The effect of soil habitat connectivity on microbial interactions, community structure and diversity:

a microcosm-based approach

Alexandra Wolf

Thesis committee:

Prof. dr. H.A.B. Wösten
Prof. dr. H.J. Laanbroek
Prof. dr. J.A. van Veen
Prof. dr. P. Baldrian
Dr. J. Falcão Salles

ISBN: 978-90-393-6145-0
Printing: Gilderprint Drukkerijen, Enschede (NL) (www.gildeprint.nl)
Layout and Cover design: agilecolor design studio/atelier (www.agilecolor.com)

The effect of soil habitat connectivity on microbial interactions, community structure and diversity:

a microcosm-based approach

Het effect van bodem verbindingen op de interactie
met microorganismen, gemeenschap structuur en diversiteit:
een microcosmos aanpak
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G. J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen
op maandag 19 mei 2014 des middags te 2.30 uur

door

Alexandra Bettina Wolf
geboren op 10 maart 1981 te Bonn, Duitsland

Promotor:
Prof. dr. G.A. Kowalchuk

This thesis was accomplished with financial support from the Vici grant of GAK from the Dutch Science Foundation (NWO) entitled “Crossing the frontiers of microbial ecology”.

Table of Contents

Chapter 1

General introduction

1.1 Soils as a microbial habitat

A large fraction of the Earth's biodiversity is contained in soils. The vast majority of this diversity is microbial with one gram of soil containing an estimated 6,000 to 50,000 bacterial species (Curtis *et al*., 2002). These diverse microbial communities contribute to a wide range of important ecosystem processes such as biogeochemical cycling, maintenance of soil structure and quality, plant productivity and pathogen control. Soils are not only diverse, but they also comprise great numbers of microbes: one gram of soil can be inhabited by up to 10 billion microorganisms (Torsvik & Øvreås, 2002). Recent advances in the application of molecular methods in microbial ecology have provided a new appreciation of the extent of soil-borne microbial diversity, but our understanding of the forces that shape and maintain this tremendous source of biodiversity still remain relatively rudimentary. Despite the huge numbers of soil-borne microbes, the internal surface area of soils is only sparsely populated (Postma & van Veen, 1990, Grundmann, 2004). This might seem contradictory at first, but the internal soil surface area is tremendous. Although this depends greatly on the soil texture, a typical 1 cm^3 of soil is approximately equivalent to 20 m^2. Even if billions of bacterial cells are distributed over this area, still only a very small fraction (less than 1 % (Young & Crawford, 2004, Young *et al*., 2008)) of the total surface area is covered by microbes. As a consequence, distances between individual cells or (micro-)colonies can be huge in relation to the tiny size of the microbes themselves (Grundmann *et al*., 2001).

Soils are highly complex and heterogeneous, consisting of solid matter (sand, silt, clay and organic matter) organized into aggregates differing in shape and size (Six *et al*., 2004, Chenu & Cosentino, 2011). The arrangement of the solid particles creates a mosaic of microhabitats with different physical and chemical properties, resulting a great multiplicity of spaces and niches (Six *et al*., 2004, Mummey *et al*., 2006, Nunan *et al*., 2007). Depending on how these particles and aggregates are stacked, a complex pore network is created within and between aggregates. Soil microbes reside in this soil pore network, and how this network is structured consequently determines the access that microbiota have to organic substrates, nutrients, oxygen, and water (Oades, 1984).

Soil pores, with their great variety in geometry and size, are, depending on the hydration status, either filled with water or air at any given time. Generally, water is held more tightly in small capillaries (Ritz & Young, 2004). Larger pores thus drain out first and tend to only be filled with water when the soil is (almost) saturated. Smaller pores, on the other hand, are mostly filled with water, except under very dry conditions. Soil water is essential for soil biota to maintain their vitality and function. Many microbial interactions with the environment, such as gaining access to nutrients, the detection of signaling molecules and motility, are mediated by the water phase, and are thereby dependent on hydraulic connectivity. Volatile compounds also play an important role in mediating

microbial interactions in soil (Effmert *et al.*, 2012). Volatiles primary diffuse through the gas-phase due to their high vapor pressure, but are active both in the water as well as gas phase, making them capable of dispersing through the whole pore network (Asensio *et al.*, 2008). Similar to soluble compounds, the diffusion of volatiles, and consequently volatile-mediated microbial interactions, is also influenced by the physicochemical properties and hydration status of the surrounding soil. This thesis focusses on hydraulic connectivity, which is determined by the extent to which water films in soil are connected. This connectivity allows cells located in distinct micro-habitats to interact and facilitates bacterial motility and nutrient diffusion, thereby impacting microbial diversity.

The connection of micro-habitats in soils depends on the soil structure (i.e. pore sizes and shape) and the hydration status. Consequently, connectivity is likely to be of far greater importance to microbial interactions than the absolute distance between microhabitats (Young & Crawford, 2004, Long & Or, 2009). Because microbial interactions are thought to be key factors controlling microbial community structure, diversity and ultimately function (Tiedje *et al.*, 2001), it is important to understand how the variety of microbial interactions are affected by habitat connectivity.

The extent of connected water films in soils can vary greatly. Most soils are generally not water-saturated, thereby imposing limitations on motility and interactions. Also, specific micro-habitats in soil have been demonstrated to select strongly for specific microbial traits. Examples include microbes in the rhizosphere, where specific root exudates and signals drive microbial community structure (Hartmann *et al.*, 2009), the mycosphere (Warmink *et al.*, 2009) and zones rich in particular nutrients that can attract soil microbes (Sengelov *et al.*, 2000, Nicolardot *et al.*, 2007, Poll *et al.*, 2010).

Unlike (most) bacteria, fungi can grow hyphae into air-filled spaces, which allows them to bridge air-gaps, making fungal spread less dependent on the water content of soil (Griffin, 1985). However, it has been shown that some motile bacteria can also cross air-gaps in unsaturated soils by moving along fungal hyphae (Kohlmeier *et al.*, 2005, Warmink & van Elsas, 2009). Fungi may thus promote bacterial distribution along hyphal “highways” in unsaturated soils.

1.2 Importance of soil microbiota: bacteria and fungi

Given their minute size, the effects of individual microbes is very small with respect to total ecosystem functioning. However, on a community level, microbes are important players, and their functioning has vital impacts at the landscape level and beyond. In fact, the activities of microbes are crucial to sustaining all terrestrial life, with major roles in the global cycling of carbon, nitrogen, sulfur and other elements (Schimel & Bennett, 2004,

Schmidt, 2006), the degradation of pesticides and toxic compounds and the formation and maintenance of soil structure (Ranjard & Richaume, 2001). Plant growth can also be promoted by bacteria; e.g. N fixation as well as through root-colonizing rhizobacteria producing phytohormones and volatiles stimulating plant growth and protecting plant roots against pathogens. Like bacteria, fungi also occupy a central role in various soil biological processes, such as biogeochemical cycling, and they are of great importance to plant health and productivity as well as soil fertility. Fungi are well adapted to the soil environment due to a variety of growth forms and trophic strategies and are practically ubiquitous in soil (0.8–16 km of hyphae per 1 2g of soil; (Finlay, 2006, Young *et al.*, 2008)). However, in soils, bacterial biomass generally exceeds fungal biomass, though the fungal:bacterial-ratio varies greatly depending on land use and soil properties with values ranging from 0.007 to 0.34 (Fierer *et al.*, 2009). The fungal mycelium facilitates growth in heterogeneous environments with a patchy distribution of resources. This growth form allows for translocation of carbon and nutrients in different directions, providing a mechanism to redistribute resources throughout the mycelial network as necessary (Boswell *et al.*, 2002). Fungi have three general trophic strategies: as plant pathogens, in symbiosis with plants and as saprotrophs. Saprotrophs play pivotal roles in decomposition of plant residues including cellulose and hemicelluloses from plant cell walls and recalcitrant compounds of woody plant litter such as lignin and lignocelluloses (Valaskova *et al.*, 2007, Baldrian, 2008).

Both fungi and bacteria can utilize a wide range of substrates, but it is generally thought that soil bacteria predominantly utilize readily degradable compounds such as root exudates, whereas fungi are the primary degraders of more recalcitrant substrates. Although bacteria and fungi often share soil habitats, they commonly occupy different niches. There is, however, also a good degree of overlap in bacterial and fungal niches and growth strategies in soil. Competition for simple substrates has also resulted in the evolution of a range of antagonistic strategies, whereas for more recalcitrant substrates, both competitive as well as mutualistic strategies have evolved (de Boer *et al.*, 2005).

Fungi are much larger in size than bacteria (typical diameter of 3–10 µm relative to 0.5–1 µm for bacteria). Consequently, fungal spread through soil occurs predominantly through macro-pores. Hattori (1988) showed that 80–90% of fungi may be restricted to larger pores, and fungi are rarely found in micro-pores, where most bacteria reside (Killham, 1994). Bacteria thus generally inhabit the more protected micro-pores, whereas fungi reside predominantly in larger pores, where they are more exposed to environmental changes such as dry-wet cycles and predation. Consequently, most fungal/bacterial interactions are likely to occur in macro-and meso-pores where bacteria and fungi co-exist.

Fungal mycelia provide a very large surface area upon which interactions can take place, and this filamentous growth form also allows fungi to simultaneously interact with different,

spatially separated organisms. Bacterial-fungal interactions impact the growth dynamics of bacteria and fungi and play a role in shaping the structure and functioning of microbial communities. Fungi have evolved mechanisms to take advantage of bacteria: e.g. some fungi are able to sense bacterial colonies and then secrete compounds to lyse the bacterial cells and then utilize the released nutrients (Barron, 1988). This strategy gives fungi a considerable advantage, especially under nutrient limited conditions. On the other hand, fungi can also be beneficial to bacteria. For example, some bacteria can feed on fungal cells (de Boer *et al.*, 2005). Furthermore, mycorrhizal fungi can translocate carbon compounds from autotrophic plants and subsequently exude them, principally in the form of trehalose, thus making them available to bacteria (Drigo *et al.*, 2010).

1.3 Drivers of soil-borne microbial community structure

Recent advances in molecular techniques continue to increase our recognition of the tremendous phylogenetic and functional diversity of soil-borne microbial communities. However, the forces that allow phylogenetically and functionally diverse soil-borne microbial communities to evolve and be maintained still remain poorly understood. Also, the ecology and life-history strategies of most microbial taxa remain unidentified (Fierer & Lennon, 2011). In order to explain how diversity develops and is maintained, it is important to identify the forces that allow diverse microbial populations to co-exist in soil. Since microbial community structure, and ultimately diversity, depends on microbial interactions, it is of major importance to understand how these interactions are affected by the factors that contribute to soil connectivity.

Soil microbial communities have generally been looked at from a human perspective; that is to say at scales that are convenient to sample and analyze. However, soil is an extremely heterogeneous environment with a great range of physical and chemical conditions especially at the very fine scales most relevant to defining microbial habitats. The sample sizes typically used in analyses of soil microbial communities, say 1 g of soil, negate a large amount of the heterogeneity present in soil. This human perspective therefore compromises our ability to describe small-scale variation in the soil environment and to link this variation with microbial community structure, function and diversity. Microbial interactions with the environment, as well as with other microbes, occur at the micro-scale. Thus, there is a distinct need to examine soil-borne microbial communities and their ecology at relevant scales.

Examining soil microbial communities at scales more relevant to their microbial inhabitants is challenging for a number of reasons. First of all, the vast majority of soil microbes remain refractory to currently available cultivation methods, and it is estimated that only a small fraction (<0.1%) of soil bacteria has been cultured to date (Hugenholtz *et al.*,

1998). The introduction of molecular methods in microbial ecology now allows for the phylogenetic description of soil-borne microbial diversity, but we still generally lack the ability to identify soil-borne microbial populations *in situ*. Thus, the coupling of identity and function still remains challenging, thereby restricting our ability to define the metabolism and interactions of the microbial populations comprising the hyper-diverse soil community.

Both cultivation-dependent and -independent methods typically destroy the soil structure of the sample, and thereby dissociate the detected microbial populations from the habitats in which they reside. It is possible to partially preserve the soil structure by picking individual aggregates for subsequent cell or nucleic acid extraction (Baileya *et al.*, 2013). However, this approach still (partially) destroys the pore network in which the microbes reside. Also, it is not possible to sample microbes from individual pores, for example in order to reveal the community composition in specific pore size classes or to investigate the effect of pore size on the communities.

Another difficulty is that the extreme complexity of soil hampers our ability to manipulate specific soil parameters independently in order to examine their impacts on the soil community or specific populations therein. For example, when connectivity is increased by increasing the water content, many other soil parameters, such as pH, salinity, osmotic pressure and nutrient levels, are altered simultaneously. Consequently, it is difficult to link the impact of these individual factors to specific microbial responses. Instead of trying to unravel the drivers of microbial soil microbial communities *in situ*, the strategy adopted in this thesis relies on the experimental manipulation of artificial soils, thereby allowing for precise and independent manipulation of specific environmental parameters of interest. By using such a strategy, many of the aforementioned difficulties in studying soil-borne microbial communities can be circumvented, allowing for a more direct examination of the impact of soil connectivity on microbial interactions, community structure, diversity and function.

1.4 Bacterial motility in soil

Soil bacteria are generally considered to not be very motile. Many bacteria are incapable of active motility and rely, like spores, on passive dispersal via the flow of water or passing invertebrates. Other bacteria possess a variety of different motility mechanisms, potentially enabling them to move actively towards nutrient sources, explore new habitats and evade predation. Passive dispersal of bacterial cells and spores obviously depends greatly on the soil hydration status and the flow of water therein (Abuashour *et al.*, 1994, Jiang *et al.*, 2006). Active bacterial motility is also greatly affected by soil moisture, as bacteria move along water films covering solid surfaces. It has been shown for example that *Pseudomonas* flagellar motility on rough surfaces is only possible in water films > 1.5 µm (Dechesne *et*

al., 2010). However, surprisingly little attention has been paid to active bacterial motility in porous media. Most research has been conducted in columns dealing with the flow of liquids and the consequent passive movement of bacteria with this flow. Such studies have generally been in the context of following the fate of genetically modified bacteria in soil or the spread of pathogens from agricultural waste to soils, surface- and groundwater (Trevors *et al*., 1990, van Elsas *et al*., 1991, Huysman & Verstraete, 1993, Abuashour *et al*., 1994). Experiments in soil columns have demonstrated that not only soil moisture, but also soil structure, influences dispersal. Studies by van Elsas and colleagues (1991) and Huysman and Verstraete (1993) also showed that increased bulk density leads to a decrease of bacterial migration to lower soil layers.

Although a wide variety of microbial mobility mechanisms is known and has been studied, it remains to be determined how these diverse abilities influence the colonization speed of different populations in soil or how colonization speed is affected by soil moisture. Also, there is little known concerning the ecological relevance of bacterial migration and its implications on microbial community structure. Thus, the work in this thesis also seeks to examine how bacteria differ in their ability to colonize new soil environments.

1.5 Aims and research questions

The main aim of the work presented in this thesis is to increase our understanding of the forces that allow for the tremendous diversity of soil-borne microbial communities, with particular focus on how soil connectivity impacts the structure, diversity and interactions within soil-borne microbial communities.

The three general research questions addressed in this thesis are as follows:

1. What are the impacts of pore size distribution and matric potential, which determine soil connectivity, on microbial resource competition and habitat utilization?
2. How does connectivity influence the structure and diversity of complex bacterial and fungal communities and is there an effect on microbial groups with different life history strategies? Do bacteria or fungi have a relative competitive advantage under specific soil conditions?
3. How does soil moisture impact the colonization potential of bacteria with different motility mechanisms?

1.6 Approach

Most laboratory experiments are typically performed in liquid media or on agar plates. This allows for a direct observation of the microbes of interest, and their (metabolic) responses to specific parameters. However, these simple environments poorly represent the complex soil environment, and microbes often behave entirely differently in these controlled conditions as compared to the porous matrices in which they occur in nature. It is therefore often not possible to extrapolate observations made in such laboratory settings to the *in situ* situation in the environment. However, as stated above, the manipulation and experimentation of microbial communities in the soil habitat is problematic. Thus, one the one hand, cultivation-based methods are too simple to mimic the true environment, while, on the other hand, soil-based approaches are too complex to allow robust interpretation.

In this thesis, I therefore chose to work with an intermediate level of complexity and realism. My approach was to perform a series of experiments in artificial soil microcosms, which allow for the independent manipulation of specific soil parameters, with the goal of unraveling the impacts of soil connectivity parameters on bacterial interactions and microbial community structure, diversity and function. I constructed and characterized a series of artificial soils differing in pore size distribution and matric potential, and consequently in connectivity between micro-habitats, using quartz sand particles of different size classes that were supplemented with different amounts of liquid. The pore size distribution of the different artificial soils was estimated from water retention curves. Depending on the question at hand, the microcosms were inoculated with individual bacterial strains, artificially constructed assemblages or natural soil microbial communities, and microbial population dynamics were tracked over time via specific plate counts, quantitative PCR and next-generation sequencing approaches. I also controlled the amounts of nutrients that were added and tracked their utilization over time, as well as microbial respiration, as a measure of microbial activity. The advantage of this relatively simple system is that many soil parameters can be held constant, so that the impact of only a single parameter, e.g. moisture or pore size, can be evaluated. At the same time, the constructed soils mimic real soil conditions, allowing for a degree of extrapolation to the environmental factors impacting the structure, diversity and functioning of actual soil communities.

1.7 Outline of the thesis

Chapter 2 provides a description of the soil habitat from a bacterial perspective and a review of the drivers of diversity of soil-borne microbial communities. Particular focus is placed on the methods and techniques that allow one to link micro-scale soil habitat descriptions with measures of microbial community structure and functioning, so as to help identify the ecological drivers of microbial diversity in soil.

Chapter 3 describes the artificial experimental soil microcosm system that I developed, and demonstrates how this system was used to study the impact of habitat connectivity on the competitive dynamics between a filamentous and a non-filamentous bacterial strain. The results show that filamentous growth is beneficial for bacteria in soils with low habitat connectivity.

Chapters 4 and 5 examine how the diversity and community structure of complex microbial communities are impacted by soil connectivity. Either complex bacterial or bacterial and fungal communities were inoculated in the artificial soil microcosms developed in Chapter 3, and community development was followed over time by phospholipid fatty acid (PLFA) analysis (Chapter 4) and next-generation sequencing of bacterial and fungal ribosomal RNA gene tags (Chapter 5).

Chapter 6 examines the differential ability of numerous bacterial populations to expand into available habitats in soil. To this end, artificial soil microcosms were again used, which allowed me to track the advance of numerous bacterial populations over time. Bacteria originating from a soil extract were inoculated in the center of sterilized microcosms, and sampling at different distances from the center was carried out over time. DNA was extracted from the resulting samples for high-throughput sequencing of 16S rRNA gene tags to compare bacterial community compositions at different distances and time points.

Chapter 7 provides a general discussion of the results presented in the thesis and examines the impacts of habitat connectivity on the development and maintenance of soil-borne microbial community structure and diversity. Furthermore, the strength and weaknesses of the approaches used in this thesis are discussed and future research priorities are identified.

Chapter 2

Micro-scale determinants of bacterial diversity in soil

Authors:
Michiel Vos, Alexandra B. Wolf, Sarah J. Jennings,
George A. Kowalchuk

Published in: *FEMS Microbiology Reviews* (2013) 37: 936-954

Abstract

Soil habitats contain vast numbers of micro-organisms and harbour a large portion of the planet's biological diversity. Although high-throughput sequencing technologies continue to advance our appreciation of this remarkable phylogenetic and functional diversity, we still have only a rudimentary understanding of the forces that allow diverse microbial populations to coexist in soils. This conspicuous knowledge gap may be partially due the human perspective from which we tend to examine soil-borne microbes. This review focusses on the highly heterogeneous soil matrix from the vantage point of individual bacteria. Methods describing micro-scale soil habitats and their inhabitants based on sieving, dissecting, and visualizing individual soil aggregates are discussed, as are microcosm-based experiments allowing the manipulation of key soil parameters. We identify how the spatial heterogeneity of soil could influence a number of ecological interactions promoting the evolution and maintenance of bacterial diversity.

One-sentence summary: Understanding the drivers of soil-borne bacterial diversity requires appreciation of the heterogeneous micro-scale environment as encountered by the bacteria themselves.

Keywords: soil heterogeneity – biodiversity – bacterial interactions – microcosm experimentation – soil ecology – soil imaging

2.1 Introduction

Soils are among the most vast (Whitman *et al.*, 1998) and biodiverse microbial habitats on our planet (Quince *et al.*, 2008). Soil-borne microorganisms play pivotal roles in an array of terrestrial ecosystem functions including nutrient cycling, sustaining plant growth, water purification, carbon storage and the maintenance of soil structure, and human reliance and pressure on these services continues to increase (Young & Crawford, 2004). Although it has been known for some time that soil communities are highly diverse (Torsvik *et al.*, 1990), the recent application of high-throughput DNA sequencing strategies in microbial ecology (e.g. Roesch *et al.*, 2007, Delmont *et al.*, 2012) have been instrumental in rekindling our appreciation of soil-borne microbial diversity. For example, more than 33,000 bacterial and archaeal taxa could be detected in just a single soil sample using a PhyloChip (Mendes *et al.*, 2011). With the help of molecular-based surveys of soil microorganisms, we are starting to gain insight into patterns of microbial diversity related to environmental pH (Lauber *et al.*, 2009), nitrogen (Fierer *et al.*, 2012), soil type (Griffiths *et al.*, 2011), moisture (Cruz-Martinez *et al.*, 2009), plant communities (Kowalchuk *et al.*, 2002), crop rotation (Lupwayi *et al.*, 1998) and human disturbance (Ge *et al.*, 2008).

Although gross trends in microbial communities can be gleaned from large-scale surveys and field-based experiments (Castro *et al.*, 2010, Kuramae *et al.*, 2012), it is clear that typical soil sampling strategies are not well suited for studies of microbial diversity and factors that drive and maintain this diversity. The typical soil sample is huge compared to the actual habitats of individual microbes, and virtually all information on the spatial location of individuals relative to each other and their resources is lost during sample processing. The destruction of spatial information hampers our ability to infer *in situ* species interactions and identify microbial niches in detail (Holden *et al.*, 2011). One would never consider ignoring spatial distribution in studies of plant or animal ecology, but, to date, this has been the standard necessity in soil microbial ecology. In essence, the typical soil microbial ecologist is confronted with a task that is similar to asking a plant ecologist to make sense out of a giant heap of plants harvested from an entire landscape. It is argued here that it is vital to appreciate the micro-scale complexity of the soil matrix, because this determines the distribution and activity of cells, their potential to interact, and ultimately how biodiversity and function can evolve and be maintained (Franklin & Mills, 2003, Grundmann, 2004, Crawford *et al.*, 2005, Nunan *et al.*, 2007, Young *et al.*, 2008, Holden *et al.*, 2011).

We propose that a large proportion of the processes that shape microbial diversity in soil occur at scales that we have to date generally failed to address. Given this gap in perception, this review aims to first present an overview of soil as a habitat from the perspective of individual bacteria. We then discuss the diverse approaches that researchers have taken to examine how the complex spatial structure of soil impacts the location, identity and

interactions of bacterial cells in soil environments. Although soil microbial communities are composed of diverse members of all three domains of life, for the sake of brevity and clarity, we here focus primarily on bacteria. Given the emerging information on micro-scale distribution of bacterial populations in soil, we discuss ecological mechanisms that may be at play in driving and maintaining bacterial diversity in soil, using insights from theory and experiments. We conclude our discussion by highlighting research opportunities and priorities that can now be addressed thanks to exciting methodological advances.

2.2 Bacterial biogeography: planet to particle

The biogeography of bacteria is a topic that first rose to prominence nearly a century ago through the Delft School of Microbiology (Baas Becking, 1934, de Wit & Bouvier, 2006, O'Malley, 2007). With advances in environmental sequencing, the topic of microbial biogeography has undergone a marked resurgence in this century (Green & Bohannan, 2006, Martiny *et al.*, 2006). It has now been well-established that microorganisms are non-randomly distributed in space; a recent meta-analysis of microbial biogeography data (Hanson *et al.*, 2012) found that approximately half of the variation in community composition could be accounted for by the effects of habitat and geographical distance, similar to data collected for plants and animals. As typical for eukaryotes, the effect of habitat on microbial biogeography was greater than that of distance. Since it is hard (if not impossible) to measure all abiotic and biotic variables that constitute 'the environment', the effect of environment might be severely underestimated when it co-varies with geographical distance. This indicates that microbes are not greatly limited by dispersal and are expected to colonize any environment in which they can thrive.

Diversity is not expected to decrease linearly from the level of the planet (containing all existing bacterial diversity) to the level of a single soil particle (containing a single microcolony). For the sake of human perspective, let's start examining bacterial distributions at the local scale of an agricultural plot. This plot will harbour numerous more or less distinct spatial niches. A whole suite of factors is expected to create environmental variation relevant to bacteria (Franklin & Mills, 2009), including plant cover (Berg & Smalla, 2009), animal activity (Singh *et al.*, 2009), wetness (Or *et al.*, 2007), fertiliser application (Fierer *et al.*, 2012), pH (Rousk *et al.*, 2010) and salinity (Rajaniemi & Allison, 2009). Bacteria are not limited by dispersal at the field scale and thus are expected to colonize any vacant niches at that scale (Bell, 2010). The finding of decreased variation in a single soil core, or even within a meter plot, thus indicates that not all niches, and not all niche specialists, that are found at the scale of the field are contained within a single sample (e.g. King *et al.*, 2010, Yergeau *et al.*, 2010). Going up in scale to an entire region, other agricultural fields will experience similar climate, soils and plant cover and can therefore

be expected to all contain roughly the same bacterial communities (Vos & Velicer, 2008). Going to even larger (continental) scales, environments can be quite different, selecting for different genotypes. These processes will result in a steep species area curve at small (below field) and large (continental) scales and a shallow species area curve at the intermediate (regional) scale (Vos & Velicer, 2008).

