Dependence of soil microbial community structure and function on land use types and management regimes

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To my Mom,
one of the strongest and most inspiring persons I know,
and my Dad,
for always believing in me.

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

‘Everything is a process. Always. Otherwise there wouldn’t be science.’

Max Erhard

CHAPTER I

SUMMARY

I. SUMMARY

Soil microbial communities are the most diverse assemblages of organisms on earth. They play key roles in nutrient cycling and help prevent soil erosion. Soil microbial communities also harbor many potentially plant-associaed microorganisms. Plant-associated microbes inhabit the rhizosphere or phyllosphere, or live endophytic within plants. Despite the increasing number of studies on both soil and plant-associated microbial communities, the response of microbial communities towards land use intensification is still not fully understood. The aim of this thesis was to provide insights into the structure, diversity and function of soil and plant-associated microbial communities by amplicon-based analyses with regard to potential abiotic, biotic and anthropogenic drivers.

The first study investigated the effect of different nucleic acid extraction methods on the abundance and diversity of 16S rRNA genes and transcripts derived from different soils. Quality and yields of nucleic acids varied considerably between the different extraction methods applied, as well as between the different soils. Furthermore, abundances of dominant soil taxa varied by a factor up to ten by applying different extraction methods. Therefore, it is of high importance to choose an extraction method that is able to reproduce diversity and composition of the soil microbial community over a range of differing soils. This is crucial when soils from the entire study area should be compared, as they might differ in their properties.

The second study presented a large-scale analysis of soil bacterial communities in temperate grasslands and forests. Therefore, 300 samples were taken in May 2011 in a joint sampling campaign of the German Biodiversity Exploratory project. Metagenomic DNA was extracted and the V3-V5 regions of the bacterial 16S rRNA gene were amplified and pyrosequenced, to assess bacterial community structure and diversity. Additionally, a functional profile was predicted based on the taxonomic profile. The bacterial community structure was driven by edaphic properties with pH as the major driver while land use intensification represented by different management regimes exhibited only minor effects. However, tree species notably affected soil bacterial community structure, with distinct bacterial communities in soils beneath broadleaved and coniferous trees. Edaphic properties were significantly different between grassland and forest soils, resulting in distinct bacterial community structures. Biogeographic variation of edaphic properties also resulted in regional patterns of bacterial community structure. Bacterial diversity was additionally strongly dependent on soil pH. Furthermore, the functional profile of the bacterial communities was

shaped by the same drivers as community structure and diversity. Because of the strong impact of soil pH, genes involved in the acid tolerance response (ATR) were in the focus of the analyses of the functional profiles. Genes involved in alkali production, biofilm formation and attributed to two component systems were more abundant in profiles from low pH soils. The functional profiles of grassland and forests soils were significantly different. The investigation of different key enzyme-encoding genes involved in nutrient cycling revealed that certain functions are either more abundant in grassland (e.g. PAH degradation, alkaline phosphatase, urease, chitinase) or forests soils (e.g. acid phosphatase, methane oxidation, nitrous oxide-reductase, nitrogenase).

The third study investigated soil bacterial and fungal communities beneath beech and spruce trees, and their changes with increasing distance to the tree trunks, soil depth and season. Community structure was driven by edaphic properties (pH, clay content) and the tree species. Seasonal changes as well as depth-related changes were observed for community structure of both bacteria and fungi. Additionally, bacterial community structure and diversity was affected by the distance from the trunk beneath spruce trees.

The following two studies synthesized the effects of land use intensification on different taxonomic groups. The fourth study investigated species abundance distributions (SADs) of 10 aboveground and belowground taxonomic groups in grasslands under different management regimes. SADs are a powerful tool to investigate community changes, as they not only capture overall changes in community structure, but also indicate whether these changes are driven by abundant or rare species. Species richness was largely unaltered by increasing land use intensification. In addition, belowground organisms (bacteria and arbuscular mycorrhizal fungi) were not significantly affected by land use intensity. The fifth study analyzed the effects on biodiversity by management regimes (even-aged or uneven-aged forests) in European beech forests. Gamma-, beta-, and alpha-diversity of 15 taxonomic groups were analyzed. Gamma diversity of bacteria and fungi as well as of plant and animals was higher in even-aged forests than in uneven-aged forests. These differences were driven by a higher beta-diversity in even-aged forests.

The last three studies focused on plant-associated microbial communities. In the sixth study bacterial endophyte communities in three agriculturally important grasses (*Lolium perenne*, *Festuca rubra* and *Dactylis glomerta*) in response to fertilization and mowing in two subsequent years (2010 and 2011) were analyzed. Diversity was highest in *D. glomerta*, and community structure was significantly shaped by the host plants. Fertilization only affected

endophytic community structure and diversity in 2010, while mowing had no effect in both years.

In studies seven and eight, a wheat/faba bean intercropping experiment investigated soil archaeal and soil and plant-associated bacterial and fungal communities, respectively. Soil archaeal but not bacterial or fungal community structure was affected by plant species and cropping regime. Bacterial and fungal community structure was similar in bulk soil and rhizosphere, and bacterial communities were distinct in the endosphere of roots and leaves. Fungal communities did not follow this trend.

In conclusion, soil microbial communities in soils are affected by edaphic properties. These effects most likely overrule effects of land use intensification. Plant-associated microbial assemblages are mainly shaped by the host plant and plant compartment. Nevertheless, agricultural management such as intercropping, alters archaeal community structure and therefore potentially affects microbial community structure on long-term basis.

CHAPTER II

GENERAL INTRODUCTION

II. GENERAL INTRODUCTION

Global ecology is currently challenged by the Anthropocene, the current, human-dominated time period (Corlett, 2015). Human influence on ecology is exhibited mainly by global warming, elevated CO_2 from fossil fuel burning and massive agriculture, or land use intensification (Sikorski, 2015). While global warming and elevated CO_2 may have positive effects on soil bacterial activity (Heimann and Reichstein, 2008; Karhu *et al*., 2014), and carbon and nitrogen fixation (He *et al*., 2014), the effects of land use intensification by means of fertilization have been shown to negatively affect soil microbial community structure and biomass (Ramirez *et al*., 2010, 2012). However, it has also been shown that neither plant invasion, vegetation clipping and nitrogen fertilization alter soil microbial community structure (Carey *et al*., 2015), while conventional versus organic farming results in distinct patterns of microbial communities (Hartmann *et al*., 2015). Therefore, anthropogenic effects on soil microbes are not yet completely unraveled. They have the potential to alter microbial community structure, diversity and function, and subsequently impact soil functioning and processes. A better understanding of microbial ecology in different systems with regard to land use intensification is essential to develop new strategies for sustainable land use and agricultural management in the future.

II.1. The soil habitat and its microbial communities

Soil as an ecosystem provides a wide range of important ecosystem services such as nutrient cycling, plant nutrition, bioremediation of pollutants, pest control and the regulation of greenhouse gases emission (Dominati *et al*., 2010). It is also a highly diverse habitat, physically as well as chemically. The soil matrix consists of mineral particles and organic matter, stabilized by the formation of aggregates with clay, silt and sand particles, the respective microbiota (Daniel, 2005) and air- and water-filled pores (Figure 1). The amount of clay, organic matter and soil moisture influences soil pH. Basic cations from humic soil substances are eventually washed out and replaced by H^+ under humid conditions, resulting in acidification. This effect can be retarded by the presence of clay minerals. Additionally, the redox potential of a soil (presence and absence of electron acceptors such as O_2, NO_3^- or Mn and Fe) can vary strongly and affect metabolic pathways such as nitrogen fixation, denitrification and methanogenesis considerably (Paul, 2014). Therefore, it also impacts microorganisms, which are dependent on these pathways for growth. Soil-inhabiting microbial communities play an important role in the global nutrient cycles. They can

contribute to the release of greenhouse gases, mineralization processes and decomposition, but can also promote plant growth. Soil conditions can change rapidly. A range of properties such as pH (Fierer and Jackson, 2006; Lauber *et al.*, 2009), carbon and nitrogen concentrations (De Vries *et al.*, 2012), and moisture (Brockett *et al.*, 2012; Cruz-Martinez *et al.*, 2009) are known to induce shifts in composition of soil microbial communities. Soil is by far the most diverse habitat with the largest community sizes of microorganisms (Daniel, 2005), with an estimate of 10^8 cells per gram (Torsvik *et al.*, 1990) and up to 8 x 10^6 species (Gans *et al.*, 2005). This can be attributed to the extreme heterogeneity of soils (reviewed by Sikorski, 2015). Microorganisms from all three kingdoms of life – *Bacteria*, *Archaea* and *Eucarya*– are common and frequently found in soil (Goldmann *et al.*, 2015; Herzog *et al.*, 2015; Schneider *et al.*, 2015). The structure of certain groups of microorganisms has been shown to directly control the rate of processes in soils (Bodelier *et al.*, 2013; Strickland *et al.*, 2009). Thus, they are of high ecological and economical importance.

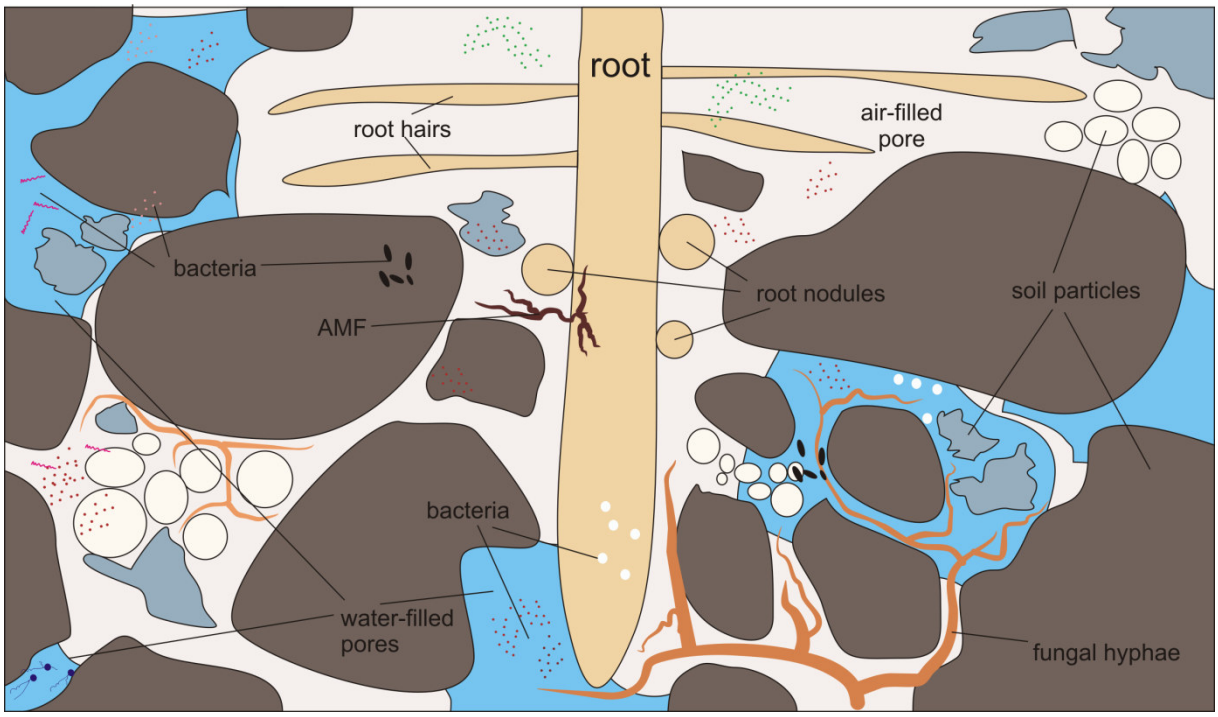

Figure 1. Generalized overview of the soil matrix with microbial habitats. The soil matrix forms by soil particles, air- and water filled pores and the respective microbiota (here bacteria and fungi; AMF: arbuscular mycorrhizal fungus). Microorganisms attach to soil particles, form biofilms on particle surfaces, or live freely in the available water films in pores and between and on the surface of soil particles. Plant roots provide additional habitats, as they can eventually be colonized by fungi and also bacteria.

II.2. Plant-associated microbial communities

Several members of the plant microbiota are known to be beneficial for their host by providing a variety of services. Bacteria promote plant growth by nitrogen fixation, phosphorus solubilization, and siderophore, phytohormone and auxin production.

Additionally, bacteria are able to produce a variety of antimicrobial compounds and thereby contribute to biological pest control and protection against pathogens (reviewed in Bulgarelli *et al*., 2013).

Plants offer different habitats for microorganisms such as the phyllosphere, rhizosphere and endosphere. The phyllosphere summarizes the aboveground plant surfaces. Microorganisms inhabiting the phyllosphere are called epiphytes (Lindow and Brandl, 2003; Vorholt, 2012). The rhizosphere was defined as the zone around the roots influenced by plants (Hiltner, 1904). Since then, the definition of the rhizosphere has been modified and includes the rhizoplane or root surface (McNear Jr, 2013). Lastly, microorganisms are able to live within the plants. This habitat is known as the endosphere and its inhabitants are called endophytes. Some endophytes are symbionts, such as nitrogen-fixing bacteria, that live in root nodules and provide nitrogen comounds to their hosts (Figure 2).

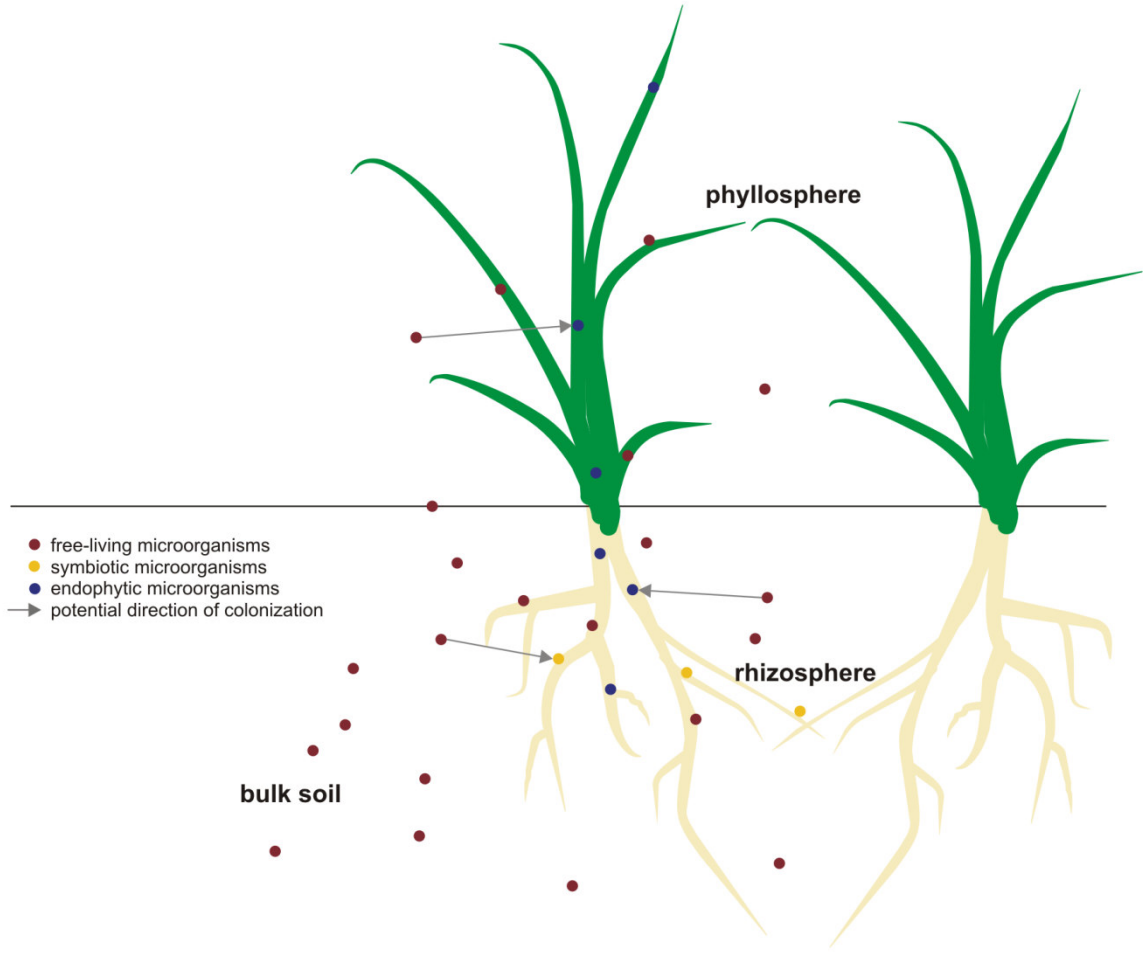

Figure 2. Microbial habitats provided by plants and different livestyles of plant-associated microorganisms. Grey arrows indicate potential directions of plant colonization by microorganisms, e.g. vio recreuitmeht from bulk soil and rhizosphere, or by plant wounding.

Endophytes are by definition microorganisms that colonize the host tissue internally without damaging the host or causing symptoms of a disease (Hallmann *et al*., 1997). Plants are suitable habitats for microorganisms as they provide nutrients and offer protection from abiotic and biotic stresses (Kowalchuk *et al*., 2010). So far, endophytic microorganisms were found in all plants that have been investigated, including grasses (Wemheuer *et al*., 2016), pine tree roots (Marupakula *et al*., 2016), oilseed rape or tomato (Nejad and Johnson, 2000). Endophytes can also colonize all plant parts including fruits, seeds, leaves, stems, tubers and roots (Hallmann *et al*., 1997; Sturz *et al*., 1997). Many endophytic bacteria are able to penetrate and colonize root tissues (Quadt-Hallmann *et al*., 1997; Reinhold-Hurek and Hurek, 1998). Additionally, plant wounding by tillage, mowing or herbivores can result in microbes entering the plant (reviewed inSiddiqui and Shaukat, 2003; Figure 2).

Endophytic communities vary among plant genotypes, between stressed and unstressed plants (Sessitsch *et al.*, 2002) as well as between developmental stages. They are dependent on the nutritional supply offered by their host plant (Hallmann *et al*., 1997). Abiotic factors such as soil parameters (altered by fertilizer and herbicide application) or crop rotation may also influence endophytic community composition (Fuentes-Ramírez *et al.*, 1999; Seghers *et al*., 2004) by altering the physiological status of the plant.

Rhizospheric microbial communities are influenced by plant species and soil type (Berg and Smalla, 2009). Microorganisms in the rhizosphere benefit from root exudates, which include organic acids, amino acids and carbohydrates (Somers *et al*., 2004) and have the potential to acidify the soil in their immediate surroundings. Several bacteria are adapted to acidic soil conditions and evolved different mechanisms to cope with low pH (Cotter and Hill, 2003; Dilworth *et al*., 2001). Root exudates may also contain secondary metabolites such as salicylic acids, jasmonic acids and chitosans (Walker *et al*., 2003), which stimulate a defense response and thereby inhibit bacterial and fungal growth. The number of bacterial cells in the rhizosphere is also reported to be higher than in bulk soil (Gamalero *et al*., 2004; Watt *et al*., 2006). Additionally, bacterial communities in the rhizosphere differ from those in the corresponding bulk soil (Marilley *et al*., 1998). The factors that directly or indirectly influence plant-associated microbial communities are numerous. However, studies analyzing the whole microbial community present in the endosphere and soil comprising different agrosystems, management regimes or land use intensities are still missing.

II.3. Biodiversity and biodiversity loss

Biodiversity loss is a problem due to its direct and indirect links to ecosystem functioning. For example, an ecosystem function in which microorganisms play important key roles is nutrient

cycling. Nutrient cycling is important for the ecosystem service of provisioning. We rely on provisioning, which can come from a variety of ecosystems, e.g. fish from fishery in aquatic and marine ecosystems and crops in agrosystems such as food, wood production systems in forests or fodder production systems in grasslands (Cardinale *et al*., 2012). Changes in ecosystem functioning can alter ecosystem services, which can have severe consequences for the world's population. This underlines the importance of biodiversity on human well-being. In their review on the impact of biodiversity loss on humanity, Cardinale *et al.* (2012) defined several ways how biodiversity loss may affect ecosystem functioning. They state that a high biodiversity supports biomass production, decomposition and recycling of nutrients as well as ecosystem stability, productiveness and multi-functionality.

The need for a thorough understanding of possible links between biodiversity, ecosystem functioning and services is evident. Human activity is known to negatively affect global biodiversity by means of different actions such as habitat conversion (forest to plantation), degradation (rainforest clear cutting) and fragmentation (highways), climate change, harvesting, and pollution (Tittensor *et al*., 2014). However, a major driver of biodiversity loss discussed in the past years is land use intensification (Newbold *et al*., 2015).

The most important land use systems in temperate zones, including Central Europe, are grasslands and forests. Both land use types occur under different management regimes along a gradient of land use intensification. In grasslands intensification occurs through fertilization, mowing and grazing in all possible combinations. In forest, the management mostly depends on the mode and interval of harvesting. In Europe, two management types are currently employed: (1) even-aged and (2) uneven-aged forests, opposed to the unmanaged forests protected as National Parks. Even-aged forests are generated by the traditional shelterwood system (Altersklassenwald). Here, a cohort of even-aged trees is replaced by a new cohort through repeated cutting over decades. The uneven-aged forests are a result of the alternative single tree selection system (Plenterwald), which is considered to be more 'close-to-nature' (Schall *et al*., Chapter III.5).

Newbold *et al*. (2015) presented a study on the effects of land use intensification on biodiversity. However, their datasets did not well represent the microbial biodiversity. Microorganisms are key players in many nutrient cycles and other ecosystem functions, and knowledge on their responses and possible adaptations towards increasing land use intensity would be important. To date, the mechanisms and extent of land use intensification influence on microbial communities and their diversity is rather limited. Since the arrival of the new millennium, several studies have focused possible effects of land use intensity on soil

microbial communities in grasslands (e.g. Carey *et al*., 2015; Tardy *et al*., 2015) and forests (e.g. Hartmann *et al*., 2014; Urbanová *et al*., 2015). In the past few years, microbial ecologists began to explore the mechanisms of land use intensification on soil microbes by employing next-generation sequencing (NGS) techniques. These technologies allow the direct sequencing of metagenomic DNA and RNA (cDNA), and 16S/18S rRNA gene or other marker gene amplicons, to gain insights into microbial community composition, diversity and function. They have been employed successfully to analyze the structure of microbial communities of various environments such as water (Wemheuer *et al*., 2014), extreme habitats (e.g. Röske *et al*., 2014; Schneider *et al*., 2013; Simon *et al*., 2009), and soil (e.g. Nacke *et al*., 2011; Uroz *et al*., 2011; Will *et al*., 2010). Still, large comparative studies employing state-of-the-art technology are lacking. Such studies would be important to understand whether land use intensification in temperate systems as shown for organisms at higher taxonomic levels is a threat to soil microbial biodiversity or not.

II.4. Study regions and frameworks

II.4.1. The German Biodiversity Exploratories

The German Biodiversity Exploratories initiative is a large-scale, long-term project to study the feedback between land use, biodiversity, and ecosystem processes in real-world ecosystems (Fischer *et al*., 2010). They comprise a set of standardized field plots in three different regions of Germany covering different management types and intensities in grasslands and forests. The Exploratories serve as a joint research platform with over 300 researchers from 68 groups of 38 different institutions involved, who study various aspects of the relationships through monitoring, comparative observation and experiments (Fischer *et al*. 2010). To gain an understanding of the interactions between land use, biodiversity and ecosystem functioning, comparative and experimental studies are required at an appropriate spatial scale (Fischer *et al*. 2010). To allow general conclusions, studies also have to cover several regions, as different regions also differ in their landscape, resources, conditions, and soil properties. All these requirements are fulfilled by the structure of the German Biodiversity Exploratories. They are situated in three different regions of Germany: (1) the UNESCO Biosphere Reserve Schorfheide-Chorin in the lowlands of Northeast Germany (Figure 1A), a young glacial landscape with many wetlands, (2) the National Park Hainich and its surrounding area, the Dün region, situated in the hilly lands of Central Germany (Figure 1B), and (3) the UNESCO Biosphere Reserve Schwäbische Alb (Figure 1C), which is

situated in the low mountain ranges of Southwest Germany (Fischer *et al*. 2010). The Exploratories not only feature a set of varying management regimes, but also cover different geographic and climatic conditions on a north-south gradient (Table 1). Land use intensity in the Exploratories ranges from low to high in both grasslands (meadows < pastures < mown pastures) and forests (unmanaged < age class < selection), which are either fertilized or non-fertilized in grasslands, and comprised of different tree species in forests (Table 1). In the past two years, the project reached the point where most initial data collections and data analyses from the first sampling campaign (2008) and also from the second campaign (2011) are finished. Now, data from the different contributing groups are available. These data enables syntheses approaches, which could provide information on how land-use intensity influences biodiversity on different trophic levels. This has been facilitated by the implementation of the land use intensity index (LUI), which is a measure for land use intensity in grasslands (Blüthgen *et al*., 2012) and the silvicultural management index (SMI), which describes land use intensity in forests (Schall and Ammer, 2013). In 2014, Allan and colleagues found that multidiversity is negatively related with land use intensity in grasslands.

However, the authors also found that interannual variation of land use intensity enhances multidiversity. Multidiversity integrated the species richness of 49 different organism groups ranging from bacteria to birds. Manning et al. (Manning *et al*., 2015) showed that management intensification in grasslands weakens associations between plant and animal taxa. Additionally, (Allan *et al*., 2015) analyzed the effects of land use intensification on ecosystem functionality. They found that biodiversity loss and changes in functional composition of communities altered several ecosystem services in grasslands. So far, comparable studies regarding forests are still missing. Furthermore, soil bacterial communities and diversity have so far only been analyzed on a limited number of plots. The general impact of land use intensity on soil bacterial communities at a large scale in different regions and different systems (grasslands and forests) is still largely unknown.

Table 1: Geological features and number of plots per land use type for each of the three exploratories. Adapted from Fischer *et al*. (2010).

		Exploratory		
		Schorfheide-Chorin	**Hainich-Dün**	**Schwäbische Alb**
Location		north-eastern Germany	central germany	south-western Germany
Size		~1300 km²	~1300 km²	~422 km²
Geology		young glacial landscape	calcareous bedrock	calcareous bedrock with karst phenomena
Human population density		23 km -1	116 km-1	285 km-1
Altitude a.s.l.		3-140 m	285-550 m	460-860 m
Annual mean temperature		8-8.5 °C	6.5-8 °C	6-7 °C
Annual mean precipitation		500-600 mm	500-800 mm	700-1000 mm
		Number of plots per land use type		
grasslands		50	50	50
Meadows	fertilized	7	7	18
	non-fertilized	11	-	4
Pastures	fertilized	-	2	2
	non-fertilized	22	18	17
Mown pastures	fertilized	3	15	9
	non-fertilized	7	8	-
forests		50	50	50
Unmanaged forest		5	13	5
Age-class forest	beech	16	24	33
	oak	7	-	-
	spruce	-	4	12
	pine	22	-	-
Selection forest		-	9	-

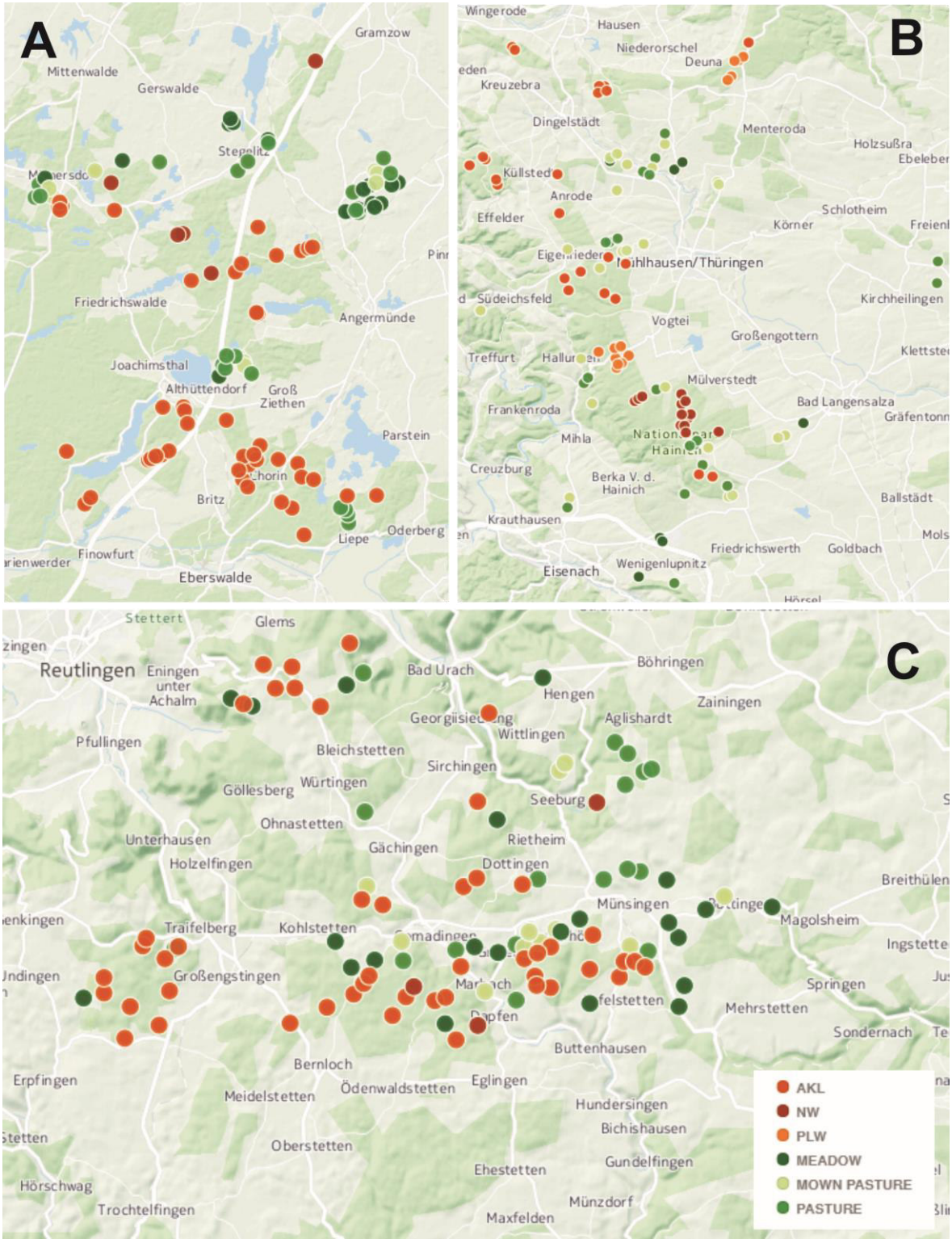

Figure 1: Maps of the three German Biodiversity Exploratories. (A) The Schorfheide-Chorin exploratory north of Eberswalde, Brandenburg, including the Biosphärenreservat. (B) The Hainich-Dün exploratory around Mühlhausen, Thuringia, including the Nationalpark Hainich. (C) The Schwäbische Alb exploratory, south-east of Reutlingen in Baden-Württemberg. Each dot is color coded by land use system (grasslands: green, forests: brown) and land use intensity (dark: low land use intensity, light: high land use intensity). The management regimes in forests are abbreviated as follows: AKL – age class forest, NW – unmanaged forest, PLW - selection forest. Complete map accessible at https://goo.gl/Ygmr04

II.4.2. The Grassland Management Experiment (GrassMan)

The Grassland Management Experiment (GrassMan) has been established as a long-term field experiment with different management intensity treatments. Initiated in 2008, it is a semi-natural, moderately species-rich grassland site in the Solling mountains in Lower Saxony, central Germany (51°44'53" N, 9°32'43" E, 490 m a. s. l.). This site has been traditionally used as an extensive pasture and meadow since the end of the 19th century (Petersen *et al.*, 2012). According to Petersen *et al.* (2012), the number of plant species ranged from 13 to 17 in 9 m^2 phytosociological relevés. The vegetation consists of a nutrient poor, moderately wet Lolio-Cynosuretum with high abundances of *Agrostis capillaris* L. and *Festuca rubra* L. (Petersen *et al.*, 2012). The dominating soil type of the experimental area is a shallow (40–60 cm), stony Haplic Cambisol (Keuter *et al.*, 2013) with a pH_{KCl} ranging from 4.18 to 5.47.

The full-factorial design of GrassMan includes two mowing frequencies (once per year in July vs. three cuttings in May, July, and September) and two fertilization treatments (none vs. NPK fertilization). The third factor aimed at varying plant diversity in the GrassMan plots. The three sward compositions (monocot-reduced, dicot-reduced, species-rich as control) were manipulated by selective herbicide applications targeting either dicots or monocots. One third of the plots remained untreated and was used as controls (species-rich). Each treatment was replicated six times resulting in 72 plots of 15 x 15 m size. The experimental layout was a Latin rectangle design, arranged in 6 rows and 12 columns, two columns forming one block (Figure 2). The distance between rows and columns was 5 m and the distance within columns 3 m.

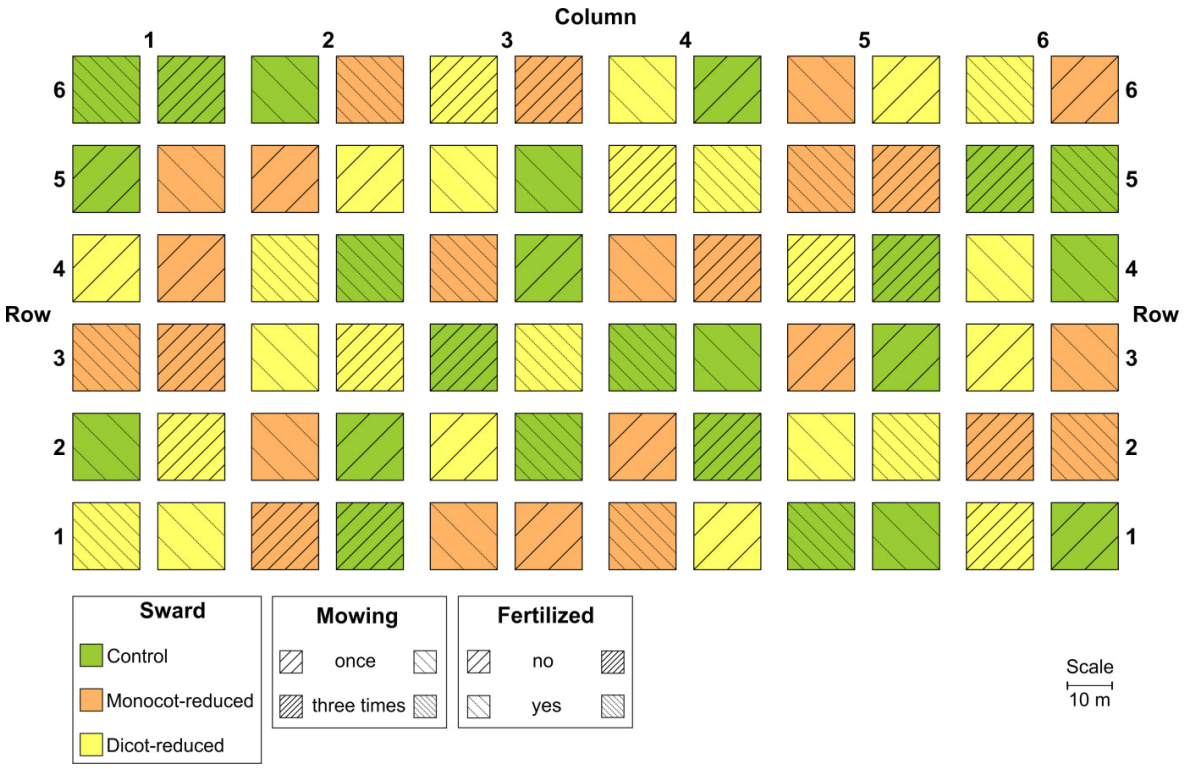

Figure 2: Experimental design of the GrassMan experimental field in the Solling Mountains in Lower Saxony, central Germany (51°44'53'' N, 9°32'43'' E, 490 m a. s. l.). The full-factorial design of this study included two mowing frequencies (mown once per year in July vs. three cuttings in May, July, and September), two fertilization treatments (no vs. NPK fertilization), and three different plant diversity levels (monocot-reduced, dicot-reduced, species-rich as control). The figure was taken from Wemheuer *et al.* (2016), after authorization from the author.

II.4.3. The *Vicia faba* L. and *Triticum aestivum* L. Intercropping Experiment (IMPAC³)

The IMPAC³ project investigates novel genotypes for mixed cropping in improved sustainable land use in arable land, grassland and forest. The overall aim is to combine agronomic, genetic, ecologic and social-economic research to gain a better understanding of the ecological functioning and societal acceptance of diversified farming systems. In 2003, Hof and Rauber found that of 344 intercropping cases 60% showed favorable mixing effects.

It is important that the intercrop components do not compete for identical ecological niches (Ofori and Stern, 1987). There is evidence that barley in intercropping mixtures has beneficial effects on the intercropping partners, or even outcompete monocultures with respect to biomass and grain yield (Rauber *et al*., 2000). An intercropping experiment was established for wheat and faba bean. Both species were grown in monocultures and in intercropping mixtures. The aim was to identify whether intercropping is beneficial for both plant species or not. Furthermore, the plant-associated microbial communities should be investigated for a detailed insight into the genotype versus environment interactions of the crop plants. Therefore, prokaryotic (*Archaea* and *Bacteria*) and eukaryotic (*Fungi*) microorganisms were analyzed belowground and aboveground.

II.5. General study aims

Nowadays, it is widely acknowledged that microbial communities play an essential role in global nutrient cycling and support ecosystem functioning. It is important to understand the factors that impact and control microbial community structure and function. Many studies have investigated the effects of environmental conditions such as pH, water and nutrient content or soil texture on microbial community composition. Anthropogenic factors, such as land use and management have been studied to a lesser extent. There are also very few large-scale comparative studies investigating the drivers of microbial community composition.

To ensure comparability between the different studies, the first part of this thesis focused on identification of a nucleic acid extraction method that is applicable to a wide range of different soils and provides in addition a high quality of nucleic acids and a recovered diversity without biasing the community composition significantly (Chapter III.1).

The main focus of this thesis was on anthropogenic effects on soil bacterial communities in grassland and forest systems under different managements in three different regions of Germany. The major aims were:

1. To investigate the influence of increasing land use intensity in grassland and forest systems on bacterial community composition, diversity and function. Therefore, 300 soil samples were taken in 2011 within the framework of the German Biodiversity Exploratories, comprising 150 grassland and 150 forest soil samples. The results are discussed in chapter III.2, III.4 and III.5.
2. To analyze changes in bacterial and fungal community composition with respect to tree species, samples were taken in 2013 and derived from spruce or beech forest plots. Four different distances to each tree were sampled in spring and autumn (Chapter III.3).

Additionally, this thesis investigated anthropogenic effects on plant-associated microbial communities. The aims of these analyses were:

3. To unravel the effect of three different perennial grasses (*Dactylis glomerata* L., *Festuca rubra* L. and *Lolium perenne* L.), fertilization and mowing on endophytic bacterial communities, discussed in Chapter III.6.
4. To elucidate how intercropping of faba bean (*Vicia faba* L.) and wheat (*Triticum aestivum* L.) changes the microbial community composition in soil and endosphere. The results are discussed in Chapter III.7. and III.8.

CHAPTER III

PUBLICATIONS

‘One should look for what *is*, and not for what one thinks should be.’

Albert Einstein

Chapter III.1.

EFFECTS OF NUCLEIC ACID EXTRACTION METHODS

III.1.

Estimates of the bacterial ribosome content and diversity in soils are significantly affected by different nucleic acid extraction methods

Running title: Effects of Nucleic Acid Extraction Methods

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I am a coauthor of this manuscript and conducted the DNA experiments and preliminary data analyses. I also commented on and discussed the manuscript with the other authors.

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Estimates of Soil Bacterial Ribosome Content and Diversity Are Significantly Affected by the Nucleic Acid Extraction Method Employed

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Modern sequencing technologies allow high-resolution analyses of total and potentially active soil microbial communities based on their DNA and RNA, respectively. In the present study, quantitative PCR and 454 pyrosequencing were used to evaluate the effects of different extraction methods on the abundance and diversity of 16S rRNA genes and transcripts recovered from three different types of soils (leptosol, stagnosol, and gleysol). The quality and yield of nucleic acids varied considerably with respect to both the applied extraction method and the analyzed type of soil. The bacterial ribosome content (calculated as the ratio of 16S rRNA transcripts to 16S rRNA genes) can serve as an indicator of the potential activity of bacterial cells and differed by 2 orders of magnitude between nucleic acid extracts obtained by the various extraction methods. Depending on the extraction method, the relative abundances of dominant soil taxa, in particular *Actinobacteria* and *Proteobacteria*, varied by a factor of up to 10. Through this systematic approach, the present study allows guidelines to be deduced for the selection of the appropriate extraction protocol according to the specific soil properties, the nucleic acid of interest, and the target organisms.

Soil is one of the most complex and diverse microbial habitats, with 1 g containing up to 10^{10} cells and 10^4 bacterial species (1, 2). While DNA sequences provide information about the total microbial community, RNA can be used to analyze the fraction of microorganisms that has the capacity to actively synthesize proteins (3–5). The ratio of bacterial 16S rRNA transcripts to 16S rRNA gene copies has been used as an indicator of the potential specific activity since it reflects the cellular ribosome content (6, 7). However, nucleic acid extraction from soils is affected by insufficient cell lysis or losses during extraction, variable reproducibility, and various effects of soil pH, clay content, and organic carbon content (8–13), and coextracted organic compounds can inhibit DNA polymerase and reverse transcriptase employed in downstream molecular analyses (14).

Direct nucleic acid extraction methods are based on the lysis of cells inside the soil matrix, whereas indirect methods commence with the isolation of bacterial cells from soil prior to nucleic acid extraction (8). Indirect methods typically yield longer nucleic acid fragments which are useful for metagenomic studies (10, 15) but often result in a significantly lower yield and diversity of nucleic acid molecules (10, 16). Existing protocols for the direct extraction of DNA and RNA from soils (13, 17–25) have partly been evaluated (19, 21, 24, 26–30), but only a few studies have compared extraction efficiencies of methods recovering both DNA and RNA (21, 24). In particular, these methods have so far not been compared with extraction protocols optimized for either DNA or RNA alone. Information about the effects of different extraction methods on the phylogenetic analysis is scant (31, 32). As a result, the implications of different extraction methods for estimates of potential bacterial activity or diversity in soils have remained largely unclear, but they are particularly relevant for studies of the interdependence of bacterial diversity, activity, and environmental parameters across physicochemically diverse soils (33, 34).

The present study employed quantitative PCR and pyrosequencing to evaluate the effects of different methods on the abundance and diversity of 16S rRNA genes and transcripts extracted from three contrasting soils and to determine essential factors for a reliable extraction of nucleic acids. The soils selected are representative for humid temperate regions worldwide and differ with respect to soil development, grain size composition, and quantity and quality of organic matter, as well as nutrient budgets.

MATERIALS AND METHODS

Study sites. Samples were collected in April 2011 from the A_h horizon (thickness between 5 and 10 cm) of representative grassland plots (AEG31, HEG4, and SEG8) of the German Biodiversity Exploratories (35; http://www.biodiversity-exploratories.de) (Table 1). The three exploratories are located in the biosphere reserve Schorfheide-Chorin (Brandenburg, northeastern Germany), the national park Hainich and its surroundings in Thuringia (central Germany), and the biosphere reserve Swabian Alb in Baden-Wuerttemberg (southwestern Germany). Plant debris, coarse roots, and pebbles were removed and the soil was passed

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TABLE 1 Origin and characteristics of topsoils sampled for nucleic acid analyses[a]

Characteristic	AEG31	HEG4	SEG8
Location	Swabian Alb, Baden-Wuerttemberg, Germany	Hainich, Thuringia, Germany	Schorfheide-Chorin, Brandenburg, Germany
Coordinates	48°25′0.0″N, 9°30′0.0″E	51°6′47.5″N, 10°26′10.3″E	53°6′50.0″N, 14°1′0.5″E
pH (H_2O)	7.7	7.6	7.7
pH (10 mM $CaCl_2$)	7.2	7.1	7.3
Water content (%, wt/wt)	45.6	44.6	31.2
Soil type	Leptosol	Stagnosol	Gleysol
Land use type	Mown pasture (sheep), unfertilized	Mown pasture (cattle), fertilized	Pasture, unfertilized
Sand (%)	2.4	6.7	13.8
Silt (%)	38.1	51.4	62.3
Clay (%)	59.5	41.9	23.9
Total carbon (g kg^{-1})	70.2	66.9	153.1
Total organic carbon (g kg^{-1})	69.6	66.9	73.2
Total nitrogen (g kg^{-1})	6.8	6.0	7.1
Soluble ammonium (mg kg^{-1})	17.4	7.6	20.3
Soluble nitrate (mg kg^{-1})	12.0	17.6	10.2
Total bacterial cells (g, wet wt^{-1})	9.9×10^9	2.5×10^{10}	3.9×10^9

[a] Values are the means from duplicate analyses. Data on soil texture, total carbon, total organic carbon, and total nitrogen are from reference 6; all other data were determined in the present study.

through a 2-mm mesh sieve. Afterwards, samples were stored at −80°C. Total soil carbon, organic carbon, total nitrogen, soluble nitrate and ammonium, and bacterial cell numbers were determined as described previously (6). The three soils selected differed with respect to soil type, texture, and carbon and nutrient contents (Table 1). SEG8 exhibited a significantly darker color than the other soils due to a high content of organic substances. All soils had near neutral and comparable pHs (Table 1).

Extraction and purification of nucleic acids. The extraction methods comprised a similar number of commercial kits (methods 1, 2, 3a, and 3b; i.e., MoBio *a*, MoBio *b*, MP, and MP) and custom protocols (methods 4, 5, 6, 7, 8; i.e., Lueders, Persoh, Petric *a*, Petric *b*, and Töwe) to assess under which conditions the more time-saving commercial kits can be applied. Also, the methods differed with respect to cell lysis conditions and subsequent purification steps in order to cover the major types of chemical treatments (Table 2). All selected extraction methods include a bead beating step for mechanical cell lysis, since this procedure represents the most efficient lysis method for soils (10, 36). In contrast to the other methods tested, the method Persoh (no. 5) includes three consecutive bead beating steps and precipitation of organic compounds by $Al_2(SO_4)_3$ and NaOH before lysis. Four of the protocols (methods 2, 4, 5, and 8; i.e., MoBio *b*, Lueders, Persoh, and Töwe) have been developed for the coextraction of DNA and RNA using phenol-chloroform-isoamyl alcohol (PCI). International Organization for Standardization (ISO) standard 11063 is based on the method published by Martin-Laurent et al. (27), was approved by the ISO, and was tested without (method Petric *a*, no. 6) or with (method Petric *b*, no. 7) subsequent purification steps (22). Each extraction protocol was tested in three technical replicates for each soil sample.

Coextracted DNA and RNA were subsequently separated using either a commercially available kit or enzymatic digestion (Fig. 1). In one approach, 50% of the volume of each individual coextract was used to separate DNA and RNA by the AllPrep DNA/RNA minikit (Qiagen, Hilden, Germany) using a modified protocol. After addition of 3.5 volumes of buffer RLT Plus (Qiagen) to the coextract, we proceeded with step 4 of the protocol (transfer of the diluted coextract to the DNA spin column). In an alternative and parallel approach, 25% of the volume of the coextract was used to obtain DNA by digestion of coextracted RNA with RNase A (DNase free; Fermentas, St. Leon-Rot, Germany) and subsequent precipitation with 0.1 volume of 3 M sodium acetate (pH 6.8) plus 2 volumes of isopropanol. The remaining 25% of the coextract was treated with DNase I (RNase free; Fermentas), and RNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of isopropanol.

Quantification of DNA and RNA. Concentrations of DNA and RNA were determined in duplicate using the Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit and the Quant-iT RiboGreen RNA assay kit (Life Technologies, Darmstadt, Germany), respectively, and a microtiter plate reader (Tecan Infinite M200; Männedorf, Switzerland). Values were corrected for background fluorescence. The quality of nucleic acids was checked via UV-visible (UV-Vis) spectroscopy (NanoDrop ND-1000; Peqlab Biotechnologie, Erlangen, Germany) based on the A_{260}/A_{280} and A_{260}/A_{230} ratios and the absorbance at 320 nm (65). Nucleic acid extracts from environmental samples with an A_{260}/A_{280} ratio above 1.75 and an A_{260}/A_{230} value above 1.65 were considered pure (21).

Reverse transcription-PCR. RNA extracts were treated with RiboLock RNase inhibitor (final concentration, 1 U μl^{-1}; Fermentas) prior to reverse transcription-PCR. For synthesis of cDNA from extracted RNA, the GoScript reverse transcription system was employed according to the protocol of the manufacturer (Promega, Madison, WI) using random hexamers.

qPCRs. Quantitative PCRs (qPCRs) were run in three (*Acidobacteria*-specific assay) or four (*Bacteria*-specific assay) replicates employing a LightCycler 480 II real-time PCR instrument (Roche, Mannheim, Germany). Bacterial and acidobacterial 16S rRNA genes and transcripts were determined using primer sets 341f and 515r (*Bacteria*-specific assay) and Acido31f and 341r (*Acidobacteria*-specific assay), respectively (see Table S14 in the supplemental material). The PCR mixture (20 μl) contained 10 μl of LightCycler 480 SYBR green I Mastermix (Roche), 0.4 mg ml^{-1} of bovine serum albumin (BSA; Roche), 0.3 μM each primer, and genomic DNA and cDNA, respectively, as the template. For calibration, almost full-length 16S rRNA gene fragments of *Edaphobacter modestus* DSM 18101^T were employed at concentrations between 10 and 10^9 copies per reaction. Sample DNA and cDNA were diluted to concentrations that yielded values in the linear range of the standard curves (6). Melting curve analysis was conducted after each run to verify product specificity. Copy numbers were calculated according to reference 37. The absence of residual DNA in RNA extracts was checked employing RNA extracts directly as the template in parallel qPCR runs. Inhibition of qPCR by coextracted inhibitory compounds was quantified by spiking DNA extracts with a defined copy number of 16S rRNA gene fragments of *E. modestus* DSM

TABLE 2 Nucleic acid extraction methods used in this study

Method no.	Method name[a]	Soil[b] (g ml^{-1})	Specific lysis conditions[c]	Detergent[c]	Vortex or bead beating conditions[d]			Method for removal of organic compounds[c]	Final purification[c]	Extraction of:		Source or reference(s)[a]
					Beads	Speed (m s^{-1})	Time (min)			DNA	RNA	
1	MoBio *a*	0.25	GTC during lysis	SDS	Garnet	1.0	10	Precipitation (CBI)	Silica column	x		Manufacturer
2	MoBio *b*	0.22	GTC, PCI during lysis	SDS	Silica carbide	1.0	15	PCI, precipitation (CBI)	Isopropanol precipitation, anion exchange	x	x	Manufacturer
3a	MP	0.33	PVP during lysis	SDS	0.1-mm silica 1.4-mm ceramic 4-mm glass	6.0	0.67	PVP, precipitation (CBI)	Silica column	x		Manufacturer
3b	MP	0.33	GTC during lysis	CBI	0.1-mm silica 1.4-mm ceramic 4-mm glass	6.0	0.67	PC, precipitation (CBI)	Isopropanol precipitation, silica column		x	Manufacturer
4	Lueders	0.36		SDS	0.1-mm zirconia/silica	6.5	0.75	PCI	PEG precipitation	x	x	20
5	Persoh	0.31	Al precipitation before lysis	SDS	0.5-mm glass 0.1-mm zirconia/silica 4-mm glass	4.0–5.5	1.5	$Al_2(SO_4)_3$ and NaOH precipitation, PCI	Isopropanol precipitation	x	x	21
6	Petric *a*	0.20	During lysis PVP, 70°C, 10 min	SDS	0.1-mm glass 2-mm glass	5.5	0.5	Precipitation Sodium acetate	Isopropanol precipitation	x		22
7	Petric *b*	0.20	During lysis PVP, 70°C, 10 min	SDS	0.1-mm glass 2-mm glass	5.5	0.5	Precipitation Sodium acetate	Isopropanol precipitation, PVPP, Sepharose 4B	x		22
8	Töwe	0.33	CTAB, PCI during lysis	CTAB, β-mercaptoethanol	0.1-mm silica 1.4-mm ceramic 4-mm glass	5.5	0.5	PCI	PEG precipitation	x	x	17, 24

[a] Named after either the manufacturer providing the instructions with the kit or after the author that published the method.
[b] Given per milliliter of extraction buffer.
[c] GTC, guanidine thiocyanate (<3%); PC, phenol-chloroform; PCI, phenol-chloroform-isoamyl alcohol; PEG, polyethylene glycol; PVP, polyvinylpyrrolidone; PVPP, polyvinylpolypyrrolidone; SDS, sodium dodecyl sulfate; CTAB, hexadecyltrimethylammonium bromide; CBI, confidential business information (the specific chemical identity and/or exact percentage of composition [concentration] is withheld as a trade secret).
[d] Except for methods MoBio *a* and MoBio *b* (which required a vortexer for bead beating), all bead beating steps were carried out using a FastPrep24 instrument (MP Biomedicals, Santa Ana, CA).

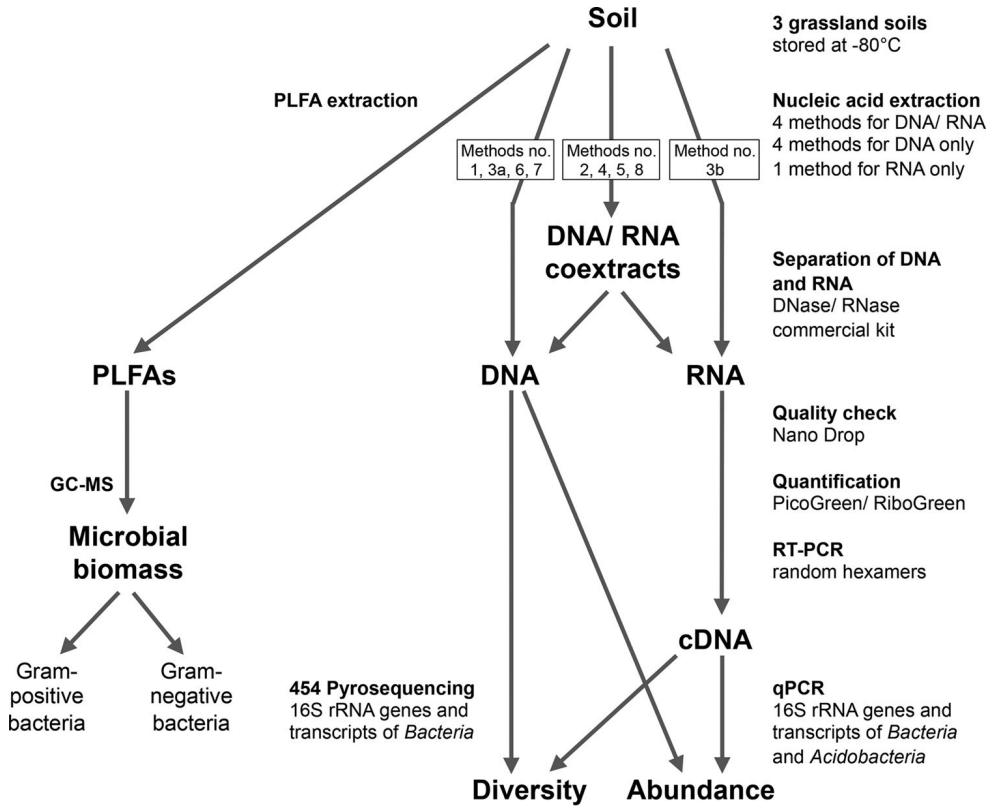

FIG 1 Methodological approach. Detailed information about the individual extraction methods is provided in Table 2.

18101^T. Inhibition factors were calculated by determining the ratio of the expected to the measured copy number.

Amplification of partial 16S rRNA genes and transcripts for pyrosequencing. DNA extracts from replicate extractions were pooled at equal amounts. Likewise, RNA extracts were pooled and used for cDNA synthesis. The V2-V3 region of 16S rRNA genes and transcript cDNA was amplified by PCR using genomic DNA and cDNA, respectively (Fig. 1). The PCR mixture (50 μl) contained 10 μl of 5-fold reaction buffer (Phusion HF buffer, Finnzymes, Espoo, Finland), 200 μM each of the four deoxynucleoside triphosphates, 5% (vol/vol) dimethyl sulfoxide (DMSO), 0.5 U of Phusion Hot Start high-fidelity DNA polymerase (Finnzymes), 10 to 200 ng of DNA as the template, and 4 μM each primer. Primer 101f containing Roche 454 pyrosequencing adaptor B and primer 530r containing a sample-specific MID (extended multiplex identifier; size, 10 nucleotides) together with Roche 454 pyrosequencing adaptor A were used for amplification (see Table S14). All samples were amplified in triplicate (PTC-200 cycler; MJ Research Inc., Watertown, MA), purified using the peqGold gel extraction kit (Peqlab Biotechnologie GmbH) as recommended by the manufacturer, and pooled at equal amounts. Quantification of PCR products was performed using the Quant-iT dsDNA BR assay kit and a Qubit fluorometer (Life Technologies). Sequences of the partial 16S rRNA genes and transcript cDNA were determined with a GS-FLX 454 pyrosequencer (Roche) and titanium chemistry as recommended by the manufacturer.

Analysis of the pyrosequencing-derived data set. The generated sequences were reassigned to single samples based on the unique MIDs. The QIIME (38) script split_libraries.py was applied to remove sequences shorter than 200 bp and sequences containing long homopolymers (>8 bp) or more than two primer mismatches. Subsequently, denoising was performed by applying the scripts denoise_wrapper.py and inflate_denoiser.py. Primer sequences remaining after running the script split_libraries.py were truncated via the program cutadapt (39). Removal of chimeras was performed by using the UCHIME program (40) and the Greengenes gold database (41) as references.

For the determination of operational taxonomic units (OTUs), we defined species and phylum level at 3 and 20% genetic distances, respectively (42). Determination of OTUs, taxonomic classification of OTUs, and the calculations of rarefaction curves, the Shannon index (43) and the Chao1 index (44) for each preprocessed data set, were performed using the QIIME 1.4 software package. Employing the QIIME script assign_taxonomy.py, preprocessed sequences were compared to the SILVA rRNA database (release 108) (45) using BLASTN. After termination of the QIIME script make_otu_table.py, a customized script was used to remove all OTUs from the OTU table that had been classified as chloroplasts. For subsequent comparative diversity analyses, the sizes of all data sets were adjusted to the same size of 6,300 sequences per DNA and RNA extract (i.e., the minimum number of available sequences that was obtained in the DNA extract of method Persoh for sample HEG4; see Table S3). These subsets of sequences were generated randomly, employing the QIIME script multiple_rarefactions.py (http://qiime.org/scripts/multiple_rarefactions.html).

Statistical analysis and graphic presentation. Statistical analysis was conducted in R (version 3.2.2 [http://www.R-project.org]). The two-sided sign test was performed using the binom.test() function with default parameters in order to assess the systematic deviation between different DNA or RNA removal methods. The two-sample *t* test was performed using the t.test() function with default parameters in order to test whether the variance among replicates obtained with commercial kits differed significantly from those of noncommercial methods. The multcomp analysis (46, 47) identified significant differences of means between any pairs of data within a multiple-group comparison (all-against-all), is suited for unequal group variances, and simultaneously controls the familywise error rate. The package vegan (48) was employed for multivariate statistical analyses. A detrended correspondence analysis of abundance data re-

vealed gradient lengths of <2, indicating that methods optimal for linear environmental gradients (such as principal-component analyses [PCA]) are suitable for the data set (49). The effects of different extraction methods and different soil samples on nucleic acid quality parameters and on phylogenetic composition were analyzed separately for DNA and RNA extractions using PCA. Data were subjected to z-transformation ($[x - \text{mean}]\ [\text{standard deviation}]^{-1}$). To analyze the effect of extraction methods on different phyla, the values obtained with all extraction methods were scaled separately by z-score transformation for each (sub)phylum-soil combination (mean = 0; standard deviation = 1). Thereby, the large differences in the order of magnitude of quality parameters or of abundances of different (sub)phyla were removed. As the z-score transformation was done separately for each soil, inherent differences in relative abundances of phyla across soils were also removed. In sum, only differences due to the different nucleic acid extraction methods were retained and subjected to PCA analysis. The *envfit*() function was used to fit soil parameters *post hoc* to the first two axes of the unconstrained PCA analysis. The R code used for statistical analysis is provided in the supplemental material. The ggplot2 package (50) was used for creation of figures.

PLFA analysis. For analysis of phospholipid fatty acids (PLFAs) (Fig. 1), 2 to 4 g of fresh soil samples was extracted following the procedure described by Frostegård et al. (51). Lipids were extracted with the Bligh and Dyer method (chloroform, methanol, and citrate buffer, 1:2:0.8 [vol/vol/vol]; pH 4) and fractionated via silica acid columns. The PLFA fraction was then subjected to alkaline methanolysis, using methylnondecanoate (19:0) as an internal standard. The generated fatty acid methyl esters (FAMEs) were identified based on their retention times employing a gas chromatography (GC) Auto System XL (PerkinElmer Corporation, St. Louis, Norwalk, CT) equipped with an HP-5 capillary column (50 m by 0.2-mm inner diameter; film thickness, 0.33 μm). To verify correct identification of FAMEs, standardized soil samples were analyzed by GC-mass spectrometry (GC-MS) with an HP 5890 series II coupled with a 5972 mass selective detector and equipped with a DB-5MS capillary column (30 m by 0.25-mm inner diameter; film thickness, 0.2 μm) (52). Bacterial biomass was estimated from the sum of i15:0, a15:0, 15:0, i16:0, 16:1ω7, i17:0, cy17:0, and cy19:0 (51, 53). The PLFAs i15:0, a15:0, i16:0, and i17:0 are indicative of Gram-positive bacteria, whereas cy17:0 and cy19:0 are characteristic of Gram-negative bacteria. The PLFA 18:2ω6,9 was used as a marker for fungal biomass.

Nucleotide sequence accession number. The sequences obtained in this study are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number SRA058915.

RESULTS

Yield and purity of extracted nucleic acids. DNA was extracted by eight and RNA by five different extraction methods (Table 2). All methods were successful for the extraction of DNA and RNA from soils HEG4 and SEG8. However, method Töwe (no. 8) reproducibly failed to extract DNA from the clay-rich soil AEG31 (Fig. 2). For the methods that extracted DNA and RNA simultaneously (methods 4, 5, and 8), we tested two different approaches for the subsequent separation of RNA and DNA (treatments with DNase and RNase or the commercial AllPrep DNA/RNA minikit; Fig. 1). Eighteen different combinations of type of nucleic acid, extraction method, and type of soil were tested (see Table S1 in the supplemental material), but none of the two methods resulted in systematically higher yields of DNA or RNA (sign test; $P > 0.05$). Therefore, DNA and RNA derived from enzymatic digestion of coextracts were selected for the following comparative analyses.

Yields of DNA and RNA differed by up to 2 and 3 orders of magnitude, respectively, depending on both the applied extraction method and the analyzed soil (Fig. 2). DNA yields ranged between 0.3 and 38.1 μg of DNA (g [wet weight]$^{-1}$) (Fig. 2A) and differed for the same soil sample by factors of 33, 65, and 107 (AEG31, HEG4, and SEG8, respectively) between the eight methods. The largest amounts of DNA were detected using the methods published by Lueders et al. (20) (no. 4) and Persoh et al. (21) (no. 5) (Fig. 2A). The variation between replicates of noncommercial methods was significantly higher than that of commercial kits (mean variances approximated 0.10 for published protocols and 0.01 for commercial kits; *t* test, $P < 0.05$) (Fig. 2A). Methods MoBio *a* (no. 1) and MP (no. 3a) were equally efficient for the extraction of DNA from AEG31 and HEG4, but method MoBio *a* proved to be more efficient for isolating DNA from the carbon-rich soil SEG8. Most notably, consistently small amounts of DNA were recovered after extraction following ISO standard extraction protocols (Petric *a* and Petric *b*; no. 6 and 7) or the PCI protocol by Töwe et al. (no. 8).

The five methods for the extraction of RNA yielded mean recoveries between 0.03 and 64.9 μg (g [wet weight]$^{-1}$) and differed by up to 3 orders of magnitude for the same soil (factors of 1,033, 67, and 21 for samples AEG31, HEG4, and SEG8, respectively) (Fig. 2B). The average amounts of extracted RNA from AEG31 and HEG4 were largest when method MoBio *b* (no. 2) was employed. In the case of SEG8, the largest amounts of RNA were recovered with method MP (no. 3b), but the variation among replicates was high. The smallest amounts of RNA were recovered from all soils when applying method Töwe.

