Changes in soil biodiversity and activity along management and climatic gradients

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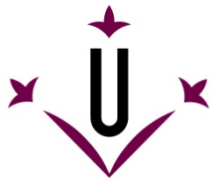

Universitat de Lleida

Changes in soil biodiversity and activity along management and climatic gradients

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“*Imagination will often carry us to worlds that never were. But without it we go nowhere.”*

Carl Sagan

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Summary

Rangelands are uncultivated areas extensively grazed by wild and domestic animals, currently threatened by land use and climatic changes. Soil microorganisms play a key role in decomposition and several ecosystem processes and the composition and function of the microbial community have been long used as indices of soil fertility.

African and European rangelands share a common anthropogenic origin, but climate and management affect them in a different way. That is why this thesis aimed to analyze the microbial community of both in order to observe the effects of some common threats from a more global perspective.

While overgrazing proved to have the most detrimental effect on the soil microbial function in Kenyan soils, a stronger effect of climate was found to affect European grasslands. Fungi and bacteria co-varied along altitudinal and climatic gradients, but the bacterial community showed a fast recovery after biological and soil physico-chemical disturbances compared to fungi.

This group of studies adds new knowledge on the structure and function of the African and European rangelands, and invite to explore new lines of research including both fungal and bacterial consortia when studying the soil microbial community.

Resum

Els anomenats “rangelands”, o pastures, són àrees sense cultivar, àmpliament pasturades per animals domèstics i salvatges, actualment amenaçats pels canvis climàtic i en l’ús del sòl. Els microorganismes del sòl tenen un paper clau tant en la descomposició com en diversos processos de l’ecosistema, fet pel qual composició i funció de la comunitat microbiana han estat utilitzats durant molt temps com a índexs de fertilitat del sòl.

Els rangelands europeus i africans comparteixen un origen antropogènic comú, però el clima i la gestió del sòl els afecten d’una manera diferent. És per això que aquesta tesi pretén analitzar la comunitat microbiana d’ambdós tipus d’ecosistemes, per tal d’observar els efectes d’algunes de les amenaces comunes des d’una perspectiva més global.

Mentre que la sobrepastura va demostrar tenir l’efecte més perjudicial sobre la funció microbiana en sòls kenyans, es va trobar un efecte més fort del clima sobre els prats europeus. Els fongs i els bacteris van covariar al llarg de gradients altitudinals i climàtics, però la comunitat bacteriana va mostrar una recuperació més ràpida després de les pertorbacions biològiques i físico-químiques del sòl, comparada amb la fúngica.

Aquest conjunt d’estudis afegeix nous coneixements sobre l’estructura i funció de les pastures africanes i europees, i convida a explorar noves línies de recerca que incloguin tant bacteris com fongs alhora d’estudiar la comunitat microbiana del sòl.

Resumen

Los llamados "rangelands", o pastos, son áreas sin cultivar, ampliamente pastoreadas por animales domésticos y salvajes, actualmente amenazadas por los cambios climático y de uso del suelo. Los microorganismos del suelo tienen un papel clave tanto en la descomposición como en diversos procesos del ecosistema, por lo que composición y función de la comunidad microbiana han sido utilizadas durante mucho tiempo como índices de fertilidad del suelo.

Los rangelands europeos y africanos comparten un origen antropogénico común, pero el clima y la gestión del suelo les afectan de una manera diferente. Es por ello que esta tesis pretende analizar la comunidad microbiana de ambos tipos de ecosistemas, a fin de observar los efectos de algunas de las amenazas comunes desde una perspectiva más global.

Mientras que el sobrepastoreo demostró tener el efecto más perjudicial sobre la función microbiana en suelos kenianos, se encontró un efecto más fuerte del clima sobre los prados europeos. Los hongos y las bacterias covariaron a lo largo de gradientes altitudinales y climáticos, pero la comunidad bacteriana mostró una recuperación más rápida después de las perturbaciones biológicas y físico-químicas del suelo, comparada con la fúngica.

Este conjunto de estudios añade nuevos conocimientos sobre la estructura y función de los rangelands africanos y europeos, e invita a explorar nuevas líneas de investigación que incluyan tanto bacterias como hongos en el estudio de la comunidad microbiana del suelo.

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Chapter 1

Introduction

1. Introduction

1.1. What are rangelands?

Rangelands are uncultivated areas extensively grazed by wild and domestic animals (Lund, 2007). They are traditionally covered by native plant communities, including grasses, shrubs and woody vegetation (Lund, 2007). Rangelands thus comprise a complex set of ecosystems including grasslands, scrublands, savannas and tundra (Lund, 2007; Kiage, 2013), covering about half of the earth's land area (Kiage, 2013) and can be found across all the continents except Antarctica (McCollum *et al.*, 2017). Although the terms rangeland and grassland have generated rather controversy, it is widely accepted that rangeland is a broader term, including diverse pastured regions where woody vegetation can be present or not, while the term grassland refers to a more biological meaning, referring only to these ecosystems covered by grasses (Blench & Sommer, 1999; Lund, 2007).

Rangelands are of global importance, not only due to their geographical extent, but also from an economic and social perspective (Lund, 2007). Rangelands provide habitat and feed resources to support domestic livestock which, in turn, provides the human population with food, manure and many other products exchangeable by cash (Milton, Dean, & Richardson, 2003; Lund, 2007; McCollum *et al.*, 2017).

The majority of rangelands both in Africa and Europe have the same anthropogenic origin, mostly derived from forest fires caused by herders (Blench & Sommer, 1999). Due to different climatic and land use

conditions, African rangelands tend towards desertification, the most severe form of land degradation affecting all land uses across Africa (Moussa, Rensburg, & Kellner, 2008). By contrast, the main losses of European grasslands and rangelands are not only due to climate change, but also to agricultural intensification as well as to land abandonment and shrub encroachment (Joyce & Wade, 1998).

Since the different types of rangelands share a common land management (grazing land), and they also share the main threats they face (land use change and climate change), in this group of studies we considered analyzing the types of rangelands most relevant in each of two different continents, Africa and Europe, in order to observe the effects of some common threats on soil fertility, biodiversity and activity from a more global perspective.

1.2. African savannas

African rangelands are dominated by savannas located in arid or semi-arid environments, covered by large extensions of grasses interrupted by trees and shrubs (Kiage, 2013). Agropastoralism is the main human activity in these areas, and human dependence on the products coming from livestock on rangelands is specially pronounced in developing countries of Africa (Talbot, 1986), leading farmers to reach unsustainable densities of livestock (Vetter, 2013). High stocking rates are considered to largely contribute to land degradation and desertification (Middleton & Thomas, 1992). Grazing intensity has been described to be the most important driver of net primary productivity

and plant cover composition, especially in semi-arid regions (Briske *et al.*, 2008). The most remarkable effects of overgrazing on the vegetation and soil are the compaction of the topsoil followed by an increase of soil temperatures, a decrease in aggregate size and stability and infiltration rates, and a reduction in soil organic matter input in the soil making soils more vulnerable to erosion (Bastida *et al.*, 2006).

Previous studies testing the effect of grazing on African rangelands emphasize that a sustainable grazing pressure is the ideal factor towards the maintenance of semi-arid rangelands (Kioko, Kiringe, & Seno, 2012). Although overgrazing is known to cause soil deterioration, and this in turn causes a decline in soil organic matter and soil biological activity, previous studies comparing plant richness between grazed and ungrazed plots found an enrichment of both plant richness and soil bulk density in the pastured plots compared with the ungrazed in Kenyan rangelands (Maitima, Mugatha, & Reid, 2009). However, in a recent review, Kiage (2013) underlined the lack of objectivity often found within those studies pointing to overgrazing as the main factor causing land degradation, emphasizing the strong impact of biophysical factors like climate, soil type and topography. Therefore, further evaluation of grazing management's effects on soil are needed (Moussa *et al.*, 2008), but also climate and other factors must be taken into consideration when studying the processes related to land degradation in African rangelands.

Figure 1.1. General aspect of different rangelands in Kenya

Other land use changes aside from grazing pressure have threatened African savannas as well, mainly agricultural intensification, resource extraction (wood, fuels...) and also land clearance by burning the area (Grace *et al.*, 2006). Because not only their socio-economical value, but also the particular biodiversity they contain and the potential to sequester carbon within other ecosystem services (Grace *et al.*, 2006), a

remarkable amount of studies have focused on African savanna conservation. During the last decades, many efforts have been done to design adequate management practices in order to improve soil quality and prevent from soil erosion and accelerated desertification (Eswaran *et al.*, 1997; Snyman, 1998; Lund, 2007; Reed, Dougill, & Taylor, 2007; Stringer & Reed, 2007). Fortunately, rangelands may recover from previous degradation due to inappropriate practices, not only from livestock management but also from agricultural intensification, if a sustainable utilization of the ecosystem is properly achieved (Kotzé & Elmarie, 2015).

Despite the scientific evidence pointing to sustainable management in rangelands, it is also essential that local policies take socio-economic history into account. A remarkable example is the ethnical group Masaai, in eastern Africa. They were initially nomadic, living exclusively on extensive grazing around large rangeland areas. Due to the progressive land adjudications, the increasing wildlife protected areas and agricultural intensification, Masaai land areas have been progressively reduced, accompanied by an increase of livestock stocking rates (Western *et al.*, 1994; Campbell *et al.*, 2000).

Figure 1.2. General aspect of different grasslands in the NE of Spain

1.3. European grasslands

Unlike African savannas, semi-natural grasslands are among the most important type of rangeland ecosystems in terms of land cover across Europe, increasing their extent since the Anthropocene (WallisDeVries,

Poschlod, & Willems, 2002; Habel *et al.*, 2013). This ecosystem is a result from the activities of humans and their livestock at low-intensity land use. After a long time of sustainable grazing management which turned grasslands into ecosystems of high biodiversity value, nowadays they are considered among the most important biodiversity hotspots around the globe (Habel *et al.*, 2013). The effects of grazing on plant species richness can be either positive or negative, depending on the type and density of livestock as well as on the whole particular environmental conditions (Olff & Ritchie, 1998).

In parallel to what is happening in African rangelands, several biotic and abiotic factors are threatening European grassland areas, being land abandonment and climate change among the main threats these grasslands face (Joyce & Wade, 1998; Hopkins & Del Prado, 2007). Because of the higher temperatures and more frequent drought periods expected within the proposed scenarios due to climatic change, the Mediterranean basin and the south of Europe are among the most vulnerable areas (Schröter *et al.*, 2005).

Due to the importance of these areas in terms of biological diversity (Kull & Zobel, 1991; Maarel & Sykes, 1993; WallisDeVries *et al.*, 2002), increasing the knowledge on the mechanisms that regulate the losses and relative abundances of the different taxonomic groups is needed for a successful management of these valuable ecosystems (Bonanomi, Incerti, & Allegrezza, 2013).

1.4. Soil microbial diversity in rangelands. An insight into the keystone species.

Soil microbial communities constitute a part of the rangeland's biota specially sensitive to changes in soil chemical and physical properties (Bardgett, Leemans, & Cook, 1997; Patra, Abbadie, & Clays-Josserand, 2005). They play a fundamental role in the stability and functioning of ecosystems, thus regulating biogeochemical processes and availability of nutrients for plants (Kotzé & Elmarie, 2015). Changes in plant cover and plant biomass are expected to affect the activity of soil microbial communities, as plant inputs are their main energy source (Moussa *et al.*, 2008). Land use change affecting plant cover then contributes to changes in microbial community composition and activity, and subsequently to those biogeochemical processes affecting soil quality (Mganga, Razavi, & Kuzyakov, 2016).

One of the paradigms which conservation biology rely on is the assumption that biodiversity promote resistance to disturbances such as drought (Tilman & Downing, 1996), and impact positively ecosystem processes (Spehn *et al.*, 2005). Fungi and bacteria are the main groups of decomposers in the soil, but their differential activity and responses to perturbations are poorly understood (Boer *et al.*, 2005).

Even within a single one of this microbial groups, a higher relative importance of particular species over others have been proposed and named "keystone species" (Mills, Soulé, & Doak, 1993). These are supposed to impact the biochemical cycles in ways that other species

cannot and fairy ring fungi are an example of such keystone species in grassland soils (Van der Wal *et al.*, 2013). Fairy ring fungi grow radially through the soil changing the soil physical and chemical properties which, in turn, affect other soil microorganisms composition and function (Shantz & Piemeisel, 1917; Edwards, 1984). This also translates into changes in plant species composition and growth (Fidanza, 2007a). These filamentous fungi have been studied as a mechanism regulating grassland's plant species diversity (Bonanomi *et al.*, 2013), as well as agents in nutrient cycling through soil organic matter decomposition (Van der Wal *et al.*, 2013).

Plants associate with a wide range of microbial symbionts (including both fungi and bacteria), and the resulting interactive effects on plant and soil communities are still poorly understood (van der Heijden *et al.*, 2016). The passage of the fairy ring fungi through grassland soils is expected to change all the belowground microenvironmental conditions (Fidanza, 2007a), thus affecting the existing pre-established synergies between plants and microbes. The detailed study of the microbial communities across fairy rings is a first step to better understand the extent to which a single fungal species can affect the surrounding plant-microbial communities. This increasing knowledge on the behaviour of the plant-microbial relationships is, in turn, essential for the development of sustainable models preventing the loss of grassland biodiversity, as a measure of soil quality preservation and against land degradation.

1.5. New horizons with improved techniques of study

1.5.1. The use of altitudinal gradients as a tool for climate change analysis

Climatic change is one of the most important factors affecting land degradation and loss of biodiversity (Schröter *et al.*, 2005). A useful way to study climatic changes are altitudinal gradients, due to the strong environmental variations over a relatively short geographical space (Körner, 2003). That is why they have been extensively used to create models that help to predict and to take measures against biodiversity losses in front of climatic changes (De Bello, Lepš, & Sebastià, 2005; Bello, Lepš, & Sebastià, 2006; Sebastià *et al.*, 2008; Bryant *et al.*, 2008). The study of grassland ecosystems along elevational and land use gradients have been mainly studied focusing on their aboveground macroorganisms but less attention has been paid to the belowground microbial community (Pellissier *et al.*, 2014). However, microbial community is known to play an essential role on biogeochemical processes (Zhang *et al.*, 2015). Therefore, understanding the changes occurring in the soil microbial community and the factors driving these changes is an essential task for understanding and predicting the response of grassland ecosystems to global climate change (Nacke *et al.*, 2011; Zhang *et al.*, 2015).

Although complex interactions have been argued to exist when studying the effects of climate on soil microbial community (Bardgett, Freeman, & Ostle, 2008), the actual biodiversity, and even less its

identification and function, are far from being discovered (Blackwell, 2011). pH has been recognized the single abiotic factor structuring the bacterial community composition at global scale (Lauber *et al.*, 2008; Rousk *et al.*, 2010; Yashiro *et al.*, 2016), but the microbial spatial pattern of distribution is still poorly understood (Bardgett *et al.*, 2008; Martiny, Eisen, & Penn, 2011; Tedersoo *et al.*, 2014).

Although there are several methodologies available to study the soil microbial community, we considered the use of enzyme activities and next generation sequencing as the most adequate methods according to our purposes.

1.5.2. Enzyme activity as soil quality indicator

Enzymes accumulate in soil mainly from microbial cells, but also to a lesser extent from animal and plant residues (Agrawal & Bahl, 1972; Tabatabai, 1982). Due to their fundamental role on nutrient cycling, the resulting soil enzyme activities are recognized as useful biological indicators of soil quality (Dick, 1994) and they have already been used as a sensitive indicator of soil stress in studies of land management and soil restoration in Africa (Badiane *et al.*, 2001).

Adolfsson (2008) focused on soil enzyme activities of African soils in a land use gradient and found an increase in several soil parameters and soil quality indicators (soil microbial biomass, C and N content, and pH) when decreasing exploitation pressures. They concluded that deforestation, agricultural intensification and overexploitation of natural resources translate into a reduction of soil microbial functional capacity.

Mganga, Razavi, & Kuzyakov (2015) studied the effects of a wide range of land uses on soil microbial activity in East Africa and found a sharp decline in the enzyme activities when increasing land use intensity, but they did not include different grazing pressures in their study. Previous studies focusing on the effect of grazing intensities on enzyme activities in grasslands around the globe found a decrease in enzyme activities under heavy grazing (Holt, 1997; Singh & Rai, 2004), but also a recovery of these activities after livestock exclusion (Yong-Zhong *et al.*, 2005). Several studies addressing the effect of livestock grazing management systems on African soil properties and land degradation showed inconclusive results or diverging conclusions (Moussa *et al.*, 2008; Kgosikoma, 2013; Kgosikoma, Mojeremane, & Harvie, 2015). Moussa *et al.* (2008) compared soil organic carbon and microbial biomass from grazed and ungrazed rangeland soils, and found no statistical significance, maybe due to the similarity in herbaceous composition between treatments. Other studies reported a loss in soil organic C and N as well as a depletion of soil microbial properties as a result of high grazing pressure in semi-arid grasslands (Su *et al.*, 2004). There is an urgent need to assess soil responses to overgrazing and rangeland degradation in order to develop sustainable land management strategies (Snyman & du Preez, 2005) and microbial parameters have proved to be a useful way to measure soil quality status under different land managements (Badiane *et al.*, 2001).

1.5.3. Next generation sequencing as a method to asses soil microbial diversity

In recent years, our understanding of the composition and distribution of the microbial communities has improved greatly due to the development and increasing availability of Next-Generation Sequencing (NGS) techniques (Zhang *et al.*, 2015). These technologies have unraveled a huge soil microbial diversity, larger than previously expected (Buée *et al.*, 2014). By targeting specific fragments of the genome, DNA metabarcoding allows for the characterization of the soil microbiota and the assessment of the taxonomic diversity of a given soil (Orgiazzi *et al.*, 2013, 2015). These data can, in turn, be related to different factors such as land use or climate change, being especially useful when studying elevational gradients.

1.6. Objectives of this thesis

The overall objective of this thesis is to increase the understanding of the relationship between biodiversity and ecosystem functioning in rangelands of Africa and Europe, and the effects of this relationship on the supply of ecosystem goods and services.

The specific objectives of the thesis are:

1) To analyze the changes in the specific and functional diversity of microorganisms in grasslands along broad climatic and land use gradients in two continents.

2) To analyze whether variations in particular plant-microorganism interactions translate into changes in ecosystem processes, by focusing on microbial keystone taxa.

Chapter 2

Effect of land use on Eastern African soils

2. Soil fertility and microbial activity change with land use and management in Eastern African ecosystems

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Abstract

We wanted to investigate the effect of land use change on soil fertility and activity in Western Africa. We used environmental data and soil parameters included in the ClimAfrica database from two countries, Kenya and Tanzania, to assess how land use and management affect soil organic carbon and fertility distribution. In addition, we conducted a detailed analysis of soil activity and fertility in three differently managed areas in Kenya: National Park of Chyulu Hills, used by wild animals only; KALRO experimental agricultural station of Kiboko, moderately grazed; heavily grazed Maasailand in the Kajiado region. Land cover and land use intensity were the main parameters explaining soil organic carbon distribution in the ClimAfrica dataset, with bedrock and soils leaving a strong footprint. Plots in the soil activity study showed idiosyncratic effects, probably related with variations in bedrock, soil type, land use history and plant functional type diversity. Nonetheless, land use intensity was the most relevant environmental factor explaining exoenzyme activity. Soils from the protected Chyulu Hills National Park in Kenya showed the highest values of soil microbial activity indicators, including microbial carbon (C) and nitrogen (N) biomass, and dissolved C and N, as well as activity of N-cycling related enzymes (peroxidase, protease and exochitinase). Soil samples from the most heavily grazed Kajiado Maasailand area showed higher inorganic N concentrations, which can be easily lost by occasional rains. In general, enzymatic activity decreased with land use intensification, except for

phenoloxidase, which was highest in the plot from the agricultural station of Kiboko including the highest plant functional type diversity in this study; it was probably associated to recalcitrant litter in this moderately grazed woody savanna. Phosphatase activity showed the lowest differences among plots, in spite of marked differences in soil phosphorus content. In conclusion, land use, intensification and management can override other environmental effects on soil fertility, organic carbon storage and exoenzymatic activity.

Keywords: exochitinase; exoglucanase; peroxidase; phenoloxidase; phosphatase; protease; soil organic carbon; soil phosphorus; soil nitrogen

2.1. Introduction

Land degradation, defined as undesirable changes in the state of land due to natural and human-made factors (Young, 1999; Stocking & Murnaghan, 2001), has been identified by numerous studies as one of Africa's major socio-economic and environmental problems (Nana-Sinkam & ECA, 1995; Ayoub, 1999; UNEP, 2006; Sulieman & Buchroithner, 2009), with consequences on food security, poverty, and environmental and political stability (Sivakumar & Ndiang'Ui, 2007). Depletion of soil organic carbon (SOC), in particular, has been recognized a major process in soil degradation, particularly in tropical environments (Nandwa, 2001; Marks *et al.*, 2009). Indeed, SOC plays a vital role in soil fertility maintenance (Palm & Myers, 1997; Marks *et al.*, 2009) as well as in soil water-holding capacity and the prevention of erosion and

desertification. In addition, terrestrial carbon sequestration is also an important component of atmospheric carbon mitigation, therefore, providing ecosystems services at the local and global scales (Marks *et al.*, 2009).

SOC content is the sum of soil organic inputs driven by plant productivity (root exudates, root and shoot turnover) or exogenous contribution (mineral fertilizers or manure), and soil organic losses via soil heterotrophic respiration, mineralization or erosion. Land use changes and management practices have the potential to affect SOC stocks in different ways (Conant *et al.*, 2017) but more information about the most adequate practices for soil fertility conservation and climate change mitigation is still needed.

In addition to SOC, soil activity is also used to monitor soil quality (Mganga *et al.*, 2016). Soil enzymes are important for catalyzing innumerable reactions necessary for life processes of micro-organisms in soils (Dahm, Szajdak, & Goliñska, 2011). Soil enzymes catalyze organic matter degradation and are critical for carbon and nutrient cycling and the formation of organic matter and soil structure (Dahm *et al.*, 2011; Nottingham *et al.*, 2016). In semi-arid grasslands, a high proportion of oxidative soil enzymes compared to hydrolytic soil enzymes could be driving high organic matter decomposition rates (Shukla & Varma, 2011).

During the last decades, many efforts have been done to design adequate management practices in order to improve soil quality and prevent soil erosion and accelerated desertification (Eswaran *et al.*, 1997; Snyman, 1998; Lund, 2007; Reed *et al.*, 2007; Stringer & Reed,

2007). Rangelands have been reported to recover from previous degradation due to inappropriate practices, not only from livestock management but also from agricultural intensification, after a sustainable utilization of the ecosystem is properly achieved (Kotzé & Elmarie, 2015). In Africa, studies in the Kilimanjaro region show how traditional agroforestry systems promote soil fertility compared with other agricultural practices, with natural forests being the ecosystems accumulating higher carbon and nitrogen (N) proportions (Mganga *et al.*, 2016). Forests also stored higher SOC stocks than other ecosystems in Western African countries in the ClimAfrica database (http://www.climafrica.net/index_en.jsp, Poch et al. 2017), while savannas held the highest SOC stocks in Eastern Africa according to the same source (Poch *et al.*, 2017).

Among African ecosystems, African rangelands are dominated by savannas located in arid or semi-arid environments, covered by large extensions of grasses interrupted by trees and shrubs (Kiage, 2013). Agropastoralism is the main human activity in these areas, and human dependence on the products coming from livestock on rangelands is especially pronounced in developing countries of Africa (Talbot, 1986), leading farmers to reach unsustainable livestock densities (Vetter, 2013). High stocking rates are considered to largely contribute to land degradation and desertification (Middleton & Thomas, 1992). Grazing intensity has been described to be the most important driver of net primary productivity and plant cover composition, especially in semi-arid regions (Briske *et al.*, 2008). Syntheses on grassland management report

that improving grassland management practices improve soil carbon stocks, and that SOC stock changes can be low between properly managed grasslands and native vegetation (Conant *et al.*, 2017); but soil C stock responses vary as a function of climate, soil, vegetation and livestock type (McSherry & Ritchie, 2013; Conant *et al.*, 2017; Zhou *et al.*, 2017). Generally speaking, grazing has been found to decrease belowground C and N pools, particularly microbial biomass C and N (Zhou *et al.*, 2017). In semi-arid areas, a decrease in SOC and total nitrogen (TN) has been found in grazed land (Wen *et al.*, 2013; Olivera *et al.*, 2014), linked to a reduction in grass cover and plant litter mass (Olivera *et al.*, 2014). Furthermore, studies suggest changes in soil enzyme activity and microbial biomass C and N with grazing, although there is not a consensus about the direction of those changes (Wen *et al.*, 2013; Olivera *et al.*, 2014; Mganga & Kuzyakov, 2014). Other studies find no differences between grazed and protected areas in Eastern Africa, suggesting that soil carbon sequestration is not well understood in drylands of Africa (Aynekulu *et al.*, 2017). Grazing intensity might be affecting the magnitude and direction of changes in belowground C and N pools and fluxes (Zhou *et al.*, 2017) and could contribute to explain the disparity of results. The most remarkable effects of overgrazing on vegetation and soil are the compaction of the topsoil followed by an increase of soil temperatures, a decrease in aggregate size and stability and infiltration rates, and a reduction in soil organic matter input to soil, making soils more vulnerable to erosion (Bastida *et al.*, 2006).

In addition to the generation of scientific evidence pointing to appropriate practices for sustainable management of African ecosystems, it is also essential for local policies to take socio-economic history into account. A remarkable example is the ethnical group Maasai, in Eastern Africa. They were initially nomadic, living exclusively on extensive cattle-grazing around large rangeland areas. Due to the progressive land adjudications, increasing wildlife-protected areas and agricultural intensification, Maasai land areas have been progressively reduced, accompanied by an increase of livestock stocking rates (Western *et al.*, 1994; Campbell *et al.*, 2000).

We wanted to investigate the effect of land use changes on soil fertility and activity in Eastern African ecosystems. We focused on the upper 10 cm soil layer, where we expect the land use effects to be more conspicuous; we also report information about the upper 30 cm soil layer. We used environmental data and soil parameters included in the ClimAfrica database (http://www.climafrica.net/index_en.jsp) from two countries, Kenya and Tanzania, to assess how land use affects soil fertility and carbon storage. In a context of climate change, the ClimAfrica project (EU VII Framework Programme) aimed at assessing the soil organic carbon stocks under different climatic conditions in African ecosystems; carry out a basic diagnostic of soil fertility; and assess the relationship between those with land cover, land use, land use intensification and management. Climate change can alter terrestrial C fluxes indirectly, by for instance acting as a driver of land use change (Falloon *et al.*, 2007). Africa could be a small C sink (Valentini *et al.*,

2014), including dry tropical cropland in Eastern Africa (Sugihara *et al.*, 2012), but emissions from land use changes in this continent contribute to a significant fraction of total emissions (Canadell, Raupach, & Houghton, 2009; Valentini *et al.*, 2014).

Low chemical fertility and low organic matter content has been recognized among the most common problems of the soils studied in ClimAfrica, and measures to preserve the quality of the medium- high-quality soils have been proposed (Poch *et al.*, 2017). Soils from Eastern Africa were acknowledged among the best soils in the ClimAfrica dataset (Poch *et al.*, 2017), and tools to evaluate and predict the evolution of soil quality in front of possible future changes in those areas are urgently needed.

In addition of an in-depth description of the soil fertility and organic carbon distribution in Eastern African ecosystems in the ClimAfrica dataset, we conducted a detailed analysis of soil fertility and microbial activity in three differently managed areas in Kenya along land use intensity gradients: the protected National Park of Chyulu Hills, grazed only by wild animals; the KALRO Arid and Range Lands Research Institute of Kiboko, managed according to sustainability criteria; and heavily grazed Maasailand in the region of Kajiado. We investigated four hydrolytic (exoglucanase, exochitinase, phosphatase, protease) and two oxidative (phenoloxidase, peroxidase) soil enzymes. Exoglucanase and phenoloxidase are considered to be linked to C cycling; exochitinase, protease and peroxidase to N cycling; phosphatase to phosphorus (P) cycling (Dalmonech, Lagomarsino, & Moscatelli, 2009; Steinauer *et al.*,

2015; Nottingham *et al.*, 2016). We wanted to assess how enzymatic activity related to the observed soil organic carbon dynamics along land use and land use intensity gradients in Eastern African savanna ecosystems.

2.2. Materials and Methods

2.2.1 Location

We selected four land covers typical from the savanna region in Eastern Africa covering a gradient of canopy density: evergreen shrubland with relatively dense woody canopy; woodland savanna with sparse woody canopy; open mesic grassland; and open cropland. We aimed at assessing soil fertility distribution, and soil conservation and function, in the main Eastern African ecosystems. We established 18 100 (in open ecosystems) or 400 m^2 (in closed shrubland savanna) plots in the ClimAfrica Case Study regions of Kenya (13 plots) and Tanzania (5 plots). In Kenya, four different locations were selected along the north-south axis in Eastern Kenia (1°48'N, 37°37'E). Three of the locations were covered mostly by savanna-type ecosystems distributed along a land use intensity gradient including: the protected Chyulu Hills National Park (Makueni County), where agricultural activities are banned and wild animals are the only grazers; the KALRO Arid and Range Lands Research Institute of Kiboko (Makueni County), protected and managed according to sustainability criteria, including medium-pressure grazing by cattle and goats; and heavily used Maasailand in the neighboring Kajiado

County, overgrazed by cattle and goats. Crops were studied in Makueni, the fourth location in Kenya (Table 2.1). Overall, the sampled regions in Kenya had an altitude of 1000 to 1200 m above sea level and presented very similar environmental conditions. Rainfall yearly distribution in the Kenya study area is bimodal, with the "long rains" falling in March to May and the "short rains" occurring from mid-October to December (Siderius & Muchena, 1977) with a mean annual precipitation (MAP) in the sampled region of 601.4 mm and a mean annual temperature (MAT) of 22.8ºC. The main soil type in the area of the surveyed spots, according to (Jones *et al.*, 2013) are Rhodic Ferralsols, but in the surveyed spots there are also Haplic Luvisols, Rhodic and Umbric Nitisols (Makueni, Kiboko); Chromic Luvisols and Haplic Vertisols (Kiboko) formed from undifferentiated gneisses; and Mollic Silandic Andosols (Chyulu Natural Park), formed from fine gravel-sized volcanic ashes and olivine basalts.

The sampled region of Ikowa in Tanzania was warmer and drier than areas sampled in Kenya, with 567 mm MAP and 23ºC MAT. Altitude was also lower than in the Kenyan plots, below 900 m above sea level. Bedrock was gneiss and biotite-rich granite. The area consists largely in dense deciduous thicket (mainly in the Ikowa hills), rangelands and rainfed croplands in floodplains and valley bottom. Main crops in the area are sorghum, millet, maize and sunflower. Livestock production is, however, the most important economic activity as well as the means for wealth and social status. Population is composed by a single ethnic indigenous group, the Gogo people, who are cultivating pastoralists. Land degradation due to overgrazing has traditionally been the main

environmental risk in the area, although firewood deforestation has also played a relevant role. All these attributes are, in general, quite typical of East African drylands systems.

Table 2.1. Location, land use, climate and summary fertility indicators of the ClimAfrica plots from Kenya (Chyulu, Kiboko, Kajiado, Makueni) and Tanzania (Ikowa) for two soil depth layers, 0-10 and 10-30 cm. Mean values are shown in the first row and 1 SE is shown below (when applicable).

Location	Land cover	Land use	MAP	MAT	MT min	MT max	Soil Depth	BD	SOCS	SOC	TN	TP	C-to-N	N-to-P	pH
Chyulu	Mesic	National Park	601	22.8	17	29	0-10 cm	1.1	3.2	3.0	0.2	4.0	14.5	0.1	7.8
	grassland	Wildlife grazing						0.0	0.2	0.2	0.0	1.6	0.9	0.0	0.2
							10-30 cm	1.2	6.4	2.7	0.2	3.3	13.7	0.1	7.6
								0.0	0.8	0.3	0.0	1.9	1.5	0.1	0.2
Chyulu	Savanna	National Park	601	22.8	17	29	0-10 cm	1.2	3.4	3.0	0.3	3.0	10.2	0.2	7.9
		Wildlife grazing						0.0	0.5	0.4	0.0	1.4	0.1	0.1	0.0
							10-30 cm	1.2	3.9	1.6	0.2	2.5	9.9	0.1	8.0
								0.0	0.8	0.4	0.0	1.1	0.1	0.1	0.1
Kiboko	Savanna	Model farm	601	22.8	17	29	0-10 cm	1.2	1.7	1.5	0.1	113.0	13.0	0.0	6.1
		Livestock						0.0	0.1	0.1	0.0	47.4	2.4	0.0	0.1
		grazing					10-30 cm	1.2	3.0	1.2	0.1	77.7	11.8	0.0	6.1
								0.0	0.2	0.1	0.0	51.2	1.4	0.0	0.1
Kajiado	Savanna	Maasai land	601	22.8	17	29	0-10 cm	1.2	1.6	1.4	0.1	139.0	10.6	0.0	6.3
		Overgrazed													
							10-30 cm	1.2	2.5	1.0	0.1	53.0	10.2	0.0	6.1

Kajiado	Shrubland	Maasai land	601	22.8	17	29	0-10 cm	1.2	1.8	1.4	0.1	43.5	10.3	0.0	6.2
		Overgrazed						0.1	0.4	0.2	0.0	24.5	0.0	0.0	0.5
							10-30 cm	1.4	3.0	1.1	0.1	53.5	14.5	0.0	6.2
								0.1	1.0	0.3	0.0	45.5	4.5	0.0	0.5
Makueni	Cropland	Maize/Cowpea	601	22.8	17	29	0-10 cm	1.2	0.7	0.6	0.1	47.5	9.4	0.0	6.6
		Manure (500)						0.0	0.0	0.0	0.0	17.5	0.4	0.0	0.3
		Ungrazed					10-30 cm	1.2	1.4	0.6	0.6	87.5	0.9	0.0	5.9
								0.0	0.0	0.0	0.0	17.5	0.0	0.0	0.0
Ikowa	Shrubland	Livestock	567	23	18	30	0-10 cm	1.2	1.6	1.4	0.1	10.2	24.5	0.0	6.0
		grazing						0.0	0.8	0.6	0.0	5.9	4.2	0.0	0.7
							10-30 cm	-	-	0.9	0.1	4.0	12.2	0.0	6.3
								-	-	0.3	0.1	1.2	7.7	0.0	0.5
Ikowa	Mesic grassland	Livestock grazing	567	23	18	30	0-10 cm	1.2	1.3	1.1	0.1	61.1	21.4	0.0	7.4

Table 2.2. Location and environmental characteristics of plots in the soil activity study in Kenya. SC: sandy clay; SL: sandy loam; C: clay; SCL: Sandy clay loam; LS: Loamy sand.

Location	Plot	Altitude	Land Cover	Texture	Bedrock	Stocking rates	Livestock	Wild herbivores
Chyulu Hills	CH-SAV	1169	Savanna	LS	Volcanic Ash	Abandoned	-	Elephants and giraffes
	CH-GRA	1076	Mesic grassland	SL	Volcanic Ash	Abandoned	-	Elephants and giraffes
Kiboko	KI-SAV	1035	Savanna	SC	Gneiss	Medium pressure	Cattle	Elephants
	KI-SAV2	1154	Savanna	C	Gneiss	Medium	Cattle	Elephants
Kajiado	KA-SAV	1045	Savanna	SCL	Gneiss	Overgrazed	Cattle	Elephants
	KA-SHR	1011	Shrubland	SC	Olivine basalts	Overgrazed	Cattle	Elephants

2.2.2. Experimental design

Sampling followed the ClimAfrica common protocol for field work developed in the ClimAfrica Case Studies. A stratified-random sampling approach for the soil site selection was used, based on land cover, bedrock, slope, aspect and basic topographic units. Overall, we established four plots on open mesic grassland; six plots on sparse woodland savanna; six plots on evergreen shrubland with relatively dense woody canopy; and two plots on rainfed crops, one on maize and the other one on cowpea. All plots were on flat areas or gentle slopes.

In addition to the ClimAfrica sampling, we carried out a detailed study to determine if there are shifts in soil fertility and microbial activity along land use intensity gradients in savanna ecosystems in Eastern Africa. Specifically, we sampled six plots in the focal areas of Kiboko, Kajiado and Chyulu in Kenya in June 2013 (Table 2.2). Land covers represented in this more detailed sampling were treeless mesic grassland (Chyulu), and savanna ranging from open woodland grassland to dense evergreen shrubland (Chyulu, Kiboko and Kajiado). Two separated plots were selected within each study location for the land use intensity comparison. In Chyulu, a shrubland savanna and a grassland were selected, dominated by *Acacia mellifera* (M. Vahl) Benth. and *Chrysopogon aucheri* (Boiss.) Stapf respectively, both on volcanic ashes (Table 2.2). The Kiboko plots were established on shrubland savanna dominated by *Acacia senegalensis* (Houtt.) Roberty on gneissic bedrock (Table 2.2). The two plots in the Kajiado region occupied by the Maasai ethnic group were both shrubland savanna with sparse to dense shrub

cover, with vegetation dominated by *Balanites aegyptiaca* Del. on gneiss, and *Acacia mellifera* on olivine basalt respectively (Table 2.2). In all three areas we intended than one plot was more intensively used than the other. At each plot, three patches of roughly 400 cm^2 were arbitrarily selected for each of the dominant plant functional types (PFT) present in each plot, the most common of which were grasses, woody legumes and non-legume forbs.