Remarkably little is known about spatial patterns of bacterial diversity below the scale of meters. Oda and colleagues found that *Rhodopseudomonas palustris* populations sampled from marsh sediments were highly similar one centimetre apart, but that this similarity rapidly decreased so that populations nine meters apart were highly disparate (Oda *et al.*, 2003). Bacteria capable of degrading the herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) were found to occur in centimeter-sized hotspots (Gonod *et al.*, 2003, Gonod *et al.*, 2006). Similarly, heavy metal contamination and bacterial metabolic potential structure were found to be structured at a scale of centimeters, (although the two were generally not correlated with each other) (Becker *et al.*, 2006).

How diversity is structured from the scale of a typical soil sample down to the scale of an individual bacterial cell is even less well understood. It is evident that bacteria are not distributed randomly or regularly within micro-samples, but that diversity is organized in patches (Ranjard & Richaume, 2001). Using geostatistical analysis (Ettema & Wardle, 2002, Franklin & Mills, 2007) on microscopic soil sections, bacterial clustering could be visualized in the range of hundreds of µm's (Nunan *et al.*, 2002, Nunan *et al.*, 2003). Patches were found to consist of a limited number of cells (Nunan *et al.*, 2003); such micro-colonies are likely produced by binary fission and are therefore genetically identical, although mutations occurring during replication would generate within-colony variation as well (Grundmann, 2004, Kraemer & Velicer, 2011). Aggregates consisting of distinctly different types of bacteria, e.g. those engaged in cross-feeding, are also expected to occur, and such clusters of morphologically distinct cells have indeed been observed (Nunan *et al.*, 2003).

2.3 Bacterial diversity at the micro-scale

It has been recognized for some time that bacterial diversity within small soil samples is very high based on studies utilizing the 16S rRNA gene marker (Vogel *et al.*, 2003, Grundmann, 2004), and this view is reaffirmed and extended by current next-generation sequencing efforts (Delmont *et al.*, 2012, Vos *et al.*, 2012). Total biological diversity is especially high when it is considered that the 16S rRNA gene is highly conserved, with an enormous amount of genomic and functional diversity hidden behind identical ribotypes. This cryptic diversity is illustrated by a collection of 78 *Myxococcus xanthus* isolates from a 16 × 16 cm soil plot that were indistinguishable based upon 16S rRNA gene

sequencing. Sequencing of three housekeeping revealed 21 genotypes (Vos & Velicer, 2006) and 45 distinct types could be distinguished based on swarming incompatibility tests (Vos & Velicer, 2009). Furthermore, these isolates exhibited extensive variation in gliding motility (Vos & Velicer, 2008), fruiting body development (Kraemer *et al.*, 2010), predation (Morgan *et al.*, 2010) and secondary metabolite production (Krug *et al.*, 2008).

Although sequencing surveys based on single marker genes greatly underestimate the number of taxa present, many of the taxa detected are expected not to be metabolically active. The abundance and small size of bacteria mean that they are prone to frequent random dispersal. A given soil patch thus will receive a periodic influx of bacterial species, many of which will not be well adapted to their new local environment. However, many microbial types can survive for extended periods of time in unfavourable habitats by entering into a dormant state. This strategy can result in an extensive 'seed bank' of microbial propagules (Lennon & Jones, 2011). Dormant cells that revive when environmental conditions turn in their favour may play an important role in the resilience of soil communities in the face of environmental changes (Prosser *et al.*, 2007). Community surveys based on actively transcribed RNA, as opposed to merely 'present' DNA sequences, are therefore likely to paint a more realistic picture of soil-borne functional diversity (Jones & Lennon, 2010). With the advent of metatranscriptomic approaches (Mitra *et al.*, 2011, Urich & Schleper, 2011) surveys of both 'diversity' and 'functional diversity' are expected to become commonplace.

2.4 Analysis of soil communities using sieving-based methods

Physical and chemical properties of soil aggregate fractions are assumed to vary with aggregate size (Scheu *et al.*, 1996, Kandeler *et al.*, 2000, Six *et al.*, 2004, Bronick & Lal, 2005). Thus, the separate analysis of different soil size fractions holds potential for gaining insight into the physical and biological differences between different soil microenvironments. Size fraction-based methods typically make no effort to preserve the pore network. However, although spatial information is lost, such approaches do have the advantage that they are low-tech and hence broadly accessible. In addition, the relatively benign treatment of samples allows for the downstream application of classical, molecular and new "-omics" methods to characterize resident communities.

Soil size fractionation is typically performed by either wet or dry sieving methods. Disadvantages of dry sieving are that aggregates are subjected to abrasive and impact forces that can affect their size and stability (Kemper & Rosenau, 1986). Also, drying during such procedures can alter microbial activity and composition (Hattori, 1988), and sieve pore sizes and visual detection limits can limit sampling to relatively large aggregates (Ranjard & Richaume, 2001). Fractionation by wet sieving and sedimentation requires

dispersion of the soil matrix in water, which has the disadvantage that larger aggregates may be disrupted, as the stability of aggregates in water is negatively correlated with size (Haynes & Swift, 1990). Another potential limitation of wet sieving methods is that it is not known to what extent bacteria are dislodged from their particle-associated habitats by wetting and mixing during the separation process.

Few studies have investigated whether microbial community structure and function differ as a function of aggregate size. Both bacterial biomass and community structure have been shown to differ among different particle-size classes (Sessitsch *et al.*, 2001). However, it is difficult to glean clear and consistent trends in the data accumulated because of methodological differences between studies, the disparity of the soils examined and natural sources in variation. A study on *Rhizobium leguminosarum* demonstrated that abundance shifted among aggregate classes in the rhizosphere over the course of a season (Mendes & Bottomley, 1998), highlighting the influence of environmental factors such as plant growth and nutrient status that vary over time on bacterial distributions. Micro-habitats also obviously vary with respect to position within an aggregate, yet we still have only a rudimentary understanding of the processes and microbial populations that inhabit the insides versus the surfaces of aggregates. One study exploited the fact that bacteria on the surface wash from aggregates more easily than bacteria on the inside, but no significant differences in community composition were found between easily dislodged and more closely bound communities (Kim *et al.*, 2008). It should be noted that this study focused on a desert soil with low organic content and rapid aggregate turnover, so these results may not be indicative of aggregate-associated communities in other soil types.

2.5 Aggregate dissection

Although technically far more challenging, the examination of individual soil aggregates offers potential advantages when it comes to teasing apart soil communities into functionally meaningful units. A variety of micro-sampling methods has been developed to facilitate the dissection of very small soil samples or individual aggregates for subsequent phenotypic or genotypic assessments (Grundmann & Normand, 2000, Dechesne *et al.*, 2003, Dechesne *et al.*, 2007). Ideally, the coordinates of each subsample are recorded to create a 3D map of genetic or functional diversity (Vogel *et al.*, 2003, Gonod *et al.*, 2006). However, even without coordinates, it is still possible to obtain valuable information, for example on whether distinct bacterial populations are spatially associated or at what scales specific functions are contained (Grundmann *et al.*, 2001, Dechesne *et al.*, 2003). Using a combined approach of micro-dissection and simulation, Grundmann and colleagues were able to reveal patches of NO_2 oxidizers in the 250 µm range (Grundmann *et al.*, 2001). A promising method of particularly high spatial resolution utilizes micro-manipulator-guided

tungsten rods 'stamped' upon a soil sample, after which microbes adhering to the 0.013 mm^2 tip surface can be dislodged by sonication and plated on agar for enumeration and identification (Dennis *et al.*, 2008). Such methods should in principle also be amenable to molecular analyses.

2.6 Visualizing soil micro-habitats and their inhabitants

To understand bacterial ecology at the level of individual cells and colonies, it is important to describe the environment accurately at very fine scales. Although geological methods typically rely on coarse-scale measures to discern general features of soil topology, several recent advances have been made in the fine-scale 3D description of soils. Besides the issues of locating micro-colonies on a comparatively very large sample surface area, a major obstacle to obtaining fine-scale structural information is the opaque nature of soil. Thus, most approaches to examine soil 3-dimensional structure of soil rely on energy sources capable of penetrating (part of) the soil matrix. One of the more recent and promising approaches in examining soil structure is the use of micro-computer tomography (µCT) (Tippkotter *et al.*, 2009, Sammartino *et al.*, 2012). This technique allows visualisation of soil structure in 3 dimensions at µm resolution, based upon the bending of x-rays by electrons in the sample. The x-rays are modified to a greater extent by elements of a higher atomic number, thereby allowing for separation by density. Current analysis has focused on the de-convolution of soil pores from the soil matrix, and occasionally the ratio between air- and water-filled soil pores (Sammartino *et al.*, 2012) (Fig. 1A).

Nuclear magnetic resonance (NMR) or Magnetic Resonance Imaging (MRI) is another potential non-destructive technique for the 3-dimensional analysis of the soil environment, especially for localizing hydrogen nuclei (i.e. water). Previously used to locate the presence of heavy-oil contamination in a soil core (in conjunction with µCT) (Nakashima *et al.*, 2011), it has the potential to locate nuclei that 'spin'; which include ^{1}H, ^{2}H, ^{13}C, ^{14}N, ^{15}N and ^{31}P (Haynes, 2012). Conventional (medical) NMR typically provides a resolution of ~3 µm (for ^{1}H), however, resolutions down to 90 nm have been reported with specially modified machines (Mamin *et al.*, 2007). Well established protocols are already in place for the conversion of data into 3D maps; NMR could therefore be a very interesting linkage technique between structure (^{1}H) and chemistry (^{13}C, ^{15}N). Both µCT and NMR are non-destructive, and can therefore be followed up with (predominantly destructive) chemical analysis methods. Four such methods are described below.

Perhaps the most well-known method for micro-scale chemical analysis is the use of ion-selective electrodes. Micro-electrodes predominately take the format of micro-glass 'needles', tipped with a membrane, the interior containing specific solutions allowing for the measurement of specific ions due to changing voltage measurements (i.e. Nitrogen-

species (De Beer *et al.*, 1997, Okabe *et al.*, 1999), Phosphorus (Wang & Bishop, 2005), Phosphate (Lee *et al.*, 2009), Oxygen (Lu & Yu, 2002) and pH (Bezbaruah & Zhang, 2002)). Multiple measurements over a specified area (controlled robotically) allow a variable-density of information. The main problems with this approach are the requirement for a relatively moist sample and the danger of the sample drying out (Kim *et al.*, 2007), tip breakage (Davenport & Jabro, 2001) and the rather coarse spatial resolution relative to individual bacteria.

Electro-dispersive X-ray spectroscopy (EDS or EDX) is a common add-on technology to scanning electron microscopy (SEM; Fig. 1B) where X-rays are directed onto a polished, resin-embedded sample to obtain information on elemental ratios. EDS allows simultaneous collection of data on (user-defined) elements with an atomic number greater than Boron (i.e. 5). The resulting grayscale intensity image can be converted to a color image with weight- and atomic percentages (Fig. 1C). The major downside of this technique is the inability to garner biological information after resin-embedding, however, depending on the resolution and staining intensity requirements, bacterial staining with an electron-dense element could possibly allow the localization of bacterial cells (Kenzaka *et al.*, 2005, Ehrhardt *et al.*, 2009, Kenzaka *et al.*, 2009).

Infra-red spectroscopy (IRS) uses the excitation of covalent bonds within the sample infrared light to yield an absorption spectrum that can be compared against libraries of known samples. This approach allows for micro-scale localization and quantification of total carbon, total nitrogen, sand and silt content (Chang *et al.*, 2001, Piccolo *et al.*, 2001, Shepherd & Walsh, 2002, McBratney *et al.*, 2006, Rossel *et al.*, 2006, Rossel *et al.*, 2006, Tatzber *et al.*, 2012). Although the use of this method to study micro-scale soil samples is still in its infancy, commercial probes for *in-situ* measurements are becoming available in different configurations (Wetterlind & Stenberg, 2010). IRS can also be very useful for inferring highly detailed patterns of soil pH (Shepherd & Walsh, 2002). Absorption at 233 nm is strongly correlated with the concentration of calcium carbonate, which in turn shows a strong positive correlation with pH (Wetterlind & Stenberg, 2010). Although this is an indirect measure, relative pH values can be examined rather accurately if proper reference measures are available.

Nano-Secondary Ion Mass Spectrometry (NanoSIMS) is a technique similar to EDS, except the primary energy source is usually Cesium (resolution 50 nm) or Oxygen ions (resolution 150 nm) that hit the sample surface and release secondary ions. These secondary ions are collected, and the energy and mass:charge ratio is measured for seven user-selected ions. The major upside of this technique is the ability to differentiate between different isotopes at ppm concentrations, allowing NanoSIMS to work hand-in-hand with stable isotope probing (Herrmann *et al.*, 2007). Similar to EDS, samples should be smooth, dry and vacuum-tolerant, effectively limiting it to resin-embedded soil (Mueller

et al., 2012) or filtered soil solutions (Herrmann *et al.*, 2007, Musat *et al.*, 2008). Recently, NanoSIMS has been used to differentiate charcoal from soil organic matter (Heister *et al.*, 2012) and determine the distribution of organic matter and ^{15}N in single intact aggregates (Mueller *et al.*, 2012, Remusat *et al.*, 2012).

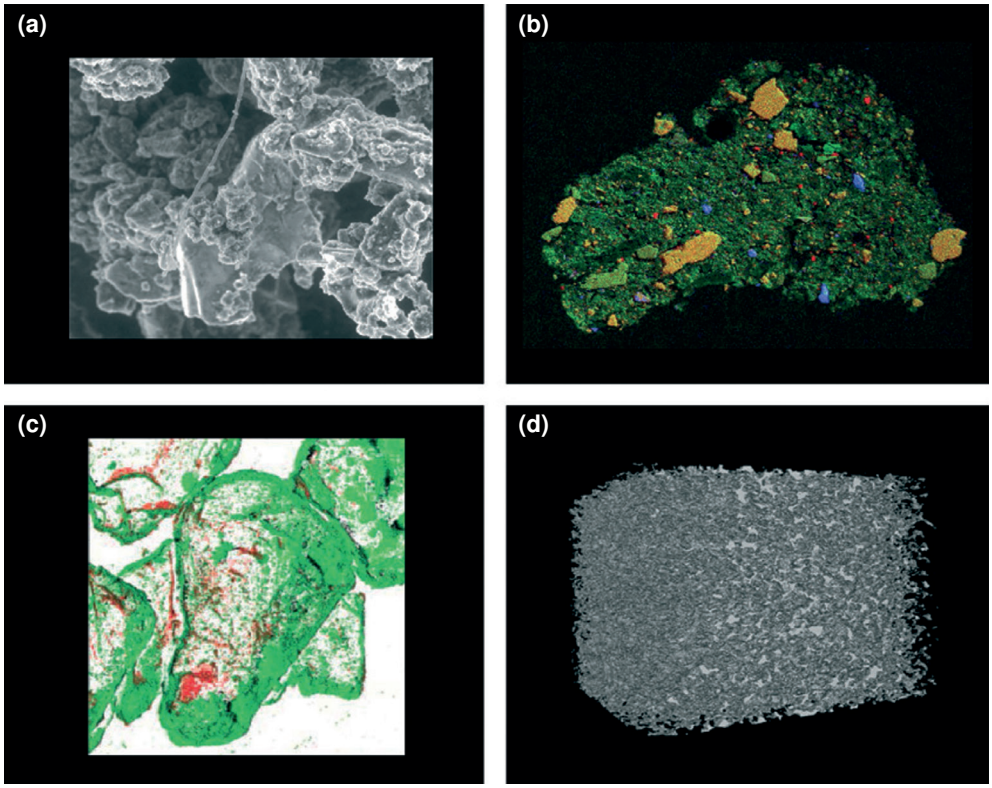


Figure 1. Examples of methods visualizing bacterial microhabitats

Panel A: fungal hypha within the complex 3-dimensional soil matrix. Image collected with FEI Quanta 200 field emission Environmental SEM from field-wet soil (SJJ unpublished data). Panel B: a composite elemental distribution map (oxygen (green), silica (red), and potassium (blue)), obtained via Energy Dispersive Spectroscopy (EDAX Pegasus EDS detector) for a resin-embedded soil aggregate (SJJ unpublished data). *Pseudomonas aeruginosa* SG81 biofilm formed on Nafion grains after live/dead staining (adapted from Leis et al. 2005). Cells with a damaged cell membrane appear red while cells with intact membranes are green. Panel D: pore network of a sand sample, obtained via micro-computed tomography (μCT) (ABW and Wilfried Otten, University of Abertay Dundee).

Perhaps even more challenging than describing the soil environment at the micro-scale is the *in situ* detection and characterization of individual bacterial cells. Some of the most promising micro-scale methods, including RAMAN (Huang *et al.*, 2010) and infra-red spectroscopy (Tatzber *et al.*, 2012), Nano-SIMS (Dekas & Orphan, 2011) and various

fluorescent *in situ* hybridization (FISH) approaches (Huang *et al.*, 2009), have so far been of limited use in soil habitats due to the intrinsic challenges associated with the soil matrix: the opaque and autofluorescent properties of soils, the highly sporadic distribution of cells (and so the need to examine many fields of view) and the difficulty in preserving the 3D integrity of samples during resin embedding (Nunan *et al.*, 2007, Eickhorst & Tippkotter, 2008).

Unfortunately, the electron density of bacteria in their native state is not discernible from the soil-water background. Direct bacterial localisation would therefore require an electron-dense stain to increase the bacterial signal. There are multiple potential methods for such an electron-dense stain, the most promising of which might be the use of a gene probe labelled with a gold particle (Kenzaka *et al.*, 2005, Ehrhardt *et al.*, 2009, Kenzaka *et al.*, 2009). Although promising, it must be kept in mind that such methods could disturb the 3D structure of the sample. In principle, it is possible to apply such staining methods to resin-embedded samples for subsequent µCT and SEM-EDS chemical mapping. This combination of methods would allow for the precise localization and visualisation of soil bacteria within the context of the chemical environment in which they reside.

2.7 Microcosm experimentation

To gain insight into ecological mechanisms, it is necessary to go beyond purely descriptive studies of soil micro-habitats and to design experiments where soil parameters can be manipulated to measure their effects on bacterial communities. To do this, a range of experimental systems are available, each trading off biological realism with the ease by which defined (a)biotic parameters can be controlled (Bronick & Lal, 2005, Guenet *et al.*, 2011). Experiments designed to test hypotheses on the ecology of soil bacteria have commonly employed microcosms filled with intact soil cores (e.g. Ruamps *et al.*, 2011), sieved soil (sterilized e.g. Wright et al., (1995) or unsterilized e.g. Chowdhury et al., (2011)) and artificial soil (e.g. Ellis, 2004, Guenet *et al.*, 2011, Pronk *et al.*, 2012). Soil microcosms allow for the manipulation of parameters such as hydration status (Wright *et al.*, 1995, Treves *et al.*, 2003), pore size distribution (Carson *et al.*, 2010) and mineral composition (Carson *et al.*, 2009), after which the effects on the resident or introduced bacterial community can be monitored by a variety of (molecular) methods.

As mentioned above, the opaque nature of soil (real or artificial) limits microscopic *in situ* observation to surfaces, or requires the use of laborious methods that preserve soil structure. An exciting development in this respect is the use of porous media consisting of transparent particles that allow for non-destructive, three-dimensional microscopic observation and tracking of spatial dynamics of biofilms and potentially even individual bacterial cells in real-time (Leis *et al.*, 2005, Ochiai *et al.*, 2010). To allow optical

transmission, it is necessary that the refractive index value of the solid particles is close to the value of the surrounding liquid. One material fulfilling this criterion is the hydrophilic, amorphous fluoropolymer Nafion (Leis *et al.*, 2005, Ochiai *et al.*, 2010). Using confocal microscopy, biofilms growing in flow cells packed with granules could be observed even throughout multiple layers of the material (Fig. 1D). This method holds great promise, as it combines relatively realistic soil conditions with three-dimensional imaging of individual cells and micro-colonies over time.

Using greater biological realism in laboratory experiments can change our understanding of important ecological processes. For instance, the co-evolutionary dynamics between *Pseudomonas* and an associated bacteriophage were found to be very different in a non-sterile soil microcosm as compared to when shaken in high-nutrient broth (Gomez & Buckling, 2011). However, depending on the question at hand, even standard agar plates can provide relevant insights into soil microbial ecology (Dechesne *et al.*, 2003). A relatively novel approach is the use of microfluidic set-ups that are completely artificial, but can provide unprecedented insights into the behaviour of individual cells in real-time (Keymer *et al.*, 2006, Keymer *et al.*, 2008, Kim *et al.*, 2008, Mannik *et al.*, 2009). Customized two-dimensional landscapes can be used to track bacterial growth, bacteria-surface interactions such as attachment and biofilm formation and evolution in real-time. However, as water-saturation is necessary for microscopic observation, experiments cannot be designed to test the impacts of different moisture conditions.

2.8 The soil habitat from a bacterial perspective

In order to understand what forces are responsible for driving microbial diversity, it is vital to view the soil habitat from the vantage point of individual bacteria. Soil is a highly complex environment of aggregated particles that create an intricate three-dimensional network of water- and air-filled pores (Oades, 1984) (Fig. 2). Aggregate formation is a key element of soil structure where clay particles and humus form micro-aggregates when their negative charge is absorbed by cations, and larger aggregates are further formed by sticky organic polymers, fungal hyphae and plant roots (Six *et al.*, 2004, Chenu & Cosentino, 2011). Micro-aggregates (<250 µm) are generally mechanically resistant, whereas macro-aggregates (>250 µm) are less stable and can be destroyed by soil management (Tisdall & Oades, 1982). Despite the extremely high total numbers of bacteria in soil, the complexity of the aggregate matrix means that the vast majority of soil surfaces are devoid of bacteria (Postma & Vanveen, 1990, Grundmann, 2004), with one estimate putting the percentage of soil surface area covered by microbes at a mere 10^{-6} (Young & Crawford, 2004).

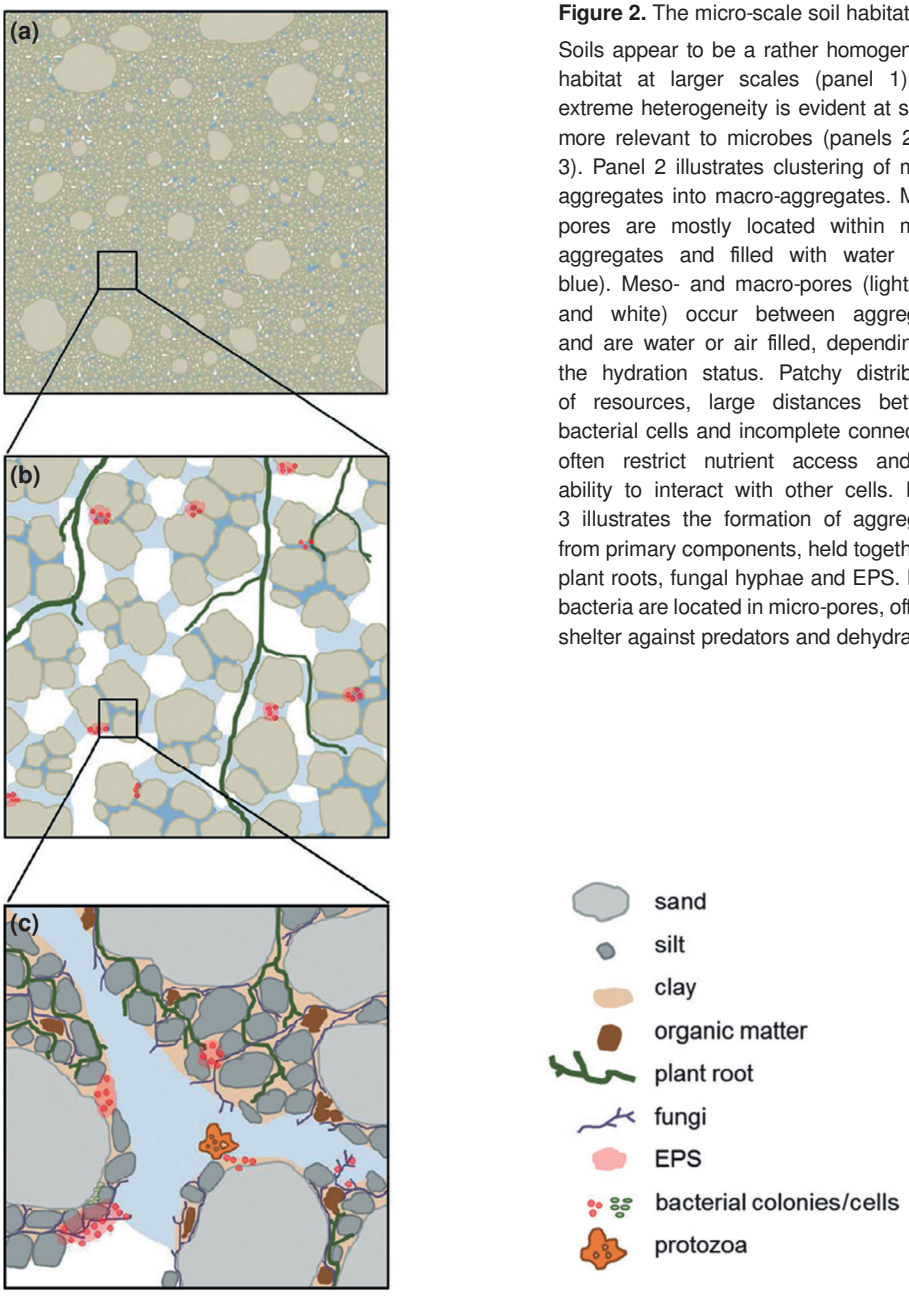


Figure 2. The micro-scale soil habitat

Soils appear to be a rather homogeneous habitat at larger scales (panel 1), but extreme heterogeneity is evident at scales more relevant to microbes (panels 2 and 3). Panel 2 illustrates clustering of micro-aggregates into macro-aggregates. Micro-pores are mostly located within micro-aggregates and filled with water (dark blue). Meso- and macro-pores (light blue and white) occur between aggregates and are water or air filled, depending on the hydration status. Patchy distribution of resources, large distances between bacterial cells and incomplete connectivity often restrict nutrient access and the ability to interact with other cells. Panel 3 illustrates the formation of aggregates from primary components, held together by plant roots, fungal hyphae and EPS. Many bacteria are located in micro-pores, offering shelter against predators and dehydration.

