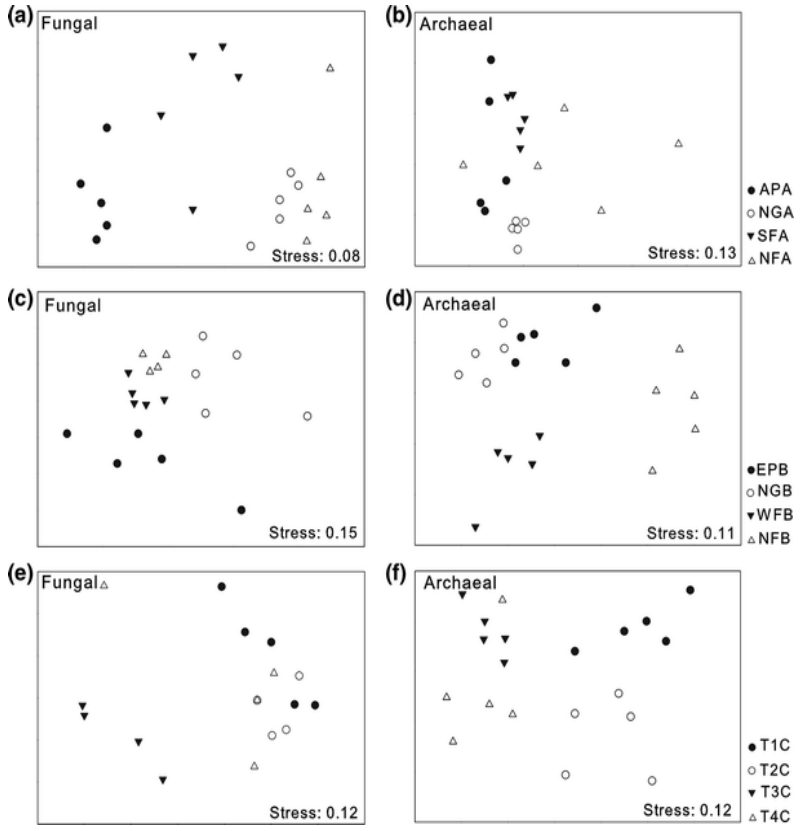

Fig. 2.2 2D Non-metric multidimensional scaling (NMDS) plots of fungal and archaeal communities structure from site A (a and b), site B (c and d) and site C (e and f) determined by RISA profiles. The stress values for all plots were <0.2 which indicate that these data were well-represented by the two-dimensional representation. APA, *Acacia* plantation; NGA, NGB, Native grassland; SFA, Soybean field; NFA, NFB, Native forest; EPB, *Eucalyptus* plantation; WFB, Watermelon field; T(1:4)C, Toposequence.

The archaeal communities were grouped according to the soil type while individual clusters of fungal communities from all different soil types were not clearly observed (Fig. 2.2). The ANOSIM test (Table 2.1) also demonstrated the high divergence between archaeal communities from different soil types. The highest dissimilarities were observed between the most distant soil samples with contrasting soil features (T1C, T4C) while the highest similarities were observed between closer soil samples (T1C, T2C and T3C, T4C).

Table 2.1 One-way analysis of similarities (ANOSIM) of soil fungal and archaeal data from twelve points encompassing two sites (A and B) with different land uses and one site (C) with different soil types from Brazilian Pampa biome

Site A			Site B			Site C		
	Fungal	Archaeal		Fungal	Archaeal		Fungal	Archaeal
Group[a]	Pairwise *R*		Group	Pairwise *R*		Group	Pairwise *R*	
APA, NGA	1.00[b]	0.54	EPB,NGB	0.57	0.34	T1C,T2C	0.53	0.66
APA,SFA	0.87	0.52	EPB,WFB	0.40	0.88	T1C,T3C	0.94	0.95
APA,NFA	1.00	0.38	EPB,NFB	0.42	0.89	T1C,T4C	0.09	0.97
NGA,SFA	0.88	1.00	NGB,WFB	0.68	0.90	T2C,T3C	0.95	0.87
NGA,NFA	0.33	0.48	NGB,NFB	0.48	0.99	T2C,T4C	0.05	0.84
SFA,NFA	0.95	0.38	WFB,NFB	0.67	0.98	T3C,T4C	0.65	0.58

[a]APA, *Acacia* plantation; NGA;NGB, Native grassland; SFA, Soybean field; NFA;NFB, Native forest; EPB, *Eucalyptus* plantation; WFB, Watermelon field; T(1:4)C, Toposequence.[b] All *p*-values were highly significant ($p < 0.001$).

Even although changes in land use have caused significant alterations in community structure of soil fungal and archaeal, showed by NMDS (Fig. 2.2) and ANOSIM test (Table 2.1), the overall network analysis (Fig. 2.3) emphasizes the existence of fungal and archaeal taxa broadly distributed among all soil samples but also reveals the presence of taxa restricted to a particular environment. The OTUs that occurred in only one land use were mainly associated with the native vegetation, especially native grassland (NGA and NGB) and between different soil types. Only the alluvial soil (T4C) presented unique fungal OTUs which probably is due to the high moisture content of the soil (Table 2.1), which is the lowest elevation of the slope.

The microbial biomass carbon content (MBC) differed significantly ($p < 0.05$) among the land uses in sites A and B, and among the soil types (site C) (Table 2.2). At site A, the native forest presented the highest amount of MBC, while at site B, the highest MBC was found at the soil under *Eucalyptus* plantation. The areas under annual cultivation, in sites A and B, presented the smallest amounts of MBC when compared to the grassland and natural forest. This trend was also observed with the qCO_2 values (Table 2.2), where the highest values were observed in annual crops. At site C, the smallest microbial biomass was found at the highest point of the toposequence (T1) and when the samples were collected in sites with smaller altitude a progressive increase in the MBC was observed, although this increment was not statistically different at 5% probability. A positive relationship can be found between soil moisture and MBC (Table S2.1 and 2.2).

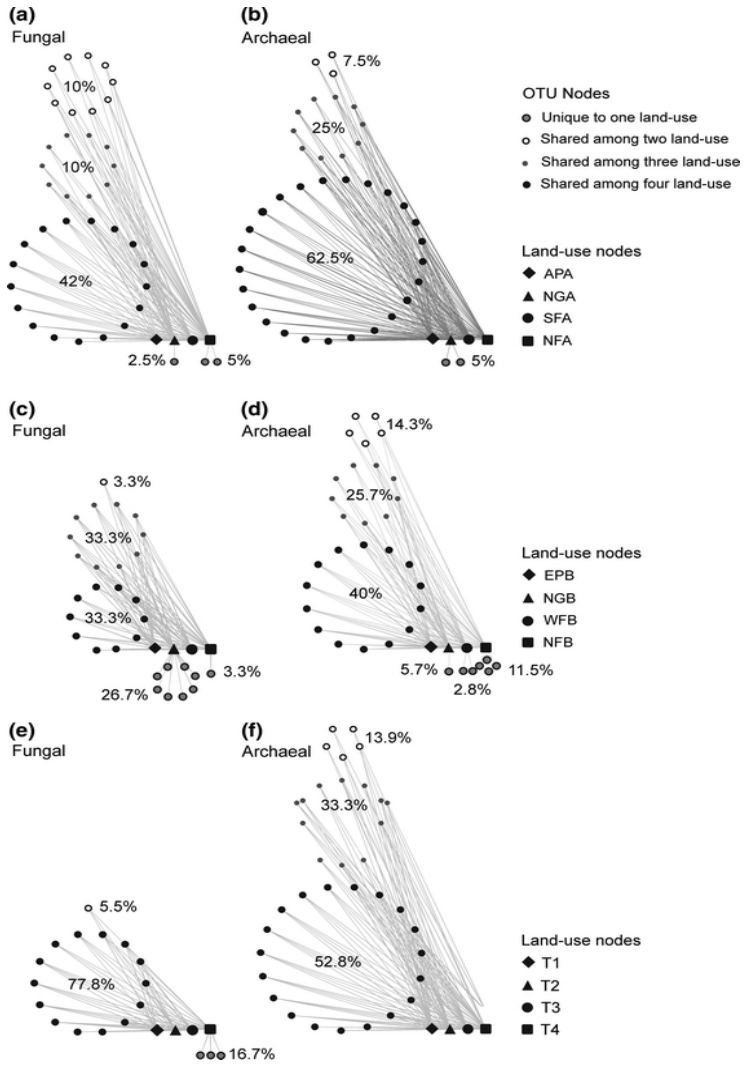

Fig. 2.3 OTU network map showing fungal and archaeal communities and OTUs shared among site A (a and b), site B (c and d) and site C (d and f) in Brazilian Pampa biome. The nodes represent land uses or soil types and fungal or archaeal OTUs shared among four, three or two land uses or fungal or archaeal OTUs unique to only one land use or soil type according to the legend.

When one OTU is found in only one node (land use or soil type) the two nodes are connected with a line (an "edge") and when one OTU is found in more than one node (land use or soil type) the OTU is connected with more than one edge. The numbers show the percentage to total of fungal and archaeal OTUs finding in each site shared or unique among land uses or soil types. APA, *Acacia* plantation; NGA, NGB, Native grassland; SFA, Soybean field; NFA, NFB, Native forest; EPB, *Eucalyptus* plantation; WFB, Watermelon field; T(1:4), Toposequence.

Table 2.2 Microbial biomass C (MBC) and metabolic quotient (qCO_2) from twelve points encompassing two sites (A and B) with different land uses and one site (C) with different soil types from Brazilian Pampa biome

Site	Land use /soil type	MBC mg $kg^{-1}$of C	qCO_2 mg mg^{-1} C-CO_2
Site A	APA[1]	33.04 b[2]	0.17 bc
	SFA	23.34 c	0.21 a
	NFA	53.35 a	0.11 b
	NGA	28.03 ab	0.08 c
Site B	EPB	90.69 a	0.04 a
	WFB	10.67 c	0.35 a
	NFB	27.52 b	0.09 a
	NGB	11.99 b	0.57 a
Site C	T1C	5.12 b	0.19 a
	T2C	16.45 a	0.28 a
	T3C	17.97 a	0.16 a
	T4C	20.43 a	0.23 a

[1]APA, *Acacia* plantation; NGA;NGB, Native grassland; SFA, Soybean field; NFA;NFB, Native forest; EPB, *Eucalyptus* plantation; WFB, Watermelon field; T(1:4)C, Toposequence. [2]Statistical analysis was performed for each site and for MBC and qCO_2 separately. Means followed by the same letter are not significantly different at the 5% level (Tukey; p <0,05).

2.4 Discussion

The fungal and archaeal communities structures were assessed by RISA (Ribosomal Intergenic Spacer Analysis) profiles. The RISA method is considered a highly reproducible fingerprint technique that allows for an easy comparison between samples (van Elsas & Boersma 2011). The method involves PCR amplification of the intergenic spacer region located between the small and large subunit rRNA genes in the rRNA operon. The intergenic spacer is extremely variable in both sequence and length (ranging from 50 bp to more than 1.5 kb) among different taxa allowing for a rapid assessment of the genetic structure of complex communities in soil environments (Borneman & Triplett 1997). It should be mentioned however that, as a molecular technique that relies upon total community DNA extraction and PCR amplification, RISA is subject to the usual systematic biases introduced by these procedures. For these reasons, any conclusions regarding the relative abundance of microbial communities represented in the RISA profiles should be carefully made. In this regard, we used the RISA profiles as a rapid survey technique in conjunction with the measurement of the overall microbial activity (microbial biomass and metabolic quotient) to verify the effect of land use change and soil type on the archaeal and fungal communities.

Changes in land use are common in many landscapes in the world and are among the factors that affect the soil archaeal and fungal community structure (Kasel et al. 2008). Another important factor that influences the microbial community is soil type, mainly by the difference in physicochemical properties (Wakelin et al. 2008; Takada Hoshino et al. 2011). In this study, we hypothesized that soil type can harbor distinct fungal and archaeal communities and land-use change alter significantly these microbial communities in the Pampa biome. To confirm our hypothesis we used two approaches to analyze the alteration in the soil fungal and archaeal

communities caused by land-use change and soil type. One approach was based on the microbial community fingerprinting, and the other on the microbial biomass and metabolic activity.

To our knowledge, this is the first report about the soil fungal and archaeal communities structure across different land uses and soil types in the Pampa biome. Our results clearly revealed that land-use changes exerted a strong influence on the community structure of soil fungal and archaeal communities in the sites analyzed. This was consistent with previous findings (Singh et al. 2009; Berthrong et al. 2009; Wakelin et al. 2008) showing that alteration in land use causes a strong effect on soil fungal and archaeal communities. The modification on the fungal community structure is mostly associated with effects of dominant vegetation type and management practices (Wakelin et al. 2008) while the shifts in archaeal community structure can be mainly associated with modification of bulk soil chemical properties caused by different land uses (Navarrete et al. 2011). We attributed these land use effects directly to the actual dominant vegetation, which could affect the soil microbial community due to the release of different carbon compounds (Berthrong et al. 2009), quantity and quality of litter inputs (Kasel et al. 2008) and allelopathic influences on microbial communities (Lorenzo et al. 2010) among other effects. Different plants in native environments release large amount of metabolites through root that might positively affect the soil microbial population (Zhang et al. 2011). In addition to direct effects of litter on microbial resources, land-use changes can indirectly influence soil microbial composition through plant-mediated changes in soil properties like aggregation, density, infiltration and evaporation of water and bioavailability of nutrients (Singh et al. 2009). Soil management practices used for the establishment of exotic forests and annual crops can modify the physicochemical properties of soil and consequently alter the soil microbial communities and ecological functions (Bissett et al. 2011; Lumini et al. 2009).

We also observed that the fungal community showed more similarity in samples of native vegetation than of introduced vegetation and the archaeal community structure showed major dissimilarity between native grassland and soybean field. These results are similar to other findings, where fungal and archaeal communities tend to be more similar in soils under natural vegetation than in arable soils, because the latter soils are exposed to frequent human disturbances strongly affecting the soil microbial community (Hossain & Sugiyama 2011; Navarrete et al. 2011).

The effect of land-use changes on fungal and archaeal communities varied according to the site analyzed. The fungal community was more influenced by land use in site A; contrary, the archaeal community structure was more altered by land use in site B. This result showed that the environmental and edaphic factors of each location could have a specific impact on the fungal and archaeal communities (Kasel et al. 2008). The low number of OTUs found in our study in *Acacia* and *Eucalyptus* afforestation show that these land usages had a greater impact on the soil fungal community than on archaeal community. Previous studies have shown that introduction of *Eucalyptus* and *Acacia* plantations in areas under natural vegetation can modify soil fungal microbial communities, mainly causing changes in the decomposer fungal community (Lauber et al. 2008; Carson et al. 2010; Lorenzo et al. 2010). The fungal communities presented a higher response due to the vegetation modification than other soil microorganisms because the characteristics of the litter can act as selective pressure towards fungal species capable of degrading specific substratum (Macdonald et al. 2009).

We found that fungal community structure was less affected by soil type than archaeal community. Similarly, previous findings reported that soil type might be less important as a primary driver of fungal community structure than other factors like soil management or land use (Wakelin et al. 2008). Apparently, the fungal communities are mainly associated with the

degradation of plant residues, suffering more intensely by the effects of shifts in vegetation type than by edaphic properties (Lorenzo et al. 2010). Fundamental differences in archaeal and fungal physiology and ecology would suggest that each group would be influenced by different factors. The archaeal communities are mainly affected by soil types and by physicochemical properties (Taketani & Tsai 2010). Chen et al. (2010) found that the population size and community structure of ammonia-oxidizing archaea is mainly determined by the soil types in flooded paddy soils, irrespective of the presence or absence of vegetation. Similarly, Takada Hoshino et al. (2011) found that the archaeal communities are strongly influenced by soil type, mainly by differences in soil physicochemical properties even under distinct management systems.

Although specific impacts on fungal and archaeal communities structure were found, different land uses and soil types shared a large number of OTUs, suggesting the existence of a core in soil fungal and archaeal communities that did not suffer significant changes related to land use changes or soil type. Microbial communities that do not change might present some degree of resistance or resilience, maintaining its structure unchanged after disturbance (Allison & Martiny 2008). Our results suggest the prevalence of a resilient core microbial community that does not suffer any change related to land-use changes, soil type or edaphic conditions. In addition to detecting the presence of a soil microbial core, we additionally found unique OTUs in natural vegetations, in sites A and B, mainly associated with the native vegetation, suggesting that land-use changes and the introduced plant species might cause the loss of some microbial communities (Carson et al. 2010). In the landscape (site C), only the alluvial soil showed unique OTUs from fungal community. This soil is located in the lower part of the toposequence and presents a moisture content above the others and can accommodate a fungal community adapted to higher water availability and reduced availability of oxygen.

To investigate the effect of land-use change and soil type on the overall microbial conditions we measured the microbial biomass and metabolic quotient at each sampling site. The results demonstrated that different land use and soil type also exerted influence on the microbial biomass carbon and the metabolic quotient. The microbial biomass controls many important functions in soil and can be used as an indicator of environmental disturbance cause by land-use changes. As well as changes found in fungal and archaeal communities in sites A and B, significant differences were also detected in the microbial biomass, indicating microbial biomass affected by the land use changes. It is well documented that the soil biomass and microbial activity (soil respiration and metabolic quotient - qCO_2) are largely determined by the most abundant and diverse group of microbes in soil *i.e.,* bacterial communities, which may be the largest contributor for biogeochemical process, such as decomposition and CO_2 efflux in soil (Hofmann & Illmer 2015; Goberna et al. 2011). Fungal communities are also actively involved in biogeochemical processes, thereby contributing significantly to soil respiration (Kant et al. 2010). The fungal abundance is positively related to microbial biomass, contributing from 52% in forest soils up to 80% in arable soils to total biomass (Susyan et al. 2011). The rate of soil respiration is highly correlated to fungal population in soil and shifts in respiration are often attributed to differences in the structure of fungal community (Aanderud et al. 2013). Although archaeal community are important in CH_4 and N dynamics (Taylor et al. 2012), the contribution of this group to soil biomass and soil respiration is less studied and largely unknown. Comparison among archaeal structure and total biomass and soil respiration have to made carefully because this group may represent only a small proportion of the community (Esperschütz et al. 2007; Ushio et al. 2013), and it is expected that this group contribute less to the soil respiration than bacterial and fungal groups. Most part of the studies so far showed no correlation of archaea with shifts in biomass or soil respiration (Goberna et al.

2011). However, the archaeal community may be important to soil respiration process at certain conditions. Han (2013) suggested that the increase in soil respiration is a process determined by the increased proliferation of microbial populations, including archaea.

Areas under natural conditions (grassland and natural forest) presented the greatest amount of MBC in both sites. This effect has already been detected in grassland and native forests (Bissett et al. 2011; DuPont et al. 2010) and might be attributed to the high plant diversity and consequent high litter deposition and release of metabolites through root turnover that might positively affect the soil microbial community (Zhang et al. 2011). Furthermore, many factors are associated with the decreased microbial biomass in croplands: soil tillage, chemical inputs (Treseder 2008), herbicide application (Singh & Ghoshal 2010), reduced inputs from plants through root exudation and rhizodeposition (Waldrop et al. 2006).

In addition, we found that soil type also can influence the MBC, because a positive correlation was found between soil moisture and MBC in site C. The results are in agreement with Bissett et al. (2011), who suggested that moisture is probably the most important factor in dry land soils determining the size of the microbial biomass. Soil water content is essential for all soil biogeochemical processes and an important factor determining microbial activity (Brockett et al. 2012). The metabolic quotient (qCO_2) is an index that expresses soil quality, representing the efficiency by which organisms use ecosystem resources (Zhang et al. 2011). High qCO_2 values indicate low C efficiency because there is a great loss of CO_2 and consequently lower amount of C incorporated into microbial biomass. In our study, the metabolic quotient differed significantly between land uses only at site A, where soybean cropland showed the highest qCO_2 value, indicating that the soil microorganisms were living under environmental stress here and natural grassland showed the lower metabolic quotient, indicating better soil quality. The change in

metabolic quotient may be due to modifications of the community of microorganisms influenced by different land uses (Bissett et al. 2011). Even with changes in fungal and archaeal communities and microbial biomass in different land uses in site B and different soil types in site C, the qCO_2 was not significantly different ($p < 0.05$). Changes in microbial composition might not affect the ecosystem process because the old and the new community can carry out the same processes even having a different microbial structure (Allison & Martiny 2008) or because the core community can maintain some of the roles played by microorganisms in the soil, since the changes detected occurred in only part of the microbial community.

2.5 Conclusion

With this study we attempted to address a simple question: Which factor, land use or soil type is responsible for the major changes in the fungal and archaeal communities in the Brazilian Pampa biome? The sampling strategy and the molecular and biochemical approaches applied here allowed us to conclude that both land-use changes and soil types are significant drivers of microbial community richness, structure as well as of soil functional capabilities. The unique OTUs occurred mainly associated with the native vegetation, suggesting that land use changes might cause the loss of some microbial groups. Nevertheless, irrespective of the land use or soil type, a large percentage of OTUs were shared among the soils, which can be indication of the existence of a soil microbial core.

2.6 Supplementary material

Table S2.1 Soil chemical and physical properties of surface soils (0-5 cm) from twelve points encompassing two sites (A and B) with different land uses and one site (C) with different soil types from Brazilian Pampa biome

Soil property	Site A				Site B				Site C			
	APA[1]	SFA	NFA	NGA	EPB	WFB	NFB	NGB	T1	T2	T3	T4
Clay (g kg^{-1})	150	150	180	120	130	135	135	85	105	95	100	90
U (%)	20.8	9.7	19.6	7.1	6.3	4.2	10.1	19.1	2.5	2.9	10.4	25.6
pH	5.2	6.1	5.4	4.3	6.3	5.1	4.5	6.4	5.4	5.0	5.2	5.9
Total C (%)	2.2	1.0	5.8	2.1	6.2	1.8	2.8	1.9	1.2	1.3	2.5	1.3
Total N (%)	0.2	0.1	0.5	0.2	0.5	0.2	0.2	0.1	0.1	0.1	0.2	0.1
P (mg L^{-1})	5.7	21.8	33.5	4.5	76.0	11.4	26.9	14.0	8.1	5.7	6.9	16.2
K (cmol$_c$ L^{-1})	0.4	0.4	0.2	0.1	0.2	0.5	0.4	0.2	0.3	0.3	0.3	0.2
Al (cmol$_c$ L^{-1})	0.3	0.4	0.1	1.2	0.0	0.3	0.5	0.0	0.1	0.3	0.2	0.0
Ca (cmol$_c$ L^{-1})	2.2	1.2	10.2	1.0	4.3	1.4	2.0	1.2	1.7	1.0	1.4	2.4
Mg (cmol$_c$ L^{-1})	0.6	0.5	1.3	0.2	1.6	0.7	0.5	0.4	0.7	0.6	0.7	1.4
CEC pH7	8.6	6.8	16.9	8.9	8.0	5.4	8.7	3.4	5.1	5.2	5.2	5.5
BS (%)	38.0	42.6	69.7	15.3	77.7	47.6	34.8	55.0	53.5	38.7	46.1	67.2
Zn (mg L^{-1})	2.6	1.6	27.2	1.3	6.9	3.6	2.8	3.0	2.1	2.1	2.1	1.6
Cu (mg L^{-1})	2.6	1.2	0.6	1.4	0.6	2.6	0.7	0.5	0.4	0.7	0.6	0.4
S (mg L^{-1})	12.2	6.0	11.5	9.2	9.3	7.9	10.4	6.2	6.8	8.2	7.5	5.0
B (mg L^{-1})	0.5	0.5	0.4	0.3	0.5	0.5	0.5	0.4	0.2	0.2	0.2	0.3

[1]APA, *Acacia* plantation; NGA, NGB, Native grassland; SFA, Soybean field; NFA, NFB, Native forest; EPB, *Eucalyptus* plantation; WFB, Watermelon field; T(1:4), Toposequence.

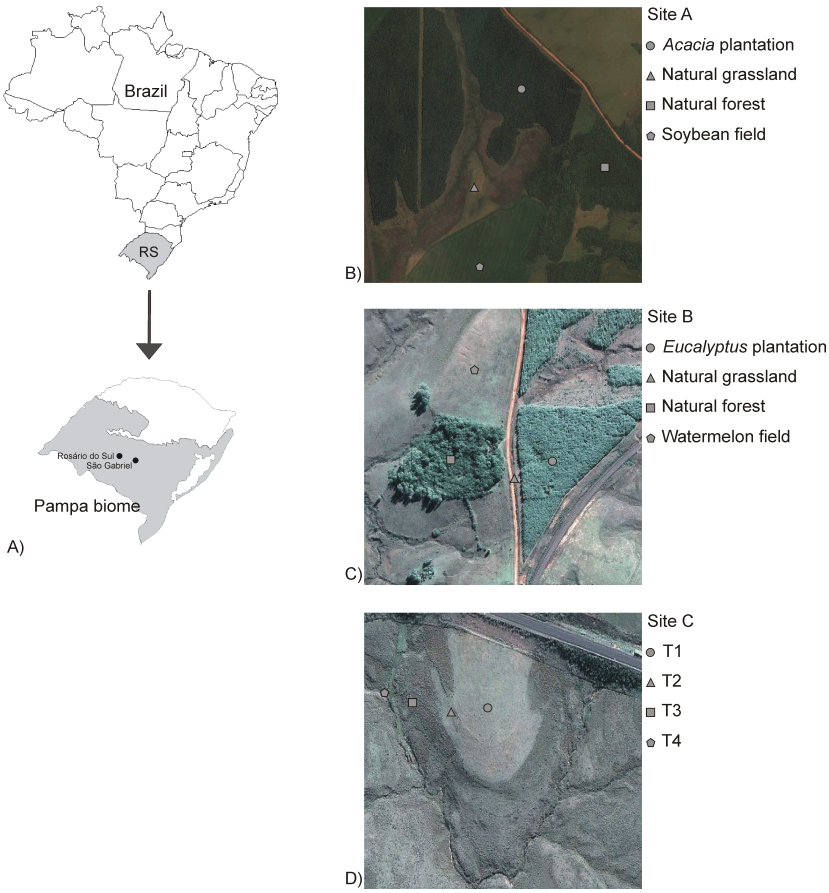

Fig. S2.1 (A) Map of Brazil and the state of Rio Grande do Sul, showing the location of Rosário do Sul and São Gabriel municipalities in the Pampa biome. (B) Site A, located in Rosário do Sul; (C) Site B, located in São Gabriel; (D) Site C, located in São Gabriel. The markers indicate points of soil sampling in different land uses and soil types.

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Chapter 3

Soil-borne bacterial structure and diversity does not reflect community activity in Pampa biome

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Abstract

The Pampa biome is considered one of the main hotspots of the world's biodiversity and it is estimated that half of its original vegetation was removed and converted to agricultural lands and tree plantations. Although an increasing amount of knowledge is being assembled regarding the response of soil bacterial communities to land-use changes, to associated plant community and to soil properties, our understanding about how these interactions affect the microbial community from the Brazilian Pampa is still poor and incomplete. In this study, we hypothesized that different land uses can harbor distinct soil bacterial communities within the same soil type. To test this hypothesis, we assessed the soil bacterial communities from four land uses within the same soil type by 454-pyrosequencing of 16S rRNA gene and by soil microbial activity analyzes. We found that the same soil type under different land uses harbor similar (but not equal) bacterial communities and the differences were controlled by many microbial taxa. No differences regarding diversity and richness between natural areas and areas under anthropogenic disturbance were detected. However, the measures of microbial activity did not converge with the 16S rRNA data supporting the idea that the coupling between functioning and composition of bacterial communities is not necessarily correlated.

Keywords

Land-use changes; 16S rRNA gene; Microbial diversity; Soil functioning

3.1 Introduction

The Pampa biome is considered one of the main hotspots of the world's biodiversity and is one of the priority areas for flora and fauna conservation (MMA 2002). Despite its importance, it is estimated that more than half of the original vegetation was removed and converted to agricultural lands and to large areas with exotic tree plantations (Pillar et al. 2009; Roesch et al. 2009). Although an increasing amount of knowledge is being assembled regarding the response of soil bacterial communities to land use systems, the associated plant community and soil properties (da C Jesus et al. 2009; Nacke et al. 2011; Kuramae et al. 2011; Kuramae et al. 2012) the understanding how land-use changes affect the microbial community from the Brazilian Pampa is still poor and incomplete. It is well known that bacterial communities are the most abundant and diverse group of soil microorganisms and exert multiple important key roles on soil, such as decomposition, biogeochemical cycles and nutrient transformations and any modifications in the microbial community caused by land-use changes might contribute for changing the ecosystem functions and soil quality (Konopka 2009).

Particularly the aboveground vegetation affects the structure, size and activity of soil microbial communities through the input of different quantities and quality of litter deposition and rhizodeposition in soil (Nacke et al. 2011). On the other hand, many studies have reported that soil properties might be considered as key factors affecting bacterial diversity and composition. Soil pH, texture (Fierer & Jackson 2006), Al, Ca, Mg, K, B and P contents (Faoro et al.

2010) are considered the major factors. Bacterial communities can also be affected by others factors *e.g,* history of land use, which was considered a stronger determinant of the composition of microbial communities than vegetation and soil properties (Lauber et al. 2008). In addition, Girvan et al. (2003) proposed that soil type is the primary determinant of the bacterial community composition in arable soils, but to date, little information is available about the ecological interaction between soil type and bacterial communities.

Nevertheless, land-use changes does not always have a significant effect on soil bacterial community. Despite major differences in soil properties and vegetation, soil microbial community was widely distributed and resilient to disturbances of the above ground vegetation (Upchurch et al. 2008; Jangid et al. 2011). This similarity might be caused by some microbial groups that show a high degree of tolerance to changes in environmental conditions, that might result in microbial communities resistant or resilient to disturbances caused by land use (Allison & Martiny 2008). The knowledge of how microbial diversity are influenced by soil management in the Brazilian Pampa may help us to understand the changes in carbon balance, energy flow, and greenhouse gas fluxes under these shifted areas and such knowledge is fundamental for the sustainable management of the soil ecosystem in this threatened hotspot of biodiversity.

Assuming that both land use and soil type affect the bacterial communities, here we performed a large-scale pyrosequencing-based analysis of the 16S rRNA gene to evaluate bacterial diversity, composition and structure from the same soil type but with different land uses. Also, we analyzed the soil microbial activity through the measurements of the microbial biomass carbon and the metabolic quotient. We hypothesized that the same soil type but under distinct land use presents distinct soil bacterial communities. In order to test our hypothesis we assessed and compared the impact of land-use changes on

soil bacterial communities from the Brazilian Pampa biome by sampling typical land uses found in this region. Our major goal was to obtain a detailed baseline description of the soil bacterial communities found in the Brazilian Pampa soils against which to compare changes in the soil microbiome caused by human activities.

3.2 Materials and Methods

3.2.1 Site description, soil sampling and soil physicochemical analyses

In order to analyze the impact caused by land-use changes on the bacterial community, soil samples were collected in a site with four typical land uses in the Pampa biome. This biome covers an area shared by Brazil, Argentina and Uruguay in the southern of South America and is characterized by typical vegetation of native grassland, with sparse shrub and tree formations (Overbeck et al. 2007). In Brazil, this biome occupy part of Rio Grande do Sul State, it has both subtropical and temperate climates with four well-characterized seasons, and was officially recognized by the Brazilian Institute of Geography and Statistics only in 2004 (IBGE 2007). To minimize the effect of climate on microbial community at each site, samples were collected at the same day (November, 2010), in adjacent areas with the same soil type (PALEUDULT, Soil Taxonomy). The samples were collected in a private land and no specific permissions were required for soil sampling. Also, our study did not involve endangered or protected species. Soil samples were collected in following land uses: NP: natural pasture (30° 00′ 38.2″ S and 54° 50′ 17.4″ W, altitude 121 m) currently used for intensive grazing of cattle, with no fertilizers input (except for the manure added by animal activity) or introduction of exotic species; NF: natural forest (30° 00′ 39.7″ S and 54° 50′ 05.6″ W, 150 m - control sample) used only for preservation of wildlife with no

fertilizers inputs and no human activity or animal influence; SF: soybean field (30° 00′ 40.3″ S and 54° 50′ 13.2″ W, 137 m) cultivated under no-tillage system on oat straw, with plants in early growth stage; and AP: 9 years old *Acacia* trees (*Acacia mearnsii* Willd.) plantation (30° 00′ 27.5″ S and 54° 50′ 10.2″ W, altitude 141 m). The land usages analyzed here are the same from site A in *Chapter 2* and published in Lupatini et al. (2013).

