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SPATIAL DISTRIBUTION AND FUNCTIONAL TRAITS OF MICROBIAL COMMUNITIES IN ARABLE SUBSOILS

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To Marco, my epic hero.

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ABBREVIATIONS

16S rRNA	Ribonucleic acid of the small prokaryotic ribosomal subunit
H_2O_d	Deionized water
H_2O_{dd}	Double-deionized water
AMF	Arbuscular mycorrhizal fungi
amoA	Ammonium monooxygenase gene
ANOVA	Analysis of variance
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
bp	Base pair
BSA	Bovine serum albumin
C_{org}	Organic carbon
C_T	Cycle threshold
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
L-DOPA	L-3,4-Dihydroxyphenylalanine
for	Forward
HSD	Honestly Significant Difference
MID	Multiplex identifier
n.d.	Not determined
NGS	Next-generation sequencing
nirK	Copper-dependent nitrite reductase gene
nirS	Cytochrome-dependent nitrite reductase gene
nosZ	Nitrous oxide reductase gene
N_{tot}	Total nitrogen
OTU	Operational taxonomic unit
PerMANOVA	Permutative multivariate analysis of variance
PCA	Principal component analysis
PCR	Polymerase chain reaction
PGP	Plant growth promotion
PGPB	Plant growth promoting bacteria
rev	Reverse
RNA	Ribonucleic acid
SIP	Stable isotope probing
SOC	Soil organic carbon
TC	Total carbon
TN	Total nitrogen
TRF	Terminal restriction fragment
Tris	Tris(hydroxymethyl)-aminomethan
qPCR	Quantitative real-time PCR
T-RFLP	Terminal restriction fragment length polymorphism

SUMMARY

Arable soils are an essential resource for food and crop production, and the role of soil microorganisms in nutrient cycling for plant growth and health is a field of active research. This thesis aims to characterize the spatial heterogeneity of subsoil microbial communities and their functional traits by examining the network of biopores that extends throughout the entire soil profile and which gives rise to “hotspots” of microbial activity in the drilosphere and rhizosphere.

We studied undisturbed subsoil, with its natural soil structure and biopore network intact, at an agriculturally managed site and in a climate chamber experiment, using excavated subsoil cores from the same field. In order to examine prokaryotic community composition, we applied fingerprint and next-generation sequencing techniques. Through the measurement of extracellular enzyme activities, quantitative DNA stable isotope probing, and quantitative real-time PCR for functional marker genes, we gathered data on the functional traits of microbes and their contributions to nutrient cycling.

Although there is a strong depth effect of reduced microbial biomass, activity, and changed community structure in bulk soil, a significantly less pronounced depth dependency has been found in the drilosphere and rhizosphere, because inputs of fresh organic matter retain their hotspot characteristics in subsoil. A key result was the spatial separation of prokaryotic phyla, which implied different life strategies as well. Bacteroidetes, Firmicutes, Proteobacteria, and to some degree Actinobacteria, which include many copiotrophic species, were predominantly found in the drilosphere, rhizosphere, and topsoil, while Acidobacteria, Gemmatimonadetes, and Nitrospirae, which mainly comprise oligotrophs, were favored in bulk soil and subsoil. We identified the main drivers for this pattern as high nutrient quantity and specific quality in the soil hotspots. Investigating extracellular enzymes, we found peroxidase to be very active in subsoil, and notably high hydrolytic enzyme activity e.g., phosphomonoesterase activity, in hotspots and in the subsoil rhizosphere of *Triticum aestivum*. DNA stable isotope probing analysis revealed that the bacteria actively utilizing plant-derived carbon in the rhizosphere changed along the soil profile. Especially in the deep rhizosphere, *Paenibacillus* (Firmicutes) and *Flavobacterium* (Bacteroidetes) appear to have key roles in carbon turnover. This highlights the importance of subsoil rhizosphere microorganisms for plant nutrition and health.

Clearly, it is worth digging deeper to unravel the complexity of soil microbial nutrient cycling and soil-microbe-plant interactions.

ZUSAMMENFASSUNG

Agrarböden sind eine unentbehrliche Ressource für die Nahrungsmittel- und Rohstoffproduktion, weswegen die Bedeutung der Bodenmikroorganismen für die Pflanzenernährung und -gesundheit ein aktueller Forschungsschwerpunkt ist. Diese Arbeit widmet sich der Charakterisierung der räumlichen Heterogenität von mikrobiellen Gemeinschaften im Unterboden sowie deren Funktionen. Hierfür wird das Netzwerk von Bioporen untersucht, welches sich über das gesamte Bodenprofil erstreckt und zu „Hotspots“ mikrobieller Aktivität – der Drilosphäre und der Rhizosphäre – führt.

Es wurde ein Unterboden einer agrarwirtschaftlich genutzten Fläche sowie ungestörte Unterbodensäulen desselben Standortes, deren natürliche Bodenstruktur und Bioporennetzwerk intakt blieben, in einem Klimakammerversuch untersucht. Um die prokaryotische Gemeinschaftsstruktur zu charakterisieren, wurden Fingerprint- und Next-Generation-Sequenzierungstechniken angewandt. Durch Messung extrazellulärer Enzymaktivitäten, quantitative stabile Isotopenmarkierung der DNA und quantitative Echtzeit-PCR an funktionellen Markergenen konnten Erkenntnisse über gewisse funktionelle Eigenschaften der Mikroorganismen und deren Bedeutung im Nährstoffkreislauf gewonnen werden.

Obwohl es einen starken Tiefeneffekt bezüglich verringerter mikrobieller Biomasse, Aktivität und veränderter Gemeinschaft im Unterboden gab, war diese Tiefenabhängigkeit in der Drilo- und Rhizosphäre weniger ausgeprägt. Dies lässt sich auf den Eintrag frischen organischen Materials in die Hotspots im Unterboden zurückführen. Eine wichtige Erkenntnis war die räumliche Trennung prokaryotischer Phyla, was auf deren unterschiedliche Lebensstrategien hinwies. Bacteroidetes, Firmicutes, Proteobacteria und einige Actinobacteria, die viele copiotrophe Arten umfassen, waren vornehmlich in der Drilosphäre, Rhizosphäre und im Oberboden abundant, wohingegen die vorwiegend oligotrophen Acidobacteria, Gemmatimonadetes und Nitrospirae vorzugsweise Bulkboden und Unterboden besiedelten. Die hohe Nährstoffmenge und besondere Nährstoffzusammensetzung in den Hotspots wurden als Ursache für diese räumlichen Ausprägungen identifiziert. Bei der Betrachtung extrazellulärer Enzymaktivität waren eine hohe Peroxidaseaktivität im Unterboden sowie hohe hydrolytische Enzymaktivitäten (z.B. Phosphomonoesterase) in den Hotspots und in der Unterbodenrhizosphäre von *Triticum aestivum* auffällig. Die stabile Isotopenmarkierung der DNA zeigte, dass entlang des Bodenprofils unterschiedliche Bakterien den pflanzenbürtigen Kohlenstoff in der Rhizosphäre nutzten. Gerade in der tiefen Rhizosphäre nahmen *Paenibacillus* (Firmicutes) und *Flavobacterium* (Bacteroidetes) eine Schlüsselrolle im Kohlenstoffumsatz ein. Dies unterstreicht die Bedeutung der Mikroorganismen in der Unterbodenrhizosphäre für die Pflanzenernährung und -gesundheit.

Tatsächlich lohnt es sich „tiefer zu graben“, um die die Komplexität des mikrobiellen Nährstoffkreislaufes im Boden und die Interaktionen von Mikroorganismen und Pflanzen aufzudecken.

List of Publications Included in This Study

Publication I (Pub I)

Fischer, D., **Uksa**, M., Tischler, W., Kautz, T., Köpke, U., and Schloter, M. (2013). Abundance of ammonia oxidizing microbes and denitrifiers in different soil horizons of an agricultural soil in relation to the cultivated crops. *Biol. Fertil. Soils* 49, 1243–1246. doi:10.1007/s00374-013-0812-8.

Publication II (Pub II)

Uksa, M., Fischer, D., Welzl, G., Kautz, T., Köpke, U., and Schloter, M. (2014). Community structure of prokaryotes and their functional potential in subsoils is more affected by spatial heterogeneity than by temporal variations. *Soil Biol. Biochem.* 75, 197–201. doi:10.1016/j.soilbio.2014.04.018.

Publication III (Pub III)

Uksa, M., Schloter, M., Kautz, T., Athmann, M., Köpke, U., and Fischer, D. (2015). Spatial variability of hydrolytic and oxidative potential enzyme activities in different subsoil compartments. *Biol. Fertil. Soils* 51, 517–521. doi:10.1007/s00374-015-0992-5.

Publication IV (Pub IV)

Uksa, M., Schloter, M., Endesfelder, D., Kublik, S., Engel, M., Kautz, T., et al. (2015). Prokaryotes in subsoil - evidence for a strong spatial separation of different phyla by analysing co-occurrence networks. *Front. Microbiol.* 6, Article 1269. doi:10.3389/fmicb.2015.01269.

Publication V (Pub V)

Uksa, M., Buegger, F., Gschwendtner, S., Lueders, T., Kublik, S., Kautz, T., et al. (2017). Bacteria utilizing plant-derived carbon in the rhizosphere of *Triticum aestivum* change in different depths of an arable soil. *Environ. Microbiol. Rep.* 9, 729–741. doi:10.1111/1758-2229.12588.

1. INTRODUCTION

Agricultural soils feed a growing world population and are an important provider of renewable resources. Unfortunately, soil pollution, soil erosion, global warming, and land consumption currently contribute both to a reduction in arable soils and in their quality. Therefore, efforts have been undertaken to investigate, understand, evaluate, and preserve this terrestrial ecosystem (Brady and Weil, 2008). Soil is one of the most complex, heterogeneous, and diverse environments on earth (Bardgett and van der Putten, 2014). It not only provides habitat for plants and soil animals but also harbors an abundant and diverse microbiome including bacteria, archaea, and fungi. Complex physical and biogeochemical processes, nutrient cycling, interactions between all soil organisms, and the importance of the soil microbiome for plant growth and crop production are subjects of numerous studies (Nannipieri et al., 2003; van der Heijden et al., 2008; Nannipieri, 2010). However, current studies are literally only “scratching the surface” of this ecosystem since most soil microbial research has focused on the upper centimeters of the soil profile. Below this topsoil horizon, the subsoil environment begins, and this work is intended to deepen our knowledge of this under-examined portion of the soil profile.

1.1 Subsoil

Subsoil research started by addressing physical, chemical, and hydrological soil properties and processes that lead to the development of observed soil profiles. In classical soil science (Bardgett, 2005; Brady and Weil, 2008; Blume et al., 2015), soil genesis is described as a slow process - up to 1000 years or more to form just 10 cm of soil. Depending on climatic conditions, parent rock material, and vegetation, site-specific depth profiles with horizons of characteristic physicochemical and biological properties are formed. Although soil profiles are highly diverse, a generalized separation of topsoil from subsoil horizons is possible:

The topsoil, often referred to as the A horizon, receives direct input of organic matter by plant litter, which is decomposed by soil animals and degraded, mineralized, and transformed by microorganisms. It is therefore rich in soil organic matter (SOM) and characterized by a dark color. In forest ecosystems, the A horizon is often covered by an O horizon, comprising mainly plant residues. In arable soil systems however, the A horizon is influenced by agricultural management practices such as tillage, which homogenizes the soil to the plow depth. Here, the topsoil can therefore be defined as the plow horizon, typically reaching down to about 30 cm under conventional tillage practices (Brady and Weil, 2008; Blume et al., 2015).

The subsoil is defined as the lower, generally humus-poor portion of the soil profile between topsoil and bedrock (Arbeitsgruppe Boden, 2006). “In arable farming systems, the term ‘subsoil’ refers to the soil beneath the tilled or formerly tilled soil horizon whereas the latter one is denoted as ‘topsoil’” (Kautz et al., 2013a). Subsoil can be subdivided into B and C horizons, the C horizon constituting the zone between the bedrock material and the B horizon. Vertical transport processes and leaching lead to depletion of compounds in the upper soil horizons and their translocation and accumulation into lower horizons (Lehmann and Schroth, 2003). Subsoils are clearly distinguishable from bedrock and aquifers.

The question of how and to what extent soil microbial communities contribute to soil development, nutrient cycling, and plant growth is a younger field of soil science (Paul, 2006; Rumpel and Kögel-Knabner, 2011; Kautz et al., 2013a; Jones et al., 2018) and was at first limited to topsoil studies. In recent decades, subsoils have received greater attention with respect to the nature of the subsoil microbial communities, as new methods in microbiology and molecular ecology have been established (Bastida et al., 2009; Pett-Ridge and Weber, 2012; Myrold and Nannipieri, 2014; Bouchez et al., 2016). The spatial distance of subsoils to the surface, soil management practices, litter deposition, and the fact that they have no direct exposure to weathering and are in closer proximity to the water table and to bedrock material results in characteristic ecosystems and ecological niches for microorganisms (Zvyagintsev, 1994; Madsen, 1995; Blume et al., 2002). So far, several studies have established a generalized picture of the contrast between topsoil and subsoil.

1) Subsoil is poor in SOM: Plant-derived C-fixation through photosynthesis, biomass production, and litter input (and the subsequent degradation of cellulose, lignin, and low molecular weight organic compounds) lead to a high SOM content in the topsoil and low in the subsoil (Lorenz and Lal, 2005). Batjes (1996) and Harrison et al. (2011) showed this was the case for different soil types at the global scale and estimated that the total C and N content between 30 cm and 100 cm soil depth was as high as that in the top 30 cm. Due to the thickness of subsoil horizons, they comprise globally about 1300 Pg carbon (Batjes, 1996).

2) Subsoil is reduced in organism abundances and microbial biomass: As a consequence of lower organic matter input into subsoil, the living biomass, *i.e.* bacteria, archaea, fungi and soil animals, is also lower (Van Gestel et al., 1992; Blume et al., 2002; Kramer et al., 2013; Müller et al., 2016; Pausch et al., 2018). Pausch et al. (2018) calculated that carbon stocks in the topsoil foodweb were three times higher than in the subsoil. Furthermore, plant root biomass and root length density rapidly decrease with soil depth (Taylor et al., 2002; Kautz et al., 2013a, 2013b).

3) Subsoil nutrient turnover rates and microbial activity are decreased: The combination of lower organic matter content and microbial biomass in subsoil leads to nutrient limitation to microbial growth and overall microbial activity. This was determined through studies measuring oxygen

respiration rates (Salome et al., 2010), decomposition rates of organic carbon sources by enzyme activities (Kramer et al., 2013; Stone et al., 2014), or incorporation rates of stable isotopes into molecular biomarkers (Blume et al., 2002; Fierer et al., 2003a, 2003c; Rumpel and Kögel-Knabner, 2011).

4) Microbial community composition is different in subsoils and their phylogenetic diversity is lower: The bacterial, archaeal, and fungal communities change with soil depth, as shown through classic cultivation, molecular fingerprint, and sequencing analyses (Watanabe et al., 2010; Eilers et al., 2012; Stroobants et al., 2014). Frequently, community structure analyses also suggest lower diversity of the microbiome in subsoil (Blume et al., 2002; Eilers et al., 2012; Lipson et al., 2013).

In summary, previous studies have drawn the picture of a reduced microbiome in subsoil with fewer functions and lower and/or delayed contributions to nutrient cycling. Aspects that are rarely considered in evaluations of the subsoil microbiome are the horizontal spatial variation of the soil, soil heterogeneity, and occurrence of microbial hotspots. As these aspects play a key role in this thesis, we will introduce heterogeneities in the next section and illustrate the most important ones in Figure 1.

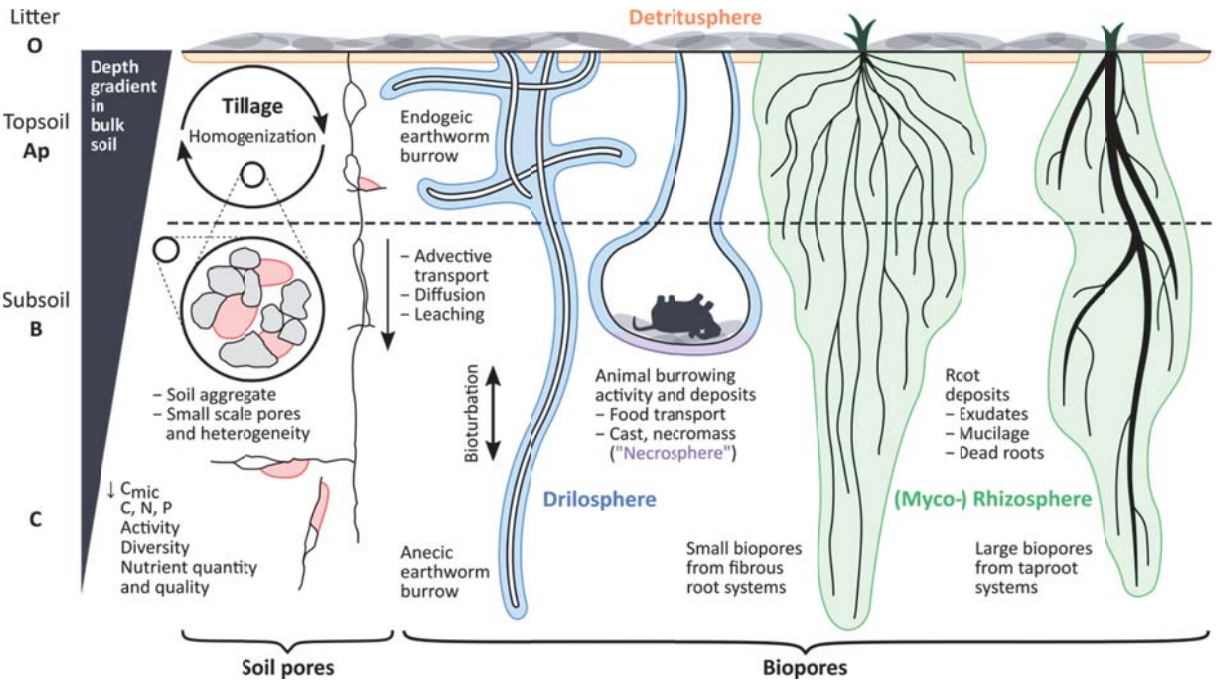

Figure 1: Soil (bio-)pores and microbial hotspots in topsoil and subsoil.

1.2 Spatial Soil Heterogeneity and Hotspots

1.2.1 Soil Heterogeneity and Biopores

Soil is one of the most heterogeneous habitats on earth (Nannipieri et al., 2003; Baker et al., 2009). Soil properties, microbial communities, and their functional traits are subject to spatial variations at different scales, from µm to km (Ettema and Wardle, 2002; Nunan et al., 2002). At the landscape and biome scale, parent material, climatic conditions, vegetation, and land use are major factors driving the development of different soil types and profiles with distinct soil properties (Brady and Weil, 2008). Global and landscape patterns of microbial communities investigated by several research groups highlighted pH as one of the major factors influencing large scale variability in microbial communities (Fierer and Jackson, 2006; Fierer et al., 2009; Bru et al., 2011).

On the µm to cm scale, water and nutrients are distributed through the subsoil via convection and diffusion, which, in turn, depend on soil texture, pore size distribution, and pore network connectivity (Brady and Weil, 2008; Blume et al., 2015). In soils with bigger pores or sandy soils, water and nutrients are rapidly leached through convective transport (Lehmann and Schroth, 2003). In contrast, smaller pores provide greater water holding capacity and clayey soils provide greater sorption capacity, thus water and nutrients are more easily retained in these soil and diffusion gains importance (Or et al., 2007). At the same time, the transport of oxygen is limited under water saturating conditions. Soils that are heterogeneous with respect to pore size and texture lead to patchy distributions of soil minerals, organic matter, and nutrients, providing diverse habitats for microbes (Ritz et al., 2004; Vos et al., 2013). Soil homogenization, *e.g.* via plowing, disturbs natural soil heterogeneity (Young and Ritz, 2000; Hobley et al., 2018).

Spatial patterns of microbial communities have been investigated at the small (µm to mm) scale (Nunan et al., 2001, 2002, 2003; Vos et al., 2013). The spatial heterogeneity of soil pores may lead to patches of considerably high microbial abundance and activity, referred to as “microbial hotspots” (Ettema and Wardle, 2002; McClain et al., 2003; Hagedorn and Bellamy, 2011; Kuzyakov and Blagodatskaya, 2015).

There are several groups of microbial hotspots that typically arise where water and/or nutrients are abundant (Kuzyakov and Blagodatskaya, 2015), cf. Figure 1. The most well-characterized microbial hotspot is the rhizosphere, the soil volume directly influenced by root activity and deposits and which can occur at various soil depths, discussed in section 1.2.3. The detritusphere is another important hotspot that develops at the interface between mineral soil and dead plant or animal residues. This includes both the thick litter layer (O horizon) at the surface and the dead roots and animals which can be found at arbitrary soil depths (Kögel-Knabner, 2002; Kuzyakov and Blagodatskaya, 2015).

Hotspots can also be found on aggregate surfaces and along soil cracks, which provide preferential water flow paths for leachates from upper soil horizons. Finally, animals transport and deposit food, soil, and feces in burrows, also giving rise to hotspots, for example the drilosphere around earthworm burrows, discussed in the following section 1.2.2.

Soil pores are mostly filled with water and air; thus are the living space for plant roots, soil animals, fungi, bacteria, and archaea. They can be roughly classified by their size as micropores (< 0.08 mm) and macropores (> 0.08 mm; (Brady and Weil, 2008)). Micropores are less prone to desiccation, whereas macropores are the first to dry out. The development and disappearance of soil pores depend on chemical and physical processes such as weathering, freeze-thawing, or drying; agricultural management practices such as tillage; and biological activity (Bronick and Lal, 2005; Blume et al., 2015). Activity of soil animals and growth of plant roots create macropores specifically referred to as “biopores” (Athmann et al., 2013).

1.2.2 Soil Hotspot – Drilosphere

Earthworms are the most well-known animals to form biopores, through their burrowing activities. In arable and grassland soil systems, biopore density can be highly variable among soil types and land uses, with from 50 to 700 biopores · m^{-2} (Don et al., 2008; Han et al., 2015a; Köpke et al., 2015). With respect to subsoil, categorization of earthworms with respect to their feeding ecology; anecic, endogeic, and epigeic species is useful (Curry and Schmidt, 2007; Pfiffner, 2014). Epigeic earthworm species, *e.g. Lumbricus rubellus*, are mainly found in compost and in the O horizon of soil feeding on plant litter; endogeic species, *e.g. Allolobophora calliginosa*, in topsoil creating a horizontal and shallow burrow network down to 30 cm; and anecic species, *e.g. Lumbricus terrestris*, found in the entire soil profile down to 4 m (Kautz et al., 2014; Pfiffner, 2014; Han et al., 2015a). The latter are therefore responsible for biopore formation in subsoils. With their bioturbation activity, earthworms significantly contribute to decomposition, soil aeration, and soil porosity (Devliegher and Verstraete, 1997; Blouin et al., 2013; Blume et al., 2015).

The lining of earthworm burrows that derives from cast deposition and repeated burrow utilization is a mantle up to about 3 mm thick, labeled the drilosphere (Brown et al., 2000; Don et al., 2008). It is darker in color compared to the surrounding bulk soil because of its enriched organic matter content, which is enriched in lignin-derived molecules and polysaccharides (Tiunov and Scheu, 1999; Andriuzzi et al., 2013; Vidal et al., 2016). Besides this, the drilosphere is characterized by its chemical composition and specific microbial communities (Tiunov and Dobrovolskaya, 2002; Aira et al., 2010; Stromberger et al., 2012). Due to observed high activities, *e.g.* of extracellular enzymes, it is considered a soil hotspot (Marhan et al., 2007; Don et al., 2008; Hoang et al., 2016). Both indigenous

and earthworm species-specific gut microbiomes shape the microbial community found in cast (Drake and Horn, 2007; Aira et al., 2010; Dallinger and Horn, 2014).

Occasionally, roots invade earthworm burrows, taking the path of least resistance for growth into deeper soil horizons (Kautz et al., 2013b). Moreover, roots benefit from exchanging water, oxygen, and nutrients at the drilosphere-burrow-interface. The interference with biopores by earthworms or plant roots gives rise to further heterogeneities (Athmann et al., 2013).

1.2.3 Soil Hotspot – Rhizosphere

When plant roots grow into the solid soil matrix, they simultaneously form pores. Root systems have varying soil penetration abilities depending on the plant species (Löfkvist et al., 2005) and can be classified according to their morphology. A "taproot system" is characterized by one thick root which normally grows vertically into the soil. From this taproot, thinner lateral roots branch off. Examples of plants with taproots are *Cichorium intybus* and *Medicago sativa*. The other typical root system, called "fibrous root system", is widely distributed among grasses; *e.g.*, *Festuca arundinacea*. There is no dominant root, but "equally ranked" roots grow from the stem, fiber-like into the soil. Differing root growth behaviors result in differently shaped biopores; those derived from taproots have wider cross sections than the biopores derived from thin roots (Perkons et al., 2014).

At the interface between root surface and soil matrix, a microhabitat is formed that is referred to as the rhizosphere. First mentioned by Hiltner (1904), the rhizosphere is one of the most intensively studied habitats; it is where microorganisms interact with each other, with the soil matrix, and with plant roots in a complex network (Bais et al., 2006; Berendsen et al., 2012; Lareen et al., 2016). Plant roots release exudates that comprise diverse organic compounds including, among others, high-molecular weight polysaccharides and proteins, low-molecular weight compounds such as amino acids, organic acids, sugars, and other secondary metabolites, and also protons (Lynch and Whipps, 1990; Haichar et al., 2014). Therefore, root exudates fulfil diverse functions as they influence water movement through the production of mucilage (Ahmed et al., 2014), increase nutrient availability and phosphorous solubilization through pH change (Jones et al., 2004), and select for specific microbes as a kind of biocontrol (Kamilova et al., 2005). For microorganisms, root exudates serve as easily available C sources (Grayston et al., 1997).

The quantity and quality of root exudates depends on several factors; *e.g.*, seasonal temperature and moisture regime, soil type and properties, plant species, physiological and developmental status of the plant and its roots (Marschner et al., 2001; Jones et al., 2004; Bais et al., 2006; Buée et al., 2009; Haichar et al., 2014; Neumann et al., 2014). During plant development, the highest exudation rates occur during early vegetative growth and again at the bolting phase until flowering, when the root

systems are experiencing maximum growth (Chaparro et al., 2013). In general, the highest exudation rates are localized at the root tips and in the root hair zones (McCully and Canny, 1985; Nguyen, 2003; Haichar et al., 2014). A plant can release up to 70% of its photosynthetically fixed carbon in the form of root exudates (Lynch and Whipps, 1990).

Besides soil type, the plant species with its distinct root exudates is a major contributing factor to the specific microbial community composition in the rhizosphere (Costa et al., 2006; Garbeva et al., 2008; Berg and Smalla, 2009; Hartmann et al., 2009; Chaparro et al., 2014). The influence of plant roots and their exudates on the surrounding soil (Hinsinger et al., 2009) and both the abundance and composition of soil organisms is called the “rhizosphere effect” (Hiltner, 1904). This term refers to the increase in nutrient quantity in the rhizosphere which in turn leads to enhanced growth and activity of a specific microbial community, The rhizosphere effect is highest within the first 2 mm from the root but diminishes with increasing distance from the root surface (Hinsinger, 1998).

In the rhizosphere microbial community, a network of competitive, pathogenic, and mutualistic interactions forms (Bais et al., 2006; Buée et al., 2009). Microbes compete with each other and with the plant for nitrogen and phosphorous compounds, which are the typical growth limiting macronutrients in this habitat (Schimel and Bennett, 2004; Kuzyakov and Xu, 2013). Furthermore, the utilization of root exudates as the carbon source leads to antagonistic interactions between microbes. In the rhizosphere, bacterial or fungal pathogens pose a huge threat to plant health and productivity (Berg et al., 2002, 2014). In this respect, mutualistic interactions between microbes and a plant can counteract pathogens or abiotic factors that would negatively influence the plant. Therefore, the term plant growth promotion (PGP) was introduced (Kloepper et al., 1980; Bashan and Holguin, 1998). Bacteria that provide such positive effects on plants are therefore called plant growth promoting bacteria (PGPB). The effect of PGP is, for example, to reduce the risk of infection by a plant pathogen due to antagonistic or competitive interactions of a PGPB with this pathogen; this is known as “biocontrol” (Kamilova et al., 2005). Biocontrol can be exercised actively or passively. Microbes can actively produce secondary metabolites, such as antibiotics, which inhibit the growth or activity of a pathogen. Passive biocontrol is manifested through greater competitiveness for nutrients by the PGPB than by the pathogen (Whipps, 2001; Berg et al., 2005). In a broader sense, PGP also includes the enhancement of plant growth by better N and P acquisition from soil or phytohormone stimulation (van der Heijden et al., 2008; Berg, 2009; Richardson et al., 2009; Chaparro et al., 2012; Sharma et al., 2013). For example, microbes can solubilize or mineralize phosphorus through production of organic acids or extracellular phosphatases. The microbes in turn benefit from the carbon released from plants in the form of root exudates.

PGP typically does not include symbiotic interactions between plants and microbes (Kloepper et al., 1989). The two most prominent examples of symbiosis – diazotrophy and mycorrhization – are briefly

described here. Plant growth is often limited by the availability of N in the forms of nitrate or ammonia. Diazotrophic bacteria are able to break the bonds of molecular dinitrogen gas, resulting in the formation of ammonia. This process is known as biological nitrogen fixation (BNF). Diazotrophs in symbiosis with plants form nodules where the oxygen-sensitive nitrogen fixation takes place. A typical nodule forming bacterium is *Rhizobium* (Wall and Favelukes, 1991). As a result, plants in these symbiotic relationships are less dependent on soil-available ammonia. As plants of the family *Leguminosaceae* (legumes; *e.g.*, *Medicago sativa*) mainly form this symbiosis, crop rotations with legumes are included to reduce nitrogen loss in agricultural systems (Peoples and Craswell, 1992). Although the symbiotic diazotrophs are intensively studied because of their importance for plant N nutrition, a significant fraction of diazotrophs in soil are free-living. They carry out BNF in soil or the rhizosphere without symbiosis with a plant; *e.*g., *Paenibacillus*, *Bacillus*, and *Cohnella* (Mavingui et al., 1992; Rosado et al., 1996; Behrendt et al., 2010; Hayat et al., 2010; Wang et al., 2012). Thus, free-living diazotrophs can provide another path to PGP.

Mycorrhizal fungi form a close symbiotic interaction with plant roots. The mycorrhizae obtain carbon sources in exchange for the nutrients phosphorous and nitrogen, and they also enhance water availability to plants, which the wide mycelium network efficiently gathers from the soil (Paul, 2006; Blume et al., 2015). In agro-ecosystems, arbuscular mycorrhizal fungi (AMF), which often are found in association with grasses and herbs, are the most important mycorrhizae for crop, especially cereal, production.

High-throughput sequencing, metagenomics, metatranscriptomics, metaproteomics, and metabolomics are methodological milestones that have expanded our investigation of the rhizosphere and the interactions between plant roots and microorganisms, providing us with a deeper understanding of the microbial diversity and complex metabolic pathways and regulation mechanisms involved (Schneider et al., 2012; Peiffer et al., 2013; Turner et al., 2013; van Dam and Bouwmeester, 2016; White et al., 2017).

1.3 Temporal Dynamics of Soil Microorganisms

Microorganisms are subject to seasonal changes (Van Gestel et al., 1992; Fuka et al., 2009; Lauber et al., 2013; Regan et al., 2014; Žifčáková et al., 2016) and disturbance effects (Fierer et al., 2003b; Evans and Wallenstein, 2014): In temperate climate zones, temperature and moisture regimes change throughout the year. Microorganisms are, in general, more active at higher temperatures and greater moisture (Nannipieri et al., 2012; Ramirez et al., 2012). Likewise, enzyme activities are sensitive to temperature (Ali et al., 2015). In cold and dry winters, lower microbial activity and growth can be

expected. However, high denitrification rates and N_2O losses can occur in winters with intense freeze-thaw cycles (Kaiser et al., 1998).

Temperature and moisture are not the only drivers of seasonal changes in microbial activity. Freezing may cause cell disruption, and dry periods increase osmotic stress and reduce overall microbial activity (Fierer et al., 2003b; Schimel et al., 2007). As a result, psychrophilic/ mesophilic/thermophilic microbes, which are adapted to cold/moderate/hot temperatures, are preferentially active under their respective preferred conditions. Dormant states and spore formation are strategies for survival during dry and very cold/hot periods. For example, Firmicutes are known to survive extreme conditions by forming endospores (Fenner et al., 2005).

In ecology, resistance is the ability of a microbial community to withstand a disturbance event; resilience is the capacity of microbes to return to their original state after a disturbance has occurred (Allison and Martiny, 2008). With respect to the temporal dynamics of a microbial community, both resistance and resilience mechanisms happen at the same time; *e.g.*, after drying and rewetting cycles, or after exposure to pollutants (Bardgett and van der Putten, 2014; Kaurin et al., 2018).

In agricultural soils, numerous additional disturbance events, such as tillage, seeding, mowing, harvest, crop rotation, and the application of pesticides or fertilizers, affect the microbial community on different time scales (Lovell et al., 1995; Fuka et al., 2009; Lauber et al., 2013). Events that trigger an increase in microbial activity are, for example, rewetting and fertilization. Such time bound phases of high microbial activity are referred to as “hot moments” (McClain et al., 2003; Hagedorn and Bellamy, 2011; Kuzyakov and Blagodatskaya, 2015). For example, N_2O emission from soil was found to peak immediately after manure fertilization (Molodovskaya et al., 2012).

1.4 Nutrient Cycling and Life Strategies of Soil Microbial Communities

Fungi, bacteria, and archaea are essential key members of the nutrient cycling community, creating, together with plants and soil animals, a complex soil food web (Paul, 2006). Soil heterogeneity and nutrient hotspots, which result in habitats of differing nutrient quality and quantity, provide physical and ecological niches for soil organisms (Scharroba et al., 2012; Rabot et al., 2018). In this respect, microbes pursue different life strategies according to available metabolic pathways, using diverse nutrient sources for growth and energy conservation (Eitinger et al., 2007).

This chapter summarizes soil microbial nutrient cycles and reviews essential functional groups together with their phylogenetic distributions.

1.4.1 Soil Carbon-Cycle

All organisms rely on carbon for growth. Through photoautotrophic C-fixation of CO_2, plants are the main carbon source in terrestrial ecosystems. Some microbial groups also have the ability to perform autotrophic C-fixation. Biomass produced by plants is deposited into the soil either through root exudates and debris, so-called "rhizodeposition", or by plant material itself; *e.g.*, dead wood and leaf litter. In addition, animal and microbial residues contribute to soil organic matter input. Such residues are composed of polymeric compounds (*e.g.*, cellulose, lignin, and chitin, but also proteins) that are decomposed, degraded, and mineralized by soil animals, such as earthworms, as well as soil and gut microbes (Paul, 2006).

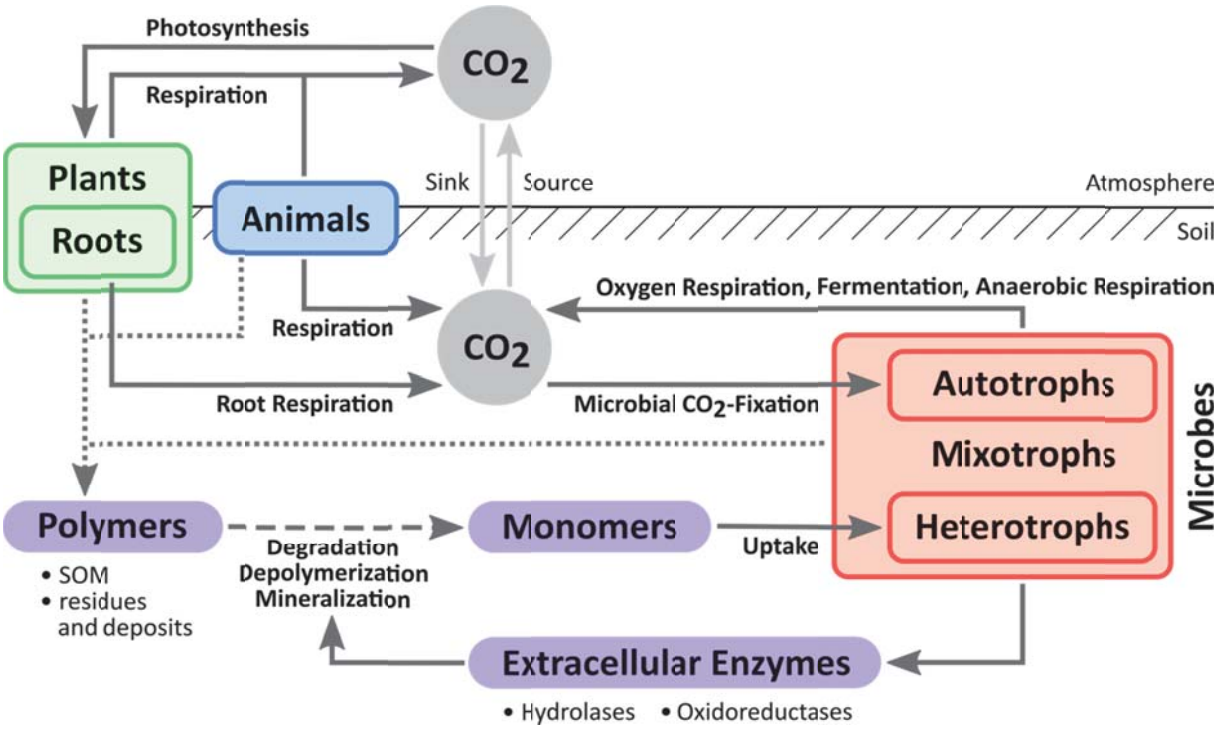

Figure 2: Organic matter degradation by soil microorganisms as part of the soil carbon cycle.

CH_4 transformation processes are not included. Examples for following compounds and enzyme classes are listed: Polymers – cellulose, hemicellulose, chitin, lignin, nucleic acids, proteins, peptides, lipids; Monomers – pentoses, hexoses, ortho-phosphate, amino acids, fatty acids; Hydrolases – β-glucosidase, cellobiohydrolase, xylosidase, chitinase, phosphomonoesterase, peptidases; Oxidoreductases – peroxidases, phenoloxidases.

In order to decompose organic matter, soil organisms produce extracellular enzymes that depolymerize organic matter into oligomers and monomers containing C, N, and P, which can be taken up directly (Nannipieri et al., 2002; Caldwell, 2005), cf. Figure 2. Cellulose is hydrolytically

degraded by brown-rot fungi and different bacteria by different enzymes: endocellulases, exocellulases, cellobiohydrolase, and β-glucosidase. Hemicellulose is cleaved by xylosidase. Lignin, in comparison to cellulose, is a rather complex and, thus, slowly degradable polymer. Its mineralization requires oxidative enzymes with radical reaction mechanisms (*e.g.*, peroxidases, phenoloxidases, and laccase) that are predominantly produced by white-rot fungi (Enoki et al., 1988). Chitin derived from fungi or animals serves as both a C and an N source and is cleaved by chitinase. Further extracellular enzymes may be produced to derive N from organic compounds; *e.g.*, proteases, peptitases, and nucleases. Phosphatases (*e.g.*, phosphomonoesterases) are released obtain P, which is often the limiting macronutrient in agroecosystems (Vitousek et al., 2010). While acid phosphomonoesterases are produced by bacteria, fungi, and plant roots, alkaline phosphomonoesterases are released only by microbes (Nannipieri et al., 2011).

Microbes take up the products of extracellular enzyme activity in order to grow and reproduce. This utilization of organic carbon substrates for growth is described as the heterotrophic life strategy.

To complete the carbon cycle, CO_2 is again released in the process of oxygen respiration, which is the common energy conservation pathway among soil microbes (Figure 2).

Soil carbon cycling is characterized by complex food web interactions between bacteria, fungi, archaea, animals, and plants. Cross-feeding between microbes is a common phenomenon (Ponomarova and Patil, 2015; Kramer et al., 2016); microbes can utilize debris or metabolites from other microbes leading to recycling of carbon compounds in soil. Nevertheless, turnover rates of carbon in soil can vary from hours to years (Kuzyakov and Gavrichkova, 2010; Rumpel and Kögel-Knabner, 2011) leading to rapid carbon loss or recalcitrant SOM pools.

For completeness, it should be mentioned that methanotrophic bacteria can exploit CH_4 as energy and carbon source, and methanogenic archaea can produce CH_4 by utilizing CO_2 as an alternative electron acceptor for anaerobic respiration (Le Mer and Roger, 2001; Serrano-Silva et al., 2014). In conclusion, soils are either CO_2/CH_4 sources or sinks and therefore especially important in global carbon cycling and climate change (Bardgett et al., 2008).

1.4.2 Soil Nitrogen-Cycle

Nitrogen is an essential macronutrient for microbial growth as it is a component of proteins and nucleic acids. It is taken up by microbes either as organic N, in the form of amino acids, or as nitrate and ammonia. Proteases and nucleases exploit and mineralize organic N compounds, so are important microbial extracellular enzymes (Schimel and Bennett, 2004). This process is called ammonification, see Figure 3. When microbes or plants take up ammonia or nitrate for biomass production, it is termed nitrogen assimilation (Nannipieri and Eldor, 2009).

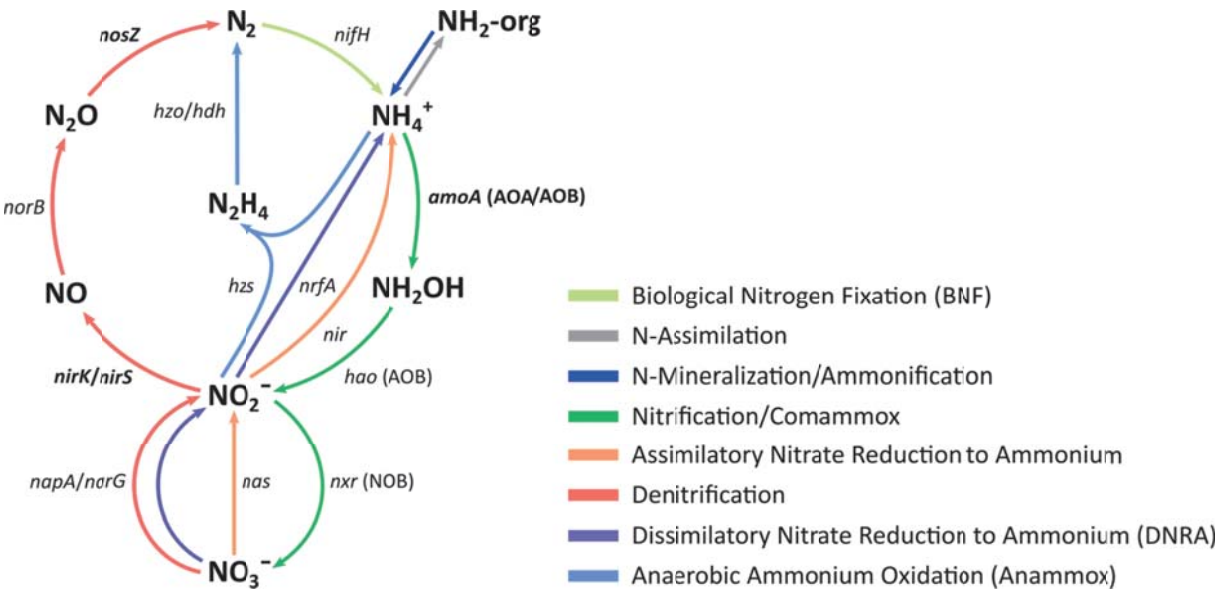

Figure 3: Soil inorganic nitrogen cycle. Arrows are labeled with enzyme-encoding genes.

AOA – Ammonia oxidizing archaea; AOB – Ammonia oxidizing bacteria; NOB – Nitrite oxidizing bacteria; Comammox – Commplete ammonium oxidation to nitrate.

A special process that makes possible the assimilation of nitrogen is BNF, which we described in the context of legumes. Dinitrogen fixation into ammonia is known only in prokaryotes and requires the enzyme nitrogenase (Eitinger et al., 2007), an oxygen sensitive enzyme found in autotrophs and heterotrophs. In addition to symbiotic representatives of α-Proteobacteria and Actinobacteria (*Rhizobium*, *Bradyrhizobium*, *Frankia*), free-living aerobic (*Azotobacter*, *Bradyrhizobium Burkholderia*, *Cyanobacterium*, *Gluconacetobacter*, *Rhizobium*) and anaerobic (*Clostridium*) isolates have been characterized (Reineke and Schönmann, 2007; Hayat et al., 2010; Reed et al., 2011; Fischer et al., 2012). The capacity for diazotrophy is an advantage in soils that are poor in nitrogen or vulnerable to nitrogen leaching.

In soil, we refer to the chemolithotrophic process of ammonia oxidation to nitrate as nitrification (Ward, 2013). It is an oxygen dependent two-step process (Hooper et al., 1997):

In the first step, ammonia is oxidized to hydroxylamine by ammonia monooxygenase encoded by the *amoA* gene (Kowalchuk and Stephen, 2001; Arp et al., 2007; Stahl and de la Torre, 2012), cf. Figure 3. Subsequently, hydroxylamine is oxidized to nitrite with the hydroxylamine dehydrogenase (*hao* gene). Interestingly, ammonia oxidizing archaea (AOA) only harbor the *amoA* gene, while ammonia oxidizing bacteria (AOB) have both *amoA* and *hao* genes (Hallam et al., 2006; Arp et al., 2007). The lack of hydroxylamine oxidoreductase in AOA possibly inhibits their activity at higher concentrations of hydroxylamine (Wu et al., 2012). AOA belong to the phylum Thaumarchaeota (*Nitrososphaera*) and

their contribution to nitrification was recently discovered (Schleper et al., 2005; Treusch et al., 2005; Tourna et al., 2011). AOB are found in β- and γ-Proteobacteria (*Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*) (Arp et al., 2007) and, to date, additionally in some Nitrospirae strains (Daims et al., 2015).

The second step of nitrification is carried out by nitrite oxidizing bacteria (NOB) through which the oxidation to nitrate is catalyzed by nitrite oxidoreductase (*nxr* gene). NOB are widely distributed at the phylum level in α-, β-, and γ-Proteobacteria (*Nitrobacter*, *Nitrospina*, *Nitrococcus*) and Nitrospirae (*Nitrospira*) (Ehrich et al., 1995; Poly et al., 2008; Lücker et al., 2010). Recently, it has been reported that complete ammonia oxidation to nitrate (comammox) can be carried out by one organism: *Nitrospira* (Pinto et al., 2015). Nitrification is commonly coupled to autotrophic carbon fixation (Xia et al., 2011), though AOA can grow mixotrophically using inorganic and organic carbon sources (Hallam et al., 2006; Jia and Conrad, 2009; Tourna et al., 2011).

In agricultural systems, fertilization with ammonia promotes nitrification and, because nitrite and nitrate sorption to the soil matrix is low, the soil is prone to leaching, resulting in nitrogen loss.

A quantitatively more severe nitrogen loss from soils is caused by denitrification. It is an anaerobic catabolic pathway, in which nitrate, serving as a terminal electron acceptor, is reduced to dinitrogen gas in a stepwise process (Zumft, 1997): Nitrate reductase (*napA*/*narG* gene) reduces nitrate to nitrite; nitrite reductase (*nirK*/*nirS* gene) catalyzes its reduction to nitric oxide; nitric oxide reductase (*nor* gene) reduces the nitric oxide to nitrous oxide; nitrous oxide reductase (*nosZ* gene) finally reduces nitrous oxide to dinitrogen gas, cf. Figure 3. The gaseous compounds, N_2O and N_2 can escape from the soil, with the former contributing to the global greenhouse effect. Denitrifiers are facultative anaerobes and phylogenetically widely distributed among microbes; *e.g.*, Archaea (*Pyrobaculum*), Proteobacteria (*Bradyrhizobium*, *Pseudomonas*, *Paracoccus*), Bacteroidetes (*Cytophaga*, *Flavobacterium*), Firmicutes (*Bacillus*), and even fungi (*Fusarium*) (Philippot, 2002; Shoun et al., 2012). Nitrate respiration provides nearly as much energy as oxygen respiration and is therefore an attractive alternative when oxygen is limited or nitrate plentiful.

Denitrifiers cannot necessarily exploit the complete denitrification pathway due to their lack of specific functional genes (Zumft, 1997; Philippot, 2002). Two types of nitrite reductases exist; however, they have only been determined to occur at the same time in one genome and only in a few cases (Jang et al., 2018): the copper-dependent nitrite reductase (Cu-Nir encoded by *nirK*) and the cytochrome-dependent enzyme (cd_1-Nir encoded by *nirS*) (Zumft, 1997; Priemé et al., 2002; Sharma et al., 2005).

Two other processes in the nitrogen cycle are used to obtain energy via anaerobic respiration; dissimilatory nitrate reduction to ammonia (DNRA), and anaerobic ammonia oxidation (anammox).

Their contributions to N-cycling in oxic, arable soils is still an area of active research (Schmidt et al., 2011; Thamdrup, 2012).

1.4.3 The Concept of Oligo- and Copiotrophic Life Strategies

As described above, soil provides spatial and temporal niches for microbial activity, but a suite of eco-physiologically meaningful functional groups is needed for complete nutrient cycling (Torsvik and Øvreås, 2002; Bardgett and van der Putten, 2014). An early eco-physiological grouping of organisms was done for plants and animals and led to the concept of r- and K-selection of the population with respect to their growth dynamics in variable or stable environments (Losos and Ricklefs, 1967; Grime, 1977; Taylor et al., 1990).

Microorganisms can accordingly be categorized into copio- and oligotrophs with respect to their growth rates and competitiveness under differing nutrient availabilities (Paul, 2006): Copiotrophic microbes react to the presence of highly available and easily accessible nutrients with rapid growth and replication (Hu et al., 1999). Due to their high nutrient uptake and carbon use efficiency, they are highly competitive against oligotrophs. For the same reason, however, their community size can undergo large temporal fluctuations. Oligotrophic microbes, in contrast, can retain a fairly stable community size at conditions of low nutrient quantity and quality including, among others, chemically stable, complex, and recalcitrant organic compounds. The energy invested in producing extracellular enzymes to access these compounds reduces their growth rate. With a focus on cell maintenance, however, they can withstand starvation periods. Furthermore, autotrophy, the ability to utilize inorganic carbon sources for growth, and auxotrophy, the inability to synthesize a necessary organic compound required for growth, are widespread metabolic characteristics among oligotrophs (Fierer, 2017).