2.2.3. Soil sampling and chemical determinations

2. 2.3.2. Soil fertility parameters of Eastern African ecosystems

In the study on soil fertility distribution in Eastern African ecosystems, measurements were carried out according to the ClimAfrica common Protocol for Field Soil Sampling and analysis (Poch *et al.*, 2017). A soil micropit of 30 x 30 x 30 cm was dug and a horizontal soil sample was extracted with a metallic cylinder (minimum volume 150 cm^3) from the center of each of two soil depths, 0-10 cm and 10-30 cm. Samples were brought to the laboratory and air-dried. A representative subsample of each fine soil sample (minimum 100 g) was separated for further analysis.

Physico-chemical characterization in the laboratory consisted of organic carbon (Walkley-Black) in the upper 10 (SOC10) and upper 30 cm (SOC30) soil layer, total nitrogen (Kjehldahl) in the upper 10 (TN10) and upper 30 cm (TN30) soil layer, extractable phosphorus (Bray) in the upper 10 (TP10) and upper 30 cm (TP30) soil layer, pH (water) in the

upper 10 (pH10) and upper 30 cm (pH30) soil layer, and particle size distribution with the hydrometer method (Page, Miller, & Keeney, 1982), including the percentage of clay, silt and sand in the upper 10 and upper 30 cm soil layer (Clay10 and Clay30; Silt10 and Silt30; and Sand10 and Sand30 respectively). Soil organic carbon stocks were calculated only for the samples from Kenya (SOCS10 and SOCS30), because no information on bulk density was available for Tanzania. We calculated the C-to-N ratio (CN) by dividing the proportion of soil organic C content by the proportion of N in the samples (CtoN10 and CtoN30 for the upper 10 and 30 cm soil layer respectively); and the N-to-P ratio (NP) by dividing TN among TP in the same soil layers (NP10 and NP30).

2.2.3.2. Microbial activity along land use intensity gradients in Kenya

To determine soil fertility and microbial activity along the land use intensity gradient study in Kenya, soil sampling took place between the 13 and 15 June 2013. Soil samples were collected to a depth of approximately 10 cm (A horizon) in all PFT patches described above. Soils were placed in non-sealed plastic bags and kept refrigerated. Once in the laboratory, all samples were carefully sieved (2 mm) and re-distributed in five 100 g subsamples. Four of them were frozen (-20ºC) and the last one stored refrigerated (+4ºC) until further processing.

Soil moisture content was determined gravimetrically by drying 5 g fresh subsamples at 105°C for 24h. Aliquots of these dry samples were used for total C and N measurement with an EA 1110 elemental analyzer

(CE Instruments, Milan, Italy) coupled to a Finnigan MAT DeltaPlus IRMS with a Finnigan MAT ConFlo II Interface (both Thermo Fisher Scientific, Waltham, MA, USA). We separated 2 g fresh soil sub-samples, extracted with 20 ml 1M KCl solution and shaken at room temperature for 30 min. Extracts were filtered (Whatman 40 ashless cellulose filter) and aliquots were immediately used for pH measurements. Extracts were stored at -20ºC before further analysis. Aliquots of these extracts were used to measure dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) by a TOC/TN analyzer (TOC-V CPH E200V/TNM-1 220V, Shimadzu, Vienna, Austria).

Ammonium and nitrate were determined colorimetrically from aliquots of the 1M KCl soil extracts. Ammonium was quantified by an indophenol dye, measuring the absorbance at 660 nm according to Kandeler & Gerber (1988); nitrate content was measured by the Vanadium (III) chloride (VC_{l3}) and Griess method and the absorbance was read at 540 nm according to (Miranda, Espey, & Wink, 2001, Tecan Infinite M200 fluorimeter, Werfen, Austria). Dissolved organic N (DON) was calculated as the difference between total dissolved and inorganic N.

Microbial biomass C (Cmic) and N (Nmic) were determined by the chloroform-fumigation extraction method (Amato & Ladd, 1988), where 2 g fresh soil sub-samples were weighed in aluminum dishes and kept in a desiccator with wet tissue papers within an ethanol-free chloroform atmosphere for 24h at room temperature. After incubation soil samples were extracted as described above. Organic carbon and total nitrogen

were measured by a TOC/TN analyzer and the difference between extracts of fumigated and unfumigated sub-samples was used to estimate Cmic and Nmic.

The potential activities of six extracellular enzymes were measured according to Kaiser *et al.* (2010). 4-Methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC) were used for substrate labelling of the hydrolytic enzymes. MUF-β-D-cellobioside was used for β -1,4-Cellobiosidase (exoglucanase), MUF-N-acetyl-β-D-glucosaminide for β-1,4-N-acetylglucosaminidase (chitinase), L-leucine-AMC for leucine aminopeptidase (protease) and MUF-phosphate for phosphatase (phosphatase) activity. Plates were incubated for 150-180 min in the dark and fluorescence was measured at 450 nm emission at an excitation at 365 nm (using a Tecan Infinite M200 fluorimeter, Werfen, Austria). Peroxidase and phenoloxidase activities were measured photometrically as described in Kaiser *et al.* (2010) using L-3,4-dihydroxyphenylalanin as a substrate. Plates were incubated for 2h and 21h and absorbance was read at 450 nm after and before incubations.

2.2.4. Data analysis

2.2.4.1. Soil fertility distribution in Eastern African ecosystems

We performed analysis of variance on six soil fertility variables from two soil layers, upper 0-10 and upper 0-30 cm: soil organic carbon content (SOC) and soil organic carbon stocks (SOCS), total N (TN), the C-to-N ratio (CN), total phosphorus (TP) and the nitrogen to phosphorus N-

to-P ratio (NP). The following environmental variables were used as fixed environmental descriptors: country (used as surrogate for climatic variables, Tanzania being warmer and drier than Kenya), land cover, land use intensity (LUI) and bedrock. In addition, we built and tested a new variable by combining land use and land use intensity (LU-LUI), with eight levels. We applied Tukey's tests for means comparison, and the Akaike Information Criterion (AIC) for model comparison. Residual analysis recommended that both SOC and SOCS be ln transformed and thus we conducted further modeling on SOCS variables in the ln scale. Bulk density in the upper 10-30 cm soil layer was not available for the Tanzanian samples, and thus SOCS in that layer (SOCS1030) could not be calculated; therefore, SOCS30 were calculated only for Kenyan soils.

We performed multivariate ordination analysis on data from the ClimAfrica database in order to assess the relative distribution of sampled plots according to soil fertility variables. We applied unconstrained Principal Component Analysis (PCA), using soil fertility parameters from the upper 10 cm soil layer. The soil fertility descriptors in the PCA were: soil organic content (SOC10); total nitrogen content (TN10); total phosphorus concentration (TP10); and the C-to-N ratio (CN10); and textural fractions and pH, which could be good predictors of soil fertility. The NP ratio did not add substantial information to the analysis and was thus removed from the analysis, reducing the number of descriptors in the PCA.

2.2.4.2. Soil fertility and exoenzymatic activity in Kenya

We applied unconstrained ordination by PCA on soil fertility and exoenzymatic activity data along land use gradients in Kenyan savanna ecosystems. In total we used 13 fertility variables: pH, water content, TN, TOC, C-to-N, DOC, Cmic, Nmic, ammonium (NH_4), nitrate (NO_3), TDN, DIN, DON; and the six analyzed soil exoenzymes: exoglucanase, exochitinase, phosphatase, protease, phenoloxidase, peroxidase. We applied backwards-forwards generalized linear models to the six exoenzymes, including as explanatory environmental variables: plot, land cover, land use intensity, bedrock, soil type, pH, soil water content and texture (using the dummy variable clayed). Land use intensity (LUI) was measured through a categorical variable with four categories: low intensity in the savanna from the Chyulu National Park, occasionally grazed by elephants and giraffes; moderate intensity in the mesic grassland in Chyulu, frequently visited by wild grazers, and in the Agricultural station of Kiboko, moderately grazed by livestock; high and very high intensity in the Maasailand of Kajiado, overgrazed by livestock, in the savanna and the evergreen shrubland respectively (Table 2). A similar semi-quantitative variable, land use pressure, ranging from 1 in lightly grazed savanna in the National Park to 4 in the most heavily grazed shrubland area in Maasailand, was also tested, but LUI performed better in the tested models. After an initial set of models developed for the six exoenzymes, we developed univariate models for each one of the environmental variables and calculated the R^2_{adj} for each explanatory

factor per enzyme. We then run another set of multivariate generalized linear models on each variable, initially including all the descriptor variables. Multivariate models were compared among them and with the best univariate models through the Akaike Information Criterion (AIC), and the anova function in R (R Development Core Team, 2008) to select the best model for each exoenzyme. We performed analysis of variance and Tukey tests for means comparison on the best single descriptors. We display boxplots of each enxoenzyme per plot, with plots ordered according to the land use intensity gradient (LUI being usually the second best single descriptor after plot) and include letters from Tukey tests results to compare plots.

All multivariate analyses were performed using CANOCO (Braak & Šmilauer, 2012). Other analyses were performed in R (R Development Core Team, 2008).

2.3. Results

2.3.1. Soil fertility distribution in Eastern African ecosystems

The highest bulk density values were found in the heavily overgrazed shrubland ecosystems in Kajiado, Maasailand (Table 2.1). The highest pH values were found in the Chyulu National Park (Table 2.1), on volcanic ash and andisols. This area also showed the highest SOCS and SOC values and lowest TP contents (Table 2.1).

The best model explaining the variability of SOCS10 distribution included land use and bedrock (Table S2.1 in Supplementary Material).

According to this model, crops held the lowest SOCS compared to other land uses ($P < 0.05$; Table 2.1, Fig. 2.1), and there was a tendency for SOCS10 to be higher in volcanic ashes than in basalt substrates ($P = 0.08152$). Models including soil organic carbon content or storage variables, and those developed for TN10, TP10 and TP30, involved those same explanatory variables (Table S2.1 in Supplementary Material). Grasslands and savannas held higher SOCS30 than crops ($P < 0.05$; Fig. 2.1). SOCS30 were higher in volcanic ashes than in other substrates, particularly gneiss ($P < 0.0001$; Table 2.1). SOC10 and SOC30 models behaved similarly to those for SOCS10 and SOCS30 respectively.

Similar to C, volcanic soils accumulated higher N contents in the upper 10 cm soil layer than other bedrocks ($P < 0.05$; Table 2.1, Fig. 2.2). Crops showed a tendency to present lower soil TN10 contents than other ecosystems (Table 2.1, Fig. 2.2). The C-to-N ratio was more closely related to land use and land use intensity factors than either N or C, but this tendency was stronger in the upper 10 cm soil layer than in-depth (Table S2.1 in Supplementary Material).

CN10 was related to land use in a complex way. Moderately grazed shrubland showed higher CN10 contents than croplands ($P = 0.0066$) and moderately grazed savannas ($P = 0.0190$). Furthermore, the best model for CN10 also included bedrock, with biotite showing higher CN10 than basalt (0.04223) and slightly higher than volcanic (0.0731). There was a tendency for Tanzanian soils to be less fertile than those from Kenya (Table 2.1). The most striking difference in soils from both countries was

a much higher CN10 ratio in Tanzania compared to Kenya ($P < 0.0001$), although CN30 was similar in both (Table 2.2; Fig. 2.2).

The best models fitting TP10 and TP30 included both bedrock and land use, however, none of the models were significant. Phosphorus content was lower in natural than in managed areas, the former located on volcanic ash (Table 2.1). Finally, bedrock was the only best predictor of the N-to-P ratio in both soil layers (Table S2.1 in Supplementary Material); volcanic bedrock held higher NP10 ratios than gneiss ($P < 0.01$) and biotite ($P < 0.05$); and NP30 than basalt ($P < 0.05$).

According to the PCA, the first PCA ordination axis of the Eastern African ClimAfrica dataset, explaining 45.8% of the sample variability, was related to a fertility gradient, partly related to land use intensity, climate and bedrock (Fig. 2.3). Samples from savannas and grasslands in the Chyulu Hills National Park in Kenya were located on the far positive end of PCA axis 1, while samples from shrublands and grasslands of Ikiwa, in Tanzania were located by the negative end (Fig. 2.3). SOC10, TN10, Sand10 and pH10 increased towards the positive side of this axis, while TP10, CN10 and Clay increased towards the negative side (Fig. 2.3). The second axis (explaining 17.7% of the variability) represented a land use gradient, with natural ecosystems with fertile soils distributed towards the negative side and crops towards the positive end of PCA axis 2 (Fig. 2.3).

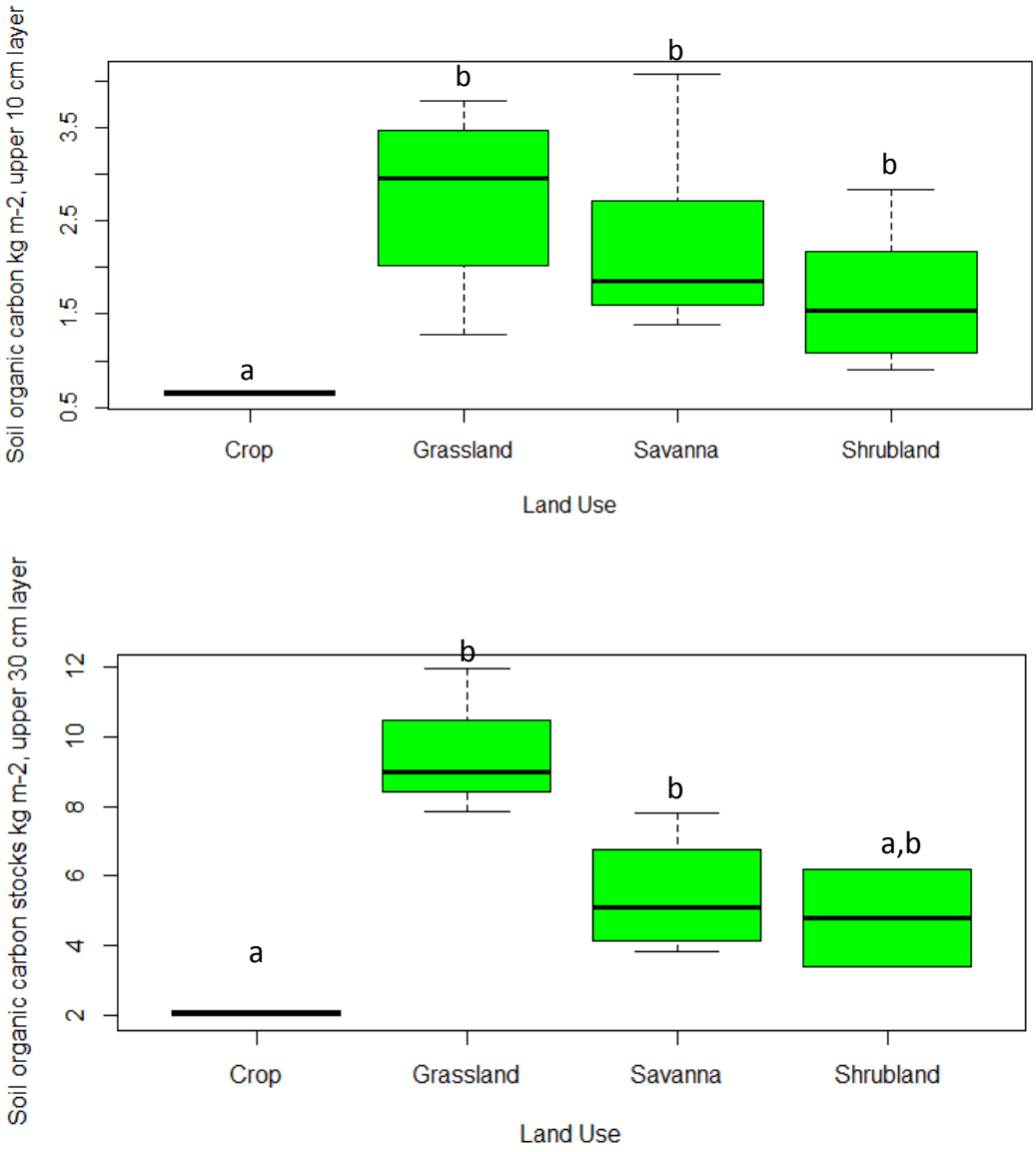


Figure 2.1. Soil organic carbon stocks distribution in four land cover types in the upper 10 cm soil layer (above) and in the upper 30 cm soil layer (below). Above, data based on all samples; below, data from Kenya only.

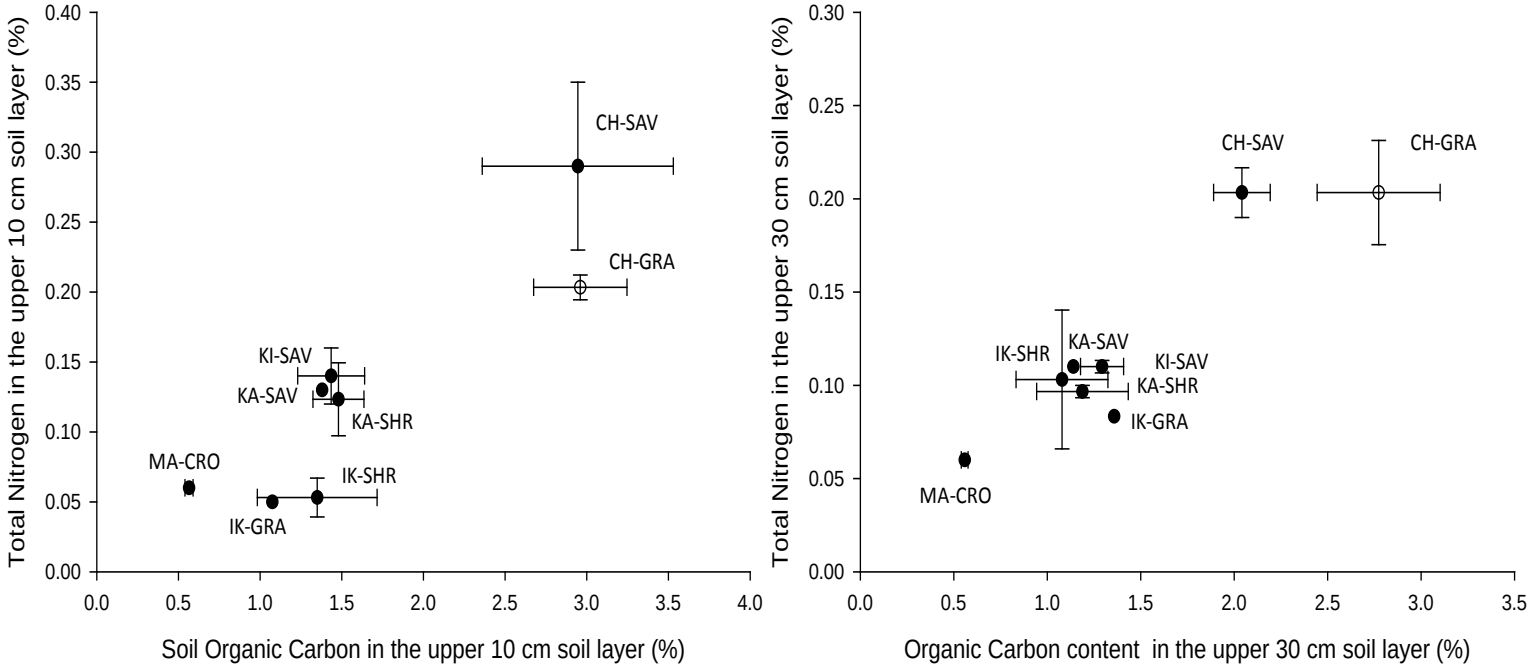


Figure 2.2. Soil total nitrogen and soil organic carbon in the upper 10 (left) and 30 (right) cm layer according to land use and location, which is a rough indicator of land use intensity. The C-to- N ratio can be estimated from the figures.

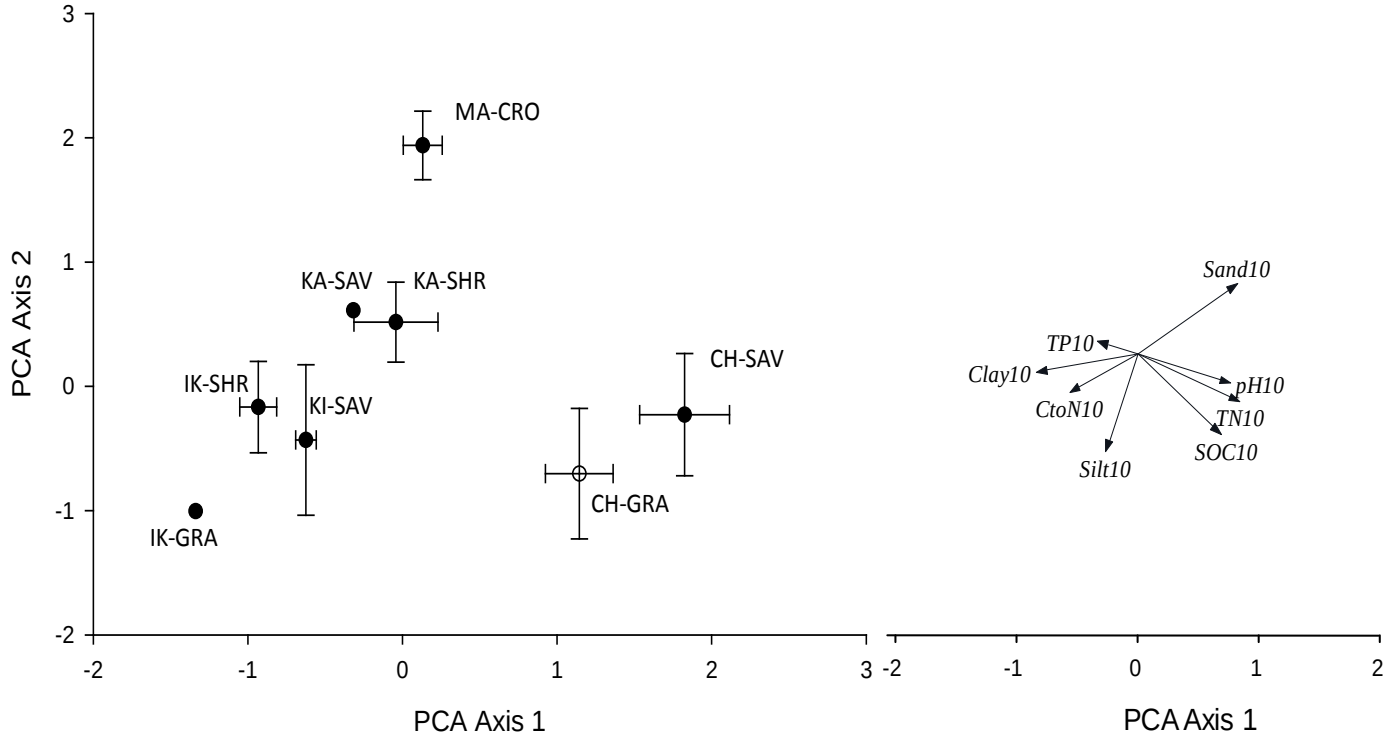
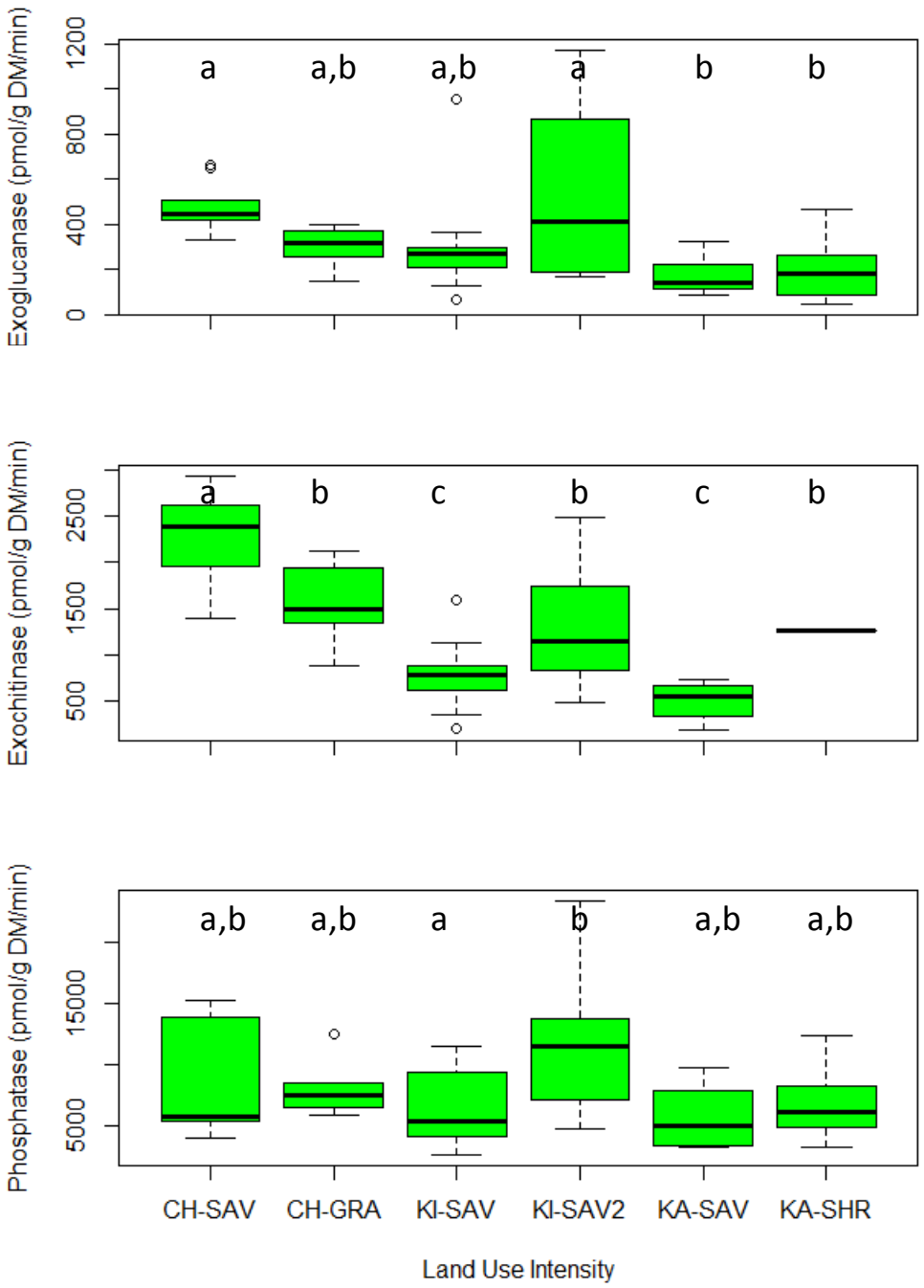


Figure 2.3. Principal Component Analysis (PCA) of the ClimAfrica data from Kenya and Tanzania, based on soil fertility factors. Left, distribution of the main ecosystems and land covers according to the first two PCA axes. Black dots represent the mean value of the samples from that particular land cover and location and whiskers indicate ±1 standard deviation. Different locations represent different land use intensities. CH-SAV: ; CH-GRA: KA-SHR: ; KA-SAV: KI-SAV: ; IK-SHR: ; IK-SAV: ; MA-CRO: . Right, distribution of the soil descriptors on the same first two PCA axes.


Exoglucanase (pmol/g DM/min)
0
400
800
1200
a
a,b
a,b
a
b
b
Exochitinase (pmol/g DM/min)
500
1500
2500
a
b
c
b
c
b
Phosphatase (pmol/g DM/min)
5000
15000
a,b
a,b
a
b
a,b
a,b
CH-SAV
CH-GRA
KI-SAV
KI-SAV2
KA-SAV
KA-SHR
Land Use Intensity

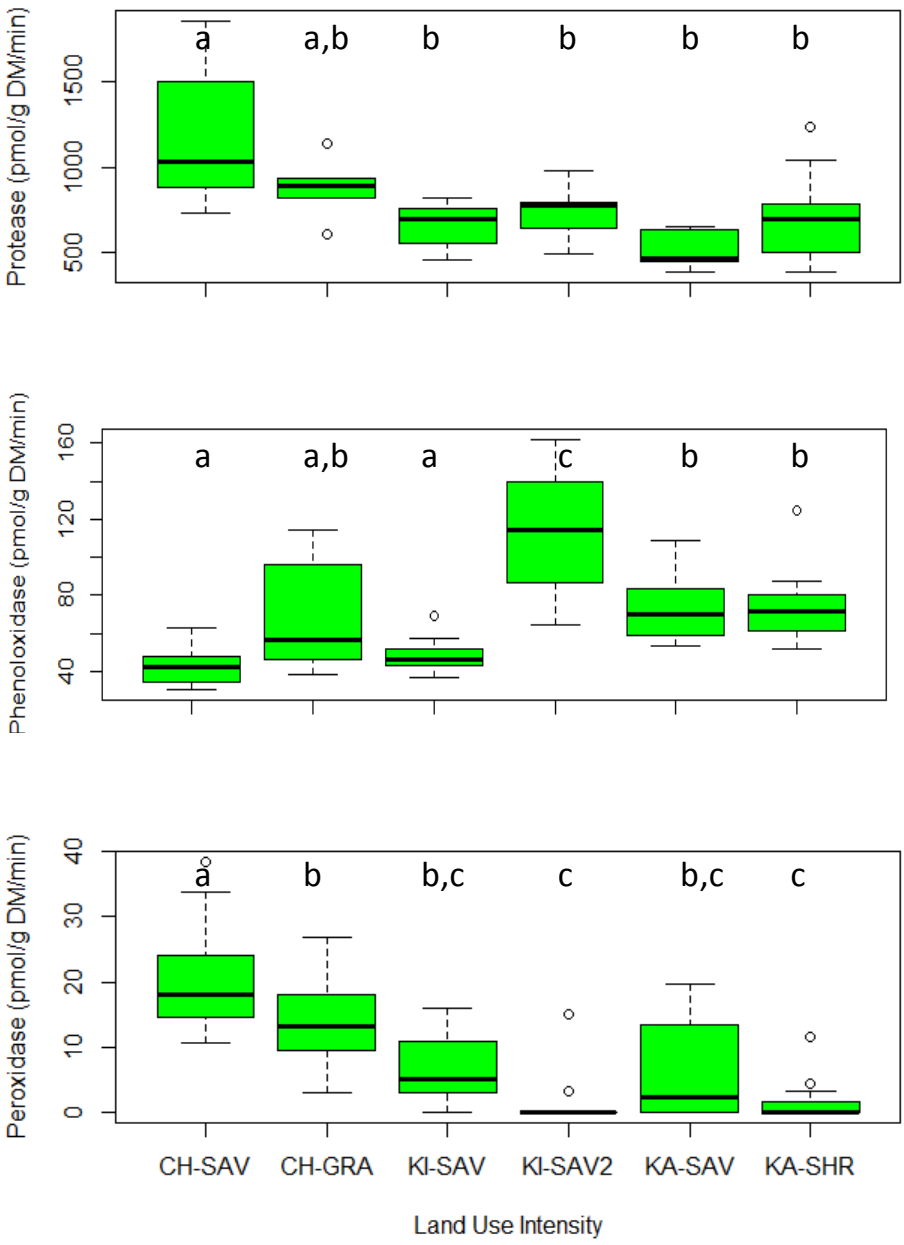


Figure 2.4. Box-plots showing the activity of six exoenzymes in the six sampled plots, distributed along the x-axis according to a land use intensity (LUI) gradient. CH-SAV: savanna from Chyulu National Park; CH-GRA: grassland from Chyulu National Park; KI-SAV and KI-SAV2: savanna plots from the agricultural station of Kiboko; KA-SHR: shrubland from the Kajiado Maasailand; KA-SAV: savanna from Kajiado.

2.3.2. Shifts in soil fertility and activity along land use intensity gradients in Kenya

Plot was the factor explaining the highest variability in exoenzymes distribution for all exoenzymes in univariate models, with LUI being the most common second explanatory single factor, followed by pH or bedrock (Table S2.2 in Supplementary Material). There was a tendency for exoglucanase, exochitinase, protease and peroxidase to decrease with land use intensity, while phenoloxidase showed a tendency to increase with intensification (Fig. 2.4). Phosphatase did not show any clear tendency with LUI (Fig. 2.4). The plot from Kiboko KI-SAV2 showed the highest variability for most exoenzymes and sometimes differed markedly from the other savanna plot in the same location and with similar soil (Fig. 2.4, Table 2.2).

Although plot was the single best predictor for all exoenzymes, plot was not included in the final models for protease and peroxidase (Table 2.3). Furthermore, local soil factors, such as soil water and most often pH, generally improved the models including plot, except for phenoloxidase, for which idiosyncratic effects were maximum (Table 2.3). Exoglucanase was better described by plot and soil humidity (Table 2.3), and exochitinase and phosphatase by plot and soil pH (Table 2.3). For protease and peroxidase, land use factors and bedrock or pH could substitute plot in the final models (Table 2.3). Most models explained around 50% of the variability of enzymatic activity, except for exoglucanase and phosphatase, which explained over one fourth (Table 2.3).

The first PCA axis, explaining the highest variability in soil fertility and activity distribution (45.27%), separated the samples from Chyulu National Park, particularly those from the savanna plot, from the rest of samples from other plots over the first PCA axis (Fig. 2.5). This axis represented a land use intensity gradient, with the heavily grazed samples from the Kajiado Maasailand area on the negative end (Fig. 2.5). The second PCA axis, explaining 15.38% of the sample variability, separated heavily grazed areas of the Kajiado area from moderately grazed savannas and grasslands, either by wild animals or livestock, with CH-SAV scarcely contributing to that axis (Fig. 2.5). Indicators of soil microbial activity, including Cmic, Nmic, DON, DOC, peroxidase activity, exochitinase, increased towards the positive side of axis 1 (Fig. 2.5), where plots less intensively used were distributed towards the negative side (Fig. 2.5). Thus, PCA axis 1 represents a gradient of increased soil activity with decreased land use intensification. On the other hand, parameters indicators of inorganic N losses, including DIN, ammonia and nitrate, increase towards the positive side of the PCA axis 2, where the heavily grazed ecosystems in Maasailand were located (Fig. 2.5). Phenoloxidase increased towards the KI-SAV2 plot (Fig. 2.5), as expected from the behavior of this enzyme (Fig. 2.4).

Table 2.3. Best model for the six exoenzymes analysed. The adjusted R^2 is an indicator of the proportion of the variability of the exoenzyme explained by the model.

Factor	Exoglucanase	Exochitinase	Phosphatase	Protease	Phenoloxidase	Peroxidase
Plot	0.001	<0.0001	0.0184	-	<0.0001	
Water	0.0747	-	-	-	-	
pH	-	0.0092	0.0023	0.0052	-	
Bedrock	-	-	-	0.0483	-	< 0.0001
LUI	-	-	-	<0.0001	-	
Land Cover	-	-	-	-	-	0.0007
R^2_{adj}	**28.96%**	**66.29%**	**26.60%**	**47.83%**	**52.74%**	**49.93%**

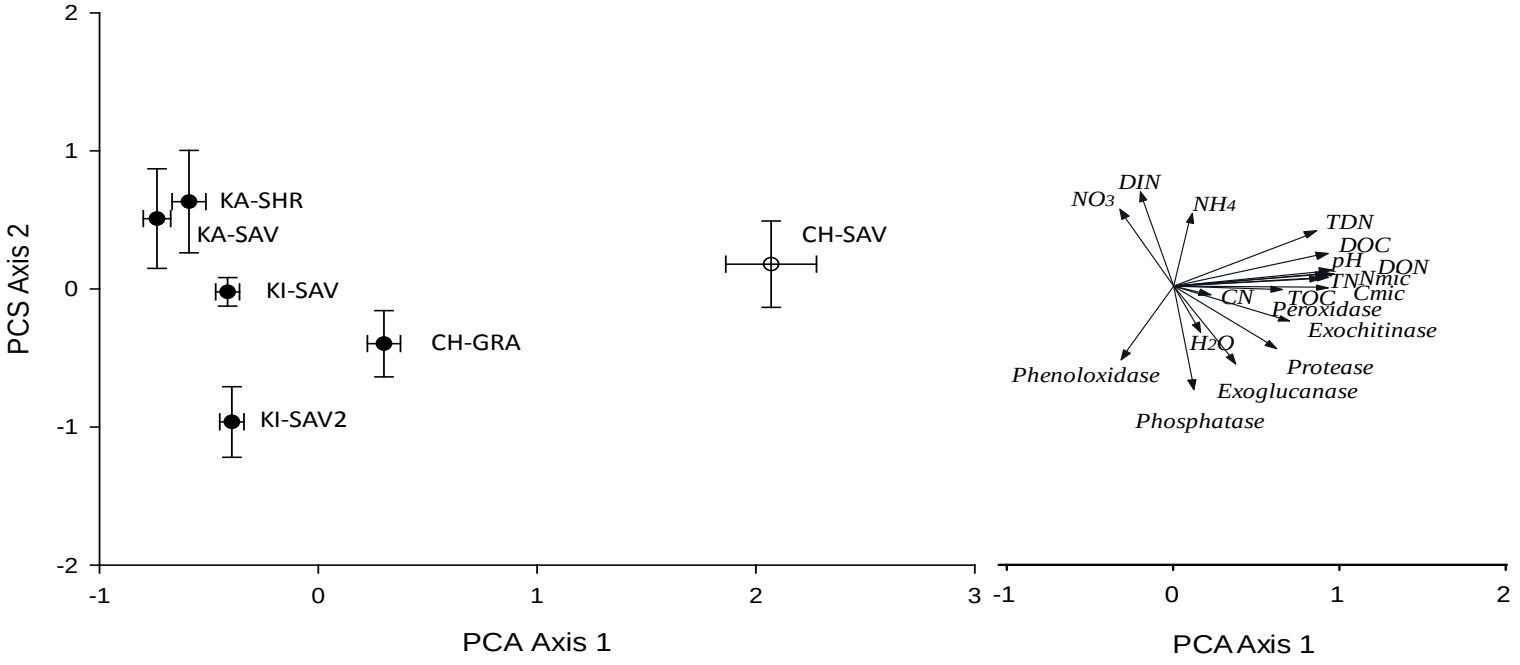


Figure 2.5. Principal Component Analysis (PCA) on samples from Kenya based on soil fertility and activity data. Left, distribution of the main ecosystems and land covers according to the first two PCA axes. Black dots represent the mean value of the samples from that particular land cover and location and whiskers indicate ±1 standard deviation. Different locations represent different land use intensities. CH-SAV: savanna from Chyulu National Park; CH-GRA: grassland from Chyulu National Park; KI-SAV and KI-SAV2: savanna plots from the agricultural station of Kiboko; KA-SHR: shrubland from the Kajiado Maasailand; KA-SAV: savanna from Kajiado. Right, distribution of the soil fertility and activity descriptors on the same first two PCA axes.