Bulk soil samples were collected following the experimental design proposed by Baker et al. (2009). The samples were taken by drawing four randomly distributed 1 m^2 squares approximately 80 m apart to each other within each land use. In each plot, composite samples were collected by taking sub-samples in every corner of the square. Soil samples were collected taking 5 cm diameter, 0-5 cm depth cores. Equal masses of sub-samples removed from cores were pooled and mixed to form four composite samples from each land use. All samples were packed on ice upon collection and transported to the laboratory and kept at -18°C up to the microbial DNA extraction and chemical analysis. From each composite sample, a subsample was removed, air dried and 2 mm mesh sieved and subjected to the chemical and physical analysis. The physicochemical analysis was performed according to the recommendations of the Brazilian Society of Soil Science (Silva 2009). To illustrate soil properties of different land use, principal component analysis (PCA) was carried out in R (Team & others 2005).

3.2.2 DNA extraction, 16S rRNA partial gene amplification and pyrosequencing

Soil DNA was extracted with the PowerSoil® DNA Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instruction with the exception that 1 g rather than 0.25 g of soil was used and the final DNA extracts were eluted into 50 µL of ultrapure H_2O rather than solution C6. DNA

concentrations were determined using NanoVue™spectrophotometer (GE Healthcare) and all DNA samples were stored at -20°C. Independent PCR reactions were performed for each soil sample with the primers 27F and 338R described in Fierer et al. (2008) for the amplification of approximately 311 base pairs of the V1-V2 region of the 16S rRNA gene. PCR reactions were carried out in triplicate with the GoTaq PCR core system (Promega, Madison, WI, USA). The mixtures contained 5 µl of 10X PCR buffer, 200 mM dNTPs, 100 mM of each primer, 2.5 U of *Taq* polymerase and approximately 100 ng of DNA template in a final volume of 50 µl. The PCR conditions were 94°C for 2 minutes, 30 cycles of 94°C for 45 seconds; 55°C for 45 seconds; and 72°C for 1 minute extension; followed by 72°C for 6 minutes. The PCR products for each of the 16 samples were purified and combined in equimolar ratios with the quantitative DNA binding method (SequalPrep Kit, Invitrogen, Carlsbad, CA, USA) for DNA pool for pyrosequencing from the A-Key adaptor. The 16S rRNA gene fragments were sequenced using 454 GS FLX Titanium (Lib-L) chemistry for unidirectional sequencing of the amplicon libraries. Barcoded primers were used to multiplex the amplicon pools in order to be sequenced together and computationally separated afterward. To do this, 8-base barcodes were added to the 5′ -end of the reverse primers using the self-correcting barcode method (Hamady et al. 2008). The primers were attached to the GS FLX Titanium Adaptor A-Key (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG -3′) and Adaptor B-Key (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′) sequences, modified for use with GS FLX Titanium emPCR Kits (Lib-L) and a two-base linker sequence was inserted between the 454 adapter and the 16S rRNA primers to reduce any effect the composite primer might have on PCR efficiency. All raw sequences were submitted to the NCBI Sequence Read Archive (SRA) under the study number SRP013204, experiment number SRX255448.

3.2.3 Processing of pyrosequencing data and taxonomic assignments

The raw sequences obtained were processed using QIIME (Caporaso et al. 2010) with default parameters. Briefly, to reducing sequencing errors and their effects, the multiplexed reads were first filtered for quality and assigned to the starting soil samples. The filtering criteria included a perfect match to the sequence barcode and primer, at least 200 bp in length, no undetermined bases, and at least 60% match to a previously determined 16S rRNA gene sequence (Hamady et al. 2008). Additionally, to identify potentially chimeric sequences, the dataset were subject to the ChimeraSlayer implemented in mothur (Schloss et al. 2009). The sequences were clustered into OTUs based on the relatedness of the sequences (97% similarity) and the representative sequences were subjected to the RDP naïve Bayesian rRNA Classifier (Wang et al. 2007), which attaches complete taxonomic information from domain to species to each sequence in the database with 80% taxonomy confidence and an e-value of 0.001. The representative set of sequences was also used to align the sequences against the Greengenes 16S rRNA database (DeSantis et al. 2006) and to build a phylogenetic tree necessary for downstream measurements.

3.2.4 Alpha- and beta-diversity analysis

For each taxonomic level (Phylum, Class, Order, Family, Genus and at 97% similarity cutoff) Good's coverage was calculated (Good 1953). To compare the similarity between bacterial communities from the soil samples we estimated the diversity of each sample using alpha Phylogenetic Diversity - PD and Rényi diversity profiles. The Phylogenetic diversity is defined and calculated as the sum of the branch-lengths of the minimal sub tree connecting the taxa in the subset (Faith 1992). This evaluation is based on a single phylogenetic tree and sensitive to the quality of the branch length and

topology. Rényi diversity profiles provide information on diversity, richness and evenness of the community. Each value of the Rényi diversity profile is based on an alpha parameter. This diversity ordering technique is preferred to ranking based on single index because rank order may change when different index are used (Tóthmérész 1995). The shape of the profile is an indication of the evenness, a horizontal profile indicates that all species have the same evenness and the less horizontal a profile is, the less evenly species are distributed.

The starting position at the left-hand side of the profile is an indication of the species richness (alpha = 0) and the diversity is ordered from high to low diversity profiles. Profiles that start at a higher level have higher richness. If the profile for one site is everywhere above the profile for another site, then this means that the site with the highest profile is the more diverse of the two and when curves for communities intersect, this mean that they cannot be ranked (Kindt & Coe 2005). For these measurements we calculated the diversity metrics for a randomly selected subset of 2,288 sequences per soil, as alpha diversity index is correlated with the number of sequences and the same number of sequences per sample is recommended (Lemos et al. 2011).

Beta-diversity was analyzed by using Principal Coordinates Analysis (PCoA) which is an ordination method based on multivariate statistical analysis that maps the samples in different dimensions and reflects the similarity of the biological communities. A matrix using the UniFrac metric (weighted and unweighted) for each pair of environments was calculated. The distances were turned into points in space with the number of dimensions one less than the number of samples. The first three principal dimensions were used to plot a three-dimensional graph that was visualized using KING (Chen et al. 2009). To test whether the results were robust to sample size we used a sequence-jackknifing technique in which the PCoA clusters were regenerated using a subset of 1,716 sequences (corresponding to about 74% of the total

number of sequences obtained in the sample with the smallest number of sequences) randomly selected from each soil for 100 replicate trials. The Jackknifed PCoA was performed using QIIME (Caporaso et al. 2010). The clusters observed in the PCoA were confirmed by a similarity percentage analysis (SIMPER) (Clarke 1993). The SIMPER analysis was performed at the family level because the Good's coverage indicated that at this taxonomic level the samples were well represented by the number of sequences obtained (see Table 3.1 in section results). The SIMPER performs pairwise comparisons of groups of sampling units and finds the average contributions of each OTU to the average overall Bray-Curtis dissimilarity between samples through the decomposition of the dissimilarity, which is calculated by the difference of abundance of each OTU in each sample. The weighted OTU table obtained as described above was transformed using log(x+1) to normalize data and the SIMPER analysis was run using R through the vegan package v.2.0-5 with a 70% cutoff (Oksanen et al. 2007).

3.2.5 Distribution of unique and shared OTUs and contribution to dissimilarity across land uses

Network-based analysis was applied to examine the OTUs (family level) shared among the soil samples. The network allows for the visualization of the OTUs that are either unique or shared by specific groups of soil samples. To obtain reliable results, this approach must have intense coverage or must be applied after removing the singletons (Lemos et al. 2011). Connections were drawn between samples and OTUs, and the network was arranged in a neat looking diagram to show the distribution of OTUs over the environments. The diagram was generated with Cytoscape (Shannon 2003) with two kinds of "nodes"; OTU-nodes and soil sample nodes. The OTUs found in only one treatment were connected by only one line ("edge") and the OTUs found in

more than one treatment were connected by more than one line.

3.2.6 Measurement of microbial metabolic activity

Three composite samples from each land use were used to measure the microbial metabolic activity. The microbial metabolic activity was estimated by measuring the microbial biomass carbon (MBC) and by calculating the metabolic quotient (qCO_2). The estimation of microbial biomass carbon was conducted by fumigation-extraction method (Vance et al. 1987) and the metabolic quotient was calculated by the ratio between the basal respiration and the microbial biomass (Stotzky 1965). Microbial biomass carbon and metabolic quotient among different land-uses were compared by Tukey's test at $p \leq 0.05$. These analyses has been described in *Chapter 2* and and published in Lupatini et al. (2013).

3.3 Results

3.3.1 Soil physicochemical properties

The physicochemical properties of soils from the four land uses are presented in the Table S3.1. Although the land uses were on the same soil type, differences in almost all edaphic properties were observed. Soils from A*cacia* plantation, soybean field and natural pasture were more similar to each other than the soil from the natural forests (Fig. S3.1 and Table S3.1). The percentage of clay in the natural forest was about 1.6-fold higher than in the other soils. Natural forest presented higher level of P, Ca, Mg, Zn nutrients, higher cation exchange capacity (CEC), higher base saturation (BS) and lower K nutrient than other sites. Potassium concentration of *Acacia* plantation, soybean field and natural forest were similar.

3.3.2 Composition and distribution of soil bacterial communities

A total of 155,195 raw sequence reads were obtained in this study. The number of high- quality sequences obtained after sequence processing in each sample and the sequence coverage are presented in Table 3.1. A total of 140,407 high-quality sequences longer than 200 bp were assigned to the Bacteria domain and 80.8% of these sequences were classified below the domain level. An average of 8,775 sequences (≥200 bases) were obtained per sample representing coverage of 99% up to family level. The coverage indicated that we could perform the following OTU-based analysis at the family level but not at lower taxonomic levels. Within the classified sequences, a total of 19 phyla were found within the samples. The dominant phyla within the samples were *Proteobacteria* (34.4%±2.3%), *Acidobacteria* (20.8%±5.2%), *Actinobacteria* (11.6%±4.0%), *Bacteroidetes* (3.5%±1.6%), *Verrucomicrobia* (3.5%±1.2%), *Firmicutes* (2.1%±0.9%), *Gemmatimonadetes* (1.2%±0.6%) and *Planctomycetes* (1.1%±0.6%). The phyla with relative abundance smaller than 1% were considered as rare. They were *BRC1, Chloroflexi, Deinococcus-Thermus, Nitrospira, OD1, OP10, OP11, TM7* and *WS3*. The phyla *BRC1* and *Deinococcus-Thermus* were found only in the natural grassland soil.

3.3.3 Soil bacterial diversity and similarity based on membership and structure

In order to identify shifts related to bacterial alpha-diversity between land uses, two different metrics to calculate bacterial diversity among samples were applied: Phylogenetic Diversity (PD) (Table 3.1) and Rényi diversity profiles (Fig. 3.1). Both measures indicated that the samples presented similar bacterial diversity. The Phylogenetic Diversity did not differ statistically between samples by the Tukey test at 5% probability error and, according to

the Rényi profiles, the samples presented similar degree of diversity, richness and evenness. Once no diversity differences were found among samples, we attempted to determine whether the land-use changes caused shifts in the structure of the bacterial communities. To assess those differences we applied a Jackknifed PCoA analysis. Four well-defined clusters were observed for both weighted (Fig. 3.2A) and unweighted (Fig. 3.2B) UniFrac distance metric according to the land use. A relatively little variation (38.4%) was explained by the first three axes with Jackknifed unweighted PCoA. On the other hand, the first three axis of the weighted Jackknifed PCoA accounted for 70% of the variation, indicating that the overall differences between the clusters were more related to the abundance of specific OTUs than to their presence or absence. In this case, the interquartile ranges (IQRs) showed that the results were robust to sample size and evenness. For both unweighted and weighted PCoA, *Acacia* plantation and soybean field was more associated to each other than to other land uses studied.

Table 3.1 Total number of sequences and coverage for taxonomic groups and 97% similarity and Phylogenetic Diversity (PD)

Land use	Sequence coverage (%)							
	Total sequences	Phylum	Class	Order	Family	Genus	3% cutoff	PD*
Acacia plantation (AP1)	8405	100	100	100	99.9	99.6	77.9	65.30[1]
Acacia plantation (AP2)	9227	100	100	99.9	99.8	99.6	80.3	60.24
Acacia plantation (AP3)	7821	100	99.9	99.9	99.8	99.7	83.3	50.90
Acacia plantation (AP4)	7133	100	99.9	99.9	99.8	99.6	77.3	62.16
Natural forest (NF1)	9983	100	100	99.9	99.9	99.5	82.4	62.47
Natural forest (NF2)	7420	100	99.9	99.9	99.8	99.5	78.4	65.19
Natural forest (NF3)	12094	100	100	100	99.9	99.6	81.4	71.51
Natural forest (NF4)	11318	100	100	100	99.9	99.7	82.4	67.32
Natural pasture (NP1)	6770	100	99.9	99.9	99.7	99.5	79.7	60.80
Natural pasture (NP2)	3949	100	99.9	99.8	99.5	98.7	70.9	64.96
Natural pasture (NP3)	2554	99.9	99.8	99.7	99.0	98.3	99.9	63.90
Natural pasture (NP4)	2288	99.9	99.7	99.6	99.3	98.2	99.9	63.81
Soybean field (SF1)	10472	100	100	100	99.9	99.6	79.8	70.28
Soybean field (SF2)	14358	100	100	99.2	99.9	99.6	81.7	71.04
Soybean field (SF3)	10248	100	100	100	99.9	99.6	78.8	69.31
Soybean field (SF4)	16367	100	100	100	99.9	99.8	87.1	61.79

* The means did not differ statistically between the samples by the Tukey test at 5% probability error. [1] The PD was calculated at the family level.

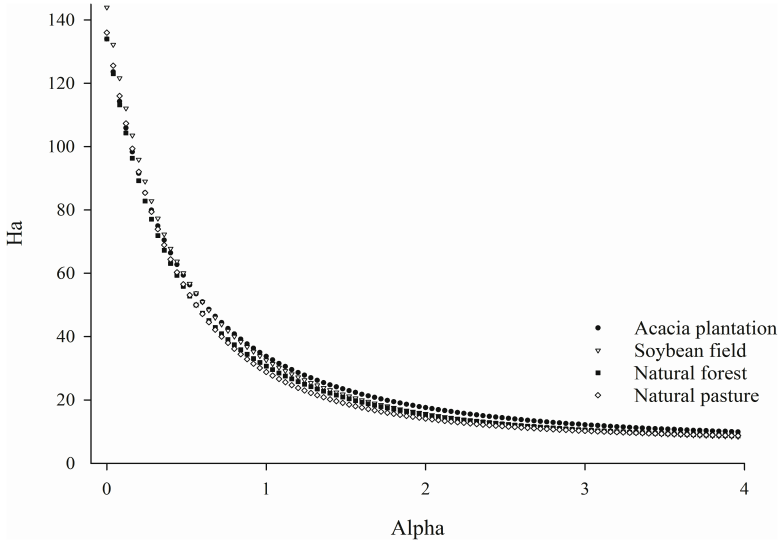

Fig. 3.1 Rényi diversity profiles of soil bacterial communities from different land uses. The x-axis shows the α value of the Rényi formula and y-axis shows Rényi diversity profiles values (Hα). α values at the scales of 0, 1, 2 are related to species richness, Shannon and Simpson diversity, respectively.

3.3.4 Impact of land use on bacterial groups

Total dissimilarity between pairs of land uses and the relative contribution of each bacteria family to the observed dissimilarity was determined by SIMPER analysis. An important component of this analysis was to identify those bacteria that were responsible for the differences observed among soil samples. The total dissimilarity among all land use pairs is shown in Fig. 3.3. On average, natural pasture presented the greatest dissimilarity (approximately 30%) among land uses; *Acacia* plantation and soybean field were the least dissimilar land uses presenting about 15% dissimilarity among

each other. The 10 most important OTUs that contributed to the community dissimilarities between the land use pairs are given in Table S3.2. Within all the bacteria family, 69% (125 OTUs out of 180) were found to consistently contribute to at least 70% of the dissimilarity in the pairwise comparisons between land uses. The SIMPER analysis also indicated that the overall differences between samples were due to a range of taxa, each contributing a relatively small percentage of the differences. Each individual bacterial family contributed not more than 2.5% of the total dissimilarity (Table S3.2).

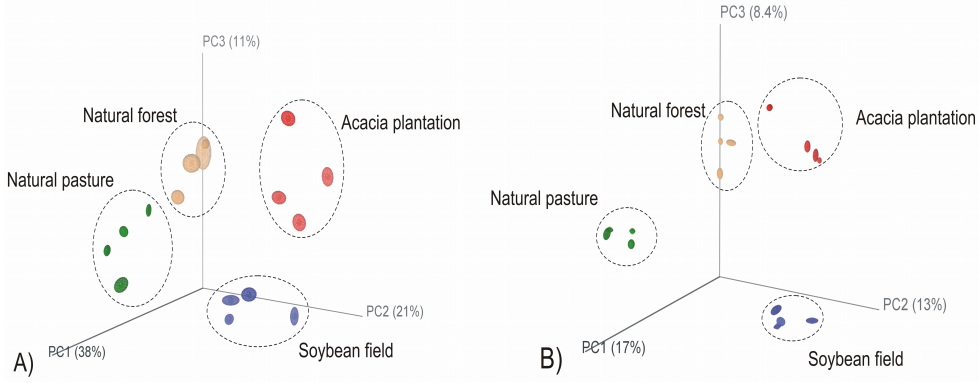

Fig. 3.2 Jackknifed Principal Coordinates plot (PCoA) depicting the clusters of bacterial communities within the soil sample from four land uses in Pampa biome. (A) weighted UniFrac distance metrics; (B) unweighted UniFrac distance metric. The clusters were generated using a subset of 1,716 sequences from each environment. The positions of the points are the average for the jackknife replicates and ellipses around points represent the interquartile range (IQR) for the 1000 jackknife replicates.

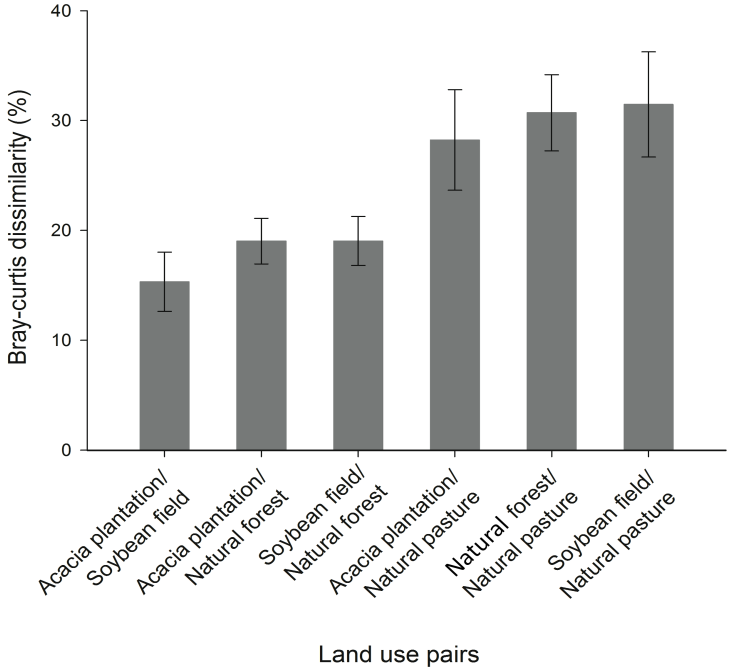

Fig. 3.3 Comparison of community structure between land use pairs by similarity percentage (SIMPER) analyses. Bars represent the standard error (*n=4*).

3.3.5 Shared microbial community

Furthermore, after analyzing the differences between soil bacterial communities, the occurrence of the bacteria families was explored using a network-like Venn diagram. Network-based analysis is used to display and analyze how OTUs are partitioned between samples. More than half of all families were present in all land uses whereas only very few families were present exclusively in a single land use (Fig. 3.4). These results were in agreement with the PCoA, which indicated that the greatest alteration caused

by land-use change was related to the difference in the abundance of bacterial OTUs instead of their presence/absence. The circles represent the bacterial OTUs.

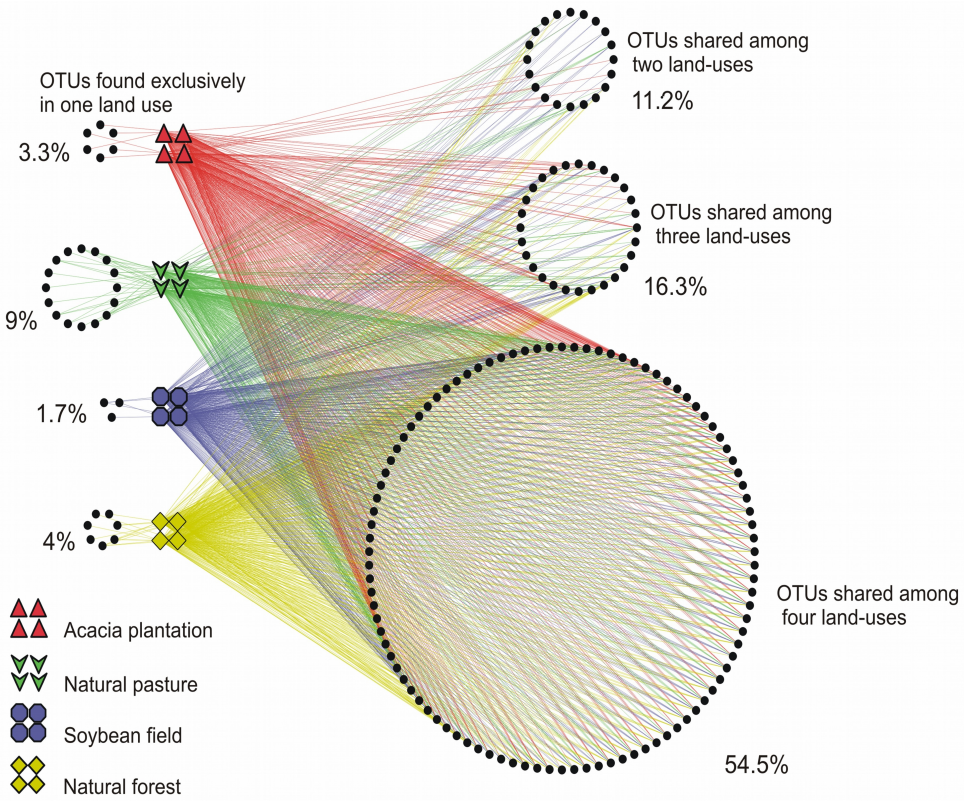

Fig. 3.4 OTU network showing OTU interactions at family level between all samples from different land uses. The "nodes" represent the bacterial family or land use. When one family is found in only one land use, the two nodes are connected with a one line (an "edge"); when one family is found in more than one land-use, the family is connected with more than one line.

3.3.6 Impact of land use on microbial biomass and activity

Microbial biomass carbon (BMC) content and metabolic quotient differed significantly ($p < 0.05$) among the land uses (Table 3.2). MBC ranged from 23.34 to 53.35 mg kg^{-1} of C and increased significantly from the human managed areas to the natural areas. Natural forest presented the highest amount of MBC (53.35 mg kg^{-1} of C) while soybean field presented the lowest amount of MBC (23.34 mg kg^{-1} of C). The highest metabolic quotient value was found in the soybean field and *Acacia* plantation while the natural areas presented smaller metabolic quotients (Table 3.2).

Table 3.2 Microbial biomass carbon (MBC) and metabolic quotient (qCO_2) from different land uses with the same soil type from the Brazilian Pampa biome

Land use	MBC mg $kg^{-1}$of C	qCO_2 mg mg^{-1} of C-CO_2
Acacia plantation	33.04 b*	0.17 bc
Soybean field	23.34 c	0.21 a
Natural forest	53.35 a	0.11 b
Natural pasture	28.03 ab	0.08 c

* Means followed by the same letter did not differ statistically between the samples by the Tukey test at 5% probability error. The results found here are the same results showed in Table 2.2 in *Chapter 2* from site A and published in Lupatini et al. (2013).

3.4 Discussion

Soil is one of the most difficult environments to work with due to its complexity, therefore there are additional methodological challenges from soil sampling to sequencing analysis (Lombard et al. 2011). Our results represent a single time point and variations in plant growth cycles and time cannot be

considered. Though seasonal dynamics might affect the microbial structure and abundance, previous studies have shown that long-term patterns within these microbial communities are expected to remain generally intact (Williams et al. 2013).

The link among plants, soil properties and below-ground communities are often described as complex drivers of the ecosystem functions and any modification of this relationship might affect the microbial structure and the ecological processes (Singh et al. 2004). With all analysis that we performed here to detect differences in soil microbial community, we found that microbial communities in the four land uses were similar, but not identical. In our study, land-use changes did not affect the soil bacterial diversity, richness and evenness when natural and non-natural areas were compared. Even using two different methods to estimate bacterial diversity, we detected no significant effect of land use on soil bacterial diversity. Furthermore, it was not possible to detect differences in species richness and evenness. However, it is also important to recognize that similarity or difference in diversity does not mean that the species identity is the same: the same diversity could be indicative that the soil bacterial communities under the influence of environmental change will gradually being replaced by another community composed by different species that survive better within the new conditions (herein called substitution hypothesis). While it is clear that plants influence microbial community structure in soil immediately adjacent to plant roots, there is conflicting evidence about plant influences in the bulk soil across individual fields (Kuramae et al. 2012). In fact the soil bacterial diversity might be relatively insensitive to different land-use changes (Jangid et al. 2011). Other factors might influence microbial community diversity acting independently and/or synergistically with the aboveground vegetation and soil chemical properties.

According to Nacke et al. (2011) and Osborne et al. (2011), shifts in

composition and structure of bacterial communities are directly determined by land use because of the differences related to the dominant plant community and soil chemical composition. The small differences found in the structure of the bacterial communities might be explained by mainly four different hypothesis: i) it is likely that the total bacterial community has been determined primarily by the soil type. Soil type has been indicated as a dominant factor driving microbial community composition, suggesting that certain characteristics of soils can lead to overall similarities and dissimilarities (Girvan et al. 2003; Suzuki et al. 2009); ii) The bacterial community is more controlled by historical contingencies (*e.g.,* prevalence of any type of vegetation, weather conditions) than by contemporary disturbances (Ge et al. 2008; Buckley & Schmidt 2001); iii) the existence of a well adapted soil bacterial community largely independent of the specific vegetation and modifications on edaphic properties related to the land use (Upchurch et al. 2008). This view is supported by some studies reporting that microbial communities are resistant to changes in plant composition (Lorenzo et al. 2010; Marshall et al. 2011) and can exhibit a great level of similarity despite some modifications in soil chemical properties (Wallenius et al. 2011). The large amount of OTUs shared between the environments of these microbial communities might suggest the presence of a core microbial community that does not suffer any change related to plant cover or soil properties promoted by land-use changes. Identifying a soil core microbiome (the suite of microbial groups shared among habitats) is crucial to appreciate the established microbial consortium, which is not usually subjected to change and, hence, possibly resistant/resilient to disturbances and a varying soil context (Shade & Handelsman 2012); iv) the apparent resilience or resistance to disturbance might be explained by cell dormancy (Jones & Lennon 2010). Although it still needs to be confirmed, the significant variation of the microbial metabolic activity observed in our experiment is consistent with this hypothesis. This

reflects the concept of “everything is everywhere” but most microorganisms may be just everywhere albeit inactive.

An important result of our study is that microbial biomass and potential activity varied across the land uses. This difference is expected because the sampling sites differed widely in terms of soil chemical properties, plant cover and management history, factors that determine microbial metabolism. While the microbial biomass controls many important functions in soil and can be used as an indicator of environmental disturbance, the metabolic quotient (qCO_2) is an index that expresses the soil quality, representing the efficiency in which organisms use the ecosystem resources (Zhang et al. 2011). Our results indicated a lack of consistent pattern between community structure and function, supporting the idea that the coupling between composition of bacterial communities and functioning is not necessarily tight. According to Frossard et al. (2012), four different outcomes are possible in such studies that contrast the structure and activity of the microbial community. One outcome is that only ecosystem function but not community structure respond to a disturbance, suggesting greater sensitivity of ecosystem function than community structure. In accordance with our conclusion, field studies that have indirectly manipulated microbial communities have not typically found evidence for strong relationships between community structure and rates of ecosystem processes (Langenheder et al. 2006). Based on that, studies on microbial assemblages need to consider similarities between communities and not only focus on dissimilarities like the majority of studies performed until now.

Although the soil bacterial community did not suffer great differentiation after removing the natural vegetation and introducing agricultural crops or silviculture, we were able to detect shifts in few specific bacterial groups in each land use. These groups were not abundant but collectively represented a large part of the differences observed among samples.

It is possible that only specific bacterial groups respond to changes in the aboveground vegetation, and these groups would have a low abundance in soils (Felske & Akkermans 1998). Since the selected sites were characterized by the same soil type and same weather conditions, we consider that the land-use changes and the new plant community may be the main factor responsible for alteration of these specific bacterial groups. These results are in line with several studies demonstrating that soil bacterial communities are driven by changes in land use, including modification in plant community and soil characteristics (da C Jesus et al. 2009). The identification of a number of specific bacterial OTUs of which distribution and abundance differ between land uses is particularly important because it provides an experimental approach linking changes in environmental characteristics to specific bacterial groups and may help to ascertain the functional roles or environmental niches occupied by microorganisms.

3.5 Supplementary information

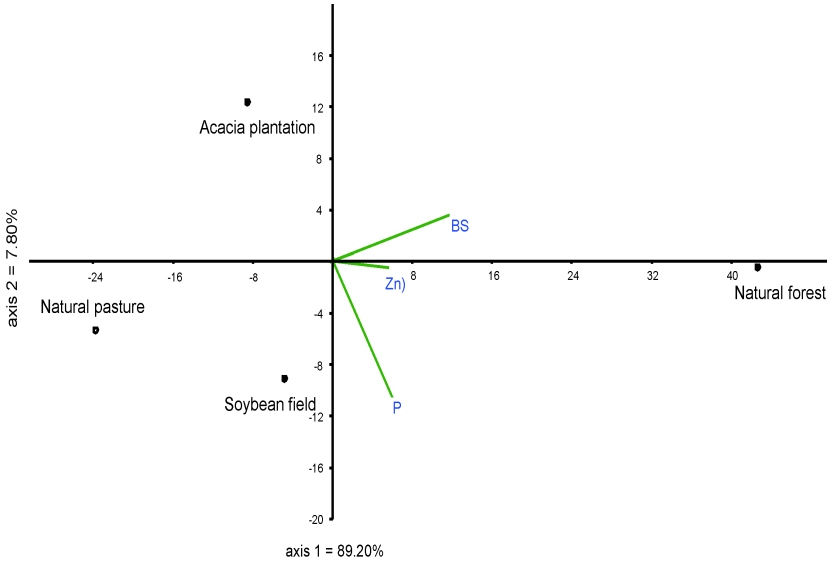

Fig. S3.1 PCA analysis of soil factors listed in Table S3.1 from four different land use (Natural forest, Natural pasture, Soybean field and *Acacia* plantation).

Table S3.1 Physical and chemical properties of subsurface soil (0 - 5cm) from different land uses upon the same soil type in the Pampa biome

Soil property	*Acacia* plantation	Soybean field	Natural forest	Natural pasture
Moisture (%)	20.8	9.7	19.6	7.1
Clay (%)	16.0	15.0	24.0	14.0
pH	6.0	6.1	6.2	5.8
P (cmolc L^{-1})	4.5	21.8	32.3	4.5
K (cmolc L^{-1})	0.35	0.40	0.20	0.08
Al (cmolc L^{-1})	0.4	0.4	0.0	1.3
Ca (cmolc L^{-1})	2.36	1.97	11.3	1.16
Mg (cmolc L^{-1})	0.73	0.51	1.42	0.19
CEC pH7	7.9	6.8	16.4	6.9
BS (%)	43.8	42.6	78.8	20.8
Zn (mg L^{-1})	1.85	1.60	25.8	1.28
Cu (mg L^{-1})	3.25	1.20	0.65	1.68
S (mg L^{-1})	12.0	6.0	9.0	7.2
B (mg L^{-1})	0.7	0.5	0.6	0.4
TOC (%)	1.00	5.02	1.77	1.43
Total N (%)	0.01	0.39	0.13	0.11

P: Phosphorus; K: Potassium; Al: Aluminum; Ca: Calcium; Mg: Magnesium; CEC: Cation exchange capacity; BS: Base saturation; Zn: Zinc; Cu: Copper; S: Sulphur; B: Boron; TOC: Total Organic Carbon; N: Total nitrogen. The results are the same showed in Table S2.1 in *Chapter 2* from site A and published in Lupatini et al. (2013).