The maximum growth rate of a microbe under conditions of nutrient excess has been found to be correlated with the number of ribosomal RNA operon copies in its genome (Klappenbach et al., 2000; Gyorfy et al., 2015). The production of proteins for growth and metabolism is limited by the number of ribosomes, whose production, in turn, depends on the availability of rRNA transcripts. A parallel transcription from several rRNA operon copies accelerates this process chain. Typically, copiotrophs have more rRNA operon copies per genome than oligotrophs (Gyorfy et al., 2015).

Nonetheless, a strict categorization into oligo- and copiotrophs is often not applicable, because further physiological traits within a microbial strain (*e.g.*, secondary metabolites such as antibiotics, or filamentous growth) can provide competitive advantages in certain circumstances or environments.

1.4.4 Functional Diversity and Phylogeny

Soils exhibit enormous phylogenetic microbial diversity; ubiquitous and abundant phyla are Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, Gemmatimonadetes, and Planctomycetes. Fierer et al. (2007) identified a strong correlation between carbon content and the relative abundance of bacteria at the phylum level, which led to an ecological classification of bacterial phyla into oligo- and copiotrophs. Typical oligotrophic phyla are Acidobacteria, Verrucomicrobia, Gemmatimonadetes, and Planctomycetes (Kuske et al., 1997; Sangwan et al., 2005; Jones et al., 2009; Ward et al., 2009; Bergmann et al., 2011; DeBruyn et al., 2011).

It became evident, however, that there is no one-to-one mapping between phylogeny and physiology of microbes (Torsvik and Øvreås, 2002). For example, denitrification and cellulose degradation are found in several branches of the phylogenetic tree while microbes of the same monophyletic group do not necessarily share the same metabolic pathways. From an evolutionary perspective, functional traits might be gained, lost, or transferred to phylogenetically unrelated groups by horizontal gene transfer (Lynd et al., 2002; Jones et al., 2008; de Vries et al., 2015). We can use marker genes to distinguish between phylogenetic origins (*e.g.*, by 16S rRNA) and functional traits (*e.g.*, by the nitrite reductase gene *nir*).

In this respect, we should also acknowledge methodological advances in molecular soil microbiology in the era of “omics” methods (Myrold and Nannipieri, 2014). Starting with high-throughput sequencing, also known as next-generation sequencing, it became possible to simultaneously detect and classify diverse soil microbial communities on the basis of PCR amplicons of 16S/18S rRNA and other marker genes. Targeted sequencing reveals the phylogeny, while the metagenomic approach, also known as shotgun sequencing, is a powerful tool for investigating functional potential and putative microbial traits at the DNA level. Additional omics methods are available and suitable for different tasks: metatranscriptomics for targeting active microbes and their gene expressions, metaproteomics for detecting the presence of proteins and enzymes associated with specific pathways, and metabolomics for unraveling the actual metabolites, which may provide the best evidence of actual turnover and metabolic pathways in soil (von Bergen et al., 2013; Johnson-Rollings et al., 2014; Hultman et al., 2015; Swenson et al., 2015). All of the above mentioned methods can also be used to identify new organisms and/or new metabolic pathways (Myrold and Nannipieri, 2014).

The interactions of a phylogenetically and functionally diverse microbial community add to soil complexity, and this work is meant to shine some light on these interactions.

1.5 Objectives of the Studies and Hypotheses

Microbial communities in arable subsoil and how they contribute to nutrient cycling and plant growth were studied within the frame of the **DFG Research Unit 1320** "Crop sequence and nutrient acquisition from the subsoil", **subproject 5** "Microbial community structure and function in different habitats of subsoils and their role in nutrient mobilization and plant growth".
The aims of this thesis are to describe and evaluate both spatial heterogeneity and temporal dynamics of the composition, functional traits, and interactions of the microbial community in subsoil and to compare these findings both to the topsoil and in relation to different plant species. The scale of soil heterogeneity investigated here is based on vertical biopores that derive from root growth or earthworm activity, thus linking topsoil and subsoil. The soil compartments created by biopores, drilosphere and rhizosphere, are the focus of these studies. We consider an arable soil system, where the performance of the microbial community is key to plant nutrition and crop productivity. Functional traits of high interest are major nutrient turnover processes, including nitrification and denitrification, and the degradation of cellulose, lignin, chitin, and organic phosphates, as well as utilization of rhizodeposits. Furthermore, we explore the diversity and composition of prokaryotes and their co-occurrence in order to gain insight into the microbial network and interactions within it. In the following, the main hypotheses of this thesis are described in detail.

Soil physicochemical and biological properties change throughout the soil profile, which we refer to as the depth effect. Biopores run vertically from the top- to the subsoil, providing pathways for preferential water and nutrient flow, as well as for direct deposition of fresh organic material into nutrient depleted subsoil horizons. Together with root and animal activity, unique habitats, *i.e.* drilosphere and rhizosphere, are developed along biopores and are hotspots of microbial activity. With these assumptions we formulated the hypothesis:

H1 The differences between bulk soil and hotspots (drilosphere and rhizosphere) in microbial communities and their functional traits are more highly developed in subsoil than in topsoil or, in other words, the soil depth effect is more pronounced in bulk soil than in microbial hotspots that developed in biopores.

We examined this hypothesis with respect to prokaryotic abundance, community composition, potential for nitrification and denitrification, and extracellular enzyme activities. For the prokaryotic community, we expected a greater overlap – or core microbiome – among topsoil compartments as compared to subsoil compartments (PUB II, III, IV, V).

The rhizosphere hotspot received special attention. Although both plant species and soil type contribute, we hypothesize that **(H1a)** soil depth has less influence on the rhizosphere bacterial community composition than does plant species (PUB II). However, due to plant and root development-dependent changes in exudation quantity and quality, we anticipated a **(H1b)** different activity pattern of rhizosphere bacteria utilizing plant-derived carbon at different soil depths (PUB V).

Given the differences in nutrient quantity and quality between bulk soil, drilosphere, and rhizosphere, and also between top- and subsoil, specific eco-physiological niches develop. We therefore propose:

H2 Autotrophic and oligotrophic life strategies are more likely to succeed in bulk subsoil, whereas copiotrophic organisms favor microbial hotspots and topsoil.

In more detail, we expected to find a spatial separation in prokaryotic community composition at the phylum level, because not all microbial traits are phylogenetically widespread. **(H2a)** Bacteroidetes, Firmicutes, and Proteobacteria are supposedly more abundant in soil hotspots, whereas Acidobacteria, Gemmatimonadetes, Nitrospirae, and Thaumarchaeota are more abundant in subsoil and bulk soil (PUB IV). Since strains of Actinobacteria and Firmicutes can grow rapidly under nutrient rich conditions, both phyla are expected to favor hotspots. With their ability to form spores, they also have an advantage in nutrient poor subsoils (PUB V).

We anticipated a different spatial pattern in extracellular enzyme classes produced by microbes: **(H2b)** Hydrolytic enzymes, which are involved in mineralization of organic matter, exhibit greater activity in topsoil and hotspots as a result of frequent nutrient deposition by earthworms, roots, and litter. In contrast, in oligotrophic bulk subsoil, oxidative enzymes, which can degrade complex or recalcitrant soil organic material, were expected to be more active (PUB III).

(H2c) Nitrification and denitrification potentials were expected to show different spatial patterns in response to both diverging N and C availabilities and differences in the redox potential of subsoil or hotspots compared to top- or bulk soil. Because nitrification is a lithotrophic process and many nitrifiers are, in addition, autotrophs/mixotrophs, they are hypothesized to be more abundant in nutrient poor environments such as bulk soil and subsoil in order to avoid competition with rapidly growing chemoheterotrophs. Denitrifiers were hypothesized to be more abundant in nutrient rich soil compartments (PUB II). In particular, nitrification and denitrification potentials are supposedly higher in the subsoil of sites where legumes grow due to increased N availability compared to sites with non-legumes (PUB I).

H3 Temporal dynamics within the microbial community and their functional traits are less pronounced in bulk subsoil than in topsoil, and, irrespective of depth, higher in the rhizosphere than in non-rhizosphere soil (PUB II).

Since climatic and anthropogenic disturbances are assumed to be delayed or mitigated before reaching the subsoil, a temporally stable bacterial community structure and nitrification/denitrification potential was expected in bulk subsoil comparted to topsoil. However, due to plant and root development-dependent changes in exudation quantity and quality, we expected greater temporal variability in bacterial community composition in the rhizosphere than any other soil compartment.

In order to support (or reject) these hypotheses, we investigated subsoils *in situ* both in a field experiment and in a controlled climate chamber using undisturbed subsoil cores from the same field site. The application of soil biological and soil molecular methods and the setup of the experiments are explained in detail in the next chapter. Chapter 3 lists and summarizes the original research articles published in the frame of the DFG Research Unit 1320. We discuss the results and draw overall conclusions in the last chapter.

2. MATERIAL AND METHODS

This section cites, summarizes and extends the methodological descriptions given in the PUBLICATIONS I-V.

2.1 Subsoil Field Experiment

Subsoil microbial communities were investigated in soil samples that were directly sampled in the field, where natural soil structure, biopore formation and plant/root development are present.

2.1.1 Study Site, Soil Characteristics, and Experiments

The study site for the field trial of this project is located at Campus Klein-Altendorf near Bonn in Nordrhein-Westfalen (Germany; 50° 37′ 21′′ N, 6° 59′ 29′′ E; Figure 4). At 150 m a.s.l, the field has a low slope gradient of 0.5-1°. According to the Köppen-Geiger classification system (Kottek et al., 2006), the area of the study site is classified as warm temperate fully humid climate with warm summer (Cfb) with a mean annual temperature of 9.6°C and an annual precipitation of 662 mm. Detailed meteorological information from the weather station at Klein-Altendorf are available since 2005 at Agrarmeteorologie Rheinland-Pfalz (www.wetter.rlp.de) and summarized in Table 2 for the sampling time points.

The soil is classified as Haplic Luvisol (Hypereutric, Siltic; Gaiser et al., 2012; IUSS Working Group WRB, 2015) that derived from loess sediments and is characterized by clay accumulation in subsoil. Previous studies determined major soil properties on the field site at different depth layers (Kautz et al., 2014; Table 1): The Ap horizon (0-27 cm) contains the highest organic carbon and nitrogen content along the soil profile and is designated as “topsoil” in this studies. Yearly conventional tillage practice on the field site forwarded its segregation from deeper soil layers. “Subsoil” depths where assigned to the Bt horizons from 41-115 cm, where the clay content is enriched during pedogenesis. Below 1.2 m soil depth, the inorganic carbon content increases rapidly being derived from carbonate rich parent material. Starting at weak alkaline to neutral pH in the topsoil, pH slightly increases with soil depth.

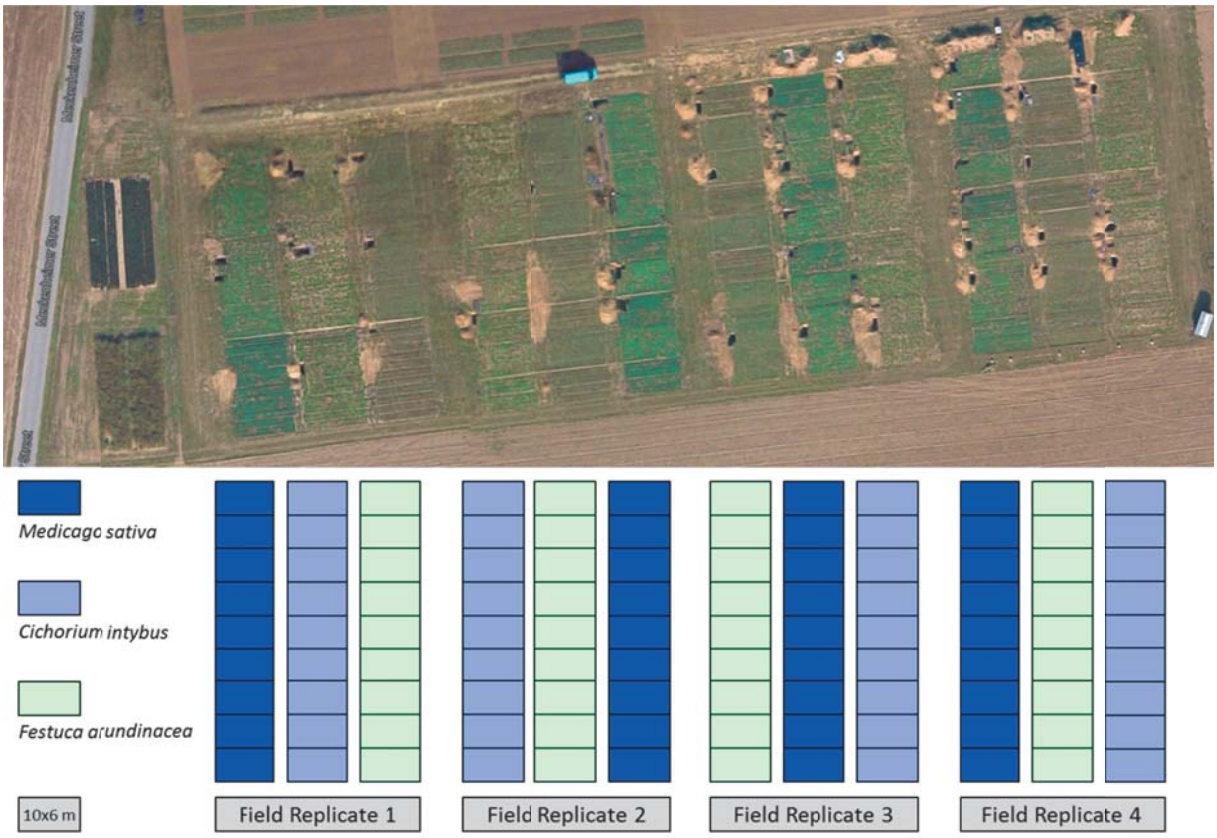

Figure 4: Aerial perspective of the study site Klein-Altendorf in 2011. Different plant species were grown as precrops according to a split-plot design. ©2016 Google, Kartendaten ©2016 GeoBasis-DE/BKG (©2009), Google.

Table 1: Soil properties at the study site Klein-Altendorf (adapted from Kautz et al., 2014).

Soil depth (cm)	Horizons (WRB)	Sand (%)	Silt (%)	Clay (%)	Soil textural class	Bulk density ($g \cdot cm^{-3}$)	pH ($CaCl_2$)	$CaCO_3$ ($g \cdot kg^{-1}$)	SOC ($g \cdot kg^{-1}$)	N_{tot} ($g \cdot kg^{-1}$)	CEC ($cmol_c \cdot kg^{-1}$)
0-27	Ap	8	77	15	SiL	1.29	6.5	<1	10.0	1.02	12.01
27-41	E/B	5	74	20	SiL	1.32	6.9	<1	4.6	0.55	11.91
41-75	Bt1	4	69	27	SiCL	1.42	6.9	<1	4.5	0.51	15.68
75-87	Bt2	4	65	30	SiCL	1.52	6.9	<1	3.9	0.50	18.48
87-115	Bt3	5	70	25	SiL	1.52	7.1	<1	2.5	0.34	15.49
115-127	Bw	5	72	23	SiL	1.46	7.3	<1	2.6	0.34	14.35
127-140+	C	8	75	13	SiL	1.47	7.4	127	n.d.	>0	n.d.

Abbreviations: WRB – World Reference Base, Ap – topsoil horizon disturbed by ploughing or other tillage practices, E – Eluvium, B – subsoil horizon, Bt – subsoil characterized by clay accumulation, Bw – weakly developed subsoil, C – intermediate horizon between subsoil and bedrock, SiL – silt loam, SiCL – silty clay loam, SOC – soil organic carbon, N_{tot} – total nitrogen, CEC – cation exchange capacity. The soil refers to a Haplic Luvisol (Hypereutric, Siltic) according to the WRB (IUSS Working Group WRB, 2015).

Table 2: Specifications of sampling time points and data collection.

Publi-cation	Data	Sampling time point	[a)]Temp. (°C)	[a)]Precip. (mm)	Soil compartment	[b)]Soil depth (cm)	Plant species growing
PUB I	qPCR: functional genes N-Cycle NH_4^+, NO_3^-	June 2010	17.3 (*16.3)	42.2 (*63.0)	Bulk soil	0-30 (Topsoil) 45-75 (Subsoil I) 75-105 (Subsoil II)	*C. intybus* *F. arundinacea* *M. sativa*
PUB II	qPCR: functional genes N-Cycle Bacterial 16S rRNA gene T-RFLP [c)]DOC, DON	April 2011, Early vegetative phase	12.7 (*8.8)	26.9 (*45.6)	Bulk soil Drilosphere Rhizosphere	10-30 (Topsoil) 60-75 (Subsoil)	*C. intybus* *F. arundinacea* *M. sativa* (3rd year)
		April 2011, Late vegetative phase	12.7 (*8.8)	26.9 (*45.6)			
PUB IV	[d)]Archaeal and bacterial 16S rRNA gene pyrosequencing (454 FLX®)	May 2011, Flowering	14.8 (*13.0)	33.2 (*56.2)			
PUB III	[i)]Potential extracellular enzyme activities	June 2012	15.7 (*16.3)	73.2 (*63.0)	Bulk soil Drilosphere Rhizosphere	10-30 (Topsoil) 45-75 (Subsoil I) 75-105 (Subsoil II)	[e)]*T. aestivum*
PUB V	-	[f)]April 2012 Monolith Excavation	9.0 (*8.8)	25.5 (*45.6)	Total soil: Disturbed topsoil Subsoil monoliths	0-20 (Topsoil) [f)]45-105 (Subsoil)	*C. intybus* *F. arundinacea* *M. sativa* (3rd year)
	^{13}C content [g)]Bacterial 16S rRNA gene T-RFLP [h)]DNA-SIP: Bacterial 16S rRNA qPCR and sequencing (MiSeq©)	[f)]December 2012 Climate Chamber Experiment	-	-	Bulk soil Rhizosphere	0-20 (Topsoil) [f)]20-50 (Subsoil U) [f)]50-80 (Subsoil L)	[e)]*T. aestivum*

* Long-term mean values at Wetterstation Bad Neuenahr-Ahrweiler (Deutscher Wetterdienst): 1951-1980

[a)] Given are the mean temperature over month and precipitation sum over month.

[b)] The description is based on the terms used in the publications. Subsoil I, Subsoil U and Subsoil in PII and PIV correspond to the same depth range: upper subsoil. Subsoil II and Subsoil L correspond to the same depth range: lower subsoil. Topsoil refers always to the depth range within 0-30 cm.

[c)] DOC and DON data were obtained from flowering developmental stage only.

[d)] Sequencing data were obtained from flowering developmental stage of *C. intybus* only.

[e)] Before *T. aestivum*, three different precrops (*C. intybus*, *F. arundinacea*, or *M. sativa*) were grown for 3 years on the field.

[f)] Subsoil monoliths for this experiment were excavated in April 2012 at 45-105 cm soil depth. This depth corresponds to 20-80 cm root depth in the greenhouse experiment due to the exclusion of the intermediate soil depth between 20-45 cm. The samples were taken after ^{13}C-CO_2 labeling of *T. aestivum* in December 2012.

[g)] T-RFLP data are only available for samples from soil cores where *M. sativa* was cultivated as a precrop.

[h)] DNA-SIP data are only available for rhizosphere samples from soil cores where *M. sativa* was cultivated as a precrop.

[i)] Namely 1,4-β-Glucosidase, Cellobiohydrolase, β-Xylosidase, 1,4-β-N-Acetylglucosaminidase, Phosphomonoesterase, Phenoloxidase, and Peroxidase.

The agricultural field extending an area of 190 × 60 m was divided into plots of 10 × 6 m. Three to four plots, which were distributed over the field and received the same treatment, were handled as true biological replicates. According to the split-plot design (Figure 4), different crop rotation systems were applied to the plots. For the experiments described here, three different precrops with contrasting root systems and nitrogen fixation abilities were cultivated for 3 years from 2009-2011, each followed by *Triticum aestivum* L. in 2012. The precrop species are *Festuca arundinacea* SCHREB. (tall fescue), *Cichorium intybus* L. (common chicory), and *Medicago sativa* L. (lucerne, alfalfa). *M. sativa* forms nodules with diazotroph Proteobacteria (Burton and Erdman, 1940) and is the only legume used in this study. Whereas *F. arundinacea* is a grass with a fibrous root system, *M. sativa* and *C. intybus* have a taproot system that strongly structures subsoil and contributes to biopore formation (Löfkvist et al., 2005; Kautz et al., 2014; Han et al., 2015a). For *M. sativa* and *C. intybus*, the density of biopores > 2 mm in 45 cm soil depth was estimated at 437 and 406 m^{-2}, respectively. In comparison, cultivation with *F. arundinacea* leads to a significant lower biopore density of 336 m^{-2} (Han et al., 2015a).

The only anecic earthworm species found on the field site is *Lumbricus terrestris* with abundances between 3-50 individuals m^{-2} determined by mustard extraction (Kautz et al., 2014; Han et al., 2015a). In addition, the endogeic earthworm species *Allolobophora calliginosa* was found on occasion (Timo Kautz, personal communication).

2.1.2 Sampling of Soil Compartments in the Field

For the different experiments, soil samples were taken directly in the field in 2010, 2011, and 2012 according to Table 2 for diverse soil chemical, microbial, and molecular analyses. The soil profile for each plot was exposed by excavation with a hydraulic shovel down to 1 m and across and area of approximately 1 × 1 m. Immediately before sampling, additional 20 cm of the profile were removed horizontally by a spate to minimize the effects of oxygen, light, and rainfall on the subsoil between excavation and sampling. Subsoil depths were defined according to soil horizons (Table 1) at 45-60 cm, 45-75 cm, and 75-105 cm. They are located in the clay-rich Bt horizons (Figure 5). The topsoil reached from 0-30 cm and represents the Ap horizon that arose from yearly conventional tillage practice. Due to high variation of soil properties between 30 cm and 45 cm at the field site, this depth was excluded from further analyses.

In addition, intact subsoil monoliths were excavated in order to label rhizosphere bacteria utilizing plant-derived carbon with ^{13}C heavy isotope (section 2.2).

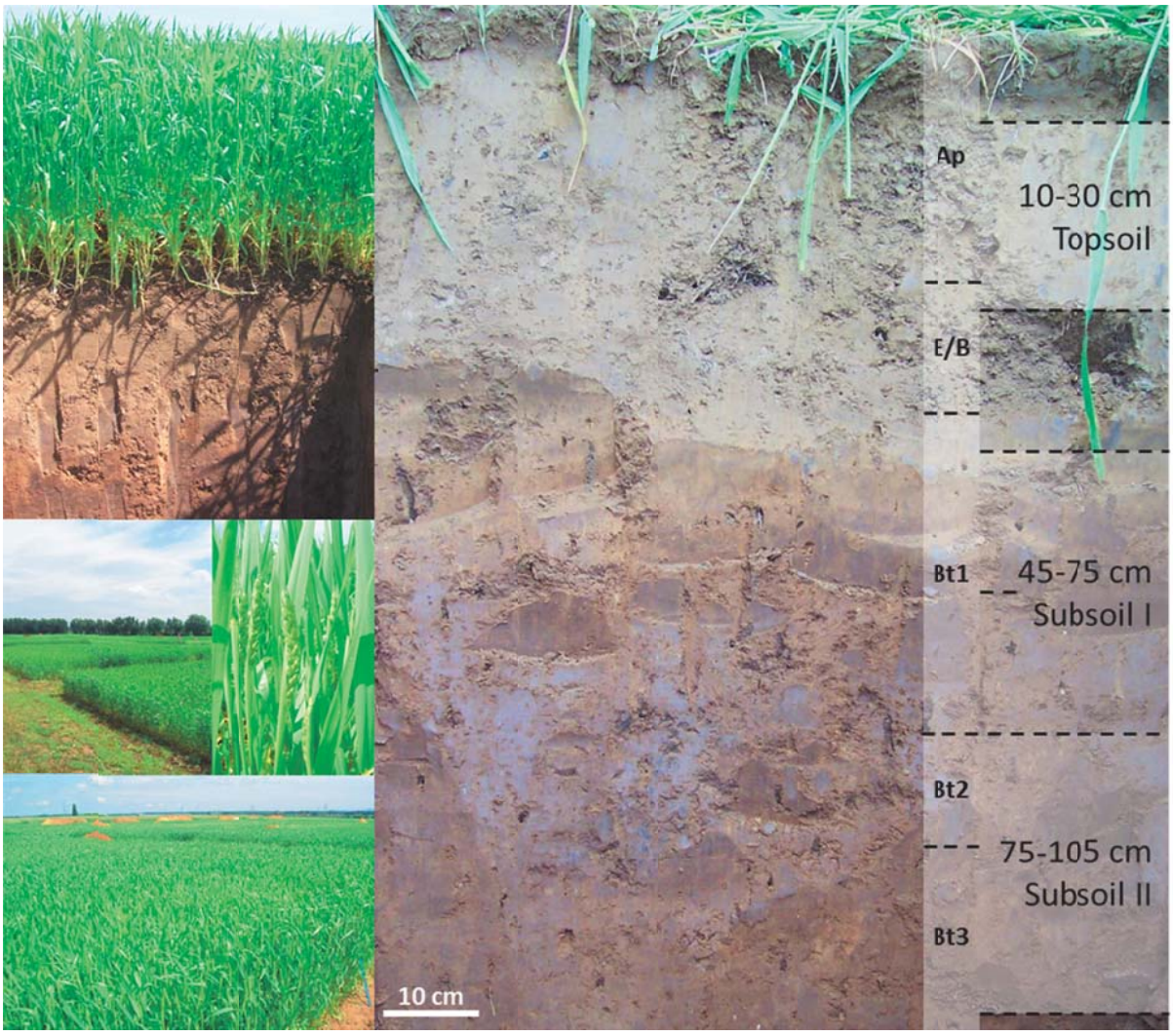

Figure 5: *T. aestivum* growing in 2012 and soil profile at Campus Klein-Altendorf. The sampling layers „Topsoil“, „Subsoil I“ and „Subsoil II“ are displayed and the soil horizons are mapped according to the depth ranges defined by Kautz et al. (2014) (Table 1).

From bottom to top, different soil compartments (Figure 6) were sampled in each depth by subsampling in the defined soil depth range – ca. 1 m horizontal and up to 20 cm vertical into the soil profile wall. All tools were sterilized by treatment with 70% EtOH before use. Sterile plastic bags, 2 ml reaction tubes or 2 ml cryotubes for -80°C storage were used for transport.

Bulk soil was sampled at a minimum distance of 1 cm to plant roots or earthworm burrows. At least 5 subsamples from different positions in each soil depth were pooled.

Earthworm burrows were distinguished from other soil pores by their round cross section and darker colored wall in comparison to the surrounding bulk soil. Furthermore, the pellet-shaped earthworm deposits lining the burrows were a good indicator in the field. It was avoided to sample loose earthworm cast, which sometimes blocked the burrows. The burrows were half-opened by a spatula

and at maximum 1 mm from the burrow wall was scraped out with a small spoon, for which the coloring was an indicator, too. From each depth layer, at least 5 burrows were used for sampling and pooled. It was avoided to sample from burrow walls where roots were simultaneously growing to exclude interference with the rhizosphere.

Rhizosphere was sampled together with the roots of the current plant species by tweezers and was defined here as maximum 2 mm adhering soil around the plant root surface. Only living roots and roots thinner than 1 mm were considered for sampling. Lignified roots were excluded.

In 2011, sampling of the soil compartments was conducted three times to investigate the temporal variability of the microbial community composition and functional traits in relation to the plant development (Table 2). This included the "early" and "late vegetative" growth phase, corresponding to BBCH 28-32 and BBCH 34-38, and the "flowering" phase (BBCH 55-65; Hack et al., 2001). The BBCH scale was developed for a consistent classification of the plant growth stages from various mono- and dicotyledonous plants and therefore extents the EC code developed for cereals. The morphological appearance of *F. arundinacea*, *C. intybus*, and *M. sativa* at the three time points is shown in Figure 7.

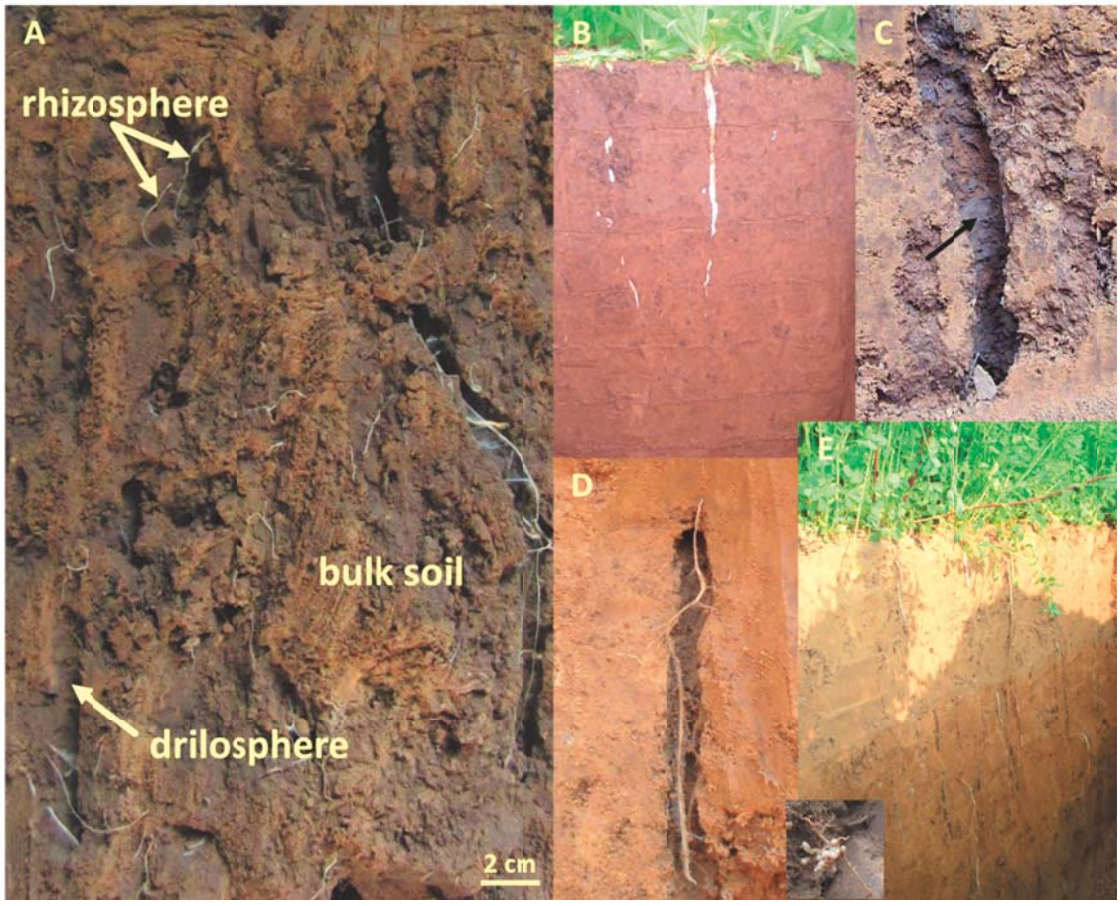

Figure 6: Biopores and soil heterogeneity.

(A) Soil compartments at 45-75 cm soil depth with *T. aestivum* growing. (B) Thick taproots of *C. intybus* forming biopores. (C) Earthworm burrow with dark-colored lining – the drilosphere (arrow). (D) Roots growing in earthworm burrows. (E) Soil profile with taproots of *M. sativa* and enlarged figure of nodules. (photographs B-E provided by Dr. Doreen Fischer).

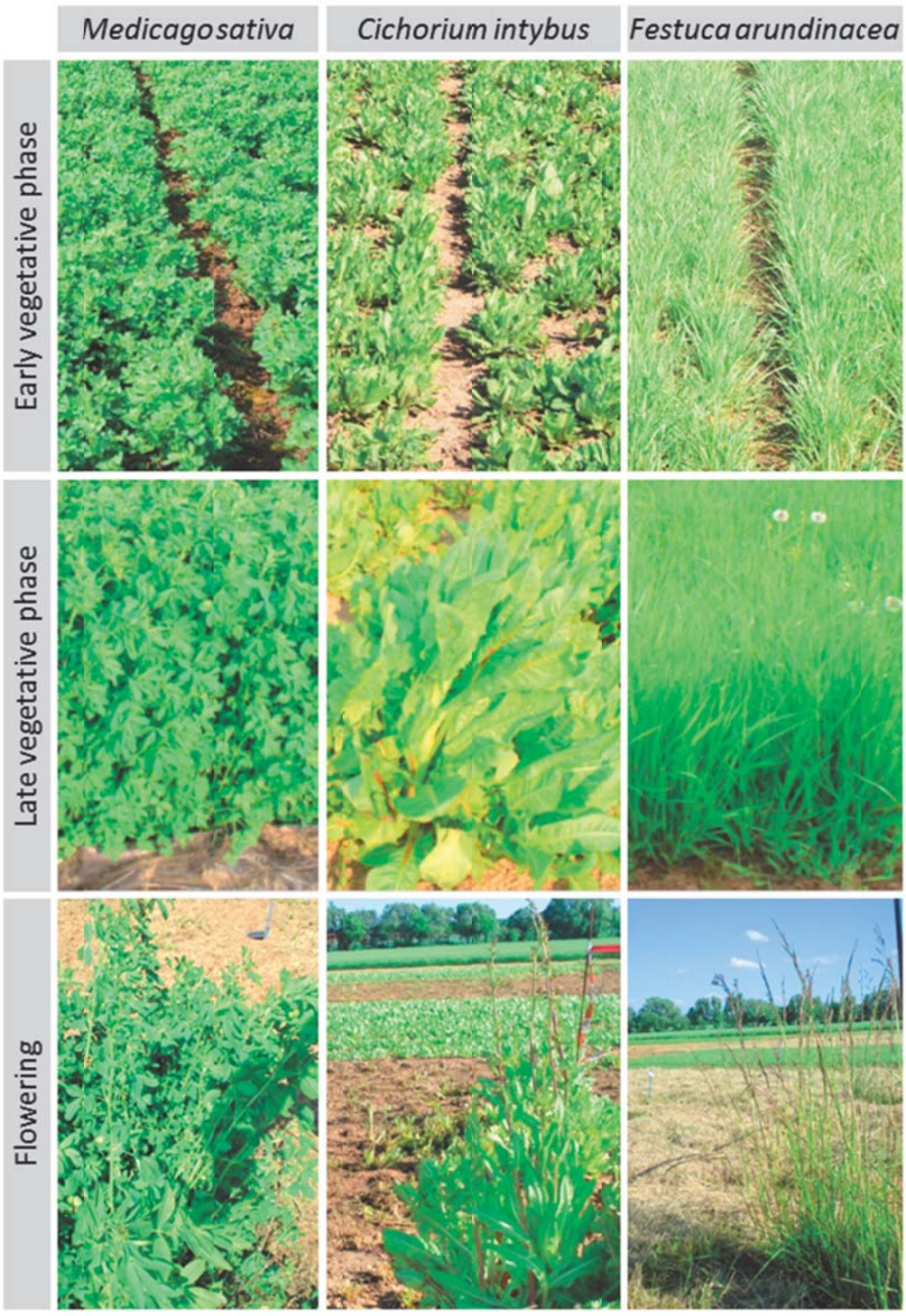

Figure 7: Precrops at different growth developmental stages in 2011. Photographs were provided by Dr. Doreen Fischer.

The soil was stored at 4°C for chloroform-fumigation and $CaCl_2$ extraction, gravimetric water content, and enzyme activity measurements, which were all carried out within 14 days after sampling. For molecular techniques, samples were frozen on dry ice directly at the field site and transferred to -80°C freezer before DNA extraction. A detailed description of downstream sample analyses are given in sections 2.3–2.8.

2.2 Stable Isotope Probing of Wheat Rhizosphere Bacteria in Undisturbed Subsoil Cores

In order to investigate the active rhizosphere bacterial community in undisturbed subsoil, an experiment was conducted in which bacteria were labeled with heavy carbon isotope ^{13}C via atmospheric CO_2 that was taken up by the plants and released as root exudates or root residues into the soil. For this, undisturbed subsoil monoliths from the field were transferred to a climate chamber to ensure controlled labeling conditions. In a quantitative DNA-SIP approach coupled with NGS, bacterial OTU relative abundance was coupled with the information about ^{13}C enrichment in the DNA.

2.2.1 Wheat Cultivation on Soil Monoliths in a Climate Chamber, ^{13}C-CO_2 Labeling, and Sampling

Twelve undisturbed subsoil monoliths were excavated (by Dr. Sascha Reth, UGT Umweltgerätetechnik GmbH, Freising, Germany) in April 2012 with a lysimetric excavation technology developed by Meißner et al. (2007) from the same agricultural field, which was used for direct field sampling (section 2.1). Three plots with different precrops were selected: *C. intybus*, *F. arundinacea*, or *M. sativa* (Table 2) and the excavation was carried out before the soil management in spring. Four subsoil cores (Ø 20 cm; 45-105 cm soil depth) were obtained from each plot with a minimum distance of 1 m between the monoliths.

A covered polystyrene box (60×180×100 cm) with a copper base plate set to 14°C was used to cool the subsoil monoliths from bottom to top continuously. Sieved topsoil from the field's Ap horizon was added on top of the subsoil cores to mimic the homogenized plough horizon between 0-20 cm. Due to inhomogeneous soil properties between 20-45 cm soil depth at the field, this depth was excluded from this study.

Triticum aestivum L. (cultivar Scirocco) was sown with a density of 350 seeds·m^{-2}. Accordingly, 11 germinated seeds were added to the topsoil of each core. Plants were cultivated with 12 light hours per day, which were provided by 4 high-pressure sodium vapour lamps (E40, 350 W). Temperature in the climate chamber oscillated between 14°C during night and 20°C during day phase. *T. aestivum* was regularly irrigated with 200 ml tap water during the 90 days of cultivation to mimic a total of 165 mm precipitation, which can be expected at the originating field site near Klein-Altendorf throughout April until June (Agrarmeteorologie Rheinland-Pfalz; www.wetter.rlp.de).

75 days after sowing, plants reached the developmental stage EC50. At this point, root exudation rates were considered to be highest (Haichar et al., 2014). The twelve soil columns were separated into three control columns and nine treatment columns. On top of all columns a polystyrene plate was placed with recesses for the soil columns and sealed with silicon to minimize gas and

temperature exchange. An aluminium frame was placed on the three control columns (58×50×104 cm) and the nine treatment columns (58×126×104 cm) and covered with an airtight plastic sheet, separately. Small ventilators and data loggers were positioned in the tents to ensure a fast air circulation and to monitor temperature and moisture.

The control tent was flushed via a membrane pump with ambient air to maintain conditions as close to nature as possible. In order to label rhizosphere bacteria utilizing plant-derived carbon, the treatment tent was supplied via a flow controller with 2.5% of ^{13}C-CO_2 (99%) in N_2 5.0 (Westfalen AG, Münster, Germany). An infrared controller was used to regulate the CO_2 concentration between 300 ppm in the light phase and 600 ppm in the dark phase during the day-night cycle. To monitor the CO_2 and $\delta^{13}C$ in the chamber atmosphere, gas samples were analyzed with a GC/IRMS-20 system (delta plus, Thermo Fisher Scientific, Dreieich, Germany). In total, 20 l of ^{13}C-CO_2 were applied to the treatment tent during 15 days (days 75-90 after sowing). Plants were watered once during the labeling period. For this purpose, the control tent was opened and closed first and the treatment tent second to avoid ^{13}C-CO_2 flow into the control tent.

After labeling, the cultivation was terminated by opening the control tents first. The plants were cut at the root-shoot transition zone to obtain the aboveground fresh biomass. It was dried at 40°C for determination of total carbon and ^{13}C content. Afterwards, the ^{13}C-CO_2 tent was opened and treated accordingly.

The twelve soil columns were first cut with an electric saw into three blocks according to the root depth: Topsoil (0-20 cm), upper subsoil U (20-50 cm) and lower subsoil L (50-80 cm). The subsoil depth ranges correspond to the field soil depths 45-75 cm and 75-105 cm due to the exclusion of soil depth 20-45 cm. The cylinder blocks were afterwards dissected longitudinally into two halves. From the exposed soil profile a representative cylinder segment was cut from the midpoint to the edge for the determination of the root biomass. For this purpose the roots were washed out of the soil with deionized water and dried. The remaining block half was used to sample bulk soil and root-rhizosphere complex with sterilized tweezers and spoons. The bulk soil was sampled with highest possible distance to the roots that increased with soil depth. Roots were sampled together with maximum 2 mm adhering rhizosphere soil around. Subsamples were taken for molecular analyses and stored at -80°C or dried at 40°C for gravimetric water content, total nitrogen, carbon and ^{13}C content analysis (Table 2). For the elemental analyses, the root-rhizosphere complex was separated in a washing step with deionized water into the root and rhizosphere compartment before drying.

2.2.2 Molecular Analyses and Quantitative DNA-SIP

DNA was extracted, as described in section 2.5, from topsoil (0-20 cm), upper and lower subsoil U (20-50 cm) and L (50-80 cm) from root-rhizosphere complex and bulk soil samples of one control column and three treatment columns, where *M. sativa* was cultivated as precrop before *T. aestivum* (Table 2). This resulted in 24 samples (4 soil columns × 3 soil depths × 2 compartments (root-rhizosphere complex and bulk soil)). A modified nucleic acid extraction procedure was applied (section 2.5).

First, terminal restriction fragment length polymorphism (T-RFLP) was conducted to compare the bacterial community fingerprints between control and ^{13}C-labeled soil columns for evaluation of the effect of the labeling treatment itself and the comparability of the four soil columns (section 2.7).

In the second step, DNA-SIP was applied based on the method described in Lueders et al. (2004) and Neufeld et al. (2007). Dependent on the ^{13}C content, DNA can be separated in a continuous chemical density gradient based on its buoyant density. The differential analysis of labeled and unlabeled sample gradients allows the determination and quantification of microbes utilizing ^{13}C substrates for growth (section 2.9.3). As ^{13}C content in bulk soil was too low to be distinct from unlabeled samples, DNA-SIP was limited to rhizosphere (4 soil columns × 3 soil depths × 1 compartment (root-rhizosphere complex) = 12 samples).

4 µg DNA was mixed with gradient buffer (0.1 M Tris-HCl pH 8, 0.1 M KCl, 1 mM EDTA) to a final volume of 1 ml and added to 5 ml CsCl solution (50 g CsCl added to 30 ml gradient buffer). The buoyant density was adjusted to 1.71 $g \cdot ml^{-1}$ based on the refractory index measured with a Reichert™ AR200™ Digital Refractometer (Thermo Fisher Scientific). The solution was loaded into a 5.1 ml polyallomer QuickSeal tube (Beckman Coulter, Krefeld, Germany) prior to isopycnic ultracentrifugation (Sorvall® Discovery™ 90SE ultracentrifuge; Thermo Fisher Scientific) in a vertical rotor (VTi 65.2; Beckman Coulter) for 36 h at 20°C and 44,500 rpm (184,000×g_{av}).

The CsCl gradient tube was punctured with a 0.4 mm needle on top and at the bottom. While UltraPure™ DNase/RNase-Free Distilled Water was replacing the solution from top at a flow rate of 1 $ml \cdot min^{-1}$, 13 fractions à ~400µl fractions were collected at the bottom of the tube. In each fraction, buoyant density was measured prior to DNA purification. 800 µl of a PEG solution (30% polyethylene glycol 6000, 1.6 M NaCl) was added and DNA was precipitated at 14,000×g for 30 min at 4°C. The supernatant was removed and the DNA was washed with 70% ice-cold EtOH (14,000×g, 15 min, 4°C). In 25 µl EB-buffer (Qiagen, Hilden, Germany) the DNA was dissolved prior to total DNA and bacterial 16S rRNA gene quantification by PicoGreen and qPCR (section 2.5 and 2.6).

Based on total DNA content, 7 consecutive fractions were chosen from each density gradient that covered >89% of the DNA. The range of the corresponding buoyant density (1.665–1.730 g·ml^{-1}) spanned the typical density range for light and heavy DNA (Lueders et al., 2004; Neufeld et al., 2007b). These 84 fractions (4 soil columns × 3 soil depths × 1 compartment (root-rhizosphere complex) × 7 density gradient fractions) were subjected to sequencing of bacterial 16S rRNA amplicons via Illumina technology (section 2.8.2).

2.3 Determination of Soil Microbial Biomass, Soil Chemical Parameters and $\delta^{13}C$

Microbial biomass (C_{mic}) and soil chemical parameters DOC, DON, NH_4^+ and NO_3^- in bulk soil were determined by chloroform-fumigation and $CaCl_2$-extraction on the basis of the methods described by Joergensen (1996) and Joergensen and Mueller (1996). With $CaCl_2$, dissolved organic carbon, nitrogen, ammonia, nitrate, and nitrite can be extracted from the soil. The pre-treatment with chloroform leads to the breaking of living cells and release of intracellular compounds. The difference between the $CaCl_2$-extractable compounds with and without fumigation therefore represents the microbial biomass.

For chloroform-fumigation, technical triplicates of 7 g bulk soil without roots and small stones, were weighed and fumigated in a desiccator with 25 ml chloroform for 24 h. After this treatment, the soil samples were extracted together with the non-fumigated triplicate samples by shaking them overhead each with 25 ml of 0.1 M $CaCl_2$ solution for 40 min. The soil suspension was filtrated through a folded filter (grade 585 ½, GE Healthcare Europe GmbH, Freiburg, Germany) and stored at -20°C before measuring. H_2O_d was used instead of the soil extracts for negative control.

NH_4^+ and NO_3^- ere photometrically determined in $CaCl_2$ extracts by ISO 56673 and ISO 13395:1996 using the Skalar 5100 Continous Flow System (SKALAR Analytic GmbH, Erkelenz, Germany). TC and TN_b in the liquid extracts were measured on the Dimatoc 2000 (Dimatec Analysentechnik GmbH, Essen, Germany) after acidification with 2 N HCl to gas out anorganic carbon in the form of carbonates which can be found especially at deeper soil horizons (Table 1). For calculation of DOC and DON the k_{EC} and k_{EN} coefficients 0.54 and 0.45 were used, respectively (Joergensen, 1996; Joergensen and Mueller, 1996).

For measurement of TC, TN and $\delta^{13}C$ in solid biological samples, the material was dried at 40°C and ground to a homogenous powder in a ball mill (Tissue Lyser II, Qiagen, Venlo, Netherlands). Aliquots between 1.5 mg and 50 mg were weighed into tin capsules (IVA Analysentechnik, Meerbusch, Germany) and analyzed via IRMS (delta V Advantage, Thermo Fisher Scientific) that was coupled to an Elemental Analyzer (Euro EA, Eurovector, Milano, Italy). Acetanilide was used as a lab standard for

calibration, whereby it was firstly calibrated against several suitable international isotope standards (IAEA; Vienna, Austria).

2.4 Potential Enzyme Activity Measurements

The activities of two classes of enzymes, expected to be at least partially extracellular located and soluble or soil particle-bound, were determined (Table 2; PUB III). For the oxidative enzymes a colorimetric enzyme assay using L-3,4-Dihydroxyphenylalanin (L-DOPA) as electron donor was used to measure phenol oxidase and peroxidase activities on the basis of Saiya-Cork et al. (2002). The hydrolytic enzymes β-glucosidase, cellobiohydrolase, xylosidase, chitinase, and phosphomonoesterase were fluorometrically measured according to Pritsch et al. (2005) by cleavage of the methylumbelliferyl group that is linked to a specific substrate.

100-400 mg bulk soil, rhizosphere, or drilosphere were weighed into 50 ml reaction tubes to prepare a soil suspension for the enzyme assays. After addition of 100 $ml \cdot g^{-1}$ H_2O_{dd}, the tubes were shaked overhead for 10 min. To dissolve the enzymes more effective, samples were sonicated for 3 min in an ice water bath. The soil suspension was filtrated through a 90 µm nylon mesh to remove bigger particles and fine roots and stored at 4°C until measurement. The same soil suspension was used for all enzyme assays, which were carried out in triplicates on 96-well microtiter plates within 24 h.

For the fluorometric enzyme assays black microtiter plates were used. 50 µl of fresh soil suspension were mixed with 100 µl of the substrate solution (Table 3) without a buffering system to start the reaction at physiological pH. H_2O_{dd} was used as a negative control instead of the soil suspension. From a 5 mM substrate stock solution prepared in 2-methoxy ethanol, the working solutions were diluted. For calibration, a stock solution of 1 mM 4-methylumbelliferone in 2-methoxy ethanol was prepared and dissolved with H_2O_{dd} to 100, 200, 300, 400, and 500 µM working solutions, corresponding to 100, 200, 300, 400, and 500 pmol per well. To determine the auto-fluorescence or fluorescence quenching of each sample, 50 µl of the suspension was mixed with 100 µl of the 300 µM 4-methylumbelliferone solution.

After substrate specific incubation time in the dark (Table Enzyme Assays), the reaction was stopped at alkaline conditions with 100 µl 1 M Tris (pH 10.7). The particles in the microtiter plates were spun down at 2420 rpm for 5 min. Fluorescence was measured by excitation at 365 nm and emission at 450 nm.

For the oxidative enzymes 150 µl of the soil suspension was mixed with 150 µl of 20 mM L-DOPA dissolved in 100 mM sodium acetate (pH 5.5). In the case of peroxidase 10 µl of 12% H_2O_2 was added. Negative controls were *(i)* 100 mM sodium acetate instead of the soil suspension to determine the auto-oxidation of the substrate and *(ii)* 100 mM sodium acetate instead of the substrates to correct

for the light absorption of compounds which derived from the soil particles. The assay was set up twice for measurement shortly after substrate addition and after 20 h incubation in the dark. The plates were spun down at 2000 rpm for 2 min. 250 µl of the particle-free supernatant were transferred to a clean microtiter plate and light attenuation was measured at 450 nm. Using the specific extinction coefficient for dopachrome ($\varepsilon = 3.6\ mM^{-1} \cdot cm^{-1}$) in the Lambert-Beer equation, concentration difference between the two time points was calculated. The peroxidase activity was estimated as the difference between oxidative activity with and without H_2O_2 addition.