2.4. Discussion

We analyzed soil fertility and activity in Eastern Africa ecosystems under a variety of environmental conditions, and found that land cover, land use intensity, bedrock and, when tested, climate, all were important factors explaining the soil variability (Fig. 4). Land use and land use intensity generally were the main factors determining soil organic carbon concentration and stocks (Table 2.1, Figs. 2.1 and 2.2), soil fertility distribution (Table 2.1, Figs. 2.2 and 2.3), and soil enzymatic activity (Table S2.2 in Supplementary Material, Figs. 2.4 and 2.5) in Eastern African ecosystems, particularly when analyzing the upper soil layer. These results agree with findings in the neighboring area of Kilimanjaro, where microbial biomass C, and exoglucanase and exochitinase activities were higher in natural compared to agricultural ecosystems (Mganga *et al.*, 2016). Similar to the Kilimanjaro region, in our study natural ecosystems held the highest NC and NT contents (Table 2.1), and croplands the lowest (Table 2.1), in spite of manure input. However, effects of land use factors were stronger for fertility parameters related to the carbon cycle than to the N or P cycles (Table S2.1 in Supplementary Materials), while for soil activity parameters, land use factors were generally the most relevant environmental drivers (Table S2.2 in Supplementary Materials).

In our study, there were confounding effects of land use with soil, determined by bedrock (Tables 2.1 and 2.2). Although the importance of parent material for soil microbial communities has been reported (Alfaro

et al., 2017), multivariate analysis and modelling showed clearly that, in our study, land use effects (land cover and LUI) were generally stronger than bedrock or pH on soil organic carbon and, in particular, fertility distribution (Table S2.1 in Supplementary Material; Figs. 2.1 and 2.3), as well as on soil activity (Table S2.2 in Supplementary Material; Figs. 2.4 and 2.5). The observed trends were stronger for the upper 10 cm soil layer, where the soil microbial activity was also measured.

The protected area of the Chyulu Hills National Park held the most pristine ecosystems in the study (Tables 2.1 and 2.2) and showed also the most fertile soils, except for P (Table 2.1, Figs. 2.2 and 2.4). Chyulu Hills is also an area occupied by volcanic soils with andic properties, that is, soils present amorphous clays with ionic exchange capacity, therefore capability to associate with the acidic radicals of soil organic matter; furthermore, they retain nitrates, which are not as easily washed as in other soils, and P, which is fixed by the amorphous clays (Table 2.1).

In addition to responses to land use and bedrock, some soil fertility and activity parameters showed idiosyncratic plot effects. The differences in soil activity between the two plots from the agricultural station of Kiboko are noteworthy. Both are found under very similar environmental conditions, savannas on gneiss moderately used. They have slightly different land use histories. Small variations in land use history have been found to generate variability in microbially-derived ecosystem functions among plots even in areas with the same bedrock and homogeneous current land cover and land use intensity (Bowles *et al.*, 2014). Furthermore, the plot showing the highest variability in soil

enzymatic activity, KI-SAV2, was also the plot containing the highest diversity of plant functional types, including legume and non-legume trees. Part of this variability and differential behavior compared to other plots could be related to this diversity. Indeed, it has been pointed out that plant diversity might be a strong control of soil microbial processes (Mitchell *et al.*, 2010; Steinauer *et al.*, 2015; De Vries *et al.*, 2015).

Phosphatase activity also showed idiosyncratic plot effects (Fig. 2.4). Contrary to expectations (Richardson & Simpson, 2011; Mganga *et al.*, 2016), phosphatase activity did not increase in the most P-limited plots (Table 2.1) neither in the plots receiving animal manure naturally, even if recent studies suggest that organic P rather than TP is the most important P fraction in predicting phosphatase activity (Margalef *et al.*, 2017). Furthermore, we did not find evidences for the reported increase in phosphatase activity with decreased P availability in some Kilimanjaro ecosystems (Mganga *et al.*, 2015), neither for high phosphatase activity on volcanic ash (Fig. 5), as found in the same area (Mganga *et al.*, 2016). Phosphatase activity is often high in volcanic soils, where clay and soil organic matter contents are abundant (Olander & Vitousek, 2000), but in our study there was no indication of this. African soils are generally phosphorus-limited, soil P deficiencies primarily resulting from either inherent low levels of soil P from parent material, or depletion of soil P by replacement of traditional systems of shifting cultivation with shorter duration unsustainable agriculture (Buresh *et al.*, 1997). We found a wide range of TP contents in our study (Table 2.1) but variations in phosphatase activity were generally low among the study plots, the

most acute differences often found between the LUI-paired plots (Fig. 2.4).

Hydrolitic enzymes, on the contrary, behaved as expected, with soil enzymatic activity decreasing with land use intensity. Activities of β-glucosidase and cellobiohydrolase (exoglucanase) increase with organic matter content, which is why they are commonly used as sensitive biological indicators of soil quality (Badiane *et al.*, 2001). Cellobiohydrolase activity (exoglucanase) frequently increases with succession, as the microbial community matures; however, it depends strongly on the substrate, being highly linked to clays and soil organic matter, to which these enzymes adhere (Olander & Vitousek, 2000). Chitinase activity (N-acetyl-b-glucosaminidase) is usually used as indicator of the presence of fungal biomass. It is correlated to the mineralization of N, playing a major role in the N cycle. It is used as an index of net N mineralization in soil (Dalmonech *et al.*, 2009).

It has been reported that oxidative enzymes are key components of the pathways involved in the breakdown of organic compounds and soil organic matter (Hassan *et al.*, 2013). The two oxidative enzymes studied are involved in lignin degradation (Burns *et al.*, 2013). However, while peroxidase decreased with land use intensity (Fig. 2.5), similarly to other N-cycling related enzymes, phenoloxidase increased (Fig. 2.5), being higher in the KI-SAV2 plot (Fig. 2.5).

The more recalcitrant is the soil organic matter, the more dependent is the availability of C, N and P on the phenoloxidase activity. Only specialized organisms have peroxidase and phenoloxidase activities, and

those who had them, have advantage over others when C sources are poor in N (Kaiser *et al.*, 2010). These enzymes are typically produced under N limiting conditions (Fog, 1988) but research has also indicated that some groups of microorganisms may produce phenoloxidases in response to high levels of inorganic N dissolved in soil (Fog, 1988; Hammel, 1997; Collins & Dobson, 1997), as is our case, where heavily grazed plots in Kaijiado Maasailand showed high levels of phenoloxidase activity (Fig. 2.5) and increased DIN levels (Fig. 2.4). Grazing can result in a reduction of litter and an increase in phenolic compounds (Olivera *et al.*, 2014). However, the highest phenoloxidase levels, with ample intra-plot variability, were found in the plant-functionally diverse KI-SAV2 plot (Fig. 2.5).

In summary, our results support an important role of land use intensity and land use change on the carbon and nutrient cycles in Eastern African ecosystems. Land use factors were key drivers of soil fertility and activity in dry savanna ecosystems, in spite of plot idiosyncratic effects and variability in bedrock and soils, and climate. Land use factors often overrode other environmental effects, particularly on carbon cycling indicators.

2.5. Acknowledgements

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Chapter 3

Microbial community across climatic gradients

3. Changes in soil microbial community composition across climatic gradients in grassland ecosystems of the Iberian Peninsula

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Abstract: Grassland ecosystems are among the most biodiverse around the world. Understanding the effects of environmental changes on the soil microbial community is essential to predict the responses of the ecosystem to the current climatic and land use changes. We focused on the microbial community through the use of Illumina metabarcoding along altitudinal and climatic gradients, analyzing the most influential factors structuring both fungal and bacterial communities. *Ascomycota* and *Actinobacteria* were the most abundant phyla in the overall survey, but profound changes in both communities were found along the environmental gradients. Whereas the temperature and humidity had the highest influence on fungal community structure, soil pH was the most important factor affecting bacteria. Fungal and bacterial communities were found to strongly co-vary along the altitudinal and

climatic gradient. The assemblages found between fungal and bacterial taxa suggest that different environmental stresses configure the interactions that are established between them. Our results offer new points of interest to deepen the knowledge on the interactions that take place between soil microorganisms, which are essential to understand the functioning of the ecosystem.

3.1. Introduction

Climate is the main factor structuring the global biodiversity around the word (Sala *et al.*, 2000; Hawkins *et al.*, 2003; Currie *et al.*, 2004). In a context of climate and land use changes, it is essential to understand the effect of those factors linked to climate on the ecosystems and on their biodiversity. This could allow the scientific community to predict the ecological responses of organisms to the global climate change (Bryant *et al.*, 2008) and thus take measures to mitigate and counteract the current and future loss of biodiversity (Kleijn *et al.*, 2011). Elevational gradients have been used extensively by ecologists because they represent indirect gradients along which multiple environmental variables are dramatically changing over short distances (De Bello *et al.*, 2005; Bello *et al.*, 2006; Fierer *et al.*, 2011; Xu *et al.*, 2014). Increasing the understanding of elevational diversity patterns may provide evidence for predicting the effects of climate change on ecosystems (Shen *et al.*, 2015). However, most of the elevational gradient surveys have focused on macroorganisms, while little is known about the

microbial distribution patterns across such gradients (Martiny *et al.*, 2006; Shen *et al.*, 2015; Wang *et al.*, 2017). Fungi and bacteria, among the main components of the soil microbiota, represent the unseen majority of soil biodiversity, and they play key roles in ecosystem processes, such as soil formation and nutrient cycling (Leininger *et al.*, 2006; Heijden & Bardgett, 2008), thus influencing a wide range of the goods and services that ecosystems provide (Barrios, 2007). Ammonia-oxidizing archaea play a key role in nitrification processes, and they seem to dominate over ammonia-oxidizing bacteria in soils (Purkhold & Pommerening-Röser, 2000; Bock & Wagner, 2001; Leininger *et al.*, 2006). However, we did not include them in this study because they comprise quantitatively a small part of the soil microbiota (Phillips, Paul, & Prosser, 2000; Hermansson & Lindgren, 2001) and nitrification processes were not the focus of this study. Previous studies on the microbiota along elevational gradients focused on a single microbial group, either bacteria (Fierer *et al.*, 2011; Singh *et al.*, 2014; Shen *et al.*, 2015) or fungi (Bahram *et al.*, 2012; Davey *et al.*, 2013; Coince *et al.*, 2014), thus leaving aside the probably abundant relationships within both microbial organisms (Bennett & Feibelman, 2001; Frey-Klett & Garbaye, 2005; Bonfante & Anca, 2009). Other studies including both microbial groups have surveyed a rather narrow elevational gradient or focusing only on high altitude ecosystems (Ritz *et al.*, 2004; Xu *et al.*, 2014; Yang *et al.*, 2014), missing an important part of the variation that elevational gradients can provide. Other studies focusing on microbial communities along environmental gradients used relatively limited

methodologies, describing communities at a very coarse levels of taxonomic resolution (Ritz *et al.*, 2004; Xu *et al.*, 2014). The current increasing availability of molecular technologies makes possible to undertake more detailed surveys of individual microbial taxa, and within them, DNA metabarcoding has proved to be an effective method for the detection of changes in microbial community composition along with changes in environmental factors (Lauber, Hamady, & Knight, 2009a; Rousk *et al.*, 2010). This has led to the recent development of new analytical procedures in order to understand the co-occurrence patterns within microbial communities (Barberán *et al.*, 2011).

Fauth *et al.* (1996) defined "assemblages" as "groups of phylogenetically related species found together within a community, not using a common resource". A hierarchy of biotic interactions and physicochemical factors regulate these assemblages at multiple spatial scales (Brosse, Arbuckle, & Townsend, 2003; Heino, Louhi, & Muotka, 2004). Identifying indicator groups across elevational gradients has potential applications into biodiversity conservation and ecosystem management planning (Backus-Freer & Pyron, 2015). Patterns of species richness along environmental gradients can be different and less pronounced than patterns of taxa composition within assemblages (Gioria, Bacaro, & Feehan, 2011). In fact, several studies showed a wide range of patterns of microbial diversity and richness in relation to elevation (Bryant *et al.*, 2008; Wang *et al.*, 2011; Fierer *et al.*, 2011). However, some studies that did not find any change in microbial richness along elevation, did find them in the structure of the microbial

community. For example, Davey *et al.* (2013) studied fungal communities associated to particular bryophyte species across alpine elevation gradients and found that OTU richness remained stable along the gradient, but distinct shifts in the fungal community composition occurred between elevation zones. Fierer *et al.* (2011) found the bacterial community composition to vary across elevational gradients, harboring each habitat type distinct bacterial communities.

Microbial assemblages have been further studied within aquatic environments (Kobori, Sullivan, & Shizuya, 1984; Shi *et al.*, 2011; Smith, Allen, & Allen, 2013; Bleijswijk, Whalen, & Duineveld, 2015). DeLong *et al.* (2006) assessed microbial plankton assemblages along the water column, and identified trends depth-depending patterns in gene content and metabolic pathway components of oceanic microbial communities. However, microbial assemblages from terrestrial ecosystems have been less studied, despite the huge effort already achieved to understand the patterns driving their biodiversity (Bardgett & Putten, 2014; Tilman, Isbell, & Cowles, 2014; Delgado-Baquerizo, Maestre, & Reich, 2016). Particularly, semi-natural grasslands harbor many more species richness than expected from their spatial extent, accumulated during millennia of low-intensity land use (Habel *et al.*, 2013). Current changes of these land uses that expanded European grasslands, along with environmental changes due to climate, are threatening these biodiverse ecosystems (WallisDeVries *et al.*, 2002). Since most of the studies on elevational gradients have focused on forest ecosystems (Fierer *et al.*, 2011; Coince *et al.*, 2014) we focus on

the soil microbial community composition of semi-natural grasslands along elevational and climatic gradients in the Iberian Peninsula. Our questions are:

1) Are fungal and bacterial communities grouped following altitudinal patterns in semi-natural grasslands?

2) Are fungal and bacterial communities driven by the same factors, which covariate across environmental gradients?

We hypothesize that microbial communities vary along altitudinal gradients, but that soil pH influence more the bacterial than the fungal community, as seen in previous studies (Rousk *et al.*, 2010). We expect a high correlation in bacterial and fungal community changes along the gradient because of the set of biotic and abiotic factors that also change linked to elevation (indirect gradients along which multiple environmental variables are dramatically changing over short distances; (Fierer *et al.*, 2011; Xu *et al.*, 2014), regardless of the differential factors that most affect each community. Finally, indicator microbial groups were expected to be found only in the most extreme environments, as ubiquitous taxa are usually found in microbial metabarcoding surveys (Barberán *et al.*, 2014).

3.2. Materials and Methods

3.2.1. Study sites

The study area distributes along climatic and altitudinal gradients (Table S3.1), including fourteen sites from the Mediterranean seaside up to the alpine vegetation belt in the Eastern Pyrenees. All sampling sites are calcareous grasslands extensively grazed either by cattle, sheep or mixed grazing. Only the site of Riart is currently cropped by species of *Vicia* and *Triticumsecale* and grazed by cattle in autum. El Prat is situated in the Mediterranean region, with the lowest altitude (5 m/asl) and dominated by species of *Galium* and *Vicia*. Monegrillo and Alguaire are the semi-arid lowland representatives, with their vegetation dominated by *Papaver rhoeas* and *Thymus vulgaris* for the former and *Hordeum vulgare* for the latter. In addition to Riart, six other locations correspond to grasslands in the montane climatic region: two sites in Pallars Jussà (henceforth Pallars 1 and Pallars 2), Besora and La Bertolina within the province of Lleida and Ballestar and Bel in Els Ports Natural Park (Tarragona). *Thymus vulgaris*, *Plantago lanceolata* and *Festuca* spp. are found within those dominant species in these locations (for more details, see Table S3.1). The Atlantic region was also sampled in the locality of Irati (two locations called Irati 1 and Irati 2), where the vegetation was dominated by *Trifolium alpinum* and *Festuca* gr. *rubra.*

3.2.2. Sampling design

The sampling took place in spring and summer of 2013 and 2014 (the latter only in Monegrillo and Alguaire), at around the vegetation peak

biomass of each site. We selected three patches per dominant plant functional type (PFT) in each sampling site, except for Riart, where six random samples were taken. This resulted in six to fifteen samples per site, depending on the functional diversity present in each location. Rings of 25 cm in diameter were defined as sampling points, vegetation inside these was cut and dry weight was estimated. Soil samples were taken to a depth of 10 cm with a 6 cm x 5 cm metal soil probe in each sampling point. A first subset of samples was kept frozen until DNA extraction and a second subset of soil samples was oven-dried at 60 ºC for 3 days and sieved through a 2 mm sieve for physical and chemical analysis.

3.2.3. Soil physical and chemical determinations

All soil chemical and physical properties were analyzed at the Applus Agroambiental, S.A. Laboratories, Lleida (Spain). Soil moisture content (H_2O) was determined gravimetrically by drying fresh soil at 105 ºC. Organic matter content (OM) was determined colorimetrically by the the Walkley and Black (1934) technique. Total nitrogen content (TN) and total carbon content (TC) were determined by the elemental autoanalyzer. Soil pH was measured in the supernatant of a 1:2.5 solid-to-liquid (g/ml) ratio suspension of soil sample in water. Electrical conductivity (EC) was determined at 25ºC from 1:2.5 solid-to-liquid (g/ml) water extracts. Labile soil phosphorous (P) was determined by the Olsen method (Olsen et al. 1954); available potassium (K), calcium (Ca), magnesium (Mg) and sodium (N) were determined spectrometrically by ammonium acetate extraction. Soil texture classification was based on

the proportions of sand, silt and clay after their gravimetrical measurement.

3.2.4. Bacterial and fungal metabarcoding

Soil samples were taken in the 0-10 cm depth soil layer with a 10 cm x 6 cm x 5 cm metal soil core in each sampling point. A total number of 133 soil samples were stabilized in a Solution C1 of MoBio's PowerSoil DNA Isolation kit during transportation to the laboratory. DNA was isolated upon arrival in the laboratory using the PowerSoil DNA isolation kit (MoBio), strictly following the manufacturer's instructions. Determination of DNA concentration was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientic).

For DNA metabarcoding library preparation, a fragment of the bacterial 16S ribosomal RNA gene of around 530 bp was amplified using the primers Bakt 341F (5' CCT ACG GGN GGC WGC AG 3') and Bakt 805R (5' GAC TAC HVG GGT ATC TAA TCC 3', Herlemann et al. 2011). In the same way, a fragment of the fungal ITS2 region of around 400 bp was amplified using the primers ITS86F (5' GTG AAT CAT CGA ATC TTT GAA 3', Turenne, Sanche, & Hoban, 1999) and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3', White et al. 1990). The primers had the Illumina adapter sequences and were tagged to their 5' end. The tags make possible to link the reads obtained during sequencing to a particular sample.

PCRs were carried out in a final volume of 25 µL, containing 2.5 µL of template DNA, 0.5 µM of the primers, 12.5 µL of Phusion DNA polymerase mix (Thermo Scientific), and ultrapure water up to 25 µL.

The reaction mixture was incubated as follows: an initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 55 °C (bacterial 16S) or 60 °C (fungal ITS2) for 20 s, 72 °C for 20 s, and a final extension step at 72 °C for 10 minutes. Despite the high number of PCR cycles used, the PCR products were checked on 1% agarose gel stained with REAL Safe (Durviz), and imaged under UV light and showed similar medium intensity bands. Negative controls that contained no DNA were included to check for contamination during library preparation.

PCR products were purified using the Mag-Bind RXNPure Plus magnetics beads (Omega Biotek), following the instructions provided by the manufacturer. After purification, the products were combined into 2 different pools (1 for the bacterial 16S samples and 1 for the fungal ITS2 samples). Then, they were quantified with the Qubit dsDNA BR Assay Kit and pooled in equimolar amounts. The purified amplicon pools were sent for sequencing in 2 different runs of the Illumina MiSeq PE300 platform. All the negative controls were included in the pools, in order to check for potential contamination. The quality of the FASTQ files was checked using the software FASTQC. A first quality-filtering step was performed using the software Geneious 8.1.8. In this step, regions from both ends of the reads with more than a 0.5% chance of an error per base were trimmed. Paired-end assembly of the forward (R1) and reverse (R2) reads was performed with FLASH (Magoč & Salzberg, 2011). The mismatch resolution in the overlapping region is accomplished by keeping the base with the higher quality score. The FASTQ files were quality filtered using the bioinformatic tool Qiime 1.9.1 (Caporaso *et al.*,

2010). DNA sequences having quality score <20 were discarded. Chimeric sequences were removed using the UCHIME algorithm (Edgar *et al.*, 2011) implemented in VSEARCH. The reference databases used were Greengenes for bacteria (DeSantis, Hugenholtz, & Larsen, 2006) and UNITE for fungi (Abarenkov, Nilsson, & Larsson, 2010). 16S and ITS2 reads were clustered into OTUs (97% identity) using the open-reference approach in Qiime. In this method, reads are clustered against a reference database and any reads which do not hit the reference sequence collection are subsequently clustered de novo. Each OTU was assigned to a microbial taxa using the BLAST algorithm (Altschul *et al.*, 1990).

Based on the resulting OTU table obtained for each sample, an additional quality-filtering was carried out: the OTUs which were present in the negative control and the OTUs represented by sequences with frequencies lower than 0.005% in the whole dataset were removed. Given the high number of resulting sequences clustered de novo, we considered unreliable those taxonomical assignments to species level. Nevertheless, we maintained the terminology of Species Hypothesis to designate those taxa discovered on identity threshold of 97% (Kõljalg *et al.*, 2013).

Finally, the rarefaction plots were constructed showing the rarefied number of OTUs defined at a 97% sequence identity threshold. When the rarefaction curves tended towards saturation, the sequencing depth was assumed to be sufficient to retrieve most of the microbial diversity.

All those samples not reaching the plateau were removed from further analysis. In addition, the percentage of coverage was calculated by the Good's method (Good & Toulmin, 1956), which is also used to check to what extent samples were adequately sampled.

3.2.5. Statistical analysis

We used canonical correspondence analysis (CCA) and redundancy analysis (RDA) to elucidate the relationships among the fungal and bacterial community distribution respectively, and a set of explanatory variables, with the program CANOCO 5 (Microcomputer Power, Ithaca, NY, USA). CCA and RDA were conducted on the MiSeq read percentages at the order level for fungi and at the class level for bacteria, with a log transformation and using the 'downweighting of rare species' option. In addition to the compositional descriptor variables, three groups of explanatory variables were included in this analysis. The first group consisted of climatic variables, including mean summer temperatures (MST), mean annual temperature (MAT), mean annual precipitation (MAP), minimum and maximum temperature (Tmin and Tmax) and the continentality index of Sebastia, CIS (MST-MAT).The second group consisted of soil variables, including pH, carbonates, proportions of particle sizes, nutrients (Ca, Mg, K, P, N and C) and electric conductivity (CE25). The third group was composed by land management variables (i.e. grazed by sheep, cattle, horses or mixed). A Monte-Carlo permutation test with 9999 permutations was used to assess the significance of the explanatory variables.

The datasets of both communities, one for bacteria and one for fungi, observed at the same sampling points, were used in a co-correspondence analysis (Co-CA, ter Braak & Schaffers, 2004a) using CANOCO 5 software. The coordinates of the Co-CA analysis were used to calculate Euclidean metric distances between microbial groups and hierarchical clustering by the Ward's method using the R software (R core Team, 2015).

3.3. Results

3.3.1. Soil microbial diversity

3.3.1.1 Illumina MiSeq run

A total of 8,372,911 fungal ITS2 (ITS) and 10,499,568 small-subunit ribosomal RNA gene (16S) amplicon sequences were obtained from the Illumina MiSeq runs. After quality filtering steps and removal of non reliable samples, 1,508,381 fungal and 1,057,510 bacterial sequences were used for further analyses. These ranged from 479 to 48,097 fungal sequences and from 1,439 to 27,832 bacterial sequences per sample.

Sequences were assigned to OTUs based on 97% sequence similarity, resulting on 1,743 fungal and 2,601 bacterial OTUs. Among these, 528 fungal OTUs were identified as Species Hypothesis (SH) and 1,215 were subsequently clustered de novo as a New Reference (NR). Regarding to bacterial OTUs, 2,066 were identified as SH while 535 OTUs were clustered as NR.

3.3.1.2. Fungal diversity

From the total ITS sequences, 1705 OTUs were classified into the kingdom fungi, from which 39 were assigned to the kingdom level, 96 OTUs to the phylum level, 198 OTUs to the class level, 356 OTUs to the order level, 245 OTUs to the family level and 771 OTUs to the genus level. Most of the fungal sequences belonged to the phylum *Ascomycota* (82.7%), followed at a great distance by *Basidiomycota* (13.4%, Table S3.2). Unidentified fungi accounted by 1,6% of the total sequence abundance and *Zygomycota* had a mean relative abundance of 0.7%. *Chytridiomycota* and *Glomeromycota* were the rarest phyla found (read relative abundance <0.2%), as expected by the low specificity of the used primers related to this taxa. At the class level, *Dothideomycetes* (28.4%), *Sordariomycetes* (21.4%), *Leotiomycetes* (12.6%) and Agaricomycetes (7.9%) showed the highest relative abundances. The top five identified fungal orders were *Pleosporales* (22.7%), *Helotiales* (8.4%), *Sordariales* (6.6%) and *Hypocreales* (6.1%) within the phylum *Ascomycota*, and *Agaricales* (5.1%) within *Basidiomycota*. All of these taxa were found with high abundances across all sampling sites.

3.3.1.3. Bacterial diversity

Taxonomic assignment of the 16S MiSeq reads resulted in 2590 OTUs into the kingdom Bacteria. From these, 1 OTU was assigned to the kingdom level, 15 OTUs to the phylum level, 169 OTUs to the class level, 795 OTUs to the order level, 1012 OTUs to the family level and 598 OTUs to the genus level. *Actinobacteria* (28.7%) was the dominant phyla found

in this study (Table S3.2), followed by *Proteobacteria* (16.7%), *Planctomycetes* (13.2%), *Acidobacteria* (12.1%), *Chloroflexi* (10.9%) and *Firmicutes* (10.2%). At the class level, *Actinobacteria* (15.6%), *Alphaproteobacteria* (11.7%) and *Bacilli* (9.9%) had the highest relative read abundances.

3.3.2. Fungal community composition and distribution

Based on the fungal community composition, the first CCA axis explained 10.8% of the variability within the distribution of soil samples. All samples separated along this first axis following a climatic gradient (Fig. 3.1). Samples belonging to the alpine and Atlantic sites were distributed on the positive side of the first axis, which was associated to an acidity, precipitation and low temperatures gradient, as well as to the predominance of grazing by cattle (including horses in the case of Niu de l'Aliga). *Coniochaetales*, *Lecanorales*, *Archaeosporales* and *Verrucariales* were found among those fungal orders characterizing this climatic zone (Figure 3.1, Table S3,3). Those fungal orders with proportions higher than 1% within all climatic zones were considered ubiquitous (Table S3,3). Within them, *Helotiales* were found with higher abundances in the alpine grasslands (18.9%). On the contrary, the also ubiquitous *Hypocreales* showed their lowest representation in these grasslands (1.3%).

Table 3.1. Conditional and simple term effects of the CCA analysis on fungal orders. The explanatory variables include climatic, edaphic and land use variables, ordered according to their percentage of variation explained.

	Conditional term effects			Simple term effects		
Variable	***% explained***	***pseudo-F***	***P(adj)***	***% explained***	***pseudo-F***	***P(adj)***
MST	9.2	12.4	<0.001	9.2	12.4	<0.001
Na	7.1	10.4	<0.001	7.2	9.5	<0.001
Clay	4.0	6.1	<0.001	5.0	6.5	<0.001
Cattle	3.7	5.8	<0.001	5.3	6.9	<0.001
Horses	3.6	6.1	<0.001	4.7	6.1	<0.001
Ca	3.1	5.1	<0.001	7.8	10.4	<0.001
Sheep	2.7	4.7	<0.001	2.5	3.2	<0.001
Gloam	2.3	4.2	<0.001	3.8	4.8	<0.001
pH	1.8	3.4	<0.001	9.0	12.1	<0.001
P	1.5	2.8	<0.001	3.6	4.7	<0.001
$CaCO_3$	1.3	2.5	<0.001	8.6	11.6	<0.001
Floam	1.3	2.6	<0.001	5.1	6.5	<0.001
Mg	1.2	2.3	<0.05	3.8	4.8	<0.001
CIS	1.2	2.2	<0.001	4.2	5.4	<0.001
MAP	0.7	1.3	0.3	8.2	11.0	<0.001

Table 3.2. Conditional and simple term effects of the RCA analysis on bacterial classes. The explanatory variables include climatic, edaphic and land use variables, ordered according to their percentage of variation explained.

	Conditional term effects			Simple term effects		
Variable	***% explained***	***pseudo-F***	***P(adj)***	***% explained***	***pseudo-F***	***P(adj)***
pH	27.1	37.3	<0.001	27.1	37.3	<0.001
Na	7.7	11.6	<0.001	7.2	7.7	<0.001
Horses	4.8	7.9	<0.001	5.8	6.2	<0.001
MAP	3.7	6.3	<0.001	25.4	34.0	<0.001
Gloam	2.7	5.3	<0.001	5.0	5.2	<0.001
K	2.7	4.8	<0.01	8.1	8.9	<0.001
MAT	2.2	4.0	<0.01	15.3	18.0	<0.001
CE25	2.1	4.2	<0.01	2.0	2.0	<0.05
Sand	1.8	3.6	<0.01	6.9	7.4	<0.001
Floam	1.2	2.4	<0.05	5.8	6.2	<0.001
CIS	1.0	2.1	0.10	9.4	10.4	<0.001
Tmax	0.8	1.8	0.19	15.1	17.7	<0.001
Ca	0.7	1.4	0.31	21.3	27.1	<0.001
$CaCO_3$	0.1	0.3	1.00	23.3	30.3	<0.001

Samples belonging to the semi-arid grasslands were distributed on the negative side of this first axis. They were thus associated to high temperatures, lower precipitation regimes and alkaline soils. Grazing by sheep was also associated to this side of the first axis (Fig. 3.1). Mean summer temperature (MST) was the most important factor associated to the distribution of fungal communities (9.2% of the explained variation, see Table 3.1). *Pleosporales*, the most ubiquitous order found in this study, peaked in the semi-arid grasslands (28.9%). The semi-arid grasslands were also characterized by a high proportion of *Pezizales*, *Teloschistales*, *Auriculariales* and *Glomerales*, among others (Fig. 3.1, Table S3.3).

The second ordination axis accounted by 8.5% of the explained variability by the CCA analysis. It constituted a gradient of continentality. Samples from coastal, Mediterranean sites were distributed on the positive side of this axis, associated with soils with high salinity values and sand fractions. By contrast, samples from continental, montane sites were distributed on the opposite direction of this second axis, associated to high CIS values and high loam and clay fractions (Fig. 3.1). The third axis explained 7% of the variability and was also associated to Na content in the soil.

Mediterranean grasslands were characterized by the fungal orders *Boletales*, *Sebacinales* and *Microascales*, as well as *Incertae Sedis* within *Sordariomycetes* (Table S3.3). Among the ubiquitous taxa, *Hypocreales* (11.5%) and Unidentified *Ascomycota* (15.6%) peaked in this climatic

zone. Differently, the montane grasslands were characterized by the orders *Geoglossales* (4.9%), *Saccharomycetales* (0.2%) and an unidentified order within the class *Lecanoromycetales* (2.1%, Table S3.3).

3.3.3. Bacterial community composition and distribution

Redundancy analysis (RDA) of the bacterial communities at the class level resulted on a first axis explaining 58.7% of the variability in the distribution of the soil samples. Similarly to that found in fungi, this first ordination axis constituted a climatic and acidity gradient (Fig. 3.2). Samples belonging to alpine and Atlantic sites located at the positive side of this axis, associated to high precipitation regimes, high total nitrogen and low soil pH. These grasslands were characterized by the highest proportion of *Bacilli* (among those ubiquitous taxa), as well as *Ktedonobacteria, Spartobacteria* and *Acidobacteriia* (Fig. 3.2, Table S3.4). Oppositely, samples from semi-arid sites distributed by the negative side of the first axis, then associated to alkaline soils and high temperatures. Montane grasslands distributed between semi-arid and alpine sites. The Semi-arid and montane grasslands accounted for the highest proportions of the ubiquitous classes *Actinobacteria*, *Phycisphaerae* and *Acidobacteria-6.* Moreover, *Rubrobacteria, Chloracidobacteria* and *Chloroflexi* peaked in this zones. The montane zone was characterized by high proportions of *Betaproteobacteria,*

Deltaproteobacteria, Thermomicrobia and *Anaerolineae* (Fig. 3.2, Table S3.4).

The second ordination axis accounted for 8.5% of the explained variability by the RDA analysis. Samples from the Mediterranean grassland were located at the positive direction of this axis, whereas samples located inland, with more continental climates, located towards the negative direction, as happened with fungi. However, the proportion of the explained variation from each of the environmental factors were different from that found in fungi (Tables 3.1 and 3.2). In contrast to that found in fungi, pH was the most important factor associated to the distribution of environmental samples based on the bacterial communities (27.1% of the explained variation). The Mediterranean grassland was characterized by the highest proportions of *Acidimicrobiia* and *Planctomycetia* (Table S3.4), among those bacterial classes present within all climatic zones (>1%). Moreover, *Gammaproteobacteria, Cytophagia, Verrucomicrobiae* and *Ignavibacteria* among other classes were found to characterize this climatic zone (Fig. 3.2, Table S3.4).

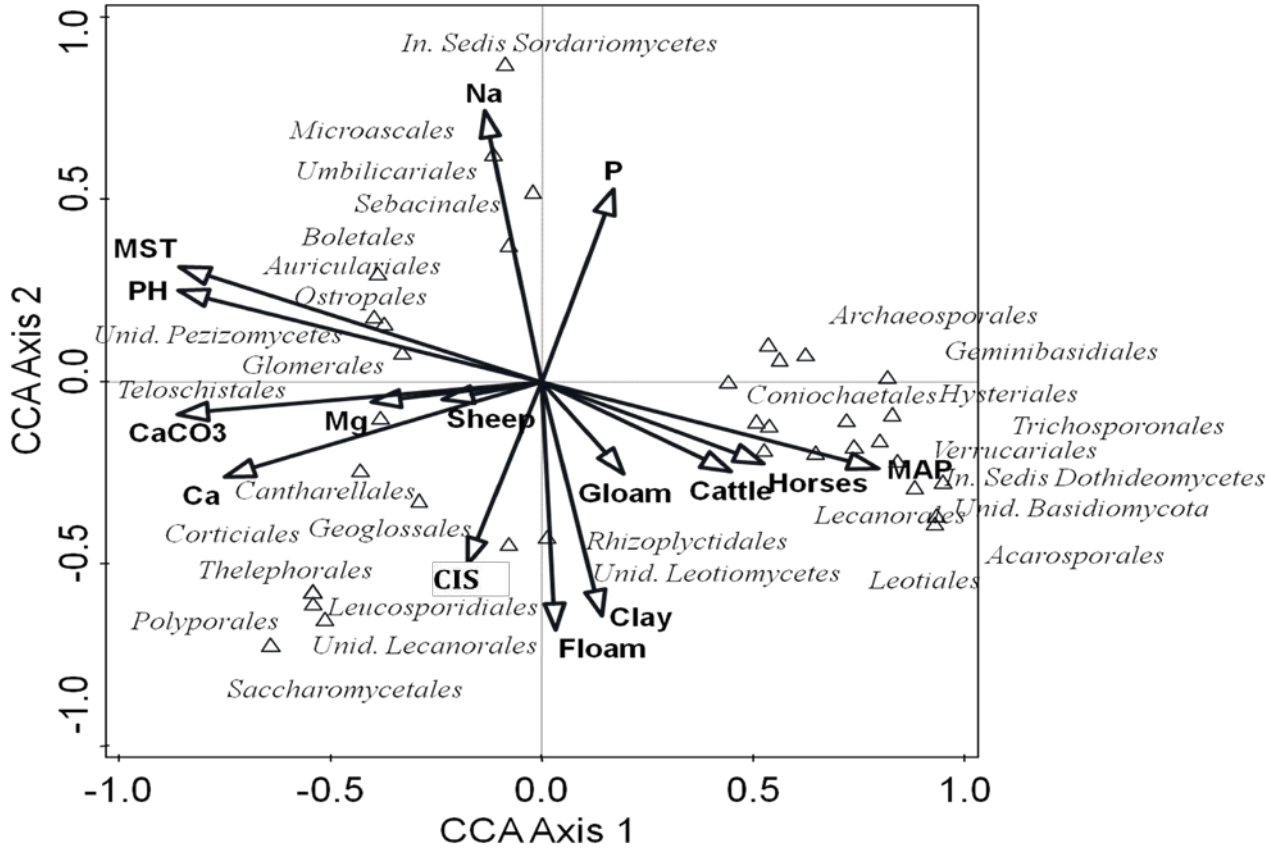

Figure 3.1. Plot of the fungal CCA representing the distribution of the fungal orders. Only the most characteristic orders of each climatic zone are represented. The direction of the main factors affecting the fungal community distribution are also represented.