Since bacteria are essentially aquatic organisms, they are reliant on those fragmented parts of the pore network that are filled with water or covered by water films for growth. Water-filled pore space and water-film thickness decrease with increasing matric potential (Young *et al.*, 2008), with large pores draining out first, followed by meso- and ultimately

micro-pores. As a typical soil contains a great diversity of pore sizes, water- and air-filled pores co-occur in close proximity (Young *et al.*, 2008). In addition to pore size, hydrophobicity (Doerr *et al.*, 2000) and roughness (Or *et al.*, 2007) of particle surfaces influence the distribution of water and thickness of associated water films in a soil. The distribution of mineral types and sizes is patchy and influences pore size distribution through stacking and binding of nutrients (Carson *et al.*, 2009). Pore size and geometry in turn determine water flow through gravity and capillary action and with it nutrient diffusion, aeration, redox potential and pH. Such environmental gradients can be steep and change rapidly over time (Or *et al.*, 2007). Bacteria in micropores are sheltered against rapid changes in overall soil hydration, but also experience limited access to fresh resources (Ranjard & Richaume, 2001). Low water content is a common cause of biological stress (van de Mortel & Halverson, 2004, Chowdhury *et al.*, 2011) to which many bacteria have adapted by producing protective extracellular polymeric substances that trap water and nutrients (Or *et al.*, 2007, Holden *et al.*, 2011). Although the role of biofilms in protection against desiccation, protozoan grazing, antibiotics, bacteriophage infection and other insults is generally appreciated, still very little is known about the importance of biofilm formation in soils (Burmølle *et al.*, 2007, Burmølle *et al.*, 2011).

2.9 Does the heterogeneity of the soil matrix promote diversity?

Spatial heterogeneity alone is known to promote diversity by creating ecological opportunity. In a famous experiment, Rainey and Travisano were able to demonstrate that a *Pseudomonas* clone propagated in an unshaken flask containing liquid broth rapidly evolved into three distinct colony types, whereas this diversification was absent in microcosms that were shaken (Rainey & Travisano, 1998). With homogenization, cells were all of the ancestral smooth type, whereas in the absence of homogenization, wrinkly morphotypes evolved to colonize the air-broth interface and fuzzy morphotypes evolved to colonize the oxygen-deprived bottom of the flasks in addition to the smooth morphotypes inhabiting the broth away from glass or air interfaces. As soil is a great deal more heterogeneous than a broth-filled microcosm, spatial heterogeneity in a multitude of abiotic parameters will undoubtedly be hugely important in the evolution of soil biodiversity.

Soils contain a great variety of nutrient forms and amounts, with the composition of organic matter being heterogeneously distributed down to the nanoscale (Lehmann *et al.*, 2008). Soil habitats thus offer great potential for resource specialization, where competition for the most profitable resources available will have the effect that the relative profitability of underutilized resources will increase in relative importance. Assuming trade-offs exist in resource utilization (i.e. organisms cannot excel metabolizing every single resource), organisms then will diversify into resource specialists (MacLean, 2005). It is important to

note that trade-offs do not only arise on the level of the resources themselves but on a multitude of other factors as well, including growth rate vs. yield and optimal temperature (Lipson *et al.*, 2009). Abiotic habitat variation is much more commonly investigated in microbial ecology studies than is biotic variation (i.e. the co-occurrence of taxa). However, it could be argued that selection exerted by interactions between different strains and species is even more important for generating diversity than is selection by the abiotic environment (Hanson *et al.*, 2012). Selection mediated by interaction with other cells can be very strong (to the death), for example between predators and prey or parasites and hosts. Moreover, biotic selection is ever-changing, as adaptations in one type will select for adaptations in the other type and vice-versa. Such co-evolutionary arms races thus have the potential to continue indefinitely.

The sparsely populated, frequently dehydrated, maze-like nature of the micro-scale soil habitat has important implications for interactions between cells (Young *et al.*, 2008). Absolute distances between cells or micro-colonies in soils are relatively large, in the range of hundreds of µm's (Grundmann *et al.*, 2001) (Fig. 2). Probably more importantly, the potential of cells to interact depends on microhabitats being connected by water films permitting growth, motility and the diffusion of nutrients and excreted products (Crawford, 1994, Long & Or, 2009). For instance, *Pseudomonas* flagellar motility has been shown to only be effective across a narrow range of moisture conditions (Dechesne *et al.*, 2010) (Dechesne et al., 2010b). In unsaturated soils, water is mostly located in corners and cracks or absorbed to solid surfaces (Tuller *et al.*, 1999) and consequently connectivity between microhabitats is generally low (Or *et al.*, 2007). In addition to hydraulic connectivity, gaseous connectivity could potentially be important in volatile-mediated interactions, but the study of this phenomenon is still in its infancy (Kai *et al.*, 2009).

The complex spatial structure of soil and the resultant non-random interactions between cells is expected to have a profound influence on the evolution and maintenance of diversity. In addition to spatial variation, temporal variation, in plant growth, animal and microbial activity and weather events, is likely to be highly important as well, as it will co-determine the lifespan of spatial niches and will also result in dispersal of its inhabitants. The range of time scales over which environmental changes act will vary from less than a second (e.g. a falling rain drop) to years (e.g. ecological succession of plant communities). An overview of a variety of dispersal mechanisms and the hypothesized scales over which they act is given in Box 1. Below, several ecological mechanisms that link micro-scale heterogeneity to bacterial diversity in soil are discussed.

Box 1. Bacterial dispersal mechanisms in soil habitats

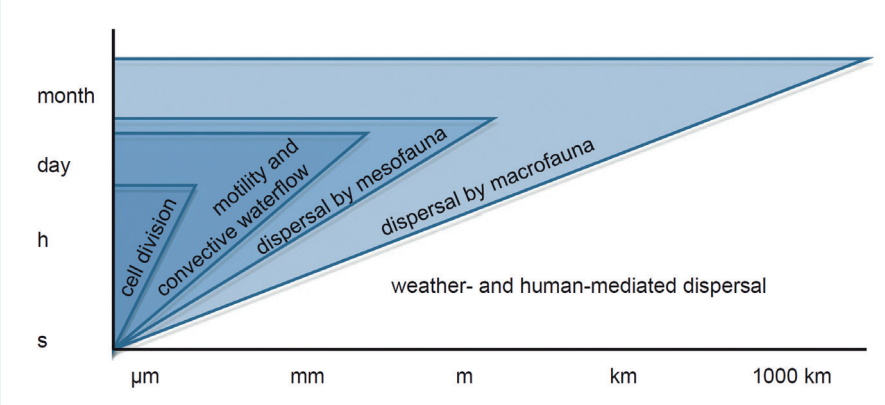


Figure 3

Dispersal to different locations in the soil matrix can be hypothesized to occur through a variety of mechanisms operating at a range of different spatial and temporal scales (Fig. 3). Both the x-axis (spatial scale) and the y-axis (temporal scale) are logarithmic. At the smallest scales, cells are 'pushed' through cell division (not shown) and experience Brownian motion. At slightly larger scales, cells disperse passively through convective water flow (Or *et al.*, 2007), or actively through active swimming or swarming over hydrated surfaces away from stresses and towards resources (Kearns, 2010). For instance, *Paenibacillus* has been found to swarm at rates up to 10.8 mm/hr on agar (Ingham *et al.*, 2011), dispersing fungal spores in the process. Non-motile bacteria must rely on passive dispersal, for example by passing invertebrates. In a very original study, it was demonstrated that earthworm movement in non-sterile microcosms not only resulted in the dispersal of bacteria, but also promoted horizontal gene transfer by bringing cells into contact with each other (Daane *et al.*, 1996). In general, habitats where different populations are brought into close proximity or contact will promote HGT (Sengeløv *et al.*, 2000).

2.10 Non-transitive fitness interactions

Most bacteria produce compounds that kill related strains (e.g. bacteriocins) or distantly related micro-organisms (e.g. polyketides) (Riley & Wertz, 2002). One of the best understood interference competition systems is that of the *E. coli col* plasmid (Kerr *et al.*, 2002). This plasmid encodes for a toxin, an immunity protein (to protect the cell from its own toxin) and a holin protein causing cell lysis. Only a small subset of clones express these genes (lysing in the process), which results in the death of surrounding cells that do not carry the plasmid. Some strains carry mutations that leave them unable to produce the toxin, while still being protected against it by the immunity protein. As they do not bear the cost of toxin production, these Resistant strains experience a growth rate advantage relative to the Killer strains. Sensitive strains do not only have an inactivated toxin gene, but also have an inactivated immunity protein, rendering them vulnerable to K cells but

allowing them to outgrow R cells. When all three strain types are allowed to grow in the well-mixed environment of a culture flask, K cells rapidly kill all S cells, after which R cells outcompete the K cells (Kerr *et al.*, 2002). In contrast, the structured environment of an agar plate allows for non-transitive fitness interactions, whereby all three strains can coexist in a game of rock-paper-scissors (K>S>R>K), a finding further supported by mathematical modelling (Kerr *et al.*, 2002).

Soil bacteria are known to possess many different combinations of toxin genes and associated resistance genes (D'Costa *et al.*, 2006, D'Costa *et al.*, 2007). The expression of such genes is highly sophisticated, and antagonistic responses can differ depending on the type of competitor that is encountered (Garbeva *et al.*, 2011). A tremendous diversity in antibiotic production and resistance was found between *Streptomyces* isolates from three random samples in a 1 m^2 grid (Davelos *et al.*, 2004). Although a consensus is emerging that antibiotics at environmental concentrations can be involved in a range of functions other than killing, such as signalling and motility (Yim *et al.*, 2007, Raaijmakers & Mazzola, 2012), it is likely that many microbial war games are played out under our feet. Such non-transitiveantagonistic interactions in the highly structured soil matrix thus have great potential for promoting diversity (Czaran *et al.*, 2002).

A recent study by Zhang and colleagues on the evolution of antibiotic resistance is of great potential relevance to our understanding of the diversity in antibiotic warfare strategies in soil (Zhang *et al.*, 2011). In this study, a microfluidic device consisting of 1200 wells connected by microchannels was inoculated with *E. coli*, and a gradient of the antibiotic ciprofloxacin was established by pumping media through nanoslits etched in the array. In contrast to culture flasks or agar plates, antibiotic resistance rapidly emerged in the microfluidic set-up. Although not explicitly demonstrated in this study, it can be hypothesized that antibiotic gradients as experienced on the chip facilitate adaptation because cells are not being killed outright, but rather are stressed, causing an elevated mutation rate (Frisch & Rosenberg, 2011). The resistant mutants that are generated are subsequently able to invade the rest of the chip. The connected microenvironments on the chip are likely to more closely resemble the gut where *E. coli* normally resides than do agar plates or culture flasks. Likewise, such a patch-like system of more or less discrete gradients of stressors seems a suitable model for soils.

Non-transitive interactions between bacteria need not be negative, as species can also positively influence each other's growth rates. Cross-feeding, feeding on metabolites excreted by others, is likely to be common in soils (Roberts *et al.*, 1993) as is the facilitation of growth of other organisms by feeding on compounds that are toxic to them. Kim and colleagues studied a system containing three different species, each performing a function key to the survival of the other two species: *Azotobacter vinelandii* (Av) producing amino acids by fixing nitrogen, *Bacillus licheniformis* (Bl) degrading penicillin and *Paenibacillus curdlanolyticus* (Pc) providing glucose by cleaving cellulose (Kim *et al.*, 2008). The three

species were not viable when cultivated in isolation and could not be co-cultivated in a well-mixed culture flask. However, all three species could coexist in individual wells of a microfluidic device connected by channels that allowed for diffusion of chemicals, but did not allow passage of cells. By varying the distance between inoculated wells, and hence the degree of metabolite exchange, the investigators could establish the optimum distance between cell types. Although the experimental set-up as well as the combination of species in this study was artificial, this study provides important insight into how different bacterial types can coexist in complex pore networks such as exist in soil.

2.11 Lack of competition

Selection can promote diversity, but, paradoxically, the absence of selection can also drive diversity by allowing maladapted types to persist. It has been proposed that inferior competitors can persevere because their spatial isolation in the complex soil matrix prevents superior competitors from outcompeting them (Zhou *et al.*, 2002, Dechesne *et al.*, 2008). A modelling approach demonstrated that a heterogeneous environment with weakly connected aqueous habitats allows for the coexistence of a weak and a strong competitor, whereas this is not the case in a wetter, better-connected environment (Long & Or, 2005, Long & Or, 2009). Similarly, two species inoculated in an artificial soil microcosm could coexist under dry conditions, but wet conditions led to the extinction of one of the species (Treves *et al.*, 2003). In an alternative experimental approach, both water potential and pore size distribution were modified in an unsterilized soil to test the impact of decreased connectivity on community diversity (Carson *et al.*, 2010). Bacterial TRFLP profiles indicated greater community diversity under drier conditions. Soil texture (modified by adding silt-and clay-sized particles that altered pore size but not mineral composition) was not found to have a significant effect on diversity, although it did affect community structure. These combined results provide evidence that low pore connectivity may contribute to the persistence of inferior competitors that would otherwise be outcompeted and disappear. It can be hypothesized that increased spatial heterogeneity can give rise to refuges where prey or hosts cannot be reached by predators or parasites, respectively. In an experimental system where bacteria and lytic bacteriophages were propagated in shaken versus static broth microcosms, coexistence was found to be more stable in the latter treatment due to the ephemeral creation of spatial structure (Brockhurst *et al.*, 2006). Amending soil with clay to decrease average pore size improved the survival of *Rhizobium* by creating protective microhabitats too small to be entered by predators (Heynen *et al.*, 1988). After manipulation of pore colonization by varying matric potential, ciliate predation on *Pseudomonas* was shown to be more efficient in larger pores (Wright *et al.*, 1995). As bacteria can act as predator (Morgan *et al.*, 2010), prey (Rosenberg *et al.*, 2009), host (Ashelford *et al.*, 2003) and parasite (Goodrich-Blair & Clarke, 2007), there is great potential for heterogeneous soil environments to promote bacterial diversity by preventing exploitation.

Bacteria excrete a whole range of extracellular molecules, involved in all aspects of their ecology, for example in communication (Stefanic *et al.*, 2012), virulence (Buttner & Bonas, 2010), predation (Konovalova *et al.*, 2010) and iron acquisition (Buckling *et al.*, 2007). As such excreted molecules can benefit not only the producing cell, but can also benefit cells that do not produce them and therefore do not carry the metabolic cost, they can be classified as public goods. Cells producing freely available molecules therefore behave as altruists and non-producers behave as cheats (West *et al.*, 2007). Evidence for bacterial cooperation and conflict has been found in a wide variety of systems (Velicer, 2003), including siderophore production (Griffin *et al.*, 2004), quorum sensing (Diggle *et al.*, 2007) and stationary phase growth (Vulic & Kolter, 2001). Cheaters have an advantage when rare as there is plenty of the public good to go around. When cheaters become more abundant, total production of public good is low and they become a victim of their own success. Such frequency dependent selection can maintain coexistence of co-operators and cheaters even in unstructured habitats (Velicer *et al.*, 2000). However, in structured habitats such as soil, coexistence of different social strategies is expected to be more stable as cheaters are only able to exploit local co-operators. The interaction between enzyme production and the physical properties of soil is expected to crucially affect cheating strategies in soil and thereby ecosystem functioning (Allison, 2005).

2.12 Discussion and Conclusions

One of the greatest challenges in soil microbial ecology is to determine what forces allow for the extremely high biodiversity observed at even very small scales (Dechesne *et al.*, 2007, Carson *et al.*, 2010). This is not merely an academic exercise, as soil function, health and remediation potential ultimately depend on the bacteria they contain, where these bacteria are located and how they interact (Nannipieri *et al.*, 2003, Dechesne *et al.*, 2010). The fine spatial heterogeneity of soils results in a complex mosaic of gradients selecting for or against bacterial growth. Spatial structure also results in non-random interactions between different cell types, allowing for non-transitive interactions whereby no type out-competes all other types. Such non-transitivity is expected to occur in multiple traits, resulting in a myriad of positive and negative interactions between strains and species (e.g. Kato *et al.*, 2005), likely to act over different scales.

Besides adaptation to the abiotic environment and adaptation to other organisms, the latter modulated by the probability of cells to come into contact, there is also an interaction between the two processes. A recent study demonstrated that the adaptation of five bacterial species to a novel abiotic environment was greatly influenced by the presence of the other species (Lawrence *et al.*, 2012). Over the course of approximately 70 generations, species in co-culture evolved faster growth rates compared to species cultured in isolation. They also showed a greater departure from ancestral resource use as

quantified by NMR spectroscopy of spent broth. Species that evolved together did not only evolve cross-feeding, but also were found to have evolved a reduced overlap in resource use (Lawrence *et al.*, 2012). Importantly, besides the effect of biotic interactions on the evolution of species diversity, this study also showed that this partitioning of resources between species can result in increased ecosystem productivity.

Although we are not certain of exact spatial and temporal scales, selective pressures will clearly vary across the soil matrix. Bacterial populations can adapt to these pressures in two main ways. First, a combination of mutation, homologous recombination and lateral gene transfer will locally generate genetic variants some of which will have higher fitness than their ancestors. Second, migration can bring in other variants from elsewhere in the matrix. How bacteria adapt to changing local conditions (be it abiotic gradients or different types of neighbouring cells) will crucially depend on the balance of these two forces. As global diversity is much higher than the diversity that can conceivably evolve anew in a small local patch of bacteria, dispersal is expected to be a very powerful force in local adaptation. However, although dispersal can act to match cells with their spatial niche, too much of it will randomize populations and decrease local adaptation (Kawecki & Ebert, 2004). In a population of *Stenotrophomonas* and associated bacteriophages in floodplain soil, phages isolated from the same soil core were found to be better at infecting bacteria from that sample than were phages isolated from soil cores five centimeters away (Vos *et al.*, 2009). This finding indicates that selection exerted by coevolving species was strong enough to shape biodiversity at a scale of centimetres (possibly extending to considerably smaller scales). It is unknown whether this local adaptation of phages to their host is caused by mutations that have locally arisen or were introduced by migration from elsewhere. This pattern of local adaptation does however demonstrate that this soil is evidently not sufficiently homogenized through flooding and cattle activity for migration to erase patterns of divergent selection at centimetre scales.

Although selection no doubt is a powerful force in determining the extent and spatial distribution of bacterial diversity, randomizing forces must play important roles too; no microbial ecologist will argue that all hundred million cells present within a cubic centimeter of soil are located precisely where they are because natural selection has neatly sorted each and every cell into their respective spatial niche. Differences in the physiologies of species could result in competitive exclusion in one particular environment, but in other environments these physiological differences may be minimal, resulting in relative species abundances to be governed primarily by chance, not selection (Alonso *et al.*, 2006, Hanson *et al.*, 2012). Disentangling the relative roles of non-random selection (niche sorting) and random dispersal (drift) in structuring microbial communities is one of the greatest challenges in microbial ecology.

Traditionally, the field of microbial ecology has been driven by technological innovation, and this is no exception for research on micro-scale bacterial interactions. Advances in

imaging technologies continue to be rapid in medical and geological sciences, and soil ecologists would be well served to keep abreast of new possibilities that may be applied to detailed descriptions of the soil environment. Similarly, next-generation DNA sequencing approaches are allowing for a quantum leap in the accumulation of rRNA gene sequence surveys and (meta)genomics (Lombard *et al.*, 2011). Although receiving far less attention than next-generation sequencing, novel high-throughput cultivation methods targeting species with specific growth requirements are equally exciting (Ferrari & Gillings, 2009, Nichols *et al.*, 2010, Vartoukian *et al.*, 2010, Ingham *et al.*, 2011). Combined with micromanipulation methods retrieving cells from individual aggregates, these cultivation methods could access both phenotypic and genotypic characteristics from cells that can be linked to specific locations in the soil matrix. Although not yet applied to soil environments, such approaches could even be taken to the level of individual cells, with single-cell sequencing holding the potential of producing (meta)genomic data one cell at a time (Woyke *et al.*, 2009). The combination of micro-scale sampling with both 'omics' methods and measurements of chemical and physical habitat characteristics (Table 1) will allow deeper insights into how soil habitats select for bacterial diversity.

Soil microcosms allow for the study of ecological interactions through manipulation of (a)biotic soil parameters and sampling over time followed by the isolation of cells, DNA, RNA or biochemical compounds. Repeated (destructive) micro-aggregate dissection from real or artificial soils housed in microcosms is also a possibility, although rarely applied. Experiments using genetic markers allowing the study of competition between different types have a long tradition in microbial ecology and could be informed by experimental evolution systems utilizing more defined microcosms that have provided many important ecological and evolutionary insights in recent years (Buckling *et al.*, 2009). Apart from allowing the study of interactions between different microbes, soil microcosms can be used to parameterize basic bacterial life history characteristics. For instance, rates of dispersal as the result of active swarming motility (Wong & Griffin, 1976), passive dispersal by convective water flow (Trevors *et al.*, 1990) or passing invertebrates (Ruddick & Williams, 1972) can be quantified.

In order to track individual cells through time, conventional microcosms need to be replaced with novel experimental set-ups. Completely artificial systems can provide fundamental new insight into microbial interactions, but it is also possible to design experiments explicitly based on real soil ecology. Consider the following scenario: micro-dissection of soil samples coupled to omics methods reveal which strains co-occur in nature. Based on genomic information, selective medium is designed (Tyson *et al.*, 2005), which in combination with microdish cultivation chips (e.g (Hesselman *et al.*, 2012)) could be used to isolate these strains. Characteristics of the local soil pore network and nutrient distribution are obtained through TEM, and a microfluidic device is designed mimicking the spatial structure of this matrix. Finally, fluorescently-labelled (or, when cell shapes

are different, even unlabelled) isolates could be inoculated on a chip filled with defined medium or soil extract to monitor activity levels as a function of pore size and water flow.

Although fundamental to progress in the field, there is more that could advance the study of micro-scale bacterial diversity than the introduction of state-of-the-art methods alone. An extensive older literature exists on the physical and hydrological complexity of soils (Stotzky, 1986, Dexter, 1988), which should be revisited in order to inform future studies. Designing microbial ecology studies based on theory developed in 'macrobial' ecology likewise will be instrumental (Prosser *et al*., 2007). For instance, the notion of a highly fragmented 'soil microscape', consisting of relatively small, more or less connected patches that are either colonized or empty, fits neatly with the concept of a metapopulation, originally developed for macro-organisms inhabiting landscapes (Hanski, 1998). Together, these efforts could help us understand what forces shape bacterial diversity at the micro-scale and more specifically inform us how natural processes are modified by human management practices. Is bacterial migration affected by tillage practices or increased human travel? Does lowered connectivity through groundwater depletion affect bacterial interactions? Are we losing soil-borne bacterial diversity and does it affect soil ecosystem services? The answers to such grand questions could well be found at the very smallest scales.

Table 1. Methods to characterize bacterial microhabitats in soil

Sampling method	Analysis method	Micro-habitat characterization	Detection bacterial presence/ activity	Single-cell resolution (~ < 2µm)
Size class fractionation	'Omics' methods*	No	Yes	Single-cell sequencing
Aggregate dissection	'Omics' methods*	No	Yes	Single-cell sequencing
	Isolation	No	Yes	No
	Phenotyping	No	Yes	No
	Micro-electrodes	Yes, chemical	Yes	Yes
	Micro-computed tomography	Yes, physical	No	No
	Nuclear magnetic resonance	Yes, chemical	Yes, with stain	No
	Electro-dispersive X-ray spectroscopy	Yes, chemical	Yes, with stain	Yes
	Infrared spectroscopy	Yes, chemical	No	No
	Nano-secondary ion mass spectrometry	Yes, chemical	Yes	Yes
	SEM + gold staining	No	Yes	No
	FISH + TEM	Yes, physical	Yes	Yes

'omics' methods*= metagenomics, tag sequencing, proteomics and transcriptomics

2.13 Acknowledgements

This work was sponsored by the Vici grant of GAK from the Dutch Science Foundation (NWO) entitled “Crossing the frontiers of microbial ecology”. MV is partly supported by investment from the European Regional Development Fund and the European Social Fund Convergence Programme for Cornwall and the Isles of Scilly to the ECEHH. We thank Wietse de Boer, William Gaze and Jennifer Martiny for helpful comments on the manuscript and Angus Buckling for helpful discussion. NIOO-KNAW manuscript # 5421.

Chapter 3

Impact of matric potential and pore size distribution on growth dynamics of filamentous and non-filamentous soil bacteria

Authors:
Alexandra B. Wolf, Michiel Vos, Wietse de Boer,
George A. Kowalchuk

Published in: *PLoS one* (2013) DOI: 10.1371/journal.pone.00836

Abstract

The filamentous growth form is an important strategy for soil microbes to bridge air-filled pores in unsaturated soils. In particular, fungi perform better than bacteria in soils during drought, a property that has been ascribed to the hyphal growth form of fungi. However, it is unknown if, and to what extent, filamentous bacteria may also display similar advantages over non-filamentous bacteria in soils with low hydraulic connectivity. In addition to allowing for microbial interactions and competition across connected micro-sites, water films also facilitate the motility of non-filamentous bacteria. To examine these issues, we constructed and characterized a series of quartz sand microcosms differing in matric potential and pore size distribution and, consequently, in connection of micro-habitats via water films. Our sand microcosms were used to examine the individual and competitive responses of a filamentous bacterium (*Streptomyces atratus*) and a motile rod-shaped bacterium (*Bacillus weihenstephanensis*) to differences in pore sizes and matric potential. The *Bacillus* strain had an initial advantage in all sand microcosms, which could be attributed to its faster growth rate. At later stages of the incubation, *Streptomyces* became dominant in microcosms with low connectivity (coarse pores and dry conditions). These data, combined with information on bacterial motility (expansion potential) across a range of pore-size and moisture conditions, suggest that, like their much larger fungal counterparts, filamentous bacteria also use this growth form to facilitate growth and expansion under conditions of low hydraulic conductivity. The sand microcosm system developed and used in this study allowed for precise manipulation of hydraulic properties and pore size distribution, thereby providing a useful approach for future examinations of how these properties influence the composition, diversity and function of soil-borne microbial communities.