2.5 DNA Extraction and Quantification

For DNA isolation from field soil samples (Table 2), the FastDNA® Spin Kit for Soil (MP Biomedicals, Eschwege, Germany) was used according to the manufacturer's instructions: In a first step, cells were lysed by shaking them with small ceramic, silica, and glass beads (lysing matrix E). The DNA is further bound at high salt concentrations to the silica column, washed and eluted with desalted, nuclease-free water. As subsoil samples contain lower amounts of DNA, the yield for all samples was increased by a second bead-beating step for 40 s and an incubation step at 55°C for 5 min before elution in addition to the standard protocol. Since roots were intact after homogenization and for simplified reading, the DNA, which was extracted from the root-rhizosphere complex, is further designated as 'rhizosphere DNA'.

DNA from bulk soil and rhizosphere samples of the ^{13}C-Labeling experiment (section 2.2; PUB V) was extracted with phenol:chloroform:isoamylalcohol (25:24:1) followed by DNA precipitation with polyethylenglycol 6000 according to a modified procedure described in Lueders et al. (2004). Compared to the FastDNA® Spin Kit for Soil, this method enabled an overall higher yield of DNA needed for subsequent CsCl density fractionation.

The quality and quantity of DNA was determined spectrometrically by NanoDrop 1000 Spectrophotometer (Peqlab, Erlangen, Germany). The quality with respect to protein and humic acid residues was determined from the ratios A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm}. For a more specific and sensitive DNA quantification necessary for library preparation in NGS and for DNA-SIP fractions, the DNA amount was determined fluorometrically by Quant-iT TM™ PicoGreen® dsDNA Assay Kit (Life technology, Darmstadt, Germany), which enabled a better DNA quantification at concentrations below 10 $ng \cdot \mu l^{-1}$. From 250-fold dilutions, samples were measured with a DNA standard ranging from 0.016 to 1 $ng \cdot \mu l^{-1}$.

Table 3: Enzyme assay conditions. Supplemented table according to PUB III. Enzyme classification and description is based on KEGG database (http://www.genome.jp/kegg/).

Enzyme name	EC number	Reaction based on KEGG database	Natural substrates	Substrate and concentration in assay	Incubation time
1,4-β-Glucosidase	3.2.1.21	Hydrolysis of 1,4-β-D-glucosidic linkages from the non-reducing terminus with release of β-D-glucose	Cellobiose	4-MU-β-D-glucopyranoside 500 µM	1 h
Cellobiohydrolase	3.2.1.91	Hydrolysis of 1,4-β-D-glucosidic linkages from the non-reducing terminus with release of cellobiose	Cellulose	4-MU-β-D-cellobioside 400 µM	2 h
β-Xylosidase	3.2.1.37	Hydrolysis of 1,4-β-D-xylans from the non-reducing terminus with release of D-xylose	1,4-β-D-xylan	4-MU-β-D-xyloside 500 µM	1 h
1,4-β-N-Acetyl-glucosaminidase (Chitinase)	3.2.1.14	Random hydrolysis of N-acetyl-β-D-glucosaminide (1,4-β-linkages)	Chitin	4-MU-N-acetyl-β-D-glucosaminide 500 µM	1 h
Phosphomonoesterase	3.1.3	Hydrolysis of phosphomonoesters with release of orthophosphate	Phosphomonoesters, Nucleotides, Phytate	4-MU-phosphate 800 µM	40 min
Alkaline phosphatase	3.1.3.1	Alkaline pH optimum			
Acid phosphatase	3.1.3.2	Acidic pH optimum			
Phenoloxidase	1.10.3	Oxido-reductase with O_2 as electron acceptor acting on diphenols as electron donor	Phenolic compounds, Lignin	L-3,4-DOPA 10 mM	20 h
Catechol oxidase	1.10.3.1	Catechol as electron donors			
Laccase	1.10.3.2	Benzendiols as electron donors, incl. hydrochinones			
Peroxidase	1.11.1	Oxido-reductase with H_2O_2 as electron acceptor	Phenolic compounds,	L-3,4-DOPA; H_2O_2	20 h
Peroxidase	1.11.1.7	Acting on phenols as electron donor	Lignin	10 mM; 0.4% (v/v)	

MU … Methylumbelliferyl group

DNA fragment size and size distribution was checked manually by electrophoresis on 1-2% agarose gels with the GeneRuler 100 bp/1 kb DNA ladders (Thermo Fisher Scientific, Braunschweig, Germany) or – for a higher precision prior to NGS library preparation – with Agilent 2100 bioanalyzer instrument using the Agilent DNA 7500 Kit (Agilent Technologies, Waldbronn, Germany).

2.6 Quantitative Real-Time PCR (qPCR)

Absolute quantification of marker genes was achieved by qPCR with target-specific primers. In this method, the dsDNA-binding fluorophore SYBR-Green is used for quantification of DNA after each PCR cycle in real time. Defined as the PCR cycle number at the same threshold fluorescence in the exponential phase of PCR, C_T values are obtained for each sample. A 10-fold dilution series in the range of 10^1-10^7 copies·µl^{-1} of a purified plasmid, which harbors the target gene of a source organism, is used as standard for calibration of the C_T values against the concentration of the respective marker gene.

Degenerated primers were used (Table 4) to quantify archaeal and bacterial 16S rRNA genes and functional marker genes for the N-cycle (PUB I, II, IV, V; Table 2). The functional marker gene *amoA* encoding the ammonia monooxygenase of either archaea (AOA) and bacteria (AOB) was used for quantification of potential ammonia oxidation. Denitrification potential was estimated by *nirK* (copper-dependent nitrite reductase gene), *nirS* (cytochrome-dependent nitrite reductase gene) or *nosZ* (nitrous oxide reductase gene).

The Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) was used for the 7300 Real-time PCR System according to Töwe et al. (2011) and Table 5. The assay with a total volume of 25 µl contained 1X Power SYBR® Green PCR Master Mix and 2 µl of DNA template or DNA standard concentration. 0.1% DEPC nuclease-free molecular grade water was used for dilutions and negative control. From DNA samples, a suitable dilution was prepared in order to prevent inhibition during the PCR reaction. On the software, the threshold line for C_T values was automatically set. The qPCR efficiency was calculated as follows and was accepted when exceeding 80%.

$$Efficiency\,[\%] = 100\% \cdot (10^{\frac{-1}{slope}} - 1)$$

Table 4: Target-specific primer sequences for qPCR, T-RFLP, and NGS.

Target Gene	Method	Primer	Target-Specific Sequence (5' → 3')	Reference	Amplicon Length	Host Organism qPCR standard
Bacterial 16S rRNA	qPCR	FP16S	GGTAGTCYAYGCMSTAAACG	(Bach et al., 2002)	263 bp	*Clavibacter michiganensis*
		RP16S	GACARCCATGCASCACCTG	(Bach et al., 2002)		DSM 463 64
	T-RFLP	[1)]FAM-Ba27f	AGAGTTTGATCCTGGCTC	(Lane, 1991)	919 bp	-
		Ba907r	CCGTCAATTCCTTTRAGTTT	(Liu et al., 1997)		
	NGS (Roche)	[2)] Ba27f	AGAGTTTGATCCTGGCTC	(Lane, 1991)	977 bp	-
		[3)] 984r	GTAAGGTTCYTCGCG	(Klindworth et al., 2013)		
	NGS (Illumina)	[4)] Ba27f	AGAGTTTGATCCTGGCTC	(Lane, 1991)	350 bp	-
		[5,6)] 357R	CTGCTGCCTYCCGTA	(Klindworth et al., 2013)		
Archaeal 16S rRNA	qPCR and NGS (Roche)	[2)] rSAf(i)	CCTAYGGGGCGCAGCAG	(Nicol et al., 2003)	618 bp	*Methanobacterium* sp.
		[3)] 985r	YCCGGCGTTGAMTCCAATT	(Bano et al., 2004)		(Timmers et al., 2012)
amoA (AOA)	qPCR	amo19F	ATGGTCTGGCTWAGACG	(Leininger et al., 2006)	624 bp	Fosmid clone 54d9
		CrenamoA16r48x	GCCATCCABCKRTANGTCCA	(Schauss et al., 2009)		(Treusch et al., 2005)
amoA (AOB)	qPCR	amoA1F	GGGGTTTCTACTGGTGGT	(Rotthauwe et al., 1997)	500 bp	*Nitrosomonas* sp.
		amoA2R	CCCCTCKGSAAAGCCTTCTTC	(Rotthauwe et al., 1997)		(Rotthauwe et al., 1997)
nirK	qPCR	nirK 876	ATYGGCGGVCAYGGCGA	(Braker et al., 1998)	164 bp	*Azospirillum irakense*
		nirK 5R	GCCTCGATCAGRTTRTGG	(Henry et al., 2004)		DSM 11586
nirS	qPCR	cd3aF	GTSAACGTSAAGGARACSGG	(Michotey et al., 2000)	413 bp	*Pseudomonas stutzeri*
		R3cd	GASTTCGGRTGSGTCTTGA	(Throbäck et al., 2004)		
nosZ	qPCR	nosZ2F	CGCRACGGCAASAAGGTSMSSGT	(Henry et al., 2006)	267 bp	*Pseudomonas fluorescens*
		nosZ2R	CAKRTGCAKSGCRTGGCAGAA	(Henry et al., 2006)		C7R12

[1)]For T-RFLP the forward primer Ba27f was 5'-labeled with 6-carboxyfluorescein (FAM).
[2)] Primer were 5'-extended with forward adapter sequence and MID (Roche, A-Key): 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-MID-target-specific sequence-3'
[3)] Primer were 5'-extended with reverse adapter sequence (Roche, B-Key): 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-target-specific sequence-3'
[4)] Primer were 5'-extended with forward adapter sequence (Illumina): 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-target-specific sequence-3'
[5)] Primer were 5'-extended with reverse adapter sequence (Illumina): 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-target-specific sequence-3'
[6)] Primer 357R is reverse complement to primer S-D-Bact-0343-a-A-15 in Klindworth et al., 2013

Table 5: Target gene-specific qPCR conditions and PCR programs.

Target Gene	Assay conditions	PCR Program			
		Cycles	Denaturation	Annealing	Elongation
Bacterial 16S rRNA	0.4 µM each primer	40	20 s, 94°C	60 s, 62°C	30 s, 72°C
Archaeal 16S rRNA	0.2 µM each primer 0.06% BSA	5 35	20 s, 94°C 20 s, 94°C	60 s, 55°C* 60 s, 50°C	30 s, 72°C 30 s, 72°C
amoA (AOA)	0.2 µM each primer 0.06% BSA	40	45 s, 94°C	45 s, 55°C	45 s, 72°C
amoA (AOB)	0.3 µM each primer 0.06% BSA	39	60 s, 94°C	60 s, 58°C	60 s, 72°C
nirK	0.2 µM each primer, 0.06% BSA, 2.5% DMSO	5 40	15 s, 95°C 15 s, 95°C	30 s, 63°C* 30 s, 58°C	30 s, 72°C 30 s, 72°C
nirS	0.2 µM each primer 0.06% BSA, 2.5% DMSO	39	45 s, 94°C	45 s, 57°C	45 s, 72°C
nosZ	0.2 µM each primer 0.06% BSA	5 40	15 s, 95°C 15 s, 95°C	30 s, 65°C* 30 s, 60°C	30 s, 72°C 30 s, 72°C

Primers are given in Table 4. Initially, DNA was initially denatured for 10-15 min at 95°C. A melt curve (15 s 95°C/30 s 60°C/15 s +1°C/15 s 95°C) was run after PCR for quality check. Fluorescence was measured after each elongation step.

*Temperature touchdown: -1°C per cycle.

2.7 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP is a PCR-based molecular fingerprint method, which is based on the phylogenetic variability of gene sequences that cause different DNA fragment sizes after restriction of the PCR amplicon with sequence-specific endonucleases. In this study, it was used to analyze the bacterial community in samples from the field and after stable isotope probing in soil monoliths (PUB II, V, Table 2).

The target gene in these studies was the bacterial 16S rRNA gene. In the first step, the 16S rRNA variable regions V1-V5 were amplified with the 6-carboxyfluorescin labeled forward primer Ba27f and the reverse primer Ba907r (Table 4). The amplicon was purified with QIAquick PCR Purification System (Qiagen, Hilden, Germany) and 100-400 ng DNA was used for the restriction with the endonuclease *Msp*I (Fermentas, St. Leon-Rot, Germany) for 15 h at 37°C overnight. The digested DNA was purified with the MinElute® Reaction Cleanup Kit (Qiagen). The TRFs were separated by electrophoresis with a POP-7 polymer and fluorometrically quantified on the 3730 DNA Analyzer (Applied Biosystems). For this, 4 ng of the digested DNA was added to 13 µl Hi-Di™ Formamide (Applied Biosystems) with a 800-fold dilution of 6-carboxy-X-rhodamine-labeled MapMarker® 1000 (Bioventures, Murfreesburo, USA) as internal standard. The mixture was pre-denaturized at 95°C for

5 min. According to Töwe et al., 2011, the conditions for the 50 cm capillary array were: 10 s injection time, 2 kV injection voltage, 66°C run temperature, and 63 min analysis time.
For the quality analysis of the electropherograms and data processing, the GeneMapper 3.5 software package (Applied Biosystems; PUB II) or Peak Scanner™ Software (Version 1.0, Applied Biosystems; PUB V) was used. Furthermore, the T-REX software (Culman et al., 2009) was applied to create integer abundance matrices with TRFs >50 bp by filtering for peak height with a threshold 1. In PUB II, TRFs below 1% in any sample were excluded for statistical analysis, whereas in PUB V further data evaluation was restricted to the 50 most abundant TRFs.

2.8 Next-Generation Sequencing (NGS)

Next-generation sequencing enables a simultaneous and fast sequencing of mixed nucleic acid sequences from metagenomic and amplicon nucleic acid libraries. There are several techniques and platforms available (reviewed in Metzker (2010)), however, key is the spatial separation of single DNA strands prior to sequencing. Several samples can be sequenced in parallel due to the use of short sequence barcodes, which are added via primers in the library preparation.

2.8.1 454-Pyrosequencing on GS FLX$^+$ (Roche)

The pyrosequencing technique developed by Roche (Mannheim, Germany) separates single DNA molecules of the 16S rRNA gene amplicon library by binding them to capture beads (one molecule per bead). The beads are encapsuled in aqueous droplets of an emulsion prior to clonal amplification, which is referred to as emulsion PCR. Subsequently, the beads are spatially separated on a microchip. During sequencing-PCR, the four nucleotides are added separately in one cycle. At the release of pyrophosphate during the DNA synthesis step, a chemical reaction is driven by the luciferase leading to chemiluminescence, which is optically detected.
From selected topsoil and subsoil DNA samples from bulk soil, drilosphere, and rhizosphere (PUB IV; Table 2) archaeal and bacterial 16S rRNA gene amplicons were sequenced on the GS FLX$^+$ instrument (Roche) following the protocol for unidirectional sequencing. The manufacturer's protocols for amplicon library preparation (June 2013), emulsion PCR, and emulsion breaking (May 2011) were followed. Target-specific primers (Table 4) amplified a 16S rRNA gene fragment covering the variable regions V1-V5 in the initial PCR. MID sequences were added only to the forward primer. FastStart™ High Fidelity PCR System (Roche) was used with 0.2 µM of each primer, 0.2 mM dNTPs and 0.3% BSA. For the archaeal 16S rRNA PCR 8% DMSO was added. 40 pg·µl^{-1} DNA for bacterial 16S rRNA or 1.2 ng·µl^{-1} for archaeal 16S rRNA gene amplification was added as template. PCR was initiated by a

heating step at 95°C for 5 min followed by 25 (bacterial 16S rRNA gene), respectively 30 (archaeal 16S rRNA gene) PCR cycles (1 min, 95°C/1 min, 50°C/1 min, 72°C) and finalized with a step at 72°C for 10 min. The amplicons were purified by NucleoSpin® Gel and PCR Cleanup Kit (Macherey-Nagel) and quantified by Quant-iT TM™ PicoGreen® dsDNA Assay Kit. The average fragment size was determined by the Agilent 2100 bioanalyzer. The amplicon libraries were unidirectionally sequenced (Lib-L) according to the manufacturer's sequencing method manual (June 2013). 91724 (178911) raw reads were obtained from the archaeal (bacterial) sequencing runs corresponding to 4064-9592 (6695-12854) reads per sample.

2.8.2 Sequencing by Bridge Amplification on MiSeq© (Illumina)

A second method separates single DNA molecules by binding them covalently to a flow cell prior to bridge amplification, which leads to clonal clusters (Illumina, CA, USA). Differently to pyrosequencing, all four nucleotides are added simultaneously during the sequencing. They are linked to different fluorogenic labels that are detected after each single nucleotide incorporation.

84 CsCl-density gradient fractions from rhizosphere DNA samples of the DNA-SIP experiment (PUB V, Table 2, section 2.2.2) were subjected to bacterial 16S rRNA gene sequencing by Illumina technology on the MiSeq® instrument. Manufacturer's protocol 'Metagenomic Sequencing Library Preparation' (Part # 15044223 Rev. B) was followed. First, variable regions V1-V2 of bacterial 16S rRNA gene were amplified in a PCR with 0.2 µM of each primer (Ba27f and 357R; Table 4) using 40 $pg \cdot \mu l^{-1}$ DNA template and NEBNext® High-Fidelity 2X PCR Master Mix (NEB, Frankfurt am Main, Germany). In triplicates, PCR was run with 27 cycles (10 s – 98°C/30 s – 60°C/30 s – 72°C). The amplicons were purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and size-separated by Agilent 2100 bioanalyzer technology (section 2.5). The Nextera® XT Index Kit v2 Set B was used for barcoding of the samples in a second PCR with 400 $pg \cdot \mu l^{-1}$ DNA template. The removal of primer dimers was achieved by excision and purification of the PCR product from agarose gels. After size determination and DNA quantification, the samples were pooled to 4 nM, each. The 'MiSeq® Reagent Kit V3 reagent Preparation Guide' (Part # 15044983 Rev. B Oct. 2013) and 'Preparing Libraries for sequencing on the MiSeq®' (Part # 15039740 Rev. D Oct. 2013) protocol were followed for preparation of the library to run. 10 pM DNA, spiked with 10% PhiX was loaded on the flow cell. A total of 11618658 raw reads were obtained corresponding to 59528-285456 reads per sample.

2.8.3 Bioinformatic Processing of Sequencing Data

Pyrosequencing data were initially processed by the gsRunProcessor v2.9 and further by the mothur software (release v.1.33.0; Schloss, 2009; Schloss et al., 2009) using the 454-SOP developed by Schloss et al. (2011): Raw sff files were initially used to generate flow files, which were trimmed within the range of 360-1050 flows and further processed to fasta files. The fasta reads were filtered to a minimum length of 150 nt and unique sequences were aligned against the SILVA reference file (release 119; Quast et al., 2013), which includes both, archaeal and bacterial 16S rRNA sequences. Aligned reads were filtered for 99% of the reads optimized for the start end of the alignment and pre-clustered. Chimeras were identified with the SILVA reference file and removed. Sequences were classified at 80% confidence level with the reference files for the RDP database (release 10; Cole et al., 2014). Mitochondrial, chloroplast, eukaryotic and unknown sequences were removed as well as bacterial or archaeal sequences for the archaeal or bacterial dataset, respectively. Distances between aligned sequences were used for clustering the sequences to OTUs with the furthest neighbor method utilizing a 0.1 cutoff. OTUs were classified at a cutoff of 80% and at 3%, 5%, or 10% dissimilarity corresponding to 97%, 95%, or 90% similarity levels. As the 'species' definition of prokaryotes at 97% similarity is a controversial topic and RDP database classifies OTUs only to the genus level, all other analyses were performed on 95% similarity level, which furthermore reduces the effect of sequencing errors. Reads from single samples were subsampled to the minimum group size (3081 for archaeal and 4815 reads for bacterial dataset). OTU abundance matrices were used for further graphical and statistical analyses (section 2.9).

The same processing was done for the MiSeq® data from the DNA-SIP experiment for bacterial 16S rRNA genes (PUB V) with following adjustments on the bases of the MiSeq® SOP by Kozich et al. (2013): Fastq raw data of paired end sequencing were initially combined to obtain contigs and subsequent fasta reads. The reads were trimmed to a length between 300-390 nt and aligned against the SILVA reference dataset, which was pre-trimmed within the positions 1044-6389 according to the used target-specific primers. Chimeras were found group-wise and against the data themselves. Subsampling was done with the minimum sample size of 30256 reads.

Raw sequencing data obtained for PUB IV (pyrosequencing of bacterial and archaeal 16S rRNA gene amplicons) are stored under the accession number SRP101445 (BioProject PRJNA293151) at the GenBank's Short Read Archive (SRA). Accordingly, MiSeq® raw sequencing data of the DNA-SIP experiment (PUB V) are stored under the accession number SRP101445 (BioProject PRJNA378229).

2.9 Statistics and Data Evaluation

Statistics and graphical illustrations shown here were obtained with R software (version 2.15.1 or 3.0.2; R Core Team, 2013).

2.9.1 Univariate and Multivariate Statistical Analyses

To determine significant differences and interactions for single variables (PUB I-V), univariate ANOVA (R package 'stats'; R Core Team, 2013) followed by post hoc Tukey's HSD test (R package 'agricolae'; de Mendiburu, 2015) with a significance level $\alpha = 0.05$ was applied. qPCR data were initially log-transformed. *P* values after multiple comparisons were corrected with the Bonferroni method.
For multivariate analysis of T-RFLP and sequencing data (PUB II, IV, V), the abundance matrix was relativized and Hellinger-transformed as recommended by Ramette (2007). qPCR data (PUB II) were log-transformed and scaled. Permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001; McArdle and Anderson, 2001) was applied using the R package 'vegan' (Oksanen et al., 2015). Principal component analysis (PCA) was conducted with the euclidean distance matrix of the data. 3D plots for PCA (PUB IV) were obtained with the R package 'scatterplot3d' (Ligges and Mächler, 2003). Heatmaps (PUB IV, V) were generated on the basis of relativized data with the R package 'gplots' (Warnes et al., 2016) using the complete linkage method for clustering of the dendrograms. For other graphical display of sequencing data in circle graphs or ternary plots, the R packages 'shape' (Soetaert, 2014) and 'vcd' (Meyer et al., 2014) were used.

2.9.2 Evaluation of 16S rRNA Gene Pyrosequencing Data and Co-occurrence Analysis

Abundance matrices of OTUs based on 16S rRNA marker genes do not necessarily reflect the prokaryotic relative cell abundance, since multiple 16S rRNA gene copy numbers per genome are common and highly variable across archaeal and bacterial phyla. According to the Ribosomal RNA Database (*rrn*DB; Stoddard et al., 2014) archaea harbor 1-4 and bacteria 1-17 16S rRNA copies per genome. To account for the taxon-specific range of multiple 16S rRNA gene copies (PUB IV), abundances of OTUs were corrected to achieve a better approximation to the 'cell abundance' for archaea and bacteria. For this, the *rrn*DB reference file ('Pan-taxa statistics for RDP taxonomy'; release 4.3.3) was used that lists mean values of observed 16S rRNA gene copies per genome for archaea and bacteria at different taxonomic levels. An 'adjusted abundance' was calculated for each OTU by dividing the absolute 16S rRNA gene abundance of this OTU by the mean 16S rRNA gene copy number per genome for the corresponding genus or nearest classifiable taxon. Furthermore,

16S rRNA gene abundance determined by qPCR was corrected in every sequenced sample to achieve a better estimate of the total prokaryotic cell abundance.

A soil-intrinsic bacterial core microbiome was identified based on samples from bulk soil, drilosphere, and rhizosphere of topsoil and subsoil. Only OTUs with reads in at least 2 of the 3 biological replicates for each soil compartment were included. Furthermore, OTUs with a variance higher than the mean relative abundance over all 18 samples were excluded.

To search for putative interactions between soil bacteria, a co-occurrence network was estimated for bacterial OTUs. For this analysis, only OTUs with >5 reads in at least 3 samples (n=18) were included. Co-occurrence between any pair of OTUs was defined by a significant correlation (P<0.05) with a correlation coefficient >0.6. Spurious correlations were minimized by estimating P values from Spearman's rank correlation according to the CCREPE method (Faust et al., 2012). OTUs were defined to be negatively correlated if the correlation coefficient was <−0.6. The corresponding co-occurrence network was derived by setting an edge between pairs of co-occurring OTUs. Clusters of co-occurring OTUs were defined by grouping OTUs with high intra-cluster connectivity and low connectivity to other OTU clusters. Microbial clusters were identified by using the Markov Dynamics clustering algorithm (Schaub et al., 2012) implemented in MATLAB®. This algorithm allowed the identification of clique-like communities within a continuous range of a parameter (i.e., Markov time), capturing dynamic characteristics of processes on the network. The number of clusters of co-occurring OTUs was determined by choosing a community number larger than two which had the longest stable assignment over a range of Markov time points.

2.9.3 Evaluation of Quantitative DNA-SIP Sequencing Data

Quantitative DNA-SIP enables the estimation of the actual incorporation of heavy isotope into the DNA of single OTUs, which can be expressed as atom fraction excess. Based on the work by Hungate et al. (2015) amplicon sequencing data and qPCR data for the same gene are combined, here for the bacterial 16S rRNA gene (PUB V). Following description of the calculation of the ^{13}C atom fraction excess for single OTUs is partially cited and modified according to Hungate et al. (2015). In a first step y_{ijk}, the abundance of OTU_i in each density gradient j in each fraction k is calculated (1), where f_{jk} (copies·µl^{-1}) is the 16S rRNA gene abundance in fraction k_j and p_{ijk} the relative abundance of OTU_i in fraction k_j.

(1) $$y_{ijk} = f_{jk} \cdot p_{ijk}$$ (copies·µl^{-1})

According to equation (2), the observed weighted average density for OTU_i in density gradient j is obtained from the $K = 7$ fractions in one gradient. x_{jk} (g·ml^{-1}) is the density in fraction k_j.

(2) $W_{ij} = \sum_{k=1}^{K} \left[x_{jk} \cdot \left(\frac{y_{ijk}}{\sum_{k=1}^{K} y_{ijk}} \right) \right]$ (g·ml^{-1})

The density shift for OTU_i is the difference between the observed weighted average density in the labeled sample gradients ($W_{ij}^{labeled}$) and the corresponding control gradient ($W_{ij}^{control}$) (3).

(3) $Z_{ij} = W_{ij}^{labeled} - W_{ij}^{control}$ (g·ml^{-1})

In parallel, a linear model was setup based on the three control sample gradients from the three depths. The weighted average mean density $W_{ij}^{control}$ for all OTUs, which were classifiable to the genus level, were averaged per depth and genus and plotted against the genomic GC content for the corresponding genus (n=234), which was obtained from the NCBI database (ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy/taxdump.tar.gz; 19.03.2016). Multiple GC content entries for single genera in the NCBI database were averaged in advance. From the linear model, GC content was calculated for OTU_{ij} in the control gradients (4).

(4) $GC_{ij} = \frac{W_{ij}^{control} - 1.66979}{0.00036}$

Based on the molecular weight of single nucleotides in the DNA ($M_A = 312.198$, $M_C = 288.174$, $M_G = 328.198$, $M_T = 303.185\ \mathrm{g \cdot mol^{-1}}$), relative GC content can be used to calculate the average molecular weight of a nucleotide in the DNA of OTU_i in the control samples.

(5) $M_{ij}^{control} = GC_{ij} \cdot \left(\frac{M_C + M_G}{2} \right) + \left(1 - GC_{ij} \right) \cdot \left(\frac{M_A + M_T}{2} \right) = GC_{ij} \cdot 0.494 + 307.692$

The final estimation of the ^{13}C atom fraction excess for OTU_i derived from following considerations: Each additional neutron increases molecular weight by 1.008665 g·mol^{-1}. Natural relative abundance of ^{13}C isotope is 1.111233 atom-%. Nucleotides A, G, and T contain 10 carbon atoms, whereas C contains only 9 carbon atoms. The average carbon content per nucleotide is therefore dependent on the GC content. When all ^{12}C carbon atoms are replaced by ^{13}C isotope (= 100 atom-% ^{13}C), a theoretical maximum average molecular weight of nucleotides (6) can therefore be expressed as follows.

(6) $M_{ij}^{\max\ 13C} = M_{ij}^{control} + 1.008665 \cdot \left[(1 - 0.01111233) \cdot \left(-0.5 \cdot GC_{ij} + 10 \right) \right]$

Given that the density shift Z_{ij} related to the control weighted average density ($W_{ij}^{control}$) equals the molecular weight increase related to the control molecular weight of the OTU_i ($M_{ij}^{control}$), molecular weight of OTU_i in the labeled samples is calculated according to equation (7).

(7) $M_{ij}^{labeled} = \left(\frac{Z_{ij}}{W_{ij}^{control}} + 1 \right) \cdot M_{ij}^{control}$ (g·mol^{-1})

Finally, ^{13}C fractional abundance for OTU_i is calculated as stated in equation (8), where relation of molecular weight in labeled, unlabeled DNA and theoretical maximum is the same as for the fractional abundance of the ^{13}C isotope.

$$A_i = \frac{M_{ij}^{labeled} - M_{ij}^{control}}{M_{ij}^{max\ 13C} - M_{ij}^{control}} \cdot (100 - 1.111233) \quad \text{(atom-\%)} \tag{8}$$

The extension of atom fraction excess values below '0' was taken as uncertainty range also for the positive measurements. Above this threshold, ^{13}C enrichments were considered as confident.

3. Summary of the Publications and Contributions

PUBLICATION I

Abundance of ammonia oxidizing microbes and denitrifiers in different soil horizons of an agricultural soil in relation to the cultivated crops

Doreen Fischer, Marie Uksa, Wolfgang Tischler, Timo Kautz, Ulrich Köpke, Michael Schloter

Brief description:

Nitrifying and denitrifying communities were examined in the top- and subsoil of an arable soil, where three plant species of varying root morphology and nitrogen uptake strategy grew: a grass with a fibrous root system and two plants with a taproot system, of which one is a legume. The abundancies of marker genes for ammonia oxidizers (archaeal and bacterial *amoA*), two types of nitrite oxidizers (*nirK* and *nirS*), and nitrous oxide oxidizers (*nosZ*) were assessed through qPCR. Overall, a significantly lower potential for nitrification and denitrification was found in subsoil compared to topsoil irrespectively of the plant species. This was shown both for absolute and relative gene abundances demonstrating not only a biomass effect but that the fractions of nitrifiers and denitrifier decrease within the microbial community with depth. Even for the legume, which evidently caused an increased nitrate and ammonia concentration in subsoil, the gene abundances decreased similarly to non-legumes. However, increased ratios of AOA to AOB and *nirK* to *nirS* revealed different ecophysiological strategies within nitrifiers and denitrifiers in the subsoil, where nutrients are limited.

Contributions:

- performed the statistical analysis and provided the figures
- contributed to the manuscript and revision

Biology and Fertility of Soils 49, 1243–1246, 2013. doi: 10.1007/s00374-013-0812-8

PUBLICATION II

Community structure of prokaryotes and their functional potential in subsoils is more affected by spatial heterogeneity than by temporal variations

Marie Uksa, Doreen Fischer, Gerhard Welzl, Timo Kautz, Ulrich Köpke, Michael Schloter

Brief description:

The bacterial community structure as a whole and selected functional groups were studied with respect to their spatial and temporal variability. We investigated the bulk soil, drilosphere, and rhizosphere of top- and one subsoil horizon during the development of three different plant species from their early vegetative growth phase until flowering. The bacterial community was analyzed by the T-RFLP fingerprint technique; nitrifiers and denitrifiers were quantified by marker genes: *amoA* (AOA and AOB), *nirK*, *nirS*, and *nosZ*. While in bulk soil a drastic change of the bacterial community structure and a decrease in richness and functional potential of nitrifiers and denitrifiers was observed between topsoil and subsoil, the depth effect in the hotspots drilosphere and rhizosphere was much less pronounced. Overall, the temporal fluctuations during plant development were far less reflected in the variation of bacterial community structure and functional potential than soil depth and compartment type. Nevertheless, the plant species was the most influential factor for the bacterial community in the rhizosphere indicating the strong interactions between the plant and the soil microbiome irrespective of soil depth.

Contributions:

- performed DNA extraction, qPCR, and T-RFLP
- conducted statistical analysis, data interpretation and did graphics
- wrote the manuscript

Soil Biology and Biochemistry 75, 197–201, 2014. doi: 10.1016/j.soilbio.2014.04.018

PUBLICATION III

Spatial variability of hydrolytic and oxidative potential enzyme activities in different subsoil compartments

Marie Uksa, Michael Schloter, Timo Kautz, Miriam Athmann, Ulrich Köpke, Doreen Fischer

Brief description:

Extracellular enzymes are produced by microorganisms to acquire nutrients (C, N, and P) from polymeric compounds. Two classes, hydrolytic and oxidative enzymes, were measured in bulk soil and the hotspots drilosphere and rhizosphere in top- and subsoil of an agricultural field site. Hydrolytic enzymes responsible for the degradation of cellulose, hemicellulose, chitin, and organic phosphomonoesters were found to be more active in topsoil and hotspots, which correlates with the high nutrient deposition by plants and animals. The vanishing depth effect in the rhizosphere can be explained by root exudation and strong interactions between microbes and plants in the frame of plant growth promotion. Remarkably, the potential phosphomonoesterase activity is highest in the rhizosphere subsoil demonstrating the high demand for P of microbes and plant roots. Peroxidase, which is needed for lignin degradation, increases in subsoil irrespectively of the soil compartment, because complex SOM is one of the primary carbon sources in this oligotrophic environment and its degradation requires oxidative enzymes. Therefore, the spatial separation of distinct enzyme classes suggests a likewise spatial separation of microbes with oligo- and copiotrophic life strategies.

Contributions:

- contributed to the planning of the experiment
- performed sampling, DNA extraction and enzyme assays
- conducted subsequent statistical analysis and data interpretation
- wrote the manuscript

Biology and Fertility of Soils 51, 517–521, 2015. doi: 10.1007/s00374-015-0992-5

PUBLICATION IV

Prokaryotes in subsoil – Evidence for a strong spatial separation of different phyla by analysing co-occurrence networks

Marie Uksa, Michael Schloter, David Endesfelder, Susanne Kublik, Marion Engel, Timo Kautz, Ulrich Köpke, Doreen Fischer

Brief description:

The spatial heterogeneity of a prokaryotic community with diverging life strategies was investigated in subsoil and soil hotspots to identify their habitat preferences and putative interactions. With 16S rRNA gene pyrosequencing and co-occurrence network analysis, we classified the archaeal and bacterial OTUs and identified clusters of co-occurring OTUs attributing them to soil compartments. The bacterial phyla Proteobacteria and Bacteroidetes represent primarily copiotrophic bacteria and were most abundant in the bulk topsoil, rhizosphere, and drilosphere cluster. The bulk subsoil cluster, in contrast, comprised a higher abundance of the bacterial phyla Acidobacteria, Actinobacteria, Gemmatimonadetes, Planctomycetes, and Verrucomicrobia, which are putative oligotrophs. The putative copiotrophic phylum Firmicutes is more abundant in the bulk subsoil than topsoil suggesting that their capability of endospore formation is an advantage in this oligotrophic environment. The archaeal community comprises almost only ammonia oxidizers and exhibits no distinct spatial separation or habitat preference in comparison to bacterial phyla. For both, archaea and bacteria, their separation into soil compartments was less pronounced in topsoil compared to subsoil, where the soil heterogeneity is not disturbed by soil management. While the archaeal community is a staple backbone, the bacterial phyla are strongly driven by hotspots and nutrient quantity and quality.

Contributions:

- contributed to the planning of the experiment
- performed qPCR and pyrosequencing
- conducted subsequent bioinformatics and statistical analysis, except co-occurrence analysis
- conducted data interpretation
- wrote the manuscript

Frontiers in Microbiology 6, article number 1269, 2015. doi: 10.3389/fmicb.2015.01269

PUBLICATION V

Bacteria utilizing plant-derived carbon in the rhizosphere of *Triticum aestivum* change in different depths of an arable soil

Marie Uksa, Franz Buegger, Silvia Gschwendtner, Tillmann Lueders, Susanne Kublik, Timo Kautz, Miriam Athmann, Ulrich Köpke, Jean Charles Munch, Michael Schloter, Doreen Fischer

Brief description:

The rhizosphere is an outstanding hotspot in subsoil, where root exudates highly influence the interaction between soil, plants, and microbes. Here, we investigate the bacterial community that utilized plant-derived organic carbon from *T. aestivum* in top- and subsoil. After labeling of the plant with ^{13}C-CO_2 bacteria involved in the C turnover were identified and classified with quantitative DNA-SIP and NGS. Besides significant differences in the bacterial community composition, also the active key players changed along soil depth in the rhizosphere, which can be explained by different root exudation quality and quantity, as well as depth dependent indigenous bulk soil microbiome. Proteobacteria were abundant and utilized the plant-derived carbon especially in the topsoil. In the upper subsoil rhizosphere, Actinobacteria seem to have a competitive advantage with respect to carbon utilization, although the overall carbon assimilation into the microbial biomass was lowest at this soil depth. Instead, Firmicutes and Bacteroidetes are important key organisms in carbon turnover of the lower subsoil rhizosphere, which has been overseen in previous studies. Plant growth promotion abilities and specific life strategies of soil bacteria determine their activity in the rhizosphere along soil depth.

Contributions:

- contributed to the planning of the experiment
- performed sampling, qPCR, T-RFLP, DNA-SIP and NGS
- conducted subsequent quantitative DNA-SIP analysis, statistics, and data interpretation
- wrote the manuscript

Environmental Microbiology Reports 9, 729–741, 2017. doi: 10.1111/1758-2229.12588

4. Discussion

4.1 Depth Effects in Bulk Soil

Soil profiles are characterized by numerous varying physical, chemical, and biological properties throughout the depth gradient. Early studies described rapid decreases in organic matter, microbial biomass, diversity, and activity below 30 cm in diverse soil ecosystems, including forests, grasslands, and arable soils. In this respect, soil habitats shape their microbial communities and microorganisms influence their surrounding environments.

In this chapter, we discuss the depth effect on microbial communities in a loess-derived Haplic Luvisol that has been conventionally cultivated for the past decades. Note that we sampled undisturbed subsoils, where soil structure, aggregation, transport processes, and natural root growth and development were retained (Luster et al., 2009; Salome et al., 2010; Han et al., 2015b).

4.1.1 Reduction and Change in the Prokaryotic Community in Subsoil

Microbial biomass is one of the fundamental soil properties used to assess soil quality and performance. It consists of archaea, bacteria, and fungi, and decreases with soil depth. We confirmed this on the basis of 16S rRNA gene abundances used as proxies for bacteria and archaea, in agreement with Eilers et al. (2012). The most obvious reason for the observed reduced microbial abundances is that organic matter and nutrient inputs into the subsoil are low, due to consumption or degradation of easy available C sources in the topsoil by microbes, and slow nutrient transport processes through the bulk soil. We sampled a Haplic Luvisol with a high clay accumulation in its subsoil; clay is known to exhibit high sorption capacity.

We were able to demonstrate that total microbial biomass decreased with depth and that diversity of bacteria and archaea were reduced as well. This agrees with results of Eilers et al. (2012) and Stroobants et al. (2014).

We found an increase in the archaeal to bacterial ratio in the nutrient depleted subsoil, which, although not highly pronounced, was similar to the findings of Eilers et al. (2012). This indicates a difference in the ecophysiological strategies of archaea and bacteria, in that they can adapt more successfully to environments with insufficient nutrients (Sims et al., 2012). This significant change in the prokaryotic community extends to the phylum level. Our sequencing revealed higher proportions of Actinobacteria, Firmicutes, Nitrospirae, and Verrucomicrobia in subsoil, whereas Acidobacteria, Proteobacteria, and Chloroflexi favored topsoil. Such phylum based community shifts have also been

described by Bergmann et al. (2011) and Eilers et al. (2012) and can be explained by differences in physiologies and life strategies, which will be addressed in section 4.3.

Another factor influencing microbial community structure is soil pH. Fierer et al. (2009b) and Lauber et al. (2009) demonstrated the importance of pH to the bacterial community at a global scale. With pH = 6.5 in the topsoil and pH = 7.0 in the subsoil at our site, the studied communities are not exposed to extremes in pH, not even a steep pH gradient between soil horizons. Nevertheless, Acidobacteria were reported to favor slightly acid conditions (Jones et al., 2009) and are present at our study site in higher abundance in the topsoil.

The distribution of small-size pores combined with soil moisture and oxygen availability, are key differentiators, besides nutrients, of microbial niche separation in bulk soil. Cell size restricts certain pore volumes and aggregates from colonization (Ruamps et al., 2011; Portillo et al., 2013). If the moisture level is too high, oxygen diffusion is limited, promoting anaerobic conditions; this takes on added importance under conditions of low soil porosity. If moisture is too low, nutrient diffusion and microbial activity in general are limited (Or et al., 2007). At our study site, the water table does not reach the investigated subsoil horizon. Therefore, overall anoxic conditions are unlikely, except in micro-niches within soil aggregates (Sexstone et al., 1985). Heavy rainfall events, which could temporarily cause waterlogging conditions such as those in wetlands and rice paddy fields, were negligible during our study period. Intensive soil management and the use of machinery can cause soil compaction and form plow pans below the plow depth, both of which contribute to waterlogging and anoxic conditions (Nawaz et al., 2013; Alaoui et al., 2018). Although soil management was undertaken at our study site, only a minor plow pan was observed. Nonetheless, methanogenic archaea were detected in higher abundance in topsoil, indicating the importance of micro-niches in aerobic soils (Shrestha et al., 2011; Angel et al., 2012).

Patchy distribution of nutrients and microbial micro-niches are characteristic of soils and increase in unhomogenized bulk subsoils (Schnecker et al., 2014); our studies often reflected this as seen in a greater variance in community composition in subsoil as compared to topsoil. In subsoils, nutrient turnover processes are therefore highly dependent on the co-localization of microbes and substrate (Nunan et al., 2001; Pinheiro et al., 2015; Preusser et al., 2017).

4.1.2 The Diminished Microbial Potential for Nutrient Cycling in Bulk Subsoil

Potential extracellular enzyme activities are indicators of turnover rates. The absolute activities of hydrolytic enzymes, which are involved in cellulose, hemicellulose, and chitin degradation, and the activity of phosphomonoesterase rapidly decrease with soil depth. This relationship is well known (Kramer et al., 2013; Stone et al., 2014) as it is primarily linked to the decreasing quantity of microbial

biomass. However, the specific hydrolytic enzyme activity per microbial biomass increases with soil depth for β-glucosidase, xylosidase, and phosphomonoesterase, indicating a high demand for C and P in subsoil. Linking extracellular enzyme activity solely to the biomass present can be misleading since enzymes can persist due to binding to SOM and clay, which prevents their leaching and decay (Paul, 2006; Nannipieri et al., 2012). Therefore, the activities of extracellular enzymes can be stable for weeks to months (Schimel et al., 2017), and this applies especially to clay-rich soils such as those at our study site.

Furthermore, oxidative enzymes are required for the degradation of complex organic matter such as lignin (Nannipieri et al., 2012). In this study, we measured phenol oxidase and peroxidase. While the phenol oxidase activity slightly decreased with depth in bulk soil, in agreement with previous studies (Schnecker et al., 2015), peroxidase seemed to be especially important in subsoil (Herold et al., 2014). Lack of labile organic substrates likely induces production of peroxidase to make the carbon of complex compounds available; it does this by improving the accessibility of reducing sugars and amino acids (Burns et al., 2013; Tian and Shi, 2014). However, we cannot exclude additional purposes of peroxidase activity; e.g., as an oxidative stress response (Sinsabaugh, 2010).

As both bacteria and fungi excrete enzymes to acquire C, P, and N, we cannot distinguish the origins of the activities measured (Mawdsley and Burns, 1994; Nannipieri et al., 2012). In the case of peroxidase though, we know that white rot fungi are the main source. As for phosphomonoesterase, bacteria, fungi, archaea, and even plant roots contribute to production of this enzyme (Ragot et al., 2015).

To differentiate between groups of organisms, functional marker genes can be targeted through the nucleic acid based analysis known as qPCR. This has successfully been established for processes of the P- and N-cycle (Bannert et al., 2011; Bergkemper et al., 2016). However, the development of group-specific primers for cellulose degradation as part of the C-cycle is challenging due to broad phylogenetic distribution, unspecific enzymes, and gene sequence diversity (de Vries et al., 2015). Here, unspecific enzymes describe enzymes with a broad substrate spectrum; e.g., β-xylosidase from *Aspergillus japonicus* can also exhibit β-glucosidase and α-L-arabinofuranosidase activities (Wakiyama et al., 2008).

For the two main pathways in the N-cycle, nitrification and denitrification, gene abundances for key enzymes were quantified via qPCR. With respect to soil depth, archaeal and bacterial *amoA*, *nirK*, *nirS*, and *nosZ* gene abundances decreased in subsoil. This decrease was again strongly linked to microbial biomass; however, when gene abundance was related to DNA content, we still found a lower proportion of the community capable of either nitrification or denitrification. A plausible

explanation is that there was simply less ammonium and nitrate in subsoil compared to topsoil (PUB I).

Overall, we would expect that nitrifiers are less affected by low organic carbon content in subsoil due to their autotrophic metabolism (Xia et al., 2011). In turn, many denitrifiers are heterotrophs and facultative anaerobes (Zumft, 1997). The former would restrict them more successful growth in topsoil, whereas the latter would have a growth advantage in anoxic subsoils or micro-niches. Note, however, that oxygen limitation in subsoil seemed not to be a major issue at our study site.

However, different carbon preference in subgroups of nitrifiers and denitrifiers seem to be reflected in the measured gene abundances: the ratio of AOA to AOB was higher in subsoil; likewise the ratio of *nirK* harboring denitrifiers to the *nirS* group was also higher in subsoil. In the literature, higher AOA to AOB ratios are described (Leininger et al., 2006; Hai et al., 2009), especially for carbon poor sites; although the mixotrophic life strategies reported for AOA (Tourna et al., 2011; Hatzenpichler, 2012) should also give them a competitive advantage in carbon rich sites. As AOB were found to be highly dependent on high ammonia concentrations (Wertz et al., 2012; Ke et al., 2013), this might explain their low abundance observed in the ammonia and carbon poor subsoil. We only measured genetic potential, which implies but is not necessarily correlated to the contribution of AOA to ammonia oxidation. Studies by Glaser et al. (2010) and Jia and Conrad (2009) showed that AOA outcompete AOB in *amoA* gene abundance, although they are significantly less involved in ammonia oxidation.

Weier et al. (1993) found a significant increase in denitrification with increased nitrate concentration, but also inhibition of the conversion of N_2O to N_2. Thus, we would expect a higher *nirS*+*nirK* to *nosZ* ratio in nitrate rich compartments. However, the ratio of nitrite reducers to N_2O reducers did not differ between the different soil depths, although nitrate concentrations were far lower in our subsoil. Again, measuring potential activities or gas fluxes would better represent denitrification rates than do gene abundances (Jahangir et al., 2012).

The ratio of *nirK*- to *nirS*-harboring nitrite reducers increased with soil depth. Previous studies have isolated both groups from different environments, which vary in organic carbon content, oxygen status, and pH, and established that *nirK* outcompetes *nirS* at carbon poor sites (Priemé et al., 2002; Sharma et al., 2005; Kandeler et al., 2006; Jones and Hallin, 2010). As is the case with nitrifiers, varying ecophysiological strategies determine abundances of subgroups of nitrite reducers in subsoil.

We have focused on the bulk soil thus far, but now we turn to the impact of biopores and hotspots in subsoil and the ways in which microbial communities and their functional traits change with depth in drilosphere and rhizosphere compartments.

4.2 Hotspots Shaping the Microbial Community and Activity in Subsoil

As outlined in chapter 4.1, nutrient limitation in subsoil is one of the main constraints on microbial biomass. Due to earthworm activity and root growth, topsoil and subsoil can be linked through biopores. These biopores make possible more rapid translocation of nutrients from topsoil to subsoil via convective transport in macropores (Massey et al., 2013). Direct nutrient deposition occurs by means of earthworm casts and root exudates, leading to the development of the drilosphere and rhizosphere hotspots (Grayston et al., 1997; Don et al., 2008; Kuzyakov and Blagodatskaya, 2015).

4.2.1 Weaker Depth Effect in the Drilosphere through Earthworm Activity

In topsoil, higher nutrient content along earthworm burrows promotes the growth of microbes, resulting in slightly higher DNA content, functional gene abundances, and activities of several hydrolytic enzymes in comparison to the bulk soil; this was shown in PUB II and PUB III. This agrees well with the results obtained by Marhan et al.(2007) and Don et al. (2008).

In bulk soil, we saw a sharp decrease in microbial biomass and functional traits with depth (PUB I, II). In the drilosphere, the steepness of this decrease was less, but the depth effect between drilosphere of the topsoil and that of the lower subsoil was still significant. However, as stated in hypothesis H1, the difference between bulk subsoil and subsoil drilosphere was much more distinct compared to topsoil, since the steady vertical transport of litter and earthworm casts (Blouin et al., 2013) resulted in an almost homogeneous drilosphere habitat.

As an exception to the depth effect, we did not find a significant depth difference in phosphomonoesterase, as described in PUB III, which indicates that there remains a need for P in subsoil. This is supported by the slight increase in specific phosphomonoesterase activity in the subsoil drilosphere. Interestingly, peroxidase activity increased with soil depth at a level comparable to that observed in bulk soil, contradicting our assumption that this enzyme was promoted by the low organic carbon content in bulk subsoil. There was fresh and labile organic matter input into the subsoil drilosphere; the function of the measured peroxidase activity is therefore a question requiring detailed further investigation. Wolters et al. (2000) concluded that high specific peroxidase activity in connection with earthworm activity drives humification and SOM stabilization. Possibly the age and utilization frequency of the sampled burrows was another factor contributing to the measured peroxidase activity. The slower turnover rates of earthworm burrows in subsoil compared to topsoil also should be considered.

In PUB IV, we looked more closely into the prokaryotic community through NGS and co-occurrence analysis; this indicated a mostly depth independent specific drilosphere community, composed

mainly of Actinobacteria, Bacteroidetes, Proteobacteria, Acidobacteria, and Verrucomicrobia. Bacteroidetes and especially the genus *Flavobacterium* are abundant bacteria that have been reported to be key organisms in the earthworm and their casts, and in animal gut systems in general (Thomas et al., 2011; Aira et al., 2015). At the phylum level, the diversity of the drilosphere microbial community is lower than in bulk soil, indicating an enrichment of a specific microbiome. This specific drilosphere microbiome results from passage of litter and soil through the gut and concomitant enrichment with earthworm gut-specific microbes. However, distinct microbial communities have been found in earthworm guts, casts, and the drilosphere (Furlong et al., 2002; Kumari et al., 2012; Dallinger and Horn, 2014). Therefore, the question arises as to which of the drilosphere bacteria were acquired from the burrow-surrounding bulk soil, and which originated from species-specific gut microbiomes. For example, in our data, the presence of Nitrospirae in the subsoil drilosphere was likely a bulk soil-derived phylum and was not enriched during gut passage. Methanogenic archaea are important members of diverse animal gut systems (Moissl-Eichinger and Huber, 2011), although a drilosphere specific methanogen community was not evident in our studies. Drake and Horn (2007) confirmed that methanogens are indeed not key members of earthworm gut systems.