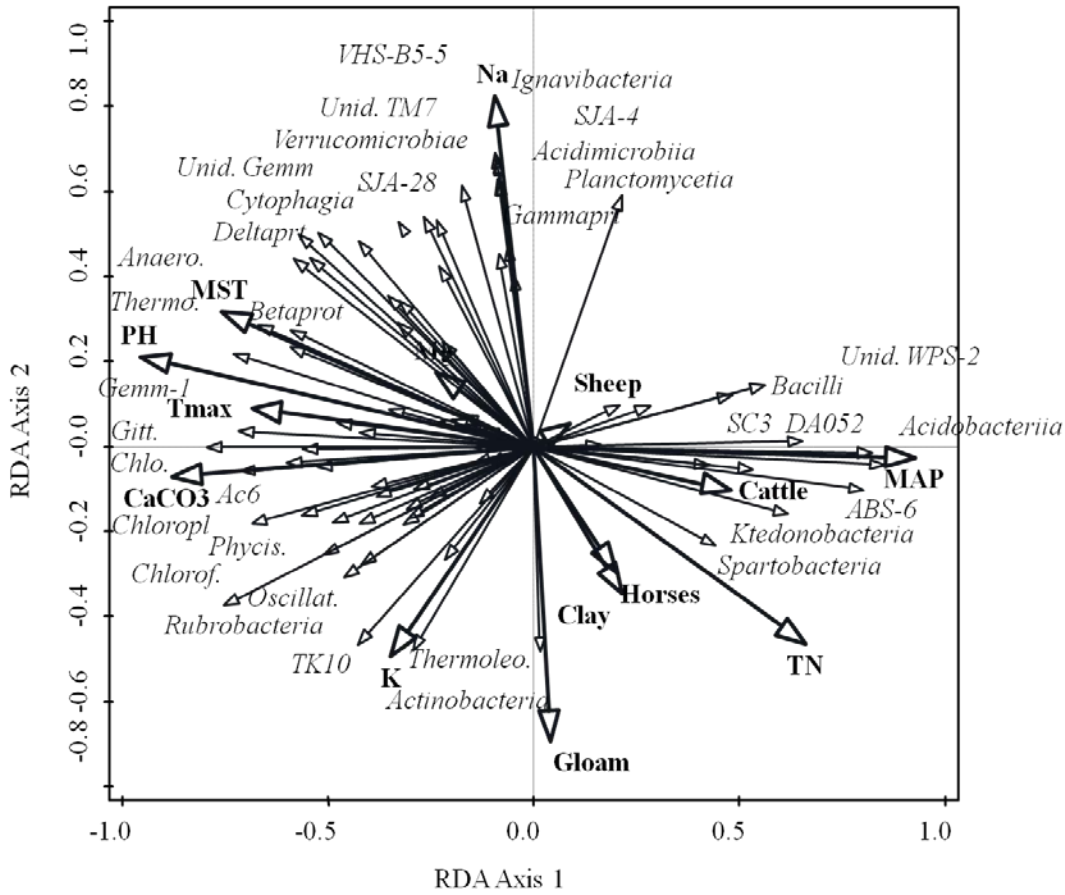

Figure 3.2. Plot of the bacterial RDA representing the distribution of the bacterial classes. Only the most characteristic classes of each climatic zone are represented. The direction of the main factors affecting the bacterial community distribution are also represented. Unid: Unidentified; Betaprt: Betaproteobacteria; Gammaprt: Gammaproteobacteria; Deltaprt: Deltaproteobacteria; Gemm: Gemmatimonadetes; Thermo: Thermomicrobia; Gitt: Gitt-GS-136; Chlo: Chloracidobacteria; Ac6: Acidobacteria-6; Chlorop: Chloroplast; Phycis: Phycisphaerae; Chlorof: Chloroflexi; Oscillat: Oscillatoriophycidae; Thermoleo: Thermoleophilia.

3.3.4. Fungal and bacterial co-variance and relationship with environmental gradients

Fungal and bacterial community co-varied along the altitudinal gradient. The common variance accounted for 5.4% of the total variation in fungal community structure and 1.22% in bacteria. Of the common variance, 9.8% was accounted by the first Co-CA axis, while the second and third axes explained 2.5% and 2.2% of the common variance, respectively. The respective Co-CA axis for bacteria and fungi were highly correlated (Spearman correlation of bacterial and fungal axis 1: r=0.8326; axis 2 : r=0.7476 and axis 3: r=0.8825). For the global microbial community, the first and second common Co-CA axes were associated to a temperature-precipitation and pH gradient, while the third axis was associated to other soil physical and chemical parameters (Fig. 3.3), including sodium content and the proportions of sand, silt and clay. Fig. 3.4 shows the distribution of samples and microbial taxa in the tridimensional space based on these three common axis.

Based on the first three axis of the Co-CA, hierarchical clustering resulted in 5 groups of microorganisms, comprising both bacteria and fungi. The most influential taxa in the distribution of samples in the tridimensional space are summarized in table S3.5. The first microbial cluster (Cluster 1) consisted in 25 fungal orders and 37 bacterial classes. Although including several ubiquitous taxa, its highest proportions were found in the semi-arid and montane grasslands (Fig. 3.4). The second microbial cluster (Cluster 2) included 35 fungal orders and 13 bacterial classes, and it was the most ubiquitous group (Fig. 3.4, Table S3.5),

related mainly to montane and some alpine grasslands, located around the origin of axes. The third microbial cluster (Cluster 3) comprised 8 fungal orders and 23 bacterial classes and they were related to the Mediterranean grassland. The fourth microbial cluster (Cluster 4) included 5 fungal orders and one single bacterial class, *Ktedonobacteria* (Table S3.5). This group peaked in the alpine grasslands. Finally, the Cluster 5 included only 5 bacterial classes, but none of the fungal orders. Although with low abundances, this group was exclusive from the Mediterranean grassland, and located at the most extreme values of the third axis (Fig. 3.4).

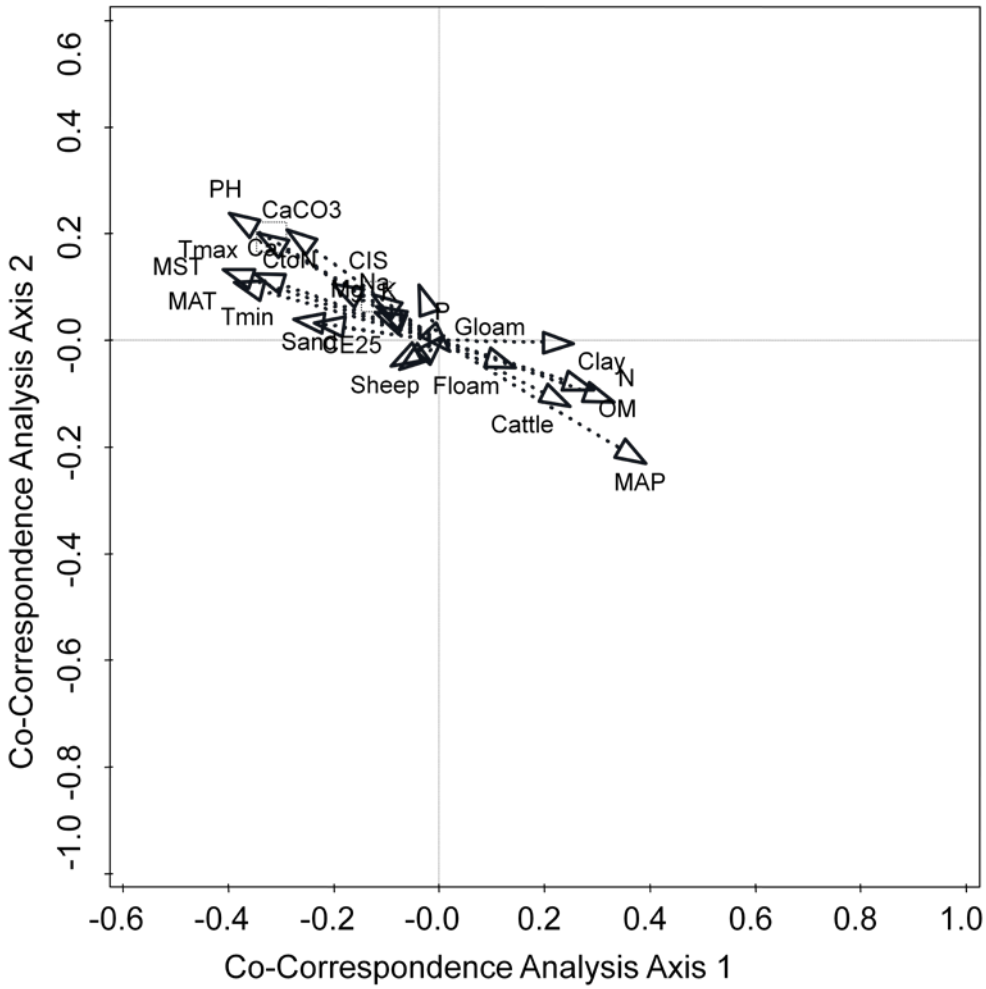

Figure 3.3. Plot of the first Co-CA axis showing the most influential environmental factors based on the microbial community composition (including fungi and bacteria).

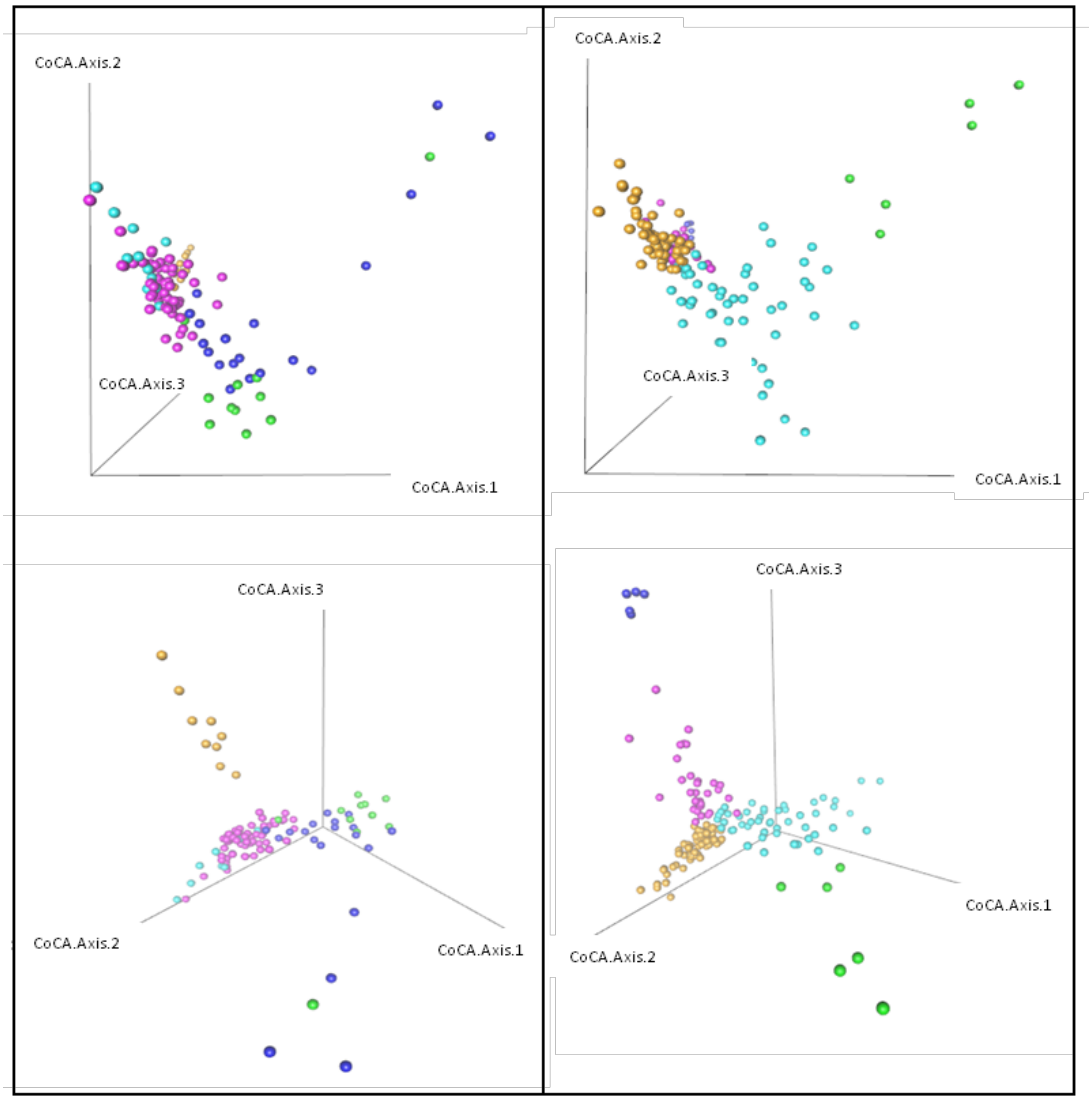

Figure 3.4. Plot of the distribution of soil samples (left) and microbial taxa (right) based on the Co-CA results. Left side: Climatic zones are represented by different colors: Yellow: Mediterranean grassland; dark-blue: Alpine grasslands; light-blue: semi-arid grasslands; purple: Montane grasslands; green: Atlantic grasslands. Right side: Different colors indicate the resulting clusters: Yellow: cluster 1; light-blue: cluster 2; purple: cluster 3; green: cluster 4; dark-blue: cluster 5.

3.4. Discussion

3.4.1. Microbial community composition

The present study focused on the composition of the fungal and bacterial communities in semi-natural grasslands, across wide environmental gradients, including altitudes from 5 to 2.479 m a.s.l. In this way, we included a fairly comprehensive set of environmental variables known to covariate with altitude, but also included different geographic areas (Table S3.1). Within the fungal community, we found a vast predominance of the phylum *Ascomycota* (Table S3.2), far above the *Basidiomycota*, as expected in this type of ecosystem (Porras-Alfaro *et al.*, 2011). Within the phylum *Ascomycota*, *Pleosporales* accounted for the highest relative abundances specially in the most arid zones (Table S3.3), as found in several studies on grassland ecosystems (Khidir *et al.*, 2010; Porras-Alfaro *et al.*, 2011). Their high abundance in arid climates may be explained because of the presence of melanin pigments in the hyphae of most of their taxa, these called dark septate fungi (DSF). These pigments confer resistance to several environmental stresses, including high temperatures and extended drought (Bell & Wheeler, 1986). The order *Helotiales*, also including several DSF, was found ubiquitously throughout the altitudinal gradients. However, they peaked in the alpine grasslands, oppositely to that found in *Pleosporales* (Table S3.3).

Different hypotheses have been proposed in microbial geographic studies, including theories considering either that every microbial taxa is

everywhere or that the environment strongly selects microbial composition, as well as intermediate views proposing that microbial community composition is a result of both past events and contemporary environmental conditions (Martiny *et al.*, 2006). We based on the definition of assemblages by Fauth *et al.* (1996), searching for those groups of taxa sharing the same environmental affinities, trying to identify the most important abiotic drivers.

3.4.2. Environmental drivers of soil fungal communities

Soil pH is known to strongly affect soil microbial communities, but when focusing only on soil fungi, previous studies found no effect or only a weak effect of pH on the overall soil fungal community (Rousk *et al.*, 2010; Bahram *et al.*, 2012), suggesting that only specific fungal taxa such as *Helotiales* and *Hypocreales* are sensitive to pH changes (Rousk *et al.*, 2010). According to our results, *Helotiales* were also found peaking in the alpine grasslands, where the pH was much lower than in any other climatic zone (Table S3.3). However, pH was not the main factor driving changes in the fungal communities (Table 3.1). Instead, mean summer temperature (MST) was the factor explaining most of the variation in fungal composition, followed by Na content. Bahram et al. (2012) studied the ectomycorrhizal (ECM) communities of forest soils along wide elevational gradients and found that MAT and MAP were significantly correlated with the ectomycorrhizal community composition. Geml *et al.* (2014) also related the first ordination axis of an ordination multivariate analysis to an elevation gradient while studying fungal communities from forest soils. Temperature has been

previously found to strongly determine the soil fungal community composition along altitudinal gradients and the suggested causes were either its direct effect on enzymatic activity or its indirect effect via host plant community or soil nutrients (Widden, 1987; Meier *et al.*, 2010). Temperature and rainfall, as the main climatic factors, show the strongest positive associations with elevational diversity patterns in animals and plants (McCain, 2009; Fierer *et al.*, 2011), and our results suggest that these climatic factors are also structuring the soil fungal communities.

We also found a strong effect of sodium content on soil fungal communities (Table 3.1), since all the samples belonging to the coastal, saline grassland distributed separately from other samples in the fungal CCA. Salinity can affect the osmotic potential of the soil solution translating in a reduced availability of water and nutrients for plants (Smith, Chen, & Chalk, 2009). The resistance of plants to stress conditions caused by an excess of salts may be enhanced by the association with fungal endophytes, which increase their tolerance to stress (Soares *et al.*, 2016). Rodriguez *et al.* (2008) exposed their 'habitat-adapted symbiosis hypothesis', where they supported that the endophytic set of fungi found in a given soil are the specific helpers that plants need for a particular type of stress. According to this hypothesis, fungi found in a saline soil are expected to provide resistance to saline stress to other cohabitant organisms. Our Mediterranean grassland had a sandy, saline soil, and *Boletales* as well as some taxa within *Sordariomycetes*, were the most characteristic taxa found there.

Ecologically, most of the taxa found within the order *Boletales* are important ECM fungi in the ecosystems and can form ECM relationships with plants of several families (Wu *et al.*, 2014). Whereas several studies focused on the role in alleviation salt stress by arbuscular mycorrhizal fungi (AMF) (Evelin, Kapoor, & Giri, 2009; Aggarwal *et al.*, 2012), only a few studies have reported an increase in the abundance of ECM in saline soils (Hrynkiewicz *et al.*, 2015). Dixon, Rao, & Garg, (1993) found that only selected ectomycorrhizal fungi seem to be able to grow and to establish symbiosis in saline soils. A general feature of *Boletaceae* are the boletoid rhizomorphs, root-like structures that enable the fungi to efficiently transport water and nutrients (Agerer, 2006). Also fungi belonging to this taxa have been reported to release particular metabolites which enable the external regulation of the osmotic equilibrium under high Na concentration (Bois *et al.*, 2006). Thus, according to the 'habitat-adapted symbiosis hypothesis' (Rodriguez *et al.*, 2008), the high abundance of *Boletaceae* almost exclusively within this saline soil could be the result of soil chemical filters (low availability of nutrients, high salinity, high pH values; Table S3.3). This contrasts to that found within all other grasslands, which distributed along the environmental gradient, suggesting that particular groups of fungi respond to different environmental filters.

Most of the samples distributed along the MST-MAP climatic gradient, represented mainly by the first CCA axis (Fig. 3.1). Fungal taxa associated to the semi-arid grasslands included *Ostropales*, *Pezizales*, *Glomerales* and *Teloschistales*, among others (Table S3.3, Fig. 3.1).

Previous studies suggest that some DSF may be related to the order *Pezizales*, thus improving plant tolerance to drought and high temperature stress (Jumpponen & Trappe, 1998). *Glomerales*, an arbuscular mycorrhizal order belonging to *Glomeromycotina*, was also found characterizing the semi-arid grasslands (Table S3.3, Fig. 3.1). This kind of fungal symbionts are commonly found in the roots of plants in arid grasslands and deserts (Trappe, 1981; Brundrett, 1991), playing an important role by increasing water uptake by plants (Allen, 1982; Cui & Nobel, 1992). Although previous studies found that the relative abundance of AMF was negatively affected by high mean temperatures and drought in grassland sites (Xiang *et al.* 2016), other studies have shown a synchronization between the fungus and the plant, in which AMF relative abundance was attributed to the response of the plant to moisture availability (Veenendaal, 1991; Veenendaal, Monnaapula, & Gilika, 1992). The lichenized orders *Teloschistales* and *Ostropales* were also found within semi-arid assemblages (Table S3.3, Fig. 3.1). Lichens are known to be highly tolerant to desiccation, and the main sources of water that lichens usually use are fog and dew. These capabilities allow them to exploit habitats under high temperatures, high solar radiation and low rainfall. Particularly, the order *Teloschistales*, here mainly represented by the family *Teloschistaceae* (data not shown) are a group of lichens especially associated to arid habitats (Gaya *et al.*, 2015). This is presumably because *Teloschistaceae* accumulate anthraquinones, secondary metabolites known to protect these organisms against high solar radiation (Gaya *et al.*, 2015).

In the other extreme of the gradient, CCA located all the samples belonging to the Atlantic, alpine and the most humid montane grasslands. All of them shared high precipitation regimes along with low temperatures and a strong decrease in soil pH. Lichenized fungi were also highly represented, as expected under extreme environmental conditions, with important proportions of the orders *Acarosporales*, *Verrucariales* and *Lecanorales* (Table S3.3, Fig. 3.1). Previous studies on alpine grasslands found an important abundance of taxa belonging to these orders in the soil, highlighting the role of particular taxa within *Lecanorales* as bioindicators of habitat variability and grazing disturbances (Prieto, Aragon, & Martinez, 2010; Rai, Upreti, & Gupta, 2012). AMF were represented in these alpine grasslands by the order *Archaeosporales* (Redecker *et al.*, 2013). Saprotrophic and pathogenic taxa were also found abundantly within fungal assemblages of the alpine grasslands, in the orders *Coniochaetales*, *Diaporthales*, *Sporidiobolales*, *Geminibasidiales* and *Leotiales* (Taylor & Hollingsworth, 2014; Tedersoo *et al.*, 2014). Fungal endophytes (either symbionts or parasites) are also found within some of these taxa (*Leotiales*, *Coniochaetales*), as expected since the endophytes represent an important soil fungal community associated with alpine plants (Schadt, Mullen, & Schmidt, 2001). Within them, the presence of melanized hyphae in DSF may protect the fungi not only against UV radiation, but also by trapping free radicals released under stressful abiotic conditions, like low temperatures. Thus, DSF could have an analogous functional role to mycorrhizal fungi towards

grass species in these alpine and subalpine grasslands (Mouhamadou *et al.*, 2011).

Finally, montane grasslands distributed close to the origin of axes in the CCA plot, indicating that they occupy an intermediate situation, specially characterized by saprotrophic taxa within the order *Geoglossales* (Tedersoo *et al.*, 2014). *Geoglossales* are commonly found in temperate grasslands around Europe, and they have been thoroughly studied because of their particular conservation significance (McHugh *et al.*, 2001).

3.4.3. Environmental drivers of soil bacterial communities

In this study, we also surveyed the bacterial community composition through the use of Illumina metabarcoding. The most abundant phyla were *Actinobacteria* and *Proteobacteria*, which are found within the nine major phyla in the available 16S rRNA libraries (Janssen, 2006). These results are in agreement with previous studies on grassland soils, where these phylogenetic groups were also found to be predominant (McCaig, Glover, & Prosser, 1999; Nacke *et al.*, 2011).

Although the first bacterial RDA axis ordered the samples according to a climatic gradient similar to that found for fungi (Fig. 3.2), one of the factors explaining most of the variation associated to the bacterial communities was pH (Table 3.2). Soil pH has been identified as a key driver of the soil bacterial community structure, having even stronger effect than climatic factors (Lauber *et al.*, 2009b; Rousk *et al.*, 2010; Barberán & Casamayor, 2014). Our results are in accordance with such

affirmations, since pH explained 27.1% of the variability associated to the bacterial community composition. Bryant *et al.* (2008a) found a decrease in the diversity of *Acidobacteria* when increasing elevation, but presumably due to changes in soil pH, which also decreased with increasing elevation. Shen *et al.* (2015) found significant differences in soil bacterial community composition across a narrow elevational gradient in the alpine tundra (2,000-2,500 m a.s.l.). They found the bacterial community composition to be correlated with soil carbon and nitrogen contents, and C/N ratio, while no effect of soil pH was detectable. They suggested that the narrow pH range and the limited variation among sites could have been masking the stronger effects of pH. In another study, Singh *et al.* (2014) found that elevation (correlated with temperature and rainfall) was the best predictor of the bacterial community composition, with pH explaining only part of the variation. In our study, pH had a wide range of variation across the environmental gradients (from 4.76 in the alpine grasslands, to 8.62 in the Mediterranean soil), but this factor was also linked to several other soil characteristics (like soil carbon and nitrogen content and soil moisture regime) that are often directly or indirectly related to soil pH (Buckman & Brady, 1961). Confounding factors are known to occur when studying elevational gradients, being the decrease in soil pH with increasing elevation one of the most relevant situations (Fierer *et al.*, 2011).

The bacterial community composition distributed the samples from semi-arid and some of the montane grasslands at the right side of the first axis. These warm and alkaline grasslands were characterized by a

set of taxa summarized in Table S3.4, mainly *Phycisphaerae*, *Rubrobacteria*, *Chloracidobacteria* and *Chloroflexi*. Previous studies found the bacterial class *Rubrobacteria* predominating in high soil temperatures and severe droughts (Holmes, Bowyer, & Holley, 2000; Davinic *et al.*, 2012). These taxa have been previously described as tolerant to gamma-radiation and desiccation (Yoshinaka, Yano, & Yamaguchi, 1973; Singleton & Furlong, 2003). Similarly, taxa belonging to the class *Chloracidobacteria* have also been found to be highly tolerant to alkaline pH and elevated temperature (Costas, Tsukatani, & Rijpstra, 2012). *Phycisphaerae*, also characterizing semi-arid grasslands, has been found to be a source of chemical substances that allow these bacteria to be resistant towards salt and temperature stresses (Jeske *et al.*, 2013).

On the other side of the gradient, bacterial community composition distributed all the samples belonging to the Atlantic and alpine grasslands. All these sites shared high rainfall, low temperatures, and similar soil properties, such as low pH values and high organic matter content. Although pH in these locations was found low (values from 4 to 6), many soil microorganisms are known to have adaptive responses to endure acidic stress (Beales, 2004). *Bacilli*, belonging to the phylum *Firmicutes*, was the most characteristic taxa of these grasslands (Table S3.4). Previous studies in acidic soils also found an active bacterial community dominated by members of *Firmicutes* and, particularly, from *Bacillaceae* (Felske, Wolterink, & Lis, 1998), as happened in the present study. *Bacillaceae* are widely distributed in natural environments, and

they are distinguished by their ability to form endospores that allow them to survive under several stresses (van Elsas, Stefanic, & Mandic-Mulec, 2015). Members within this taxon have been found to correlate with acidic soils (Zhalnina *et al.*, 2015). In the same way than in our study, previous studies found a negative correlation of *Ktedonobacteria* (phylum *Chloroflexi*) with pH, almost disappearing in samples with a pH > 7. However, in spite of their acidic preferences, little more is known about this lineage (Kim *et al.*, 2015). Also specific from these locations was the class *Acidobacteriia*, which is also known to predominate under low pH conditions (Sait & Hugenholtz, 2002).

Similarly to that found for the fungal CCA, the second ordination axis of the bacterial RDA was related to sodium content, which explained 7.7% of the bacterial community composition and characterized all the samples from the Mediterranean grassland (Table 3.2). In fact, the saline boundary is one of the most important evolutionary barriers structuring biological communities, and it has been previously reported for both bacteria (Lozupone & Knight, 2007) and archaea (Auguet, Barberan, & Casamayor, 2010). Our Mediterranean grassland was the most differentiated of the study, being located on the coast and having high salinity, alkaline pH and sandy texture. This soils were characterized by high proportions of classes known to be halophylic (Table S3.4). Previous studies on saline sediments also found a predominance of the class *Gammaproteobacteria* within the phylum *Proteobacteria* (Sun *et al.*, 2013), which is also known to be highly resistant to solar radiation. *Planctomycetia*, belonging to the phylum *Planctomycetes*, has also been

found in hipersaline habitats (Andrade *et al.*, 2017) and various soil types, like sandy soils like our study site (Zhou *et al.*, 2003). *Cytophagia*, belonging to *Bacteroidetes*, was also characterizing this grassland. The phylum *Bacteroidetes* is specially important in aquatic environment, including marine and freshwater sediments (Barberán & Casamayor, 2010; Tian, Wang, & Hu, 2010) as well as in solar salterns (Benlloch *et al.*, 2002; Boujelben *et al.*, 2012) and in continental saline areas (Monegros, Casamayor *et al.*, 2013).

3.4.4. Common drivers of soil microbial communities

In a previous study, Lozupone & Knight (2007) reported that salinity was the most influential factor driving the global microbial community composition, rather than pH, temperature extremes, or other physical and chemical factors. In our study, both bacterial and fungal community compositions separated all the saline samples apart from all those non-saline, even when having similar pH values. However, sodium content explained only 7.7% and 7.1% of the bacterial and fungal community compositions respectively, being the second most important factor in both cases. On the other hand, there was a differential effect of pH on fungal and bacterial communities, being much stronger over bacterial taxa distribution (Tables 3.1 and 3.2). Although pH has been proved to strongly influence the overall community composition (Hartman *et al.*, 2008; Lauber *et al.*, 2009b), Rousk *et al.* (2010b) found a far weaker influence of this factor on fungi, probably due to their known tolerance to a wider range of pH values than bacteria. Furthermore, it should be taken into account that pH is the result of a combined set of soil

characteristics including salinity, nutrient availability and soil moisture(Lauber *et al.*, 2009a), which in turn depend on climatic factors. In fact, previous studies reporting a strong effect of pH on fungal community, could not distinguish the effect of the climatic variables from those of soil chemistry because of the high correlation (Coince *et al.*, 2014) and others suggested that this effect may be an indirect effect of the differential competition from the dynamic bacterial communities along the pH gradient (Rousk *et al.*, 2010).

In order to improve the knowledge about the co-variation between fungal and bacterial communities, we applied co-correspondence analysis (Co-CA, ter Braak & Schaffers, 2004). This method finds the maximum co-variance between the sample positions in bacterial and fungal spaces, identifying the most important axes common in both communities (Johannesen *et al.*, 2017). These concordance among microbial assemblages reveals interactions among them (Santoul, Soulard, & Figuerola, 2004). Thus, taxa that most contributes to these common axes are strongly associated, showing similar responses to environmental gradients (Kilgour & Barton, 1999; Infante *et al.*, 2009). Previous studies applied Co-CA to study the best predictors of the microbial community composition. Mitchell *et al.* (2010) included plant species composition and soil chemical variables, and found the vegetation composition to predict microbial community composition at least as well as soil chemical variables. By contrast, Chow *et al.* (2014) found this method unable to estimate bacterial community variance from virus and protistan community data. Valverde *et al.* (2015) found

that the cyanobacterial community composition alone explained an important proportion of the variation in the heterotrophic bacterial community composition.

In spite of their societal and economical relevance, little is known about the fungal-bacterial interactions in soils, specially from the point of view of degradation processes, co-existence mechanisms and phytopathogens (Boer *et al.*, 2005). In our study, we found a strong correlation between bacterial and fungal communities (r=0.83, r=0.74 and r=0.88 for the first three axis). This suggests that both communities are probably responding to combinations of factors co-varying along the altitudinal gradients, which are not easily measurable (Ritz *et al.*, 2004).

When taking into account both communities, samples distributed along the environmental gradient over the first and second axes, with the same separation of the saline soil along the third axis as found when including only one of the communities (Figs. 3.3 and 3.4). As shown in Fig. 3.4, the clusters including both bacterial and fungal assemblages based on the Co-CA axes revealed a differential stress-tolerances between clusters. Thus, Cluster 2 differentiated microbial assemblages found in alpine and subalpine grasslands and other ubiquitous taxa, but Cluster 4 included only the taxa characterizing the alpine (and some Atlantic) grasslands. The same happened along the salinity gradient, where microbes clustered in a first group of halophylous and ubiquitous taxa (Cluster 3), but the Cluster 5 consisted of only bacterial classes, presumably highly tolerant to saline stress and alkaline pH.

Each one of these groups represents an starting point from which the study of the relations between the fungi and the bacteria that coexist in the same environmental conditions can be undertaken. For example, in the Cluster 3, found most abundantly in the Mediterranean, saline grassland, we found *Boletales* and *Gammaproteobacteria* (Table S3.5). As indicated above, *Boletales* contain most of the taxa forming EM, while *Gammaproteobacteria* includes the family *Pseudomonadaceae*. The genus *Pseudomonas* is known to resist under extreme conditions (Christner *et al.*, 2003), but also to have a beneficial role in the establishment of EMC symbiosis, in the so-called "mycorrhizal helper bacteria"(MBH; GARBAYE, 1994). Then, the fungus promotes plant growth conferring tolerance to abiotic stresses (here high salinity in a sandy soil; Begum & Tamilselvi, 2016) and the associated bacteria enhance the symbiosis establishment, as well as protection against various phytopathogens (Frey-Klett, Garbaye, & Tarkka, 2007). On the other hand, when focusing on Cluster 4 (Table S3.5), we found several fungal taxa associated to the alpine grasslands, including the lichenized *Lecanorales*, while only one single bacterial class (*Ktedonobacteria*). It is well known the antibiotic activity of the secondary metabolites produced by lichens. Particularly, several taxa belonging to *Lecanorales* have been studied because of their antimicrobial activity with clinical purposes (Bellio *et al.*, 2015). This could be an explanation for that only a bacterial class was found in these grassland soils (Table S3.5).

3.5. Conclusions

In this Illumina metabarcoding assay, we unveiled how the soil microbial community composition changed along the environmental gradients explored. We found particular assemblages of bacterial and fungal taxa which were known to have adaptations to each particular set of environmental stresses, highlighting the usefulness of this methodology to find taxa with particular capabilities. Furthermore, bacterial and fungal communities co-varied strongly along the gradient. A thorough search within the resulting groups in this study might reveal associations previously unknown. Although soil chemical factors (particularly soil pH) seemed to influence more the bacterial than the fungal community, a set of factors are known to co-variate with altitude, and the responses of both microbial consortia seem to be interlinked. Although more in-depth research is needed, our results provide a set of potential indicators for predicting the changes in microbial community composition in a context of global climatic change.

3.6. Acknowledgements

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Chapter 4

Fungal community of fairy rings

4. Metabarcoding analysis of fungal communities across fairy rings in a montane grassland

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ABSTRACT: Increasing numbers of fungal species have been described recently from semi-natural grassland soils, rising the conservation interest of these species-rich habitat. Here, we characterize the soil fungal community composition in a montane grassland of the Eastern Pyrenees through the use of Illumina metabarcoding, and provide an assessment of the differences in the fungal community composition along transects across six fairy rings. A total of 711,299 MiSeq reads were obtained and 483 to 894 operational taxonomic units (OTUs) at 97% identity level were observed per soil sample. The dominant taxa in

the grassland were ascomycetes belonging to Pleosporales. The first axis of a multivariate ordination analysis (CCA) on fungal orders indicated a clear differentiation of the fungal community composition outside of the rings compared to the ring areas, showing high abundances of Pleosporales and Eurotiales, whereas all other zones inside the fairy rings showed high abundances of Agaricales. We found varying saprophytic taxa associated to the studied rings, including the genera *Clavaria*, *Psathyrella*, *Tricholoma*, *Amanita* and *Lycoperdon*. The results of this metabarcoding analysis highlight the importance of particular keystone taxa in the structuring of fungal communities and their effect upon grassland fungal communities.