The different extraction methods also produced various qualities of DNA and RNA (see Fig. S1 in the supplemental material). The DNA extracts obtained with method MoBio *a* (no. 1) exhibited high A_{260}/A_{280} and A_{260}/A_{230} ratios and low A_{320} values for all soils, indicating that this commercial kit produces high-quality DNA irrespective of the specific properties of the soil. The ISO standard extraction protocol produced measurable DNA extracts of medium quality only after purification using polyvinylpyrrolidone and Sepharose 4B columns (method Petric *b*; no. 7). The protocol by Lueders et al. (no. 4) resulted in RNA extracts with low contamination. The commercial kit MoBio *b* (no. 2) produced high-purity RNA for soils AEG31 and HEG4 but contaminated RNA for soil SEG8, which is characterized by a high organic matter content. The commercial kits from MP (no. 3a and 3b) produced very low A_{260}/A_{230} values for all soils.

Abundance of 16S rRNA genes and transcripts. Due to the dilution of DNA and RNA for qPCR and reverse transcription, respectively, inhibition of qPCR was negligible (inhibition factors ranged between 0.94 and 1.00). However, the different extraction procedures left different amounts of residual DNA in the RNA extracts as determined by direct qPCR of bacterial 16S rRNA genes in the RNA extracts (see Table S2 in the supplemental material). Thus, an additional DNase treatment of RNA extracts obtained with commercial kits is mandatory.

The mean copy numbers of bacterial 16S rRNA genes recovered from the soils ranged between 5.8×10^7 and 3.8×10^{10} copies (g [wet weight]$^{-1}$) (Fig. 2A). Mean acidobacterial gene copy numbers ranged between 5.6×10^5 and 2.8×10^8 copies (g [wet weight]$^{-1}$). The multcomp test demonstrated that the extraction methods produce significantly different results. The highest bacterial copy numbers for AEG31 and HEG4 and second highest values for SEG8 were detected in DNA isolated with method MP. The protocol by Persoh et al. also yielded high gene copy numbers

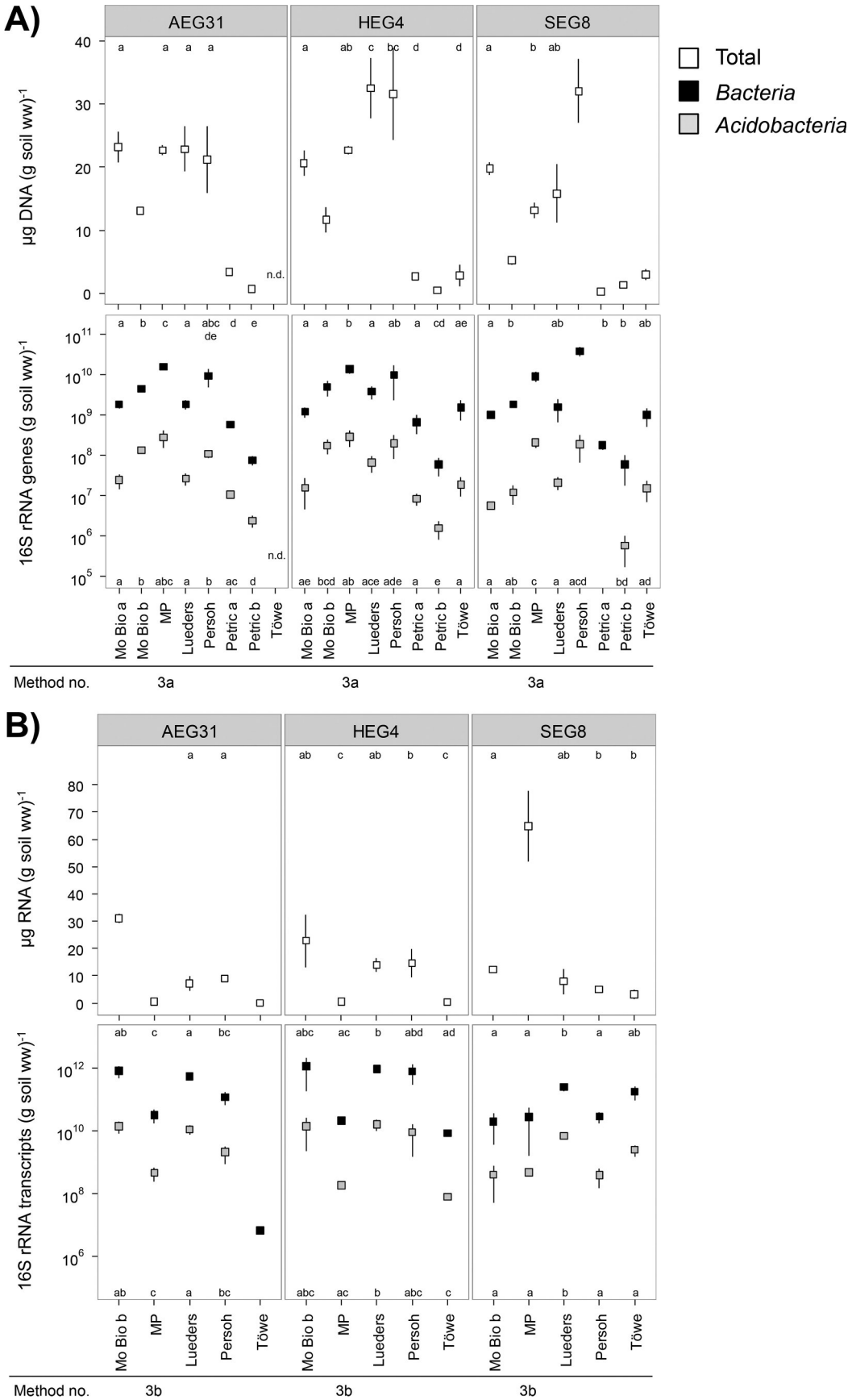

FIG 2 Yield of total DNA and copies of 16S rRNA genes (A) and yield of total RNA and copies of 16S rRNA transcripts (B). The squares (total DNA or total RNA, white squares; *Bacteria*, black squares; *Acidobacteria*, gray squares) represent mean values for the three extracts per soil sample (±standard deviations). All values are means from replicate qPCR analyses (*Bacteria*, $n = 4$; *Acidobacteria*, $n = 3$). Methods that do not share a letter are significantly different (multcomp, $P < 0.05$). Concentrations of DNA and RNA were determined in duplicates, using PicoGreen and RiboGreen, respectively. No results are shown for AEG31 using method Töwe (no. 8) since extraction of DNA from this soil failed with this method. Other missing values were under the detection limit of the qPCR assay.

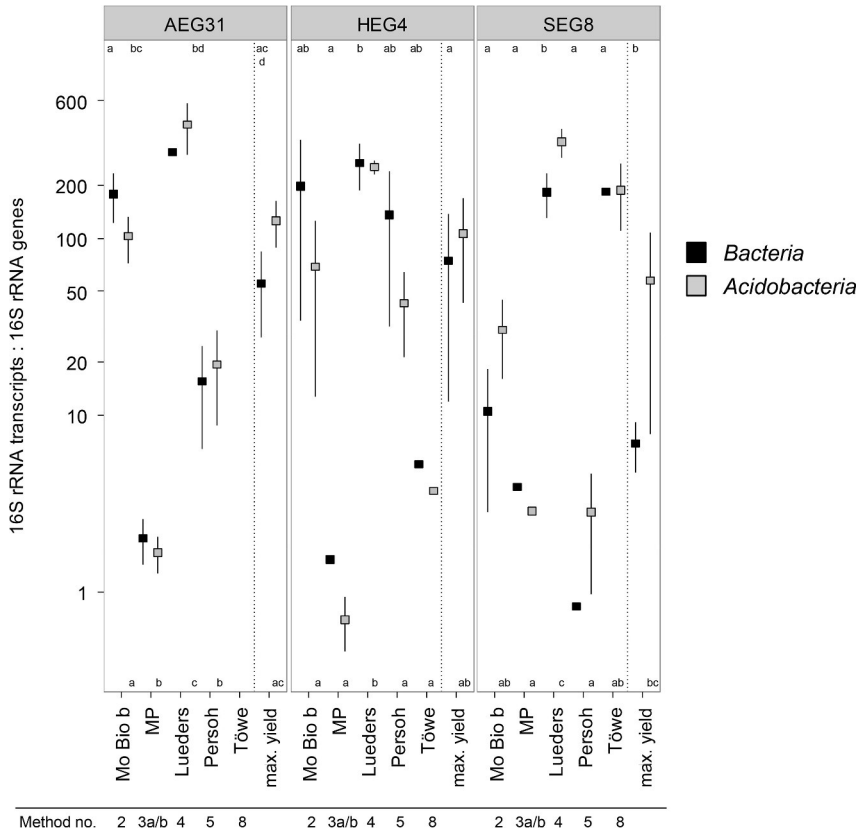

FIG 3 Ratio of 16S rRNA transcripts to 16S rRNA genes as an indicator of specific activity for *Bacteria* (black squares) and *Acidobacteria* (gray squares). The squares represent mean values for the three extracts per soil sample (±standard deviations). Values are means from replicate qPCR analyses (*Bacteria*, $n = 4$; *Acidobacteria*, $n = 3$). Ratios calculated from methods yielding the maximum transcript or gene copies (denoted "max. yield") were calculated based on the methods that yielded the highest copy numbers per soil, nucleic acid, and taxon. The *y* axis is logarithmic but shows absolute values. Methods that do not share a letter are significantly different (multcomp, $P < 0.05$). No results are shown for AEG31 using method Töwe (no. 8) since extraction of DNA from this soil failed with this method.

for all three soils. The lowest gene copy numbers were obtained with the ISO standard extraction protocol (Petric *a* and Petric *b*).

Mean values for bacterial transcript numbers ranged between 6.6×10^6 and 1.1×10^{12} copies (g [wet weight]$^{-1}$) (Fig. 2B). For acidobacterial transcripts, mean values ranged between 7.8×10^4 and 1.6×10^{10} copies (g [wet weight]$^{-1}$). Method MoBio *b* (no. 2) yielded the highest bacterial transcript numbers for AEG31 and HEG4, but the transcript numbers for SEG8 were the lowest. In contrast, method Lueders et al. (20) yielded the highest bacterial transcript numbers for SEG8 and the second highest results for AEG31 and HEG4.

For the three soils investigated in the present study, the ratio of 16S rRNA transcripts to 16S rRNA genes ranged between 0.5 and 353.3 for *Bacteria* and between 0.5 and 595.5 for *Acidobacteria* and was strongly affected by the particular extraction method used (Fig. 3). The mean bacterial and acidobacterial ratios for the same soil sample differed by more than 2 orders of magnitude between the extraction methods. Most extraction methods produced high variations within extraction replicates (higher than factor 10). In contrast, ratios were reproducible for all three soils using method Lueders (no. 4) (Fig. 3).

Based on the methods that yielded highest copy numbers per soil, nucleic acid, and taxon, we calculated maximum yield ratios from values generated with the optimum extraction methods for DNA and RNA, respectively ("max. yield" in Fig. 3). Maximum ratios ranged between 3.5 and 121.0 for *Bacteria* and between 18.4 and 175.5 for *Acidobacteria*.

Bacterial diversity detected in different extracts. No PCR product for the V2-V3 region was obtained from two of the 23 DNA extracts, namely, those obtained from soil sample SEG8 with the ISO standard extraction 11063 (Petric *a*, no. 6) and the ISO standard extraction 11063 with purification (Petric *b*, no. 7), probably due to their low quality (see Fig. S1). Amplification of cDNA derived from RNA extracts was successful for all 15 samples. A total of 801,118 reads was generated. After preprocessing, including quality filtering and denoising, 481,572 sequences with an average length of 380 bp were analyzed further (see Tables S3 and S4).

Rarefaction curves, richness, and diversity were calculated for OTUs defined at sequence divergence levels of 3 and 20% (see Fig. S2 and S3 and Tables S5 and S6). Comparison of the rarefaction analyses with the number of OTUs determined by the Chao1 richness estimator revealed that 56 to 94% (at a genetic distance of 20%) and 22 to 69% (at a genetic distance of 3%) of the estimated richness were covered by our survey. At 20% sequence divergence, the number of DNA-based OTUs obtained from rarefaction analysis ranged between 89 and 244 (see Table S5) and varied by factors of 1.3 (AEG31), 2.5 (HEG4), and 1.4 (SEG8) between extraction methods. In order to focus on extraction method-dependent variations, richness estimates (no. of OTUs determined by rar-

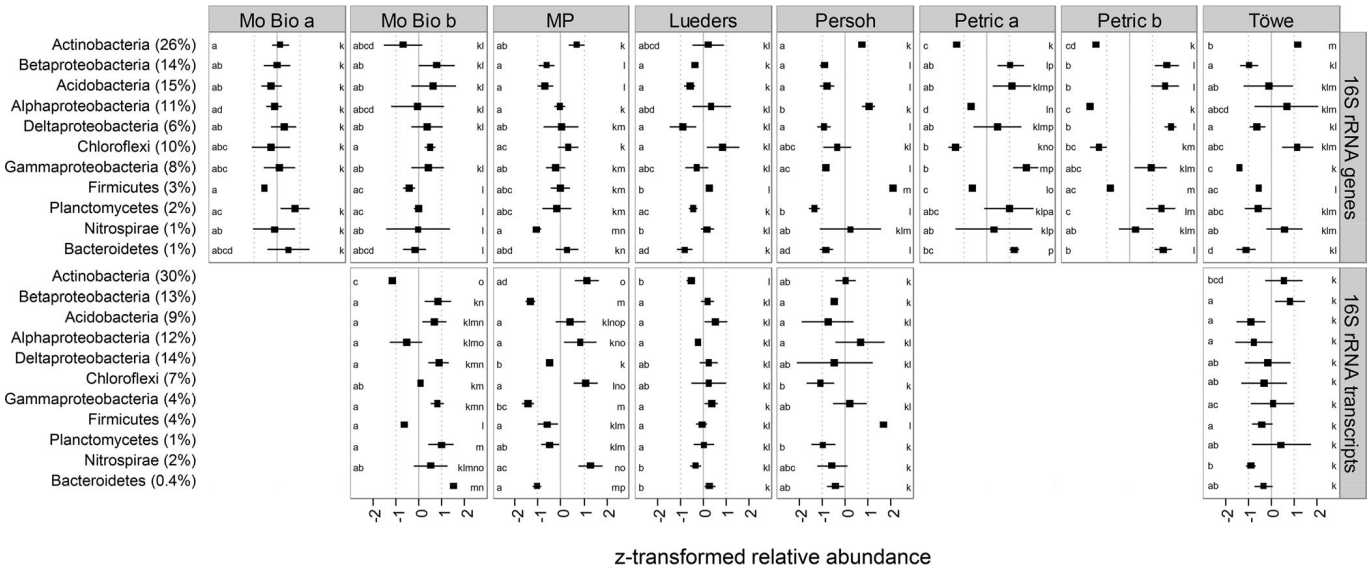

FIG 4 z-transformed relative abundances of abundant phyla and proteobacterial classes detected in DNA and RNA extracts. Data were subjected to z-transformation ($[x - \text{mean}]\ [\text{standard deviation}]^{-1}$). After z-transformation, values around 0 approximate the mean value of all extraction methods and the standard deviation between all methods equals 1. Negative values are indicative of a lower fraction of a taxonomic group, and positive values are indicative of a higher fraction, compared to the means of all eight (DNA) and five (RNA) methods, respectively. The squares represent mean values of the three soil samples (±standard deviations). Mean relative abundances over all samples are provided after the taxon name. Horizontally spread letters from a to d shown in the left-hand side of each block denote statistically significant differences between the extraction methods for a given phylum. Methods that do not share a letter from a to d, per phylum, are significantly different (multcomp, $P < 0.05$). Vertically spread letters ranging from k to p shown in the right-hand side of each block denote statistically significant differences between phyla, per extraction method. Phyla that do not share a letter from k to p, per soil, are significantly different (multcomp, $P < 0.05$).

efaction and by Chao1 and Shannon index H′) were subjected to z-transformation (see Fig. S3). At 3% and 20% genetic distances, method Petric *a* (no. 6) yielded the lowest Shannon indices. The number of detected OTUs obtained from the RNA-based sequence analysis ranged between 135 and 282 at a genetic distance of 20% (see Table S6) and differed by factors of 1.5, 1.4, and 1.6 for the soils AEG31, HEG4, and SEG8, respectively. Methods MoBio *b* (no. 2) and Lueders (no. 4) consistently yielded higher values for all diversity estimates (see Fig. S3B and Table S6).

Bacterial 16S rRNA sequences were affiliated with 24 bacterial phyla and 17 candidate divisions (see Tables S7 to S12). The relative abundances of individual bacterial phyla were strongly affected by the nucleic acid extraction method used. For example, the relative abundance of *Actinobacteria* and *Betaproteobacteria* in HEG4 ranged between 4.5 and 43.5% and 7.1 and 33.9%, respectively (see Table S8). Some methods (e.g., methods MoBio *a* [no. 1] and MP [no. 3a] for DNA and method Lueders [no. 4] for RNA) revealed abundances that are very similar to the mean abundances of any abundant taxonomic group, while other methods resulted in significantly higher or lower fractions of distinct taxonomic groups (Fig. 4). Many abundant taxonomic groups occurred less frequently in DNA and RNA extracted with method Persoh (no. 5) than with other methods, whereas *Firmicutes* were more abundant (Fig. 4). Abundance values obtained with methods Petric *a*, Petric *b*, and Töwe (no. 6, 7, and 8) deviated strongly from the mean abundances of all methods.

From the extraction methods that yielded the highest copy numbers per soil and nucleic acid, we determined optimized ratios of RNA-based relative abundances to DNA-based relative abundances for the 11 most abundant phyla and proteobacterial groups (Table 3). The highest ratios (up to 3.8) were obtained for *Deltaproteobacteria*, *Betaproteobacteria*, and *Planctomycetes*, and low ratios (0.3 to 0.8) were obtained for *Firmicutes* and *Bacteroidetes*.

The analysis of soil phospholipid fatty acids is a nucleic acid-independent method to determine the microbial biomass in soils (54) and was therefore used for an independent assessment of the soil microbial community in the studied soils. Based on the analysis of phospholipid fatty acids (see Table S13 in the supplemental

TABLE 3 Optimized relative abundance ratios (RNA-based relative abundance to DNA-based relative abundance) for the 11 most abundant phyla and proteobacterial classes[a]

Phylum	Ratio in soil sample		
	AEG31, leptosol (Swabian Alb)[b]	HEG4, stagnosol (Hainich)[b]	SEG8, gleysol (Schorfheide-Chorin)[c]
Actinobacteria	0.6	0.6	0.8
Betaproteobacteria	1.8	1.6	1.4
Acidobacteria	1.1	0.8	0.9
Alphaproteobacteria	1.2	1.0	0.7
Deltaproteobacteria	3.2	3.8	2.5
Chloroflexi	0.7	0.7	0.7
Gammaproteobacteria	1.1	0.6	1.1
Firmicutes	0.3	0.7	0.6
Planctomycetes	1.9	1.4	2.2
Nitrospirae	1.5	1.7	1.5
Bacteroidetes	0.5	0.4	0.8

[a] Ratios were calculated based on extraction methods that yielded highest bacterial copy numbers per soil and nucleic acid.
[b] Based on methods MP (no. 3a) for DNA and MoBio *b* (no. 2) for RNA.
[c] Based on methods Lueders (no. 4) for DNA and Persoh (no. 5) for RNA.

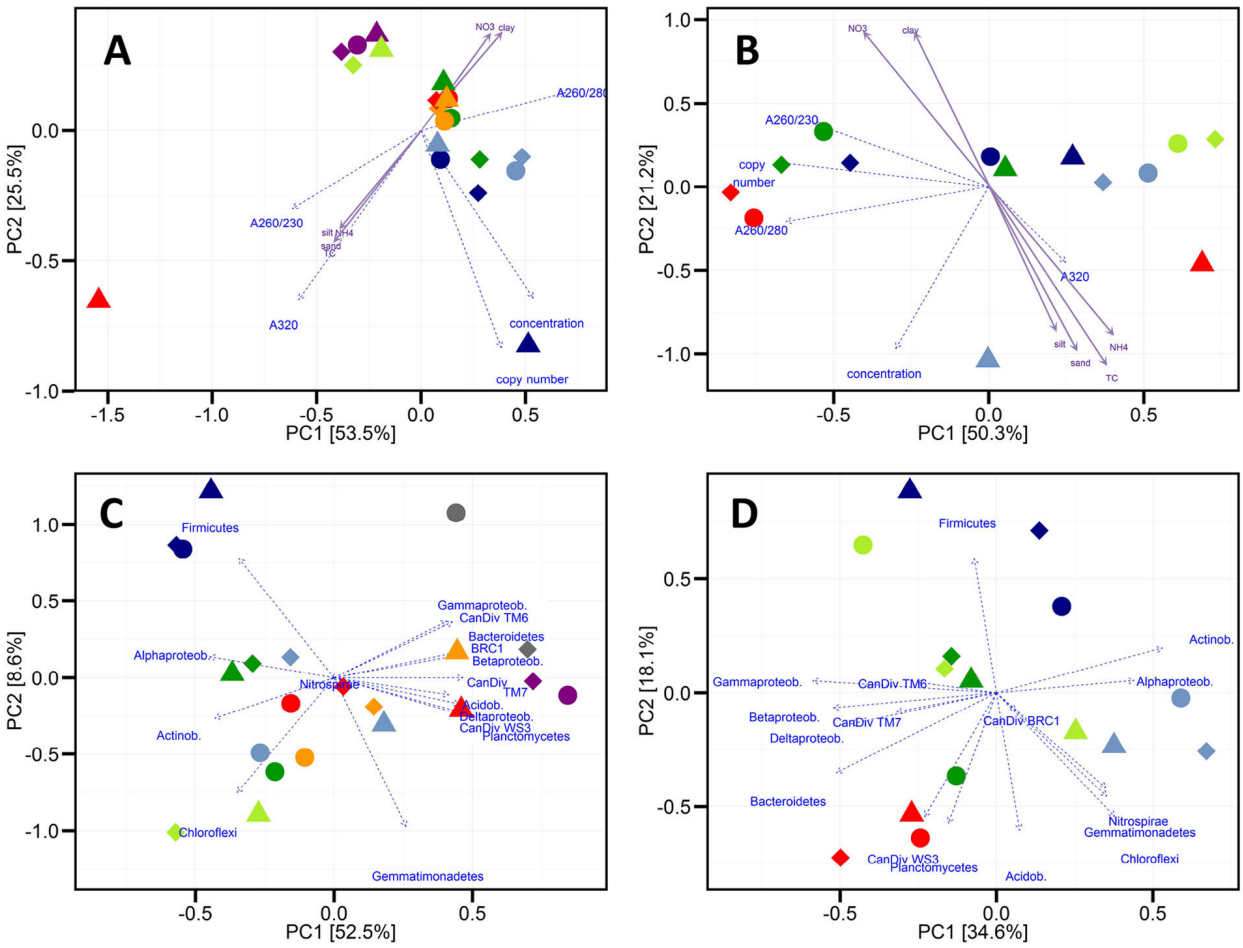

FIG 5 Unconstrained principal-component analysis of z-transformed concentration, copy number, and quality values of DNA extracts (A), of RNA extracts (B), and of relative abundances of 16 abundant phyla and proteobacterial classes detected in DNA extracts (C) and RNA extracts (D). Blue labels and dashed lines give scores of the dependent variables. Numerical variables of the soils were fitted *post hoc* onto the principal components of quality parameters of nucleic acid extracts in panels A and B and are shown as purple and straight arrows. Data for method Petric *a* (no. 6) are not included in panel A since measurements of quality parameters failed for undiluted extracts from HEG4 and SEG8 due to the large amounts of contaminants (see Fig. S1 in the supplemental material); method Petric *a* yielded low concentrations and 16S rRNA gene copy numbers, similar to method Töwe, and method Töwe did not yield detecable amounts of DNA from the clay-rich soil AEG31 (Fig. 2A). Data on the phylogenetic composition in panels C and D were z-transformed for each (sub)phylum-soil combination and hence are independent of the *a priori* differences in the composition of soil bacterial communities in the different soil types. Only (sub)phyla with relative abundances of >0.5% in more than half of the extracts were included in this analysis.

material), the ratio of Gram-positive to Gram-negative bacterial biomass approximated 3 for all three soils. Due to the different numbers of signature molecules for Gram-positive and Gram-negative organisms, this ratio is a rough estimate and is mainly used for comparison between different soil samples (55). Based on the analysis of 16S rRNA genes, the ratios of most abundant Gram-positive taxa (i.e., *Actinobacteria* and *Firmicutes*) to most abundant Gram-negative taxa (i.e., *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, and *Proteobacteria*) were highly variable and depended on the extraction method (see Tables S7 to S12). The highest ratios were detected with method Persoh (no. 5; ratio, 0.5 to 1.1), whereas the lowest ratios were obtained with the ISO standard extraction protocol (no. 6 and 7; ratio, 0.1). The ISO standard extraction protocol was particularly less efficient in extracting DNA from Gram-positive bacteria than other extraction methods.

Multivariate statistical analysis. z-transformed values of concentration, copy number, and the three quality parameters (A_{260}/A_{230}, A_{260}/A_{280}, and A_{320}) were used to assess the effects of extraction methods and soil parameters on the quality and quantity of nucleic acids recovered. A large fraction of variation was explained by the first and second principal components (in Fig. 5A and B, 79 and 71.5%, respectively). Methods Petric *b* and Töwe (no. 7 and 8) were distinct from all other methods in that they consistently recovered the smallest amount of DNA and amplifiable 16S rRNA genes from all soils. The results of the remaining 5 extraction methods were more similar in most cases, with the notable exception of MoBio *b* extracts from soil SEG8, which had distinctly high A_{320} and low A_{260}/A_{280} values, and the Persoh extracts from SEG8, which were characterized by a combination of high values of A_{320}, DNA concentration, and amplifiable 16S rRNA genes (Fig. 5A). Standard parameters that differed significantly between the three soils (Table 1) were fitted *post hoc* onto the principal components, but none of the numerical variables of the soils showed a significant correlation with the two principal components (all *P* values were >0.06).

Most RNA extracts differed with respect to principal component 1 (PC1), which was largely determined by A_{260}/A_{230}, A_{260}/A_{280}, and 16S rRNA copy numbers. The most unfavorable combination of these quality parameters was found for the Töwe extraction method (Fig. 5B). A pronounced influence of the soil type was apparent for the three methods MoBio *b*, Lueders, and Persoh (no. 2, 4, and 5), which consistently yielded the most unfavorable values for the three quality parameters in extracts generated from soil SEG8. However, this effect of soil type could not be related to any of the standard soil parameters tested, similar to the DNA extracts.

Unconstrained PCA of the scaled abundances of 16 (sub)phyla revealed inherent differences in the microbial community composition in DNA and RNA extracts from different soils (see Fig. S4A and B). The community composition of the soils as determined in DNA extracts separated according to PC2 and due to the relative abundances of *Alphaproteobacteria*, *Gemmatimonadetes*, and *Nitrospirae* (see Fig. S4A). Of the soil parameters tested, nitrate and ammonia concentrations were significantly correlated with PC1 and PC2 ($P \leq 0.01$, envfit function in vegan). In unconstrained PCA, the bacterial community composition determined in RNA extracts did not show clear patterns for the different extraction protocols but yielded significant correlations ($0.017 < P < 0.047$) of community composition with the soil parameters total carbon, nitrate concentration, and sand content (see Fig. S4B).

After removing the effects of the inherently different bacterial community compositions of the different soils by appropriate scaling, PCA still revealed a clear effect of the different extraction methods on the community composition determined (Fig. 5C and D). Notably, method Persoh (no. 5) recovered a higher fraction of *Firmicutes* in DNA and RNA extracts than the other methods. While methods Lueders (no. 4) and Töwe (no. 8) recovered larger fractions of *Alphaproteobacteria*, *Actinobacteria*, and *Chloroflexi* in DNA extracts, method Petric *b* (no. 7) had distinctly higher fractions of *Beta-*, *Gamma-*, and *Deltaproteobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Planctomycetes* (Fig. 5C). With the exception of method Töwe, the phylogenetic compositions were most similar in RNA extracts obtained by the same method (Fig. 5D). Method MP (no. 3b) selected for *Alphaproteobacteria*, *Actinobacteria*, *Nitrospirae*, *Gemmatimonadetes*, and *Chloroflexi*, whereas method MoBio *b* (no. 2) yielded higher abundances of *Planctomycetes* and candidate division WS3.

DISCUSSION

Though certain parameters of the different protocols cannot be compared on a quantitative basis or are simply kept confidential by commercial suppliers (Table 2), several factors affecting nucleic acid extraction were identified.

Factors determining the extraction and amplification efficiency of nucleic acids. Considering the bacterial numbers in the soils {1.2×10^9 to 2.5×10^{10} cells (g [wet weight]$^{-1}$)} (Table 1) and the average genome size of soil bacteria (4.7 Mb [56]), 1 g of soil is expected to contain up to 127 μg of bacterial DNA, while the actual amount obtained in our study ranged between 0.3 and 38.1 μg of DNA (g [wet weight]$^{-1}$), suggesting that some extraction protocols clearly fail to recover DNA from the majority of bacterial cells present in the soils even if coextracted eukaryotic DNA is not accounted for. Slow-growing cells of the soil bacterium *Streptomyces coelicolor* A3 (15) and *Escherichia coli* B/r contain 31 and 20 fg of RNA cell^{-1}, respectively (57). Whereas the RNA content of soil bacteria *in situ* has not been determined, marine and estuarine bacteria contain, on average, 9.4 fg of RNA cell^{-1} (7). Accordingly, the amount of extracted bacterial RNA in the three exploratory soils would be expected to exceed 11.3 to 235 μg (g [wet weight]$^{-1}$), whereas the actual recovery of RNA ranged between 0.03 and 64.9 μg (g [wet weight]$^{-1}$).

Systematically low yields of DNA and 16S rRNA gene copy numbers were obtained with methods Petric *a*, Petric *b*, and Töwe (no. 6, 7, and 8). In particular, method Töwe failed to extract detectable amounts of DNA from the clay-rich soil AEG31. This method was the only one employing hexadecyltrimethylammonium bromide (CTAB) as a detergent. At pH values above 5, low extraction efficiencies for clay-rich soils (9, 13) are caused by the strong binding of the polyvalent anion DNA to negatively charged clay minerals via divalent cations (58). Our results corroborate those of a previous study showing that SDS and Tris-HCl yielded larger DNA and RNA amounts than lysis buffers containing CTAB (21). In contrast, the low performance of method Petric *a* can be attributed to the liberation of loosely bound organic matter (indicated by the high A_{320}), most likely through high-temperature lysis and the lack of an efficient subsequent purification. Method Petric *b* comprises two additional final purification steps which, however, did not improve the DNA yield. Obviously, the strong contamination of extracts by organic compounds interfered with purification and hence resulted in a low recovery of DNA. Thus, the two ISO standard extraction protocols are not suitable for recovering DNA at high yields, at least from the three types of soils investigated in the present study.

Methods Persoh and MoBio *b* differed pronouncedly from all others by the high A_{320} values that were specifically observed in DNA extracts from soil SEG8. MoBio *b* extracts from this soil also exhibited lower A_{260}/A_{280} values, whereas method Persoh recovered significantly larger amounts of DNA and amplifiable 16S rRNA genes. Soil SEG8 had the lowest clay and highest total organic carbon (TOC) contents. The larger amount of organic compounds liberated by extraction may be due to a much lower binding strength of organic compounds to the sand and silt fractions prevailing in SEG8. Method MoBio *b* differed from all other methods in employing a combination of guanidine thiocyanate, a strong chaotropic agent, and phenol-chloroform-isoamyl alcohol. Apparently, this combination resulted in the undesired extraction of organic compounds. In contrast, method Persoh encompasses a unique precipitation step that is highly efficient in removing the liberated organic compounds. Method Persoh (no. 5) is thus clearly superior with respect to yield and purity and hence should be chosen when extracting DNA from soils that contain loosely bound organic compounds. According to our analysis, method MP (no. 3a) represents the second most robust method for PCR-based analysis of DNA from soil.

The available commercial kits for RNA extraction do not offer a higher reproducibility than noncommercial protocols. Based on our multivariate analysis, organic compounds coextracted from soil SEG8 systematically interfered with the isolation of RNA for the three methods Lueders, Persoh, and MoBio *b*. Similar to the case with DNA, method MoBio *b* yielded the largest amounts of organic compounds and hence should not be applied to soils containing large amounts of loosely bound organic compounds. Based on their generally low performance, methods Töwe and MP cannot be recommended for the extraction of RNA from soils.

Factors affecting diversity analyses. Some of the extraction methods tested have been reported to extract bacterial, archaeal, and fungal DNA with different efficiencies (23). Our results demonstrate that the 9 different protocols actually result in a distinct phylogenetic composition of the nucleic acids recovered. This substantiates previous findings that were based on low-resolution fingerprint analyses (24, 26, 27).

A comparison with the results of the independent assessment of the soil microbial community through the analysis of the phospholipid fatty acid composition indicates that the different protocols differ with respect to extraction efficiency of Gram-positive bacteria, which are known to be more resistant to cell lysis than Gram-negative bacteria (9). The consistently higher fraction of *Firmicutes* recovered in DNA as well as RNA extracts obtained with the Persoh method suggests that a significantly higher efficiency of cell disruption of *Firmicutes* is achieved by the extended bead beating of this method. The ISO standard extraction protocol (methods Petric *a* and *b*) had a lower efficiency of extracting DNA from Gram-positive bacteria than did all other extraction methods. Notably, methods Lueders, Persoh, and Töwe, and partially also MP, recovered higher percentages of *Alphaproteobacteria*, *Actinobacteria*, and *Chloroflexi* in the DNA extracts. All 4 methods employ a phenol-chloroform step that might affect the efficiency of cell lysis for these groups.

Effects of extraction protocols on the determination of the cellular ribosome content. The cellular ribosome content is proportional to the specific growth rate in various bacteria (4, 59, 60) and has been employed as an indicator of the potential activity of bacterial cells (6, 7). Although the cellular ribosome content is affected by nutrient limitation (4), dormancy (3), or species-specific differences in *rrn* operon and genome numbers (59), it still provides valuable information on the status of bacterial cells *in situ*. Based on our results, conclusions about the ribosome content of bacterial taxa critically depend on the extraction method used. For example, application of method MP (no. 3a and 3b) would lead to the conclusion that the complete bacterial community is inactive (ratios approximate a value of 1) in all three soils, while a high ribosome content (ratios between 200 and 300) was determined using method Lueders (no. 4).

During exponential growth of *Escherichia coli*, the maximum ratio of rRNA transcripts to genes is 2,006, as calculated from published ribosome numbers per cell, the *rrn* operon copy number, and ploidy (number of genomes) per cell (61). The much lower values determined for the three exploratory soils by the optimum combination of extraction method data could be caused by a large fraction of physiologically inactive soil bacteria (62) and/or a low average physiological activity of the bacterial cells. The highest ratios (up to 3.8) were obtained for *Deltaproteobacteria*, *Betaproteobacteria*, and *Planctomycetes*, suggesting that these taxonomic groups harbor many species which are potentially active in the soil. In contrast, low ratios (0.3 to 0.8) for *Firmicutes* and *Bacteroidetes* suggest that only a small fraction of these taxa is potentially active in the studied soil. Interestingly, mean maximum-yield ratios for *Acidobacteria* were always higher than those for *Bacteria* as a whole in all three soils, indicating that *Acidobacteria* on average and as a group have a higher ribosome content than the average of soil bacteria in these three soils. These differences in the ribosome content between *Acidobacteria* and total *Bacteria* become even more pronounced when considering the low average *rrn* operon number of *Acidobacteria* (1.5) compared to that of total *Bacteria* (4.6) (63).

Conclusions. Our comparative analysis demonstrates that the choice of extraction protocols specifically affects the quality and quantity as well as the phylogenetic composition of the resulting DNA and RNA extracts. Notably, and despite the advantages of method standardization in general (64), a single standardized protocol for nucleic acid extraction that would be equally efficient for different soil types, target organisms, and nucleic acid types does not exist. However, several guidelines for the selection of appropriate extraction protocols can be deduced (1). CTAB is inferior to SDS to overcome the quantitative adsorption of DNA to clay minerals (2). The extraction from soils containing large amounts of loosely bound organic matter requires phenol-chloroform-isoamyl alcohol or ion-exchange columns for efficient purification steps that cannot compensated for by the addition of PVP (3). Guanidine thiocyanate should be avoided when large amounts of organic matter would be coextracted; instead, organic contaminants should be precipitated in an initial step (4). Extending the bead beating beyond the period usually employed improves cell lysis of Gram-positive soil bacteria.

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Chapter III.2.

DRIVERS OF SOIL BACTERIA

III.2.

Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests

Running title: Drivers of Soil Bacteria

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Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests

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Soil bacteria provide a large range of ecosystem services such as nutrient cycling. Despite their important role in soil systems, compositional and functional responses of bacterial communities to different land use and management regimes are not fully understood. Here, we assessed soil bacterial communities in 150 forest and 150 grassland soils derived from three German regions by pyrotag sequencing of 16S rRNA genes. Land use type (forest and grassland) and soil edaphic properties strongly affected bacterial community structure and function, whereas management regime had a minor effect. In addition, a separation of soil bacterial communities by sampling region was encountered. Soil pH was the best predictor for bacterial community structure, diversity and function. The application of multinomial log-linear models revealed distinct responses of abundant bacterial groups towards pH. Predicted functional profiles revealed that differences in land use not only select for distinct bacterial populations but also for specific functional traits. The combination of 16S rRNA data and corresponding functional profiles provided comprehensive insights into compositional and functional adaptations to changing environmental conditions associated with differences in land use and management.

Soil bacteria play an important role in biogeochemical cycles[1,2]. They control soil processes such as decomposition[3] and mineralization, including the associated release of greenhouse gases such as carbon dioxide (CO_2), nitrous oxide (N_2O), and methane (CH_4)[4,5] into the atmosphere. Moreover, several soil bacteria promote plant growth and productivity[2,6]. As soil represents a highly dynamic and complex environment, bacterial communities living in this ecosystem are influenced by a multitude of different biotic and abiotic factors. Previous studies showed that soil pH is a major driver of these communities[7–9]. Lauber and colleagues[8] observed that the overall bacterial community composition in different soils from across South and North America was significantly correlated with soil pH. This was confirmed by a study of bacterial communities in German grassland and forest soils[9]. Other studies investigating the effect of edaphic parameters on soil bacteria found that these communities were influenced by the availability of nutrients such as carbon, nitrogen[10,11], and soil moisture in grasslands[12] and forests[13].

In recent years, the impact of land use intensification on bacterial community diversity and composition, e.g. by fertilization in grasslands, has been frequently investigated[14–17]. In a study by Herzog *et al.*[15], composition and diversity of entire and active bacterial communities were altered by fertilizer application. Lauber *et al.*[16] analyzed soil bacterial communities across different land use types such as grasslands and forests. For soil bacteria in forest systems, soil disturbance and organic matter removal[18,19] as well as the dominant tree species[20] have been shown to influence community composition. This provides evidence that land use intensification can alter soil bacterial community composition. However, most studies have focused on a limited number of soil samples in one region. Therefore, the response of bacterial communities in grasslands and forests to land use intensification and

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Land use	Exploratory	n	pH	C:N ratio	Gravimetric water content (%)	Clay (g kg^{-1})	Silt (g kg^{-1})	Sand (g kg^{-1})
Forest	All plots	150	4.5 ± 1.1^{A}	13.8 ± 3.2^{A}	33.5 ± 18.1	289.0 ± 203.3^{A}	440.5 ± 247.1	67.5 ± 386.7
	Schorfheide-Chorin	50	3.4 ± 0.1^{a}	18.1 ± 2.8^{a}	12.0 ± 4.3^{a}	48.5 ± 18.9^{a}	74.0 ± 49.2^{a}	875.0 ± 60.6^{a}
	Hainich-Dün	50	4.6 ± 0.9^{b}	12.8 ± 1.1^{b}	33.5 ± 6.4^{b}	307.0 ± 99.3^{b}	634.5 ± 95.6^{b}	54.5 ± 17.5^{b}
	Schwäbische Alb	50	5.2 ± 0.8^{b}	12.9 ± 0.9^{b}	52.5 ± 10.0^{c}	501.0 ± 104.8^{c}	445.0 ± 107.6^{c}	42.5 ± 46.0^{b}
Grassland	All plots	150	6.7 ± 0.7^{B}	10.3 ± 0.9^{B}	31.5 ± 39.4	425.0 ± 192.4^{B}	418.0 ± 159.3	74.5 ± 228.2
	Schorfheide-Chorin	50	6.4 ± 0.9^{a}	10.4 ± 1.1^{a}	54.5 ± 60.5^{a}	159.5 ± 87.0^{a}	317.0 ± 191.7^{a}	489.5 ± 220.8^{a}
	Hainich-Dün	50	7.1 ± 0.9^{b}	10.1 ± 0.5^{b}	22.0 ± 5.4^{b}	452.0 ± 130.3^{b}	489.5 ± 122.7^{b}	53.5 ± 23.1^{b}
	Schwäbische Alb	50	6.2 ± 0.5^{a}	10.2 ± 0.7^{b}	41.0 ± 11.1^{a}	571.0 ± 134.0^{c}	$386.0\ 114.6^{a}$	41.0 ± 45.0^{b}

Table 1. Edaphic properties among different land uses and exploratories (median ± SD). Significant differences between study regions are indicated by lowercase letters and between forest and grassland by capital letters according to Dunn's test ($P < 0.05$).

environmental changes is not yet fully understood. Large comparative studies are required to unravel the diverse interactions between bacteria and their environments, and how changes in community composition might reflect changes in bacterial functioning.

The aim of the present study was to identify key drivers of bacterial community composition, diversity, and functions in forest and grassland soils. In addition, we aimed at clarifying in which way soil bacterial communities respond to management regime, and if changes are merely a product of the edaphic properties. In this study, 300 soil samples were taken from the three German Biodiversity Exploratories Schorfheide-Chorin, Hainich-Dün and the Schwäbische Alb[21]. Two previous studies focusing on subsets of samples taken in the Biodiversity Exploratories showed that bacterial diversity was influenced by land use intensity[22] and land use type[9]. Bacterial communities were assessed by pyrotag sequencing targeting the bacterial 16S rRNA gene. Additionally, functional profiles were calculated from obtained 16S rRNA gene data[23]. We focused on three main hypotheses: (1) soil bacterial communities exhibit distinct biogeographic patterns, (2) respond differently to soil conditions and land use intensification, and (3) bacterial community composition, diversity and functioning are shaped in a similar way within the same land use system.

Results and Discussion

General characteristics of the soil samples. Soil samples showed significant differences with respect to soil texture and edaphic properties (Table 1, Supplementary Material Tables S1 and S2). Forest soils were more acidic, had a higher C:N ratio and smaller clay amount than grassland soils. Forest soil samples derived from the different exploratories exhibited significant differences in all measured edaphic properties. The Schorfheide-Chorin forest soils were more acid and had higher C:N ratios compared to the Hainich-Dün and Schwäbische Alb soils, which did not differ significantly. In addition, Schorfheide-Chorin forest soils also exhibited the lowest gravimetric water content, clay and silt amount of all exploratories.

Grassland soil samples derived from the different exploratories also exhibited significant differences between all measured edaphic properties. The Hainich-Dün grasslands soil had the highest pH values, lowest gravimetric water content and highest silt amount compared to the Schorfheide-Chorin and Schwäbische Alb soil, which did not differ significantly. The Schorfheide-Chorin grassland soils exhibited the highest C:N ratio and sand amount compared to the other two exploratories. Clay amount was lowest in the Schorfheide-Chorin grassland soils, followed by the Hainich-Dün soils. The highest clay amounts were determined for the Schwäbische Alb grassland soils. Significant differences in soil parameters between the different management regimes were not recorded (ANOVA, $P > 0.5$ in all cases).

Soil bacterial communities. Composition and diversity of soil bacterial communities were assessed by pyrotag sequencing of 16S rRNA genes. After quality filtering, denoising, and removal of potential chimeras and non-bacterial sequences, approximately 2,700,000 high quality sequences with an average read length of 525 bp were obtained for further analyses. All sequences were classified below phylum level. Based on richness estimator data (Michaelis-Menten fit; Supplementary Material Table S3) 78–88% of the operational taxonomic units (OTUs) at 80% identity (phylum level) and 27–55% of the OTUs at 97% identity (species level) were covered by the surveying effort (for rarefaction curves, see Supplementary Material Figs S1 and S2).

Obtained sequences clustered into 203,530 OTUs (97% identity) and were assigned to 51 bacterial phyla, 574 orders and 1,215 families. The dominant phyla and proteobacterial classes (>1% of all sequences across all samples) were *Actinobacteria* (23.75% ± 8.55%), *Alphaproteobacteria* (20.43% ± 5.21%), *Acidobacteria* (18.39%% ± 9.19%), *Deltaproteobacteria* (7.22% ± 2.84%). *Bacteroidetes* (5.15% ± 2.60%), *Chloroflexi* (5.09% ± 2.10%), *Betaproteobacteria* (4.64% ± 2.38%), *Gammaproteobacteria* (4.32% ± 1.23%), *Gemmatimonadetes* (1.88% ± 0.92%), *Firmicutes* (1.18% ± 3.20%), and *Nitrospirae* (1.14% ± 1.10%). These phylogenetic groups were present in all samples and accounted for more than 95% of all sequences analyzed in this study (Fig. 1). These results are consistent with previous studies on grasslands[24] and temperate beech forests[25]. The most abundant phylotype (3.99% ± 2.44) is an uncultured member of the Subgroup 6 of the *Acidobacteria*. The five most abundant phylotypes that could be assigned to a genus are *Bradyrhizobium* (2.66% ± 1.45%), *Candidatus Solibacter* (2.00% ± 1.86%), *Haliangium* (1.39% ± 0.74%), *Variibacter* (1.36% ± 0.58%) and *Gaiella* (1.34% ± 1.31%) of all sequences, respectively.

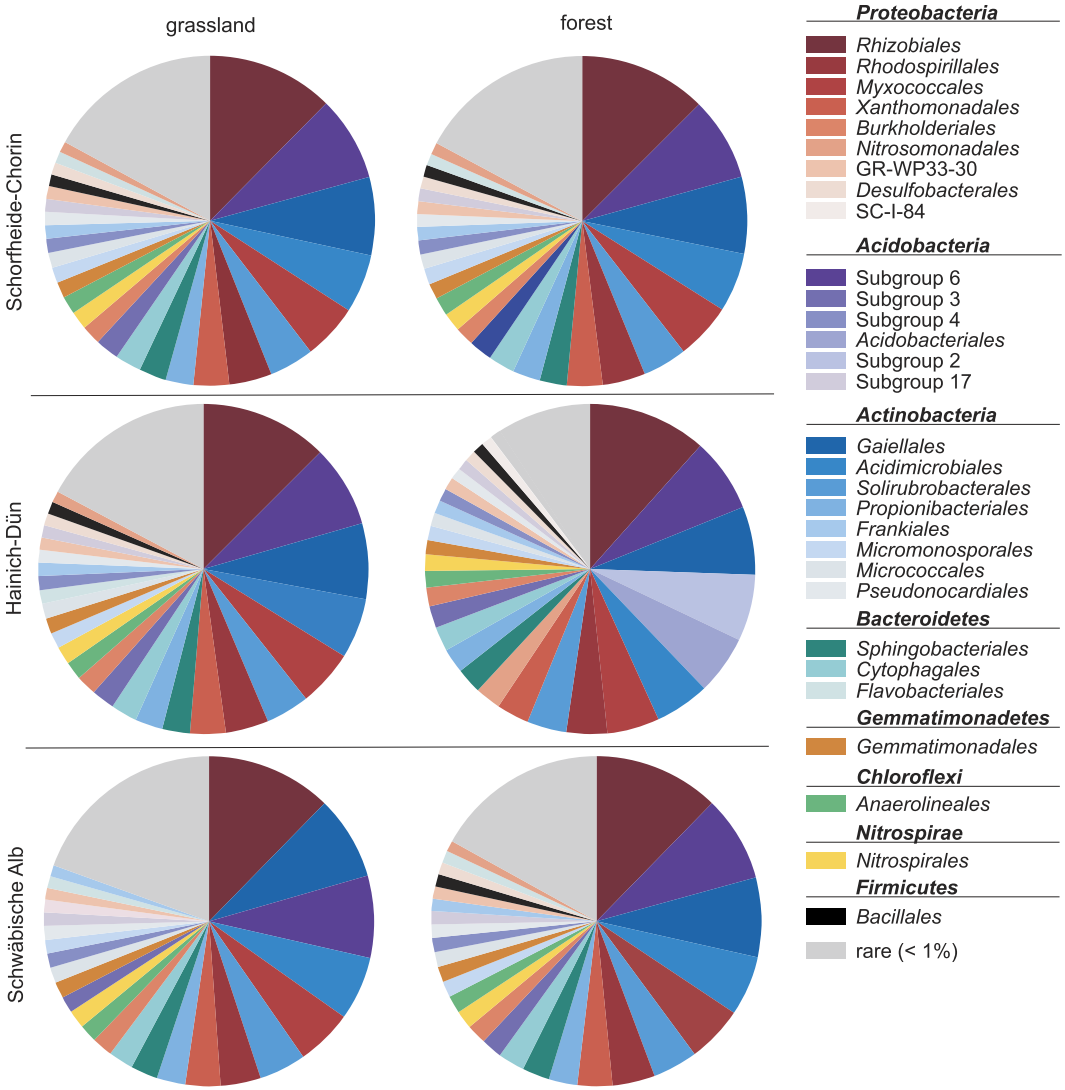

Figure 1. Abundances of bacterial orders in Schorfheide-Chorin, Hainich-Dün and Schwäbische Alb grassland and forest soils. Mean abundances of the most abundant bacterial orders (>1% of the total bacterial community) for each exploratory and land use are given. Rare: sum of bacterial orders contributing <1% to the total bacterial community per exploratory.

Biogeographic variations of soil bacterial diversity and community composition. Diversity (represented by the Shannon index H') and community structure of soil bacteria (PERMANOVA, $P < 0.001$) differed between the three Biodiversity Exploratories. The Hainich-Dün exploratory harbored the most diverse bacterial community ($H' = 10.22$) compared to Schorfheide-Chorin ($H' = 9.72$) and the Schwäbische Alb ($H' = 9.92$). Furthermore, grassland soils are significantly more diverse than forest soils ($H' = 10.12$ and $H' = 9.48$, respectively, with $P < 0.001$), which supports previous findings of Nacke and colleagues[9], who reported that bacterial communities were more diverse in grasslands at phylum level. As samples derived from forests soils were more acidic than grassland soil samples ($P < 0.001$), the difference in pH might explain the difference in diversity (Table 1).

The most dominant bacterial orders of the complete dataset differed in their distribution across the three exploratories. These differences most likely arose from differrences in edaphic properties in the exploratories. Therefore, we tested for correlation of environmental factors by NMDS analysis based on Bray-Curtis dissimilarities. Fitting the edaphic properties to the ordination revealed the pH as the strongest driver of the community. Additional canonical correspondence analysis (CCA) using pH as constrain showed that pH explains 26% of the variation in community structure ($P < 0.001$, Supplementary Figure S3). We additionally found a separation of soil bacterial communities by sampling region (PERMANOVA, $P < 0.001$) and the two land use types grassland and forest (PERMANOVA, $P < 0.001$) (Supplementary Figure S4). Therefore, we were interested in a detailed analysis of the factors driving the changes in the structure of bacterial communities in each exploratory. We further split the data between grasslands and forests due to the strong separation between the community structure of both land use types.

Key drivers of bacterial communities. To identify the key drivers of soil bacterial community structure for each land use type in each exploratory, we performed NMDS analysis for the six subsets. The soil pH was the only property, which affected the community structure in each subset (Fig. 2). Another property influencing the community structure in grasslands and forests was soil texture (amount of clay, sand and/or silt), which represents pore size, water and gas fluxes, and nutrient availability[26,27]. Moreover, soil texture is important for niche separation and protection from predation[28].

In grassland soils, the C:N ratio influenced bacterial community structure in the Schwäbische Alb and Schorfheide-Chorin, but not in the Hainich-Dün. This is supported by a PFLA-based study on soil bacterial communities, in which edaphic properties such as soil texture, pH, and C and N concentration were involved in structuring soil bacterial communities[10]. The land use intensity index (LUI) was only correlated with the Schwäbische Alb grassland community. However, the LUI only accounts for the amount and not for the source of fertilization. In the Schwäbische Alb grasslands, most plots received organic fertilizer (manure, dung), whereas fertilization in the Hainich-Dün and Schorfheide-Chorin was predominated by mineral fertilizer application. These findings support a recent study, in which soil microbial communities of farming systems receiving organic fertilizer were different compared to those of conventional, minerally fertilized systems and control soils[29]. In agreement with Geisseler and Scow[30], clear trends suggesting bacterial community structural shifts due to long-term mineral fertilizer application, were not found in our survey.

In forest soils, the tree species was correlated with bacterial community structure in all exploratories, while the silvicultural management index (SMI) only significantly influenced the community structure in the Schorfheide-Chorin (Fig. 2). Soil bacterial communities under broadleaved (*Fagus* and *Quercus*) and coniferous (*Pinus* and *Picea*) trees formed distinct patterns. This is in accordance with results of previous studies[9,20]. Nacke *et al.*[9] analyzed a subset of soil samples derived from the Schwäbische Alb and found that the bacterial community structure was different under beech (*Fagus*) and spruce (*Picea*). This is consistent with a study comparing bacterial communities under coniferous and broadleaved trees[20]. We did not observe a difference between the two broadleaved tree species, although differences in soil community structure between broadleaved trees have been described for *Fagus* versus *Tilia* and *Acer*[31]. These effects might be partly due to the reduced soil acidification and higher turnover rates of the leaf litter of *Tilia* and *Acer*[32]. Coniferous tree species such as spruce (*Picea abies*) and pine (*Pinus sylvestris*) are known to significantly decrease the soil pH (reviewed in ref. 33) due to the special chemical structure of evergreen litter or capture of atmospheric acidic compounds[34]. This would result in an indirect pH effect on soil bacteria. Additionally, this might be one of the reasons why tree species play an important role in the structuring of bacterial communities in all forest samples analyzed.

According to our hypothesis that bacterial community structure and diversity would be affected in similar ways under the same land use, we compared the bacterial diversity, represented by the Shannon index (H'), between the different management regimes (Supplementary Material Table S4). Differences in diversity were detected for the tree species in the Schwäbische Alb and Schorfheide-Chorin.

Interestingly, the management regimes in grasslands (meadow, pasture, mown pasture) and forests (unmanaged forest, age-lass forest, selection forest) exhibited no significant effect on bacterial diversity (PERMANOVA, $P < 0.05$). This is in contrast to a previous study by Will *et al.*[22], who found a higher bacterial diversity in grassland soils of low land use intensity in the Hainich-Dün. In contrast, Tardy *et al.*[17] investigated bacterial diversity along gradients of land use intensity and observed the highest bacterial diversity in moderately managed soils. The authors suggest that this effect is related to the stress response of the bacterial community. In highly stressed environments, as under high land use intensity, diversity decreases due to the dominance of competitive species and competitive exclusion, while in unstressed environments diversity decreases due to the dominance of adapted species through selection. In accordance with our hypothesis, we could find soil conditions such as pH that consistently drive bacterial community structure as well as diversity, while management regimes and therefore land use intensity have no significant influence. In addition, we could show that pH is the best predictor of bacterial communities.

Bacterial functioning in grassland and forest soils. We further hypothesized that bacterial functioning was driven in a similar manner as bacterial community structure and diversity. To clarify this hypothesis, we focused on pathways involved in the cycling of carbon, nitrogen, phosphorus, and sulfur (Fig. 3) and compared the relative abundances of key enzyme-encoding genes between the two land uses grassland and forest. Abundances of the enzyme-encoding genes were derived from a novel bioinformatic tool Taxa4Fun[23]. Tax4Fun transforms the SILVA-based OTUs into a taxonomic profile of KEGG organisms, which is normalized by the 16S rRNA copy number (obtained from NCBI genome annotations). As soils harbor unknown or uncultured organisms, not all 16S sequences can be mapped to KEGG organisms. Spearman correlation analysis of functional profiles derived from whole metagenome sequencing and profiles deduced from 16S rRNA gene sequences revealed a median of the correlation coefficient of 0.8706 for soils[23]. This indicated that Tax4Fun provides a good approximation to functional profiles obtained from metagenomic shotgun sequencing approaches. This is especially valuable to deduce functional profiles for a large number of samples derived from complex environments, as achieving representative coverage for each sample of a large sample set by metagenome shotgun sequencing would be a daunting task.

Most key enzyme-encoding genes involved in the cycling of C, N, S, and P are either more abundant in grassland or forest soils (Mann-Whitney test, $P < 0.05$, Supplementary Material Table S5). For example, genes that encode acid phosphatases were observed at 1.4-fold higher abundances in the functional profile of the forest soils than in the grassland soils, while alkaline phosphatases showed the opposite trend. We assume that this effect could be attributed to the difference in pH between the land use types, as we showed that pH is the best predictor for bacterial communities. The genes encoding urease were 1.2-fold more abundant in the grassland. The availability of urea was higher in the grassland samples, as these are partly fertilized with manure or dung or were

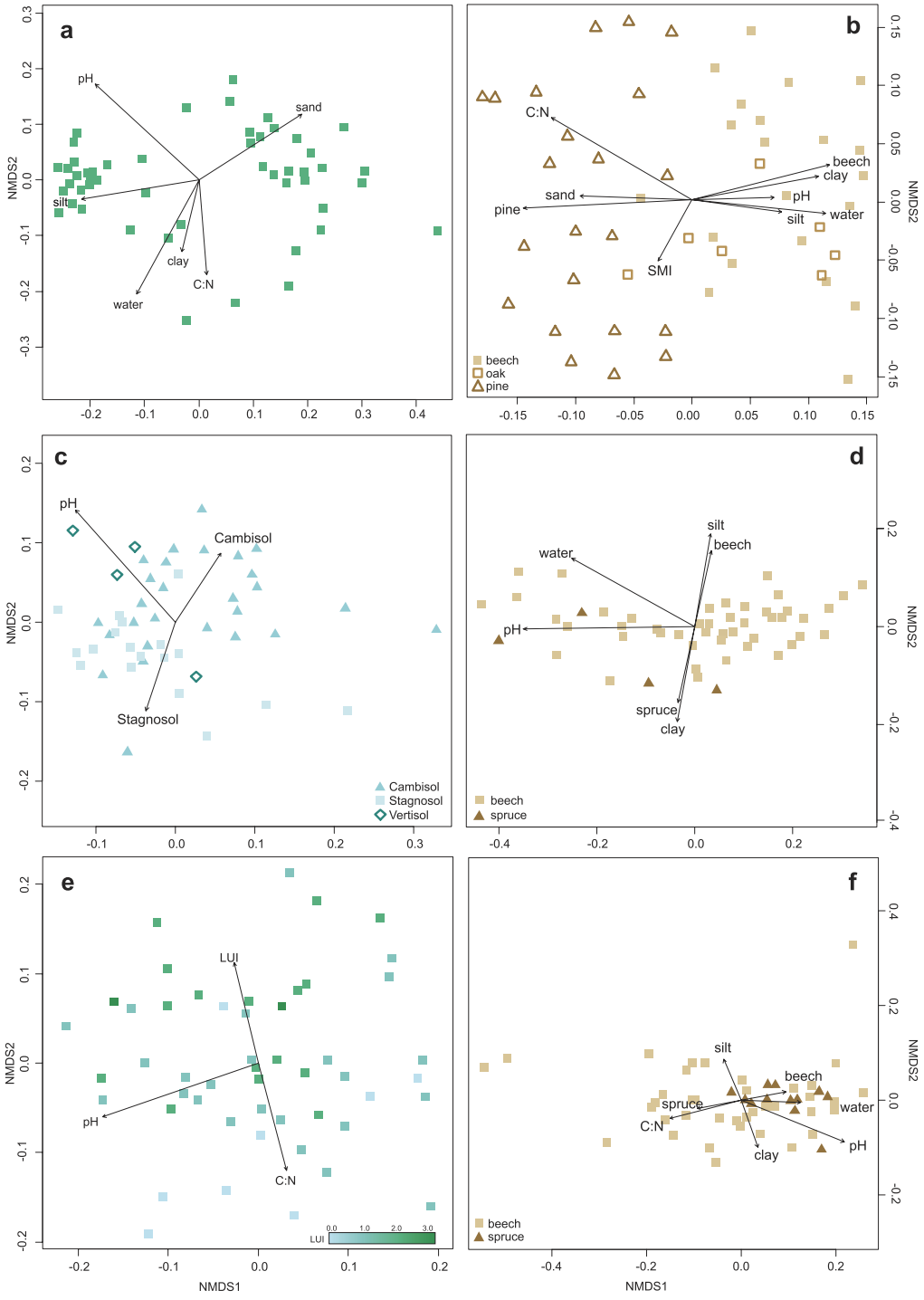

Figure 2. NMDS plots split by region and land use. NMDS plots based on Bray Curtis dissimilarities of grassland (**a,c,e**) and forest (**b,d,f**) bacterial communities. Environmental parameters that are significantly ($P < 0.05$) correlated are indicated as arrows (C:N: carbon: nitrogen ratio; water: gravimetric water content; sand: sand amount; silt: silt amount; clay: clay amount; LUI: land use intensity index in grasslands; SMI: silvicultural management index in forests). (**a**) Schorfheide-Chorin grassland samples; (**b**) Schorfheide-Chorin forest samples; (**c**) Hainich-Dün grassland samples; (**d**) Hainich-Dün forest samples; (**e**) Schwäbische Alb grassland samples; (**f**) Schwäbische Alb forest samples. Note that the NMDS axes have different scales for each ordination.

grazed by animals. Chitinase genes also showed a 1.2-fold higher abundance in grasslands compared to forest soils. This might result from the higher abundance of *Actinobacteria* in grassland soils, as this group is known

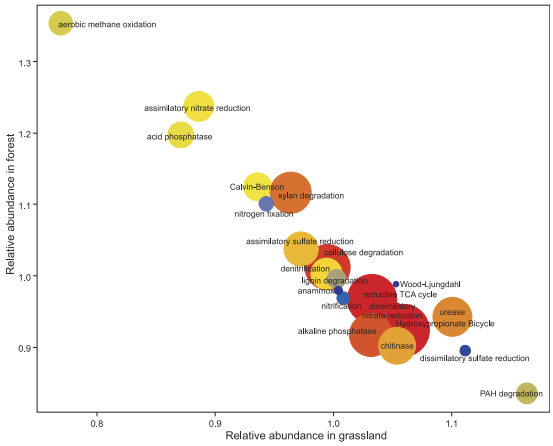

Figure 3. Relative abundances of key enzymes in grassland and forest. Key genes for nitrogen, sulfur, and methane metabolism, carbon fixation pathways, cellulose, xylan, lignin and polyaromatic-hydrocarbon (PAH) degradation, acid and alkaline phosphatases and urease were combined. Their mean abundance (relative to the mean in the complete dataset) in grasslands soil was plotted against the mean abundance in forest soils. Size and color of the circles indicate the mean abundance in the complete dataset. Low abundance: small blue circles; medium abundance: medium yellow circles; high abundance: large red circles. The enzymes included in the analysis are given in Supplementary Material Table S5.

to harbor a high number of chitinase genes[35]. Genes involved in polyaromatic hydrocarbon (PAH, here lignin) degradation are more abundant in grasslands. In forest systems, this process is primarily performed by lignino-lytic fungi (mainly saprotrophic basidiomycetes), which are able to degrade wooden biomass[36]. One key enzyme for aerobic methane oxidation, methanol dehydrogenase, was notably more abundant in forest soils. Methane oxidation in forest soils is the largest biological sink for atmospheric methane[4] and therefore plays a critical role in the flux of this greenhouse gas. Additionally, nitrous-oxide (N_2O) reductase, which catalyzes the last step in denitrification and reduces N_2O to N_2, is also more abundant in forest soils (data not shown). These results indicate that temperate forest ecosystems not only play a crucial role in the regulation and removal of methane, but also of the greenhouse gas nitrous oxide.

Interestingly, the key enzyme of nitrogen fixation, the nitrogenase, is less abundant in grassland than in forest soils. In this study, only bulk soil was sampled and therefore presumably only free-living nitrogen-fixing bacteria could be detected. It is possible, that nitrogen fixation by free-living bacteria plays a greater role in forest systems, whereas symbiotic and rhizospheric bacteria, which were not covered by the study, carry out the major part of nitrogen fixation in grassland systems.

The obtained results suggest that the different land uses grassland and forest not only select for distinct bacterial populations, but also for specific functional traits within their bacterial communities. As the grasslands and forests analyzed in the present study are long-term established systems, it would be interesting to evaluate if a similar adaptation is also present in younger systems.

Soil pH is the best predictor of bacterial communities. In the present study, pH was the only factor, which influenced the bacterial community regardless of exploratory and land use. Furthermore, it not only affected bacterial community structure, but also the functional profile of the soil bacteria. As already mentioned, CCA analysis revealed that pH explains 26% of total variance in the community profile (Supplementary Figure S3). Thus, the pH was the strongest predictor for bacterial community structure.