Table S3.2 Comparison of community structure between land use pairs on the same soil type by similarity percentage (SIMPER) analyses

Closest bacterial relative	Contribution to dissimilarity	Proportion of all sequences	
		***Acacia* plantation**	**Soybean Field**
	%		
Proteobacteria;Gammaproteobacteria;Legionellales; Coxiellaceae	1.7	0.04	0.16
Bacteroidetes;Sphingobacteria;Sphingobacteriales; Other	1.7	0.17	0.47
Proteobacteria;Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae	1.69	0.02	0.11
Proteobacteria;Gammaproteobacteria; Enterobacteriales;Enterobacteriaceae	1.55	0.16	0.01
Proteobacteria;Alphaproteobacteria;Rhodospirillales; Rhodospirillaceae	1.48	0.01	0.07
Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae	1.4	0.29	0.79
Bacteroidetes;Other;Other;Other	1.29	0.20	0.33
Actinobacteria;Actinobacteria;Solirubrobacterales; Patulibacteraceae	1.22	0.06	0.01
Proteobacteria;Betaproteobacteria;Other;Other	1.21	0.85	1.72
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Sinobacteraceae	1.2	0.19	0.07
		***Acacia* plantation**	**Natural forest**
Acidobacteria;Acidobacteria_Gp25;Gp25;Other	2.23	<0.01	0.16
Acidobacteria;Acidobacteria_Gp17;Gp17;Other	2.15	<0.01	0.17
Acidobacteria;Acidobacteria_Gp11;Gp11;Other	2.09	<0.01	0.12
Bacteroidetes;Flavobacteria;Flavobacteriales; Flavobacteriaceae	1.91	0.05	0.39
Acidobacteria;Acidobacteria_Gp15;Gp15;Other	1.85	<0.01	0.09
Acidobacteria;Acidobacteria_Gp22;Gp22;Other	1.78	<0.01	0.08
Actinobacteria;Actinobacteria;Actinomycetales; Geodermatophilaceae	1.76	0.10	<0.01
Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae	1.71	0.29	1.81
Actinobacteria;Actinobacteria;Actinomycetales; Propionibacteriaceae	1.61	0.12	0.65
Bacteroidetes;Other;Other;Other	1.53	0.20	0.70
		Soybean field	**Natural forest**

Actinobacteria;Actinobacteria;Actinomycetales; Geodermatophilaceae	2.42	0.24	<0.01
Proteobacteria;Alphaproteobacteria;Rhizobiales; Methylobacteriaceae	2.12	0.18	<0.01
Acidobacteria;Acidobacteria_Gp25;Gp25;Other	2.06	<0.01	0.16
Acidobacteria;Acidobacteria_Gp11;Gp11;Other	1.93	<0.01	0.12
Proteobacteria;Betaproteobacteria; Nitrosomonadales; Nitrosomonadaceae	1.7	0.09	<0.01
Acidobacteria;Acidobacteria_Gp22;Gp22;Other	1.64	<0.01	0.08
Acidobacteria;Acidobacteria_Gp17;Gp17;Other	1.64	0.01	0.17
Proteobacteria;Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae	1.62	0.11	0.01
Bacteria_incertae_sedis;Ktedonobacteria; Ktedonobacterales;Ktedonobacteraceae	1.54	0.59	0.10
Firmicutes;Bacilli;Bacillales;Other	1.48	0.39	0.06

		***Acacia* plantation**	**Natural grassland**
Proteobacteria;Deltaproteobacteria; Desulfuromonadales;Geobacteraceae	2.05	<0.01	0.75
Actinobacteria;Actinobacteria;Solirubrobacterales; Solirubrobacteraceae	1.95	0.40	0.02
Chloroflexi;Anaerolineae;Anaerolineales; Anaerolineaceae	1.93	<0.01	0.78
Actinobacteria;Actinobacteria;Actinomycetales; Intrasporangiaceae	1.73	0.57	0.06
Actinobacteria;Actinobacteria;Actinomycetales; Pseudonocardiaceae	1.62	0.19	0.01
Acidobacteria;Acidobacteria_Gp4;Gp4;Other	1.58	1.38	0.44
Proteobacteria;Gammaproteobacteria; Enterobacteriales;Enterobacteriaceae	1.5	0.16	<0.01
Actinobacteria;Actinobacteria;Solirubrobacterales; Conexibacteraceae	1.45	0.14	0.01
Actinobacteria;Actinobacteria;Actinomycetales; Nocardioidaceae	1.45	0.57	0.08
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Sinobacteraceae	1.45	0.19	0.01

		Soybean field	**Natural grassland**
Acidobacteria;Acidobacteria_Gp4;Gp4;Other	1.9	2.77	0.44
Bacteroidetes;Sphingobacteria;Sphingobacteriales; Other	1.72	0.47	0.02
Actinobacteria;Actinobacteria;Actinomycetales; Intrasporangiaceae	1.65	0.63	0.06
Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae	1.56	0.79	0.16

Proteobacteria;Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae	1.47	5.58	1.42
Actinobacteria;Actinobacteria;Solirubrobacterales; Solirubrobacteraceae	1.45	0.17	0.02
Actinobacteria;Actinobacteria;Actinomycetales; Propionibacteriaceae	1.44	0.16	0.01
Actinobacteria;Actinobacteria;Actinomycetales; Pseudonocardiaceae	1.41	0.14	0.01
Proteobacteria;Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae	1.37	0.11	<0.01
Verrucomicrobia;Spartobacteria; Spartobacteria_genera_incertae_sedis;Other	1.37	2.25	0.74

		Natural forest	**Natural grassland**
Actinobacteria;Actinobacteria;Actinomycetales; Propionibacteriaceae	2.31	0.65	0.01
Acidobacteria;Acidobacteria_Gp4;Gp4;Other	2.12	3.65	0.44
Bacteroidetes;Flavobacteria;Flavobacteriales; Flavobacteriaceae	2.11	0.39	<0.01
Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae	2.08	1.81	0.16
Actinobacteria;Actinobacteria;Solirubrobacterales; Solirubrobacteraceae	1.9	0.24	0.02
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Sinobacteraceae	1.81	0.33	0.01
Bacteroidetes;Sphingobacteria;Sphingobacteriales; Other	1.61	0.37	0.02
Acidobacteria;Acidobacteria_Gp25;Gp25;Other	1.58	0.16	<0.01
Acidobacteria;Acidobacteria_Gp6;Gp6;Other	1.5	6.39	1.36
Actinobacteria;Actinobacteria; Acidimicrobidae_incertae_sedis;Ilumatobacter	1.46	0.06	<0.01

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Chapter 4

Shifts in soil bacterial community after eight years of land-use change

Authors

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Abstract

The interaction between plants, soil and microorganisms is considered to be the major driver of ecosystem functions and any modification of plant cover and/or soil properties might affect the microbial structure, which, in turn, will influence ecological processes. Assuming that land-use changes are the major drivers of soil bacterial diversity and structure, it can be postulated that changes in plant cover causes significant shifts in soil bacterial community composition. To address this issue, this study used 16S rRNA pyrosequencing to detect differences in diversity, composition and/or relative abundance of bacterial taxa from an area covered by pristine forest, as well as eight-year-old grassland surrounded by the same forest. It was shown that a total of 69% of the operational taxonomic units (OTUs) were shared between sites. Overall, forest and grassland samples presented the same diversity and the clustering analysis did not show the occurrence of very distinctive bacterial communities between sites. However, 11 OTUs were detected in statistically significantly higher abundance in the forest samples but in lower abundance in the grassland samples, whereas 12 OTUs occurred in statistically significantly higher abundance in the grassland samples but in lower abundance in the forest samples. The results illustrated that the history of land use might influence present-day community structure.

Keywords

Pyrosequencing; Bacterial diversity; Grassland; Afforestation

4.1 Introduction

Soils are considered to be the most diverse microbial habitats on Earth. However, little is known about how environmental changes affect the microbiota and its functions (Fierer et al. 2007; Liebich et al. 2006). Land-use changes and agricultural management are major causes of biodiversity loss with negative consequences for the environment (Balvanera et al. 2006; Doran & Zeiss 2000; Navarrete et al. 2010). Changes in composition or species diversity of above-ground communities can affect the composition and function of below-ground communities and vice versa (van der Heijden et al. 2008). Particularly, changes in the above-ground vegetation affect the size, activity and composition of soil microbial communities (da C Jesus et al. 2009; Nüsslein & Tiedje 1999). Nevertheless, agricultural practices do not always deplete soil bacterial diversity, since shifts in microbial diversity and structure caused by different land-use changes may have a positive, negative or neutral impact (Singh et al. 2004). According to Jangid et al. (2011), microbial communities in relatively pristine deciduous forest and long-term mowed grassland soils were very similar, despite major differences in soil properties and vegetation.

Although changes in soil properties due to continuous cultivation appear to be a slow process, any land-use change can possibly cause a disturbance, which in turn might affect soil microbial communities. According to Allison and Martiny (2008), there are three potential impacts caused by disturbance. After disturbance, the microbial composition might be resistant and not change, might be altered and rapidly return to the original composition

(resilient) or might remain altered, which would imply a functional change. It was assumed in this study that recent changes in plant cover would not cause major changes in soil properties and that soil properties are the major drivers of soil bacterial diversity and structure. Therefore, the hypothesis was that the soil bacterial community from a pristine forest would not be different from the soil bacterial community from a cultivated grassland surrounded by the forest in the first years of cultivation.

Within this context, the aim of this work was to investigate bacterial communities from distinct land uses, and address the following question: what is the contribution of plant community composition on bacterial community patterns in the first years after land-use change? An area covered by pristine forest soil was analyzed in conjunction with eight-year-old grassland surrounded by this forest. The area had low human activity, no inputs of fertilizers (except for the manure added by animal activity) and a very low animal influence, which was ideal for testing the effect of plant cover removal on soil bacterial communities. The diversity and composition of bacterial taxa was analyzed by high throughput pyrosequencing of 16S rRNA gene amplified from DNA extracted directly from the soil samples.

4.2 Materials and methods

4.2.1 Site description and soil sampling

The sampling site was located in the Pampa biome, which has both subtropical and temperate climates with four well-characterized seasons. The climate is the most important factor determining the soil and vegetation. The soil in the major part of the region had an extremely sandy texture due to its sedimentary rook origin (Roesch et al. 2009; Tornquist et al. 2009). The dominant vegetation in this biome is grassland. However, there is a presence of forest formations limited mainly to gallery forests along rivers and forest

formations surrounded by natural grassland (Overbeck et al. 2007; Roesch et al. 2009). Not only the natural grassland is under land-use changes, but also the forests are converted for anthropogenic uses such as pasture or crop fields (Costella et al. 2013). The site analyzed here consisted of pristine forest (gallery forest) and eight-year-old grassland surrounded by the same forest (Table 4.1). The grassland is this study resulted from the deforestation of a small area (5,500 m^2) inside the forest for a non-commercial cattle settlement (Fig. S4.1). No fertilizers, except for the manure added by animal activity, were added to the soil and no exotic plants were introduced.

The soil samples were taken by drawing four randomly distributed 1 m^2 plots per land use (Baker et al. 2009) and a composite sample was collected by taking samples in every corner of the square. Equal amounts of sub-samples from cores were pooled and mixed to compose four samples from the native forest and four samples from the grassland. Bulk soil samples were collected by taking 5 cm diameter, 0 - 10 cm depth cores during the spring of 2010 and stored at -18°C until DNA extraction and chemical analyses were performed. For the soil chemical analyses, the four replicates from each land use were combined. The soil pH was determined in water (1:1 soil to water ratio) and the concentrations of Ca, Mg, Al, K, Na, P, total nitrogen, NH_4^+, NO_3^- + NO_2^- and total organic carbon (TOC) were quantified according to (Embrapa 1997). The dissolved organic carbon (DOC) was quantified according to Bartlett & Ross (1988). DNA was isolated from at least 1g of soil using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. After DNA extraction, samples were purified with the DNeasy Tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions, and the total DNA concentration was quantified using the NanoVue spectrophotometer (GE Healthcare, Harriet, USA).

4.2.2 16S rRNA gene amplification and pyrosequencing

The 16S rRNA gene fragments were sequenced using 454 GS FLX Titanium (Lib-L) chemistry for unidirectional sequencing of the amplicon libraries. Barcoded primers allow for combining amplicons of multiple samples into one amplicon library and, furthermore, enable the computational separation of the samples after the sequencing run. To do this, 8-base barcodes were added to the 5′-end of the reverse primers using the self-correcting barcode method of Hamady et al. (2008). The primers were attached to the GS FLX Titanium Adaptor A-Key (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) and Adaptor B-Key (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′) sequences, modified for use with GS FLX Titanium emPCR Kits (Lib-L) and a 2-base linker sequence that was inserted between the 454 adapter and the 16S rRNA primers in order to reduce any effect the composite primer might have had on PCR efficiency. A total of eight independent PCR reactions were performed for each composite soil sample with the universal primers 27F and 338R for the amplification of the V1-V2 region of the 16S rRNA gene. PCR was performed with the GoTaq PCR core system (Promega, Madison, WI, USA). The mixtures contained 5 µL of 10x PCR buffer, 200 mM dNTPs, 100 mM of each primer, 2.5 U of *Taq* polymerase and approximately 100 ng of DNA template in a final volume of 50 µL. The PCR conditions were 94°C for 2 min, 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for a 1 min extension, followed by 72°C for 6 min. The PCR products were purified and combined in equimolar ratios with the quantitative DNA binding method (SequalPrep Kit, Invitrogen, Carlsbad, CA, USA) in order to create a DNA pool that was further used for pyrosequencing from the A-Key adaptor. All raw sequences were submitted to the NCBI Sequence Read Archive (SRA) under the accession number SRA013204.1.

4.2.3 Processing of pyrosequencing data and statistical analyses

The raw sequences obtained were processed using the Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al. 2010) with the default parameters. Briefly, bacterial sequences were first quality trimmed by removing short sequences (<200 bp), sequences that presented low average quality scores (<25), sequences that did not present a perfect match to the sequence barcode and primer, sequences that presented more than two undetermined bases (Hamady et al. 2008). Additionally, to identify potential chimeric sequences, the dataset was subject to the ChimeraSlayer implemented in mothur (Schloss et al. 2009). After removing low-quality sequences, the multiplexed reads were assigned to the corresponding soil samples based on their barcodes. Bacterial sequences were grouped into operational taxonomic units (OTUs) using a 97% identity threshold and the most abundant sequence from each OTU was selected as a representative sequence for that OTU. Afterwards, the sequences were taxonomically classified using the RDP naïve Bayesian rRNA Classifier (Wang et al. 2007), which assigns complete taxonomic information from domain to species to each sequence in the database with 80% taxonomy confidence and an e-value of 0.001. Good's coverage was calculated for each taxonomic level (Phylum, Class, Order, Family and Genus) (Good 1953). The representative set of sequences was also used for aligning the sequences against a reference database and to build a phylogenetic tree necessary for downstream measurements. These taxonomic assignments were used to build an OTU table, which was a matrix of OTU abundance for each sample with meaningful taxonomic identifiers for each OTU. The total number of sequences obtained from the native forest and the grassland are shown in Table 4.2.

4.2.4 Measurement of differences between the bacterial communities

To explore the similarities and differences between the two sites tested, jackknifed principal coordinates analysis (PCoA) and a hierarchical clustering analysis were performed in order to find clusters of similar groups of samples. PCoA is an ordination method based on multivariate statistical analysis that maps the samples in different dimensions and reflects the similarity of the biological communities. A matrix using the UniFrac metrics (weighted and unweighted) for each pair of sites was calculated. The distances were turned into points in space with the number of dimensions one less than the number of samples. The first three principal dimensions, which usually contain most of the variation found in the samples, were used to plot a three-dimensional graph that illustrated the distribution of soils according to their similarity. To test whether the results were robust for sample size, a sequence-jackknifing technique was used in which the PCoA clusters were regenerated using a subset of 600 sequences randomly selected from each soil for 100 replicate trials, and this was used to create a graph made up of the mean values obtained. Ellipses were drawn around the mean values representing the interquartile ranges (measurement of statistical dispersion obtained by sequencing jackknifing). If the ellipses are small, the same result would likely be achieved with a different set of sequences from the same site, but if the ellipses are large a different result might be expected. Furthermore, a hierarchical cluster tree was constructed on the basis of the distance matrix calculated by the unweighted UniFrac algorithm. To assess the uncertainty in hierarchical cluster analysis 1000 bootstrap re-samplings were computed. The jackknifed PCoA and the hierarchical cluster analysis were performed using QIIME (Caporaso et al. 2010).

To compare the diversity between bacterial communities from the soil

samples, the diversity of each sample was estimated using the Shannon-Weaver index (Shannon 2001) and Faith's index of phylogenetic diversity (Faith 1992). For these measurements, the diversity metrics were calculated for a randomly selected subset of 12,393 sequences per soil, as alpha diversity indexes are correlated with the number of sequences collected (Lemos et al. 2011). To find which OTUs were abundantly different between the two sites a chi-square test (based on 50,000 Monte Carlo iterations) was calculated in order to obtain a *p*-value for the null hypothesis that there was no difference between all possible pairwise combinations of soil samples from the native forest and the grassland. The *p*-values (≤0.01) were ordered and processed in order to find a false discovery rate (FDR) less than or equal to 1%. The test was performed using the OTU table summarized at the genus level with the sub-sampled number of sequences (12,393 sequences) for each sample in PANGEA (Giongo et al. 2010).

4.3 Results

4.3.1 Vegetation and soil chemical analysis

The most common native tree species that were found in the forest belonged to the families of *Boraginaceae, Euphorbiaceae, Fabaceae, Lauraceae, Malvaceae, Meliaceae, Myrtaceae,* and *Rutaceae*. The most dominant grass species found in the grassland belonged to the *Poaceae* family. The number of plant families indicated greater plant diversity in the forest and a dominance of a single family in the grassland. The location, altitude and soil chemical analyses are presented in Table 4.1. The pH and sodium content did not differ between soils from the native forest and those from the grassland. All the other variables measured presented higher contents in the native forest than in the grassland, except for K that was greater in the grassland than in the forest. Some nutrients, such as P and NO_3^- + NO_2^- were found to be at least 1.5-fold

higher in the native forest than in the grassland. The total organic carbon was 2.2-fold higher in the native forest than in the grassland.

4.3.2 Assessment of taxon distribution and bacterial diversity

After filtering the reads by base quality and removing reads smaller than 200 bases, a total of 170,046 sequences were obtained from the eight soil samples collected in the native forest and the grassland from the Pampa biome. From all samples, 127,238 (74.83%) were classified below the domain level. The number of high quality sequences per sample varied from 12,393 to 37,225 and the average number of sequences per sample was 21,256 (Table 4.2). The classified sequences were affiliated to 20 bacterial phyla but only eight phyla were found at a relative abundance of greater than 1% (Fig. 4.1 A), and ten phyla were found at a relative abundance smaller than 1% (Fig. 4.1 B). The major phylogenetic groups did not differ between forest and grassland soil samples.

Table 4.1 Location, altitude and soil chemical analyses of native forest and grassland soils from Brazilian Pampa biome

	Native forest	Grassland
Coordinates	30° 24′ 09.3″ S 53° 52′ 59.1″ W	30° 24′ 08.9″ S 50° 53′ 05.9″ W
Altitude (m)	616	616
pH	5.8	5.6
Ca + Mg (cmolc kg^{-1})	39.0	23.2
Al (cmolc kg^{-1})	0.50	0.13
Na (cmolc kg^{-1})	0.014	0.014
K (cmolc kg^{-1})	0.6	1.0
P (cmolc kg^{-1})	39	12
Total N (%)	0.76	0.40
$NH4^+$ (mg kg^{-1})	180	120
$NO3^-$ + $NO2^-$ (mg kg^{-1})	102	30.8
Total organic carbon (%)	7.3	3.3
Dissolved organic carbon (g kg^{-1})	0.24	0.21

To identify shifts in bacterial diversity between the forest and the grassland, two diversity indices, the Shannon-Weaver index and the phylogenetic diversity (PD) index, were calculated. For the calculations, a random subset of sequences (12,393 per sample) was sampled in order to correct for the differences between samples related to the sampling coverage (Table 4.2). Overall, forest samples and grassland samples presented the same diversity. The Shannon index ranged from 10.01 to 10.58 for samples from the forest and from 10.18 to 10.53 for samples from the grassland.

The average Shannon diversity index revealed no significant

differences between site according to the Tukey's test at a 5% probability error. The PD index ranged from 145.37 to 172.23 for samples from the forest, and from 141.84 to 165.02 for samples from the grassland. Although the average PD index was larger in the samples from the forest, the Tukey's range test at a 5% probability error revealed no significant differences between sites.

Table 4.2 Total number of sequences, Good's coverage and diversity index

	Forest				Grassland			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Total nº. of sequence	16,337	16,994	37,225	17,240	12,393	14,328	25,797	29,732
	Sequence coverage (%)							
Phylum	99.98	99.99	99.99	99.98	100	100	100	100
Order	99.96	99.96	99.99	99.97	99.97	99.99	99.99	99.98
Class	99.93	99.95	99.98	99.95	99.97	99.97	99.98	99.97
Family	99.85	99.91	99.96	99.91	99.90	99.87	99.96	99.95
Genus	99.67	99.76	99.85	99.77	99.69	99.69	99.86	99.87
3% dissimilarity cutoff	85.36	83.05	90.95	85.55	84.77	82.39	89.75	90.50
	Diversity index							
[a] **Phylogenetic diversity**	154.5	172.3	145.4	148.0	141.8	165.0	143.8	144.2
Shannon	10.3	10.6	10.0	10.6	10.2	10.5	10.4	10.2

[a] All samples were sub-sampled to 12,393 sequences prior to diversity index calculations. The average phylogenetic diversity for the forest samples was 155.0 and for the grassland samples it was 148.7. The average Shannon diversity index for the forest samples was 10.4 and for the grassland samples 10.3. The means did not differ statistically between the forest samples and the grassland samples by the Tukey test at a 5% probability error.

4.3.3 Similarity between communities based on membership and structure

The weighted and unweighted PCoA analyzes (Fig. 4.2A and B) did not show the occurrence of very distinct groups of soil bacterial communities. In addition, the analysis of microbial communities using hierarchical cluster analysis showed that the bacterial communities from the same site (forest or grassland) were more similar to each other than bacterial communities in different sites, as observed by the two highly supported clusters made up of samples from the forest soil and the grassland soil (Fig. 4.3).

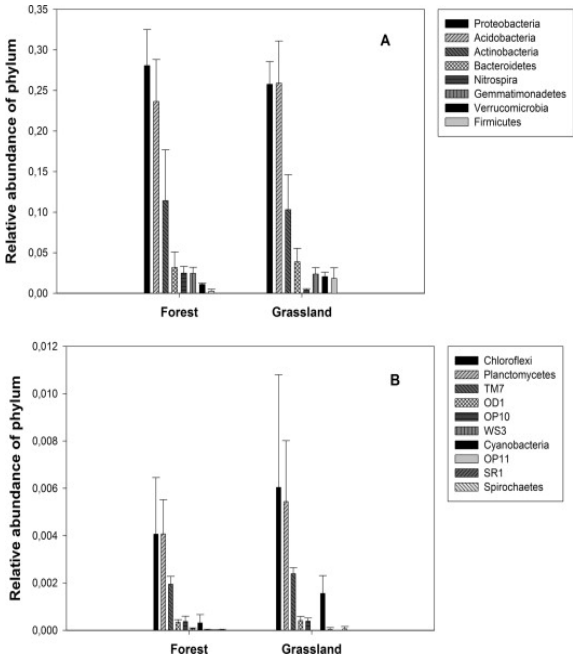

Fig. 4.1 Relative abundance of phyla for each soil library. (A) represents the relative abundance greater than 1%; (B) represents the relative abundance smaller than 1%. Bars represent the standard error ($n = 4$).

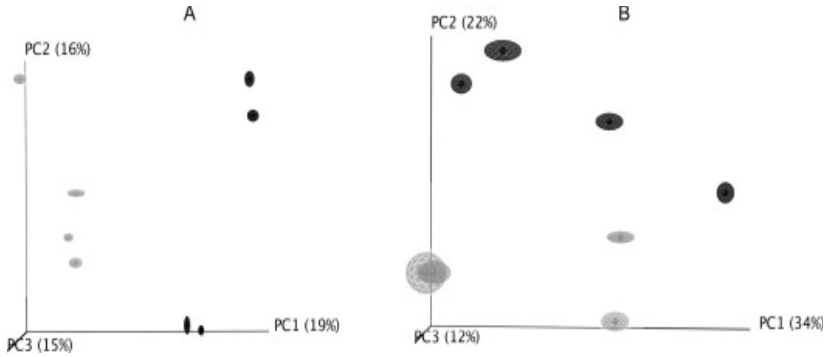

Fig. 4.2 Jackknifed PCoA plots with (A) unweighted UniFrac distance metric, which accounts for presence/absence of taxa and (B) weighted UniFrac distance metric, which accounts for changes in the relative abundance of taxa. The clusters were generated using a subset of 600 sequences from each site for 100 replicate trials. The positions of the points are the average for the jackknife replicates and ellipses were drawn around the mean values to represent the IQRs. Dark grey - grassland and light grey - natural forest.

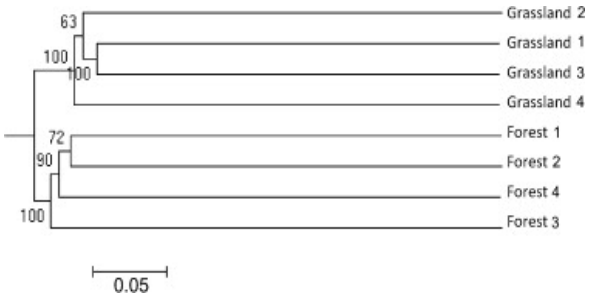

Fig. 4.3 Hierarchical cluster constructed on the basis of the distance matrix calculated by the unweighted UniFrac algorithm. Numbers at branch points indicate the percentage of 1000 bootstrap re-samplings.

4.3.4 Co-occurrence of OTUs among soil samples

An important component of this analysis was to identify those bacteria that were responsible for the differences observed between forest and grassland soil samples. To determine the OTUs that were statistically different between sites, an exact chi-square test was performed. On the basis of the test, only eleven OTUs were found to be in higher abundance in the forest samples but in lower abundance in the grassland samples (Table 4.3). The unclassified Bacteria also presented different abundances between the sites. A total of seven unclassified OTUs presented higher abundances in the native forest, while two unclassified OTUs showed higher abundance in the grassland. Among those sequences that could not be classified to known taxa, the OTUs 5084, 6116 and 4424 presented the greatest difference between forest and grassland.

Table 4.3 List of the closest bacterial relatives of operational taxonomic units (OTUs) whose abundances differed statistically ($p \leq 0.01$; FDR ≤ 0.01) between forest and grassland soils

	% of all forest sequences	% of all grassland sequences	Fold difference
[a]Classifiable OTUs with greater abundance in the native forest			
Acidobacteria;Acidobacteria;Gp6;Gp6	15.26	11.26	1.4
Acidobacteria;Acidobacteria;Gp22;Gp22	0.61	0.28	2.2
Actinobacteria;Actinobacteria;Acidimicrobiales	0.59	0.21	2.8
Actinobacteria;Actinobacteria;Actinomycetales; Micromonosporaceae	5.11	2.88	1.8

	% of all forest sequences	% of all grassland sequences	Fold difference
Actinobacteria;Actinobacteria;Actinomycetales; Mycobacteriaceae;Mycobacterium	0.71	0.41	1.8
Actinobacteria;Actinobacteria;Actinomycetales; Propionibacteriaceae;Microlunatus	1.24	0.82	1.5
Nitrospira;Nitrospira;Nitrospirales; Nitrospiraceae;Nitrospira	3.91	0.57	6.9
Proteobacteria	5.02	3.57	1.4
Proteobacteria;Alphaproteobacteria	5.44	3.42	1.6
Proteobacteria;Alphaproteobacteria;Rhizobiales	14.57	10.70	1.4
Proteobacteria;Deltaproteobacteria	4.36	3.37	1.3
Classifiable OTUs with greater abundance in the grassland			
Acidobacteria;Acidobacteria;Gp1;Gp1	1.73	5.83	3.4
Acidobacteria;Acidobacteria;Gp4;Gp4	8.48	10.36	1.2
Actinobacteria;Actinobacteria;Actinomycetales	4.56	5.91	1.3
Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Terrimonas	1.14	1.87	1.6
Chloroflexi;Anaerolineae;Anaerolineales; Anaerolineaceae	0.51	0.78	1.5
Firmicutes;Bacilli;Bacillales;Bacillaceae; Bacillus	0.38	2.74	7.2
Planctomycetes;Planctomycetacia;	0.26	0.42	1.6

	% of all forest sequences	% of all grassland sequences	Fold difference
Planctomycetales;Planctomycetaceae			
Proteobacteria;Alphaproteobacteria; Rhodospirillales	0.15	0.45	3.0
Proteobacteria;Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae	0.41	0.69	1.7
Proteobacteria;Betaproteobacteria	2.63	3.76	1.4
Verrucomicrobia;Spartobacteria; Spartobacteria;genera;incertaesedis	0.58	1.04	1.8
Verrucomicrobia;Subdivision3; Subdivision3 genera incertae sedis	0.62	1.36	2.2
Unclassified			
OTUs with greater abundance in the native forest			
Bacteria 1137	0.081	0.006	1.3
Bacteria 3541	0.020	0.002	1.0
Bacteria 4424	2.058	0.056	3.6
Bacteria 5084	0.141	0.002	7.0
Bacteria 5735	0.061	0.004	1.5
Bacteria 5785	0.121	0.010	1.2
Bacteria 6116	0.101	0.002	5.0
OTUs with greater abundance in the grassland			
Bacteria 2294	0.726	0.107	1.5

	% of all forest sequences	% of all grassland sequences	Fold difference
Bacteria 2768	0.020	0.008	4.0

[a] Each OTU was classified at the highest taxonomic level with 80% taxonomy confidence and an *e*-value of 0.001. The unclassified Bacteria correspond to an OTU that did not match any of the sequences in the database according to the criteria mentioned above.

The analysis of the OTUs that were partitioned between samples showed that most of the taxa were shared between forest and grassland (69%). However, 16.4% of the taxa were found only in the forest soil and 14.6% only in the grassland. The exclusive OTUs from each site are shown in Table S4.1. The abundance of a genus was analyzed statistically to provide support for the analysis of shared OTUs using a t-test on QIIME. The OTUs found exclusively in the forest samples belonged to the genera *Sphingobium*, *Methylotenera* and *Pedobacter*, and to the phylum *WS3*. The OTUs found exclusively in the grassland samples belonged to the genera *Dechloromonas*, *Zoogloea* and *Geobacter*.

4.4 Discussion

As microorganisms play key roles in nutrient cycling and other important functions in soils, the shifts in microbial communities caused by land-use changes might directly affect the functioning of ecosystems, such as biogeochemical cycles (Berthrong et al. 2009). In this study, differences in diversity, composition and/or relative abundance of bacterial taxa were tested from bulk soil samples collected in two sites: pristine forest and eight-years-old

grassland resulted by the deforestation of a small area inside the forest. The area chosen for sampling was ideal for testing the effect of removing plant cover on soil bacterial communities, since it presented low human activity, no inputs of fertilizers and a very low animal influence. As the samples were taken in one single period of time (during the spring) it is important to mention that the results obtained represent a "*snapshot*" of the microbial community status, and temporal variations in plant growth and seasonal fluctuations are not considered.