4.1. Introduction

Since the beginning of the 20th century, semi-natural grasslands have seriously decreased in terms of land cover across Europe due to land use changes, being increasingly restricted to remote areas in most European countries (Heilmann-Clausen & Vesterholt, 2008). Agricultural intensification on one hand, and the decreasing number of grazing animals on the other, have led to the inclusion of grasslands within endangered habitats (Newton *et al.*, 2003; Heilmann-Clausen & Vesterholt, 2008). The high biodiversity present in these ecosystems has been studied especially from the viewpoint of animals and plants (Kahmen, Poschlod, & Schreiber, 2002; WallisDeVries *et al.*, 2002; Hodgson *et al.*, 2011; Howland *et al.*, 2016), whereas the kingdom fungi

started to be included in conservation programs only recently (Dahlberg, Genney, & Heilmann-Clausen, 2010). Decreases in fungal species occurring in these habitats during the last decades motivated the establishment of conservation measures (Moore, 2001; Griffith *et al.*, 2013). Rotheroe *et al.*, 1996 proposed the "CHEG profile" for the quantification of a set of taxa typical of nutrient poor "waxcap" grasslands, including grassland species of Clavariaceae ('fairy clubs'), the genus *Hygrocybe* ("waxcaps"), Entolomataceae ("pink gills") and Geoglossaceae ("earth tongues"). Since then, members of the genera *Dermoloma, Camarophyllopsis* and *Porpoloma* (Griffith *et al.*, 2013) have been also included in order to qualify grasslands in terms of their conservation value, being then sometimes referred collectively as CHEGD fungi (Griffith, Easton, & Jones, 2002). Nevertheless, basidiocarps are ephemeral and their occurrence depends on the specific combination of environmental factors (Miller, Grand, & Tredway, 2011; Griffith *et al.*, 2013). Recently, Next-generation DNA sequencing (NGS) techniques have been developed, and increasing amounts of data on the whole fungal community are being recorded, regardless of basidiocarp occurrence (Miller *et al.*, 2011; Geml *et al.*, 2014a). This has allowed the scientific community to detect an inconceivable diversity not only of macrofungi, but also of microfungi in grassland soils, especially those fungi belonging to the phylum Ascomycota often associated to plant roots (Lumini *et al.*, 2010; Geml *et al.*, 2014a). However, most of the studies have focused on arid and semiarid grasslands (Khidir *et al.*, 2010; Martínez-García *et al.*, 2011; Porras-Alfaro *et al.*, 2011; Wehner *et al.*,

2014; Büntgen *et al.*, 2015), while fungal diversity in temperate grasslands has been less frequently considered (Brodie, Edwards, & Clipson, 2003).

Within the overall fungal diversity in grassland soils, fairy ring fungi are considered "keystone" species, because of their strong impact on niche creation and nutrient cycling (Bonanomi *et al.*, 2013; Van der Wal *et al.*, 2013). These formations have been attributed to the radial growth of the mycelia from saprophytic Basidiomycota (Vargas, 1993; York, 1998). Based on the geometry of the mycelial nutrient source, Gregory (1982) divided the fairy rings into "free" and "tethered". The former are caused by saprophytic fungi growing in open areas such as semi-natural grasslands and golf courts, while the latter are caused by ectomycorrhizal fungi growing in association with trees. Based on the effects on the vegetation, Shantz & Piemeisel (1917) classified the rings into three types. Type-I rings are those that kill the grass or seriously damage it, typical from *Marasmius oreades* (Bolt.) Fr. Type-II rings only stimulate the vegetation at the margin of the ring, being *Tricholoma* spp. and *Lycoperdon* spp. examples of this ring type (Gregory, 1982). Finally, type-III rings are formed by *Hygrocybe* and *Panaeolus* spp. among others, and appear as a ring of fruit bodies that causes no visual effect on the vegetation (Griffith & Roderick, 2008). The impact of various fungi on the vegetation may be highly variable depending on the weather conditions and plant species, specially for those fungi causing type I and II rings (Shantz & Piemeisel, 1917; Terashima, Fukiharu, & Fujiie, 2004). For example, hand-watering of turf grasses have been used

to prevent type-II rings from turning into type-I rings (Fidanza, 2007b). Most of the studies on fairy rings have focused on the effect of mycelial development on soil properties, plant growth and microbial community (Bonanomi *et al.*, 2013), whereas only a few studies focus on fungal communities using NGS technologies (Miller *et al.*, 2011; Kim *et al.*, 2013).

In this study, we focus on the soil fungal community composition of a montane grassland located in the North-Eastern Pyrenees in the Iberian Peninsula, where we recently detected rings of vegetation growing vigorously. Although no basidiocarp occurrence have been described so far by local people in the studied fairy rings, species belonging to Tricholomataceae are usually found in grasslands adjacent to our study site. Thus, the aims of the present study are i) To describe the fungal community composition of a montane grassland using Illumina MiSeq from amplified markers, focusing particularly on those fungi belonging to Ascomycota and Basidiomycota; ii) Characterizing the fungal community across the different visible zones of the fairy rings; and iii) Identifying those possible macrofungi causing the ring effects on the vegetation, on the basis of the results provided by this technique.

We hypothesize that one single Basidiomycete is responsible for the fairy ring formation, presumably a species known to form type-II rings, based on the visible effects on the vegetation in the form of a perceptible ring of outgrown plant biomass. We expect to find high relative abundances of a single species in those zones around the vigorous vegetation, as found in other studies (Kim *et al.*, 2013), as well

as differences in the fungal community composition in the different zones across the rings, given the spatial heterogeneity in decomposition processes and niche formation reported to occur by other studies (Van der Wal *et al.*, 2013).

4.2. Material and methods

4.2.1. Site and experimental design

The study was conducted in La Bertolina, a pastured grassland located in Pla de Busa, in the Eastern Pyrenees (42°05'56" N, 1°39'40" E), 1276 m.a.s.l. La Bertolina has a mean annual temperature of 8.7 ºC and a mean annual precipitation of 954.8 mm (ACDC, http://www.opengis.uab.cat/acdc/catala/cartografia.htm, accessed 2015; Ninyerola, Pons, & Roure, 2007). The bedrock is limestone with a high stoniness of polygenic conglomerates (ICGC, http://betaportal.icgc.cat/visor/client_utfgrid_geo.html, geologic map 1:50000 BG50M_v1r1, 2007, accessed 2015). This grassland is extensively grazed by cattle (0.44 LSU ha^{-1}) from May-June to November. In this montane meso-xerophytic grassland, the vegetation is dominated by grasses (*Festuca arundinacea* Schreb., *Poa bulbosa* L., *Dactylis glomerata* L.), although legumes and other forbs are also commonly found (*Plantago lanceolata* L., *Trifolium pratense* L., *Medicago lupulina* L., etc.).

The survey was conducted in May 2015, because it is when the rings in the vegetation are more visible. Previous studies in this grassland area made possible the geo-referencing of fairy rings from 2013 aerial images (data not shown). Two ring sizes were selected, large and small ("L" and "S" respectively), with three replicates per size. Selection of the rings was made taking into consideration the distance from other rings in order to avoid interferences, and also the continuity of the ring, avoiding those rings that were incomplete whenever possible. Five sampling points were designated in a radial transect across each ring: 1) In the center of the ring ("Center"); 2) In the geo-referenced 2013 ring zone ("Ring13"); 3) In the 2015 dark-green vegetation zone ("Ring15"); 4) In the area immediately outside of the ring ("Front") and 5) outside the ring, without visible ring influence (> 2m), ("Outside"). Circular plots of 25 cm in diameter were defined as sampling points.

4.2.2. Soil sampling and fungal metabarcoding

Soil samples were taken in the 0-10 cm depth soil layer with a 10 cm x 6 cm x 5 cm metal soil core in each sampling point. A total number of 30 soil samples were stabilized in a Solution C1 of MoBio's PowerSoil DNA Isolation kit during transportation to the laboratory. DNA was isolated upon arrival to the laboratory using the PowerSoil DNA isolation kit (MoBio), strictly following the manufacturer's instructions. Determination of DNA concentration was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientic).

A fragment of the fungal ITS2 region of around 400 bp was amplified using the primers ITS86F (5' GTGAAT CATCGAATCTTT GAA 3', Turenne, Sanche, & Hoban, 1999) and ITS4 (5' TCCTCCGCTTATTGATATGC 3', White *et al.*, 1990). The primers had the Illumina adapter sequences and were tagged to their 5' end. The tags make it possible to link the reads obtained during sequencing to a particular sample.

PCRs were carried out in a final volume of 25 µL, containing 2.5 µL of template DNA, 0.5 µM of the primers, 12.5 µL of Phusion DNA polymerase mix (Thermo Scientific), and ultrapure water up to 25 µL. The reaction mixture was incubated as follows: an initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s, and a final extension step at 72 °C for 10 minutes. Despite the high number of PCR cycles used, the PCR products were checked on 1% agarose gel and showed similar medium intensity bands. The tags required for multiplexing different libraries in the same sequencing pool were attached in a second PCR round with identical conditions and only 5 cycles. The libraries were run on a 1% agarose gel stained with REAL Safe (Durviz), and imaged under UV light. Negative controls that contained no DNA were included to check for contamination during library preparation.

PCR products were purified using the Mag-Bind RXNPure Plus magnetics beads (Omega Biotek), following the instructions provided by the manufacturer. Then, they were quantified with the Qubit dsDNA BR Assay Kit and pooled in equimolar amounts. The equimolar pool was sequenced in a fraction of an Illumina MiSeq PE300 run. The negative

controls were included in the pool, in order to check for potential contamination. The quality of the FASTQ files was checked using the software FASTQC. A first quality-filtering step was performed using the software Geneious 8.1.8. In this step, regions from both ends of the reads with more than a 0.5% chance of an error per base were trimmed. Paired-end assembly of the forward (R1) and reverse (R2) reads was performed with FLASH (Magoč & Salzberg, 2011). The mismatch resolution in the overlapping region is accomplished by keeping the base with the higher quality score.

The FASTQ files were quality filtered using the bioinformatic tool Qiime 1.9.1 (Caporaso *et al.*, 2010). DNA sequences having quality score <20 were discarded. Chimeric sequences were removed using the UCHIME algorithm (Edgar *et al.*, 2011) implemented in VSEARCH, using UNITE as a reference database (Abarenkov *et al.*, 2010). ITS2 reads were clustered into OTUs using the open-reference approach in Qiime. In this method, reads are clustered against a reference database and any reads which do not hit the reference sequence collection are subsequently clustered de novo. Each OTU was assigned to a fungal taxa using the BLAST algorithm (Altschul *et al.*, 1990).

Based on the resulting OTU table obtained for each sample, an additional quality-filtering was carried out: the OTUs which were present in the negative control and the OTUs represented by sequences with frequencies lower than 0.005% in the whole dataset were removed. Given the high number of resulting sequences clustered de novo, we considered unreliable those taxonomical assignments to species level.

Nevertheless, we maintained the terminology of Species Hypothesis to designate those taxa discovered on identity threshold of 97% (Kõljalg *et al.*, 2013).

Finally, the rarefaction plots were constructed showing the rarefied number of OTUs defined at a 97% sequence identity threshold. When the rarefaction curves tended towards saturation, the sequencing depth was assumed to be sufficient to retrieve most of the fungal diversity. In addition, the percentage of coverage was calculated by the Good's method (Good & Toulmin, 1956), which is also used to check to what extent samples were adequately sampled.

In order to identify a core mycobiome, we included all those OTUs present across all samples without accounting for their relative abundance (Shade & Handelsman, 2012). Finally, as fairy rings are commonly expected to be caused by filamentous saprophytic fungi belonging to Basidiomycota (Vargas, 1993; York, 1998), we focus on the order Agaricales to describe those OTUs associated to ring formation.

4.2.3. Statistical analysis

CANOCO 5 (Microcomputer Power, Ithaca, NY, USA) was used for multivariate ordination analyses. The effect of the different zones across the fairy rings (Center, Ring13, Ring15, Front and Outside) was studied by Canonical Corrrespondence Analysis (CCA), both at the species and order level. The significance of the explanatory variables included in the CCAs was evaluated by Monte Carlo permutation tests (999 permutations). All ordination analyses were based on arcs-sine

transformed and normalized fungal proportions. Since the high stochastic variation of fungal communities in individual sampling sites might have hidden the differences among “ring zones”, the variable “sampling site” was partialled out by defining it as a covariate and as a permutation block.

All other data analyses were performed using the R software (R core Team, 2015). We assessed differences on the relative abundance of the most abundant phyla, orders and genera between rings, ring size and ring zones by linear model analysis and Tukey post-hoc tests. Differences between zones were tested based on the sampling classification (Centre, Ring13, Ring15, Front and Outside) and also on the reclassification of these categories into “Outside the ring”, which included the “Outside” category and “Inside the ring” which included all other categories.

4.3. Results

4.3.1. Sequencing output and taxonomic assignment

From a total of 3,895,522 MiSeq reads, 711,299 reads were available for taxonomical assignment after quality filtering. The number of validated reads ranged from 102,154 to 5,951 per sample. In total, we found 1,146 OTUs, ranging from 483 to 894 OTUs per soil sample at 97% identity level. From those, 268 hit the reference sequence collection and 878 were subsequently clustered de novo. Rarefaction curves for the overall sampling show how we reached the plateau for most of the samples, indicating that we retrieved most of the fungal diversity (Fig.

4.1). Each resulting OTU was classified from the phylum down to the genus level. The proportion of sequences that remained unidentified (no blast hit) was 0.7% (27 OTUs), while 1.3% of the fungal sequences (23 OTUs) were assigned to unclassified fungi. 497 OTUs could be identified to the genus level, 146 to the family level, 298 to the order level, 105 to the class level and 50 OTUs only to the phylum level. From the whole Basidiomycota set of reads, 36.8% hit the reference database, while it only happened for 22.1% of Ascomycota reads.

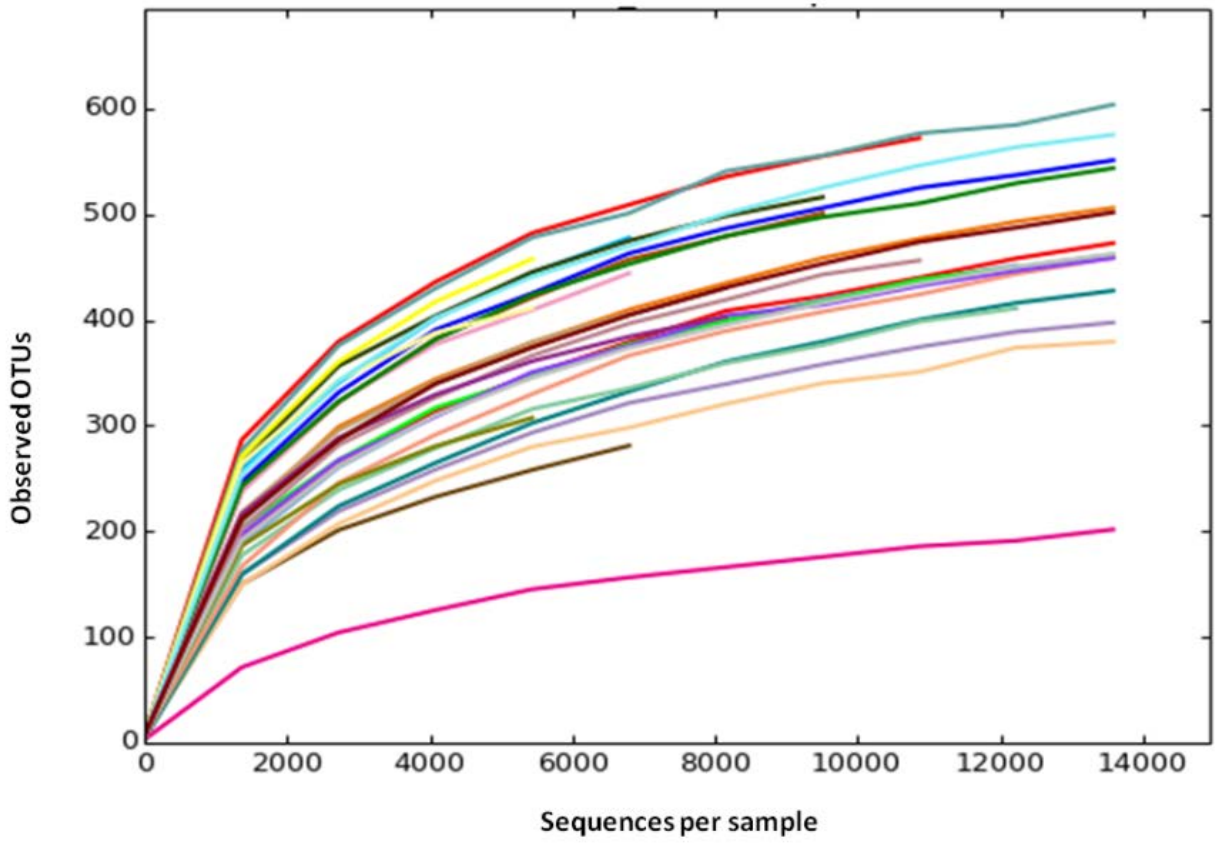

Figure 4.1. OTU accumulation curves per sample. Different colors correspond to the samples in the study.

4.3.2. Fungal community composition

In this section we provide a description of the general taxonomic composition of the fungal community of our montane grassland, followed by a characterization of the core mycobiome OTUs (the ensemble of OTUs present in all samples); we finally describe the occurrence found in this study of those taxa included within CHEGD species, used for the characterization of grasslands for habitat conservation purposes.

Taxonomic composition was firstly analyzed at the phylum level (Table 4.1). Ascomycota dominated the fungal community and accounted for an average of 79.2% of the total read abundance (974 OTUs), while Basidiomycota represented the 18.1% (106 OTUs). Much less abundant were Zygomycota (0.4%, 6 OTUs) and Glomeromycota (0.1%, 7 OTUs), while Chytridiomycota was represented by only 2 OTUs.

The most common known orders within Ascomycota were Pleosporales (22.3%), Eurotiales (9.6%), Helotiales (8.3%), Hypocreales (6.8%), Sordariales (4.4%) and Geoglossales (4.1%) while Agaricales dominated within Basidiomycota (13.6%). Details in the composition and taxonomic identification are shown in Table 4.1.

Table 4.1. General composition of the fungal community in La Bertolina. % SH: Percentage of OTUs assigned to Species Hypothesis; % NR: Percentage of New Referenced OTUs.

	Relative abundance (%)	**Number of OTUs**	% SH	% NR
Phylum				
No blast hit	0.7	27		
Ascomycota	79.2	974	22.1	77.9
Basidiomycota	18.1	106	36.8	63.2
Chytridiomycota	0	2	50	50
Glomeromycota	0.1	7	42.9	57.1
Incertae sedis	0.1	1	0	100
Zygomycota	0.4	6	83.3	16.7
Unknown	1.3	23	21.7	78.3
Common orders				
Pleosporales	22.3	269	22.7	77.3
Agaricales	13.6	56	30.4	69.6
Eurotiales	9.6	94	2.1	97.9
Helotiales	8.3	98	24.5	75.5
Hypocreales	6.8	154	29.2	70.8
Unidentified Dothideomycetes	5.4	47	12.8	87.2
Unidentified Ascomycota	5.3	73	54.8	45.2
Sordariales	4.4	58	41.4	58.6
Geoglossales	4.1	8	0	100
Tremellales	3.3	21	52.4	47.6

Within those OTUs identified to the genus level, the highest relative abundances were for *Clavaria* (8.7%), *Psathyrella* (1.9%) and *Cryptococcus* (3%) within phylum Basidiomycota, and *Geoglossum* (4.1%), *Tetracladium* (4%) and *Paraphoma* (2.4%) within phylum Ascomycota.

The core mycobiome was composed by 41 OTUs, representing an average read abundances of 25.1% (Table 4.2). Within these, Ascomycota was the predominant phylum (37 OTUs), followed by Basidiomycota (3 OTU), and finally a single OTU was assigned to an unidentified fungal phylum. Pleosporales was the dominant order (18 OTUs). Sixteen genera belonging to five orders of this core mycobiome were identified within the phylum Ascomycota, whereas only three OTUs belonging to the genus *Cryptococcus* were shared within Basidiomycota (Tremellomycetes). Average percentages of reads per sample and standard deviation are shown in Table 4.2.

Taxa included within CHEGD species represented 33 OTUs (13.8% of the reads), among them *Hygrocybe* spp. (0.56% mean read abundance; 2 OTUs), *Entoloma* spp. (0.31%; 10 OTUs), *Geoglossum* spp. (4.1%; 8 OTUs) and Clavariaceae spp. (8.8%; 13 OTUs). From these OTUs, New.Ref.OTU2 (*Geoglossum* sp.) was a commonly found member shared by all the samples (1.8%). Tricholomataceae, often associated to waxcap grassland communities, accounted for 1.2% of the average read abundance (5 OTUs).

Table 4.2. Shared OTUs among all soil samples (core mycobiome).

Order	Taxon	OTU ID	Mean %	SD
Botryosphaeriales	*Camarosporium* sp.	SH186959.07FU_KF742571_reps	0.54	1.38
Geoglossales	*Geoglossum* sp.	New.ReferenceOTU2	1.79	2.13
Helotiales	*Tetracladium* sp.	SH204309.07FU_HM036615_reps	2.57	2.38
	Articulospora sp.	SH179785.07FU_GU722054_reps	0.44	0.6
	Helotiales sp.	New.ReferenceOTU37	0.40	0.55
	Rhexocercosporidium sp.	SH186786.07FU_AF487895_refs	0.22	0.26
Hypocreales	*Ilyonectria* sp.	SH238190.07FU_JF735320_refs	0.35	0.22
	Clonostachys sp.	SH182678.07FU_AF358233_refs	0.35	0.32
	Fusarium sp.	SH443332.07FU_U61678_refs	0.20	0.24
	Nectriaceae sp.	SH175278.07FU_AB520304_reps	0.14	0.12
Onygenales	Onygenales sp.	SH197967.07FU_HG327917_reps	0.10	0.14
Pleosporales	*Paraphoma* sp.	SH211090.07FU_KF800312_reps	2.10	2.15
	Darksidea sp.	SH186943.07FU_EU144635_reps	1.98	1.97
	Sporormiaceae sp.	SH184181.07FU_GU910719_reps	1.15	1.54
	Pleosporales sp.	SH200448.07FU_KJ188720_reps	0.67	0.82
	Pleosporaceae sp.	SH216786.07FU_EU144461_reps	0.49	0.59
	Pyrenochaeta sp.	New.ReferenceOTU13	0.45	0.43
	Paraphaeosphaeria sp.	New.ReferenceOTU15	0.42	0.49
	Pleosporales sp.	SH182990.07FU_JX043150_reps	0.38	0.44
	Pleosporales sp.	New.ReferenceOTU43	0.37	0.75
	Pyrenochaeta sp.	New.ReferenceOTU9579	0.35	0.29
	Pyrenochaetopsis sp.	New.ReferenceOTU21	0.34	0.46
	Pleosporales sp.	SH192113.07FU_KJ555145_reps	0.27	0.21
	Pyrenochaeta sp.	SH217882.07FU_JF506840_reps	0.26	0.3
	Preussia sp.	New.ReferenceOTU56	0.26	0.42
	Paraphoma sp.	SH208741.07FU_HQ649827_reps	0.25	0.19
	Sporormiella sp.	SH215533.07FU_GU910617_reps	0.25	0.3
	Pyrenochaeta sp.	New.ReferenceOTU3584	0.15	0.17
	Chalastospora sp.	SH199530.07FU_FJ839608_refs	0.10	0.07
Tremellales	*Cryptococcus* sp.	SH181628.07FU_AF444469_refs	1.20	2.07
	Cryptococcus sp.	SH181630.07FU_AJ581050_refs	0.53	0.93
	Cryptococcus sp.	SH203797.07FU_EU266559_reps	0.48	0.89
unidentified	Dothideomycetes sp.	SH219124.07FU_EU480250_reps	2.09	3.72
	Dothideomycetes sp.	SH207018.07FU_EU490036_reps	0.93	1.07
	Sordariomycetes sp.	SH175275.07FU_GU055572_reps	0.61	0.55
	Ascomycota sp.	SH198116.07FU_FJ708609_reps	0.42	0.5
	Dothideomycetes sp.	New.ReferenceOTU61	0.33	0.79
	Dothideomycetes sp.	New.ReferenceOTU8206	0.32	0.58
	Ascomycota sp.	SH182984.07FU_KF385325_reps	0.22	0.32
	Fungi sp.	New.ReferenceOTU35	0.16	0.15
Xylariales	Xylariales sp.	New.ReferenceOTU10	0.48	0.6

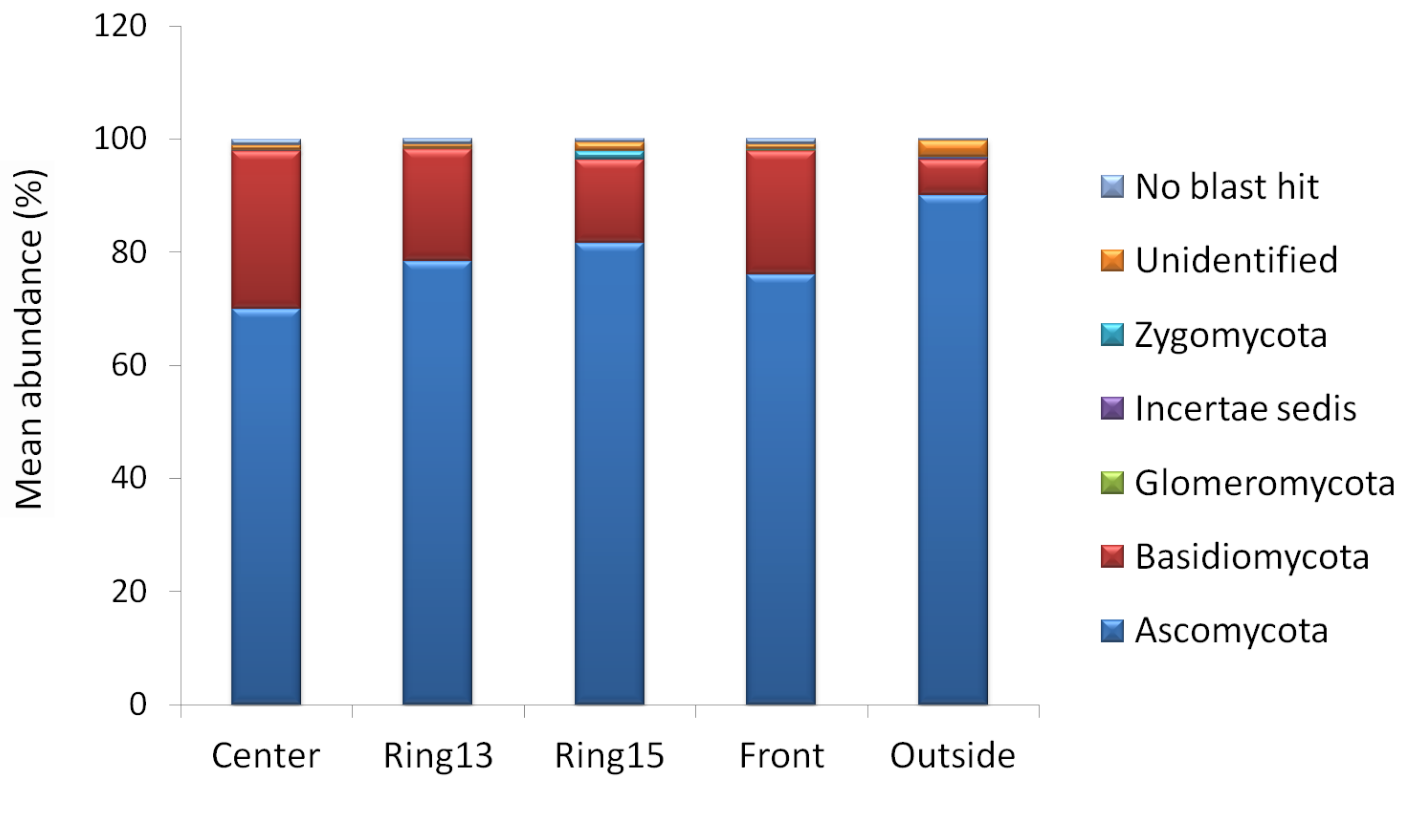

Figure 4.2. Phyla composition across fairy rings (x 100).

Explanatory variables related to ring, ring spatial pattern and ring size accounted for 18.4% of the total variation and all axes were marginally statistically significant (P = 0.063 testing the first axis and P = 0.055 testing all axis). The first CCA axis, accounting for the maximum variability explained by a single axis (10.29% of the total variability), separates the Outside zone from the fairy rings zones (P = 0.011, pseudo-F = 2.9). Within those zones, Ring15 is the closest to Outside in terms of composition, and Inside, the most differentiated (P = 0.073, pseudo-F = 1.7) (Fig. 4.4). The second CCA axis (explaining 5.24% of the total variability), separates Ring15 from the rest. Among those most abundant orders whose frequency was used in the CCA (Fig. 4.3), Agaricales were less frequent outside the ring (1.5% relative abundance, F = 3.7, P = 0.065), in comparison with all the zones inside the ring, peaking in the Center zone (25.5%). Hypocreales showed also lower frequencies outside the ring (2.3%, F = 24.3, P < 0.01) than in the ring zones. The opposite pattern was observed for Pleosporales and Eurotiales, both more abundant outside the ring (33.5%, F = 4.4, P = 0.045 and 18.6%, F = 3.3, P = 0.082, respectively) than in any other zone inside the ring.

At the genus level, we found few statistically significant differences between zones due to the high variability between replicates. The genus *Clavaria* featured all the zones inside the ring (Fig. 4.5), accounting for 6.5% to 19.9% of the mean read abundance per zone and peaking in the

Center zone (19.4%, Standard Deviation = 25). *Psathyrella* was characteristic of the Ring13 zone (P < 0.07, 9.4%, SD = 14.9), while *Tetracladium* (P < 0.06, 7.1%, SD = 5.8) and *Geoglossum* (8.4%, SD = 5.6) peaked in the Ring15 and Front zones respectively. *Geoglossum* was also de most abundant identified genus in the Outside zone (4.1%, SD = 5.6) although this zone was characterized by OTUs identified to order or higher levels, mainly belonging to Pleosporales and Eurotiales (Figs. 4.3 and 4.4).

4.3.3. Ring-associated OTUs

We compared the studied rings and assessed the common patterns among them at the OTU level. OTUs belonging to Ascomycota showed high abundances across all zones and all rings, but those from Basidiomycota peaked inside the areas affected by the fairy ring (Fig. 4.2). Agaricales was the order within Basidiomycota most commonly associated with fairy rings formation (Fig. 4.3 and 4.4). From this order, some taxa within the family Clavariaceae showed the highest relative abundances inside the rings while strongly decreasing towards the Outside zones. Specifically, we found two OTUs identified within the genus *Clavaria*, New.Ref.OTU1 and New.Ref.OTU758, whose relative abundances pointed towards a strong link with those zones inside the fairy rings (Table 4.3). New.Ref.OTU6, identified within the genus *Psathyrella*, had a single peak in the Ring13 zone (P < 0.05, Fig. 4.5, Table 4.3), whereas New.Ref.OTU48 and New.Ref.OTU46, assigned to Tricholomataceae, showed the highest relative abundance in the Center

and Front zones of some of the rings (Table 4.3). Other OTUs assigned to Agaricales showed higher abundances inside the rings than outside, including *Hygrocybe*, *Lycoperdon*, *Cyathus*, *Entoloma* and *Amanita* (Table 4.3).

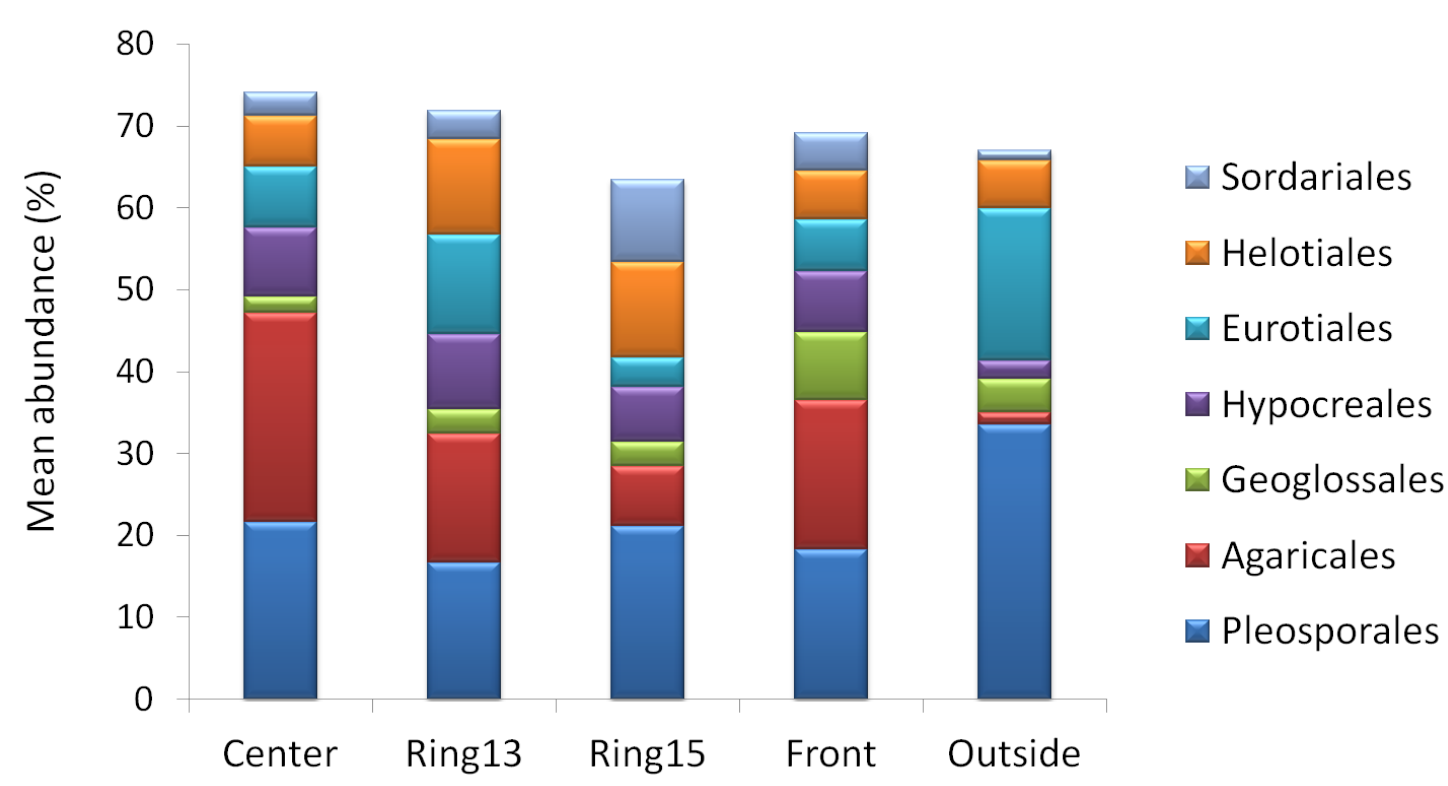

Figure 4.3. Composition of common identified orders per zone.

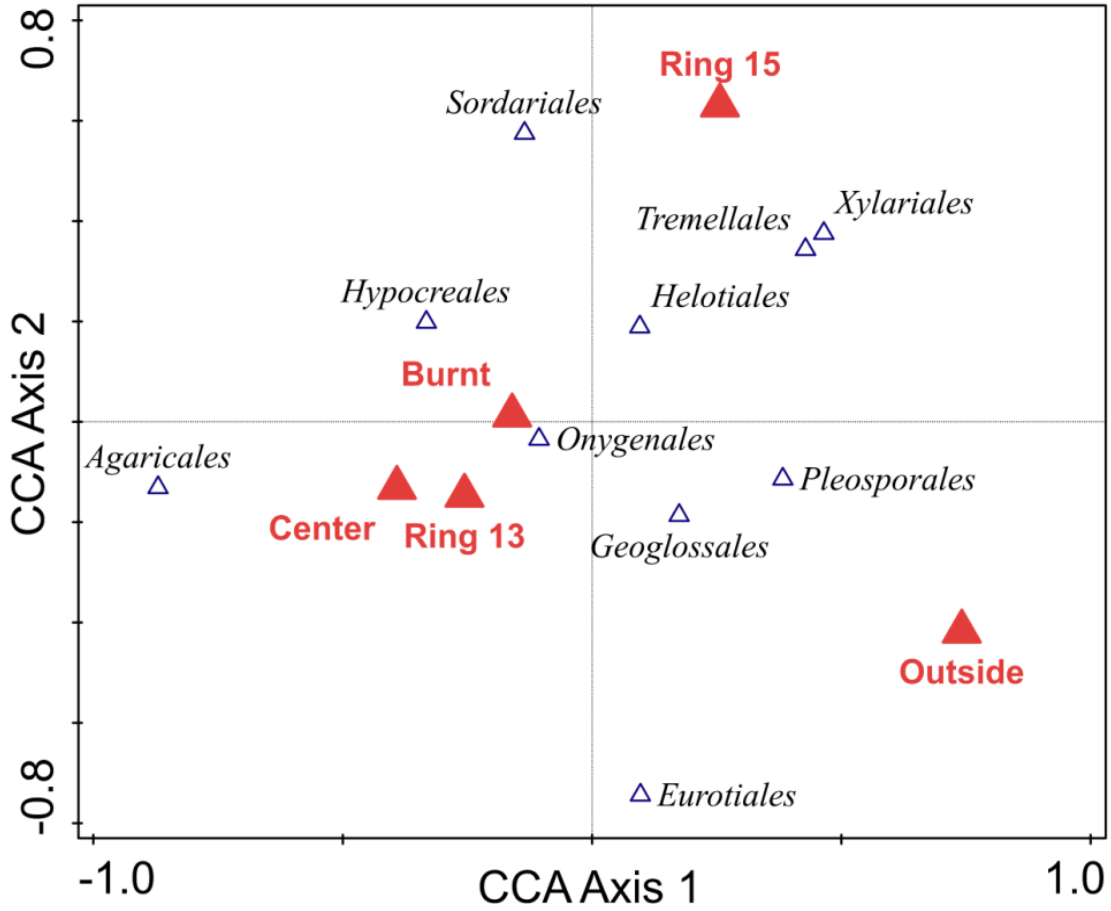

Figure 4.4. CCA showing differences in the fungal community composition to the order level across fairy ring.