We hypothesized that bacterial community structure and functioning would be shaped in a similar manner. Environmental correlations with the Tax4Fun-derived functional profile were tested by NMDS based on Bray-Curtis dissimilarities (Fig. 4). The results are similar to those obtained for the community structure. The pH played an important role in shaping the functional profile and explained 32% of the variance (tested by CCA, $P < 0.001$, Supplementary Figure S5). This supports our hypothesis that structure and functions of bacterial communities are shaped by similar mechanisms. The functional profile also showed a separation between grassland and forest systems.

Additionally, we found that pH is the strongest predictor of soil bacterial diversity ($P < 0.001$, $R^2 = 0.4$) (Fig. 5). It has already been shown that diversity of soil bacterial communities in the exploratories is positively correlated with pH[9,22]. However, our results indicate a more complex relationship between pH and diversity. Diversity was lowest at low pH, then increased and appeared to be stable between pH 5 and 7 and increases again under slightly alkaline conditions. This is in contrast to Fierer and Jackson[7] and Lauber *et al.*[8], who described a peak of soil bacterial diversity in near neutral soils.

Multinomial regression models revealed multiple responses of bacterial orders to soil pH. To better understand the complex relationship of single bacterial groups and soil pH, we applied multinomial regression models on the 30 most abundant orders of the dataset (Supplementary Material Figure S6). Four general

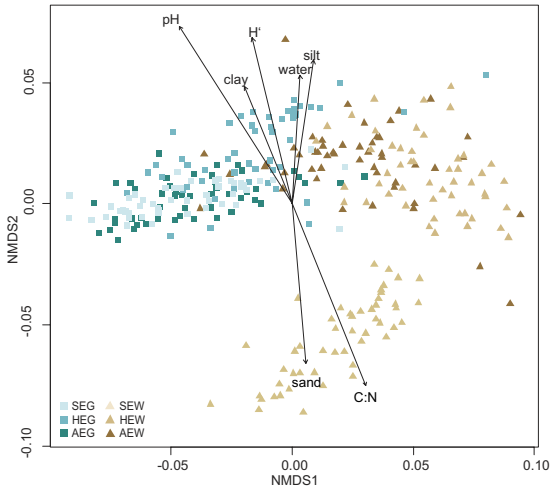

Figure 4. NMDS based on Bray Curtis dissimilarities of the functional profile. Statistically significant correlations of soil characteristics (C:N: carbon: nitrogen ratio; water: gravimetric water content; sand: sand amount; silt: silt amount; clay: clay amount) and the Shannon index (H') were indicated by arrows. Grassland soil samples are represented by brown squares, forest samples by green triangles. Samples from different regions are distinguished by color shading (SEG: Schorfheide-Chorin grassland; SEW: Schorfheide-Chorin forest; HEG: Hainich-Dün grassland; HEW: Hainich-Dün forest; AEG: Schwäbische Alb grassland; AEW: Schwäbische Alb forest).

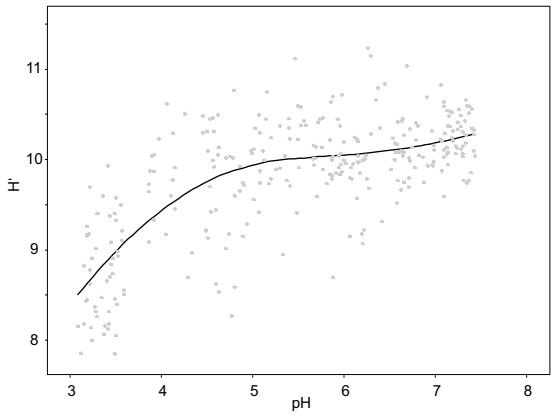

Figure 5. Relationship between soil bacterial diversity, represented by the Shannon index (H') and soil pH. Points indicate observed Shannon indices for each sample, while the line represents the non-linear cubit regression fitted to the data (adjusted $R^2 = 0.5337$, $P < 0.001$).

responses were observed: (1) decrease in abundance with increasing pH (*Acidobacteriales*, acidobacterial subgroup 3, *Frankiales, Corynebacteriales*), (2) increase in abundance with increasing pH (acidobacterial subgroup 6, *Gaiellales*, *Acidimicrobiales, Propionibacteriales*), (3) narrow pH range with high abundance (*Rhizobiales*, *Rhodospirillales*), and (4) relatively constant abundance across pH range (*Bacillales*, *Gemmatimonadales, Sphingobacteriales*) (Fig. 6). In their publication on niche theory, Austin and Smith[37] described pH as a direct physiological gradient acting on organisms, resulting in unimodal, or skewed unimodal response curves restricted by growth limiting conditions at one end, and competition at the other end. This is supported by our observation of few highly abundant orders at low pH and many less abundant orders in near neutral soils. The ability to grow at low pH values is known as ATR (acid tolerance response) and confers a competitive advantage compared to other bacteria in soils.

To test which mechanisms are involved in acid tolerance of soil bacteria, we chose those genes reported to be involved in acid tolerance in *Rhizobia*[38] and Gram positive bacteria[39] that were present in the functional profile. Additionally, we analyzed the genes present of the KEGG pathway for biosynthesis of unsaturated fatty acids (ko01040) as well as 3-trans-2-decenoyl isomerase. This enzyme is involved in the generation of unsaturated fatty acids and was shown to increase acid tolerance in *Streptococcus mutans* by changing cell membrane composition[40]. We found that the genes for biosynthesis of unsaturated fatty acids were highly abundant in low pH samples (pH 3–4), while decenoyl isomerase did not follow this trend (Fig. 7). Therefore, this gene might not be generally involved in acid tolerance in soil. Additionally, most genes involved in alkali production, two

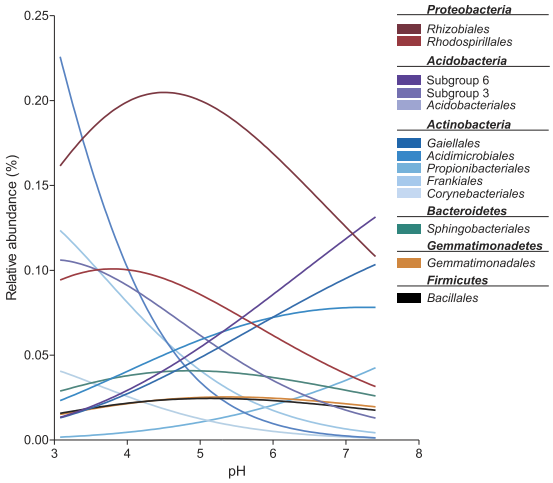

Figure 6. Response curves of selected bacterial orders towards pH. Each line represents the predicted abundance changes along the measured pH gradient, based on predictions derived from multinomial regression models. A detailed version of this graph including the 30 most abundant orders is available as Supplementary Material Figure S4.

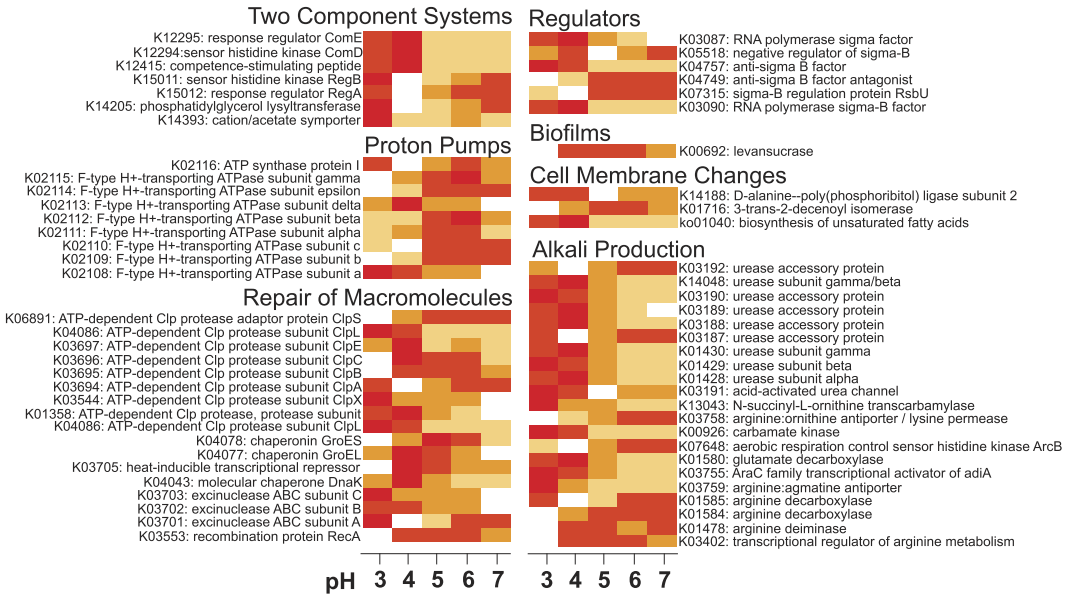

Figure 7. Heatmap based on mean abundances of genes putatively involved in ATR. Only genes with KEGG orthologs and present in the functional profile are shown. The KEGG pathway for biosynthesis of unsaturated fatty acids is included, also on the basis of the genes with KEGG orthologs in the functional profile. White: low relative abundance; yellow: mean relative abundance; red: high relative abundance.

component systems and repair of macromolecules were more abundant in the low pH samples compared to more neutral samples. Several genes involved in DNA repair were probably also involved in the ATR of soil-inhabiting bacteria, as well as levansucrase, a gene involved in biofilm formation. Our results suggest that bacteria can apply an active mechanism to cope with stressful pH conditions. Alkali production increases the pH in the immediate environment, improving bacterial survival chances. Additionally, macromolecule repair-enzymes protect and repair DNA and proteins, and bacteria seem to enhance pH tolerance by altering their cell wall components or protect themselves within biofilms.

Conclusion

During the last years, several studies targeting soil microbial communities and their driving forces came to the same conclusion that soil pH is the major driver of bacterial communities. This statement, however, falls short as it provides no direct answer about the complex interaction of soil bacteria with pH. We showed that soil bacteria respond differently to changing pH conditions, being adapted to certain pH ranges or even stable over a broad pH range. Obtained data suggest that this adaptation is attributed to different mechanisms including

alkali production and alteration of cell wall components. In addition to soil pH, it is generally assumed that land use intensity drives bacterial community composition and diversity. However, the present study demonstrated that land use intensity plays a minor role, or that its effect is concealed by the tree species effect in forest. Biogeographic variations and the corresponding changing edaphic properties resulted in distinct patterns of soil bacteria, which explains regional differences and also the distinct patterns of bacterial communities in grasslands and forests. This is in line with our first and second hypothesis.

Large comparative studies are required to unravel the diverse interactions between bacteria and their environments, and how changes in community structure might reflect changes in bacterial functioning. With a total of 300 samples representing different land uses and gradients of land use intensity, this study provides comprehensive insights into soil bacterial communities present in temperate systems. Taking the enormous size and diversity of soil microbial communities into account, functional information on soil bacterial communities has been limited as it was so far mainly derived from small-scale comparative metagenomic approaches with a rather low coverage. However, the ability to focus on functional genes and enzymes offers novel insights in the nutrient cycling potential of soil bacterial communities. Consequently, the application of novel bioinformatic and statistical approaches, such as Tax4Fun and multinomial log-linear models, in microbial ecology resulted in a more holistic understanding of the links between bacteria and their environment.

Materials and Methods

Study regions. The present study was conducted as part of the German Biodiversity Exploratories initiative, which is a project investigating large-scale and long-term relationships of biodiversity and land use in Central European grasslands and forests[21]. Its unique design allows detailed analysis of bacterial communities along a regional north-south gradient in Germany. The study is based on 300 plots in three study regions (exploratories). They are located in the Schorfheide-Chorin, the Hainich-Dün and the Schwäbische Alb. Each study region covers the land use types forest and grassland. Grassland plots are 50 m × 50 m and forest plots are 100 m × 100 m in size.

The grassland land use intensity-gradient was represented by three different management regimes (meadows, pastures and mown pastures) that are non-fertilized or fertilized. Fertilization always represents higher land use intensity. The land use intensity index (LUI[41]) combines and equally weights the three components of land use in grasslands: (1) fertilization, (2) mowing, and (3) grazing. To account for interannual variation in management practices, the LUI was calculated from 2006 (start of the experiment) to 2011 (sampling year) (Supplementary Table S1). It is therefore used as an index for long-term management and thereby allows the evaluation of long-term effects on bacterial communities.

In forests, the land use intensity-gradient was represented by different forest management systems (age class forest, selection forest and unmanaged forest). Additionally, forest plots were dominated by one of the following tree species: (1) European beech (*Fagus sylvatica*), (2) sessile/pedunculate oak (*Quercus petrea*/*Quercus robur*), (3) Scots pine (*Pinus sylvestris*) or (4) Norway spruce (*Picea abies*). The silvicultural management index (SMI) was used to assess the impact of management intensity in forest systems (Supplementary Table S1). This index integrates three characteristics of forest stands: (1) tree species, (2) stand age and (3) aboveground, living and dead wooden biomass[42]. Detailed information on land use, the applied management, dominant tree species, soil type and fertilization for every experimental plot is provided in Supplementary Material Table S2.

Sampling and soil properties. Soil samples were collected from all 300 experimental plots in May 2011. In brief, plots were sampled along two 36 m transects in forests and along two 18 m transects in grasslands. The top 10 cm of the soil layer were taken from 14 locations along the two transects in each plot with a split tube auger of 5 cm diameter. At forest sites, the litter layer was removed with a metal frame (15 × 15 cm) prior to sampling. The soil cores were pooled and sieved to remove stones >0.5 cm and roots.

Ten grams of the pooled soil samples were used to determine the gravimetric water content, which represents the water content of the respective sample at the sampling time. The subsamples were weighted and dried at 105 °C to a constant weight. Air-dried soil samples sieved to <2 mm were used for the determination of soil texture, soil pH, and carbon (C) and nitrogen (N) concentrations as described previously[43]. Detailed information on soil characteristics is given in Supplementary Material Table S1.

DNA extraction, amplification of 16S rRNA genes and pyrosequencing. Total microbial community DNA was isolated from approximately 0.25 g soil per sample using the MoBio Power Soil DNA isolation kit (MoBio laboratories, Carlsbad, CA, USA) following the manufacturer's recommendations. This method was recently shown to perform equally well over a range of different soils[44]. It produces similar amounts of DNA and 16S rRNA gene copies for each soil tested and does not overestimate any of the abundant phyla detected throughout the soils. Therefore, extraction biases were limited and comparability given for all DNA extractions. DNA concentrations were quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA) as recommended by the manufacturer.

The V3-V5 region of the 16S rRNA gene was amplified by PCR. The PCR reaction mixture (50 μl) contained 10 μl 5-fold reaction buffer, 200 μM of each of the four deoxyribonucleoside triphosphates, 2% DMSO, 2% BSA, 0.2 μM of each of the primers, 0.5 U of Phusion High fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA) and approximately 50 ng of isolated DNA as template. The V3-V5 region was amplified with the following set of primers containing the Roche 454 pyrosequencing adaptors and a unique MID per sample (underlined): V3for 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-TACGGRAGGCAGCAG-3′[45] and V5rev 5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-MID-CCGTCAATTCMTTTGAGT-3′[46]. The following thermal cycling scheme was used: initial denaturation at 98 °C for 3 min, 25 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s followed by a final extension at 72 °C for 10 min. All samples were amplified in triplicate, pooled in equal amounts and purified by gel electrophoresis using peqGOLD

Gel Extraction kit as recommended by the manufacturer (Peqlab Biotechnologie GmbH, Erlangen, Germany). PCR products were quantified using the Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Invitrogen GmbH, Karlsruhe, Germany) as recommended by the manufacturer. The Göttingen Genomics Laboratory determined the 16S rRNA gene sequences employing the Roche GS-FLX+ pyrosequencer with Titanium chemistry (Roche, Mannheim, Germany).

Analysis of pyrosequencing data. Pyrosequencing-derived 16S rRNA gene sequences were processed using the QIIME software package version 1.8[47]. Following the extraction of raw data, reads shorter than 300 bp, with long homopolymer stretches (>8 bp), or primer mismatches (>3) were removed. Subsequently, sequences were denoised employing Acacia version 1.53b[48]. Cutadapt[49] was employed to truncate remaining primer sequences. Chimeric sequences were removed using UCHIME implemented in USEARCH version (8.0.1623) first in de novo and subsequently in reference mode using the SILVA SSURef 123 NR database as reference database[50,51]. Afterwards, processed sequences were clustered with UCLUST version 1.2.22q in operational taxonomic units (OTUs) at 97% and 80% genetic identity representing species and phylum level, respectively[52]. OTUs were classified by BLAST alignment against the most recent SILVA database (see above). Rarefaction curves, alpha diversity indices (Chao1, Shannon, Simpson) and Michaelis-Menten-Fit were determined using QIIME according to Wemheuer *et al.*[53]. The analysis was performed by using 5,311 sequences per sample (Supplementary Material Table S3). Non-metric multidimensional scaling plots were generated based on Bray Curtis dissimilarities or weighed UniFrac distances in R using the metaMDS function to visualize differences in bacterial community composition.

Statistical analyses. All statistical analyses were conducted employing R version 3.1[54]. The results of all statistical tests were regarded significant with $P \leq 0.05$, and only significant results are shown and described throughout the manuscript. The median is used throughout the manuscript instead of the mean value, except stated otherwise. For all statistical analysis, the dataset calculated for 97% identity (species level) was used.

The Mann-Whitney-test and non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) were used due to the non-normal distribution of the data. They were performed to test for differences in soil parameters and bacterial diversity between land use systems, exploratories and management regimes. The effects of environmental parameters onto the variance of bacterial communities were analyzed using the *envfit* function as described previously[55]. Canonical correspondence analysis (CCA) on single soil properties was carried out using the *cca* function and subsequently tested for significance applying the *permu.test* function with 1000 permutations. All these functions are contained in the vegan package[56]. Response curves of bacterial orders toward pH were calculated employing a multinomial log-linear model (function *multinom* contained in the nnet package).

Functional profiles were predicted from obtained 16S rRNA gene data using Tax4Fun[23]. Genes involved in acid tolerance (ATR) and encoding key enzymes in nutrient cycling were identified in the resulting profiles using their KEGG orthologs. The heatmap, based on the ATR-involved genes was calculated using the *heatmap.2* function of the gplots package[57]. Differences in the abundances of key genes involved in nutrient cycling were analyzed employing the Mann-Whitney test in R. The mean abundances of genes in grasslands and forests (relative to mean abundance in complete dataset) were plotted against each other using *ggplot* of the ggplot2 package[58].

Sequence data deposition. Sequence data were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number SRP065604.

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Author Contributions

R.D. and H.N. conceived the study and planned the experiments. K.K. and V.K. performed the experiments. K.K. and B.W. analyzed data. I.S. and M.S. contributed data. K.K., B.W., F.W. and R.D. wrote the manuscript. All authors interpreted the results and reviewed the manuscript.

Additional Information

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Chapter III.3

TREE SPECIES EFFECTS ON SOIL MICROBIAL COMMUNITIES

III.3.

Manuscript ready for submission

Diversity and structure of soil microbial communities under European beech and Norway spruce at fine spatial scale in contrasting seasons

Running title: Tree Species Effects on Soil Microbial Communities

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TREE SPECIES EFFECTS ON SOIL MICROBIAL COMMUNITIES

Abstract

The complex interaction between trees and soil microbes in forests as well as its inherent seasonal and spatial variation is barely understood. In this study, we analyzed the effects of major European tree species (*Fagus sylvatica* L. and *Picea abies* (L.) Karst) on soil bacterial and fungal communities. Mineral soil samples were collected from different depths (0-10 cm, 10-20 cm) and different horizontal distances towards beech or spruce trunks (0.5, 1.5, 2.5, 3.5 m) in early summer and autumn. We assessed diversity as well as composition of soil bacterial and fungal communities based on 16S and ITS rDNA sequences. At a genetic distance of 3%, 23,727 bacterial and 1,336 fungal OTUs were detected. Diversity of both, bacteria and fungi, under beech and spruce was driven by abiotic soil properties (such as soil pH and clay contents). Seasonal effects on microbial diversity were not found and the distance from the tree trunk was only relevant for the diversity of bacteria under spruce ($P<0.01$). Community composition of bacteria and fungi was significantly affected by tree species. Different ectomycorrhizal fungi (e.g. *Tylospora*) which are known to establish mutualistic associations with plant roots showed a tree species preference. Moreover, bacterial and fungal community composition showed spatial and seasonal shifts in soil surrounding beech and spruce. These shifts were partly a result of changes in nutrient availability, as organic carbon content decreased with increasing soil depth. With respect to horizontal distances from tree trunks, overall bacterial community composition showed significant variations under spruce trees. These variations might be partly due to changes in root impacts horizontally through soil profiles in the study area.

Introduction

Earth currently harbors approximately three trillion trees and only one gram of soil can contain billions of microbial cells (Rosselló-Mora and Amann, 2001; Crowther *et al.*, 2015). The effect of trees on bacterial and fungal microorganisms in forest soils, however, comprising many taxa involved in decomposition of plant litter as well as deadwood, is still poorly understood. Trees substantially impact soil physical, chemical and biological properties by species-specific leaf and root litter inputs, root architecture, root exudates, and nutrient uptake (Priha and Smolander, 1999; Saetre, 1999, Augusto *et al.*, 2002; Ayres *et al.*, 2009; Cesarz *et al.*, 2013). Furthermore, canopy structure and stem flow affect moisture and nutrient level in soil located close to tree trunks (Johnson and Lehmann, 2006). As a

consequence of direct or indirect tree impacts, changes in the spatial distribution of microbes, vertically through the soil profile as well as horizontally, can occur (Saetre and Bååth, 2000; Ettema and Wardle, 2002).

European beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) Karst) represent dominant forest trees in Central Europe (Hanewinkel *et al.*, 2012; Cesarz *et al.*, 2013). It has been indicated that these tree species cause soil microbial community shifts, e.g., by modification of soil characteristics such as pH or carbon stocks (Lejon *et al.*, 2005; Schulp *et al*., 2008; Thoms *et al.*, 2010; Nacke *et al.*, 2011). Since the 19th century, reforestation of devastated forest sites using Norway spruce has been very common in Central Europe (Berger and Berger, 2012). Replacement of beech by spruce species is associated with changes in humus form, acidity and soil structure (Berger and Berger, 2012). Root system and exudation differs significantly between beech and spruce species. Spruce is typically shallow rooted, whereas beech is able to acquire nutrients from a wide range of soil horizons through its deep root system (so called 'base-pump'). The released root exudates can vary between *Fagus sylvatica* L. and *Picea abies* (L.) Karst, e.g., seasonally, in quantity as well as in composition (Geßler *et al.*, 1998; Fender *et al*., 2013) and potentially affect microbial processes such as respiration (Cesarz *et al.*, 2013). Besides roots, also crowns of broad-leaved trees undergo seasonal changes. As a consequence of litterfall, fresh organic matter accumulates on forest floor in autumn and early winter. Thus, substantial seasonal changes in nutrient availability for microorganisms occur under beech trees. The constitution as well as decomposability of leaf and needle litter originating from European beech and Norway spruce, respectively, varies significantly (Priha and Smolander, 1997). Components of needle litter from Norway spruce such as waxes and phenolic compounds are highly recalcitrant towards biological degradation, whereas beech leaf litter contains higher amounts of more easily decomposable water-soluble substances (Nykvist, 1963; Priha and Smolander, 1997). However, *Fagus sylvatica* L. litter decomposition rates are slower than those of other deciduous tree species including *Acer* spp., *Carpinus betulus* L., *Fraxinus excelsior* L. and *Tilia* spp. litter (Jacob *et al*., 2010). European beech and Norway spruce also differ in the magnitude of stemflow. In beech stands, the stemflow water contributes 5-20% to the annual soil water input (Koch and Matzner 1993, Johnson and Lehmann, 2006). Stemflow in conifer forests is much lower (often below 1%) compared to beech forests due to differences in branch angle, specific surface roughness of branches and bark (Johnson and Lehmann, 2006). Koch and Matzner (1993) analyzed the main chemical soil properties in a European beech and Norway spruce forest as influenced by the distance from the stem basis. Under a 144-year old beech forest,

pH values of the whole soil profile (0-50 cm; Dystric Cambisol) were decreased in 10 cm and 40 cm distance from the stem basis compared to the soil pH in >4 m distance from the stem basis. A similar effect could not be demonstrated under a 70-year-old Norway spruce forest. However, Koch and Matzner (1993) could not find any consistent significant effect for C and N concentrations as well as the CN ratio in relation to the distance to the stem basis.

A number of available studies describing drivers of soil microbial diversity and community structure have focused on the impacts of agricultural plants, whereas effects of different tree species have been rarely explored (Priha and Smolander, 1997; Acosta-Martínez *et al.*, 2008; Haichar *et al.*, 2008; Li *et al.*, 2012; Wubet *et al.*, 2012; Urbanová *et al.*, 2015). An improved knowledge about tree impacts is essential to evaluate the importance of beech and spruce in shaping forest soil ecosystem characteristics. Currently, detailed information on soil bacterial and fungal community composition and the factors altering it in beech and spruce dominated forests is lacking. In many previous studies, methods providing coarse phylogenetic information were used to identify effects of tree species on soil microbial communities. Differences in the genetic structures of soil bacterial and fungal communities in spruce, Douglas-fir, oak and beech plots in France were indicated by automated ribosomal intergenic spacer analysis (ARISA) profiles (Lejon *et al.*, 2005). Furthermore, Jiang *et al.* (2011) found distinct bacterial and fungal communities in soil beneath broad-leaved and coniferous species based on DGGE profiles. More recently, Tedersoo and colleagues (2015) analyzed pyrosequencing-derived ITS sequences to assess the effects of tree diversity on fungi, protists and meiofauna inhabiting Estonian and Finnish forest soil. Results indicated that compared to the effects of individual tree species and soil parameters, tree diversity *per se* had a minor influence on the taxonomic richness of soil biota (Tedersoo *et al.*, 2016). In addition, based on amplicon pyrosequencing data, significant effects of tree species dominating study areas in the Czech Republic on soil bacterial and fungal community composition were reported by Urbánova *et al*. (2015).

In this study, we applied pyrosequencing of the V3-V5 16S and the ITS rDNA gene region to assess diversity as well as composition of soil bacterial and fungal communities, respectively, under European beech and Norway spruce. Since the life cycle of microbes inhabiting forest soils can be strongly affected by seasons through changes in abiotic and biotic factors (Thoms and Gleixner, 2013), samples collected in early summer and autumn were analyzed. Furthermore, to determine spatial tree effects, soil removed from different depths and horizontal distances towards tree trunks was considered within this survey. A total

of 128 soil samples, allowing robust statistical analysis of tree effects on soil microbial communities, were collected under beech and spruce trees.

To our knowledge, this is the first survey combining seasonal and fine spatial scale to investigate tree impacts on diversity as well as composition of soil bacterial and fungal communities. We aim to verify (1) that soil microbial diversity and community composition show a strong host species effect and (2) that tree architecture determines seasonal and spatial patterns in soil microbial diversity and community composition.

Materials and Methods

Sampling, soil properties and DNA extraction

All soil samples were derived from a beech (*Fagus sylvatica* L.) and a spruce (*Picea abies* L. (Karst)) forest site located in the Hainich-Dün region in Germany (Fischer *et al.*, 2010). The age of the trees at both sites ranged between 50 to 65 years. The mineral soil was sampled in 0---10 cm and 10-20 cm depth using split tubes with a diameter of 4.8 cm (Eijkelkamp Agrisearch Equipment, Giesbeck, Netherlands). Mineral soil samples were taken from different horizontal distances (0.5, 1.5, 2.5 and 3.5 m) towards the trunks of four randomly selected trees per site (see Figure 1). Sampling was performed during two seasons, early summer and autumn. We applied a paired sampling approach, meaning that the sampling positions in autumn were <30 cm away from the sampling points in early summer. Detailed information on location of selected trees and sampling points can be found in Supplementary Table S1. A total of 128 soil samples (2 sites x 2 soil depths x 4 trees x 4 horizontal sampling distances x 2 seasons), were immediately sieved <4 mm mesh size in the field and homogenized. One aliquot of each sample was frozen (-20°C) for nucleic acids extraction, and another aliquot was air-dried and sieved to <2 mm for soil chemical analyses.

The pH values were measured in duplicate in the supernatant of 1:2.5 mixtures of soil and 0.01 M $CaCl_2$ with a glass electrode. Additionally, the gravimetric water content of the air-dried soil was determined. A previous study by Wäldchen *et al.* (2012) showed that the gravimetric water content of air-dried soil is well suited to predict clay contents in our study area. For simplification purposes the 'estimated clay contents' according to Wäldchen *et al.* (2012) are designated as 'clay contents' in the following sections. The remaining soil was ground to <100 µm. Ground samples were analyzed for total carbon (C) and nitrogen (N) by dry combustion with the CN analyzer 'Vario Max' (Elementar Analysensysteme GmbH, Hanau, Germany). Inorganic carbon (IC) concentrations were determined with the same

analyzer after the ignition of samples for 16 hours at 450°C. The organic carbon concentrations equaled the differences between total carbon concentration and inorganic carbon concentration.

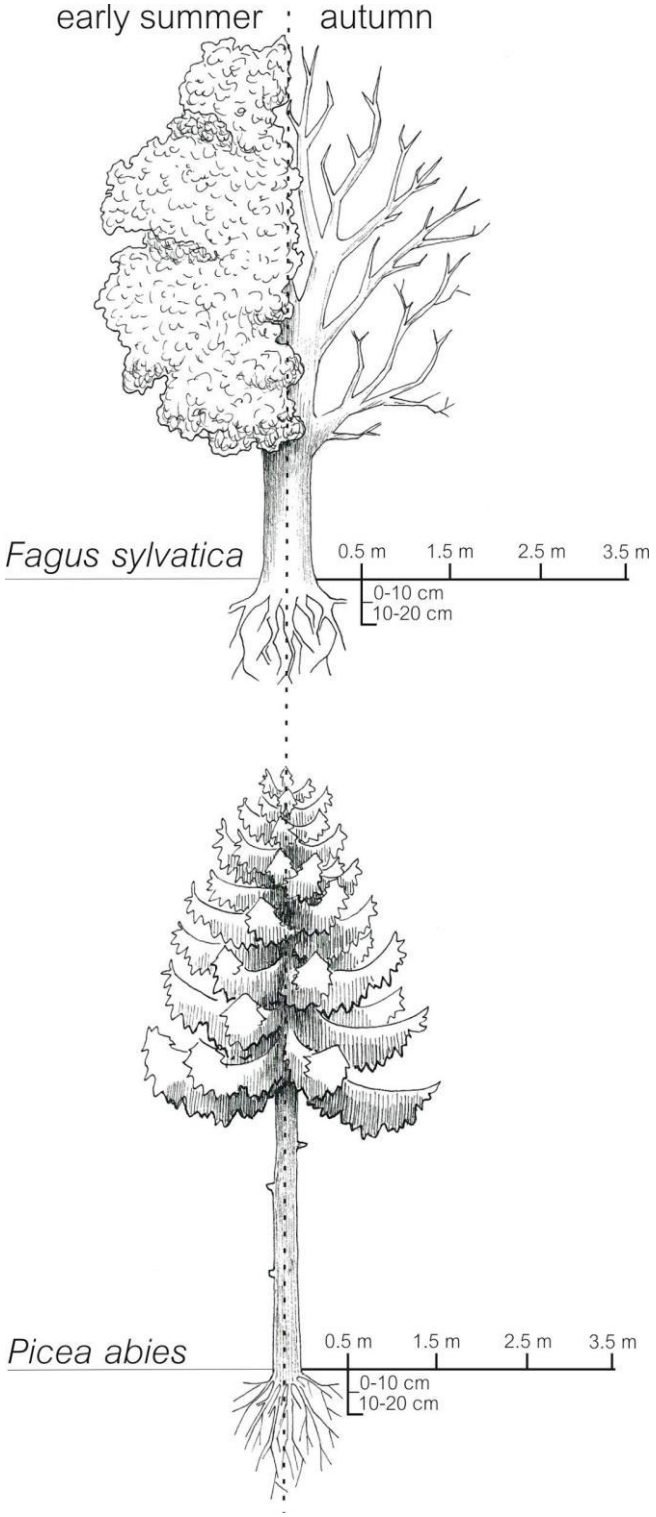

Figure 1. Sampling design: In early summer and autumn 2012 samples were taken in 0.5 m, 1.5 m, 2.5 m and 3.5m distance from the tree trunks of four European beech and four Norway spruce trees (tree replicates); at all sampling points soil samples from 0-10 cm and 10-20 cm, were taken.

Total microbial community DNA was extracted from approximately 2 g of frozen soil per sample using the PowerSoil total RNA isolation kit, the PowerSoil DNA elution accessory kit, and the PowerClean DNA Clean-Up kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instruction. DNA concentrations were quantified using a NanoDrop UV-Vis spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol.

Amplification and pyrosequencing of partial 16S rRNA genes and ITS rDNA

The V3-V5 region of bacterial 16S rRNA genes was amplified by PCR. The PCR reaction mixture (50 µl) contained 10 µl fivefold reaction buffer (Phusion HF buffer, Thermo Fisher Scientific Inc., Germany), 200 µM of each of the four deoxynucleoside triphosphates, 5% DMSO, 1 U Phusion high fidelity DNA polymerase (Thermo Fisher Scientific Inc.), approximately 25 ng DNA as template, and 4 µM of each of the primers. Primers used were 343F (Liu *et al*., 2007) containing a sample-specific MID (Extended Multiplex Identifier, size: ten nucleotides) and the Roche 454 pyrosequencing adaptor A (underlined), and 907R (Cuesta Garrote *et al*., 2011) containing Roche 454 pyrosequencing adaptor B (underlined) (343F, 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-TACGGRAGGCAGCAG-3'; 907R, 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCGTCAATTCMTTTGAGT-3'). The PCR reactions were initiated at 98 °C (2 min), followed by 25 cycles of 98 °C (45 sec), 58 °C (45 sec) and 72 °C (40 sec), and ended with incubation at 72°C for 5 min.

To produce fungal ITS rDNA amplicon libraries primer ITS1F (Gardes and Bruns, 1993) containing Roche 454 pyrosequencing adaptor B and a sample-specific MID, as well as primer ITS4 (White *et al.*, 1990) containing Roche 454 pyrosequencing adaptor A have been used (see also Wubet *et al,*. 2012). The PCR reactions were performed in a total volume of 50 µl reaction mix containing 1 µl DNA template (7-15 ng), 25 µl Go Taq Green Master mix (Promega, Mannheim, Germany) and 1 µl 25 pmol of each of the ITS region-specific primers. Touchdown PCR conditions as described by Wubet *et al.* (2012) were used to amplify fungal ITS rDNA.

All samples were amplified in triplicate, purified using the peqGold gel extraction kit (Peqlab Biotechnologie GmbH) and the Qiagen gel extraction kit (Qiagen, Hilden, Germany) as recommended by the manufacturer, and pooled in equal amounts. Quantification of PCR products was performed using the Quant-iT dsDNA BR assay kit and a Qubit fluorometer (Life Technologies GmbH, Karlsruhe, Germany). Sequences of partial 16S rRNA genes and fungal ITS rDNA were decoded at the Göttingen Genomics Laboratory and the Department of

Soil Ecology (UFZ-Helmholtz Centre for Environmental Research, Halle, Germany), respectively, by using a Roche GS-FLX 454 pyrosequencer (Roche, Mannheim, Germany) and Titanium chemistry as recommended by the manufacturer.

Pyrosequencing data processing and analysis

All generated sequences were reassigned to single samples based on the different MIDs. Bacterial 16S rRNA gene sequence datasets were preprocessed as described by Broszat *et al.* (2014). Uclust (Edgar, 2010), implemented in QIIME (Caporaso *et al*., 2010), was used to determine bacterial OTUs at a genetic distance of 3%. To taxonomically classify OTUs, partial 16S rRNA gene sequences were compared with the SILVA SSU database release 119 (Pruesse *et al*., 2007). OTUs classified as chloroplast or mitochondrion as well as unclassified OTUs were removed from 16S rRNA gene sequence datasets.

Fungal ITS rDNA sequence datasets were preprocessed as described by Goldmann *et al.* (2015). Cd-hit (Li and Godzik, 2006) was applied to determine fungal OTUs at 3% genetic distance. To identify fungi and taxonomically classify OTUs, ITS rDNA sequences were queried against the UNITE database (Kõljalg *et al.*, 2013) by using the classify.seq comment as implemented in MOTHUR (Schloss *et al.*, 2009). After discarding unclassified OTUs, BLASTn search (e.g. Johnson *et al.*, 2008) against the NCBI GenBank database (Benson *et al.*, 2015) was subjected to improve taxonimical resolution.

Bacterial and fungal OTUs comprising only one or two sequences (singleton and doubleton OTUs) were removed from the datasets. The number of analyzed sequences per sample can have an effect on the predicted number of OTUs (Morales *et al*., 2009). Therefore, OTU-based comparisons were performed at the same level of surveying effort (bacteria: 2,540 sequences per sample; fungi: 1,996 sequences per sample). OTUs identified at a genetic distance of 3% were used to calculate the Shannon index (Shannon, 1948).

Statistical analyses

The response of main soil characteristics (e.g. CN ratio, clay content) to soil depth, season and distance from the tree trunk was assessed for both study areas separately by analysis of covariance (ANCOVA) using the aov command of the R-package stats. The random effects of the four sampling transects per study area were considered in the analysis by including them as a factor in our linear models (tree replicate). Additional ANCOVA's were calculated to identify the major drivers of bacterial and fungal shannon diversities. In these analyses, we considered tree replicate, soil pH, estimated clay contents, soil depth, season and distance

from the tree trunk as explanatory variables. We additionally considered all two way interactions with soil pH. Normal distribution of residuals and equality of variances were tested with the diagnostic plots offered by the statistical software R.

To assess the effect of removing rare OTUs on the bacterial and fungal community composition, respectively, we calculated non-metric multidimensional scaling (NMDS) ordination with 20 random starts from the complete datasets and the datasets without singletons and doubletons. In order to test the congruence between ordinations, we used the Procrustes analysis and its protest command (Peres-Neto *et al.*, 2006) implemented in the vegan package (Oksanen *et al*., 2012) of R (R Development Core Team, 2015). Significance of congruence was tested using a Monte Carlo procedure with 999 permutations. We found that neither bacterial nor fungal community composition was affected by the presence or absence of rare OTUs (bacteria: Procrustes correlation coefficient = 0.991, $P<0.001$; fungi: Procrustes correlation coefficient = 0.986, $P<0.001$). Thus, as mentioned above, singleton and doubleton OTUs were not considered with respect to amplicon sequence-based analysis of bacterial and fungal communities. Furthermore, to improve comparability of datasets and avoid inclusion of potential artificial sequences resulting from amplicon generation and pyrosequencing bias, singletons should be removed (Zhou *et al*., 2011).

The effect of tree species on soil bacterial and fungal community composition, respectively, was visualized using principal coordinates analysis plots generated with emperor (Vázquez-Baeza *et al*., 2013).

In order to test the effects of tree replicate, soil pH, clay content, soil depth, sampling season and horizontal distance away from tree trunk on bacterial and fungal community composition, we performed multivariate analysis of variance (MANOVA) under usage of the adonis command of the R-package vegan (Oksanen *et al*., 2012) based on weighted UniFrac (Lozupone *et al*., 2011) distance matrices. These analyses were conducted for the microbial communities under each tree species individually.

To further identify individual taxa strongly associated with a specific tree species, season or spatial position in soil, the multipatt algorithm and the IndVal function in the R package indicspecies (De Cáceres and Legendre, 2009) has been used based on relative abundance of bacterial genera and fungal OTUs, respectively. The PAST statistical package (Hammer *et al*., 2001) was used for the performance of Mann-Whitney U test and Spearman's rank correlations. We applied Mann-Whitney U test to identify dominant genera showing significant differences in relative abundance between soil surrounding beech and spruce, seasons, upper (0-10 cm) and lower (10-20 cm) mineral soil increments or different soil

sampling distances from tree trunks. Spearman's rank correlations were used to correlate relative abundances of dominant genera with soil parameters.

Accession numbers

The 16S rRNA gene and ITS rDNA sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under study accession numbers SRP040766 and SRP044665, respectively.

Results and Discussion

General characteristics of soil samples and amplicon datasets

Both forest stands grow on lime stone which is covered with a loess layer of variable thickness. The loess layer is thinner at the spruce than at the beech forest site. Therefore, pH values in 0-10 cm depth ranged between 3.1 and 5.9 at the spruce site and between 3.7 and 4.4 at the beech site (Table 1, Table S2). At 5 out of 32 sampling locations in the spruce forest the pH in 0-10 cm depth was >5.5 indicating that the loess layer was less pronounced or absent and that the parent material mainly originated from lime stone. In 10-20 cm depth the average pH increased by 0.9 units in the spruce stand whereas it decreased by 0.2 units in the beech stand which is again a result of the lower loess layer thickness in the spruce compared to the beech stand. This was confirmed by the clay content (0-10 cm) which was with 388±15.2 g kg^{-1} (mean±standard error) on average higher at the spruce than at the beech site with 276±4.4 g kg^{-1}. The soils contained in 0-10 cm depth on average 32.6±2.3 g kg^{-1} and 26.2±0.8 g kg^{-1} organic C in the spruce and beech stand, respectively. The organic C concentrations decreased with depth. Organic C concentrations in 0-10 cm were strongly related to estimated clay contents ($r=0.79$, $P<0.001$). Due to colinearity between clay contents, organic C concentrations, and CN ratios we only included clay contents in the following statistical analyses.

Table 1. Basic soil properties at the beech and spruce site.

		Beech	Spruce
pH [-]	0-10 cm	4.0 ± 0.0	4.0 ± 0.2
	10-20 cm	3.8 ± 0.0	4.9 ± 0.2
Clay Content [g kg^{-1}]	0-10 cm	276 ± 4.4	388 ± 15.2
	10-20 cm	249 ± 4.2	380 ± 14.0
Organic Carbon [g kg-1]	0-10 cm	26.2 ± 0.77	32.6 ± 2.30
	10-20 cm	14.5 ± 0.55	15.1 ± 0.65
CN ratio [-]	0-10 cm	12.0 ± 0.10	14.8 ± 0.27
	10-20 cm	11.0 ± 0.11	11.0 ± 0.20

Data on diversity as well as composition of soil bacterial and fungal communities was obtained by pyrosequencing of 16S rRNA genes and the ITS region, respectively. Amplicon data preprocessing yielded a total of 864,096 (bacteria) and 255,488 (fungi) high-quality sequences with an average length of 464 and 300 bp, respectively. At a genetic distance of 3%, 23,727 bacterial and 1,336 fungal OTUs were identified across all analyzed soil samples (singleton- and doubleton-OTUs were not considered). After subsampling (see Materials and Methods section), the number of OTUs per individual soil sample ranged from 505 to 1,440 (bacteria) and 45 to 191 (fungi).

Microbial diversity under beech and spruce trees

In our study areas, the Shannon index of soil bacteria was higher under beech (6.28±0.02) than under spruce (6.05±0.15) (Figure 2). In contrast, the Shannon index of soil fungi was higher under spruce (2.55±0.06) than under beech (2.26±0.10). The Shannon index of both, bacteria and fungi, was mainly controlled by soil pH (P<0.001) (Table 2). Only the diversity of soil fungi under spruce was not significantly affected by pH. The pH, however, was significantly different for the four tree replicates in the spruce forest (Table S2) which significantly affected diversity and which were considered first in the linear model. A positive effect of soil pH on soil bacterial diversity and metabolic richness in temperate forest soils was also shown by Jeanbille *et al.* (2016). They underline that this could be a result of the release of aluminium to soil solution in acid soils. The aluminium toxicity could partly explain the sharp decline in bacterial diversity at pH values <4 in our study areas. The pH effect on soil fungi is in contrast to a previous large scale study on fungal diversity across

Germany, where general soil properties did not correlate significantly with either general fungal OTU richness or fungal Shannon diversity (Goldmann *et al*., 2015). The previous study, however, already showed that ectomycorrhizal fungal OTU richness and Shannon diversity is positively related with pH and negatively with C/N ratio. It seems that the factors driving fungal diversity strongly depend on spatial scale and parent material of the respective studies. In addition to pH, bacterial and fungal diversity responded to soil texture that determines soil moisture, the organic C content and the nutrient status of soils. Carson *et al*. 2010 showed that bacterial diversity was highest at a ≤56% water-filled pore space and they suggest that pore connectivity provides a principle to explain high diversities in soil (Carson *et al*., 2010). Our study did not show any significant effect of soil depth on bacterial and fungal diversity. This is in contrast to previous studies where bacterial and fungal Shannon diversity was decreasing with soil depth (Eilers *et al.*, 2012; López-Mondéjar *et al.*, 2015, Voříšková *et al.*, 2014). Our results show that the previously shown decrease in bacterial and fungal diversity with depth could be related to changes in soil pH or clay content with depth. There was also no direct effect of season on bacterial and fungal diversity (Table 2). In accordance with this result, Kuffner *et al.* (2012) identified no statistically significant seasonal differences in bacterial diversity when analyzing mineral soil (top 5 cm) derived from temperate forest dominated by Norway spruce. Voříšková *et al.* (2014) analyzed seasonal effects on soil fungal communities in deciduous forest (temperate oak forest). They found that summer communities were marginally more diverse than winter communities, but similar to our results, they detected no significant differences between summer and autumn samples. An effect of distance was found for bacterial diversity under spruce and not at all for fungal diversity. Former studies displayed bacterial clustering within small spatial scales (see review by Vos *et al*., 2013). Patchy distribution as well as colony forming might explain the significant relations between bacterial diversity and distance from spruce trunks. Certain fungi (e.g.ectomycorrhizal fungi) tend to form dense networks of hyphae which can be widely extended around host trees (Agerer, 2001; Kluber *et al*., 2010). Such fungal mats seem to maintain constant fungal diversity across distances under both beech and spruce.

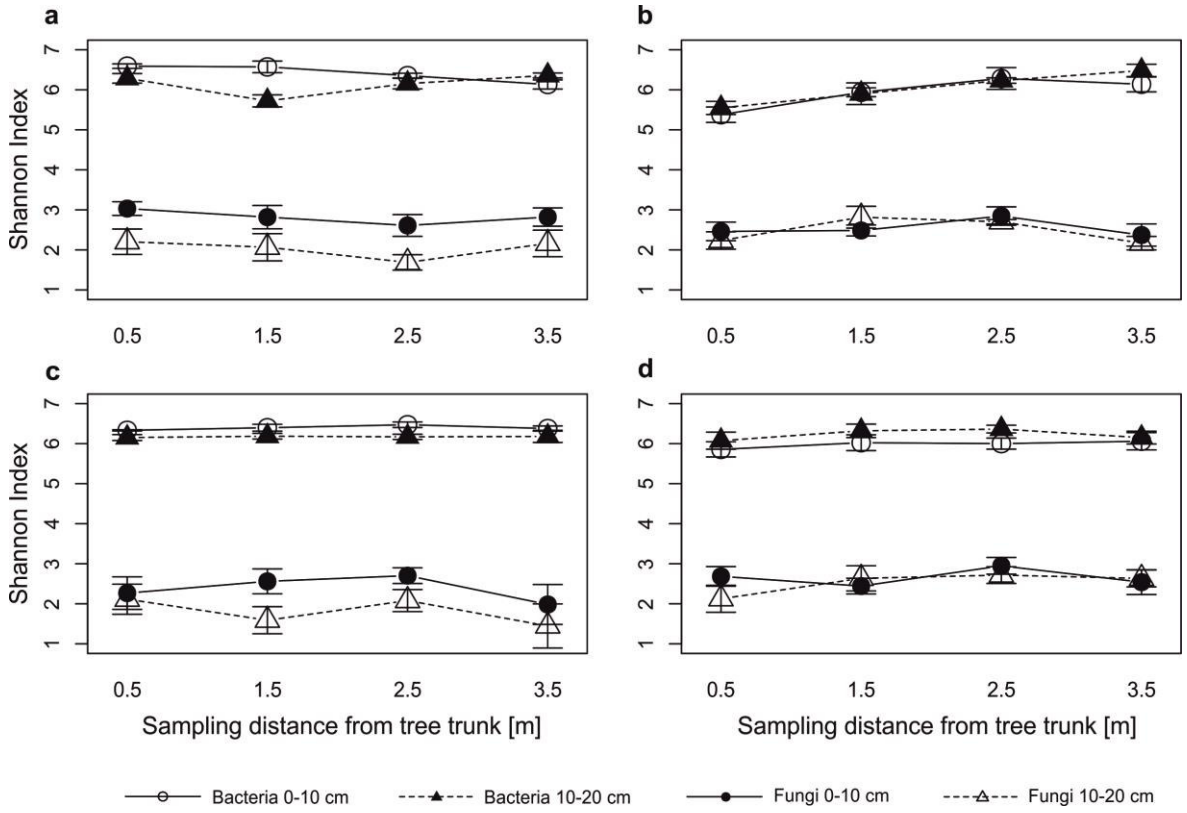

Figure 2. Line plots showing soil bacterial and fungal diversity as assessed by Shannon index at 3% genetic distance under beech in a) early summer, c) autumn and under spruce in b) early summer d) autumn.

Table 2. Analysis of covariance shannon diversities of bacteria and fungi in the beech and spruce stands with tree replicate, pH, clay content, soil depth, season und distance as response variable. Explanatory variables are given in rows in the order of entering the analysis. This table presents degrees of freedom (df), mean squares (MS) and F-values. Significant results are indicated by *P<0.05, **P<0.01, ***P<0.001.

		Beech				**Spruce**			
		bacterial diversity		fungal diversity		bacterial diversity		fungal diversity	
	df	MS	F	MS	F	MS	F	MS	F
Tree replicate	3	0.03	0.6	1.50	4.4**	0.45	3.7*	0.71	3.9*
pH	1	0.79	14.1***	6.70	19.6***	1.82	15.0***	0.24	1.3
Clay	1	0.24	4.3*	2.63	7.7**	0.24	2.0	0.95	5.2 *
Depth	1	0.16	2.9	0.62	1.8	0.02	0.2	0.47	2.5
Season	1	0.02	0.4	1.09	3.2	0.03	0.2	0.02	0.1
Distance	1	0.01	0.2	0.29	0.8	1.26	10.4**	0.01	0.1
pH: Replicate	3	0.05	1.0	0.29	0.8	0.13	1.1	0.08	0.4
pH: Clay	1	0.02	0.3	0.12	0.4	0.10	0.8	0.40	2.2
pH: Depth	1	0.01	0.3	0.37	1.1	0.02	0.2	0.60	3.3
pH: Season	1	0.10	1.7	0.00	0.0	0.38	3.1	0.05	0.3
pH: Distance	1	0.12	2.1	0.94	2.7	0.00	0.0	0.24	1.3
Residuals	48	0.06		0.34		0.12		0.18	

Soil bacterial and fungal community profiles

The bacterial phyla and proteobacterial classes detected in each individual soil sample comprised *Acidobacteria (average relative abundance:* 40.7±0.8%), *Alphaproteobacteria* (20.5±0.4%), *Actinobacteria* (9.4±0.3%), *Gammaproteobacteria* (5.8±0.2%), *Chloroflexi*,, (4.8±0.2%), *Gemmatimonadetes* (4.4±0.2%), *Deltaproteobacteria* (3.8±0.2%), *Betaproteobacteria* (3.3±0.1%), *Bacteroidetes* (2.1±0.1%) and candidate division WPS-2 (1.5±0.1%) (Figure 3). These taxonomic groups were also encountered in recent DNA- as well as RNA-based microbial analyses of other forest soils (Fierer *et al*., 2012; Nacke *et al*., 2014; DeAngelis *et al.*, 2015, Schneider *et al.*, 2015). The detected phyla and proteobacterial classes were reported in similar relative abundances in a meta-analysis of soil-derived 16S rRNA gene libraries (Janssen, 2006).

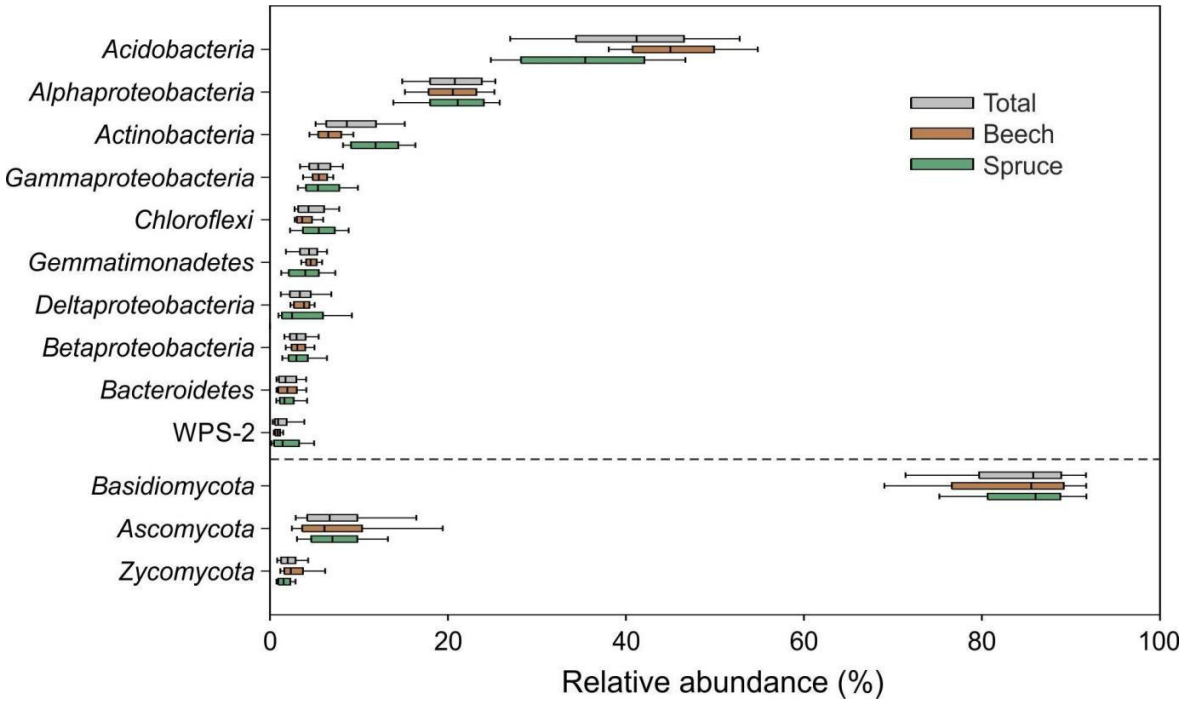

Figure 3. Box-and-whiskers plot showing relative abundances of bacterial and fungal phyla as well as proteobacterial classes detected in each of the analyzed 128 soil samples. Relative abundances of taxa across all samples (grey color) as well as separately with respect to soil surrounding beech (brown color) and spruce (green color) are depicted. The dashed line separates relative abundances of bacterial and fungal taxa.

With respect to genus level-based analysis of amplicon data, bacteria previously isolated from roots (e.g, *Rhizomicrobium*) (Ueki *et al*., 2010) as well as forest soil (e.g., *Reyranella*) (Kim *et al*., 2013), known to form symbiotic relationships with trees (e.g., *Bradyrhizobium*) (Ferro *et al*., 2000) or utilizing plant material (e.g., *Acidothermus*, *Mucilaginibacter*, and *Sorangium*) (Mohagheghi *et al*., 1986; Pankratov *et al*., 2007; Schneiker *et al*., 2007) were detected in high relative abundance (Figure 4). *Acidobacteria* represent the most abundant phylum in our study. Subgroups 2 (average relative abundance: 14.1±0.6%), 1 (11.1±0.5%), 3 (10.1±0.3%), and 6 (2.8%±0.3%) showed the highest average relative abundance among acidobacterial representatives. All other subgroups (4, 5, 7, 9, 10, 11, 12, 13, 15, 17, 18, 20, 22, 25, and 26) showed average relative abundances < 1%. Recently, López-Mondéjar *et al.* (2015) also reported that subgroups 1, 2, and 3 of the *Acidobacteria* were most abundant when analyzing temperate deciduous forest soil. Currently, little is known about ecological functions of *Acidobacteria* in soil. Although the number of *Acidobacteria* isolates is increasing (George *et al*., 2011; García-Fraile *et al*., 2015; Pascual *et al*., 2015), many subgroups (e.g., subgroup 2) lack cultured representatives which could be used to study metabolic functions. It can be assumed that acidobacterial taxa substantially contribute to decomposition of plant-derived biopolymers, cell walls of fungi, and exoskeletons of arthropods in forest soils as genomic

and culture characteristics of subgroup 1 and 3 strains revealed utilization of cellulose, hemicellulose and chitin as carbon source (Ward *et al*., 2009; García-Fraile *et al*., 2015).

The fungal phyla Basidiomycota, Ascomycota and Zygomycota represent on average 87.7±0.7%, 8.9±0.6% and 2.5±0.2% of the relative abundance of all fungi found in this study (Figure 3). The primer pairs applied in this study are known to favor Asco- and Basidiomycota (Bellemain *et al*., 2010) which might explain the high amount of detected basidiomycotous OTUs. Ectomycorrhizal fungi, which are common and abundant in temperate forest ecosystems (Tedersoo *et al*., 2010) were also dominated by members of the Basidiomycota followed by Ascomycota in our study.

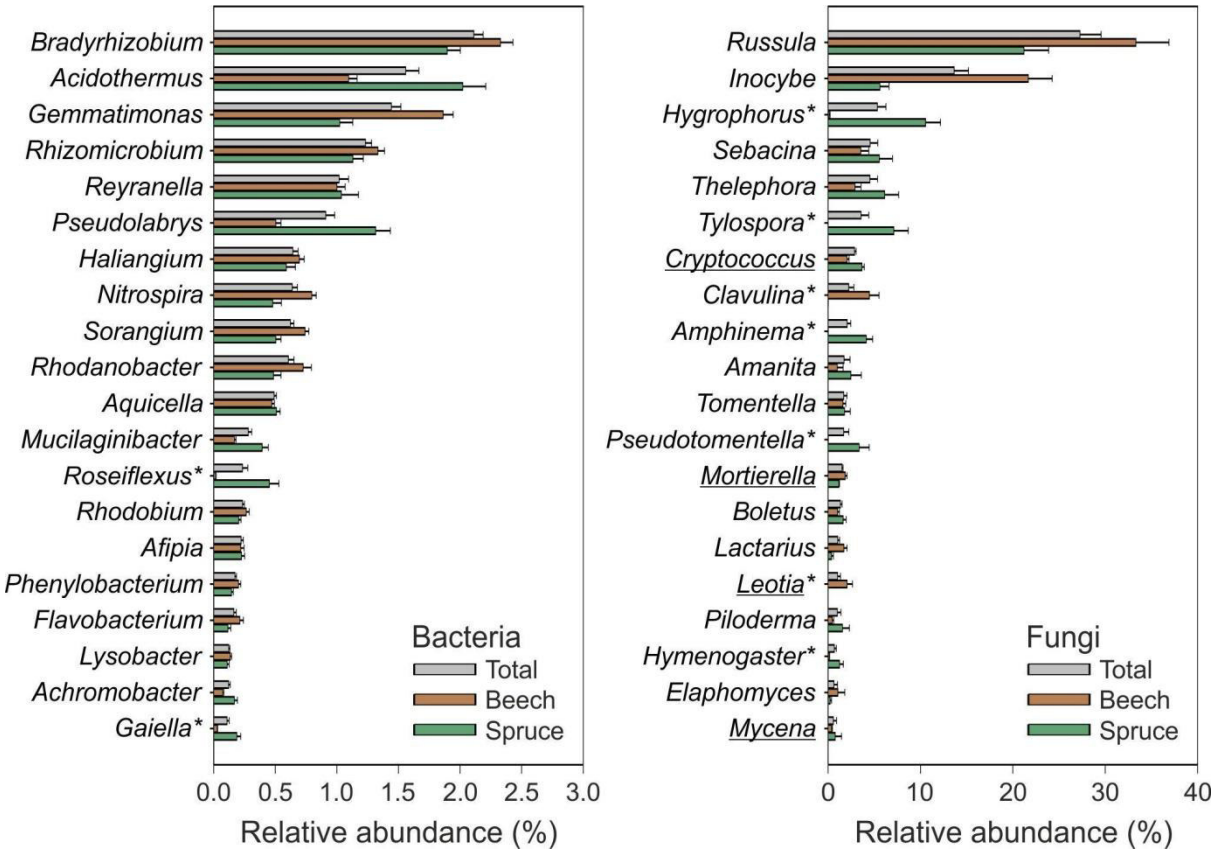

Figure 4. Relative abundances of dominant bacterial and fungal genera detected in the analyzed soil samples. The data represent mean values and standard errors of relative abundances with respect to the twenty most abundant bacterial and fungal genera, respectively. Acidobacteria were analyzed at the subgroup level and therefore not considered within this figure. Relative abundances of taxa across all samples (grey color) as well as separately with respect to soil surrounding beech (brown color) and spruce (green color) are depicted. Asterisks indicate taxa showing an at least five-fold difference in mean relative abundance between spruce and beech as well as $P < 0.001$ regarding Mann-Whitney U test. Underlined taxa: saprotrophic fungi (all other depicted fungal genera represent ectomycorrhizal fungi).

Among the 20 most abundant fungal genera, 16 are known to be ectomycorrhizal fungi (Figure 4) (Tedersoo *et al*., 2010). *Russula* and *Inocybe* are widely distributed mutualists (Kirk *et al*., 2008) and were highly abundant in the analyzed soil samples. The soil related yeast *Cryptococcus*, as well as the multi-cellular *Mortierella*, *Leotia* and *Mycena* were also

detected in high relative abundance. These fungi are reported to contribute to decay processes and live as saprotrophic fungi in humus, dead wood or plant litter (Wang *et al*., 2006, Yurkov *et al.,* 2011, Haňáčková *et al*., 2015).

Similar to Shannon diversity, microbial community composition was significantly affected by pH and clay content (Table 3). Several previous studies identified soil pH as a major driver of soil bacterial community composition across different regions and land use types (e.g., Lauber *et al*., 2009; Kaiser *et al*. submitted). In accordance with our results, pH also explained a substantial fraction of variance in microbial community composition within other deciduous and coniferous forest soils (Lauber *et al.*, 2009; Thoms *et al*., 2010; Goldmann *et al*., 2015).

Table 3. Multivariate analysis of variance based on weighted UniFrac distances with tree replicate, pH, clay content, soil depth, season and distance as response variable. Explanatory variables are given in rows in the order of entering the analysis. This table presents degrees of freedom (df), mean squares (MS) and R^2-values. Significant results are indicated by *P<0.05, **P<0.01, ***P<0.001.

		Beech				Spruce			
		bacterial community		fungal community		bacterial community		fungal community	
	df	MS	R^2	MS	R^2	MS	R^2	MS	R^2
Tree replicate	3	0.03	0.06*	1.64	0.23***	0.2	0.17***	2.23	0.32***
pH	1	0.22	0.17***	0.68	0.03**	0.38	0.11***	0.99	0.05***
Clay	1	0.09	0.07***	0.52	0.02*	0.05	0.01	0.45	0.02**
Depth	1	0.04	0.03**	0.33	0.02	0.13	0.04*	0.32	0.02
Season	1	0.08	0.06***	0.44	0.02*	0.08	0.02	0.43	0.02*
Distance	1	0.02	0.02	0.62	0.03*	0.25	0.07***	0.46	0.02**
pH:Replicate	3	0.01	0.03	0.34	0.05	0.06	0.05*	0.36	0.05***
pH:Clay	1	0.01	0.01	0.1	0.01	0.04	0.01	0.41	0.02*
pH:Depth	1	0.02	0.02	0.14	0.01	0.05	0.01	0.3	0.01
pH:Season	1	0.01	0.01	0.17	0.01	0.23	0.07***	0.32	0.02
pH:Distance	1	0.02	0.01	0.35	0.02	0.02	0.01	0.29	0.01
Residuals	48	0.01	0.51	0.25	0.56	0.03	0.43	0.19	0.43

Tree species effects on microbial community composition

Principal coordinates analysis based on weighted UniFrac distances revealed that samples collected under beech and spruce tend to cluster separately and, thus, indicates similarity in bacterial and fungal community composition in soils from the same forest site (Figure 5). Differences in relative abundances of microbial taxa could be identified between soil under beech and spruce (Figure 4). Furthermore, indicator microorganisms for soil surrounding

beech or spruce were identified by considering individual bacterial and fungal OTUs comprising at least 10 sequences (Table S3).

Acidobacteria subgroups 2, 3, 7, 10, 12, and 13 showed higher relative abundances in soil under beech than in soil under spruce, whereas the opposite trend was found for *Acidobacteria* subgroups 11, 18, and 25. Moreover, the majority of OTUs representing indicators at the beech site were affiliated to *Acidobacteria* (mainly subgroup 2) (Table S3a). This is partly a result of the different pH levels in the two forest stands. The relative abundances of subgroups 2 and 13 decreased with increasing soil pH whereas those of 11 and 18 were positively correlated with pH ($P < 0.001$). Similar correlations between relative abundances of these subgroups and pH were found in soils from across North and South America as well as the Hoosfield strip at Rothamsted research (UK) (Jones *et al*., 2009; Rousk *et al*., 2010).