Keywords: filamentous bacteria – soil matric potential – pore size distribution – micro-habitats – *Streptomyces atratus* – *Bacillus weihenstephanensis*

3.1 Introduction

Soils are highly heterogeneous systems, containing a wide range of micro-habitats and environmental gradients (Horn *et al.*, 1994). This extreme heterogeneity, at a variety of spatial scales (Crawford *et al.*, 2005, Lehmann *et al.*, 2008), offers a large potential for niche differentiation and may be an important factor in realizing the tremendous diversity of microbial communities in soil (Young *et al.*, 2008). Bacteria are distributed heterogeneously within the soil matrix, and distances between individual cells and micro-colonies are often very large in comparison to the size of bacterial cells. Whether resources can be accessed by a given organism depends on the distance between the microhabitats, and, perhaps more importantly, on the level of connectivity between these microhabitats via water films (Young *et al.*, 2008). Soil connectivity depends on the geometry of the pore network, which impacts the distribution of soil water, as well as the hydration status (Or *et al.*, 2007). As the growth of many bacterial species is dependent on the availability of soluble organic compounds, the distribution of cells and the hydraulic connectivity of soil micro-habitats will also have great implications for competitive interactions.

Bacteria are essentially aquatic organisms, as they rely on water for functioning and require water-filled pores or water films for passive and/or active motility (Vos *et al.*, 2013). Given their ability to bridge air-filled gaps via their hyphae, filamentous fungi may have distinct advantages over many bacteria in unsaturated soils (Yuste *et al.*, 2011). At low matric potential, fungi can explore micro-habitats that appear not to be accessible to most bacteria (Griffin, 1985), and this may explain the observations that fungal activity often exceeds bacteria activity under these conditions (Anderson & Domsch, 1973, Wilson & Griffin, 1975, Faegri *et al.*, 1977). Experimental work with *Rhizoctonia solani* confronted with different ratios of air- and water-filled pore volumes in sand provides evidence that having a large fraction of air-filled pores stimulates fungal spread in soils (Otten *et al.*, 1999). Most soil-bacterial cell morphologies (e.g. rods, cocci, spirals, etc.) are not adapted to bridging the air-filled spaces that occur in non-saturated soils. However, although bacteria are unable to cross air-filled pores on their own, it has been shown that some motile bacteria can move along fungal hyphae (so-called fungal highways) (Kohlmeier *et al.*, 2005). Also, it has been demonstrated that some bacterial strains, have the ability to co-migrate with other bacteria along fungal hyphae (Warmink & van Elsas, 2009a, Warmink *et al.*, 2011). Fungal hyphae may thus promote the distribution of motile bacteria in unsaturated soils.

Filamentous actinomycetes represent an exception within the bacterial domain, providing a morphological bridge between bacteria and filamentous fungi (Griffin, 1985), and although they are much smaller than fungi, their filamentous growth form could provide similar advantages for the exploration of unsaturated soils. The natural habitat of most actinomycetes is soil, where they typically comprise 1 to 20% of the culturable community

(Trujillo, 2001). *Streptomyces* is the most abundant genus and encompasses key players in the decomposition of soil organic matter due to the ability to produce a large array of extracellular enzymes such as chitinases, cellulases and hemicellulases (Schrempf, 2007). Streptomycetes are also known for producing a vast array of antibiotics, some of which are valuable in medicine and agriculture (Watve *et al*., 2001).

We hypothesized that actinomycetes might possess "fungal-like" characteristics with respect to their exploitation of less well connected soils, thereby being able to out-compete non-filamentous bacteria under low connectivity conditions. To address this hypothesis, we investigated the competitive ability of a filamentous bacterium (*Streptomyces atratus*) versus a non-filamentous Gram-positive bacterium (*Bacillus weihenstephanensis*) across a series of defined environmental conditions varying in pore size distribution, moisture and habitat connectivity. The population sizes of the two strains were subsequently tracked over time. In line with our hypothesis, we predicted that *Streptomyces* would have a competitive advantage under conditions of low connectivity and that *Bacillus* would perform best in more well-connected habitat matrices.

3.2 Material and Methods

3.2.1 Bacterial strains

We used two soil isolates, *Bacillus weihenstephanensis* AW02 (NCBI submission ID 1572885) and *Streptomyces atratus* AW01 (NCBI submission ID 1572838), both isolated from the Park Grass Experiment at Rothamsted Research, plot 3 (nil) in August 2009. These strains were chosen because they both represent Gram positive soil bacteria that co-occur in soil and process alternative growth and soil exploration strategies. Both strains were isolated from the same single soil aggregate, which was dispersed in phosphate buffer (pH 6.5), shaken for 30 min followed by 2x1 min sonication. Diluted soil suspension was plated on soil suspension agar prepared from soil taken outside the experimental plot. The soil suspension agar was prepared by weighing 100 g air-dried soil in 900 mL phosphate buffer (pH 6.5), shaking for 30 min, sonicating (Branson 5210 ultrasonic bath) twice for 1 min and subsequent filter-sterilization through 0.2 µm pore size; per liter suspension, 15 g agar (Merck) was added. The strains were identified by 16S rRNA gene sequencing, and these sequences have been deposited under NCBI accession numbers JX944825 and JX944824 for *B. weihenstephanensis* AW02 and *S. atratus* AW01, respectively. To determine growth curves of both strains in liquid medium, six replicates of each strain were grown in 96 well plates in 10% tryptic soy broth (TSB) (Oxoid), and the OD at 600 nm was measured over a period of 20h. Measurements were taken every 20 min, and the 96-well plate was shaken for 2 min before each measurement.

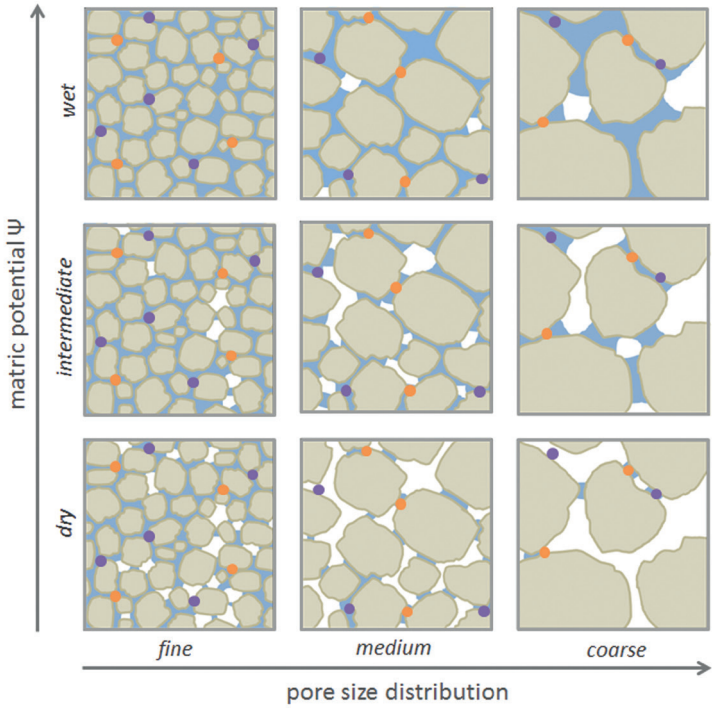


Figure 1. Schematic diagram of the experimental setup. Three matric potentials were combined with three sand particle size fractions with different pore size distributions, giving a total of nine treatments. In each box, the appropriate gravimetric water content of each treatment is indicated. Grain size, water distribution and bacterial cells (orange and purple) are indicated for illustrative purposes and are not based on actual microscopic visualization. Habitat connectivity decreases with decreasing matric potential and increasing pore size. In well-connected soils, (e.g. the treatments "wet" and/or "fine pores"), bacterial species (orange and purple) often inhabit connected microhabitats/pore spaces, thereby allowing for completive interactions. Under less-connected conditions (e.g. low matric potential "dry" and coarse pores), microhabitats are discontinuous, thereby reducing competitive bacterial interactions.

3.2.2 Construction of microcosms

Sand microcosms were constructed using quartz sand particles of different size distributions, obtained by milling (Retsch Mortar Mill RM 200) acid-washed sea sand (Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany) for 10 min followed by fractionation of the particles into size classes by sieving. Particles of different size fractions were used to create three distinct textures: "fine" (sand particles 53-106 µm), "medium" (106-212 µm), and "coarse" (212-425 µm), thereby creating a range of pore size classes. For the competition experiments, microcosms were established in 100 mL glass vials to which 10 g quartz sand of one of the three particle size fractions was added. For the motility experiments, microcosms were established in glass petri dishes to which 50 g of quartz sand of the different grain size fractions was added. Microcosms were sterilized

by autoclaving followed by oven-drying prior to use. To prevent moisture loss, the glass vials were closed with screw cap lids, and the petri dishes were sealed with 2 layers of parafilm after inoculation. The moisture content was set to matric potentials of -10 (“wet”), -20 (“intermediate”) and -50 kPa (“dry”) (see next section). In total, sand microcosms with all nine combinations of three different pore size distributions and three different moisture regimes were established (Fig. 1) with three replicates per treatment and inoculant per time point. Microcosms were weighed after inoculation and at the end of the experiment to confirm that there was no moisture loss.

3.2.3 Water retention curves and pore size distribution

Water is retained in soils largely due to matric forces (adhesive forces between water and solids) in pores and interconnecting pore necks. At saturation, all pores are filled with water, and the matric potential, Ψ, is zero. When the water content decreases, large pores empty first because water is held less tightly adhered to solids in larger capillaries than in smaller ones. The matric potential is more negative when water is adhered more strongly and is thus lower in smaller pores than in larger ones. Consequently, the water content of a soil at a given matric potential depends on the distribution of the pore sizes (diameters). The relationship between matric potential, Ψ, and pore neck diameter, d, is given by the equation Ψ [-kPa] = 300 / d [µm] (Nimmo, 2004). Based on this equation, the pore size distribution of a soil can be calculated from the water retention curve (WRC) (Danielson & Sutherland, 1986). The WRC describes the matric potential – water content relationship and can be determined by draining a saturated soil and determining the water content at a given matric potential (Papendick & Campbell, 1981).

Practically, the WRCs of the 3 sand particle size fractions were determined using a pressure plate (15 bar ceramic plate extractor Cat.#1500 and 5 bar ceramic plate extractor Cat.#1600 by Soilmoisture Equipment Corp., Santa Barbara CA,; for pressures of -1580 and -300 kPa) and a ceramic suction table (pF laboratory station, ecoTech Umwelt-Meßsysteme, Germany for pressures -50, -30, -20, -10, -5.3, and -3.7 kPa). Aluminum cylinders with a diameter of approximately 3 cm and a height of 5 cm were completely filled for the suction table and to 1 cm height for the pressure plate. Samples were wetted until saturated, before being drained with the respective apparatus. The decrease in the water content during drainage was calculated from the loss of water volume. All analyses were performed in triplicate. From nine experimentally determined data points (water content at a given matric potential), WRC was determined for each sand particle size fraction according to the model of Van Genuchten (van Genuchten *et al.*, 1991) in RETC version 6.02. The bulk density of the sand particles in the cylinders was comparable to that used in the microcosms, allowing one to assume that the pore size distribution in the sand microcosms can also be calculated from the water retention curves.

3.2.4 Motility measurements

The motility rates of both strains across the range of sand microcosm were measured using a method that was developed to determine expansion of bacteria in soils (Wong & Griffin, 1976). Microcosms were established in glass petri dishes, sterilized by autoclaving and oven-drying, and adjusted to the different matric potentials by adding the appropriate volumes of liquid growth medium (10%TSB) and inoculated at the center of the petri dish with 5 µL overnight culture of *B. weihenstephanensis* or *S. atratus*. After 5h, 23h and 47h, bacterial expansion was determined by sampling with a multi-pronged sampler which measures expansion in 4 directions (Fig. S1). The prongs (spaced equally at 2 mm intervals) were first pushed approximately 5 mm into the sand and then onto a TSB agar plate. The soil layer in the petri dish was approximately 1 cm thick; by sampling this way we avoided sampling from the water-film which formed at the sand matrix-petri-dish interface at the bottom of the petri dish. After three days of incubation of the TSB agar plates the transfer of bacteria by prongs was determined and the expansion in the sand microcosm was calculated. All measurements were performed in duplicate, resulting in a total of 8 measurements per treatment and time point (2 replicates, 4 measured directions), of which the mean was used for statistical analysis. A Three-Way ANOVA was performed in SigmaPlot (version 12.3) to test for the effects of pore size distribution (independent variable 1), matric potential (independent variable 2) and time (independent variable 3), on the expansion of both *Bacillus* and *Streptomyces* (radius of extension of each strain = dependent variable).

3.2.5 Competition experiments

10% TSB was used as growth medium and microcosms were incubated at 20 °C, with all treatments performed in triplicate. Different treatments had different total amounts of nutrients since we have chosen to use similar nutrient concentrations in the liquid phase for all 9 texture-moisture combinations. The alternative to have all combinations with same absolute amount of nutrients would have resulted in strongly different concentrations of nutrients in the liquid phase, which will result in different osmotic pressures. Microcosms were inoculated either with the *Streptomyces* and *Bacillus* strain as pure cultures (10^5 cells/g soil; based on colony-forming unit (CFU) counts) or with both strains in a 1:1 ratio with 10^5 cells/g soil of each strain. The inoculation was performed by adding the appropriate numbers of bacterial cells to the nutrient solution, which was subsequently added to the sand microcosms. Microcosms were tilted, after which the inoculum was pipetted on the bottom of the glass vial. Tilting the microcosms back ensured homogenization of the inoculum . A total of 405 microcosms were constructed, representing 9 treatments, 3 inoculums, 3 replicates, and 5 time points. Sampling was performed destructively after 0, 3, 6, 9, and 12 days by adding phosphate buffer (10 mM, pH 6.5) to a total volume of

10 mL to each microcosm, and cells were suspended by shaking the microcosms for 30 min, followed by sonication (Branson 5210 ultrasonic bath) twice for 1 min. One mL of the resulting supernatant was sampled to make serial dilutions, which were spread on 10% TSB agar plates for the determination of CFU of both strains. As the two strains differ in their colony morphology, they could be easily distinguished on agar plates. A three-way ANOVA was performed in SigmaPlot to test for a possible effect of pore size distribution (independent variable 1), matric potential (independent variable 2) and time (independent variable 3), on the ratio between *Bacillus* and *Streptomyces* (B/S-ratio = dependent variable), which we used as indicator of the competitive strength of the strains. To test the effect of pore size distribution (as generated by the different sand particle sizes), matric potential and the presence of the competitor (*Streptomyces*) on cell densities of *Bacillus* (dependent variable), a three-way ANOVA was performed for each time point (except the starting point T0= 0d) using the following parameters: pore size distribution (independent variable 1), matric potential (independent variable 2) and inoculant (independent variable 3). A series of three-way ANOVAs was performed to test the effect of pore size distribution, matric potential and the presence of the competitor (*Bacillus*) on cell densities of the *Streptomyces* (dependent variable).

3.2.6 Antagonism assay

As many *Streptomyces* are known to produce antibacterial compounds (Baltz & 2007), we also tested for a possible antagonistic effect of *Streptomyces* against *Bacillus*, by performing an agar overlay assay. 2 µL of an overnight culture of *Streptomyces* was spotted on the surface of a 10% TSB agar plate and incubating at 20°C until growth could be observed. The plate was then overlaid with 8 mL 10% TSB soft agar seeded with 50 µL of an overnight culture of *Bacillus*. After incubation, the plates were examined for zones of inhibition.

3.3 Results and Discussion

3.3.1 Characterization of the artificial soil microcosms: water retention curves

The water retention curves were characterized by the van Genuchten model giving the constants displayed in Table S1 (van Genuchten *et al.*, 1991). The pore size distributions of each sand size fraction (as shown in Fig. 2) was calculated from the water retention curves (Fig. S2). Because the relationship between matric potential Ψ and pore neck diameter d is given by the equation Ψ [-kPa] = 300 / d [µm], we could predict which pore sizes would theoretically be filled with water at a specific matric potential (shown in Fig. 2).

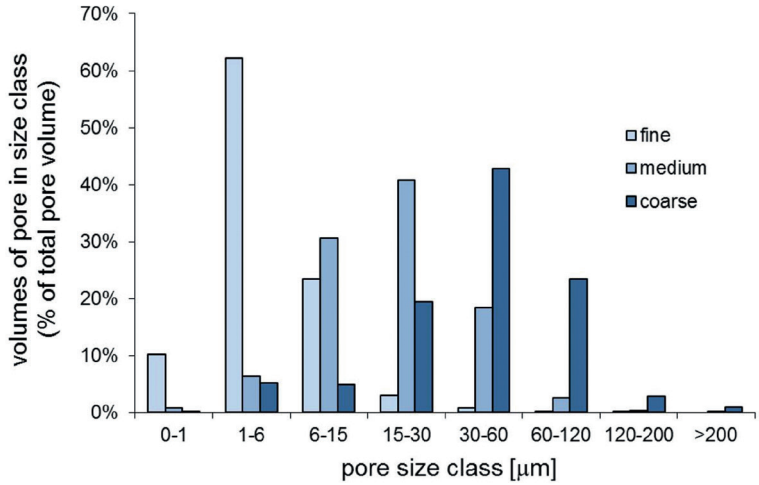


Figure 2. Pore size distribution of the three sand size fractions (fine, medium, coarse). Boxes indicate water-filled pores at different matric potentials, i.e. in the "dry" treatments all pores with a pore-neck diameter <= 6 µm are filled with water, in the "intermediate" treatments <= 15 µm and in the "wet" treatments <= 30 µm, respectively. Pores above these pore-neck diameters are filled with air.

3.3.2 Motility

The tests for the expansion ability of the strains in sand microcosms revealed that *Bacillus* displayed greater expansion in sand than *Streptomyces* except for the dry medium and -coarse sand sizes, where expansion of both strains was nearly equal (Fig. 3). This may be attributed to the faster growth rate of *Bacillus* as compared to that of *Streptomyces* as observed for cultures in liquid 10%TSB (Fig. S3). The expansion rate of *Bacillus* was significantly affected by pore size distribution ($p<0.001$) as well as by matric potential ($p<0.001$) (Tab. 1). It was fastest in the most connected sand fractions (fine and wet) (Fig. 3). There was also a significant interaction effect of matric potential and pore size distribution on the expansion of *Bacillus* ($p<0.001$). As hydraulic connectivity is determined by the interplay of hydration status and pore geometry, this demonstrates that habitat connectivity impacts the expansion of *Bacillus*. An explanation for *Bacillus* being more affected by matric potential and pore size is that motility of this strain relies exclusively on the presence of water-filled pores and water films on solid surfaces. The expansion rate of *Streptomyces* was also significantly affected by pore size distribution ($p<0.001$) and was greatest in the fine sand fraction (Fig. 3). Unlike *Bacillus*, the expansion rate of *Streptomyces* was not significantly affected by matric potential ($p=0.066$). Overall, matric potential and pore size distribution had a smaller effect on the expansion of *Streptomyces* than *Bacillus.* We attributed this to the hyphal growth form of *Streptomyces* that allows it to spread through air-filled pores and makes it less dependent on water for its motility.

Table 1. Three-way ANOVA of factors affection expansion of bacteria in sand microcosms.

	Streptomyces			*Bacillus*		
Source of Variation	DF	F	P	DF	F	P
pore size distribution	2	56.5	**<0.001**	2	68	**<0.001**
matric potential	2	2.8	*0.066*	2	109.4	**<0.001**
time	3	249.5	**<0.001**	2	431.7	**<0.001**
PSD x MP	4	0.8	0.558	4	2	*0.095*
PSD x T	6	17.3	**<0.001**	4	69.4	**<0.001**
MP x T	6	0.8	0.55	4	19.2	**<0.001**
PDS x MP x T	12	0.6	0.883	8	2	*0.051*
Residual	252			189		
Total	287			215		

P values <0.05 (in bold) were regarded as statistically significant and p values < 0.1 (in italic) display a non-significant trend towards significance.

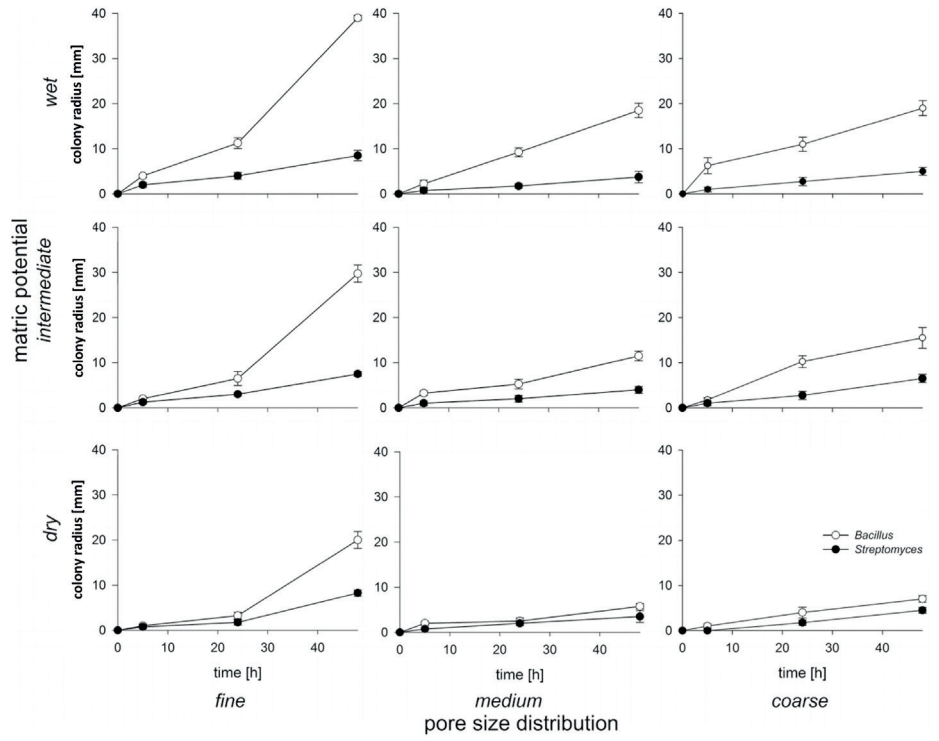


Figure 3. Expansion of *Streptomyces* and *Bacillus* inoculated individually in nine different combinations of pore size distribution and matric potential. Each data point represents the mean of 2 replicate microcosms with 4 measurements per microcosm. Error bars show the standard deviation.

3.3.3 Population dynamics

In the mixed inoculated microcosms, the rod-shaped *Bacillus* exhibited greater growth than the filamentous *Streptomyces* during the first three days of incubation in all treatments (Fig. 4). This result is in accordance with the faster growth of this *Bacillus* strain in liquid cultures (Fig 3S). In less-connected conditions (medium and coarse sand combined with dry and intermediate matric potential), *Streptomyces* caught up and ultimately reached higher cell numbers than *Bacillus* (Fig. 4) after 12 days of microcosm incubation. In wetter treatments (see Fig. 1), the numbers of *Bacillus* significantly exceeded the numbers of *Streptomyces* during the whole incubation period: *Bacillus* outnumbered *Streptomyces* by 52-, 22- and 1.6-times in the wet-fine, wet-medium and wet-coarse treatments, respectively, at day 12. In less-connected soils (coarse and dry), *Streptomyces* cells outnumbered *Bacillus* cells at the end of the experiment, e.g. the medium-dry and coarse-intermediate soils had approximately 1.7 and 2.2 times more *Streptomyces* cells than *Bacillus* cells at day 12, respectively.

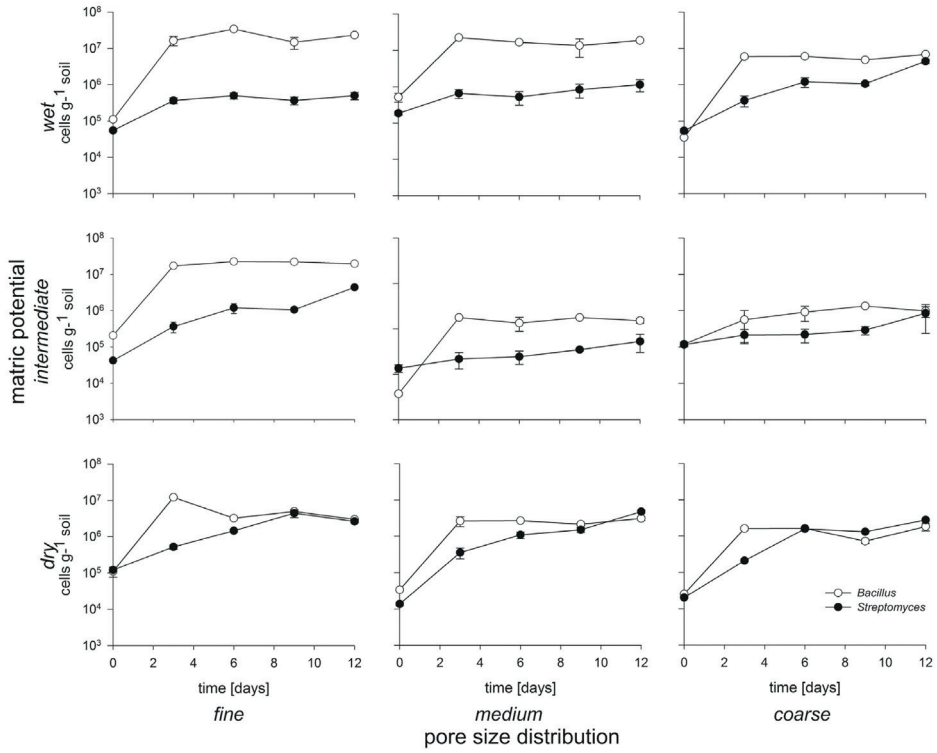


Figure 4. Population dynamics of *Streptomyces* and *Bacillus* competing in nine different combinations of pore size distribution and matric potential. The x-axis is time in days and the y-axis is cell number (g drywt soil) on a log-scale. Each data point is the mean of three microcosm replicates. Error bars show the standard deviation.