We found that the replacement of forest for grassland reduce most of the soil chemical properties measured. In agreement with our results, many researchers reported that the conversion of natural forest to other forms of land use (*e.g.*, crop fields or pasture) lead to a reduction in soil chemical properties such as organic content, N and Ca, suggesting that continuous use of soil for anthropogenic purposes might be responsible for deterioration in soil quality (Braimoh & Vlek 2004). Due the removal of forest, soil attributes change as a consequence of the loss of input from forest litter, increase rates of organic matter decomposition and nutrient depletion caused by prolonged nutrient mining without sufficient replenishment of nutrients and of nutrient losses by soil erosion (Tan et al. 2005; Bringhurst & Jordan 2015). Yet, the magnitude of these changes vary with land cover, land management intensity and time (Houghton et al., 1999; Kizilkaya & Dengiz 2010). Geissen et al. (2009) studied the effect of land-use changes after 15 years and did not detect chemical soil degradation but did detect severe compaction of soils under permanent pasture. In certain ecosystems, the effect of agricultural practices on soil properties (*e.g.*, forestland replaced by cropland and pasture) was clearly detected only 50 years after the land-use change (Kizilkaya & Dengiz 2010).

Our approach was based on pyrosequencing of the 16S rRNA genes amplified from microbial DNA extracted directly from four soil samples from each site. This approach is considered to present high levels of robustness and

resolution (Liu et al. 2007; Lozupone & Knight 2007). However, it should be mentioned that an incomplete dataset was examined and other factors, not assessed in this study, such as biases at the steps of DNA extraction, PCR amplification, primer choice and sequencing, might present some degree of interference in the results obtained. Nevertheless, as the sequencing errors were removed, these biases were unlikely to have missed many taxa that could have resulted in the main findings being incorrect. The selection of primers is still under debate among researchers, since no primer is truly "universal" and the choice of a reference database and taxonomy can have a dramatic impact on the resulting classification accuracy (Soergel et al. 2012). The most widely used PCR primer sets span hypervariable regions V1-V3 but *in silico* tests have revealed that primers designed for amplification of this region underestimate the richness because they neglect candidate divisions (Winsley et al. 2012). On the other hand, *in silico* predictions may not reflect the real performance of the primers. The V3-V4 region, for example, has shown high-simulated accuracy and good classification consistency but the set of primers designed to amplify this region has been proven to produce biases caused by amplification of artifacts arising from the combination of these two specific V3-forward and V4-reverse primers (Claesson et al. 2010). Although we are aware that our primer choice excluded some phyla during amplification, mainly *Verrucomicrobia* (Bergmann et al. 2011), and that the barcoded primers used might be a source of bias (Berry et al. 2011), we opted to amplify the DNA with the 27F and 338R primers and performed the sequencing through the reverse end. This was because this set of primers generate low rates of PCR artifacts and the sequences produced provided relatively good cluster recovery even for short (≤250 bases) pyrosequencing reads (Liu et al. 2007). Thus, PCR primers rarely amplify all bacterial members of a community and any PCR-based approach is likely to miss some bacterial groups or at least underestimate the abundance of some bacterial taxa. Although microbial surveys are always limited by these

practical problems, it is possible to obtain robust comparisons across samples when the data analysis is conducted in a consistent manner (Bent & Forney 2008; Hamady et al. 2008).

To detect relevant bacterial patterns within our samples, the datasets of 16 rRNA sequences were analyzed using phylogenetic- and taxon-based approaches. The methods based on phylogeny are useful for exploring similarities and differences based on a phylogenetic tree, while OTU-based approaches need a rigid OTU definition based on a cutoff distance. Since there are no accepted dissimilarity cutoffs for the different microbial taxonomic levels, the 3% dissimilarity clustering threshold proposed by Kunin et al. (2010) was used. According to the authors, diversity estimates are grossly overestimated when clustering thresholds are higher than 97% identity. Therefore, phylogenetic parental sequences can be grouped differently than those based on OTU identification. In this regard, two different metrics were applied to calculate bacterial diversity among samples: the Shannon diversity index (H′) and the phylogenetic diversity index (PD). Shannon index is an OTU-based analysis and measures the average degree of uncertainty in predicting to what species an individual chosen at random from a collection of S species and N individuals will belong. The value increases as the number of species increases and as the distribution of individuals among the species becomes even (Ludwig 1988). The phylogenetic diversity is defined and calculated as the sum of the branch-lengths of the minimal subtree connecting the taxa in the subset (Faith 1992). This evaluation is based on a single phylogenetic tree and is sensitive to the quality of the branch length and topology. Another problem associated with measurements of microbial diversity using diversity indexes is related to uneven sequence sampling. Diversity index values increase with sample size and make normalization of the number of sequences in all samples crucial. Within this work the calculations of both diversity indexes mentioned above were performed with sub-samples of 12,393

sequences. This reduced the bias associated with the sample size and allowed for a better comparison between the samples.

Generally, in agricultural systems with low vegetative diversity and high xenobiotic inputs, overall species diversity may be reduced to a bottleneck, from which species diversification is possible, albeit from a limited number of phyla (Roesch et al. 2009). Agricultural practices such as tillage, application of pesticides and nutrients, machinery traffic used for the soil and crops management, modify the physical and chemical properties of soil and, consequently, alter soil microbial diversity and ecological functions (Bissett et al. 2011; Lozupone & Knight 2007). In the same line, Hossain and Sugiyama (2011) suggested that soils exposed to frequent human disturbances might show modification of microbial structure or reduction of the microbial diversity. Contrary, we found that the removal of forest did not reduce the bacterial diversity. In general, our findings are in agreement with other studies, in which deforestation or low plant diversity due the result of forest conversion for pasture did not necessarily lead to a reduction of the bacterial diversity (da C Jesus et al. 2009; Ding et al. 2013). Deforestation might not always be reflected in loss of microbial community diversity likely may be explained due to the large presence of inactive bacterial cells in soils detected by DNA-based methods (Lennon & Jones 2011). Recently, Fierer and Lennon (2011) revised the concepts on generation and maintenance of diversity in microbial communities. Dormancy "refers to an organism's ability to enter a reversible state of low metabolic activity when faced with unfavorable environmental conditions" (Lennon & Jones 2011) and it may work as a microbial seed bank that helps to maintain the high levels of microbial biodiversity observed in nearly all ecosystems (Jones & Lennon 2010). As our approach was not sensitive to microbial activity, the metabolic status of our samples was unclear. However, following the concept of a seed bank proposed by Lennon and Jones (2011), the similarity in diversity levels of our soil samples may reflect a

reservoir of biodiversity that could potentially be resuscitated in the future under different environmental conditions.

The interaction between plants, soil and microorganisms is the driver of ecosystem functions and a disturbance might affect the microbial structure, which, in turn, will influence the soil processes and ecological functions (Singh et al. 2004). In our experiment, the disturbance was constant but relatively recent (eight years), and before removing the plant cover the same soil microbial diversity and structure was expected to be found. According to Miki et al. (2010), a change in the composition of a plant community leads to a change in the litter quality that in turn alters the nutrient cycling process and resultant soil conditions. Due to differences in vegetation composition, a clear discrimination between the microbial diversity and community structure from the forest and the grassland soils would be expected (Mitchell et al. 2010). However, a large overlap (69% of shared OTUs) was found between both microbial communities and no clear discrimination between them. Analysis of shared OTUs would be reasonable only when the sequencing coverage was enough to detect most of the OTUs present (90% or more), since the power for detecting overlapping species from multiple environments is strongly related to the sequencing intensity (Lemos et al. 2011). To circumvent the problem associated with detection of overlapping taxa, we first calculated how well each sample was representative for the bacterial community. The data summarized at the genus level provided reasonable coverage (greater than 99%), therefore the analysis of bacterial genera that were either unique or shared by specific soil samples was sensitive enough to detect the changes in the number of sequences, as well as the presence/absence of taxonomic units.

According to Martiny et al. (2006), the present-day community structure may have been driven by historical events (*e.g.*, prevalence of any type of vegetation, weather conditions) that might influence this particular community structure. Although the soil bacterial community did not suffer

great alteration after removing the natural forest, we were able to detect shifts related to specific bacterial groups. A total of 11 OTUs were found in statistically significant higher abundance in the forest samples but in lower abundance in the grassland samples. The *Nitrospira* genus, for example, was found in greater numbers in the forest than in the grassland. On the other hand, 12 bacterial taxa were found in higher abundance in the grassland samples but in lower abundance in the forest samples. This observation might be indicative that soil bacterial communities under the influence of environmental change will gradually be replaced by another community composed of different species that survive better with the new conditions. The bacterial community structure might change with time and, without any significant changes in soil properties, the plant cover will be the major driver of bacterial diversity and structure, as proposed by Mitchell et al. (2010). In addition, the new bacterial community may be functionally equivalent to the original one even if it has a different structure (Allison & Martiny 2008). The results obtained by other studies indicate that a greater degree of disturbance would be necessary to cause major shifts in microbial diversity and structure for the soil tested in this work. The results suggest the prevalence of a resilient microbial community less influenced by plant cover in which the history of land use might influence present-day community structure.

4.5 Supplementary material

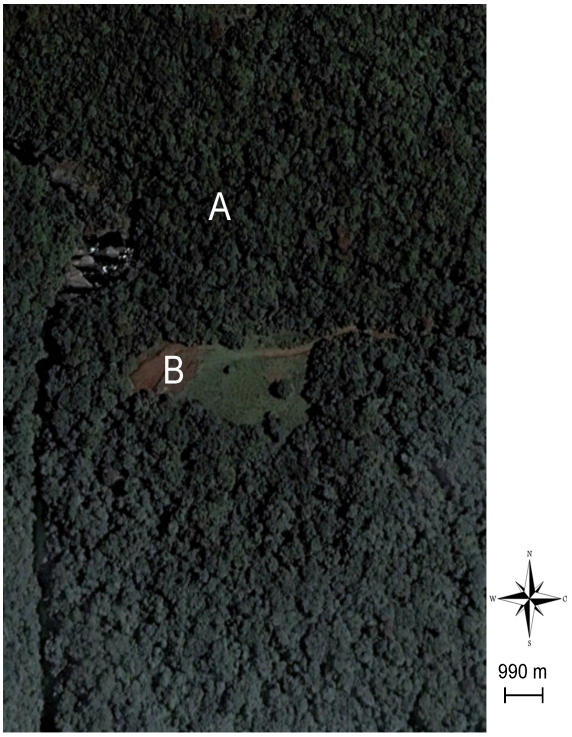

Fig. S4.1 (A) Pristine forest and (B) eight-years-old grassland resulted by the deforestation of a small area (5,500 m^2) inside the forest.

Table S4.1 List of the closest bacterial relative of Operational Taxonomic Unities (OTUs) uniquely found in native forest or grassland.

Native Forest	**Grassland**
Actinobacteria;Actinobacteria; Actinomycetales;Segniliparaceae; Segniliparus	*Firmicutes;Clostridia*
Bacteroidetes;Bacteroidia;Bacteroidales	*Proteobacteria;Gammaproteobacteria; Xanthomonadales*
Bacteroidetes;Bacteroidia;Bacteroidales; Porphyromonadaceae	*Firmicutes;Bacilli;Bacillales; Bacillaceae;Lysinibacillus*
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Xanthomonadaceae; Thermomonas	*Proteobacteria;Deltaproteobacteria; Bdellovibrionales;Bacteriovoracaceae; Bacteriovorax*
Proteobacteria;Betaproteobacteria; Burkholderiales;Comamonadaceae; Hylemonella	*Actinobacteria;Actinobacteria; Actinomycetales; Promicromonosporaceae;Xylanimonas*
SR1;SR1_genera_incertae_sedis	*Proteobacteria;Betaproteobacteria; Burkholderiales;Alcaligenaceae; Azohydromonas*
Actinobacteria;Actinobacteria; Actinomycetales;Nocardiaceae;Nocardia	*Chloroflexi;Anaerolineae;Anaerolineale; Anaerolineaceae;Anaerolinea*
Planctomycetes;Planctomycetacia; Planctomycetales;Planctomycetaceae; Pirellula	*Proteobacteria;Alphaproteobacteria; Rhodospirillales;Rhodospirillaceae; Azospirillum*
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Xanthomonadaceae; Stenotrophomonas	*Actinobacteria;Actinobacteria; Actinomycetales;Streptosporangiaceae; Sphaerisporangium*
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Sinobacteraceae;Nevskia	*Proteobacteria;Alphaproteobacteria; Rhizobiales;Methylobacteriaceae; Methylobacterium*
Actinobacteria;Actinobacteria; Actinomycetales;Bogoriellaceae;Georgenia	*Proteobacteria;Alphaproteobacteria; Rhizobiales;Bradyrhizobiaceae; Balneimonas*
Firmicutes;Clostridia;Clostridiales; Ruminococcaceae	*Proteobacteria;Gammaproteobacteria; Enterobacteriales;Enterobacteriaceae; Pantoea*
Proteobacteria;Gammaproteobacteria; Pseudomonadales;Pseudomonadaceae; Cellvibrio	*Proteobacteria;Alphaproteobacteria; Rhodospirillales;Rhodospirillaceae; Magnetospirillum*
Actinobacteria;Actinobacteria; Actinomycetales;Cellulomonadaceae	*Proteobacteria;Deltaproteobacteria; Desulfuromonadales*
Bacteroidetes;Flavobacteria;Flavobacteriales; Cryomorphaceae	*Proteobacteria;Betaproteobacteria; Burkholderiales;Comamonadacea*
Proteobacteria;Deltaproteobacteria;	*Proteobacteria;Deltaproteobacteria;*

Native Forest	**Grassland**
Myxococcales;Phaselicystidaceae; Phaselicystis	*Desulfuromonadales;Geobacteraceae*
Proteobacteria;Betaproteobacteria; Methylophilales;Methylophilaceae; Methylophilus	*Proteobacteria;Deltaproteobacteria; Desulfobacterales;Desulfobulbaceae; Desulfobulbus*
Actinobacteria;Actinobacteria; Actinomycetales;Promicromonosporaceae; Promicromonospora	*Actinobacteria;Actinobacteria; Actinomycetales;Actinosynnemataceae; Lechevalieria*
Actinobacteria;Actinobacteria; Actinomycetales;Nocardiaceae;Williamsia	*Actinobacteria;Actinobacteria; Actinomycetales;Geodermatophilaceae; Modestobacter*
Proteobacteria;Deltaproteobacteria; Myxococcales;Nannocystaceae;Nannocystis	*Actinobacteria;Actinobacteria; Actinomycetales;Propionibacteriaceae; Friedmanniella*
Bacteroidetes;Sphingobacteria; Sphingobacteriales;Cytophagaceae; Dyadobacter	*Proteobacteria;Alphaproteobacteria; Rhizobiales;Methylobacteriaceae; Microvirga*
Firmicutes;Bacilli	*Proteobacteria;Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae;Sphingosinicella*
Proteobacteria;Alphaproteobacteria; Rhizobiales;Hyphomicrobiaceae;Devosia	*Proteobacteria;Betaproteobacteria; Burkholderiales;Burkholderiaceae; Chitinimonas*
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Xanthomonadaceae; Dokdonella	*Proteobacteria;Betaproteobacteria; Burkholderiales;Comamonadaceae; Roseateles*
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Xanthomonadaceae; Dokdonell;Verrucomicrobia;Opitutae	*Proteobacteria;Deltaproteobacteria; Myxococcales;Myxococcaceae*
Actinobacteria;Actinobacteria; Actinomycetales;Micromonosporaceae; Rugosimonospora	*Actinobacteria;Actinobacteria; Actinomycetales;Geodermatophilaceae; Blastococcus*
Actinobacteria;Actinobacteria; Actinomycetales;Nocardiaceae; Smaragdicoccus	*Proteobacteria;Betaproteobacteria; Rhodocyclales;Rhodocyclaceae;Zoogloea*
Actinobacteria;Actinobacteria; Actinomycetales;Cryptosporangiaceae; Cryptosporangium	*Acidobacteria;Acidobacteria_Gp19; Gp19*
Actinobacteria;Actinobacteria; Actinomycetales;Streptomycetaceae; Streptacidiphilus	*Planctomycetes;Planctomycetacia; Planctomycetales;Planctomycetaceae; Gemmata*
Bacteroidetes;Sphingobacteria; Sphingobacteriales;Cytophagaceae;Emticicia	*Proteobacteria;Alphaproteobacteria; Rhodospirillales;Rhodospirillaceae; Defluviicoccus*
Chloroflexi;Anaerolineae;Anaerolineales;	*Firmicutes;Bacilli;Bacillales;*

Native Forest	Grassland
Anaerolineaceae;Longilinea	*Paenibacillaceae;Brevibacillus*
Proteobacteria;Betaproteobacteria; Burkholderiales;Comamonadaceae; Comamonas	*Firmicutes;Clostridia;Clostridiales; Peptococcaceae;Desulfosporosinus*
Bacteria;Proteobacteria;Alphaproteobacteria; Caulobacterales;Caulobacteraceae; Brevundimonas	*Proteobacteria;Alphaproteobacteria; Rhizobiales;Rhizobiaceae;Kaistia*
Proteobacteria;Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Paracoccus	*Proteobacteria;Alphaproteobacteria; Rhodobacterales;Rhodobacteraceae; Pannonibacter*
Proteobacteria;Gammaproteobacteria; Xanthomonadales;Xanthomonadaceae; Pseudoxanthomonas	*Proteobacteria;Gammaproteobacteria; Enterobacteriales;Enterobacteriaceae; Serratia*
Proteobacteria;Deltaproteobacteria; Myxococcales;Cystobacteraceae;Hyalangium	*Verrucomicrobia;Verrucomicrobiae; Verrucomicrobiales; Verrucomicrobiaceae;Luteolibacter*
Proteobacteria;Alphaproteobacteria; Rhizobiales;Phyllobacteriaceae;Aminobacter	*Firmicutes;Clostridia;Clostridiales; Clostridiaceae;Clostridium*
Proteobacteria;Alphaproteobacteria; Rhodospirillales;Acetobacteraceae; Roseomonas	*Cyanobacteria;Cyanobacteria*
Chloroflexi;Chloroflexi	*Chloroflexi;Thermomicrobia; Sphaerobacterales;Sphaerobacteraceae; Sphaerobacter*
Bacteroidetes;Sphingobacteria;Sphingobacte riales;Sphingobacteriaceae;Pedobacter	*Actinobacteria;Actinobacteria; Actinomycetales;Nocardioidaceae; Actinopolymorpha*
WS3;WS3_genera_incertae_sedis	*Actinobacteria;Actinobacteria; Actinomycetales;Nocardiaceae; Rhodococcus*
Bacteroidetes;Sphingobacteria;Sphingobacte riales;Cytophagaceae;Cytophaga	*Actinobacteria;Actinobacteria; Actinomycetales;Micromonosporaceae; Planosporangium*
Proteobacteria;Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae; Sphingobium	*Actinobacteria;Actinobacteria; Actinomycetales;Nakamurellaceae; Nakamurella*
Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Filimonas	*Proteobacteria;Betaproteobacteria; Rhodocyclales;Rhodocyclaceae; Dechloromonas*
Acidobacteria;Acidobacteria_Gp9;Gp9	*Proteobacteria;Deltaproteobacteria; Desulfuromonadales;Geobacteraceae; Geobacter*
Proteobacteria;Deltaproteobacteria; Myxococcales;Haliangiaceae;Haliangium	*Verrucomicrobia;Verrucomicrobiae; Verrucomicrobiales; Verrucomicrobiaceae;Haloferula*

Native Forest	**Grassland**
Proteobacteria;Betaproteobacteria; Burkholderiales;Oxalobacteraceae; Janthinobacterium	*Proteobacteria;Alphaproteobacteria; Rhodospirillales;Rhodospirillaceae; Skermanella*
Proteobacteria;Alphaproteobacteria; Rhizobiales;Xanthobacteraceae; Azorhizobium	*Proteobacteria;Alphaproteobacteria; Sphingomonadales;Erythrobacteraceae*
Bacteroidetes;Sphingobacteria; Sphingobacteriales;Flammeovirgaceae	*Firmicutes;Bacilli;Bacillales; Bacillales_incertae_sedis;Solibacillus*
Actinobacteria;Actinobacteria; Actinomycetales;Micromonosporaceae; Catellatospora	
Acidobacteria;Holophagae;Holophagales; Holophagaceae	
Bacteroidetes;Flavobacteria;Flavobacteriales; Flavobacteriaceae;Chryseobacterium	
Proteobacteria;Deltaproteobacteria; Myxococcales;Cystobacteraceae;Stigmatella	
Proteobacteria;Alphaproteobacteria; Rhodospirillales;Rhodospirillaceae; Telmatospirillum	
Proteobacteria;Betaproteobacteria; Methylophilales;Methylophilaceae; Methylotenera	

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Chapter 5

Moisture is more important than temperature to shape bacterial and archaeal communities in subtropical grassland

Authors

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To be submited

Abstract

It is generally accepted that moisture and temperature play key roles in the assemblage and functioning of microbial communities in soil. However, how seasonal variations regulate the role of niche- versus neutral-based processes during the assemblage of these communities has not been examined in the light of the coexisting active and dormant communities in native subtropical grassland. We carried out an experiment in a well-controlled microcosm system, in which we investigated the individual and combined effects of moisture and temperature mimicking winter and summer in a subtropical grassland. The relative size of the active and dormant populations was assessed using the rRNA:rDNA ratio after direct extraction from soil. Moisture was the most important factor influencing community diversity and structure, with a larger effect on the active, dormant and total community than temperature. The dormant community comprises the largest proportion of microbial diversity in these soils, and mainly reflects on the diversity and structure pattern of the total community. Active and dormant communities were controlled by the same assemblage rules and the relative influence of underlying assembly mechanisms are moisture-dependent, with niche-based mechanisms being more influential in communities under stress at dry conditions. The results of this study provide new informations regarding seasonal variations on microbial communities in soil and how the coexisting active and dormant communities are shaped in a native subtropical grassland soil.

Keywords

16S rRNA; Assembly process; Niche and neutral process; Microbial ecology; Phylogenetic structure; Dormancy

5.1 Introduction

Grasslands in subtropical and temperate regions are exposed to large seasonal variations in rainfall and air temperature, which has considerable influence on the moisture and temperature status of the soil (Deng et al. 2012), thereby strongly affecting key processes such as soil organic matter decomposition, plant growth and nutrient turnover in terrestrial environments (Stark & Firestone 1995; Karhu et al. 2014). Microbes are the key drivers of these processes and generally, temperature positively correlates with microbial activity and is a key controlling factor regulating microbial functioning in soil (Lipson 2007). Changes in temperature may also influence the structure of bacterial communities (Prevost-Boure et al. 2011). Water content is an important regulator of gaseous diffusion processes as well as of biological activity in the soil matrix, with maximum aerobic microbial activity occurring at moisture levels of around 70% of water-holding capacity. Low water content inhibits microbial activity for instance by restricting substrate supply and selecting for only species adapted to survive under these conditions (Stark & Firestone 1995; Valverde et al. 2014).

Although water content and temperature are well-known regulators of the composition and function of microbial communities (Bell et al. 2008; Brockett et al. 2012), it is only poorly understood how microbial communities respond to moisture and temperature variations in a subtropical grassland ecosystem. Large seasonal variability may select for stable communities adapted to inter- and intra- annual changes (Stres et al. 2008). Microbial communities in these systems are said to remain intact and not linked to

seasonal changes (Williams et al. 2013). This apparent stability might be explained by the presence of a large but inactive, dormant, pool of microorganisms in soil (Smit et al. 2001) which may mask the shifts in the composition of the active community. Usually the active fraction is a minor fraction of the total microbial community, which is more dependent on the variation in the availability of substrates and climatic fluctuations (McMahon et al. 2011). Thus, habitat selective factors may exert differential effects on active and dormant communities. Hence, the relevant question is if active and dormant communities show different responses to seasonal changes and to what extent those patterns are reflected in the composition of the total microbial community.

Although studies have shown that both niche- and neutral-based process are important in structuring microbial communities, the question how seasonal variations regulate the importance of niche- and neutral-based processes is not yet answered (Stegen et al. 2012). Studies have demonstrated that habitat characteristics create conditions that favor or disfavor the relative importance of these assembly processes (Ferrenberg et al. 2013). For example, niche-based processes through environmental selection are more plausible to shape microbial communities in highly stressed habitats (Wang et al. 2013), such as at environmental disturbances promoted by drought (Chase 2007). Contrarily, in more benign environments (or less disturbed habitats, *e.g.,* higher productivity or "wet" habitats) microbial communities are more likely to be shaped by neutral processes (Valverde et al. 2014). According to the the phylogenetic conservatism theory (Webb et al. 2002), phylogenetic clustering can be interpreted in terms of niche-based processes, where only phylogenetically closely related taxa co-occur at specific environmental filtering conditions, whereas no phylogenetic clustering or overdispersion is the opposite pattern and indicate that neutral mechanisms are the dominant assembly processes, generating communities with more variable species composition

(Horner-Devine & Bohannan 2006; Jones & Hallin 2010). However, the contribution of niche- *vs* neutral-based assembly processes has not yet been examined in the light of the coexistence of active and dormant microbial communities under the influence of seasonal climatic variations in soil.

In the present study, we aimed to obtain a better understanding of how seasonal variations of moisture and temperature affect the shaping of coexisting active and dormant bacterial and archaeal communities and what the dominant assemblage rules are operating at these circumstances. We sequenced 16S rDNA and 16 rRNA sampled from the soil of a well-controlled microcosm system to investigate the individual and combined effects of moisture and temperature, mimicking winter and summer conditions in a native subtropical grassland, on the composition of the active, dormant and total microbial communities. The lower moisture content and high temperature of the experiment are typical for the dry summer conditions and the higher moisture content and low temperature are typical for winter conditions. First, we examined what proportion of the community is metabolically active (or dormant) at a given moment using the rRNA:rDNA ratio and we measured the diversity of both fractions in order to get insight in the main contributors to the diversity of the total community. Second, we analyzed to what extent moisture, temperature and their interactions affect the structuring mechanisms of active and dormant communities in order to show how this determines the dynamics of the total community. Therefore, we tested the relative importance of the niche- and neutral-based processes by comparing phylogenetic turnover followed by phylogenetic relatedness, which provide information about the underlying assemblage processes along the climatic regimes tested here. We hypothesized that the active community will be more sensitive to changes in moisture and temperature than the dormant community, and the latter community will be more determinative for the patterns observed in the total community. We also hypothesized that niche-based processes would become

more important for bacterial and archaeal community assembly at the extreme conditions examined here.

5.2 Material and methods

5.2.1 Microcosm experimental design and soil sampling

The microcosm experiment was designed to mimic the natural climate conditions over the year in a subtropical grassland in a Brazilian Pampa biome by applying different combinations of soil moisture and temperature. The soil used in the experiment was sampled from a site which is characterized by four well-defined seasons with seasonal variation of soil moisture and temperature. Soil cores were collected from the upper soil layer (0-20 cm) in autumn (May, 2013) of a native grassland (29° 45' S, 53° 45' W) used for cattle grazing, with no input of fertilizers besides the animal manure.

The microcosms consisted of undisturbed soil blocks of 15 x 20 x 25 cm placed into a pot with the same dimensions. The cores were collected and kept at the same sampling temperature and moisture (28°C and 20%) for a maximum 6 h before the microcosms were set up. In order to know the boundaries of the moisture variation we determined the permanent wilting point using water potential psychrometry equipment (Klute & others 2003) and the field capacity in a sand suction column (Reinert & Reichert 2006).

The experimental design included three replicates for all treatments. Based on the information on the permanent wilting point and field capacity, three levels of soil moisture were applied *i.e.,* 8%, 16% and 23% (w/w), which were kept constant during the whole experiment by adding sterile distilled water. The microcosms were submitted to different temperatures (10°C, 20°C and 30°C) within the three above mentioned soil moistures treatments during 20 days. The temperatures chosen were based on previous measurements in soil over the year in the same grassland area from where the soil cores were

collected (Fig. S5.1). Samples were taken after 20 days of incubation from the top layer (0 - 5 cm) of the microcosm systems and kept at -80°C until total RNA and DNA extraction.

5.2.2 Co-extraction of soil DNA and RNA

From each sample 2g of soil was used for simultaneous total RNA and DNA isolation using the RNA PowerSoil kit and the PowerSoil®DNA Elution Accessory Kit (MoBio laboratories, Inc., Carlsbad, CA, USA) following the manufacture instructions. RNA and DNA quantities and quality were determined using NanoVue™spectrophotometer (GE Healthcare). The residual DNA from RNA samples was removed via DNase treatment (TURBO DNA-free™ Kit, Life Technologies, Carlsbad, CA, USA). The total RNA was synthesized to complementary DNA (cDNA) using random hexamer with Maxima H Minus First Strand cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA). Resulting DNA and cDNA were used as templates for amplification of the 16S rRNA.

5.2.3 16S rDNA and rRNA amplification and sequencing

The composition of the bacterial and archaeal communities was determined based on partial 16S rDNA and 16S rRNA (V4 region) sequences directly amplified using bacterial/archaeal primer set 515F/806R (Caporaso et al. 2011) from DNA and RNA (cDNA) obtained for each replicate per treatment. The PCR reactions were performed in triplicates with the KAPA HiFi HotStart ReadyMix (2X). The thermocycling conditions were as follows: initial denaturing of 95°C for 3 min, 25 cycles of 98°C for 20 s, annealing temperature of 60°C (20 s) and 72°C for 15 minutes extension, followed by 72°C for 1 minute. Reaction mixtures contained about 100 ng of DNA or cDNA templates, 0.5 µL of each primer (100 mM of each primer), 12.5 µL of 2X KAPA HiFi

HotStart ReadyMix and water to complete the final volume to 25 µL.

The PCR products of each sample were purified with AMPure XP PCR Purification Kit (Agencourt), quantified with Qubit Fluorometer (Invitrogen) and combined in equimolar ratios. The DNA fragments were sequenced using Ion Torrent™ semiconductor technology for unidirectional sequencing of the amplicon libraries. Barcoded primers were used to multiplex the amplicon pools in order to sequence together and computationally separated afterwards. The barcode of 12 bases was added to primer 806R and unidirectional sequencing was performed from the A-key adapter. A two-base linker sequence was inserted between the adapter and the 16S rDNA primers to reduce any effect of the composite primer on PCR efficiency. Ion OneTouch™ 2 System and Ion PGM™ Template OT2 400 Kit Template were used for library preparation and the sequencing was performed using Ion PGM™ Sequencing 400 kit on Ion PGM™ System using Ion 314™ Chip v2.

5.2.4 16S rDNA and rRNA data analysis

The 16S rDNA and rRNA raw sequences were analyzed following the recommendations of the Brazilian Microbiome Project (Pylro et al. 2014). Briefly, the OTU file table was built using the UPARSE pipeline (Edgar 2013). The reads were truncated at 200 bp and quality filtered using a maximum expected errors value of 0.5 (meaning that on average one nucleotide in every two sequences is incorrect). Filtered reads were dereplicated and unique sequences (singletons) were removed. These sequences were clustered into OTUs at 97% similarity cutoff following the UPARSE pipeline, which incorporates chimera checking into this step, and representative sequences for each phylotype. After clustering, the sequences were aligned and classified using the SILVA reference database (release SSU_Ref_119) with a confidence threshold of 50% (Claesson et al. 2009) using mothur (Schloss et al. 2009). The

representative sequence of each OTU was used to construct a dendrogram using a distance matrix with relaxed neighbor joining (RNJ) algorithm in clearcut (Sheneman et al. 2006) available in mothur.

5.2.5 Statistical analysis

The BIOM file was imported into the R environment (Team & others 2005) using the phyloseq package (McMurdie & Holmes 2013). Good's coverage estimator (Good 1953) was calculated for 97% similarity cutoff in order to assess if the number of sequences obtained represented the entire community. For activity-based comparisons (potential activity), active and dormant OTUs were identified based on comparison of the relative recovery of 16S rRNA and 16S rDNA following Jones and Lennon (2010), where OTU's with rRNA:rDNA > 1 was scored as active, and any OTU with rRNA:rDNA < 1 was scored as dormant (Jones & Lennon 2010; Breidenbach & Conrad 2015; DeAngelis et al. 2010). Members of the 16S rDNA data set not represented in the 16S rRNA data set were also included in the dormant community. Total microbial community was analyzed using the rDNA data set, which includes both dormant and potentially active microbial species.