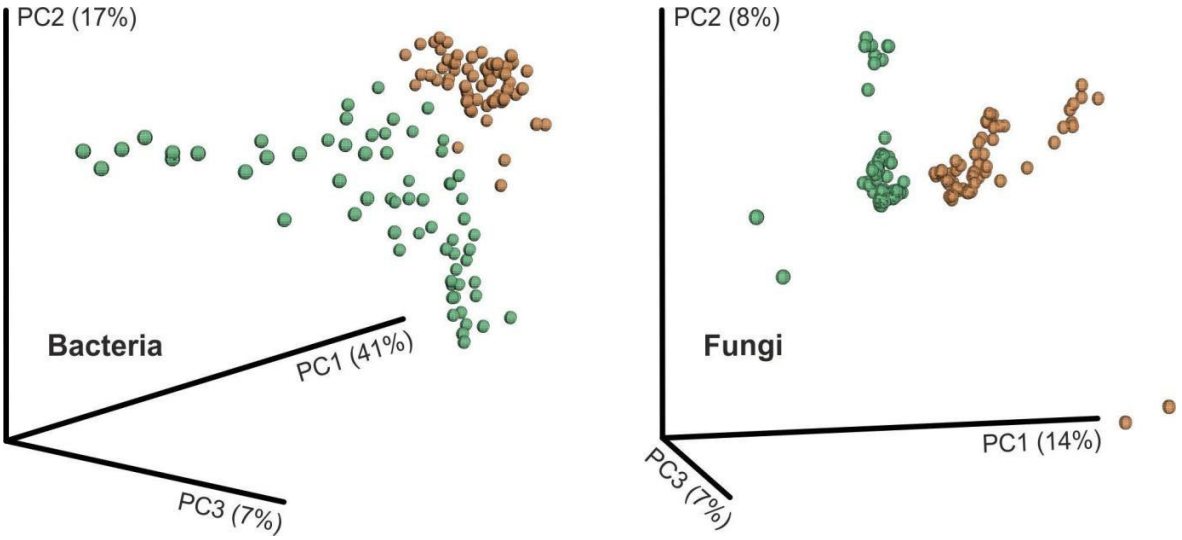

Figure 5. Principal coordinates analysis plots based on weighted UniFrac distances calculated at 3% genetic distance. Brown circles represent samples derived from beech surrounding soil and samples derived from spruce surrounding soil are depicted as green circles.

With respect to dominant bacterial genera, *Gaiella* and *Roseiflexus* showed more than five-fold higher relative abundances in soil under spruce than under beech (Figure 4). As several potential plant compound breakdown genes have been identified in *Chloroflexi* (Hug *et al*., 2013; Houghton *et al*., 2015), it is possible that *Roseiflexus* (a member of the *Chloroflexi*) plays an important role in decomposition of spruce litter. Information on abundance and function of *Gaiella* representatives in forest soil ecosystems is rare. The so far only cultured representative of the genus *Gaiella*, *Gaiella occulta* (recovered from a deep mineral aquifer in Portugal), shows optimal growth within a pH range of 6.5 to 7.5, but no growth occurs at pH 5.0 (Albuquerque *et al*., 2011). In our study, highest relative abundances of *Gaiella* were

detected in soils under spruce exhibiting pH values < 5.0 (1.1 and 1.2% relative abundance in soil with a pH of 4.2 and 4.5, respectively).

Recent studies pointed out that forest vegetation (in particular dominant tree species) is important for distribution of mutualistic and saprotrophic fungi (Lauber *et al*., 2008; Aleklett and Hart, 2013). The fungal genera *Hygrophorus*, *Tylospora*, *Amphinema*, *Hymenogaster* and *Pseudotomentella* showed more than fivefold higher relative abundance in soil under spruce than in soil surrounding beech, whereas the opposite trend was found for *Clavulina* and *Leotia* (Figure 4). Except *Leotia*, all of these genera represent ectomycorrhizal fungi which establish mutualistic associations with plant roots (Smith and Read, 2008) and can show tree species preferences. Thoms et al. (2010) presume that specific direct interactions are most reasonable for mycorrhizal fungi which showed significant correlations with percentage of *Fagus* and *Tilia* litter within their survey. In accordance with our study, Goldmann *et al*. (2015) and Miyamoto *et al*. (2015) reported that *Tylospora* shows a preference for coniferous trees.

For both tree species eight fungal OTUs were identified as potential indicators (Table S3c and d). OTU38 (*Mortierella elongata*) *a*nd OTU99, a *Trichoderma* species, were indicators for saprotrophic keyplayers under beech. To our knowledge, genera of these fungi were described ubiquitous (Wuczkowski *et al*., 2003; Nagy *et al*., 2011). The same can be said for the most abundant OTU of our dataset – *Russula cyanoxantha* which is known to be widespread ectomycorrhizal fungus not just under beech (Grebenc and Kraigher, 2007). In contrast, the ECM fungus *Xerocomus chrysenteron* (Otu0054) is known to have a preference for beech (Shi *et al*., 2002). Indicator species for spruce were three OTUs classified as saprotrophic fungi. *Exophiala* (OTU75) was already described as fungal genus decaying leafs in rain forests (Polishook *et al*., 1996) or as rhizospheric fungus in temperate sites (Summerbell, 2005). Another two *Penicillium* OTUs (OTU94 and OTU116) were identified as saprotrophic indicators for spruce. Already 1980, Johansson and Marklund reported *Penicillium* to be antagonistic to *Fomes*, a well-known fungus infecting spruce trees (Schmidt, 2013). The indicative ECM under spruce, *Hygrophorus* (OTU5 and OTU8) as well as *Amphinema* (OTU19), were abundant and already described for spruce ecosystems (Scattolin *et al*., 2008; Velmala *et al*., 2013).

Spatial and seasonal variability of microbial community composition under beech and spruce

Bacterial community composition varied significantly with depth under beech and spruce (Table 3). Previous surveys based on DGGE analysis as well as Sanger sequencing and

pyrosequencing of 16S rRNA genes also revealed differences in bacterial community composition between topsoils and subsoils (Hansel *et al*., 2008; Eilers *et al*., 2012; Huang *et al*., 2013). This is a result of changes in soil characteristics such as organic C or N concentrations along soil profiles (Hansel *et al*., 2008; Will *et al*., 2010). Here, we found that relative abundances of the dominant genus *Gaiella* were negatively correlated with organic C concentration ($P < 0.001$) and higher in 10-20 cm depth than in 0-10 cm depth. The relative abundance of the dominant bacterial genus *Mucilaginibacter* also showed variations with soil depth. It was higher in 0-10 cm depth than in10-20 cm depth ($P < 0.001$). Different *Mucilaginibacter* representatives are capable of pectin, xylan, and laminarin degradation (Pankratov *et al*., 2007). In addition, *Mucilaginibacter* has been previously associated with cellulose decomposition based on Stable Isotope Probing (Štursová *et al*., 2012). Leaf litter contains high amounts of the plant cell wall components xylan, pectin, and cellulose, and enters the upper mineral soil first. This might explain the increased relative abundance of *Mucilaginibacter* in upper (0-10 cm) mineral soil layers.

Recently, McGuire *et al*. (2013) found discrete fungal communities in different soil horizons in boreal and tropical forest. This can be explained by changing carbon and nutrient contents in combination with decay abilities (McGuire *et al*., 2010; Prescott, 2010). Assumtions that fungal taxa underlay similar mechanisms in temperate forests were not verified by our results (Table 3). However, the detected saprotrophic fungi tend to be associated with the upper (0-10 cm depth) mineral soil layers which are rich in organic C (Figure S1). Additionally, the indicator species analysis identified mainly saprotrophic OTUs in the upper 10 cm of studied soil. Influenced by the litter layer, the upper 10 cm show a high heterogeneity and competition for space and nutrient availability is strongly given (Kadowaki *et al*., 2013). Yet, ECM fungal taxa are connected to plant roots and receive carbon through this mutualistic connection (Smith and Read 2008). Therefore, ECM fungi are not C-limited and may colonize deeper soil layers (McGuire *et al*. 2013). Accordingly, in this study ECM fungi were abundant within 0-10 cm as well as 10-20 cm soil depth (Figure S1).

A significant seasonal effect on bacterial community composition in soil under beech ($P < 0.001$) (Table 3) has been revealed by MANOVA. We found that genera belonging to the *Rhizobiales* (*Bradyrhizobium* and *Rhodobium*) showed significantly higher relative abundance within soil under beech in autumn than in early summer ($P < 0.001$). Recently, López-Mondéjar *et al*. (2015) reported that bacterial communities undergo seasonal changes in mineral soil of a *Quercus petraea* (Matt.) Liebl forest. They assume that seasonal differences in the activity of tree roots are a major driver of soil bacterial community composition in

deciduous forest. As *Rhizobiales* are known to interact with plants, seasonal root impacts might affect their abundance in temperate deciduous forest. Furthermore, seasonal shifts in soil moisture and temperature may also affect bacterial community composition in the analyzed soil (Kaiser *et al*., 2010).

Seasonal impacts on fungi as found in this study (beech: $P = 0.05$; spruce: $P = 0.011$), were reported previously (e.g. Buée *et al*., 2005; Moll *et al*., 2015; Vargas-Gastelum *et al*., 2015). In contrast to our findings Voříšková *et al*. (2014) detected no significant seasonal effect on fungal community composition within mineral soil in oak forests. Nevertheless, in the litter horizon, which was not analyzed in our study, seasonal changes in fungal community composition were identified in other studies (Voříšková *et al*., 2014). These changes are associated with the input of nutrients from fresh litter which occurs in temperate deciduous forests each autumn (Voříšková *et al*., 2014). Despite, Stevenson *et al*. (2014) found that sampling season had a higher impact on bacterial than on fungal communities when analyzing soil derived from a region with temperate climate in New Zealand.

Spatial horizontal variations of overall bacterial community composition were significant in soil under spruce ($P < 0.001$). It is known that spatial distribution of soil microbes can reflect the zone of influence and positioning of individual trees in forests (Saetre and Bååth 2000; Ettema and Wardle 2002). Changes in root impacts horizontally in the tree surrounding might alter occurrence and abundance of bacteria under the selected gymnosperm trees. We found that relative abundances of the dominant bacterial genus *Nitrospira* were significantly higher at 3.5 than at 0.5 m horizontal distance toward spruce trunks. Furthermore, a *Nitrospirales* OTU was identified as indicator for horizontal distances of 2.5 and 3.5 m toward spruce trunks (Table S3b). N demand of spruce trees in summer and autumn is mainly met by uptake of N compounds from soil and subsequent transport of reduced N from the roots to the shoot via transpiration stream (Weber *et al*., 1998). Due to a negative relationship between fine root biomass and distance to spruce trunk (steep decrease of fine root biomass at distances >2 m toward spruce trunk) (Petritan *et al*., 2011), uptake of N compounds via roots might be more pronounced in soil located close to the analyzed coniferous tree trunks. This potentially explains the spatial horizontal variations in occurrence of nitrifying bacteria belonging to *Nitrospirales* under spruce. Under beech trees, the relative abundance of *Pseudolabrys* differed significantly between 0.5 and 3.5 m horizontal soil sampling distance from trunks. Higher relative abundances were detected in soil located close to tree trunks. This effect was recorded with respect to both analyzed soil depths ($P < 0.05$). The taxon *Pseudolabrys*, representing one of the most abundant genera detected in this study (see Figure 4), belongs to

the *Rhizobiales* which are known to interact with plants (Erlacher *et al*., 2015). A higher root density may be a major reason for high relative abundance of *Pseudolabrys* in soil located close to beech trunks.

Fungal community composition differs significantly at different horizontal distances away from tree trunks (Table 3). Previous studies revealed an increase in pH away from trunks of different tree species (Koch and Matzner, 1993; Branco *et al*., 2013) in conjunction with changing appearances of single fungal species (Branco *et al*., 2013). The significant correlation between distance and pH found under spruce (Table S2) might be explanatory for the different fungal communities. However, C/N ratio and clay content were related to changing distances under spruce, whereas additionally organic carbon and nitrogen content correlate with distance under beech (Table S2). These differences in soil chemistry at different distances away from tree trunks could be additional accountable for changes in fungal community composition. Branco *et al.* (2013) reported that water stem flow and through fall account also for the differences in fungal community structure. Spatial heterogeneity under spruce can arise for instance by canopy gap formation (Bardgett, 2005) or decrease of fine root biomass (Petritan *et al*., 2011), too. A missing canopy at 3.5 m horizontal distance could alter litter fall or soil temperature and consequently soil fungal communities.

Conclusion

Our study showed that beech and spruce trees strongly shaped the community composition of soil bacteria and fungi in temperate forests. Tree species-specific preferences with respect to bacterial and fungal genera such as *Gaiella*, *Roseiflexus* or *Hygrophorus* and *Clavulina* were identified. In contrast to community composition, bacterial and fungal diversity were not significantly affected by tree species, but mainly controlled by abiotic soil properties such as soil pH and clay contents.

Trees also have manifold impacts on the seasonal and spatial distribution of microorganisms. In our study, both, bacterial diversity and community composition were strongly affected by horizontal distance towards spruce trunks, probably because of higher fine root biomass near the spruce trunks. Indicator species analyses showed a vertical variation with a higher importance of saprotrophic bacterial and fungal taxa in the upper soil layer (0-10 cm) compared to the soil in 0-20 cm. Overall, our results indicate that trees influence the spatial variation of bacteria and fungi mainly by their above and belowground litter inputs and not through their diverse patterns in stemflow.

Thus, additional studies considering root architecture and exudation patterns, and the influence of tree canopy on the spatial distribution of leaf litter fall are necessary to further elucidate interactions between trees and soil microbes.

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TREE SPECIES EFFECTS ON SOIL MICROBIAL COMMUNITIES

SUPPLEMENTARY INFORMATION

Supplementary figures and tables are provided along with the electronic version of this thesis (on DVD), under the following paths:

Figure S1:	Supplementary Information/Chapter III.3/Figure S1.docx
Table S1:	Supplementary Information/Chapter III.3/ Table S1.docx
Table S2:	Supplementary Information/Chapter III.3/ Table S2.docx
Table S3:	Supplementary Information/Chapter III.3/ Table S3.doc

CHAPTER III.4

GROUP-SPECIFIC EFFECTS OF GRASSLAND LAND USE ON SADS

III.4.

Submitted to Agriculture, Ecosystem & Environment

Contrasting effects of grassland management modes on species abundance distributions of multiple groups

Running headline: Group-specific Effects of Grassland Land Use on SADs

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Author's contributions:

NKS & WWW conceived and developed the idea for the manuscript. NKS, TL & WWW refined the intellectual content and scope. NS conducted all analyses and wrote the first draft. FB, NB, SB, RD, MMG, KJ, **KK**, JM, DP, SCR, SS, IS, CW, MW, SW, TW organized or conducted data collection in the field and commented on the manuscript.

Summary

Intensive land use is a major cause of biodiversity loss, but most studies employ simple diversity measures, and analyses of other community attributes are only recently gaining attention. Species abundance distributions (SADs) are one of such community attributes. Their analysis can not only reveal changes in the overall abundance structure within a community but also indicate whether those changes are driven by the abundant or the rare species.

We tested the effect of grassland management intensity (including the land-use modes fertilization, mowing, grazing and a combined measure of land-use intensity) on species richness and SADs for three belowground (arbuscular mycorrhizal fungi, prokaryotes and insect larvae) and seven aboveground groups (vascular plants, bryophytes and lichens; arthropod herbivores; arthropod pollinators; bats and birds). Three descriptors of SADs were evaluated: general shape (abundance decay rate), proportion of rare species (rarity) and proportional abundance of the commonest species (dominance).

Across groups, species richness was largely unaffected by intensification and only decreased with increasing mowing intensity. Of the three SAD descriptors, only the abundance decay rate became steeper with increasing land-use intensity across groups. This change was driven by a decrease in rarity among plants, which were the only group individually reacting to combined land-use intensity. Among the individual groups, bats and birds showed the most differentiated changes in rarity with effects of grazing being positive and effects of fertilization being negative. Decay rate and dominance in insect larvae were affected by mowing and grazing intensity.

Effects of land-use intensity on abundance distributions were not consistent between different groups or land-use modes. Therefore, analyses of individual land-use modes are needed to understand the mechanisms behind overall effects. We also caution against the use of single taxa as surrogates of diversity in other groups. Results suggest that SADs are widely applicable to investigate global and regional changes in terrestrial ecosystems, since effects on dominant and rare species can be clearly disentangled.

GROUP-SPECIFIC EFFECTS OF GRASSLAND LAND USE ON SADS

Introduction

Intensification of land use is one of the main global drivers of biodiversity decline (Vitousek, 1994; Newbold *et al.,* 2015). Many studies on the effect of land-use intensity on biodiversity rely however on single diversity measures, such as species richness or abundance-weighted indices, such as Shannon or Simpson.

While synthetic indices like Shannon or Simpson are clearly useful and incorporate species abundances, they are often used as indicators of changes in diversity without an interpretation of the underlying changes in abundance. The information on species abundances can be exploited in more detail by analyzing species abundance distribution (SADs). In contrast to diversity measures, SADs give information about how the composition of a community changes along a gradient such as land-use intensity (Simons *et al*., 2016). As two communities with identical diversity can differ markedly in their abundance structure, species composition or functional diversity, much progress has been made towards understanding such differences. For instance by analyzing beta diversity to distinguish species turnover (i.e. changes in composition) from nestedness (Baselga, 2010; Solar *et al*., 2015), or by calculating mean functional traits and functional trait diversity (Birkhofer *et al*., 2015; Simons *et al*., In press). However, species-abundance distributions (SADs) have seldom been used to assess the effects of global or regional changes on species assemblages (McGill *et al,*. 2007; Simons *et al*., 2015). This is surprising, given that the typical abundance structure of species communities with few dominant and many rare species had been recognized as a fundamental principle in ecology already in the 1930s (Motomura, 1932).

SADs can be quantified by the slope of a regression between the logarithm of species abundances and species ranks (Figure 1). Variations in this slope among communities reflect differences in the numerical hierarchy of species in a community, which in turn often reflects their competitive ranking. In addition, SADs contain information on the dominance structure of a community (i.e. the relative abundance of the most abundant species) and on the proportion of rare species in a community (i.e. the length of the SAD tail) (Figure 1). With those three descriptors of SADs, one can first estimate the overall change in the abundance structure of communities along environmental gradients (by changes in the SAD's steepness) and then disentangle the two possible mechanisms (i.e. change in dominance vs. change among the rare species) which can lead to a change in the overall shape. Previous studies have shown that land-use intensity in grassland has more pronounced effects on rare than on common species (Allan *et al*., 2014), indicating that land-use intensity can have variable

effects on different portions of an SAD. Unawareness of such differences can substantially bias our expectations or conclusions on how land use affects community resistance and resilience, interactions within communities, ecosystem functions or ecosystem services (Naeem and Wright, 2003; Cadotte *et al*., 2011; Wood *et al*., 2015).

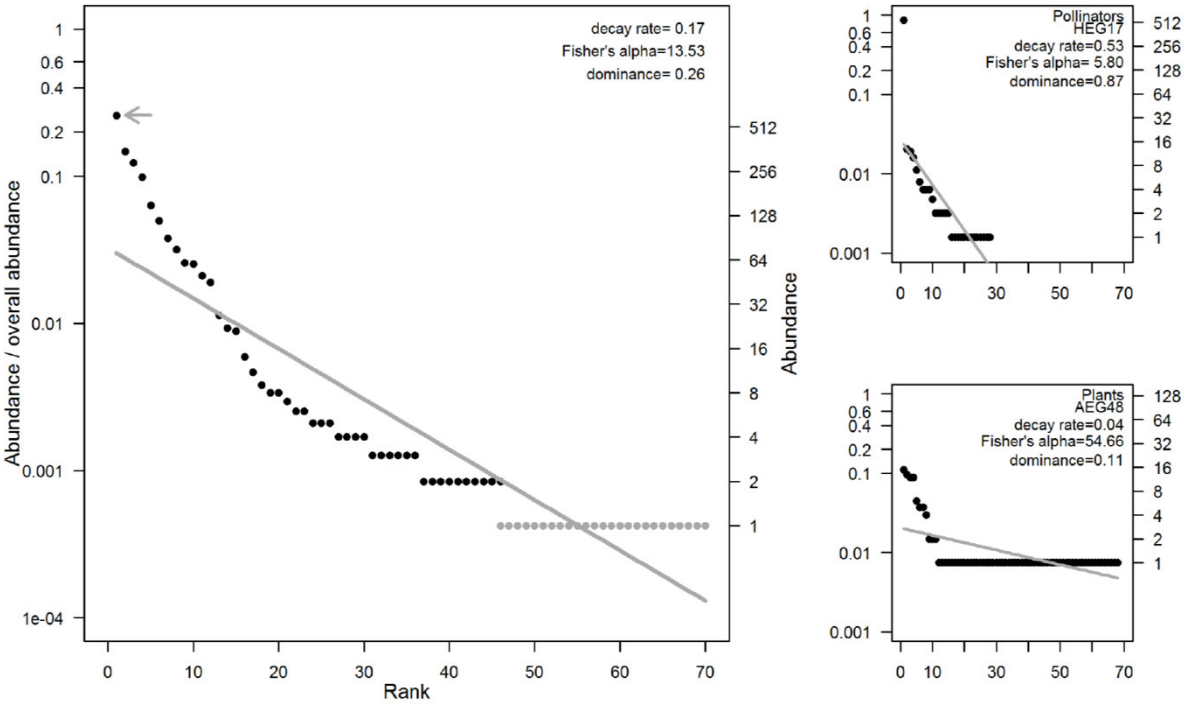

Figure 1: Conceptual figure of the three SAD descriptors (left graph) and examples of species-abundance distributions from our data set (right graphs). The abundance decay rate indicates the steepness of the distribution (indicated by the grey solid line). Berger-Parkers dominance is a descriptor of the dominance of the most common species and calculated as the relative abundance of this commonest species in relation to the overall abundance (indicated by the grey arrow). Fisher's alpha is a descriptor of the proportion of rare species (indicated by the grey points). The upper right graph shows data from pollinators which were sampled on an unfertilized, grazed grassland in the region Hainich-Dün and was selected as it shows the highest abundance decay rate in a community with more than 20 species. The lower right graph shows data from plants (i.e. vascular plants, bryophytes and lichens) which were sampled on an unfertilized, grazed grassland in the region Schwäbische Alb and was selected as it shows the lowest abundance decay rate among the communities with a similar number of species as the other examples.

In a recent study, Simons *et al.* (2015) found that land-use intensity leads to steeper abundance distributions in arthropod communities by way of an increase in dominance. The effect on dominance was mainly driven by fertilization, whereas other land-use modes, i.e. grazing and mowing, affected the number of rare species. The different grassland land-use modes -grazing, mowing or fertilization- were also found to have distinct effects on the species richness of plants (Socher *et al*., 2013) and to have opposing effects on the functional diversity of arthropods (Simons *et al*., In press). Differentiated effects of land-use modes have also been shown in forests, where structural properties and structural complexity of individual stands showed a variety of effects on species abundance patterns of different taxa (Jung *et al.,*

2012; Gossner *et al*., 2014). Despite differences between single modes, compound measures of land-use intensity are useful to compare effects across ecosystems, landscapes or management approaches. They sometimes are also effective predictors of species richness across taxonomic groups (Gossner *et al*., 2014). Nonetheless, it is important to disentangle the effects of single land-use modes on diversity in order to better understand the mechanisms behind the loss of biodiversity in managed landscapes.

By disentangling the effects of different land-use modes on abundance structures in grassland arthropods and by comparing the two mechanisms behind changes in SADs, Simons *et al*. (2015) showed that intensive land use (especially fertilization) increases the dominance within communities. While we have surely gained valuable new insights from this study, we should not assume that changes in other taxonomic groups are driven by the same mechanisms. In fact, effects are likely to differ among some taxonomic groups and converge between others. Therefore, the effects of land-use intensity on species-abundance distributions should be assessed on the widest possible range of functionally relevant organisms. We collected abundance data from ten taxonomic and/or functional groups which were sampled on the same plots along a gradient of grassland land-use intensity to answer the following questions:

1. Does land-use intensity increase the steepness of the species-abundance distribution in all groups?
2. Are changes in SAD steepness driven by changes in the dominance of the most abundant species or by changes in the number of rare species?
3. Do the strength of land-use intensity effects and the mechanisms behind the observed changes differ between groups?
4. Do different land-use modes have similar or divergent effects on SAD descriptors?

Material and Methods

Study system and land use

The study was conducted within the large-scale and long-term Biodiversity Exploratory project (Fischer *et al*., 2010), which comprises three regions in Germany: (1) the UNESCO Biosphere Reserve Schorfheide-Chorin in the North-East (53°02' N 13°83' E, about 1300 km² in size, 3–140 m a.s.l.), (2) the National Park Hainich and its surrounding areas in Central Germany (51°20' N 10°41' E, about 1300 km², 285–550 m a.s.l.), and (3) the UNESCO Biosphere Reserve Schwäbische Alb in the Swabian Jura in the South-West (48°43' N 9°37' E, about 422 km², 460–860 m a.s.l.). In each of the three regions, 50 plots of 50 m × 50 m size were selected on managed grasslands to cover the entire range of land-use intensity and land-use modes representative for these regions (see Fischer *et al*., 2010, for details). All grasslands are continually managed by farmers as meadows (only mown), pastures (only grazed) or mown pastures (mown and grazed), which are either unfertilized or fertilized.
Land-use intensity on each plot was assessed yearly through standardized questionnaires since 2006. Mowing intensity was expressed as the number of cutting events per year. Grazing intensity was represented by the standardized number of grazer individuals (cattle, sheep and/or horses) per hectare times the number of days the plots were grazed per year. Fertilization intensity includes nitrogen amounts from chemical fertilizer, manure or slurry per hectare (see Blüthgen *et al*., 2012, for a more detailed description). Intensities of the three land-use modes were standardized by dividing the values by the corresponding mean from the respective region and then combined into a standardized index of land-use intensity (LUI) by summing the resulting values for the three modes and taking the square-root to achieve more evenly distributed data (Blüthgen *et al*., 2012). We used the mean LUI and the mean intensity of the single land-use modes over three years (2006-2008) to better represent long-term land use.

Biodiversity sampling

We compiled biodiversity data from ten taxonomic and/or functional groups for which richness and abundance data was available, comprising three belowground groups (soil prokaryotes, arbuscular mycorrhizal fungi and insect larvae) and seven aboveground groups (vascular plants, bryophytes, lichens, herbivorous arthropods, pollinators, bats and birds). All taxa within each group were sampled within the same assessment, i.e. with the same method on different subplots within the 50 m × 50 m plots.

Belowground groups were sampled from soil cores. *Prokaryotes* and *arbuscular mycorrhizal fungi (AMF)* were sampled from 14 soil cores (40 cm length, 5 cm diameter) per plot, taken in May 2011. All soil cores per plot were homogenized and combined into one sample per plot (see also Solly *et al*., 2014). Total microbial DNA was isolated from soils using a MoBioPowerSoil DNA Isolation Kit. Operational taxonomic units (OTUs) of prokaryotes were determined to species level based on pyrosequenced V3-V5 regions of the 16S rRNA gene using the QIIME software package version 1.8 (Caporaso *et al*., 2010). The NS31-AM1 fragment of the fungal 18S rDNA was amplified using arbuscular mycorrhizal fungal specific primers (Morris *et al.,* 2013) and sequenced using a Genome Sequencer FLX+ 454 System. The reads were quality filtered using MOTHUR (Schloss *et al*., 2009) and classified using the MaarjaM AMF reference database (Opik *et al.*, 2010). Detailed description of the data processing is presented in Supplementary Information (Appendix S1). *Insect larvae* were extracted from an additional soil core per plot (5 cm depth, 20 cm diameter) sampled in April 2011, by means of a heat/moisture gradient in the cores (Kempson *et al.*, 1963) over a period of eight days. Extracted larvae were stored in 70 % ethanol until identification to family level (Stehr, 1991; 2005). Although belowground taxa were sampled two years after the aboveground taxa and are likely affected by the land use in the years 2009 and 2010, we used the 2006-2008 index for the entire dataset. Given that the combined land-use intensity over this triennium is highly correlated with the combined land-use intensity from 2009 to 2011 ($F_{1,148}$=746.26, p<0.001; R^2=0.83; slope=0.99±0.04) the choice of index should not affect the results.

Vascular plants, *bryophytes* and *lichens* were assessed in 4 m × 4 m subplots in each plot in early summer 2008. Species abundances were estimated as percentage of ground cover. For details on vascular plant sampling see Socher *et al.* (2012) and for bryophyte and lichen sampling Müller *et al.* (2012). Vascular plants, bryophytes and lichen were then combined as one group (hereafter referred to as "plants"), because of low species richness of bryophytes and lichens on some plots. *Herbivorous arthropods* were sampled twice, in June and August 2008, by sweep-netting with a total of 60 double-sweeps in transects along three plot borders (Simons *et al.* 2014). Only twice-sampled plots were analyzed and data from the two samples were pooled. Hemiptera: Cicadina (Cicadomorpha, Fulgoromorpha), Hemiptera: Heteroptera, Coleoptera and Orthoptera were determined to species level. Only adult individuals and herbivorous species were included in the analysis. The assignment of feeding guilds followed Gossner *et al.*, (2015). Abundances of *pollinators* were assessed in 2008 during peak flowering (May to August). On 31 plots, no flowering plants were observed at the

time of visit, mainly due to grazing or mowing. On the remaining plots, 162 surveys were conducted (Alb: 63; Hainich: 51; Schorfheide: 48) in total, sampling 29 plots up to four times as pollinator composition changes during the flowering period. Each survey covered a transect area of 200 m × 3 m three times during six hours of morning and afternoon sampling. Only insects posed directly in the center of the flowers while seemingly feeding on pollen or nectar were caught with an insect net or with help of an exhauster. Insects resting on petals were not taken into consideration. *Bird* species and their abundances were scored by standardized audio-visual point-counts for five periods of 5 minutes per point count, locality and season (Renner *et al.*, 2014). Since abundances in each year were very low, we combined counts from the years 2008 to 2012. *Bats* were assessed with standardized acoustic surveys (Jung *et al.,* 2012) between June and September in the years 2008 to 2012. Acoustic monitoring does not allow the identification of individuals, therefore we used the cumulative number of species presence records per plot within the five year study period as a measure of abundance. Bats and birds were analyzed together because of low species richness of the individual groups in some plots.

We used different levels of taxonomic resolution (species, family or operational taxonomic unit) depending on the taxon. Hence, taxonomic richness instead of species richness is used to describe effects on diversity. Individuals which could not be identified to species (plants, aboveground arthropods) or family level (belowground insect larvae) were excluded from the analysis.

Land-use effects on SAD descriptors

As species-abundance distributions (SADs) can only be calculated above a minimum number of species and individuals, we excluded plots with fewer than three taxonomic units or fewer than five counts overall (5 individuals or 5% cover) per group. Six plots were excluded for insect larvae, three plots for birds plus bats, two plots for AMF and one plot for pollinators. All other groups were sampled with at least five counts and at least three taxonomic units on all plots. For each group and plot, we counted the overall number and abundance of taxonomic units (species, families, OTUs) to which we fitted species abundance distributions. All analyses were conducted in R v.3.1.2 (R Core Team, 2014).

Three descriptors were extracted from the SADs: abundance decay rate to describe the overall shape of the SAD, the Berger-Parker index to describe dominance, and the standardized value of Fisher's alpha to describe rarity. The abundance decay rate was calculated from the geometric series, or niche pre-emption model (Motomura, 1932), in which

the expected abundance n of a species i is defined by the total number of individuals N of all species, the estimated abundance decay rate r per rank and by a constant factor C (defined by r and the number of species S) (McGill, 2011):

$$n_i = N\,C\,r\,(1-r)^{i-1} \text{ with } C=[1-(1-r)S]^{-1}$$

Although the above model includes two parameters (r and C), it can be treated as a one-parameter model, because C is defined by r (Oksanen *et al.*, 2012). The abundance decay rate r is the fitted parameter and represents the slope of the model. We extracted the abundance decay rate r from the niche pre-emption model using the R package 'vegan' (Oksanen *et al.*, 2012). The dominance d (May, 1975) and Fisher's alpha were extracted from the log-series distribution (Fisher *et al.*, 1943) fitted with the R package 'sads' (Inacio Prado and Dantas Miranda, 2013). Dominance d, or Berger-Parker Index (May, 1975), is calculated as the count of the most abundant taxonomic unit (N1) divided by the total count over all taxonomic units (N). The value for Fisher's alpha was corrected by the number of taxonomic units (alpha/S). Restriction to plots for which SAD descriptors were obtained for all groups would have almost halved the number of observations (to 87 of 150 plots), therefore missing data from individual groups were coded as NA (not available) in the dataset. The number of plots with data for each group are shown in Table 1.

The effect of land-use intensity on each of the three descriptors was analyzed with linear mixed effect models within the R package 'lmerTest' (Kuznetsova *et al*., 2014). Each model included an interaction between land-use intensity (one of the three modes or all three combined) and the groups as fixed effects. Both the abundance decay rate and dominance were positively correlated with the number of taxonomic units (richness), hence we used the residuals from linear models between richness and abundance decay rate or dominance, respectively. We were not interested in estimating the differences in effects between regions, instead we allowed random variation of slopes and intercepts between regions within the groups. Therefore, we included two random effects in the model:

response ~Land use*Group+ (1|Group:Region)+(0+LUI|Group:Region)

Table 1: Overall number of taxa and counts as well as average and extreme values per plot in each sampled group. Taxonomic resolution is organizational taxonomic units on species level (3% genetic divergence) for procaryotes and arbuscular mycorrhizal fungi (AMF), family for insect larvae and species for other groups. Each group can include several taxonomic groups, but each group was assessed with the same method (see Method section for more detail). Nr plots indicates the number of plots which were sampled/on which the group was recorded.

Group	Richness				Abundance*				Nr. plots	Reference
	total	min	mean	max	total	min	mean	max		
Prokaryotes	7017	599	778.41	1149	807,188	3482	5381.25	9983	150/150	1
AMF	270	1	29.30	67	33,849	4	225.66	546	150/150	1
Insect larvae	31	1	5.85	11	5,075	1	33.83	120	150/150	1
Vascular plants	269	9	24.45	57		63%	132%	195%	145/145	2
Bryophytes	50	1	2.71	14		1%	12%	133%	146/144	1
Lichens	63	1	9.64	25		1%	11%	32%	145/14	1
Herbivores	382	9	29.50	55	54,181	33	389.79	2690	139/139	2
Pollinators	739	2	39.64	95	25,309	3	212.68	662	119/119	3
Bats	9	1	3.73	7	1,200	1	8.00	24	150/148	1
Birds	85	1	5.78	25	2,993	1	19.95	239	150/144	4

* for prokaryotes: number of detected sequences; for vascular plants, bryophytes and lichens: % cover.
1: data published in the Supplementary Material of this publication. 2: data published in Simons etal. (2014) PLoS One 9:e107033. 3: data published in Weiner etal. (2014) Ecology 95:466-474. 4: data published in Renner etal. (2014) PLoS One 9:e112347

Including two random effects in the model ensured that the variation in slopes and variation in intercepts were uncorrelated. If one of the two random effects did show zero variation, it was excluded from the model. The overall effects of land-use intensity, group and their interaction on the three descriptors were tested for significance using F-values from ANOVA with Satterthwaite approximation for degrees of freedom. For the overall effect for land-use intensity the groups are ignored, hence one intercept and one slope across all data points is calculated. A significant effect of group indicates that intercepts differ between groups. The interaction term between land-use intensity and group tests if slopes differ between groups but does not show which of the groups differ from each other. Differences in intercepts and slopes between the groups were tested for significance using t-Tests with the same approximation for degrees of freedom. Note however that differences are only tested for each group against the first group (i.e. the reference or control). In our analyses, soil prokaryotes are the reference group, hence a significant t-Test for plants would show that the intercept of plants differs significantly from the intercept of soil prokaryotes. Equivalently, a significant t-Test for the interaction between land-use intensity and plants would show that the slope of plants is significantly different from the slope of soil prokaryotes. If intercept or slope of soil prokaryotes differ significantly from zero and none of the t-Tests with the other groups is significant, one can infer that intercepts and slopes are significantly different from zero in all

groups. Both F-tests and t-tests were run in the R package 'lmerTest' (Kuznetsova *et al.*, 2014).

Results

Across all groups and all 150 plots, 84,565 individuals of insects (below-and aboveground) and 4,193 individuals of vertebrates together with 841,037 sequences of soil prokaryote and arbuscular mycorrhizal fungi DNA were recorded, which represent 7,287 organizational taxonomic units of prokaryotes and mycorrhiza, 31 families of insect larvae, 1,121 species of insects and 94 vertebrate species. Additionally, 382 species of vascular plants, bryophytes and lichens were recorded (Table 1).

Table 2: Linear mixed effect model results (F-values) for the effect of land-use intensity (LUI), and the three land-use modes on taxonomic richness and SAD descriptors. Land-use modes are grazing (Graz), fertilization (Fert) and mowing (Mow) intensity. SAD (species-abundance distribution) descriptors are abundance decay rate (for the general shape of the SAD), corrected Fisher's alpha (corrected for the number of taxa, i.e. equivalent to proportion of rare species) and Berger-Parker dominance (the proportional abundance of the most abundant taxon). Models included random variation of slopes and intercepts between regions within groups (for full statistics see Tables S1-S8). For abundance decay rate and dominance, residuals were taken from linear models with taxonomic richness, to correct for effects of taxonomic richness on those two descriptors. Significance levels are based on F-values, calculated by a type III analysis of variance with Satterthwaite approximation for degrees of freedom within the 'lmerTest' package in R (Kuznetsova et al. 2014). Number of observations: 966; number of groups: 7, number of regions: 3. ***: p<0.001 / **: p<0.01 / *: p<0.05.

	Taxonomic richness	Abundance decay rate	Fisher's alpha	Dominance
Land-use intensity	0.38	7.63 *	1.20	3.21
Group	300.02 ***	6.84 ***	5.59 **	2.93 *
LUI:Group	0.56	2.30	3.85 **	1.10
Fertilization intensity	0.23	7.53 *	2.76	5.92 *
Group	219.58 ***	8.22 ***	20.87 ***	3.84 **
Fert:Group	0.06	0.93	2.80 *	1.46
Mowing intensity	1.71	3.39	3.86	1.56
Group	320.28 ***	3.47 **	9.95 ***	1.81
Mow:Group	0.69	2.01	0.81	1.50
Grazing intensity	0.00	0.61	0.36	0.84
Group	332.26 ***	44.80 ***	12.10 ***	4.83 **
Graz:Group	0.06	3.78 **	2.94	3.16 **

We found no overall effect of combined land-use intensity on taxonomic richness (Table 2) and effects among individual groups were also not significant (Figure S1A & Table S1). Among the single land-use modes, mowing intensity did not show an overall effect on taxonomic richness (Table 2) but negative effects for individual groups (Figure S1B, Table S2). Both fertilization and grazing intensity did not show significant overall effects on taxonomic richness, and also did not influence the richness of the individual groups (Table S3-S4, & Figure S1C-D).

Land-use intensity effects on SAD descriptors

The combined land-use intensity showed a significant but weak positive overall effect on abundance decay rate (Table 2, Table S5). Among the individual groups, the slope of soil prokaryotes (i.e. the reference group) was not significantly different from zero and only plants had a significantly greater slope (Figure 2A, Table S5). However, this significant difference did not lead to a significant interaction between land-use intensity and group (Table 1). Hence, the overall effect can also be driven by differences in the average abundance decay rate across groups with increasing land-use intensity. In fact, insect larvae had a significantly higher average abundance decay rate (indicated by significant differences in the groups' intercepts), and plants had significantly lower abundance decay rates than soil prokaryotes (Table S5 & Figure 2A). We found no overall effect of combined land-use intensity on neither dominance nor rarity (Table 2). However, we found a significant interaction between the combined land-use intensity and the groups for rarity (Table 2). This interaction was driven by a significant decrease in rarity for plants with increasing combined land-use intensity (Figure 2B & Table S6). The combined land-use intensity did neither have an overall significant effect nor an effect on dominance for the individual groups (Figure 2C, Table S7).
In summary, plants showed the clearest response such that rarity decreased with increasing combined land-use intensity. The combined land-use intensity increased the steepness of the abundance distributions across groups, mainly due to the effect on plants and the differences in the average level of abundance decay rates.

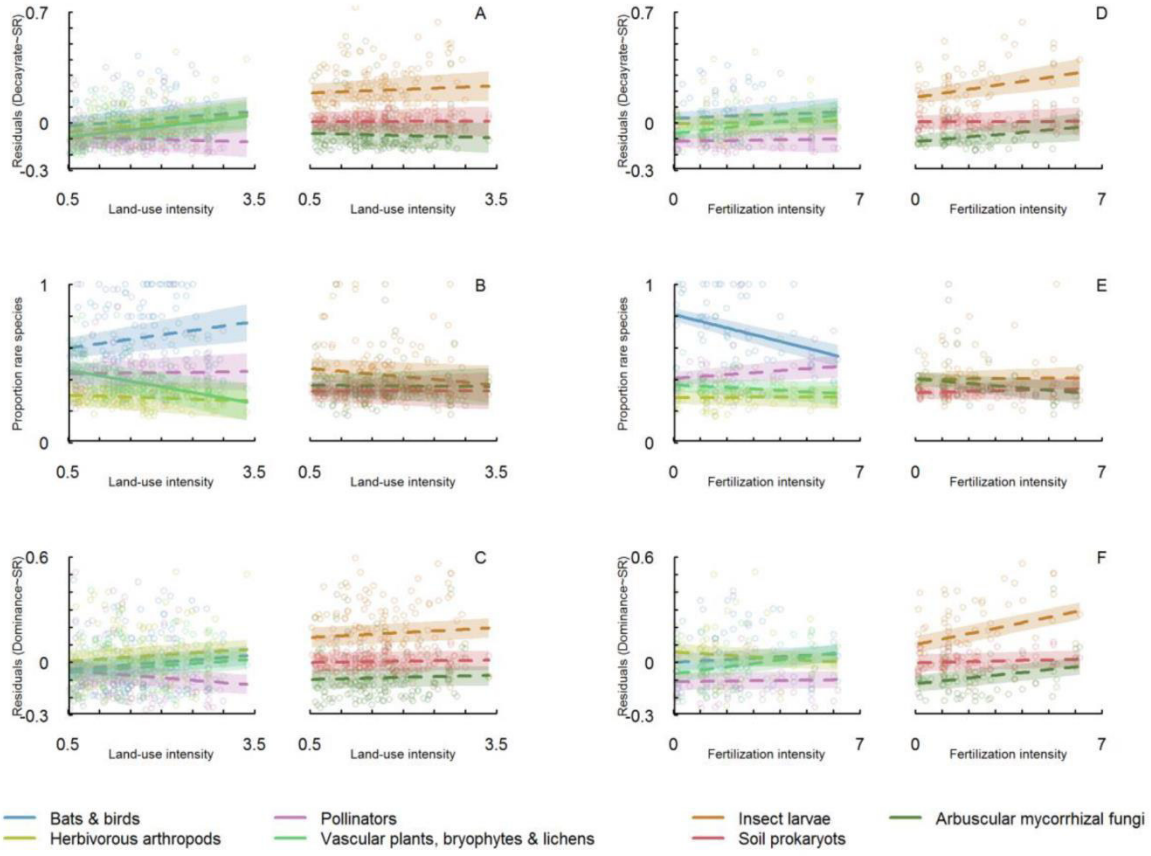

Figure 2: Effects of combined land-use intensity (A-C) and fertilization intensity (D-F) on the three descriptors of the species-abundance curves. The seven groups are indicated by different colors. Lines show the predicted values from linear mixed effect models with the shaded area indicating the standard deviation of the slope and intercept between regions within groups. For abundance decay rate and dominance, mixed effect models were calculated for residuals taken from linear models between taxonomic richness and abundance decay rate and dominance, respectively to estimate the effect of land-use intensity independent of the effects of taxonomic richness. Solid lines indicate significant ($p<0.05$) interactions between the respective group and the slope from post-hoc tests conducted on the linear mixed effect models. Dashed lines indicate non-significant post-hoc tests.

Effects of land-use mode on SAD descriptors

We found a significant but weak positive overall effect of fertilization intensity on abundance decay rate (Table 2, Table S8), but none of the individual groups showed significant changes in decay rate (Table S8, Figure 2D). The analysis for rarity showed a significant interaction between fertilization intensity and group, driven by a significant decrease in vertebrate rarity (Table S9 & Figure 2E) with increasing fertilization. Fertilization intensity had an overall effect on dominance across groups but no effect on the dominance in individual groups (Table S10, Figure 2F).

Mowing intensity showed no overall effect on abundance decay rate (Table 2). Among the individual groups, insect larvae showed a significantly greater slope for abundance decay

rate than the soil prokaryotes, for which slope was not different from zero (Table S11 & Figure 3A). However, this did not lead to a significant interaction between mowing intensity and group (Table S11). Mowing intensity did not show an overall or group-specific effect on rarity (Table S12, Figure S3B). While mowing intensity did not have an effect on dominance across groups (Table 2), insect larvae showed a significantly greater slope for dominance than the reference group (Table S13, Figure S3C).

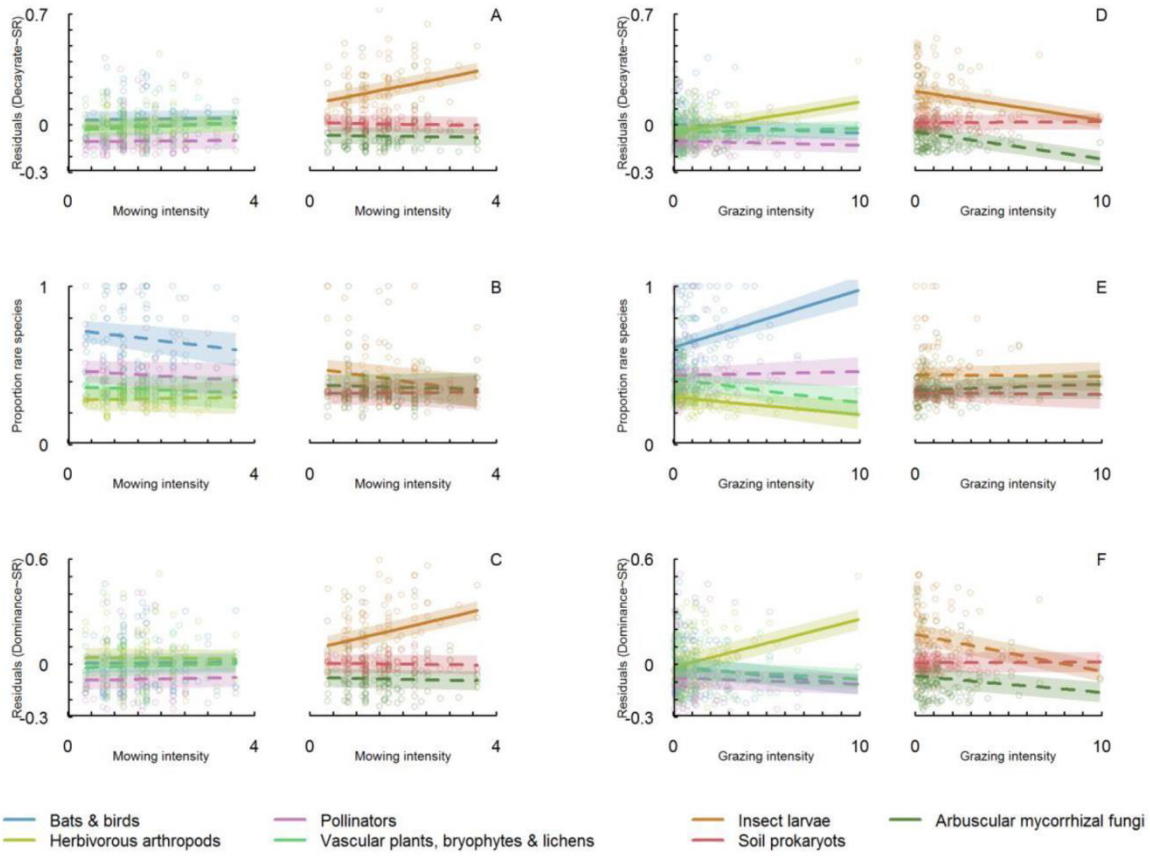

Figure 3: Effects of mowing (A-C) and grazing (D-F) intensity on the three descriptors of the pecies-abundance curves. The seven groups are indicated by different colors. Lines show the predicted values from linear mixed effect models with the shaded area indicating the standard deviation of the slope and intercept between regions within groups. For abundance decay rate and dominance, mixed effect models were calculated for residuals taken from linear models between taxonomic richness and abundance decay rate and dominance, respectively to estimate the effect of land-use independent of effects of taxonomic richness. Solid lines indicate significant ($p<0.05$) interactions between the respective group and the slope from post-hoc tests conducted on the linear mixed effect models. Dashed lines indicate non-significant post-hoc tests.

Grazing intensity did not show an overall effect on abundance decay rate but the model results showed a significant interaction between grazing intensity and the groups (Table 2). This interaction was driven by a significant decrease in the abundance decay rate of insect larvae and a significant increase in abundance decay rate of herbivorous arthropods with increasing grazing intensity (Table S14, Figure 3D). While grazing intensity showed no

significant overall effect on rarity, vertebrates had a significantly greater slope for rarity than soil prokaryotes (Table S15, Figure 3E). As for abundance decay rate, grazing intensity showed a significant interaction with group for its effect on dominance (Table 2), driven by a significantly greater slope for herbivorous arthropods compared to the reference group (Table S16, Figure 3F). The effect of grazing intensity on abundance decay rate and dominance of insect larvae and herbivorous arthropods were strongly influenced by one plot with a very high grazing intensity (compare to results without the most extreme grazing value in Table S17-Table S20).

In summary, we found that only fertilization intensity shows the same effect on abundance decay rate across groups as the combined land-use intensity. Instead, different land-use modes show distinct effects on abundance decay rate and rarity for individual groups.

Discussion

The combined land-use intensity affected species richness and descriptors of abundance structures only in plants. Regarding differences between groups and land-use modes, rarity of vertebrates showed the most differentiated reaction with effects of grazing being opposite to effects of fertilization. Across individual land-use modes, effects on dominance were mostly consistent with the effects on abundance decay rate, indicating that changes in decay rates are often driven by changes in dominance.

Species-abundance distributions as indicators of changes in communities

As well as abundance-corrected diversity indices, species-abundance distributions (SADs) can be used to compare changes in communities even if they are sampled by different methods or intensities, because the abundance decay rate is calculated from relative abundances. However, both the abundance decay rate and Fisher's alpha are strongly related to species richness, hence it is important to correct both values for species richness when more than one dataset is compared. When analyzing effects across different groups, the correct interpretation of the statistical results is also crucial. Effects across groups can either be driven by significant effects in all individual groups (i.e. individual groups show the same effect direction and similar effect strength) or by a shift in the occurrence of groups with different average values along the gradient (i.e. values of individual groups do not change significantly). An overall significant effect of land-use intensity on the abundance decay rate

and missing effects among individual groups in our results indicate that the abundance distributions of the individual groups did not change with increasing land-use intensity, but a higher number of groups with steep abundance distributions was sampled under intensive land use than under extensive land use. Effects of single land-use modes on the abundance decay rate in single groups were more often accompanied by an effect on dominance than by an effect on rarity. This indicates that the change in abundance decay rate is mostly caused by an increase in the relative abundance of the most abundant taxon (species or equivalent). The close link between abundance decay rate and dominance suggests that a simple index such as the Berger-Parker dominance is a useful index for rapid community structure assessments.

Responses in different groups

This study includes belowground taxa that are often ignored in multi-group biodiversity studies due to difficulties in sampling, even though they play an important role for many ecosystem processes (Blossey and Hunt-Joshi, 2003). Here they were sufficiently well represented to allow the comparative assessment of response of above and below-ground groups to land-use modes and intensification.

The average abundance decay rate and dominance differed more strongly among belowground groups than among aboveground groups. Even though the taxonomic diversity of prokaryotes and arbuscular mycorrhizal fungi was much higher than those of insect larvae, these differences were not responsible for the differences within the belowground groups, as both values were corrected for taxonomic richness. Among the belowground groups, arbuscular mycorrhizal fungi showed the lowest dominance and shallow, i.e. even, abundance curves. Several other studies have found that species abundance structures of arbuscular mycorrhizal fungi fit better to lognormal or broken stick models than geometric models (Dumbrell *et al*., 2010, Unterseher *et al*., 2011, Moebius-Clune *et al*., 2013). McGill *et al*. (2007) nicely summarizes that "[the] geometric model predicts extremely uneven abundances, broken stick […] extremely even abundances [and] lognormal and logseries are intermediate with distinct predictions about the proportions of very rare species – high in logseries, low in lognormal", hence arbuscular mycorrhizal fungi communities show generally more even distributions, which are characterized by many rare species rather than strong dominance. One notable exception is the study by Moebius-Clune *et al*. (2013), which found a very pronounced dominance of the top ranked taxon. However, this study was conducted in maize fields and might therefore represent a very specific type of community. The generality of even distributions in soil microbes, particularly the prokaryotes and arbuscular mycorrhizal fungi

across our plots (i.e. the absence of a land-use effect), might also be due to the ubiquitous distribution of the majority of the dominant OTU's of these microbes in this ecosystem. A lack of strong land-use effects on the community structure of arbuscular mycorrhizal fungi was also found across a range of agricultural soils in Switzerland (Jansa *et al.*, 2014). In contrast to the soil microbes, insect larvae abundance distributions showed high dominance of few families and were more similar to the abundance structures of above-ground groups. This is not surprising, as insect larvae spend only a part of their life cycle below ground, in their adult stages they are found aboveground. We might hence conclude that there is a fundamental difference in the abundance structures of below- and aboveground groups which deserves further exploration.

None of the individual belowground groups, and only plants among aboveground groups, showed a significant reaction to the combined land-use intensity. The increase in the steepness of the abundance distributions and the decrease in the proportion of rare species among plants is not surprising as more intense grassland management aims at increasing productivity, thereby fostering fast-growing and highly competitive plants (Gaujour *et al.*, 2012). This in turn suppresses the growth of less-competitive species, i.e. mostly herbs which comprise the majority of rare species (Socher *et al.*, 2013). While the proportion of rare plant species decreased with increasing land-use intensity, the relative abundance of the most abundant species (i.e. dominance) did not change. This indicates that increased nutrient availability does not only promote one species, but several of the more abundant species.

Differences between land-use modes

As intensification of land-use intensity in grasslands generally serves the purpose of increasing biomass production, one might expect similar effects with an increase in the intensity of individual land-use modes and a strong effect of a combined index of land-use intensity. In particular, because both grazing and mowing reduce above-ground plant biomass, and fertilization intensity is known to be associated with higher mowing intensity (Blüthgen *et al.*, 2012). In clear contrast to this expectation, we did not find stronger effects of the combined land-use intensity compared to the single land-use modes across groups and even contrasting effects among the single land-use modes.

Insect larvae showed steeper abundance distributions with increasing mowing intensity, driven by an increase in dominance. The three families with the highest relative abundance were either *Bibionidae*, *Staphylinidae* or *Cecidomyiidae* (they comprised between 80 % and 96 % of all individuals on plots with high mowing frequency). Larval abundance has been

found to increase with the percentage cover of bare soil, and facilitation of larval development due to elevated soil temperature has been suggested as underlying mechanism (Sonnemann, *pers. comm.*). Percentage cover of bare soil and thus soil temperature also increases after mowing. While optimal soil temperatures should benefit all larvae, positive effects are expected to be most pronounced in dominant families, which may exhibit a more r strategic life history, and for which season and other environmental factors are apparently also optimal at time of sampling. While mowing increases soil temperature evenly across a grassland, grazing creates patches of dry-warm and cold-moist soil. This increase in diversity of niches may lead to the reduced steepness of the abundance distribution in insect larvae.

For vertebrates (i.e. bats & birds), a higher level of grazing intensity entailed a higher proportion of rare species, while the proportion of rare species decreased with increasing fertilization intensity. Possible mechanisms behind the negative effect of fertilization intensity could be a more homogeneous vegetation structure which is not attractive for ground-breeding birds. Ground-breeding birds such as the Eurasian skylark (*Alauda arvensis*) have been found to be very sensitive to agricultural activities in grasslands (Donald *et al*,. 2002). A homogenous canopy structure also lacks taller plants (e.g. thistles) which are used by different birds as stalking aids. Grazing however leads to heterogeneous and patchy vegetation including spots of bare soil (important as dust baths for some birds) or water holes. The feces of the grazing animals might also increase the abundance of insects (dung beetles, flies, etc.) as food source. Those can hide less well in the short vegetation and are hence easily accessible for birds and bats. Additionally, the dominance of herbivorous arthropods increased with increasing grazing intensity, indicating that resource availability for carnivorous birds increases. All those factors should lead to a decreased competition for resources on grazed sites, which allows more species to persist ('More Individuals Hypothesis'; Srivastava and Lawton, 1998). Despite those possible local-scale factors, birds and bats are generally more affected by heterogeneity on the landscape scale (e.g. structural elements such as hedges, forest patches or old buildings). An analysis on the direct and indirect effect of land use across different scales on bats has indeed found that land-use changes at larger spatial scales are more important than the local land use (Treitler *et al.,* submitted, *pers. communication* K. Jung). Due to their high mobility, bats and birds can easily evade to alternative sites when the local land-use intensity changes. Thus their use as biodiversity surrogates or indicators for local- and plot-scale effects of land use in European grassland systems concerning the diversity of other taxa is limited. The use of birds as surrogates should hence be limited to ecologically very similar groups only. Several other

authors (Lawton *et al*., 1998; Billeter *et al*., 2007; Dormann *et al*., 2007; Gossner *et al*., 2013) have already cautioned against the use of single indicator taxa for management or conservation strategies and our results strengthen their argument.

Conclusions

We showed that the intensification of different land-use modes has variable effects on community abundance structures and that effects are not consistent between taxonomic groups. Hence, effects of individual land-use modes on diversity should be considered as well as the effect of overall intensification. We also caution against the use of single taxa or trophic groups as indicators of overall biodiversity changes because we did not find consistent patterns of land-use effects across groups. A negative effect of intensive land use on diversity is mostly not contested (Sala *et al*., 2000; Newbold *et al*., 2015), its effects on the structure of communities are however still not considered by default. Changes in abundances within communities are however important for ecosystem production, function and services as those are more strongly affected by species abundances than by species numbers (e.g. Soliveres *et al*., in press). While rare species are generally considered to be not as important for ecosystem functions as common species (one reason why Shannon or other indices basically ignore rare species, depending on the Hill coefficient), they are important for biodiversity conservation. Therefore, we recommend to include species abundance distributions in the standard toolkit of biodiversity studies in terrestrial habitats. They are easy to use, comparable across groups and disentangle effects on common and rare species.

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SUPPLEMENTARY INFORMATION

Supplementary figures and tables are provided along with the electronic version of this thesis (on DVD), under the following paths:

Appendix S1:	Supplementary Information/Chapter III.4/Appendix S1.docx
References:	Supplementary Information/Chapter III.4/References.docx
Figure S1:	Supplementary Information/Chapter III.4/Figure S1.docx
Table S1:	Supplementary Information/Chapter III.4/ Table S1.docx
Table S2:	Supplementary Information/Chapter III.4/ Table S2.docx
Table S3:	Supplementary Information/Chapter III.4/ Table S3.doc
Table S4:	Supplementary Information/Chapter III.4/ Table S4.docx
Table S5:	Supplementary Information/Chapter III.4/ Table S5.docx
Table S6:	Supplementary Information/Chapter III.4/ Table S6.doc
Table S7:	Supplementary Information/Chapter III.4/ Table S7.docx
Table S8:	Supplementary Information/Chapter III.4/ Table S8.docx
Table S9:	Supplementary Information/Chapter III.4/ Table S9.doc
Table S10:	Supplementary Information/Chapter III.4/ Table S10.docx
Table S11:	Supplementary Information/Chapter III.4/ Table S11.docx
Table S12:	Supplementary Information/Chapter III.4/ Table S12.doc
Table S13:	Supplementary Information/Chapter III.4/ Table S13.docx
Table S14:	Supplementary Information/Chapter III.4/ Table S14.docx
Table S15:	Supplementary Information/Chapter III.4/ Table S15.doc
Table S16:	Supplementary Information/Chapter III.4/ Table S16.docx
Table S17:	Supplementary Information/Chapter III.4/ Table S17.doc
Table S18:	Supplementary Information/Chapter III.4/ Table S18.docx
Table S19:	Supplementary Information/Chapter III.4/ Table S19.docx
Table S20:	Supplementary Information/Chapter III.4/ Table S20.doc

Chapter III.5.

FOREST MANAGEMENT AND BIODIVERSITY

III.5.

Manuscript ready for submission

Unforeseeable effects of forest management systems on biodiversity of temperate European beech forests – habitat heterogeneity dilution

Runnging title: Forest Management and Biodiversity

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PS and CA designed research, PS, MMG and CA analyzed the data and outlined the manuscript, PS, MMG, SH and CA wrote the paper. SBo, DP, KJ, E-DS and TW contributed to writing. MMG, SBo, SBl, SBö, VB, RD, KG, **KK**, ML, JM, JO, EP, DP, KJ, SR, JS, M.Ts., MTü, BW, TW, FB, MF and WWW contributed data. SBl, SBö, VB, RD, KG, **KK**, ML, JM, JO, EP, SR, JS, MTs, MTü, BW, FB, MF and WWW commented on the manuscript.

Abstract

The need for forest management approaches that integrate commodity production and biodiversity conservation has increasingly been recognized over the last decades. While there is much evidence that biodiversity benefits from replacing clear-cuts by retention tree approaches, knowledge on the effects of spatially more fine-grained management systems, such as single tree selection cutting, on biodiversity is scarce. Nevertheless, finer-grained systems leading to uneven-aged forests are advocated because they are believed to mimic natural small scale gap dynamics of many temperate forests and to promote structural complexity. Here we tested how even-aged forests resulting from shelterwood cuttings and uneven-aged forests affect biodiversity of 15 taxonomic groups. We compared gamma-, beta-, and alpha-diversity in a region where both management systems co-occur. Gamma-diversities of animals, plants, fungi and bacteria were higher in even-aged than in uneven-aged forests, also when focusing on forest specialist species. Differences were driven by a higher beta-diversity in even-aged forests. In addition, both management systems showed limited complementarity in species composition at the landscape scale as the percentage of exclusive species was low in uneven-aged compared to even-aged forests. Our results suggest that coarse-grained heterogeneity of forest landscapes provided by differently aged tree cohorts is more important for biodiversity than fine-grained heterogeneity within uneven-aged stands. Fine-grained heterogeneity may dilute the variability of environmental conditions within age-cohorts in forests. The results question the recent trend towards a replacement of even-aged management in European temperate forests and suggest habitat-heterogeneity-dilution as the mechanism linking the habitat-heterogeneity-hypothesis and the intermediate-disturbance-hypothesis in temperate forests.

Significance Statement

Forest management seeks for approaches which integrate production goals and nature conservation to avoid further biodiversity loss. In Central Europe single tree selection cutting is strongly promoted at the expense of even-aged forest management because it is considered to better preserve biodiversity due to enhanced within stand heterogeneity. In contrast to this expectation, we show that biodiversity across multiple trophic groups including bacteria, fungi, plants and animals consistently benefits from the even-aged forest management. Dilution of habitat-heterogeneity was most likely the cause for the lower biodiversity in

uneven-aged forests. Our findings have important implications for the management of temperate forests as they contradict current forest policy and conservation strategies.

Introduction

Around the world new forest management and conservation approaches are discussed in order to better integrate production goals and nature conservation (Messier *et al*., 2015). Interest in approaches other than the classical even-aged management system has greatly increased in many parts of the world over the last decades (Puettmann *et al*., 2015). The alternative approaches, ranging from the 'close-to-nature' forestry in Europe to the 'new forestry' ecosystem management approach in North America, emphasize not only commodity production but address equally other objectives, such as intrinsic ecosystem values or maintaining species and structural diversity (Puettmann *et al*., 2015). The basic question, whether or not alternative silvicultural approaches can promote biodiversity by simultaneously improving harvest revenues, soil protection, resilience and recreational value, to name just a few, is the same in North America and Central Europe. However, the systems traditionally applied differ strongly between the two continents (Figure 1 *A* and *B*). In temperate North America the traditional clear-cut system is contrasted by the alternative retention tree approach (Lindenmayer *et al.*, 2012; Gustafsson *et al*., 2010). In Central Europe the shelterwood system, in which a cohort of even-aged trees is replaced by a new evenly aged cohort through repeated cuttings over decades, is contrasted by the alternative single tree selection system, where single trees are harvested on a rather fine grain resulting in uneven-aged forests (Pommerening *et al*., 2004; Figure 1 *A* and *B*). Both alternative approaches attempt to ensure habitat continuity (Grove *et al*., 2002; Fritz *et al*., 2008). There is much evidence that biodiversity generally benefits from the retention tree approach when compared to clear-cutting (Lindenmayer *et al*., 2012; Fedrowitz *et al*., 2014; Vanderwel *et al*., 2007; Figure 1 B). Knowledge on the effects of the fine-grained approach on biodiversity in comparison to traditional management is, however, scarce. Nevertheless, all over temperate Europe, fine-grained systems are advocated over the traditional shelterwood system (Pro Silva, 2012). This is because the fine-grained approach is assumed to mimic small scale gap dynamics of natural European beech (*Fagus sylvatica*) forests (Hobi *et al*., 2015), a forest type which would dominate across Central Europe and which has traditionally been managed without clear-cutting. Single tree selection is therefore considered as a 'close-to-nature' management strategy, even though it has limited resemblance to natural forest dynamics

(O'Hara *et al*., 2016)). It was shown that even fine-grained management may affect particular taxonomic groups negatively when compared to no management (Paillet *et al*., 2010; Bässler *et al*., 2014; Birkhofer *et al*., 2012), but it is generally assumed to facilitate alpha-biodiversity (Klopfer and MacArthur, 1960; Carey *et al*., 1999; Brunet *et al*., 2010) by enhancing within stand horizontal and vertical structural complexity and by promoting a high diversity of microhabitats and thus niches for species.