Overall, the shared microbiome between bulk soil and drilosphere is higher in topsoil compared to subsoil. Remarkably, in PUB II and III, the drilosphere bacterial community was extremely heterogeneous, for several possible reasons. Information on the age of earthworm burrows and frequency of their use at the time of sampling was not available, but these factors affect the microbial community (Tiunov and Scheu, 2000). Some burrows sampled could have been blocked by casts and therefore disconnected to the pore system (Pagenkemper et al., 2015). In other burrows, roots had grown in, which could have led to a mixing of the drilosphere and rhizosphere microbiomes. In addition to the anecic earthworm species *Lumbricus terrestris*, the endogeic species *Allolobophora calliginosa* was present (T. Kautz, personal communication) and contributed to the sampled topsoil drilosphere.

Sampling of the drilosphere was done on plots with *F. arundinacea*, *C. intybus*, or *M. sativa* growing (PUB II). Although an effect on earthworm gut community composition can result from their feeding on litter from different plant species (Knapp et al., 2009), in our experiment plant species had no definite effect on the drilosphere community. Studies in which samples were taken directly before and after mowing or plowing would be needed to support or reject our assertion.

In summary, earthworm burrows can counteract depth effects and result in drilosphere hotspot in subsoil, confirming hypothesis H1. This is plausible, as frequent nutrient inputs from fresh organic matter occur in the burrows.

4.2.2 Weakest Depth Effect in the Rhizosphere through Root Exudation

Similar to what was observed in the drilosphere, PUB II showed that DNA content and functional gene abundances were higher in the rhizosphere than in bulk soil and that the depth effect was less pronounced or even absent. Root exudation increases the availability of labile organic compounds (Lynch and Whipps, 1990; Haichar et al., 2014) that promote microbes not only in topsoil but also in subsoil. In comparison with the drilosphere, the hotspot effect of the rhizosphere is even higher. This can be explained by the continuous influence of the root on surrounding soil in contrast to intermittent cast deposition and usage of earthworm burrows, and differences in the quality of organic carbon released. In particular, the hydrolytic enzyme activities investigated in PUB III were up to two orders of magnitude higher than in bulk soil and drilosphere, an observation also described by Ai et al. (2012) and Brzostek et al. (2013).

When different activities across soil depth were considered, an interesting observation occurred in our soil profile: Hydrolytic enzyme activities were lowest in the upper subsoil, whereas in the lower subsoil they were as high as in topsoil. High activities in the lower subsoil rhizosphere can be explained by the fact that root exudation is likely to be highest there, because the exudation rate is highest in young roots and root tips (McCully and Canny, 1985), which dominate in the lower subsoil. In the case of phosphomonoesterase, we observed a continuous increase in its activity with soil depth in the rhizosphere. The enzyme activity of phosphomonoesterase contributed by plant-derived acid phosphomonoesterases reflected the high demand for P by microbes and plants in lower subsoil. It has been recognized by Kautz et al. (2013a) that subsoil is mainly a source for P and less for C and N. As described for bulk soil and the drilosphere, the role of peroxidase activity increase in the subsoil rhizosphere has yet to be evaluated. Overall, the rhizosphere is a “super-hotspot” with respect to microbial activity in subsoil.

Plant species significantly shapes the bacterial community in the rhizosphere and is even more influential than soil depth, as stated in hypothesis H1a. In PUB II, IV, and PUB V, different communities were found in association with *F. arundinacea*, *M. sativa*, *C. intybus*, and *T. aestivum*. For the latter two, NGS data was available, indicating the highest abundances of Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes at the phylum level. The role of those groups in PGP is widely accepted (Garbeva et al., 2008; Haichar et al., 2008, 2012; Buée et al., 2009; Hartmann et al., 2009) and involves interactions of rhizosphere microbes with each other and plant roots. This includes nutrient acquisition from the surrounding bulk soil through extracellular enzymes, organic acids, siderophore production (Aranda et al., 2011; Ofek et al., 2012; Madhaiyan et al., 2013; Lampis et al., 2015), hormonal stimulation (Kuffner et al., 2010; Hanak et al., 2014; Kielak et al., 2016), and biocontrol (Bashan and Holguin, 1998).

Our analysis in PUB IV identified a specific rhizosphere cluster of co-occurring OTUs in the rhizosphere of *C. intybus*, and several negative interactions with OTUs in other soil compartments. Such co-occurrences/-exclusions imply positive/negative interactions and indicate the key organisms involved. However, the underlying mechanisms remain to be identified by other methods, such as RNA-based studies, metagenomic, metaproteomic, and metabolomic analyses, as well as classic cultivation and soil imaging techniques (Casida, 1983; Compant et al., 2005; Shih et al., 2014; Franzosa et al., 2015; Schwarz et al., 2018).

For example, Actinobacteria are known to produce secondary metabolites that inhibit the growth of other microbes (Basilio et al., 2003; Viaene et al., 2016); this is one underlying mechanism for the negative interactions we measured in our study. Interestingly, OTUs of the same genus, for example *Streptomyces*, were often negatively correlated, indicating antagonistic interactions and functional redundancy in the respective soil compartment or at depth. Production of antibiotics, in addition to competition for nutrients, is an important regulatory mechanism for controlling plant pathogens (Haesler et al., 2014; Hamedi and Mohammadipanah, 2014); also Proteobacteria, for example *Pseudomonas*, take part in this process (Haichar et al., 2012; Choi et al., 2015).

In comparison to bulk soil, archaea were not more abundant in the rhizosphere hotspot. This agrees with the fact that 95% are putative nitrifiers, which grow autotrophically or mixotrophically, and therefore cannot compete with the fast growing heterotrophs, which feed on root exudates (Tourna et al., 2011). The dominance of Thaumarchaeota/AOA in the archaeal community in soils is a common observation (Bates et al., 2011; Eilers et al., 2012).

As our studies focused on prokaryotes, the importance of fungi (Malik et al., 2015), and especially mycorrhizae (Hafner et al., 2014), could not be covered. Sosa-Hernández et al. (2017) showed that subsoil rhizosphere, drilosphere and bulk soils harbor specific AMF communities that provide plants with nutrients, especially phosphorus. The term “mycorrhizosphere” was introduced to acknowledge the importance of mycorrhizae in plant nutrition (Timonen and Marschner, 2006).

Analogous to the drilosphere, rhizosphere bacteria can come either from the surrounding bulk soil or the plant itself; i.e., seed-born origin (Philippot et al., 2013). The fact that the same plant variety can have different bacterial communities when growing in different soil types demonstrates the recruitment of microbes from the soil (Garbeva et al., 2008; Berg and Smalla, 2009). This is linked not only to the soil indigenous microbiome, but also to the varying chemical composition of root exudates as a result of different substrates (Neumann et al., 2014). Generally, one finds a functionally redundant rhizosphere microbiome for one plant species (Lemanceau et al., 2017). However, a core plant microbiome contains obligate co-existing microbes that are essential for plant development and health (Chaparro et al., 2012; Mendes et al., 2013). Therefore, they are found not only in the rhizosphere but often as root endophytes (Lundberg et al., 2012; Berg et al., 2014), which are

transferred via seeds to the next generation (Truyens et al., 2015). Differences in soil properties and root exudate qualities in top- and subsoil led, therefore, to significant differences in the rhizosphere community found in our studies. Still, top- and subsoil rhizosphere share more OTUs then bulk soil and rhizosphere in comparison.

In summary, we confirmed hypothesis H1 by showing that the depth effect in the rhizosphere for both microbial communities and their functional traits is far less distinct than that of the bulk soil. Moreover, plant species determines the rhizosphere bacterial community far more than the depth effect, as hypothesized in H1a.

Having assessed the potential of the microbial community, we now focus on the contribution of specific rhizosphere bacteria to the turnover of plant-derived carbon at different soil depths, addressing hypothesis H1b in PUB V. Using quantitative DNA-SIP of rhizosphere bacteria, we have been able to not only estimate the relative abundances of bacterial OTUs, but also to calculate the ^{13}C content in the DNA for each OTU, which correlates with the utilization of plant-derived carbon. We studied undisturbed subsoil cores, which provided a natural soil matrix for root growth.

The activity patterns of bacteria utilizing plant-derived carbon change with soil depth in the rhizosphere, and this was detectable at the phylum level. Generally, Proteo- and Actinobacteria utilize the carbon at all depths, though Actinobacteria exhibit a higher ^{13}C enrichment in the upper subsoil, whereas Proteobacteria are comparatively less enriched. Donn et al. (2015) measured a shift from Proteobacteria to Actinobacteria during *T. aestivum* development indicating a functional transition of the rhizosphere microbiome from nutrient acquisition around young roots towards biocontrol at later stages of root and plant development. In the lower subsoil, Bacteroidetes and Firmicutes play an extraordinary role in carbon turnover. Although abundant, Nitrospirae and Gemmatimonadetes did not contribute to the turnover of plant-derived carbon in the rhizosphere, and Acidobacteria only to a minor degree in topsoil. These patterns reflect the overall microbial life strategies of bacterial phyla (Xia et al., 2011) that are discussed in detail in section 4.3.

The existence of functional redundancy among bacteria, outlined throughout this section, was clear, as OTUs of the same genus, for example *Streptomyces*, exhibited diverging relative abundances and ^{13}C enrichment values at different soil depths.

The quantitative DNA-SIP method considers all relevant consecutive fractions of the DNA density gradient for NGS, not only pre-selected heavy and light fractions. Therefore, it is possible to derive a density shift in the DNA that is independent of the genomic GC content (Buckley et al., 2007; Hungate et al., 2015). Thus we can detect enriched bacteria with either high or low genomic GC content (Actinobacteria and Bacteroidetes/Firmicutes, respectively), whose abundances would not occur in the same heavy DNA gradient fraction (Neufeld et al., 2007a; Uhlik et al., 2009). In our study, we

identified Actinobacteria, Bacteroidetes, and Firmicutes for the first time as having concurrent key roles in the turnover of *T. aestivum* derived C in both subsoil and in topsoil (Haichar et al., 2008; Ai et al., 2015).

With respect to overall ^{13}C enrichment in the DNA, the subsoil rhizosphere is a key hotspot for carbon nutrient cycling. A surprising observation was the overall lower ^{13}C enrichment in the upper compared to the lower subsoil. A similar relationship was found for hydrolytic enzyme activities in the rhizosphere. Again, increased exudation by young roots at the sampling point in the lower subsoil would explain the high microbial activity there. In a study by (Mendez-Millan et al., 2012), a similar increase for wheat-derived biomarkers in lower subsoil horizons was observed.

Interpretation of DNA-SIP data is limited by the phenomenon of cross-feeding (Seth and Taga, 2014). The discrimination between primary utilizers of plant-derived carbon and secondary consumers of ^{13}C metabolites is non-trivial (Neufeld et al., 2007a, 2007c). Those that directly take up carbon substrates from the root are, in general, more likely to exhibit higher ^{13}C content in their DNA, because there is less dilution of the ^{13}C. More frequent sampling and shorter labeling periods could filter out the cross-feeding effects to some extent (Coyotzi et al., 2016). As we sampled only at one time point and considered a single plant species, a more general validation of the identities of key bacteria awaits further confirmation.

RNA based methods are more suited to reflect the active community at the sampling time point (Blagodatskaya and Kuzyakov, 2013). In order to detect the ^{13}C utilizing microbes more directly, which means not only after growth and replication, RNA-SIP is an alternative (Rettedal and Brözel, 2015; Kramer et al., 2016). Although the RNA quantity correlates with microbial activity, it is only a weak indicator of microbial abundance.

In summary, we confirmed hypothesis H1b by showing that identities of the key actors in the turnover of plant-derived carbon are distinct at different soil depths within the rhizosphere.

4.2.3 Subsoil Heterogeneity Derived from Biopore Hotspots

The last two sections emphasized the drilosphere and rhizosphere as key hotspots in the subsoil, where a distinct community is responsible for the measured high nutrient turnover and activity rates. There are earthworm-specific, plant species-specific, and subsoil-specific communities. Therefore, not only nutrient inputs matter but also the quality of substrates deposited in the subsoil (Scharroba et al., 2012).

Although we observed heterogeneity of soil microbes at different depths and in hotspots, it should be asked whether there are ubiquitous microbes irrespective of a compartment's properties. In PUB IV, we summarize these as the soil-intrinsic core microbiome (Shade and Handelsman, 2012). Through

co-occurrence and cluster analysis, we showed that the bacterial bulk topsoil community cluster matches well with the core microbiome. The community of bacteria and archaea shared between bulk soil, drilosphere, and rhizosphere compartments is larger in topsoil than in subsoil, confirming hypothesis H1. While there is a distinct compartmental specialization of bacteria, archaea exhibit a stable backbone (Bates et al., 2011) and do not respond so drastically to high nutrient content in soil hotspots (Wu et al., 2011). However, Pereira e Silva et al. (2012) detected greater temporal fluctuation in archaea compared to bacteria and fungi in response to N fertilization, and this is related to archaeal ammonia oxidation.

To definitively evaluate the overall contribution of subsoil hotspots to nutrient cycling, burrow and root densities, which distinguish the volumes of drilosphere and rhizosphere, respectively, must be taken into account. Actually, given the high root length density in topsoil it is questionable whether there is any distinct bulk topsoil which is not influenced by roots. Due to the decreased biopore and root densities in subsoil (Kautz et al., 2013b; Perkons et al., 2014), there may be much lower total turnover rates in the subsoil compared to topsoil, even if hotspots are included. Soil type, biopore density, and soil pore network connectivity determine hotspot abundance and thus the contribution of subsoil to total nutrient cycling. Heitkötter and Marschner (2018) estimated through zymography that most of the total subsoil enzyme activity is condensed in <1 to 10% of the soil volume. To estimate the hotspot volume in subsoil, modelling or 3D imaging, for example with XRCT analysis, can provide tomographic insights (Hinsinger et al., 2005; Pagenkemper et al., 2013; Schlüter et al., 2014). Unfortunately, subsequent measurement of microbial activities and community structure in these destructively sampled soil compartments cannot be done because of the impact of X-rays on microbes (Fischer et al., 2013). However, X-ray intensity and exposure time can be reduced to negligible effects (Schmidt et al., 2015).

It has been shown that environmental factors influence the soil microbial community at multiple spatial scales (Franklin and Mills, 2003; Kim et al., 2015; Ali et al., 2018). In this study, we sampled at the mm to cm scale, where the convective transport of water and nutrients as well as the presence of soil animals and roots drive both vertical and horizontal heterogeneity of microbes and their functional traits (Ettema and Wardle, 2002). Although not resolved, additional effects at the µm to mm scale, such as diffusion, microbial mobility, and cell-cell interactions, contribute to this heterogeneity (Nunan et al., 2002; Or et al., 2007). Soil type, land use, and soil management are the operative influences at large scales (Ettema and Wardle, 2002); we considered these as given system properties for measurements in our studies. Effects on biopores and heterogeneity of microbial communities in drilosphere and rhizosphere hotspots at such scales are, therefore, sources of systematic error.

All in all, carbon quantity and quality are determining factors for all microbes and at all scales, resulting in the development of diverse life strategies that are adapted to the respective nutrient regimes.

4.3 Diverging Microbial Life Strategies in Subsoils and Hotspots

The incredibly high phylogenetic diversity of soil microbes necessitates a classification based on their physiology and life strategy (Torsvik and Øvreås, 2002). The general partitioning of heterotrophic microbes into oligo- and copiotrophs describes preferences for carbon substrates of different qualities, which in turn is related to their growth rates. Autotrophic microbes, e.g., nitrifiers, which are independent of organic carbon sources, are often concomitant photo- or chemolithotroph and use other electron donors. Anaerobic conditions constrain alternative terminal electron acceptors, so that denitrifiers rely on nitrate/nitrite/N_2O. However, the production of biomass through autotrophy and/or anaerobic metabolism requires more energy than chemoorganoheterotrophy, which uses oxygen for respiration (Eitinger et al., 2007). Associated with this, they also exhibit a reduced growth rate.

At nutrient rich sites, high metabolic diversity and antibiotic production is advantageous in the competition for nutrients. Stress and fluctuating conditions lead to the promotion of tolerant and specialized groups; e.g., those that form spores to overcome dry or nutrient deficient periods.

A complete and detailed review is given in Fierer (2017).

4.3.1 Spatial Separation of Oligotrophic and Copiotrophic Prokaryotes in Subsoil and Hotspots

At the phylum level, Fierer et al. (2007) described bacteria as somewhat classify-able into oligo- and copiotrophs. β-Proteobacteria and Bacteroidetes were described as typical copiotrophs, whereas Acidobacteria are oligotrophs (Foesel et al., 2014). However, a clear correlation of Actinobacteria, Firmicutes, and α-Proteobacteria with carbon degradation was not found.

Similar to Fierer et al. (2007), we found indications of a separation of prokaryotic phyla into copio- and oligotrophs in PUB IV. We confirmed hypothesis H2a, since Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria were found to be abundant and active in hotspots and in topsoil, while Acidobacteria, Nitrospirae, Gemmatimonadetes, Planctomycetes, and Verrucomicrobia preferred bulk and subsoil. Bacteria belonging to Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria were, interestingly, easily cultivatable strains from soil samples (Janssen, 2006), which is typical for copiotrophs (Fierer et al., 2007, 2012). They grow rapidly on easily available carbon sources and exhibit a diverse metabolic potential, making them highly competitive in carbon- and

nitrogen-rich environments, not only in the drilosphere and rhizosphere (Bernard et al., 2007; Fierer et al., 2012). Bacteroidetes and Firmicutes have, in general, higher 16S rRNA gene copy numbers per genome (Stoddard et al., 2014) which enable them to grow rapidly. We know that Firmicutes and Actinobacteria can form endo- or exospores, respectively, enabling dormancy during dry and nutrient deficient periods. This may be why we find those groups so strongly involved in subsoil rhizosphere carbon turnover. Acidobacteria, Nitrospirae, Gemmatimonadetes, Verrucomicrobia, Planctomycetes, and Thaumarchaeota (all putative oligotrophs; Strous et al., 1999; Zhang et al., 2003; Bergmann et al., 2011; Davis et al., 2011), were only recognized as abundant soil microbes by NGS. Except for Nitrospirae and Thaumarchaeota (both nitrifiers), their functional roles and interactions with the other microbes remains ambiguous and is a subject of current research. Their classification into oligotrophs is supported by their low 16S rRNA gene copy numbers per genome (Stoddard et al., 2014), which also limits their maximum growth rates as compared to copiotrophs (Klappenbach et al., 2000; Davis et al., 2011; Goldfarb et al., 2011).

We found Chloroflexi predominantly in topsoil, which makes sense, as this group needs light for photosynthesis. Interestingly, they belong to the drilosphere co-occurring OTU cluster, indicating the importance of litter transport from the surface through topsoil to subsoil by earthworms.

We must use caution with the phylum based ecological classification of bacteria, which becomes apparent when looking at Actinobacteria and Proteobacteria. Both groups are composed of OTUs exhibiting highly diverse patterns of abundance and activity in different soil compartments and depths. As there is a high variance with respect to metabolisms, life strategies, and 16S rRNA gene copy numbers per genome in both phyla, they likely comprise both oligo- and copiotrophs (Thompson et al., 1992; Goldfarb et al., 2011; Mau et al., 2014).

In summary, we confirmed hypothesis H2a by finding a trend toward greater abundances of copiotrophs in hotspots in topsoil, while oligotroph abundances were greater in bulk soil and subsoil.

However, the phylum based classification from 16S rRNA gene based amplicon NGS only indicates physiology and life strategy. Some metabolic pathways, for example cellulose degradation and denitrification, are phylogenetically widely distributed (Zumft, 1997; Berlemont and Martiny, 2016). In contrast, nitrification and methanogenesis are characteristic of very specific phylogenetic groups (Woese et al., 1978; Garcia et al., 2000; Purkhold et al., 2000; Treusch et al., 2005). Even with metagenome sequencing, the functional roles of assigned genes and taxa remain to be verified by RNA-, protein- and/or cultivation-based methods (Maloney et al., 1997). Furthermore, our selection of primers did not cover all target organisms (Baker et al., 2003) and the database used is biased towards easily cultivatable strains, so we underestimated the diversity and abundance of oligotrophs in our soil. Another constraint of 16S rRNA gene based amplicon NGS is variation in the operon copy numbers per genome in prokaryotes (Stoddard et al., 2014). As a consequence, our relative gene

abundances did not necessarily reflect cell abundances, as was shown in PUB IV. This limitation means that we additionality underestimated oligotrophic strains, as they tend to harbor only one to two copy numbers. On the other hand, Firmicutes and Bacteroidetes, comprising up to 16 operon copies, were overestimated with respect to their cell abundances. Although there are biases in NGS, this method is, to date, the best for capturing the entire diverse soil microbial community.

Different sequencing technologies and algorithms for bioinformatic processing and the multitude of available databases yield different outputs concerning the lengths and error rates of sequences, OTU clustering, and classifications (Loman et al., 2012; Werner et al., 2012; Plummer and Twin, 2015). In our study, we used pyrosequencing (Roche technology), sequencing by clustering, and bridge amplification (Illumina technology) once, each of which required different target specific reverse primers. This must be kept in mind when comparing the data from publications PUB IV and PUB V. For example, Firmicutes seemed to be more important in the rhizosphere of *T. aestivum*, while their abundance is lower in PUB IV, where we applied Illumina sequencing technology. However, the reason for this could also be the different plant species (*C. intybus*) used in this experiment.

We are aware that such strict categorization into oligo- and copiotrophs is a simplification that cannot possibly do justice to the actual microbial diversity. Fierer (2017) and Malik et al. (2018) extended the bacterial separation into oligo- and copiotrophs on the basis of Grime's competitor-stress tolerator-ruderal framework applied to soil bacterial heterotrophs. Applying this concept to our phyla, we found: Actinobacteria and to some extent Proteobacteria are competitors; Acidobacteria, Gemmatimonadetes, Verrucomicrobia, and Chloroflexi are the stress tolerators; Bacteroidetes, Firmicutes, and to some extent Proteobacteria are ruderals. Using this concept, copiotrophs were separated mainly according to their ability to react to disturbances such as drying and rewetting. This framework could be extended to account for metabolic pathways by microbes involved in the N-cycle, and to characterize microbial capacities to conserve energy.

Focusing on specific microbial traits, we continue to discuss the spatial separation of microbial life strategies into soil compartments in the following sections.

4.3.2 Extracellular Enzyme Activities Reflect Microbial Life Strategies

Extracellular enzyme production is key for soil microbes to acquire C, N, and P from polymeric and complex sources (Sinsabaugh et al., 2008). In PUB III, we found a very distinct spatial separation of hydrolytic and oxidative enzymes. Hydrolytic enzyme activities were correlated with nutrient quantity and therefore were especially high in hotspots and topsoil, as also found by Sinsabaugh et al. (2008); peroxidase, however, exhibited an opposite pattern.

As discussed earlier, oxidative enzymes are a strategy for utilizing nutrients of low quality and high complexity (Kleber, 2010). Peroxidases and phenoloxidases have been shown to be involved in oxidative stress responses, synthesis of secondary compounds, and humification (Sinsabaugh, 2010). Thus, their role in SOM/lignin degradation in our subsoil remains ambiguous; compare section 4.1.2. The patterns of hydrolytic and oxidative enzyme activity we identified reflect the spatial separation of oligo- and copiotrophs and thus we can confirm hypothesis H2b.

The enzyme activities we measured may originate from different organisms; however, additional organisms can benefit from monomeric substrates that have been enzymatically cleaved off. In soil ecology, microbes that do not produce enzymes themselves but take up the enzyme products, and therefore save energy, are referred to as “cheaters” (Escalante et al., 2015). In soil, it is predicted that higher enzyme costs under conditions of nutrient limitation favor cheaters, while low enzyme diffusion rates in a heterogeneous soil matrix favor enzyme producers (Allison, 2005).

The origins of enzymes are, therefore, hard to track, but combined metagenomic, metatranscriptomic, and metaproteomic analyses can resolve the identities of key enzyme producers in soil (Nannipieri et al., 2012). This was first done with chitinase by Johnson-Rollings et al. (2014), who found only one genus responsible for overall chitinase activity in their study.

With respect to polymeric carbohydrate degradation, data obtained by metagenomic analysis has indicated distinct patterns of glycoside hydrolysis under conventional and minimum tillage conditions (de Vries et al., 2015). We therefore assume different microbial communities are responsible for enzyme production found in topsoil, subsoil, and hotspots.

4.3.3 Contrasts in Ecophysiological Strategies of Nitrifiers and Denitrifiers

Nitrification and denitrification correspond with contrasting microbial life strategies. Nitrifiers can employ autotrophic C-fixation, and they need oxygen for ammonia/nitrite oxidation. Denitrifiers, in contrast, are typical heterotrophs using nitrate/nitrite/N_2O as alternative electron acceptors in anaerobic respiration. Therefore, we assumed in hypothesis H2c that nitrifiers would favor bulk soil to avoid competition with heterotrophic denitrifiers, which in turn were expected to be more abundant in hotspots.

From the functional gene abundance data alone, we cannot conclude that there is a niche separation of nitrifiers and denitrifiers regarding distinct soil compartments or between top- and subsoil (PUB I and PUB II). This was surprising, because we found lower nitrate and ammonium concentrations in the subsoil and lower organic carbon in bulk and subsoil. Nevertheless, actual activity rates may differ from gene abundances (Subbarao et al., 2006; Xu et al., 2013). We have additional information from NGS data for nitrifiers, due to their distinct phylogenetic distribution, which can be found in PUB IV.

Typical AOB and NOB from Proteobacteria were not found in our data set at all. Thaumarchaeota (AOA) contributed more to the hotspot and topsoil microbial communities than to bulk soil or subsoil communities. The bacterial phylum Nitrospirae (NOB) was more abundant in bulk soil and subsoil, opposite that of Thaumarchaeota. A negative spatial correlation or potential niche separation for these two groups, Thaumarchaeota and Nitrospirae, was detected, and suggests spatial preferences for ammonia and nitrite oxidizers. In contrast, Stempfhuber et al. (2016) reported a positive correlation between AOA and *Nitrospira* at the plot scale. Nitrospirae, first thought to be only NOB, were recently found to perform complete ammonia oxidation to nitrate (comammox) themselves (Daims et al., 2015; van Kessel et al., 2015). If they indeed function as ammonia oxidizers, spatial separation of AOA and comammox bacteria at our site becomes plausible due to competition.

The ratio of archaeal to bacterial *amoA* was inverse that of Thaumarchaeota to putative comammox Nitrospirae, in disagreement with our 16S rRNA gene-based sequencing data. We explain this by the fact that not all Nitrospirae are targeted by the applied primers for bacterial *amoA* (Pjevac et al., 2017) and not all of them are comammox bacteria, so do not harbor *amoA* genes (Daims et al., 2015). Incorrect classification or incomplete detection of nitrifiers may also have been due to insufficient 16S rRNA gene targeting primers or incomplete databases. Nonetheless, spatial separation of AOA and AOB due to different ecophysiologies of these groups has been demonstrated (Hatzenpichler, 2012; Stempfhuber et al., 2014). Several studies have highlighted the mixotrophic life strategy of AOA that suggests their competitive advantage over AOB in hotspots and topsoil (Leininger et al., 2006; Tourna et al., 2011), which is supported by our sequencing data.

A widely discussed question is whether AOA or AOB contribute more to ammonia oxidation (Wu et al., 2011). Although the total abundance of AOA exceeds that of AOB (Leininger et al., 2006; Schauss et al., 2009), AOB is reported to be more active (Di et al., 2010).

With respect to denitrification, our discussion was restricted to functional gene abundances, as we could not name the taxa identified by 16S rRNA gene sequencing due to the wide phylogenetic distribution of denitrifiers. Different ecophysiological strategies of *nirK* and *nirS* harboring denitrifiers was evident; the ratio of *nirK* to *nirS* increased in bulk soil and subsoil, which contain less carbon. This indicates a somewhat oligotrophic life strategy by organisms harboring *nirK* strains or a more copiotrophic life strategy by those with *nirS* strains. Several studies have discussed differences in carbon requirements, oxygen level preferences, and pH among denitrifiers (Priemé et al., 2002; Yuan et al., 2012; Novinscak et al., 2013; Barrett et al., 2016). Likewise, the nitrite reductases encoded by *nirK* or *nirS* exhibit different kinetic parameters, biochemical properties, and are underlain by different expression regulation systems. Quantitative mRNA based analyses would be needed to clarify responses of specific denitrifier communities to carbon, nitrogen, and redox conditions.

We demonstrated that different groups of ammonia oxidizers and nitrite reducers exhibit contrasting preferences for subsoil and hotspots, though not as expected in hypothesis H2c and with conflicting results from functional gene abundances and high-throughput sequencing.

Here, we focused on nitrification and denitrification. Other pathways of the N-cycle, such as DNRA, anammox, or BNF were not in the scope of this work. Because anoxic conditions are limited at our site, strictly anaerobic metabolic strategies such as sulfate reduction and methanogenesis were considered to be of minor importance (Fogt et al., 2019). Nevertheless, evidence of DNRA in subsoil hotspots with low molecular weight carbon sources has emerged (Schmidt et al., 2011).

BNF is a process that enhances overall soil nitrogen availability; intercropping with legumes is therefore commonly used to counteract massive N losses in arable soils. In PUB I, we compared the performance of the legume *M. sativa* with two non-legumes and assessed their effects on the microbial community and its potential for nitrification and denitrification. We detected increased ammonia and nitrate concentrations in subsoils on plots where *M. sativa* had been growing. However, this did not increase any of the nitrification or denitrification gene abundances we investigated, contrary to our expectations. Therefore, we must reject this aspect of hypothesis H2c. In contrast, Zhao et al. (2017) reported a decreasing trend in denitrification potential with legume cropping. Numerous studies investigating nitrification and denitrification in topsoil have highlighted the importance of legumes, fertilization, and soil carbon content on nitrogen cycling communities and activities (Sharma et al., 2005; Ollivier et al., 2011). The potential of both pathways, however, does not necessarily reflect actual activity.

4.4 Plant Development Impacts Microbial Community Less than Spatial Heterogeneity

As is the case for spatial variability, temporal variation can be measured at different scales. In this study we considered the impact of plant development from the early vegetative growth phase until flowering, a duration of six weeks (PUB II). Overall, temporal fluctuations in bacterial community composition or the potential for nitrification and denitrification were minor compared with observed variability related to soil depth or compartment type.

Interestingly, when activities were considered, temporal fluctuations in microbially driven processes exceeded spatial variations, for example in the case of N_2O emissions (Imer et al., 2013) or soil enzyme activities (Spohn and Kuzyakov, 2014).

In PUB II, T-RFLP and PCA analyses of bacterial 16S rRNA genes revealed that the variance that could be explained by temporal dynamics was higher in bulk soil than in rhizosphere or drilosphere communities. Root exudation quantity and quality did not change enough that it could be detected in community structure. Meanwhile, environmental shifts, such as slight temperature and precipitation

increases in May, can help explain the variability found in bulk soil, though the subsoil community was, surprisingly, as much affected as the topsoil community. It is well known that temperature and soil moisture are major constraints on microbial activity. Therefore, seasonal changes can induce shifts in a microbial community and its active key members (Smalla et al., 2001; Kramer et al., 2013; Žifčáková et al., 2016), but which was not covered in our study.

Functional gene abundances related to the N-cycle displosed, irrespective of soil depth and compartment, a higher proportion of *nirK* in the early vegetative phase and an increase in archaeal *amoA* during flowering, but no clear trends for *nirS*, *nosZ*, or bacterial *amoA* genes (PUB II). Explanations for the observed responses are discussed in other studies, not all of them in agreement; these include seasonal changes in temperature, soil moisture, plant development, root exudation, fertilization, and agricultural management (Hai et al., 2009; Glaser et al., 2010; Pereira e Silva et al., 2012; Chaparro et al., 2013, 2014; Novinscak et al., 2013; Regan et al., 2017). All of these factors affect the nitrifying and denitrifying microbial communities differently according to their life strategies.

In summary, temporal variations during early plant development in spring are of minor importance to the bacterial community and functional genes compared with the soil compartment. Contrary to our expectations, the variations found were not higher in the rhizosphere than in bulk soil and there was no significant difference found with respect to soil depth, so we must reject hypothesis H3.

It is also the case that microbial activity and growth/abundances change at different temporal scales. While rewetting or fertilization can induce gene transcription by soil microbes within minutes to hours (up to a few days), the growth/replication/biomass response in the soil only becomes visible on a time scale of days to months (Bælum et al., 2008; Barnard et al., 2013). As a consequence, abundances of respective functional gene groups may only be detected at later time points than the hot moments of microbial activity actually occurred (Blagodatskaya and Kuzyakov, 2013). For example, increased archaeal AOA abundances during flowering could possibly reflect increased activity of AOA at an earlier plant developmental phase, rather than being triggered by flowering itself. RNA based estimates are therefore more precise temporal indicators of the activity of functionally meaningful microbial groups.

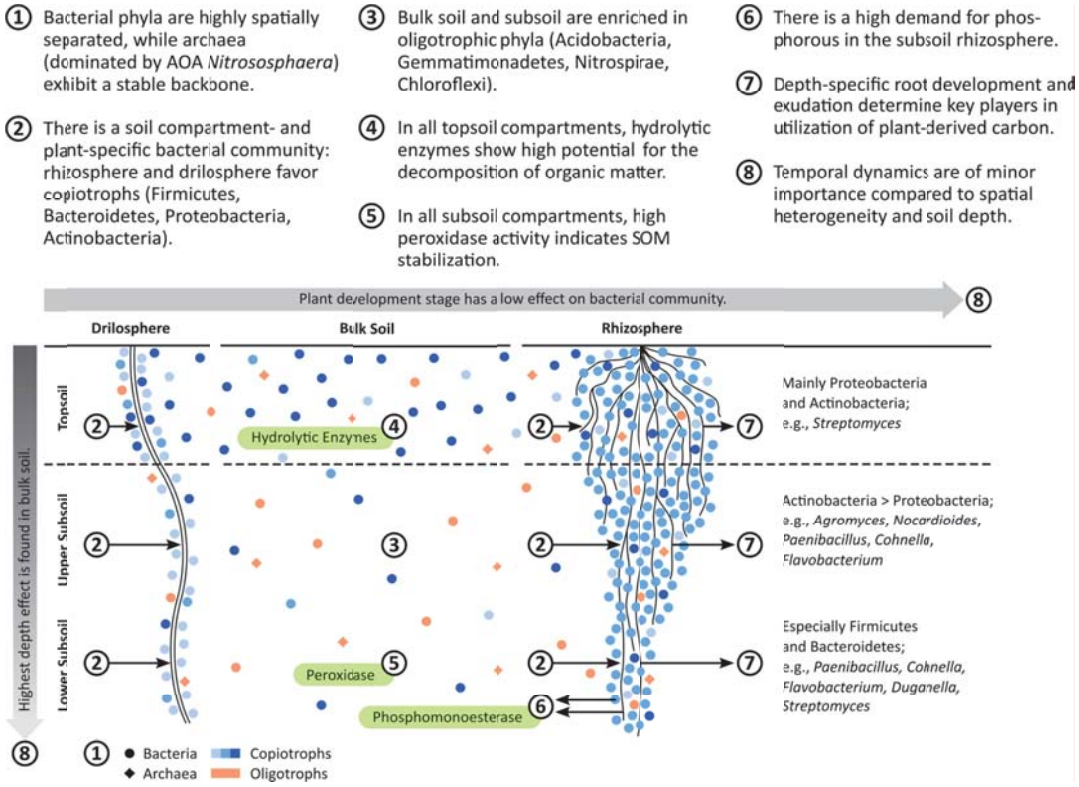

Figure 8: Summary of the key findings – microbial communities in subsoil and hotspots.

4.5 Outlook

We are aware that this work serves mainly as a case study, because just one soil type at a single site was its focus. Here, we investigated an agricultural site with fertile and relatively nutrient rich topsoil and subsoil with high clay accumulation. We found the subsoil played a significant role in nutrient cycling. It would be especially valuable to study the importance of the subsoil for plant health and crop yield when nutrient poor topsoils are cultivated, and where we would expect the subsoil to be of major importance. Studying more sandy subsoils, on the other hand, where greater leaching effects and reduced sorption capacity play a role, we would see diminished importance of subsoil.

Additionally, it is of great interest to learn whether agricultural management can and, if so, to what extent, shape the subsoil and its microbial communities over the long term (Schneider et al., 2017). The use of plants with different root systems has been shown to influence soil pore structure (Pagenkemper et al., 2013), although functional microbial traits did not appear to be much affected. One would also need to research the effects of different tillage practices and fertilization strategies, which have been, to date, restricted to topsoil and shallow subsoil studies. A special case is that of rice paddy fields, where anaerobic conditions influence the microbial community and nutrient cycling in a drastically different way.

Nonetheless, on a global scale, climatic conditions determine the role of subsoils. Microbial communities and their activities are enormously and differently influenced by the climate in deserts, tropics, and the tundra for example, while our study was conducted in a warm temperate humid climate.

It is a complex and methodologically challenging task to elucidate microbial interactions and their underlying mechanisms and drivers in the soil environment. The observed patterns of microbial abundances and activities, as well as the co-occurrence analysis, gave us a only first glimpse into this complexity. In order to deepen our understanding of mutualistic and antagonistic interactions and food web networks, other methods, such as metagenome/metatranscriptome/metaproteome/metabolome, and soil imaging, are needed (Shih et al., 2014; Franzosa et al., 2015; Haichar et al., 2016; Jansson and Hofmockel, 2018). More broadly, fungi and animals who also significantly contribute to nutrient cycling must be included (Kramer et al., 2016).

More importantly, such analyses must achieve resolution on smaller spatial and temporal scales than considered here, because microbes interact with each other and the soil on the nm to µm scale.

The abundance of oligotrophs in subsoil became apparent to us in this study . Due to their slow growth rate and other substrate spectra, however, their activity was not detected with ^{13}C-based SIP,

in comparison to copiotrophs. Substrate-independent labeling with $H_2{}^{18}O$ could therefore provide an alternative approach (Hungate et al., 2015; Spohn et al., 2016).

In order to temporally resolve microbial activities and interactions, it would be advisable to collect data on hourly to daily intervals. Extended sampling throughout the season or over years would, in turn, be essential to elucidate long term effects in subsoil. This has particular importance in carbon and nitrogen turnover models, providing them with robust estimates of subsoil microbial abundancies, activities, and turnover rates (Högberg et al., 2007). Finally, we would like to estimate the extent to which subsoils respond to or influence global nutrient cycling and climate change.

4.6 Conclusions

In this thesis, we have shed some light on the complex topic of microbial communities in arable subsoils and hotspots. Figure 8 illustrates the key findings that are summarized in this section.

To maintain soil structure and the soil pore network, we directly sampled in the field and also used undisturbed subsoil cores from this site. We applied cultivation-independent molecular analyses, including NGS and DNA-SIP, to elucidate diverse soil microbial communities.

Our data support the importance of the drilosphere and rhizosphere in subsoil as significant compartments of microbial abundance, activity, and nutrient turnover.

We observed that biopores can compensate for the strong depth effect typically found in bulk soil and are indeed the source of hotspots in subsoil (Figure 8). Also, we found that the degree of soil heterogeneity is much higher in subsoil compared to topsoil, due not only to plowing/homogenization in topsoil but also to greater distances between biopores in subsoil. Furthermore, the origin of biopores matters; in the drilosphere and rhizosphere very distinct microbial communities develop.

We showed that the spatial distribution of nutrients is one of the strongest influences contributing to niche separation of microbial phyla and their functional traits in subsoil and soil compartments. In comparison to bacteria, archaea form a stable backbone throughout soil depth and compartments. In bulk subsoil, microbes relying on oligotrophic life strategies are widely distributed, whereas in topsoil and biopore hotspots, fresh inputs of organic matter promote copiotrophic life strategies. This could be shown even at the phylum level. Nitrifiers and denitrifiers with different eco-physiological strategies prefer, analogous to oligo- and copiotrophs, subsoils or hotspots.

Extracellular enzymes mirror the organic matter turnover of oligo- and copiotrophs in different soil compartments. Hydrolytic enzymes exhibited high potential in topsoil and especially rhizosphere hotspots, while peroxidase activity was high in bulk subsoil, indicating local SOM stabilization. In the

rhizosphere subsoil, high phosphomonoesterase activity implied a high demand for phosphorus by roots and microbes.

Key players involved in the turnover of plant derived carbon changed with soil depth. In the subsoil rhizosphere these were Firmicutes (*Paenibacillus*, *Cohnella*), Bacteroidetes (*Flavobacterium*), Proteobacteria (*Duganella*), and Actinobacteria (*Streptomyces*, *Agromyces*), see Figure 8.

Remarkably, temporal dynamics were negligible drivers of microbial community composition in comparison to the spatial heterogeneity that primarily determined their structure. We did, however, only measure abundances to elucidate the hot moments of functionally meaningful microbial groups. Finally, it is worth digging deeper to appreciate the importance of microbial activity in subsoils.

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APPENDIX

A LIST OF PUBLICATIONS

List of publications included in this study

Fischer, D., **Uksa**, M., Tischler, W., Kautz, T., Köpke, U., and Schloter, M. (**2013**). Abundance of ammonia oxidizing microbes and denitrifiers in different soil horizons of an agricultural soil in relation to the cultivated crops. *Biol. Fertil.* Soils 49, 1243–1246. doi:10.1007/s00374-013-0812-8.

Uksa, M., Fischer, D., Welzl, G., Kautz, T., Köpke, U., and Schloter, M. (**2014**). Community structure of prokaryotes and their functional potential in subsoils is more affected by spatial heterogeneity than by temporal variations. *Soil Biol. Biochem.* 75, 197–201. doi:10.1016/j.soilbio.2014.04.018.

Uksa, M., Schloter, M., Kautz, T., Athmann, M., Köpke, U., and Fischer, D. (**2015**). Spatial variability of hydrolytic and oxidative potential enzyme activities in different subsoil compartments. *Biol. Fertil. Soils* 51, 517–521. doi:10.1007/s00374-015-0992-5.

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List of publications related to this study

Sosa-Hernández, M. A., Roy, J., Hempel, S., Kautz, T., Köpke, U., **Uksa**, M., Schloter, M., Caruso, T., and Rillig, M. C. (**2018**). Subsoil arbuscular mycorrhizal fungal communities in arable soil differ from those in topsoil. *Soil Biol. Biochem.* 117, 83–86. doi:10.1016/j.soilbio.2017.11.009.

List of publications related to other research areas

Talk, A., Kublik, S., **Uksa**, M., Engel, M., Berghahn, R., Welzl, G., Schloter, M., and Mohr, S. (**2016**). Effects of multiple but low pesticide loads on aquatic fungal communities colonizing leaf litter. *J. Environ. Sci.* 46, 116–125. doi:10.1016/j.jes.2015.11.028.

Kunze, C., Bommer, M., Hagen, W. R., **Uksa**, M., Dobbek, H., Schubert, T., and Diekert, G. (**2017**). Cobamide-mediated enzymatic reductive dehalogenation via long-range electron transfer. *Nat. Commun.* 8, Article 15858. doi:10.1038/ncomms15858.

B PUBLICATION I

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SHORT COMMUNICATION

Abundance of ammonia oxidizing microbes and denitrifiers in different soil horizons of an agricultural soil in relation to the cultivated crops

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Abstract The role of subsoils and their microbial communities for the nutrient supply for plants is to a large extent unknown, especially in comparison to well investigated topsoil layers. Therefore, in this study, the influence of three different plant species with different rooting systems and different N uptake strategies on ammonium and nitrate levels and microbial communities involved in ammonia oxidation and denitrification was investigated in different soil horizons. Overall, our results show a higher genetic potential for both processes in topsoils than in subsoils independent of the present plant. Although we found accumulation of N in top and subsoils in plots with legumes, we could not observe an impact of the higher nitrate content on the genetic potential of denitrification and ammonia oxidation. However, differences in the ratios of ammonia oxidizing archaea to bacteria and also between denitrifying bacteria harboring genes for copper- (*nirK*) or cytochrome- (*nirS*) dependent nitrite reductase in top and subsoil samples reveal different ecophysiologies of microbes involved in N turnover in top and subsoil habitats.

Keywords Nitrite reduction · N_2O reduction · Ammonia oxidation · Microbial community · Arable soil · Subsoil · Root morphology

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Introduction

Although N is one of the most abundant elements on earth, it is highly limiting the growth of most biota, as it occurs mainly as dinitrogen gas or is fixed in organic compounds. Only a limited number of specialized microorganisms are able to transform dinitrogen gas into ammonia or catalyze the degradation of proteins and other polymeric substances containing N into amino acids, thus forming compounds that can be easily utilized by most plants, animals, and microorganisms (Hooper and Johnson 1999; Nannipieri and Paul 2009). Not surprisingly, in soils, which are low in bioavailable N, plants and microbes compete for ammonia and nitrate (Kaye and Hart 1997), resulting in reduced plant growth and performance (Schimel and Bennett 2004). Therefore, a better understanding of the soil microbiome and its contribution to N turnover in soils is essential to improve crop quality and yield, mainly if organic or low fertilizer regimes are used (Avrahami and Bohannan 2003; Wu et al. 2011; Chaparro et al. 2012).

Many studies in the past investigated N turnover, and the abundance, respectively, the activity of the associated microbial communities in topsoils. Within this area, a large number of biotic as well as abiotic factors have been identified that drive processes related to N mineralization, N fixation, nitrification, or denitrification—including soil texture, soil pH, pest management, temperature, and water content (for review see Ollivier et al. 2011). However, the N turnover in subsoils was mostly ignored. This is in contrast to current strategies of plant breeders who try to improve root development of many crops to make them more tolerant to stressors like drought (Varshney et al. 2011; Purushothaman et al. 2013) and to enhance nutrient mobilization from deeper soil layers. Thus, the aim of this study was to investigate the abundance of selected nitrifiers and denitrifiers in topsoil compared to

subsoil in a plot experiment, where plants with different root morphology as well as different N uptake strategies were grown. The ammonium monooxygenase gene (*amoA*) served as a marker for ammonia oxidizing bacteria and archaea. For denitrifiers, the abundance of the nitrite reductase genes *nirS* and *nirK* as well as the N_2O reductase gene (*nosZ*) was measured. We postulated that in legume-based systems, based on an increased availability of N mainly in subsoils, abundance of ammonia oxidizers as well as of denitrifiers is higher compared to systems where nonlegumes are grown.

Materials and methods

Soil samples were taken from a plot experiment at campus Klein–Altendorf near Bonn, Germany (50°37′21″N, 6°59′29″E), where different precrops typically used in agriculture were grown: *Festuca arundinacea* Schreb. with a rooting system characterized as a fibrous root system, and *Cichorium intybus* L. and legume *Medicago sativa* L. both with a tap root system. For each crop, four replicated plots were set up using a randomized split-plot design. Soil samples were taken in early summer 2010 at the flowering stage (plant development stage BBCH 63–69) from three plots with a soil auger at three different depths: topsoil (0–30 cm), subsoil I (45–75 cm), and subsoil II (75–105 cm) and treated as true replicates. To reduce the spatial heterogeneity of the plots, five samples per plot were taken and pooled. The soil samples were stored at −20 °C for further analysis. The soil has been classified as haplic luvisol (IUSS Working Group WRB 2006). More details on the experiment and selected soil characteristics can be found in Gaiser et al. (2012).

DNA of soil samples was extracted with FastDNA® spin kit for soil (MP Biomedicals, France) according to manufacturer's instructions. DNA-content was quantified via spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, USA). Quantitative real time assays of *nirK*, *nirS*, *nosZ*, and *amoA* (AOA and AOB) were conducted on a 7300 real time PCR system (Applied Biosystems, Germany) using SYBR Green according to Töwe et al. (2010). Ammonium and nitrate were analyzed photometrically, according to ISO 56673 and ISO 13395:1996 in soil extracts with 0.01 M $CaCl_2$. Statistics were performed using univariate and multivariate ANOVA.

Results and discussion

Nitrate-N was higher in samples derived from the plots where *Medicago* was grown compared to plots planted with *Festuca* and *Cichorium* (Table 1). A clear gradient of nitrate-N concentrations from topsoil to subsoil was only visible in the plots where *Festuca* and *Cichorium* were grown. Interestingly, in plots with *Medicago*, equal amounts of nitrate-N in topsoil (0–30 cm) and subsoil (45–105 cm) were measured. Ammonium-N was low in topsoil and subsoil samples and independent from the cultivated plant species (Table 1). Clear differences between topsoil and subsoil samples were obtained for the abundance of all investigated functional groups of microbes related to the amount of soil independent from the plant species. Data did not change, when the obtained values were based on the amount of the extracted DNA (data not shown). Therefore, results from all plots related to one soil depth were pooled to obtain a more robust statistical analysis (Fig. 1). In all cases, abundance for ammonia oxidizers as well as nitrite and N_2O reducers was higher in the samples derived from topsoil, indicating a higher potential for the respective processes in this compartment. As expected, the ratio between archaeal and bacterial ammonia oxidizers increased with soil depth, confirming earlier observations of Leininger et al. (2006). Despite the lower amounts of bioavailable C in subsoils (data not shown), the ratio of *nirK* to *nirS* harboring nitrite oxidizers decreased with soil depth. Surprisingly, the ratio of nitrite reducers to N_2O reducers did not differ between the different soil depths, despite the significant differences found in nitrate availability mainly in the plots with nonlegumes. This is in contradiction to data published by Weier et al. (1993), who found a significant increase in denitrification with increased nitrate concentration but also inhibition of the conversion of N_2O to N_2. Even in the subsoil samples from the plots with the legumes where nitrate concentrations in subsoil was comparably high as in the topsoil layer, no increase in gene copy numbers of *nirK*, *nirS*, and *nosZ* was recorded, compared to the nonlegumes.