The effect of soil moisture, temperature and their interactions on shaping the active and dormant proportions of the microbial community were assessed by two-way analysis of variance (ANOVA) with repeated measures using *aov* in the stats package (Team & others 2005). ANOVA residuals were plotted to determine the normal distribution and further confirmed by the Shapiro-Wilk W test ($p > 0.05$) using *shapiro.test* in the stats package. When the differences were significant, they were further analyzed using a post-hoc test by the *HSD.test* in the agricolae package (de Mendiburu 2014). For the estimation of alpha diversity and richness, the data set was rarefied to 4,167 sequences per sample and three different approaches were employed: (a)

community richness was calculated by Chao1's estimator, which weights total and rare species; and (b) compositional diversity was assessed by applying the Shannon diversity index considering the number and abundance of species (*estimate_richness* function in phyloseq package); and (c) phylogenetic diversity was calculated by Faith's phylogenetic diversity index (PD, Faith 1992) incorporating phylogenetic distances between species (*pd* function in picante package (Kembel et al. 2010)). Similar to the evaluation of the effects of moisture and temperatures on the fractions of the microbial community, the diversity index were analyzed using two-way analysis of variance (ANOVA) with repeated measures after plotting the residuals and confirming the normality of the data using the Shapiro-Wilk W test ($p > 0.05$). When the differences were significant, they were further analyzed using the post-hoc test by the *HSD.test*.

To test the hypothesis that environmental factors (moisture, temperature or the interaction of both) shape the active, dormant and total community, the 16S rRNA:rDNA ratio obtained from the entire data set was used to construct a compositional dissimilarity matrix generated by Bray-Curtis. The matrix were ordinated by Principal Components Analysis (PCoA) using *ordinate* in the phyloseq package and the variance partitioning was calculated using the two-way permutational multivariate analysis of variance (PERMANOVA (Anderson 2001)) model based on Bray-Curtis using *adonis* function in the vegan package (Oksanen et al. 2007). A heatmap with the most important OTUs (based on the rRNA:rDNA ratio) responsible for the differences between moisture and temperature regimes was obtained using *heatmap.2* in the gplots package (Warnes et al. 2015). The OTUs were identified using the similarity percentage (SIMPER) analysis calculated using the *simper* function in the vegan package. The distance between samples was calculated by the *vegdist* function using the Bray-Curtis method from the vegan package and the samples were clustered using the *hclust* function with

the “average” agglomeration method from the stats package. A similarity profile permutation test (SIMPROF) was used to identify significant groups of samples at 5% significance level with 10,000 simulations calculated by the *simprof* function using the clustsig package (Whitaker & Christman 2014).

We examined the difference in phylogenetic turnover (phylogenetic β-diversity) among the moisture and temperature regimes using the unweighted UniFrac metric (Lozupone & Knight 2005) associated with PCoA and PERMANOVA to provide insight into how moisture and temperature correlates with the clustering of communities. The same rarefied data as used for the calculation of the diversity index were used here. As we detected that moisture was most important to separate the communities, we quantified the Net Relatedness Index (NRI) (Webb et al. 2002) to depict phylogenetic community structures, using *ses.mdp* in the picante package along the moistures regimes. The NRI captures the influence of deeper phylogenetic nodes (*e.g.*, Phyla, Class) to the complete community tree. For NRI, positive values indicate clustered (=niche-based process), while negative values indicate overdispersed phylogenetic structures (=neutral-based process). The phylogenetic structuring of a community might also be random, also indicating neutral-based processes (Kembel & Hubbell 2006). To evaluate the degree of non-random phylogenetic community structuring, taxa were randomized across the tips of the phylogeny (null.model = “taxa.labels”) with 999 permutation shuffling taxa among soil moisture. Two-tailed *p*-values were obtained by comparing values for NRI with those from the distributions of random communities. Assemblages with $p < 0.025$ were considered significantly clustered, and assemblages with $p > 0.975$ were considered significantly overdispersed. We then used ANOVA followed by a *post hoc* test using the function *HSD.test* in the agricolae package after verifying the normal distribution of the residuals and correlation analysis using the *cor.test* in the base package (Team & others 2005) to examine the relationship between clustering values and moistures regimes.

5.3 Results

5.3.1 Sequence data and community coverage

After quality filtering, a total of 899,265 sequences was obtained (509,086 for 16S rDNA and 390,179 for 16S rRNA) with an average of 12,665 sequences (200 bases in length and maximum expected error of 0.5) obtained per sample representing 82% up to 96% sequencing coverage (Good's coverage) at 97% similarity cutoff for the OTU definition (Table S5.1). Those results indicated that the numbers of sequences from each library were representative for the communities measured.

5.3.2 Taxonomic and diversity patterns of active and dormant communities

The dormant fraction contributed around 70% of total community regardless of moisture, temperature or their interactions (ANOVA, $p > 0.05$) (Fig. S5.2). In addition, we observed strong taxonomic differences in the distribution of active and dormant OTUs (Fig. 5.1 and Table S5.2). Specifically, 78% of *Lentisphaerae* OTUs and 55% of the *Thaumarchaeota* OTUs were scored as active, whereas *Thermodesulfobacteria* and almost all of the Candidate divisions OP3, BRC1, WS3 and TM7 OTUs were scored as dormant (less than 12% of active OTUs). Further, a group of 12 phyla were classified as dormant (100% of OTUs dormant, rRNA:rDNA < 1) at all soil conditions (Fig. 5.1 and Table S5.2). Moisture and temperature or their interactions only had significant effects on the fraction of active OTUs of a few phyla, *i.e., Chlamydiae*, *Thaumarchaeota*, *Caldiserica*, *Spirochaetae* and BHI80-139 (ANOVA, $p < 0.05$).

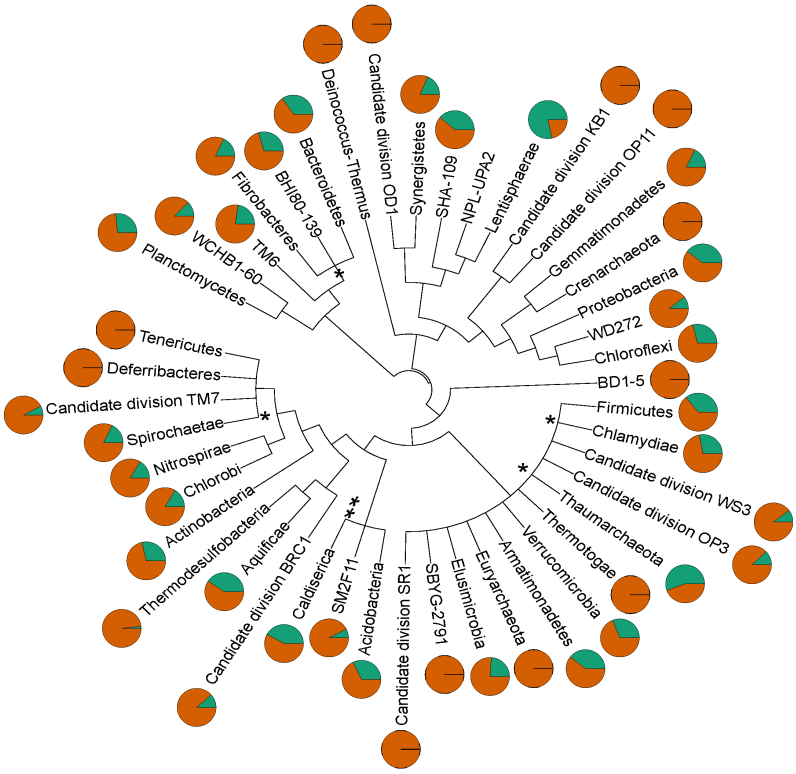

Fig. 5.1 Distribution and representation of the proportion of OTUs scored as active and dormant within phyla. Green represents the active fraction of OTUs while orange represents the dormant fraction within phyla. * Proportion of active *vs* dormant OTUs within major phyla significantly influenced by moisture or ** moisture x temperature ($p < 0.01$).

The active fraction of the community showed lower richness (Chao1) and diversity (PD) compared to the dormant fraction and the total microbial communities (Fig. 5.2). Moisture was the most important predictor of diversity, with Chao1, Shannon and PD values differing significantly across moisture

regimes (two-way ANOVA, $p < 0.05$; 0.01; 0.001). Overall, the communities revealed lower richness and diversity values in low water content (8%) than in high moisture regimes (23%) when assessed by tukey-test ($p < 0.01$). Only the PD diversity for the total community was not affected neither by moisture or temperature. Temperature nor the interaction between moisture and temperature affected the richness and diversity values (two-way ANOVA; $p > 0.05$; Fig. 5.2); only the diversity of total community (Chao1) was slightly affected by temperature ($p < 0.1$) within 8% and 16% moisture regime, with low values of diversity in 30°C.

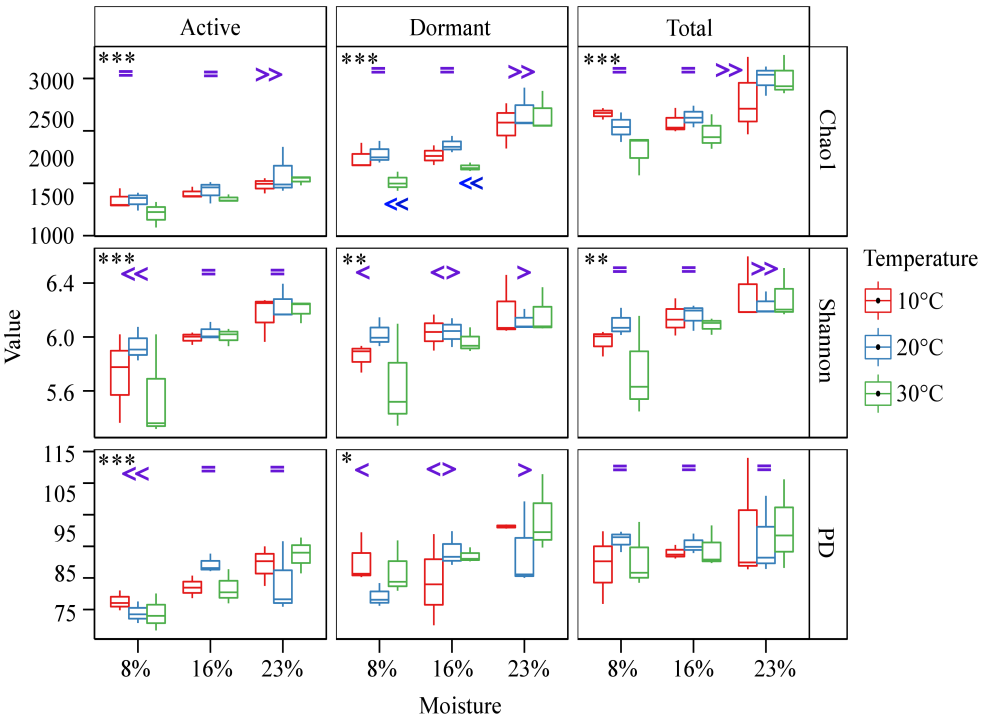

Fig. 5.2 Richness and diversity estimators of bacterial and archaeal communities. Purple symbols of ">", ">>" indicate significantly higher values than other one or two moisture regimes, respectively, in pairwise tukey-test; symbols of "<", "<<" indicate significantly lower values than other one or two moisture regimes, respectively; "<>" symbol indicate no difference between the higher or the lower diversity in moisture regimes; blue symbols "<<" indicate significantly lower values than other two temperatures within moisture regime ($p < 0.1$); the "=" symbols indicate no significant difference ($p > 0.05$). *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ indicate the level of significance by ANOVA test for moisture level.

5.3.3 Moisture is more important as driver of the structuring and potential activity of bacterial and archaeal communities

Overall, there were significant differences in community structure at OTU level between the soil conditions applied here. The ordination and variance analysis carried out by using Bray-Curtis dissimilarity suggested that soil moisture was the main driver shaping the community structure (Fig. 5.3). The structure of the total community was more influenced by moisture (PERMANOVA; pseudo-F = 4.72, R^2 = 0.28, $p < 0.001$) than the dormant (PERMANOVA; pseudo-F = 4.22, R^2 = 0.26, $p < 0.001$) and the active one (PERMANOVA; pseudo-F = 3.77, R^2 = 0.21, $p < 0.001$). To a smaller degree than moisture, temperature (PERMANOVA; pseudo-F = 1.77, R^2 = 0.09, $p < 0.001$) and interactive effect of moisture and temperature (PERMANOVA; pseudo-F = 1.85, R^2 = 0.20, $p < 0.001$) were also a good predictors of the structure of the active community (Fig. 5.3). Neither temperature nor the interaction between moisture and temperature affect the total and dormant community (PERMANOVA; $p > 0.05$).

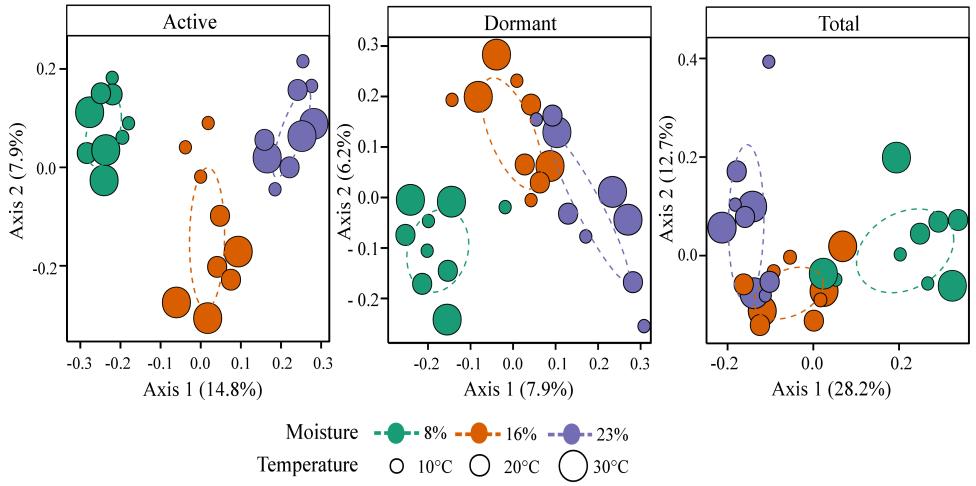

Fig. 5.3 Principal coordinates analysis (PCoA) ordination based on Bray-Curtis dissimilarity of microbial community structure. The variations explained by the first two axes are indicated on the graphs. Centroid and color of each ellipse are defined by the distribution and standard deviation of points in the defined group (here drawn around the moisture regimes, the most important predictor of the community structure) representing 95% confidence intervals for the standard error of the average of scores for each group.

As the rRNA:rDNA ratio indicate the potential activity of OTUs, a heatmap in conjunction with the cluster analysis was constructed allowing for the visualization of the main OTUs responsible for the patterns found for the active community in Fig. 5.3, so, to understand how moisture and temperature change the potential activity of the members of a community. Most OTUs belonged to broad phylogenetic groups commonly found in soil under these conditions, *i.e., Proteobacteria, Actinobacteria, Verrucomicrobia* and *Planctomycetes* (see Fig. 5.1). However, based on the heatmap and SIMPROF, the potential activity of OTUs changed substantially between moisture regimes, which explains the differences found in the clustering analysis (Fig. 5.4), where samples from 8% and 16% are clustered separately from the 23%

moisture regime, and only the temperature 30°C clustered separated from 10°C and 20°C within 23% moisture regime. In addition, the potential activity of these OTUs can vary in different ways between moisture conditions, such as a group of OTUs wich increases the potential activity with increasing water content *e.g., Sorangium*, *Flavisolibacter,* in opposite to another group, which increased the activity at reduced water content *e.g., Chthoniobacter, Acidimicrobium* and *Conexibacter* (Fig. 5.4). Data also show that a small number of OTUs, *e.g., Phenylobacterium, Pedomicrobium* and *Candidatus Solibacter* are resistant to variations in moisture and temperature being always active at all conditions (Fig. 5.4).

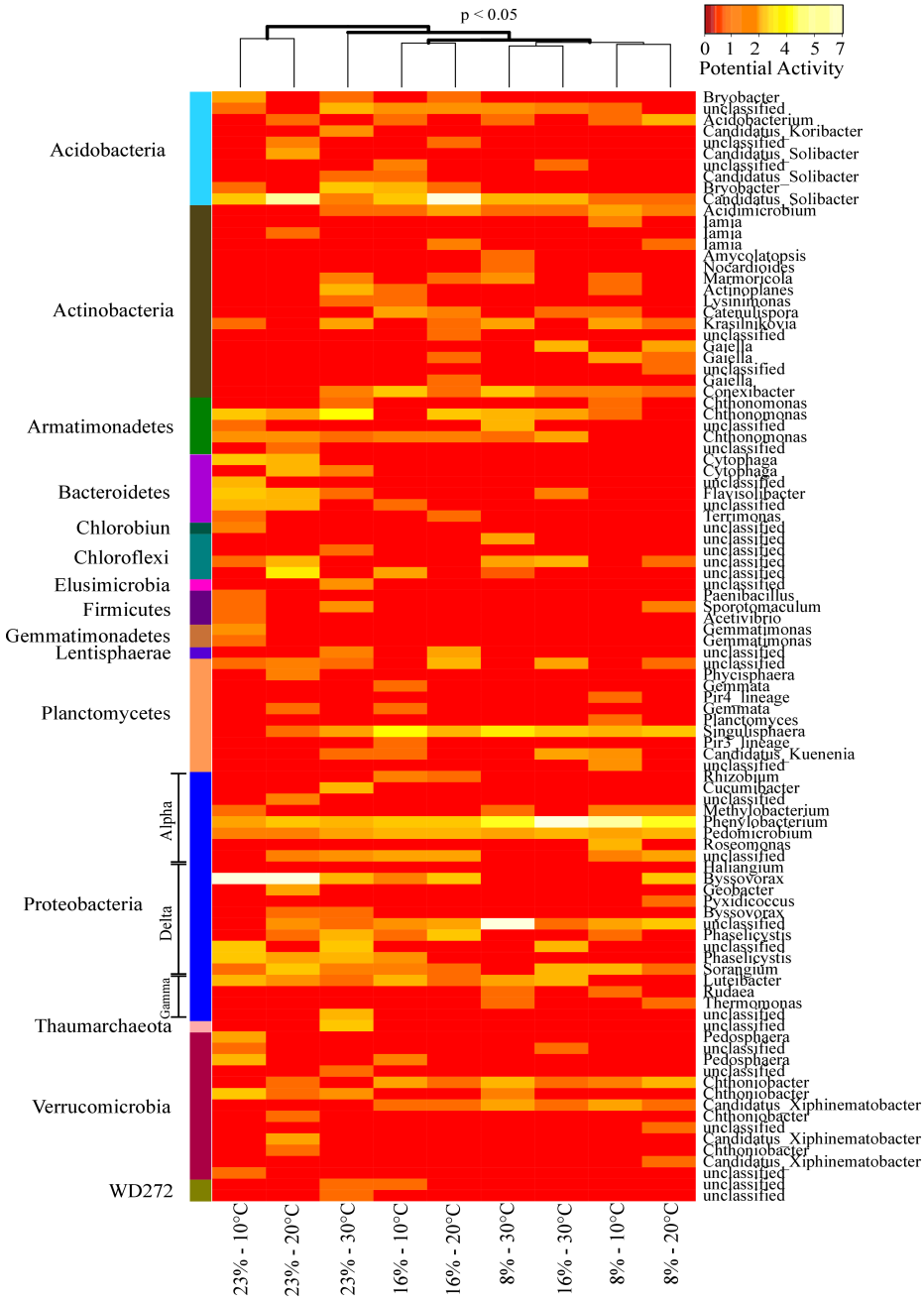
p < 0.05
0 1 2 4 5 7
Potential Activity
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chlorobiun
Chloroflexi
Elusimicrobia
Firmicutes
Gemmatimonadetes
Lentisphaerae
Planctomycetes
Proteobacteria
Alpha
Delta
Gamma
Thaumarchaeota
Verrucomicrobia
WD272
Bryobacter
unclassified
Acidobacterium
Candidatus_Koribacter
unclassified
Candidatus_Solibacter
unclassified
Candidatus_Solibacter
Bryobacter
Candidatus_Solibacter
Acidimicrobium
Iamia
Iamia
Iamia
Amycolatopsis
Nocardioides
Marmoricola
Actinoplanes
Lysinimonas
Catenulispora
Krasilnikovia
unclassified
Gaiella
Gaiella
unclassified
Gaiella
Conexibacter
Chthonomonas
Chthonomonas
unclassified
Chthonomonas
unclassified
Cytophaga
Cytophaga
unclassified
Flavisolibacter
unclassified
Terrimonas
unclassified
unclassified
unclassified
unclassified
unclassified
unclassified
Paenibacillus
Sporotomaculum
Acetivibrio
Gemmatimonas
Gemmatimonas
unclassified
unclassified
Phycisphaera
Gemmata
Pir4_lineage
Gemmata
Planctomyces
Singulisphaera
Pir3_lineage
Candidatus_Kuenenia
unclassified
Rhizobium
Cucumibacter
unclassified
Methylobacterium
Phenylobacterium
Pedomicrobium
Roseomonas
unclassified
Haliangium
Byssovorax
Geobacter
Pyxidicoccus
Byssovorax
unclassified
Phaselicystis
unclassified
Phaselicystis
Sorangium
Luteibacter
Rudaea
Thermomonas
unclassified
unclassified
Pedosphaera
unclassified
Pedosphaera
unclassified
Chthoniobacter
Chthoniobacter
Candidatus_Xiphinematobacter
Chthoniobacter
unclassified
Candidatus_Xiphinematobacter
Chthoniobacter
Candidatus_Xiphinematobacter
unclassified
unclassified
unclassified
23% - 10°C
23% - 20°C
23% - 30°C
16% - 10°C
16% - 20°C
8% - 30°C
16% - 30°C
8% - 10°C
8% - 20°C

Fig. 5.4 Heatmap and accompanying cluster analyses (x-axis) based on Bray-Curtis distance showing the variation in active community across moistures and temperature regimes. In the dendrogram, the thicker line represents groups that are significantly differentiated based on a SIMPROF analysis ($p < 0.05$). Each OTU is colored in shades from light yellow (high potential activity) to red (low potential activity) as shown in the color bar (above right) based on 16S rRNA:rDNA ratio. Only the most important OTUs responsible for the differences in the patterns in the active community in Fig. 5.3 are depicted here. The OTUs are classified at genus level in the y-axis (right) and phyla level (left).

5.3.4 Differences in the processes operating at the assemblage of the active and dormant communities

We detected consistent separation of bacterial and archaeal community clusters measured using unweighted UniFrac, suggesting that differences in community relatedness occurred along the moisture gradient (Fig. S5.3). Again we proved that moisture had the larger effect on the composition of the communities, with a greater effect on the composition of the active community (PERMANOVA; pseudo-F = 12.5, $R^2 = 0.29$, $p < 0.001$) than on the dormant and total communities, both equally affected (PERMANOVA; pseudo-F = 3.25, $R^2 = 0.21$, $p < 0.001$). Temperature and its interaction with moisture had a significant effect, but smaller than moisture, on the active community (PERMANOVA; temperature, pseudo-F = 6.70, $R^2 = 0.16$; moisture *vs* temperature, pseudo-F = 7.32, $R^2 = 0.33$, $p < 0.001$); it had no significant effect on the structure of the dormant and total community (Fig. S5.3). As moisture was the most important factor structuring both the active, the dormant and the total community, we evaluated the clustering, random and overdispersion patterns over the moisture regimes. The phylogenetic structure at deeper phylogenetic nodes (*e.g.,* Phyla, Class) of the microbial community (NRI) tended towards significant clustering at low water content (8%), whereas it became

random with a tendency to overdispersion (negative values) with the increase in moisture content (Fig. 5.5). The shift in phylogenetic clustering to random patterns along a gradient of soil moisture regimes was rather strong (Active, r = -0.66, $p < 0.001$; Dormant, r = - 0.77, $p < 0.001$; Total, r = 0.71, $p < 0.001$), with more than 88% of the community being significantly clustered at 8% water content and 14% at 23% moisture regime (Fig. 5.5). This implies that taxa in communities at low water content (8%) are on average more closely related than taxa in communities at high moisture regimes (Fig. 5.5). No community groups more distantly related than expected (overdispersion) were detected (Fig. 5.5).

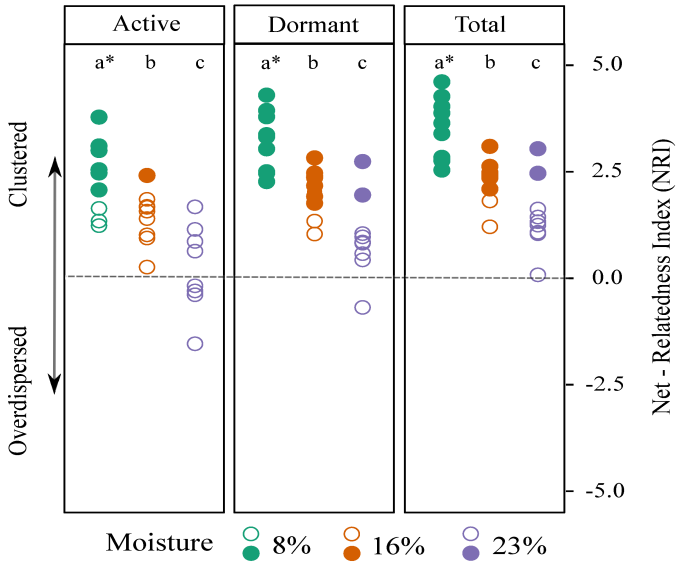

Fig. 5.5 Variation in phylogenetic structure along the moisture gradient as measured with Net Relatedness Index (NRI). Positive values indicate phylogenetic clustering, and negative values indicate phylogenetic overdispersion. Observed community structures unlikely to arise by chance (groups less distantly related than expected) are depicted by solid symbols. * Different letters indicate that NRI values differed from one moisture regime to another in phylogenetic structure ($p < 0.05$).

5.4 Discussion

Water content and temperature are two of the most important environmental factors regulating composition and activity of microbial communities in soils (Bell et al. 2008). However, our understanding of how microbial communities respond to these environmental factors is limited due the high heterogeneity of environmental conditions in the field which may mask the explicit influence of theses factors on microbial assemblages (Srivastava et al. 2004). Microcosm-based models offer a simplified system to study specific environmental factors in isolation or defined combinations so to provide an insight in the ecological rules shaping microbial communities without the interference of unwanted external factors (Ellis 2004; Jessup et al. 2004). However, microcosm conditions may not always accurately reflect the field conditions, resulting in oversimplification of natural community responses to environmental fluctuations in space and time (Coelho et al. 2013). The aim of this study was to increase our knowledge of the effect of winter and summer conditions on the bacterial and archaeal communities in a subtropical grassland by incubating soil cores at combinations of three moistures and three temperatures regimes mimicking the average climatic conditions at the Brazilian Pampa biome.

One should realize that characterization of active and dormant microbial communities based on a PCR approach is not without biases. Biases may be due to differential DNA and RNA extraction efficiencies, multiple rRNA operons, variations attributable to the cDNA tranformation (Stenström et al. 2001) and deviating levels of RNA in dormant cells (Rastogi et al. 2009; Blazewicz et al. 2013). Nevertheless, there is sufficient evidence that the rRNA:rDNA ratio is a suitable parameter for quantifying the potential activity of communities in soil and so to discriminate active and dormant fractions (DeAngelis & Firestone 2012; Jones & Lennon 2010; Campbell et al. 2011;

Blagodatskaya & Kuzyakov 2013). So, we used the 16S rRNA:rDNA ratio as a proxy to assess the active and dormant communities. Also the sequencing approach that we applied, *i.e.* Ion Torrent sequencing, is prone to errors. The difficulty in interpreting homopolymeric regions and failures in generating full-length reads may lead to inacurrate OTU identification and so to inadequate estimates of the composition of microbial communities (Salipante et al. 2014). However, stringent filtering and accurate clustering algorithms can help to limit the effects of sequencing errors (Edgar 2013; Pylro et al. 2014).

Although culture-independent studies of microbial communities have been carried out in grassland soil, this study is the first to have done so with the aim to compare the impact of environmental factors on the coexisting active and dormant fractions within a microbial community, and to infer community assembly processes which may differ between these communities in the context of seasonal variation in moisture and temperature regimes. The vast majority of the microbial community in our soils seems to be constantly dormant and only a relatively small portion of the community is active. Thus, it appears that, in soils under strong seasonal variations, as in subtropical environments, dormancy or the ability to enter in a low state of reduced metabolic activity is phylogenetically widespread and is used as a life history strategy for the majority of microorganisms (Jones & Lennon 2010). In general the soil environment seems to maintain the largest proportion of dormant cells among natural ecosystems, which is suggested to be a reason for the high levels of diversity within bacterial communities in soil (Torsvik et al. 1996; Lennon & Jones 2011), as it may prevent the extinction of microbial species at extreme environmental stress. Furthermore, specific environmental cues at local-scale, distinct ecophysiology and niche differentiation may contribute to control dormancy differently for each population, which explains the variable distribution of dormancy among different microbial lineages and the shifts in the ratio of rRNA to rDNA of individual members (Schostag et al. 2015).

Moisture was the most important factor, with respect to its influence on community diversity, structure and potential activity at the OTU level for active, dormant and total communities. Overall, our results are consistent with previous studies performed in environments such as sediments (Valverde et al. 2014) and forest soils (Bouskill et al. 2012) which showed that water content plays an important role in the composition and diversity of microbial communities over seasons. We did not measure the oxygen concentration, solute concentration and water potential (Ψ) which are directly related to shifts in soil moisture. Each of these factors has been shown to affect microbial community composition and are said to be predictors of microbial structure (Bouskill et al. 2012). So, we cannot rule out that one or more of these factors are also of importance for the structuring of the communities in our soils rather than moisture *per se*. Temperature has long been recognized to be a significant determinant for the composition and physiology of microorganisms at global scales (Lipson 2007; Ding et al. 2015). However, the results of this study showed that temperature appears to be of less importance at local scales, particularly in subtropical ecosystems where the community might contain a widely adaptive (*e.g.,* functional plasticity and dormancy) capacity to large variations in temperature (Jangid et al. 2011). Microbial communities from subtropical and temperate regions generally have a broad tolerance to large fluctuations in temperature compared with tropical (micro)organisms that are adapted to little seasonal variability (Hahn & Pockl 2005; Schindlbacher et al. 2011; Wallenstein & Hall 2011).

One of the main aims of this study was to assess the impact of seasonal changes on microbial community assembly processes and whether active and dormant communities are controlled by the same rules. Although phylogenetic turnover (β-diversity) provides information about the main factors promoting species shifts between communities, phylogenetic relatedness along environmental gradients helps to unravel the role of assembly processes and

environmental filtering (Jones & Hallin 2010; Wang et al. 2013). Interestingly, active and dormant communities behaved in a similar manner along moisture gradients and were controlled by the same assembly processes, despite being potentially different in the controlling biological traits and so in responses to environmental forces, as predicted in others reports (Baldrian et al. 2012; Zhang et al. 2014). Our results suggest that the relative influence of niche and neutral processes may vary along the moisture gradient, with niche-based mechanisms being more influential in communities under stress, *i.e.,* at dry conditions. At low water content, as in 8% moisture treatment (being close to permanent wilting point), which normally occurs in summer, phylogenetic clustering may be the rule, resulting in lower diversity and richness due to habitat selection (Zhou et al. 2002; Treves et al. 2003). It is likely that the greater environmental stress created by dry conditions filtered out those taxa that did not have the capabilities of conferring resistance against dry conditions (Evans & Wallenstein 2013). Hence, the phylogenetic clustering indicates that the community was able to persist under dry conditions, irrespective of their metabolic status, which may be the result of long-term evolutionary adaptation and *kin selection* to overcome harsh conditions caused by low water content (Placella et al. 2012; Arora 2013; Manzoni et al. 2014). On the other hand, at high water content (16% and 23% moisture) there is a decline in phylogenetic clustering with a tendency to overdispersion, indicating that the structuring of this community will be more influenced by neutral-based process. In the presence of abundant water supply, dispersion through connected water channels may cause random distribution of species creating more divergent community compositions (Chase 2007), while heterogeneities in substrate supply and water potential status may lead to distinct niches increasing the diversity and richness at these conditions and decreasing the importance of the habitat filtering (Valverde et al. 2014).