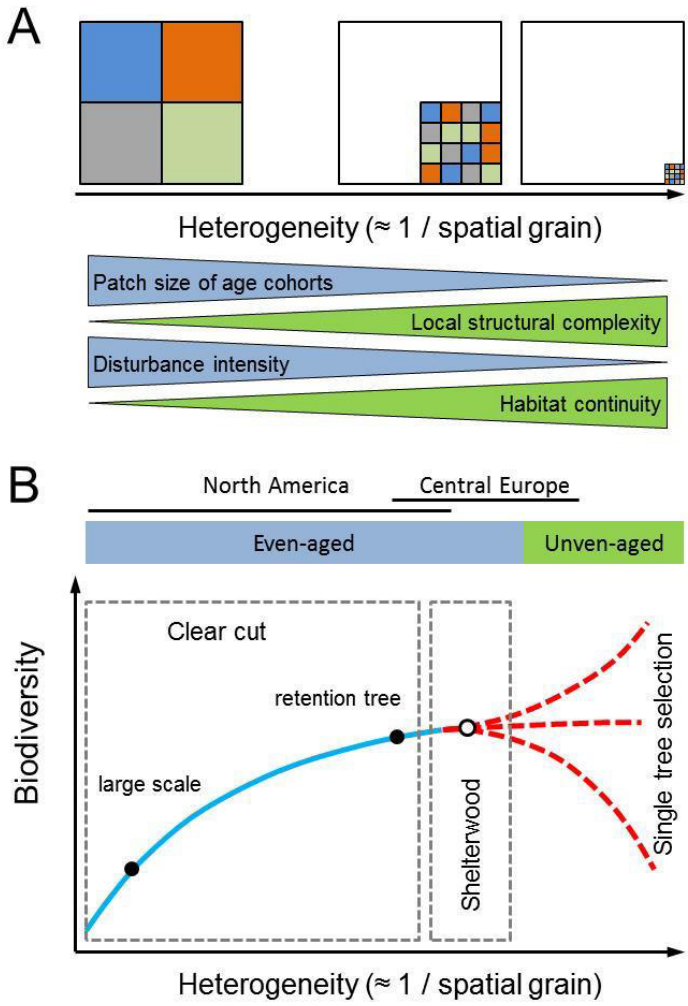

Figure 1. Conceptual framework of possible effects of spatial grain on biodiversity resulting from different forest management systems. (*A*) Forest management systems basically differ in the patch size of cohorts of even-aged trees resulting in different structural complexity on the confined area of a forest stand. Man-made (or natural) disturbance is the driver of spatial grain and also affects local habitat continuity. (*B*) North America and Central Europe differ in the most frequently applied final harvest approaches. While age-class forest management resulting from clear-cuts with and without retention trees is mainly applied across temperate North America, clear-cuts are abandoned in Central Europe. In European temperate deciduous forests age-class forests traditionally result from shelterwood cuttings over 30 to 40 y. The blue line, indicating an increase in biodiversity with increasing spatial heterogeneity, is supported by recent findings from retention tree approaches reporting positive effects for biodiversity. The red dashed line indicates the potential biodiversity effect of forest management systems which further increase spatial heterogeneity, e.g. by single tree selection resulting in uneven-aged forests.

FOREST MANAGEMENT AND BIODIVERSITY

The assumption of facilitating alpha-biodiversity by increasing within stand heterogeneity is based on a main principle of community ecology that suggests a positive relationship between habitat heterogeneity and species diversity (habitat-heterogeneity-hypothesis; MacArthur and MacArthur, 1961; Wilson, 2000). Thus, increasing heterogeneity within stands by fine-grained management, e.g. by creating high variation in tree diameter, height and age is expected to promote biodiversity (Pro Silva, 2012). However, the area-heterogeneity-tradeoff-hypothesis challenges the generality of the positive heterogeneity-diversity relationship (Allouche *et al*., 2012; Stein *et al*., 2014). It is suggested that within a fixed space an increase in habitat heterogeneity reduces the average amount of effective area available for an individual species and will increase its likelihood of extinction. Within a confined area the heterogeneity-diversity relationship may therefore be rather unimodal (Chocron *et al*., 2015; Figure 1 *B*).

Which of these mechanisms are more important in temperate European forest landscapes that are subject to conversion of traditional to alternative forest management is unclear. While the traditional coarse-grained shelterwood cutting results in a coarse heterogeneity of different age-cohorts that represent different forest developmental phases across the landscape, the currently favored fine-grained single-tree selection approach maximizes small-scale vertical and horizontal complexity within a single stand (Figure 1 *A*). Although alpha-diversity, particularly of forest specialist species is expected to increase with decreasing grain size of heterogeneity, the potential outcome for biodiversity at the landscape scale is still an open and challenging question (Figure 1 *B*.). Ideal study sites to test the two contradicting hypotheses are forests that have already been managed for decades in contrasting grain size at a large spatial scale.

We investigated the effect of coarse- and fine-grained heterogeneity as a result of different forest management systems (FMS) on alpha-, beta- and gamma-diversity of 15 taxonomic groups by using presence/absence data collected within the framework of the Biodiversity Exploratories (Fischer *et al*., 2010). The ‘Hainich’ -Exploratory in Central Germany allows a direct comparison of even-aged (EA, N = 17, traditional) and uneven-aged (UEA, N = 13, alternative) forest management systems under comparable climatic and edaphic conditions and a similar spatial arrangement between plots within a region (Table S1). The uneven-aged beech forests of ‘Hainich’ are exceptional in extent and continuity for broadleaved forests of the temperate zone. Whereas uneven-aged forest management is applied in mixed mountain forests of beech, fir, and spruce in several European countries

(Schütz, 2001), its application to broadleaved forests in the 'Hainich' is to our knowledge unique across the Holarctic.

In addition, the 'Hainich'-Exploratory comprises unmanaged (UNM, N = 13, reference) forests (Table S1). However, in Central Europe we face the situation that unmanaged forests are generally scarce and confined to distinct areas (e.g. National parks). In our study region UNM are spatially blocked within the Hainich National Park. The closer distance between UNM plots may affect alpha- and beta-diversity. Therefore, we present results on gamma-diversity for EA and UEA using UNM as a reference, whereas alpha- and beta-diversity is contrasted only between EA and UEA (but see results for EA, UEA and UNM in SI).

The structural differences of EA, UEA and UNM might support complementary of species assemblages. Thus, a mix of FMS within a forest landscape is believed to enhance landscape scale biodiversity (Redon *et al*., 2014). By randomly combining plots (two- and threefold combinations of EA, UEA and UNM), we analyzed if mixing of FMS indeed increases gamma-diversity. To disentangle complementarity further, we analyzed the fraction of exclusive species in each FMS. We considered total species per FMS and the subgroups of forest specialists (narrow niche) and non-forest specialists (wide niche).

We used sample-size based rarefaction and extrapolation to compare gamma-diversity for Hill-numbers ${}^{0}D$ (species richness), ${}^{1}D$ (Shannon diversity) and ${}^{2}D$ (Simpson diversity), which successively increase weighting of abundant species (Chao *et al*., 2014), between FMS and a two- and threefold mixtures of FMS. Beta-diversity was quantified as (multi-site) species turnover (Baselga, 2012).

Methods

Study sites and forest management.

The study forests are located in central Germany, federal state of Thuringia, along the forested hill chains of Hainich, Westerwald, and Dün (51° 02' 45'' N to 51° 22' 12'' N, 10° 12' 28'' E to 10° 32' 03'' E), and are part of the Biodiversity Exploratories project (Fischer *et al*., 2010). The geological surface is Triassic limestone, locally covered by periglacial loess. Natural vegetation is a mesophytic deciduous forest dominated by *Fagus sylvatica* on nutrient-rich soils, with only minor contributions of admixed tree species (*Fraxinus excelsior*, *Acer pseudoplatanus*, *Carpinus betulus*, *Tilia* sp., *Ulmus* sp.) in mid and late successional stages (Bohn *et al*., 2004). Study forests were chosen to represent prevalent forest

management systems (FMS) of the region, which are even-aged (EA, N = 17), uneven-aged (UEA, N = 13) and unmanaged (UNM, N = 13). In avoiding clear-cuts and species change the even-aged and the uneven-aged FMS are variants of continuous cover forestry that vary only in the grain of management operations. Plots (100 m x 100 m in size) of different FMS are separated at minimum 3 km to factor out species spillover between FMS. Characteristics of the environment, spatial arrangement and stand structure of the FMS are listed in Table S1.

The even-aged forests are managed as stands of about 4 ha in size with a rotation of 120 to 140 years following regeneration through seeds from shelterwood trees. Proportional to the share on rotation we selected plots from the developmental phases of thicket, pole wood, immature timber, mature timber, and thicket with shelterwood (3, 3, 4, 4, and 3 plots, respectively). This traditional system of beech forestry has become increasingly uncommon during the last decades and is being replaced by finer-grained regeneration systems that rely on final harvest of single trees (so called target diameter harvest spanning about 3 decades), groups of trees, and, infrequently, of small areas (canopy opening up to 1000 m^2). In fact, current good practice guidelines of forest management strongly discourage the shelterwood system being claimed ‘not natural’, ‘too large in scale’, ‘too high in disturbance intensity’, and ‘homogenizing within stand variability’ (FSC Working Group Germany, 2012). However, in our study area the classical shelterwood management system is still implemented. Uneven-aged forests are managed by single tree harvest and thinning. This single tree selection system is traceable back 140 y at Hainich and 250 y at Dün sites (Wäldchen *et al*., 2013). Unmanaged forest plots are located in the Hainich National Park mainly within the UNESCO World Heritage Site “Primeval Beech Forests of the Carpathians and the Ancient Beech Forests of Germany”. In the 19^{th} century these forests have been managed as coppice with standards for firewood and timber, and then underwent a transformation process into high forests for several decades. Time since abandonment of management is 20 y (5 plots) to 70 y (8 plots).

Environmental conditions and spatial structure of plots are comparable for EA and UEA, while UNM are located at lower elevation in closer distance (Table S1). Additionally, for UNM we found no relationship between distance and compositional dissimilarity (Table S6). This missing effect of distance in UNM may not only affect beta-diversity negatively, but gamma-diversity as well. Alpha-diversity of mobile taxa, in contrast, may be enhanced. Therefore we report results for UNM in SI, but do not contrast unmanaged forests with managed forests for alpha- and beta-diversity.

Species community data.

Arthropods, bryophytes, and lichens were sampled in 2008 and vascular plants in 2009. Birds and bats were assessed in 2009 and 2010, dead-wood fungi in 2010 and 2011, and ectomycorrhizal fungi (DNA) and bacteria (RNA and DNA) in 2011. Selected sampling methods adequately represent the local community within a taxon. Two pitfall traps and four flight-interception traps (two in the understorey and two in the canopy) were used for arthropod sampling. Arthropods were then separated into spiders, harvestmen including pseudoscorpions, beetles, hymenopterans, lacewings and true bugs. Vascular plants, bryophytes, lichens, and dead-wood fungi were sampled in 20 m × 20 m quadrats in the center of each plot. Birds were monitored by the number of singing males, and bats by their flight activities. Assessment of belowground taxa (Ectomycorrhizal fungi DNA, Bacteria RNA/DNA) is based on soil samples from a sampling campaign for microbial analysis. All taxa cover a broad spectrum of functional groups, but are also assumed to respond in different ways to forest management, driven by variation in mobility and structural and microclimatic habitat requirements.

Statistical analyses.

To compare species gamma-diversity for the 15 taxonomic groups between FMS we used a methods framework published recently (Chao *et al*., 2014). This 'diversity accumulation curve' framework extended methods for rarefaction and extrapolation of species richness (species accumulation curve) (Colwell *et al*., 2012). It a) provides estimators for inter- and extrapolation of higher order Hill numbers (Jost, 2006), b) allows to estimate sample completeness (Chao *et al*., 2012) and by this enables a sample-coverage-based estimation, and c) uses a bootstrapping method for constructing confidence intervals around Hill numbers based on the unconditional variance (Colwell *et al*., 2012). This facilitates the comparison of multiple assemblages (Figure S2). Hill numbers ${}^{q}D$ quantify diversity in units of equivalent numbers of equally abundant species by increasingly weighting abundance with the order of diversity q. Diversities with orders <1 disproportionally favor rare species, at order 1 species are weighted proportionally to their frequency in the samples, while all orders >1 disproportionally favor common species. This allows us to analyze the effects of FMS on the diversity of rare and common species within one framework. We estimated species diversity curves for orders 0, 1, and 2: ${}^{0}D$ species richness, ${}^{1}D$ the exponential of Shannon's entropy, ${}^{2}D$ the inverse of Simpson's concentration, for all species of taxonomic groups, forest specialists, and non-forest specialists (SI Methods).

Diversity of even-aged and uneven-aged FMS was compared for a range of base sample sizes (BSS) to assess the robustness of the findings. As BSS we used minimum reference sample size (Min. RSS, i.e. classical rarefaction), maximum reference sample size (Max. RSS, extrapolation of UEA and UNM to the number of samples of EA), and an intermediate value (extrapolation of UEA and UNM and rarefaction of EA) (Table S2). We did not extrapolate beyond max. RSS to factor out differences in the quality of species pool estimates between FMS and taxa, which can affect the shape of the accumulation curve when extrapolating. This was observed for species poor taxonomic groups due to a more random occurrence of singleton species. In Figures 2 and 3 we generally present results of max. RSS, except for bats, harvestmen, lacewings, and lichens. For these taxa, we present results for rarefaction (min. RSS), as estimated pool size (Chao's estimator) of a single FMS exceeded the pool size of the combined FMSs (bats in EA, harvestmen, lacewings, and lichens in UNM). Note that neither the trend nor the significance of the findings was affected by this conservative presentation of results. Significantly different estimates are indicated by pairwise non-overlapping 95% confidence intervals, obtained by bootstrapping based on 200 replications.

Differently managed forests may host different species assemblages, so that landscape scale species diversity increases when mixing management types due to complementarity. To assess complementarity of EA and UEA, we analyzed gamma-diversity for hypothetical mixed forest landscapes composed of 50% EA and 50% UEA plots, using the same approach as for single FMS. Based on 500 resamplings of 9 plots per FMS (out of 17 for EA and 13 for UEA) gamma-diversity of mixed assemblages was quantified by rarefaction, and characterized by mean and 95% quantile (Table S2). Other combinations (two- and threefold) of FMS were analyzed analogously. We identified complementarity when diversity of the mixed system exceeded the diversity of the richer assemblage (Edwards *et al*., 2014). The partial contribution of alpha- and beta-diversity to gamma-diversity is controlled by the plot level sampling effort. Generally, with increasing sampling effort, e.g. more traps or more frequent bird monitoring, alpha-diversity increases on the cost of beta-diversity (as a larger percentage of the species pool is already found within plots). This is why we used alpha- and beta-diversity to compare the structure of assemblages in forests managed differently rather than to characterize the assemblages in absolute terms. Beta-diversity share on gamma diversity was quantified as species turnover, which, other than species nestedness and total beta, is the beta component that effectively contributes to gamma-diversity. This facilitates comparisons between FMS that are unbiased by nestedness and richness differences of

samples within an assemblage. Choosing between the two approaches to quantify species turnover, pairwise site dissimilarity and multiple-site dissimilarity, we selected multiple-site dissimilarity, because the mean of pairwise dissimilarities is a biased estimator for assemblages (Baselga, 2012). However, variability of multiple-site dissimilarities can be assessed only by resampling. Therefore, we quantified beta-diversity as multiple-site turnover component of Jaccard dissimilarity (Baselga, 2012) based on 200 resamplings of 9 plots per FMS (out of 17 for EA and 13 for UEA) (Table S3, Fig. 1 D, E, F). For testing differences between EA and UEA we used pairwise comparison of resamplings (i.e. for two-sided $p < 0.05$ at least 196 of 200 comparisons showed larger values for one FMS) (Baselga and Orne, 2012), and for indicating variability the 90% quantile. Alpha-diversity was measured as species richness and tested for differences between EA and UEA using ANOVA (Table S4, Figure 1 G, H, I).

Complementarity of FMS and exclusiveness of species in FMS are linked, since there is no complementarity without exclusive species. However, complementarity does not quantify the fraction of exclusive species in FMS. As number of plots per FMS differed, we determined exclusive species by resampling. Based on 500 resamplings of 9 plots per FMS (out of 17 for EA and 13 for UEA) we quantified the fraction of exclusive species per FMS, relating exclusive species to species richness per resampling (Table S5, Figure 3). Differences between EA and UEA were assessed by pairwise comparison (i.e. for two-sided $p < 0.05$ at least 488 of 500 resamplings showed larger values for one FMS). Note that the percentage of exclusive species characterizes resamplings (N = 9) rather than 'true' species assemblages of FMS. However, differences between FMS are indicative.

For analyzing gamma-diversity the package 'iNEXT' version 2.0 (Chao *et al*., 2014) and for beta-diversity the package 'betapart' version 1.3 (Baselga *et al*., 2012) were used in R version 3.1.2 (R Core Team, 2014).

Results and Discussion

Gamma-diversity (^{0}D, ^{1}D and ^{2}D) was higher in EA compared to UEA (up to 77%) for six of the 15 taxonomic groups, at least for one of the Hill-numbers, while nine groups showed no difference (Figure 2). Spiders, beetles and vascular plants showed consistently higher diversities in EA (^{0}D, ^{1}D and ^{2}D). The consistent response of taxa across Hill-numbers suggests that higher gamma-diversity in EA is robust against weighting of rare and abundant species. This pattern was confirmed when focusing on forest specialists among spiders,

beetles and vascular plants (Table S2). This is an important finding as it is often argued that higher diversities in EA forests is a consequence of an invasion of common and disturbance tolerant non-forest specialist species, especially in plants (Boch *et al*., 2013). The results suggest that coarse-grained heterogeneity provided by developmental phases of EA is more important for biodiversity than the fine-grained heterogeneity of UEA within stands.

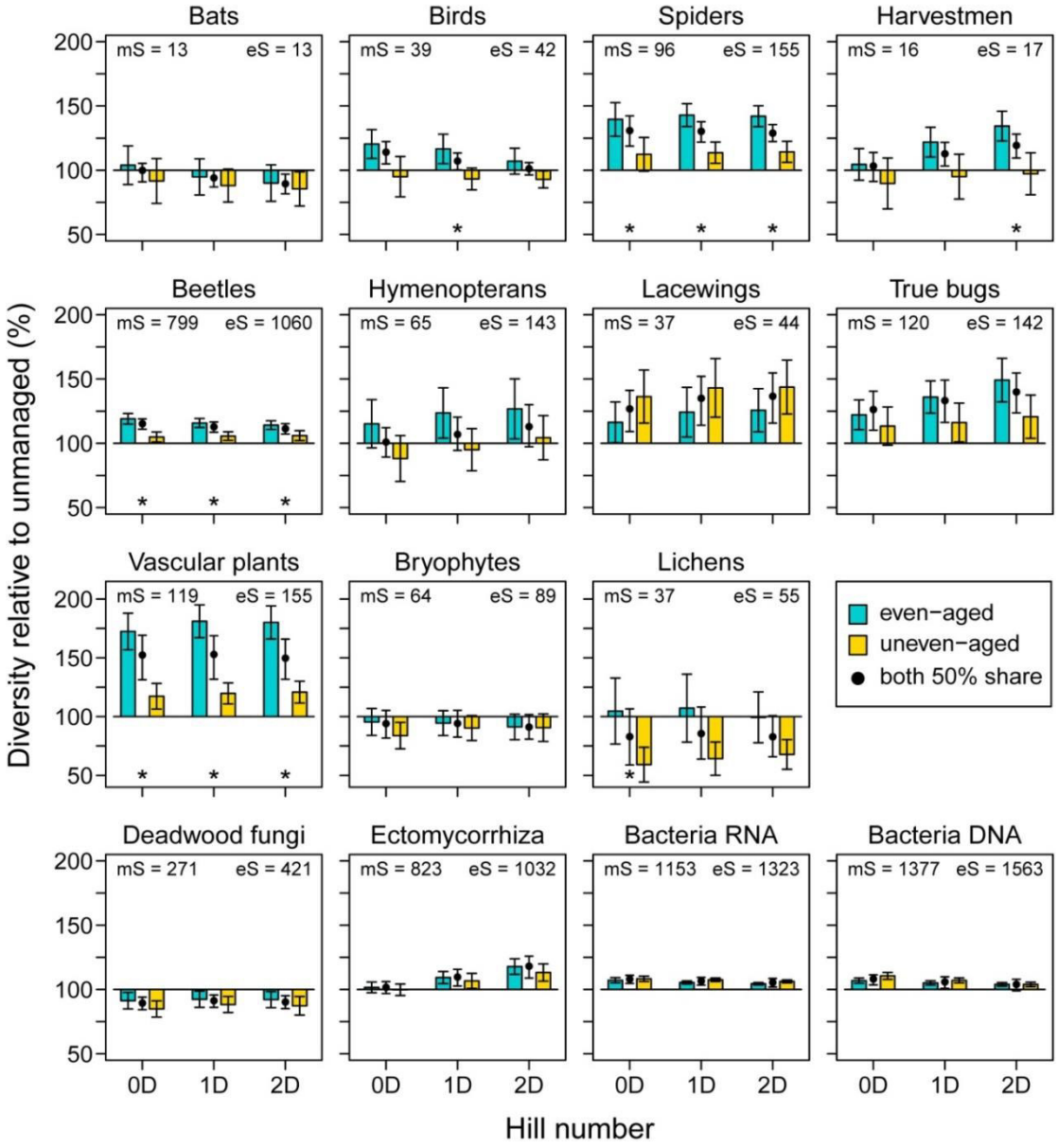

Figure 2. Gamma-diversity of forest management systems for 15 taxonomic groups. Even-aged (EA), uneven-aged (UEA) and equal proportion mixtures of both (dots) were related to unmanaged forests (Table S2). Diversity was quantified for Hill-numbers ^{0}D, ^{1}D, and ^{2}D, which increasingly weight abundance of species, using sample-size based rarefaction and extrapolation to factor out differences in sample size. * $p < 0.05$ significant difference between EA and UEA. We found higher diversities only for EA (compared to UEA) irrespective of weighting of rare and abundant species. Consistently higher diversities across Hill-numbers for EA were observed for the species rich taxa of spiders, beetles and vascular plants. Species assemblages of EA and UEA (as assessed by equal proportion mixtures) were not complementary for the vast majority of taxa. Rather, UEA comprised a subset of the assemblage of EA. Measured (mS) and estimated species richness (eS, Chao's incidence estimator) of the pooled data indicate the completeness of sampling.

Thereby it is important to keep in mind that the removal of mature even-aged European beech forests through shelterwood cuttings lasts at least 20 to 40 y, i.e. the forest area is never cleared. The observed loss of biodiversity with a decreasing grain-size of forest management suggests that mechanisms of the area-heterogeneity-tradeoff are acting in UEA. This would imply that alpha-diversity is negatively affected in UEA through species extinctions by insufficient size of suitable area (Carnicer *et al*., 2013).

However, when focusing on species richness (^{0}D) we found no overall effect of FMS on alpha-diversity across taxonomic groups (Figure 3 *A*, Table S4). The lower gamma-diversity can therefore not be explained by an area-heterogeneity-tradeoff. Birds, regarding total species as well as forests specialists, even showed a higher diversity in UEA (Figure 3 *A*, *D*), in accordance with the habitat-heterogeneity-hypothesis, as firstly demonstrated by MacArthur & MacArthur (1961). However, as only birds responded positively to fine-grained management, support for the habitat-heterogeneity-hypothesis is also rather weak. With respect to the young developmental phases of EA, the weak management effects on alpha-diversity detected here oppose other studies (Cale *et al*., 2013; Gao *et al*., 2014), that showed a dampening effect of young homogenous forests on alpha-diversity. When disentangling the components of gamma-diversity further, we found that mainly beta-diversity is affected by grain-size of forest management. Besides an overall 4% reduction, five individual taxa (spiders, birds, hymenopterans, vascular plants, and lichens) showed lower beta-diversity in UEA compared to EA (Figure 3 *B*, Table S3). The response of forest specialists was even higher (-10%) and was found in three of the six taxa for which we could distinguish forest specialists (Figure 3 *E*). Beta-diversity is not only lower in UEA but is also driven by different factors compared to EA. Matrix correlations revealed that species turnover in UEA is related to geographic distance between plots for a variety of taxa (vascular plants, beetles, and spiders; Table S6) rather than to environmental conditions (Table S7, Table S8). However, geographic distance reflects only physical properties of the study area, which should also affect the similarly distributed EA plots (Table S1). In EA distance was most likely masked by the higher dissimilarity between forest developmental phases. In this FMS microclimate within plots (expressed as daily temperature range) explained turnover of beetles, spiders and birds (Table S7) as previously found for saproxylic beetles (Müller *et al*., 2015).

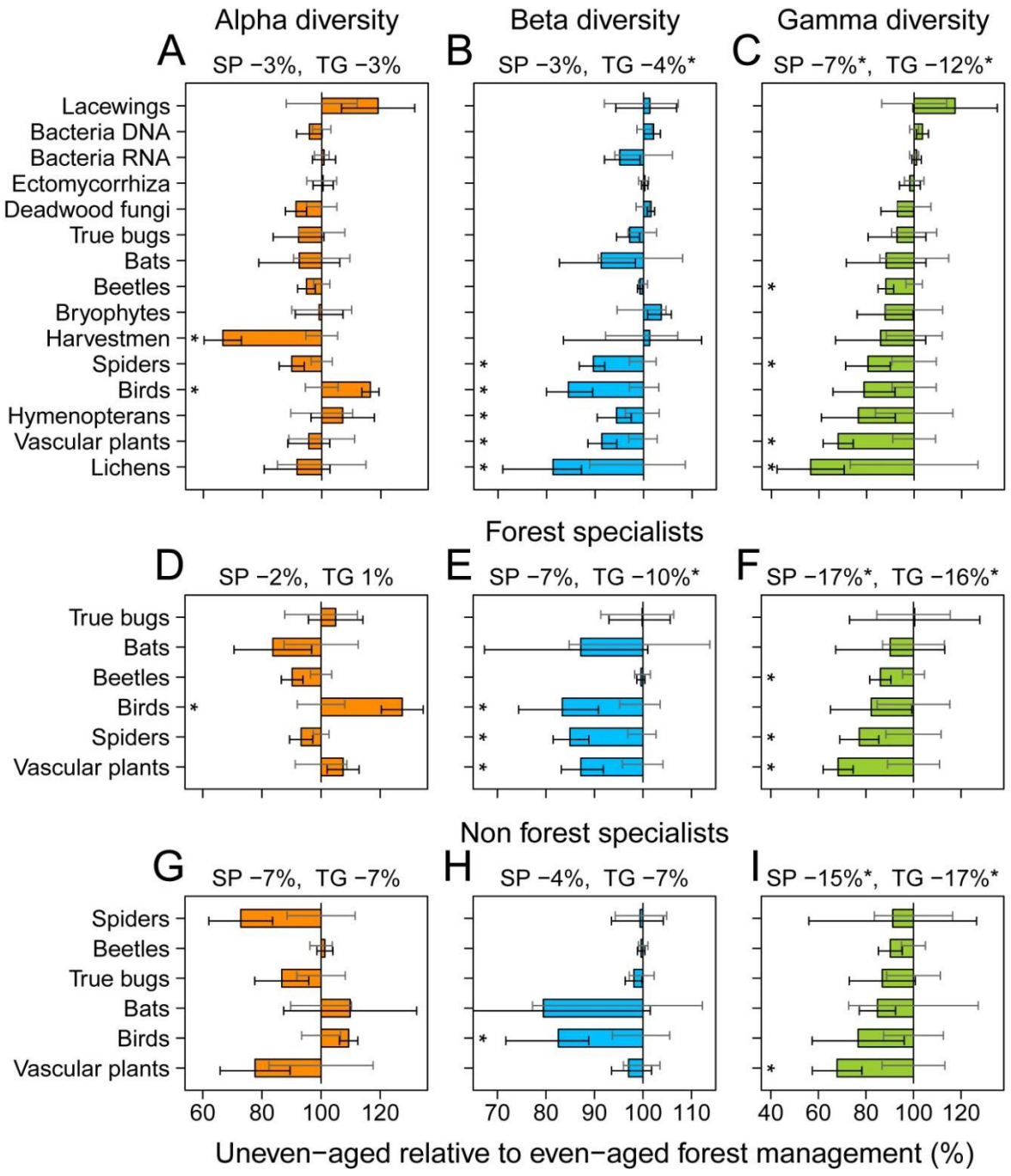

Figure 3. Alpha- (*A*, *D*, *G*), beta- (*B*, *E*, *H*) and gamma- (*C*, *F*, *I*) diversity of even-aged and uneven-aged forest management systems for 15 taxonomic groups. We distinguished all species of a taxon (*A-C*) and groups of forest (*D-F*) and of non-forest specialist species (*G-I*). Diversity of the uneven-aged (UEA) was standardized to the even-aged forest management system (EA) accounting for differences in sample size (Table S2, S3, S4). The overall response is given for taxonomic groups (TG) and species (SP, square root weighting of species number). Error bars (UEA: black, EA: grey) indicate 95% confidence interval for gamma- and alpha-diversity, and 90% quantile for beta-diversity. * $p < 0.05$ significant difference between even-aged and uneven-aged forests. Gamma-diversity (*C*, *F*, *I*) was analyzed using sample-size-based rarefaction and extrapolation. Confidence intervals were obtained by bootstrapping based on 200 replications. Beta-diversity (*B*, *E*, *H*) was measured as multiple-site beta turnover component of Jaccard dissimilarity and quantified using 200 resamplings of 9 plots per FMS (out of 17 for EA and 13 for UEA). Differences between EA and UEA (at least 196 of 200 comparisons show larger values in one FMS) and 90% quantiles are based on resamplings. Alpha-diversity (*A*, *D*, *G*) was analyzed using ANOVA.

In EA the daily temperature amplitude during the vegetation period is elevated and more variable between plots than in UEA and UNM. This can be explained by a more variable canopy height and canopy cover of developmental phases. The highest amplitudes were observed in thickets (daily range: 10 ± 1.1 °C, daily maximum: 19.0 ± 0.9 °C) while the mature timber phase resembled the microclimate of UEA and UNM (Table S1).

Intermingling of age-cohorts within a stand with decreasing grain size results in increasing heterogeneity of differently aged and sized neighboring trees. This is commonly described as high structural complexity. However, our results reveal that this does not result in 'effective' habitat heterogeneity, as different properties of habitats (provided by tree age-cohorts) in terms of environmental factors and resources (e.g. temperature, radiation, dead wood) are diluted by fine-grained intermingling (Table S1). This habitat-heterogeneity-dilution, therefore, explains our findings on beta- and gamma-diversity more coherently than the habitat-heterogeneity-hypothesis or an area-heterogeneity-tradeoff.

When compared with UNM eight groups showed higher gamma-diversity in EA with consistency across Hill-numbers for spiders, beetles, vascular plants, bacteria RNA and bacteria DNA. In UEA five taxa were more (consistently across Hill-numbers for bacteria RNA and DNA) and two less divers (lichens ^{0}D, ^{2}D; deadwood fungi ^{0}D) compared to UNM (Table S3). Gamma-diversity of forest specialists among spiders, beetles and vascular plants was more divers across Hill-numbers in EA compared to UNM, whereas no differences were found between UEA and UNM. This suggests that in Central European landscapes which were intensively managed during historic times, today's unmanaged forests are still too young (maximum time of no management within the 'Hainich'-Exploratory is 70 y) to develop a structural complexity that provides a niche diversity comparable to primeval forests.

The question arises whether by mixing of different management regimes gamma-diversity can be increased at the landscape scale due to complementarity in species assemblages (Redon *et al.*, 2014). Only for true bugs (^{0}D of total species driven by non-forest specialists) and ectomycorrhizal fungi (subtle increase in ^{0}D, ^{1}D and ^{2}D) a higher gamma-diversity was achieved by mixing EA and UEA (total species Figure 2, Table S2; forest specialists Table S2). For the other taxa complementarity between EA and UEA was too low to enhance landscape scale biodiversity. Moreover, the limited complementarity indicates that UEA comprised mainly a subset of the species assemblage of EA. We consider these findings even more robust as they were consistent for the species rich taxa of vascular plants, beetles and spiders, independent of abundance weighting (^{0}D, ^{1}D and ^{2}D), irrespective of the degree of linkage to forests, and across trophic levels (producers, consumers and predators).

Mixing of EA and UNM resulted in higher gamma-diversity of forest specialist birds (^{0}D, ^{1}D and ^{2}D), harvestmen (^{0}D), forest specialist beetles (^{2}D) bryophytes (^{0}D and ^{1}D), lichens (^{2}D) and ectomycorrhizal fungi (^{0}D) (Table S2). However, the effect of mixing is very limited for all of these taxa except ectomycorrhizal fungi (i.e. less than 1 species equivalent). In ectomycorrhizal fungi complementarity may be promoted by higher tree species richness and lower dominance of European beech in UNM (Table S1), which both are legacies of the former coppice-with-standards management within the Hainich National Park.

Complementarity between UEA and UNM was higher as for both combinations with EA. Gamma-diversity in a mixed UEA and UNM landscape was higher for birds (^{0}D), bats (^{0}D of total species and forest specialists), spiders (^{0}D of total species, and ^{0}D and ^{1}D of forest specialists), beetles (^{0}D and ^{1}D of total species, ^{0}D, ^{1}D and ^{2}D of forest specialists), forest specialist true bugs (^{2}D), vascular plants (^{0}D and ^{1}D of total species and forest specialists), and ectomycorrhizal fungi (^{0}D) compared to landscapes composed of UEA or UNM only (Table S2). However, the resulting gamma-diversity of mixing UEA and UNM was always lower than in a landscape composed only of EA.

A mixture of all three FMS showed no overall increase in gamma-diversity. We found that a landscape with equal proportions of EA, UEA und UNM generally showed lower gamma-diversity than a landscape composed only of EA or of EA and UNM (except for ^{0}D of lacewings). Thus, biodiversity across taxa, irrespective of forest linkage, is probably best maintained by traditional coarse-grained forest management (EA) combined with unmanaged areas (highest diversity of bryophytes and deadwood fungi in UNM, and highest forest specialist bird diversity when UNM mixed with EA).

The higher diversity in EA compared to UEA is driven by a larger proportion of exclusive species (Figure S1 *A*, *B*, *C*). This was consistent in trend and significance for total species (significant for birds, spiders, harvestmen and vascular plants) and the subgroups of forest specialists (spiders and vascular plants), and non-forest specialists (birds and spiders). Thus, our results cannot be ascribed to the occurrence of disturbance indicators only (Boch *et al*., 2013), but are rather driven by the habitat-heterogeneity of forest developmental phases within EA. Thereby, the early developmental phases most likely promote species of early-successional forest stages that were shown to be important for biodiversity (Swanson *et al*., 2010), but are missing in UEA. The late developmental phases of the EA on the other hand seem to even provide a more suitable habitat for forest specialists than UEA. Hence, the proportion of exclusive species also suffered from a dilution of habitat-heterogeneity. Based on our findings we suggest habitat-heterogeneity-dilution as the mechanism linking the

habitat-heterogeneity-hypothesis and the intermediate-disturbance-hypothesis (Figure 1, Roxburgh *et al*., 2004) in temperate forests.

Globally forest management shifts from clear-cuts with a complete tree removal at a large scale towards silvicultural approaches which include retention trees or cuttings which are carried out at a smaller scale and with lower intensities (Puettmann *et al*., 2015, Lindenmayer *et al*., 2012). In conclusion, our findings show that biodiversity is sensitive to different types of forest management even when clear-cuts are avoided. Moreover, our study highlights that biodiversity responds positively to forest management if silviculture creates a variety of environmental conditions at the landscape scale (Sebek *et al*., 2015). Against the background of the framework presented in Figure 1 we conclude from existing knowledge and from our results that both extremes, large-scale clear-cuts and single tree selection cuttings, result in low heterogeneity at the landscape scale compared to systems acting at intermediate spatial grains. In contrast, maximizing fine grained within stand heterogeneity seems to have no additional positive effect on biodiversity. We therefore support recent views that nature-based silviculture should vary across time and at larger spatial scale (O'Hara *et al*., 2016). Moreover, we question the current trend towards a replacement of even-aged management by the single tree selection system, which we consider to be driven by beliefs rather than by evidence. Additionally, we argue that studies focusing on alpha-diversity only may be misleading because compositional variability between plots is ignored (Gossner *et al*., 2013).

Our findings have not only important implications for forest management and nature conservation but also point out the large impact of natural dynamics on species diversity. Recent large scale inventories in primeval European beech forests in the Carpathians revealed that forest structure was mainly driven by small-scale disturbances (Hobi *et al*., 2015). Within the more oceanic distribution of European beech, the natural disturbance regime may be more intense. This is supported by studies reporting severe storm events in old-growth forests across oceanic parts of the temperate zone (D'Amato and Orwig, 2008; Yamashita *et al*., 2002; Nagel *et al*., 2014). We therefore assume that, in contrast to eastern European forests, primeval Atlantic European beech forests had shown some coarse-grained structural heterogeneity. If so, this natural dynamic could nicely be mimicked by traditional even-aged forest management combined with forest reserves.

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SUPPLEMENTARY INFORMATION

Supplementary figures and tables are provided along with the electronic version of this thesis (on DVD), under the following paths:

SI Methods:	Supplementary Information/Chapter III.5/SI Methods.docx
SI References:	Supplementary Information/Chapter III.5/SI References.docx
Figure S1:	Supplementary Information/Chapter III.5/Figure S1.docx
Figure S2:	Supplementary Information/Chapter III.5/Figure S2.docx
Table S1:	Supplementary Information/Chapter III.5/ Table S1.docx
Table S2:	Supplementary Information/Chapter III.5/ Table S2.docx
Table S3:	Supplementary Information/Chapter III.5/ Table S3.doc
Table S4:	Supplementary Information/Chapter III.5/ Table S4.docx
Table S5:	Supplementary Information/Chapter III.5/ Table S5.docx
Table S6:	Supplementary Information/Chapter III.5/ Table S6.doc
Table S7:	Supplementary Information/Chapter III.5/ Table S7.docx
Table S8:	Supplementary Information/Chapter III.5/ Table S8.docx

CHAPTER III.6

BACTERIAL ENDOPHYTES IN IMPORTANT GRASS SPECIES

III.6.

For submission to Frontiers in Microbiology (Specialty Section: Plant Biotic Interactions)

Grasses are not equal green: the impact of management regimes on bacterial endophyte community composition and function differs with grass species

Running title: Bacterial endophytes in important grass species

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All authors were involved in revision of the manuscript and provided commentary on the results.

BACTERIAL ENDOPHYTES IN IMPORTANT GRASS SPECIES

Abstract

Despite the important role of endophytic bacteria for plant growth and health, our understanding of the complex interactions between these bacteria, their host plant and agricultural practices is still limited. To investigate the influence of fertilizer application and mowing frequency on bacterial endophytes in three agricultural important grass species, plant samples were collected over two consecutive years (2010 and 2011). Samples were taken on the GRASSMAN experimental field which was set up to address these particular questions in a multidisciplinary approach. Structural responses were assessed by pyrotag sequencing of 16S rRNA genes. Bacterial communities were dominated by different bacterial genera including *Pseudomonas* and *Stenotrophomas* which are known as plant growth-promoting bacteria. Fertilizer application resulted in grass species-specific responses of bacterial endophyte communities, whereas mowing frequency affected bacterial communities only in combination with fertilization and grass species. We identified several indicator species being highly associated with grass species and fertilizer application and thus most likely responsible for the observed differences in community composition. Obtained data suggests that the applied regimes may directly influence bacterial endophyte communities through the modification of plant physiology as grass-specific responses towards fertilizer application were visible. In addition, we predicted artificial metagenomes from obtained 16S rRNA gene data to study functional response as well. Genes involved in plant growth promotion, i.e., nitrogenases, differed in their abundances between plants and also between the different management regimes. However, the grass species itself rather than the applied management regimes explained most of the observed variance in the investigated datasets as the response of bacterial endophytes towards agricultural practices is plant species dependent. Our study provides novel insights into the complex interaction of endophytes and their host plants and thus might be useful for the future development of a sustainable agricultural production.

Introduction

Endophytic bacteria have been found in a wide range of plants and comprised of many bacterial genera and species (Kobayashi and Palumbo, 2000; Hardoim *et al*., 2015). They can promote plant's growth and/or resistance to diseases and environmental stress conditions by a

variety of mechanisms (Sturz *et al*., 1999; Lodewyckx *et al*., 2002; Ryan *et al*., 2008) including the fixation of atmospheric nitrogen (Stoltzfus *et al*., 1997; Reinhold-Hurek and Hurek, 1998) or the production of antibiotics and phytohormones (Rosenblueth and Martinez-Romero, 2006; Ryan *et al*., 2008; Compant *et al*., 2010). Moreover, it is widely recognized that endophytic bacteria can be used for a variety of applications in agricultural cropping systems (Kobayashi and Palumbo, 2000; Sturz *et al*., 2000; Senthilkumar *et al*., 2011). However, our understanding of the complex interactions between bacterial endophytes, their host plants and agricultural practices is still limited.

Recent studies showed that bacterial endophytic communities in species within the *Poaceae* family were strongly influenced by fertilizer application (e.g., *Robinson et al*., 2015; Rodríguez-Blanco *et al*., 2015; Wemheuer *et al*., 2016). In a study on the effect of nitrogen fertilization on nitrogen-fixing (diazotrophic) bacteria of two maize genotypes, fertilization increased the number of endophytic diazotrophs in roots and stems (Rodríguez-Blanco *et al*., 2015). Tan *et al*. (2003) found a rapid change of the diazotrophic population structure in rice roots within 15 days after fertilizer application. Nonetheless, most previous research focused on the effect of fertilizer application on diazotrophic and/or root endophytic bacteria in a single grass species (Fuentes-Ramírez *et al*., 1999; Tan *et al*., 2003; Seghers *et al*., 2004; Prakamhang *et al*., 2009; Rodríguez-Blanco *et al*., 2015). Consequently, the impact of different agricultural practices on the entire bacterial community in aerial plant parts has not been investigated so far. Moreover, large comparative studies on structural and functional changes of endophyte communities in different plant species towards agricultural practices are still missing.

Hence, we investigated the influence of management regimes on bacterial endophyte communities in the agricultural important grass species *Dactylis glomerata* L., *Festuca rubra* L. and *Lolium perenne* L. We have chosen these three grass species because they differ in their indicator values such as tolerance against mowing (Dierschke and Briemle, 2002). In a previous study, we analyzed the effect of fertilizer application and different mowing frequencies on the bacterial endophyte communities in these grass species by denaturing gradient gel electrophoresis (DGGE) (Wemheuer *et al*., 2016). Although DGGE fingerprints revealed that management regimes influenced the bacterial endophyte communities in a plant species-specific manner, the phylogenetic resolution of this approach is limited. As a consequence, we applied pyrotag sequencing targeting the 16S rRNA gene to gain deeper insights into structural changes of the endophyte communities. In addition, functional predictions (artificial metagenomes) were calculated from obtained 16S rRNA data using

Tax4Fun (Aßhauer *et al*., 2015) to investigate functional changes as response to applied management regimes. We focused on three main hypotheses: (1) the three grass species show differences in endophyte diversity and exhibit distinct endophytic communities as they differ in their physiological traits and in their recruitment strategy, (2) applied management regimes affect the diversity and community structure of bacterial endophytes in a grass species-specific manner as these grass species differ in their response to environmental conditions, and (3) although structural changes of endophytic communities occur, community functions are unaffected by agricultural practices

Materials and Methods

Sampling

Sampling was performed as described previously (Wemheuer *et al*., 2016). In brief, aerial plant material of *L. perenne*, *F. rubra*, and *D. glomerata* was collected on 19th September 2010 and 12th September 2011 from the Grassland Management Experiment (GrassMan). The experimental design included four treatments (no fertilization and mown once or thrice per year, respectively, and fertilization with nitrogen/phosphorous/potassium (NPK) and mown once or thrice per year, respectively). For a detailed description of the design and the applied management regimes see Wemheuer et al. (2016) and Petersen et al. (2012). Three samples per treatment and grass species were taken in both sampling years with one exception: only two *L. perenne* samples were collected in 2010 due to the lack of this plant species on the study site. In total, 71 plant samples were analyzed in this study (Supplementary Table S1). One sample comprised ten individual plants. Collected plants did not show obvious disease symptoms such as leaf spots, chlorosis, or other types of pathogen-induced lesions. The plant samples were immediately cooled down (below 4°C) and transported to the laboratory. During the study period, precipitation and mean temperature were 93.6 mm and 11.42°C in September 2010 and 54.75 mm and 14.75°C in September 2011, respectively.

Surface sterilization and extraction of total community DNA

Surface sterilization of collected plant material and the control of this process were performed as described in Wemheuer *et al.* (2016). Further analysis confirmed that the surface sterilization was effective in eliminating both cultivable as well as noncultivable epiphytic bacteria as well as potential DNA traces from the plant surfaces (data not shown). The

surface-sterilized plant material was ground to a fine powder in liquid nitrogen using an autoclaved mortar and pestle. Ground tissue powder aliquots were subsequently stored at -20°C until DNA extraction. Total microbial community DNA was extracted employing the peqGOLD Plant DNA Mini Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions with two modifications as described previously (Wemheuer *et al.*, 2016).

Amplification of the 16S rRNA genes

Bacterial endophyte communities were assessed by a nested PCR approach targeting the 16S rRNA gene. For details of the first PCR reaction mixture and the thermal cycling scheme see (Wemheuer *et al*., 2016). In brief, the primers 799f (AACMGGATTAGATACCCKG) (Chelius and Triplett, 2001) and 1492R (GCYTACCTTGTTACGACTT) (Lane, 1991) were used in the first PCR to suppress co-amplification of plant chloroplast 16S rRNA genes (Chelius and Triplett, 2001). PCR amplification resulted in two PCR products: a mitochondrial product with approximately 1.1 kbp and a bacterial product of approximately 735 bp. Genomic DNA of *Bacillus licheniformis* DSM13 was used as template in the positive control to select for the bacterial product. Three independent PCRs were performed per sample. Bacterial-specific bands were purified using the peqGOLD Gel Extraction Kit (Peqlab) according to the manufacturer's instructions, quantified using a Nanodrop (ND-1000) (Peqlab) and subjected to the nested PCR reaction (one per triplicate). The V6-V8 region of the 16S rRNA gene was amplified with primers containing the Roche 454 pyrosequencing adaptors and key as well as one unique MID per sample (underlined): F968 5′-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-$(dN)_{16}$- AACGCGAAGAACCTTAC -3′ and R1401 5′-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-CGGTGTGTACAAGACCC -3′ (Nübel *et al*., 1996). The PCR reaction (25 µl) contained 5 µl of five-fold Phusion HF buffer, 200 µM of each of the four deoxynucleoside triphosphates, 4 µM of each primer, 2 U of Phusion high fidelity hot start DNA polymerase (Thermo Scientific, Waltham, MA, USA) and approximately 10 ng of the PCR product as template. The following thermal cycling scheme was used: initial denaturation at 98°C for 30 s, 30 cycles of denaturation at 98°C for 15 s, annealing at 53°C for 30 s, followed by extension at 72°C for 30 s. The final extension was carried out at 72°C for 2 min. Negative controls were performed using the reaction mixture without template. Obtained PCR products were controlled for appropriate size and subsequently purified using the peqGOLD Gel Extraction Kit (Peqlab) as recommended by the manufacturer. Quantification of the PCR products was performed using the Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Thermo

Scientific) as recommended by the manufacturer. Purified PCR products from the three independent PCRs were subsequently pooled in equal amounts. The Göttingen Genomics Laboratory determined the 16S rRNA gene sequences employing the Roche GS-FLX+ pyrosequencer with Titanium chemistry (Roche, Mannheim, Germany).

Processing of pyrosequencing derived data

Generated 16S rRNA datasets were processed and further analysed employing the QIIME 1.8.0 software package (Caporaso *et al*., 2010) and other tools. Short sequences (< 250bp,) with long homopolymer stretches (> 8bp) and too many primer mismatches (> 3 bp) were removed prior to denoising with Acacia version 1.53b (Bragg *et al*., 2012). Remaining reverse primer sequences were truncated employing cutadapt (version 1.0) (Martin, 2011). Chimeric sequences were removed with Usearch (version 7.0.190, Edgar, 2010) and sequences of all samples were subsequently joined and clustered in operational taxonomic units (OTUs) at 3% genetic distance as described previously (Wemheuer *et al*., 2015) (Supplementary Table S2). To determine taxonomy, a consensus sequence for each OTU was classified by BLAST alignment against the Silva SSURef 119 NR database using QIIME (Camacho *et al*., 2009*;* Quast *et al*., 2013). Rarefaction curves, alpha diversity indices (Richness, Chao1 and Shannon,) were determined using in R (version 3.1.2; R Developmental Core Team, 2013) using the vegan package (Supplementary Table S3). In addition, the drc package was used in R to determine the Michaelis-Menten-Fit. Functional profiles were predicted from obtained 16S rRNA data using Tax4Fun (Asshauer *et al*., 2015) with short read mode disabled (Supplementary Table S4).

Statistical analysis

The impact of applied management regimes as well as putative differences between the three investigated grass species and the two sampling years was statistically analyzed using R. For this purpose, ordination plots (NMDS*;* non-metric multidimensional scaling) for community structures were generated based on Bray-Curtis dissimilarities using the *metaMDS* function within the vegan package (Oksanen *et al*., 2013). Furthermore, the impact of the applied treatment was evaluated using the envfit function as described by Wietz *et al*. (2015). Differences in richness and diversity were analyzed by pairwise t test with Bonferroni-corrected P values. Changes were considered significant at $P \leq 0.05$. Plots for community functions were generated by redundancy analysis (RDA) using the Tax4Fun profiles. Samples taken in 2010 and 2011 were analyzed separately to overcome temporal pseudoreplication.

To further identify the bacterial assemblages associated with the different grasses, correlation-based indicator species analysis was performed using *multipatt* (indicSpecies) (De Cáceres and Legendre, 2009). The abundance of all OTUs affiliated to the same genus were summarized prior to analysis. Low abundant taxa representing less than 0.01% of the whole community were excluded from the analysis as they tend to be unique and erroneously may be declared as indicator species. In total, 393 bacterial taxa (from a total of 729 on genus level) were taken into consideration. For visualization, a bipartite network was generated using the three grasses per treatment, i.e., each grass as either fertilized or non-fertilized, as source nodes, and the genera as target nodes. All taxa with a possible association were visualized but only those with significant ($P \leq 0.05$) associations were identified in the networks. Network generation was performed using the *edge-weighted spring embedded layout* algorithm in *Cytoscape* (Shannon *et al*., 2003), with the edge weight corresponding to the association strength of each genus with each treatment.

Nucleotide sequence accession numbers

Sequence data are deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number SRA419370.

Results and discussion

Bacterial endophyte community is dominated by a few abundant phyla

The response of bacterial endophytic communities in three agriculturally important grass species to different fertilization and mowing regimes was assessed by pyrotag sequencing of 16S rRNA genes. After data processing, more than 57,000 high-quality sequences with approx. 711 sequences per sample were used to analyze endophyte community structure and diversity (Supplementary Table S2). Calculated rarefaction curves (data not shown) as well as diversity indices (see Supplementary Table S3) revealed that a major fraction (>50%) of the bacterial community was recovered by the surveying effort.

Obtained 16S rRNA gene sequences were clustered into 7,192 bacterial OTUs (Supplementary Table S2). We calculated an average number of 62 OTUs per plant sample at the same surveying effort (Supplementary Table S2). Seven abundant phyla including *Proteobacteria*, *Actinobacteria* and *Firmicutes* were present in all samples and accounted for more than 96% of all sequences analyzed in this study (Figure 1) *Proteobacteria* (76.10%)

were predominant across all samples. This is in line with previous studies of bacterial endophytes (see Table 1 in (Hardoim *et al*., 2015) Sequences assigned to this phylum mainly belonged to *Betaproteobacteria* (40.86%) followed by *Gammaproteobacteria* (22.09%), *Alphaproteobacteria* (11.13%) and *Deltaproteobacteria* (2.01%). *Actinobacteria* (6.22%) and *Firmicutes* (6.02%) were the second and the third most abundant bacterial phyla, respectively. Other identified phyla that were present in all samples were *Bacteroidetes* (4.63%), *Acidobacteria* (1.74%), and *Deinococcus-Thermus* (1.23%). samples were *Bacteroidetes* (4.63%), *Acidobacteria* (1.74%), and *Deinococcus-Thermus* (1.23%). These findings are in agreement with previous studies of bacterial endophytes in different grass species (Sessitsch *et al*., 2012; Bulgarelli *et al*., 2015; Maropola *et al*., 2015; Robinson *et al*., 2015) as well as in leaves of other plant species including *Arabidopsis* and *Solanum* (Bodenhausen *et al*., 2013; Romero *et al*., 2014).

At genus level, *Massilia* (11.49%) was predominant across all samples (Figure 1). Members of this genus are typical bacteria in rhizosphere and soil as well as root colonizers (Nagy *et al*., 2005; Zhang *et al*., 2006; Ofek *et al*., 2012). Moreover, this genus was detected in the phyllosphere of tomato and crofton weed (Enya *et al*., 2007; Zhou *et al*., 2010) as well as in soybean stems (Ikeda *et al*., 2009) and sweet pepper shoots (Rasche *et al*., 2006). In a previous study on bacterial communities associated with roots and leaves of *Arabidopsis*, *Massilia* was prevalent in both samples (Bodenhausen *et al*., 2013). Isolates of *Massilia* are able to reduce nitrate (Zhang *et al*., 2006) suggesting the important role of this genus in the soil nitrogen cycle. Moreover, some *Massilia* isolates exhibited *in vitro* attributes related to plant growth promotion, such as Indole-3-acetic acid (IAA) production (Kuffner *et al*., 2010). In another study, an isolate of *Massilia* showed *in vitro* antagonism towards the pathogen *Phytophthora infestans* (Weinert *et al*., 2010). Other abundant genera observed in this study were *Pseudomonas* (10.0%), *Limnohabitans* (5.02%), *Acidovorax* (4.51%), *Rhodanobacter* (3.42%), *Rhizobium* (2.63%) and *Methylobacterium* (1.86%).

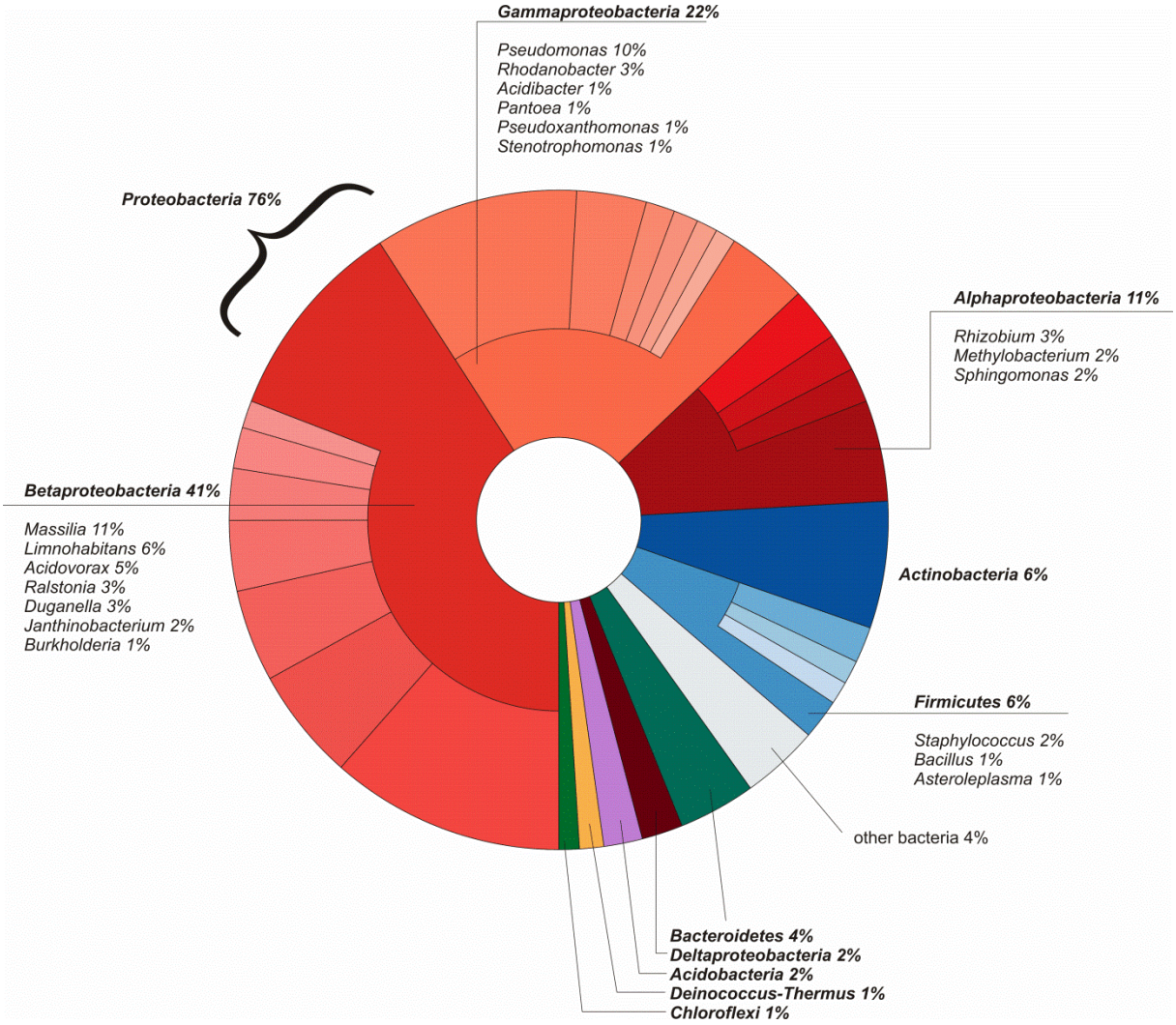

Figure 1. Abundant bacterial phyla and corresponding classes in the three investigated grass species as revealed by pyrotag sequencing. Only groups with an average abundance >1% in at least one of the investigated grass species are shown. Taxonomy is derived from the SILVA SSURef 119 NR database.

The most commonly observed endophytic genera such as *Massilia*, *Pseudomonas*, *Bacillus*, and *Rhizobium* are also members of common soil bacterial communities (Lodewyckx *et al.*, 2002). As consequence, some authors suggest that the endophyte microbiome is a subset of the rhizosphere or soil (e.g., (Seghers *et al.*, 2004; Gottel *et al.*, 2011). Moreover, genera such as *Pseudomonas*, *Rhizobium*, *Stenotrophomonas*, and *Burkholderia* are well-known for their plant growth-promoting functions and/or for the production of secondary metabolites including antibiotics or antifungal compounds (Lodewyckx *et al.*, 2002; Lugtenberg *et al.*, 2002; Glick, 2012). In addition, genera including *Bacillus*, *Burkholderia*, *Pseudomonas*, and *Rhizobium* are reported as the most significant phosphate solubilizing bacteria (Bhattacharyya and Jha, 2012). As these bacteria play an important role for plant growth and, thus, in

agriculture, it is of crucial importance to analyze the influence of management regimes on endophytic communities.

Grass species harbor distinct bacterial endophyte communities

We compared bacterial diversity (represented by the Shannon index H') and richness (number of observed OTUs) with respect to the three grass species (Supplementary Table S3). Interestingly, we detected differences between both sampling years. In 2011, richness and diversity of bacterial endophytes in *L. perenne* were significantly higher compared to those of *D. glomerata*, whereas no significant effect of grass species was recorded in 2010 (data not shown). To analyze putative diversity patterns of endophytic communities with regard to grass species and applied management regimes, community profiles were analyzed by NMDS. We found distinct bacterial communities in 2011 and 2010 (Figure 2) although this result derived from pseudoreplicated data. The observed differences might be attributed to the community structure in *D. glomerata* which was more divers compared to those of the other two grass species.

Further statistical analysis revealed that plant species significantly influenced endophytic community composition in 2011 (Table 1). This finding is in accordance with a previous study of McInroy and Kloepper (1995) who found differences in the bacterial endophyte population in field-grown sweet corn and cotton grown side by side. They suggested that internal plant niches are colonized by a wide range of bacteria. Different plant hosts also differed in their ability to be colonized by the same bacteria (Rosenblueth and Martinez-Romero, 2006). Moreover, different plant species vary in their biochemical composition, which may affect bacterial endophyte community (Hallmann and Berg, 2006). According to Hallmann (2006), the differences in bacterial endophytic community structures between different plant species growing next to each other can only be explained by plant species-specific selection mechanisms.

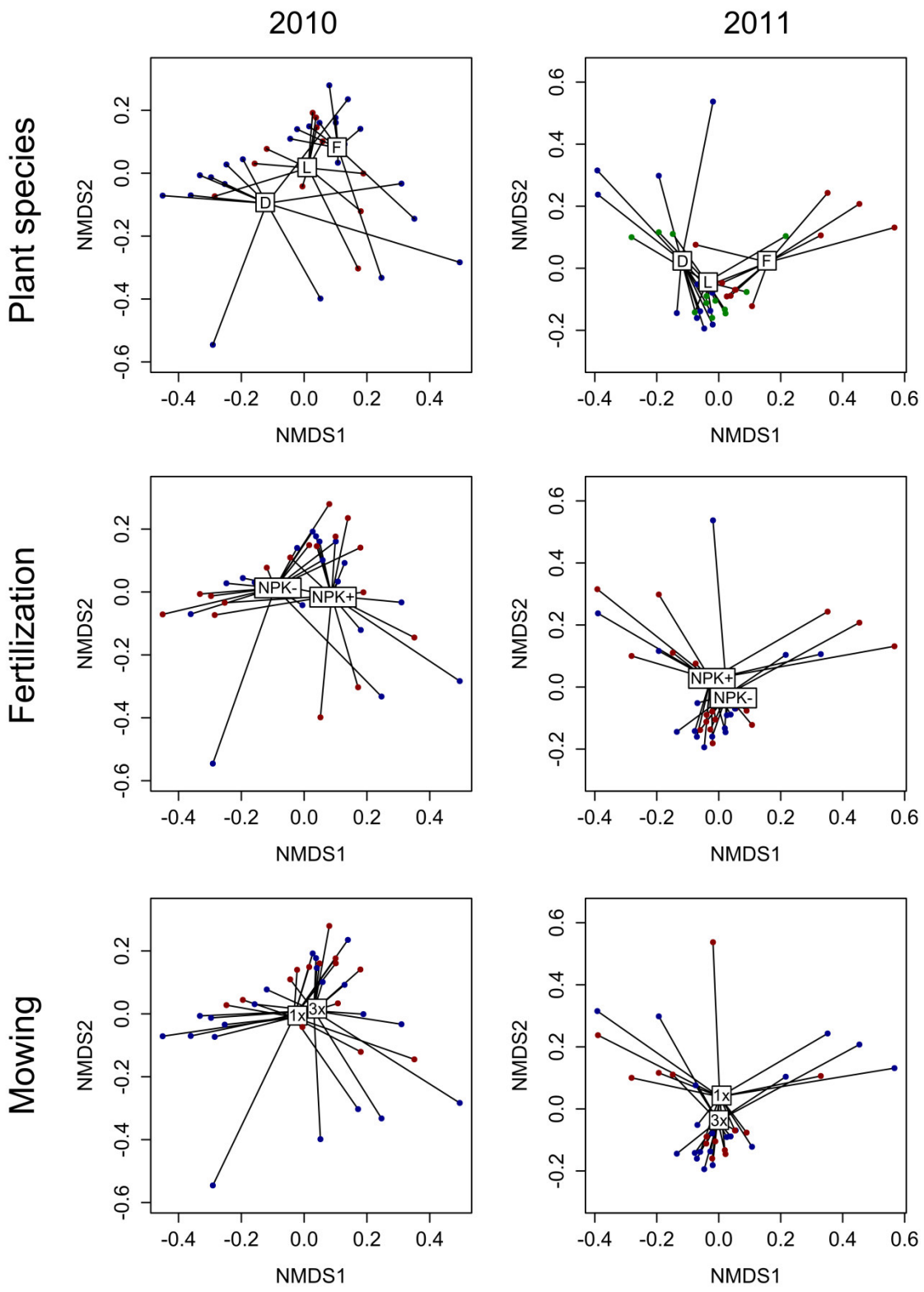

Figure 2. NMDS ordination of bacterial communities. Ordination of communities is based on Bray-Curtis dissimilarities. Samples were linked according to plant species, fertilization and mowing regime separately for 2010 and 2011, to identify potential drivers of community structure. D: Dactylis glomerata; L: Lolium perenne; F: Festuca rubra; NPK-: without fertilization; NPK+: with fertilization (sodium, phosphate and potassium); 1x: mown once; 3x: mown thrice.