These results indicate that the abundance of nitrite and N_2O reducers in subsoils is not limited by the presence of nitrate. Therefore, it might be postulated that significant lower amounts of DOC in subsoils are the drivers for the low abundance of denitrifiers in subsoil. This is in contrast to topsoils where denitrification is mainly driven by the availability of oxygen and not limited by the presence of DOC (Sharma et al. 2005). For ammonia oxidizers, the reduced copy numbers in subsoils might be related to an increased competition for ammonia between plants and microbes for ammonia, as overall concentrations in deeper soil horizons were low and cannot be easily increased by, e.g., fertilization due to the reduced mobility of ammonia in soil. Although the importance of the dissimilatory nitrate reduction to ammonium, also known as DNRA, for soil ecosystems has been proven recently (Schmidt et al. 2011) in our study, obviously, this process plays only a minor role, which might be a result of the still high availability of oxygen also in subsoils as well as the lack of low molecular weight C sources. The increased ratio of ammonia oxidizing archaea to ammonia oxidizing bacteria is a result of the very

Table 1 Ammonium-N and nitrate-N content in soil samples b.d.l, below detection limit (1 mg/kg dw)

Plant	Depth	NH_4^+-N (mg/kg dw)	STD	NO_3^--N (mg/kg dw)	STD
Festuca	Topsoil	0.85	0.53	1.89	1.14
Medicago	Topsoil	0.90	0.37	7.18	3.28
Cichorium	Topsoil	1.28	1.24	10.43	15.58
Festuca	Subsoil I	0.16	0.03	b.d.l	
Medicago	Subsoil I	0.18	0.04	6.36	3.65
Cichorium	Subsoil I	0.17	0.06	b.d.l	
Festuca	Subsoil II	0.09	0.02	b.d.l	
Medicago	Subsoil II	0.17	0.04	4.92	1.77
Cichorium	Subsoil II	0.15	0.01	b.d.l	

flexible ecophysiology of ammonia oxidizing archaea, which are able to use also other N sources than ammonia or could shift to a heterotrophic lifestyle (Tourna et al. 2011). However, the slow growing rates of AOA probably do not allow a significant increase in abundance despite the lacking of AOB in subsoils.

In this study, we measured the abundance of selected functional groups of microbes, which represent the genetic potential of a soil for a particular turnover process. However, this data cannot be linked directly to in situ turnover rates. Thus, one of the major goals for future studies will be the assessment of activity pattern of the corresponding groups of microbes by performing mRNA based analysis. This, however, will require different sampling strategies mainly to address the dynamics in time and space. Overall, our data are only based on one time point during the vegetation period. Although we assume a less dynamic system in subsoils compared to topsoils in respect to microbial communities, there is the need to integrate also other time points during the vegetation period to generalize the presented data even for DNA-based studies. However, whereas an increase of the sampling frequency for top soils is easy to achieve for subsoils, we have to face the problem of "destructive sampling" and a lasting perforation of the plots up to a depth of 1 m, resulting in, e.g., preferential flow phenomena and other artifacts.

In soils with very high clay content and low oxygen availability in the subsoils, the situation might be different, and processes like denitrification might become more pronounced. Also questions related to the diversity of denitrifiers between topsoil and subsoil need to be further addressed,

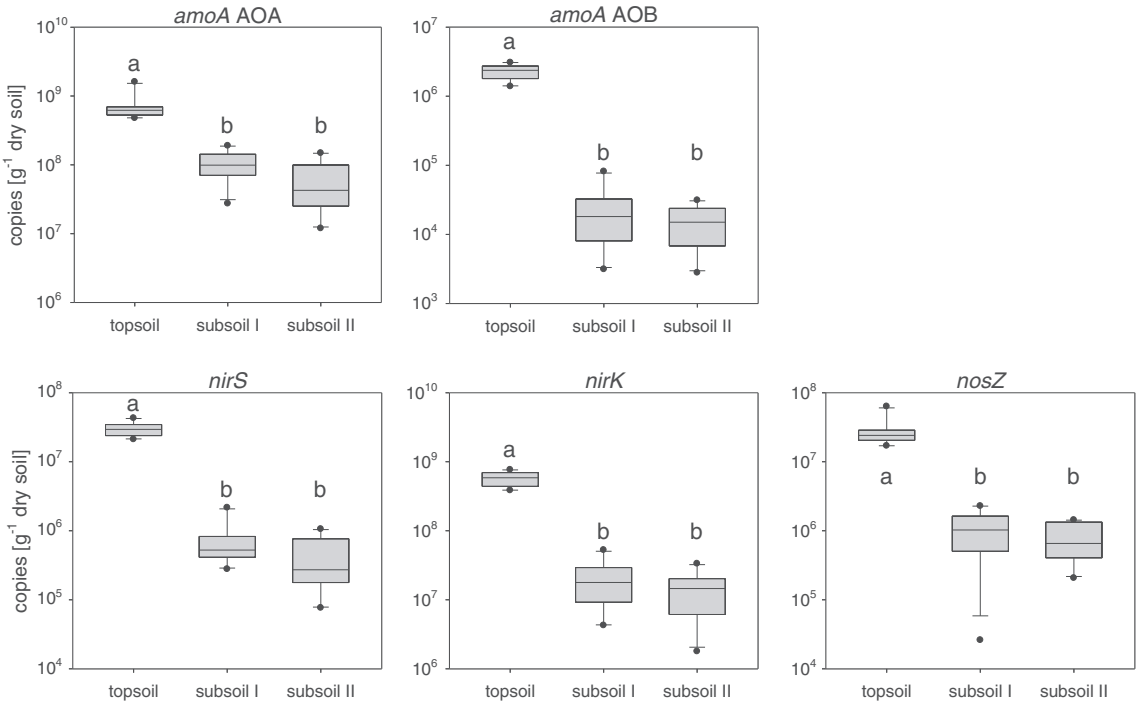

Fig. 1 Gene copy numbers of the ammonia monooxygenase gene (*amoA*) of archaea (AOA) and bacteria (AOB), the nitrite reductase genes *nirS* and *nirK* and the N_2O reductase gene *nosZ* in topsoil and subsoil samples, data from different plants were pooled (n=9), different letters indicate significant differences (ANOVA $p<0.05$), soil depth: Topsoil 0–30 cm, subsoil I 45–75 cm, and subsoil II 75–105 cm

which may then give also a light on the very stable ratio of nitrite reducers to N_2O reducers comparing different soil horizons.

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Short communication

Community structure of prokaryotes and their functional potential in subsoils is more affected by spatial heterogeneity than by temporal variations

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ABSTRACT

Spatial and temporal dynamics of microbial community structure and function in subsoils have been rarely studied in the past. In this paper we present data on how bacterial communities as well as selected functional groups of microbes change in the rhizosphere, the drilosphere, and in bulk soil over time in topsoil as well as in subsoil. We show that the overall richness of bacteria and abundance of nitrifiers and denitrifiers decreases in bulk soil with soil depth. However, these effects were not or to a much lower degree observed in the rhizosphere and the drilosphere. Temporal fluctuations contributed by far less than spatial factors to the dynamics of bacterial communities and abundance of nitrifiers and denitrifiers in all compartments independent from the soil depth.

Structure and function of microbial communities in soils are highly dynamic over time and space (Fuka et al., 2009). This is indicated by the concept of "hotspots" and "hot moments" (McClain et al., 2003; Hagedorn and Bellamy, 2011). Therefore it is not surprising that much research has been done in the past in order to identify the pattern of microbial heterogeneity in soil and to identify abiotic and biotic drivers. The rhizosphere has been identified as a hotspot for microbial activity due to the secretion of root exudates (Marschner et al., 2001; Garbeva et al., 2008). The interface between plant litter and soil, called the detritusphere, can be considered as another focus point for microbes (Schulz et al., 2012) due to the presence of large amounts of nutrients directly after litterfall. Besides plants, soil animals also form hotspots for microbial activity in soil. For example several studies have indicated that the coating of earthworm channels, called the drilosphere, harbours a large number of microbes, which differ significantly in number and ecophysiology from those of the bulk soil (Dallinger and Horn, 2013). Besides the spatial pattern of heterogeneity, also the shifts of microbial communities over time have been of great interest. Next to the seasonal variations in temperature and moisture regime, the plant developmental stage as well as changes in the litter quality during the decomposition processes highly influence the microbial community structure and shifts in functionality (Molodovskaya et al., 2012; Lauber et al., 2013; Shade et al., 2013).

Despite the fact that subsoil systems have been identified as an important reservoir for nutrients in the last decade and thus will play a pronounced role in the future for sustainable plant production (Blume et al., 2002; Eilers et al., 2012; Fischer et al., 2013), the identification of hotspots and hot moments in subsoils has been so far mostly neglected. Especially in subsoil hotspots might be of a great importance as structural elements and nutritional pools for plant roots and microbes. However, in fact it is still unclear if the dynamics of microbes over time in deeper soil layers are comparable to those in topsoils or if the topsoil acts as a buffer and shifts over time are far less significant. Also, the role of plants and soil animals in the formation of hotspots in subsoils is still poorly understood.

Here we present data from a study where spatial and temporal heterogeneity patterns of soil microbes in top- and subsoils have been investigated in an agricultural field, which was cultivated with

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Table 1
Statistical evaluation of bacterial community structure and abundance of nitrifiers and denitrifiers by PerMANOVA. Significance code: $^{***}P \leq 0.001$, $^{**}P \leq 0.010$, $^{*}P \leq 0.050$. The plant development stage is indicated by week 14, 17, and 20, corresponding to early vegetative phase (BBCH 28-32), late vegetative phase (BBCH 34-38), and flowering (BBCH 55-65), respectively.

Factor	Data subset	16S rRNA gene diversity	Functional gene abundance g^{-1} dry matter
Compartment	Total	0.001***	0.001***
	Topsoil	0.001***	0.001***
	Subsoil	0.001***	0.001***
	Week 14	0.001***	0.001***
	Week 17	0.001***	0.001***
	Week 20	0.001***	0.001***
	C. intybus	0.001***	0.001***
	F. arundinacea	0.001***	0.001***
	M. sativa	0.001***	0.001***
Depth	Total	0.001***	0.001***
	Bulk soil	0.001***	0.001***
	Drilosphere	0.003**	0.001***
	Rhizosphere	0.023*	0.001***
	Week 14	0.001***	0.001***
	Week 17	0.001***	0.001***
	Week 20	0.001***	0.001***
	C. intybus	0.001***	0.001***
	F. arundinacea	0.001***	0.001***
	M. sativa	0.001***	0.001***
Vegetation state	Total	0.001***	0.001***
	Bulk soil	0.002**	0.001***
	Drilosphere	0.021*	0.001***
	Rhizosphere	0.039*	0.001***
	Topsoil	0.004**	0.001***
	Subsoil	0.008**	0.001***
	C. intybus	0.035*	0.001***
	F. arundinacea	0.013*	0.001***
	M. sativa	0.140	0.001***
	Bulk topsoil	0.001***	0.001***
	Bulk subsoil	0.001***	0.001***
	Drilosphere topsoil	0.069	0.001***
	Drilosphere subsoil	0.128	0.001***
	Rhizosphere topsoil	0.061	0.001***
	Rhizosphere subsoil	0.090	0.001***
Plant species	Total	0.001***	0.025*
	Bulk soil	0.104	0.301
	Drilosphere	0.012*	0.002**
	Rhizosphere	0.001***	0.357
	Topsoil	0.001***	0.043*
	Subsoil	0.001***	0.267
	Week 14	0.001***	0.086
	Week 17	0.001***	0.236
	Week 20	0.001***	0.286
	Bulk topsoil	0.001***	0.032*
	Bulk subsoil	0.414	0.252
	Drilosphere topsoil	0.006**	0.040*
	Drilosphere subsoil	0.065	0.001***
	Rhizosphere topsoil	0.001***	0.368
	Rhizosphere subsoil	0.001***	0.295
Compartment × depth	Total	0.001***	0.001***
Compartment × vegetation state	Total	0.001***	0.002**
Compartment × plant species	Total	0.001***	0.053
Depth × vegetation state	Total	0.081	0.005**
Depth × plant species	Total	0.004**	0.650
Vegetation state × plant species	Total	0.337	0.718

different crops with diverging root morphology. Besides the overall bacterial diversity, we measured copy numbers of selected functional genes (*nirS*, *nirK*, *nosZ* and *amoA*) per g dry soil, which were used as proxy for the abundance of nitrifiers and denitrifiers. Samples from three soil compartments were analysed (bulk soil, drilosphere, and rhizosphere). We hypothesized that in deeper soil layers differences in microbial community structure and function between hotspots like the drilosphere or rhizosphere and bulk soil become more pronounced than in the topsoil. Vice versa in topsoils temporal dynamics are higher than in subsoils due to the impact of abiotic factors like temperature and precipitation.

Soil samples were taken from a plot experiment which has been performed at campus Klein – Altendorf near Bonn, Germany, where *Festuca arundinacea* Schreb. with a rooting system characterized as a fibrous root system, *Cichorium intybus* L. and legume *Medicago sativa* L., both with a tap root system, were grown. In total 9 plots (each 10 × 6 m), located on the same field, were used in this study (3 plots per plant), which were randomly distributed according to a split-plot design and sampled separately in 2011. Per plot 5 subsamples (each 1 g) of bulk soil, drilosphere, and rhizosphere were taken from topsoil (10–30 cm) and subsoil (60–75 cm) and pooled, respectively. A sterilized spoon or tweezer was used to

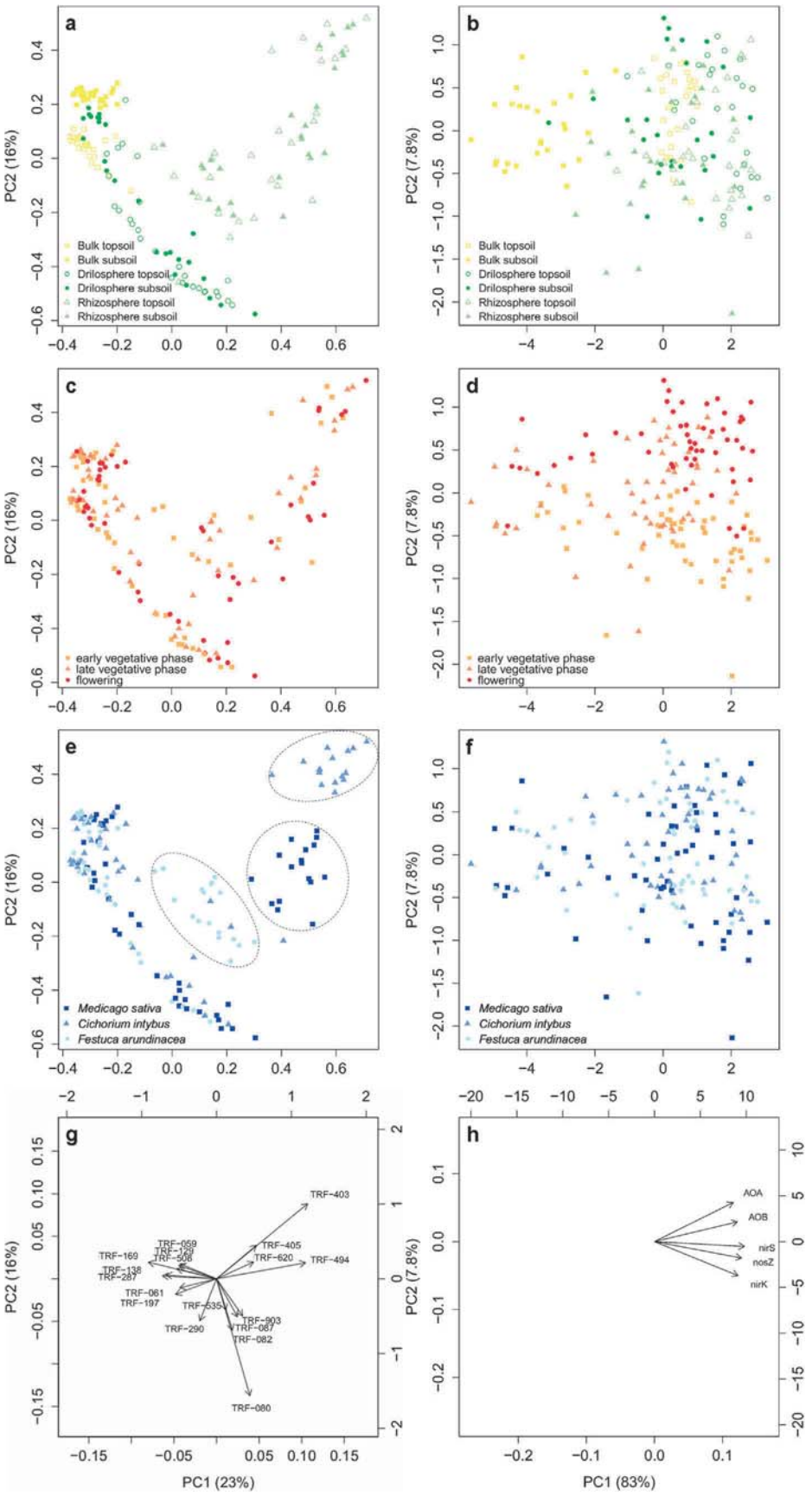

Fig. 1. Principal component analysis of 16S rRNA gene based bacterial community structure (1a,c,e,g) and abundance of nitrifiers and denitrifiers based on selected marker genes (1b,d,f,h) coloured in relation to depth and compartment type (1a,b), vegetation state (1c,d), or plant species (1e,f). The plant development stage is indicated by week 14, 17, and 20, corresponding to early vegetative phase (BBCH 28-32), late vegetative phase (BBCH 34-38), and flowering (BBCH 55-65). Data sets were Hellinger- (tRFLP data) or log-transformed (abundance of nitrifiers and denitrifiers). Fig. 1g and h indicate the loadings of the variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sample bulk soil, drilosphere, which was defined as maximal 1 mm coating of earthworm holes, and roots together with maximal 2 mm adhering rhizosphere soil. The sampling was repeated at three different time points during the vegetation period ("early vegetative phase", "late vegetative phase" and "flowering", corresponding to the plant development stages BBCH 28-32, BBCH 34-38, and BBCH 55-65, according to Hack et al., 2001). The soil has been classified as haplic luvisol (after IUSS Working Group WRB 2006). More details on the experiment and selected soil characteristics can be found in Gaiser et al. (2012). The soil samples were stored at 4 °C for biochemical – respectively −80 °C for further molecular analysis.

Dissolved organic carbon (DOC) and microbial biomass (C_{mic}) were measured according to Joergensen (1996). The bacterial community structure was assessed using 16S rRNA gene fingerprinting, based on terminal restriction fragment length polymorphism (tRFLP; for details see supplemental material). The quantification of nitrifiers and denitrifiers was performed on the basis of the abundance of the bacterial and archaeal ammonium monooxygenase gene (*amoA*) respectively both types of nitrite reductase genes (*nirS* and *nirK*) and the N_2O reductase (*nosZ*) by quantitative real time PCR. As denitrifiers and nitrifiers can harbour more than one gene copy of *nirS*, *nirK* or *nosZ* (up to 2–3 copies per cell; Jones et al., 2008; Sanford et al., 2012) or *amoA* (2.5 copies archaeal *amoA* per cell in average; Trias et al., 2012) the gene copy numbers per g dry soil do not necessarily represent the exact abundance of the respective organism. However, we use the gene abundance as a proxy for the abundance of nitrifiers or denitrifiers.

A principal component analysis and a permutational multivariate analysis were used for data evaluation. For further details see supplemental material.

A combined analysis of all data revealed significant interactions between all factors investigated (depth, compartment type, time, plant species) both for the bacterial community structure and the abundance of nitrifiers and denitrifiers (Table 1; Fig. 1).

Factor I – soil depth: Topsoil and subsoil differed significantly, when bulk soil samples were compared ($P \leq 0.001$; Fig. 1a,b). This difference is mainly related to (1) the reduced diversity as indicated by the lower number of TRFs (Table S1) and to (2) the lower abundance of nitrifiers and denitrifiers found in bulk soil samples from subsoil (Fig. S1). The trend did not change when gene copy numbers were related to ng of extracted DNA instead of gram of soil. This demonstrates that the lower gene abundance is not only a result of the lower biomass found in bulk subsoil (Fig. S2), but that in microbial biomass the relative contribution of nitrifiers and denitrifiers also decreases with soil depth, confirming the results of Fischer et al. (2013). The pronounced "depth effect" found in bulk soil samples could not be confirmed for the drilosphere and rhizosphere (Fig. 1a,b; S1). For these two compartments differences between subsoil and topsoil were low both when bacterial community structure and function was analysed. This suggests an importance of the nutrient input into subsoil hotspots due to root exudation or earthworm casts (Mendez-Millan et al., 2012).

Factor II – soil compartment: The compartment had a high influence on the bacterial community structure (Fig. 1a). For the abundance of nitrifiers and denitrifiers this effect became more pronounced when subsoil was analysed (Fig. 1b; S1) as here a higher difference between rhizosphere respectively drilosphere and bulk soil was observed compared to the topsoil. This indicates an importance of hotspots especially in subsoils for the nitrogen turnover.

Factor III – temporal dynamics: Compared to depth and compartment type, the temporal variations had a lower impact on the bacterial community structure (Table 1, Fig. 1c). Interestingly, only bacterial communities of the bulk soil changed significantly over time both in topsoil and subsoil (Table 1). Blume et al. (2002) also showed differences of microbial community both in bulk subsoil and topsoil depending on season and soil chemical properties.

In contrast to the bacterial community structure, the abundance of nitrifiers and denitrifiers changed over time in all compartments (Fig. 1d). These differences could mainly be explained by the dynamics in abundance of the *nirK* gene which was higher at the early vegetative phase and the higher archaeal *amoA* gene abundance during flowering (Fig. 1h, S1). These results are in agreement with many studies describing responses of microbial functional groups to temperature and water regime due to seasonal fluctuations or to carbon and nitrogen variation in relation to the plant development stage and root exudates, although the mechanisms and main drivers are still controversy (Hai et al., 2009; Glaser et al., 2010; Chaparro et al., 2013). Only the abundance of *nirS* did not change significantly over time (Fig. 1h). This observation supports earlier findings by Novinscak et al. (2013) who could also show only a slight decrease of *nirS* in the rhizosphere at the end of the growing season. Interestingly, in the drilosphere, dynamics in time were similar pronounced like in the rhizosphere.

Factor IV – plant species: The bacterial community structure was significantly influenced by the plant species in the rhizosphere ($P \leq 0.001$) and drilosphere ($P = 0.012$), but not in the bulk soil ($P = 0.104$; Table 1, Fig. 1e). Especially for *C. intybus* and *M. sativa* unique rhizosphere bacterial communities could be detected, which were missing in bulk soil or drilosphere (Fig. 1g). A high abundance of rhizobia in the rhizosphere and in the nodules of *M. sativa* (Wall and Favelukes, 1991) as well as the presence of fluorescent pseudomonades in the rhizosphere of *C. intybus* (Van Outryve et al., 1988) was reported. Those microbes may contribute to the differences in bacterial communities observed in this study. The plant species also influenced the abundance of nitrifiers in the rhizosphere significantly but did not influence the abundance of denitrifiers ($P = 0.001$ vs. $P = 0.213$). This is in accordance with Xu et al. (2013), who found a difference for the nitrification potential but not for the denitrification potential in the rhizosphere. The plant species-dependent ability and variability of nitrification inhibition supports our results (Subbarao et al., 2006).

Overall, as postulated, the differences between compartments which are known to have higher microbial activity like rhizosphere and drilosphere compared to bulk soil were higher in samples obtained from subsoil compared to topsoil. This was true for the bacterial community structure as well as for the abundance of nitrifiers and denitrifiers indicating a higher contribution of hotspots to microbial nutrient turnover in deeper soil layers. In comparison to hotspots, temporal variation had a minor contribution to the dynamics of bacterial community structure and abundance of nitrifiers and denitrifiers both in topsoil and subsoil. However in 2011 during the sampling period in April and May precipitation and temperature did not change significantly (Agrarmeteorologie Rheinland-Pfalz; www.wetter.rlp.de), which could explain the low dynamics in time at least partly.

The presented data demonstrate that rhizosphere and drilosphere can be considered as important hotspots for potential microbial activities in subsoils. Further RNA-based studies in the future are needed to prove under which conditions microbes make use of this potential and induce nutrient turnover in this compartments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.04.018.

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Supplementary Information

Supplementary Material and Methods

DNA was extracted with FastDNA® Spin Kit for Soil (MP Biomedicals, Eschwege, Germany). The protocol has been modified by a second bead beating of the sample, and an additional incubation at 55°C for 5 min before DNA elution, to increase the DNA amount. DNA content was measured via spectrophotometer NanoDrop® ND-1000 (Thermo Fisher Scientific, Waltham, USA).

Quantitative real-time PCR (qPCR) of the archaeal and bacterial ammonium monooxygenase gene (*amoA* AOA and *amoA* AOB) for nitrification potential and of the denitrification genes for nitrite reductase (copper-dependent *nirK*, cytochrome-harbouring *nirS*) and nitrous oxide reductase (*nosZ*) was conducted. The 7300 Real-Time PCR System (Applied Biosystems®, Darmstadt, Germany) was used with Power SYBR® Green PCR Master Mix (Applied Biosystems®, Darmstadt, Germany) according to Töwe et al. (2010). Thermal profiles have been modified for *amoA* AOB (40 cycles: 94°C – 1 min/58°C – 1 min/72°C – 1 min), *nirS* (40 cycles: 95°C – 45 s/57°C – 45 s/72°C – 45 s), and *nosZ* (5 cycles: 95°C – 15 s/65°C – 30 s/72°C – 30 s; 40 cycles: 95°C – 15 s/60°C – 30 s/72°C – 30 s). The qPCR efficiency for all genes was higher than 80%. Based on results from pre-experiments an inhibition of the PCR was not observed (data not shown).

16S rRNA gene terminal restriction fragment length polymorphism (tRFLP) was conducted using 6-carboxyfluorescein (FAM)-labeled primer Ba27f (Liu et al., 1997) and the primer Ba907r (Lane, 1991) targeting bacteria. The PCR was carried out using TopTaq™ DNA Polymerase (Qiagen, Hilden, Germany) with the program as follows: 5 min - 95°C/27 cycles [45 s – 95°C/45 s – 58°C/45 s – 72°C]/5 min – 72°C. Amplicons were purified by the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and thereupon restricted with *Msp*I (Fermentas, St. Leon-Rot, Germany) for 15 h at 37°C. Digested 16S rRNA gene

fragments were purified by the MinElute® Reaction Cleanup Kit (Qiagen, Hilden, Germany). One µl containing 4 ng of the product was added to 13 µl of Hi-Di™ Formamide (Applied Biosystems®, Darmstadt, Germany), which contained a 800-fold dilution of a 6-carboxy-X-rhodamine-labeled MapMarker® 1000 (Bio-Ventures, Murfreesboro, USA). After denaturation at 95°C for 5 min fragments were size-separated and quantified using the 3730 DNA Analyzer (Applied Biosystems®, Darmstadt, Germany) according to Töwe et al. (2011): "Electrophoresis was performed with POP-7 polymer in a 50 cm capillary array under the following conditions: 10 s injection time, 2 kV injection voltage, 7 kV run voltage, 66 °C run temperature, and 63 min analysis time. Electropherograms were analyzed using the GeneMapper 3.5 software package (Applied Biosystems, Germany)".

Statistics were performed using the R 2.15.1 software. qPCR data have been logarithmized and scaled. tRFLP electropherograms were processed by removing all fragments smaller than 50 bp and applying the T-REX Software (Culman et al., 2009) by filtering for peak hights with the threshold 1. After relativization, complete TRFs were deleted from dataset without any values above 1%. Data have been Hellinger-transformed as recommended by Ramette (2007). Complementary, for the processed qPCR and tRFLP data a principal component analysis and a permutational multivariate analysis of variance (Anderson, 2001; McArdle and Anderson, 2001) using the euclidean distance as a distance matrix was conducted. The interpretation for tRFLP data showed the same results when the calculation is based on the presence instead of the abundance of fragments using a 0-1 matrix (data not shown).

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Supplementary Tables:

Table S1: Number of different terminal restriction fragments (TRFs) found in soil compartments and their comparison between topsoil and subsoil by t-test.

Compartment	Depth	No. TRFs ± standard deviation	*P* value (t-test)
bulk soil	topsoil	51.6 ± 10.4	0.005
	subsoil	43.6 ± 17.6	
drilosphere	topsoil	52.2 ± 10.7	0.993
	subsoil	51.8 ± 14.1	
rhizosphere	topsoil	49.7 ± 15.3	0.923
	subsoil	49.7 ± 16.5	

Supplementary Figures:

Figure S1: Gene abundances of *amoA* AOA (a, b), *amoA*, AOB (c, d), *nirK* (e, f), *nirS* (g, h), *nosZ* (i, j) based on dried soil (a, c, e, g, i) or extracted DNA (b, d, f, h, j). The plant development stage is indicated by week 14, 17, and 20, corresponding to early vegetative phase (BBCH 28-32), late vegetative phase (BBCH 34-38), and flowering (BBCH 55-65).

Figure S2: Dissolved organic carbon (DOC) and microbial biomass (C_{mic}) measured in bulk topsoil and bulk subsoil of the different treatments at the flowering (BBCH 55-65). * indicates significant differences calculated with ANOVA ($P < 0.050$).

Figure S1

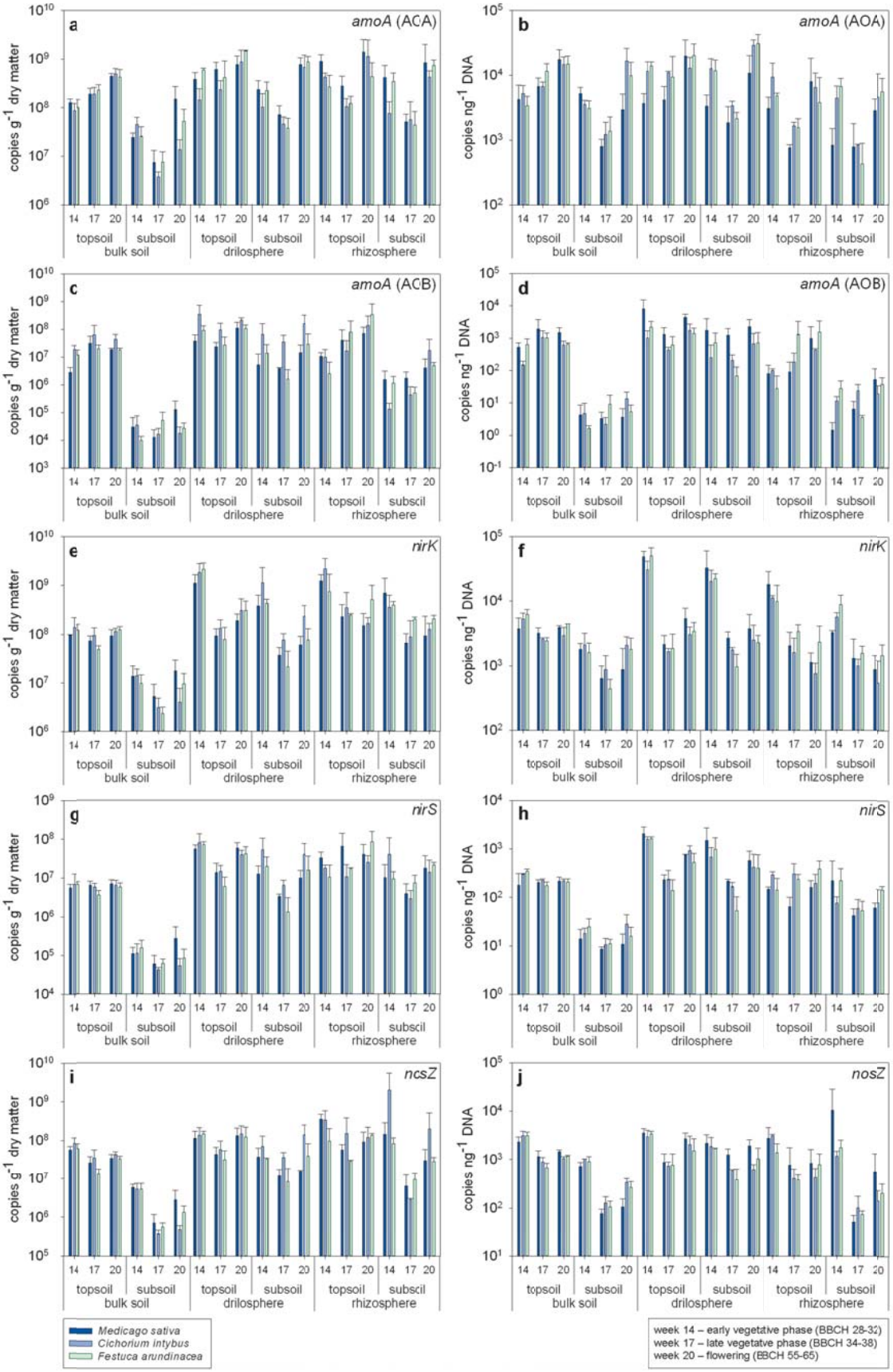
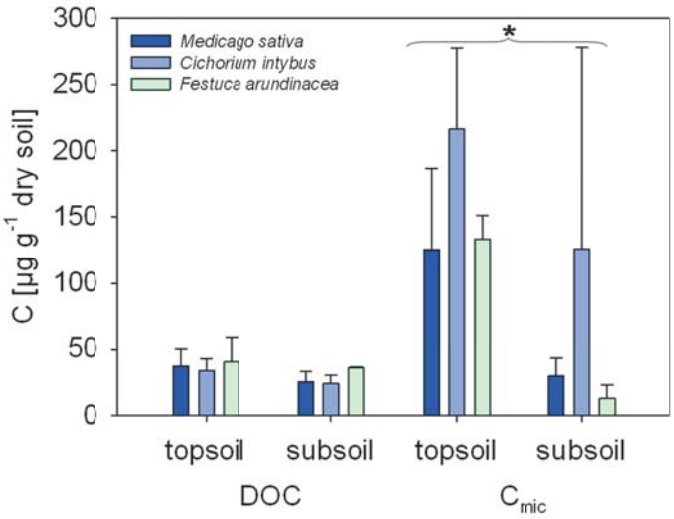

a
amoA (ACA)
b
amoA (AOA)
c
amoA (ACB)
d
amoA (AOB)
e
nirK
f
nirK
g
nirS
h
nirS
i
ncsZ
j
nosZ
copies g-1 dry matter
copies ng-1 DNA
14 17 20
topsoil
subsoil
bulk soil
drilosphere
rhizosphere
Medicago sativa
Cichorium intybus
Festuca arundinacea
week 14 – early vegetative phase (BBCH 28-32)
week 17 – late vegetative phase (BBCH 34-38)
week 20 – flowering (BBCH 55-65)

Figure S2


300
250
200
150
100
50
0
C [µg g-1 dry soil]
Medicago sativa
Cichorium intybus
Festuce arundinacea
*
topsoil
subsoil
topsoil
subsoil
DOC
Cmic

D PUBLICATION III

Biol Fertil Soils (2015) 51:517–521
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SHORT COMMUNICATION

Spatial variability of hydrolytic and oxidative potential enzyme activities in different subsoil compartments

Marie Uksa · Michael Schloter · Timo Kautz · Miriam Athmann · Ulrich Köpke · Doreen Fischer

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Abstract The spatial heterogeneity of nutrient turnover in subsoils has been rarely studied in the past, although drilosphere and rhizosphere are found to be important microbial hotspots in this oligotrophic environment. In this study, we measured different potential enzyme activities in different soil compartments of subsoil and topsoil. It could be shown that the activities of hydrolases, which cleave readily available organic substrates, are significantly higher in samples from the drilosphere and rhizosphere both in topsoil and subsoil. In bulk soil, hydrolase activities decrease with depth. In contrast, oxidative enzymes, which are involved in the decay of recalcitrant organic material, are released from the microbial community especially in the bulk fraction of subsoil. This emphasizes the importance of subsoil for nutrient acquisition and gives evidence for a distinct spatial separation of microbes with diverging lifestyles.

Keywords Soil enzymes · Spatial heterogeneity · Microbial hotspots · Drilosphere · Rhizosphere · Bulk soil

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Introduction

The microbial community and activity in subsoils below the plough layer received little attention compared to topsoil, although subsoil resources are enormous and thus important for nutrient acquisition of plants and microbes (Batjes 1996; Harrison et al. 2011). Previous studies emphasized the rapid decrease of microbial biomass, microbial diversity and enzyme activities with depth in mineral soils, where readily available substrates get limited (Fuka et al. 2008; Eilers et al. 2012; Stone et al. 2014). In addition, ecophysiological strategies of microbes in subsoil are different compared to topsoil due to changing soil parameters, which is indicated by Fischer et al. (2013) for nitrifying and denitrifying microbial communities.

However, former studies focused on bulk soil and did not include the spatial subsoil heterogeneity, which is caused for example by anecic earthworms and deep-rooting plants. It has been postulated that the hotspots drilosphere and rhizosphere with their distinct microbial communities (Marschner et al. 2001; Berg and Smalla 2009; Stromberger et al. 2012) can enhance the overall genetic potential in subsoil. This has been partly proven for the diversity of bacterial communities and the abundance of functional genes related to nitrification and denitrification, which are comparable in the topsoil and subsoil hotspots, although steep gradients were found in bulk soil samples (Uksa et al. 2014). However, whether these observations hold true for other microbial traits is still an open question.

Thus, in this paper, we describe the spatial heterogeneity and localisation of potential enzyme activities in different subsoil compartments, which is still an open field in soil enzymology (Nannipieri et al. 2012). Potential enzyme activities serve as proxies for organic matter degradation and indicate the microbial nutrient demand for carbon, nitrogen and phosphorus (Caldwell 2005; Sinsabaugh et al. 2008; Moorhead et al. 2012). Readily degradable substrates such as cellulose

or proteins are depolymerized mainly by hydrolases, whereas oxidative enzymes are involved in the decay of recalcitrant compounds (Burns et al. 2013). We measured potential enzyme activities of a set of hydrolases and oxidative enzymes in different subsoil compartments of an agricultural field and compared them to topsoil. We hypothesize diverging spatial heterogeneity patterns for different classes of enzymes in subsoil as a result of lower substrate availability and quality changes: hydrolase activities are expected to be higher at subsoil drilosphere and rhizosphere, where organic material is frequently deposited due to earthworm activity and root exudation (Grayston et al. 1997; Don et al. 2008; Andriuzzi et al. 2013). In contrast, oxidative enzyme activities are assumed to be more important in bulk subsoil, where fresh substrates are rarely available and recalcitrant organic material accumulates due to slow turnover rates (Rumpel and Kögel-Knabner 2011).

Materials and methods

Soil samples were obtained from a total of 12 plots (10×6 m each) of an agricultural field trial at Campus Klein-Altendorf near Bonn (Germany; 50° 37′ 21″ N, 6° 59′ 29″ E) in June 2012. The growing crop *Triticum aestivum* L. was at the developmental stage EC 58–59 (end of ear emergence; Zadoks et al. 1974). At this time point, highest response of enzyme activities due to root exudation is expected. From 2009 to 2011, three different precrops (four replicates each) were cultivated on the same plots: *Festuca arundinacea* Schreb. with a rooting system characterized as a fibrous root system and *Cichorium intybus* L. and legume *Medicago sativa* L., both with a taproot system known to strongly structure soil (Löfkvist et al. 2005). Further details regarding the soil, characterized as haplic luvisol, can be found in the publication of Gaiser et al. (2012).

From each plot, the soil compartments, bulk soil, drilosphere and rhizosphere were obtained from topsoil (10–30 cm), the upper subsoil (45–75 cm) and deep subsoil (75–105 cm) vertical to the exposed soil profile. The drilosphere was defined as maximal 1-mm coating of the earthworm burrows and the rhizosphere as maximal 2-mm adhering soil around the root. At least five subsamples per plot were pooled, stored at 4 °C and analysed within 10 days after sampling. For DNA extraction, parallel samples were stored at −80 °C.

Soil water content was determined by drying the sample at 105 °C until constant mass. The content of microbial carbon (C_{mic}) was determined in bulk soil by chloroform fumigation and $CaCl_2$ extraction according to Joergensen (1996) and Joergensen and Mueller (1996). As the method requires high amounts of soil material, it was not applicable to drilosphere and rhizosphere. Therefore, the microbial biomass in each compartment was estimated by the DNA content (Renella et al. 2006; Gangneux et al. 2011), which correlated well to C_{mic} in bulk soil (data not shown). DNA was extracted from samples stored at −80 °C using the FastDNA® Spin Kit for Soil (MP Biomedicals, Eschwege, Germany). The method was modified by a second bead beating for 40 s and an incubation at 55 °C for 5 min before elution to enhance DNA yield. DNA concentration was measured with a NanoDrop 1000 Spectrophotometer (PeqLab, Erlangen, Germany).

The enzyme assays used in this study include intracellular, extracellular and soil-bound enzyme pools (Burns 1982). Activities of β-1,4-glucosidase, cellobiohydrolase, β-xylosidase, chitinase and phosphomonoesterase were measured using 4-methylumbelliferone-labelled fluorogenic substrates according to Pritsch et al. (2005). The assay did not include a buffering system, which enabled the simultaneous measurement of acid and alkaline phosphomonoesterase. Activities of phenoloxidase and peroxidase were determined with the substrates L-3,4-dihydroxyphenylalanine (DOPA) and H_2O_2 according to Saiya-Cork et al. (2002). Potential enzyme activities were related to gramme dry soil for absolute activity or to DNA content as a proxy for specific activity.

Statistical significance was tested by factorial ANOVA followed by Tukey's honest significant difference (HSD) test using the R software (R Core Team 2013). For further details on methods, see supplemental material.

Results and discussion

The used precrops affected neither enzyme activities nor the microbial biomass as measured by the DNA content (data not shown). This confirms previous data from Fischer et al. (2013) and Uksa et al. (2014) that despite the differences in root morphology and nutrient allocation, obviously the influence of the precrop can be considered as minor compared to the soil depth or compartment type. Therefore, data from the different precrops were combined for a more robust statistical analysis and clear illustration of enzyme activities and microbial biomass in soil compartments of topsoil and subsoil (Fig. 1, S1).

Hydrolase activities As expected, the spatial distribution of hydrolase activities positively correlates with the availability of fresh organic matter, which is deposited in topsoil and at the hotspots drilosphere and rhizosphere due to plant litter input, fertilization, root exudates and earthworm cast (Fig. 1a–e). In the topsoil, higher hydrolase activities were measured in the drilosphere and in the rhizosphere as compared to bulk topsoil ($P \leq 0.001$). These differences between compartments become even more pronounced in the subsoil. Hydrolase activities in the bulk soil sharply declined already in the upper subsoil (45–75 cm), whereas the depth effect for the drilosphere and rhizosphere was far less pronounced, which increased the differences between bulk soil and the hotspots in the subsoil.

Interestingly, lowest hydrolase activities in the rhizosphere were measured in the upper subsoil (45–75 cm) and not in the

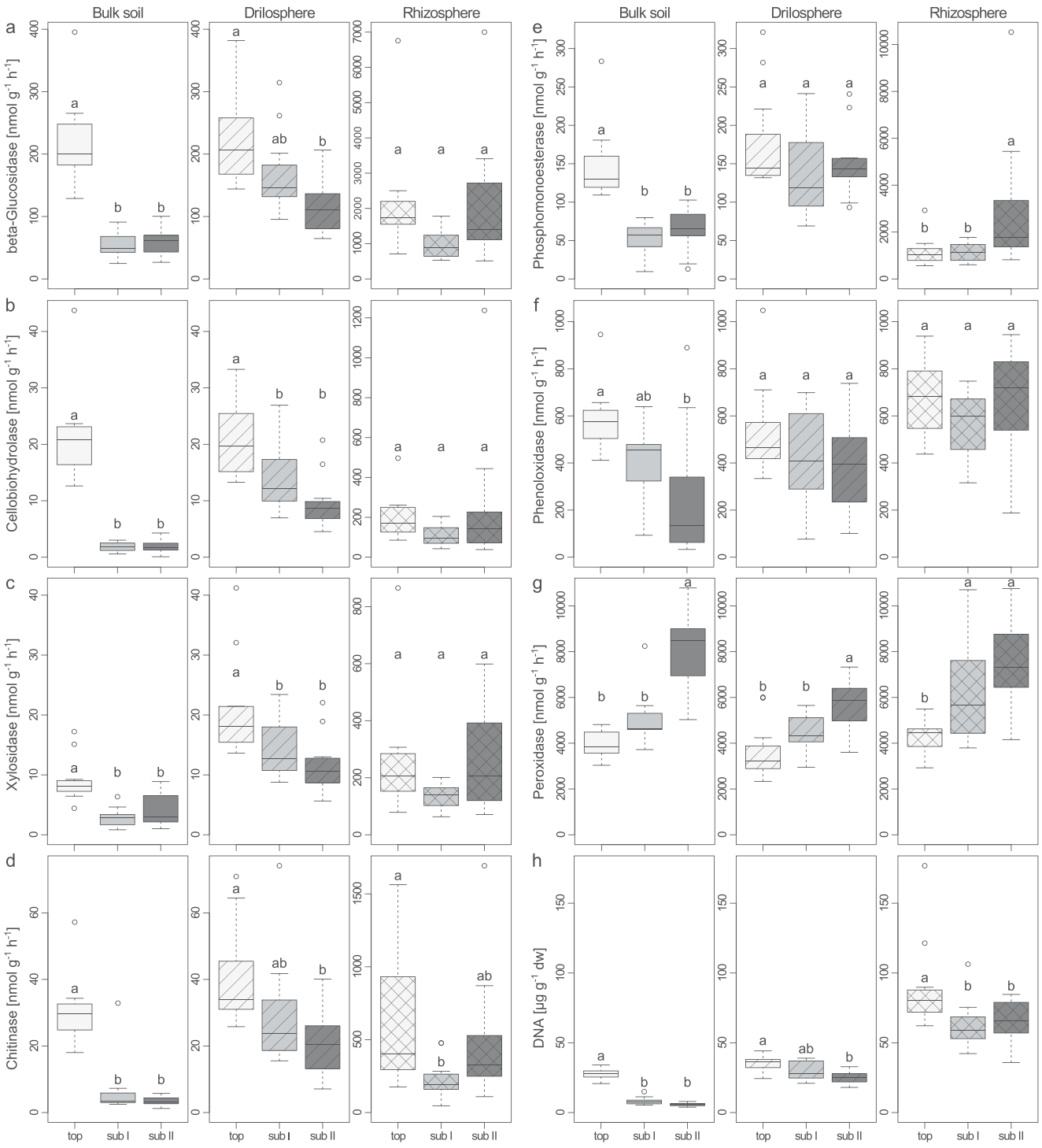

Fig. 1 Potential enzyme activities and DNA content—**a** beta-glucosidase, **b** cellobiohydrolase, **c** xylosidase, **d** chitinase, **e** phosphomonoesterase, **f** phenoloxidase, **g** peroxidase, **h** DNA—in different soil compartments along the soil profile. Different letters indicate significant differences ($P \leq 0.05$; $n = 12$). *top* topsoil (10–30 cm), *sub I* upper subsoil (45–75 cm), *sub II* deep subsoil (75–105 cm)

deep subsoil (75–105 cm). Possibly, roots are fast growing through the upper subsoil in order to acquire nutrients and water in deeper soil horizons. This is supported by a study on the same field site by Kautz et al. (2013b) showing that in the upper subsoil, Bt-layer roots were preferentially growing in biopores, where the mechanic resistance is lower than that in the bulk soil. As younger roots release more root exudates (McCully and Canny 1985), this may trigger the increased hydrolase activities in the deep subsoil. However, enzymes in the rhizosphere are produced both by microbes

and the plant. Thus the origin of enzymes in the rhizosphere still needs to be clarified by using DNA-based methods in future studies (Nannipieri et al. 2012).

Phosphomonoesterase activity was significantly higher in the deep subsoil rhizosphere compared to both topsoil and the upper subsoil. Therefore, our data indicate that subsoil is a more intensely used P source compared to topsoil, as suggested also by Kautz et al. (2013a). It is frequently reported that phosphatase activities do not necessarily correlate with relative P availability and can be suppressed by inorganic P addition because C and N availability might as well regulate phosphatase activity (Nannipieri et al. 1978; Olander and Vitousek 2000; Sinsabaugh et al. 2008). Again like for hydrolases also here, the role of plant-derived phosphomonoesterase versus those produced by microbes needs to be addressed in the future.

The specific hydrolase activities (as indicated by the ratio between hydrolase activities and the amount of extracted DNA) are shown in Fig. S1a–e. Here for β-glucosidase, xylosidase and phosphomonoesterase in bulk soil significantly higher specific activities were observed in subsoil than in topsoil. This might be caused either by a higher enzyme production per cell, a higher portion of microorganisms releasing these enzymes, or a different community synthesizing more or more efficient enzymes in the subsoil (Kramer et al. 2013). A very distinct bacterial community fingerprint found in bulk subsoil compared to topsoil at our field site (Uksa et al. 2014) supports the idea of a microbial community well adapted to oligotrophic conditions in the bulk subsoil.

Oxidative enzyme activities The overall differences of phenoloxidase activity in different soil depths and between the hotspots respectively bulk soil were far less pronounced compared to the hydrolase activities (Fig. 1f). Completely different pattern in comparison to other enzymes was observed for peroxidase: The peroxidase activity increased with depth in all soil compartments (Fig. 1g). In addition, significantly higher specific phenoloxidase and peroxidase activities (as indicated by the ratio between enzymatic activity and the amount of extracted DNA) were found in bulk soil compared to the hotspots in all depth layers ($P \leq 0.013$; Fig. S1f, g).

This indicates a release of oxidative enzymes when substrate concentration is generally low and recalcitrant organic matter with long residence times has a larger proportion, which is to be expected in bulk subsoil (Shindo and Kuwatsuka 1976; Rumpel and Kögel-Knabner 2011). Overall our data nicely confirms the idea that soil peroxidase regulates organic matter decomposition through improving the accessibility of reducing sugars and amino acids (Tian and Shi 2014). However, the role of phenoloxidases and peroxidases is not as clear as for the hydrolases, because they are produced also for other purposes like oxidative stress response, detoxification of phenolic compounds or defence (Sinsabaugh 2010; Nannipieri et al. 2012).