In conclusion, in this study we aimed to evaluate how seasonal

variations of moisture and temperature affect the coexisting active and dormant microbial communities and what the dominant assemblage rules are operating in these processes. We hypothesized that the active community will be more sensitive to changes in moisture and temperature than the dormant community is, and the latter community will be more determinative for the patterns observed in the total community. This hypothesis was justified by the data which showed that the active community is more affected by moisture as well as by temperature, whereas the dormant and the total community structure were affected solely by moisture. Although both fractions are determinative to the structure of the total community, the dormant community comprises an important source of microbial diversity, and better reflects to the diversity and structure patterns of the total community. Furthermore, we hypothesized that niche-based processes would become more important for microbial community assemblage at the extreme conditions examined here and that the active community is more influenced by niche-based process. We showed that active and dormant communities are controlled by the same rules and the relative influences of underlying assembly mechanisms are moisture-dependent, with niche-based mechanisms being more influential in communities under stress at dry conditions. This is an interesting result that may have a strong impact on the conceptualization in studies linking ecological processes to community assembly of active and dormant communities.

5.5 Supplementary Material

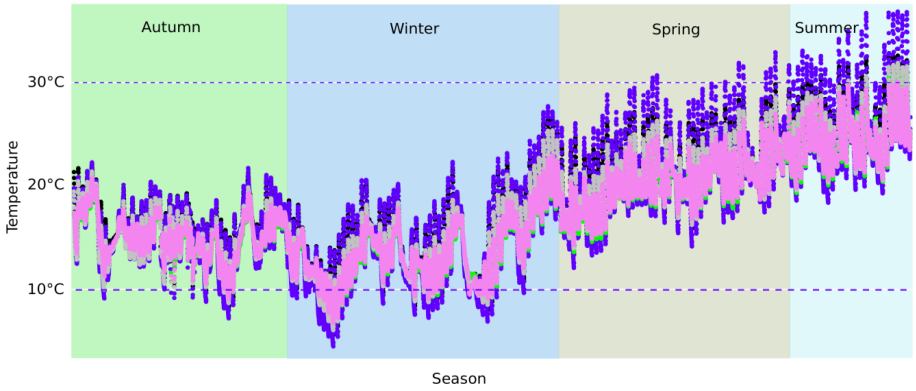

Fig. S5.1 Soil temperature variation over one year (from April/2014 to March/2015) every 30 minutes with thermopar (copper-constantan) sensors installed at top layer (0 - 5 cm) connected to a multiplexer (Campbell Scientific of AM25T ™) and the control and recording the values done by an electronic data logger (Logger). The horizontal blue lines indicate the upper and lower temperatures choose to set up the experiment.

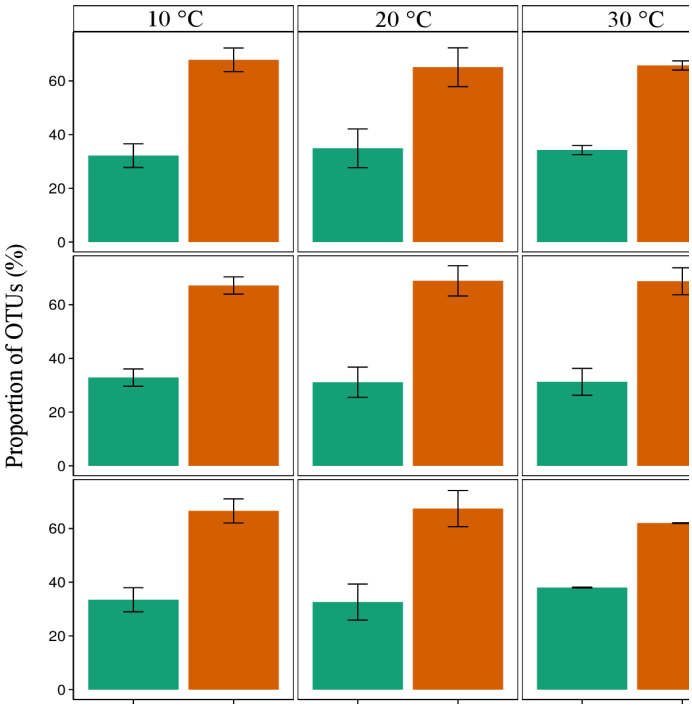

Fig. S5.2 Proportion of active and dormant communities across a gradient of soil moisture and temperature. Error bars represent the standard deviation (n = *3*).

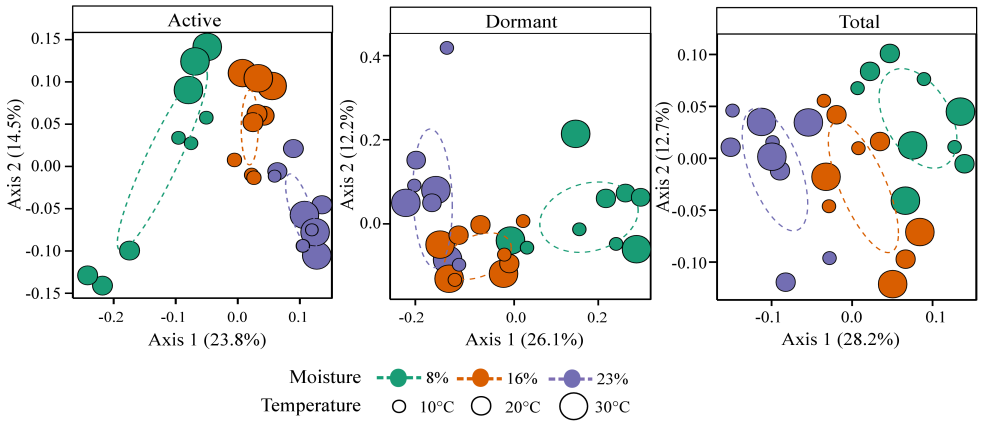

Fig. S5.3 Principal coordinates analysis (PcoA) ordination based on phylogenetic dissimilarity using unweighted UniFrac. Each point represents an individual sample, with colors indicating moistures and symbols indicating temperatures. The variations explained by the first two axes are shown. Centroid and color of each ellipse are defined by the distribution and standard deviation of points in the defined group representing 95% confidence intervals for the standard error of the average of scores for each moisture condition.

Table S5.1 Sample ID, treatment, total number of sequences and Good's coverage using DNA- and RNA-based approach

Sample ID	Treatment	Total number of sequences*	Good`s coverage**
DNA-based approach			
1	23% x 30°C	18806	0.94
2	8% x 20°C	16799	0.95
5	23% x 10°C	18955	0.96
6	23% x 30°C	17740	0.95
7	8% x 10°C	16737	0.95
9	23% x 30°C	19486	0.95
10	16% x 10°C	17968	0.95
11	16% x 20°C	17930	0.95
12	23% x 30°C	21271	0.95
13	23% x 10°C	21168	0.95
14	8% x 10°C	19648	0.95
16	16% x 10°C	22854	0.96
17	23% x 20°C	19078	0.95
18	23% x 10°C	19364	0.94
19	23% x 20°C	9615	0.92
20	8% x 30°C	9314	0.93
21	8% x 20°C	6813	0.91
23	8% x 30°C	15900	0.95
24	16% x 20°C	7480	0.90
28	16% x 30°C	8513	0.92
29	16% x 30°C	7496	0.91
30	8% x 10°C	7096	0.89
34	23% x 10°C	10308	0.92
36	23% x 20°C	8577	0.90
RNA-based approach			
37	23% x 30°C	9308	0.92
38	8% x 20°C	6835	0.89
40	23% x 20°C	14381	0.93

Sample ID	Treatment	Total number of sequences*	Good`s coverage**
41	16% x 10°C	11790	0.94
42	16% x 30°C	6908	0.90
43	8% x 10°C	10148	0.92
44	8% x 20°C	9820	0.92
45	23% x 30°C	12673	0.93
46	16% x 10°C	5578	0.88
47	16% x 20°C	8225	0.90
48	23% x 20°C	7729	0.89
49	23% x 10°C	12095	0.92
50	8% x 10°C	21194	0.95
52	16% x 10°C	30150	0.96
53	23% x 20°C	7370	0.88
54	23% x 10°C	14526	0.93
55	16% x 20°C	13932	0.94
56	8% x 30°C	6196	0.89
57	8% x 20°C	8294	0.91
59	8% x 30°C	9838	0.92
60	16% x 20°C	10452	0.91
62	8% x 30°C	6526	0.88
63	20%, 20°C	16164	0.94
64	16% x 30°C	10171	0.92
65	16% x 30°C	11155	0.94
66	8% x 10°C	4639	0.81
70	23% x 10°C	8290	0.91
72	23% x 20 °C	28090	0.96

* The sequences classified as Eukaryota Domain (similarity < 50%) and *Cyanobacteria* phylum were removed to calculate the number of sequences and Good's coverage.

** Good's coverage was calculated using the formula: (1 - (singletons/individuals)) × 100.

Table S5.2 Variation in proportion of active OTUs within phyla

Phylum	**Active proportion (%)**
Lentisphaerae	78.4 (26.5)[1]
Thaumarchaeota	55.5 (36.9)*
Caldiserica	42.0 (26.8)**
Aquificae	40.7 (48.1)
Proteobacteria	39.7 (8.0)
Armatimonadetes	39.4 (12.5)
SHA-109	38.8 (26.5)
Firmicutes	36.4 (18.1)
Bacteroidetes	34.7 (10.9)
WD272	33.2 (19.2)
Acidobacteria	32.4 (7.2)
Verrucomicrobia	31.17 (8.1)
BHI80-139	29.6 (46.5)*
Chloroflexi	28.9 (9.3)
Actinobacteria	28.5 (10.1)
Chlamydiae	27.9 (36.9)*
Planctomycetes	26.5 (10.9)
Elusimicrobia	23.9 (15.2)
TM6	22.4 (26.6)
Nitrospirae	21.1 (26.1)
Synergistetes	18.5 (24.6)
Fibrobacteres	17.9 (31.0)

Phylum	**Active proportion (%)**
Spirochaeta	17.9 (35.5)*
Gemmatimonadetes	17.8 (10.9)
Chlorobi	16.3 (28.5)
WCHB1-60	12.6 (21.0)
Candidate division OP3	12.1 (27.9)
Candidate division BRC1	11.6 (26.0)
Candidate division WS3	10.35 (21.7)
Candidate division TM7	7.7 (10.2)
Thermodesulfobacteria	1.8 (9.6)

[1] Values were calculated across all moisture and temperatures regimes and values between brackets represent the standard deviation ($n = 36$). * and ** represent proportion influenced ($p < 0.05$) by moisture and moisture x temperature, respectively. The temperature alone did not have an effect on the proportion of active community within phyla. Phyla considered as dormant (100% of the OTUs with rRNA:rDNA gene ratio < 1) were omitted here (they are: *Thermotogae*, *Euryarchaeota*, SBYG - 2791, Candidate division SR1, *Deferribacteres*, *Tenericutes*, BD1-5, *Deinococcus-Thermus*, NPL-UPA2, Candidate division KB1, Candidate division OP11 and Candidate division OD1).

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Chapter 6

Network topology reveals high connectance levels and few key microbial genera within soils

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Abstract

Microbes have a central role in soil global biogeochemical processes, yet specific microbe-microbe relationships are largely unknown. Analytical approaches such as network analysis may shed new light on understanding of microbial ecological processes. We investigated the soil bacterial community interactions through cultivation-independent methods in several land uses common in two Brazilian biomes. Using correlation network analysis we identified bacterial genera that presented important microbial associations within the soil community. The associations revealed non-randomly structured microbial communities and clusters of operational taxonomic units (OTUs) that reflected relevant bacterial relationships. Possible keystone genera were found in each soil. The more interactive genera were also more abundant but, within those genera, the abundance was not related to taxon importance as measured by the Betweenness Centrality (BC). Most of the soil bacterial genera were important to the overall connectance of the network, whereas only few genera played a key role as connectors, mainly belonged to phyla *Proteobacteria* and *Actinobacteria*. Finally it was observed that soils of each land use contained a different set of keystone genera. Taking into account that species interactions could be more important to soil processes than species richness and abundance, especially in complex ecosystems, this approach might represent a step forward in microbial ecology beyond the conventional studies microbial ecology.

Keywords

Microbial ecology; Network analysis approach; Keystone species; Microbe-microbe interactions

6.1 Introduction

Understanding the interactions among different taxa within a soil microbial community and their responses to environmental changes is a central goal in microbial ecology and important to better explore the complexity of soil processes. Soil microbial ecologists have borrowed several ecological theories from macroecology, including competitive strategies (Prosser et al. 2007) and biogeography (Griffiths et al. 2011). Most of the statistical techniques adapted to microbial systems have been used to test these theories, but they are focused on simple patterns of alpha and beta diversity to answer fundamental ecological questions (*e.g.,* to understand how different soil management types affect the bacterial community diversity and composition). On the other hand, interactions among associated taxa could contribute more to the understanding of ecosystem processes and functions than species diversity in soil environmental processes (Zhou et al. 2011).

Within a microbial community, interactions can be visualized as ecological networks, in which interactive taxa are linked together, either directly or indirectly through intermediate species. The study of network systems has received great attention in the last years, mainly as result of the increasing possibilities to obtain and analyze large data sets (Borgatti et al. 2009; Barthélemy 2004). These methods have been applied to the study of various biological contexts including the microbiota in human microbiome (Duran-Pinedo et al. 2011; Faust & Raes 2012), cancer (Choi et al. 2005), food webs (Estrada 2007), marine microbial community (Steele et al. 2011), and

recently this technique has been used to better understand soil microbial processes by examining complex interactions among microbes (Prasad et al. 2011). This approach can truly be applied to large soil microbial data sets offering new insights into the microbial community structure and the ecological rules guiding community assembly (Barberán et al. 2011).

The network analysis could be essential to explain several fundamental questions still unclear in microbial ecology. A good example is related to the presence or not of keystone species. The concept of keystone species was introduced in microbial ecology (Steele et al. 2011; Pester et al. 2010) and to date the identification of keystone taxa or populations is a critical issue in soil microbial ecology given the extreme complexity, high diversity, and uncultivated status of the larger portion of the community (Zhou et al. 2011). Keystone species are important to maintain the function of the microbial community and their extinction might lead to community fragmentation (Martín Gonzalez et al. 2010). Another important issue that network analysis could address is the importance of the abundance of taxa for supporting the structure and function of the soil microbial community. So far, most studies described in literature have focused on the dominant species in soil ecosystems (Campbell & Kirchman, 2013). However, low abundant taxa could be of particular importance for ecosystem functioning despite their low abundance and therefore some of them may be considered as keystones (Rafrafi et al. 2013).

In order to gain understanding on the organization of a complex microbial community, here we used correlation network analysis to study the soil microbial organization. Specifically we addressed the following questions: (i) Is it possible to detect keystone bacterial taxa in soils? (ii) If yes, are the keystone taxa exclusive to each land use or are they the same in most land uses? (iii) Are the most abundant taxa more important to connect distinct operational taxonomic units (OTUs) and maintain the structure of microbial

interactions in soil? To answer those questions we performed a large-scale pyrosequencing-based analysis of the 16S rRNA gene on replicate samples from two biomes in Brazil and implemented microbial ecological network analysis to examine how the microbial community members interact with each other and which members are important to support the microbial community structure.

6.2 Materials and Methods

6.2.1 Sampling sites and sample collection

To analyze soil bacterial community interactions, soil sampling were performed within two biomes in Brazil: one site was located within the Pampa biome which covers an area shared by Brazil, Argentina, and Uruguay in the southern of South America and is characterized by typical vegetation of native grassland, with sparse shrub and tree formations (Overbeck et al. 2007). The soils from this biome came from two sites. The first site is described in *Chapter 3* (site A - Lupatini et al., 2013) and the same data obtained from sequencing was re-analyzed here. Soil samples were collected in areas with four different land uses: natural pasture (30° 00′ 38.2″ S and 54° 50′ 17.4″ W) - currently used for grazing of cattle; native forest (30° 00′ 39.7″ S and 54° 50′ 05.6″ W) - used only for preservation of wildlife; soybean field (30° 00′ 40.3″ S and 54° 50′ 13.2″ W) - cultivated under no-tillage system on oat straw; 9-years-old *Acacia* tree plantation (*Acacia mearnsii* Willd.) (30° 00′ 27.5″ S and 54° 50′ 10.2″ W) (for more details about areas and sampling see Lupatini et al. (2013) - raw sequences were submitted to the NCBI Sequence Read Archive under the study number SRP013204, experiment number SRX255448). The second site is described in *Chapter 4*, and the same data was used. From this site, the soil samples were collected from a natural forest (30° 24′ 09.3″ S and 53° 52′ 59.1″ W) and 8-years-old pasture (30° 24′ 08.9″ S and 50° 53′ 05.9″ W) used for grazing of cattle (more details about areas and soil sampling in Suleiman et al.

(2013). The sequences were submitted to the NCBI Sequence Read Archive under the study number SRP013204, experiment number SRX148308 Composite samples (four sub-samples per sampling point) were collected during the spring of 2010 by taking 5 cm diameter, 0 - 5 cm depth cores. Equal masses of sub-samples removed from cores were pooled and mixed. Four biological repetitions were taken per each land use. DNA was isolated from at least 1 g of soil using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions.

The second sampling site was located within the Cerrado biome, which is a representative biome in central Brazil and the second largest biome in species diversity of South America. Cerrado has similar characteristics with Pampa biome, composed mostly by grasslands and small areas of forest formations (Oliveira & Marquis 2002). This ecosystem is considered an hotspot in the world, characterized by high diversity of endemic plants (Myers et al. 2000). Similar to Pampa biome, during the last decades Cerrado biome also undergoes processes of land-use changes and has been rapidly converted into pastures and agricultural lands (Carvalho et al. 2009), which made this biome to be one of the most threatened ecosystems in the world with more than 40% of the natural cover converted to other uses (Sano et al. 2009; Lapola et al. 2013). The soil sampling at Cerrado biome was carried out in a natural forest (19° 20′ 41″ S and 48° 00′ 58″ W); 20-years-old pasture used for grassing (19° 20′ 42″ S and 48° 05′ 22″ W); 15-years-old sugarcane field (19° 20′ 43″ S and 48° 05′ 49″ W); and *Pinus* plantation (19° 04′ 39″ S and 48° 10′ 19″ W) (for more details about areas and sampling see Rampelotto et al. (2013)). The sequences were submitted to the NCBI Sequence Read Archive under the study number SRP017965, experiment number SRX217724). Each soil sample was taken as a cut out measuring 30 × 20 × 5 cm (L × W × D). Four subsamples were collected randomly within this cut out and were passed through a 3.35 mm sieve. Genomic DNA was extracted from 250 mg of soil sample using Soil DNA

Isolation Kit (Norgen, Canada) as described by the manufacturer.

6.2.2 16S rRNA gene amplification and pyrosequencing

The 16S rRNA gene fragments were sequenced using 454 GS FLX Titanium (Lib-L) chemistry for unidirectional sequencing of the amplicon libraries. Barcoded primers allow for combining amplicons of multiple samples into one amplicon library and, furthermore, enable the computational separation of the samples after the sequencing run. Independent PCR reactions were performed for each soil sample to amplify the V1-V2 region (311 nucleotides) with the primers 27F and 338R. The primers were attached to the GS FLX Titanium Adaptor A-Key (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) and Adaptor B-Key (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′) sequences, modified for use with GS FLX Titanium Em PCR Kits (Lib-L) and a two-base linker sequence was inserted between the 454 adapter and the 16S rRNA primers to reduce any effect the composite primer might have on PCR efficiency. PCR reactions were carried out in triplicate with the GoTaq PCR core system (Promega, Madison, WI, USA). The mixtures contained 5 µl of 10x PCR buffer, 200 mM dNTPs, 100 mM of each primer, 2.5 U of Taq polymerase, and approximately 100 ng of DNA template in a final volume of 50 µl. The PCR conditions were 94°C for 2 min, 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min extension, followed by 72°C for 6 min. The PCR products were purified and combined in equimolar ratios with the quantitative DNA binding method (SequalPrep Kit, Invitrogen, Carlsbad, CA, USA) to create a DNA pool for pyrosequencing on a Roche GS-FLX 454 (Roche Applied Science, Branford, CT, USA).

6.2.3 Sequence processing and network analysis

The raw sequences were processed using mothur v.1.30.2 (Schloss et al. 2009). Briefly, the multiplexed reads were filtered for quality and assigned to corresponding soil samples. The filtering criteria removed any sequence, of which the longest homopolymer was greater than 8 nucleotides, contained ambiguous base call, had more than one mismatch to the barcode sequence, more than two mismatches to the primer sequence and was smaller than 200 bases in length. In addition, the sequences were trimmed by using a moving window of 50 bases long and average quality score higher than 30. The dataset was simplified by obtaining a non-redundant set of sequences that were further aligned against the SILVA reference alignment (http://www.arb-silva.de/). Finally, to reduce sequencing noise a pre-clustering step was applied (Huse et al. 2010) and the chimeric sequences were checked by chimera.slayer. For network analysis, the OTUs were grouped at genus level and only those genera with more than five sequences were considered in the following analysis. The choice for the genus level aimed to generate consistent OTUs for subsequent analyses based on correlations. This approach also circumvents the potential taxonomic misclassification due to sequencing bias. Since the networks comprised a set of shared taxa within a soil, the bacterial genera represented by zero sequences in a sample were excluded from data analysis.

Associations between the microbial communities were examined by calculating all possible Pearson rank correlations between bacterial genera. A valid interaction event was considered to be a robust correlation if the Pearson correlation coefficient (r) was either equal or greater than 0.9 or - 0.9 and statistically significant *p*-value (equal or smaller than 0.05 - calculated as the proportion of the r-values generated from randomized data larger than the Pearson correlation coefficient that was calculated from the original data). To describe the topology of the resulting networks, a set of measures (average

clustering coefficient, average path length, and modularity) were calculated (Newman 2006). The network structure was explored and visualized with the interactive platform Gephi (Bastian et al. 2009) using directed network (where edges have direction) and Fruchterman - Reingold layout.

To determine whether our webs were not random networks and really represented the actual bacterial interactions in soil, we compared random networks of equal size (same number of nodes and edges) to the networks obtained by this study. One thousand random networks were calculated by the Erdös-Rényi model and from each random network, values of average clustering coefficient, average path length and modularity were calculated. This approach is based on using a fixed number of links to connect randomly chosen nodes and serves as point of reference against which our real biological networks might be compared (Vick-Majors et al. 2014). The proportion of those values larger than values calculated based on the original data were computed to get a p-value for the null hypothesis that the networks were obtained at random. To measure the relative importance (how influential a taxon is within a network) of each taxon within the network we calculated two measures of centrality: Betweenness Centrality (BC) and Closeness Centrality (CC) (Martín Gonzalez et al. 2010; Freeman 1978). BC counts the fraction of shortest paths going through a given bacterial taxon to another. The BC of a taxon in a network reflects the importance of control that the taxon exerts over the interactions of other group of taxa in the network (Vick-Majors et al. 2014). CC denotes the proximity of a node to all other nodes in the network quantifying how many steps away genus *i* is from all others in the web. Taxa with high CC are likely to have a pronounced effect on microbial community because it can rapidly affect other species in a community (Martín Gonzalez et al. 2010). Finally, to identify possible patterns between taxon abundance vs. CC or BC we use dispersion graphs to describe the relationship between these pair of variables.

6.3 Results

The number of high-quality sequences obtained after sequence processing in each sample and the sequence coverage is presented in Table S6.1. An average of 12,164 sequences (≥ 200 bases and ≥ 30 quality score) were obtained per sample. The smallest sequence coverage at the genus level was 94% however most of the samples presented a sequence coverage of 99%. The coverage indicated that the number of sequences obtained from each soil sample was sufficient to reveal most of the taxonomic units at genus level.

Based on the global network statistics presented in Table 6.1 and irrespective of the biome or land use studied only a small portion of OTUs (9.16 to 21.8%) showed positive or negative interactions with other members of the soil bacterial community. Those interactive OTUs were the most abundant ones making up about 68 to 92% of the total number of OTUs found in the soils. The proportion of positive correlations were variable according to the land use and ranged from 22.3% (soybean field from Pampa biome) to 54.7% (sugarcane field from Cerrado). On average, the number of negative correlations was higher than the number of positive correlations in most land uses tested (Table 6.1).

Based on the high BC score few possible keystone taxa were detected (Fig. 6.1A - 6.5A). The OTUs considered keystone species (depicted as nodes with larger sizes in the network) mainly belonged to different genera of the phyla *Proteobacteria* and *Actinobacteria*. OTUs belonging to *Chloroflexi*, *Bacteroidetes* and *Firmicutes* were also characterized as keystone taxa. These keystone taxa were not the same among or within biomes and appeared to be unique to each sampling location. The five keystone genera selected by the greatest value of BC from each of the soil sites are presented on Table 6.2. Based on the CC ranking, a larger number of OTUs were identified as highly important (high CC) for connectance of the microbial network since the values

of CC did not present a high variation among the OTUs. No keystone genera were detected by this measurement denoting similar proximity of all genera within the network (Fig. 6.1B - 6.5B).

Table 6.1 Global network statistics for microbial association networks from land uses in Pampa and Cerrado biome

Biome	**Pampa**						**Cerrado**			
	Site A				***Site B***					
Land use	***Acacia* plantation**	**Soybean field**	**Natural forest**	**Natural pasture**	**Natural forest**	**Natural pasture**	**Sugarcane field**	***Pinus* plantation**	**Natural forest**	**Pasture**
Total number of OTUs[1]	724	611	807	780	912	900	755	629	714	748
Number of nodes or OTUs[2]	107(14.8*)	56(9.16)	154(19.1)	146(17.7)	197(21.6)	197(21.8)	107(14.2)	70(11.1)	121(16.9)	84(11.2)
Total N°. of significant correlations	718	148	1499	1048	2715	2389	1730	703	2257	1079
No. of significant positive correlations	353 (49.2**)	33(22.3)	586(39.1)	535(51.1)	1030(37.9)	1117(46.7)	946(54.7)	296(42.1)	817(36.2)	426(39.5)
No. of significant negative correlations	365(50.8)	115(77.7)	913(60.9)	513(48.9)	1685(62.1)	1272(53.3)	784(45.3)	407(57.9)	1440(63.8)	653(60.5)
Avg. Clustering Coefficient	0.30	0.27	0.31	0.29	0.31	0.31	0.38	0.40	0.38	0.38
Random Avg. Clustering Coefficient	0.12	0.11	0.12	0.11	0.14	0.12	0.304	0.29	0.31	0.31
Avg. Path Length	2.42	1.88	2.68	2.75	2.72	2.75	2.226	2.15	2.13	2.13
Random Avg. Path Length	2.03	2.55	2.01	2.06	1.87	0.12	1.695	1.71	1.69	1.69
Modularity	0.48	0.63	0.55	0.61	0.49	0.52	0.439	0.53	0.44	0.5
Random Modularity	0.21	0.34	0.52	0.53	1.51	1.92	1.111	0.14	0.11	0.12

[1] All OTUs presented in soil samples in Pampa and Cerrado biomes. [2] Only the OTUs selected by $r \geq \pm 0.9$, $p < 0.05$.* The numbers in parentheses indicate the percentage of OTUs that show interactions in relation to total number of OTUs found in land uses or ** in relation to total number of significant correlations between OTUs in a network.

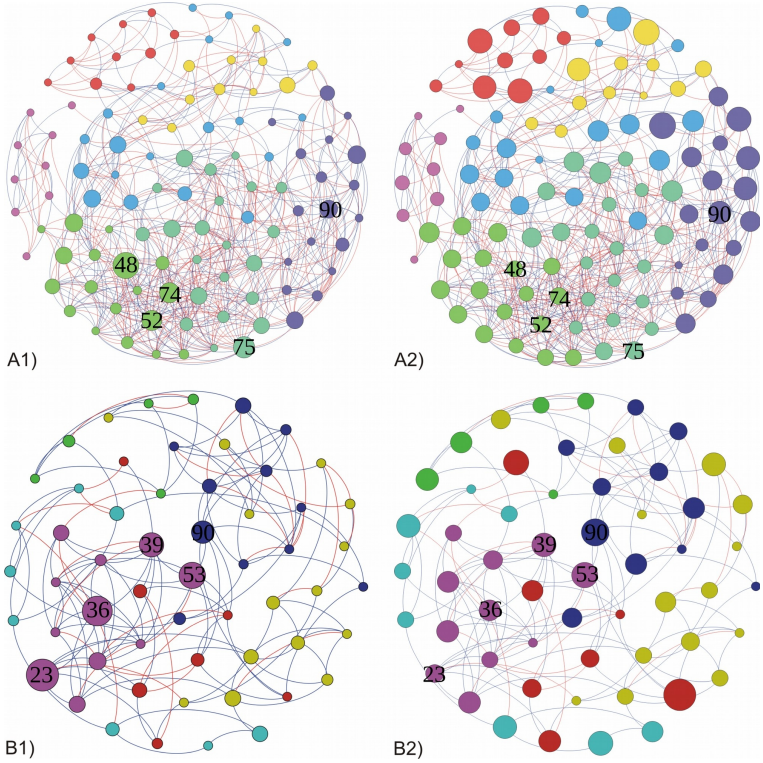

Fig. 6.1 Network interactions of soil bacterial genera found in *Acacia* plantation (A1, A2) and in the Soybean plantation (B1, B2) from site A on Pampa biome. A connection stands for a strong Pearson's correlation ($r \geq \pm 0.9$ and p-value ≤ 0.05). Each circle (usually called node) represents a bacterial genus and the sizes of the circles are proportional to the value of betweenness centrality in (A1) and (B1). In (A2) and (B2) the sizes of the circles are proportional to value of closeness centrality. Lines connecting two bacterial genera represent the interactions between them. Blue lines represent the positive correlations and red lines represent negative significant correlations. The colors of the circles represent the bacterial modules. For clarity, the OTU's identity was omitted and only key nodes were depicted by the OTU number.

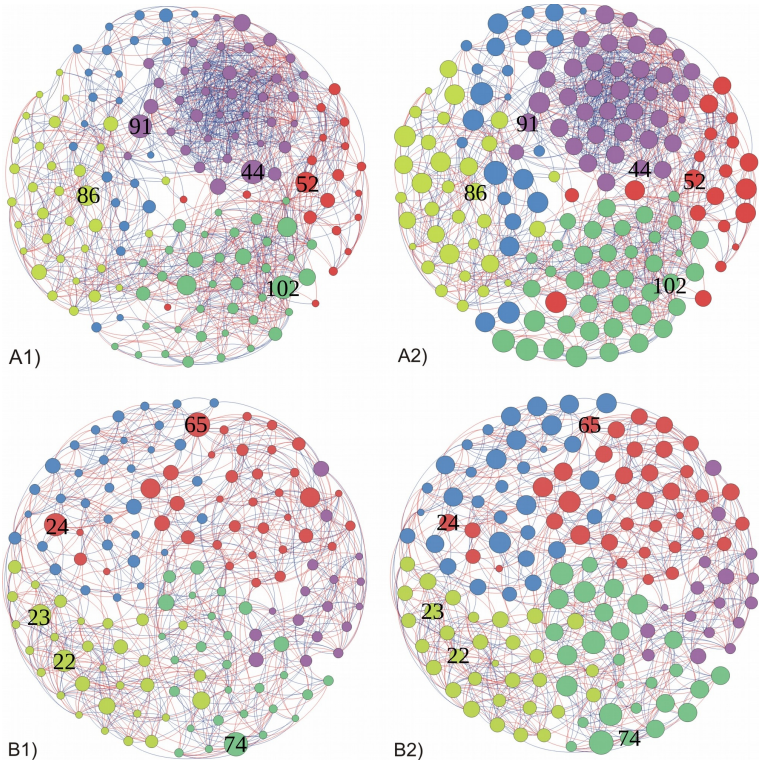

Fig. 6.2 Network interactions of soil bacterial genera found in the Natural forest (A1, A2) and in the Natural pasture (B1, B2) from site A on Pampa biome. A connection stands for a strong Pearson's correlation ($r \geq \pm 0.9$ and p-value ≤ 0.05). Each circle (usually called node) represents a bacterial genus and the sizes of the circles are proportional to the value of betweenness centrality in (A1) and (B1). In (A2) and (B2) the sizes of the circles are proportional to value of closeness centrality. Lines connecting two bacterial genera represent the interactions between them. Blue lines represent the positive correlations and red lines represent negative significant correlations. The colors of the circles represent the bacterial modules. For clarity, the OTU's identity was omitted and only key nodes were depicted by the OTU number.