Table 1. Results of the envfit analysis for the full 16S rRNA dataset and the predicted functional profiles. Significant ($P \leq 0.05$) and marginal significant ($P \leq 0.10$) parameters are underlined or written in italics, respectively.

Management regime	2010		2011	
	r2	P	r2	P
	Community composition			
Plant	0.095	0.176	0.265	0.001
Fertilization	0.119	0.013	0.005	0.848
Mowing	0.007	0.795	0.003	0.895
Plant + Fertilization	0.346	0.007	0.321	0.010
Plant + Mowing	0.168	0.351	0.318	0.006
Fertilization + Mowing	0.145	0.122	0.068	0.581
Plant + Fertilization + Mowing	0.561	0.005	0.526	0.006
	Community function			
Plant	0.075	0.272	0.212	0.001
Fertilization	0.006	0.847	0.003	0.907
Mowing	0.037	0.32	*0.069*	*0.061*
Plant + Fertilization	0.160	0.404	0.240	0.036
Plant + Mowing	0.141	0.506	0.328	0.002
Fertilization + Mowing	0.112	0.265	0.089	0.403
Plant + Fertilization + Mowing	0.340	0.371	0.469	0.022

Bacterial community composition and diversity is influenced by fertilization but not by mowing frequency

We did not find any influence of mowing frequency on bacterial richness and diversity in both sampling years (Supplementary Table S5). Interestingly, no significant effect of fertilizer application on bacterial richness or diversity was observed in 2011, while richness and diversity were significantly reduced on fertilized plots in 2010. Similarly, a higher diazotrophic bacterial diversity in the roots of rice cultivated in unfertilized and previously uncultivated soil than in paddy soil amended with nitrogen fertilizer were recorded by Prakamhang *et al*. (2009). In contrast to this, Rodriguez-Blanco *et al*. (2015) found that N-fertilization increased both the diversity and richness of diazotrophic bacteria in roots and stems of maize. However, only the diazotrophic endophyte communities were investigated in these studies.

To analyze the effect of applied management regimes onto bacterial community structures, community profiles were analyzed by multivariate analysis. Interestingly, the endophyte communities build distinct clusters as response to fertilizer application in 2010 but not in 2011 while mowing treatments had no impact on community structure in both sampling years (Figure 2). The statistical analysis further revealed that the interaction of plant species with

fertilization explained approximately 34% or 32% of the variance in 2010 and 2011, respectively. Although mowing frequencies did not have any significant impact on community structure, more than 50% of the variance in the dataset is explained by a combination of plant species, fertilizer application, and mowing frequency (Table 1).

We further evaluated the impact of applied management regimes on bacterial communities for the three grass species separately. Statistical analysis revealed that fertilizer application and mowing frequencies significantly affected bacterial community composition in *D. glomerata* in 2010 explaining more than 60% of the variance (Table 2). Although not significant, a similar result was observed for *L. perenne* in 2011. Whereas only 10% of the variance was explained by fertilizer application and mowing solely, its combination resulted in more than 58% explained variance. Interestingly, we detected no impact of applied management regimes on community functions in *D. glomerata* whereas both management regimes affected community functions in both, *F. rubra* and *L perenne.*

Table 2. Results of the envfit analysis for the different plant species. Significant correlations ($P \leq 0.05$) are underlined. (Fert. = Fertilization)

Management	*Dactylis glomerata*				*Festuca rubra*				*Lolium perenne*			
	2010		2011		2010		2011		2010		2011	
regime	r2	P	r2	P	r2	P	r2	P	r2	P	r2	P
Community composition												
Fert.	0.4029	0.007	0.1999	0.16	0	0.495	0.1779	0.167	0.1226	0.289	0.1015	0.343
Mowing	0.0905	0.425	0.0858	0.222	0	0.906	0.0156	0.869	0.0301	0.728	0.0917	0.405
Fert.+ Mowing	0.6016	0.008	0.3142	0.368	0.2002	0.285	0.3812	0.189	0.1561	0.793	*0.5827*	*0.073*
Community function												
Fert.	0.0259	0.992	0.054	0.662	0.0521	0.761	0.0424	0.668	0.0643	0.761	0.1629	0.174
Mowing	0.0547	0.851	0.1049	0.274	0.0342	0.883	0.167	0.156	*0.1715*	*0.084*	0.0574	0.632
Fert. + Mowing	0.1659	0.976	0.2627	0.498	0.1738	0.881	0.4937	0.049	0.3032	0.429	0.5315	0.026

It has been proposed that the bacterial endophyte community is a subset of the rhizosphere and/or root-associated bacterial community as many of the bacterial endophytes originated from the corresponding rhizosphere (Germida *et al*., 1998; Sessitsch *et al*., 2002; Gottel *et al*., 2011). As consequence, factors shaping the soil community will also influence the endophyte community. In a recent study on the effect of fertilizer application on soil bacterial communities conducted at the GRASSMAN experimental field, soil bacterial communities were strongly influenced by fertilization (Herzog *et al*., 2015).

Effects of fertilizer application on endophytes as observed here are in line with previous studies (Tan *et al*., 2003; Prakamhang *et al*., 2009; Wemheuer *et al*., 2016). Endophytic

populations in cotton roots are affected by application of nitrogen-containing chitin as an organic amendment (Hallmann *et al*., 1999). According to Tan *et al*. (2003), a rapid change of both the population and the activity of nitrogen-fixing bacteria in rice roots was observed within 15 days after N-fertilization. However, most previous research focused on the effect of fertilizer application or on diazotrophic and/or root endophytic bacteria in a single grass species (Fuentes-Ramírez *et al*., 1999; Tan *et al*., 2003; Seghers *et al*., 2004; Prakamhang *et al*., 2009; Rodríguez-Blanco *et al*., 2015).

As the recorded effects on endophytic communities were different between the three grass species examined in this study, it is most likely that also the grasses are affected differently by management regimes. The grass species investigated in this study differ in their indicator values such as tolerance against mowing or grazing (Dierschke and Briemle, 2002). Both *D. glomerata* and *L. perenne* have a higher tolerance against mowing than *F. rubra*. In contrast, *L. perenne* has a higher indicator value for nitrogen compared to the other two grass species. Tan *et al*. (2003) showed that environmental conditions strongly influenced the diazotrophic endophytic community structure in rice roots. During the study period, precipitation and mean temperature differed between the two sampling years which might affect the endophyte communities as well.

Identification of indicator species with regard to grass species and management regimes revealed differences

To identify bacterial taxa most likely responsible for the observed differences between the investigated plant species, we performed an indicator species analysis to identify genera significantly associated with one or all plant species. This analysis is based on the relative frequency (occurrence in a certain plant species) and relative average abundance. Taxa which are shared between two of the grass species are not shown. Approximately 20% of the OTUs did not show significant differences in relative abundance and frequency (Figure 3). This core community is represented by 142 members across the bacterial phyla. However, several taxa were only associated with one of the three grasses. Although approximately 10.3% of all bacterial taxa were exclusively associated with *F. rubra*, only the genera *Pseudoclavibacter* and *Luteimonas* were significantly associated with this grass species. Recently, *Pseudoclavibacter endophyticus* was isolated from healthy roots of *Glycyrrhiza uralensis* (Li *et al*., 2016). Several members of this genus were isolated from rhizosphere soil (Kim and Jung, 2009; Du *et al*., 2015). In a study on the microbiome of lettuce, *Luteimonas* was found as abundant genus in the rhizosphere (Erlacher *et al*., 2015). Several members of this genus

were isolated from the rhizosphere of different plant species (Cheng *et al*., 2015; Ngo and Yin, 2016), whereas the recently-described *Luteimonas cucumeris sp. nov*, was isolated from cucumber leaves (Sun *et al*., 2012). As this isolate was able to reduce nitrogen, *Luteimonas* might play an important role in the nitrogen cycle.

Interestingly, only the genus *Streptococcus* was significantly associated with *D. glomerata*, whereas 23 genera including *Neorhizobium*, *Gaiella* and *Dyella* were significantly associated with *L. perenne*. These findings are in line with the study of Zinniel *et al*. (2002) who showed that different agronomic crops and prairie plant species harbor a significant variation of indigenous bacterial endophytes although there was a lack of strict specificity. However, only isolated endophytes were investigated in this study. Members of the genera *Neorhizobium* and *Dyella* are interesting due to their ability to promote the growth of the plants. *Dyella ginsengisoli* has originally been isolated from a ginseng field in South Korea (Jung *et al*., 2009), but was also observed as endophyte in rice seeds (Hardoim *et al*., 2012). The *Dyella ginsengisoli* strain ATSB10 exhibited 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and was able to solubilize inorganic phosphate (Anandham *et al*., 2008). In addition, this strain has been reported to increase the root length of canola seedlings by 145%. In a recent study about effects of *Neorhizobium huautlense* strain T1-17 on hot pepper, the fruit biomass was increased (Chen *et al*., 2016). Genome analysis of ten *Neorhizobium galegae* strains revealed plant growth promoting properties of several strains (Österman *et al*., 2015).

In the present study, all three grass species investigated were associated with a wide range of endophytic species, a core community (Figure 3). This is in line with a previous study showing a core community of bacterial endophytes in different maize species (Johnston-Monje and Raizada, 2011). As the three grass species investigated in the present study shared approximately 20% of all taxa (on genus level), there must be additional factors besides plant species-specific selection mechanisms. There are different lifestyle strategies of endophytic species as previously described (Gaiero *et al*., 2013; Hardoim *et al*., 2015). Many bacteria are obligate endophytes which are strictly bound to life inside plants during their entire lifespan (Hardoim *et al*., 2008). This might also determine the core community as observed in the present study.

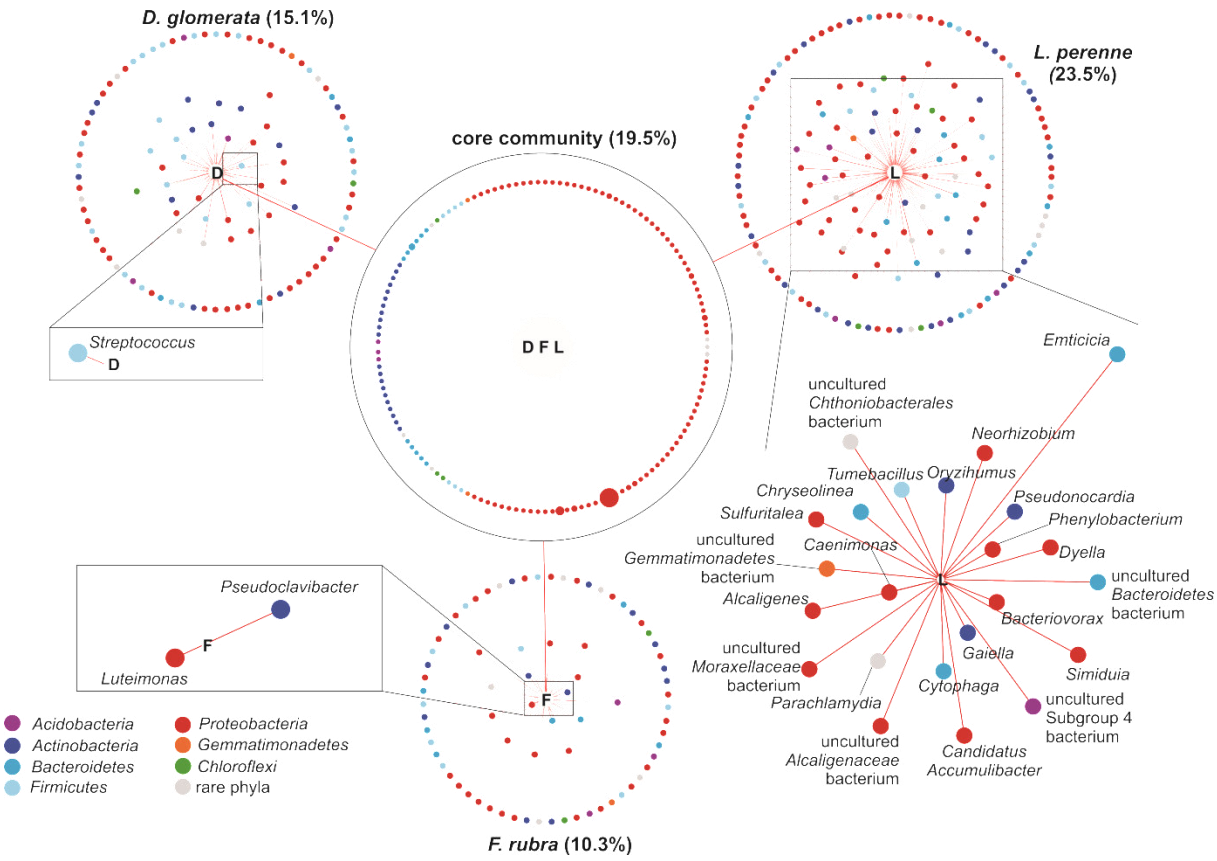

Figure 3. Number of taxa (genus level) shared between the three grass species). Circular layouts represent all associated taxa, while significant associations are enlarged for each plant. Node size corresponds to the relative abundance of each taxon in the whole dataset.Only uniquely associated taxa or those associated with all three plant species (designated as core community) are shown.

As mentioned earlier, plants vary in their biochemical composition which might explain differences in the bacterial endophytic community (Hallmann and Berg, 2006). Hallmann *et al.* (1999) suggested that changes in plant physiology may result in the development of distinct bacterial endophytic communities. Endophytic bacteria rely on the nutritional supply offered by their host plant. As consequence, any factor influencing the nutritional or physiological status of the host plant may consequently have an influence on the endophytic community in the plant (Hallmann *et al*., 1997; Fuentes-Ramírez *et al*., 1999). Several factors, such as temperature or precipitation, have a direct effect on the plant physiology and thus an indirect impact on the colonization and the survival of bacteria in the endosphere (Hallmann *et al*., 1997; Hardoim *et al*., 2012).

As only fertilizer application had a strong effect on the structure of the endophyte community in the three grass species, we carried out the indicator analysis under the two fertilization regimes. Interestingly, 18 of the 393 genera analyzed showed a significant association (Figure 4). Additionally, the associated bacterial taxa differed in the grass species according to the

treatment. The only exceptions were *Bacteriovorax* and *Caenimonas* which were significantly associated with *L. perenne* regardless of the treatment. In the fertilized treatment, the only significantly associated genus *Sporocytophaga* was shared with *L. perenne*. Regarding the non-fertilized treatments, *Limnobacter* and *Oryzihumus* were associated with *L. perenne*, while *Geobacter*, *Telmatobacter* and a *Solirubrobacterales* member were associated with *D. glomerata*. In the fertilized plants, *Tepidimonas*, *Schlegelella* and *Anoxybacillus* were associated with *D. glomerata*. In addition, *Tumebacillus*, *Gaiella* and *Planomicrobium* showed unique associations with *L. perenne* plants growing on fertilized plots.

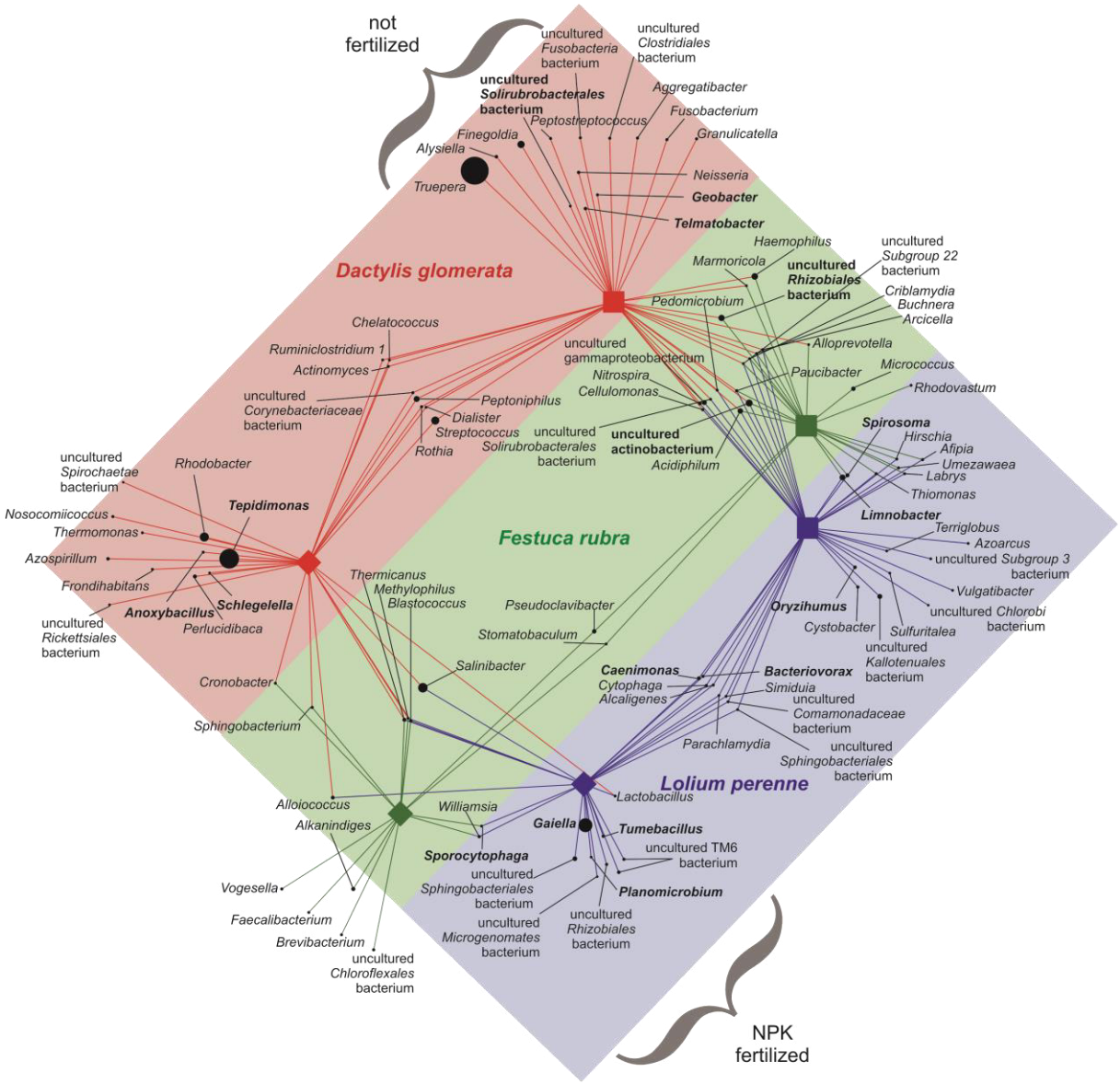

Figure 4. Bipartite association network of taxa with different treatments. Node size corresponds to the relative abundance of each taxon in the whole dataset. Significantly associated taxa are given in bold.

Functional profiles of endophytic bacteria

To investigate the impact of applied management regimes on endophyte functioning, artificial metagenomes were calculated from 16S rRNA gene data using Tax4Fun (Asshauer *et al.*, 2015). Statistical analysis revealed that functional profiles were affected by the host plant in 2011 but not in 2010 (Figure 5). A combination of the applied treatments with plant species showed significant correlations with calculated functional profiles as well (Table 1). We further focused on genes involved in plant growth promotion, i.e., nitrilase, amidases or nitrogenase (Figure 6) as well as on genes involved in the nitrogen cycle. These genes differed in their abundances between plants and also between the different treatments (Figure 6). This is supported by the statistical analysis (Table 3). Fertilizer application significantly affected the relative abundance of 523 genes in the functional profile of endophytes in *D. glomerata* in 2010, but only 20 genes in 2011. For *L. perenne*, a similar response was found. Mowing influenced the functional profiles of endophytes in *L. perenne* in 2011 and in *F. rubra* in 2010.

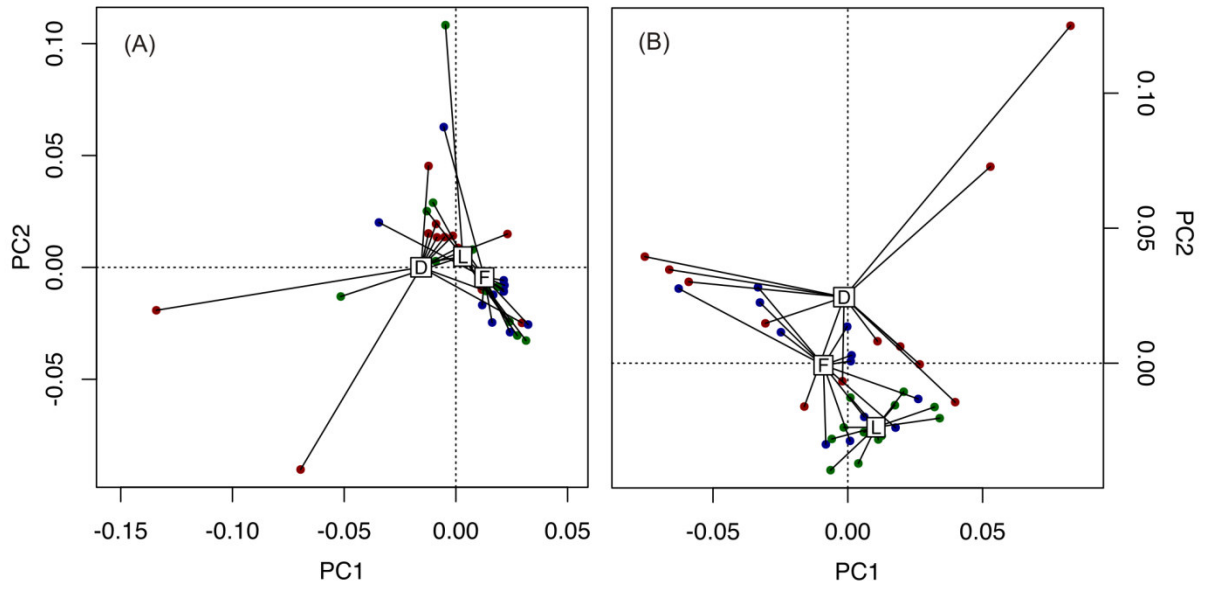

Figure 5. NMDS ordination of the functional profile based on Bray-Curtis dissimilarities. (A) Functional profiles of the endophytic communities of the different plants (L: *Lolium perenne*; F: *Festuca rubra*; D: *Dactylis glomerata*) in 2010 and (B) 2011.

Moreover, most genes were found in higher abundances in *D. glomerata* compared to the other two grass species (Figure 5). In addition, genes involved in plant growth promotion such as amidase or nitrilase were found in higher abundances in *D. glomerata* samples of fertilized plots mown once year, whereas genes of the nitrogen cycle were more abundant in *D. glomerata* samples mown three times a year compared to those mown once a year.

Table 3. Number of genes significantly influenced by applied management regimes. The number in brackets refers to the percentage of genes influenced divided by the total number of genes predicted from 16S rRNA data (n = 6408). Fert. = Fertilization.

Management regime	*Dactylis glomerata*				*Festuca rubra*				*Lolium perenne*			
	2010		*2011*		*2010*		*2011*		*2010*		*2011*	
Fert.	523	(8.16%)	20	(0.31%)	48	(0.75%)	41	(0.64%)	128	(2.00%)	280	(4.37%)
Mowing	151	(2.36%)	353	(5.51%)	94	(1.47%)	756	(11.8%)	783	(12.2%)	202	(3.15%)
Fert.+ Mowing	180	(2.81%)	37	(0.58%)	98	(1.53%)	577	(9.00%)	33	(0.51%)	1492	(23.3%)

In *F. rubra*, the abundance of nitrilase genes did not differ between the treatments. In contrast to this, higher average abundances were observed in *L. perenne* and *D. glomerata* samples of unfertilized plots mown one a year or fertilized plots mown once a year, respectively. This observation was also true for most of the genes investigated including the ACC deaminase and the amidase. The ACC deaminase is involved in stress alleviation in plants (Hardoim *et al*., 2015). ACC is a precursor of ethylene, which is a key regulator of the colonization of plant tissue by bacteria (Hardoim *et al*., 2008) and inhibits the nodule formation in legumes (Rocha *et al*., 2007). Nitrilases and amidases have been reported to play a role in plant hormone production (Hardoim *et al*., 2015). Nitrilases are further involved in the utilization of nitrogen compounds and in detoxification (as reviewed in (Howden and Preston, 2009). Howden *et al*. (2009) found a nitrilase from *Pseudomonas fluorescens* to be involved in the detoxification of cyano-compounds. Nitrogenase plays an important role in endophytic communities as it is putatively involved in the fixation of atmospheric nitrogen. It was also found to be significantly more abundant in endophytes compared to phytopathogens, suggesting its important role in enhancing plant productivity under nitrogen limitation (Hardoim *et al*., 2015).

However, it remains unclear why the genes encoding these enzymes were more abundant in *D. glomerata* under fertilization and mown once a year, as well as in *L. perenne* without fertilization and mown once a year. Similar results were obtained when investigating genes involved in plant colonization and establishment (Supplementary Figure S1, Supplementary Table S5). These genes differed in their abundances between plants with higher abundances observed in *D. glomerata*. Moreover, they differed in *L. perenne* and *D. glomerata* between the treatments investigated, while most of them were relatively stable in *F. rubra*. It is further not clear, why the abundance of genes seems generally lower in this grass species. So far, our knowledge about functional changes of endophyte communities as response to management regimes in different grass species is still limited

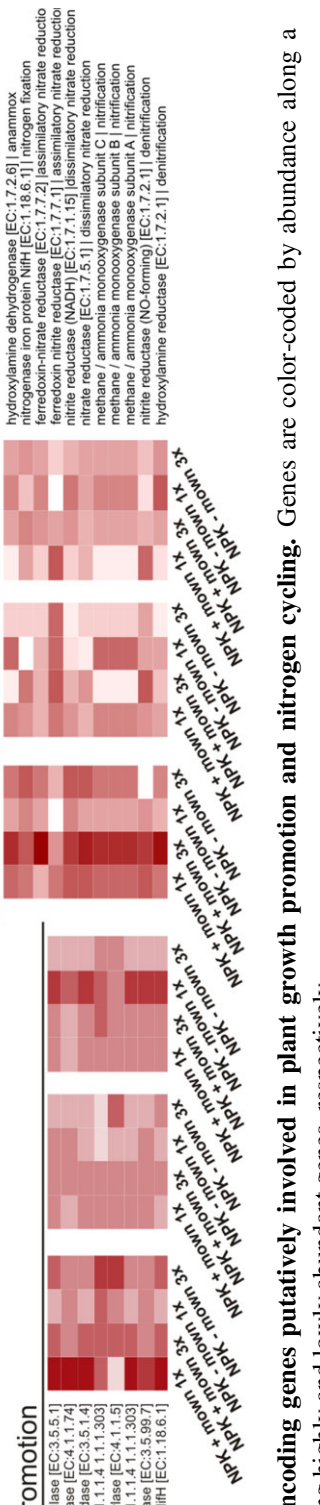

Figure 6. Heatmap of enzyme-encoding genes putatively involved in plant growth promotion and nitrogen cycling. Genes are color-coded by abundance along a gradient of red to white, representing highly and lowly abundant genes, respectively.

Conclusion

The majority of studies on endophyte diversity with respect to management regimes examined the effect of only one management regime in one single year, or focused on culturable endophytes or one functional group only. In this study, the influence of different management regimes on three agricultural important grass species was investigated in two consecutive years by high-throughput sequencing. According to our hypothesis (1), the three grass species show differences in endophyte diversity and exhibit distinct endophytic communities. However, approximately 20% of the taxa analyzed were shared between the grass species. Our results demonstrate further that the influence of management varied between the applied management regimes. Fertilizer application had a strong effect on richness and community composition, whereas mowing frequency had no significant effect. However, the combination of mowing, fertilization and plant species explained more than 50% of the variance of community composition observed. Interestingly, the effect of different management regimes is dependent on the host plant which is in line with hypothesis (2). Functional analysis revealed that the abundance of genes involved in plant growth promotion differed between the three grass species investigated. Moreover, the abundances of genes encoding for many enzymes such as amidase or nitrogenase were affected by the applied management regimes. At this stage we cannot determine if management regimes directly influence the endophyte community or indirectly through the modification of plant physiology. Nevertheless, this study provides first insights into structural and functional changes of endophyte communities in three agricultural important grass species as response to combined fertilizer application and mowing regimes. More studies targeting the influence of management regimes in combination with the impact of sampling year and plant species are required to unravel the diversity of interactions between endophytic bacteria, plant species and management regimes.

Acknowledgements

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SUPPLEMENTARY INFORMATION

Supplementary figures and tables are provided along with the electronic version of this thesis (on DVD), under the following paths:

Figure S1:	Supplementary Information/Chapter III.6/Figure S1.docx
Table S1:	Supplementary Information/Chapter III.5/ Table S1 - Sampling Data.xlsx
Table S2:	Supplementary Information/Chapter III.5/ Table S2 – OUT Table.xlsx
Table S3:	Supplementary Information/Chapter III.5/ Table S3 – AlphaDiversity.xlsx
Table S4:	Supplementary Information/Chapter III.5/ Table S4 – FunctionalProfile.xlsx
Table S5:	Supplementary Information/Chapter III.5/ Table S5 – Statistics Richness.docx

Chapter III.7.

SOIL ARCHAEAL COMMUNITIES UNDER DIFFERENT CROPPING REGIMES

III.7.

Submitted to Archaea

Changes of soil archaeal community structure and diversity in response to intercropping of wheat (*Triticum aestivum* L.) and faba bean (*Vicia faba* L.)

Running title: Soil Archaeal Communities under Different Cropping Regimes

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Experiments: SG

Data analysis: SG, KK, FW

Wrote the manuscript: SG, KK, FW

SOIL ARCHAEAL COMMUNITIES UNDER DIFFERENT CROPPING REGIMES

Abstract

Multiple cropping systems provide many economic and ecological advantages including increased plant productivity. Although being important for nutrient cycling, the response of soil archaeal communities towards these cropping systems has been rarely studied. Here we investigated the effect of crop species and cropping system on soil archaeal communities in a greenhouse experiment. For this purpose, faba bean and wheat plants were grown under four different cropping regimes (monoculture wheat, monoculture bean, row or mixed intercropping of bean and wheat). Soil archaeal community structure and diversity were assessed by sequencing of 16S rRNA gene amplicons. The highest archaeal diversity was observed in soil samples of row intercropping. Archaeal community structures between monocultures and intercropping regimes exhibited their own distinct community patterns which were attributed to community structures under row intercropping. In addition, we identified several indicator species being highly associated with crop species or cropping regimes and thus most likely responsible for the observed differences in community structure. For example, one species affiliated to the Marine Group II belonging to the *Thermoplasmatales* was associated with row intercropping. Obtained data underline the importance of soil archaea in agriculture and identify the complex interactions between cropping systems, archaeal communities and crop species.

Introduction

Archaea are found in a wide range of habitats including grassland, rainforest or agricultural soils (Etto *et al.,*. 2012; Gattinger *et al.,* 2007; Chroňáková *et al.,* 2015; Schneider *et al.,* 2015). As these microorganisms play a key role in biogeochemical cycling on earth (reviewed in Offre *et al*., 2013), it is important to identify the main drivers forming archaeal communities in soil ecosystems. Previous studies showed that archaeal communities are affected by various factors such as plant species (Mao *et al*., 2011; Mao *et al.,* 2013; Zhang *et al.,* 2015a) or landscapes (Schneider *et al.,* 2015). Moreover, fertilizer application influenced soil archaeal communities (Gattinger *et al.,* 2007; Dorr de Quadros *et al.,* 2012; Schneider *et al.,* 2015).

Intensive agricultural systems including monocultures have clearly negative environmental impacts such as the loss of biodiversity (Matson *et al.,* 1997). Conversely, multiple (or mixed) cropping systems provide enhanced ecological and economical services

including a better control of diseases, pests and weeds (Francis, 1989; Paulsen *et al.,* 2006; Winter *et al*., 2014). Moreover, these cropping systems enhance plant productivity by improving efficient use of light, nutrients and water (Francis, 1989; Malézieux *et al.,* 2009), most probably through (positive) interspecific interaction in the rhizosphere (Li *et al.,* 1999; Zhang *et al.,* 2004; Inal *et al.,* 2007). Multiple cropping systems are very diverse and defined as the growing of two or more crops on the same field in one year (Francis, 1986). Crop plants in these cropping systems do not compete for the same ecological niches as they ideally differ in uptake and utilization of nutrients and in the time of their major demands on the environmental resources (Willey, 1979; Jensen, 1996; Malézieux *et al.,* 2009).

Previous studies reported that the diversity and community composition of soil archaea was influenced by multiple cropping systems such as intercropping or crop rotation (Breidenbach *et al.,* 2015; Liu *et al.,* 2015; Zhang *et al.,* 2015a). In a study of the methanogenic archaeal community in a paddy field soil, differences in abundance and composition of this community in response to a rice-soybean crop rotation were observed (Liu *et al.,* 2015). This is consistent with a study of Breidenbach *et al.* (2015) analysing the effect of crop rotation between rice and maize by 454 pyrosequencing of archaeal 16S rRNA gene and 16S rRNA. They found the community composition to be altered in the rotational fields. For example, the abundance of aerobic *Thaumarchaeota* increased, whereas the abundance of anaerobic methanogenic lineages decreased. In contrast to these results, crop rotation had only a minor effect on archaeal communities under field conditions (Watanabe *et al.,* 2006; Scavino *et al.,* 2013). However, our knowledge on structural changes of the entire archaeal community in response to multiple cropping systems and crop species is still rather limited, as most previous research focused only on ammonia-oxidizing or (Mao *et al*., 2011; Wang *et al*., 2012; Mao *et al.,* 2013) or methanogenic archaea (Watanabe *et al.,* 2006; Gattinger *et al.,* 2007; Liu *et al.,* 2015).

The aim of the present study was to investigate the response of soil archaeal community diversity and composition to different cropping systems as part of the IMPAC^3 project ("Novel genotypes for mixed cropping allow for improved sustainable land use across arable land, grassland and woodland"). To assess structural changes of the soil archaeal community, the two agricultural important crop species common wheat (*Triticum aestivum* L.) and faba bean (*Vicia faba* L.) were grown in monoculture or as mixture. The two component crops were grown simultaneously in rows (row intercropping) or with no distinct row arrangement (mixed intercropping) as defined by Andrews and Kassam (1976). The entire archaeal community was examined by sequencing of 16S rRNA genes and subsequent analysis of

obtained sequencing data. We hypothesized (1) that intercropping of bean and wheat results in an increased wheat biomass and in turn to a decreased bean biomass. In addition, we expected (2) crop species and cropping regimes affect community structure and diversity of soil archaea. We further hypothesized (3) that that species richness and diversity are higher in multiple cropping systems compared to monocultures.

Materials and Methods

Experimental design

To investigate the effect of cropping system and crop species on the entire archaeal community, a greenhouse experiment was conducted in summer 2015. Faba bean (genotype: Hiverna) and wheat (genotype: Hybery) were cultured in monoculture or as mixture in polypropylene containers (Semadeni, Eurobehälter, LogiLine ® SGL Boden, 600 x 400 x 212 mm), containing 75% sterile commercial plant substrate (Fruhstorfer Erde Typ T25; N: 200-300 mg L^{-1}, P_2O_5: 200-300 mg L^{-1}; Hawita Gruppe GmbH Vechta, Germany) and 25% sand. This plant substrate is a peaty soil with a pH ($CaCl_2$) of 5.5 to 6.5. For monocropping systems, twenty faba bean (FBM) or eighty wheat (WM) plants per container were grown in rows, respectively (Figure 1). In multiple cropping systems, approximately forty wheat and ten faba bean plants per container were grown in rows (row intercropping; RI) or with no distinct row arrangement (mixed intercropping; MI). The four different cropping regimes (monoculture wheat, monoculture bean, row or mixed intercropping of bean and wheat) were replicated five times in a randomized design. All plants were daily irrigated. To increase nutrient-limitation as well as intra- and interspecies interactions between the plants, no fertilization regime was applied.

Sampling and edaphic parameters

Soil samples were collected after a growing period of four weeks. We sampled the bulk soil, defined as root-free soil around the crops, and the rhizosphere soil, defined as soil tightly adhering to the roots. In the two intercropping regimes, bulk and rhizosphere soil samples of the two crop species were pooled for each container. All samples were frozen and stored at -20°C. For determination of soil properties, subsamples were dried at 60°C for two days and sieved to < 2mm. Soil organic carbon (C) and total nitrogen (N) concentrations from all dried subsamples were determined using a LECO TruSpec CN analyzer (Leco Copr., St. Joseph, MI). The gravimetric soil water content (%) of all soil samples was calculated from

oven-dried subsamples. Soil pH values were measured as follows: 2 g soil of each container and crop species were mixed with 5 mL PCR grade water. After incubation for 24 hours, pH_{Water} was measured in the supernatant with a glass electrode. Subsequently, 0.37 g KCl was added and pH_{KCl} was measured. Detailed information on soil characteristics is given in the supplemental material (Table S1).

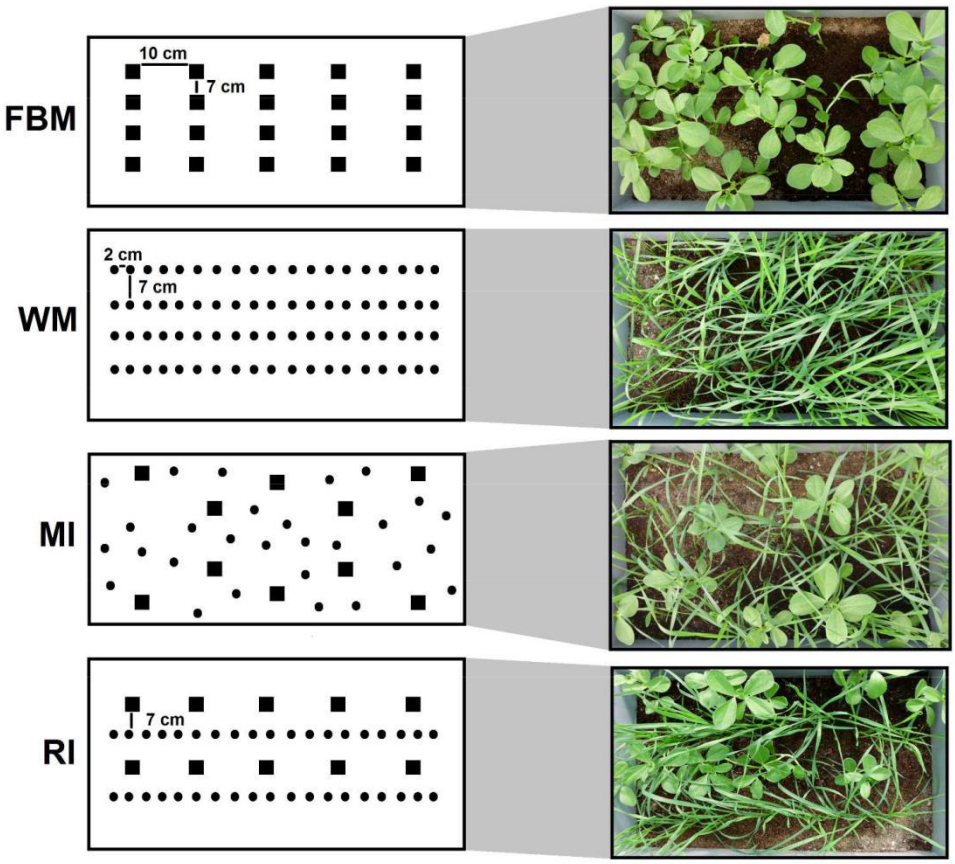

Figure 1. Study design. The 4 different cropping systems are shown (from top to bottom): faba bean in monoculture (FBM), wheat in monoculture (WM), mixed intercropping (MI) and row intercropping (RI) of faba bean and wheat.

Crop biomass and height

Aboveground as well as below-ground plant material of the two crop species were harvested separately for each container. Aboveground (shoots, leaves) and root biomass for each crop species and each container were measured. In addition, the heights of approximately 10 faba bean and 20 wheat plants in intercropping regimes and approximately 20 plants of monocropped faba bean and wheat plants were measured. Ten wheat and five bean plants which did not show any obvious disease symptoms were randomly selected from

each container for molecular analysis. For determination of water content, ten wheat and five faba bean plants per container were weighted and subsequently oven-dried at 60°C for 48 h and re-weighted.

Extraction of environmental DNA and amplification of archaeal 16S rRNA genes

DNA was extracted from 0.125 g bulk or rhizosphere soil using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., USA) according to the manufacture's protocol. DNA concentrations were quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA) according to the manufacturer's protocol.

To assess archaeal community structures, the V4-V5 region of the 16S rRNA was amplified by PCR. The PCR reaction (25 µL) contained: 5 µL of five-fold Phusion GC buffer (Thermo Scientific, Waltham, MA, USA), 200 µM of each of the four deoxynucleoside triphosphates, 4 µM of each primer, 1.5 mM $MgCl_2$, 2.5% DMSO, 1 U of Phusion High Fidelity Hot Start DNA polymerase (Thermo Scientific) and approximately 10 ng of DNA. The following thermal cycling scheme was used: initial denaturation at 98°C for 30 s and 30 cycles of 10 s at 98°C, 30 s at 63°C and 15 s at 72°C. The final extension was carried out at 72°C for 2 min. The initial annealing temperature of 63°C was reduced 1°C per cycle for the next 10 cycles and maintained at the final annealing temperature of 53°C for the remaining 20 cycles. Negative controls were performed by incubating the reaction mixture without template. The V4-V5 region was amplified with the following set of primers containing MiSeq adaptor (underlined): (Miseq_Arch_for5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GGTGBCAGCCGCCGCGGTAA -3' and Miseq_Arch_rev 5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-CCCGCCAATTYCTTTAAG -3'). Primer were designed *de novo* using all aligned archaeal sequences from the most recent, non-redundant SILVA (SSURef 123) database (Quast *et al.,* 2013). After removal of all vertical gaps, Shannon values were determined in a sliding window of 20 bp in 1 bp-steps along the sequence to identify highly conserved regions (low Shannon values). Subsequently, primers targeting these regions were designed using the proportional distribution of each nucleotide at each position.

Two independent PCR reactions were performed for rhizosphere as well as for bulk soil samples of each container. Obtained PCR products were purified using the peqGOLD Gel Extraction kit (Peqlab) and subsequently quantified using the Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Invitrogen) as recommended by the manufacturer. The four purified PCR products per container were subsequently pooled in equal amounts. The Göttingen

Genomics Laboratory determined the sequences of the partial 16S rRNA genes employing the MiSeq Sequencing platform and the MiSeq Reagent Kit v3 as recommended by the manufacturer (Illumina, San Diego, USA).

Processing and analysis of datasets

Generated 16S rRNA gene and rRNA datasets were processed with Usearch version 8.0.1623 (Edgar, 2010): Paired-end reads were merged and quality-filtered. Filtering included the removal of reads shorter than 350 bp. Processed sequences of all samples were joined and clustered in operational taxonomic units (OTUs) at 3% genetic divergence using the UPARSE algorithm implemented in USEARCH. A *de novo* chimera removal was included in the clustering step. Afterwards, putative chimeric sequences were removed using UCHIME in reference mode with the most recent SILVA database (Silva SSURef 123 NR) as reference data set (Camacho *et al.*, 2009). Subsequently, processed sequences were remapped on OTU sequences to calculate the distribution of each OTU in every sample. In addition, OTU sequence were taxonomically classified by BLAST alignment against the most recent SILVA database (see above) using QIIME (Caporaso *et al.,* 2010). All non-archaeal OTUs were removed (Supplementary Table S1). Alpha diversity indices (Supplementary Table S2) and rarefaction curves (Supplementary Figure S1) were calculated with QIIME version 1.9 as described previously (Wemheuer *et al.*, 2014).

Statistical analysis

All statistical analyses were conducted employing R (version 3.1.2; R Development Core Team, 2014). Differences were considered as statistically significant with $P \leq 0.05$. Environmental parameters were tested for normality using the Shapiro test and for variance homogeneity using the Levene test within the car package. Correlation between cropping types and soil pH as well as aboveground and root biomass were subsequently tested by ANOVA and Kruskal-Wallis test, respectively. Ordination plots (NMDS; non-metric multidimensional scaling) were calculated using the metaMDS function within the vegan package (Oksanen *et al.,* 2013) based on Bray-Curtis dissimilarities. The impact of cropping system on archaeal community structure was tested using the envfit function as described previously (Wietz *et al.,* 2015). Indicator species were identified using the multipatt function within the IndicSpecies package (De Cáceres and Legendre, 2009).

Sequence data deposition

Sequence data were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number SRA347195.

Results and Discussion

Soil properties

In this study, the influence of cropping system and regime on archaeal communities in soil was assessed. Therefore, bulk and rhizosphere soil samples from four different cropping regimes (monoculture wheat, monoculture bean, row or mixed cropping of bean and wheat) were collected. Several soil properties from soil samples including total N and C content, C:N ratio, water content, as well as soil pH were determined (Table 1, Table S1 in the Supplements). The C:N ratios ranged between 43.5 and 53.2 with the highest C:N ratio in rhizosphere soil samples of wheat in cropping regime MI (Table S1 in the Supplements). The lowest and highest C:N ratios in bulk soil samples were observed in cropping regimes WM and MI, respectively (Table 1). As the C:N ratio explains the ability to use soil carbon and nitrogen for microbial processes such as the decomposition of soil organic matter (Wardle, 1992), it is an indicator of soil microbial activity (He *et al*., 1997). Statistical analysis revealed that C:N ratios in bulk as well as in rhizosphere soil samples did not differ significantly among the four cropping regimes with one exception: the C:N ratio in bulk soil samples of the cropping regime MI were significantly higher compared to the other cropping regimes (Table 1).

The soil pH values were relatively constant among all soil samples (pH_{water} = 6.82±0.13; pH_{KCl} = 6.55±0.09) with no significant differences between the four cropping regimes (data not shown). Water content varied between 61.6 and 86.3% with the lowest and the highest water content in bulk as well as rhizosphere soil samples of WM and FBM, respectively (Table 1, Table S1 in the Supplements). In addition, the water content in rhizosphere soil samples of WM was significantly lower compared to the other three cropping regimes (Table 1). We tested further if one of the edaphic parameters influenced the richness or the diversity of soil archaeal communities. No influence of water content, C:N ratio and soil pH on diversity and richness was detected (data not shown).

Plant growth and biomass

To analyse the effect of cropping system and regime on plant growth and yield, aboveground as well as belowground biomass were recorded. Although not been statically significant, a higher aboveground biomass of wheat plants was observed in intercropping systems compared to the monocultures (Figure 2). This is in line with recent studies in legume-cereal intercropping systems (e.g., Li *et al.,* 1999; Li *et al.,* 2001; Zhang *et al,.* 2004; Zhang *et al,.* 2015b). Zhang *et al.* (2004) found an increased maize yield in a maize/faba bean intercropping system due to an interspecific facilitation in phosphorus and nitrogen (N) uptake. In a study of Inal *et al.* (2007), the shoot yield of intercropped maize and intercropped peanut was lower compared to monoculture plants. The authors suggested that this effect mainly results from root development shortage by associated maize or competition between the plants. In mixtures combining a cereal and a legume, cereals are more competitive than legumes in taking up N from the soil due to faster root development and demand (Corre-Hellou et al., 2006) which could explain the findings in the present study. However, Inal et al. (2007) observed a better nutrition of intercropped maize and peanut with several micronutrients. Recently, it was concluded that th beneficial effects of intercropping crop yields and/or nutrient supply resulted mainly from (positive) interspecific interactions in the rhizosphere (Li *et al.,* 1999; Li *et al.,* 2001; Zhang *et al.,* 2004; Inal *et al.,* 2007).

Interestingly, intercropped plants had a higher average root biomass when grown in rows (RI) compared to those grown with no distinct row arrangement (MI) or in monocultures (Figure 2). In addition, shoot/root ratio for faba bean decreased from monoculture over mixed intercropping to row intercropping becoming significant between FBM and RI (data not shown). This indicates a higher root biomass for faba bean in RI which in turn correspondsto increased environmental stress (Eghball and Maranville, 1993).

Table 1: Edaphic parameters. Due to the low amount of sampling material, pH was not determined in rhizosphere samples. Different letters indicate significant differences with $P < 0.05$.

	Bulk soil						*Rhizosphere*							
							Faba bean				*Wheat*			
	pH H_2O	***pH KCl***	***Moisture [%]***	***C_{total} [%]***	***N_{total} [%]***	***C:N ratio***	***Moisture [%]***	***C_{total} [%]***	***N_{total} [%]***	***C:N ratio***	***Moisture [%]***	***C_{total} [%]***	***N_{total} [%]***	***C:N ratio***
FBM	6.82±0.16 a	6.55±0.13 a	31.22±6.25 a	8.42±2.35 a	0.18±0.05 a	46.33±1.09 a	34.43±3.16 a	10.50±1.02 a	0.22±0.03 a	48.24±1.68 a				
WM	6.80±0.11 a	6.57±0.05 a	18.14±4.03 c	6.33±0.54 a	0.14±0.01 a	45.11±1.26 a					22.33±2.99 a	10.86±1.66 a	0.22±0.03 a	50.29±2.25 a
RI	6.76±0.18 a	6.51±0.09 a	21.54±3.61 b,c	7.68±2.15 a	0.17±0.04 a	46.47±1.08 a	29.12±4.11 a	13.76±2.63 a	0.27±0.05 a	50.13±1.07 a	26.44±3.56 a	12.08±2.26 a	0.24±0.05 a	50.92±1.22 a
MI	6.83±0.13 a	6.56±0.09 a	24.99±2.19 a,b,c	7.83±1.03 a	0.17±0.02 a	47.41±0.37 b	26.39±6.33 a	11.89±2.17 a	0.24±0.05 a	49.99±2.20 a	24.86±6.14 a	9.48±1.55 a	0.18±0.03 a	51.43±1.55 a

Abbreviations: C_{total}, total soil organic carbon; N_{total}, total soil nitrogen; FBM, faba bean in monoculture; WM, wheat in monoculture; MI, mixed intercropping; RI, row intercropping.

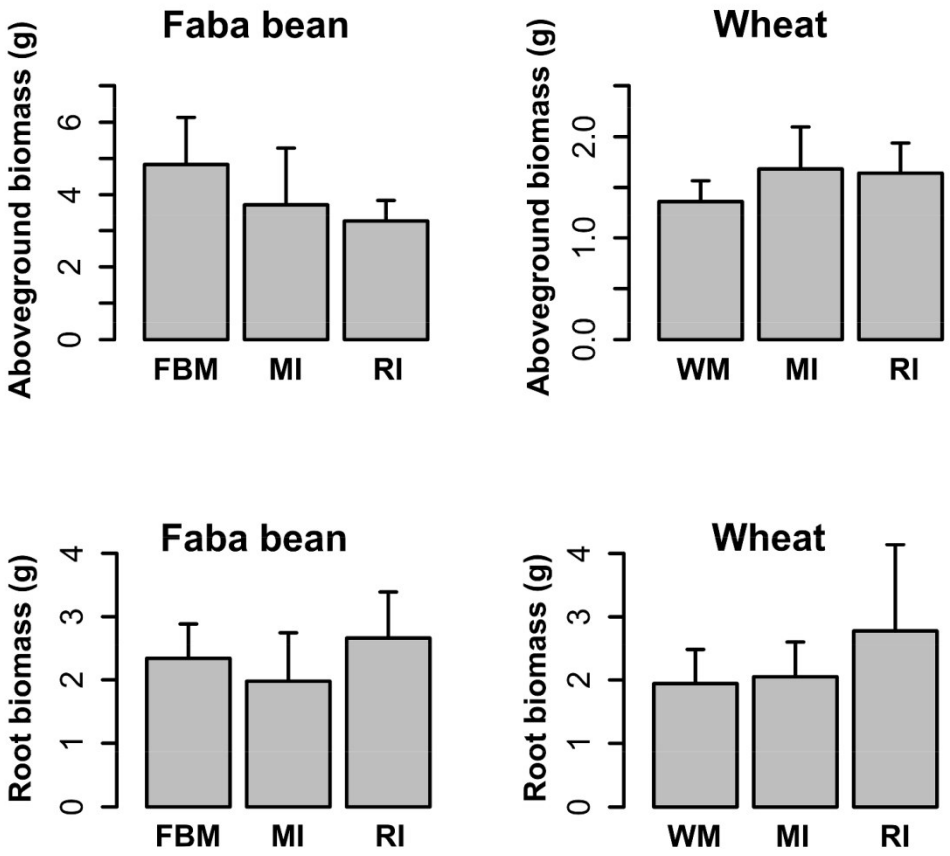

Figure 2. Average aboveground and root biomass per plant of faba bean (FBM) and wheat (WM) in monocultures as well as in mixed (MI) and row intercropping (RI).

Our results might be related to interspecific competition and facilitation that act on the crop plants in intercropping systems simultaneously (Li *et al*., 1999; Eghball and Maranville, 1993; Zhang and Li, 2003; Ghosh *et al*., 2006). Ghosh *et al*. (2006) showed that biological activities as well as several above- and belowground growth components were improved in intercropped sorghum but reduced in intercropped soybean indicating interspecies competition between the crops. In a study on low-input legume-cereal intercropping systems, the interaction between aboveground and belowground competition varied among species from negative to positive synergy and additivity (Mariotti *et al*., 2009). The authors concluded that the sowing time of the components and/or the design of the intercropping system should be modified to reduce the competition and to enhance facilitation effects.

General characteristics of archaeal community structure

To analyse and compare community structure and diversity of the soil archaeal communities, DNA was extracted from soil samples of faba bean and wheat plants grown in monoculture or in mixture. Subsequently, archaeal community composition and diversity were assessed by

amplicon-based sequencing of the V4-V5 region of the 16S rRNA gene. Following quality filtering, denoising, and removal of potential chimeras and non-archaeal sequences, 47,998 high quality sequences with an average read length of 396 bp were used for analyzing archaeal community structures and diversity. Obtained sequences were clustered into 164 OTUs. Calculated rarefaction curves at 3% genetic divergence (species level) revealed that the majority of the archaeal community was covered by the surveying effort (Figure 3). In addition, calculated coverage was 78.3% (Supplementary Table S3).

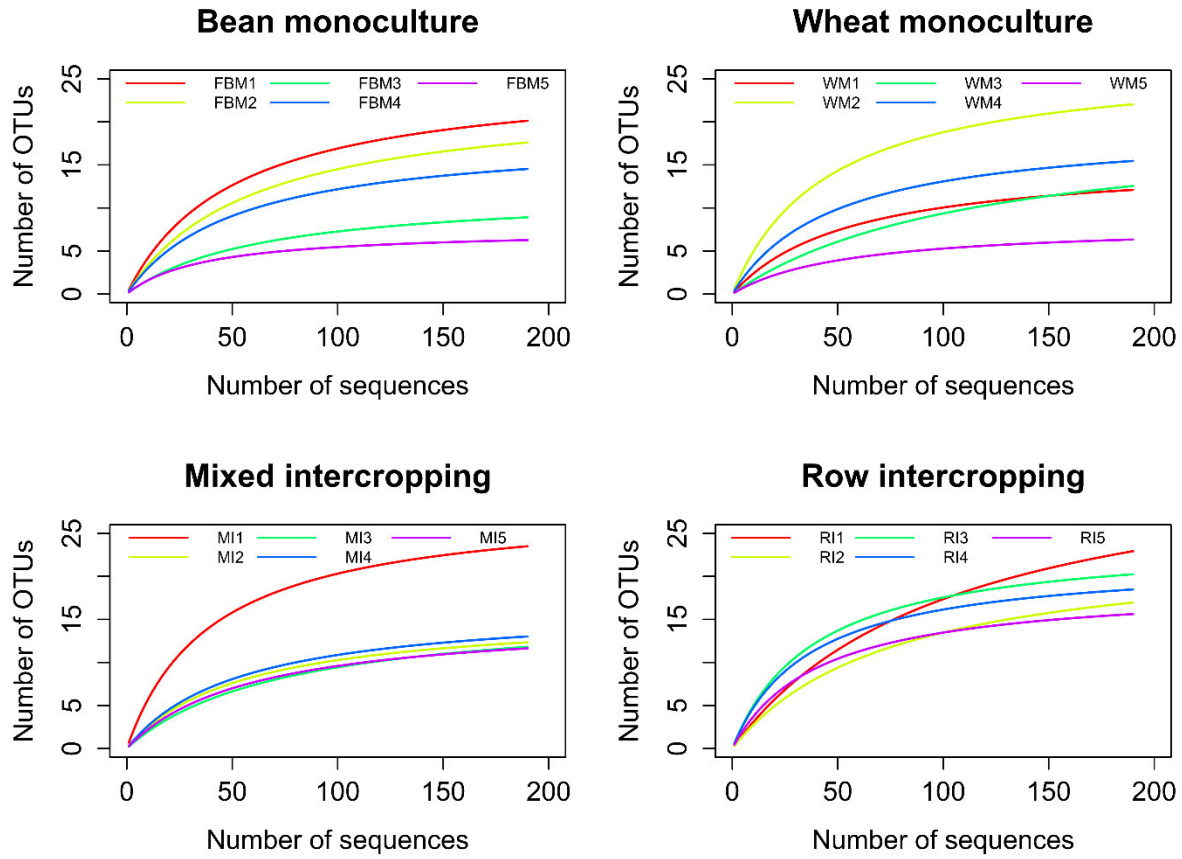

Figure 3: Rarefaction curves for five samples per cropping regime. Faba bean in monoculture (**FBM1-5**), wheat in monoculture (**WM1-5**) as well as faba bean and wheat in mixed (**MI1-5**) or row intercropping (**RI1-5**) are depicted.

Thaumarchaeota were identified as the dominant archaeal phylum across all samples (55.7%) followed by *Woesearchaeota* (DHVEG-6) (26.7%), Euryarchaeota (15.8%) and the Miscellaneous Crenarchaeotic Group (1.7%) (Figure 4). *Thaumarchaeota* were dominated by members of the Soil Crenarchaeotic Group and the terrestrial group. Within the *Euryarchaeota*, *Methanobacteria* were predominant, followed by *Thermoplasmata* and *Methanomicrobia.* These results were roughly in agreement with previous studies showing that *Thaumarchaeota*, the Miscellaneous Crenarchaeotic Group, and *Euryarchaeota* are common in a variety of different soils such as grassland, rainforest or agricultural soils (Etto

et al., 2012; Chroňáková *et al.,* 2015; Schneider *et al.,* 2015). In a previous study on the influence of outdoor cattle husbandry on archaeal communities in four different grassland soils, *Thaumarchaeota*, *Euryarchaeota* as well as the Miscellaneous Crenarchaeotic Group were detected (Chroňáková *et al.,* 2015). This is in line with the results of Schneider *et al.* (2015) who analysed the impact of rainforest transformation to intensively managed crop systems on prokaryotes. They found that the archaeal community was mainly represented by *Thaumarchaeota* as well as *Euryarchaeota* and in lower abundance by the Miscellaneous Crenarchaeotic Group.

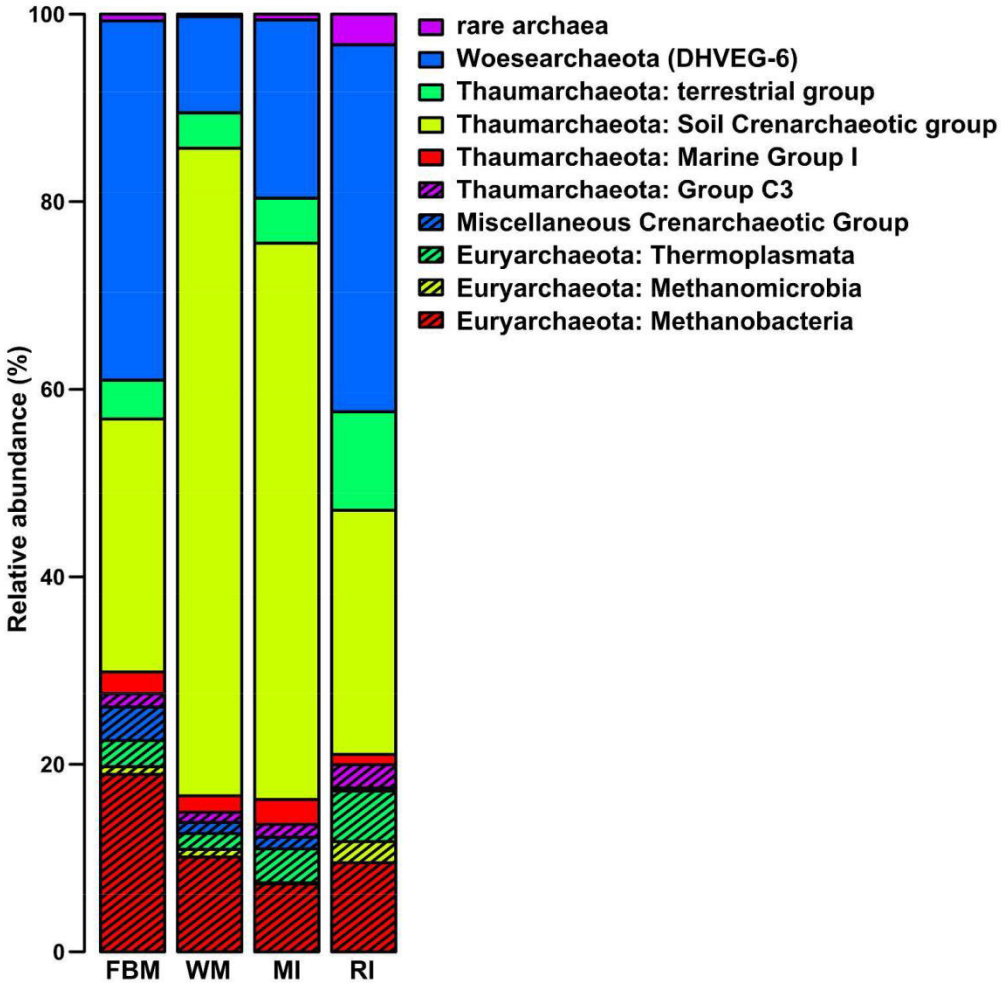

Figure 4. Average composition of the archeal community under the four cropping regimes faba bean monoculture (FBM), wheat monoculture (WM) as well as mixed (MI) and row intercropping (RI).

The occurrence of *Woesearchaeota* in soil samples of the present study is interesting, as members of this phylum dominated the surface waters of oligotrophic lakes (Ortiz-Alvarez and Casamayor, 2015) as well as the surface layer (0-3 cm) sediment of Lake Taihu (Fan and Xing, 2016). So far, most known archaeal genomes of this phylum are sediment- or groundwater-associated (Castelle *et al.,* 2015). As *Woesearchaeota* are a relatively young

phylum, knowledge about ecology and distribution of this phylum in other ecosystems such as soil is still rather limited.

Diversity of archaeal community is altered by cropping system but not by crop species

The diversity and richness of the archaeal communities did not differ between individually cropped wheat and faba bean plants (Supplementary Table S3). In contrast, Dorr de Quadros *et al.* (2012) investigated the archaeal and bacterial community in three legumes-cereal crop rotation systems in a subtropical Acrisol and found that the microbial diversity was higher in soil samples which were cultivated with cereals only. In two recent studies, the cultivation of different bioenergy crops significantly changed the abundance and diversity of ammonia-oxidizing, N-fixing and denitrifying organisms in soils (Mao, *et al*., 2011; 2013). The contrasting findings could be explained by the longer growth period of plants on experimental field sites, whereas a four-week greenhouse experiment was conducted in this study.

The archaeal richness in soil samples of the RI regime was higher than in soil samples derived from the other cropping regimes (Table S3 in the Supplements). However, statistical analysis revealed that the richness of soil archaeal communities was not affected by the different cropping regimes (data not shown). This finding might be related to the growth period of the plant in the present study which is supported by a previous study of Hargreaves *et al*. (2012). The authors observed that the cropping system influenced the diversity of bacteria and archaea only in July when plants are fully grown. Previous studies showed that a better growth of intercropped plants could improve the quantity and quality of root exudates and the turnover of root biomass, which benefits the growth of microorganisms in the rhizosphere (Chu *et al.,* 2007; Yang *et al.*, 2007). Compared to RI, plants in MI and in monoculture were grown in closer vicinity to other plants of the same and/or the other crop species, resulting in higher intra- and intraspecific competition, respectively. This could have influenced the nutrient availability and thus the archaeal diversity in soil. However, still little is known about the effect of multiple cropping systems and crop species on archaeal diversity as most previous studies focused only on ammonia-acidizing archaea (e.g., Mao *et al*., 2011; 2013).