Conclusion

In conclusion, the spatial heterogeneity of enzyme activities in subsoil is highly pronounced and different for distinct classes of enzymes. Rhizosphere and drilosphere are significant hotspots of hydrolase activities as a result of higher substrate availability in these compartments that leads to an increased microbial biomass, but also due to the expression of these enzymes by plants and microbes at the plant soil interface. Thus these hotspots are important for the nutrient turnover of readily degradable substrates especially in subsoil. In contrast, oxidative enzyme activities involved in the decay of recalcitrant organic matter are synthesized by the microbial community especially in the bulk subsoil. This supports the assumption of a pronounced spatial separation of microbes with diverging lifestyles in subsoil. Further molecular studies are needed to assess the microbial diversity and key organisms behind the functional redundancy of enzyme activities and to understand the regulatory and ecological mechanisms behind the production of enzymes in soil.

Acknowledgments This work was funded by the German Research Foundation within the Research Unit 1320 (MU831/21-1). We are grateful for the technical assistance by Arsin Sabunchi and Gudrun Hufnagel and thank all the anonymous reviewers for their insightful discussions and productive comments.

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Supplemental material

Supplemental material and methods – enzyme assays

For enzyme activity measurements, soil suspensions of bulk soil, drilosphere and rhizosphere samples were prepared by shaking 0.1-0.4 g in 100 volumes *A. bidest.* for 10 min. After sonication for 3 min on ice the suspension was filtered through a 90 µm nylon mesh and stored at 4°C. Enzymatic measurements were carried out within 24 h with three analytical triplicates on 96-well microtiter plates and with the assay conditions shown in Table S1.

For the hydrolase activity measurements 50 µl of the soil suspension was incubated with 100 µl of the substrate working solution while the negative control contained *A. bidest.* instead of the suspension. Each plate contained a calibration with 4-methylumbelliferone (0, 100, 200, 300, 400, 500 pmol per well). In addition, the fluorescence of 50 µl soil suspension with 300 pmol 4-methylumbelliferone in 150 µl was measured to determine the auto-fluorescence or fluorescence quenching of each sample. Incubation at 21°C in the dark started with the substrate addition and was stopped according to Table S1 with 100 µl 1 M Tris (pH 10.7). The black 96-well plates were spun down (2420 rpm, 5 min) before measurement on the fluorometer: extinction 365 nm, emission 450 nm.

For the phenoloxidase and peroxidase assay 150 µl of the soil suspension was added to 150 µl 20 mM DOPA dissolved in 100 mM sodium acetate (pH 5.5). 10 µl of H_2O_2 (12 % in *A. bidest.*) was added to the peroxidase measurement. Controls were: (1) 150 µl soil suspension with 150 µl 100 mM sodium acetate, (2) 150 µl 100 mM sodium acetate with substrates, (3) 300 µl 100 mM sodium acetate. Shortly after substrate addition and 20 h incubation in the dark, plates were spun down (2000 rpm; 2 min) and the particle free supernatant was measured in 96 well-plates at 450 nm on a plate reader. The extinction coefficient ε used for calculation is 3.6 mM^{-1} cm^{-1} for dopachrome at 450 nm. Peroxidase

activity was calculated as the difference between the activities measured with H_2O_2 and without H_2O_2.

Table S1: Enzymatic assay conditions for hydrolases and oxidative enzymes.

Enzyme	EC number	Substrate	Assay concentration	Incubation time
β-1,4-Glucosidase	3.2.1.21	4-MU-β-D-glucopyranoside	500 µM	1 h
Cellobiohydrolase	3.2.1.91	4-MU-β-D-cellobioside	400 µM	2 h
β-Xylosidase	3.2.1.37	4-MU-β-D-xyloside	500 µM	1 h
β-1,4-N-Acetylglucosaminidase (Chitinase)	3.2.1.14	4-MU-N-acetyl-β-D-glucosaminide	500 µM	1 h
Phosphomonoesterase	3.1.3	4-MU-phosphate	800 µM	40 min
Phenoloxidase	1.10.3	L-3,4-Dihydroxyphenylalanine (DOPA)	10 mM	20 h
Peroxidase	1.11.1	L-3,4-Dihydroxyphenylalanine (DOPA); H_2O_2	10 mM; 0.4 %	20 h

MU … Methylumbelliferyl

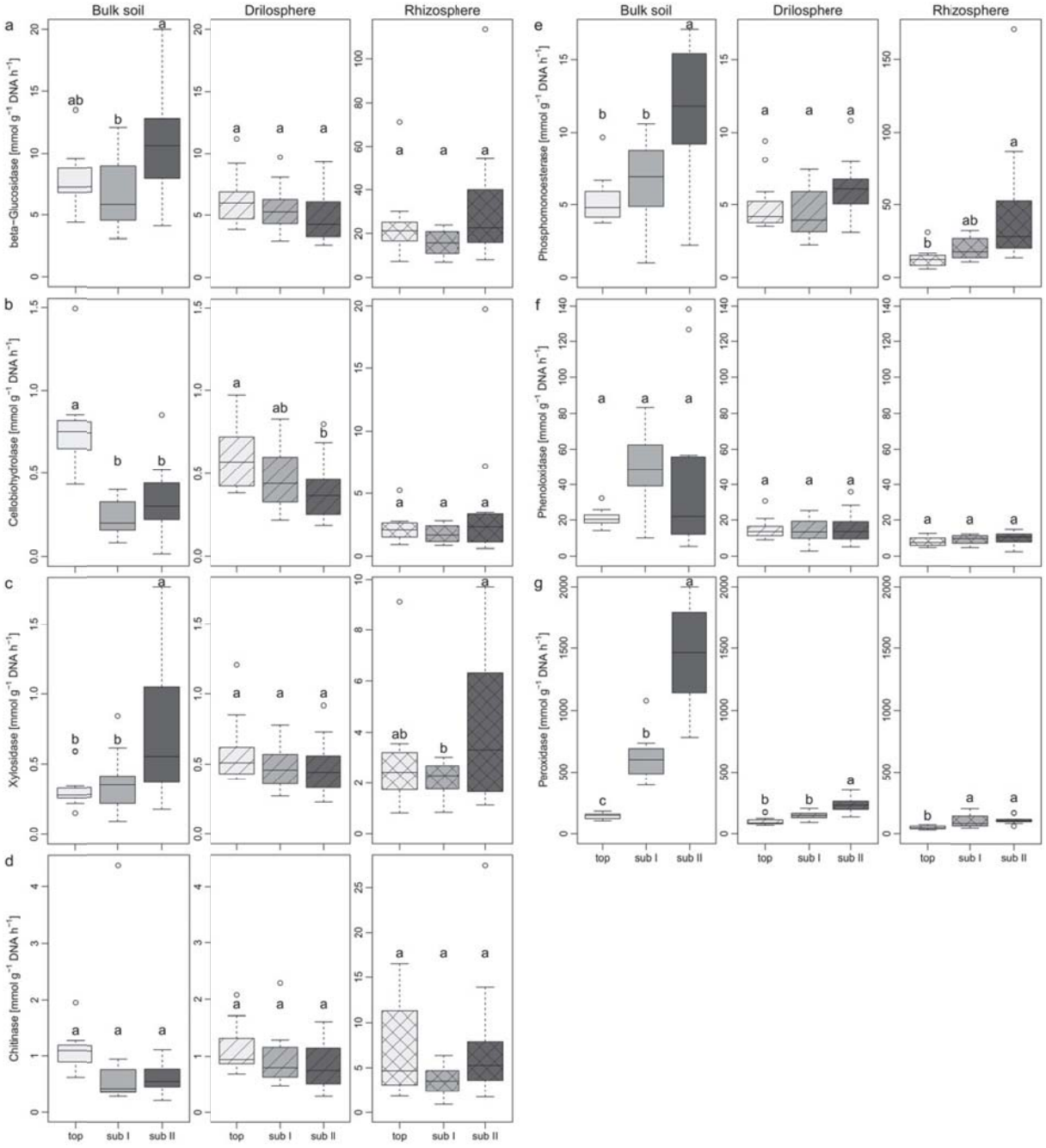

Fig. S1 Specific potential enzyme activities in different soil compartments along the soil profile. Different letters indicate significant differences ($P \leq 0.05$; $n=12$). *top* topsoil (10–30 cm), *sub I* upper subsoil (45–75 cm), *sub II* deep subsoil (75–105 cm).

E PUBLICATION IV

frontiers in Microbiology

ORIGINAL RESEARCH
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Prokaryotes in Subsoil—Evidence for a Strong Spatial Separation of Different Phyla by Analysing Co-occurrence Networks

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Uksa M, Schloter M, Endesfelder D, Kublik S, Engel M, Kautz T, Köpke U and Fischer D (2015) Prokaryotes in Subsoil—Evidence for a Strong Spatial Separation of Different Phyla by Analysing Co-occurrence Networks. Front. Microbiol. 6:1269. doi: 10.3389/fmicb.2015.01269

Microbial communities in soil provide a wide range of ecosystem services. On the small scale, nutrient rich hotspots in soil developed from the activities of animals or plants are important drivers for the composition of microbial communities and their functional patterns. However, in subsoil, the spatial heterogeneity of microbes with differing lifestyles has been rarely considered so far. In this study, the phylogenetic composition of the bacterial and archaeal microbiome based on 16S rRNA gene pyrosequencing was investigated in the soil compartments bulk soil, drilosphere, and rhizosphere in top- and in the subsoil of an agricultural field. With co-occurrence network analysis, the spatial separation of typically oligotrophic and copiotrophic microbes was assessed. Four bacterial clusters were identified and attributed to bulk topsoil, bulk subsoil, drilosphere, and rhizosphere. The bacterial phyla Proteobacteria and Bacteroidetes, representing mostly copiotrophic bacteria, were affiliated mainly to the rhizosphere and drilosphere—both in topsoil and subsoil. Acidobacteria, Actinobacteria, Gemmatimonadetes, Planctomycetes, and Verrucomicrobia, bacterial phyla which harbor many oligotrophic bacteria, were the most abundant groups in bulk subsoil. The bacterial core microbiome in this soil was estimated to cover 7.6% of the bacterial sequencing reads including both oligotrophic and copiotrophic bacteria. In contrast the archaeal core microbiome includes 56% of the overall archaeal diversity. Thus, the spatial variability of nutrient quality and quantity strongly shapes the bacterial community composition and their interaction in subsoil, whereas archaea build a stable backbone of the soil prokaryotes due to their low variability in the different soil compartments.

Keywords: subsoil, drilosphere, rhizosphere, bacterial diversity, core microbiome, co-occurrence

INTRODUCTION

Soils are known as hotspots for biodiversity. Moreover soils provide a wide range of ecosystem services including nutrient cycling, carbon sequestration, safeguarding of water resources and plant growth promotion (van der Heijden et al., 2008; Berg, 2009; Bardgett and van der Putten, 2014). In contrast to the microbiome of topsoil, which has been well studied in the last decades, focussing on microbial community structure and function as well as plant-microbe interactions (Berg and Smalla, 2009), microbes below the plow horizon so far are poorly investigated. The general opinion implies a decrease of abundance, diversity and activity of bacteria, fungi and archaea with soil depth as a result of the more oligotrophic conditions present in deeper soil layers; consequently it is assumed that the contribution of the subsoil microbiome to the overall turnover of nutrients in soil is low (Fuka et al., 2009; Eilers et al., 2012; Stone et al., 2014). However, these observations are biased by the fact that small-scale spatial heterogeneity of microbes in subsoils has received almost no attention and the presence of hotspots in subsoils, which may change the described low microbial activity in subsoils, has been mostly overlooked (Nunan et al., 2003; Vos et al., 2013).

Commonly hotspots in subsoils are mainly connected to vertical biopores, which are formed by earthworms or thick tap roots (Kuzyakov and Blagodatskaya, 2015). These biopores are characterized by relatively high nutrient input due to plant exudates in the rhizosphere (Neumann et al., 2014) or cast deposition of earthworms and their coating in the drilosphere (Andriuzzi et al., 2013). As microbial community composition is linked to substrate quantity and quality (Marschner et al., 2001; Aira et al., 2010; Stromberger et al., 2012) a pronounced spatial heterogeneity of microbes with differing lifestyle in subsoils can be assumed. This has been partly confirmed by DNA based fingerprint analyses of bacterial community structure. Here differences between bulk soil, drilosphere, and rhizosphere communities in subsoil were more pronounced in subsoil compared to topsoil (Uksa et al., 2014). These differences induced a high spatial variability of potential enzyme activities in the investigated subsoil compartments (Uksa et al., 2015). However, still data is missing on microbial network structures in the different subsoil compartments and the related ecophysiology of the microbiomes.

In this study we analyzed archaeal and bacterial community composition based on barcoding of 16S rRNA after PCR amplification of DNA directly extracted from bulk soil, drilosphere and rhizosphere of top- and subsoil samples from an agricultural field planted with the fodder crop *Cichorium intybus*. This plant species is known to strongly structure soils by the formation of thick biopores also in the subsoil (Löfkvist et al., 2005; Kautz et al., 2014). We analyzed network structures and co-occurrence pattern in the different compartments. We adressedthe question whether for top- and subsoils a specific set of co-occurring microbes can be identified independent from the spatial variability in each soil layer or if each hotspot (rhizosphere or drilosphere) harbors a set of co-occuring microbes independent from soil depth. The latter would emphasize a selection of microbiomes by earthworms or plants (Berg and Smalla, 2009). In addition the number of shared microbes and the size of the core microbiome in topsoil and subsoil was estimated. Based on our previous results (Uksa et al., 2014) we hypothesized that in topsoils the number of shared OTUs between the different compartments bulk soil, drilosphere and rhizosphere is higher as compared to subsoils.

MATERIALS AND METHODS

Experimental Field Site and Soil Sampling

Soil samples were obtained from three separated plots (each 10×6 m) of an agricultural field at Klein-Altendorf (Germany; 50°37′21" N, 6°59′29" E) in May 2011 and treated as true replicates. At the month of sampling the mean temperature was 14.8°C and mean daily precipitation was 33.2 mm (Agrarmeteorologie Rheinland-Pfalz; www.wetter.rlp.de). *Cichorium intybus* L. was grown on the field for the third year; at the sampling time point plants were in the early flowering stage. *C. intybus* has a tap root system and thus forms large sized biopores, which significantly structure the soil (Löfkvist et al., 2005). The soil has been classified as Haplic Luvisol and characterized by a silty clay loam texture with clay accumulation in the subsoil between 45 and 95 cm (Gaiser et al., 2012).

For soil sampling, one soil pit per plot with a size of $1 \times 1 \times 1$ m was excavated using a hydraulic shovel. Before sampling about 5 cm per side wall were carefully removed by a spade. From the profiles, the bulk soil, the drilosphere and the rhizosphere were sampled both in topsoil (10–30 cm) and subsoil (60–75 cm). One millimeter coatings around earthworm burrows of 0.4–1.2 cm were considered as drilosphere and scraped out with a small sterile spoon. Roots were sampled from the soil profiles together with maximal 2 mm adhering soil by using sterile tweezers. The adhering soil was referred as rhizosphere. Soil with no roots and earthworm channels was defined as bulk soil. At least 5 subsamples for each compartment were pooled from each profile, transported on dry ice and stored at −80°C before DNA extraction.

DNA Extraction and Quantitative Real-time PCR of 16S rRNA Genes

DNA was extracted using the FastDNA® Spin Kit for Soil (MP Biomedicals, Eschwege, Germany) following the manufacturer's protocol. To enhance DNA yield, an additional bead beating step for 40 s and an incubation step at 55°C for 5 min before elution was performed. NanoDrop 1000 Spectrophotometer (PeqLab, Erlangen, Germany) was used for DNA quality assessment by measurement A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} ratios. The DNA concentration was determined from 250-fold dilutions using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Darmstadt, Germany) with a detection range from 0.016 to 1 ng·μl^{-1}.

Abundance of bacterial and archaeal 16S rRNA genes was quantified by real-time PCR using a 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) and the Power SYBR® Green PCR Master Mix (Applied Biosystems)

following the protocol described by Töwe et al. (2010). Primers rSAf(i) (Nicol et al., 2003) and 985r (Bano et al., 2004) were used for archaeal 16S rRNA gene amplification, whereas bacterial 16S rRNA gene copy numbers were quantified with primers FP16S and RP16S (Bach et al., 2002) at a final concentration of 0.2 or 0.4 μM, respectively. According to an *in silico* analysis using the Genomatix software, version November 2012 (www.genomatrix.de), the archaeal primer pair covered representatives of Thaumarchaeota, Euryarchaeota and Crenarchaeota and thus could be considered as universal. Cloned 16S rRNA genes from *Methanobacterium* sp. (Timmers et al., 2012) and *Clavibacter michiganensis* subsp. *michiganensis* (DSM 46364) were used as qPCR standards for archaea and bacteria respectively. The DNA template was 128-fold diluted to avoid inhibition as tested in pre-experiments (data not shown). To increase efficiency of archaeal real-time PCR, 0.06% BSA was added to the master mix. For the amplification of bacterial 16S rRNA genes 40 PCR cycles (95°C—20 s, 62°C—1 min, 72°C—30 s) were performed; for amplification of the archaeal 16S rRNA genes 5 PCR cycles (95°C—20 s, 55°C—1 min, 72°C—30 s, lowering the annealing temperature for 1°C each cycle) followed by 35 PCR cycles with 50°C annealing temperature were performed. PCR efficiency was 85% for archaeal, and 92% for bacterial 16S rRNA gene amplification.

Barcoded Pyrosequencing and Data Processing

PCR amplicons of bacterial and archaeal 16S rRNA genes were sequenced using the 454 GS FLX+ instrument (Roche, Penzberg, Germany) following the manufacturer's protocols for amplicon library preparation (version June 2013) and emPCR amplification (version May 2011) with primers for unidirectional sequencing (Lib-L) and the XL+ Kit (version June 2013).

The specific primer sequences for bacterial 16S rRNA genes were 27F (5′-AGAGTTTGATCMTGGCTC-3′; *E. coli* position 8-25; Lane, 1991) and 984r (5′-GTAAGGTTCYTCGCG-3′; *E. coli* position 970-985; Klindworth et al., 2013). For archaeal 16S rRNA genes, the primer pair rSAf(i) (5′-CCTAYGGGGCGCAGCAG-3′; *E. coli* position 341–357; Nicol et al., 2003) and 958r (5′-YCCGGCGTTGAMTCCAATT-3′; *E. coli* position 940–958; Bano et al., 2004) was used.

Following the 454 sequencing guidelines for unidirectional sequencing, primer sequences were extended by the adapter sequences A and B for forward and reverse primers respectively; in addition the forward primer was labeled with a multiplex indices (MID). PCR reaction was performed with FastStart™ High Fidelity PCR System (Roche). To improve PCR efficiency 0.3% BSA was added; for the amplification of the archaeal 16S rRNA gene in addition 8% DMSO, as suggested by Timmers et al. (2012), was added. For amplification 1 ng DNA (bacterial 16S rRNA gene) respectively 30 ng (archaeal 16S rRNA gene) was used as template. PCR was initiated by a heating step to 95°C for 5 min followed by 25 (bacterial 16S rRNA gene) respectively 30 (archaeal 16S rRNA gene) cycles (95°C for 1 min, 50°C for 1 min and 72°C for 1 min) followed by a final extension at 72°C for 10 min.

Three PCR amplicons for each sample were pooled and purified with the NucleoSpin® Gel and PCR cleanup Kit (MACHEREY-NAGEL, Düren, Germany). The final DNA amount of the amplicon libraries was determined with Quant-iT™ PicoGreen® dsDNA Assay Kit as mentioned above. The average fragment size was measured with Agilent 2100 bioanalyzer instrument using the Agilent DNA 7500 Kit (Agilent Technologies, Waldbronn, Germany). The final sequencing run was performed according to the manufacturer's protocol and initial data processing was performed using gsRunProcessor v2.9.

Data processing of raw flowgrams was carried out with mothur (release v.1.33.0; Schloss et al., 2009) following the 454 SOP by Schloss et al. (2011). The SILVA reference file, comprising of bacterial, archaeal, and eukaryotic rRNA sequences of the small subunit (release 119; Quast et al., 2013) was used for alignment and chimera removal. Sequences were classified with the RDP database (release 10; Cole et al., 2014), which included both bacterial and archaeal 16S rRNA sequences, at 80% confidence level. OTUs were assigned by clustering at 95 and 90% similarity level. Pyrosequencing data sets were deposited at GenBank's Short Read Archive under the following accession number: PRJNA293151 (BioProject).

Statistical Analyses

Statistical analysis and graphic illustrations were computed with the R software (version 3.0.2; R Core Team, 2013) and the packages "agricolae" (de Mendiburu, 2014), "scatterplot3d" (Ligges and Mächler, 2003), "shape" (Soetaert, 2014), "stats" (R Core Team, 2013), "vcd" (Meyer et al., 2014), and "vegan" (Oksanen et al., 2015). Reads were subsampled according to the minimum number of reads per sample (3081 archaeal/4815 bacterial sequences). Richness, rarefaction and Shannon diversity index were calculated on the basis of 90% similarity level, as rarefractures analysis indicated full coverage at this level. As the "species" definition of prokaryotes at =97% similarity is still a controversial topic and RDP database classifies OTUs only down to 95% which corresponds to the genus level, all other analyses were performed on this similarity level. Prior to multivariate analysis with PerMANOVA, relative abundance data was Hellinger-transformed (Ramette, 2007). Significant differences within single OTUs were tested by ANOVA followed by posthoc Tukey-HSD test ($\alpha = 0.05$). Bonferroni test was used for adjustment of *P*-values after multiple comparisons.

As the copy number of 16S rRNA genes highly varies across bacterial and archaeal genomes, 16S rRNA gene abundance data was adjusted according to the Ribosomal RNA Database (*rrn*DB; Stoddard et al., 2014) by using the "Pan-taxa statistics for RDP taxonomy" file (release 4.3.3). To obtain the adjusted abundance for each OTU, the absolute abundance of 16S rRNA reads were divided by the mean copy number of 16S rRNA genes per genome for the corresponding genus or nearest classifiable level. The resulting discrepancy between 16S rRNA gene abundance and adjusted abundance in every sequenced sample was used to correct the total 16S rRNA gene abundance determined by qPCR.

All OTUs with a minimum of 6 reads in at least 3 samples were considered for the estimation of microbial co-occurrence networks. Co-occurrence between any pair of OTUs was defined

by a significant correlation ($P < 0.05$) with a correlation coefficient >0.6. The corresponding co-occurrence network was derived by setting an edge between pairs of co-occurring OTUs. To analyse spurious correlations caused by the compositional structure of the relative abundance, the CCREPE (Faust et al., 2012) method was used to estimate *P*-values from Spearman's rank correlation coefficients. Clusters of co-occurring OTUs were defined from the resulting co-occurrence network by grouping OTUs with high intra-cluster connectivity and low connectivity to other OTU clusters. Microbial clusters were identified by using the Markov Dynamics clustering algorithm (Schaub et al., 2012) implemented in MATLAB®. This algorithm allowed the identification of clique-like communities within a continuous range of a parameter (i.e., Markov time), capturing dynamic characteristics of processes on the network. The number of clusters of co-occurring OTUs was determined by choosing a community number larger than two which had the longest stable assignment over a range of Markov time points. Similarly to positive correlations, OTUs were defined to be negatively correlated if the correlation coefficient was < -0.6.

RESULTS

Abundance of 16S rRNA Genes from Archaea and Bacteria in Different Soil Compartments of Top- and Subsoil

Microbial bbiomass was estimated by the amount of extracted DNA and related to soil dry weight (Figure S1; Gangneux et al., 2011). As expected, highest amounts of DNA were extracted from rhizosphere samples; DNA concentrations in the drilosphere were lower but still higher than in bulk soil ($P = 0.001$). Whereas no significant differences were found in DNA concentrations comparing rhizosphere samples from the top- and the subsoil, for bulk soil and drilosphere significant lower DNA concentrations were measured in subsoil as compared to topsoil ($P = 0.005$, $P = 0.011$).

QPCR analysis revealed 10^7-10^9 archaeal and 10^8-10^{12} bacterial 16S rRNA copies g^{-1} dry weight. For all soil compartments in topsoil and subsoil, bacterial 16S rRNA gene copy numbers were higher compared to their archaea counterpart ($P < 0.001$). Ratios of bacterial to archaeal 16S rRNA gene copy numbers were in the range of 20–380 (**Figure 1A**), which corresponds to a proportion of 0.3–4.8% of archaeal 16S rRNA genes. Significantly higher ratios were found in the rhizosphere of the subsoil ($P < 0.001$), but no differences were observed between topsoil and subsoil within each soil compartment. The results did not change, when 16S rRNA gene abundance was corrected for the varying 16S rRNA gene copy numbers per genome (data not shown).

Comparison of Archaeal and Bacterial Richness and Diversity in Different Soil Compartments of Top- and Subsoil

The used barcoding approach resulted after de-multiplexing in 81388 (archaea) and 160768 (bacteria) flowgrams. After data trimming and de-noising, 80,151 and 158,567 high quality reads with an average length of 568 and 545 bp were obtained for archaea and bacteria, respectively. 8.1 and 1.3% of the reads were removed as chimeric sequences from the archaeal and bacterial dataset. One thousand and twenty-two sequences derived from chloroplasts in the bacterial dataset and were not included in downstream analysis. Also unknown sequences (21 archaeal and 3 bacterial reads) were not further processed. Sequences were analyzed on the level of 90 and 95% similarity and subsampled according to the minimum sample size in each dataset. Singletons were not excluded from the analysis, as they were not evenly distributed across the samples and variation between the six soil compartments exceeded the overall variation (Figure S2).

Richness of bacteria and archaea was estimated on a level of 90% similarity, where coverage was highest and expected effects of singletons derived from sequencing errors were lowest (Figure S3). Overall, bacterial richness and diversity was significantly higher compared to archaea. Interestingly, rarefaction curves showed significant higher richness in the topsoil for bacteria ($P < 0.001$), but a higher richness in the subsoil for archaea ($P = 0.001$). Nevertheless, Shannon diversity indices were for both, archaea and bacteria, higher in the topsoil ($P = 0.026$, $P < 0.001$; **Figures 1B,C**, Figure S4). In the subsoil only for bacteria differences between the soil compartments were found. In the drilosphere the highest diversity based on the Shannon index was observed ($P = 0.009$). Interestingly in this compartment the effect of soil depth for both archaea and bacteria was lowest.

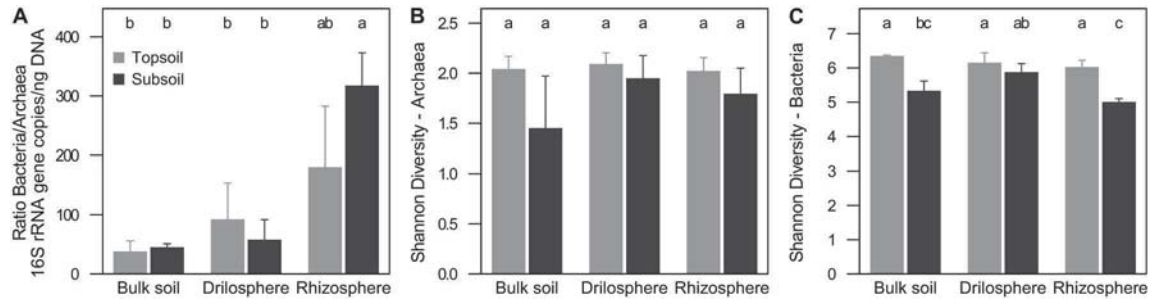

FIGURE 1 | Relation of 16S rRNA copies between archaea and bacteria (A) and Shannon diversity index at 90% similarity level of archaea (B) and bacteria (C) in soil compartments of topsoil and subsoil. Different letters indicate significant differences ($P \leq 0.05$).

Soil Depth and Compartment-specific Microbes as Revealed by Community Composition

At 95% similarity level, clustering revealed 614 archaeal and 9425 bacterial OTUs. They were analyzed in the first step by PCA to investigate how the six compartments differ in their community compositions: the first three components are plotted in **Figure 2**. A clear separation between topsoil and subsoil could be detected for archaea only (PerMANOVA: $P = 0.001$), whereas the compartments bulk soil, drilosphere and rhizosphere showed no significant differences ($P = 0.489$). In contrast to these findings, a clear difference for compartments as well as for soil depth ($P = 0.001$, each) was found for bacteria. Variation between the replicates was lowest in bulk samples from topsoil for both, the bacterial and archaeal dataset.

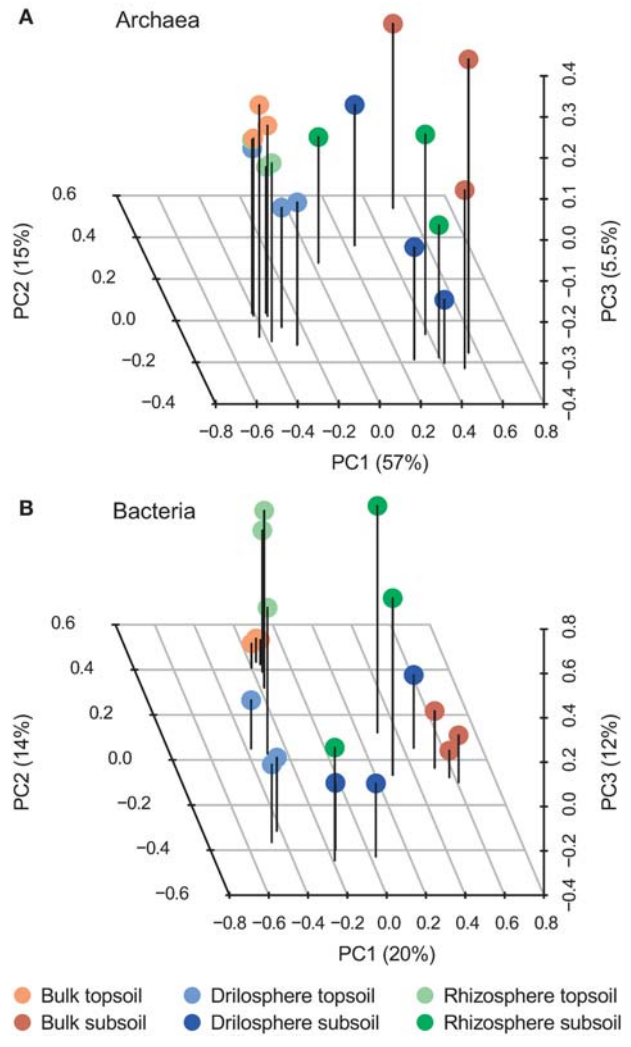

FIGURE 2 | Principal component analysis (PCA) of archaeal (A) and bacterial (B) OTUs based on 16S rRNA gene amplicons at 95% similarity level. The first three components from the PCA of relative, Hellinger-transformed data are shown.

Archaeal communities were dominated by the genus *Nitrosophaera* with a relative abundance of 90–99% for all six compartments (Figures S5, S6). This genus is known as an ammonium-oxidizing archaeon and the only abundant genus found in our dataset for the phylum Thaumarchaeota. Some OTUs classified as *Nitrososphaera* were significantly higher abundant in topsoil (9), whereas others dominate in subsoil (4). Euryarchaeota was the second phylum detected, being higher abundant in subsoil ($P = 0.048$). Most OTUs belonging to Euryarchaeota could not be classified further, except the methanogen *Methanosarcina* which was significantly higher abundant in the topsoil ($P = 0.001$) especially in the drilosphere and rhizosphere with >0.3% of all reads, whereas relative abundance in subsoil was lower (0.06%). Ternary plots for archaeal OTUs indicated for top- and subsoil (**Figures 3A,B**) that drilosphere and rhizosphere did not harbor "specialized" OTUs, which would be located at the respective tip area of the ternary plot. Only for bulk soil, specialized archaea were found, when top- and subsoil were compared, which were classified as Euryarchaeota. However, the majority of the archaeal OTUs was located at the middle of the ternary plot, harboring mainly Thaumarchaeota including 20 ubiquitous OTUs (all *Nitrososphaera*), that were present in all samples and contributed to 56% of the reads analyzed.

The bacterial community analyses revealed 21 bacterial phyla present, although only for 10 phyla relative abundance in all six investigated soil compartments was >0.5% (Figure S7): Actinobacteria (29–43%), Bacteroidetes (5–32%), Proteobacteria (10–24%), Acidobacteria (4–18%), Verrucomicrobia (3.4–5.6%), Planctomycetes (2–3.9%), Nitrospirae (0.3–3.5%), Firmicutes (1.5–2.8%), Gemmatimonadetes (0.4–2%), and Chloroflexi (0.2–0.9%). Acidobacteria, Gemmatimonadetes, and Planctomycetes were significantly higher abundant in bulk soil. In contrast to bulk soil, rhizosphere and drilosphere harbored a higher portion of Bacteriodetes. Proteobacteria in turn were typically found as major parts of the rhizosphere community. Besides the compartment type, also depth related differences were present on the phylum level. For topsoil only the low abundant phylum Chloroflexi was significantly increased, whereas in subsoil samples bacterial community harbored more Actinobacteria, Nitrospirae and Verrucomicrobia. For the latter phylum only for bulk soil and drilosphere significant differences were found. In addition, unclassified bacterial OTUs on phylum level (3–12%) were higher abundant in bulk samples from subsoil. Interestingly, Firmicutes did not show significant differences between the compartments. Data are summarized as ternary plots (**Figures 3C,D**).

To identify a bacterial "intrinsic core microbiome" only OTUs at the level of 95% homology were selected, which were present in at least 2 of the 3 biological replicates for each of the six soil compartments and where the standard deviation did not exceed the mean value of the relative abundance to enable a low variation between the samples. This resulted in 52 both rare and abundant OTUs, that contributed in sum to 7.6% of the reads analyzed (**Figures 4**, S5). All abundant phyla were represented in the core microbiome with the majority of Actinobacteria

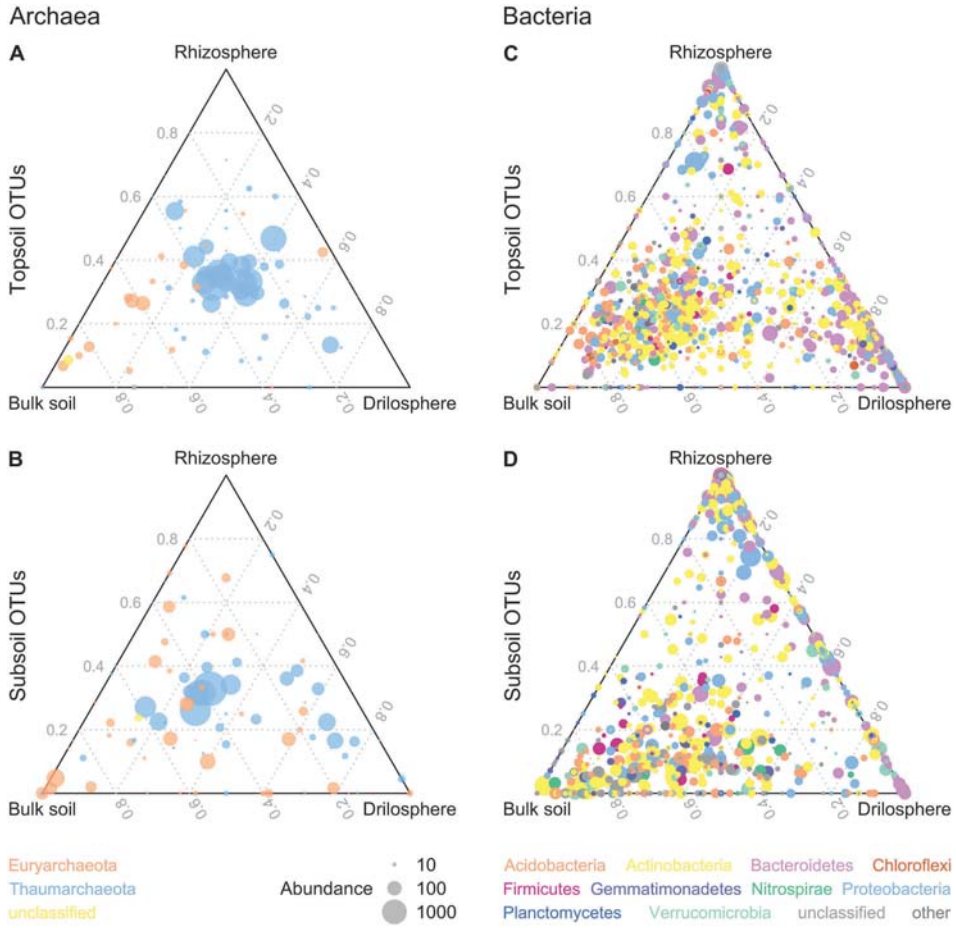

FIGURE 3 | Distribution of archaeal (A,B) and bacterial (C,D) OTUs between soil compartments in ternary plots. OTUs with a higher abundance in topsoil or subsoil are displayed in **(A,C)** or **(B,D)**. Similarity level is 95% and only OTUs with a minimum absolute abundance of 5 are shown. The size of the dots represents the absolute abundance of one OTU.

and Proteobacteria accounting for 35 and 39% of the reads, respectively.

Besides the overall core microbiome, the core microbiomes were analyzed separately for topsoil and subsoil using the same criteria as described above. The bacterial topsoil core microbiome shared 4.3% of all OTUs between bulk soil, drilosphere and rhizosphere, which corresponds to 27% of the reads from the topsoil. In contrast, the bacterial subsoil core microbiome shared only 2% of OTUs, which accounted 16% of the reads. The same procedure for the archaeal dataset revealed an increased core microbiome as compared to bacteria both in topsoil and subsoil, but again, the subsoil archaeal core microbiome shared between bulk soil, drilosphere and rhizosphere (7% OTUs accounting for 69% of the reads) was smaller than in topsoil, where 11% of the OTUs were detected in all compartments, which represented 93% of the reads.

Clusters of Co-occurring Bacterial OTUs

Co-occurrence analysis of bacteria at 95% similarity level resulted in the identification of four clusters of co-occurring OTUs (**Figure 5**) that could be attributed to the different soil compartments and depths as revealed by clustering (Figure S8). In the dendrogram, replicates of bulk topsoil and bulk subsoil clustered closer together than samples obtained from rhizosphere and drilosphere, which emphasizes the high variability of those compartments. The clusters were further named "rhizosphere cluster R," "drilosphere cluster D," "bulk topsoil cluster Bt," and "bulk subsoil cluster Bs" and reflect the significant differences found for the overall community composition between the six soil compartments (**Figures 5A–D**).

Remarkably, the clusters Bt and Bs shared 28 and 11 OTUs of the 52 OTUs of the core microbiome, respectively, but no OTUs were shared between the core microbiome and the hotspot clusters D and R. In the ternary plot, Bt and Bs clusters were located in the bulk soil-orientated middle area, whereas D and R clusters were more located at the tips of the triangle, where "specialized" OTUs were expected (Figure S9). Many phylogenetic lineages and genera were shared between the four clusters, although they were represented

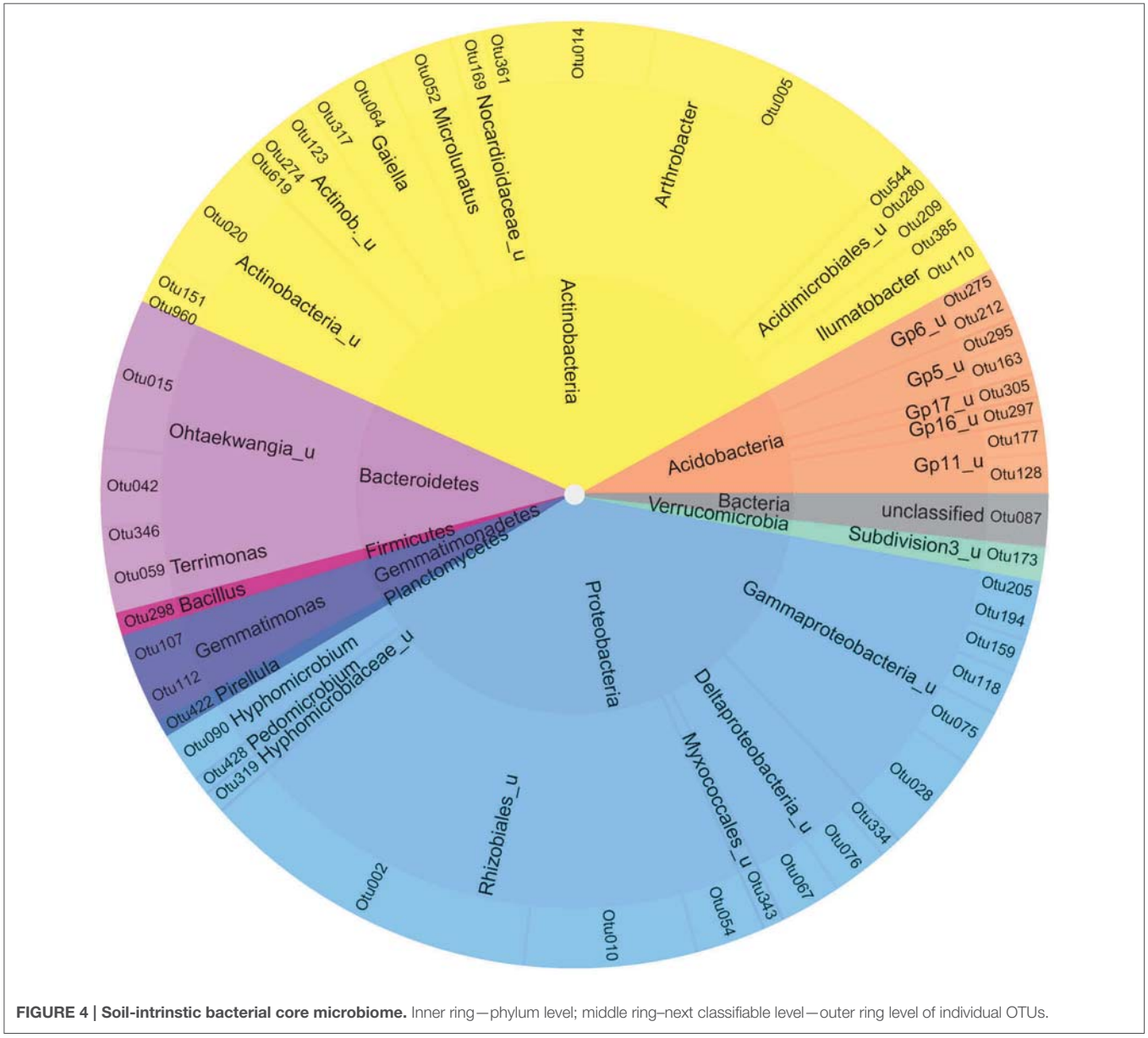

FIGURE 4 | Soil-intrinstic bacterial core microbiome. Inner ring—phylum level; middle ring–next classifiable level—outer ring level of individual OTUs.

by different OTUs, especially *Nocardioidaceae* (Actinobacteria), *Ohtaekwangia* (Bacteroidetes), *Chitinophagaceae* (Bacteroidetes), and Gammaproteobacteria including *Steroidobacter*.

Bs clusters were characterized by the dominance of Actinobacteria (54%). Also low abundant phyla and unclassified bacteria were highly represented in Bs cluster. Acidobacteria were highly abundant in Bt and Bs clusters, and were represented by 4–6 classes. Interestingly, the four major lineages of Verrucomicrobia were restricted to one cluster each: Spartobacteria to Bs, Subdivision3 (Verrucomicrobiae) to Bt, and *Opitutus* (Opitutae) and *Luteliobacter* (Verrucomicrobiae) to D. Similar distribution pattern were observed for the phylum of Fermicutes: The genera *Bacillus*, *Cohnella,* and *Paenibacillus* were typical for the Bs cluster, whereas *Clostridiaceae* were part of the Bt cluster.

In contrast to the bulk soil clusters, D and R clusters harbored many specialized OTUs and lineages. Interestingly, drilosphere and rhizosphere shared more OTUs in subsoil (**Figure 3D**). The D cluster was dominated by Bacteroidetes with 43%. The R cluster was the smallest and harbored only Actinobacteria, Bacteroidetes and Proteobacteria. Especially the high proportion of Proteobacteria distinguished the R cluster from the others.

OTUs of a cluster that negatively correlated with most OTUs from other clusters, are listed in **Table 1**. In this respect the genera *Ilumatobacter*, *Gaiella*, *Marmoricola*, and *Steroidobacter* were of high interest. Each of these genera harbored different OTUs that are linked to different clusters and contributed strongly to the negative correlations between them. Acidobacterial OTUs distinguished the Bt and Bs clusters from each other as well as clusters D and R. *Flavobacterium*, again was a key genus in the

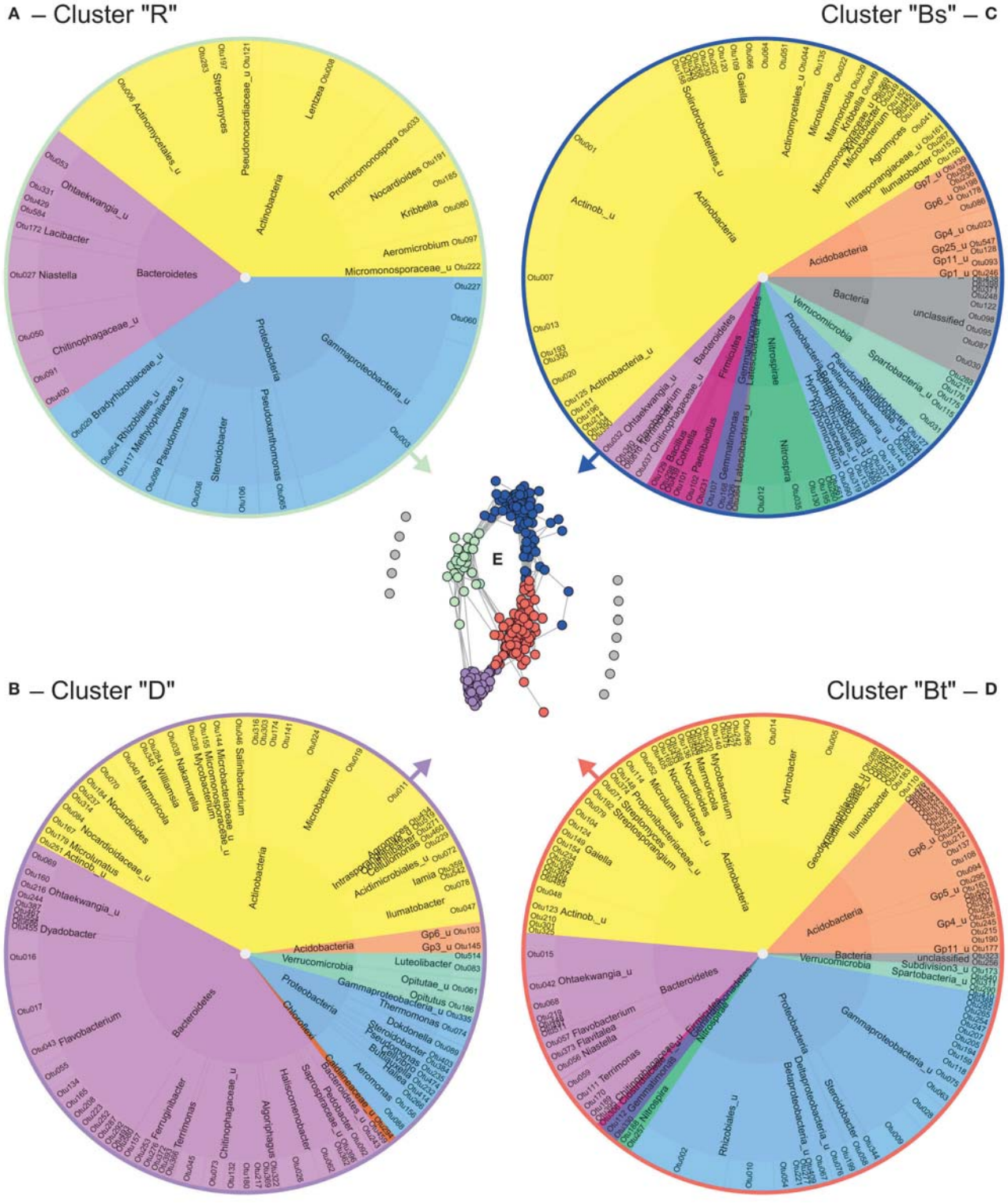

FIGURE 5 | Formation and composition of bacterial clusters of co-occurring OTUs (A–D) as revealed by network analysis (E). Each dot in the network represents one OTU at 95% similarity level and each connecting line a positive correlation with Spearman's rank correlation coefficient >0.6. For the gray colored OTUs in the network no positive correlations were found. Inner ring—phylum level; middle ring—genus or nearest classifiable level. R, rhizosphere; D, drilosphere; Bs, bulk subsoil; Bt, bulk topsoil.

TABLE 1 | Negative correlations between clusters of co-occurring OTUs.

	Cluster Bt	Cluster Bs	Cluster D	Cluster R
Bt	-	Gp4_u Otu215; Gp6_u Otu394 *Marmoricola* Otu406 *Microlunatus* Otu114 Actinobacteria_u Otu048, Otu301 Ohtaekwangia_u Otu068 *Clostridiaceae*_1_u Otu306 *Steroidobacter* Otu344 Gammaproteobacteria_u Otu254	Gp6_u Otu275	*Ilumatobacter* Otu413 *Gaiella* Otu367
Bs	*Ilumatobacter* Otu267 *Agromyces* Otu041, Otu420 *Marmoricola* Otu329 *Gaiella* Otu066, Otu109 Bacteria_u Otu098, Otu248	-	Gp11_u Otu128 *Ilumatobacter* Otu153 *Gaiella* Otu120 Latescibacteria_u Otu364	Gp6_u Otu236 Actinobacteria_u Otu020 Bacteria_u Otu030, Otu371
D	*Microbacterium* Otu141 *Salinibacterium* Otu046 *Marmoricola* Otu040 *Nocardioidaceae*_u Otu167 *Flavobacterium* Otu017, Otu287, Otu580 *Aeromonas* Otu088 *Buttiauxella* Otu232 *Luteolibacter* Otu083, Otu514	*Ilumatobacter* Otu078 *Microbacteriaceae*_u Otu144 *Microlunatus* Otu179 *Ferruginibacter* Otu253 *Terrimonas* Otu366 *Chitinophagaceae*_u Otu073	-	*Flavobacterium* Otu016, Otu252, Otu493 *Ferruginibacter* Otu157, Otu253 Bacteroidetes_u Otu459
R	*Aeromicrobium* Otu097 *Streptomyces* Otu283	*Bradyrhizobiaceae*_u Otu029	*Steroidobacter* Otu106	-

The most important OTUs of each cluster (row) responsible for minimal 20% of all negative correlations to another cluster (column) are listed. R, rhizosphere; D, drilosphere; Bs, bulk subsoil; Bt, bulk topsoil.