4.4. Discussion

4.4.1. Fungal community composition in La Bertolina

Since the development of massive sequencing techniques, fungal surveys focus not only on basidiocarp occurrence, but also on the whole fungal diversity existing in the soil. Thus, evidencing the potential of using high-throughput DNA sequencing of soil samples for fungal biodiversity and habitat conservation (Geml *et al.*, 2014a).

It has been highlighted the need for enlarging those databases and make them available to the scientific community for the identification of sequenced taxa. As it happened with several previous studies (Toju, Sato, & Tanabe, 2014; Menkis *et al.*, 2015; Urbina *et al.*, 2015), only 268 OTUs from the total 1146 identified in our results matched the reference database. The remaining 878 OTUs were then clustered de novo, suggesting the magnitude of the unknown part of the kingdom fungi (O'Brien *et al.*, 2005; Taylor *et al.*, 2013). A high number of OTUs were identified to order or higher taxonomic levels, hindering the ecological interpretation of the overall fungal diversity found in this study. The variability in the number of OTUs between samples (from 483 to 894) underlines the fact that conclusions derived from diversity results rely on the sampling depth (Unterseher *et al.*, 2011). In our study, rarefaction curves not only showed a sufficient depth for the majority of the samples but also highlighted the huge spatial variability within fungal richness between samples even within a single grassland (Fig. 4.1).

Unlike boreal and temperate forests, where Basidiomycota is the dominant phylum (O'Brien *et al.*, 2005), grassland soils are known to be colonized mainly by Ascomycota, specially those grasslands located in semi-arid zones (Porras-Alfaro *et al.*, 2011). In this temperate grassland survey, we found a higher proportion of Ascomycota than Basidiomycota in the New Reference sequences, highlighting the huge Ascomycota diversity that remains undescribed. Porras-Alfaro *et al.* (2011) found similar proportions of these phyla in a semiarid grassland (86% Ascomycota, while in the present survey those represented 79% of the reads). Within the latter, Pleosporales was the dominant group. It has been said that arid and semiarid grasslands can be hotspots of pleosporalean diversity (Porras-Alfaro *et al.*, 2011) and our results suggest the same trend in montane grasslands. Integrated by fungal endophytes, epiphytes, plant parasites, lichenicolous fungi, saprophytes and coprophilous fungi (Zhang *et al.*, 2009), the order Pleosporales comprise almost a forth part of the overall sequenced OTUs in this study. Representatives of the genera *Darksidea, Paraphoma, Preussia and Pyrenochaeta* among others, were abundantly found in all samples (Table 4.2). All these genera contain species described as fungal endophytes (Arenal, Platas, & Peláez, 2007; Khidir *et al.*, 2010; Zhang *et al.*, 2012; de Gruyter *et al.*, 2013; Hyde *et al.*, 2013; Knapp *et al.*, 2015). It has been proposed the linkage between fungal endophyte abundance and environmental stresses (Knapp, Pintye, & Kovács, 2012) in which fungal endophytes activity would allow host plants access to nitrogen and phosphorus in environments with nutrient rich organic pools (Khidir

et al., 2010). This could be an explanation for their abundance in a pasture, where organic inputs from livestock depositions are frequent. Fairy ring zones are also known to be inputs of organic nutrients, when the mycelia decomposes and releases all previous acquired nutrients (Fidanza, Colbaugh, & Davis, 2000).

4.4.2. Core Mycobiome

Identifying those organisms shared by all samples from a particular habitat is essential to decoding the ecology of fungal consortia (Shade & Handelsman, 2012). We found that almost half of the OTUs forming the core belonged to Pleosporales (Table 4.2). This include genera known to play as endophytes but also as plant pathogens, such as species belonging to the genus *Fusarium* (LeBlanc, Kinkel, & Kistler, 2014) and *Rhexocercosporidium* (Tedersoo *et al.*, 2014). Coprophilous species have also been reported within some genera found in the core, such as *Sporormiella* and *Preussia* (Arenal *et al.*, 2007), as expected on a pasture. Nevertheless, most of the taxa common to all soil samples contained generalist Ascomycota generally found ubiquitously across different environmental conditions, such as species belonging to the genus *Tetracladium* (Moll *et al.*, 2016). *Geoglossum* spp., shared by all samples and commonly listed within CHEGD species, showed a dominant role in this grassland community, along with *Clavaria* spp., both supposed to display an unknown biotrophic lifestyle (Birkebak *et al.*, 2013).

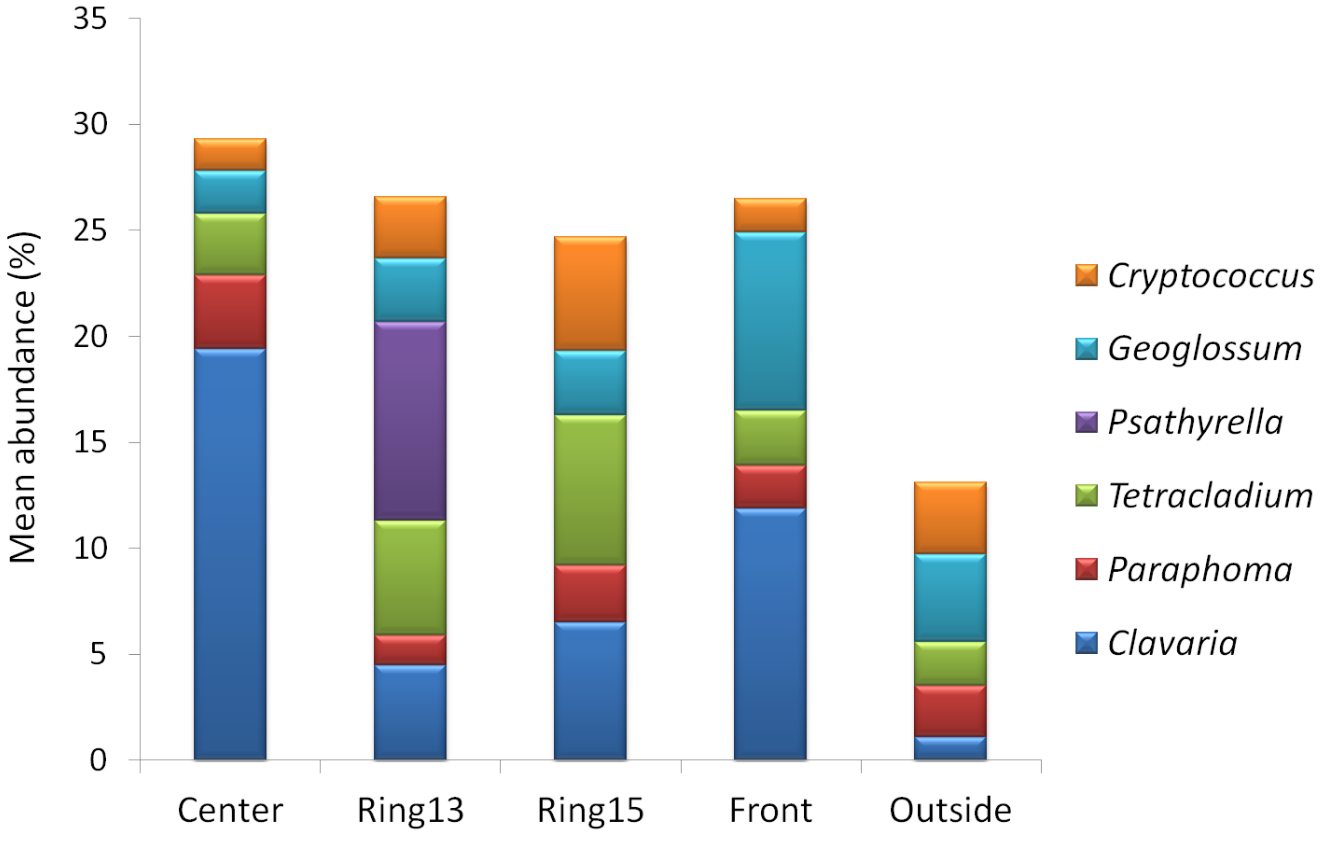

Figure 4.5. Distribution of the most abundant identified genera per zone.

Table 4.3. Ring-associated OTUs within Agaricales. Mean read proportions per zone.

Ring	Center		Ring13		Ring15		Front		Outside	
	Taxa	%	Taxa	%	Taxa	%	Taxa	%	Taxa	%
S1	*Hygrocybe* sp.	16.4	*Psathyrella* sp.	23.2	*Clavaria* sp.	3.5	*Clavaria* sp.	37.3	Tricholomataceae sp.	0.2
	Tricholoma sp.	11.7	*Clavaria* sp.	7.9	*Entoloma* sp.	0.5	*Clavaria* sp.	15.5	*Clavaria* sp.	0.0
	Clavaria sp.	11.3	*Clavaria* sp.	3.0	*Clavaria* sp.	0.3	*Clavaria* sp.	0.5	*Clavaria* sp.	0.0
S2	*Clavaria* sp.	1.2	*Psathyrella* sp.	33.2	*Clavaria* sp.	0.0	Agaricales sp.	0.5	Agaricales sp.	0.0
	Clavaria sp.	0.6	Agaricales sp.	0.2	*Entoloma* sp.	0.0	*Amanita* sp.	0.2	Tricholomataceae sp.	0.0
	Clavaria sp.	0.5	*Tubaria* sp.	0.2	Clavariaceae sp.	0.0	Tricholomataceae sp.	0.2	*Tubaria* sp.	0.0
S3	*Clavaria* sp.	58.7	*Cyathus* sp.	2.4	*Clavaria* sp.	0.6	Tricholomataceae sp.	21.5	*Clavaria* sp.	5.7
	Clavaria sp.	1.1	*Clavaria* sp.	0.8	*Amanita* sp.	0.5	*Clavaria* sp.	4.2	*Amanita* sp.	0.7
	Clavariaceae sp.	1.0	*Entoloma* sp.	0.5	*Entoloma* sp.	0.4	*Tricholoma* sp.	0.6	*Clavaria* sp.	0.1
L1	*Clavaria* sp.	0.6	*Entoloma* sp.	0.5	*Clavaria* sp.	0.2	*Arrhenia* sp.	0.7	*Entoloma* sp.	0.1
	Clavariaceae sp.	0.3	*Entoloma* sp.	0.4	*Clavaria* sp.	0.1	Clavariaceae sp.	0.3	Tricholomataceae sp.	0.1
	Entoloma sp.	0.3	*Entoloma* sp.	0.2	*Entoloma* sp.	0.1	*Mycena* sp.	0.2	*Entoloma* sp.	0.1
L2	*Clavaria* sp.	1.5	*Entoloma* sp.	2.1	*Lycoperdon* sp.	0.3	*Lycoperdon* sp.	1.5	*Clavaria* sp.	0.3
	Lycoperdon sp.	0.8	*Lycoperdon* sp.	1.0	*Entoloma* sp.	0.1	*Lycoperdon* sp.	0.1	*Entoloma* sp.	0.1
	Entoloma sp.	0.3	Clavariaceae sp.	0.4	*Psathyrella* sp.	0.1	*Entoloma* sp.	0.1	*Entoloma* sp.	0.0
L3	*Clavaria sp.*	33.5	*Clavaria* sp.	14.2	*Clavaria* sp.	20.1	*Clavaria* sp.	12.2	*Clavaria* sp.	0.3
	Amanita sp.	0.3	*Amanita* sp.	0.5	*Clavaria* sp.	13.7	*Coprinopsis* sp.	5.5	*Entoloma* sp.	0.1
	Coprinopsis sp.	0.2	Agaricales sp.	0.3	*Amanita* sp.	0.5	*Coprinellus* sp.	1.3	*Amanita* sp.	0.1

4.4.3. Fungal community composition across fairy ring zones

Few studies have used NGS technologies in a comparative study of the fungal communities in fairy rings, and those who did it, focused mainly on tethered rings (Gregory, 1982) of ectomycorrizal edible fungi (Kim *et al.*, 2013) or free rings of golf courts (Miller *et al.*, 2011), due to their economical value. Miller *et al.* (2011) implemented a combination of traditional and DNA-based methodologies to characterize the causal fungi of fairy rings on golf courts and found that sequencing of the internal transcribed spacer (ITS) region may be the most effective and rapid procedure for dealing with fairy ring species description from soil samples.

It is expected that fungal communities in pastures present an Ascomycota/Basidiomycota ratio higher than other ecosystems (Tedersoo *et al.*, 2014), given the smaller proportion of ectomycorrhizal fungi in the soil. However, a strong increase in Basidiomycota proportion was found in all zones inside the rings. This was in accordance with the results found by (Kim *et al.*, 2013), who described a vast predominance of Tricholomataceae (Basidiomycota) in the ring zone of *Tricholoma matsutake*.

According to our expectations, soil fungal communities inside the ring did not resembled those from outside the ring (Fig. 4.4). Agaricales peaked in the inside zones Center, Ring13 and Front, suggesting their relationship with the ring formation. However, the Ring15 zone showed a reduction in Agaricales whereas Pleosporales and Helotiales increased (Fig. 4.3), suggesting that the establishment of associations between

fungal endophytes and plants could be mediating the absorption of nutrients from organic material in that zone where vegetation looked more developed. A well differentiated community was found in the Outside zone, mainly because of the strong decrease in the relative abundance of Agaricales, and the high predominance of Pleosporales and Eurotiales.

4.4.4. CHEGD and fairy ring species

We discuss the significance of the CHEGD species for fungal diversity and habitat conservation in our grassland and provide some candidates for fairy ring formation.

Current changes in land uses have motivated old, natural or semi-natural, often pastured grasslands to be considered an endangered habitat. Those extensively managed grasslands represent the specific habitat for about 400 fungal species (Griffith *et al.*, 2013). Indices of habitat quality and Red Lists have been proposed across Europe from surveys of basidiocarps, such as the CHEGD profile (Rotheroe *et al.*, 1996; Griffith *et al.*, 2013), proposed to quantify a set of taxa commonly found in semi-natural grasslands. Several assessments have focused on fungal diversity around North-Western Europe, as are the cases of Scotland (Newton *et al.*, 2003), Northern Ireland (McHugh *et al.*, 2001) and Wales (Rotheroe *et al.*, 1996; Rotheroe, 2001), including several CHEGD species in the Red Lists of these countries. To our knowledge, this is the first survey in the Iberian Peninsula quantifying CHEGD fungi through massive sequencing techniques. We found 38 taxa (2 *Hygrocybe*, 10 *Entoloma*, 13 *Clavaria*, 8 *Geoglossum* and 5 unidentified

Clavariaceae) accounting for 13.8% of the average read abundance. Although not a majority proportion, our results of fungal community composition across zones suggest that some of them play a key role in the interspecific interactions that take place within fairy rings.

Parker-Rhodes (1955) stated that when fairy rings from the same species meet, the intersected portions are obliterated. Intersections between different species could result in different options, including bilateral extinction as the most likely outcome in very closely related species. Since the rings in the study site always disappeared when they intersect (data not shown), we could assume that species forming those rings were from the same species, or closely related ones.

We found 18 OTUs belonging to the family Clavariaceae, 13 of them identified as *Clavaria* sp. This genus showed high relative abundances in most of the visible ring zones and inside the rings, while low relative abundances were found outside (Table 4.3), suggesting a strong link of this genus with vegetation rings. Previous studies (Ramsbottom, 1953; Smith, Jackson, & Woolhouse, 1989) pointed out the occurrence of *Clavaria* spp. in association to ring formation in pastures, but the ecology of this genus remains unclear. Mycorrhizal associations have been described in *Clavaria argillacea* (Englander & Hull, 1980), while Mitchel (2006) pointed out that Clavariaceae, jointly with *Hygrocybe* spp., could be playing a role as deep humic decayers, based on their isotopic signals. Subsequent research evidenced that the genus *Clavaria* was not included within the ligninolytic Clavariaceae, and that an unknown biotrophic nutritional mode was suitable for those non-

ligninolytic Clavariaceae (Griffith *et al.*, 2002; Tedersoo, May, & Smith, 2010; Seitzman *et al.*, 2011; Birkebak *et al.*, 2013). It is commonly accepted that some taxa with mycorrhizal morphologies show enzymatic patterns able to degrade recalcitrant compounds. This fact suggests that the ^{15}N enrichment found in *Clavaria* spp. in some studies (Lilleskov, Hobbie, & Fahey, 2002; Read & Perez-Moreno, 2003; Birkebak *et al.*, 2013) could be due to a nutritional mode based on organic nitrogen from host plants, in exchange with that N obtained from the decomposition of highly recalcitrant organic matter.

Studies upon the effect of herbicides on *Hygrocybe* spp. fructification supported the idea of a biotrophic strategy in this genus, all pointing to a relationship with grasses (Griffith *et al.*, 2014). This is a genus known to form type-III fairy rings, without visible effects on vegetation, contrasting with those type-I and type-II rings, caused by saprophytic basidiomycota (Griffith & Roderick, 2008). *Clavaria* spp. are not usually associated to ring formation (but see Ramsbottom, 1953; Smith *et al.*, 1989), but they are often included among common grassland species due to their shared ecological requirements. Due to the looseness of their mycelia in the soil, it has been proposed that these two taxa could mix together within a single ring, in contrast to the antagonistic interactions described in type-I and type-II rings (Dowson, Rayner, & Boddy, 1989; Griffith *et al.*, 2014). Nonetheless, the fairy rings of the present survey showed clear effects on the vegetation growth, so that a saprophytic basidiomycota could be expected to be responsible for their formation.

New.ReferenceOTU6 OTU, identified as *Psathyrella* sp., is the basidiomycota with highest relative abundances inside the ring zones after *Clavaria* spp. *Psathyrella* is a genus clearly saprophytic (Hobbie, Weber, & Trappe, 2001), which serves as either primary or secondary decomposers in terrestrial ecosystems (Singer & Smith, 1973). Its activity inside the rings could be promoting the growth of vegetation due to the release of nutrients in this zone (Nelson, 2008).

Tricholoma sp. (New.ReferenceOTU48), was found in high proportion inside two rings, S1 and S3, peaking in the Front zone and almost disappearing outside (Table 4.3). This genus has representatives known to cause fairy rings, especially those ectomycorrhizal species. Two ecological types have been distinguished within this genus: a first group forming mycorrhizae with trees, and a second group, cellulose decomposer, currently included into other genera (Gregory, 1982). Within the latter there is *Tricholoma georgii* (L.), today known as *Calocybe gambosa* (Fr.) Donk. This species has been described by local people in the fields adjacent to our study site, but it has never been seen right in the study site. Belonging to Tricholomataceae, it might be an erroneous taxonomic assignment of de novo clustering due to the high similarity within sequences of these related taxa. *Calocybe gambosa* is a common species in calcareous grasslands, often forming type-II fairy rings (Ramsbottom, 1953), those in which vegetation growth can be observed in the inner zone of mycelial front (Vargas, 1993). These type of rings have been treated with nitrogen additions to hide the effects on vegetation of fungal decomposition and nitrogen enrichment on soil in

golf courses (Nelson, 2008). Nitrogen release could be associated to the high abundances found of taxa including fungal endophytes in the Ring15 zone, fungi that could be associated to grasses enhancing nutrient absorption and promoting plant growth.

Entoloma spp. and *Lycoperdon* sp. were also found in higher abundances inside the ring than Outside (Table 4.3), peaking in the visible ring zones (Ring15 and Front). Particularly, one of the rings (L2) showed relevant relative abundances of an OTU identified as *Lycoperdon* sp. Miller *et al.* (2011) used DNA-based methodologies to determine fungal community across fairy rings and *Lycoperdon* spp. were the most relevant ring forming representatives. It is known to form type-II rings in grasslands and pastures, and its basidiocarps could be easily unnoticed by local people. *Entoloma* sp. was found in all samples, peaking inside the ring L2. Being a saprophytic genus usually found along with CHEGD species in pastures (Mitchel, 2006), it is also known to form fairy rings in this habitat. *Amanita* sp. and *Cyathus* sp. were also found in higher abundances inside the rings than outside.

There are about 60 mushroom species which can grow in the fairy ring pattern (Kirk & Lang, 2008). Within those Basidiomycota found in the Front zones, where it was expected to be the mycelia causing the ring, *Tricholoma* spp. and *Clavaria* spp. seem to be the most likely responsible species of these fairy rings. The former is supported by descriptions given by local people about adjacent zones to the study site, but we could not explain their low relative abundances in most of

the rings. The latter would require further studies regarding its ecological strategies.

It is tempting to hypothesize that *Clavaria* spp. was taking advantage of the nitrogen enrichment and new niche formation by the ring fungus, to establish mutualistic relations with those opportunistic plant species growing inside the ring. Taxa identified into the genera *Psathyrella*, *Amanita*, *Entoloma*, *Cyathus* and *Lycoperdon* could be causing the rings with interspecific intersections resulting in bilateral extinction, but low proportions of all of them suggest that they are ring-associated fungi decomposing the resulting organic material after ring formation.

4. 5. Acknowledgements

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Chapter 5

Bacterial community of fairy rings

5. Insights into the changes of the soil bacterial community across fairy rings using environmental DNA metabarcoding

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ABSTRACT: Fairy ring fungi are considered keystone species in grasslands due to their strong impact on soil physical and chemical properties. However, their effects on the bacterial community composition have been barely studied. Here, we analyze these effects through the use of Illumina metabarcoding and provide a description of the soil bacterial diversity across fairy rings in a semi-natural grassland. A total of 254,135 MiSeq reads and 405 to 1,444 operational taxonomic units (OTUs) were observed per soil sample. The dominant taxa in the grassland were *Firmicutes* driven by *Bacilli*. We found strong changes in the stimulation zone of the fairy rings; the most relevant were a

reduction in the bacterial richness and an increase in taxa belonging to *Firmicutes*. No differences were found between the inside and outside zones of the rings, suggesting a fast recovery of the bacterial community after a biological disturbance.

5.1. Introduction

Calcareous grasslands are among the most important ecosystems worldwide in terms of biological diversity, including a large set of rare species from various taxonomic groups (Kull & Zobel, 1991; Maarel & Sykes, 1993; WallisDeVries *et al.*, 2002; Bonanomi *et al.*, 2012). Changes in land uses have resulted into the loss of many semi-natural grasslands across Europe. Increasing knowledge on the mechanisms that regulate the coexistence and relative abundance of the different taxonomic groups is needed for a successful management of these valuable ecosystems (Bonanomi *et al.*, 2013).

Among the whole biodiversity existing in semi-natural grasslands, saprophytic basidiomycete fungi causing fairy rings have been described as keystone species (Van der Wal *et al.*, 2013). Growing radially through the soil with the eventual formation of fruiting bodies, these fungi also become evident aboveground because of their effect on the vegetation.

Based on the symptoms on vegetation, Shantz & Piemeisel (1917) divided fairy rings into three types: type I rings are characterized by causing severe symptoms or plant death; type II rings exhibit arcs of greener plants with luxuriant growth; and the occurrence of rings of

basidiocarps without any effect on vegetation is referred as type III. Type II can appear and go mysteriously, but they can also turn into type I rings, as the dead zone characterizing type I generally does not appear until summer (Dernoeden, 2002).

The enhancement in vegetation growth in type I and II rings is attributed to the breakdown of the soil organic matter by the saprophytic fungus. In this process, parts of the protein material are changed into compounds of nitrogen readily available to higher plants, which then exhibit a luxuriant growth in this zone. Once dead, resulting vegetal material will, in turn, serve as a food supply for the fairy ring or other saprophytic fungi growing in the soil (Shantz & Piemeisel, 1917).

Fungi also modify soil aggregation and other soil physical and chemical properties such as pH and humidity, resulting in shifts in the soil bacterial community of the stimulation zone (Bonanomi *et al.*, 2012; Caesar-TonThat *et al.*, 2013). Understanding those changes induced by the interaction of the fairy ring fungi on the bacterial communities is crucial to understand the mechanisms that promote the productivity of semi-natural grasslands (Caesar-TonThat *et al.*, 2013). Although microbial changes across fairy ring zones have been previously studied (Ohara & Hamada, 1967; Vaario *et al.*, 2011; Wang *et al.*, 2015), only a few studies have used Next-generation DNA sequencing (NGS) methods to characterize the changes in the composition of bacterial communities (Miller *et al.*, 2011; Kim *et al.*, 2014b). Mechanisms underlying the negative effects of fairy ring fungi on vegetation, such as soil

hydrophobicity and release of phytotoxins have been further documented (Gramss, Voigt, & Bergmann, 2005; Rillig, 2005; Fidanza, 2007a; Bonanomi *et al.*, 2012). However, changes on the diversity and composition of the bacterial community related to the enhancement of vegetation growth on type II fairy rings have received less attention (Caesar-TonThat *et al.*, 2013).

In this study, we focused on the soil microbial community of a pastured grassland of the North-Eastern Pyrenees in the Iberian Peninsula. Rings of luxuriant growth of vegetation were visible without any occurrence of basidiocarps. In a previous study on this grassland (Marí et al. in rev.), we described the fungal community of these fairy rings through the use of Illumina metabarcoding. Although we could not find a unique and unequivocal causal fungal species, we showed that the fungal community composition changed towards a higher basidiomycota/ascomycota ratio after the fungal front passage. These changes remained stable towards the center of the ring and significantly different from the outside zones. As we could not detect any significant increase in dead plant biomass in the zone adjacent to the luxuriant growth, we considered these fairy rings to be of type II.

Thus, the aims of this study were a) To characterize the bacterial diversity and community composition in this montane, pastured, semi-natural grassland, b) To describe those changes in the bacterial community composition across the fairy ring visible zones. We hypothesize that the fungi would affect the soil bacterial community

composition indirectly by changing the physical and chemical properties of the soil. We expected an acidification of the soil beneath the stimulation zone, but no changes of soil moisture given the unseen negative effects on the vegetation. We hypothesize similar changes in the bacterial major groups inside of the rings, in parallel to that found in fungal communities.

5.2. Materials and methods

5.2.1. Study site and experimental design

The study was conducted in the area of pastured grassland called La Bertolina, in the Eastern Pyrenees (42°05'56" N, 1°39'40" E), 1276 m.a.s.l. This area was previously cropped and annually tilled until 1998, after about 40 years of cereal cultivation. The mean annual temperature of this area is 8.7 ºC and the mean annual precipitation 954.8 mm (ACDC, http://www.opengis.uab.cat/acdc/catala/cartografia.htm, accessed 2015; Ninyerola, Pons, & Roure, 2007). The texture of the soil is sandy loam with a limestone bedrock with a high stoniness of polygenic conglomerates (ICGC, http://betaportal.icgc.cat/visor/client_utfgrid_geo.html, geologic map 1:50000 BG50M_v1r1, 2007, accessed 2015). This grassland is extensively grazed by cattle (0.44 LSU ha^{-1}) from spring to autumn. The vegetation is dominated by grasses (*Festuca arundinacea* Schreb., *Poa bulbosa* L., *Dactylis glomerata* L.), although *Trifolium pratense* L.,

Plantago lanceolata L., and other forbs are also common in this grassland.

Sampling strategy was conducted as described in Marí et al. (in rev.). Briefly, in May 2015, when the rings were more visible, six rings were selected, on the basis of their situation in order to avoid intersections with other adjacent rings. These rings had diameters ranging from 2.8 to 7 meters. A radial transect was designated across each ring, with five circular sampling points of 25 cm in diameter: 1) In the center of the ring ("Center"); 2) In the geo-referenced 2013 ring zone ("Ring13"); 3) In the 2015 dark-green vegetation zone ("Ring15"); 4) In the area immediately outside of the ring ("Front") and 5) outside the ring (> 2m), without visible ring influence ("Outside").

5.2.2. Soil sampling and bacterial Illumina metabarcoding

A total number of 30 soil samples were taken in the 0-10 cm depth soil layer with a 10 cm x 6 cm x 5 cm metal soil core in each sampling point. DNA was isolated upon arrival to the laboratory using the PowerSoil DNA isolation kit (MoBio), strictly following the manufacturer's instructions. Determination of DNA concentration was performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientic).

For DNA metabarcoding library preparation, a fragment of the bacterial 16S ribosomal RNA gene of around 530 bp was amplified using the primers Bakt 341F (5' CCT ACG GGN GGC WGC AG 3') and Bakt 805R (5' GAC TAC HVG GGT ATC TAA TCC 3', Herlemann *et al.*, 2011). The

primers had the Illumina adapter sequences and were tagged to their 5' end. The tags make possible to link the reads obtained during sequencing to a particular sample.

PCRs were carried out in a final volume of 25 µL, containing 2.5 µL of template DNA, 0.5 µM of the primers, 12.5 µL of Phusion DNA polymerase mix (Thermo Scientific), and ultrapure water up to 25 µL. The reaction mixture was incubated with an initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 55 °C for 20 s, 72 °C for 20 s, and a final extension step at 72 °C for 10 minutes. Despite the high number of PCR cycles used, the PCR products were checked on 1% agarose gel stained with REAL Safe (Durviz), and imaged under UV light and showed similar medium intensity bands. Negative controls that contained no DNA were included to check for contamination during library preparation.

PCR products were purified using the Mag-Bind RXNPure Plus magnetics beads (Omega Biotek), following the instructions provided by the manufacturer. Then, they were quantified with the Qubit dsDNA BR Assay Kit and pooled in equimolar amounts. The purified amplicon pools were sent for sequencing to the Illumina MiSeq PE300 platform. All the negative controls were included in the pools, in order to check for potential contamination. The quality of the FASTQ files was checked using the software FASTQC. A first quality-filtering step was performed using the software Geneious 8.1.8. In this step, regions from both ends of the reads with more than a 0.5% chance of an error per base were

trimmed. Paired-end assembly of the forward (R1) and reverse (R2) reads was performed with FLASH (Magoč & Salzberg, 2011). The mismatch resolution in the overlapping region is accomplished by keeping the base with the higher quality score. The FASTQ files were quality filtered using the bioinformatic tool Qiime 1.9.1 (Caporaso *et al.*, 2010). DNA sequences having quality score <20 were discarded. Chimeric sequences were removed using the UCHIME algorithm (Edgar *et al.*, 2011) implemented in VSEARCH. The reference database used was Greengenes (DeSantis *et al.*, 2006). 16S reads were clustered into OTUs using the open-reference approach in Qiime. In this method, reads are clustered against a reference database and any reads which do not hit the reference sequence collection are subsequently clustered de novo. Each OTU was assigned to a microbial taxa using the BLAST algorithm (Altschul *et al.*, 1990). Based on the resulting OTU table obtained for each sample, the OTUs which were present in the negative control and the OTUs represented by sequences with frequencies lower than 0.005% in the whole dataset were removed.

Finally, the rarefaction plots were constructed showing the rarefied number of OTUs defined at a 97% sequence identity threshold, assuming a sufficient sequencing depth when the rarefaction curves tended towards saturation. All those samples not reaching the plateau were removed from further analysis. Additionally, the percentage of coverage was calculated by the Good's method (Good & Toulmin, 1956). As diversity is correlated to the number of sequences collected, we randomly selected a subset of 1566 sequences per soil sample to

determine and compare the bacterial OTU richness. This was calculated both by measuring the number of detected OTUs per sample and by using the Chao1 estimator (Colwell & Coddington, 1994; Chao & Shen, 2003), which calculates the estimated true species richness by taking into account the number of rare species to extrapolate the number of undiscovered ones.

5.2.3. Statistical analyses

The following data analysis were performed with the R software (R Development Core Team 2008). Bacterial phyla and classes, with read abundances higher than 1%, were the main data analyzed. Families and genera with read abundances higher than 0.5% were also analyzed. One-way ANOVA followed by Tukey's multiple comparison test (Sokal and Rohlf, 1995) were used to test for significant differences among the means of the relative read abundances of each taxa across ring zones. OTU richness and the diversity index Chao1 were also tested using one-way ANOVA to detect differences across ring zones.

We applied multivariate analysis to the grassland samples using as descriptors the relative abundances of the OTUs obtained from the Illumina metabarcoding data set. In particular, we used redundancy analysis (RDA), including ring size, the zone across the rings, soil pH and moisture as independent explanatory variables. The interactions between factors were also tested. A forward selection procedure and Monte Carlo permutation test based on 999 random permutations were used to determine the significance of the experimental variables

explaining the observed variance in the composition of the bacterial communities. The CANOCO 5 software package (Microcomputer Power, Ithaca, NY, USA) was used to perform multivariate analysis.

5.3. Results

5.3.1. Sequencing results of bacterial communities

A total of 254,135 filtered reads and 2,067 OTUs were obtained from thirty samples through Illumina MiSeq sequencing analysis. From 2,035 to 18,865 reads were found per sample, with a number of OTUs ranging from 405 to 1,444. As shown in Figure 5.1, rarefaction curves tended to approach the plateau for most of the samples, indicating that the sequencing depth was enough to retrieve most of the bacterial diversity.

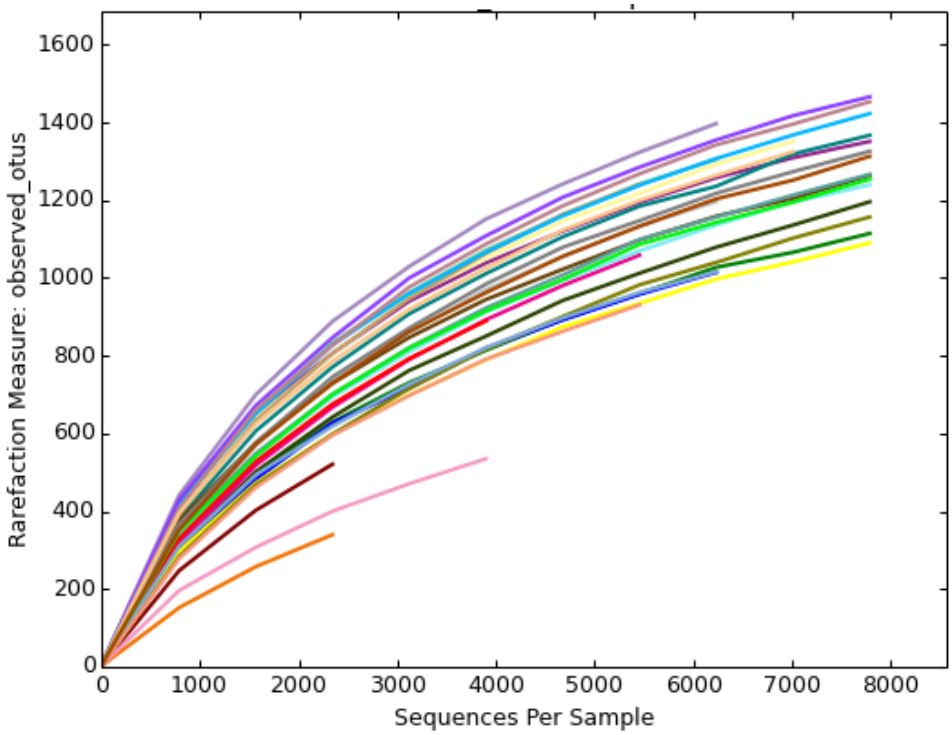

Figure 5.1. Rarefaction curves for the overall sampling.

5.3.2. Bacterial community composition in La Bertolina

Those sequences that could not be classified into any known bacterial kingdom represented the 0.2% of reads, and were categorized as Unassigned. The remaining 99.8% of bacterial reads were assigned into 17 different phyla, 62 classes, 88 orders, 108 families and 98 genera. 761 OTUs were not categorized at the family or genus level. The most abundant phyla found across all thirty samples were *Firmicutes* (38.9% of mean read abundance), *Actinobacteria* (21.1%), *Proteobacteria* (13.2%), *Acidobacteria* (9.6%) and *Planctomycetes* (7.1%). Less abundant were *Chloroflexi* (4.9%), *Bacteroidetes* (1.8%), *Gemmatimonadetes* (1.3%) and *Verrucomicrobia* (1%). The remaining phyla were joined together into "Other phyla" for further analyses.

Increasing the phylogenetic resolution, the bacterial community was dominated by the class *Bacilli* within the phylum *Firmicutes* (38.6%). This class was mainly dominated by the order *Bacillales*, and it had an OTU classified into the family *Bacillaceae* and another OTU assigned as *Bacillus* sp., which accounted for 30.8% and 4.1% of the main reads per sample respectively.

The class *Actinobacteria* was dominant within the phylum *Actinobacteria* (12.4%). The most abundant order was *Actinomycetales* (12.1%) whereas at the genus level, *Mycobacterium* (1.9%), *Pseudoconardia* (1.5%) and *Rubrobacter* (1%) were the most relevant identified taxa. Within the phylum *Proteobacteria, Alphaproteobacteria* was the dominant class (8.4%, mainly constituted by *Rhizobiales*), and

the genera *Rhodoplanes* (1.6%) and *Skermanella* (1%) were the most abundant identified taxa. The class *Acidobacteria-6* (5.9%) was dominant within the phylum *Acidobacteria*, (predominantly the order *iii1-15,* 5.8%), as happened with the class *Planctomycetia* (4.6%) within the phylum *Planctomycetes* (dominated by the order *Gemmatales*, 2.4%).

5.3.3. Changes in bacterial community across fairy rings and environmental gradients

The first and second axis of the RDA explained 12.9% and 5.8% of the bacterial community composition variation respectively (Fig. 5.8). Most of the samples distributed at the negative side of the first axis, which was related to a gradient of OTU richness. By contrast, samples belonging to the Ring15 zone located at the positive side of this axis. Samples belonging to all ring zones distributed along the second RDA axis, which was associated to the diameter of the ring (Fig. 5.8).