We found that the population dynamics of the filamentous *Streptomyces* and the rod-shaped *Bacillus* strains were influenced by pore size distribution ($p<0.001$) and matric potential ($p<0.001$), as well as the interaction between pore size distribution and matric potential ($p=0.005$) (Tab. 2). *Bacillus* cell numbers were not affected by the presence of the *Streptomyces* strain at 3 out of 4 time points, whereas *Streptomyces* was affected by the presence of *Bacillus* at all time points ($p<0.001$ for all time points; Tab. 3). In line with our hypothesis, the filamentous *Streptomyces* performed best when connectivity was low (coarse pores and dry conditions). Due to its faster growth rate, we anticipated that *Bacillus* would be more successful than *Streptomyces* in the early stages of the experiment. Such a pattern was indeed observed, with *Bacillus* cells outnumbering *Streptomyces* cells after three days of incubation in all treatments (Fig. 4). As the experiment progressed, the *Streptomyces* strain outcompeted *Bacillus* in poorly-connected soils, probably because of the ability of *Streptomyces* to exploit new microhabitats which may still contain nutrients that are inaccessible to the *Bacillus* strain. These results indicate that the hyphal growth may provide a benefit in less-connected matrices by giving the organism access to nutrient patches that cannot be reached by non-hyphal organisms.

Table 2. Three-way ANOVA of factors affecting *Bacillus/Streptomyces* (B/S) ratios.

Source of Variation	DF	F	P
pore size distribution (PSD)	2	12.0	**<0.001**
matric potential (MP)	2	18.8	**<0.001**
time (T)	3	2.7	*0.053*
PSD x MP	4	4.1	**0.005**
PSD x T	6	0.6	0.752
MP x T	6	1.1	0.367
PSD x MP x T	12	0.9	0.573
Residual	72		
Total	107		

The ratio between cell densities of *Bacillus* and *Streptomyces* (B/S-ratio) was used as a measure of competitive strength. P values <0.05 (in bold) were regarded as statistically significant and p values < 0.1 (in italic) display a non-significant trend towards significance.

The treatments in which *Bacillus* had the greatest competitive advantage coincided with the treatments where this strain also had the greatest advantage in motility (Fig. 3 and 4, Tab. 1, 2, and 3). Treatments in which *Streptomyces* could catch up with or outnumber *Bacillus* were those where the differences in expansion rate between *Bacillus* and *Streptomyces* were smallest. This suggests that motility may have been a particularly important factor in determining the outcome of the competition in the sand microcosms.

Table 3. Three-way ANOVAs of factors affecting population densities of *Bacillus* and *Streptomyces* strains after the given periods of incubation.

		Bacillus								*Streptomyces*							
Source of Variation / time [d]		**3**		**6**		**9**		**12**		**3**		**6**		**9**		**12**	
	DF	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
pore size distribution	2	63.4	<0.001	203.5	**<0.001**	51.1	**<0.001**	315.4	**<0.001**	17.0	**<0.001**	39.9	**<0.001**	17.2	**<0.001**	47.7	**<0.001**
matric potential	2	19.2	<0.001	152.9	**<0.001**	26.3	**<0.001**	346.7	**<0.001**	37.9	**<0.001**	94.8	**<0.001**	8.8	**<0.001**	37.6	**<0.001**
Inoculant (I)	1	1.6	0.220	0.3	0.617	4.5	**0.042**	0.2	0.667	76.6	**<0.001**	314.1	**<0.001**	60.7	**<0.001**	162.1	**<0.001**
PSD x MP	4	13.5	<0.001	43.7	**<0.001**	29.5	**<0.001**	82.5	**<0.001**	19.0	**<0.001**	72.3	**<0.001**	3.5	**0.017**	15.2	**<0.001**
PSD x I	2	1.2	0.300	1.2	0.303	1.3	0.279	19.9	**<0.001**	17.2	**<0.001**	38.4	**<0.001**	14.9	**<0.001**	46.8	**<0.001**
MP x I	2	10.5	<0.001	0.9	0.420	3.0	*0.063*	8.5	**<0.001**	32.7	**<0.001**	87.0	**<0.001**	9.0	**<0.001**	30.8	**<0.001**
PSD x MP x I	4	3.4	0.018	1.9	0.136	4.6	**0.004**	12.8	**<0.001**	16.0	**<0.001**	73.7	**<0.001**	4.1	**0.007**	14.9	**<0.001**
Residual	36																
Total	53																

P values <0.05 (in bold) were regarded as statistically significant and p values < 0.1 (in italic) display a non-significant trend towards significance.

Increased motility in well-connected soils also may enable *Bacillus* to more readily colonize new habitats. Remus-Emsermann and colleagues (Remus-Emsermann, 2012) found that the level of pre-colonization of leaf surfaces affected the establishment of a secondary colonizer. Similarly, in the well-connected soil microcosms, microsites are likely to be pre-colonized by the faster-growing and more motile *Bacillus* strain, which may then hamper subsequent colonization by the *Streptomyces* strain.

Overall, the patterns observed in our competition experiments could generally be explained by differences in growth rate, motility and growth form. Although we attributed the relative success of *Streptomyces* in the least connected artificial soil microcosms to its ability to produce hyphae, its ability to produce toxin may also have played a role in its interaction with *Bacillus*. The antagonism assay indicated the production of an inhibiting compound by *Streptomyces*, as zones of inhibition around colonies of *Streptomyces* were observed in our *Bacillus* soft-agar overlay experiment (Fig. S4). Although toxin production was indicated in our soft-agar overlay assay, the growth dynamics observed in our study did not seem to indicate any effects of toxin production under the conditions used. Tracking toxin levels and examining the impacts of soil connectivity on toxin-mediated antagonistic interactions remain interesting issues for future research in bacterial competition.

The hyphal growth form of bacteria has been recognized to be superior over non-filamentous growth forms for the degradation of insoluble polymers such as cellulose and chitin in soils, as hyphae can grow along polymer chains and penetrate into these structures (Mccarthy & Williams, 1992). However, our results indicate another advantage of the hyphal growth of actinomycetes, namely that it enables these organisms span air-filled pores to access nutrients that cannot be accessed by non-filamentous bacteria. This may allow for the co-existence of filamentous and non-filamentous bacteria in soil. Hence, both specialization (e.g. polymer degradation) and habitat exploitation abilities of actinomycetes may contribute to the maintenance of microbial diversity.

Despite the potential importance of soil characteristics that impact habitat connectivity on interactions between (individual) microbes, and thereby ultimately on soil biodiversity, relatively few studies have investigated how soil structure and connectivity affect competition between soil microorganisms. Treves et al. (Treves *et al.*, 2003) introduced two bacterial species, *Ralstonia eutropha* and *Sphingomonas sp.*, competing for a single resource into sand microcosms with different matric potentials and found that both strains could co-exist under dry conditions, but not under wet conditions. The authors concluded that spatial isolation created by low moisture content could contribute to the structuring of soil microbial communities. Similarly, Carson et al. (Carson *et al.*, 2010) provided evidence that low pore connectivity caused by low water potential could increase the richness and diversity of a complex bacterial community in soil. The observation that soil pore size can impact community composition was also made by Ruamps et

al. (Ruamps *et al.*, 2011) who used ^{13}C-labelled fructose and PLFA to track differential responses related to pore size classes. Whereas these studies focused separately on either the effects of moisture content or pore size, we developed and characterized an artificial sand microcosm system that allows for the independent manipulation of both moisture and pore size distribution, thereby allowing us to address the individual impact of these factors as well their interaction. Such systems should prove useful in helping to disentangle the impacts of various microbial interactions and soil parameters on shaping soil-borne microbial diversity. In contrast to true soils, this system has the advantage that soil structure parameters can be precisely defined and reproduced. The experimental conditions used in our microcosm experiments provided a wide and realistic range of soil pore sizes (Vreeken-Buijs *et al.*, 1998) and matric potentials (Chowdhury *et al.*, 2011). Although we used this system specifically to investigate interactions between filamentous and non-filamentous bacteria, it holds the potential to facilitate the examination of other organismal interactions in soil, such as chemical signaling and quorum sensing, grazing and resource competition.

3.4 Acknowledgements

The authors thank Hans van Veen for helpful suggestions on the experimental design. NIOO-KNAW manuscript # 5535.

3.5 Supporting information

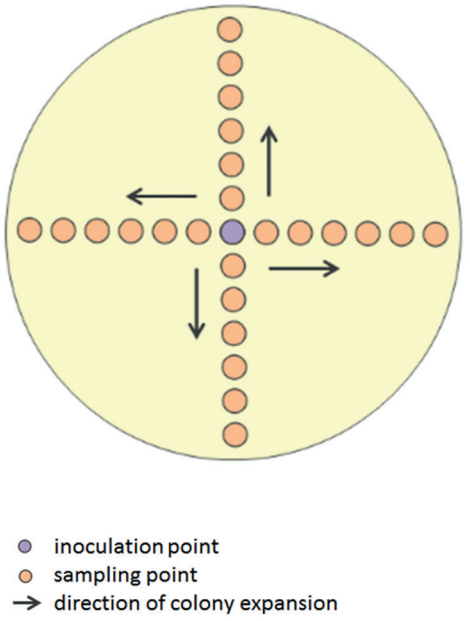


Figure S1. Schematic design for measurements of bacterial motility. Microcosms containing sand with different pore size distributions and matric potentials were established in glass petri dishes. The microcosms were inoculated in the middle with an overnight culture of either *Streptomyces* or *Bacillus* (=inoculation point). A multi-pronged sampling device was used at 24 and 48 h to measure the bacterial expansion in four directions by transferring bacterial cells with the prongs from defined distances (=sampling points) onto agar plates where colony formation was observed.

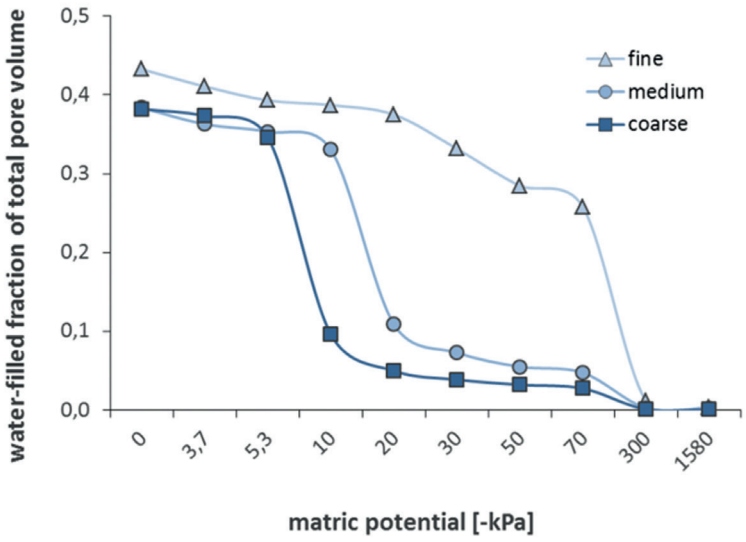


Figure S2. Water retention curves of the 3 sand fractions used in the experiments, showing the water-filled pore space at each matric potential.

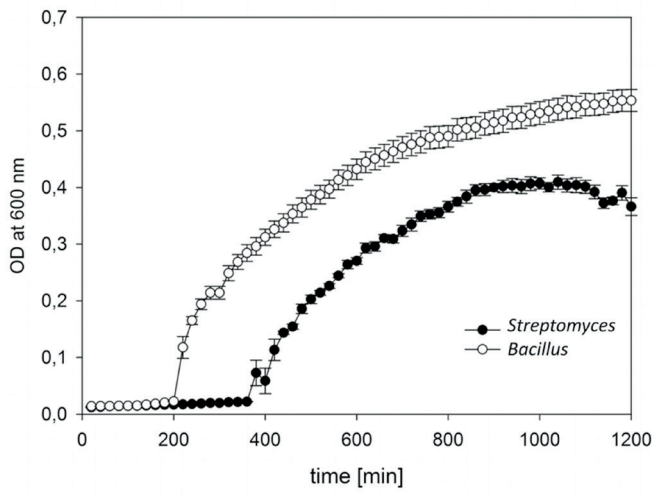


Figure S3. Growth curves of *Bacillus weihenstephanensis* and *Streptomyces atratus* in 10% tryptic soy broth (n=6). Error bars represent the standard error of the mean.

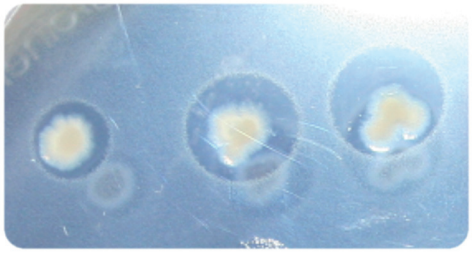


Figure S4. Antagonism assay of *Streptomyces* colonies overlaid with *Bacillus* in soft-agar. Zones of inhibition around the colonies indicate the production of an inhibiting compound by *Streptomyces*.

Table S1. Constants of water retention curves according to the model of van Genuchten as expressed by the equation $S_e = [1+(\alpha\ h)n]^{-m}$ with $S_e = (\theta_h - \theta_r)/(\theta_s - \theta_r)$ and $m = 1-(1/n)$. θ_h is the soil water content ($cm^3\ cm^{-3}$) at the suction h (cm), θ_r and θ_s are the residual and saturated soil water contents ($cm^3\ cm^{-3}$). S_e is the effective saturation; the parameters α, m, and n are empirical and determined by a best-fit procedure; α is a parameter related to the inverse of the air entry suction (cm^{-1}), n is a dimensionless curve shape parameter and s is the slope of θ_h (van Genuchten, 1980).

soil type	S	α	n	total porosity [%]
fine	0.401	0.002	2.378	40.11
medium	0.379	0.008	2.964	37.88
coarse	0.375	0.017	3.252	37.49

Chapter 4

Soil microbial community responses to particle size and moisture content

Authors:
Alexandra B. Wolf, Johannes Rousk, Wietse de Boer, George A. Kowalchuk

Abstract

Soil-borne microbial communities harbor a vast biodiversity, and their compositions are to a large extent driven by the physical structure of soil and micro-habitat connectivity within the soil matrix. However, relatively little is known about how soil pore size distribution and matric potential, which collectively determine micro-habitat connectivity, impact the relative success of different microbial populations in soil. To examine this issue, we conducted a series of microcosm experiments in which a natural microbial community were introduced into quartz sand environments differing in pore size distribution and matric potential. Microbial respiration and biomass production were tracked over time, and the microbial community composition determined using Phospholipid Fatty Acid (PLFA) analysis after 5 and 14 days. We hypothesized that the connectivity of the habitat matrix would influence the microbial community structure independent of the means by which it was manipulated – *i.e.* either by changing pore size distribution or matric potential. Specifically, we hypothesized that fungi would enjoy a relative advantage over bacteria in poorly connected environmental matrices due to their ability to bridge air-gaps, and that shifts would occur within bacterial PFLA profiles in response to habitat connectivity. We observed the highest production of bacterial biomass in soils with a large fraction of fine pores, but there was no effect of matric potential on bacterial biomass. Fungal biomass was highest in systems with a large fraction of coarse pores and low matric potential. The ratio of bacterial to fungal PLFAs was affected by both pore size and matric potential with fungi having a relative advantage over bacteria in more poorly connected soils (dry and coarse pores), and bacteria enjoying a relative advantage in well-connected soils (wet and fine pores). Furthermore, both bacterial and fungal community structure, as well as the bacterial:fungal ratio were more strongly affected by pore size distribution than matric potential. In total, our results highlight the importance of physical connectivity in shaping microbial communities.

Keywords: soil microbial communities – bacterial:fungal ratios – Phospholipid Fatty Acids – ergosterol

4.1 Introduction

Bacteria and fungi have developed different strategies to adapt to the heterogeneous soil environment. Both fungi and bacteria can utilize a wide range of substrates, but soil bacteria are generally thought of as the primarily degraders of simple substrates such as root exudates, whereas fungi predominantly degrade more recalcitrant substrates. Competition for simple substrates between bacteria and fungi has resulted in the development of antagonistic strategies, whereas for more recalcitrant substrates, both competitive as well as mutualistic strategies have evolved (de Boer *et al.*, 2005). Bacteria and fungi frequently share soil habitats, but they commonly occupy different, albeit often overlapping, niches. Hattori (1988) showed that 80–90% of fungi occur in larger pores and fungi are rarely found in micro-pores, where most bacteria reside (Killham, 1994). Bacteria thus mostly inhabit the more protected micro-pores, which provide protection from disturbances such as predation and dryness, whereas fungi reside predominantly in larger pores where they are more exposed to environmental changes such as dry-wet cycles and predation. Consequently, most fungal/bacterial interactions are likely to occur in macro-and meso-pores where bacteria and fungi co-exist.

Access to resources depends on how these are distributed within the soil matrix and the ability of microorganisms to physically encounter these resources. Simple, soluble nutrients can diffuse towards microorganisms via soil water. Alternatively, microorganisms can passively and/or actively move towards nutrients. Different microbial populations have evolved disparate life-history and growth strategies to deal with the barriers encountered in the physical soil matrix, and it is to be expected that changes in soil conditions will differentially select for and against such strategies (see Chapter 6). Bacteria do not only require water to sustain their viability, function and growth, but they also depend on water to gain access to nutrients. Passive dispersal of bacterial cells and spores obviously depends on the soil hydration status and the flow of water through the soil matrix (Abuashour *et al.*, 1994, Jiang *et al.*, 2006). Also, active bacterial motility is greatly restricted by soil moisture. For example, it has been shown both experimentally and via simulations that surface roughness and hydration status greatly impact bacterial motility, and that active motility of a *Pseudomonas putida* strain was only possible within a surprisingly narrow range of matric potentials (Dechesne *et al.*, 2010a, Dechesne *et al.*, 2010b, Wang & Or, 2010). Like bacteria, fungi also require water for their growth and functioning. However, the fungal hyphal growth form is fundamentally different from the most common growth forms of bacteria *i.e.* rods and cocci. This growth pattern provides an extremely useful adaption to the complex soil habitat. Hyphae generally grow along solid surfaces while absorbing nutrients from the substratum, which can then be redistributed throughout the mycelium. This growth pattern is well adapted to the patchy nutrient distribution typically encountered in soil environments. Fungal hyphae are also capable of growing into air (Falconer *et*

al., 2007, Falconer *et al.*, 2012), which allows them to bridge air-gaps, making fungal expansion less dependent on water films than that of non-filamentous bacteria (Yuste *et al.*, 2011). These two properties allow fungi to forage efficiently in soil environments via dense hyphal growth in rich patches and sparse hyphal highways across nutrient poor patches (Ritz & Young, 2004). There is evidence that fungal spread is affected by the geometry of the pore network. Otten and colleagues (2004) observed that *Rhizoctonia solani* grew preferentially along, as opposed to across, the surface of macro-pores, suggesting a metabolic cost of bridging air-gaps. Also, fungal invasion is affected by soil porosity, pore volume and pore connectivity (Pajor *et al.*, 2010). Overall, the hyphal growth seems to provide fungi with several advantages in soil habitats. However, in soils with a high level of disturbance, such as tilled agricultural systems, the advantages provided by fungal hyphae may be nullified due to tissue damage, something that generally does not affect bacteria (Hendrix, 1986).

Because bacteria are generally more reliant on connected water films to obtain resources than fungi, there is reason to believe that certain soil moisture conditions are more favourable for bacteria as opposed to fungi and vice versa. Corollary to this, the ratio of bacterial to fungal biomass would be expected to change in response to soil grain size and moisture content, as these factors impact the proportion of water-filled habitats in soil. Several field and greenhouse studies have addressed the effect of changing moisture content on the bacterial:fungal ratio (e.g. Frey *et al.*, 1999, Reichardt *et al.*, 2001, DeGrood *et al.*, 2005, Stromberger *et al.*, 2007), but no consensus response to these factors has yet emerged, possibly indicating system-specific effects on bacteria and fungi (Strickland & Rousk, 2010). Most studies on the impact of moisture on bacterial:fungal ratios have been carried out in the context of drying and rewetting (Gordon *et al.*, 2008, Bapiri *et al.*, 2010), but little is known regarding how the soil hydration status influences resource competition of bacteria and fungi and consequently bacterial:fungal ratios. The complex nature of soils makes it difficult to manipulate specific soil parameters independently and to deduce the relative importance of these parameters on the microbial community. For example, soil connectivity can be manipulated by changing the moisture content or by changing the soil texture. Also, in changing for instance moisture content by adding or removing soil water, other parameters such as salinity, osmotic pressure, pH and nutrient levels are also impacted, making it difficult to determine the relative importance of moisture itself on the community. Similarly, soil texture can be manipulated by adding solid particles such as sand or clay. However, this results in changes numerous physico-chemical properties of the soil as well.

In the current study, we sought to examine the impact of habitat connectivity on soil microbial communities in a system that allowed for independent manipulation of matric potential and pore size distribution. To this end, we inoculated a series of quartz sand microcosms representing a range in these parameters with natural soil microbial

communities and tracked microbial community structure over time by PLFA analysis. Our main objectives were to determine the effect of pore size distribution and matric potential, and consequently habitat connectivity, on fungal and bacterial biomass and community structures. We hypothesized that fungi would have a relative advantage over non-filamentous bacteria in poorly connected habitats due to their ability to bridge air-filled pores, while bacterial would be hold an advantage in more well-connected systems.

4.2 Material and Methods

4.2.1 Preparation and inoculation of microcosms

Soil-like microcosms were constructed using quartz sand particles of different sizes, obtained by milling acid-washed sea-sand (Honeywell Speciality Chemicals Seelze GmbH, Seelze, Germany) for 10 min followed by fractionation of the particles into size classes by sieving as described by Wolf *et al.* (2013). These different size fractions were used to create microcosms of different textures: “fine” (sand particles 53-106 µm), “medium” (106-212 µm), and “coarse” (212-425 µm), thereby creating a range of pore sizes. By using fine sand particles mainly small pores are created, medium-sized particles create pores of medium size and coarse particles create mainly large pores. Treatment names therefore refer to both particle and pore sizes (see Wolf *et al.*, (2013)) for a description of the pore size distribution of the different treatments). Microcosms were established in 50 mL serological glass vials with rubber stoppers to which 10 g quartz sand of the respective particle size range was added. Microcosms were sterilized by autoclaving followed by oven-drying. The moisture content was set to matric potentials of -10 (“wet”), -20 (“intermediate”) and -50 kPa (“dry”) as described in (Wolf *et al.*, 2013). Each particle size was combined with each matric potential, resulting in 9 treatments.

Artificial root exudate (ARE; C/N ratio 10.4) was used as growth medium and prepared according to Baudoin *et al.* (2003) with modification of the pH to 5.8. ARE stock solution contained 18,4 mM glucose, 18,4 mM fructose, 9,2 mM saccharose, 4,6 mM citric acid, 9,2 mM lactic acid, 6,9 mM succinic acid, 18,4 mM L-serine, 11 mM L-glutamic acid, 18,4 mM L-alanine and 10 mM KH2P04. The stock solution was adjusted to pH 5.8 with 5N NaOH, filter sterilized, aliquoted in 40 ml portions and stored at -20°C. The working solution was prepared by mixing stock solution with 10 mM phosphate buffer (pH 5.8) at a 1:2 ratio. Each microcosm was inoculated by mixing the sand with 100 µL soil suspension. The soil suspension inoculum was prepared by dispersing 50 g field wet soil collected from a former arable field site located near Ede, the Netherlands (52°04′N, 5°45′E; see van der Putten *et al.* (2000)) for a detailed description of the soil characteristics) in 450 mL phosphate buffer (pH 5.8) by shaking for 30 min and sonicating (Branson 5210

ultrasonic bath) twice for 1 min. We let soil particles settle and then used the supernatant as inoculum. Five 2 mL aliquots of the supernatant inoculum were sampled, cells spun down for 2 min at 10,000 rpm, and frozen at -80°C until subsequent analysis (see below). The appropriate amounts of liquid (see Tab. S1 for gravimetric water content and amount of C added to each microcosm) and inoculum were added to each microcosm, which were then incubated at 20°C until sampling. Sampling points were based on data of a pilot experiment in which CO_2 concentration was tracked over time in the different treatments (Fig. S1). Sampling was performed destructively after 5 days (T1= active community) and after 14 days (T2= long term effect), and samples were stored at -80°C until further analysis.

As different amounts of liquid were added to each treatment, the total nutrient input was also different between treatments (Tab. S1). Alternatively, the same amounts of nutrients per dry weight of sand could be added. However, this would lead to drastic differences in osmotic pressure, which has been shown to affect the bacterial:fungal ratio (Chowdhury *et al.*, 2011a, Chowdhury *et al.*, 2011b), as well as very high nutrient concentrations in the dry treatments. All microcosms were nutrient deprived within five days of inoculation based upon residual glucose measurements (see below and Fig. S2).