Crop species and cropping system altered the structure of soil archaeal communities

Crop species as well as cropping regime influenced the archaeal community structure as well as the abundance of archaeal phyla and taxa (Figure 4). For example, the Miscellaneous

Crenarchaeotic Group was more abundant in FBM than in the other cropping regimes. Legumes such as faba bean are well-known for their symbiosis with rhizobia which can improve soil fertility such as N content (Zahran, 1999). Thus, we suggest that members of this group favour nutrient rich soils. This is supported by the results of Chroňáková *et al.* (2015) who observed high abundances of the Miscellaneous Crenarchaeotic group in a nutrient rich, cattle-influenced grassland soil. In contrast to this, the Miscellaneous Crenarchaeotic Group was more abundant in managed soils with lower C:N ratios than in soils of the other land-use systems such as rainforests (Schneider *et al.,* 2015). Members of the Miscellaneous Crenarchaeotic Group have been found in a variety of different marine and continental habitats including subsurface ecosystems, soils or hydrothermal vents (as reviewed in Kubo *et al.,* 2012). However, the ecological role of the Miscellaneous Crenarchaeotic Group is almost unknown.

Thaumarchaeota was the most abundant archaeal phylum in soil samples of cropping regimes WM and MI (75.8 and 68.6 %, respectively). Members of the *Thaumarchaeota* are widely distributed in moderate environments including soil (Nicol *et al*., 2003; Chroňáková *et al.,* 2015; Schneider *et al.,* 2015) and marine waters (Wemheuer *et al.,* 2012), but are also found in extreme environments such as hot springs (Wemheuer *et al.,* 2013) or hypersaline lakes (Schneider *et al.,* 2013). In the last years, the phylum *Thaumarchaeota* gained more attention as it comprises all known ammonia-oxidizing archaea. Thus, this phylum plays an important role in the global N and C cycles (Pester *et al*., 2011). As only a few members of the *Thaumarchaeota* have been cultivated (Stahl and De la Torre, 2012), our knowledge of their ecological role in soil ecosystems is limited. However, all cultivated *Thaumarchaeota* are adapted to low concentrations of substrates, i.e. ammonia (Stieglmeier *et al.,* 2014). This could explain the predominance of this phylum in the cropping regimes WM and MI due to the higher demand of wheat for N resulting in lower N concentrations. This is in agreement with the study of Chan *et al.* (2013). Here, the abundance of ammonia-oxidizing archaea was higher in maize than in alfalfa cultivation. The authors suggested that this is probably related to the high affinity of ammonia-oxidizing archaea for ammonia because the N availability was limited in maize cultivation due to the high N demand of maize. As we applied no fertilizer during the experiment, this fact could have also play a role for the archaeal community changes observed in this study.

Members of the *Euryarchaeota* were detected in higher abundances in soil of cropping regimes FBM and RI than in WM and MI. A comparison of this phylum at class level revealed that the relative abundance of *Methanobacteria* was higher in FMB, whereas

Thermoplasmata and *Methanomicrobia* increased in soil of RI. Members of the euryarchaeotal classes *Methanomicrobia* and *Methanobacteria* are known as methanogens (Garrity *et al.,* 2001) and are involved in sulfate reduction and the carbon cycle through methanogenesis in anoxic marine sediments (Lyimo *et al.,* 2009). *Thermoplasmata* have been found in a wide range of environments such as soils and marine sediments (reviewed in Paul *et al.,* 2012). Recently, members of this class have been detected as methylotrophic methanogens in bovine rumen with methylamines as their major energy and carbon sources, indicating their high potential as target in future strategies to mitigate methane emissions from ruminant livestock (Poulsen *et al.,* 2013). Moreover, several species of the phylum *Thermoplasmata* play an important role in iron and sulphur cycling (Edwards *et al.,* 2000). Thus, they may substantially improve the nutrient status of plants in RI which, in turns, resulted in a higher diversity of archaea as observed in the present study.

Interestingly, *Woesearchaeota* were the most abundant archaeal group in soil samples of FBM and RI. As mentioned above, genomes of this phylum are sediment- or groundwater-associated (Castelle *et al.,* 2015). The AR20 genome is the first complete genome of this phylum. Metabolic reconstruction indicates that most of the core biosynthetic pathways are partial or absent, such as glycolysis. However, the AR20 genome encodes genes for the synthesis of several traits indicating a symbiotic or parasitic lifestyle. It is possible that members of this phylum form close mutualistic relationships with faba bean as it is known for symbiotic bacteria (rhizobia).

Ordination analysis revealed that the cropping system (monoculture versus multiple cropping) affected the archaeal community composition as plants grown in monoculture regimes displayed a significant different community pattern compared to those grown under intercropping regimes (Figure 5A). We suggest that these changes are mainly attributed to the archaeal communities in RI regimes, which are different to community structures observed in monocultures and MI (Figure 5B). Similar results were observed in two previous studies (Wang *et al*., 2012; Breidenbach *et al.,* 2015). Wang *et al.* (2012) analysed the effect of different cropping systems (monocultures vs. mixed cultures), crop species and soil type on the microbial community structure in three different soils by denaturing gradient gel electrophoresis. They found that ammonia-oxidizing-archaea and N_2-fixer community composition were strongly influenced by crop species and cropping system indicating that these groups are quite sensitive to environmental conditions. Recently, Breidenbach *et al.* (2015) observed that crop rotation affected the archaeal community composition. In contrast, Chan *et al.* (2013) found only minor cropping effects on soil archaeal gene population

dynamics. This is in accordance with a recent study showing that crop rotation influenced archaeal communities only slightly (Scavino *et al.,* 2013; Watanabe *et al*., 2006).

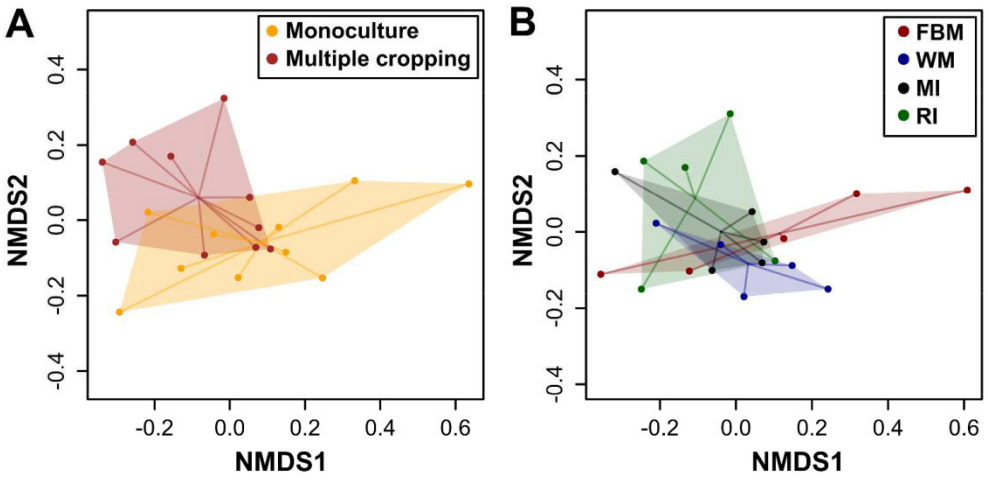

Figure 5. Effect of cropping system (A) and cropping regime (B) on archeal community structures.

Ordination analysis further revealed that there were no significant differences between archaeal community compositions of faba bean and wheat growing in monoculture (Figure 5B). This might be related to the short growing period. Zhang *et al*. (2015) found that growth stages of plants shaped the community compositions as well as in the abundance of ammonia-oxidizing archaea in the rhizospheres of faba bean and maize plants. Moreover, the authors detected an effect of plant species on these archaea. In contrast to this, Fan *et al*. (2011) showed that plant species did not significantly change the community structure of ammonia-oxidizing archaea. In another study, the ammonia-oxidizing archaeal communities were not affected by three different bioenergy crop species (Mao *et al*., 2013). Plant species effects on archaeal communities were detected in previous studies on two wetland plants (Llirós *et al*., 2014) and two contrasting vascular plants (Cadillo-Quiroz *et al*., 2010). Mao *et al*. (2011) showed that the planting of bioenergy crop species altered the archaeal community as well as N-cycling genes in soil.

Although we could not detect any statistical influence of crop species growing in monoculture on archaeal community structure, several taxa differed in their abundance. We suggest two possible explanations for these effects. First, as plant species release specific root exudates and thereby select a specific community, the simultaneous growing of two or more plant species will result in a higher number and variety of root exudates. This was shown in two previous studies (Wu *et al*., 2009; Lamb *et al*., 2011). Wu *et al*. (2009) found that the composition of the archaeal community in a paddy field was affected by different rice cultivars, which varied in their quantity of organic root exudates. Lamb *et al*. (2011) observed

that plant species richness had direct positive effects on archaeal *amoA* abundance. They suggested that with increasing complexity of the plant community more and different root exudates are distributed, which results in higher numbers of microbial niches in soil.

Secondly, the influence of plant species on archaeal communities can be related to differences in plant-specific traits, including rooting depth as well as litter quantity and quality (Gregory, 2006). Other reasons are secondary effects of plant species on soil pH and nutrient levels (Wardle, 1992; Lynch and Whipps, 1991), which might result in archaeal community compositional changes. Inal *et al*. (2007) found that the rhizosphere was modified by the roots of intercropped maize and peanut plants, which improved the availability of phosphorus (P) and iron (Fe). Consequently, crops growing in multiple cropping systems interact with each other and the environment in an information feedback loop: the environment affects the plants, and the plants affect the environment (Malézieux *et al*., 2009). This is supported by the results of the present study as plants of faba bean and wheat under different cropping regimes interact with each other and their environment including soil archaeal communities in diverse ways.

To identify potential indicators for the two crop species faba bean or wheat, cropping system (monoculture versus multiple cropping) and the four cropping regimes (FBM, WM, RI or MI), multipattern analysis was applied. Several OTUs had significant indicator potential and were therefore strongly associated with a certain cropping regime (Table 2). Interestingly, no OTU was significantly associated with monoculture wheat plants, while several OTUs including *Methanobacterium* sp. and *Methanomassilicoccus* sp. were associated with monoculture faba bean (Table 2). *Methanobacterium* sp. and *Methanomassiliicoccus* sp. are known as anaerobic methanogenic archaea. *Methanobacterium* was isolated from sewage sludge and is also capably of autotrophy/ autotrophic growth (Zeikus and Wolee, 1972), whereas *Methanomassiliicoccus luminyensis* (order *Methanomassiliicoccales)* was first isolated from human faeces (Dridi *et al*., 2012). Interestingly, members of the *Methanomassiliicoccales* have an energy metabolism distinct from other methanogens (Söllinger *et al*., 2015). However, two uncultured archaeal OTUs were even better indicators for faba bean and could be found in each soil in which only faba bean was grown (B=1 in both cases). They belonged to the *Woesearcheota* and the Miscellaneous Crenarchaeotic Group. Furthermore, a member of the *Thermoplasmatales* Marine Group II could be identified as a good indicator for multiple cropping systems, and was additionally present in all RI soils.

Table 2. Results of the indicator species analysis showing potential indicative archaeal OTUs for the different crop species, cropping systems and cropping regimes. Statistical values: A - species only occurs in sites of this group, B - all sites of this group harbour this species.

Indicator group	*archeal OTU*	*A*	*B*
Crop species			
FBM	uncultured *Woesearcheota* archeon	0.9632	1
	uncultured crenarcheote (misc. crenarcheaotic group)	0.8754	1
	uncultured thaumarcheaon	0.7826	0.8
	Methanomassiliicoccus sp.	0.9787	0.6
	Methanobacterium sp.	0.8333	0.6
Multiple cropping	uncultured *Thaumarcheota* terrestrial group crenarcheaote	0.9083	0.9333
Cropping system (Mono vs. Multiple)			
Multiple cropping	*Thermoplasmatales* Marine Group II archeaon	0.9153	0.7
Cropping regime (FBM, WM, MI and RI)			
FBM	uncultured *Woesearcheota* archeon	0.9325	1
	uncultured crenarcheote (misc. crenarcheaotic group)	0.8075	1
	Methanomassiliicoccus sp.	0.9583	0.6
RI	*Thermoplasmatales* Marine Group II archeaon	0.7119	1
	uncultured *Thermoplasmatales* archeaon	0.9444	0.6
FBM, MI and RI	uncultured *Thaumarcheota* terrestrial group crenarcheaote	0.9228	0.9333

Conclusion

Due to the prevalent role of archaea in biogeochemical cycling and thus in agriculture, it is important to assess structural responses of these communities to crop species and cropping systems. In this study, the archaeal community in soil of wheat and bean grown in monoculture or as multiple cropping system were investigated. In addition, aboveground and root biomass were measured. The biomass of the two crop species did not significantly vary between the four different cropping regimes, although the root biomass of monoculture wheat plants tended to be higher than that of intercropped wheat plants. This is in contrast to our hypothesis (1) that intercropping would result in higher biomass of wheat plants and in turn to a decreased bean biomass. According to our hypothesis (2), crop species as well as cropping regimes influenced the archaeal community structure and/or the abundance of several taxa. Moreover, we found a clear separation of soil archaeal communities by monoculture and multiple cropping system. Contrary to our hypothesis (2), diversity and richness of the archaeal communities did not differ between individually cropped wheat and faba bean plants. In contrast to this, we observed a higher diversity in row intercropping systems than in monoculture or mixed intercropping which is partly in line with our hypothesis (3). We suggest that this is the result of the intra- and interspecific interactions between plants, edaphic parameters, and archaeal communities. In the near future, we will analyse the response of soil archaea to cropping systems under field conditions. This will enable us to better understand the ecology and functions of soil archaea in intercropping systems.

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SOIL ARCHAEAL COMMUNITIES UNDER DIFFERENT CROPPING REGIMES

SUPPLEMENTARY INFORMATION

Supplementary figures and tables are provided along with the electronic version of this thesis (on DVD), under the following paths:

Table S1:	Supplementary Information/Chapter III.7/ Table S1.docx
Table S2:	Supplementary Information/Chapter III.7/ Table S2.docx
Table S3:	Supplementary Information/Chapter III.7/ Table S3.doc

CHAPTER III.8.

MICROBIAL COMMUNITIES UNDER CROPPING REGIMES

III.8.

For submission to Frontiers in Microbiology (Specialty Section: Plant Biotic Interactions)

Differences between soil and endophytic microbial diversity of wheat (*Triticum aestivum* L.) and faba bean (*Vicia faba* L.) in monoculture and intercropping

Running title: Microbial communities under cropping regimes

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MICROBIAL COMMUNITIES UNDER CROPPING REGIMES

Abstract

Multiple cropping systems provide many economic and ecological advantages. They have been shown to enhance plant productivity and offer great potential in sustainable intensification of agriculture. Thus, it is of crucial importance to understand the response of microbial communities towards these cropping systems. Bacterial and fungal soil communities are known to be influenced by cropping system or by crop plant, but little is known about the effects of multiple cropping systems on endophytic communities and the interactions between these communities. Here, we studied bacterial and fungal communities in bulk and rhizosphere soils as well as in the roots and leaves of wheat and faba bean plants growing in monoculture and in two multiple cropping regimes. Cropping regime and crop plant did not affect microbial community composition, but microbial communities were different between the different sampling locations investigated. This effect was more pronounced for bacteria than fungi, probably due to the active mode of colonization employed by fungi. Multipattern analyses revealed that differences in bacterial community composition between plant compartments are most likely caused by proteobacterial endophytes strongly associated with either leaves or roots. Co-occurrence networks analysis showed strong negative interactions between fungal OTUs, indicating strong competition and probably niche adaptation by specialized fungal taxa. In addition, differences in the richness and diversity between the crop plants were recorded. To our knowledge, this is one of the first studies investigating the bacterial and fungal community in rhizosphere, soil and endosphere with regard to multiple cropping by using high-throughput next generation sequencing.

Introduction

In the last decades, multiple (or mixed) cropping systems have come into the focus of interest due to their potential for a sustainable intensification of agriculture (Vandermeer, 1992). They provide enhanced ecological and economical services such as a reduction of pest damages and herbivore density (Francis, 1986; Letourneau *et al.*, 2011; Winter *et al.*, 2014). In addition, multiple cropping systems enhance plant productivity by improving a more efficient use of available resources (Hauggaard-Nielsen and Jensen, 2005; Aziz *et al.*, 2015). Recently, it was suggested that an enhanced yield may also be the result of (positive) interspecific interactions in the rhizosphere (Li *et al.*, 1999; Inal *et al.*, 2007) or of changes in microbial and chemical properties in the soil (Song *et al.*, 2007b).

Microorganisms including bacteria and fungi play essential roles in biogeochemical cycling and thus, ecosystem functioning (Ellouze *et al.*, 2014*;* van der Heijden and Hartmann, 2016). They

have been found in almost every environment such as the plant microbiome (Lugtenberg and Kamilova, 2009; Turner *et al.*, 2013a; Berg *et al.*, 2014; van der Heijden and Hartmann, 2016). Many bacteria and fungi in the plant's rhizosphere and endosphere are beneficial to plant nutrition acquisition, health and growth (Kent and Triplett, 2002; Lugtenberg and Kamilova, 2009; Philippot *et al.*, 2013). These microorganisms may also alleviate abiotic stress conditions of their host plants (Malinowski and Belesky, 2000; De Zelicourt *et al.*, 2013; Timmusk *et al.*, 2014). In addition, they can enhance resistance of the host plant against biotic stressors such as herbivores or pathogens (Siddiqui and Shaukat, 2003; Dematheis *et al.*, 2013; Vidal and Jaber, 2015). Therefore, these microorganisms play an important role in agricultural production.

In the last years, the number of studies investigating the effect of agricultural practices such as cropping systems on the plant microbiome in endo- and rhizosphere has increased (e.g. Wang *et al.*, 2012; Murugan and Kumar, 2013; Nettles *et al.*, 2016; Taheri *et al.*, 2016). Song *et al.* (2007a) analyzed ammonia-oxidizing bacteria in the rhizosphere of intercropped wheat, maize and faba bean using denaturing gradient gel electrophoresis (DGGE). They found that the community composition of these bacteria differed between intercropping systems and monocultures. However, most previous research focused only on microorganism in the rhizosphere and/ or on ammonia-oxidizing bacteria (Song *et al.*, 2007a; Zhang *et al.*, 2015; Li *et al.*, 2016). So far, the interactions between fungal and bacterial communities in soil, rhizosphere and endosphere in the agricultural important crop species under different cropping systems have not been studied simultaneously using high-throughput next generation sequencing.

Hence, the aim of the present study was to investigate structural and functional responses of fungal and bacterial communities in soil, rhizosphere and endosphere towards different cropping systems. This experiment is part of the IMPAC^3 project ("Novel genotypes for mixed cropping allow for improved sustainable land use across arable land, grassland and woodland"). To assess structural changes of the fungal and bacterial communities, the two agricultural important crop species common wheat (*Triticum aestivum* L.) and faba bean (*Vicia faba* L.) were grown in monoculture or in two intercropping regimes as defined by Andrews and Kassam (1976): without any arrangement (mixed intercropping) or in distinct rows (row intercropping).

Bacterial and fungal communities in bulk and rhizosphere soil as well as in aerial and root endosphere were examined using Illumina (MiSeq) sequencing targeting the bacterial 16S rRNA gene and the fungal internal transcribed spacer (ITS), respectively. In addition, we used this unique dataset to calculate functional profiles with Tax4Fun (Aßhauer *et al.*, 2015). Additionally, fungal OTUs were parsed into ecological guilds using FUNGuild (Nguyen *et al.*, 2016). We hypothesized (1) that crop species as well as cropping regime affect microbial community and diversity. In addition, we expected (2) that microbial communities in bulk and rhizosphere soil as well as in the

endosphere exhibit distinct microbial patterns. We further hypothesized (3) that factors altering the community composition in soil and endosphere will also affect microbial functioning. However, we assumed (4) that these effects would be more pronounced in endophyte communities in roots compared to those in aerial plant parts as well as in microbial communities in rhizosphere soil compared to bulk soil.

Material and Methods

Experimental design

To examine the influence of different cropping systems on the entire fungal and bacterial community in soil and endosphere, a semi-open greenhouse experiment with plants growing under normal diel illumination conditions was conducted in summer 2015. The two crop species faba bean (genotype: Hiverna) and wheat (genotype: Hybery) were cultured in monoculture or as mixture in polypropylene containers (Semadeni, Eurobehälter, LogiLine ® SGL Boden, 600 x 400 x 212 mm), containing 75% sterile commercial plant substrate (Fruhstorfer Erde Typ T25*;* N: 200-300 mg L^{-1}, P_2O_5: 200-300 mg L^{-1}*;* Hawita Gruppe GmbH Vechta, Germany) and 25% sand. This plant substrate is a peaty soil with a pH ($CaCl_2$) of 5.5 to 6.5. For monocropping systems, twenty faba bean (FBM) or eighty wheat (WM) plants per container were grown in rows. In multiple cropping systems, forty wheat and ten faba bean plants per container were grown either in distinct rows (row intercropping*;* RI) or without any arrangement (mixed intercropping*;* MI). The four different cropping regimes were replicated five times in a randomized design. All plants were irrigated daily. To increase nutrient-limitation as well as intra- and interspecies interactions between the plants, no fertilization was applied.

Soil sampling and edaphic parameters

Soil samples were collected after a growing period of four weeks. We sampled the rhizosphere soil, defined as soil tightly adhering to the roots, and the bulk soil, defined as root-free soil around the crops. In the two intercropping treatments, bulk soil samples of the two crop species were pooled for each container. All soil samples were frozen and stored at -20°C. For determination of soil properties, subsamples were dried at 60°C for two days and sieved to < 2mm. Soil organic carbon (C) and total nitrogen (N) concentrations from all dried subsamples were determined using a LECO TruSpec CN analyzer (Leco Copr., St. Joseph, MI). The gravimetric soil water content (%) of all soil samples was calculated from oven-dried subsamples. Soil pH values were measured as follows: 2 g soil of each container and crop species were mixed with 5 mL PCR grade water. After

incubation for 24 hours, pH_{Water} was measured in the supernatant with a glass electrode. Subsequently, 0.37 g KCl was added and pH_{KCl} was measured. Detailed information on soil characteristics is given in the Supplementary Table S1.

Sampling and surface sterilization of plant material

Above- as well as belowground plant material of the two crop species were harvested separately for each container. Aboveground (shoots, leaves) and root biomass for each crop species and each container were measured. In addition, the heights of approximately 10 faba bean and 20 wheat plants in intercropping regimes and approximately 20 plants of monocropped faba bean and wheat plants were measured. For determination of water content in aerial plant parts, ten wheat and five faba bean plants without roots per container were weighted and subsequently oven-dried at 60°C for 48 h and re-weighted. For detailed information see Supplementary Table S2. Ten wheat and five bean plants which did not show any obvious disease symptoms such as leaf spots were randomly selected from each container for molecular analysis. Plant material derived from the same container and plant species was pooled prior to surface sterilization.

Aerial plant parts (shoots and leaves) were surface-sterilized by serial washing in 70% ethanol for 1 min, 2% sodium hypochlorite for 30 sec and 70% ethanol for 1 min, followed by two times immersion in sterile, distilled water for 30 sec and once in sterile, Diethylpyrocarbonate (DEPC)-treated water. Surface sterilization of roots were performed according to Sun *et al.* (2008) with slight modifications. In this study, 2% sodium hypochlorite (2%) and sterile, DEPC-treated water was used. To control the success of the applied disinfection process, aliquots of the water used in the final wash step were plated on common laboratory media plates, i.e., Luria-Bertani-Agar and potato dextrose agar, respectively. The plates were incubated in the dark at 25°C for at least one week. No growth of microorganisms was observed. In addition, water from the same aliquots was subjected to PCR targeting the bacterial 16S rRNA gene and ITS region of fungal rDNA. No amplification of 16S rRNA gene or ITS region was detected (data not shown). Thus, these results confirmed that the surface sterilization was successful in eliminating cultivable as well as non-cultivable epiphytic bacteria and fungi as well as potential DNA traces from the plant surfaces. Surface-sterilized plant material was ground to a fine powder in liquid nitrogen using an autoclaved mortar and pestle. Aliquots of the obtained powder were stored at -20°C until DNA extraction.

Extraction of total community DNA

Total endophytic microbial community DNA of aerial plant parts (referred to as leaves or leaf samples, for simplification) as well as roots was extracted employing the peqGOLD Plant DNA Mini Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions with two

modifications as described previously (Wemheuer *et al.*, 2016). Total environmantal DNA of rhizosphere as well as bulk soil samples was extracted employing the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., USA) according to the manufacture's protocol. The concentration of DNA extracts was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Amplification of the 16S rRNA gene

Bacterial endophyte and soil communities were assessed by a nested PCR approach targeting the 16S rRNA gene. For details of the first PCR reaction mixture and the thermal cycling scheme see (Wemheuer *et al.*, 2016). In brief, the primers 799f (5′-AACMGGATTAGATACCCKG-3′) (Chelius and Triplett, 2001) and 1492R (5′-GCYTACCTTGTTACGACTT-3′) (Lane, 1991) were used in the first PCR to suppress co-amplification of chloroplast-derived 16S rRNA genes (Chelius and Triplett, 2001). PCR amplification resulted in two PCR products: a bacterial product of approximately 735 bp and a mitochondrial product with approximately 1.1 kbp. Genomic DNA of *Bacillus licheniformis* DSM13 was used as template in the positive control to select for the bacterial product. Obtained PCR products were subjected to nested PCR.

The V6-V8 region of the 16S rRNA gene was amplified with the primers 968F and 1401R (Nübel *et al.*, 1996) containing MiSeq adaptors (underlined) (MiSeq-968F 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACGCGAAGAACCTTAC-3′*;* MiSeq-1401R 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGGTGTGTACAAGACCC-3′). The PCR reaction (25 µl) contained 5 µl of five-fold Phusion HF buffer, 200 µM of each of the four deoxynucleoside triphosphates, 4 µM of each primer, 1 U of Phusion high fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA) and approximately 50 ng of the PCR product as template. Negative controls were performed by using the reaction mixture without template. The following thermal cycling scheme was used: initial denaturation at 98°C for 30 s, 30 cycles of denaturation at 98°C for 15 s, annealing at 53°C for 30 s, followed by extension at 72°C for 30 s. The final extension was carried out at 72°C for 2 min. Three independent PCRs were performed per sample. Obtained PCR products per sample were controlled for appropriate size, pooled in equal amounts, and purified using the peqGOLD Gel Extraction Kit (Peqlab). Quantification of the PCR products was performed using the Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Thermo Scientific) as recommended by the manufacturer.

Amplification of the ITS region

The fungal communities in soil and endosphere were assessed by a nested PCR approach targeting the ITS region. In the first PCR, the primers ITS1-F_KYO2 (5′-

TAGAGGAAGTAAAAGTCGTAA-3′) (Toju *et al.*, 2012) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990) were used. The PCR reaction mixture (25 µl) contained: 5 µl of five-fold Phusion GC buffer, 200 µM of each of the four deoxynucleoside triphosphates, 4 µM of each primer, 5% DMSO, 25 mM $MgCl_2$, 0.5 U of Phusion High Fidelity Hot Start DNA polymerase (Thermo Scientific) and approximately 10 ng DNA sample as template. Negative controls were performed by using the reaction mixture without template. The following thermal cycle scheme was utilized: initial denaturation at 98°C for 30 seconds followed by 6 cycles of denaturation at 98°C for 15 s, annealing at 53°C for 30 s decreasing 0.5°C in each cycle, followed by extension at 72°C for 30 s and 29 cycles of denaturation at 98°C for 15 s, annealing at 50°C for 30 s, followed by extension at 72°C for 30 s. The final extension was carried out at 72°C for 2 min. Obtained PCR products were subjected to nested PCR.

The ITS2 region was amplified with the primers ITS3_KYO2 (Toju *et al.*, 2012) and ITS4 (White *et al.*, 1990) containing the MiSeq adaptors (underlined) were used: MiSeq-ITS3_KYO2 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATGAAGAACGYAGYRAA-3′) and MiSeq-ITS4 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC -3′). Purification and quantification were performed as described for bacterial PCR products. Three independent PCRs were performed per sample and obtained PCR products were pooled in equal amounts.

Processing and analysis of bacterial and fungal datasets

Generated datasets was processed with Usearch version 8.0.1623 (Edgar, 2010). Paired-end reads were merged and quality-filtered. Filtering included the removal of reads shorter than 300 bp. Processed sequences of all samples were joined and clustered in operational taxonomic units (OTUs) at 3% genetic divergence using the UPARSE algorithm implemented in USEARCH. A *de novo* chimera removal was included in the clustering step. Afterwards, putative chimeric sequences were removed using UCHIME in reference mode with the most recent RDP training set (version 15) as reference data set (Cole *et al.*, 2009) for bacteria and the most recent uchime reference data (version 7) obtained from the UNITE database for fungi, respectively. Afterwards, OTU sequences were taxonomically classified using QIIME (Caporaso *et al.*, 2010) by BLAST alignment against the most recent SILVA database (SILVA SSURef 123 NR) and the latest QIIME release of the UNITE database (version 7), respectively. All non-bacterial or non-fungal OTUs were removed from the respective datasets. Subsequently, processed sequences were mapped on OTU sequences to calculate the distribution and abundance of each OTU in every sample. Alpha diversity indices

(Supplementary Tables S3 and S4) and rarefaction curves were calculated in R (version 3.2.3) (R Development Core Team, 2013) using the vegan package (Oksanen *et al.*, 2013).

Statistical analysis

All statistical analyses were conducted in R, with the exception of the statistics on fungal thriophic guilds, which was carried out in SigmaPlot (version 11.0, Systat Software GmbH, Erkrath, Germany). Differences were considered as statistically significant with $P \leq 0.05$. Non-metric multidimensional scaling plots (NMDS) were calculated using the metaMDS function within the vegan package based on Bray-Curtis dissimilarities to visualize differences in community composition separately for bacteria and fungi. The impact of cropping system on bacterial and fungal community structure was tested using the envfit function as described previously (Wietz *et al.*, 2015).

To analyze possible effects of sampling location on richness and diversity, ANOVA with error (Crawley, 2007) were conducted. A pairwise t-test with Bonferroni correction was applied to test for differences between the cropping systems. Differences in richness and diversity with regard to cropping regime were tested by ANOVA and Kruskal-Wallis test, respectively. Prior to analysis, alpha diversity indices were tested for normality using the Shapiro test and for variance homogeneity using the Levene test within the car package. Differences between single treatments were tested either by pairwise t test or Wilcoxon test using Bonferroni corrected p values. Correlation between cropping types and soil pH as well as aboveground and root biomass were tested by ANOVA and Kruskal-Wallis test, respectively. Prior to analysis, environmental parameters as well as alpha diversity indices were tested for normality using the Shapiro test and for variance homogeneity using the Levene test within the car package. Differences between single treatments were tested either by pairwise t test or Wilcoxon test using Bonferroni corrected p values. Functional profiles for bacterial communities were predicted from obtained 16S rRNA gene data using Tax4Fun (Aßhauer *et al.*, 2015). In addition, we used this unique dataset to parse fungal genera into ecological guilds using FUNGuild according to Nguyen *et al.* (2016).

To identify potential indicators for each cropping regime and associations with the sampling locations, multipattern analyses were applied at genus level. Therefore, all fungal and bacterial genera (178 and 881, respectively) which were found with more than 0.5% abundance in the respective sampling locations were used. Therefore, *multipatt* from the IndicSpecies (De Cáceres and Legendre, 2009) package was applied. The resulting indicator values for a particular cropping system, as well as the associations strengths (that is, point biserial coefficients *R*) with a particular compartment were corrected for unequal sample size using the function *IndVal.g* and *r.g*, respectively (Tichy and Chytry, 2006). As a single taxon can occupy a certain niche in several

cropping systems, and be present in more than just one of the compartments in the endosphere and soil, it is necessary to consider all possible combinations to detect these associations (De Cáceres *et al.*, 2010).

Correlation-based co-occurrence networks were additionally constructed for each of the sampling locations to gain a more detailed insight into the possible interactions between fungi and bacteria in the soil and endosphere. Therefore, bacterial and fungal OTU tables were combined (bulk soil, n = 20*;* rhizosphere, n = 27*;* root, n = 15*;* leaf, n = 18). To enhance reliability of the co-occurrence patterns, only taxa present in >50% of the samples were taken into analysis. Pairwise correlation coefficients were calculated using *cor* in R. Positive correlation coefficients ($r > 0.6$ for bulk soil, rhizosphere and roots, $r > 0.3$ for leaves) were considered as two taxa co-occurring, or cooperation between the two taxa. Negative correlation coefficients ($r < -0.6$ for bulk soil, rhizosphere and roots, $r < -0.3$ for leaves) were considered as two taxa avoiding each other, or competition between the two taxa. Network visualization was performed in Cytoscape version 3.2.0 (Shannon *et al.*, 2003). The *Spring Embedded Edge-Weighed Layout* was employed for both bipartite and co-occurrence networks.

Results and Discussion

Soil characteristics and growth of faba bean and wheat plants

Several soil (Table 1, Supplementary Table S1) and plant properties (Table 2, Supplementary Table S2) were determined. Soil pH values were constant among all soil samples (pH_{water} = 6.82±0.13*;* pH_{KCl} = 6.55±0.09) with no significant differences between the four cropping regimes. Water content varied between 61.6 and 86.3%. Statistical analysis revealed that the water content in rhizosphere soil samples of WM was significantly lower compared to the other three cropping regimes. Moreover, soil C and N content in bulk as well as in rhizosphere soil samples did not differ significantly among the four cropping regimes. However, the C:N ratio in bulk soil samples of the cropping regime MI were significantly higher compared to the other cropping regimes. The C:N ratio explains the ability to use soil carbon and nitrogen for microbial processes such as the decomposition of soil organic matter (Wardle, 1992). As consequence, it is an indicator of soil microbial activity (He *et al.*, 1997). The results of the present study indicate that the soil microbial activity in all soil samples with exception of bulk soil sample MI did not differ significantly among the four cropping regimes.

Table 1: Edaphic parameters for bulk soil and rhizopshere, respectively. Different letters indicate significant differences with $P < 0.05$.

	Moisture (%)	*C_{total} (%)*	*N_{total} (%)*	*C:N ratio*
		Bulk soil		
FBM	31.22±6.25a	8.42±2.35a	0.18±0.05a	46.33±1.09a
WM	18.14±4.03c	6.33±0.54a	0.14±0.01a	45.11±1.26a
RI	21.54±3.61b,c	7.68±2.15a	0.17±0.04a	46.47±1.08a
MI	24.99±2.19a,b,c	7.83±1.03a	0.17±0.02a	47.41±0.37b
		Faba bean rhizosphere		
FBM	34.43±3.16a	10.50±1.02a	0.22±0.03a	48.24±1.68a
RI	29.12±4.11a	13.76±2.63a	0.27±0.05a	50.13±1.07a
MI	26.39±6.33a	11.89±2.17a	0.24±0.05a	49.99±2.20a
		Wheat rhizosphere		
FBM	22.33±2.99a	10.86±1.66a	0.22±0.03a	50.29±2.25a
RI	26.44±3.56a	12.08±2.26a	0.24±0.05a	50.92±1.22a
MI	24.86±6.14a	9.48±1.55a	0.18±0.03a	51.43±1.55a

Abbreviations: ***C_{total}***, total soil organic carbon*;* ***N_{total}***, total soil nitrogen*;* FBM, faba bean in monoculture*;* WM, wheat in monoculture*;* MI, mixed intercropping*;* RI, row intercropping.

To analyze the effect of cropping system and regime on plant growth and yield, aboveground as well as root biomass were recorded. The heights of the two crop species did not differ between the different cropping regimes (Table 2, Supplementary Table S2). Nonetheless, we found a higher aboveground biomass of wheat plants in intercropping systems, whereas the aboveground biomass of bean plants growing in the two intercropping regimes was lower compared to the monocultures. However, this was not statistically significant which might be related to the short growing period of the plants. Similar results for increased yields of the cereal crop were observed in a maize/faba bean intercropping system. The authors suggest that these results are caused by interspecific facilitation in phosphorus and nitrogen uptake. This is in line with other studies concluding that the beneficial effects of intercropping crop yields and/or nutrient supply resulted mainly from (positive) interspecific interactions in the rhizosphere (Vandermeer, 1992*;* Li *et al.*, 1999).

Table 2. Plant growth characteristics. Different letters indicate statistically significant differences with $P < 0.05$ between groups. The above- and belowground biomass per plant (g) is shown. For height, approximately 10 faba bean and 20 wheat plants in intercropping regimes and approximately 20 plants of monocropped faba bean and wheat plants were measured.

	Height (cm)	*Aboveground biomass (g)*	*Water content (%)*	*Root biomass (g)*	*shoot/root ratio*
Faba bean					
FBM	21.40±1.84a	4.83±1.16a	90.47±0.55a	2.34±0.49a	2.11±0.52a
RI	18.80±1.69a	3.27±0.52a	87.92±1.66b	2.66±0.65b	1.29±0.32b
MI	19.26±2.68a	3.72±1.40a	87.10±2.49b	1.98±0.69a	1.92±0.43ab
Wheat					
WM	38.78±1.12a	1.36±0.18a	84.58±4.01a	1.94±0.48a	0.76±0.25a
RI	40.30±3.00a	1.64±0.27a	82.4±2.02a	2.78±1.22b	0.71±0.29a
MI	39.76±1.40a	1.68±0.37a	84.72±1.12a	2.05±0.49a	0.86±0.22a

Abbreviations: FBM, faba bean in monoculture; WM, wheat in monoculture; MI, mixed intercropping; RI, row intercropping.

A higher average root biomass was observed for intercropped plants grown in rows (RI) compared to those grown in monocultures or with no distinct row arrangement (MI) (Table 2). The shoot/root ratio for faba bean decreased from monoculture over intercropping regime MI to RI. Further analysis revealed that the differences observed for faba bean in cropping regimes FBM and RI were statistically significant (data not shown). According to Eghball and Maranville (1993), environmental stresses increase the relative weight of roots compared to shoots. The results of the present study indicate that wheat had a higher competetive availibitly than faba bean growing in cropping regime RI. This finding is in line with previous studies showing that interspecific competition and facilitation act on the crop plants simultaneously in intercropping systems (Li *et al.*, 1999; Zhang and Li, 2003; Ghosh *et al.*, 2006). In mixtures of cereal and legume crops, cereals are more competitive than legumes in taking up N from the soil due to their faster root development and a higher N demand (Corre-Hellou *et al.;* Hauggaard-Nielsen *et al.*, 2001). Recently, it was concluded that the time of sowing or planting of the component crops and/or the design of the intercropping system should be modified to reduce the competition and to enhance facilitation effects (Mariotti *et al.*, 2009; Aziz *et al.*, 2015).

Bacterial and fungal communities are dominated by a few phyla

Sequencing of bacterial 16S rRNA and fungal ITS gene amplicons from soil and endosphere samples resulted in 815.423 and 146.660 high-quality reads for bacteria and fungi, respectively. These sequences grouped into 3.994 bacterial and 567 fungal OTUs. Calculated rarefaction curves at 3% genetic distance (species level) revealed that the majority of fungal and bacterial community

was recovered by the surveying effort (data not shown). Richness (number of observed OTUs) and diversity (Shannon indices) for bacterial communities ranged from to 5.8 to 258.1 and 0.36 to 4.37, respectively (Supplementary Table S3). For fungal communities, richness and Shannon indices varied between 3.2 and 23.4 and between 0.52 and 2.83 (Supplementary Table S4). The high deviance observed for bacterial and fungal richness derived from the different sampling locations analyzed. The lowest and highest bacterial richness was observed for endophytes in aerial parts of monoculture wheat plants and in bulk soil of MI, respectively (Table 3). In addition, the lowest and highest fungal richness was found in roots and aerial parts of monoculture faba bean, respectively (Table 3).

Across all samples, *Proteobacteria* (81.2±17.8%), *Firmicutes* (5.5±15.5%), *Actinobacteria* (5.2±6.7%), *Bacteroidetes* (3.0±3.3%) and *Acidobacteria* (1.3±1.7%) were the most abundant bacterial phyla (Figure 1). This is in line with previous studies investigating bacterial communities in bulk soil, rhizosphere and roots of barley (Bulgarelli *et al.*, 2013) as well as in root and leaves of wheat (Robinson *et al.*, 2015). Similar results were obtained for bacterial communities in soil and rhizosphere samples of different cereal crops and legumes (Turner *et al.*, 2013b) and in soil of winter wheat-rice as well as winter wheat-maize cropping systems (Zhao *et al.*, 2014). In the present study, the bacterial orders *Xanthomonadales* and *Burkholderiales* dominated the bulk/rhizosphere soil and endophytic samples, respectively. At genus level, *Rhodanobacter* (*Xanthomonadales*) dominated bulk soil and rhizosphere samples (61% and 59%, respectively). *Ralstonia* was the most abundant member of the *Burkolderiales* in leaf and root samples (15% and 24%, respectively). One member of this genus, *R. solanacearum*, was previously described as pathogen involved in eggplant wilt by colonization of the vascular vessels (Vasse *et al.*, 1995).

Table 3. Bacterial and fungal richness and diversity. Diversity is expressed as Shannon values and richness as the number of observed OTUs. Different letters indicate statistically significant differences between groups.

	Bacteria		*Fungi*	
	Richness	**Diversity**	**Richness**	**Diversity**
		Bulk soil		
FBM	95.00±25.01a	1.90±0.54a	14.76±2.25ab	2.18±0.25a
WM	67.60±55.80a	1.58±0.80ab	18.42±1.34ac	2.50±0.07a
RI	103.60±27.26a	1.95±0.49a	16.20±1.30abc	2.31±0.13a
MI	111.20±71.33a	2.00±1.04b	16.82±1.18abc	2.41±0.10a
		Faba bean rhizosphere		
FBM	225.90±46.40a	4.20±0.85a	18.40±1.95a	2.52±0.14a
RI	94.20±55.43a	2.31±0.85a	18.68±3.87a	2.52±0.32a
MI	131.20±19.56a	2.11±0.40a	16.80±2.10a	2.32±0.17a
		Wheat rhizosphere		
WM	70.60±30.07a	1.46±0.59a	18.54±2.91ab	2.52±0.24a
RI	103.00±36.43ab	1.88±0.57a	14.78±0.71ab	2.14±0.13b
MI	172.80±21.05b	3.45±0.54a	21.13±0.67ac	2.66±0.02a
		Faba bean roots		
FBM	122.60±0a	3.24±0a	13.77±4.52a	2.08±0.53a
RI	53.20±28.93a	1.26±0.56a	18.50±0a	2.28±0a
MI	14.00±34.36a	0.36±1.11a	18.20±0.80a	2.47±0.08a
		Wheat roots		
WM	82.20±16.78a	2.09±0.34a	15.10±7.46a	2.03±0.89a
RI	126.50±30.36a	2.89±0.49a	18.50±1.09a	2.37±0.03a
MI	80.80±28.87a	1.47±0.73a	18.24±2.66a	2.32±0.26a
		Faba bean aerial parts		
FBM	19.40±5.67a	0.68±0.58a	22.00±0a	2.74±0a
RI	37.90±7.95a	2.13±0.46a	15.83±2.70a	2.03±0.29a
MI	23.80±0a	1.32±0a	12.90±0a	2.00±0a
		Wheat aerial parts		
WM	5.80±8.93a	1.09±0.62a	16.04±1.17a	2.34±0.09a
RI	12.70±2.44a	1.77±0.32a	15.96±3.42a	2.34±0.31a
MI	34.30±9.00a	2.39±0.31a	16.73±2.49a	2.35±0.24a

Abbreviations: FBM, faba bean in monoculture; WM, wheat in monoculture; MI, mixed intercropping; RI, row intercropping.

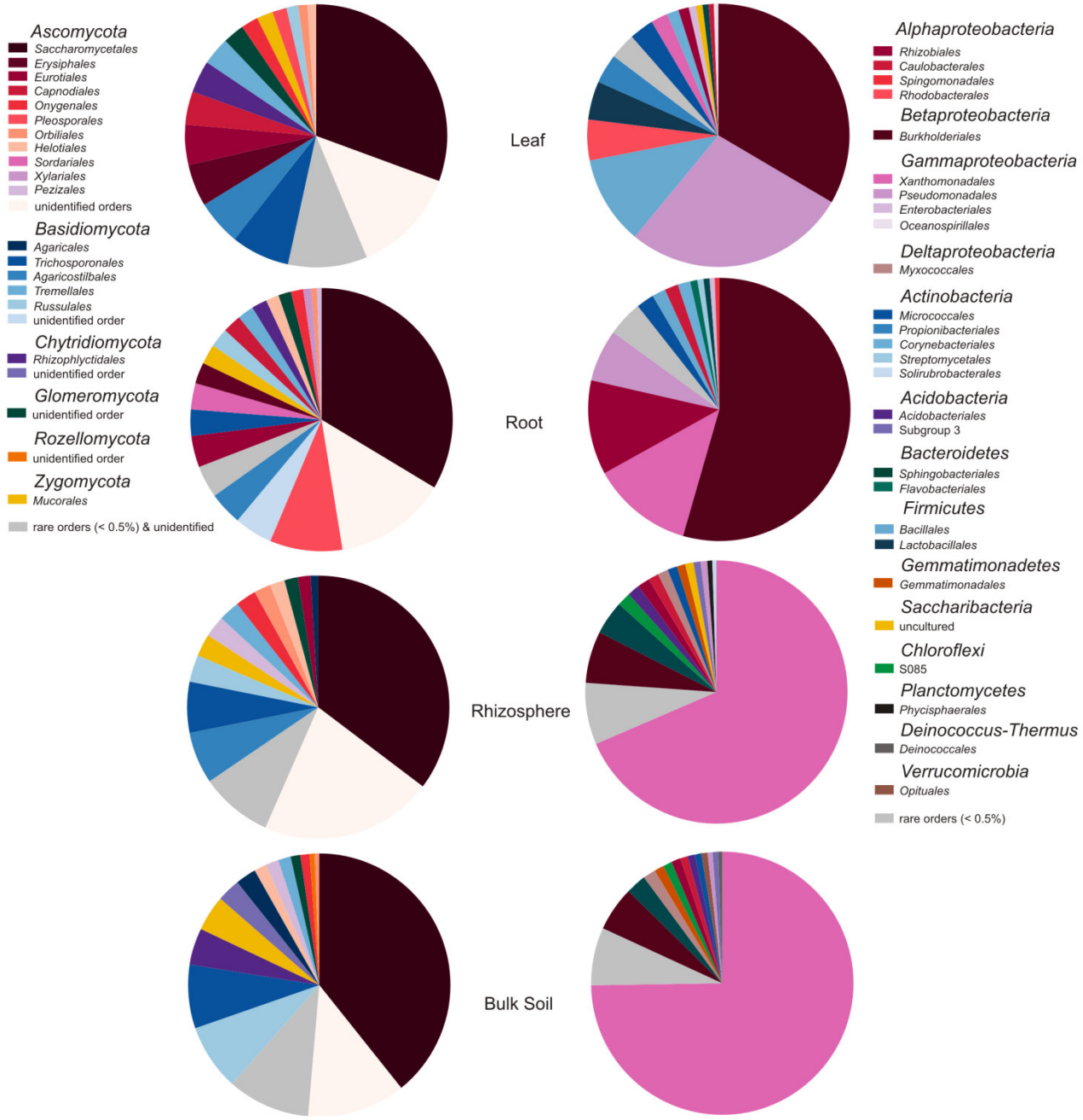

Figure 1. Relative abundances of bacterial and fungal orders, derived from the different sampling locations. All order representing more than 0.5% oft he total community per location are shown. All orders >0.5% were considered rare.

Fungi were represented by the abundant phyla *Ascomycota* (66.0±12.1%), *Basidiomycota* (19.6±10.1%), *Chytridiomycota* (3.1±6.0%) and *Glomeromycota* (1.8±2.9%) (Figure 1). This is in accordance with previous studies (Shakya *et al.*, 2013*;* Penton *et al.*, 2014*;* Chen *et al.*, 2015). Penton *et al.* (2014) analyzed fungal communities in soils of agricultural fields under continuous wheat cropping and found that *Ascomycota*, *Basidiomycota*, and *Chytridiomycota* dominated the fungal communities. In the present study, the *Saccharomycetales* (*Ascomycota*) dominated all soil and endosphere samples. In contrast to this, the endophytic fungal community in rice sprouts and roots were dominated by the order *Eurotiales* and *Hypocreales*, respectively (Wang *et al.*, 2016).

Diversity and richness of fungal and bacterial communities are driven in a different way

We analyzed the effect of cropping regimes on diversity and richness of fungal and bacterial communities in all sampling locations (endosphere and soil samples) separately to avoid pseudoreplication. Statistical analysis revealed that the richness and diversity of bacterial communities in bulk soil as well as the richness in rhizosphere soil of wheat plants were significantly affected by the applied cropping regimes (Table 3). This is in accordance with a study of Yang *et al.* (2016). They investigated the bacterial community in rhizosphere soil of ten common spring crops in North China under different cropping systems and found that the bacterial diversity was influenced by crop species as well as cropping system. In contrast to this, different crop rotations had only a minor influence on bacterial diversity. The authors suggest that this is related to the previous fallow period (Silva *et al.*, 2013).

In addition, the richness and diversity of fungi in rhizosphere samples of wheat as well as the richness of fungi in bulk soil samples was significantly affected by cropping regimes. This is in line with previous studies (e.g., Chen *et al.*, 2015*;* LeBlanc *et al.*, 2015). In a study about the impact of cropping systems on fungal communities in soil, fungal richness and diversity of these communities was increased by crop rotation (Chen *et al.*, 2015). This is in line with a study of Manici and Caputo (2009). Here, a higher fungal diversity in fields with crop rotation compared to fields intensively cultivated with potatoes for many years. Similar results were found by Xiong *et al.* (2016) investigating effects of long-term vanilla monocropping of fungal communities in bulk and rhizosphere soils.

Composition of fungal and bacterial communities is not affected by crop species and cropping regime but by sampling location

To identify the influence of the different cropping systems on microbial community composition, NMDS analyses were performed separately for the bacterial and fungal communities (Figure 2 A and B). Neither bacterial nor fungal communities showed a clustering with respect to the different cropping regimes. In contrast to this, the bacterial endophytes in roots of intercropped soybean and maize plants were significantly affected by intercropping (Zhang *et al.*, 2011). Similar results were observed for fungal communities in the endosphere of durum wheat roots (Taheri *et al.*, 2016).

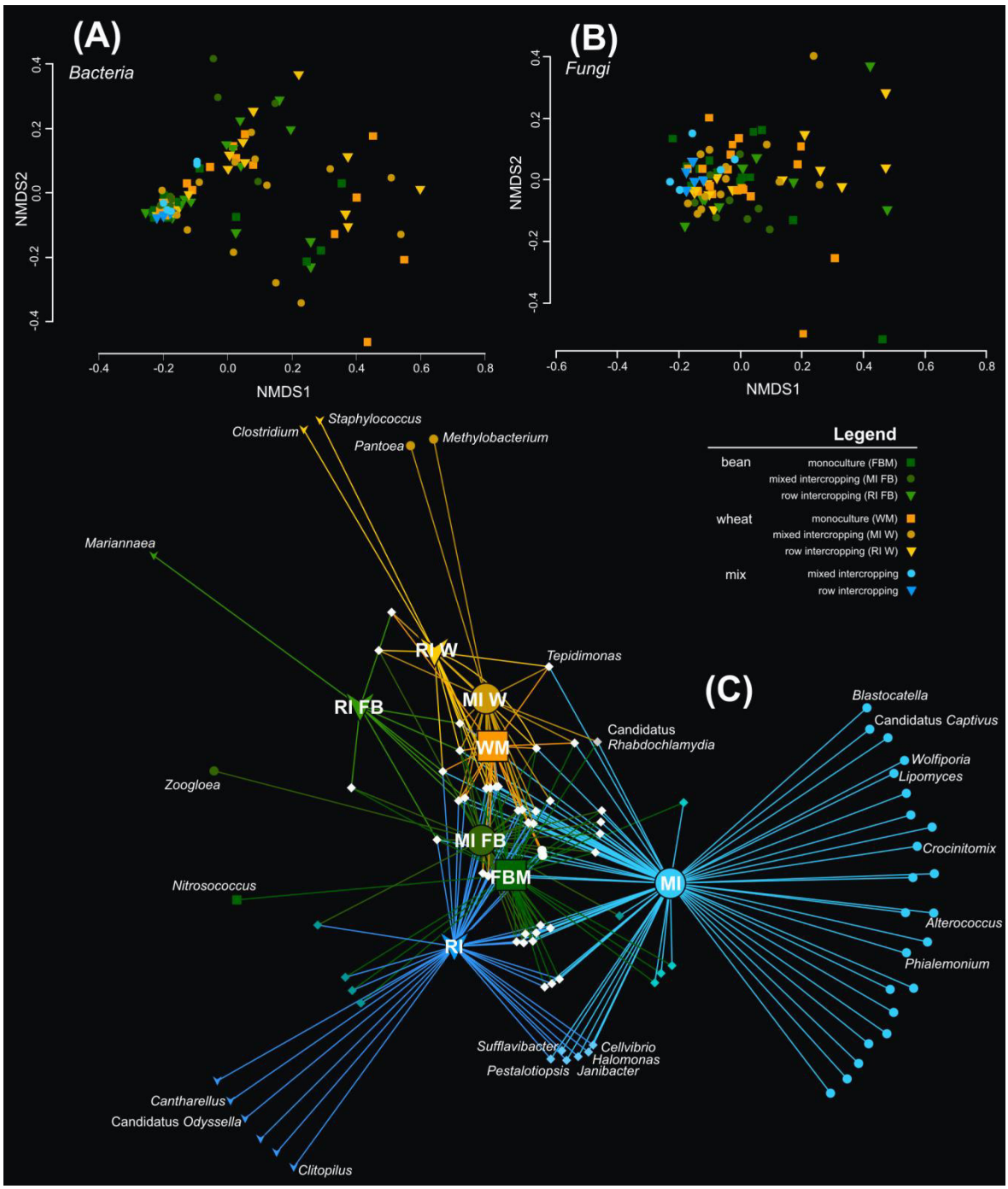

Figure 2. Microbial community response toward the cropping systems. NMDS ordination of bacterial (A) and fungal (B) communities color coded by the respective cropping regime. Ordination is based on Bray-Burtis dissimilarities between samples.The bipartite network based on indicator values (C) of the 92 significantly associated OTUs is also color coded by cropping regime. Cropping regimes and OTUs associated with only one cropping regime are color coded with respect to A and B. The edge-weighed spring-embedded algorithm pulled together OTUs with similar associations and cropping regimes with similar structure. White nodes represent multi-regime cross-combinations. OTUs that could be assigned to a genus and that are associated with only one or two cropping regimes are indicated. FBM: all samples derived from faba bean monoculture; WM: all samples derived from wheat monoculture; MI FB: faba bean endospheric samples from mixed intercropping; RI FB: faba bean endopheric samples from row intercropping; MI W: wheat endospheric samples from mixed intercropping; RI W: wheat endospheric samples from row intercropping; MI: soil and rhizosphere samples from mixed intercropping; RI: soil and rhizosphere samples from row intercropping.

Contrasting results for effects of cropping systems on soil and rhizosphere microbial communities were observed in previous studies. Navarro-Noya *et al.* (2013) found that wheat-maize rotation and monoculture had no effect on the bacterial community composition in soil. In contrast, fungal and bacterial communities in paddy soils (Jiang *et al.*, 2016) or in bulk soil samples (Suzuki *et al.*, 2012) were altered by crop rotation systems. In the last mentioned study, however, the effect was strong only for fungal communities, while the bacterial communities were mainly affected by soil properties. In a previous study about the effect of soil type and cropping system on fungal and bacterial communities in the rhizosphere of legumes and wheat, cropping system had only little effect on these communities (Wang *et al.*, 2012). Similar findings were obtained in a study on soil bacterial communities in winter wheat–rice (WR) and winter wheat–maize (WM) cropping systems derived from five locations (Zhao *et al.*, 2014). Here, the effect of crop rotation was only low, but significant. Other studies showed that different cropping practices changed microbial communities in roots and/or soil (Manici and Caputo, 2009*;* Zhang *et al.*, 2011*;* Yang *et al.*, 2016). Yang *et al.* (2016) found that the rhizosphere bacterial community composition was influenced by cropping system as well as crop species.

In the present study, crop species did not affect bacterial and fungal community composition in soil and endosphere. This is not in accordance with recent studies showing that different plant species harbor distinct endophytic communities (Gange *et al.*, 2007*;* Gaiero *et al.*, 2013*;* Bonito *et al.*, 2014*;* Wemheuer *et al.*, 2016) as well as rhizosphere and/or bulk soil microbial communities (Kent and Triplett, 2002*;* Berg and Smalla, 2009*;* Turner *et al.*, 2013b*;* Pii *et al.*, 2016). In a study investigating the effect of different plant species such as bean or clover and soil type on microbial communities, plant species had the strongest effect in soil as well as in plant-associated habitats rhizosphere and rhizoplane (Wieland *et al.*, 2001). Mouhamadou *et al.* (2013) showed that fungal communities in bulk and rhizosphere soil of two perennial grass species were affected by the grass species investigated. However, in a previous study investigating the leaf endophytic fungal communities of different trees, no distinct communities were identified for individual tree species (Cannon and Simmons, 2002).

We suggest that the missing effects of intercropping and crop species on bacterial and fungal communities are most likely attributed to the short growth period. It is possible that effects of both intercropping and plant species will only become evident after a longer growth period. This finding is in accordance with a study on rhizosphere ammonia-oxidizing bacteria under different intercropping systems analyzed by DGGE. Here, intercropping had a strong effect on these bacteria at anthesis, but was less pronounced at the seedling stage of the two crops (Song *et al.*, 2007a). Wang *et al.* (2012) observed distinct DGGE-patterns for fungal communities in the rhizosphere of

intercropped wheat and faba bean plants collected during the flowering period. This might have favored the shifts in microbial community composition observed in their study.

Since the cropping regime did not seem to have an influence on microbial community composition, we next distinguished between the different sampling locations (bulk/rhizosphere soil and leaf/root endosphere). Bacterial samples derived from the soil samples clustered closely and therefore contained very similar structured communities (Figure 3 A). There was no difference between bulk soil and rhizosphere bacterial communities, which is in contrast to previous findings (Costa *et al.*, 2006; Jin *et al.*, 2014; Mendes *et al.*, 2014; Sugiyama *et al.*, 2014). In the present study, the endosphere samples derived from roots and aerial plant parts (leaves/shoots) formed two additional clusters, indicating distinct community patterns in the respective plant compartments. This is consistent with previous observations for several plant species such as wheat (Robinson *et al.*, 2015), cacti (Fonseca-García *et al.*, 2016), or rice (Edwards *et al.*, 2015).

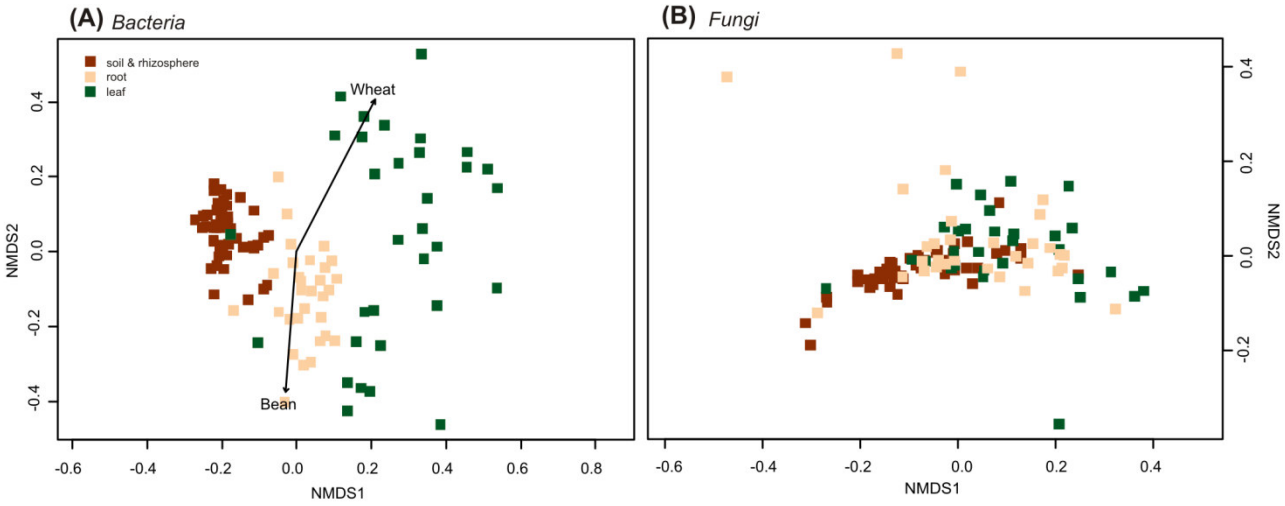

Figure 3. NMDS ordination of bacterial (A) and fungal (B) communities. This figure represents Figure 2 A and B, but color-coded by the respective sampling locations.

Fungal community structure, however, does not seem to be influenced by sampling location and did not cluster with respect to endosphere or soil (Figure 3 B). The findings in the present study are not in line with a previous study showing that plant compartment was the principal driver of fungal community composition in endosphere, phyllosphere and soil (Fonseca-García *et al.*, 2016). Mouhamadou *et al.* (2013) found that fungal communities in soil differed between bulk and rhizosphere soil samples of the two investigated grass species. In another study of fungal communities in roots and shoots of perennial forbs, a high degree of plant organ specificity was detected (Wearn *et al.*, 2012). In contrast to the studies mentioned above, Coleman-Derr *et al.* (2016) observed that composition of prokaryotic communities of different *Agave* species was primarily determined by the plant compartment, while the composition of fungal communities was mainly influenced by the biogeography of the host species.

Indicator analysis revealed differences in soil communities between the cropping regimes

A bipartite network was used to visualize the indicative potential of taxa (on genus level) for one or a combination of cropping systems (Figure 2 C). The bipartite network differed from the NMDS ordinations (Figure 2 A and B). The most striking difference is the distinct spacing of the MI and RI soil and rhizosphere samples (given in blue). While the bacterial and fungal community composition between both systems is very similar, there are a number of indicators uniquely associated with cropping regimes RI or MI. This suggests that the cropping regime might not substantially alter the community composition in general, but is represented by a number of uniquely associated taxa. On the contrary, endophytic samples derived from both intercropping regimes do not seem to select for such specialists. However, this might results from the fact that endophytic communities are not as homogenous as soil and rhizosphere communities. The samples of cropping regimes MI and RI have the highest number of associated taxa, while no taxon is exclusively associated with wheat monoculture, and only one taxon (*Nitrosococcus*) is exclusively associated with faba bean monoculture. This is interesting as the oxidation of ammonia to nitrite is carried out only a few bacteria such as *Nitrosococcus* (Purkhold *et al.*, 2000). Legumes including faba bean form symbiotic relationships with rhizobia allowing them to fix atmospheric nitrogen (Zahran, 1999).

Influence of sampling location on the microbial community interactions

A second bipartite association network was constructed to visualize the association of taxa with different sampling locations. This time, the bipartite network strongly resembled the NMDS ordination plots, by recovering the two major discriminants which is the sampling location and the endosphere versus soil (Figure 4). Endosphere and bulk soil shared a high number of associated taxa which are mainly bacteria. This finding is reflected by a very similar bacterial community structure in the soil samples in general. Additionally, 278 of significantly associated taxa are bacteria and only 31 belong to the fungi, suggesting that fungi are more competitive to each other than bacteria. However, three fungal taxa were associated with the aboveground endosphere: the basidiomycete *Cuniculitrema*, an unidentified *Glomeromycete*, and *Penicillium*. Members of this genus are known as ubiquitous soil fungi and important in the food industry where they are used to produce enzymes and other macromolecules (Rodriguez Couto and Sanroman, 2006) and are also able to produce antibiotics (Yang *et al.*, 2008). *Malassezia* (*Basidiomycota*) and the two ascomycetes *Alternaria* and *Monographella* are the only fungal taxa significantly associated with roots that could be assigned to a genus. While *Malassezia* is a saprotroph (Findley, *et al.* 2013), both *Alternaria* and *Monographella* are described as pathothrophs (Tedersoo *et al.* 2014).