The explanatory variables included explained 57% of the community variation. The interaction between Zone (Ring15) and Diameter was the factor explaining most of the variation (7.7 %, Table 5.1), as shown in the first RDA axis, whereas pH and Diameter were associated to the second RDA axis, explaining 6.6% and 6.5% of the total variation respectively. Humidity was also significant, associated to the second axis explaining 5.1% of the total variation. By contrast, when looking at the RDA conditional term effects, we did not find a significant effect of either pH or soil humidity (Table 5.1).

Table 5.1: Simple and conditional term effects of the RDA analysis. "% explained" shows the percentage of variability explained by each variable.

Simple Term Effects				Conditional Term Effects			
Factor	**Explains %**	**pseudo-F**	**P(adj)**	**Factor**	**Explains %**	**pseudo-F**	**P(adj)**
Zone.Ring15 *Diameter	7.7	2.3	0.03	Zone.Ring15* Diameter	7.7	2.3	0.03
pH	6.6	2	0.03	Diameter	5.7	1.8	0.02
Diameter	6.5	2	0.03	Ring.Ring6	4.3	1.4	0.25
Ring.Ring4	5.7	1.7	0.07	Ring.Ring3	4.2	1.3	0.25
Zone.Ring15	5.2	1.5	0.1	Zone.Ring15	3.7	1.2	0.45
Humidity	5.1	1.5	0.1	Ring.Ring2	3.5	1.1	0.56
Ring.Ring2	5	1.5	0.1	Zone.Outside *Diameter	3.3	1.1	0.63
Ring.Ring1	4.6	1.4	0.13	Zone.Outside	3.5	1.1	0.56
Ring.Ring5	3.7	1.1	0.5	Ring.Ring5	3.2	1.1	0.7

Indeed, we did not find any significant differences in soil pH and humidity across ring zones ($p > 0.7$, Fig. S5.2). Soil pH (Fig. S5.1) ranged between 7.45 in a soil sample from the center of the ring, and 8.14 in two samples from the Front zone, with an average value per zone ranging from 7.86 (sd: 0.2) in the Center zone to 7.98 (sd:0.1) Outside, without any significant difference ($p > 0.7$). Soil moisture ranged between 6.5% (sd: 1.8) in the Ring15 zone and 7.5% (sd: 1.3) Outside.

We found significant differences in the OTU richness estimators across fairy ring zones for a sampling depth of 1566 sequences per sample (Fig. 5.7). The number of observed OTUs had a tendency to decrease towards the Ring15 zone (mean=439.4, sd= 138.39) compared to Outside (593.6, sd: 62.1, $p<0.1$) and to a lesser extend to Center (572.2, sd:83.7, $p=0.14$). The same tendency was observed for the Chao 1 richness estimator, whose lowest values were also found in the Ring15 zone (970.1, sd:265.8) and the highest in the Outside zone (1223.4, sd;96.6, $p=0.13$) and Center zone (1180.6, sd: 148.8, $p=0.27$). The RDA plot also shows how only a few OTUs are associated to the Ring15 zone (Fig. 5.8). Although the phylum distribution across all five fairy ring zones shared a similar pattern (Fig. 5.2), we found a gradual differentiation in the composition of the bacterial community towards the Ring15 zone. This was the case for the most abundant phylum across all sampling zones, *Firmicutes*, which showed significantly higher proportions in the Ring15 zone (54%, sd: 19, $p<0.1$), compared to Center (32.9%, sd:11.1) and Outside (31.9%, sd:14.1).

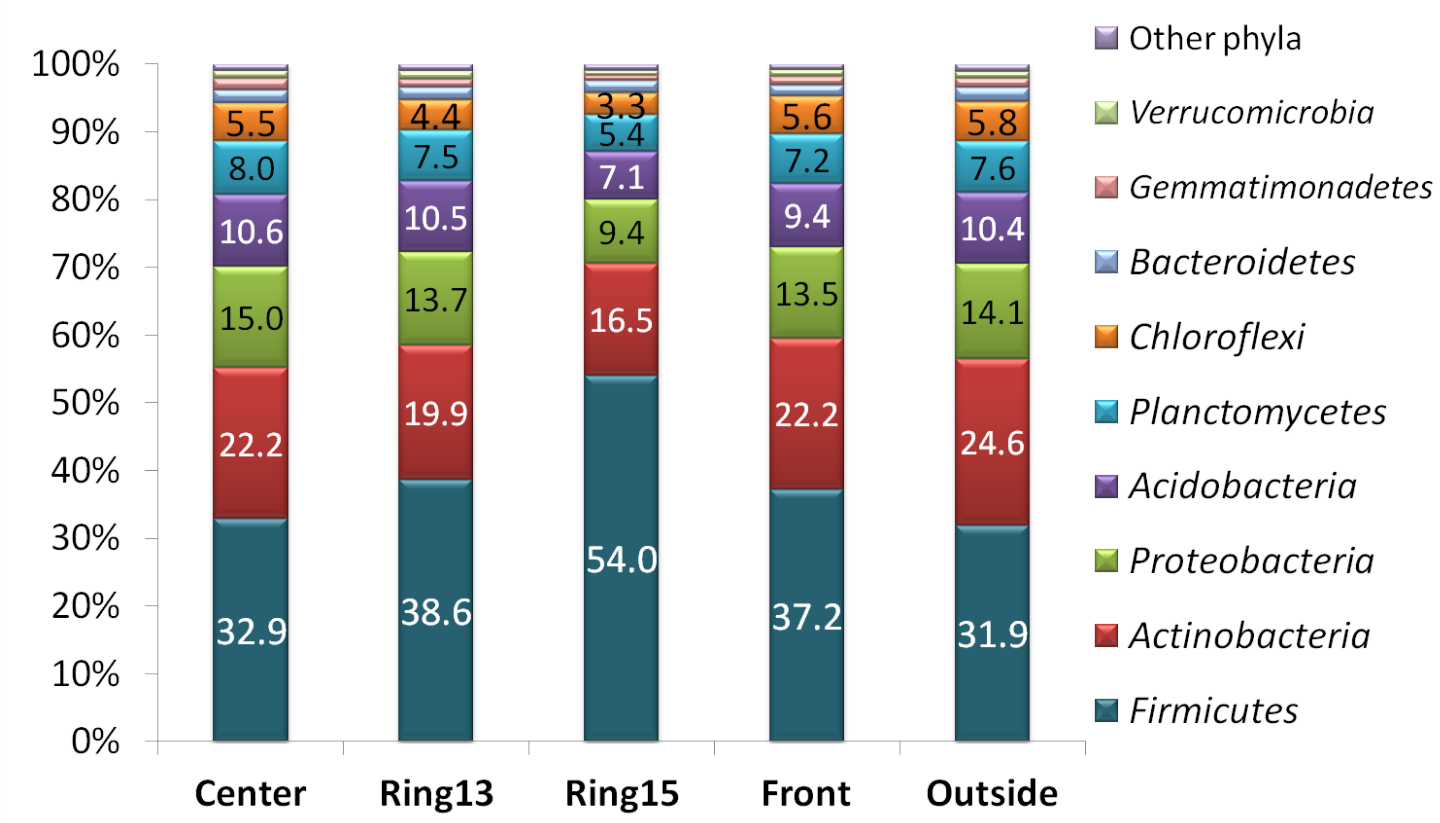

Figure 5.2. Phyla composition across zones

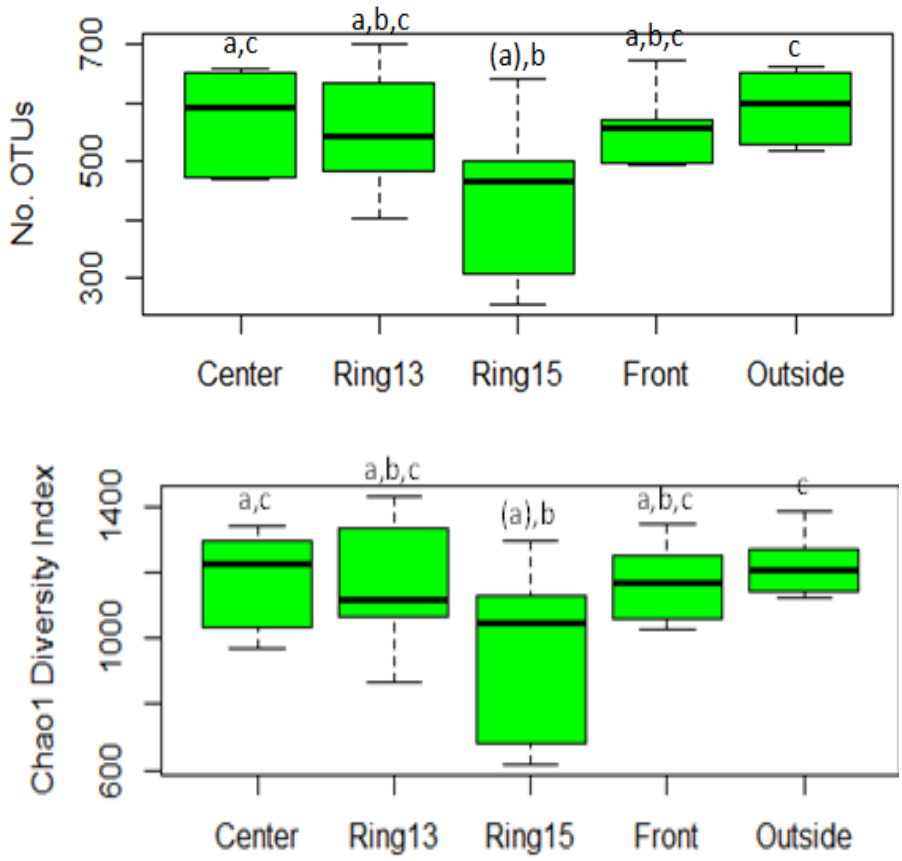

Figure 5.7. Diversity estimators across zones.

Conversely, the less abundant phylum *Chloroflexi* had significantly lower abundances in the Ring15 zone (3.3%, sd:1.2) respect to Center (5.5%, sd: 1.6, $p < 0.1$), Front (5.6%, sd:2.1, $p < 0.1$) and specially lower than Outside (5.8%, sd: 1.2, $p < 0.05$). In the same way, *Gemmatimonadetes* had the lowest abundances in the Ring15 zone (0.8%, sd: 0.4) but the highest in the Center zone (1.6%, sd: 0.6, $p < 0.05$).

The same trend to decrease in the Ring 15 zone was found with the phyla Actinobacteria (16.5%, sd: 8.5 in the Ring15, 24.6%, sd: 12.5 Outside), *Proteobacteria* (9.4%, sd: 5.5 in Ring15; 15%, sd: 3.7 in Center and 14.1%, sd:4.2, Outside), *Acidobacteria* (7.1%, sd:3.3 in Ring15; 10.6%, sd:2.8 in Center and 0.4%, sd:2, Outside) and *Planctomycetes* (5.4%, sd: 2.3 in Ring15; 8%, sd: 1.7 in Center and 7.6%, sd: 2.1, Outside).

We also analyzed the bacterial class composition across fairy ring zones, and we found a strong differentiation towards the Ring15 zone (Fig. 5.3). *Bacilli*, the most abundant class within the phylum *Firmicutes*, was higher in Ring15 (53.8%, sd:19, $p < 0.1$) compared to Center (32.5%, sd: 11.2) and Outside (31.6%, sd: 14). All the other differences happened the other way around. Thus, *Acidobaceria-6* and *Deltaproteobacteria* were significantly lower in Ring15 than in most of the other zones (3.6%, sd:2, $p < 0.1$ and 1%, sd:0.5, $p < 0.05$, respectively). The same trend was found in *Planctomycetia* within the phylum *Planctomycetes* (3.2%, sd:1.3 in Ring15; 5.2%, sd:1.3 in Center and 5.1%, sd:1.1 Outside); in *Acidimicrobiia* (1.9%, sd:1.3 in Ring15; 3.3%, sd: 1.3 in Center and 3.4%, sd: 1.4 Outside) and in *Betaproteobacteria* (1.1%, sd: 0.7 in Ring15 while 2.2%, sd: 1.2 Outside).

The majority of the 108 families identified showed the same decreasing pattern towards the Ring15 zone, with the exceptions of *Bacillaceae* (51.6%, sd:17.6, $p < 0.05$) and *Pseudomonadaceae* (0.3%, sd: 0.4, $p < 0.1$), both higher in the Ring15 zone with respect to the Outside

of the rings (26.6%, sd:15.9 and 0.05%, sd:0.05, respectively; Figs. 5.4 and 5.5).

Among the most abundant identified genera (>0.5% of the total reads), we found lower abundances in the Ring15 zone of *Rhodoplanes* (1%, sd:0.7, $p < 0.1$) and of *Gemmata* (0.6%, sd 0.2, $p<0.05$), specially compared to Center (2.1%,sd 0.7 and 1.1%, 0.3 respectively). The same trend was found in the genera *Mycobacterium*, *Skermanella*, *Rubrobacter* and *Catellatospora* (Fig. 5.6).

Finally, when going down to the OTU level, we found a few taxa associated to the Ring15 zone (Fig. 5.8), all of them belonging to the genus *Bacillus*, but without any possible identification to the species level.

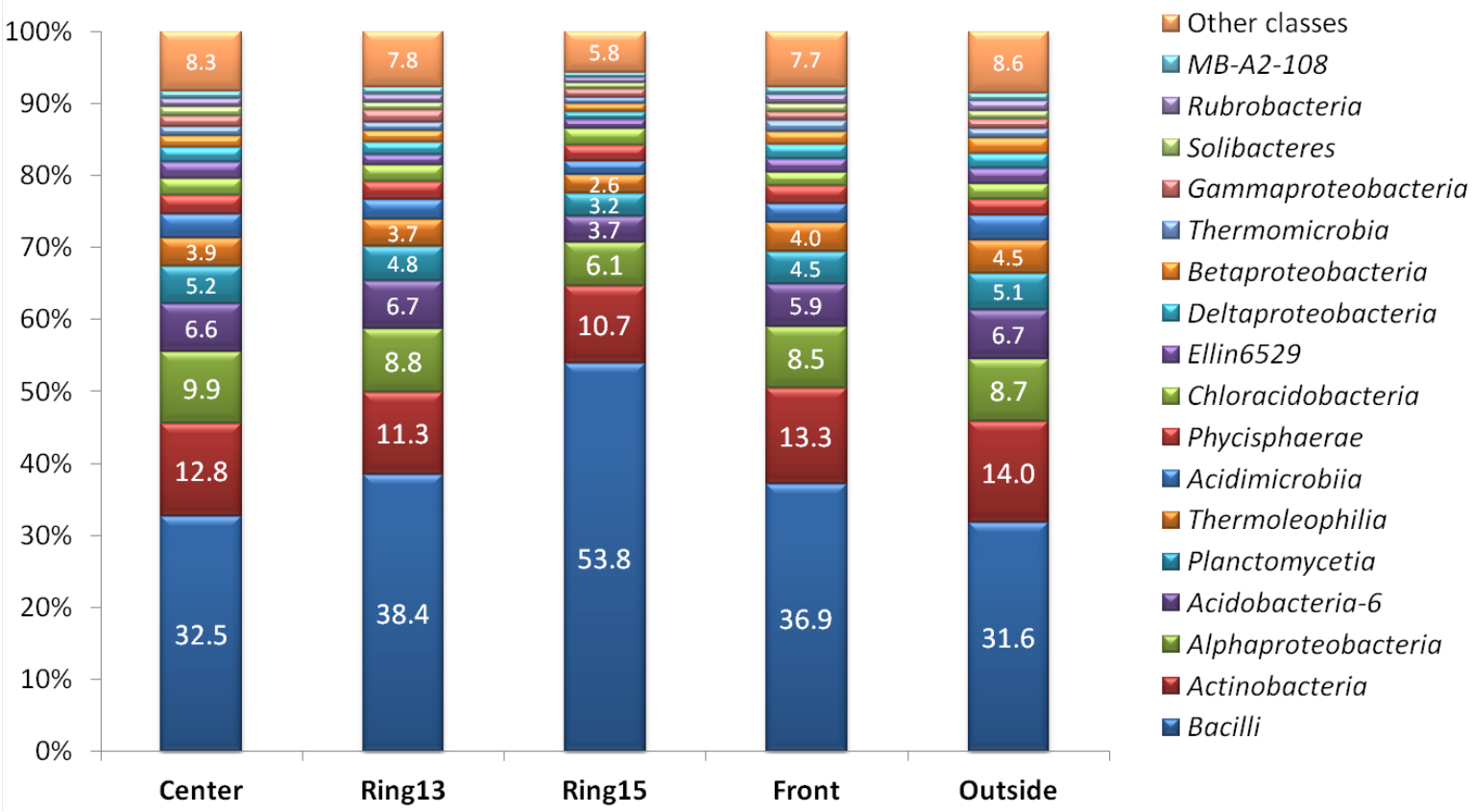

Figure 5.3. Classes composition across zones

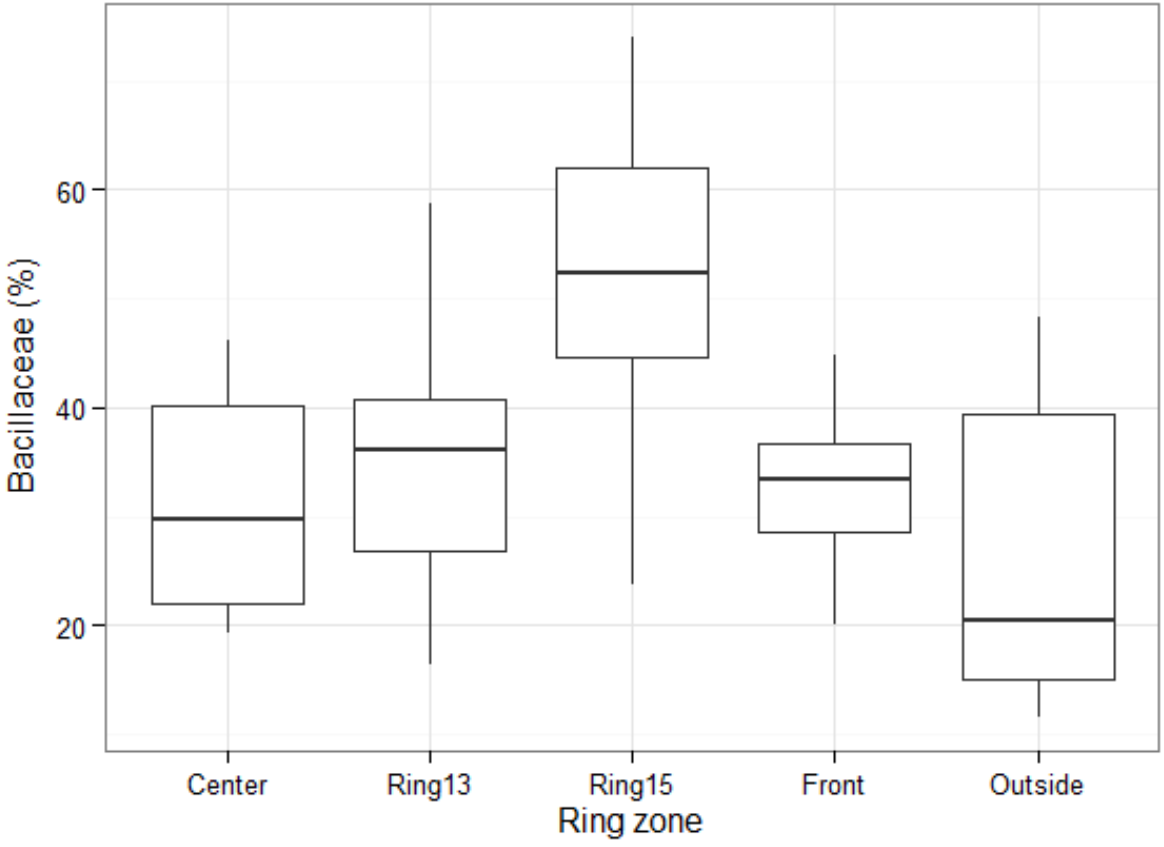

Figure 5.4. Proportion of the family *Bacillaceae* across zones.

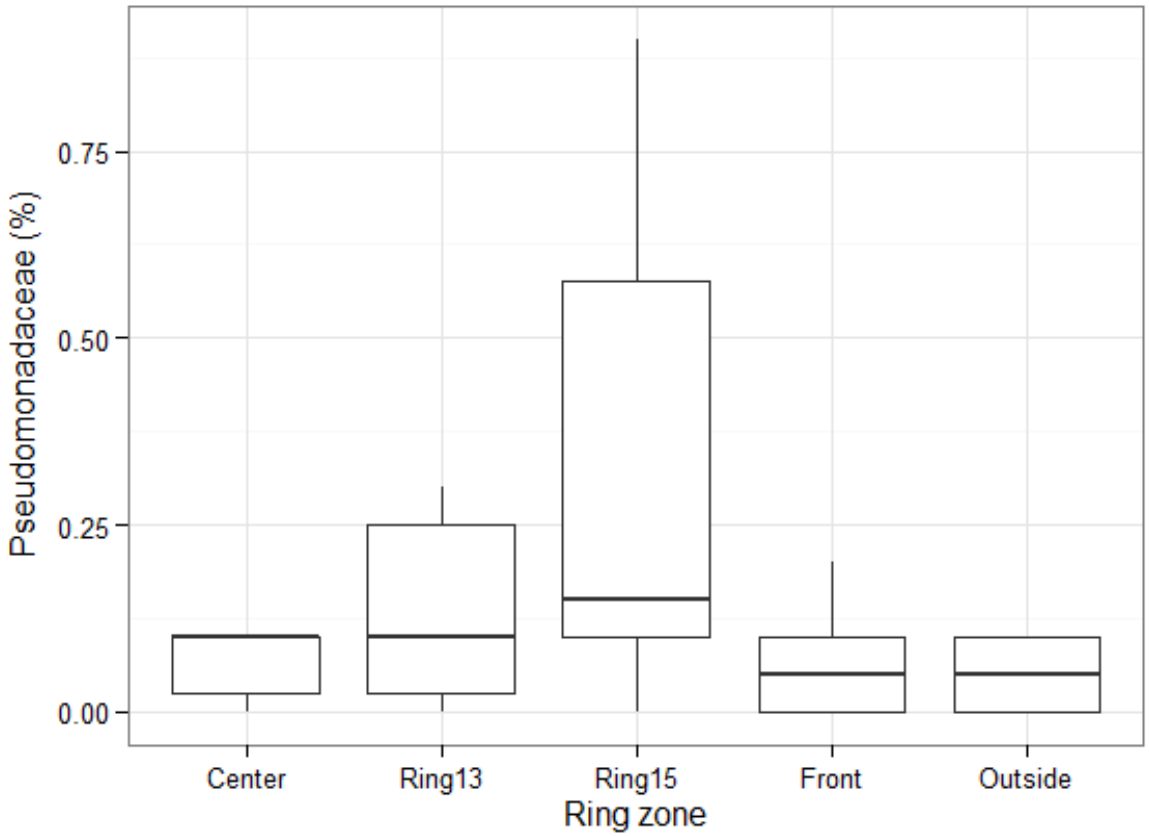

Figure 5.5. Proportion of the family *Pseudomonadaceae* across ring zones.

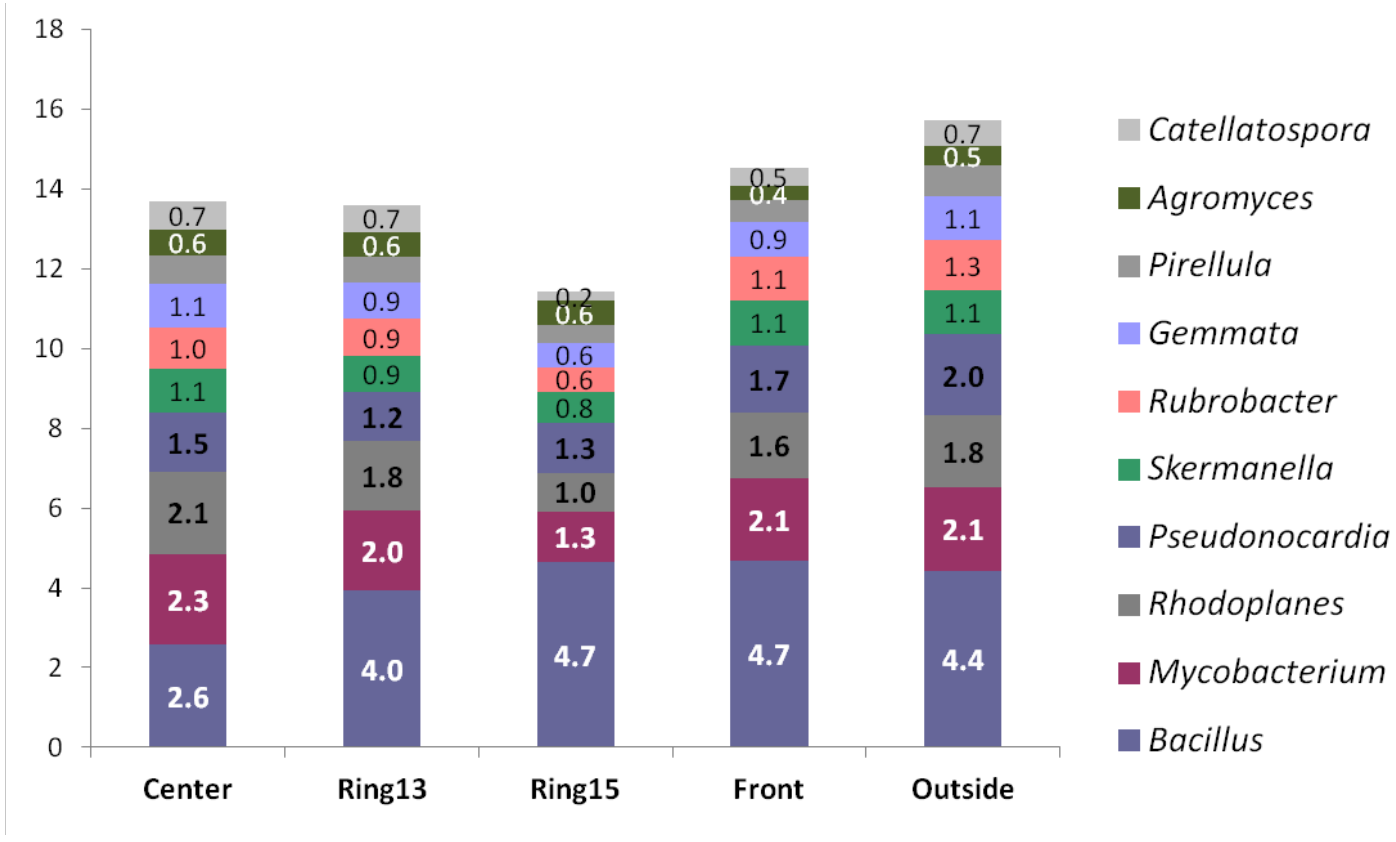

Figure 5.6. Proportions of the most abundant identified genera (>0.5%).

5.4. Discussion

Most of the bacterial 16S rRNA gene libraries carried out so far in soils are dominated by the same nine major phyla (Janssen, 2006) and these are what we found in this study. In particular, we found *Firmicutes* (driven by *Bacilli*), followed by *Actinobacteria* (driven by *Actinobacteria* class), as the dominant phyla in this soil (Fig. 5.2). Contrary to our findings, several studies on grassland, cropland or other soils dominated by grasses, revealed a predominance of *Acidobacteria*, *Proteobacteria* and *Actinobacteria* when using 454 pyrosequencing of the soil bacterial libraries (Eilers *et al.*, 2010; Will *et al.*, 2010; Nacke *et al.*, 2011; Shange *et al.*, 2012; Kim *et al.*, 2014a; Pan *et al.*, 2014). In contrast, Kuramae *et al.*, 2010 found similar results to ours when studying a semi-natural chalk grassland after the abandonment of intensive agriculture by using different molecular methodologies, where *Firmicutes* and *Actinobacteria* were the most abundant phyla. Lienhard *et al.* (2014) also found an increase in *Firmicutes* in grasslands under tillage when compared to natural pastures, using a 454 pyrosequencing approach. This suggests that the main community composition could still be an inheritance of the previous agricultural use in our study site until about 19 years ago. In fact, it is known the strong impact of agricultural practices on the microbial community composition, due to the induced changes in the physical and chemical characteristics of the soil (Govaerts *et al.*, 2007) and our results support that this influence may remain stable for several years after cessation of cropping.

The predominance of *Firmicutes* has been related to different stress factors, such as the snow cover during the colder winter months (Scavino *et al.*, 2013), the drought episodes during summer (Grönemeyer *et al.*, 2012) or also due to agricultural practices like tillage (Lienhard *et al.*, 2014). This is due to their ability to form endospores, which make bacteria resistant to extreme temperatures and enhance their survival under stress conditions (Riesenman & Nicholson, 2000; Filippidou *et al.*, 2016). In particular, OTUs belonging to the order *Bacillales* were found with the highest relative abundances and *Bacillus* (within *Firmicutes*), which is also reported to grow at temperatures below 0 °C (Druce & Thomas, 1970), was the most abundant taxa identified at the genus level in this study (Fig. 5.6). *Actinobacteria* was the second most abundant phyla in this study (Fig. 5.2). Decreasing abundances have been found under tillage management (Lienhard *et al.*, 2014), which has been explained by the particular filamentous morphology of *Actinobacteria*, that confers them an special sensitivity to physical disturbances (Stackebrandt, Rainey, & Ward-Rainey, 1997). However, although *Actinobacteria* includes many common taxa well adapted to environmental stresses (Grönemeyer *et al.*, 2012), their high abundances could be attributed to the grazing history of the site after cessation of cropping as they are commonly described as K strategists, well represented in non-disturbed sites (Acosta-Martínez *et al.*, 2008; Lienhard *et al.*, 2014).

Fungal degradation of lignocellulose and organic N results in lower pH and enhanced solubilization of nutrients, which become readily available

for plants (Kreisel, 1961; Gramss *et al.*, 2005). Fairy ring fungi have been described to acidify the soil beneath the stimulation zone (Gramss *et al.*, 2005; Bonanomi *et al.*, 2012; Kim *et al.*, 2014b), and the composition of the bacterial community is known to be strongly influenced by this factor (Lauber *et al.*, 2008; Rousk *et al.*, 2010; Lanzén *et al.*, 2015). Indeed, our results highlighted soil pH as the second most important factor explaining the variation in the bacterial community composition at the OTU level across fairy ring zones. However, the fact that pH was not significant when considering the conditional term effects (Table 5.1) suggests that other factors in addition to soil pH must change across ring zones and therefore ring zone was a better explanatory variable of bacterial community than soil pH. Furthermore, soil pH in this calcareous grassland ranged between 7.45 and 8.14, values significantly higher than those found in the soils in other studies (Gramss *et al.*, 2005; Bonanomi *et al.*, 2012; Kim *et al.*, 2014b). High carbonate content in calcareous soils provide a strong buffering capacity making these soils relatively resistant to acidification (Brady & Weil, 2008; Kirk, Bellamy, & Lark, 2010). This fact could be counteracting the biochemical effects of the microbial activity beneath the stimulation zone.

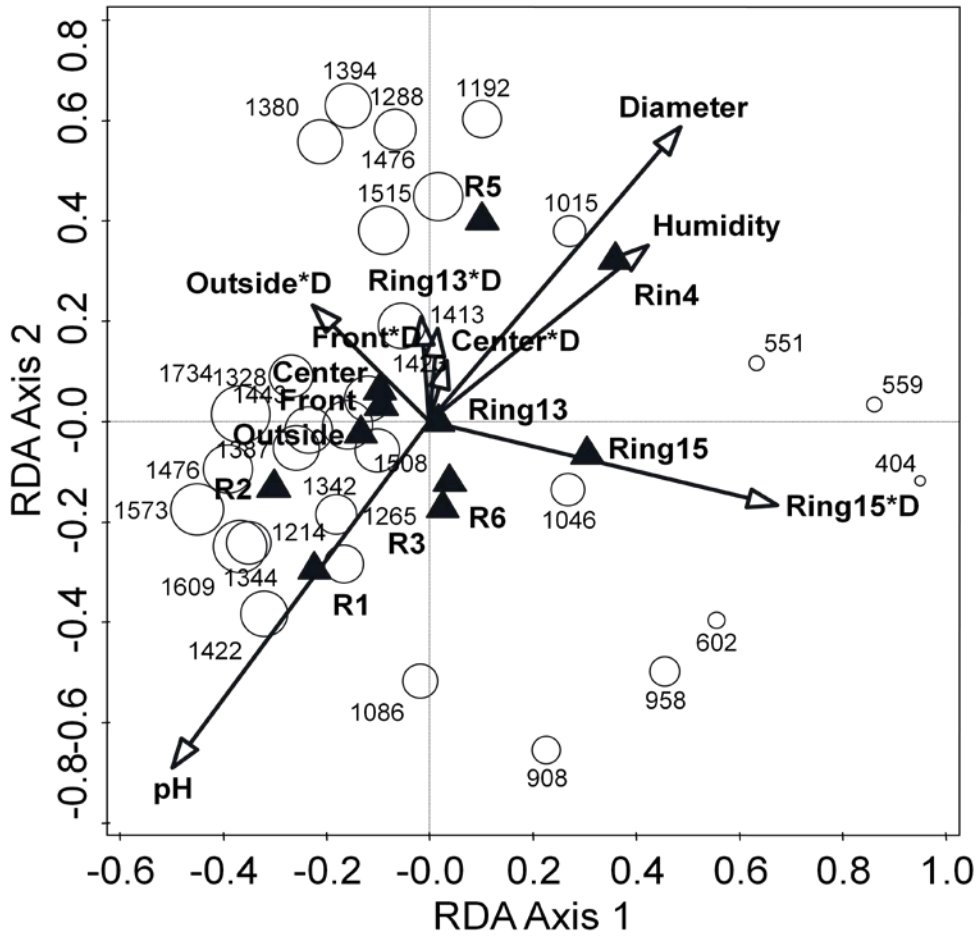

Figure 5.8. RDA plot including the relationship between the composition of the bacterial community at the OTU level with soil factors, ring diameter and ring zone. The size of the circles is proportional to the OTU richness.

The quantity and quality of soil organic matter (SOM) shows a wide oscillation between different land uses and plant community compositions (Breulmann *et al.*, 2014). Changes in the availability of nutrients through SOM mineralization are mainly determined by the soil microbial community, and in turn, its composition and function depend on the organic inputs to the soil (Bardgett, 2005). Although pastured

grasslands have been considered copiotrophic environments because of the continuous inputs of organic matter (Shange *et al.*, 2012), land management, climate and grazing pressure determine the resulting soil fertility of a specific soil (Haynes & Williams, 1993). Fungal decomposition beneath fairy rings is usually associated to an enhanced nutrient availability for plants (Edwards, 1984). According to the copiotroph/oligotroph ecological division of soil bacteria, changes in nutrient availability are expected to translate into changes in the proportion of the different strategists (Fierer, Bradford, & Jackson, 2007). High availability of nutrients in the soil beneath the Ring15 or stimulation zone is expected to favour copiotrophic, fast-growing bacterial taxa, while reducing the abundances of the oligotroph, slow-growing taxa (Fierer *et al.*, 2007; Leff *et al.*, 2015). In this study we performed a comparative metabarcoding analysis of the soil bacterial communities across the zones of six fairy rings, and we found a strong increase of *Bacilli* within the phylum *Firmicutes* beneath the Ring15 zone. The same increase in *Firmicutes* (particularly in the genus *Bacillus*) was also found by Oh *et al.* (2016) in *T. matsutake* fairy rings. This phylum is commonly included into copiotrophic groups that grow fast under high availability of nutrients (Fidanza, 2007b). Bonanomi *et al.* (2012) suggested that fungal crossing could result in a reduction of the total bacterial populations, in favor of a few selected species of bacteria. This is what we found in this study, where the increase in the relative abundance of *Bacilli* was accompanied by a reduction in most of the other taxa, including *Actinobacteria* and *α-Proteobacteria* (Fierer *et al.*,

2007; Shange *et al.*, 2012), which are also considered copiotrophic but lacking the stress resistance mechanisms found within *Firmicutes*.

It is estimated that mycorrhizal symbioses are found in about 85% of plant species, and their establishment is influenced by other soil microbes, specially bacteria (Rigamonte, Pylro, & Duarte, 2010). Those bacteria promoting the establishment and function of mycorrhizal symbioses are known as Mycorrhization Helper Bacteria (MHB, Garbaye, 1994). Taxa belonging to *Proteobacteria*, *Firmicutes* and *Actinomycetes* have been included within MHB, and they contribute to mobilize soil nutrients and to protect plant roots against pathogens (Fitter & Garbaye, 1994; Garbaye, 1994; Frey-Klett, Garbaye, & Tarkka, 2007). Although with minority abundances (less than 1% of the total amount of reads), *Pseudomonadaceae* also peaked in the Ring15 zone (Fig. 5.5). Several taxa within this family are found in high relative abundances and compete successfully with other microbes in nutrient-rich substrates (Overbeek & Elsas, 1997; Artursson, Finlay, & Jansson, 2006). They adhere to soil particles, roots and fungal hyphae, having a beneficial role in mycorrhizal establishment and plant growth (Garbaye, 1994; Artursson *et al.*, 2006; Edgar *et al.*, 2011). Furthermore, species within the genus *Pseudomonas* have been found to enhance the yield of saprotrophic fungi cultures (Young, Chu, & Young, 2012). The visible enhancement of plant growth could thus be a result not only of the enhanced availability of nutrients by the fairy ring fungus, but also by a complex network of synergies including bacterial taxa.