4.2.2 CO_2-, glucose- and pH measurements

At days 0, 1, 2, 3, 4, 5, 6, 9 and 14 of the incubations, the headspace CO_2 concentrations in the microcosms were measured in 3 randomly selected replicates for each treatment. Approximately 25 µL of headspace volume was sampled with a Pressure-Lok® syringe (Vici Precision Sampling) and analyzed by gas chromatography-thermal conductivity detection analysis (TraceGC Gas analyser, Thermo Scientific). Reference CO_2 gas (1,200 ppm, Westfalen AG) was used in order to calculate CO_2 concentrations.

At T0, T1 and T2, glucose measurements were performed on the microcosm samples using the Glucose (GO) Assay Kit (Sigma-Aldrich). Glucose was extracted from 1 g material by adding 2 mL deionised water and shaking at 200 rpm for 1 h. The solution was then filtered through 0.2 µm filter to clarify the solution and glucose concentrations were determined following the manufacturer's protocol. We did not measure the concentration of other sugars, amino- and organic acids, but used glucose as a proxy of nutrient levels.

The pH of the microcosm samples was measured at T1 and T2 with a glass electrode using a 1:5 sand-to-water ratio.

4.2.3 PLFA analysis

We analysed the PLFA profiles with the aim of quantifying the dynamics of bacterial and fungal biomass in response to the different treatments. PLFA analyses were performed on 1 g of frozen soil according to Frostegard *et al.* (1993) with modifications described by Nilsson *et al.*, (2007). An internal standard (methyl nonadecanoate fatty acid, 19:0) was added before the methylation step. The PLFAs chosen to indicate bacterial biomass were a15:0, 15:0, i16:0, 16:1ω7c, 10Me16:0, a17:0 cy17:0, a17:0, 18:1ω7 and cy19:0, while PLFA 18:2ω6,9 was used to indicate fungal biomass (Frostegard & Baath, 1996). The bacterial:fungal biomass ratio was calculated from the sum of the bacterial specific biomarkers and the fungal specific biomarker.

4.2.4 Ergosterol measurements

Ergosterol was used as an estimator of fungal biomass and was extracted as described by Gong *et al.* (2001). Briefly, 3 g of moist sand sample (n= 2 per treatment) were added to a 20 ml polyethylene scintillation vial containing 4 g of acid-washed glass beads (2 g of 250–500 µm diameter and 2 g of 1000 µm diameter). After the addition of 6 ml methanol, the vial was vortexed for 10 s and subsequently shaken intensively for 1 h on a bench-top shaker at room temperature. The sand mixture was allowed to precipitate for 15 min before a 1.5 ml aliquot of the supernatant was transferred into an Eppendorf microfuge tube and centrifuged for 10 min at 11,000 rpm. The supernatant was then filtered through a 0.2 µm PTFE syringe filter and loaded for HPLC analysis.

4.2.5 Statistical analysis

To assess the effects of particle size and matric potential and the interaction between these two factors on the abundances of bacterial and fungal PLFAs (in nmol PLFA/g(dw)), as well as the ratio between fungal and bacterial biomass, we compared bacterial and fungal PLFAs and the bacterial:fungal ratio across the different treatments by performing two-way ANOVAs with Bonferroni pair-wise comparisons post-hoc tests in R (v 2.14.1; www.r-project.org). Variation partitioning analysis (Borcard *et al.*, 1992, Legendre *et al.*, 2012) was performed to test and determine the likelihood of the factors pore size, matric potential and time in explaining patterns in the community structure. The PLFA composition (mol-% of the 18 most abundant PLFAs) was analyzed by principal component analysis (PCA) after standardizing to unit variance, and redundancy analysis (RDA) was used to compare microbial community structures from different treatments based on PLFAs and to test the effect of the different treatment levels, as implemented in Canoco 5 (Microcomputer Power, Ithaca, NY).

4.3 Results

4.3.1 CO_2-, glucose- and pH measurements

The microbial communities started producing CO_2 at approximately day 2, and CO_2 concentrations in the headspace of microcosms plateaued after about 6 days in most treatments (Fig. S1). Calculations of the percentage of originally added C that was respired show total C respiration of 13-21% after 5 days and 14-23% after 14 days (Tab. S1). The percentage of C that was respired was significantly higher in fine treatments than in coarse treatments (P= 0.016), but there were no differences among different matric potentials. Glucose was depleted in all treatments by day 5 (Fig. S2).

The pH in the microcosms at T1 and T2 ranged from approximately 6.2 to 8.7, with pH negatively correlated with increasing particle size (P< 0.001; Fig. S3). There was no effect of matric potential on the pH.

4.3.2 Total bacterial PLFAs

Bacterial PLFA amounts differed significantly across particle size treatments at both time points (P< 0.001 at T1 and P= 0.001 at T2), with PLFA concentrations being significantly higher in microcosms containing the smallest sand particles (P< 0.001; Tab. S2, S3 and Fig. 1). Also, matric potential had significant effect on bacterial PLFA concentrations (P< 0.001 at T1 and P= 0.041 at T2), with greater values at higher moisture contents (Tab. S2, S3 and Fig. 1).

4.3.3 Fungal PLFAs and ergosterol measurements

At T1, fungal PLFA amounts differed significantly across particle size treatments (P= 0.007) with the highest concentrations found in coarse sand particle microcosms (Tab. S2, S3, S4 and Fig. 1). However, at T2 there was no statistically significant effect of particle size (P= 0.562). At both time points, fungal PLFA concentrations differed significantly across matric potentials (P_{T1} = 0.009 and P_{T2}= 0.006) with fungal PLFA concentration being higher in dry and intermediate treatments than in wet treatments (Tab. S2, S3, S4 and Fig. 1).

At T1, ergosterol concentrations were below the detection limit in microcosms containing fine and medium-sized sand (Tab. 1). However, ergosterol could be detected in all microcosms for the coarse particle treatment. Ergosterol concentrations differed significantly across particle size treatments at both time points (P< 0.001, Fig. 2 and Tab. S5), with ergosterol concentrations being significantly lower in microcosms containing fine and medium sand particles (Tab. S6), but there were no differences among different levels of matric potential at either time point (Tab. S6).

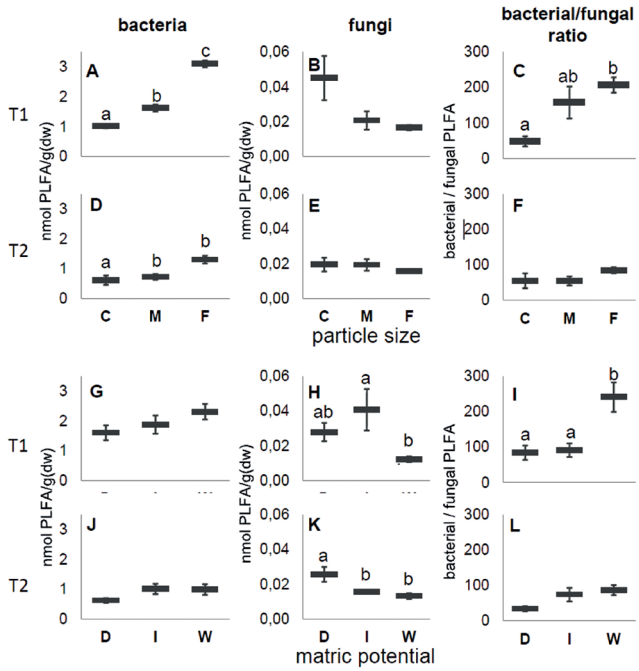


Figure 1. Graphs depicting the abundance (nmol PLFA per g(dw)) of the sum of 9 bacterial specific PLFAs (= bacterial) and one fungi specific PLFA (= fungal), as well as the bacterial/fungal ratio, which was calculated from the bacterial and fungal specific PLFAs biomarkers in the fungal-bacterial experiment at the different pore sizes (A-F) and moisture concentrations (G-L) at T1 and T2. Error bars represent the standard errors. Different letters (in A, C, D, H, I and K) indicate statistical differences based on Bonferroni ($P < 0.05$), no significant differences were found in B, E, F, G, and J). C= coarse, M= medium, F= fine pores; D= dry, I= intermediate, W= wet matric potential.

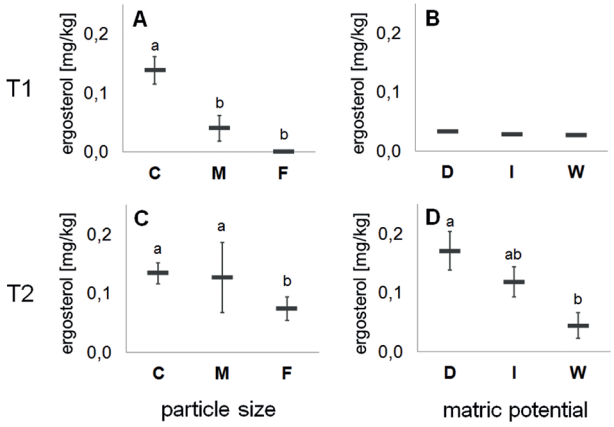


Figure 2. Graphics depicting the ergosterol concentrations (mg/kg soil) in the different treatments at different particle sizes (panel A and C) and matric potentials (panels B and C) at T1 and T2. Error bars represent the standard errors. Different letters indicate statistical differences based on Bonferroni ($P< 0.05$). At T1, ergosterol concentrations were higher in the coarse pores than in medium and fine, and at T2 concentrations were higher in medium and coarse than in fine. There was no statistically significant effect

of matric potential at T1, however at T2, ergosterol concentrations were higher in dry treatments than in wet treatments. C= coarse, M= medium, F= fine pores; D= dry, I= intermediate, W= wet matric potential.

4.3.4 Bacterial:fungal ratio

A 2-way ANOVA showed a significant effect of both pore size and matric potential on the ratio of bacterial to fungal PLFAs, as well as pore size and matric potential at T1, while at T2 only the matric potential had a significant effect (Tab. S2 and Fig. 1). Post-hoc comparisons of the individual response variables revealed relatively higher bacterial biomass in fine and wet treatments at T1, whereas fungal biomass was relatively higher in coarse and medium and dry and intermediate treatments (Tab. S3). At T2, no statistically significance of bacteria:fungi ratio was found across treatments.

Table 1. Ergosterol concentrations in mg per kg soil the different treatments at T1 and T2. C= coarse, M= medium, F= fine pores; D= dry, I= intermediate, W= wet matric potential; T1= 5 days, T2= 14 days.

pore	matric potential	T1 ergosterol [mg/kg]	se	T2 ergosterol [mg/kg]	se
F	W	0.000	NA	0.040	0
F	I	0.000	NA	0.060	0
F	D	0.000	NA	0.121	0.01
M	W	0.000	NA	0.000	NA
M	I	0.080	0.02	0.129	0.00
M	D	0.000	NA	0.250	0.01
C	W	0.080	0.01	0.093	0.01
C	I	0.179	0.01	0.167	0.02
C	D	0.127	0.02	0.142	0.01

4.3.5 PLFA composition

Variation partitioning analysis revealed that both particle size and matric potential explained a significant portion of the variation in total PLFA patterns ($P_{particle\ size}$= 0.002 and $P_{matric\ potential}$= 0.002). Although both factors were significant in terms of explaining variation obtained in the total PLFA composition, pore size explained 41.5% of the total variation and matric potential only 8.8%. This suggests that overall pore size has a greater impact in structuring the microbial community than matric potential. For both T1 and T2, the total PLFA composition in treatments with fine particles cluster together on left side in the PCA plot, whereas samples associated with intermediate particle size group together around the center and coarse on the right side (Fig. 3). While the clustering of samples in treatments with fine particle sizes was very pronounced, samples showed overlap in PLFA composition in treatments with intermediate and coarse particles. No clear clustering of samples with similar matric potential could be found. A very similar pattern was observed in PCA plots using only bacteria specific PLFAs (Fig. S4). For T1, forward selection in the redundancy analysis

(Fig. 4) showed that total PLFA patterns were significantly correlated with particle size (all P= 0.002) and wet and intermediate matric potential (P_{wet}= 0.02 and $P_{intermediate}$= 0.006). Most of the variation was explained by the fine particle size (32.5%), whereas medium and coarse particle sizes each explained 10.4% of the variation. Wet matric potential explained 12.4% of the total variation and intermediate matric potential 4.0%. Similarly, at T2 PLFA patterns were significantly correlated with fine, medium and coarse particle size (all P= 0.002) and wet and intermediate matric potential (P_{wet}= 0.02 and $P_{intermediate}$= 0.018), with fine explaining 32.5% and medium and coarse each explaining 11% of the variation. Wet and intermediate treatments explained 8.5% and 3.1% of the variation, respectively (Fig. 4).

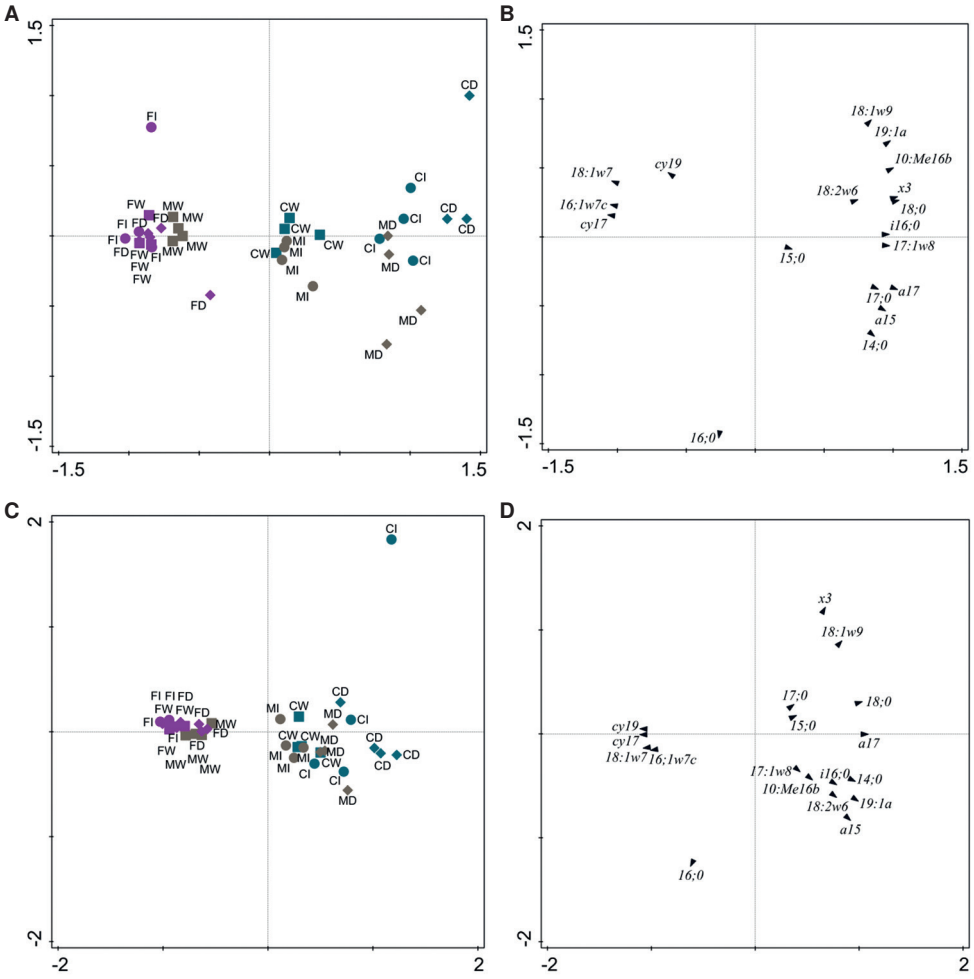


Figure 3. PCA plots of PLFA patterns (relative abundances) of T1 (panel A) and T2 (panel C). Panel A and C depict the sample scores and panel B and D depict the species scores. Principal component analysis (PCA) revealed substantial differences in PLFA patterns among treatments. The first principal component (PC1) accounted for 66.3% (T1) and 53.6% (T2) of the total sample variance and the second principal component (PC2) explained 8.9% (T1) and 12.8% (T2) respectively.

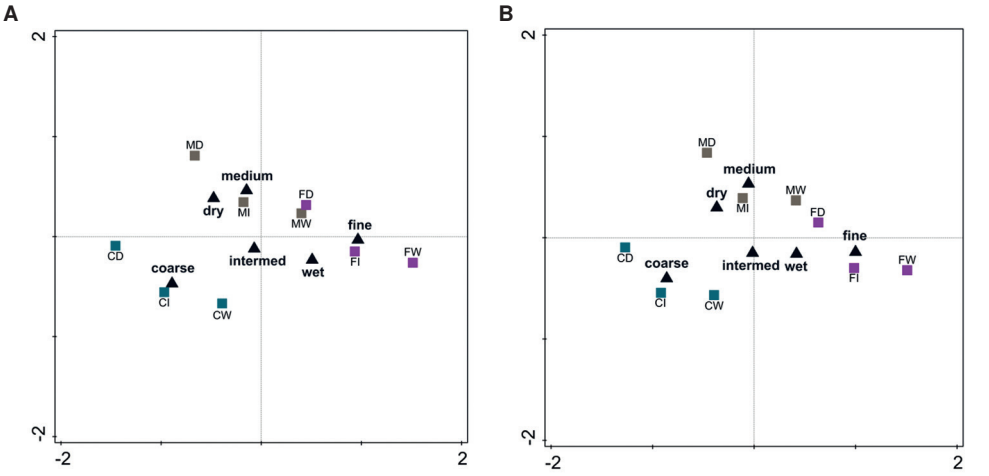


Figure 4. RDA plots of PLFA patterns of T1 (panel A) and T2 (panel B). Symmetric scaling of ordination scores, species scores were divided by standard deviation. Species were centered. Black triangles represent the centroid of each environmental variable (treatment) that explains a significant proportion of the variation. Sample codes are as follows: C= coarse, M= medium, F= fine pores; D= dry, I= intermediate, W= wet matric potential. At T1, the effect of pore size explains a significant proportion of the variation in the data set, with a significantly correlated between fine, medium and coarse treatments (all P= 0.002) and the PLFA patterns. Also, the matric potential impacts the community composition with a significant correlation between wet (P= 0.002) and intermediate (P= 0.006) and the PLFA patterns. Likewise, at T2, PLFA patterns are significantly correlated with fine, medium, coarse (all P= 0.002) and wet (P= 0.002) ant intermediate (P= 0.018) treatments.

4.4 Discussion

In this study, we investigated how connectivity, as determined by pore size distribution and matric potential, influences bacterial and fungal biomass, bacterial:fungal ratios and PLFA patterns over time in a microcosm experiment. We used quartz sand particles of three different size classes to create treatments with different pore size distributions, fine, medium and coarse. We combined these different particle sizes with three different matric potentials (“dry”, “intermediate” and “wet”) in a factorial design, resulting in 9 treatments of different levels of habitat connectivity (Wolf *et al.*, 2013). Coarse soils contain mostly large pores that are only water-filled at the highest matric potentials and are consequently poorly connected compared to fine soils, especially at high matric potential. Results demonstrate that overall the impact of particle size on the PLFA composition was greater than the impact of matric potential, as determined by variation partitioning analysis. We found that bacterial density was strongly affected by particle size, with highest biomass in fine treatments, whereas the effect of matric potential was much less pronounced. Both PLFA and ergosterol data show that fungal biomass increases with increasing particle

size and decreasing moisture. It should be noted that in our experimental design more nutrients have been added in the wetter treatments, as we have chosen to have similar nutrient concentrations in the "soil solution" for all treatments. Although this may impact the community development, it is clear that nutrient-limiting conditions were already encountered in all microcosms as of day 5 (Fig. S4).

The ratio between bacterial and fungal PLFAs was influenced by both particle size and matric potential, the two factors that determine habitat connectivity. These patterns, taken together with the interaction of pore size and matric potential, demonstrate that bacteria performed better in well-connected environments whereas fungi performed better in more poorly connected treatments. We therefore conclude that pore size and matric potential both have an impact on the relative abundance of fungi and bacteria.

The ratio of bacterial to fungal PLFAs was significantly affected by both pore size and matric potential, and there was also an interacting effect of pore size and matric potential (Tab. S2 and Fig. 1). Bacteria had the greatest relative advantage in fine and wet, thus well-connected, treatments, whereas fungi had a relative advantage in coarse and medium as well as dry and intermediate, thus less-connected, treatments. The advantage of bacteria over fungi in well-connected soils was especially pronounced at T1. This strong, initial advantage for bacteria may also be associated with the greater availability of nutrients and resulting microbial activity in the initial stages of the experiments (Figs. S1 & S2). Our observation that fungi had a relative advantage over bacteria in dry as compared to wet conditions is consistent with results from previous studies documenting that fungi are generally more resistant to desiccation than bacteria (Bapiri *et al.*, 2010, Barnard *et al.*, 2013). Furthermore, in well-connected habitats, bacteria may be faster in consuming nutrients, whereas in poorly connected soils fungi may get access to micro-habitats that do not contain any bacteria and cannot accessed by them. Previous work has demonstrated that substrate rich conditions result in an increase of fungal biomass relative to bacterial biomass (Griffiths *et al.*, 1999, Reischke *et al.*, 2014). However, in our study the most substrate rich treatments (fine and wet) yielded the highest bacterial:fungal ratios, which supports our interpretation that the observed relative decrease of the bacterial:fungal ratio in coarse and dry soils can indeed be attributed to particle size and matric potential and not differences in substrate concentrations.

Besides bacteria and fungi being differently impacted by particle size and moisture, differences in the bacterial:fungal ratio in different treatments may also be the result of competitive interactions between bacteria and fungi. It is known that bacteria and fungi influence each other in various ways. Experiments comparing fungal growth in soil in the presence and absence of bacteria have demonstrated increased fungal growth in the absence of bacteria, which could be mostly attributed to exploitation competition (Rousk *et al.*, 2008). However, interference competition can also be an important type of interaction

between bacteria and fungi. For instance, there are indications that soil bacterial community composition is strongly influencing the production of fungistasic compounds (Garbeva et al., 2011b). In addition, it has been demonstrated that interspecific bacterial competition triggers the production of broad-spectrum antibiotics that have also inhibitory effects on fungi (Garbeva & de Boer, 2009, Garbeva *et al.*, 2011b).

Overall, we found that pore size had a greater impact than matric potential on the PLFA community profiles and the bacterial:fungal ratio (Figs. 1, 2 and Tab. S4). Coarse-grained soils contain a greater proportion of large pores that remain air-filled at all but the highest matric potentials, with water being held in crevices and gaps as disconnected water films, providing conditions that are more favourable to the hyphal growth form of fungi as compared to non-filamentous bacteria. Also, fungal and bacterial species differ in their ability to resist desiccation, and shifts in community composition may reflect this imposed selection, as evidenced by the clear shifts in bacterial PLFA patterns in response to connectivity parameters (Fig. S4).

The pH in our microcosms at T1 and T2 was between approximately 7 and 8 at T1 and T2, with the highest pH in fine-wet treatments and the lowest in coarse-dry treatments (Fig. S3), possibly due to higher cation-exchange-capacity in fine soils because of increased surface area. Previous work by Rousk *et al.* (2011) has documented that the relative importance of fungi as compared to bacteria decreases with increasing pH. Thus, pH could have contributed to the relatively greater presence of fungi in the less connected microcosms, where the pH was slightly lower. Although previous work has examined a much larger discrepancy in pH over much longer time-scales, pH cannot be ruled out as a factor contributing to the observed community differences. Future experiments designed to manipulate pH specifically and independently would be very helpful in disentangling the role of pH from other soil properties, which to date has mostly been studied via survey-based approaches in which multiple parameters co-vary with pH (Rousk *et al.*, 2010).

In our study, bacteria performed best in fine pore-dominated microcosms. In natural soils, most bacteria inhabit micro-pores (< 10 **µ**m), which provide protection against desiccation and predators (Ranjard & Richaume, 2001). We found that fungi performed best in coarse pores (at both time points) and dry conditions (only at T2). Several previous studies have aimed at elucidating the effect of matric potential on fungal biomass; however no clear consensus has yet emerged. Reichardt (2001) and DeGrood and colleagues (2005) observed a relative increase in fungal biomass in field and greenhouse studies with decreasing matric potential, whereas others have reported a decrease of fungal biomass in field experiments (Frey *et al.*, 1999, Stromberger *et al.*, 2007, Williams & Rice, 2007). These contrasting outcomes are most likely attributed to the different soil types examined (and largely uncharacterised soil properties in these soils) and different fungal species present, which may differ in their tolerance to low matric potentials (Harris, 1980, McLean

& Huhta, 2000, Klamer & Hedlund, 2004). In our system, we only manipulated matric potential and pore size distribution, independently, while keeping other soil parameters constant and also, the "starting community" was the same in all treatments. However, future studies are needed that identify how specific fungal and bacterial species respond to different soil moistures, e.g. by combining experiments with phylogenetic characterization of the community to link these species to known traits such as drought resistance.

4.5 Acknowledgements

We thank Anne Steenbergh for assistance with the CO_2 measurements and Iris Chardon for conducting the ergosterol measurements.