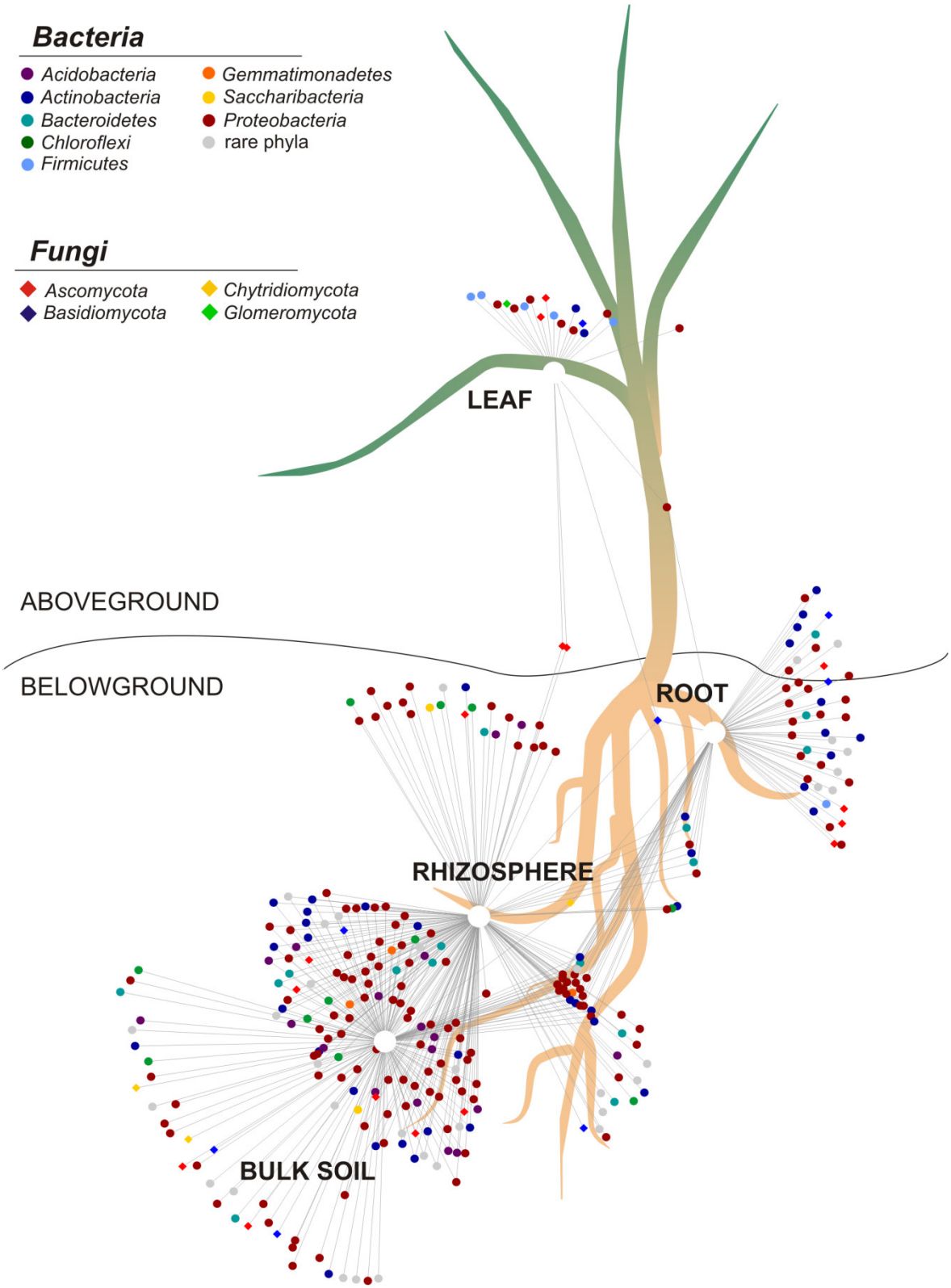

Figure 4: Bipartite association network. Positive associations between leaf, root, rhizosphere and bulk soil and 305 significantly associated OTUs are shown. The edge-weighed spring embedded algorithm pulled together OTUs with similar associations and compartments with similar structure.

We further analyzed the response of taxonomic groups to the different sampling locations, employing co-correlation networks. These networks can identify how uniformly a group responds to a specific influence. Among the most populated phyla, the network density for *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Basidiomycota* was the highest while it was more dispersed for *Proteobacteria*, *Chloroflexi*, *Verrucomicrobia* and *Ascomycetes* (Figure 5). Interestingly, the proteobacterial network was highly reflecting the influence of soil and endosphere, respectively. Additionally, *Proteobacteria* seem to contribute most to the separation of leaves from roots. This is in line with other studies that found *Proteobacteria* to constitute the main part of endophytic communities (Bulgarelli *et al.*, 2013*;* Romero *et al.*, 2014*;* Hardoim *et al.*, 2015*;* Robinson *et al.*, 2015). *Bacteroidetes* and *Actinobacteria* in contrast, separate the roots, while *Chloroflexi* seem to contribute most to the separation of the rhizospheric samples. *Ascomycota* and *Basidiomycota* are the only taxonomic groups where OTUs does negatively influence each other, which resulted in further separation between leaf and roots.

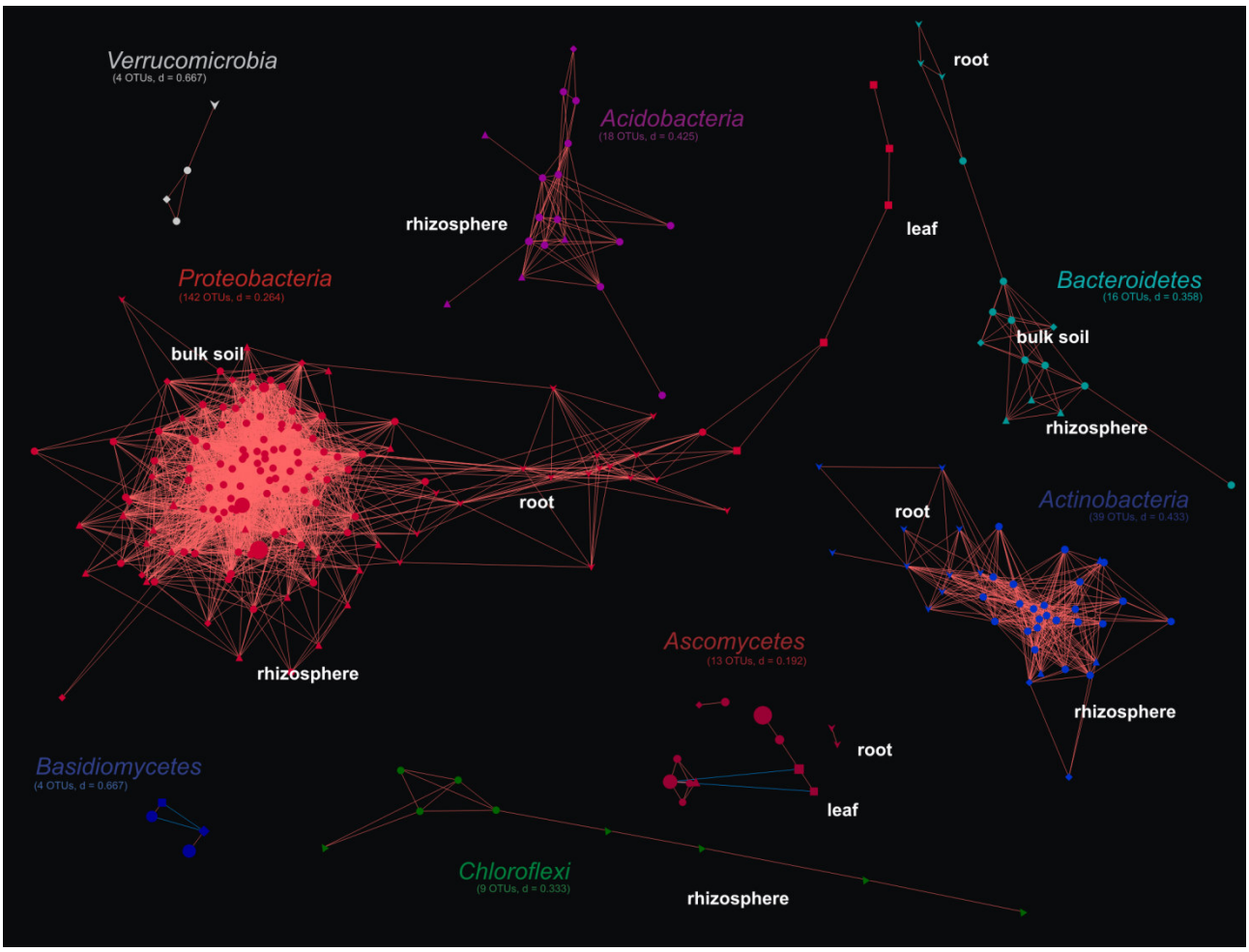

Figure 5. Co-correlation networks calculated for the significantly (P < 0.05) associated OTUs of the most populated phyla. Nodes correspond to OTUs and their size to the average abundance of each OTU across the dataset. Edges represent positive (red) or negative (blue) correlations between pairs of OTUs (R > 0.3 or R <-0.3 for positive and negative correlations, respectively). Again, the edge-weighed spring embedded algorithm pulled together strongly correlated OTUs. Strong clustering of OTUs indicated that most OTUs of this cluster showed a similar response (in most cases a positive correlation). Network density (d) calculated for each network represents the number of significant co-correlations divided by all possible co-correlations, that is, higher density represents more uniform response. Symbol coding indicates association to one specific compartments (squares = eaves*;* V = roots*;* triagles = rhizosphere*;* diamonds = bulk soil). OTUs associated with more than one compartments are circular and clusters are labeled with the approximate compartment association.

Bacterial and fungal co-occurrence along the growth axis

Bacterial communities differed with respect to the sampling location, and whether they stem from the endosphere or the soil. Fungal communities did not follow this pattern. However, it is important not only to consider the differing environmental parameters, such as the cropping regime or plant species, or even the different compartments. Bacteria and fungi co-occur in microbial communities and could be found in the endosphere as well as in the soil samples. We further elucidated, how the co-occurrence patterns of bacteria and fungi change along the growth axis of a plant. Co-occurrence can always be a hint at cooperation, while a negative relationship between OTUs can hint at avoidance or competition. The correlation-based co-occurrence networks constructed for each

compartment were very different (Figure 6 for endosphere, Figure 7 for soil and rhizosphere). They not only differed with respect to the number of nodes and edges (which to some degree reflected the diversity of each compartment), they also varied with respect to type of interactions.

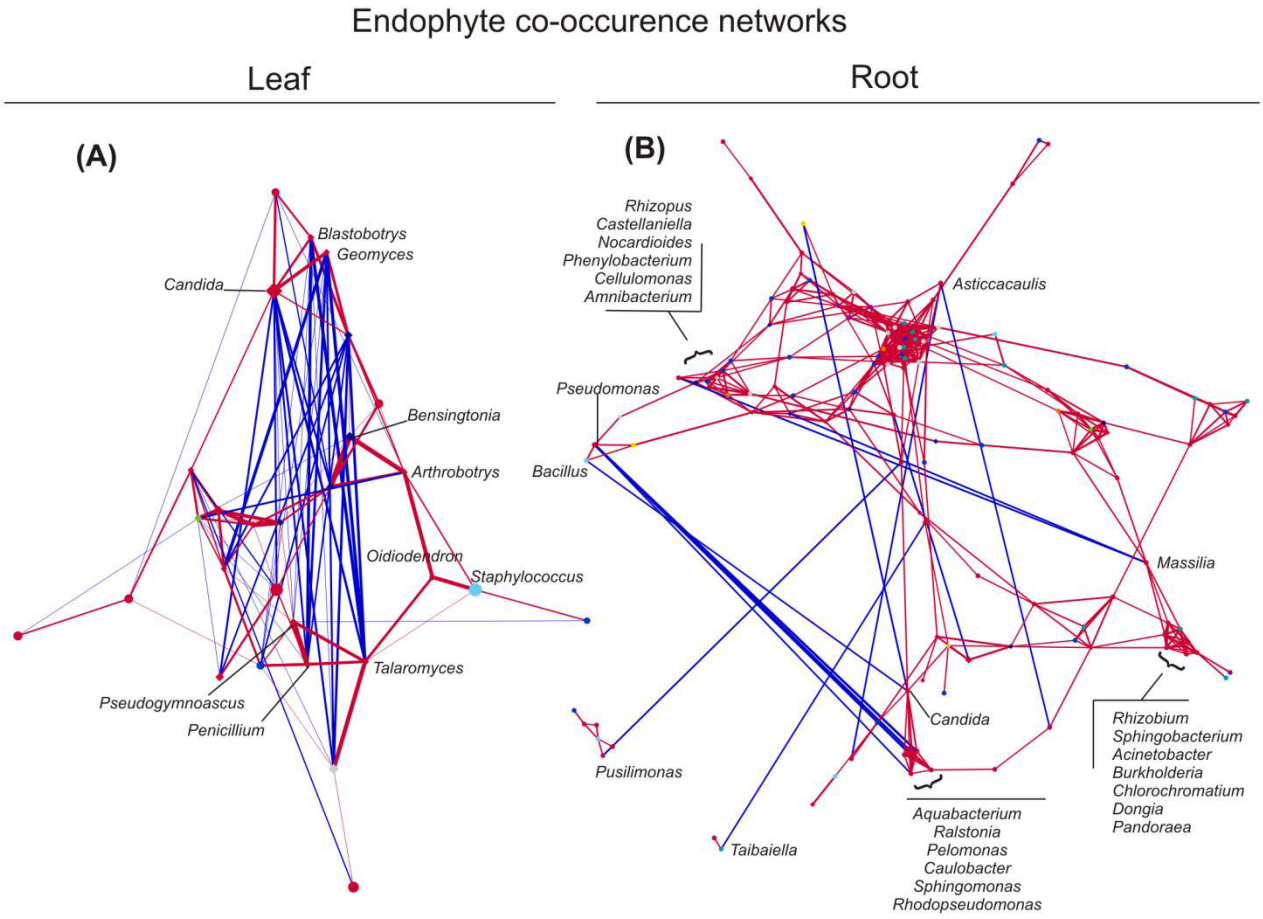

Figure 6. Co-occurrence networks of fungal and bacterial OTUs in the endosphere. Node color indicates respective phylum per OTU as provided in Figure x. Node size is scaled to the mean relative abundance of each node per compartment. Edges indicate co-occurrence or cooperation relationships in red, avoidance or competition relationships are in blue. Edge width is scaled to the correlation coefficient of each pair of OTUs. Bacterial OTUs are given as circular nodes, fungal nodes are diamond shaped.

A striking feature of the leaf network (Figure 6 A) is the number of negative correlation, which occurred almost exclusively between fungal OTUs, which would suggest a strong intra-phylum competition. Possibly, they occupy different niches within the leaves. *Candida* is the most abundant fungal OTU in leaves, *Streptococcus* the most abundant bacterial OTU. They seem to be better adapted to the leaf habitat. Furthermore, the strong negative relation of *Rhodobacter,* and to a lesser extent *Xanthomonas,* in both bulk soil and rhizosphere are interesting (Figure 7 A). *Rhodanobacter* is also by far the most abundant phylotype in the soil samples, which might be reflected by the negative interactions. Possibly, *Rhodanobacter* is enriched in the potting soil that is used. Therefore, the negatively related clusters might reflect the soil and rhizosphere communities that are starting to establish themselves. To our knowledge, this study provides the first analysis of

fungal and bacterial co-occurrence patterns in different sampling locations and plant compartments. Therefore, knowledge on the interactions is still very limited.

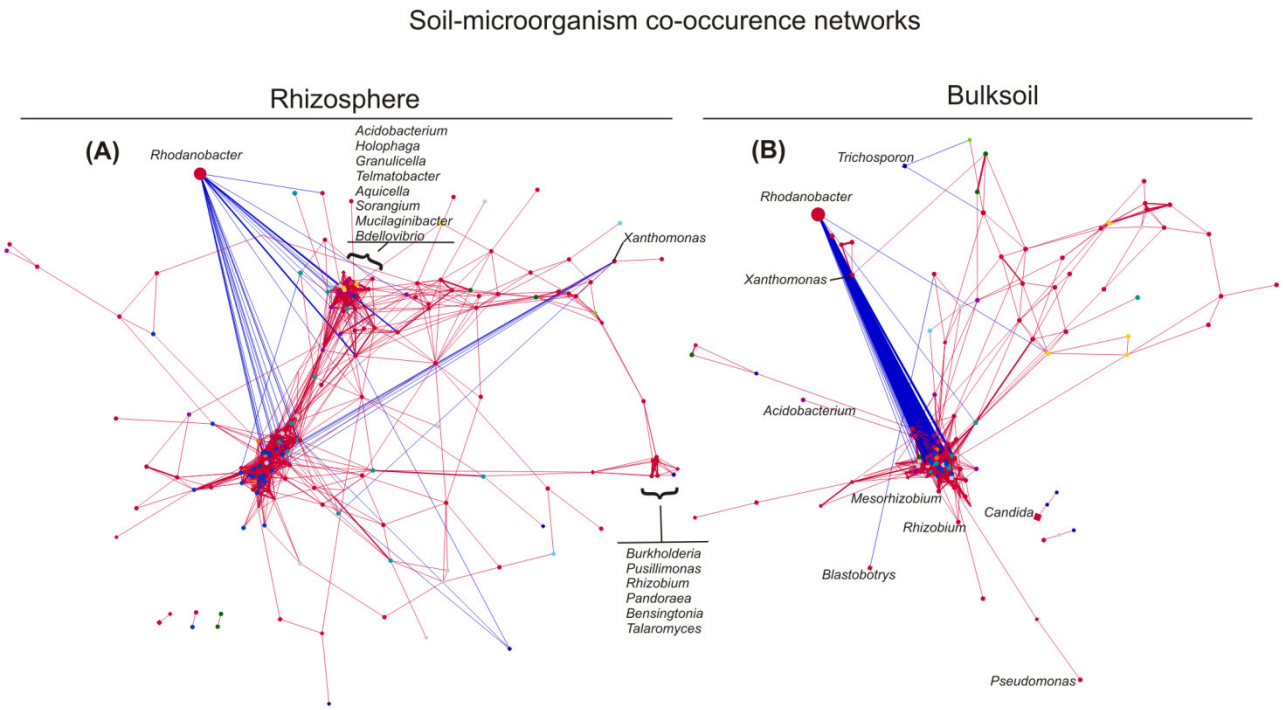

Figure 7. Co-occurrence networks of fungal and bacterial OTUs in the soil. Color-coding, node sizes and edge-weighting according to Figure 6.

Cropping regime, crop species, and sampling location influenced the functioning of bacterial and fungal communities

To gain a closer look into the ecological roles of fungal community members, we used FUNGuild to classify fungal species by their trophic modes to gain an insight into their lifestyle (Nguyen *et al.*, 2016)e. While 80 OTUs went unmatched, 158 were parsed into an ecological guild. The community composition based on the trophic modes was compared between the different treatments (Table 4). The abundances between the different cropping systems and plants did not vary significantly, except for pathothroph-saprotrophic fungi. They showed a higher abundance in WM- and, accordingly, wheat-derived samples.

Table 4. Relative abundances of fungal trophic guilds (mean ± SD). The abundances are shown for the different cropping regimes, sampling locations and the two crop specoes. Letters indicate significant differences with $P < 0.05$ between the treatment groups as revealed by Dunn's and Man-Whitney test.

Treatment	*n*	*Pathotrophs (%)*	*Saptrotrophs (%)*	*Symbiotrophs (%)*
		Cropping regime		
FBM	19	6 ± 13	14 ± 20	1 ± 2
WM	20	7 ± 20	13 ± 10	2 ± 2
RI	35	7 ± 16	16 ± 16	2 ± 4
MI	34	5 ± 11	16 ± 14	3 ± 4
		Sampling location		
Bulk soil	20	0.3 ± 0.5^{abc}	5.7 ± 3.8^{a}	1.3 ± 1.3
Rhizosphere soil	29	0.5 ± 1.7^{a}	19.4 ± 16.3^{b}	1.7 ± 2.7
Root	29	10.8 ± 19.1^{b}	15.3 ± 16.9^{abc}	1.9 ± 3.5
Aerial parts	30	10.4 ± 19.1^{bc}	17.1 ± 13.9^{bc}	2.8 ± 0.8
		Crop species		
Faba bean	49	4.8 ± 11.0	18.1 ± 18.3	2.1 ± 3.6
Wheat	49	8.3 ± 18.9	14.1 ± 11.3	1.9 ± 3.1

Abbreviations: FBM, faba bean in monoculture; WM, wheat in monoculture; MI, mixed intercropping; RI, row intercropping.

Between the different plant parts as well as rhizosphere and bulk soil, the community composition varied more strongly. Firstly, endophytic fungal communities contained significantly more pathogens than rhizosphere samples. This is not surprising, as pathogens co-evolve with their hosts and are specialized in host colonization by secreting effector molecules. Thereby, they interfere with plant hormone synthesis or plant defense (Lo Presti *et al.*, 2015). Secondly, the pathotroph-saprotroph-symbiotrophic group showed significant differences in abundance, especially between rhizosphere and roots. In general, this group was more abundant in bulk soil and rhizosphere samples than in endosphere samples. Lastly, saprotrophic fungi dominated the fungal community in all samples. They were significantly more abundant in rhizosphere compared to bulk soil samples. Additionally, they were also highly abundant in endosphere samples. This might be explained by the fact that saprotrophic fungi are generally defined to receive nutrients by break down of dead host cells (Nguyen *et al.*, 2016). Saprotrophic fungi have already been described to dominate the endophytic fungal community of potato roots derived from rotational fields (Manici and Caputo, 2009). Our results show that the recruitment of different trophic guilds of fungi is influenced by the sampling location and, consequently, explains the differences found for the community profiles (NMDS).

In addition, functional profiles were predicted for bacterial communities and the abundances of key enzymes of the nitrogen metabolism were compared (Figure 8) because nitrogen is a major driver of bacterial communities in the endosphere (Rodríguez-Blanco *et al.*, 2015; Wemheuer *et al.*, 2016) as well as soil (Herzog *et al.*, 2015; Li *et al.*, 2016). In general, the abundances of these key enzymes differed between the sampling locations endosphere vs. soil and between the cropping regimes monocultures (FBM and WM) vs. intercropping. More precisely, the highest abundances of almost all key enzymes were observed in FBM samples. Nitrogenase, the enzyme catalyzing the nitrogen-fixing step, was found in highest abundance in the faba bean monoculture samples, being slightly more abundant in the soil samples. Legumes such as faba bean are well-known for their symbiosis with rhizobia. Biological nitrogen fixation can improve nitrogen content in soils (Peoples *et al.*, 1995*;* Zahran, 1999) which might result in higher abundances of key enzymes including the nitrogenase. However, for wheat monoculture and samples from intercropping, the nitrogenase gene was more abundant in the endosphere compared to the soil communities. Biological nitrogen fixation by endophytic bacteria were observed in leaves of several plants such as crop species (James, 2000; Burbano *et al.*, 2011; Carvalho *et al.*, 2014) or trees including *Pinus flexilis* (Moyes *et al.*, 2016). The findings observed in this study indicates that nitrogen fixation plays a greater role in the endosphere as expected.

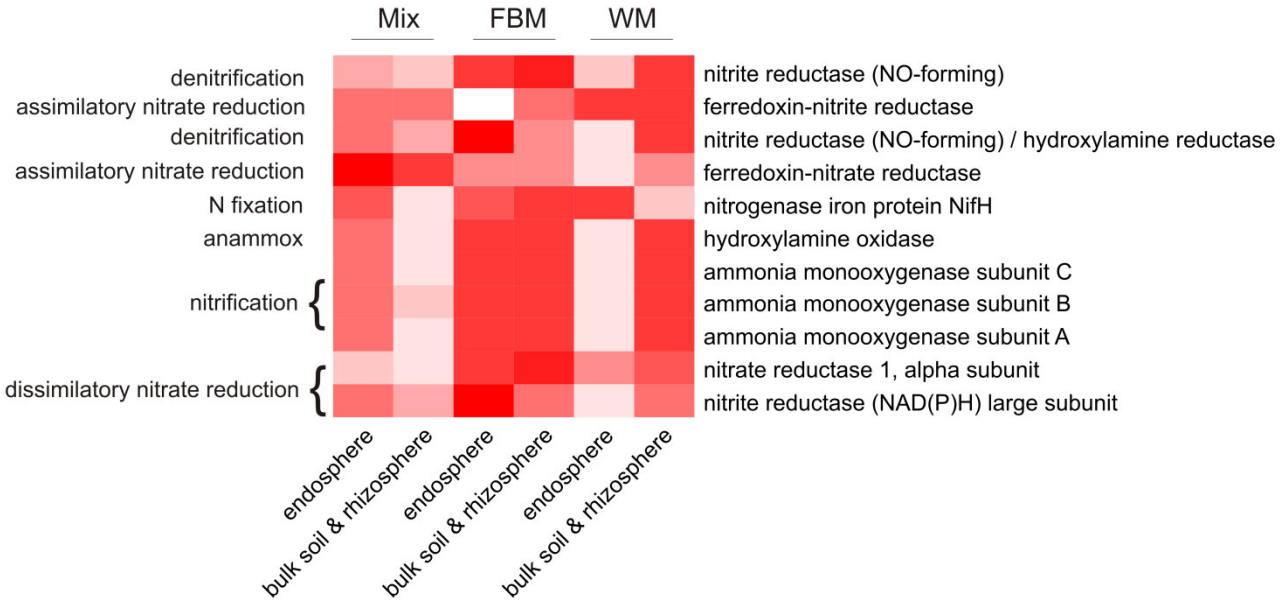

Figure 8. Key-enzyme encoding genes involved in nitrogen cycling. For each subpathway one or more key-enzyme encoding gene(s) is giving for samples derived from intercropping (mix), faba bean monoculture (FBM) and wheat monoculture (WM). Samples were distinguished between endosphere and bulk soil and rhizosphere. Genes are color-coded by abundance along a gradient of red to white, representing highly and lowly abundant genes, respectively.

In addition, most enzymes were more abundant in wheat samples derived from soil, when compared to the wheat endosphere samples. In the samples derived from intercropping, however, this trend was reversed: all key enzyme were more (or equally) abundant in the endosphere compared to the

soil samples. This indicates an effect of cropping regime and crop species on N cycling bacteria although the composition of bacterial communities was not affected by these factors. However, more studies are required to analyze not only changes of microbial community diversity and composition, but also the functioning of these communities with regard to cropping regime, sampling location as well as crop species.

Conclusion

Although multiple cropping systems provide many economic and ecological advantages, our knowledge about the impact of these systems on microbial communities is still rather limited. Nonetheless, it is of crucial importance to understand this impact as multiple cropping offers a great potential in the sustainable intensification of agriculture. The present study provides first insights into the complex response of bacterial and fungal communities in soil and endosphere of wheat and faba bean plants towards the different cropping regimes by using high-throughput next generation sequencing. Crop species did not affect bacterial and fungal community composition in soil and endosphere, while differences in the richness and diversity between the two crop plants were recorded. In addition, cropping regimes influenced the microbial diversity and richness in soil and rhizosphere, but had no effect on community composition. This is only partly in line with our hypothesis (1) that crop species as well as cropping regime affect microbial community and diversity. We observed differences between soil and endophytic communities in roots and aerial parts which is in line with our hypothesis (2). However, this observation was more pronounced for bacteria than fungi. The differences in bacterial community composition between plant compartments are most likely caused by proteobacterial endophytes strongly associated with either aerial parts or roots. Functional classification of fungal taxa revealed differences between soil and endosphere which supports the hypothesis (3). Moreover, strong negative interactions between fungal OTUs were detected indicating strong competition and probably niche adaptation by specialized fungal taxa. Contrary to our hypothesis (4), the effects of crop species and cropping regime on diversity, composition, and functioning were similar in the soil communities as well as in the two endophytic communities. Obtained data generated the basis for further research on the complex interaction of management practices and their impact on soil and plant-associated microbial communities and their functioning.

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SUPPLEMENTARY INFORMATION

Supplementary figures and tables are provided along with the electronic version of this thesis (on DVD), under the following paths:

Table S1:	Supplementary Information/Chapter III.8/ Table S1.xlsx
Table S2:	Supplementary Information/Chapter III.8/ Table S2.xslx
Table S3:	Supplementary Information/Chapter III.8/ Table S3.xlsx
Table S4:	Supplementary Information/Chapter III.8/ Table S3.xlsx

CHAPTER IV

GENERAL DISCUSSION

‘Education is what remains after you have forgotten everything you learned in school.’

Albert Einstein

IV. GENERAL DISCUSSION

Microorganisms are the most abundant and diverse group of organisms. Especially *Bacteria* and *Archaea* can be found in every environment investigated so far, even in those that are completely life-threatening from an anthropogenic view. They thrive in hot springs and deep sea vents, volcanic sites and the perpetual ice of Antarctica (Mientus *et al.*, 2013*;* Montross *et al.*, 2014*;* Urich *et al.*, 2014*;* Wemheuer *et al.*, 2013). Microorganisms are major drivers of biogeochemical cycles (Lengeler *et al.*, 1999*;* Martinko and Madigan, 2005) and important players in ecosystem structuring (Azam and Malfatti, 2007). The sampling and classification of microorganisms is, however, challenging due to their small size. Additionally, only a very small amount of the actual diversity of prokaryotic microorganisms can be cultivated by using standard cultivation approaches.

The mechanisms that shape microbial community structure, influence their functioning and act upon the environment are still not fully understood. Microbial community analyses are mostly based on taxonomic marker gene analysis, e.g. 16S rRNA genes sequences, which are used to classify the organisms present in a sample. Therefore, the environmental DNA (or RNA) needs to be extracted. This is challenging, especially with soil samples (Lombard *et al.*, 2011). Varying effects of soil pH (Sagova-Mareckova *et al.*, 2008), clay (Novinscak *et al.*, 2011) and organic carbon content can compromise extraction, as compounds such as humic acids are known to inhibit DNA polymerase and reverse transcriptase that are often employed in downstream analyses (Tebbe and Vahjen, 1993). Consequently, generation of datasets comprising a wide variety of different soil types raise the necessity of an extraction protocol that (1) produces high purity nucleic acids from different soil types, (2) yields long nucleic acid fragments (3) is representative for the microbial diversity in a sample. The application of the same extraction method is crucial and a prerequisite for comparative analyses between samples within a study. Therefore, the first part of this thesis focused on comparing various DNA and RNA extraction methods (Chapter III.1). The MoBio Power Soil DNA isolation kit perform equally well over the range of tested soils (Wüst *et al.*, 2016). It produced similar amounts of DNA and 16S rRNA gene copies for each soil and did not overestimate any of the abundant phyla detected.

The main focus of this thesis was the analysis of diversity and ecology of terrestrial microbial assemblages in different environments. The focus was on the response of the microbial communities towards anthropogenic factors such as land use type and intensification. In particular, the effects of land use intensity on soil bacterial community

diversity, structure and function in soils of grasslands and forests in Germany (Chapter III.2, III.4 and III.5) and in the endosphere of grasses (Chapter III.6) were investigated. In addition, the response of soil microbial communities toward different tree species (Chapter III.3) was analyzed. Finally, soil and endophytic microbial communities in an intercropping experiment with faba bean and wheat (Chapter III.7 and Chapter III.8) were investigated.

IV.1. TERRESTRIAL MICROBES

IV.1.1. Soil bacterial communities and their response to land use intensity

The main study of this thesis (Chapter III.2) focused on investigating the effect of land use type and intensity on soil bacterial communities in forests and grasslands. In forests, land use intensification occurs through different factors. First, there are different management regimes. These management regimes are unmanaged forests, age class forests (clear cutting at regular intervals) and selection forests (single tree removal). Age class and selection forests are different in the strength of disturbances (soil compaction), mostly due to differing harvesting intervals. Unmanaged forests are natural, undisturbed forests from which neither dead wood nor other organic matter is removed. Furthermore, forests differ in the dominant tree species and stand age. Replacing natural beech stands by the faster growing species spruce indicates land use intensification by selection of tree species. Furthermore, tree species selection and stand age affect the susceptibility of a stand to disturbances such as wind throw and pathogens. This results in the need of a higher management intensity to reduce the risk of stand loss. In 2013 (Schall and Ammer, 2013) implemented a silvicultural management index (SMI), to integrate all these components and facilitate the investigation of land use intensification in forests.

In grasslands, there are also different factors contributing to land use intensification. The management regimes are meadow, pasture and mown pasture. They already integrate mowing and grazing, two of the intensification factors. Additionally, plots of each management are either fertilized or unfertilized. Blüthgen and colleagues (Blüthgen *et al.*, 2012) introduced the land-use intensity index (LUI) to assess land use intensity in grasslands. It weights each of the three factors equally taking into account the amount of nitrogen applied by fertilization (kg nitrogen ha^{-1} y^{-1}), the mowing frequency (cuts per year) and the grazing intensity (livestock units days of grazing ha^{-1} y^{-1}).

Bacterial diversity and community composition were generally unaffected by increasing land use intensity in both systems. However, a significant correlation of the SMI

with forest soil bacterial communities in the Schorfheide-Chorin could be found. The SMI did not affect the bacterial community structure in forest soil in the Hainich-Dün or Schwäbische Alb. This could be attributed to the high variance of SMI within the pine stands in the Schorfheide-Chorin, where also the tree species-effect is most pronounced. No differences between the management regimes could be detected. Strikingly, the tree species was an important driver of bacterial community structure in forests. The effect of tree species clearly distinguished between soil bacterial communities from coniferous or broadleaved forest soils. Several studies focused on the effects of land use intensification through manipulation of tree species, soil compaction or harvesting and organic matter removal on soil microbial communities (Table 1). In all studies taking the effect of tree species into account, an effect on the microbial community could be found. Again, this separation was mainly between coniferous and broadleaved tree species, although distinct microbial communities under different types of broadleaved trees are reported (Urbanová *et al.*, 2015). The effect of tree species is most likely an indirect one, as coniferous trees are known to acidify their surrounding soils significantly (Hornung, 1985). A detailed analysis of the tree species-related effects on microbial communities (Chapter III.3) showed the presence of distinct microbial communities in soil of beech and spruce dominated forests. The diversity of bacteria was higher in beech forest soils compared to spruce forest. The opposite was detected for fungal diversity. Additionally, the results indicated that community composition was affected by the distance from spruce tree trunks. This effect was not detected in beech dominated forest soils and is likely due to the very different root systems. While spruce trees are typically shallow rooted, beech trees develop a deep reaching root system and can acquire nutrients over a range of soil horizons. Spruce roots would therefore more strongly influence the upper soil layers around the tree than compared to beech. Upper soil layers favored saprotrophic bacterial and fungal taxa, which are usually connected with organic matter breakdown (Edwards and Zak, 2010*;* Lindahl *et al.*, 2007*;* Pankratov *et al.*, 2007*;* Stursová *et al.*, 2012). Furthermore, microbial community was impacted by season, an effect that was significant in beech forests, but not in spruce forests. Seasonality in soil microbial communities has been reported previously for bacteria in oak dominated forests (López-Mondéjar *et al.*, 2015), and fungi in beech forest soils (Buée *et al.*, 2005). Interestingly, this study also provided evidence for distinct microbial community profiles under single trees, which has been previously reported for fungi in *Pinus muricata* forest soils (Branco *et al.*, 2013).

Although the studied gradient of land use intensification ranged from unmanaged forests to age class forests and selection forests an impact of management on soil bacterial

community structure and diversity was not detected. Furthermore, gamma diversity of 15 taxonomic groups in even-aged and uneven-aged forests was not significantly impacted by the two forest systems (Chapter III.5). The management regimes differ in their rates of soil disturbance by compaction through harvesting and organic matter removal. Both factors influence microbial communities (Hartmann *et al.*, 2012*;* Hartmann *et al.*, 2014), but the effect on soil bacterial communities was not as pronounced as on soil mycorrhiza.

Table x: Studies on land use intensification in grasslands and forests. Studies contained within this thesis are given in bold.

Reference	Method	Type of land use intensification	Samples	Effect on	Effect?
FOREST					
Lejon *et al.*, 2005	ARISA	tree species	16	community composition	yes
He *et al.*, 2006	16S RNA clone libraries	dead wood removal / management	12	community composition / diversity	no
Axelrood *et al.*, 2002	16S RNA clone libraries	soil compaction	6	bacterial community composition	yes
Lin *et al.*, 2011a	16S RNA clone libraries	native forest / plantation*;* tree species	9	community composition / diversity	yes
Lin *et al.*, 2011b	16S RNA clone libraries	disturbance (native/plantation)		community composition / diversity	yes
Selvam *et al.*, 2010	16S RNA clone libraries	tree species		diversity	yes
Upchurch *et al.*, 2008	16S RNA libraries	forest age	9	diversity	yes
Hackl *et al.*, 2004	TRFLP / 16S sequencing	tree species / mixed stands	60	bacterial community composition	yes
Felsmann *et al.*, 2015	TRFLP / 16S sequencing	tree species	9	community composition / diversity	yes
Myers *et al.*, 2001	PLFA	tree species		community composition	yes
Thoms *et al.*, 2010	PLFA	tree species diversity	27	community composition	yes
Busse *et al.*, 2006	PLFA	compaction / organic matter removal / weed control		community composition	yes
Siira-Pietikäinen *et al.*, 2001	PLFA	harvesting		community composition	yes (clear felling)
Chatterjee *et al.*, 2008	PLFA	harvesting	162	community composition	yes
Brockett *et al.*, 2012	PLFA	tree species	63	community composition / enzyme activities	some
Hackl *et al.*, 2005	PLFA	tree species /mixed stands	240	community composition	yes

Urbanová *et al.*, 2015	pyrosequencing	tree species	42	community composition	yes
Hartmann *et al.*, 2012	pyrosequencing	organic matter removal / compaction	81	community composition	yes
Nacke *et al.*, 2011	pyrosequening	tree species	9	community composition/diversity	yes
Hartmann *et al.*, 2014	pyrosequencing	compaction	9	community composition / diversity	yes
Nacke *et al.* Chapter III.3	**pyrosequencing**	**tree species**	**128**	**community composition**	**yes**
Kaiser *et al.* Chapter III.2	**pyrosequencing**	**tree species / organic matter removal / compaction**	**150**	**community composition / diversity**	**yes**
GRASSLAND					
Kennedy *et al.*, 2005b	ARISA	unimproved/semi-improved	70	community composition	yes
McCaig *et al.*, 2001	16S RNA clone libraries /DGGE	unimproved/semi improved /improved grassland	9	diversity / community composition	yes
Kuramae *et al.*, 2012	DGGE / microchip /real time PCR	natural grassland/arable field, pasture; deciduous vs. Pine forest	125	community composition	no
Clegg *et al.*, 2003	DGGE/PLFA	N fertilized /soil drainage	12	community composition	yes
Jangid *et al.*, 2010	PLFA / 16S RNA clone libraries	grassland restoration at different timepoints	30	community composition	yes
Steenwerth *et al.*, 2002	PLFA	agriculture/ annual/perennial grassland	42	community composition	yes
Yeates *et al.*, 1997	PLFA	conventional/organic grassland management		community composition	no
Grayston *et al.*, 2001	PLFA	unimproved/semi-improved/improved grassland	18	community composition/microbial biomass	yes
Ingram *et al.*, 2008	PLFA	no grazing/heavy grazing		community composition	yes
Patra *et al.*, 2008	PLFA	intensive/extensive management		community composition	yes
Herold *et al.*, 2014	PLFA	fertilization / mowing / grazing	27	community composition	no
Kennedy *et al.*, 2005a	TRFLP	unimproved/semi-improved	30	community composition	yes
Sayer *et al.*, 2013	TRFLP	unimproved/improved/restored	9	community composition	yes
Thomson *et al.*, 2015	TRFLP, pyrosequencing	unimproved/improved; short-term/long-term abandoned	55	community composition	yes
Will *et al.*, 2010	pyrosequencing	fertilization / mowing / grazing	9	diversity	yes
Hartmann *et al.*, 2015	pyrosequencing	conventional / organic farming	80	community composition / diversity	yes

Nacke *et al.*, 2011	pyrosequening	fertilization / mowing / grazing	9	community composition/diversity	no
Tardy *et al.*, 2015	pyrosequencing	low / medium / high land use intesity	162	diversity	yes
Carey *et al.*, 2015	MiSeq	fertilization / mowing	40	community composition / diversity	no
Kaiser *et al.* Chapter III.2	**pyrosequencing**	**fertilization / mowing / grazing**	**150**	**community composition / diversity**	**no**

In the grassland systems investigated within this thesis, a small land use intensification effect on soil microbial communities was recorded. The land use intensity index (LUI, Blüthgen *et al.* 2012) was significantly correlated with the microbial community structure in grasslands in the Schwäbische Alb, but not in the Hainich-Dün or Schorfheide-Chorin. The effect most likely results from stronger organic fertilization in the Schwäbische Alb. Grasslands in the Hainich-Dün and Schorfheide-Chorin predominantly receive inorganic fertilizers. A recent study by Hartmann and colleagues (2015) described distinct microbial communities for conventional and organic farming systems. None of the other factors (grazing or mowing) resulted in an effect on bacterial community composition or diversity. Several studies investigated the influence of grassland management on soil microbial communities and showed that management altered bacterial community composition (Table 1). However, previous studies carried out within the Biodiversity Exploratories also could not verify an effect of land use intensity in grasslands (Herold *et al.*, 2014*;* Nacke *et al.*, 2011), except for Will *et al.* (2010) who showed the highest bacterial diversity in non-fertilized meadows. Additionally, a recent study focused on species-abundance distributions of 10 above- and belowground taxonomic groups (Chapter III.4). Belowground microorganisms (represented by bacteria and arbuscular mycorrhizal fungi) were not affected by land use intensification, or mowing, grazing, and fertilization. Generally, species richness was largely unaltered by land use intensity (Chapter III.4). Tardy *et al.* (2015) encountered that bacterial diversity was highest in moderately managed soils. They investigated the effects along a gradient of management intensity in three different regions. It was suggested that this is a result of the stress response of the bacterial communities. In highly stressed environments diversity decreases due to the dominance of competitive species and competitive exclusion whereas in unstressed environments diversity decreases due to the dominance of adapted species through selection. In conclusion, land use intensification has no general effect on soil microbial community composition or diversity within the Biodiversity Exploratory

framework. However, it is very difficult to compare the results of this thesis with studies that have not been carried out within the Biodiversity Exploratories. This is due to the fact that land use and management per se are not standardized definition. Thus, the definitions of land use and management vary considerably between studies. As far as it could be inferred from the publications summarized in Table 1, land use intensification was analyzed within a similar range as covered by the Biodiversity Exploratories. Additionally, it has to be noted that most of these studies applied different community profiling techniques such as TRFLP (Liu *et al.*, 1997) or DGGE (Muyzer *et al.*, 1993), or PLFA. None of these techniques provides such a high level of taxonomic resolution as NGS-based sequencing and analysis of 16S rRNA gene amplicons (Prosser *et al.*, 2010).

IV.1.2.Soil bacterial communities in response to environmental parameters

While land use intensification had no significant effect on microbial community composition and diversity, both differed between the two land use types grassland and forest and the corresponding sampling regions (Chapter III.2). Soil pH was identified as the strongest driver of bacterial communities in grasslands and forests (Chapter III.2). It also exhibited a strong impact on bacterial and fungal communities in beech and spruce forests (Chapter III.3). This pH effect reflects the separation of community composition between grasslands and forests and the effect of tree species (coniferous vs. broadleaved). Forest soil samples were generally more acidic compared to grassland soil samples, while samples from coniferous forests were more acidic than samples derived from broadleaved forests. Soil pH further explains the higher bacterial diversity in grassland soils compared to forest soils. Soil pH was previously described as a strong driver of microbial community composition at the continental scale (Fierer and Jackson, 2006). Additionally, factors such as the C:N ratio and the gravimetric water content were shown to drive community composition to some extent, which is also in line with previous studies (Brockett *et al.*, 2012*;* Cederlund *et al.*, 2014).

This study went beyond the scope of previous studies by not only identifying drivers of bacterial communities but also by testing the response of the most abundant bacterial groups toward pH. The responses differed between groups*;* some showed preference for very acidic conditions (high abundance at low pH), whereas others were almost unaffected by pH (relatively stable abundance over the studied pH range). Effects on certain bacterial groups have previously been investigated by correlation analysis (Nacke *et al.*, 2011*;* Naether *et al.*, 2012). However, relations between organisms and environmental gradients more likely result

in unimodal or skewed unimodal response curves restricted by growth limiting conditions and competition (Austin and Smith, 1990). Furthermore, the results provided insights into the acid tolerance (ATR) or adaptation to acidic conditions of bacteria in soils (Chapter III.2). An important system obviously employed by soil bacteria are proton pumps, which enable an active control of intracellular pH values. Enzymes involved in the arginine metabolism (especially arginine deiminase) also seem to be more abundant in low pH soils, indicating a role of alkali production in soil bacterial ATR. This system has been described for several soil-dwelling bacteria (*Pseudomonas*, *Bacillus*, *Mycoplasma*) as well as oral bacteria (Cunin *et al.*, 1986). A possible mechanism is that arginine is actively transported into the cell (e.g. by arginine:ornithine antiporters) where alkaline compounds (such as NH_3) are produced (e.g. by arginine deiminase) that might help stabilize intracellular pH. Levansucrase, an enzyme, which is involved in exopolysaccharide formation, was highly abundant in low pH soil samples. Bacteria therefore might also employ biofilm formation to protect themselves from stressful acidic conditions, as has previously been shown for *Lysteria monocytogenes* (Oh and Marshall, 1996).

In conclusion, a major factor driving microbial communities is the soil pH. Bacteria respond strongly toward pH and several groups seem to occupy certain ‘pH niches’, where they reach highest abundances. Lastly, the here reported study (Chapter III.2) presents evidence for a genomic adaptation of soil bacteria against acidic conditions.

IV.1.3. Bacterial functioning in grasslands and forests

As grassland and forest differ in their soil microbial community compositions, the functions carried out by bacteria and fungi might also differ between these ecosystems. Metagenomic approaches to unravel the functional potential of soils have been carried out before (e.g. Delmont *et al.*, 2012*;* Fierer *et al.*, 2012a*;* Fierer *et al.*, 2012b*;* Pan *et al.*, 2014). These studies unravel bacterial functions to the level of metabolic subsystems from the SEED database (Overbeek *et al.*, 2014). Other studies focused only on specialized enzymes such as carbohydrate-active enzymes (CAZymes) (Cardenas *et al.*, 2015), or directly targeted enzymes of interest (Baldrian *et al.*, 2012). Both approaches cannot unravel the whole potential of bacterial contribution to nutrient cycling. The application of metagenomic shotgun sequencing of soil DNA samples has several drawbacks. Soils are enormously diverse and therefore have a very high genomic diversity. This makes it almost impossible to capture the whole genomic information of a sample. Further, lots of information from shotgun

metagenomic sequencing cannot be annotated (Fierer *et al.*, 2012b). Within this study, a bioinformatic approach was applied and functional profiles predicted on the basis of 16S rRNA genes with a newly developed tool called Tax4Fun (Aßhauer *et al.*, 2015). This approach is much more time- and cost-effective, and Tax4Fun has been shown to predict highly accurate functional profiles from 16S rRNA data for a range of samples including soils. It further provides detailed information on the level of KEGG pathway or KO genes. Genes encoding key enzymes for important metabolic pathways involved in nutrient (nitrogen, phosphate, carbon and sulfur) cycling were either more abundant in grassland or forest. It has been suggested, that methanotrophic bacteria in forest soils are the largest sink of atmospheric methane (Kolb, 2009). The results within this study suggest that the functional potential for aerobic methane oxidation and nitrous-oxide reduction is indeed higher in forest soils. This suggests that forest soils play an important role in the regulation and removal of atmospheric greenhouse gases such as methane and nitrous oxide. The functional potential for acid phosphatases is higher in more acidic forest soils, while alkaline phosphatases are potentially more abundant in grasslands. The genes encoding urease were 1.2-fold more abundant in the grassland profile. The availability of urea was higher in the grassland samples, as these are partly fertilized with manure or dung or were grazed by animals. Chitinase genes also showed a 1.2-fold higher abundance in grasslands compared to forest soils. This might result from the higher abundance of *Actinobacteria* in grassland soils as this group is known to harbor a high number of chitinase genes (Bai *et al.*, 2014). Genes involved in polyaromatic hydrocarbon (PAH) degradation are more abundant in grasslands. Possibly, this degradation is carried out by bacteria in grassland systems, while in forest systems this process is taken over by ligninolytic fungi (Hammel, 1995), which were not analyzed within this study.

To date, this is the first approach to distinguish multiple functions from different ecosystems based on single genes. The results of this thesis suggested distinct functional traits for grassland and forest systems and further indicate a certain functional ‘adaptation’ to the respective environment. As the grasslands and forests within the present study are long-term established systems, it would be interesting to evaluate if a similar ‘adaptation’ on the genetic level is also present in younger systems. The results further are based on a predictive metagenome and not an actual one. Therefore, metagenomic approaches targeting the respective key genes, or metagenomic shotgun sequencing will be necessary to verify these findings.

In conclusion, bacterial communities in German grasslands and forests form distinct patterns and are driven by edaphic parameters. Land use intensification does not significantly alter community composition or diversity. This could be attributed to a general resistance or resilience of soil bacterial communities toward anthropogenic influences. However, it is also important to keep in mind the range of factors, which are investigated for their effects. As suggested by (Brockett *et al.*, 2012), 'the range of (...) values (…) may not have been as great (…) relative to the range of other site factors (…), to display the expected relationships.' Optimally, the factors causing a certain effect that should be investigated should be the only factors allowed to range, while all other factors should be held constant. Naturally, it is hard to control for stable or similar conditions in the field or in natural systems in general. Therefore, the presence or absence of effects should always be seen in relation to the range of factors causing them.

IV.2. PLANT-ASSOCIATED MICROBIAL COMMUNITIES

Soil-inhabiting microorganisms are often also associated with plants. This is especially true for the rhizosphere, the part of the soil immediately influenced by plant roots. Bacterial nitrogen fixers are often associated with or live in symbiosis with roots of several plants. Also fungi form close associations or symbioses with plants. Arbuscular mycorrhizal fungi actively penetrate their host plants cells, while ectomycorrhizal fungi form a non-invasive symbiosis with their host plants. Despite root-associated, rhizospheric microorganisms, also epiphytes (living in the phyllosphere*;* Vorholt, 2012) and endophytes (living inside the plant without causing disease*;* Hallmann *et al.*, 1997) are closely associated with plants. These organisms are thought to be beneficial to their host plants by providing various functions such as productions of antimicrobial compounds and biological pest control (Bulgarelli *et al.*, 2013).

Within this thesis, three studies focused on plant-associated microbial communities. The first study focused on bacterial endophytes in three agricultural important grasses: (1) *Lolium perenne* L., (2) *Festuca rubra* L. and (3) *Dactylis glomerata* L. In the design of this study, impact of fertilization and mowing frequency was analyzed with regard to plant species-specific endophytic communities in 2010 and 2011. Bacterial community composition was significantly shaped by the respective host plant, and diversity was higher in *D. glomerata*. The impact of fertilization and mowing on community composition and diversity varied between the sampling times without a clear trend. Diversity and community composition were influenced by fertilization only in 2010 whereas no effect of mowing frequency was recorded in either year. It is possible that fertilization and mowing exhibit indirect effects on the endophytic communities by changing the physiological status of plants.

Fuentes-Ramírez and colleagues (1999) observed reduced colonization of sugarcane by *Gluconacetobacter diazotrophicus* after nitrogen-fertilization. They concluded that fertilization altered the plant physiological state, which interfered with the interactions between plant and endophyte. A similar hypothesis was raised by (Hallmann *et al.*, 1999), who observed an impact of organic amendments on endophytic communities in crops. However, the effects of fertilization and mowing on endophytic communities analyzed in Chapter III.6 are not consistent at both sampling times. Possibly, other factors such annual mean temperature or precipitation might interfere and additionally alter the physiological states of the plants, as these factors cannot be controlled in a field study.

The last two studies within this thesis analyzed soil and plant-associated microbial communities in an intercropping experiment with faba bean (*Vicia faba* L.) and wheat (*Triticum aestivum* L.). The impact of plant monocultures and mixed cultures on soil archaeal communities (Chapter III.7) and on bacterial and fungal communities in soil, rhizosphere and endosphere (Chapter III.8) was investigated. Intercropping has previously been shown to produce beneficial effects for the mixing partners, as long as the intercropping partners do not occupy identical ecological niches (Ofori and Stern, 1987). In Chapter III.6, three different grass species significantly affected bacterial community composition. In the intercropping experiment however, plant species did only affect bacterial endophytic communities, but not the community composition of the rhizospheric or soil microbial communities. Plant species-specific endophytic communities have been indicated by previous studies (Berg and Smalla, 2009). There is also evidence that bacterial and fungal communities in the rhizophere are influenced by intercropping of faba bean and wheat (Song *et al.*, 2007*;* Wang *et al.*, 2012). These experiments however were carried out over a growing period of up to three years. The 4 weeks growth period in the presented experiment might have been too short for specific bacterial and fungal communities to establish. This additionally suggests that endophytic communities are mainly shaped by recruitment through the host plant. It has been suggested that endophytic communities are partly inherited from the parent generation (Ralphs *et al.* 2011, Uchitel *et al.* 2011). However, this inherited part of the endophytic community did not seem to play a strong role in shaping the community composition in wheat and faba bean, especially with respect to the fungal endophytes. Possibly, the effect will only become apparent after longer host-microbe adaptation periods. Nevertheless, soil archaeal community composition was affected by plant species and cropping regime (Chapter III.7). Bacterial and fungal communities were not altered by cropping regime, regardless if they derived from bulk

soil, rhizosphere or endosphere (Chapter III.8). Only microbial diversity and richness of bulk soil and rhizosphere samples was affected by the cropping regimes. Previous studies showed that ammonia-oxidizing archaea were affected by crop species (wheat, faba bean, white lupin) and cropping regime (Wang *et al.* 2012) and that crop rotation also affected archaeal community composition (Breidenbach *et al.* 2015). This in contrast to other studies suggesting that cropping effects and crop rotation have only minor effects on archaeal communities (Chan *et al.* 2013, Scavino *et al.* 2013).

It is unclear, why soil archaeal communities are influenced by cropping regime in the present experiment but bacteria and fungi are not. This might be due to the low diversity of soil-inhabiting archaea. However, little is known about the ecological roles of archaea in soils, and it is possible that they have faster adaptation systems or are more susceptible to changing environmental conditions.

While cropping regime did not influence bacterial and fungal communities in this experiment, the origin of the samples (leaf, root, rhizosphere or bulk soil) was the major driver of microbial community composition. This effect was more pronounced for bacteria but also detectable for fungi. Bulk soil and rhizospheric communities were very similar but endophytic communities differed. Bacterial endophyte communities varied more strongly in their composition between root and leaf samples, while this effect was only weakly detected for fungi. Fungi can actively colonize their plants host tissue, but bacteria are more restricted to passive dispersal inside the plant. This might contribute to the similar fungal endophytic communities.

In conclusion, it could be shown that anthropogenic influences alter plant-associated microbial communities and result in plant species-specific patterns of endophytic communities. Furthermore, the results suggest that endophytic communities are no result of inheritance but rely more on recruitment of microbes by the host plants. Additionally, archaeal communities in soils were shown to be more susceptible to changes in cropping regime than bacterial or fungal communities.

IV.3. Concluding remarks and outlook

The majority of studies within this thesis investigated the diversity, structure and function of microbial communities under changing environmental conditions. Our knowledge about the response of microbial communities towards changing conditions such as altered soil properties or land use intensification, are still limited.

In summary, drivers of microbial community structure and diversity subsequently affect microbial functioning (Figure 4). Soil microbial communities are primarily shaped by edaphic properties (pH, soil texture, nutrients, moisture*;* Chapter III.2 and III.3). They are additionally affected by plants, e.g. the dominant tree species in forests (Chapter III.2 and III.3) or crop species (faba bean vs. wheat*;* Chapter III.7). Plant-associated microbial communities living in the rhizosphere and endosphere are also affected by their corresponding host plants (Chapter III.6), or the sampled plant compartments (Chapter III.8). Land use intensification only exhibited minor effects on microbial community structure in grassland and forest soils (Chapter III.2, III.4, III.5). Similarly, endophytic bacterial communities only responded weakly to fertilization and mowing (Chapter III.6). In grassland and forest soils, bacterial functioning followed the same drivers as bacterial community structure and diversity (Chapter III.2) and functioning of endophytic bacterial communities differed between different grasses (Chapter III.6). Furthermore, grassland and forest soils selects for certain bacterial traits (e.g. greenhouse gas regulation in forest soils, urease and alkaline phosphatase in grassland soils*;* Figure 4).

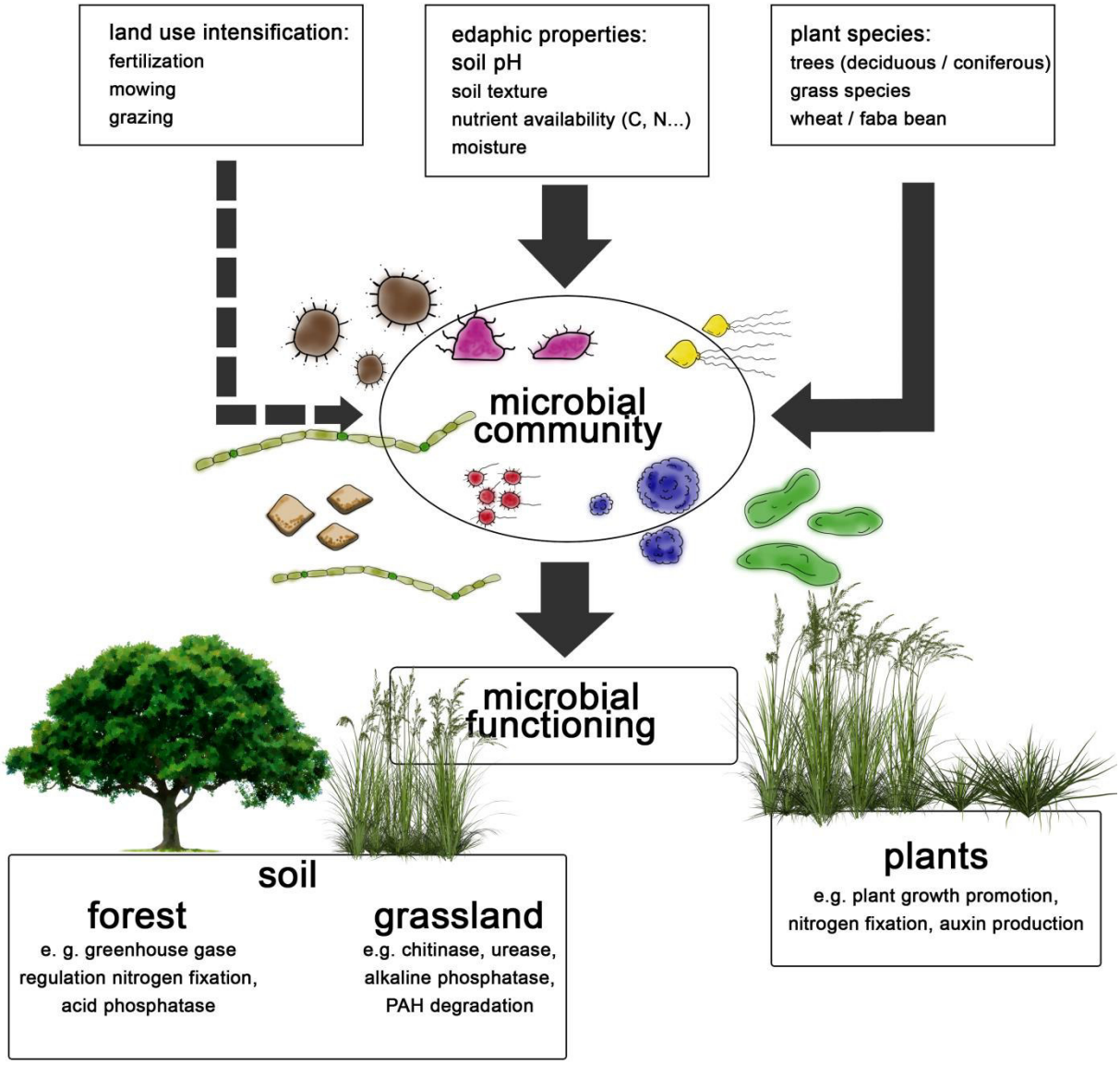

Figure 4. Drivers of microbial communities and microbial functioning as identified within this thesis. Drivers of soil and plant-associated microbial community structure and diversity subsequently drive microbial functioning in forest and grassland soils (Chapter III.2) and endophyte functioning within different grasses (Chapter III.6).

In summary, it was found that soil microbial communities are driven primarily by edaphic properties, while plant-associated microbial communities are primarily driven by their respective host plants. Land use intensification, as the anthropogenic factor under analysis, had minor effects on the communities. However, this effect was not consistently recovered for all studies, or samples under analysis. This suggests that land use intensification will only affect microbial communities if it significantly alters edaphic factors such as nutrient concentrations and availability or the plants physiological status (e.g. by extensive fertilization), which was not the case in any of the studies presented within this thesis.

Therefore, microbial communities are well adapted to their respective environments and resistant to land use intensification practiced in temperate grassland and forest systems.

The results provided within this thesis contribute to our understanding of drivers and ecological roles of microbial community structures in soils and in association with plants. The results also suggest that the application of novel statistical and bioinformatic tools has the potential to broaden our knowledge on microbial communities, by providing new insights into the relationships between microbes and their environments. However, it will be necessary to investigate not only the total microbial communities, which was the aim of this thesis, but also the active fraction (RNA-based analyses) to fully unravel reasons for structural changes of microbial communities. Metagenomic and metatranscriptomic approaches as well as enzymatic assays will aid in drawing a comprehensive picture of the ecological roles that bacteria, fungi and archaea play in soils, the rhizosphere and in association with plants.

CHAPTER V

GENERAL REFERENCES

V. GENERAL REFERENCES

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CHAPTER VI

APPENDIX

VI. APPENDIX

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‘There is no formula for success except, perhaps, an unconditional acceptance of life and what it brings.’

- Arthur Rubinstein

APPENDIX

CURRICULUM VITAE

Name:	Kristin Kaiser
Date of Birth:	2nd March, 1987
Place of Birth:	Melsungen, Germany

Education

Since 06/2012	Georg-August-University Göttingen, Germany PhD thesis at the Department of Genomic and Applied Microbiology at the Institute of Microbiology and Genetics Topic: **Dependence of soil microbial community structure and function on land use types and management regimes**. (as part of the German Biodiversity Exploratories)
10/2009 - 03/2012	Georg-August-University Göttingen, Germany Master of Science program „Microbiology and Biochemistry“ Master thesis: **Functional and phylogenetic analyses of soil microbial communities derived from the German Biodiversity Exploratory Hainich**.
10/2006 - 09/2009	Georg-August-University Göttingen, Germany Biology in the Bachelor of Science program Bachelor thesis: **The influence of transcription onto the reversion of the *gudB*-allele in *Bacillus subtilis*** (Der Einfluss der Transkription auf die Reversion des *gudB*-Allels in *Bacillus subtilis*)
2003 - 2006	Secondary school, Geschwister-Scholl Schule in Melsungen, Germany Qualification: A levels
1999 - 2003	Secondary school, Gymnasium at the Gesamtschule in Melsungen, Germany
1997 – 1999	Transition stage, Gesamtschule in Melsungen, Germany
1993 - 1997	Primary school in Melsungen, Germany

Practical experiences

2010	Supervision of Microbiology practical courses for bachelor students
2010	Labrotation, Institute for Microbiology and Genetics, General Microbiology, Structural and functional analysis of microbial consortia
2011	Labrotation, Institute for Microbiology and Genetics, General and Applied microbiology, Cloning and heterologous expression of putative cellulase genes
2012 – 2013	Supervision of Master Thesis 'Metagenomics of Deadwood' (by Carl Foest)
2013	Supervision of Master Thesis 'Assessment and exploitation of bacterial and functional diversity in soils of the Biodiversity Exploratory Hainich' (by Vera Korolkov)

Scientific Meetings and Method Courses

2012	4th International Congress of the European Soil Sciences Societies Eurosoil 2012, Bari, Italy
2013	10th Annual Assembly of the German Biodiversity Exploratories, Mainz, Germany
2013	Annual Conference of the Association for General and Applied Microbiology (VAAM), Bremen, Germany
2013	International Proteomics Summer School in Greifswald, Germany
2013	2nd International Thünen Symposium on Soil Metagenomics, Workshop on Bioinformatic tools for soil microbiologists, Braunschweig, Germany
2016	Workshop on SEM modeling, Agroecology department, University of Göttingen

APPENDIX

LIST OF PUBLICATIONS

Wüst P, Nacke H, **Kaiser K**, Marhan S, Sikorski J, Kandeler E, *et al.* (2016). Estimates of the bacterial ribosome content and diversity in soils are significantly affected by different nucleic acid extraction methods. Appl Environ Microb 82:2595-2607.

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THESIS DECLARATIONS

Declaration of plagiarism

I hereby confirm that I have written this doctoral thesis independently. I have not used other sources or facilities others than the ones mentioned in the chapters. Moreover, I have not used unauthorized assistance and have not submitted this thesis previously in any form for another degree at any institution or university.

Kristin Kaiser
Göttingen, May 2016