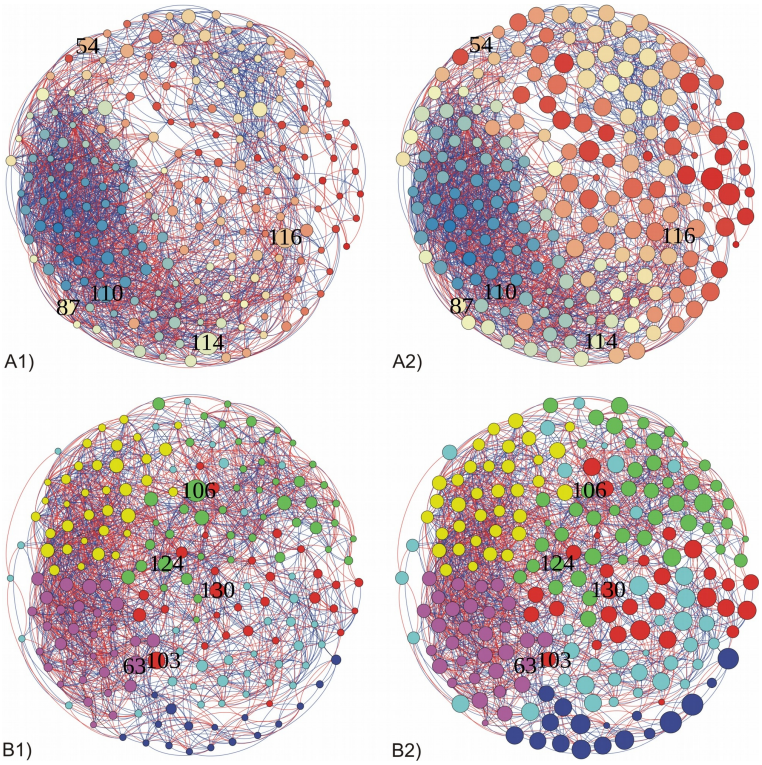

Fig. 6.3 Network interactions of soil bacterial genera found in Natural forest (A1, A2) and in the Natural pasture (B1, B2) from site B, on Pampa biome. A connection stands for a strong Pearson's correlation ($r \geq \pm 0.9$ and p-value ≤ 0.05). Each circle (usually called node) represents a bacterial genus and the sizes of the circles are proportional to the value of betweenness centrality in (A1) and (B1). In (A2) and (B2) the sizes of the circles are proportional to value of closeness centrality. Lines connecting two bacterial genera represent the interactions between them. Blue lines represent the positive correlations and red lines represent negative significant correlations. The colors of the circles represent the bacterial modules. For clarity, the OTU's identity was omitted and only key nodes were depicted by the OTU number.

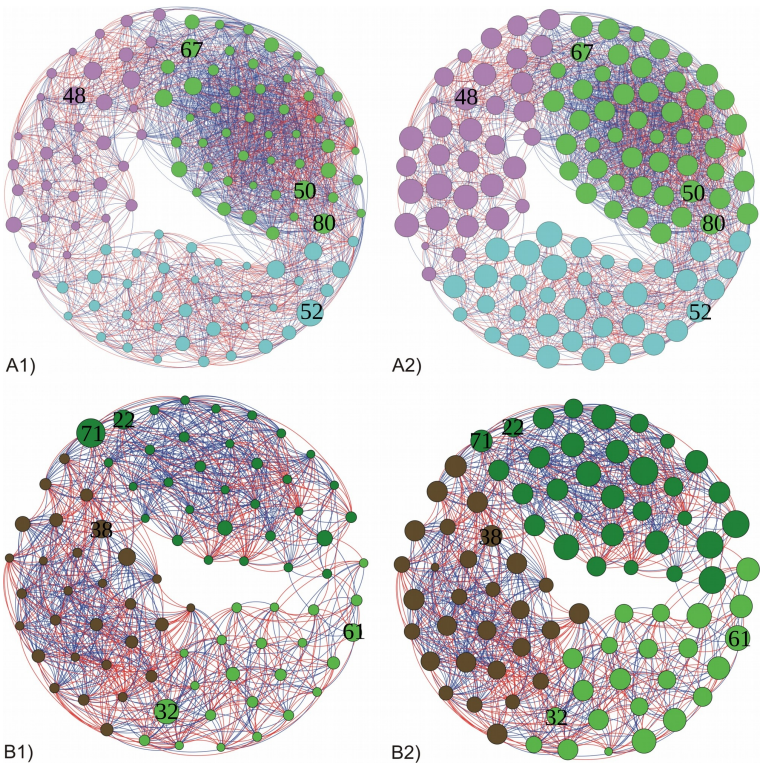

Fig. 6.4 Network interactions of soil bacterial genera found in Natural forest (A1, A2) and in the Pasture (B1, B2) on Cerrado biome. A connection stands for a strong Pearson's correlation ($r \geq \pm 0.9$ and p-value ≤ 0.05). Each circle (usually called node) represents a bacterial genus and the sizes of the circles are proportional to the value of betweenness centrality in (A1) and (B1). In (A2) and (B2) the sizes of the circles are proportional to value of closeness centrality. Lines connecting two bacterial genera represent the interactions between them. Blue lines represent the positive correlations and red lines represent negative significant correlations. The colors of the circles represent the bacterial modules. For clarity, the OTU's identity was omitted and only key nodes were depicted by the OTU number.

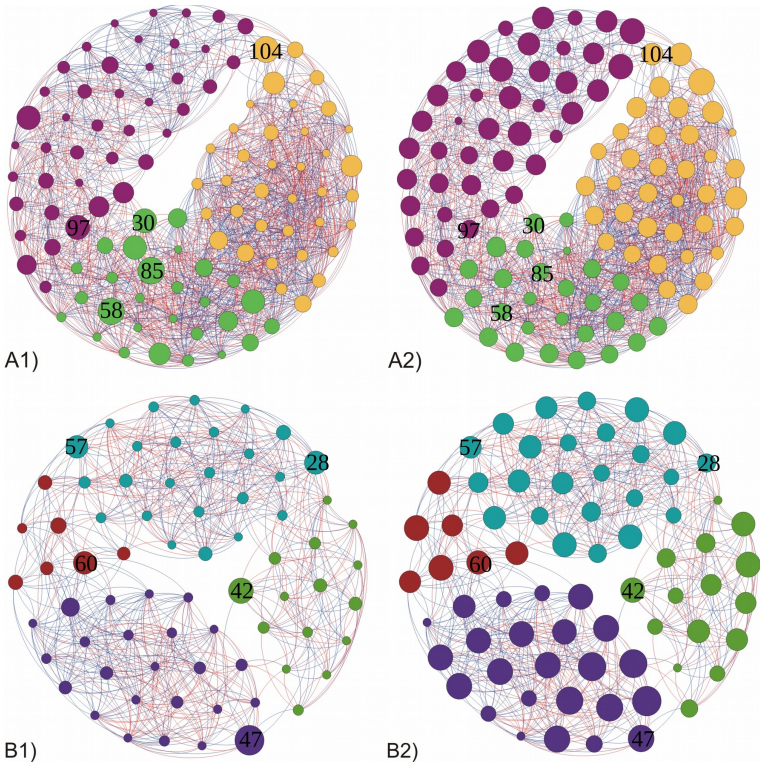

Fig. 6.5 Network interactions of soil bacterial genera found in Sugarcane (A1, A2) and in the *Pinus* plantation (B1, B2) on Cerrado biome. A connection stands for a strong Pearson's correlation ($r \geq \pm 0.9$ and p-value ≤ 0.05). Each circle (usually called node) represents a bacterial genus and the sizes of the circles are proportional to the value of betweenness centrality in (A1) and (B1). In (A2) and (B2) the sizes of the circles are proportional to value of closeness centrality. Lines connecting two bacterial genera represent the interactions between them. Blue lines represent the positive correlations and red lines represent negative significant correlations. The colors of the circles represent the bacterial modules. For clarity, the OTU's identity was omitted and only key nodes were depicted by the OTU number.

Table 6.2 The five genera selected by the greatest values of Betweenness Centrality (BC) found in each of the sampling sites. Values of closeness centrality (CC), relative abundance and taxonomic informations are also showed on the table

Id	BC	CC	Relative Abundance (%)	Taxonomy
PAMPA BIOME - SITE A				
Acacia plantation				
Otu048	167.86	1.74	0.41	*Firmicutes;Clostridia; Thermoanaerobacterales; Thermoanaerobacteraceae; Desulfovirgula*
Otu075	124.91	2.07	0.28	*Proteobacteria;Alphaproteobacteria; Rhizobiales;Bradyrhizobiaceae; Agromonas*
Otu074	113.81	1.93	0.28	*Proteobacteria;Gammaproteobacteria; Xanthomonadales;Xanthomonadaceae; Luteimonas*
Otu052	113.40	1.70	0.39	*Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Segetibacter*
Otu090	94.85	3.11	0.22	*Actinobacteria;Actinobacteria; Actinomycetales;Streptosporangiaceae; Thermopolyspora*
Soybean Field				
Otu023	31.83	1.25	0.87	*Acidobacteria;Acidobacteria_Gp7; unclassified;unclassified;unclassified*
Otu036	28.67	1.67	0.46	*Proteobacteria;Alphaproteobacteria; Rhizobiales;Beijerinckiaceae; Methylocapsa*
Otu053	23.83	2.33	0.34	*Proteobacteria;Betaproteobacteria; Methylophilales;Methylophilaceae; Methylotenera*
Otu039	21.00	2.08	0.42	*Proteobacteria;Gammaproteobacteria; Xanthomonadales;Xanthomonadaceae; Luteimonas*
Otu090	18.50	2.57	0.20	*Proteobacteria;Alphaproteobacteria; Rhodospirillales;Acetobacteraceae; Acidicaldus*

Id	BC	CC	Relative Abundance (%)	Taxonomy
Natural Forest				
Otu044	373.99	2.14	0.49	*Proteobacteria;Alphaproteobacteria; Rhodospirillales;Rhodospirillaceae; Caenispirillum*
Otu091	360.95	2.64	0.22	*Actinobacteria;Actinobacteria; Actinomycetales;Nocardioidaceae; Pimelobacter*
Otu102	360.62	2.39	0.18	*Firmicutes;Clostridia;Clostridiales; Lachnospiraceae;Catonella*
Otu052	305.71	2.44	0.41	*Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Terrimonas*
Otu086	291.67	2.39	0.23	*Actinobacteria;Actinobacteria; Actinomycetales;Geodermatophilaceae; Blastococcus*
Natural pasture				
Otu065	253.60	2.79	0.31	*Proteobacteria;Gammaproteobacteria; Thiotrichales;Piscirickettsiaceae; Sulfurivirga*
Otu074	246.91	2.28	0.26	*Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Lacibacter*
Otu024	232.90	2.77	0.89	*Firmicutes;Clostridia;Clostridiales; Ruminococcaceae;Ethanoligenens*
Otu022	218.44	2.00	0.92	*Thermodesulfobacteria; Thermodesulfobacteriales; Thermodesulfobacteriaceae; Caldimicrobium*
Otu023	210.93	2.56	0.90	*Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Terrimonas*
PAMPA BIOME - SITE B				
Natural Forest				
Otu114	756.52	2.34	0.17	*Actinobacteria;Actinobacteria; Acidimicrobiales;Acidimicrobiaceae; Ferrimicrobium*
Otu116	630.83	2.82	0.16	*Proteobacteria;Betaproteobacteria; Burkholderiales; Burkholderiales_incertae_sedis;*

Id	BC	CC	Relative Abundance (%)	Taxonomy
Otu110	479.81	1.91	0.17	*Thiomonas*
Otu054	446.16	2.26	0.34	*Actinobacteria;Actinobacteria; Actinomycetales;Micromonosporaceae; Asanoa*
Otu087	444.01	2.44	0.21	*Acidobacteria;Acidobacteria_Gp22; unclassified;unclassified;unclassified*
				Acidobacteria;Acidobacteria_Gp11; unclassified;unclassified;unclassified
Natural Pasture				
Otu106	580.47	2.24	0.34	*Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Flavisolibacter*
Otu063	437.57	2.66	0.30	*Actinobacteria;Actinobacteria; Actinomycetales;Microbacteriaceae; Microterricola*
Otu103	403.97	2.27	0.19	*Proteobacteria;Gammaproteobacteria; Chromatiales;Chromatiaceae; Nitrosococcus*
Otu124	332.86	2.05	0.14	*Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Lacibacter*
Otu130	330.74	2.00	0.13	*Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Segetibacter*
CERRADO BIOME				
Sugarcane Field				
Otu085	182.27	1.72	0.23	*Proteobacteria;Betaproteobacteria; Burkholderiales;Oxalobacteraceae; Undibacterium*
Otu058	179.22	1.74	0.35	*Proteobacteria;Betaproteobacteria; Burkholderiales;Comamonadaceae; Rhodoferax*
Otu104	172.96	2.65	0.18	*Actinobacteria;Actinobacteria; Actinomycetales;Sporichthyaceae; Sporichthya*
Otu097	157.86	1.96	0.18	*Proteobacteria;Alphaproteobacteria; Caulobacterales;Caulobacteraceae; Caulobacter*
Otu030	155.65	1.86	0.59	*Actinobacteria;Actinobacteria;*

Id	BC	CC	Relative Abundance (%)	Taxonomy
				Actinomycetales;Kineosporiaceae; Kineosporia
Pinus Plantation				
Otu047	180.34	2.52	0.39	*Actinobacteria;Actinobacteria; Actinomycetales;Sporichthyaceae; Sporichthya*
Otu042	142.37	2.24	0.47	*Proteobacteria;Betaproteobacteria; Burkholderiales;Oxalobacteraceae; Duganella*
Otu028	124.23	1.40	0.71	*Bacteroidetes;Sphingobacteria; Sphingobacteriales;Chitinophagaceae; Terrimonas*
Otu060	120.65	2.09	0.28	*Proteobacteria;Alphaproteobacteria_order_incertae_sedis; Alphaproteobacteria_incertae_sedis; Elioraea*
Otu057	119.63	1.87	0.30	*Proteobacteria;Betaproteobacteria; Burkholderiales;Oxalobacteraceae; Janthinobacterium*
Natural Forest				
Otu052	276.09	2.17	0.24	*Actinobacteria;Actinobacteria; Acidimicrobiales;Acidimicrobiaceae; Acidimicrobium*
Otu080	180.84	2.03	0.13	*Actinobacteria;Actinobacteria; Actinomycetales;Acidothermaceae; Acidothermus*
Otu050	172.54	2.26	0.25	*Proteobacteria;Alphaproteobacteria; Rhizobiales;Beijerinckiaceae; Methylovirgula*
Otu067	159.45	1.85	0.18	*Proteobacteria;Alphaproteobacteria_order_incertae_sedis; Alphaproteobacteria_incertae_sedis; Elioraea*
Otu048	156.83	2.36	0.27	*Acidobacteria;Acidobacteria_Gp4; unclassified;unclassified;unclassified*
Natural Pasture				
Otu071	259.25	2.20	0.21	*Planctomycetes;Planctomycetacia; Planctomycetales;Planctomycetaceae; Zavarzinella*

Id	BC	CC	Relative Abundance (%)	Taxonomy
Otu032	208.29	1.47	0.50	*Bacteroidetes;Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae;Mucilaginibacter*
Otu022	139.44	1.70	1.02	*Proteobacteria;Betaproteobacteria; Burkholderiales; Burkholderiales_incertae_sedis; Methylibium*
Otu038	121.18	2.07	0.37	*Proteobacteria;Gammaproteobaceria; Oceanospirillales;Litoricolaceae; Litoricola*
Otu061	120.52	2.68	0.25	*Proteobacteria;Alphaproteobacteria; Rhodospirillales;Acetobacteraceae; Rhodovarius*

Studies in soil microbial ecology suggest that abundant microorganisms might have high impact on microbial structure and function. To understand how taxon abundance and the centrality measures are related, a dispersion graph with the relative abundance of all OTUs *vs*. the values of betweenness and closeness was constructed (Fig. 6.6). Despite the abundance of genera seems to be an important parameter that defines the interactions between taxonomic members of the soil bacterial community, the diagrams indicates that there is no strong relation between taxon abundance and centrality measures. However, it is possible to note that few abundant taxa presented a slight tendency to have high values of CC (Fig. 6.6B).

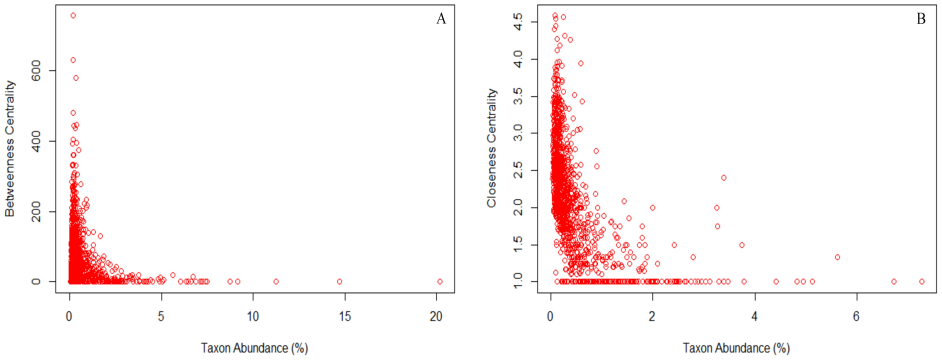

Fig. 6.6 Relationship between taxon relative abundance from the total number of OTUs at genus level found in different land uses in Pampa and Cerrado biome and betweenness (A) and closeness (B) centrality.

6.4 Discussion

In this study, we focused on microbial community associations within two ecologically important biomes in Brazil. We collected soil samples from a set of biological replicates, allowing us to detect patterns on ecological interactions using network analyses, which describe who is present and who affects whom positively or negatively. Positive correlations between microbial populations suggest the occurrence of a mutualistic interaction while negative correlations might suggest the presence of competition for hosts or predatory relationships between microorganisms (Steele et al. 2011). Those interactions are strongly linked to important soil processes. For instance, a mutualistic relationship between ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) is essential to the stability of the nitrification process, a key reaction of the global nitrogen cycle (Graham et al. 2007). On the other hand, species of *Myxobacteria* are a group of micropredator bacteria metabolically

active in the soil ecosystems that play a key role in the turnover of carbon (Lueders et al. 2006).

Every approach presents positive and negative aspects. Before continuing the discussion, it is appropriate to consider some limitations of this work in order to better interpret the results: (i) unlike other studies, only correlations with $r \geq \pm 0.9$ ($p \leq 0.05$) were used to generate the networks. According to Taylor (1990) the correlation coefficient (a linear association between two variables) is an abstract measure and does not give a direct precise interpretation. Low values of r do not explain or account for significant variation in the value of the dependent variable. Conservative cutoffs increase the confidence of detecting only strong interactions; less stringent cutoffs decrease the reliability of the results; (ii) PCR-based and massive sequencing techniques introduce biases related to primer mismatches, insertion/deletion (indels) sequencing errors, and chimeric PCR artifacts which can affect the interpretations of microbial community structure and diversity (Pinto and Raskin, 2012); (iii) the copy number of the 16S rRNA gene varies greatly per bacterial genome (from one in many species up to 15 in some bacteria) and these differences induce errors in relative abundance measurements (Klappenbach 2001); (iv) the network analysis is considered an OTU-based approach since it relies on detection of correlation between taxonomic unities. According to Lemos et al. (2011), in order to apply such an approach, a large sampling intensity (coverage ≥ 90%) is needed to get reliable results. Datasets with low number of sequences are likely to present a low sequence coverage that in turn will make it more unlikely to find OTU correlations; (v) finally, another drawback related to microbial network constructions is the faulty prediction of a relationship between two taxa since interspecies interactions might be affected by third-party organisms in prokaryotic ecosystems (Haruta et al. 2009). Within this study, we attempted to overcome these biases as much as possible. Although those biases may not be neglected, considering the high

levels of robustness and resolution of our methodology, the low variation among replicates from each land use and the quality of the results, we believe these biases were minimized.

Linking the structure of microbial communities to soil ecosystem functioning has been a challenge in ecology. The extent, specificity, and stability of microbial associations are difficult to assess systematically in the environment (Chaffron et al. 2010). However, co-occurrence network analysis (primarily based on statistically significant tests of correlation) were successfully applied to at least partially solve this problem (Barberán et al. 2011; Faust & Raes 2012; Friedman & Alm 2012; Gilbert et al. 2012; Rodriguez-Lanetty et al. 2013). Studies on ecosystem functioning are traditionally limited to measurements of changes in species diversity and composition limiting our ability to link the structure of communities to the function of natural ecosystems (Philippot et al. 2013; Rudolf & Rasmussen 2013). An important benefit of networks to study microbial ecology is the ability to understand which organisms are most important in maintaining the structure and interactions of microbial communities in soils. Due to the choice of a linear model to describe how the taxa of a soil microbial community interact with each other, the network analysis allows only for the detection of positive and/or negative interactions. While we acknowledge that not all correlations between bacterial genera found in this study might be valid, empirical evidence that correlated microbial species might actually be interactive was already demonstrated. Duran-Pinedo et al. (2011) provided an important evidence of accuracy and usefulness of this kind of analysis by isolating a not-yet-cultivated organism based on the network analysis results. The authors showed that network analysis could facilitate the cultivation of a previously uncultivated organism (*Tannerella* sp. OT286) and proved that certain species that did not grow in artificial media alone could form colonies in the presence of other microorganisms. Due to the limitations of this approach,

here we adopted the term "theoretical" network association to express the positive and/or negative interactions between soil microbial genera (for an extensive revision about the difficulties and pitfalls about the use of network inference to assess microbial interactions see Faust & Raes (2012)). The application of theoretical network modeling to microbial ecological networks provide insight into the complex organization levels of microbes and identify key microbial populations or key functional genes in soil ecosystems. Using theoretical network modeling, it was shown that the structure of the networks under ambient and elevated CO_2 levels was substantially different in terms of network topology, node overlap, module preservation, and network hubs, suggesting that the network interactions among different phylogenetic groups/populations were markedly changed (Zhou et al. 2011).

In this study we attempted to answer three fundamental questions: (i) Is it possible to detect keystone bacterial taxa in soils? (ii) If yes, are the keystone taxa exclusive to each land use or are they the same in most land uses? (iii) Are the most abundant taxa more important to connect distinct OTUs and to maintain the structure of microbial interactions in soil? We have shown that network approach can indeed identify putative keystone within a microbial community and each land use contained a different set of keystone genera. The more interactive genera were more abundant within a community but, within those genera, the abundance was not necessarily related to taxon importance. Many approaches attempted to detect different aspects of network topology and thus provided different information for better understanding how the microbial communities are arranged in the soil. The effective center (or centers) of a network, also called "hubs" might represent keystones species as predicted from network theory (Montoya et al. 2006). However, the network structure is very complex and there is no unifying approach for identifying such hubs. A number of studies have been performed using the degree centrality to identify hubs in networks but we decided to use BC and CC because the degree

is a local quantity which does not explain the importance of a node in the network (Barthélemy 2004). Our analysis of centrality illustrates that most of soil bacterial taxa are important to the overall connectance of the network (presented high CC), whereas only few taxa play a key role as connectors (presented high BC). Collectively, the impact of these keys species on total community is shaped by the repertoire of interactions with other members, with one important observation: if these highly connected nodes are lost, the network - representing a picture of the association patterns resembling inter-dependencies within members in a community - would change dramatically. (Montoya et al., 2006). Eiler et al. (2012) also detected numerous phylogenetic groups with high number of associations, which may represent groups with particular strong interdependencies. They suggested that in a highly complex environment, like soil, there may be hundreds of such keystones species, which play an exceptionally important role in determining the structure and function of ecosystems. Rudolf and Rasmussen (2013) showed that differences in food network structure were significantly correlated with changes in all ecosystem processes.

The most widely used definition for keystone species is one "whose impact on its community or ecosystem is large, and disproportionately large relative to its abundance" (Power et al. 1996). According to our network analysis, only a fraction of the total number of OTUs presented either positive or negative interactions (Table 6.1) and the more interactive taxa were also found in larger abundance within the soil samples. On the other hand, the abundance of the interactive taxa did not present any relationship with the two measures of centrality applied in this study (see Fig. 6.6). Recently, Campbell and Kirchman (2013) and Zhang et al. (2013) suggested that abundant and easily detectable organisms might have a high impact on microbial structure, function, and nutrient cycling. Our network analysis corroborated such findings, but, the role of less-abundant organisms is not easily understood and

might not be neglected. Less abundant members of the soil microbial community may contribute significantly to biogeochemical processes participating in key processes such as sulfate reduction and nitrification (Pester et al. 2010; Nieder & Benbi 2008). In addition, these rare or less abundant microorganisms might act as keystone species in complex soil bacterial communities and could serve as a reservoir of genetic and functional diversity and buffer ecosystems against species loss or environmental change (Brown et al. 2009). Finally, it was observed that each land use presented a different set of keystone genera.

In this study, we investigated the microbe associations in complex microbial soil ecosystems applying systems biology principles. Such approach is essential to explain the persistence of microbial species in constantly changing ecosystems, and the tolerance of current ecosystems to natural gains and losses of species as well as their vulnerability to unnaturally inflated extinction rates (Montoya et al. 2006). The visualization of microbial networks allowed us to detect microbial hubs, which are key microbes that let us comprehend the complex microbial systems in which they are found. Ultimately, such network models will be able to predict the outcome of community alterations and the effects of perturbations. Although exploring such ecological networks is essential to a better understanding of microbial ecology, more investigations are needed to circumvent important methodological limitations such as prediction of a relationship between two genera through inference of correlations. The technique will benefit from the incorporation of a less simplistic model that take into account not only the relationship between two microbial genera but also the effect of third-party microorganisms in the system and random processes. The network approach proves to be valuable to practical community-level conservation biology and represents a step forward in microbial ecology beyond the conventional studies of microbial richness and abundance.

6.5 Supplementary Material

Table S6.1 Total number of high-quality sequences and sequencing coverage for taxonomic genus level in land uses in Pampa and Cerrado biomes

Land use/biome	Total sequences	Coverage genus level
Pampa biome		
Site A		
Acacia plantation 1	8380	0.98
Acacia plantation 2	9083	0.98
Acacia plantation 3	7802	0.98
Acacia pantation 4	7327	0.98
Natural forest 1	9262	0.98
Natural forest 2	12684	0.99
Natural forest 3	9083	0.98
Natural forest 4	14654	0.99
Natural pasture 1	8971	0.98
Natural pasture 2	6665	0.98
Natural pasture 3	10798	0.98
Natural pasture 4	10384	0.99
Soybean field 1	6412	0.98
Soybean field 2	3686	0.96
Soybean field 3	2407	0.95
Soybean field 4	2276	0.94
Site B		
Natural forest 1	14516	0.99
Natural forest 2	14884	0.99
Natural forest 3	34724	0.99
Natural forest 4	16223	0.99
Natural pasture 1	11543	0.99
Natural pasture 2	13143	0.99
Natural pasture 3	23388	0.99
Natural pasture 4	27167	0.99
Cerrado biome		
Sugarcane field 1	13213	0.99
Sugarcane field 2	13923	0.99
Sugarcane field 3	14347	0.99
Natural forest 1	13216	0.99
Natural forest 2	13921	0.99
Natural forest 3	14347	0.99
Pinus plantation 1	13209	0.99
Pinus plantation 2	13918	0.99
Pinus plantation 3	14347	0.99
Pasture 1	5291	0.99

Land use/biome	Total sequences	Coverage genus level
Pasture 2	14800	0.99
Pasture 3	7935	0.99

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Chapter 7
General discussion

The aim of the research described in this thesis was to assess the impact of land-use changes in conjunction with soil type and seasonal climatic variations on the composition, diversity and dynamics of the soil microbiome in the Pampa ecosystem. To reach this goal, microbial communities across different soil types and land use systems were analyzed. Furthermore, a microcosm experiment mimicking seasonal natural variation in soil moisture and temperature was performed. I used a combined approach of molecular fingerprinting, next-generation sequencing and network approaches to assess the soil microbiome.

In this chapter, I will discuss:

a) the methodology used to explore the total microbial community, including sampling strategy, molecular fingerprinting techniques, high-throughput sequencing, the separation of active and dormant populations, and network approach, which allows to assess microbial co-occurrence and keystone species;

b) the importance of soil type and soil climate to shape the structure of microbial communities and to determine their diversity;

c) how land-use changes through the removal of natural vegetation (grassland and forest) and introduction of anthropogenic uses may affect the diversity, structure and function of microbiome of the Pampa ecosystem;

d) ideas and directions for future studies on land-use changes in the Pampa ecosystem.

7.1 Methodological approaches: assessing the total microbial community in soil

The field studies in *Chapters 2, 3, 4* and *6* can be classified as observational in which randomization is restricted solely to selecting samples from the population of interest and no manipulation of experimental conditions is performed. The approach used in this experimental design sometimes generates controversy among the scientific community as it indicates a specific limitation regarding the statistical concept of repetitions. Some researchers argue that this type of experiment cannot have true landscape level of replication but rather pseudoreplication and, therefore, it would not be a statistically valid experimental design for testing hypothesis. This view gained popularity ever since the work published by Hurlbert (1984), whose released his review and critique to ecologists, in matters of misconceptions in experimental design and statistical treatments emphasizing the need of genuine replication. However, after the publication of this study, the critical reevaluation of pseudoreplication has been discussed in a significant number of scientific articles. Hargrove and Pickering (1992) argue that, landscape-level experiments are often not possible and the nature of landscape-scale studies precludes replications in the way they are constructed in classical experimentation, which requires true replicates (Hurlbert 1984; 2009; Krebs 1999). However, Schank and Koehnle (2009) support the idea that pseudoreplication is a pseudoproblem that sets impossible standards for the majority of the experimental designs and analyses of experiments. My experiments, as case study, provided an unique opportunity to investigate the effects of land-use changes on microbial communities in areas that are otherwise nearly identical in terms of physiography and microclimate. Thus, the sampling strategy used in *Chapters 2, 3, 4* and *6* supported my conclusions on the effects of land-use changes on soil microbial community in the Pampa

biome at a local scale.

Cultured-based methods provided the base for the knowledge on microbial identification, physiology and ecology (Handelsman 2004). Not discarding the importance of standard culture techniques for investigation of the ecology of microbial communities (Bevivino et al. 2014), it is noteworthy that they are biased in their evaluation of microbial diversity. These methods select a certain set of microorganisms which are easily cultivable (*e.g., Proteobacteria, Firmicutes*) rather than the ones, that are abundant in soil and other ecosystems, but difficult to culture (*e.g.*, *Acidobacteria*) (George et al. 2011). Based on the most recent classification schemes, there are 30 phyla in the domain Bacteria and 5 phyla in the Archaea domain with cultivated representatives (Euzéby 1997). Thus, as the total number of phyla reaches more than 52 (Rappé & Giovannoni 2003) (candidate phyla included) many major groups cannot be cultured and are known solely via cultured independent methods (called candidate phyla) (Hugenholtz et al. 1998). These facts provide sufficient evidence that methods that circumvent the need for cultivation are preferable for the assessment of microbial communities in a highly diverse and complex ecosystem such as the soil. Aiming to obtain thorough understanding of the soil microbiome, I therefore chose to survey the soil microbial community in all of the work described in this thesis using uncultured-based approaches.

After amplification by PCR, a variety of *fingerprinting* techniques are available for analysis of microbial communities (van Elsas & Boersma 2011). In *Chapter 2*, I used the RISA approach (Ribosomal Intergenic Spacer Analysis - (Borneman & Triplett 1997), to get insight into how soil type and land-use changes shift the structure and diversity of the archaeal and fungal communities. Based on length heterogeneity of the ITS (Internal Transcribed Spacers) region, the comparison of samples showed that each soil type or land use was characterized by a specific community profile, demonstrating the

effectiveness of this method to detect differences in the structure and diversity of the microbial community from different ecological niches. However, though this approach is suitable to study community shifts induced by land-use changes and soil types (as in *Chapter 2*), archaeal and fungal diversity and composition need to be explored in more details in order to obtain full understanding of the impact of these factors. The complexity of microbial communities often hampers the detection of subtle changes by *fingerprinting* approaches, since these methods do not provide taxonomic identities, and do not allow for the detection of populations present at low abundance (Ranjard et al. 2001; van Elsas & Boersma 2011). Although diversity indices based on molecular *fingerprinting* are generally much lower than based on high-throughput sequencing methods (Jami et al. 2014; Chen et al. 2015), both approaches are capable of recovering highly comparable trends related to shifts in community diversity and structure pointing to similar conclusions about the processes shaping microbial communities (Pilloni et al. 2012; Castro-Carrera et al. 2014; Verbruggen et al. 2012; Xia & Jia 2014; Cleary et al. 2012).