Several studies on fairy rings have focused on the effect of fungi on plant growth (Edwards, 1984, 1988; Terashima *et al.*, 2004) and plant diversity (Bonanomi *et al.*, 2012). However, only a few studies have focused on the effect of fairy ring fungi on the microbial diversity, and those who did, often were limited to particular groups of microbes and resulted in divergent conclusions (Smith & Rupps, 1978; Gramss *et al.*, 2005; Lian *et al.*, 2006; Bonanomi *et al.*, 2012). The effects of fairy rings on plant and bacterial diversity and richness could be caused by an increase in the availability of mineral nitrogen (in both forms, $N\text{-}NO_3$ and $N\text{-}NH_4$+) in the stimulation zone (Bonanomi *et al.*, 2012). Previous studies reported a decrease in plant species richness in the stimulation zone of *Agaricus campestris* fairy rings, presumably due to the increase in the available nitrogen (Bonanomi *et al.*, 2012). However, previous studies focusing on the effect of nitrogen enrichment on the bacterial community found contrasting results. Campbell *et al.* (2010) showed a decrease in bacterial OTU richness across fertilization treatments, whereas Fierer *et al.* (2012) did not find a significant effect. Kim *et al.*, 2014 studied the bacterial community across *Tricholoma matsutake* fairy rings and they did not find any significant differences in OTU richness neither. Conversely, in a recent pyrosequencing approach, Oh *et al.* (2016) found a reduction in bacterial diversity in the ring zone of *T. matsutake*. In the same line, in this study we found a reduction in the bacterial OTU richness in the stimulation zone or Ring15, although a recovery was recorded towards the center of the ring (Figs. 5.7, 5.8). This reduction was linked to an increase of some taxa known as

copiotrophs (see above), and a reduction in other genera. Among them, *Gemmata* and *Catellatospora* showed a reduction in the stimulation zone. Both have been previously reported to decrease their relative abundance in nitrogen application treatments (Shang & Yi, 2015), suggesting that nitrogen enrichment could be one of the drivers of the changes in the bacterial community across fairy rings. However, the disparity of results between different studies suggest a high spatial variability in the effect of nitrogen on the bacterial community (Fierer *et al.*, 2012). Further studies including the here missing *Archaea* should also be developed, because of its relevant role in the nitrogen cycle (Cabello & Roldan, 2004).

Among those taxa identified at the genus level, we also found a reduction of *Rhodoplanes* in the Ring15 zone. This is a facultative phototroph genus (Hiraishi & Ueda, 1994) characterized by their capacity to complete denitrification (Santana *et al.*, 2016), although sandy loam soils like that of our study site are usually well aerated and therefore unlikely to create the anaerobic conditions required for denitrification under normal conditions (Skiba, Smithy, & Fowler, 1993). A previous study on the bacterial communities associated to different Boletales (Uroz *et al.*, 2012) showed a reduction in the sequences related to *Rhodoplanes* in the ectomycorrhizosphere when comparing with the surrounding bulk soil. This supports the idea that specific additional relationships are established beneath the Ring15 zone between bacteria, fungi and plant roots.

In a previous study, Marí *et al.* (in rev.) found an enrichment in Basidiomycota versus Ascomycota taxa towards the inner zone of these fairy rings. In contrast, in the present study bacterial community did not change the predominance of the main groups at the phylum level across ring zones (Fig. 5.2). The only changes were in the proportions of the most abundant taxa and only beneath the Ring15 or stimulation zone. Conversely to that found in fungi, where differences in community composition where found across all zones inside the fairy rings compared to outside, almost the same proportions of bacterial taxa where found both inside and outside of the rings. This fast recovery of bacterial proportions and richness towards the inside of the ring suggests a high degree of resilience of the soil bacterial community after this biotic perturbation.

5.5. Conclusions

In conclusion, in this study we contributed to the knowledge of the soil bacterial communities across fairy rings by using the 16S bacterial Illumina metabarcoding in a pastured grassland. We found strong changes in bacterial phyla proportions across the fairy rings, showing an increase in the relative abundance of selected taxa belonging to *Bacilli* (*Firmicutes*) towards the stimulation zone at the expense of a decrease of the rest of the bacterial populations (*Rhodoplanes*, *Gemmata* and *Catellatospora* among others). However, the bacterial communities recovered towards the inside of the rings almost up to the same proportions that those found in the outside zones, pointing to a higher

resilience of bacterial communities to physical and chemical perturbations than previously found in fungal communities. The decrease in OTU richness also found in this zone contrasts with previous studies using various methodologies, suggesting the low awareness of the processes taking place as the fungal front advances through the soil. Further studies including the whole soil biota are needed to unravel the relationships between above and belowground communities across fairy rings in grassland pastures and other economically valuable environments.

5.6. Acknowledgements

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Chapter 6

General discussion

6. General discussion

6.1. Methodological considerations

The results of this thesis highlight the need to better understand the factors affecting the distribution of the soil microorganisms, in order to better predict the responses of the ecosystems to the changing climate and land use. Also, understanding the interactions among particular groups of species, plant-microorganism as well as fungal-bacterial relationships, become increasingly essential when aiming to increase knowledge on the ecosystem functioning.

This thesis aimed to analyze the changes in microbial patterns along climatic and land use gradients in two continents. On the one hand, microbial biodiversity of the African rangelands was analyzed from a functional point of view, given the urgency in the undertake of measures against desertification and recovery of land fertility. On the other hand, European rangelands were studied through the use of Illumina metabarcoding, in order to deepen knowledge on the factors determining the structure of the microbial community as well as in the interactions among microbial consortia.

The metabarcoding approaches include PCR amplification of the targeted regions, in this thesis from the fungal ITS2 region and bacterial 16S ribosomal RNA gene; this is followed by next-generation sequencing (NGS) and by the alignment of the sequenced fragments to known genomic databases in order to relate them to specific taxa (Coissac, Riaz,

& Puillandre, 2012; Links & Chaban, 2013). As in all PCR-based methods, the biases that the different steps of this methodology can introduce into the natural abundances of the sequences must be taken into account, especially when we treat the data quantitatively (V. Wintzingerode, Göbel, & Stackebrandt, 1997).

DNA extraction procedures are possible sources of bias, due to the variable resistance to cell lysis that the different organisms studied can present. This is followed by the efficiency of the extraction method, which may also vary depending on the composition of the sample (Casamayor *et al.*, 2002).

One of the main sources of bias are the use of "universal" primers, due to differences in primer specificity that may overestimate some taxa over others (Schmalenberger, Schwieger, & Tebbe, 2001). This can cause taxa with a low representation in the original DNA to become more abundant in the final results. As a result, this bias prevents from correctly inferring the abundance of species in the original DNA sample. In fact, previous studies testing the results obtained with different methodologies based on the PCR conclude that when using different primer sets, much more diverging results are obtained (Casamayor *et al.*, 2002).

Additional biases are introduced on account of the different efficiency of the amplification reaction (Suzuki & Giovannoni, 1996) and due to read losses during DNA libraries preparation (Pawluczyk *et al.*, 2015). A relevant loss of data has been recorded in Illumina metabarcoding

studies through the different filtering steps; however, because of the higher sequencing depth of Illumina compared to other metabarcoding procedures (such as 454 pyrosequencing), this method allow us dealing with read losses and still characterize the target organisms in sufficient depth (Schmidt *et al.*, 2013). Moreover, we performed repeated PCR with multiple annealing temperatures, which is known to facilitate the recovery of OTU richness (Schmidt *et al.*, 2013).

In spite of all the limitations mentioned above, it is expected that, within the same study, PCR biases go always in the same direction. Therefore, it is possible to compare how the abundance of a given taxon varies across different samples, but special care should be taken when comparing results from different studies (Schloss, Gevers, & Westcott, 2011; Schmidt *et al.*, 2013; Geisen *et al.*, 2015).

On the other hand, some eukaryotes may also have several copies of the targeted gene (Zhu *et al.*, 2005) causing overestimates of some taxa over others when analyzing mixed environmental samples (Medinger *et al.*, 2010; Gong *et al.*, 2013). In our studies, we chose the primer pair ITS86F / ITS4 for fungal metabarcoding; these primers have been described as selectively specific for the Ascomycetes, Basidiomycetes and Zygomycetes fungal clades (Turenne *et al.*, 1999) and they proved to be suitable for the analysis of the soil fungal community, obtaining a low incidence of repeat sequences (Vancov *et al.*, 2009).

Additional sources of bias are the steps of sequence alignment followed by the application of a clustering algorithm, which allow the

establishment of barcode clusters of a determined genetic similarity referred as species or OTUs (Hebert *et al.*, 2003, 2004). The diversity of alternative algorithms used for this steps, as well as the poor representation of microbial strains in culture collections, translate into an incomplete characterization of the microbial community (Andreakis *et al.*, 2015). The high amount of reads corresponding to previously uncultured, unidentified clones, impose a severe limitation in the ecological interpretation of our results.

6.2. Effects of land use in African rangeland soils

Continuous grazing results in a reduction of vegetation cover, a decrease in the organic matter accumulation in the ecosystem as well as in the soil microbial activities (Yong-Zhong *et al.*, 2005). Enzyme activities and microbial biomass have been used as soil quality indicators (Badiane *et al.*, 2001; Pabst *et al.*, 2015). When characterizing the effect of land use and land use intensity in Eastern African rangelands, we found them to be the most important factors determining various parameters of soil quality (Table 2.1, Figs. 2.2 - 2.6). Within them, we found a strong decrease in several of the microbial parameters with increasing land use pressure, specially in the hydrolytic enzymes, known to be sensitive indicators of soil quality (Badiane *et al.*, 2001, Fig. 2.4). The only exception that increased at high grazing pressures (Fig. 2.4) was found in phenoloxidase activity, and it was not surprising since this enzyme often do not correlate with the other enzymes (Sinsabaugh, 2010). As indicated by several enzyme activities, C and N cycling where

favored under light grazing pressure (Badiane *et al.*, 2001; Lagomarsino, Grego, & Kandeler, 2012). The enrichment in inorganic N found under high grazing regimes was accompanied by a reduction in microbial biomass, soil respiration and soil organic matter, and these results are according to that found in previous studies (DeForest *et al.*, 2004; Zeglin *et al.*, 2007; Treseder, 2008).

Previous studies on the effect of grazing on soil properties indicated an increase in microbial biomass and nutrient availability due to nitrogen enrichment by urine and dung (Tu *et al.*, 2014). Our results indicate that extensive grazing, under low and intermediate pressures, result in an enhancement of soil quality and microbial parameters, whereas overgrazing caused the opposite effects (Table 2.1, Fig. 2.5). This highlights the importance of appropriate design of management strategies, including a range of stocking rates which can be predicted on the basis of the herein measured soil biological parameters.

6.3. Microbial communities across European climatic and land use gradients

6.3.1. Climatic factors

Altitudinal and climatic gradients are often used in biological studies because of the wide range of climatic conditions and biotic turnover over short distances (Bryant *et al.*, 2008; Yashiro *et al.*, 2016). Although most of these studies have focused on animals and plants (Terborgh, 1977; Loiselle & Blake, 1991; Patterson, Pacheco, & Solari, 1996; Bhattarai & Vetaas, 2004; Bello *et al.*, 2006; Frei *et al.*, 2014), there is an

increasing interest on understanding the elevational patterns that microbial communities follow (Bryant *et al.*, 2008; Fierer *et al.*, 2011). However, due to the particular geology and land use history, each mountain ecosystem shapes differently its local above and belowground communities (Yashiro *et al.*, 2016). The outcome is the establishment of a unique and complex network of interactions, which will determine the resulting goods and services that each ecosystem will provide. In spite of the microbial abundance, microbial diversity and the key role that microbes play in ecosystem functioning, their responses to the climatic changes found along elevational gradients remain controversial (Bryant *et al.*, 2008; Yashiro *et al.*, 2016).

The results presented here sustain the effectiveness of the Illumina MiSeq sequencing on the biogeographical study of fungal and bacterial communities. Their combination with ordination methods and subsequent clustering revealed fungal-bacterial associations as well as their environmental affinities. In line with previous studies, bacterial community composition changed according to soil pH, supporting the generalized consideration that pH is the main driver of this microbial group (Lauber *et al.*, 2009b; Rousk *et al.*, 2010). By contrast, fungi have been found to be tolerant to wider pH ranges than bacteria (Bradley, Drijber, & Knops, 2006; Rousk, Brookes, & Bååth, 2011) and the results of this thesis corroborate this tolerance. Instead, a climatic gradient, driven by temperature and moisture regime, was found to be the major driver of the changes in soil fungal community. Meier *et al.* (2010)

measured the sensitivity of net fungal growth to the temperature using an elevation gradient in forest, and found a significant effect, due to changes in the dynamics of the enzymes involved in wood decomposition. They also found strong changes in the fungal community composition along the gradient, but it was identified as a succession of wood-decomposing fungal community in parallel to the changes in tree species composition with increasing elevation (Kulhánková, Béguiristain, & Moukoumi, 2006). It would be necessary to include a complete survey of the vegetation in the studied plots in order to better understand this indirect effect of the temperature on the fungal community.

6.3.2. Land use factors

Different land uses were included in our altitudinal gradient. However, similarly to climate, they also showed confounding effects, since cattle and horses are usually linked to colder and more humid grasslands, while sheep are found on lower altitudes (Leifeld *et al.*, 2015). Moderate grazing is necessary for the formation and maintenance of the grassland plant communities, but the plant community composition is strongly determined not only by the grazing pressure, but also by the herbivore behaviour (Yunusbaev, Musina, & Suyundukov, 2003; Loucougaray, Bonis, & Bouzille, 2004; Boschi & Baur, 2007; Sebastià *et al.*, 2008). While sheep and horses are known to feed on a wider spectrum of plant species than cattle (Zotov & Erizhev, 2000), sheep grazing is known to be more destructive for the grassland stands (Yunusbaev *et al.*, 2003). Our results showed the effect of cattle and

horse grazing in the same direction than MAP, while the effect of sheep was linked to higher temperatures and low rainfall regimes (Figs. 3.1 - 3.3). Therefore, changes in vegetation composition are expected to happen both due to the effect of differential grazing and climatic conditions along the elevation gradient, resulting in changes in the associated microbial communities.

6.4. Co-variation and interactions between fungi and bacteria

Plant communities have been suggested to drive the complex spatial patterns in soil microbial communities due to the highly diverse microhabitats that they form in the soil (Wardle, Bardgett, & Klironomos, 2004; Millard & Singh, 2010). Soil microbes, in turn, carry out a large number of processes that influence the composition of the plant community, resulting in a complete network of synergies between the above and the belowground community (Zak *et al.*, 2003; Wardle *et al.*, 2004), including both the bacterial and the fungal domain. However, many studies carried out on soil microbial communities only included one of these groups, in spite of the key role of the interactions taking place within them in ecosystem processes (Wardle, 2006). Through the use of Co-correspondence analysis (ter Braak & Schaffers, 2004), our results showed a high correlation among the fungal and bacterial community composition across the altitudinal gradient, reaffirming the effect of climate as a main driver of the soil microbial community (Castro, Classen, & Austin, 2010; Frey *et al.*, 2013), either via direct

effects (temperature and moisture) or indirect effects (soil chemical properties and vegetation composition).

Deepening knowledge of the fungal-bacterial interactions in soils is interesting from several points of view (Boer *et al.*, 2005), such as the understanding of the ecological processes linked to grassland management. In this group of studies we showed how the combination of Illumina metabarcoding of fungal and bacterial communities and Co-Correspondence analysis (ter Braak & Schaffers, 2004) allows finding those groups sharing specific environmental requirements. For example, we found *Ktedonobacteria* clustered together with different lichenized taxa (i.e. *Lecanorales* and *Verrucariales*, Table S3.5), predominating under alpine conditions (Tables S3.3 and S3.4). Both of these fungal and bacterial taxa have been found under extreme environments (Semenova *et al.*, 2015; Kim *et al.*, 2015), and the genus *Ktedonobacter* was previously found among the bacterial community associated to lichens and mosses in an alpine grassland (Navarro-Noya *et al.*, 2014). In fact, previous studies evidenced the specific selection of bacterial taxa associated with lichen symbiosis by the lichen (Grube *et al.*, 2009; Bates, Cropsey, & Caporaso, 2011). Several types of interactions and niche differentiation have been described between fungi and bacteria, including mutualism, competition and selective inhibition or favor (Boer *et al.*, 2005). For example, some saprotrophic *Basidiomycota* belonging to *Agaricales* are known to produce antibiotics that inhibit Gram-positive bacteria but not some strains of *Pseudomonas* (Sidorova &

Velikanov, 2000). In this thesis, the effect of saprotrophic agaricales growing in the grassland soil on the bacterial community composition was assessed (Chapter 5), and the family *Pseudomonadaceae* (belonging to *Proteobacteria*) was within the few bacterial taxa increasing its abundance after the fungal passage (Fig. 5.5). This supports the theory that selective inhibitors are released by the fungus, as a mechanism for selection of the antibiotic-resistant bacterial community (Linderman & Paulitz, 1990; Olsson, Chalot, & Bååth, 1996). *Pseudomonadaceae*, along with *Bacillaceae* (belonging to *Firmicutes*), both increased their relative abundance in the stimulation zone of the fairy rings, and both contain species known to be mycorrhiza helper bacteria (MBH, Frey-Klett & Garbaye, 2005). This suggests that the enhancement of plant growth in this zone may be attributed not only to the expected release of nutrients by the fairy ring fungus (Stone & Thorp, 1971; Edwards, 1988) but also as a result of the interactions between plants, MBH and other associated fungi. Although little is known about the interactions existing among the belowground biota, our results suggest a complexity of the processes even higher than expected.

6.5. The resilience of the microbial community

In addition to the strong correlation in the changes of the bacterial and fungal communities along our climatic gradient, in the Chapters 4 and 5 of this thesis we showed different responses of both consortia to local biotic perturbations. When observing the effect of the fairy ring fungal passage, bacterial communities showed a fast recovery to the

same proportions of taxa, while the fungal community maintained the changes towards the center of the ring.

Although an increasing body of literature regarding resistance and resilience of the soil microbial communities is currently available, only a few studies used high throughput sequencing methods describing the microbial community structure (Griffiths *et al.*, 2013). Within these who did, Lekberg *et al.* (2012) found an unexpectedly high arbuscular mycorrhizal (AM) community resilience after physical disturbance. They suggested a dominance of AM fungi having a high degree of tolerance to disturbance. On the other hand, Girvan *et al.* (2005) also found a fast recovery of the bacterial community composition after soil chemical perturbations, suggesting an adaptation by the surviving communities to perturbation. Our results are in accordance with this fast recovery of the bacterial community, since the passage of the fairy ring fungi is known to alter both physical and chemical properties of the soil and the community composition showed almost no changes two years later (Figs. 5.2 - 5.6). This is in accordance with our results along the altitudinal gradient, where highly adapted communities were found under each type of stress measured, suggesting a strong adaptability of the bacterial communities to the ongoing changes in the soil environment. By contrast, the soil fungal community across fairy rings changed towards an enrichment on taxa belonging to Basidiomycota, over the entire ring surface (Fig. 4.2). Our results suggest a view of the fairy ring fungi growing radially that could be seen as a wave in

expansion, causing a cascade of effects on fungal biodiversity in its path. This contrasts with the results of Kim *et al.* (2013) in *Tricholoma matsutake* rings, which showed an enrichment in Basidiomycota in the ring zone, but decreasing again towards the center of the ring. Further long-term studies would be needed on the fungal community composition inside the fairy rings in order to understand whether this changes become a new steady state, or the enrichment in Basidiomycota vanishes with the disappearance of the ring. At any rate, fairy ring fungi have proved to be a keystone species in grassland ecosystems, as they trigger profound changes in the spatial heterogeneity of decomposition processes (Van der Wal *et al.*, 2013).

Chapter 7

Conclusions

7. Conclusions

- Land use and land use intensity were the most important factors determining soil organic carbon concentration and stocks, soil fertility distribution, and soil enzymatic activity in Western African ecosystems.

- The reduction in the activity of the hydrolytic enzymes with land use intensity confirmed its effectiveness as biological indicators of soil quality in Western African ecosystems.

-Illumina metabarcoding proved to be a useful method for the characterization of the bacterial and fungal communities across different climates and land use changes.

-Soil microbial community composition changed along altitudinal and climatic gradients in grassland ecosystems. The bacterial community structure was mostly influenced by local soil chemical factors (particularly soil pH and salinity), whereas regional climatic factors (temperature and rainfall) had a stronger influence on the fungal community.

- The bacterial and fungal communities co-varied strongly across the climatic gradients. Co-Correspondence analysis proved to be a useful way to find particular assemblages of bacterial and fungal taxa sharing similar environmental requirements or adaptations to particular sets of environmental stresses.

- Fungal species causing fairy rings were responsible of profound changes in the overall soil fungal community composition. They caused

an enrichment of saprotrophic Basidiomycota inside of the rings compared to the outside communities, which were richer in Ascomycota.

- We could not identify a single fungal species causing the visible effects of the fairy rings in the studied grassland; several Basidiomycota species could be forming rings simultaneously.

- Fairy ring fungi caused strong changes in the soil bacterial community composition in the stimulation zone of the rings. This changes were characterized by an increase in the relative proportions of *Bacillaceae* (*Firmicutes*) and *Pseudomonadaceae* (*Proteobacteria*).

- The bacterial community showed a fast recovery after the fairy ring passage, because no differences were found between the bacterial community composition inside and outside of the rings. This indicates a higher resilience of the bacterial community than that of the fungal community in front of the biotic disturbance that a fairy ring implicates.

Annex: Supplementary materials

Table S2.1. Environmental variables included in the best model of each soil organic carbon and fertility variable in the Eastern African dataset from Kenya and Tanzania. LU-LUI is a combined 8-level index encompassing land use and land use intensity.

Variable	Bedrock	Land Use	LU-LUI
SOCS10	x	x	
SOCS30	x	x	
SOC10	x	x	
SOC30	x	x	
TN10	x	x	
TN30	x	-	
CN10	x		x
CN30[a]		-	x
TP10[a]	x	x	
TP30[a]	x	x	
NP10	x		
NP30	x		

[a]Non-significant model

Table S2.2. Explanatory power (%) of four single environmental variables on six exoenzyme activity distributions. LUI, land use intensity.

Enzyme	Plot	LUI	pH	Bedrock
Exoglucanase	25.6	15.86	0	7.29
Exochitinase	61.9	51.35	33.97	46.06
Phosphatase	12.56	0	0	0
Protease	39.36	36.93	23.79	32.72
Phenoloxidase	52.74	9.54	11.77	6.57
Peroxidase	50.85	41.34	40.92	46.78

Table S3.1. General characteristics of the study sites. MAT: mean annual temperature; MAP: mean annual precipitation.

Climatic region	Location	Geographical region	Altitude (m.asl)	MAT (ºC)	MAP (mm)	Management	Dominant species (pendent)
Semi-Arid	Monegrillo	Ebro depression	657	12.6	225.2	sheep	*Papaver rhoeas, Thymus vulgaris*
	Alguaire	Ebro depression	334	13.9	430.5	sheep	*Hordeum vulgare*
Mediterranean	El Prat	Llobregat delta	5	15.6	664.9	sheep	*Galium* sp., *Vicia* sp.
Montane	Pallars 2	Central Pyrenees	622	11.9	666.4	sheep	*Thymus vulgaris*
	Ballestar	Ports de Besseit	636	13.6	714.9	cattle & sheep	*Medicago* sp., *Santolina chamaecyparissus*
	Besora	Eastern Pyrenees	712	11.7	720.5	cattle	*Plantago lanceolata, Bromus hordeaceus*
	Pallars 1	Central Pyrenees	937	10.4	775	sheep	*Festuca* sp.
	Bel	Ports de Besseit	860	12.3	800.8	cattle & sheep	*Thymus vulgaris, Eryngium campestre*
	Pla de Riart	Eastern Pyrenees	1019	10	824.1	cropland with cattle	*Vicia* sp., *Triticumsecale* sp.
	La Bertolina	Eastern Pyrenees	1276	8.7	954.8	cattle	*Festuca arundinacea, Trifolium repens*
Alpine	Castellar de N'Hug	Eastern Pyrenees	1850	5.4	1199	cattle & sheep	*Festuca rubra, Carex caryophyllea, Endressia pyrenaica*
	Niu de l'Àliga	Eastern Pyrenees	2479	2.4	1302.1	cattle & horses	*Festuca nigrescens, Trifolium alpinum, Carex caryophyllea*
Atlantic	Irati 1	Atlantic	1064	9.5	1413.4	cattle & sheep	*Trifolium alpinum, Festuca* gr. *rubr*
	Irati 2	Atlantic	1014	10.6	1594.2	cattle & sheep	*Trifolium alpinum, Festuca* gr. *rubr*

Table S3.2. Microbial community composition. Main taxa found across climatic gradients in grasslands. Mean (%): proportion of the overall MiSeq reads.

Fungal phylum	Mean (%)	Bacterial phylum	Mean (%)
Ascomycota	82.7	*Actinobacteria*	28.7
Basidiomycota	13.6	*Proteobacteria*	16.7
Chytridiomycota	0.1	*Planctomycetes*	13.2
Glomeromycota	0.1	*Acidobacteria*	12.1
Zygomycota	0.7	*Chloroflexi*	10.9
Unid. Fungi	1.6	*Firmicutes*	10.2
No blast hit	1.1	*Verrucomicrobia*	2.8

Fungal class	Mean (%)	Bacterial class	Mean (%)
Dothideomycetes	28.4	*Actinobacteria*	15.6
Sordariomycetes	21.4	*Alphaproteobacteria*	11.7
Leotiomycetes	12.6	*Bacilli*	9.9
Agaricomycetes	7.9	*Phycisphaerae*	7
Unid. *Ascomycota*	6.4	*Planctomycetia*	6.1
Tremellomycetes	5.1	*Acidobacteria-6*	5.2
Geoglossomycetes	4	*Thermoleophilia*	5.1
Eurotiomycetes	4	*Acidimicrobiia*	4.3
Lecanoromycetes	2.8	*Ktedonobacteria*	3.5
Pezizomycetes	1.9	*Chloracidobacteria*	3.2
Unidentified Fungi	1.6	*Rubrobacteria*	2.9
No blast hit	1.1	*Solibacteres*	2.2

Table S3.3. Fungal community composition at the order level in terms of the percentage of MiSeq reads per climatic zone. Only ubiquitous taxa (with higher read abundances than 1% at any zone) and the characteristic orders per climatic zone are included.

	Fungal order	Climatic zone				
		Mediterranean	Semi-Arid	Montane	Alpine	Atlantic
Ubiquitous	*Pleosporales*	17.1	28.9	26.5	16.6	20.4
	Helotiales	2.1	3.0	5.2	18.9	16.5
	Unid. *Ascomycota*	15.6	9.0	6.1	6.4	4.0
	Sordariales	4.7	10.4	5.8	3.2	16.1
	Hypocreales	11.5	4.9	8.2	1.3	2.6
Characteristic of particular climatic zones	*Acarosporales*	0.0	0.0	0.0	0.0	0.0
	Archaeosporales	0.0	0.0	0.0	0.0	0.5
	Auriculariales	0.0	1.1	0.3	0.0	0.0
	Boletales	14.0	0.2	1.1	0.2	0.0
	Cantharellales	0.0	0.1	0.1	0.0	0.0
	Coniochaetales	0.0	0.0	0.1	8.0	3.0
	Corticiales	0.0	0.2	0.1	0.0	0.0
	Geminibasidiales	0.0	0.0	0.0	2.0	0.2
	Geoglossales	0.0	0.8	4.9	0.3	6.5
	Glomerales	0.0	0.3	0.0	0.0	0.0
	Hysteriales	0.0	0.0	0.0	0.4	0.2
	In. Sedis Dothideomycetes	0.0	0.0	0.0	0.2	0.0
	In. Sedis Sordariomycetes	17.2	0.2	0.4	0.6	0.3
	Lecanorales	0.0	0.0	0.0	3.3	0.0
	Leotiales	0.0	0.0	0.0	0.5	0.0
	Leucosporidiales	0.0	0.0	0.1	0.0	0.0
	Microascales	0.1	0.0	0.1	0.0	0.0
	Ostropales	0.0	0.0	0.0	0.0	0.0
	Pezizales	0.1	3.1	2.4	0.1	1.7
	Polyporales	0.0	0.0	0.1	0.0	0.0
	Rhizophlyctidales	0.0	0.0	0.2	0.1	0.0
	Saccharomycetales	0.0	0.0	0.2	0.0	0.0
	Sebacinales	1.6	0.1	0.2	0.1	0.1
	Teloschistales	0.0	2.3	0.6	0.0	0.0
	Thelephorales	0.0	0.0	0.1	0.0	0.0
	Trichosporonales	0.0	0.0	0.0	2.0	1.7
	Umbilicariales	0.0	0.0	0.1	0.0	0.0
	Unid. *Basidiomycota*	0.0	0.0	0.0	0.1	0.0
	Unid. *Lecanoromycetes*	0.0	0.0	2.1	0.0	0.2
	Unid. *Leotiomycetes*	0.0	1.0	3.7	4.1	0.8
	Unid. *Pezizomycetes*	0.0	0.1	0.0	0.0	0.0
	Verrucariales	0.0	0.0	0.0	0.1	0.0

Table S3.4. Bacterial community composition at the class level in terms of the percentage of MiSeq reads per climatic zone. Only ubiquitous classes (with higher read abundances than 1% at any zone) and the characteristic classes per climatic zone are included.

	Bacterial class	Climatic zone				
		Mediterranean	Semi-Arid	Montane	Alpine	Atlantic
Ubiquitous	*Actinobacteria*	7.8	17.0	16.3	20.4	11.8
	Alphaproteobacteria	10.5	11.1	10.5	12.9	13.7
	Bacilli	5.6	1.2	9.2	10.9	24.1
	Phycisphaerae	4.0	11.4	8.2	4.4	1.8
	Planctomycetia	12.8	4.9	4.9	6.3	7.7
	Acidobacteria-6	3.9	7.8	7.0	1.1	1.3
	Thermoleophilia	1.5	7.4	5.1	5.7	1.9
	Acidimicrobiia	13.0	2.7	3.3	4.4	2.6
Characteristic of particular climatic zones	*ABS-6*	0.0	0.0	0.0	0.3	0.8
	Acidobacteriia	0.0	0.0	0.0	1.6	5.7
	Anaerolineae	6.1	0.9	1.9	0.3	0.2
	Betaproteobacteria	2.8	2.3	2.5	0.5	0.6
	Chloracidobacteria	2.3	3.2	5.0	0.7	0.6
	Chloroflexi	0.2	0.5	0.7	0.1	0.1
	Chloroplast	0.1	0.8	0.2	0.0	0.0
	Cytophagia	2.0	0.7	0.7	0.0	0.3
	DA052	0.0	0.0	0.0	0.4	1.2
	Deltaproteobacteria	3.9	2.2	2.0	0.6	0.9
	Gammaproteobacteria	3.6	0.9	1.5	0.4	1.0
	Gemm-1	1.2	1.5	0.9	0.2	0.2
	Gitt-GS-136	0.2	0.3	0.2	0.1	0.1
	Ignavibacteria	0.1	0.0	0.0	0.0	0.0
	Ktedonobacteria	0.0	0.0	0.1	12.8	12.7
	Oscillatoriophycideae	0.0	0.2	0.1	0.0	0.0
	Rubrobacteria	0.2	4.6	4.7	0.0	0.1
	SC3	0.0	0.0	0.0	0.1	0.3
	SJA-4	0.1	0.0	0.0	0.0	0.0
	Spartobacteria	0.2	0.4	1.9	5.5	2.0
	Thermomicrobia	4.0	3.9	1.9	0.4	0.4
	TK10	0.1	0.8	0.5	0.4	0.3
	Unid.*Gemmatimonadetes*	0.2	0.0	0.0	0.0	0.0
	Unid.*TM7*	0.2	0.0	0.1	0.0	0.0
	Unid.*WPS-2*	0.0	0.0	0.0	0.1	0.6
	Verrucomicrobiae	0.2	0.0	0.1	0.0	0.0
	VHS-B5-50	0.1	0.0	0.0	0.0	0.0

Table S3.5. Taxa resulting from the hierarchical clustering of the Co-CA axis. Only the most relevant coordinates are shown.

	Fungal order	Bacterial class	
Cluster 1	*Auriculariales*	*Sva0725*	*Oscillatoriophycideae*
	Xylariales	*Rubrobacteria*	*Unid.FBP*
	Teloschistales	*0319-6E2*	*Gemmatimonadetes-3*
	Pyrenulales	*Armatimonadia*	*Gemmatimonadetes-5*
	Glomerales	*Chthonomonadetes*	
	Ostropales	*Unid. Chlorobi*	
	Unid. *Pezizales*	*P2-11E*	
	Unid. *Exobasidiomycetes*	*Chloroplast*	
Cluster 2	*Sordariales*	*ABS-6*	
	Diaportales	*Acidobacteriia*	
	Trichosporonales	*DA052*	
	Unid. *Orbiliomycetes*	*Bacteroidia*	
	Sporidiobolales	*Bacilli*	
	Hysteriales	*Clostridia*	
	Tubeufiales	*SC3*	
	Leucosporidiales	*Spartobacteria*	
	Archaeosporales		
	In. Sed. Dothideomycetes		
	Venturiales		
	Rhytismatales		
	Geastrales		
Cluster 3	*Boletales*	*PAUC37f*	*Unid.Gemmatimonadetes*
	In. Sed. Sordariomycetes	*Acidimicrobiia*	*Gemm-2*
	Sebacinales	*Cytophagia*	*Nitrospira*
	Polyporales	*Flavobacteriia*	*OM190*
		Anaerolineae	*Planctomycetia*
		S085	*Gammaproteobacteria*
		SAR202	*Unid.TM7*
		TK17	*Verrucomicrobiae*
Cluster 4	*Lecanorales*	*Ktedonobacteria*	
	Geminibasidiales		
	Verrucariales		
	Acarosporales		
	Unid. *Basidiomycota*		
Cluster 5		*BSV26*	
		Ignavibacteria	
		SJA-28	
		VHS-B5-50	
		SJA-4	

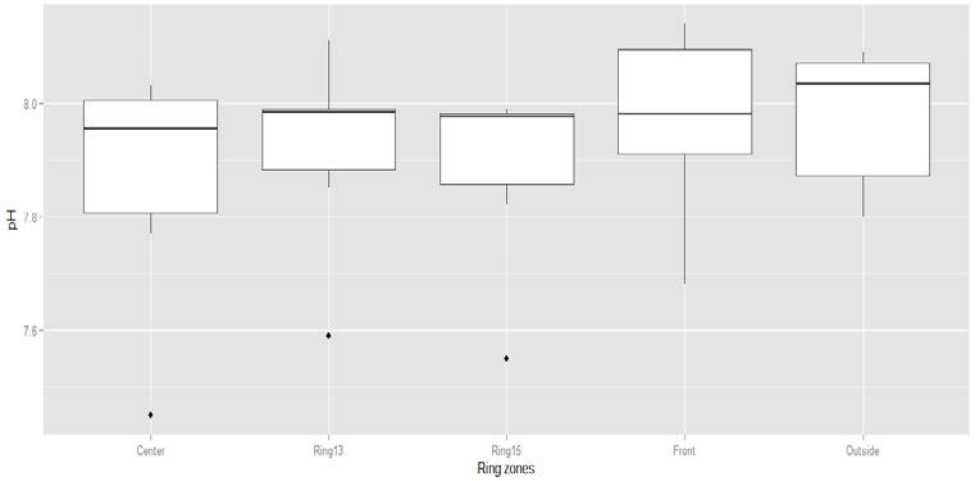

Figure S5.1. Soil pH values across ring zones.

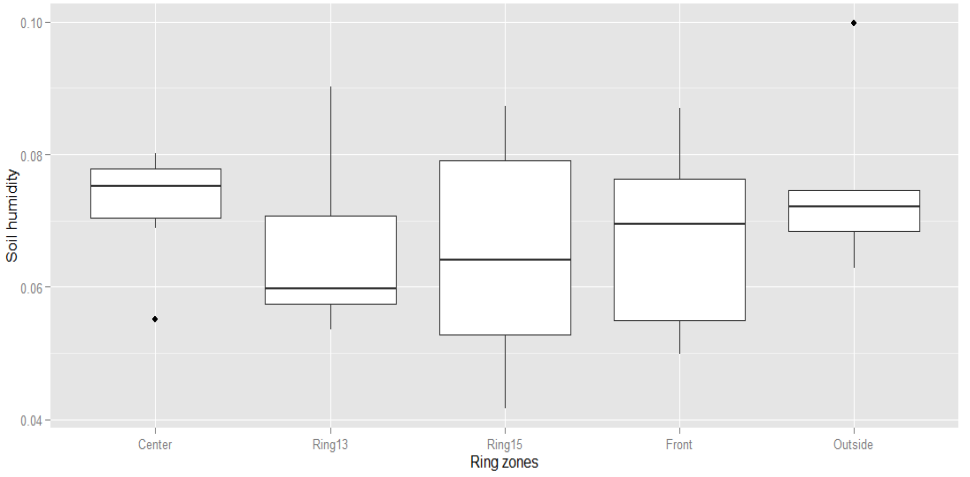

Figure S5.2. Soil humidity across ring zones.

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