4.6 Supplementary information

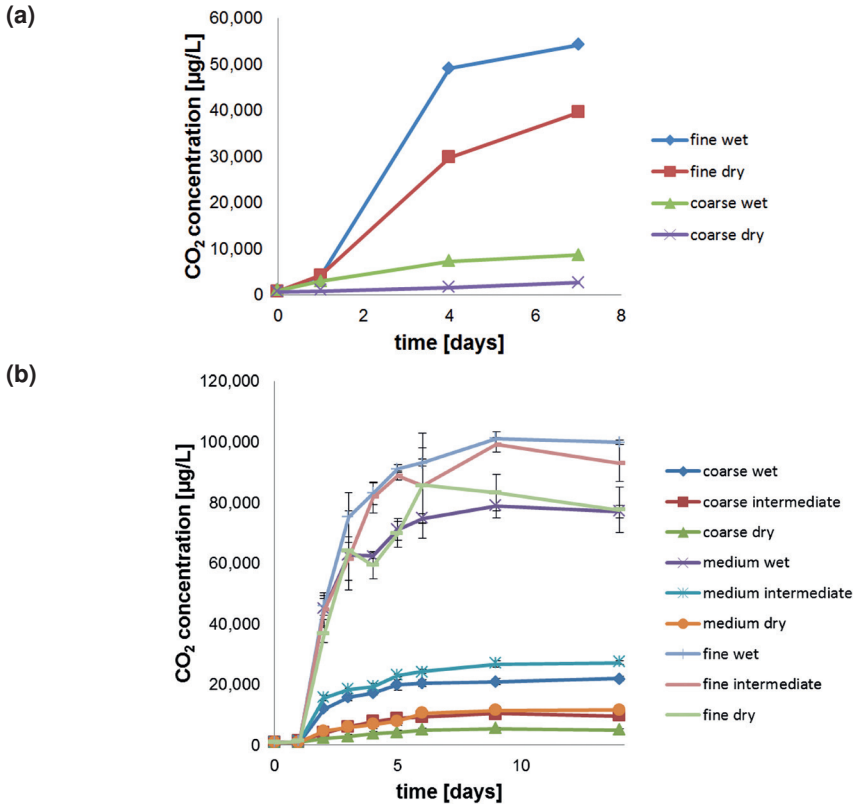


Figure S1. CO_2 concentrations in the headspace of the microcosms in the pilot study (n=1) (a) and fungal-bacterial experiment (n=3) (b) . Error bars in (b) and (c) depict the standard error.

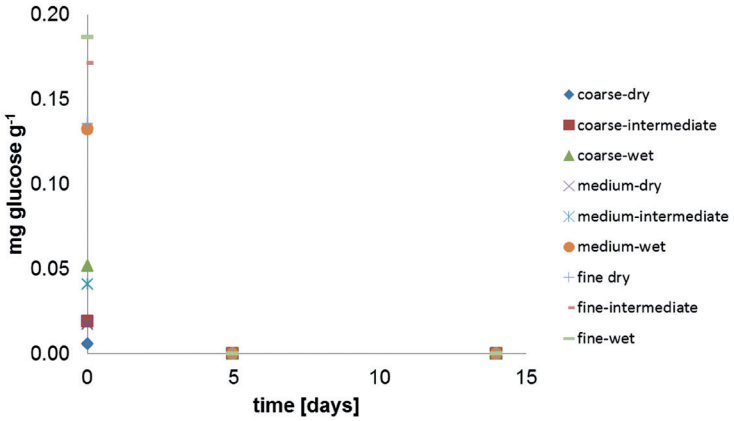


Figure S2. Glucose concentrations at T0, T1 and T2 in the different treatments in the fungal-bacterial experiment (n=2).

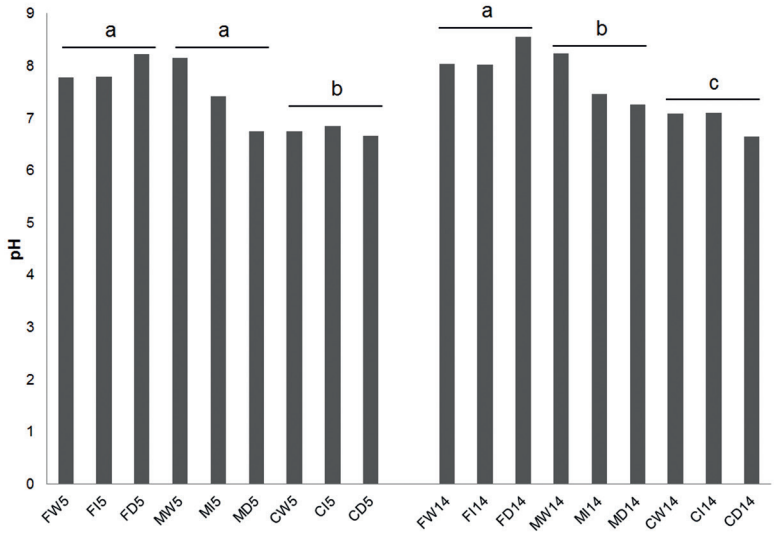


Figure S3. pH measurements in the sand microcosms at T1= 5 days and T2= 14 days (n= 2). Treatments with different letters indicate significantly differences (bonferroni, P< 0.05). C= coarse, M= medium, F= fine pores; D= dry, I= intermediate, W= wet matric potential.

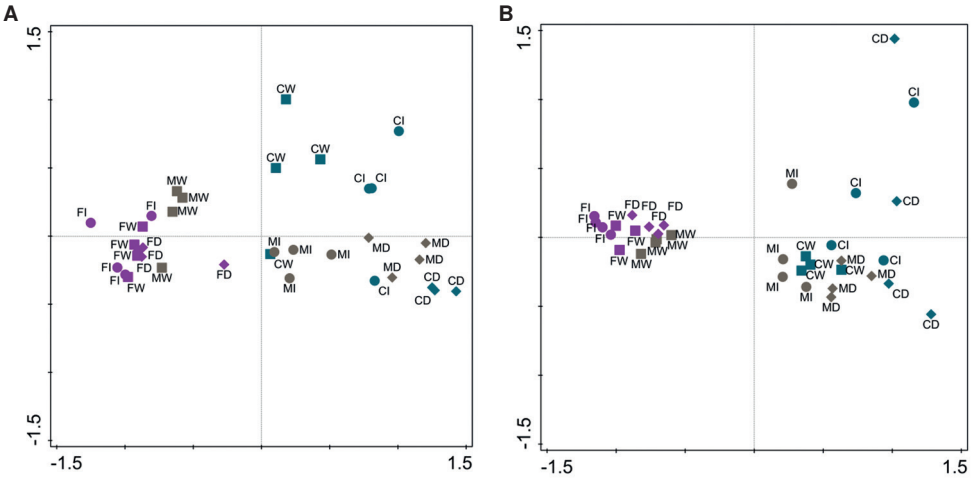


Figure S4. PCA of only bacterial PLFAs at T1 (panel A) and T2 (panel B). The first component explains 71.3% (T1) and 64.7% (T2) respectively, and the second component explains 11.2% (T1) and 13.6% (T2).

Table S1. Gravimetric water content, C addition per g of soil, total C that was measured in the microcosm headspace and percentage of originally added C that was respired at T1 and T2 days in the different treatments in the bacterial and fungal-bacterial experiment.

			bacterial experiment				Fungal-bacterial experiment			
treatment	gravimetric H_2O content	C addition per g soil [mg]	total C in microcosm headspace [mg]		% of C respired		total C in microcosm headspace [mg]		% of C respired	
			T1	T2	T1	T2	T1	T2	T1	T2
FW	25,7%	0,600	1,16	1,36	19,33	22,60	1,24	1,36	20,73	22,72
FI	24,9%	0,581	0,81	0,85	13,98	14,66	1,21	1,27	20,87	21,84
FD	18,9%	0,441	0,89	0,96	20,25	21,86	0,95	1,06	21,51	23,99
MW	20,2%	0,471	1,04	1,04	21,98	22,10	0,97	1,05	20,59	22,31
MI	6,7%	0,156	0,24	0,35	15,62	22,24	0,31	0,37	20,10	23,79
MD	3,4%	0,079	0,07	0,12	9,26	15,19	0,11	0,16	13,71	20,20
CW	5,9%	0,138	0,22	0,28	15,80	20,09	0,27	0,30	19,69	21,68
CI	3,5%	0,082	0,06	0,10	7,85	11,85	0,12	0,13	14,66	15,85
CD	2,0%	0,047	0,03	0,05	6,38	9,95	0,06	0,07	12,56	14,17

Table S2 Mean relative abundance (molar % of the total PLFA concentrations) (standard deviation in parentheses, n= 4) of individual biomarker PLFAs. The sum of 8 bacterial specific PLFAs (1) (= bacterial%) was used as indicator of bacterial biomass, and PLFA 18:2ω6,9 (2) was used as an indicator for fungal biomass (fungal%). The bacterial:fungal ratio was calculated from the abundances of bacterial and fungal specific PLFAs (in nmol per g(dw)).

	time [d]																	
	5																	
	matric potential																	
	D		I		W		D		I		W		D		I		W	
	pore size																	
	C						M						F					
	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se
14;0	3.0	0.01	3.1	0.00	1.8	0.00	3.4	0.01	2.1	0.00	0.9	0.00	3.4	0.01	1.1	0.00	0.8	0.00
a15 (1)	4.0	0.00	3.6	0.00	3.1	0.00	5.7	0.00	3.6	0.00	1.1	0.00	5.7	0.00	1.3	0.00	0.9	0.00
15;0 (1)	1.2	0.00	5.2	0.01	5.4	0.02	1.6	0.00	0.4	0.00	2.8	0.01	1.6	0.00	1.7	0.01	0.8	0.00
i16;0 (1)	2.0	0.00	1.5	0.00	0.5	0.00	1.2	0.00	0.7	0.00	0.3	0.00	1.2	0.00	0.3	0.00	0.2	0.00
16;1ω7c (1)	3.0	0.00	6.0	0.00	10.8	0.01	7.4	0.01	11.9	0.01	18.3	0.00	7.4	0.01	20.4	0.02	19.9	0.01
16;0	22.1	0.02	25.8	0.01	26.7	0.00	29.5	0.03	30.9	0.01	29.6	0.00	29.5	0.03	24.2	0.06	30.9	0.00
10:Me16b (1)	10.2	0.01	5.1	0.00	4.1	0.00	5.1	0.01	4.0	0.00	2.1	0.00	5.1	0.01	0.1	0.00	0.1	0.00
a17	2.7	0.00	2.3	0.00	1.6	0.00	3.1	0.00	1.9	0.00	0.7	0.00	3.1	0.00	0.7	0.00	0.7	0.00
17:1ω8	2.4	0.00	2.0	0.00	1.5	0.00	1.9	0.00	1.1	0.00	0.6	0.00	1.9	0.00	0.6	0.00	0.7	0.00
cy17 (1)	4.1	0.00	7.5	0.01	14.4	0.00	7.3	0.01	12.3	0.00	17.6	0.00	7.3	0.01	24.0	0.02	21.0	0.00
17;0	1.8	0.00	2.1	0.00	1.4	0.00	3.1	0.00	1.5	0.00	0.8	0.00	3.1	0.00	0.7	0.00	0.8	0.00
x3	7.2	0.00	6.8	0.01	4.5	0.00	5.0	0.00	3.8	0.00	1.6	0.00	5.0	0.00	0.7	0.00	0.7	0.00
18:2ω6,9 (2)	1.6	0.00	3.5	0.01	0.6	0.00	1.4	0.00	0.5	0.00	0.1	0.00	1.4	0.00	0.2	0.00	0.1	0.00
18:1ω9	5.4	0.02	4.0	0.00	2.4	0.00	2.6	0.00	2.3	0.00	0.9	0.00	2.6	0.00	0.8	0.00	1.6	0.01
18:1ω7 (1)	3.6	0.00	4.7	0.00	8.4	0.00	5.3	0.01	9.0	0.01	14.1	0.00	5.3	0.01	16.0	0.02	14.2	0.00
18;0	20.2	0.02	12.5	0.01	9.6	0.00	13.0	0.00	11.2	0.01	5.3	0.00	13.0	0.00	3.8	0.00	3.4	0.00
19:1a	3.6	0.00	2.2	0.00	1.7	0.00	1.9	0.00	1.4	0.00	0.8	0.00	1.9	0.00	0.5	0.00	0.5	0.00
cy19 (1)	2.0	0.00	1.9	0.00	1.7	0.00	1.5	0.00	1.6	0.00	2.4	0.00	1.5	0.00	3.0	0.00	2.6	0.00
total PLFAs in nmolPLFA/g(dw)	1.73	0.12	2.39	0.14	3.12	0.22	2.79	0.36	3.62	0.32	7.10	0.40	9.15	0.67	10.75	0.90	11.35	0.46
bacterial PLFAs in nmolPLFA/g(dw)	0.82	0.09	0.97	0.06	1.19	0.11	1.15	0.11	1.33	0.13	2.40	0.15	2.64	0.15	3.34	0.13	3.33	0.11
fungal PLFAs in nmolPLFA/g(dw)	0.03	0.01	0.09	0.03	0.02	0.00	0.04	0.01	0.02	0.00	0.01	0.00	0.02	0.00	0.02	0.00	0.01	0.00
bacterial/fungal PLFAs	28.92	9.65	11.31	2.30	69.83	27.79	30.81	8.76	78.85	48.23	308.69	117.55	147.65	51.06	167.75	55.63	277.63	82.43

	time [d]																	
	14																	
	matric potential																	
	D		I		W		D		I		W		D		I		W	
	pore size																	
	C						M						F					
	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se	PLFA	se
14;0	4.5	0.00	4.6	0.01	1.6	0.00	2.9	0.00	3.4	0.00	0.6	0.00	0.7	0.00	0.4	0.00	0.7	0.00
a15 (1)	5.7	0.01	5.0	0.01	2.7	0.00	7.3	0.01	5.4	0.00	1.5	0.00	2.1	0.00	1.2	0.00	2.1	0.00
15;0 (1)	14.3	0.08	8.9	0.07	0.4	0.00	1.3	0.00	5.4	0.04	0.3	0.00	0.5	0.00	0.4	0.00	0.5	0.00
i16;0 (1)	1.5	0.00	1.9	0.00	0.5	0.00	1.3	0.00	0.8	0.00	0.3	0.00	0.7	0.00	0.3	0.00	0.7	0.00
16;1ω7c (1)	2.7	0.01	3.1	0.00	11.9	0.01	6.2	0.00	7.5	0.00	11.4	0.01	8.0	0.01	10.1	0.00	8.0	0.01
16;0	23.1	0.01	23.5	0.05	30.8	0.00	28.6	0.02	28.4	0.02	30.6	0.01	29.0	0.00	29.5	0.00	29.0	0.00
10:Me16b (1)	2.0	0.01	0.6	0.00	0.6	0.00	1.8	0.01	1.8	0.00	0.7	0.00	0.5	0.00	0.3	0.00	0.5	0.00
a17	4.2	0.01	3.8	0.00	1.2	0.00	3.6	0.00	2.9	0.00	0.8	0.00	0.8	0.00	0.4	0.00	0.8	0.00
17:1ω8	1.4	0.00	1.0	0.00	0.3	0.00	4.0	0.02	0.7	0.00	0.4	0.00	0.5	0.00	0.3	0.00	0.5	0.00
cy17 (1)	6.4	0.01	9.8	0.03	26.5	0.01	9.2	0.02	14.8	0.01	29.9	0.01	32.8	0.01	32.5	0.01	32.8	0.01
17;0	1.0	0.00	1.2	0.00	0.5	0.00	3.3	0.02	1.4	0.00	0.5	0.00	0.7	0.00	0.5	0.00	0.7	0.00
x3	7.2	0.01	12.5	0.07	1.4	0.00	4.6	0.00	3.0	0.01	1.2	0.00	1.2	0.00	0.6	0.00	1.2	0.00
18:2ω6,9 (2)	3.1	0.01	0.9	0.00	0.2	0.00	2.0	0.00	1.0	0.00	0.2	0.00	0.4	0.00	0.3	0.00	0.4	0.00
18:1ω9	4.7	0.01	6.4	0.03	1.9	0.01	4.2	0.00	3.3	0.00	0.8	0.00	1.2	0.00	0.8	0.00	1.2	0.00
18:1ω7 (1)	4.0	0.01	3.3	0.01	10.9	0.01	6.3	0.01	7.8	0.00	11.5	0.01	10.1	0.00	13.1	0.00	10.1	0.00
18;0	8.4	0.01	9.0	0.02	3.0	0.00	8.2	0.00	7.3	0.01	2.3	0.00	3.2	0.00	1.9	0.00	3.2	0.00
19:1a	4.5	0.01	3.0	0.00	1.0	0.00	3.3	0.00	2.8	0.00	0.8	0.00	1.4	0.00	0.9	0.00	1.4	0.00
cy19 (1)	1.0	0.01	1.6	0.00	1.7	0.00	2.1	0.00	2.5	0.00	6.0	0.00	6.1	0.00	6.6	0.00	6.1	0.00
total PLFAs in nmolPLFA/g(dw)	1.04	0.14	3.06	1.71	1.48	0.29	1.55	0.18	1.70	0.17	4.04	0.89	4.01	0.92	6.76	0.78	7.12	1.34
bacterial PLFAs in nmolPLFA/g(dw)	0.45	0.12	0.97	0.46	0.42	0.07	0.53	0.05	0.61	0.12	1.03	0.23	0.88	0.14	1.44	0.11	1.68	0.25
fungal PLFAs in nmolPLFA/g(dw)	0.03	0.01	0.01	0.00	0.01	0.00	0.03	0.01	0.02	0.00	0.01	0.00	0.01	0.00	0.02	0.00	0.02	0.00
bacterial/fungal PLFAs	14.42	12.07	71.48	223.42	30.38	21.70	17.03	7.81	36.87	148.90	102.84	91.4	60.61	81.89	87.87	55.40	101.60	122.91

Table S3. Results of a series of two-way ANOVAs (with the 2 factors: particle size (PS) and matric potential (MP)), performed for bacterial PLFAs, fungal PLFAs (in nmolPLFA/g(dw)) and the bacterial:fungal PLFA ratio.

					T1				
		bacterial PLFAs			fungal PLFAs			bacterial/fungal PLFAs	
source of variation	df	F value	P	df	F value	P	df	F value	P
particle size size	2	234.9	< 0.001*	2	6.1	0.007*	2	16.2	< 0.001*
matric potential	2	31.3	< 0.001*	2	5.6	0.009*	2	21.0	< 0.001*
PS x MP	4	6.9	< 0.001*	4	4.3	0.008*	4	3.6	0.018*
Residuals	26			26			26		

					T2				
		bacterial PLFAs			fungal PLFAs			bacterial/fungal PLFAs	
source of variation	df	F value	P	df	F value	P	df	F value	P
particle size	2	9.2	0.001*	2	0.6	0.562	2	1.4	0.263
matric potential	2	3.6	0.041*	2	6.3	0.006*	2	3.8	0.036*
PS x MP	4	2.0	0.132	4	2.1	0.105	4	1.3	0.300
Residuals	26			26			26		

* indicates statistical significance (P values <0.05).

Table S4. Adjusted P values of the PLFA composition from post-hoc comparison according to Bonferroni correction for bacterial PLFAs, fungal PLFAs (in nmolPLFA/g(dw)) and the bacterial:fungal PLFA ratio.

		T1			T2		
factor		bacterial	fungal	bacterial/ fungal	bacterial	fungal	bacterial/ fungal
particle size	F-C	< 0.001*	0.054	0.004*	0.004*	1	0.590
	M-C	0.008*	0.121	0.068	1	1	1
	M-F	< 0.001*	1	0.829	0.017*	1	0.570
matric potential	I-D	1	0.820	1	0.250	0.047*	0.194
	W-D	0.280	0.560	0.003*	0.320	0.013*	0.058
	W-I	0.860	0.050*	0.004*	1	1	1

* indicates statistical significance (P values <0.05).

Table S5. Results of a series of two-way ANOVAs (with the 2 factors: particle size (PS) and matric potential (MP)), performed for ergosterol concentrations (in mg/kg).

	T1			T2		
source of variation	**df**	**F value**	**P**	**df**	**F value**	**P**
particle size	2	79.3	<0.001*	2	55.1	<0.001*
matric potential	2	10.3	0.004*	2	6.1	0.019*
PS x MP	4	17.9	<0.001*	4	8.6	0.003*
Residuals	10			10		

* indicates statistical significance (P values <0.05).

Table S6. Adjusted P values of the PLFA composition from post-hoc comparison according to Bonferroni correction for ergosterol concentrations (in mg/kg).

factor		**T1**	**T2**
particle size	F-C	<0.001*	<0.001*
	M-C	0.044*	0.028*
	M-F	0.140	0.066*
matric potential	I-D	1	1
	W-D	1	1
	W-I	1	1

* indicates statistical significance (P values <0.05).

Chapter 5

Habitat connectivity has contrasting impacts on bacterial and fungal diversity

Authors:
Alexandra B. Wolf, Wietse de Boer, Sarah J. Jennings,
Annemieke van der Wal, George A. Kowalchuk

Abstract

Soils harbour extremely diverse microbial communities that are involved in sustaining the life functions of terrestrial ecosystems. However, little is still known about the factors that allow for the maintenance of such highly diverse and functionally redundant communities in soil habitats. We hypothesized that decreasing habitat connectivity would allow for the maintenance of greater bacterial diversity due to greater physical separation of potentially competing populations. Given their ability to form hyphae and thereby bridge air-filled pores, fungi were not expected to exhibit such an inverse relationship between habitat connectivity and diversity. We examined the role of micro-habitat connectivity, as influenced by pore size distribution and matric potential, on bacterial and fungal diversity via a series of microcosms experiments in which these factors were systematically and independently regulated. Quartz sand microcosms were inoculated with soil suspensions containing bacteria and fungi or only bacteria, and community development was tracked over time by pyrosequencing of PCR-amplified fungal ITS regions and bacterial 16S rRNA genes. Both bacterial and fungal community compositions were affected by matrix properties, with grain size having a greater impact than moisture content. Bacteria and fungi, however, exhibited contrasting patterns of biodiversity across our connectivity gradient. Habitat connectivity was inversely correlated with bacterial diversity yet positively correlated with fungal diversity. Highly abundant groups of bacteria and fungi exhibited a range of responses to habitat connectivity, potentially related to their life-history strategies and abilities to resist desiccation. The presence of fungi also generally increased bacterial diversity and decreased bacterial community variability. In total, reduced habitat connectivity, as typically observed in non-saturated soils, was demonstrated to facilitate the maintenance of bacterial diversity.

Keywords: soil microbial communities – habitat connectivity – bacterial diversity – fungal diversity – high-throughput tag sequencing

5.1 Introduction

Soil habitats contain an unparalleled microbial diversity, which is of crucial importance to ecosystem functioning and sustaining terrestrial life. In fact, soils contain greater biodiversity than classical competition exclusion theories would predict (Hutchinson, 1961). However, the mechanisms that allow for this diversity to be maintained are poorly understood. Microbes constantly compete for resources, which has resulted in the evolution of a great variety of microbial interactions, and such interactions can impact community structure and ultimately diversity (Tiedje *et al.*, 2001). Although some microbial interactions are mediated via signals transmitted via the gaseous phase (*i.e.* via volatile compounds), most interactions related to resource competition occur in the aqueous phase. The 3-D geometry of soils and the distribution of water throughout the heterogeneous soil landscape therefore determine the extent to which microbial populations can interact. However, relatively few studies have examined how the physical structure of soil habitats contributes to the maintenance of microbial biodiversity (Vos *et al.*, 2013).

Most bacteria rely on water to gain access to nutrients, either by nutrient diffusion through water films or via active and/or passive motility towards nutrient sources (Abuashour *et al.*, 1994, Jiang *et al.*, 2006). In contrast, many fungi can grow hyphae into air-filled spaces, thereby allowing them to bridge air-gaps that separate different micro-habitats. Fungal exploration of soil habitats is therefore less dependent on the water content of soil (Griffin, 1985). Thus, hydraulic connectivity differentially impacts the ability of different organisms to interact and might be of far greater importance in determining microbial community interactions than the absolute distance between micro-habitats.

Habitat connectivity is determined by both the soil structure (pore geometry) and hydration status (as described in detail in Chapter 3). Low connectivity and habitat fragmentation has the potential to physically separate inferior species from superior competitors, thereby contributing to the maintenance of diversity (Hughes Martiny *et al.*, 2006). Under prolonged periods of increased connectivity, previously sheltered populations may lose this isolated status, allowing more competitive species to subsequently out-compete them. Soil moisture, coarseness and pore size distribution have been suggested to impact soil-borne microbial diversity, with lower soil connectivity generally judged to support higher levels of diversity (Carson *et al.*, 2010, Chau *et al.*, 2011). However, specific manipulation of connectivity often involves confounding effects, such as simultaneous manipulation of other soil parameters and physico-chemical soil properties, and diversity measures have typically been restricted to coarse methods of community examination, such as T-RFLP. Thus, direct and detailed experimental evidence for a relationship between soil micro-habitat connectivity and microbial diversity is still lacking.

Overall, there is no consensus whether fungi or bacteria are more susceptible to (changes in) soil moisture. It has been suggested that fungi are generally better adapted to dry conditions (e.g. Yuste *et al.*, 2011) and, as shown in Chapter 4, the hyphal growth form provides fungi with a relative advantage in poorly connected soils, due to their ability to bridge air-gaps. However, it has also been proposed that fungi will respond less to changes in moisture than bacteria because their chitinous cell walls make them generally more resistant and resilient to changes in moisture and temperature (Holland & Coleman, 1987). Alternatively, Frey and colleagues (1999) found a positive correlation of fungal biomass and fungal:bacterial ratios with soil moisture, while bacterial biomass remained relatively constant.

Due to the complex nature of most soils, it is difficult to manipulate specific soil parameters independently, thereby hampering efforts to deduce the relative importance of specific parameters related to soil connectivity on the microbial community. Soil connectivity can either be manipulated by changing the moisture content or by changing the soil structure. However, other parameters such as salinity, osmotic pressure, pH, nutrient levels are also changed when manipulating these factors, confounding subsequent interpretations related to the impact of soil connectivity on the community. Another option is to manipulate the pore size distribution, e.g. by adding solid particles such as sand or clay. However, this will change the physico-chemical properties of the soil. In the current study, we therefore used a well-defined microcosm system allowing for precise and independent manipulation of pore size distribution and matric potential, and consequently connectivity, without changing other soil parameters.