D cluster that negatively correlated especially with OTUs from Bt and R clusters. *Aeromicrobium* accounted for most negative correlations of the R cluster with the Bt cluster.

DISCUSSION

Variation within Soil Compartments on the Plot Scale

As shown in the PCA (**Figure 2**), the variation of both archaeal and bacterial communities was much lower in topsoil than in subsoil. The more homogeneous topsoil on the plot scale is a result of plowing and the high root density at the time point of sampling. Furthermore, overall the higher nutrient status in topsoil compared to subsoil might have induced lower gradients between the soil compartments. Thus, the variation between and within the soil compartments in subsoil were increased as a result of longer distances between hotspots and less disturbance from outside. These observations differ from non-managed ecosystems. For example, Eilers et al. (2012) showed a higher variation in topsoil compared to subsoils, when microbial communities of a forest soil where compared. Overall the drilosphere and rhizosphere communities in general shared more abundant OTUs in the subsoil as compared to the topsoil (**Figure 3D**). A possible explanation is that roots grow into earthworm burrows and *vice versa* earthworms invade biopores developed from decaying roots.

Archaea—a Small, but Stable Backbone of Prokaryotic Communities in the Soil

Archaea were in all analyzed samples part of the soil prokaryotic community independent from spatial heterogeneity and depth. Their proportion in this study compared to bacteria is comparable to other studies where the microbiome of bulk soils has been analyzed (Bates et al., 2011; Pereira e Silva et al., 2012). Although their abundance based on 16S rRNA gene copies was below 5% in all samples, the highly abundant genus *Nitrososphaera* was a core genus and most likely strongly relevant for nitrification, as no OTUs indicative for ammonium oxidizing bacterial genera like *Nitrosomonas*, *Nitrispina*, or *Nitrosococcus* were identified. Especially in subsoil the dominance of only a few *Nitrososphaera* OTUs reflected the higher richness of archaeal communities as compared to topsoil. The common occurrence of *Nitrososphaera* in soils and their contribution to ammonium oxidation has been intensively investigated (Schauss et al., 2009; Tourna et al., 2011). A pronounced bias of the used archaeal 16S rRNA gene primers toward *Nitrososphaera* could be excluded, as the relation of the major archaeal taxa remained constant in a metagenome analysis after direct sequencing of the same soil samples (data not shown).

In the more oligotrophic environments of bulk soil, overall more archaeal 16S copies were detected and in particular unclassified OTUs from the Euryarchaeota increased. This points to an overall oligotrophic strategy of Euryarchaeota and is backed up by the higher archaeal richness which was observed

in subsoil samples. Only the anaerobic methanogenic archaeon *Methanosarcina* was found in the copiotrophic environments of drilosphere and rhizosphere topsoil. As a residue of earthworm activity, the origin of this prokaryote might be the gut microbiome of invertebrates. However, also the assimilation of straw-derived carbon in the rhizosphere was shown for *Methanosarcina* (Shrestha et al., 2011) making it quite likely that microbes from the earthworm gut can survive in soil.

The Soil Intrinsic Core Microbiome of Bacteria

The definition of a core microbiome is still a challenging task (Shade and Handelsman, 2012). A "soil core microbiome" can be found by comparison of different soils, but this often neglects the spatial heterogeneity both on the horizontal and vertical axis and only gives information about the specific soil compartment investigated. Therefore, the attempt here was to identify an "intrinsic soil core microbiome" that can be interpreted as a backbone of a specific soil type, regardless of its depth and spatial heterogeneity. This study gives evidence that on phylum level the cluster Bt, mostly affiliated to the bulk topsoil, is indeed a good representation of the soil intrinsic core microbiome over horizontal and vertical gradients as it includes most phyla and groups found generally in soils (Stroobants et al., 2014).

Co-occurrence analysis revealed a well-defined microbial cluster in subsoil which clearly differs from the other clusters. The actinobacterial dominance in the Bs cluster suggests a high potential for secondary metabolism in subsoil that needs to be investigated further, as over 50% of the Actinobacteria could not be further classified. Their potential for plant growth promotion, mainly biocontrol of phytopathogens, (Haesler et al., 2014; Hamedi and Mohammadipanah, 2014) as well as for the degradation of recalcitrant carbon, which is typically found in deeper soil layers (Rumpel and Kögel-Knabner, 2011) might be immense. The abundance of Acidobacteria, which are reported as slow-growing microbes (Foesel et al., 2014), as well as members of Actinobacteria, Chloroflexi, and Gemmatimonadetes (Zhang et al., 2003; Davis et al., 2011), and endospore-forming Firmicutes in this cluster might explain the lower microbial activity in subsoil (Kramer et al., 2013; Stone et al., 2014; Uksa et al., 2015). The genus *Nitrospira* which was highly abundant in the Bs cluster is the possible complement to *Nitrososphaera* for the nitrification processes in this soil compartment, as no other known nitrite-oxidizing bacteria like *Nitrobacter, Nitrospina*, or *Nitrococcus* could be detected.

The co-occurring OTUs which were typical for drilosphere and rhizosphere indicated specialized microbial clusters with low overlaps to the bulk soil clusters. These OTUs could not been clustered according to soil depth like shown for the bulk soil (**Figure 2**), probably due to their vertical expansion in the biopores and nutrient input via earthworm cast and root exudates. The relatively high accessibility of nutrients therefore favors copiotrophic microbes and those interacting with earthworms and plants.

The high abundance of Bacteroidetes has been found in the driolosphere cluster, which can be explained nicely by the high abundance of this group of bacteria in the gut microbiome of invertebrates and earthworm cast (Furlong et al., 2002). Besides Bacteroidetes, also Proteobacteria are an essential part of the earthworm associated microbiome, like the genus*Aeromonas* which was specifically detected in earthworm cast (Kumari et al., 2012).

In the rhizosphere, a high interaction of Proteobacteria is well accepted (Berg and Smalla, 2009; Hartmann et al., 2009; Haichar et al., 2012; Lundberg et al., 2012). Those bacterial groups are more copiotrophic and able to grow fast on easy available nutrient (Fierer et al., 2007, 2012; Thomas et al., 2011).

Some antagonistic relationships which can be found in literature were confirmed in this study by negative correlations (Casida, 1983) and pointed out that not only nutrient availability but also the origin are relevant. Furthermore, OTUs from the same lineage or even the same genus (e.g., *Gaiella*, *Steroidobacter*, *Ilumatobacter*, *Ohtaekwangia*) are found to be negatively correlated and are therefore members to different clusters. These findings indicate antagonistic interaction or competition also on the species or ecotype/strain level and the presence of redundant phylogenetic lineages within differing soil compartments.

CONCLUSION

In this study, pronounced differences in bacterial and archaeal community structure in relation to soil depth and hotspots have been described. We identified an intrinsic soil core microbiome, that shows high similarity to the bulk topsoil cluster, which is frequently analyzed in studies, where samples from different compartments are taken together or homogenized However, specific soil communities and phylogenetic lineages become visible at different depths or hotspots, when sampling was performed at smaller scales without mixing or homogenization of different compartments. These observed differences could berelated to the nutrient availability, nutrient quality (Fuka et al., 2008) and the presence of soil invertebrates or plants. However, this study is based on one time point during the vegetation only and one soil type. It must be clarified in future studies whether the observed response pattern is also valid in different soil profiles, e.g., sandy soils, and at other time points of plant growth, or at phases were plant residues in terms of litter or moisture regimes play a major role in soil carbon dynamics.

We could confirm that more putative copiotrophs are present in the hotspots like rhizosphere amd drilosphere as compared to bulk soil and that the proportion of putative oligotrophs increases mainly in bulk soil. Furthermore, the nutrient rich hotspots drilosphere and rhizosphere form distinct bacterial communities with many putative antagonistic interactions. As expected, the size of the archaeal core—microbiome shared between the soil different soil compartments is larger as compared to the bacterial core—microbiome, which indicates a lower specialization of archaea toward copiotrophic lifestyle. However, we could also show that in subsoil the shared microbiomes between bulk soil and the hotspots decreased.

Supported by enzyme studies (Uksa et al., 2015) and culture-based approaches (Maloney et al., 1997), oligotrophic

organisms might be functional important for the turnover of recalcitrant material in the bulk subsoil, whereas in hotspots copiotrophic microbes contribute to the rapid decay of fresh organic matter. Therefore, the question arises, how oligotrophic and copiotrophic microbial patches in the subsoil interact with each other.

A general ecological classification of microbes based on the phylogeny however cannot be made, despite some bacterial phyla are mainly considered as oligotrophs or copiotrophs as they show correlation mainly with the carbon content (Fierer et al., 2007). In fact, the copy numbers of 16S rRNA genes per genome, which can vary highly within bacteria and archaea, is a better indication for the microbial lifestyle, as copiotrophic prokaryotes have the tendency to harbor more 16S rRNA gene copies compared to slow-growing organisms (Stoddard et al., 2014). Thus, the spatial distribution of microbes postulated to have an oligotrophic or copiotrophic lifestyle in this study must be confirmed on the basis of metagenome, metatranscriptome, and metabolome studies in the future.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01269

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

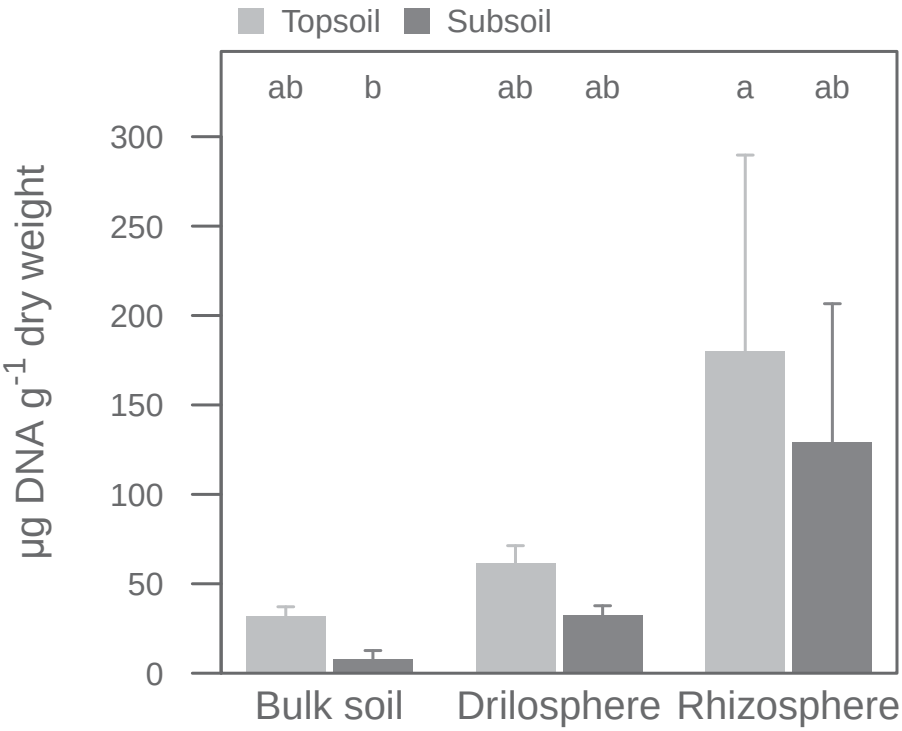

Figure S1: Biomass in different soil compartments and depth layers as estimated by DNA content.

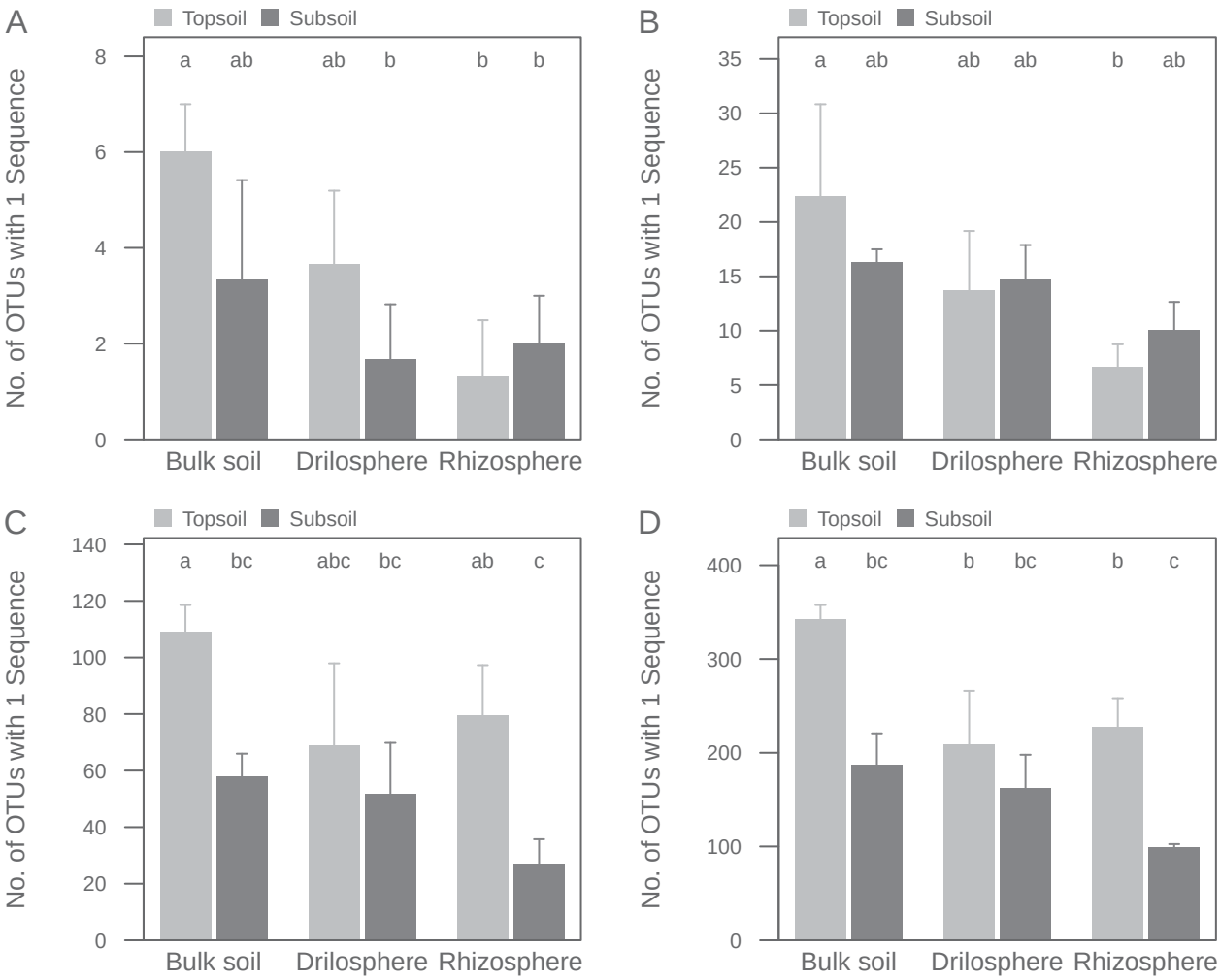

Figure S2: Count of singletons from archaeal (A, B) and bacterial (C, D) OTUs at 90% (A, C) and 95% (B, D) similarity level. Different letters indicate significant differences ($P \leq 0.05$).

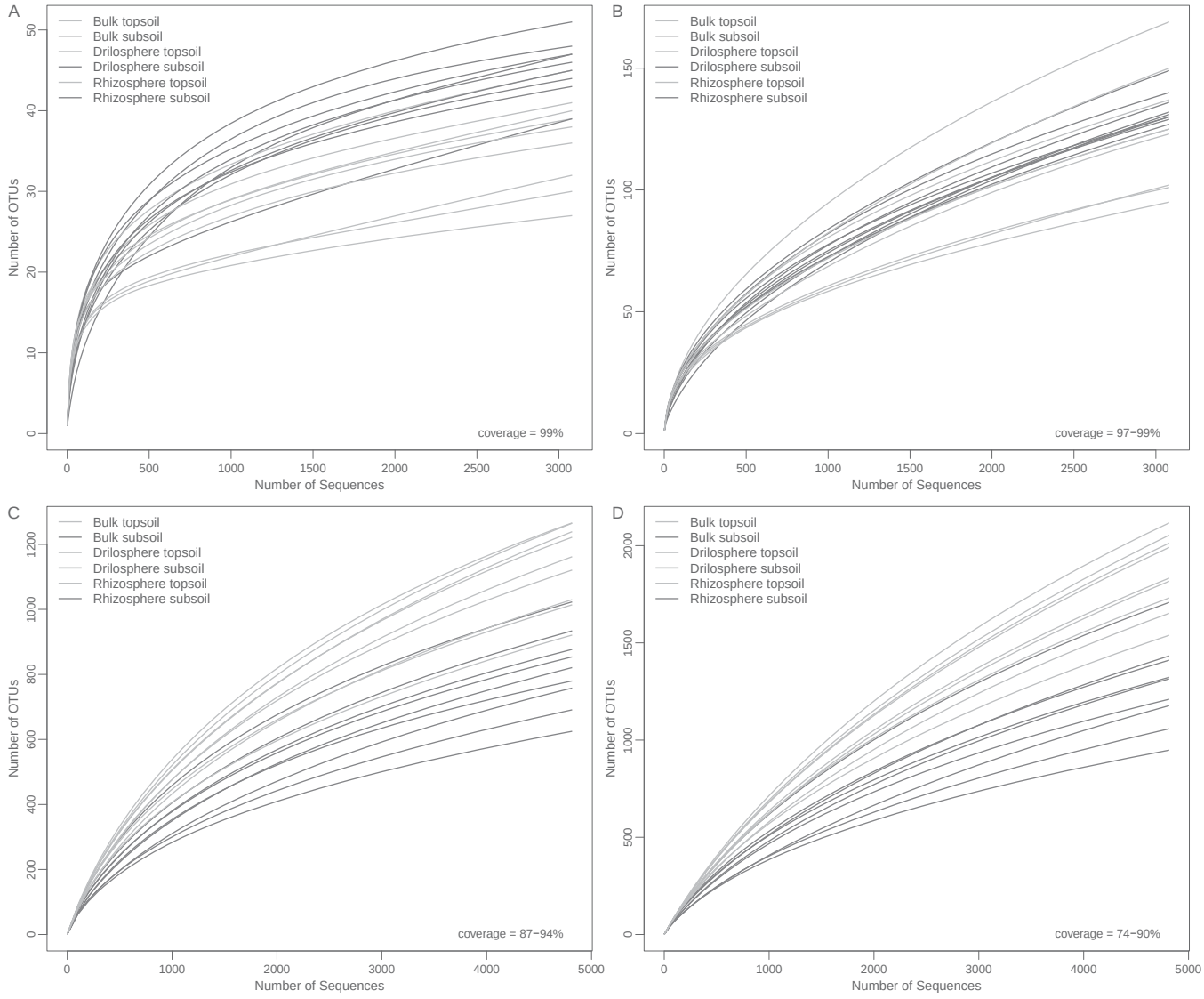

Figure S3: Rarefaction curves of archaeal (A, B) and bacterial (C, D) OTUs at 90% (A, C) and 95% (B, D) similarity level.

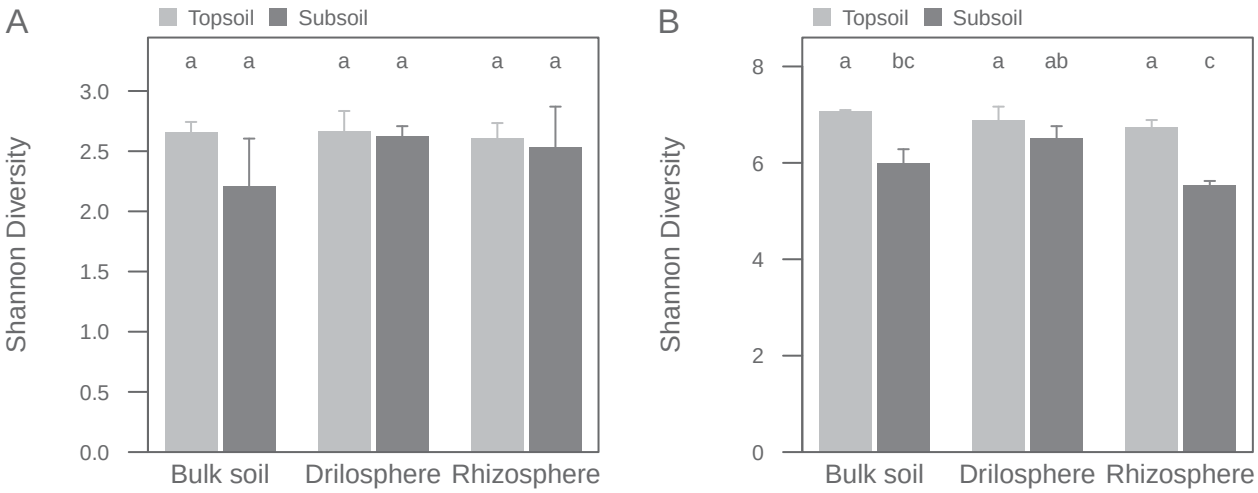

Figure S4: Shannon diversity index at 95% similarity level of archaea (A) and bacteria (B) in soil compartments of topsoil and subsoil. Different letters indicate significant differences ($P \leq 0.05$).

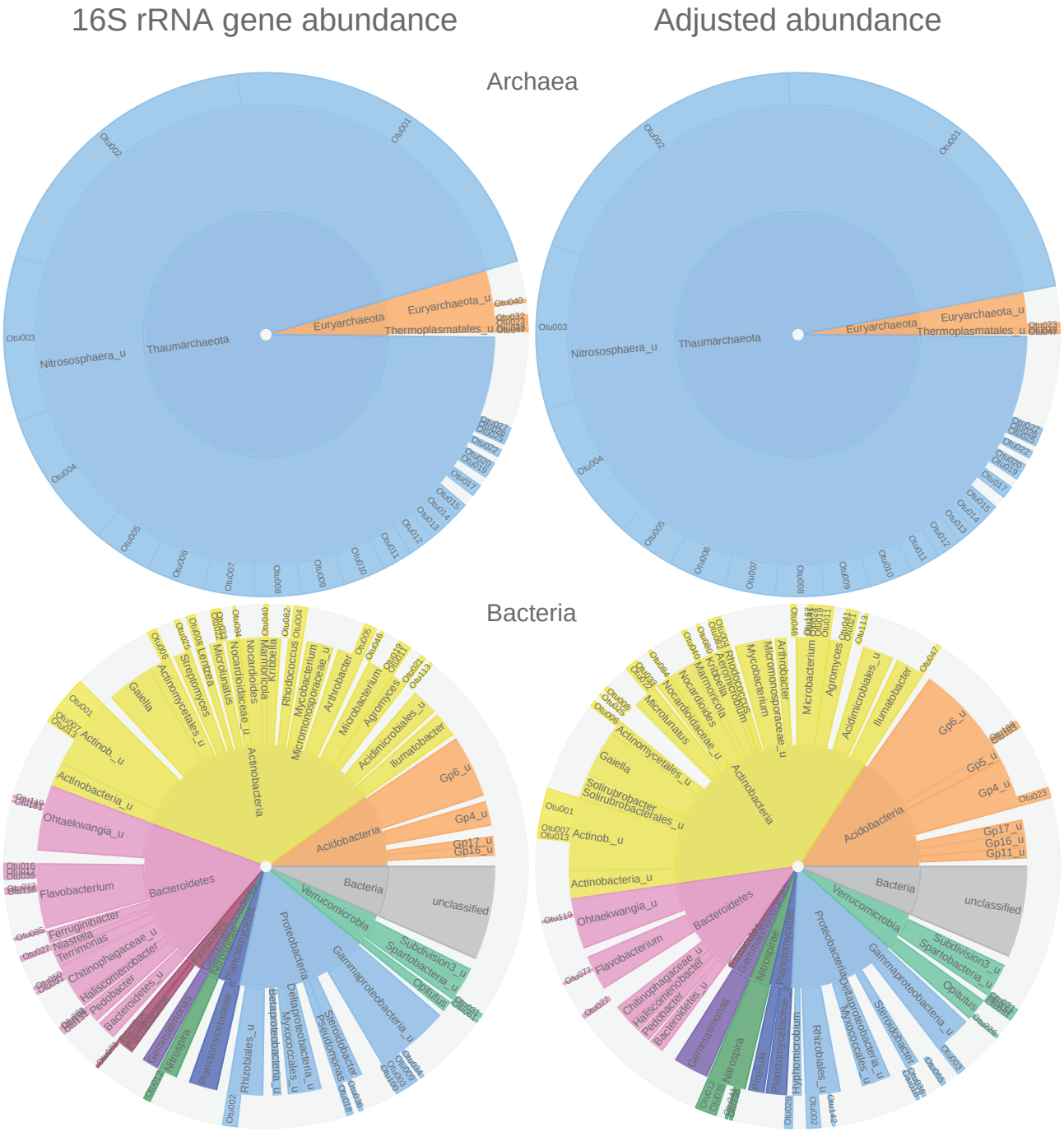

Figure S5: Estimation of the real abundance of archaea and bacteria from 16S rRNA gene copy numbers over all samples. Adjustment of the abundance was performed by information of varying 16S rRNA gene copy numbers per genome at *rrn*DB. OTUs were clustered at 95% similarity level and only OTUs with a relative abundance ≥ 1.5% in at least one of all samples are displayed. Inner ring - phylum level; middle ring - genus or nearest classifiable level.

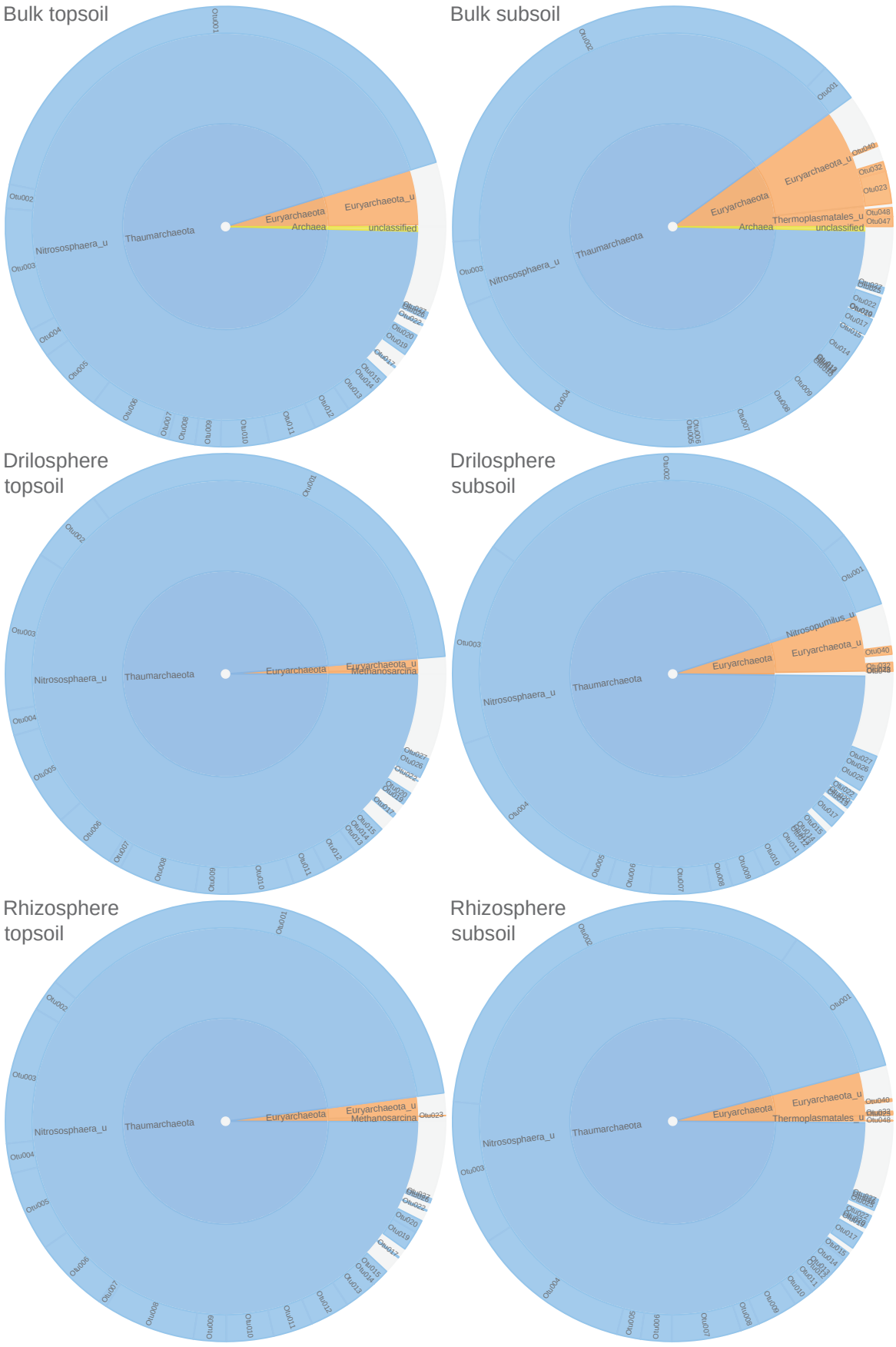

Figure S6: Archaeal community composition in different soil compartments of topsoil and subsoil. OTUs were clustered at 95% similarity level and only OTUs with a relative abundance ≥ 1.5% in at least one sample are displayed. Taxonomic groups below a relative abundance of 0.2% within each pie are not colored. Inner ring - phylum level; middle ring - genus or nearest classifiable level.

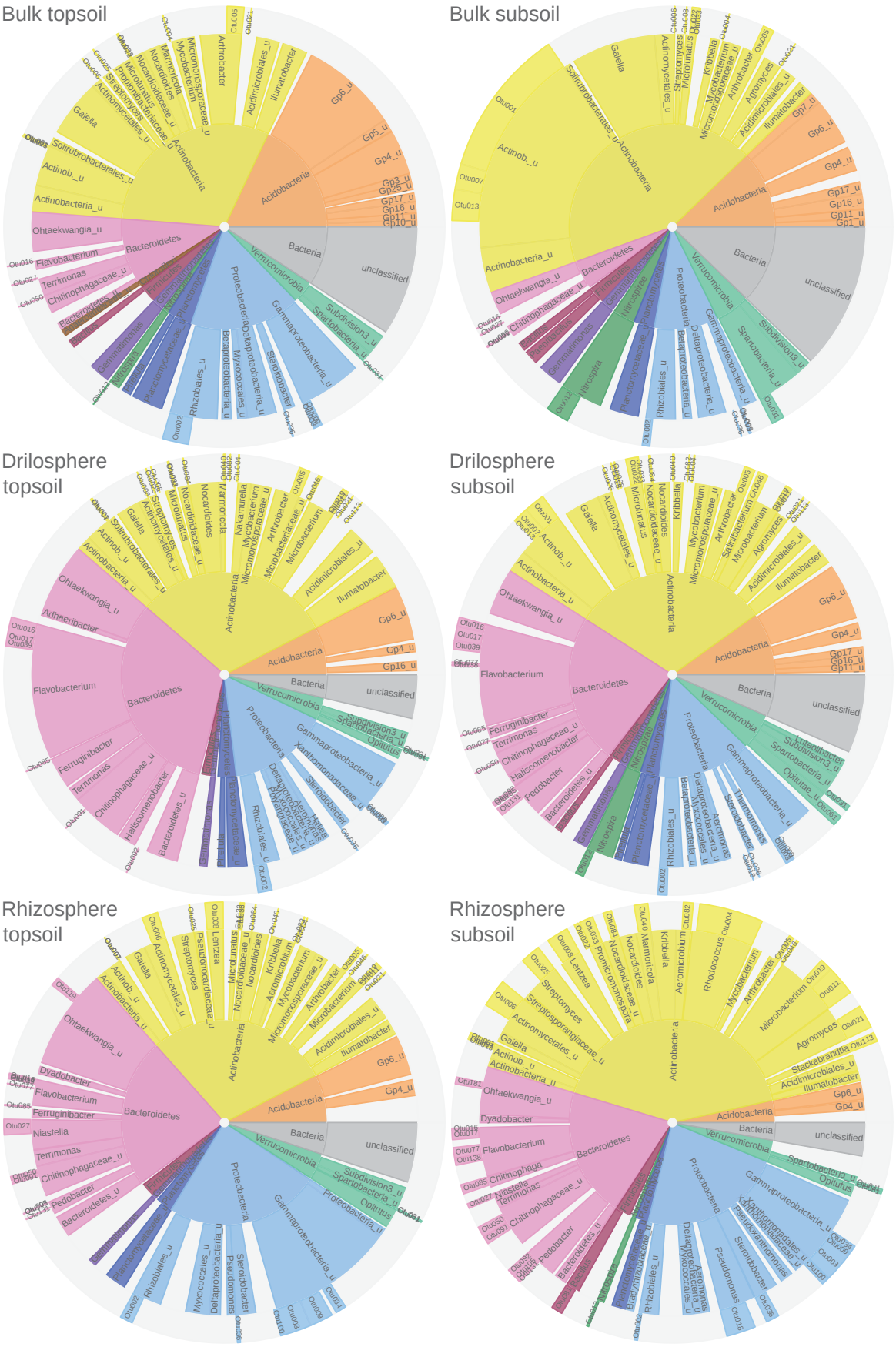

Figure S7: Bacterial community composition in different soil compartments of topsoil and subsoil. OTUs were clustered at 95% similarity level and only OTUs with a relative abundance ≥ 1.5% in at least one sample are displayed. Taxonomic groups below a relative abundance of 0.5% within each pie are not colored. Inner ring - phylum level; middle ring - genus or nearest classifiable level.

Figure S8: Dendrogram and cluster analysis of bacterial OTUs at 95% similarity level. The OTUs are ordered from top to bottom according to their (1) affiliation to clusters of co-occurring OTUs and their (2) relative abundance. Only OTUs with an abundance >5 in minimal 3 samples are included. Color key of the heatmap is scaled within each OTU to the mean relative abundance.

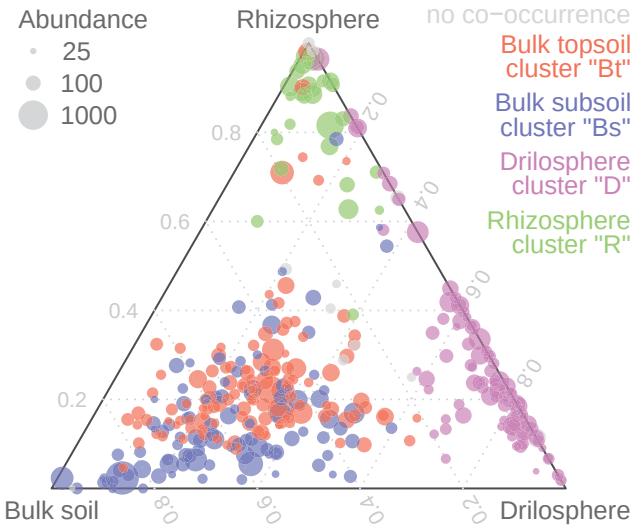

Figure S9: Distribution of bacterial OTUs between soil compartments and their affiliation to clusters of co-occurring OTUs according to cluster analysis at 95% similarity level.

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Bacteria utilizing plant-derived carbon in the rhizosphere of *Triticum aestivum* change in different depths of an arable soil

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Summary

Root exudates shape microbial communities at the plant-soil interface. Here we compared bacterial communities that utilize plant-derived carbon in the rhizosphere of wheat in different soil depths, including topsoil, as well as two subsoil layers up to 1 m depth. The experiment was performed in a greenhouse using soil monoliths with intact soil structure taken from an agricultural field. To identify bacteria utilizing plant-derived carbon, ^{13}C-CO_2 labelling of plants was performed for two weeks at the EC50 stage, followed by isopycnic density gradient centrifugation of extracted DNA from the rhizosphere combined with 16S rRNA gene-based amplicon sequencing. Our findings suggest substantially different bacterial key players and interaction mechanisms between plants and bacteria utilizing plant-derived carbon in the rhizosphere of subsoils and topsoil. Among the three soil depths, clear differences were found in ^{13}C enrichment pattern across abundant operational taxonomic units (OTUs). Whereas, OTUs linked to Proteobacteria were enriched in ^{13}C mainly in the topsoil, in both subsoil layers OTUs related to *Cohnella, Paenibacillus, Flavobacterium* showed a clear ^{13}C signal, indicating an important, so far overseen role of Firmicutes and Bacteriodetes in the subsoil rhizosphere.

Introduction

The microbiome of the rhizosphere has been considered as an important driver of functions contributing to plant health, growth and yield (Berg *et al*., 2014). Thus, microbes in this compartment have been intensively studied in the last decades and enormous efforts have been made to unravel the complex processes taking place (Berendsen *et al*., 2012; Philippot *et al*., 2013; Lareen *et al*., 2016). Today, it is well accepted that bacteria promote plant growth mainly via nutrient mobilization from the soil, phytohormone production, stimulation of the plant immune system or biocontrol of phytopathogens (Berg, 2009; Hartmann *et al*., 2009). Studies on defined bacterial components have emphasized the special relevance of Proteobacteria (*Pseudomonas, Rhizobium, Burkholderia, Lysobacter*), Actinobacteria (*Streptomyces*), Bacteroidetes (*Flavobacterium, Cytophaga*) and Firmicutes (*Bacillus, Paenibacillus*) in the rhizosphere of diverse plant species (Haichar *et al*., 2008; 2012; Buée *et al*., 2009). Also other microbes colonizing the rhizosphere, mainly fungi, are considered to influence plant health (Malik *et al*., 2015), although their plant growth promoting abilities in nonmycorrhizal interactions are far less investigated.

Today, it is obvious that the microbial community composition of the rhizosphere is mainly driven by the plant species, the plant development stage and the soil type (Marschner *et al*., 2001; Berg and Smalla, 2009). A

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major mechanism how plants select for their specific microbiomes belowground is the specific composition of root exudates, which are used by microbes as an easily available carbon source (Haichar *et al*., 2008). The quality and quantity of exudates varies with changes in soil physical and chemical parameters, plant developmental status and at different root zones (Jones *et al*., 2004; Haichar *et al*., 2008; 2014; Chaparro *et al*., 2014). Highest root exudation rates were measured close to the root tip and during plant growth until flowering (Lynch and Whipps, 1990; Haichar *et al*., 2014; Neumann *et al*., 2014).

However, although previous studies have investigated the nature and composition of rhizosphere microbes in detail, the transferability of findings to natural plant-microbe-soil systems may not always be straightforward due to the following considerations: (i) experiments are often conducted with disturbed or sieved soils, where the soil structure and compartments have been homogenized, thus, neglecting the influence of small-scale soil heterogeneity on root development (Luster *et al*., 2009; Han *et al*., 2015), (ii) studies are often limited to nutrient rich topsoil, although roots of agricultural crops can easily grow down to 2 m (Kautz *et al*., 2013; Perkons *et al*., 2014) and soil depth is recognized as a further important driver of soil microbial community composition (Berg and Smalla, 2009; Scharroba *et al*., 2012) and (iii) rhizosphere microbiomes are often investigated at the level of presence or the relative abundance of taxa, but not their direct involvement in rhizosphere carbon flows.

In this study, we investigated bacteria utilizing plant-derived carbon in the rhizosphere of *Triticum aestivum* in different soil depths. To reach our goals, we investigated intact soil columns planted with wheat over a soil depth of 1 m. Thus, the natural covariation of soil structure, pore network and root developmental stage over depth was conserved. We applied ^{13}C-CO_2 fumigation to the plant shortly before sampling and used DNA-based stable isotope probing (SIP) combined with barcoding of the 16S rRNA gene amplicons by high throughput sequencing to reveal a high resolution of key rhizosphere bacteria utilizing plant-derived carbon (Haichar *et al*., 2016).

Based on recent measurements of hydrolytic enzyme activities in the rhizosphere of wheat using samples from the same field (Uksa *et al*., 2015b), we hypothesize that the degree of substrate assimilation and microbial activity in the subsoil rhizosphere is comparable to that of the topsoil rhizosphere. However, as a prestudy with soil from the same field trial demonstrated a substantial change of abundant bacterial phyla from topsoil to subsoil with a decrease of Proteobacteria and an increase of Firmicutes (Uksa *et al*., 2015a), we postulate, that rhizosphere bacteria, which utilize the plant-derived carbon, will differ in the different soil depths under investigation. As most microbes colonizing the rhizosphere are acquired from the soil microbiome, we expect a dominance of Firmicutes in the subsoil utilizing plant-derived carbon in the subsoil rhizosphere, whereas in topsoil a dominance of Proteobacteria occurs.

Results

^{13}C distribution pattern in the different soil and plant compartments

At the end of the experimental period (90 days), *Triticum aestivum* formed a dense rooting network in all three analyzed soil depths. However, a sharp decrease of root biomass with soil depth (ANOVA; $P < 0.001$) was clearly visible (Supporting Information Fig. S1). The labelling of the plant with ^{13}C-CO_2 resulted in a significant ($P < 0.001$) ^{13}C enrichment in the plant shoot biomass (46.4 atom-%) as well as in the rhizosphere (6.1 atom-%) and bulk soil (1.3 atom-%) independently of the soil depth (Supporting Information Fig. S1).

Bacterial community composition and ^{13}C enrichment of OTUs in the rhizosphere at different soil depths

Seven consecutive fractions of each DNA gradient known to span the range of buoyant densities (BDs) typical for light and heavy DNA were selected for downstream analyses. 16S rRNA gene-targeted qPCR indicated elevated

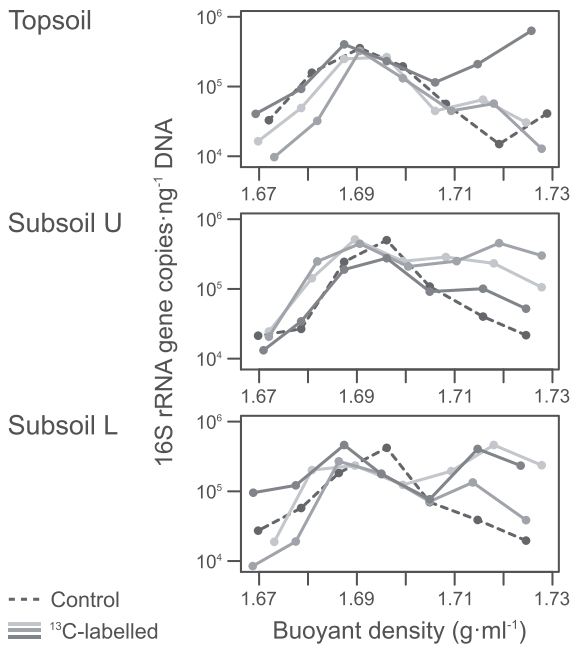

Fig. 1. 16S rRNA gene abundance in CsCl-gradient fractions. Seven consecutive fractions are displayed, which were selected for barcoded 16S rRNA gene amplicon sequencing. Topsoil (0 cm–20 cm); Subsoil U (upper subsoil, 20 cm–50 cm); Subsoil L (lower subsoil, 50 cm–80 cm).

gene counts in heavy fractions of ^{13}C-labelled treatments compared to the controls, that is, at BDs of 1.715 g·ml^{-1} CsCl or higher (Fig. 1). This was a first indication of the successful incorporation of ^{13}C-label into the DNA of rhizosphere microbiomes in our experiment.

The selected fractions were subjected to sequencing of bacterial 16S rRNA gene amplicons. After quality filtering 2 541 504 reads were obtained from all fractions, resulting in 29 188 OTUs at a level of 95% similarity. For further analysis, reads from all samples were rarefied to 30 256 reads per sample.

In a first analysis, sequencing data from the seven fractions per sample were combined by weighing the relative abundance of each OTU according to the proportion of 16S rRNA gene abundance in each fraction. With this, we first compared overall depth-resolved rhizosphere communities, without differentiation between OTUs of labelled and control samples. Richness and Shannon diversity (*H*) were significantly higher in rhizosphere samples of the topsoil (ANOVA; $P < 0.001$; $H = 7.47$) compared to subsoil U ($H = 6.36$) and subsoil L ($H = 6.31$). Clustering of the samples and relative abundance of the 100 most abundant OTUs are displayed in Fig. 2. The relative abundance of bacterial phyla is provided in Supporting Information Table S1 and Fig. S2. As expected the following phyla were major parts of the bacterial community: Actinobacteria (18%–44%), Proteobacteria (15%–27% [42%–63% Beta-, 12%–23% Gamma-, 11%–21% Deltaproteobacteria]), Acidobacteria (8%–21%), Firmicutes (3%–16%), Bacteroidetes (3%–14%), Nitrospirae (1%–3%) and Gemmatimonadetes (1%–2%). Already at the phylum level, significant differences were found between the soil depths: a significantly higher abundance was observed for Acidobacteria in topsoil (ANOVA; $P = 0.001$), for Actinobacteria in subsoil U ($P = 0.004$) and for Nitrospirae in subsoil L ($P = 0.020$). In addition, the overall relative abundance of Proteobacteria was reduced in the rhizosphere of the upper subsoil (subsoil U; $P = 0.001$). As expected, Firmicutes were generally more abundant in the lower subsoil rhizosphere. Similar to the T-RFLP fingerprints (Supporting Information results and Supporting Information Fig. S3), variations between the four soil columns increased with soil depth. Whereas topsoil and subsoil U samples formed condensed clusters, the subsoil L bacterial community exhibited considerable variability mainly due to the occurrence of single, highly abundant OTUs in only one or two samples, for example, Nocardiaceae, *Achromobacter*, *Microbacterium*, *Flavobacterium*, *Pedobacter*, *Janthinobacterium* or *Steroidobacter* (Fig. 2).

In a second step, ^{13}C enrichment was estimated for bacterial OTUs as an indication of their direct involvement in carbon flow at the plant-soil interface of different soil depths, (Fig. 3A–C). Labelling was inferred via taxon-specific buoyant density shifts and interpreted as ^{13}C atom-% enrichment (Hungate *et al.*, 2015). Up to 35 ^{13}C atom-% enrichment were observed for specific OTUs, while the uncertainty thresholds increased with soil depth (topsoil – 1.0; subsoil U – 5.2; subsoil L – 16.0 ^{13}C atom-%). ^{13}C enrichment values were lower in average in the upper subsoil U compared to topsoil or subsoil L. However, overall relative abundance of ^{13}C-enriched OTUs was found to be highest in subsoil U and subsoil L rhizosphere. Among the three soil depths, clear differences were found in the ^{13}C enrichment pattern across abundant OTUs. Enrichment of ^{13}C was highly pronounced for OTUs related to *Cohnella*, *Paenibacillus*, *Flavobacterium* and Chitinophagaceae in subsoil U and especially subsoil L. These OTUs also were of high relative abundance compared to the topsoil. For OTUs classified as Actinobacteria, for example, *Agromyces*, *Arthrobacter*, *Glycomyces*, *Kitatospora*, *Lentzea* and *Promicromonospora*, both, their relative abundance and ^{13}C-labelling were highest in the upper subsoil. In turn, reads which could be assigned to *Streptomyces* spp. were ^{13}C-enriched and highly abundant in all depths. Interestingly, different OTUs related to *Streptomyces* were contributing to this observation in different soil depths (Figs 2 and 3).

In contrast to Actinobacteria, proteobacterial OTUs were generally less abundant and had a lower ^{13}C-atom fraction excess in the subsoil U rhizosphere compared to the other soil depths. The most important proteobacterial OTU was closely related to *Duganella*, which appeared very important in rhizosphere of subsoil L. Other ^{13}C-enriched Proteobacteria were identified as *Ideonella*, *Lysobacter*, *Massilia*, *Polaromonas*, *Pseudoxanthomonas*, *Steroidobacter* and *Variovorax* showing varying abundance and ^{13}C enrichment in dependency to soil depth.

Labelling of Acidobacteria was apparent only in topsoil. Here, the Gp4 class exhibited considerable ^{13}C enrichment in one OTU. The phyla Nitrospirae and Gemmatimonadetes, as well as most unclassified OTUs, showed no relevant ^{13}C enrichment and their relative contribution to the microbial community was rather low.