The high-throughput sequencing allows for significant steps forward in our understanding of complex microbial communities due to the efficiency and unbiased nature of the sequencing (Margulies et al. 2005; Lauber et al. 2009; Rodrigues et al. 2013). The massive sequencing enables the assessment of the microbiome from the top to the bottom, *i.e.,* from the most abundant species going down into the rare biosphere (Pester et al. 2010; Lynch & Neufeld 2015). As the main objective of this study was to obtain a deep understanding and taxonomic identification of the members of the microbiome, I surveyed the microbial community in *Chapters 3, 4, 5* and *6* using next-generation sequencing. Through the chapters, it is possible to see the evolution of sequencing technologies. In 2005, a breakthrough in massive DNA sequencing was announced (Margulies et al. 2005), introducing the pyrosequencing method associated with barcode indexing (Hamady et al. 2008) (used in *Chapters 3*, *4*

and *6*). In 2010, Ion Torrent approaches became available (used in *Chapter 5*), in which base-sequence composition is determined by measuring pH variation, thereby reducing the complexity of the equipment and the cost of sequencing reactions (Whiteley et al. 2012), but with the same accuracy to assess the microbial community as other sequencing methods (Yergeau et al. 2012).

Since the next-generating sequencing technologies are very recent, sequence processing and analysis approaches are still in progress and may fail by artifacts introduced by amplification and sequencing errors (Schloss et al. 2011). The most popular bioinformatic pipelines for sequence data processing, *i.e.*, QIIME (Caporaso et al. 2010) (used in *Chapters 3* and *4*) and mothur (Schloss et al. 2009) (used in *Chapter 6*) provide algorithms for reducing artifacts including steps such as quality filtering, denoising, chimera filtering and clustering (Quince et al. 2011). However, many biases and spurious OTUs often remain, confounding inferences of community structure and diversity (Bokulich et al. 2012). Recently, a new package has received considerable attention. The UPARSE pipeline (used in *Chapter 5*) generates a number of operational taxonomic units (OTUs) consistently closer to the expected number of species in a community (in comparison with a "mock" community), as compared with pipelines recommended by QIMME and mothur, in which the number of OTUs, often, far exceeds the number of real species (Kunin et al. 2010; Edgar 2013).

Based on evidence that the rRNA content correlates well with growth rate and activity (Fegatella et al. 1998; Muttray & Mohn 1999; Campbell et al. 2011), calculating the rRNA:rDNA ratio is an effective approach to differentiate the response of the active and dormant fractions of a microbial community to environmental variations (DeAngelis and Firestone, 2012; Hugoni et al. 2013; Schostag et al. 2015). This approach has been proved to be sensitive to detect the dynamics of the active community rather than the single assessment by rDNA (Dlott et al. 2015) and to predict the contribution of

dormant cells to the maintenance of microbial diversity (DeAngelis et al. 2010; Jones & Lennon 2010). In *Chapter* 5, I showed that habitat selective factors may exert different effects on active and dormant communities as the rRNA:rDNA ratio differed along moisture and temperature variations in soil, which shows the potential of this approach to get an accurate picture of the dynamics within microbial communities. However, more studies with cultured representatives of the microbial communities in different ecosystems are needed to further confirm the usefulness of this approach and the functional relevance that it represents (Dlott et al. 2015).

When properly constructed, network analyses linked to ecological questions can reveal co-occurrence patterns and keystone species ("hubs") thus providing a robust tool to develop and test hypotheses on within-community processes. As an alternative approach to previous studies described in this thesis, where I checked for shifts in microbial diversity and abundance, in *Chapter 6*, I explored the potential of network approaches to predict keystone species and examine microbial interactions in a range of natural and anthropogenic land usages. First of all, the network topology may provide answers to questions about the level of connectance of microbial members of the network, *i.e.,* the proportion of possible interactions between species that are realized in a specific time and space frame (Bissett et al. 2014). The resultant network topology in *Chapter 6* conforms to the scale-free and small-world model (Chaffron et al. 2010), where most of the nodes (representing microbial genera) are not neighbors (small degree, *i.e.,* few connections between near-by nodes) but most of them can be reached from another one by a small number of connections (positive and negative links between nodes). The level of connectivity, assessed by closeness centrality (denote the distance of one node to another one) indicated that most soil bacterial members were important to the connectance of the whole microbial community, making the microbial community robust to changes, which may explain the resistance of the soil

microbiome to disturbances (Montoya et al. 2006).

Analysis of the networks in *Chapter 6* also highlighted that each land-use system presented a different and specific set of potential key species mainly belonging to different genera of the phyla *Proteobacteria, Actinobacteria*, *Chloroflexi*, *Bacteroidetes,* and *Firmicutes.* These key members of the microbial communities may act as the main intermediaries between microbial groups - they may have fewer connections, but mediate more group associations. The keystones have a large impact on community composition shaping the interactions with other members and could be more important to soil processes than species richness and abundance *per se*, especially in soil ecosystems. Eiler et al. (2012) also detected numerous "hubs", representing phylogenetic groups with strong interdependencies with other taxa. They suggest that in highly complex environments, like the soil, there may be hundreds of such keystone species. If important members in a community get lost by anthropogenic disturbances, the patterns of the microbial interactions would change dramatically and important soil process may be altered (Zhou et al. 2011; Montoya et al., 2006; Steele et al. 2011; Berry & Widder 2014).

7.2 Soil type and climate: overriding determinants of the soil microbiome

Previous studies have found soil type as one of the most important factors determining the structure and functioning of soil microbial communities (Girvan et al. 2003; Suzuki et al. 2009; Takada Hoshino et al. 2011). Bossio et al. (1998), ranked the impact of various environmental variables governing the composition of microbial communities, with soil type ranked as the most important variable explaining the abundance of microbial groups, followed by time (representing different seasons) of sampling, farming operation, management system and spatial variation in the field.

Results from my study showed that soil type and associated soil properties may, indeed, be important explanatory factor for the structure of archaeal and fungal communities (*Chapter 2*). The data obtained from different soils along the toposequence also indicate that archaeal and fungal communities may be differentially controlled by soil type likely due to physiological and ecological contrasts between prokaryotic and eukaryotic groups (Wakelin et al. 2008; Pereira e Silva et al. 2012). While archaeal communities are largely influenced by soil type and associated chemical properties (Taketani & Tsai 2010; Chen et al. 2010; Takada Hoshino et al. 2011), fungal communities are less dependent of soil type and more dependent of environmental changes, such as land-use changes (*Chapter 2*), fertilization and management intensity as shown earlier by Oehl et al. (2010) and Suzuki et al. (2009).

Distinct bacterial and fungal communities have been associated with soils of varying pH (Lauber et al. 2009), moisture (Rogers & Tate 2001), P content (Faoro et al. 2010) and texture (Girvan et al. 2003). Overall, as described in *Chapter 2*, the soil properties did not vary consistently with soil type in the toposequence (*e.g.*, pH around 5 and clay around 95-100 g kg^{-1}). However, soil moisture ranged from well-drained to saturated conditions and also P-content was different between soil types, which, therefore, may be the most important factors explaining the divergence in community structure along the toposequence. Similarly to our results, also Rogers and Tate (2001) found that the topographic position can influence microbial community structure and activity through effects on moisture and vegetation along a toposequence.

Yet, as detected in *Chapter 3,* the bacterial community in the same soil type was not affected much after removing the natural vegetation and introducing agricultural crops and/or exotic tree plantation. Each soil type may harbor a specific bacterial community able to grow and proliferate in the particular environment of specific combinations of chemical and physical

characteristics (Delmont et al. 2014). Therefore, it may have a large buffering capacity to conserve its diversity against the impact of land usages or management practices (Wieland et al. 2001; Reeve et al. 2010).

One of the likely explanations of this resistance of the soil microbiome structure and functioning to disturbance is the existence of a large and dominant inactive fraction within the soil microbial community. In *Chapter 5*, I showed that, indeed, the dormant community comprises an important source of microbial diversity in these soils, reflecting in the diversity patterns of the total community. In environments exposed to strong fluctuations, as in my soils, dormancy or the ability to enter a state of reduced metabolic activity is supposed to be a life history strategy for the majority of microorganisms, preventing the extinction of microbial species (Jones & Lennon 2010).

The active community is more affected by moisture as well as by temperature, whereas the dormant and the total community were affected solely by moisture, indicating the dominance of water content regulating the diversity, structure and composition of microbial assemblages in soil. Overall, our results are consistent with previous studies showing that water content plays an important role in the composition and diversity of microbial communities over seasons (Valverde et al. 2014; Waldrop & Firestone 2006; Bouskill et al. 2012). Temperature has long been recognized to be a determinant for the microbial assemblages at global scales (Lipson 2007; Ding et al. 2015). However, temperature appears to be of less importance at local scales, particularly in subtropical and temperate ecosystems where the community might contain a widely adaptive (*e.g.,* functional plasticity and dormancy) capacity to variations in temperature as compared to tropical organisms that are adapted to little seasonal variability (Bardgett et al. 1999; Schindlbacher et al. 2011; Wallenstein & Hall 2011).

Despite being potentially different in biological traits, active and dormant communities are controlled by the same assemblage process along

moisture regimes. My results suggest that the relative influence of niche and neutral processes vary along the moistures gradient, with niche-based mechanisms being more influential at low water content, *i.e.,* at greater environmental stress. At low water content (8% moisture), phylogenetic clustering may be the rule, resulting in lower diversity due to habitat selection (Valverde et al. 2014). At high water content (16% and 23% moisture), there is a decline in phylogenetic clustering, indicating that this community is more influenced by random or neutral-based processes. It has been demonstrated, indeed, that in more benign environments (*e.g.*, "wet" habitats), the presence of abundant water and heterogeneities in substrate supply may lead to distinct niches increasing the diversity and richness and decreasing the importance of the habitat filtering (Chase 2007; Valverde et al. 2014).

7.3 Effects of land-use changes on the soil microbiome

7.3.1 Diversity

In the last decade, the loss of biodiversity has become a global concern as more and more evidences becomes available showing that it will negatively affect the functionality of the ecosystem (Wagg et al. 2014), such as nutrient cycling for plant growth (Bartelt-Ryser et al. 2005), plant productivity and soil health (*e.g.*, disease suppression) (Mazzola 2004), being key to a sustainable society. For soil ecosystems, recent global predictions indicate that grassland ecosystems likely will experience the greatest change in biodiversity because of large scale land-use changes being the main drivers of biodiversity losses (Sala et al. 2000; Souza et al. 2013). Not only above-ground, but also below-ground organisms are affected severely by land-use changes, as shown earlier (Lorenzo et al. 2010; Carbonetto et al. 2014).

Because most soils with agricultural crops and exotic tree plantations receive considerable chemical inputs which change directly or indirectly the

soil environment, it is conceivable that microbial diversity in these soils will be altered and potentially reduced compared to the native soils of the Pampa ecosystem (Cardinale et al. 2012; Carbonetto et al. 2014). Moreover, studies linking microbial assemblages and above-ground plant communities provide empirical support to the prediction that a decrease in plant diversity will lead to a decrease of microbial diversity (Lange et al. 2015). However, contrary to these predictions, in *Chapters 2, 3* and *4*, I demonstrated that relatively short-term disturbances (*i.e.,* less than 15 years of changes in land usage through removal of grassland and forest vegetation) of plant diversity and composition did not deplete archaeal and bacterial richness and diversity. This was in line with earlier observations that in agriculture sites or in sites with low vegetation diversity (generally as a result of converting a natural site into agricultural land) bacterial diversity was not reduced as compared to native sites (da C Jesus et al. 2009; Ding et al. 2013). The clearest decrease in diversity was observed for the soil fungal communities at *Eucalyptus* and *Acacia* plantations (*Chapter 2*). The differences in quality and quantity of litter compounds generated by these two exotic species may be the main factors acting as selective pressure towards specific fungal species, such as specific decomposers, capable of degrading specific substrates (Macdonald et al. 2009; Lauber et al. 2013). Furthermore, the decrease of light availability, decrease in microhabitat heterogeneity, and the proliferation of strong competitors may also have contributed to the observed decrease in fungal richness (Jacquemyn et al. 2003). It has been suggested that the fungal community recovers slowly after disturbances, because these microorganisms tend to be less resistant and resilient to disturbances than *e.g*., bacteria are (Hedlund et al. 2004; de Vries et al. 2012).

However, it is also important to recognize that similarity in diversity or richness does not mean that species composition is the same: the same diversity, but different microbial structure and composition, as found in this

studies, might indicate that, under the influence of environmental changes, microbial communities have gradually been replaced by another community composed of species with different abundances and phylogenetic relatedness that survive better under the new conditions (which I called substitution hypothesis in *Chapter 3*). Moreover, the apparent diversity stability to disturbance might be explained by microbial dormancy. Only a fraction of the bacterial community appeared to be metabolically active (*Chapter 5*) and the large dormant portion of microorganisms may contribute to diversity stability via generation of a long-lived seed bank (Chesson 2000) and niche complementation (intraspecific differences in resource and habitat use by active and dormant members) (Cordero & Polz 2014).

7.3.2 Structure and core microbial community

Environmental disturbances caused by land-use changes such as changes in nutrient availability (Bardgett et al. 1999), pH (da C Jesus et al. 2009), soil density (Jiao et al. 2012), soil temperature (Zogg et al. 1997) and moisture (Drenovsky et al. 2010) lead to alteration in the soil microbiome composition and functioning in grasslands (Wakelin et al. 2013). Based on the differences between natural (*e.g.,* grassland and forest) and anthropogenic (*e.g.,* soybean and *Eucalyptus*) usages and associated management I expected a strong effect on the microbial structure associated with the shifts in soil properties. However, this was not the case for all land uses examined here. The results described in *Chapter*s *2*, *3* and *4*, indicate that the differences in microbial community structures of the land use systems evaluated here were primarily based on differences in relative abundance of specific microbial groups rather than on their presence/absence (*Chapters 3* and *4*).

Relatively short-term disturbances as in the studies described here may not always drive strong alterations in microbial structure. Marshall et al.

(2011) already indicated that microbial communities are relatively insensitive to short-term changes in plant composition, as they may respond only gradually to associated changes in rhizodeposition, litter quantity and quality and soil properties. The apparent stability of the microbial community structure as observed in *Chapters 2*, *3* and *4*, have also been reported previously by Araújo et al. (2013). The current community structure may have been determined largely by historical events (*e.g.,* prevalence of vegetation type, weather conditions as well as soil type) rather than by the current disturbances (Martiny et al. 2006). It is also possible that the apparent stability of the soil microbiome is conferred by the intra-community interactions and the presence of microbial key species (*Chapter 6*), as argued by some authors (Bissett et al. 2013; Peura et al. 2015).

Although a large fraction of the soil microbiome did not show great shifts, alterations in the abundance of specific microbial groups were detected (*Chapters 3* and *4*). These groups were not dominant but collectively represented a considerable part of the differences observed among samples. According to Bissett et al. (2013), this pattern occurs because the degree of resistance and resilience differs among microbial groups and may vary according to (i) the sensibility to environmental disturbances, *e.g., Acidobacteria* may be sensitive to alteration in Ca and Mg - (*Chapter 2*) (Navarrete et al. 2013), (ii) the effects of these disturbances on other organisms which microorganisms interact with *e.g.,* rhizobia are linked with the presence of leguminous plants in the soybean field (*Chapter* 3) (Sugiyama et al. 2014), (iii) differences in ecological functions *e.g.,* higher concentration of NH_4^+ in natural forest (*Chapter 3*) may have favored the growth and activity of the *Nitrospira* group, resulting in alteration of the nitrification process, as depicted by the higher amounts of NO_3^- + NO_2^- in soils (Schramm et al. 1998; Meyer et al. 2013) and, (iv) their abundance, *e.g.,* rare taxa are more sensitive to changes, since they are more vulnerable to extinction than abundant ones in

the short term disturbances caused by human activities (Gaston 2008).

I detected a large overlap of microbial taxa between soil types and land usages within sites, suggesting the existence of a resistant or even resilient "core microbiome" that did not suffer any changes related to shifts in soil properties or plant cover (*Chapters 2, 3* and *4*). The core microbiome can be defined as the fraction of microorganism shared among habitats and presumed to play key roles in ecosystem functioning (Shade & Handelsman 2012). Since the soil microbiome is the result of thousands of years of soil formation processes, climate dynamics and vegetation developments (Tarlera et al. 2008; Berg & Smalla 2009), it is plausible that the current microbiome is comprised of a core of species completely adapted to the prevailing natural environmental conditions of the Pampa ecosystem and largely resistance against short- or even longer- term anthropogenic disturbances of the biotic and abiotic ecosystem properties (Yasir et al. 2015; Montecchia et al. 2015).

Similar to the findings described in this thesis, Montecchia et al. (2015) detected the existence of a core microbiome, composed of a large proportion of OTUs persistent at three land usages (forest, short- and long-term agriculture), and resistant to disturbances caused by changes in land use. Similarly, Orgiazzi et al. (2013) found ubiquitous fungal taxa present in different ecosystems (soil types and land usages), which they classified as generalist fungi, with oligotrophic and chitinolytic abilities, again suggesting the presence of a stable core adapted to nutrient-poor soil conditions and with the ability to exploit organic resources broadly distributed in soils. However, the function and importance of the core microbiome is far away from being understood as only few studies have been directed to the understanding of this stable compartment of the microbial community in soil (Bacci et al. 2015; Orgiazzi et al. 2013). Nevertheless, the core microbiome may become a vital strategic factor in the development of sustainable agricultural practices in future *e.g.*, for management of plant diseases, in a similar way as proposed in some reports as

"transfer therapy" realized in gut and rhizosphere (Gopal et al. 2013; Mendes & Raaijmakers 2015). Even though microbial diversity and structure can be shifted by ecosystem disturbances, the core microbiome would allow for maintaining at least some of the key soil functions after conversion of natural vegetation to agricultural land or tree plantations (Montecchia et al. 2015).

7.3.3 Relationship between structure and functions of microbial communities

The measures of broad-scale functions, such as biomass production and potential microbial activity (based on the metabolic quotient - qCO_2) did not converge with the 16S rDNA sequence (*Chapters 3*, bacterial and archaeal communities) and ITS profile (*Chapter 2*, archaeal and fungal community) data, supporting the idea that structure and functioning of microbial communities are not necessarily correlated. There are several reasons for the contrasting patterns in structure and functioning of microbial communities as the result of land use changes. One is that only function but not community structure responds to a disturbance (Frossard et al. 2012). Another reason, confirmed here, and by others (Allison & Martiny 2008; Bowen et al. 2011), is that once a certain level of microbial diversity is reached in soil under either natural or anthropogenic conditions, all key functions exist within the different communities, and so differently structured communities may exert similar functions. Also functions may be limited by other factors such as water content and temperature which do not directly affect significantly composition or diversity (Bissett et al. 2011). And in case of intensive management, as in agricultural lands and tree plantations, there may be a time lag in the structural responses of the microbial community while functional responses may be faster (Berga et al. 2012).

7.4 Final conclusions and future directions

I showed that changes in land usages by the removal of natural vegetation and introduction of crops and exotic trees do not always lead to a reduction of microbial diversity or to extreme modifications in community structure and composition. Yet I showed that specific microbial groups, key species and patterns of microbial co-occurrence were sensitive to anthropogenic impacts, but how these shifts influence the soil ecosystem functioning remains obscure. It was not possible to assess all combination of crops and exotic tree plantations on the Pampa ecosystem, but even although no potential high risk level on the Pampa ecosystem was observed, we should be concerned about the long-term impacts of disturbances and how it will influence soil microbial diversity and, as a consequence, functioning.

Several factors may act as drivers of communities in a natural ecosystem, including soil type, land usages and seasonal climatic variations. To better understand the emergent patterns of microbial structuring and functioning observed here, ecological aspects of the soil microbiome such as the level of resistance/resilience, physiological status and microbe-microbe interactions in combination with the type, level and time of perturbations have to be considered in more details. Bacteria seem not to be the most responsive group of microorganisms to short-term disturbances, whereas the fungal community showed to be more sensitive to variations in the above ground plant community, which calls for deeper investigations in this and others disturbance sensitive groups.

The studies described here are useful for identification of the ecological processes that link biodiversity and ecosystem services, assisting in future restoration, monitoring programs and to meet policy objectives regarding the consequence of changes in the Pampa ecosystem. Future studies should address how the patterns of microbial community dynamics within the Pampa

biome and other grasslands are linked to key ecosystem processes such as nutrient cycling, plant growth promotion, and overall ecosystem health. In order to better investigate the potential threats of land-use changes, the development of a more integrative and multidisciplinary research approach encompassing biotic and abiotic ecosystem processes combined with analyses of "meta-omics" processes (metagenome, transcriptomics, proteomics and metabolomics) will improve our ability to decipher how environmental traits moderate changes in the soil microbiome and the above-below ground feed-back mechanisms operating in the Pampa biome.

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Summary

Pampa biome is a grassland ecosystem considered to be a hotspot of the world's biodiversity, where subtropical climatic conditions prevail, with hot summers and cold winters. The climate in association with the geologic material, creates a natural condition highly sensitive to natural and anthropogenic disturbances. This biome has experienced disturbances through land-use changes; more than half of the original vegetation has been converted for human usage. Microbes are essential for soil stability and functioning and any disturbance will lead to modifications of the diversity and composition of microbial communities, which, in turn, influence the functionality of ecosystems. **The main aim of this thesis** was to assess the impact of land-use changes in conjunction with soil type and seasonal climatic variations on the diversity, composition and dynamics of the soil microbiome in the Pampa ecosystem.

Different approaches were applied in the current studies including molecular fingerprinting, next-generation sequencing and network approaches in field and well-controlled experiments. The results described in *Chapter 2* showed that land use systems and soil type are both drivers of fungal and archaeal diversity and community structure, and also result in shifts in microbial biomass and metabolic activity. The microbial community structure was associated with the different soil types, but the differences were more evident for the archaeal than for the fungal community. The fungal community was more sensitive to the type of land use, with a reduction of diversity observed at *Eucalyptus* and *Acacia* plantations.

As a continuation of the work described in *Chapter 2*, in *Chapter 3*, I assessed the response of bacterial communities using 16S high-throughput sequencing to land-use changes in a landscape with natural (grassland and forest) and anthropogenic land uses (soybean field and *Acacia* plantation). Both composition and diversity of the bacterial community were not impacted much after removal of the natural vegetation and introduction of agricultural crops

or exotic tree plantation. Remarkably, in the research described in both chapters, the measures of broad-scale functions did not converge with the archaeal, bacterial and fungal structures, evidencing that functioning and structure of microbial communities are not necessarily linked.

In order to find out how short-term disturbances in plant composition influence the diversity and composition of bacterial communities, the communities in soils of a forest (high plant diversity) and a grassland (low plant diversity), resulted from the replacement of the forest, were evaluated (*Chapter 4*). The bacterial diversity as well the composition of the community were not different between forest and pasture indicating that short-term shifts in plant diversity and composition does not drive strong alterations in microbial structure; only shifts in specific microbial groups were detected. Besides, in correspondence with previous chapters (*Chapter 2* and *3*), a large overlap of microbial taxa between land usages were detected. These findings indicate the presence of a stable core microbiome resistant to anthropogenic disturbances.

I also tested the diversity and structure, as well as the dynamics of active and dormant communities in response to seasonal climatic variations in a microcosm system, mimicking winter and summer, (*Chapter 5*). The active community was affected by moisture as well as by temperature, whereas the dormant and the total community were affected solely by moisture. The dormant community comprises the larger proportion of microbial diversity and mainly reflects on the pattern of the total community. Active and dormant communities were controlled by the same assemblage rules, which were moisture-dependent, with niche-based mechanisms being more influential under dry conditions and random processes at high water content.

As an alternative approach to previous studies, the potential of network approaches to predict keystone species and examine microbial interactions in natural and anthropogenic land usages were explored and the results are

described in *Chapter 6*. The network topology conforms to the scale-free and small-world model, where most of the nodes are not neighbors but can be reached from another one by a small number of connections. The results highlighted that each land-use system has a different and specific set of putative key species mainly belonging to *Proteobacteria, Actinobacteria*, *Chloroflexi*, *Bacteroidetes, and Firmicutes*, which might play a role intermediating microbial groups associations.

This study presents an integrated analysis and provides new insights in the diversity, composition, associations within and assembly rules of the soil microbiome and have shown the potential ecological importance for ecosystem stability in Pampa biome.

Samenvatting

De Pampa is een grasland ecosysteem dat beschouwd wordt als een van de biodiversiteit hot spots waar een subtropisch klimaat heerst met hete zomers en koude winters. Het klimaat en de geologie van het gebied creëren een natuurlijk system dat uiterst gevoelig is voor natuurlijke en antropogene verstoringen. Verstoringen van het Pampa ecosysteem hebben onder meer plaats gevonden door veranderingen van het landgebruik; meer dan de helft van alle vegetatie is veranderd ten behoeve van menselijk gebruik. Micro-organismen zijn essentieel voor de stabiliteit en het functioneren van het bodem ecosysteem en elke verstoring van het ecosysteem leidt tot veranderingen in de microbiële diversiteit en de samenstelling van de microbiële gemeenschap, wat, op zijn beurt, de functionaliteit van het ecosysteem zal beïnvloeden. Het doel van het onderzoek dat in dit proefschrift beschreven is, was het vaststellen van het effect van veranderingen in het land gebruik in samenhang met bodem type en klimatologische seizoen variatie op de diversiteit, samenstelling en dynamiek van het microbiële gemeenschap in de bodem van het Pampa ecosysteem.

Verschillende benaderingen zijn hier toegepast waaronder moleculaire fingerprint methoden, Next-Generation Sequencing en netwerk analyses in veld en goed gecontroleerde laboratorium experimenten. De resultaten die in *Hoofdstuk 2* zijn beschreven, laten zien dat land gebruik en bodem type beiden bepalend zijn voor de diversiteit en structuur van de schimmel en Archaea gemeenschappen en ook van invloed zijn op de microbiële biomassa en metabolische activiteit. De structuur van de microbiële gemeenschap was verschillend voor verschillende bodem typen, maar de verschillen waren duidelijker voor de Archaea gemeenschap dan voor de schimmel gemeenschap. De schimmel gemeenschap was meer gevoelig voor het soort land gebruik, waarbij een reductie in de diversiteit werd geconstateerd bij *Eucalyptus* en *Acacia* plantages.

Als vervolg op het werk, dat beschreven is in *Hoofdstuk 2*, is in *Hoofdstuk 3* het onderzoek beschreven, dat ik heb verricht om de respons van de bacteriële gemeenschap op veranderingen in het landgebruik in een landschap met natuurlijk (grasland en bos) en antropogeen landgebruik (soja en *Acacia* plantages) te bepalen met behulp van 16S rDNA High-Throughput Sequencing. Noch de samenstelling noch de diversiteit van de bacteriële gemeenschap waren veel veranderd na verwijdering van de natuurlijke vegetatie en de introductie van de landbouwgewassen en de exotische boom plantages. Opmerkelijk in beide hoofdstukken is, dat de overall functies niet samenhingen met de structuur van de Archaea, bacteriële en schimmel gemeenschappen, wat er op duidt de er geen koppeling is tussen functioneren en structuur.

In *Hoofdstuk 4* is onderzoek aan een bos (hoge plant dichtheid) en een grasland (lage plant dichtheid), dat verkregen was door vervanging van het bos, beschreven dat er op gericht was om uit te vinden in welke mate korte termijn verstoringen van de vegetatie de diversiteit en de samenstelling van de bacteriële gemeenschap beïnvloedden. De bacteriële diversiteit en de samenstelling van de gemeenschap waren niet verschillend voor de bos en grasland bodems wat aangeeft dat korte termijn veranderingen in planten diversiteit en samenstelling niet leidt tot sterke veranderingen in de structuur van de microbiële gemeenschap; er werden alleen veranderingen in specifieke microbiële groepen waargenomen. Verder werd er, evenals in het werk dat in de *Hoofstukken 2* en *3* is beschreven, een grote mate van overlap in microbiële taxa tussen land gebruik typen, gevonden. Dit duidt op het bestaan van een kern microbioom dat resistent is tegen antropogene verstoringen.

Ik heb ook de diversiteit en de structuur van de microbiële gemeenschap als mede de dynamiek van de actieve en inactieve gemeenschapsfracties, onderzocht in relatie tot de klimatologische seizoen variatie in een microkosmos systeem waarin met name zomer en winter

condities werden nagebootst; dat onderzoek is beschreven in *Hoofdstuk 5*. De actieve fractie werd beïnvloed door zowel het bodemvochtgehalte als de temperatuur, terwijl de inactieve fractie en de totale gemeenschap alleen door het bodemvochtgehalte werden beïnvloed. De inactieve fractie omvatte een groter deel van de totale microbiële diversiteit en weerspiegelt in sterkere mate de patronen die werden gevonden voor de totale gemeenschap dan de actieve fractie. Actieve en inactieve gemeenschappen waren samengesteld volgens dezelfde assemblage modellen, die bodemvocht afhankelijk waren en onder droge condities meer bepaald werden door niche-gebaseerde regels en bij hogere vochtgehaltes meer door random processen.

Als alternatieve benadering ten opzichte van voorafgaande studies heb ik de mogelijkheden van netwerk benaderingen onderzocht voor het voorspellen van de aanwezigheid van *keystone* soorten en het onderzoeken van microbiële interacties in ecosystemen met natuurlijk en antropogeen land gebruik (*Hoofdstuk 6*). De netwerk topologie van de systemen, die ik onderzocht heb, komt overeen met zogenaamde *scale-free* en *small-world* modellen, waar de meeste van de knooppunten geen buren zijn, maar zijn verbonden met andere via een klein aantal connecties. De resultaten van dit onderzoek laten zien dat elk landgebruik systeem een verschillende, specifieke, set aan *keystone* soorten heeft, die vooral behoren tot de *Proteobacteria, Actinobacteria*, *Chloroflexi*, *Bacteroidetes,* en *Firmicutes*, die een rol spelen als intermediair tussen verschillende microbiële groepen.

Het onderzoek, dat in dit proefschrift beschreven is, is een integrale analyse en verschaft nieuw inzicht in de diversiteit, samenstelling en samenhang van de microbiële gemeenschap en de regels volgens welke de bodem gemeenschap wordt gevormd. De hier beschreven bevindingen dragen bij tot een beter begrip van de microbiële respons op natuurlijke en antropogene verstoringen in een subtropische ecosysteem en van het potentiele belang van micro-organismen voor de stabiliteit van Pampa ecosystemen.

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Manoeli Lupatini
2015.12

Curriculum vitae

Manoeli Lupatini was born on 30th of June1985 in Espumoso, Rio Grande do Sul, Brazil. In 2002, she finished her secondary education at "Instituto Estadual de Educação Dr. Ruy Piégas Silveira", in Espumoso. In 2004, she started the Bachelor in Agronomy at Federal University of Santa Maria, Santa Maria, Brazil. On the first year of bachelor, after showing interest in two main research topics, Soil Science and Microbial Ecology, she started an undergraduate project studying the mycorrhizal fungi and the association with plant community under the supervision of Dr. Z. I. Antoniolli. In her secondary project, in the same laboratory, she performed research studying the potential of microorganisms isolated from soil and rhizosphere to promote the plant growth. In 2012, she continued with her MSc study in Soil Science at Federal University of Santa Maria. For her masters thesis, she did research on soil microbial ecology based on advanced molecular techniques using bioinformatic tools and statistical approaches under the supervision of Dr. R.J.S. Jacques and Dr. L.F.W. Roesch. For this work, she received a grant from "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)". In 2012, she started her PhD project "Microbiome dynamics in Pampa soils" described in this thesis in collaboration with Federal University of Santa Maria, Federal University of Pampa and Netherlands Institute of Ecology (NIOO/KNAW) under the supervision of J.A. van Veen and Dr. E. Kuramae.

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