Discussion

Unravelling soil-microbe-plant interactions in undisturbed subsoil

Soil depth is a factor which is still rarely considered despite the fact that roots grow deep into subsoil. As with depth soil properties change and the bulk soil microbial community composition changes drastically towards oligotrophic, slow-growing microbes (Eilers *et al.*, 2012; Uksa *et al.*, 2015a), mechanisms of


Count
0
200
400
600
0
0.04
Value
Phylum
Acidobacteria
Gp11_u_OTU0078
Gp16_u_OTU0873
Gp4_u_OTU1310
Gp7_u_OTU0627
Gp4_u_OTU0074
Gp4_u_OTU0599
Actinobacteria
Streptomyces_OTU0119
Kitasatospora_OTU1003
Streptomyces_OTU0035
Promicromonospora_OTU0076
Streptomyces_OTU0037
Lentzea_OTU0042
Streptomyces_OTU0141
Agromyces_OTU0097
Gaiella_OTU0423
Pseudonocardiaceae_u_OTU1004
Glycomyces_OTU0861
Nocardiaceae_u_OTU0570
Streptomyces_OTU0106
Glycomyces_OTU0601
Gaiella_OTU0125
Streptomyces_OTU0096
Glycomyces_OTU0611
Actinomycetales_u_OTU0148
Streptomyces_OTU0094
Arthrobacter_OTU2604
Agromyces_OTU0580
Agromyces_OTU1542
Promicromonospora_OTU0342
Streptomycetaceae_u_OTU0085
Lentzea_OTU0271
Streptomyces_OTU0232
Agromyces_OTU0192
Propionibacteriaceae_u_OTU1445
Gaiella_OTU0140
Microbacterium_OTU2668
Microbacteriaceae_u_OTU0612
Actinomycetales_u_OTU1707
Pseudonocardiaceae_u_OTU2292
Promicromonospora_OTU0854
Pseudonocardiaceae_u_OTU0481
Streptomyces_OTU1012
Streptomycetaceae_u_OTU2889
Streptomyces_OTU2544
Microbacteriaceae_u_OTU1925
Microbacteriaceae_u_OTU6990
Microbacteriaceae_u_OTU3453
Bacteroidetes
Flavobacterium_OTU0394
Chitinophagaceae_u_OTU9599
Chitinophagaceae_u_OTU0066
Flavobacterium_OTU0040
Pedobacter_OTU0552
Flavobacterium_OTU0549
Flavobacterium_OTU0635
Flavobacterium_OTU0613
Flavobacterium_OTU1673
Chitinophaga_OTU5428
Chitinophagaceae_u_OTU0590
Chitinophaga_OTU1072
Firmicutes
Paenibacillus_OTU0006
Cohnella_OTU0010
Paenibacillus_OTU0011
Paenibacillus_OTU0005
Paenibacillaceae_1_u_OTU0001
Cohnella_OTU0124
Paenibacillus_OTU0053
Gemmatimonadetes
Gemmatimonas_OTU0536
Nitrospirae
Nitrospira_OTU0181
Nitrospira_OTU0054
Nitrospira_OTU2337
Nitrospira_OTU0298
Proteobacteria
Duganella_OTU0009
Duganella_OTU0056
Oxalobacteraceae_u_OTU0116
Variovorax_OTU0013
Janthinobacterium_OTU0105
Xanthomonadaceae_u_OTU0205
Proteobacteria_u_OTU4803
Oxalobacteraceae_u_OTU0033
Comamonadaceae_u_OTU0079
Variovorax_OTU0082
Massilia_OTU0216
Proteobacteria_u_OTU0258
Achromobacter_OTU0485
Variovorax_OTU0199
Betaproteobacteria_u_OTU0067
Ideonella_OTU0870
Steroidobacter_OTU0507
Acidovorax_OTU0812
Pseudoxanthomonas_OTU0872
Polaromonas_OTU0117
Hydrogenophaga_OTU0603
Steroidobacter_OTU1663
unclassified
Bacteria_u_OTU0034
Bacteria_u_OTU0284
Bacteria_u_OTU0917
Bacteria_u_OTU0309
Bacteria_u_OTU0027
Bacteria_u_OTU1885
Bacteria_u_OTU0168
Top 13C-lab 2
Top 13C-lab 1
Top control
Top 13C-lab 3
Sub L 13C-lab 1
Sub L 13C-lab 3
Sub L 13C-lab 2
Sub L control
Sub U control
Sub U 13C-lab 1
Sub U 13C-lab 2
Sub U 13C-lab 3

Fig. 2. Bacterial community composition in control and ^{13}C-labelled rhizosphere samples.
Sequencing reads in gradient fractions were combined on the basis of weighted relative abundances. The 100 most abundant OTUs were selected for clustering and ordered from top to bottom first by their phylum affiliation and secondly, by the mean relative abundance across all samples. PERMANOVA revealed significant differences between soil depths ($P = 0.001$). Top – Topsoil (0 cm–20 cm); Sub U – upper subsoil (20 cm–50 cm); Sub L – lower subsoil (50 cm–80 cm); ^{13}C-lab – plants were labelled with ^{13}C-CO_2; control – no labelling; u – unclassified at 80% cutoff.

interactions between the plant root and the surrounding microbes and soil will be affected as well. There are still methodological limitations that impede direct ^{13}C labelling in the field and sampling down to subsoil. The use of undisturbed subsoil columns incubated under greenhouse conditions may be a good compromise. With undisturbed subsoil – overlaid by homogenized topsoil which mimics a ploughing event – we could preserve the naturally developed soil profile and its spatial heterogeneity including bulk density, soil structure, soil pore network, earthworm burrows, biogeochemical gradients as well as microbial community distribution patterns and niche-separation. Root growth, root development and deposition of root exudates were therefore as close to natural conditions as possible. The separation or colocalization of microbes and substrates has been shown to be critical for carbon turnover on a small scale (Pinheiro *et al*., 2015) and is a so far underestimated factor in studies using homogenized soil solely.

Detection of carbon utilizing microbial pools via quantitative DNA-SIP

In this study, we investigated the bacterial communities utilizing plant-derived carbon in the rhizosphere of topsoil and subsoil of wheat. For this aim, DNA-SIP was used as a powerful method to detect and quantify microbes that directly or indirectly take up ^{13}C-labelled carbon provided by plants (Haichar *et al*., 2016).

The relatively long time span of our labelling experiment, which was needed to obtain sufficient amounts of ^{13}C labelled carbon, needed for subsequent SIP analysis, in the microbial DNA pool (15 d), made it difficult to differentiate between primary exudate consumers or secondary metabolite or biomass consumers in the investigated soils. Crossfeeding in natural food-webs can complicate the interpretation of SIP data and time-resolved SIP analyses can help to overcome this caveat (Coyotzi *et al*., 2016). In our present analyses, microbes with a higher ^{13}C enrichment were considered more likely to be primary consumers of root exudates, however, the simultaneous presence of slow-growing primary consumers can also not be excluded (Haichar *et al*., 2008; Rettedal and Brözel, 2015).

Previous studies have emphasized the importance of sufficient ^{13}C-labelling for successful separation of heavy and light DNA and to resolve labelling from GC-effects (Neufeld *et al*., 2007a; Uhlik *et al*., 2009). Due to the simultaneous contribution of ^{13}C enrichment and GC content to the buoyant density of DNA, unlabelled genomes with high GC content may be found in the same gradient fractions as low-GC DNA with a high ^{13}C enrichment (Buckley *et al*., 2007). In our present approach, this potential caveat was circumvented by high throughput-sequencing of 16S rRNA genes across all relevant gradient fractions, including ^{13}C treatments and unlabelled controls. Thus, we could (i) achieve a high phylogenetic resolution of labelled microbial taxa, (ii) define taxon-specific buoyant density shifts and (iii) infer from that the degree of ^{13}C-labelling for single OTUs (Hungate *et al*., 2015). At the same time, we are aware that the use of only one ^{12}C-control column vs. triplicate ^{13}C-labelled treatment columns per depth compromises a strictly quantitative and statistical evaluation of labelling effects.

The key players – rhizosphere bacteria growing on plant-derived carbon in different soil depths and their putative plant growth promotion abilities

As proposed, results indicated that both on phylum and genus level, utilization patterns of plant-derived carbon were highly dependent on soil depth. However, for all soil depths the dominance of ^{13}C-enriched genera attributed to Proteobacteria, Actinobacteria, Bacteroidetes or Firmicutes pointed towards an import role of fast growing root-exudate metabolizing bacteria. Particularly within the aforementioned phyla, bacteria have been isolated from diverse rhizosphere soils and characterized as fast growing microbes in the presence of labile carbon sources (Fierer *et al*., 2007; 2012; Ramirez *et al*., 2012). Nevertheless, the spectrum of physiological traits and lifestyles within a defined phylum can still be rather diverse (Goldfarb *et al*., 2011). *Arthrobacter* for example was originally reported to be oligotrophic (Thompson *et al*., 1992) but has been repeatedly identified in SIP studies to consume labile sugars in soils (Mau *et al*., 2014; Kramer *et al*., 2016). Also in our study, this taxon was highly ^{13}C-enriched in the subsoil U. In contrast, reads from, for example, *Nitrosospira* showed almost no atom fraction excess in all depths, which was not surprising, as this genus is known as an autotrophic nitrifier (Xia *et al*., 2011). Also Acidobacteria and Gemmatimonadetes were almost not ^{13}C-labelled and therefore less involved in the turnover of plant-derived carbon. This result is in accordance with the expectation that most

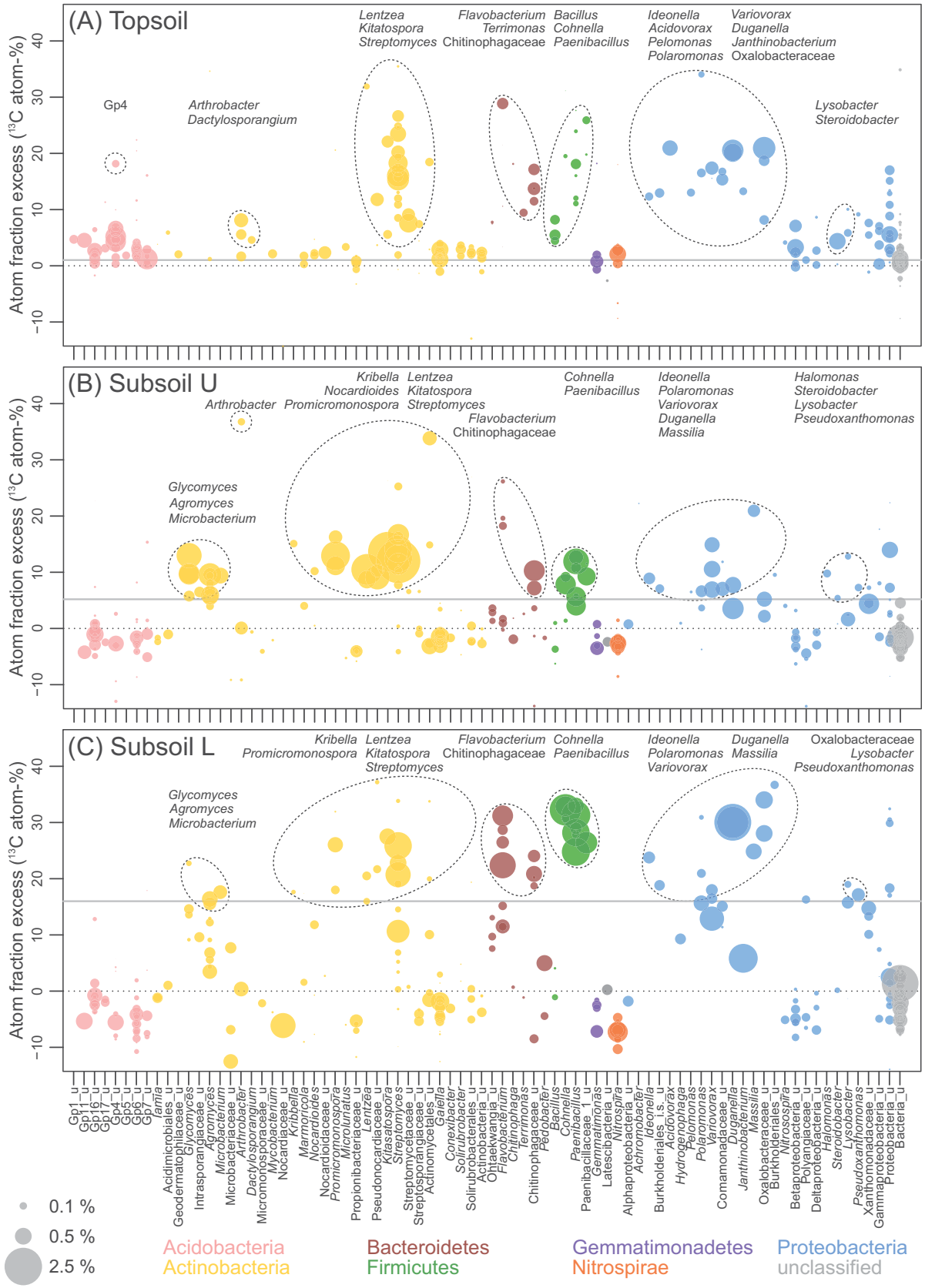

Fig. 3. ^{13}C enrichment of bacterial OTUs in different soil depths.
The mean density shift – expressed as ^{13}C atom fraction excess – for each OTU between the three labelled samples and the control ($n = 3$) was plotted according to its phylogenetic affiliation. OTUs with a minimum relative abundance of 0.1% in at least one sample were selected. Spot sizes represent the OTU mean relative abundance in the control and the three labelled samples ($n = 4$). The threshold of uncertainty (continuous line) was set according to negative values of OTUs with a relative abundance $>$ 0.1%. Dashed circles include important OTUs above the threshold of uncertainty for easier recognition.
A. Topsoil (0 cm–20 cm).
B. Subsoil U (upper subsoil, 20 cm–50 cm).
C. Subsoil L (lower subsoil, 50 cm–80 cm).

bacteria of those phyla are oligotrophic (Zhang *et al.*, 2003; Jones *et al.*, 2009; Foesel *et al.*, 2014). Generally, atom fraction excess variation within phyla and even within OTUs affiliated to the same genus was still high. Especially for the genus *Streptomyces*, different OTUs showed not only different relative abundances over depth, but also a high variation in ^{13}C-labelling indicating distinct growth rates and substrate usage spectra.

Actinobacteria and *Streptomyces* as well as *Duganella* and *Janthinobacterium*, all observed in our study in different soil depths with differing ^{13}C labelling intensity, are known for their ability to produce secondary metabolites with antimicrobial activities (Basilio *et al.*, 2003; Choi *et al.*, 2015; Viaene *et al.*, 2016). Many of these taxa are discussed in the context of biocontrol (Haesler *et al.*, 2014). Labelling intensities of these taxa varied strongly over depth, suggesting that key players involved in plant protection from phytopathogens in the rhizosphere significantly differed over depth in our study. Donn *et al.* (2015) also observed a shift from *Proteobacteria* to *Actinobacteria* during wheat development, but at a larger temporal scale not focusing on roots of the same plant. They found *Oxalobacteraceae* and *Pseudomonadaceae* to be abundant at younger parts of roots, whereas at older parts or at senescent roots *Micromonospora* species and other Actinobacteria were enriched.

Also other microbes possibly related to plant growth promoting functions showed similar variability over depth. For example, strains of *Massilia*, *Duganella*, *Variovorax* and *Pseudoxanthomonas* are known to produce siderophores (Aranda *et al.*, 2011; Ofek *et al.*, 2012; Madhaiyan *et al.*, 2013; Lampis *et al.*, 2015). These taxa were especially ^{13}C-labelled in the subsoil L rhizosphere, possibly providing an additional positive effect on deeper wheat roots in terms of iron and phosphorous supply (Sharma *et al.*, 2013). This is consistent with our previous report of highest phosphatase activities in deeper rhizosphere from the same site (Uksa *et al.*, 2015b).

In the aforementioned study (Uksa *et al.*, 2015b) also glycoside hydrolase activities were measured in the wheat rhizosphere from topsoil, subsoil U as well as subsoil L and were generally lower in the upper subsoil as compared to topsoil and the lower subsoil. Possibly, hydrolase activities in the lower subsoil are induced by easy available hydrocarbons released from young roots to increase further carbon mobilization from the surrounding bulk soil. A putative producer of glycoside hydrolases in the subsoil L rhizosphere may be *Flavobacterium*. Bacteria of this genus are copiotrophs, living on easy available substrates and were found to induce hydrolase activities in the wheat rhizosphere in former studies (Thompson *et al.*, 1992; Mawdsley and Burns, 1994; Heijnen *et al.*, 1995). Furthermore, at the early plant vegetative growth phase – as in our study – *Flavobacterium* is more abundant whereas Sphingobacteria take over at later plant developmental stages (Donn *et al.*, 2015). This may explain, why this family did not show up in our study, although other studies of the wheat rhizosphere found them in higher abundance (Haichar *et al.*, 2008; Stroobants *et al.*, 2014).

Similarly, members of the genera *Paenibacillus*, *Bacillus* and *Cohnella* spp. (all Firmicutes) are known as free-living diazotrophs (Mavingui *et al.*, 1992; Rosado *et al.*, 1996; Behrendt *et al.*, 2010; Wang *et al.*, 2012). In our studies, these genera were of highest abundance and showed highest ^{13}C enrichment not only in subsoil rhizosphere (this study) but also generally in subsoils (Uksa *et al.*, 2015a). In contrast, an apparent lack of labelled Firmicutes with possible nitrogen fixing abilities in topsoil may indicate sufficient nitrogen supply or functional redundancy by other lineages there.

Finally, also the production of phytohormones such as indol acetic acid might play a role for plant growth promotion in the deeper subsoil L, where roots are predominantly young. For *Massilia*, *Janthinobacterium*, *Arthrobacter* (Kuffner *et al.*, 2010), *Paenibacillus* (Hanak *et al.*, 2014) and even an acidobacterial strain (Kielak *et al.*, 2016) IAA production was documented. Different mechanisms of plant growth promotion might fall together in single species and other bacteria in turn benefit from those mutualistic relationships.

Lack of ^{13}C enrichment in bacterial DNA in the upper subsoil

Interestingly, OTUs in the upper soil depth (subsoil U) showed a lower atom fraction excess on average when compared with the deeper subsoil L, although the overall ^{13}C content of the soil was not lower in the rhizosphere of subsoil U. In addition, total carbon content increased

in the rhizosphere with soil depth (data not shown). The following scenario – based on the depth and age-differential release of root exudates preferably in young roots (Haichar *et al*., 2014) – could explain this observation: During labelling, root exudation could have been highest in the subsoil L, because average root age is expected to be lowest in the deepest soil and root exudation is expected to be highest in the early root developmental stage (Neumann *et al*., 2014). In the upper subsoil U, root exudation could already have been gradually reduced as a result of increased average root age when labelling with ^{13}C-CO_2 occurred. These assumptions are supported by a previous study on the same soil and soil depths in the field (Uksa *et al*., 2015b), where potential hydrolytic enzyme activities in the rhizosphere showed a similar decrease in the upper subsoil U. The limitation of readily available carbon sources such as root exudates in this soil depth at this explicit time point of sampling can explain the gap and is supported by the high abundance of Actinobacteria, for example, *Arthrobacter*, which can compete at nutrient limiting conditions.

Conclusion

We were able to show that, dependent on soil depth, distinct patterns of bacteria utilizing plant-derived carbon occur that indicate shifts in plant growth promoting bacteria already at the phylum level. The composition of root exudates, the surrounding indigenous microbial community or other soil properties at specific soil depths are major drivers of the observed patterns, while their specific contributions remain unclear.

As postulated, the degree of assimilation of plant-derived carbon by single bacterial taxa in the rhizosphere of subsoil L is similar to the topsoil. Furthermore, the so far underestimated role of Firmicutes and Bacteroidetes as important bacteria, which utilize plant-derived carbon in the subsoil, is an outstanding result adding to other related findings from the wheat rhizosphere and residues (Bernard *et al*., 2007; Ai *et al*., 2015). This first investigation of the microbial communities, which utilize plant-derived carbon in an undisturbed subsoil via DNA-SIP shows that it is worth to take a 'deeper' look into the rhizosphere, otherwise carbon turnover processes and key players might be overlooked or underestimated. The here presented study, like most of the studies in the past, focuses on the analysis of the bacterial part of the microbiome. Nevertheless, as indicated in the introduction, also other microbes like for examle fungi can contribute to plant health. Thus, we propose to implement those organisms into future studies on the role of plant-derived carbon, in order to fully illucidate the influence of root exudates on the rhizosphere microbiome.

Experimental procedures

Soil properties and soil core excavation

The soil used for this study originated from an arable field at Campus Klein-Altendorf near Bonn (Germany, 50°37′21″ N, 6°59′29″ E) and has been classified as Haplic Luvisol. The Ap horizon (topsoil, 0–20 cm) has been classified as a silt loam with a pH 6.5 and was influenced by conventional tillage. Subsoil horizons Bt1 (upper subsoil U, 45–75 cm) and Bt2 (lower subsoil L, 75–105 cm) are characterized by a high bulk density and clay accumulation (silty clay loam) with pH values of 6.9; total carbon and nitrogen decreased with depth. The intermediate E/B horizon (20–45 cm) varies highly in the field and is, therefore, excluded from this study. For further details about soil properties consult Gaiser *et al*. (2012) and Kautz *et al*. (2014). In April 2012, before soil management and cultivation started, 12 undisturbed subsoil monoliths from 45 to 105 cm soil depth and 20 cm in diameter were obtained with a lysimeter excavation technology (Meißner *et al*., 2007). The distance between the monoliths taken at the field was set to 1 m. The soil columns were deposited in a covered polystyrene box (60 × 180 × 100 cm) on a copper plate. The plate was set to 14°C in order to cool the soil from the bottom continuously. To simulate the disturbed plough horizon, the undisturbed subsoil cores were covered with a 20 cm thick layer of homogenized, sieved topsoil (Ap horizon) from the same field. As the soil depth between 20 cm and 45 cm were excluded from this study, subsoil U and L (45 cm–75 cm and 75 cm–105 cm) refer to the root depths 20 cm–50 cm and 50 cm–80 cm, respectively.

Wheat cultivation, ^{13}C-CO_2 labelling and sampling

Eleven germinated seeds of *Triticum aestivum* L. (cultivar Scirocco) were sown in the topsoil which is equivalent to a seeding density of 350 seeds per m^2 typically used in the area of the sampled soil. Seventy-five days after sowing plants had reached the developmental stage EC50 providing the highest root exudation rates (Haichar *et al*., 2014) and were labelled with ^{13}C-CO_2 (for details see the Supporting Information).

After labelling, the soil columns were vertically dissected into three blocks using an electric saw: topsoil (0 cm–20 cm), upper subsoil (U, 20 cm–50 cm) and lower subsoil (L, 50 cm–80 cm). The subsoil from 20 cm to 80 cm corresponded to the field soil depth of 45 cm–105 cm. Each block was cut longitudinally into two halves. For the determination of the root biomass a representative cylinder segment was cut along the whole height of each block half from the midpoint to the edge. The roots for the determination of the root biomass were washed with

deionized water. From the second half of each block, roots with adhering soil within max 2 mm distance to the root surface were sampled with sterile tweezers and designated as root-rhizosphere-complex. Bulk soil was sampled with a sterile spoon with highest possible distance to the roots which increased from topsoil to subsoil. Therefore, the effect of root exudation on designated bulk soil samples from topsoil cannot be totally excluded. For DNA extraction, the root-rhizosphere complex and bulk soil material was stored at −80°C until further analysis. For dry weight and carbon measurement of the shoot and roots, rhizosphere and bulk soil, the sample material was dried at 40°C.

Microbial analysis

Details on the described experimental procedures can be found in the Supporting Information. The analyses were limited to one control and three treatment soil columns resulting in 24 samples [4 soil columns × 3 soil depth × 2 compartments (root-rhizosphere complex and bulk soil)]. DNA was extracted from samples using a modified nucleic acid extraction method according to Lueders *et al*. (2004). Since roots were intact after homogenization for simplified reading, we further designated the DNA, which was extracted from the root-rhizosphere complex, as 'rhizosphere DNA'.

In order to verify that the four soil columns used for DNA-SIP and 16S rRNA sequencing are comparable regarding their overall bacterial community composition, terminal restriction fragment length polymorphism (T-RFLP) was performed as a preanalysis. A detailed description and results can be found in the Supporting Information and Supporting Information Fig. S3.

DNA-SIP was performed on the basis of density gradient centrifugation and fractionation according to Lueders *et al*. (2004) and Neufeld *et al*. (2007b). Due to insufficient ^{13}C enrichment in the bulk soil (Supporting Information Fig. S1), density gradient centrifugation was limited to rhizosphere DNA.

For sequencing of bacterial 16S rRNA genes, seven consecutive fractions that contained sufficient DNA amounts for downstream molecular analyses were chosen from each CsCl-gradient resulting in 84 samples [4 columns × 3 soil depth × 1 compartment (rhizosphere) × 7 fractions]. Bacterial 16S rRNA gene abundance was determined in each of the fractions by quantitative real-time PCR using the 7300 Real-Time PCR System and the Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) following the protocol described by Töwe *et al*. (2010). Barcoded amplicon sequencing was performed using the Illumina MiSeq platform (Illumina, USA). A total of 11 618 658 reads were obtained which is equivalent to 59 528 – 285 456 reads per sample.

Fastq files were processed and the sequencing reads filtered using Mothur software [release v.1.33.0; Schloss *et al*. (2009)] according to the SOP by (Schloss *et al*., 2011). For the alignment and removal of chimeras and plant-derived 16S rRNA gene sequences from chloroplasts and mitochondria, the SILVA reference file (release 119) [Quast *et al*. (2013)] was used. The RDP database (release 10) [Cole *et al*. (2014)] was the reference for classification of OTUs, which were found at 95% similarity clustering of the reads using the furthest neighboring method. Raw read sequences can be found at GenBank's Short Read Archive (SRA) under the accession number SRP101445 (BioProject PRJNA378229).

The ^{13}C enrichment for each OTU was determined on the basis of the publication by Hungate *et al*. (2015), where a detailed description and formulas can be found. First, the weighted average mean density was calculated for each OTU across the seven fractions in each gradient. In this study, an intrinsic correlation of the GC content to the density has been performed. OTUs from the control samples, which could be classified at the genus level, were summed up and the weighted average density was calculated from the seven fractions accordingly for each genus in each depth. If available, the corresponding genomic GC content from the NCBI database (ftp.ncbi.nlm.nih.gov/pub/taxonomy/taxdump.tar.gz; 19.03.2016) was assigned to each genus taxon found in the unlabelled control datasets, which resulted in 234 data points. Multiple GC content entries for single genera in the NCBI database were averaged in advance. The correlation between average mean density and GC content was expressed in a linear model (Supporting Information Fig. S4), which was used to determine the GC content for each OTU in the control samples.

To calculate the increase of ^{13}C content for each OTU, the density shift between the control and the ^{13}C-labelled sample in the corresponding soil depth was determined as the difference of the weighted mean average densities. The GC content of each OTU served as a basis to calculate the increase in molecular weight of the DNA by the density shift and thus the ^{13}C enrichment, which is expressed as atom fraction excess. The extension of atom fraction excess values below '0' was taken as uncertainty range also for the positive measurements. Above this threshold, ^{13}C enrichments were considered as confident.

Significant differences for single variables – root biomass, ^{13}C content, Shannon diversity and relative abundance of bacterial phyla – were calculated with univariate analysis of variance (ANOVA, R package 'stats', R Core Team (2013)). Square root transformed relative abundance data from 16S rRNA gene sequencing and T-RFLP

were used to compute permutational multivariate analysis of variance [PERMANOVA, R package 'vegan', Oksanen *et al*. (2015)]. Heatmaps are based on the same data (R package 'gplots', Warnes *et al*. (2016)). For clustering of the dendrograms, the complete linkage method was applied on the Euclidean distance matrix.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Root biomass along soil depth (A) and ^{13}C enrichment in the plant shoot (B), rhizosphere (C) and bulk soil (D). Different letters indicate significant differences ($P \leq 0.05$). Topsoil (0-20 cm); Subsoil U (upper subsoil, 20-50 cm); Subsoil L (lower subsoil, 50-80 cm).

Fig. S2. Relative abundance of bacterial phyla in different soil depths. Data are obtained from Table S1. Topsoil (0-20 cm); Subsoil U (upper subsoil, 20-50 cm); Subsoil L (lower subsoil, 50-80 cm).

Fig. S3. 16S rRNA gene T-RFLP fingerprint of rhizosphere and bulk soil DNA samples before density fractionation. In the heatmap, TRFs with a minimum relative abundance of 1.5% in at least one sample are ordered from top to bottom according to their mean relative abundance. Top – Topsoil (0-20 cm); Sub U – Upper Subsoil (20-50 cm); Sub L – Lower Subsoil (50-80 cm); ^{13}C-lab – plants were labelled with ^{13}C-CO_2; control – no labelling. The TRF number indicates the length of the restriction fragment in bp.

Fig. S4. Internal calibration of buoyant density and genomic GC content. The weighted mean densities of classifiable genera in the control samples ($n = 234$) are set into relation of genomic GC content information of the same taxa from NCBI genome database. The colour of each dot represents the phylum affiliation of the genus.

Table S1: Relative abundance of bacterial phyla in different soil depths. Topsoil (0-20 cm); Subsoil U (upper subsoil, 20-50 cm); Subsoil L (lower subsoil, 50-80 cm). Significant differences between soil depths were calculated with univariate ANOVA ($P < 0.05$) followed by HSD post hoc test.

Supplementary Information

Supplementary Material and Methods

Plant cultivation and labelling with ^{13}C-CO_2

The plants were cultivated in a climate chamber with 12 h light per day provided by 4 high-pressure sodium vapour lamps (E40, 350 W). The air temperature oscillated between 14°C during night and 20°C during day. Throughout the 90 days of cultivation the plants were irrigated regularly with 200 ml tap water to an equivalent of 165 mm precipitation, which can be expected at the field site near Klein-Altendorf from April until June (Agrarmeteorologie Rheinland-Pfalz; www.wetter.rlp.de).

At the time point of ^{13}C-CO_2 labelling a polystyrene plate with recesses for the soil columns was placed on top of the polystyrene box and sealed with silicone at the interspaces to minimise temperature and gas exchange. An airtight tent was set up above the three control (58×50×104 cm) and nine treatment columns (58×126×104 cm), separately. To provide an atmosphere as close to natural conditions as possible, the control columns were flushed with ambient air with a membrane pump, whereas the treatment columns were supplied via a flow controller with a gas comprising 2.5% of ^{13}C-CO_2 (99%) in N_2 5.0 (Westfalen AG, Münster, Germany). For regulating the CO_2 concentration an infrared controller was used. CO_2 concentration and ^{13}C content of chamber atmosphere were measured with a GC/IRMS-system (delta plus, Thermo Fisher Scientific, Dreieich, Germany). The CO_2 concentration ranged between 300 ppm in the light phase and 600 ppm in the dark phase during the day-night cycle. During 15 days of labelling, in total 20 l of ^{13}C-CO_2 were applied. Within this time, plants were watered once by opening the control tent first and afterwards the ^{13}C-CO_2 tent to avoid gas exchange with the control tent.

After, the experiment was terminated and the soil columns were dissected. To avoid 13C-CO2

uptake via the control plants, the control tent was opened first and the aboveground plant biomass was cut at the root-shoot transition zone before the ^{13}C-CO_2 tent was opened to proceed accordingly.

^{13}C measurements of plant and soil materials

Solid material was ground to a homogenous powder using a ball mill (Tissue Lyser II, Qiagen, Venlo, Netherlands). Aliquots were transferred into tin capsules (IVA Analysentechnik, Meerbusch, Germany) and total carbon content and ^{13}C abundance was determined with an IRMS (delta V Advantage, Thermo Fisher Scientific) coupled to an Elemental Analyzer (Euro EA, Eurovector, Milano, Italy). For calibration, a lab standard (acetanilide) was used, which was calibrated itself against several suitable international isotope standards (IAEA; Vienna, Austria).

Isopycnic centrifugation, gradient fractionation, and DNA purification from rhizosphere samples

From each sample, 4 µg DNA – filled up to 1 ml with gradient buffer (GB; 0.1 M Tris-HCl pH 8, 0.1 M KCl, 1 mM EDTA) – was mixed with 5 ml CsCl solution (50 g CsCl added to 30 ml GB) and adjusted to a buoyant density of 1.71 $g \cdot ml^{-1}$. The buoyant density was calculated from refractory index measured with Reichert™ AR200™ Digital Refractometer (Thermo Fisher Scientific). 5.1 ml polyallomer QuickSeal tubes (Beckman Coulter, Krefeld, Germany) were loaded and placed into a vertical VTi 65.2 Rotor (Beckman Coulter). Ultracentrifugation was carried out in Sorvall® Discovery™ 90SE ultracentrifuge (Thermo Fisher Scientific) for 36 h at 20°C and 44,500 rpm ($184{,}000_{gav}$).

After centrifugation, the CsCl gradient solution was fractionated from bottom to top by puncturing the tube with a 0.4 mm needle and replacing the solution at the top with UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific) via a syringe

pump. 13 fractions à ~400µl were obtained at a flow rate of 1 ml·min^{-1}. Buoyant densities (BDs) were measured using a refractometer as described previously (Lueders *et al.* 2004). DNA was purified from the fractions by adding 800 µl of a PEG solution (30% polyethylene glycol 6000, 1.6 M NaCl) and centrifugation at 14.000×g for 30 min at 4°C. The supernatant was removed and the DNA was washed with 70% ice-cold EtOH (14.000×g, 15 min, 4°C). The DNA was dissolved in 25 µl EB-buffer (Qiagen, Hilden, Germany). The quantity of DNA in each fraction was determined from 250-fold dilutions using the Quant-iT™ PicoGreen dsDNA Assay Kit (Life Technologies, Darmstadt, Germany) with a detection range from 0.016–1 ng·µl^{-1} (data not shown). Over the 13 fractions from each gradient, >89% of total DNA was recovered in 7 consecutive fractions between BDs of 1.665 and 1.730 g·ml^{-1}. These fractions span the range of BDs typical for light and heavy DNA (Lueders *et al.* 2004) and Neufeld *et al.* (2007b)) and were chosen for subsequent 16S rRNA gene-based qPCR and amplicon sequencing.

16S rRNA Gene Barcoding

The library preparation of the 84 samples and sequencing was carried out by following the '16S Metagenomic Sequencing Library Preparation' protocol (Part # 15044223 Rev. B). In the first PCR step a 16S rRNA gene fragment across the variable regions V1-V2 was amplified with the primers Ba27f (Liu *et al.*, 1997) and S-D-Bact-0343-a-A-15 (357R; Klindworth *et al.* (2013)). The target-specific primers (bold) were adapted to the corresponding forward and reverse overhang adapter sequences: 5′– TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG **AGA GTT TGA TCC TGG CTC** –3′ and 5′– GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**CT GCT GCC TYC CGT A** –3′. PCR was carried out in triplicates with 40 pg·µl^{-1} DNA template, 0.2 µM of each primer and NEBNext® High-Fidelity 2X PCR Master Mix (NEB, Frankfurt am Main, Germany). The PCR program was optimised to 27 cycles (98°C – 10s / 60°C – 30s / 72°C – 30s). The

triplicate amplicons were pooled and purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). The DNA quality and fragment size was validated with Agilent 2100 bioanalyzer instrument using the Agilent DNA 7500 Kit (Agilent Technologies, Waldbronn, Germany).

Indexing of the PCR products was carried out with Nextera® XT Index Kit v2 Set B, NEBNext® High-Fidelity 2X PCR Master Mix and 400 $pg \cdot \mu l^{-1}$ DNA. To enhance amplicon quality, the PCR product was purified by agarose gel electrophoresis and narrow band excision prior to gel clean-up and fragment size determination. The amplicons were pooled to 4 nM each and loaded on the MiSeq® sequencer following the 'MiSeq® Reagent Kit V3 reagent Preparation Guide' (Part # 15044983 Rev. B Oct. 2013) and 'Preparing Libraries for sequencing on the MiSeq®' (Part # 15039740 Rev. D Oct. 2013) protocols. 10 pM DNA was loaded on the flow cell and 10% PhiX was spiked to the sample

Terminal restriction fragment length polymorphism (T-RFLP)

Three of the labelled soil columns and one control column were subjected to community fingerprinting prior to DNA-SIP. In order to verify that these columns are comparable regarding their bacterial community composition, T-RFLP-fingerprint of the 16S rRNA gene fragments was performed on bulk soil and rhizosphere samples over all three soil depths.

The T-RFLP method is described in detail in Töwe *et al.* (2011) and Uksa *et al.* (2014). The primers Ba27f (Liu *et al.*, 1997) and Ba907r (Lane, 1991) were used to target the bacterial 16S rRNA gene. Endonuclease *Msp*I (Fermentas, St. Leon-Rot, Germany) was used to digest the amplicon. T-RFLP electropherograms were processed with Peak Scanner™ Software (Version 1.0, Applied Biosystems) and T-REX software (Culman *et al.*, 2009). Terminal restriction fragments (TRFs) below 50 bp were removed and the analysis was based on peak height by filtering with the threshold 1. Relative abundance data of the 50 most abundant TRFs were used for the generation of heatmaps in R and to compute PERMANOVA.

Supplementary Results

Total bacterial community fingerprinting

Bacterial 16S rRNA T-RFLP fingerprints in the bulk soil and rhizosphere from one control column and three labelled columns in three soil depths are displayed in Figure S4. Considering the 50 most abundant TRFs, like expected, the most pronounced differences were found when bulk soil and rhizosphere were compared (PERMANOVA; $P = 0.001$). However, also significant differences between the three investigated soil layers were visible, when the same compartment (bulk soil: $P = 0.001$; rhizosphere: $P = 0.001$) was analysed, although there were no significant differences between the upper and lower subsoil in each compartment, respectively (subsoil U and L in rhizosphere: $P = 0.065$, and in bulk soil: $P = 0.057$). Overall, bacterial communities from topsoil of all soil columns clustered closely together for both compartments. For samples from subsoil U and subsoil L, clear clusters were not observed and bacterial communities showed a high variability, possibly reflecting the higher natural habitat variability in the undisturbed subsoil cores.

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Supplementary Figures and Tables

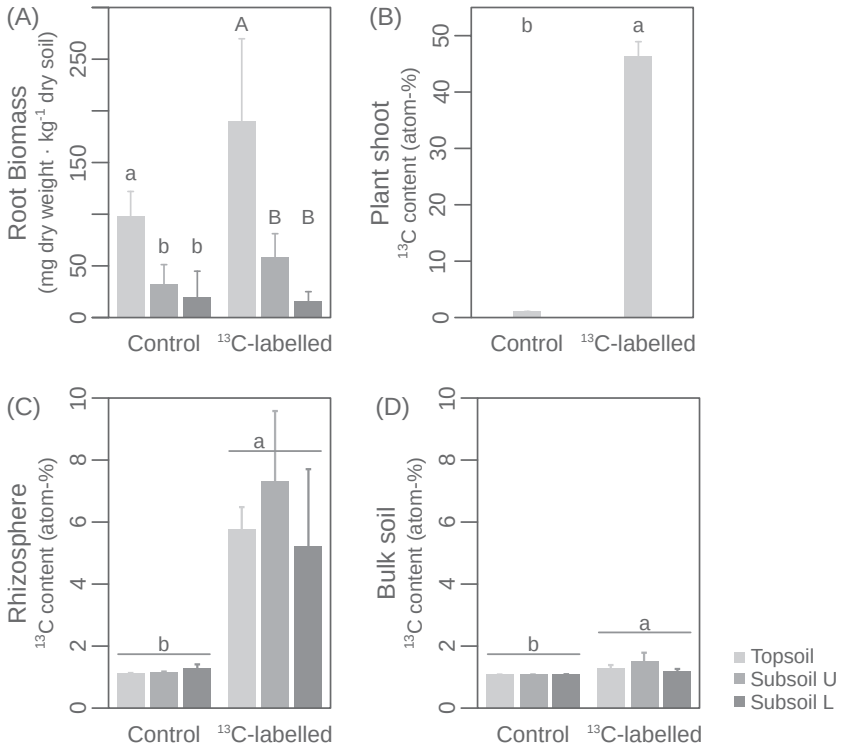

Figure S1: Root biomass along soil depth (A) and ^{13}C enrichment in the plant shoot (B), rhizosphere (C) and bulk soil (D). Different letters indicate significant differences ($P \leq 0.05$). Topsoil (0-20 cm); Subsoil U (upper subsoil, 20-50 cm); Subsoil L (lower subsoil, 50-80 cm).

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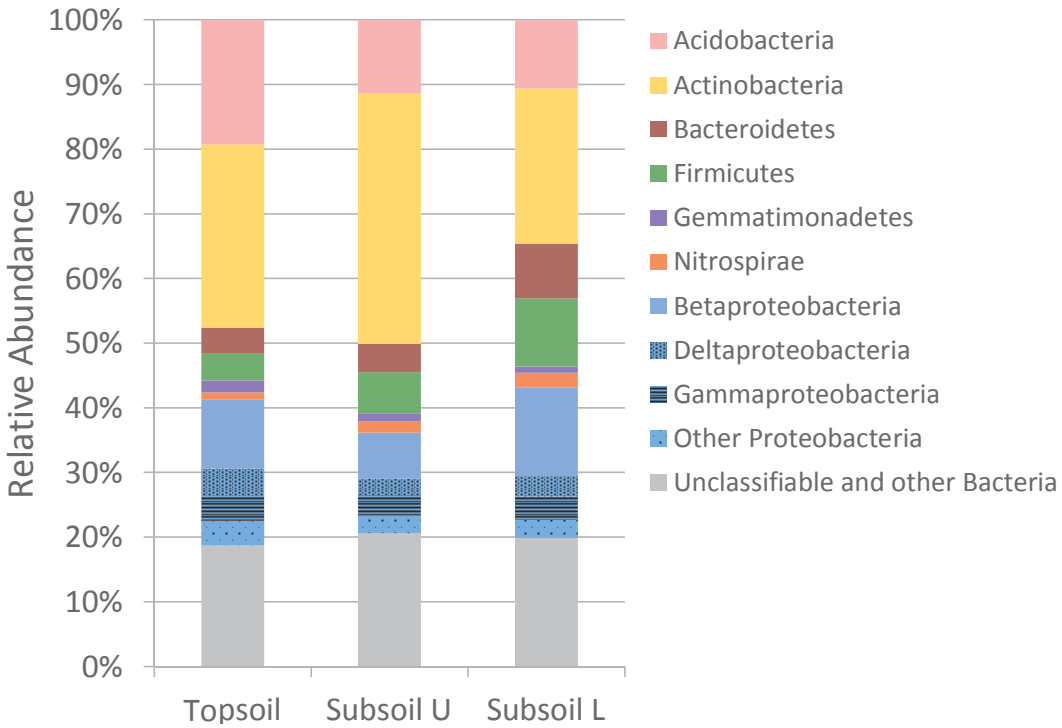
Phylum	Class	Topsoil				Subsoil U				Subsoil L			
		Control	^{13}C-labelled			Control	^{13}C-labelled			Control	^{13}C-labelled		
			replicate 1	replicate 2	replicate 3		replicate 1	replicate 2	replicate 3		replicate 1	replicate 2	replicate 3
Acidobacteria		18.34	20. 2	20.90	16.85	9.28	10.21	12.98	12.61	8.05	11.81	13.85	8.67
Actinobacteria		32.10	25.56	26.84	28.91	44.00	41.04	36.18	34.06	30.87	18.05	19.50	27.39
Bacteroidetes		3.70	3.39	2.50	6.50	5.35	4.26	4.38	3.53	13.73	6.10	5.56	8.71
Firmicutes		4.45	4.80	3.62	3.66	8.53	6.04	5.22	5.54	2.69	14.62	8.64	16.06
Gemmatimonadetes		1.57	1.84	2.31	1.53	0.93	1.45	1.28	1.44	0.99	1.58	0.76	0.61
Nitrospirae		0.94	1.33	1.19	1.04	1.23	1.85	2.00	1.84	1.24	2.54	2.82	2.52
Proteobacteria		20.61	21.84	22.10	25.78	16.84	15.57	14.63	15.04	27.40	21.95	22.05	21.65
	β-Proteobacteria	9.74	9.27	9.67	14.10	7.80	7.83	6.49	6.34	15.08	12.68	13.36	13.61
	δ-Proteobacteria	4.06	4.61	4.58	3.78	2.80	2.56	2.47	2.44	3.97	3.15	3.00	2.35
	γ-Proteobacteria	3.21	4.07	4.20	3.74	3.79	2.93	2.86	3.35	5.31	3.21	2.58	3.36
	Other	3.60	3.88	3.66	4.15	2.45	2.24	2.81	2.92	3.03	2.90	3.10	2.34
Other		0.33	0.39	0.36	0.29	0.36	0.40	0.52	0.48	0.60	0.75	0.73	0.69
Unclassified Bacteria		17.98	20.04	20.17	15.45	13.47	19.17	22.82	25.47	14.43	22.60	26.10	13.69

Phylum	Class	Topsoil	Subsoil U	Subsoil L	ANOVA	HSD Posthoc		
		Mean	Mean	Mean	Soil Depth	Topsoil	Subsoil U	Subsoil L
					P value			
Acidobacteria		19.23	11.27	10.59	0.001***	a	b	b
Actinobacteria		28.35	38.82	23.95	0.004**	b	a	b
Bacteroidetes		4.02	4.38	8.53	0.049	a	a	a
Firmicutes		4.13	6.33	10.50	0.093	a	a	a
Gemmatimonadetes		1.81	1.28	0.98	0.025*	a	ab	b
Nitrospirae		1.12	1.73	2.28	0.020*	b	ab	a
Proteobacteria		22.58	15.52	23.26	0.001***	a	b	a
	β-Proteobacteria	10.69	7.12	13.68	0.001***	b	c	a
	δ-Proteobacteria	4.26	2.57	3.12	0.002**	a	b	b
	γ-Proteobacteria	3.80	3.23	3.62	0.580	a	a	a
	Other	3.82	2.61	2.84	0.001***	a	b	b
Other		0.34	0.44	0.69	<0.001***	b	b	a
Unclassified Bacteria		18.41	20.23	19.20	0.867	a	a	a



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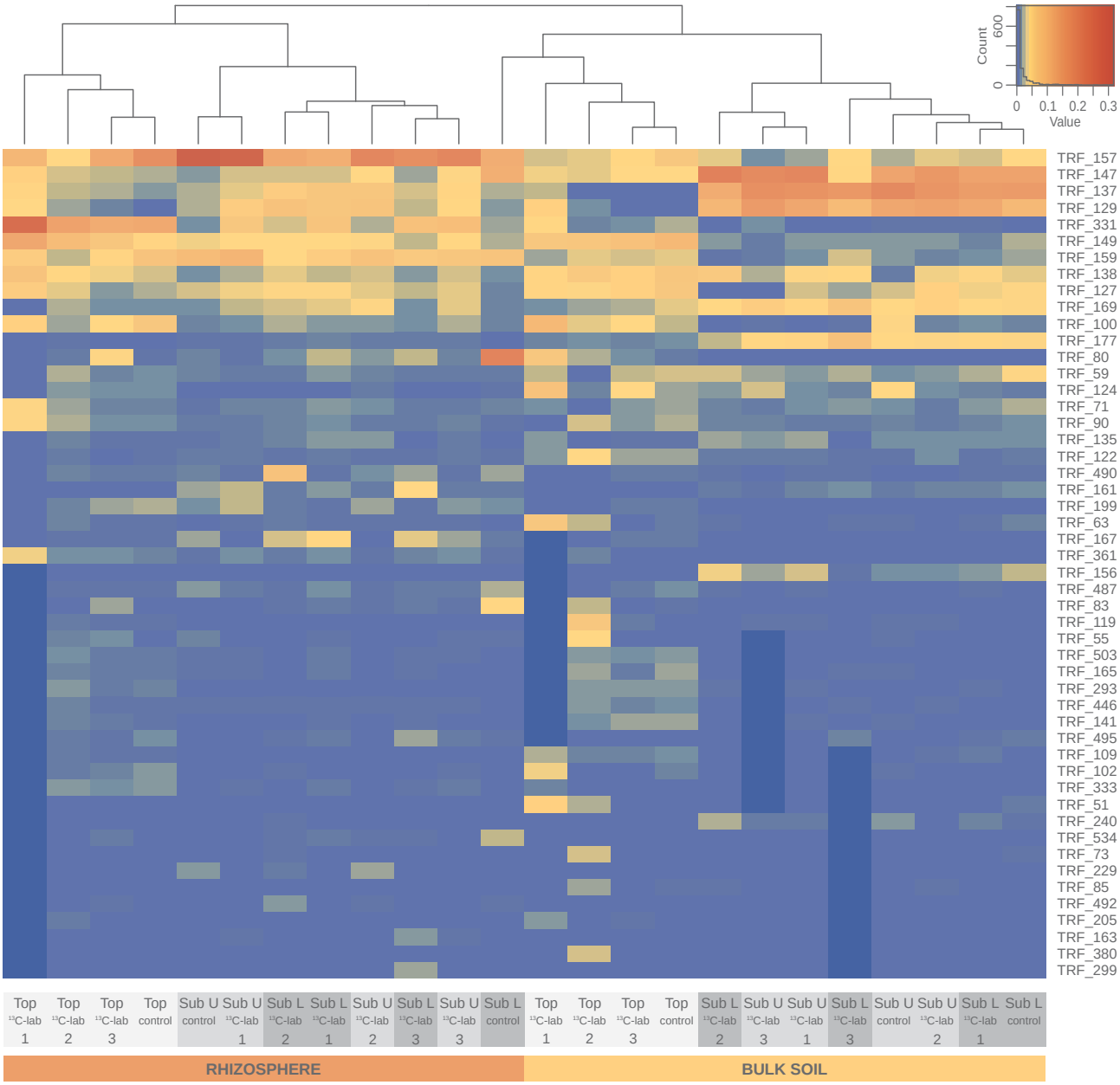

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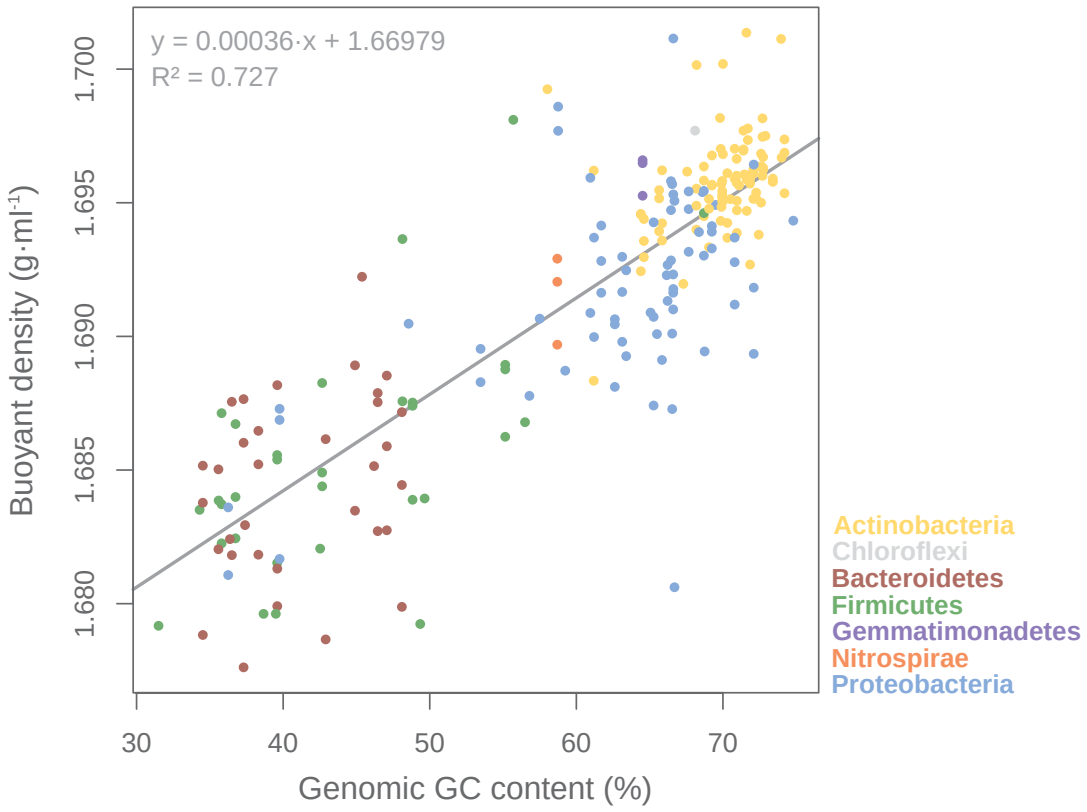

Figure S5: Internal calibration of buoyant density and genomic GC content. The weighted mean densities of classifiable genera in the control samples (n = 234) are set into relation of genomic GC content information of the same taxa from NCBI genome database. The colour of each dot represents the phylum affiliation of the genus.

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