Our objective was to investigate the effect of habitat connectivity, as determined by pore size distribution and matric potential, on the structure and diversity of bacterial and fungal communities. Since lower connectivity should reduce bacterial competition, we hypothesized that low hydraulic connectivity would allow for the co-existence of otherwise competing species resulting in greater diversity. We expected the diversity of the fungal community to be less affected by habitat connectivity due to their ability to bridge air-gaps. Furthermore, we expected treatments with different moisture levels to select for different bacterial and fungal communities because of variance in tolerance to drought and moisture stress. Our experiment consisted of two parts: in the “bacterial experiment” we introduced only bacteria into the microcosms and in the “fungal-bacterial experiment” we co-inoculated the microcosms with both fungi and bacteria. This experimental setup allowed us to investigate the bacterial and fungal community structure as affected by habitat connectivity, as well as to determine the effect of fungi on the bacterial community. Our study design consisted of a series of microcosm experiments with treatments of different connectivity levels (high moisture and fine pores = high connectivity and low moisture and coarse pores = low connectivity) in conjunction with a next-generation sequencing approaches to analyse the bacterial and fungal community structures and diversity over time.

5.2 Material and Methods

5.2.1 Preparation and inoculation of microcosms

Soil-like microcosms were constructed using quartz sand particles of different sizes, obtained by milling acid washed sea sand (Honeywell Speciality Chemicals Seelze GmbH, Seelze, Germany) for 10 min followed by fractionation of the particles into size classes by sieving as described by Wolf *et al.* (2013). These particles of different size fractions were used to create three distinct soil types: "fine" (sand particles 53-106 µm), "medium" (106-212 µm), and "coarse" (212-425 µm), thereby creating a range of pore size classes across the different artificial soils. Microcosms were established in 50 mL serological glass vials sealed with rubber stoppers to which 10 g quartz sand of the respective particle size range was added. Microcosms were sterilized by autoclaving followed by oven-drying. The moisture content was set to matric potentials of -10 ("wet"), -20 ("intermediate") and -50 kPa ("dry"). Each particle size class was combined with each matric potential in a full factorial design, resulting in 9 treatments.

Artificial root exudate (ARE) pH 5.8 (composition of ARE stock solution: 18.4 mM glucose, 18.4 mM fructose, 9.2 mM saccharose, 4.6 mM citric acid, 9.2 mM lactic acid, 6.9 mM succinic acid, 18.4 mM L-serine, 11 mM L-glutamic acid and 18.4 mM L-alanine (C/N ratio 10.4) and 10 mM KH_2P0_4, pH adjusted to 5.8 with 5N NaOH; the stock solution was filter sterilized, aliquoted in 40 ml portions and stored at -20°C; working solution was prepared by mixing 1 mL stock solution with 2 ml of 10 mM phosphate buffer (pH 5.8) after Baudoin *et al.* (2003) with modification of the pH was used as growth medium and microcosms were incubated at 20°C. Each microcosm was inoculated with 100 µL soil suspension corresponding to approximately 10^3 bacterial cells in the bacterial inoculum as determined by plate counting and incubated at 20°C until destructive sampling at 5 and 14 days.

The soil suspension inoculum was prepared by dispersing 50 g field wet soil collected from a former arable field site located near Ede, the Netherlands (52°04′N, 5°45′E; see van der Putten *et al.* (2000) for a detailed description of the soil characteristics) in 450 mL phosphate buffer (pH 5.8) by shaking for 30 min and sonicating (Branson 5210 ultrasonic bath) twice for 1 min. We let solid particles settle and then used the supernatant as inoculum. For the bacterial community experiment, containing only bacteria and no fungi, hereafter referred to as the bacterial experiment, the suspension was filtered sequentially through filters with successively smaller pore diameter (11, 8, 6 and 3 µm) to remove soil particles and the fungal community (Whatman filter papers 1 Cat No 1001-150, 102-150, 1003-150, and Whatman Cellulose Nitrate Membrane Filters 7193-002). For the experiment with bacteria and fungi, hereafter referred to as fungal-bacterial experiment, we used the unfiltered supernatant as inoculum.

The soil suspension was serial diluted and plated on 10% tryptic soy broth agar plates (Oxoid) to determine CFUs of the inoculum (3 replicates). Two mL suspension (inoculum) were sampled, cells were spun down, frozen, DNA extracted to determine bacterial community of the inoculum (5 replicates for the bacterial inoculum and the fungal-bacterial inoculum each). Sampling was performed destructively after inoculation (T0), after 5 days (T1) (4 replicates) and after 14 days (T2) (4 replicates) and samples were stored at -80°C until further analysis. Sampling points were based on data of a pilot experiment in which CO_2 concentration was tracked over time in the different treatments (see Fig. S1); T1= active community, T2= stationary phase community.

All 5 replicates of the extracted DNA from the inoculum needed to be pooled in order to obtain enough DNA for PCR amplification. For comparison, we also extracted and sequenced the DNA from the soil that was used to prepare the inoculum (n= 5). The time point T0 was omitted from the analysis as we could not obtain sufficient amounts of DNA from these very low microbial biomass samples.

5.2.2 CO_2- and glucose measurements

At days 0, 1, 2, 3, 4, 5, 6, 9 and 14 of the incubations, the headspace CO_2 concentrations in the microcosms were measured in 3 randomly selected replicates for each treatment. Approximately 25 µL of headspace volume was sampled with a Pressure-Lok® syringe (Vici Precision Sampling) and examined by gas chromatography-thermal conductivity detection analysis (TraceGC Gas analyser, Thermo Scientific). Reference CO_2 gas (1,200 ppm, Westfalen AG) was used in order to calculate CO_2 concentrations.

At T1 and T2, glucose measurements were performed on the artificial soil samples using the Glucose (GO) Assay Kit (Sigma-Aldrich) in order to estimate the microbial consumption of glucose. Glucose was extracted from 1 g microcosm material by adding 2 mL deionised water and shaking at 200 rpm for 1 h. The solution was then filtered through 0.2 µm filter to clarify the solution. Glucose concentrations were determined following the manufacturer's protocol.

5.2.3 Molecular analyses: DNA extractions and bacterial 16S rRNA gene and fungal ITS region amplification

The total genomic DNA was extracted from 0.25 g soil (n= 5), the bacterial and the fungal-bacterial inoculum (each n= 5), and 0.25 g artificial soil sample from a total number of 154 microcosms (2 experiments x 9 treatments x 4 replicates x 2 time points + 2 x 5 inoculum) using the MO BIO PowerSoil DNA isolation kit following the manufacturer's protocol with the modification of heating the sample to 60°C for 10 min after the addition of solution C1, and using simultaneous addition of 100 µL each of solutions C2 and C3.

A portion of the 16S rRNA gene spanning the V3 region was amplified from the extracted DNA using bacterial primers previously described (Bartram *et al.*, 2011). PCR amplifications were performed using 25 µL 2x Phusion Master Mix Finnzymes, 2.5 µL forward primer, 2.5 µL reverse primer, ~5 ng/ µL DNA template, filled up to 50 µL with H_2O in a thermal cycler (C1000 Touch™ Thermal Cycler, Bio-Rad; conditions: 2 min at 98°C, 25 cycles of 10 s 98°C, 30 s 50°C, 15 s 72°C, followed by 7 min at 72°C). The PCR products were excised from 1.3% agarose gels (Certified PCR Low Melt Agarose, Bio Rad Laboraties) and purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. PCR amplicons of each sample were pooled at approximately equal amounts and sequenced on the Illumina HighSeq 2000 platform, by BGI.

Part of the fungal 5.8S gene region and the internal transcribed spacer 2 (ITS) region was amplified with the primer pair ITS1F and ITS4 (Ihrmark *et al.*, 2012). PCR amplification was conducted in a thermal cycler (C1000 Touch™ Thermal Cycler, Bio-Rad) in 50 µL reactions [0.25 ng μL^{-1} template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, primers at 200 nM, 0.025 U μL^{-1} polymerase in buffer, 5 min at 94 °C; 35 cycles of (30 s at 94°C; 30 s at 55°C; 30 s at 72°C); 7 min at 72°C]. The PCR products were verified on 1.5 % agarose gel and then purified and subjected to 454-sequencing by Macrogene.

5.2.4 Sequence analysis: Bacterial sequences

Illumina HiSeq 2000 150 cycle paired-end sequencing returned a total of 36,369,216 raw sequences from 115 samples (2 experiments á 9 treatments x 4 replicates x 2 time points + 5 inoculum resulting in a total of 149 samples, of which 115 were successfully processed) sequenced over two lanes. The sequences were quality filtered (minimum q:25, no N's), and a consensus sequence prepared with PandaSeq (threshold: 0.9, minimum overlap: 50, length: 173-200 bases) (Masella *et al.*, 2012). An OTU (Operational Taxonomical Unit) table was prepared in QIIME (Caporaso *et al.*, 2010) with the pick_subsampled_reference_otus_through_otu_table.py (minimum sequences per otu: 50, otu-picking method: uclust_ref (Edgar, 2010) pipeline, using the SILVA 108 rRNA reference database (Quast *et al.*, 2013) with the two lanes run in series. Sequences were aligned (Caporaso *et al.*, 2010) and taxonomy assigned using RDP classifier 2.2 (Wang *et al.*, 2007). All downstream analyses utilised an OTU table rarefied to 10,000 sequences.

5.2.5 Fungal sequences

A total of 1,481,726 partial ITS2 gene sequences were obtained from 63 samples (9 treatments x 4 replicates x 2 time points + 5x inoculum resulting in a total 77 samples, of which 63 were successfully processed) and transferred to the Galaxy interface (Goecks *et al.*, 2010), de-multiplexed and further analyzed with the QIIME pipeline version 1.6. Sequences with a minimum length of 200 bp, a maximum of 6 ambiguous bases, 6

homopolymer runs, zero primer mismatches, a maximum of 1.5 errors in the barcode sequence and passed a quality score window of 50 were binned according to sample id and the barcodes were removed. Then, the Qiime denoiser version 1.6.0 (Reeder & Knight, 2010) was used to correct for sequencing errors and chimeras were removed by IME (Edgar, 2010). Sequences were aligned by Muscle (Caporaso *et al.*, 2010) and UCLUST (Edgar, 2010) was used to assign sequences to OTUs, using a minimum sequence identity cutoff of 97%. From all OTU clusters the most abundant sequence was selected as a representative for taxonomy assignment by using the UNITE / Qiime 12_11 reference database with a minimum identity value of 75%. Singletons were excluded from the data set and the OTU table was rarefied to 2,000 sequences.

5.2.6 Statistical analysis

To assess the effects of particle size and matric potential on the bacterial and fungal communities, we compared bacterial and fungal OTU richness and diversity across the different treatments. First, OTU richness was estimated using Chao-1 estimation (Chao, 1984), and diversity was estimated using the Shannon's H index in PAST 2.17 (Hammer *et al.*, 2001). We then performed two-way ANOVAs followed by bonferroni correction in R (v 2.14.1; www.r-project.org) to determine how richness and diversity of fungal and bacterial communities were affected by particle size and matric potential, as well as to test for interactive effects between these two factors. Principal component analysis (PCA) was used to compare microbial community structures from different treatments based on fungal as well as bacterial sequencing data. Redundancy analysis (RDA) was used to test if the compositions of bacterial and fungal communities were related to particle size and matric potential. Significance of canonical axes was assessed by the forward approach using Monte Carlo permutation tests under the reduced model (Legendre et al., 2011). PCA and RDA were performed in Canoco version 5 (Microcomputer Power, Ithaca, NY).

5.3 Results

5.3.1 CO_2- and glucose measurements

In the bacterial experiment, CO_2 production started approximately at day 4, whereas in the fungal-bacterial experiment CO_2 production began at day 2 (Fig. S1). CO_2 concentrations in the headspace of microcosms plateaued at day 6 in most treatments in both the bacterial as well as the fungal-bacterial experiment (Fig. S1). Calculations of the percentage of originally added C that was respired showed that after 5 days between 6 and 19% percent was respired in the bacterial experiment and 13 and 21% C in the bacterial fungal experiment. After 14 days, these values were 10-23% and 14-23%, for the bacterial and

bacterial fungal experiments, respectively (Table S1). In the bacterial experiment, there were no significant differences in respired C between particle sizes (P= 0.162), matric potential (P= 0.098) and time (P= 0.200). In the fungal-bacterial experiment, however, significantly more C was respired in the fine as compared to coarse treatments (P= 0.016), but, like in the bacterial experiment, there was no difference between different matric potentials (P= 0.29) and time points (P= 0.16). Overall, more C was respired in the fungal-bacterial experiment than in the bacterial experiment (P= 0.037).

Both in the bacterial and the fungal-bacterial experiment, glucose levels were depleted in all treatments by day 6 (Fig. S2). We did not measure other sugars or amino- and organic acids, using glucose as a proxy of total nutrient levels.

5.3.2 Sequencing: Soil and inoculum

The bacterial and fungal taxonomic compositions of the soil used to prepare the inoculum, as well as that of the inocula of the bacterial and fungal-bacterial experiments, are given in Fig. S3. No statistical significant differences in bacterial diversity (Shannon's H) or richness (Chao1) was observed between the soil and the inoculum (all P= 1). Likewise, no statistically significant differences in fungal diversity (Shannon's H) and richness (Chao1) were observed between the soil and the inoculum ($P_{diversity}$= 0.754 and $P_{richness}$= 0.734).

5.3.3 Fungal and bacterial community composition and diversity

In the fungal-bacterial experiment, principal component analysis (PCA) of log transformed data revealed differences in fungal community composition across the different treatments. At T1, the fungal community composition in treatments with fine particle sizes clustered together on the left side in the PCA plot, whereas fungal community composition in treatments with coarse particle sizes grouped together on the right side in the PCA plot (Fig. 1). Samples in treatments with intermediate particle sizes showed overlap in fungal community composition across treatments of different particle sizes. For T2, a grouping based on coarse particles sized could be observed, but there was no clear grouping related to medium and fine particle sizes (Fig. 1). For both time points, no clear clustering of samples was apparent in relation to matric potential. For T1, forward selection in the Redundancy Analysis showed that the fungal community composition was significantly correlated with fine particle sizes (P= 0.002) and wet matric potentials (P= 0.002). For T2, the fungal community composition was again significantly correlated with fine particle sizes (P= 0.008) and wet matric potentials (P= 0.004) (Fig. A8). PCA also revealed differences in bacterial community composition among treatments in the fungal-bacterial experiment (Fig. 1). For T1, samples in treatments with fine and medium particles showed overlap in bacterial composition and clustered together on the left side in the PCA plot, whereas bacterial community composition from the coarse treatments grouped together

on the right side in the PCA plot (Fig. 1). For T2, the same grouping of treatments could be observed. For both time points, no clear clustering of samples with similar matric potential could be found. For T1, forward selection in the Redundancy Analysis showed that the bacterial community composition was significantly correlated with coarse particle sizes (P= 0.002) and “dry” matric potentials (P= 0.002). For T2, bacterial community composition was significantly correlated with coarse (P= 0.002) and medium particle sizes (P= 0.002) (Fig. S8).

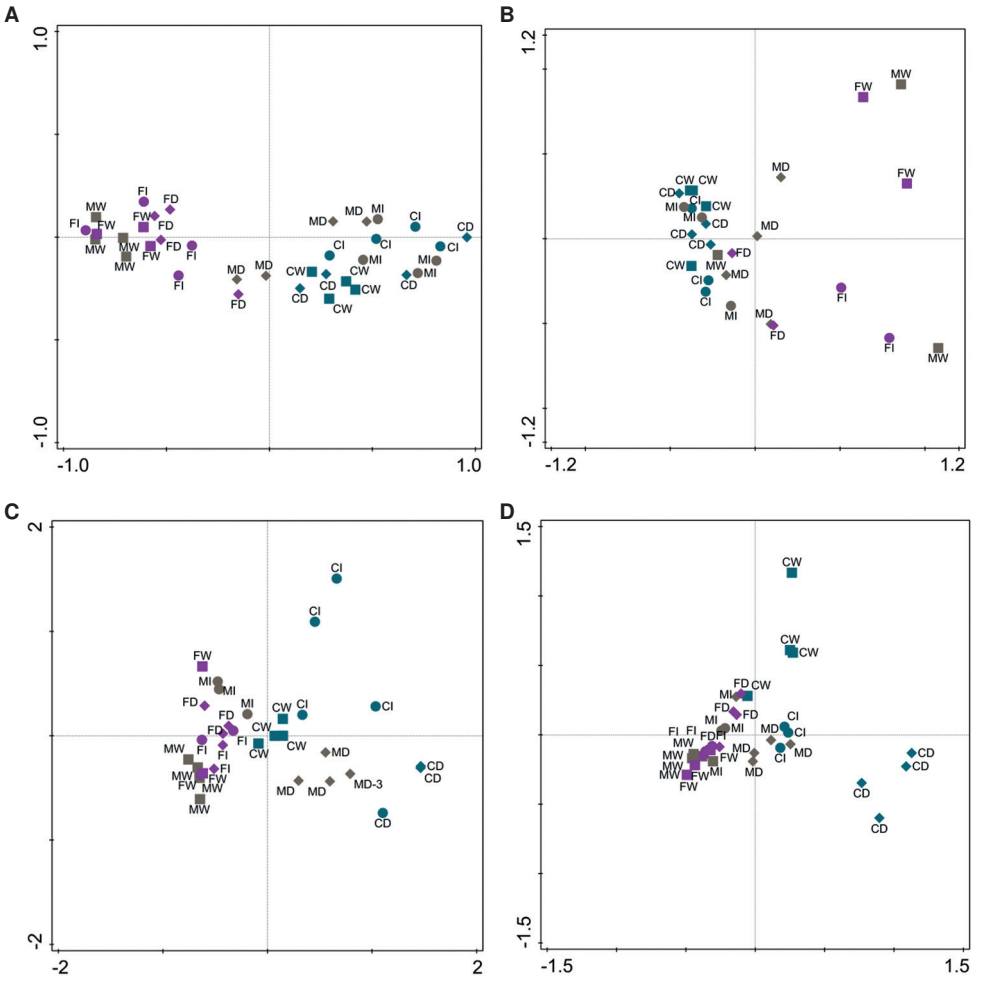


Figure 1. PCA plots of fungal species (A and B) and bacterial phyla (C and D) in the fungal-bacterial experiment at T1 (A and C) and T2 (B and D) as determined by pyrosequencing. A: fungi T1 PC1: 32.6%, PC2: 16.3%, B: fungi T1 PC1: 21.2%, PC2: 14.4%, C: bacteria T1 PC1: 43.5%, PC2: 21.8%, D: bacteria at T2: PC1 18.9%, PC2 10.1%.

A comparison of fungal OTU richness (Chao1) and Shannon diversity by ANOVA followed by bonferroni correction revealed that both the richness and diversity of the fungal communities was greatest in the fine particle treatments. Matric potential had no effect on the richness of fungal communities (Figs. 2, 3 and Tab.1, S2 and S3). At T1, fungal diversity was greater in the wet treatments than in the intermediate and dry treatments, but at T2 there was no significant effect of matric potential on fungal diversity (Figs. 2, 3 and Tab.1, S2 and S3). A comparison of bacterial OTU richness (Chao1) and Shannon diversity by ANOVA followed by bonferroni correction revealed greater bacterial diversity and richness in the coarse particle treatments as compared to the medium- and fine particle ones (Tab. S3). There was no effect of matric potential on diversity, but bacterial richness was greatest in dry treatments at T1, whereas at T2 there was no effect of matric potential on diversity (Figs. 2 and 3). Overall, particle size affected both the fungal and bacterial communities more than matric potential.

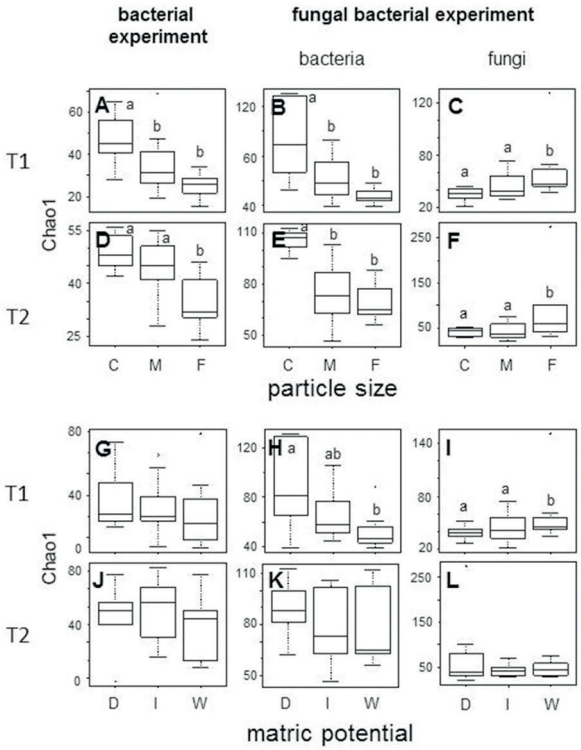


Figure 2. Bacterial and fungal richness (Chao1) in relation to pore size and moisture at T1 and T2 for the bacterial (left-most column of figures) and the fungal-bacterial experiment. The different particle sizes are given by C= coarse, M= medium and F= fine, and moisture levels are given as D= dry, I= intermediate and W= wet. The median is indicated at the midpoint, the hinges indicate the upper and lower quantities, and the lines represent the spread. Statistically different values within a panel are denoted by different letters.

A comparison of the most abundant OTUs among treatments by ANOVA followed by bonferroni correction showed that specific fungal and bacterial OTUs were affected differently by different particle sizes (Tab. S4 and S5). For instance, *Trichoderma hamatum* and zygomycete sp. were more abundant in coarse treatments, whereas *Fusarium oxysporum* was most abundant in fine treatments (Fig. 4). Similarly, bacterial OTUs showed variation across our particle size treatments, with some OTUs being more abundant in medium and fine treatments, whereas others being more abundant in coarse soils (Fig. 5). For most fungal and bacterial OTUs, there was no significant difference among matric potentials (Figs. 4 and 5). Thus, particle size again appeared to have a larger effect on the relative density of the most abundant fungal and bacterial populations.

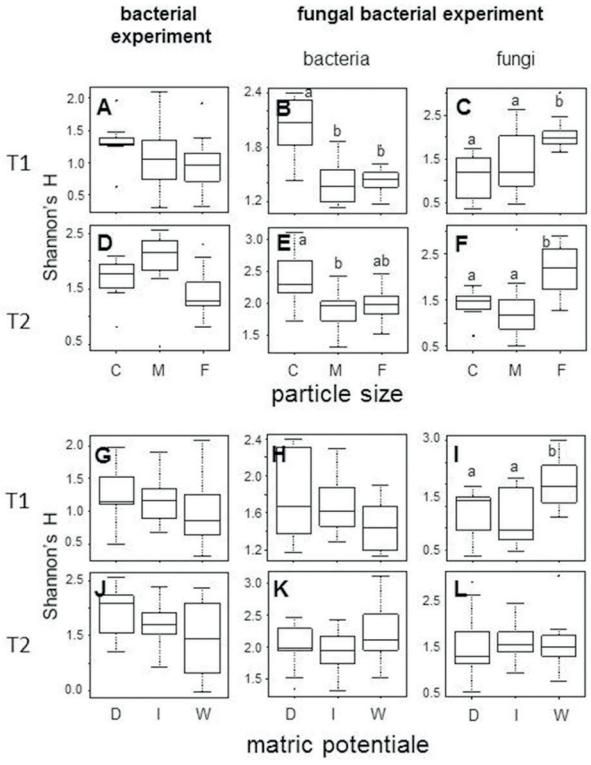


Figure 3. Bacterial and fungal diversity (Shannon's H) in relation to particle size and matric potential at T1 and T2 in the bacterial and the fungal-bacterial experiment. The different particle sizes are given by C= coarse, M= medium and F= fine, and matric potentials are given as D= dry, I= intermediate and W= wet. The median is indicated at the midpoint, the hinges indicate the upper and lower quantities, and the lines represent the spread. Statistically different values within a panel are denoted by different letters.

In comparison to the fungal-bacterial experiment, the impacts of connectivity parameters on the bacterial community composition, as revealed by PCA, were less pronounced In the bacterial experiment (Fig. 6). For T1, forward selection in the Redundancy Analysis

showed that the bacterial community composition was significantly correlated with coarse particle sizes (P= 0.01) and "wet" matric potentials (P= 0.022). For T2, bacterial community composition was significantly correlated with fine particles (P= 0.01) and "dry" treatments (P= 0.002) (Fig. S9).

Table 1. Richness and diversity indices (Chao1 and Shannon index) for bacterial OTUs of soil, inoculum and treatments in bacterial as well as bacterial fungal experiment. The different particle sizes are given by C= coarse, M= medium and F= fine, and matric potentials are given as D= dry, I= intermediate and W= wet.

				Chao-1		Shannon_H	
	particle size	matric potential	time [days]	average	standard error	average	standard error
soil	NA	NA	0	195.25	2.65	3.43	0.05
inoculum bac exp	NA	NA	0	153.00	NA	2.97	NA
inoculum fung-bac exp	NA	NA	0	135.67	3.34	2.98	0.11
bacterial exp	F	W	5	25.75	5.85	1.09	0.37
	F	W	14	30.00	3.30	0.80	0.13
	F	I	5	27.00	3.34	1.01	0.13
	F	I	14	34.00	2.03	1.33	0.15
	F	D	5	26.75	0.65	1.18	0.25
	F	D	14	36.67	5.36	1.61	0.21
	M	W	5	35.75	9.83	1.02	0.33
	M	W	14	42.00	3.57	2.10	0.49
	M	I	5	49.33	2.33	2.02	0.16
	M	I	14	29.75	1.95	1.19	0.22
	M	D	5	40.00	3.37	1.11	0.15
	M	D	14	49.00	3.54	2.40	0.12
	C	W	5	37.00	4.03	1.06	0.17
	C	W	14	46.67	3.03	1.50	0.31
	C	I	5	51.67	3.81	1.35	0.05
	C	I	14	50.75	1.98	1.77	0.11
	C	D	5	65.00	NA	1.97	NA
	C	D	14	NA	NA	NA	NA
fungal-bacterial exp	F	W	5	43.33	2.33	1.47	0.02
	F	W	14	62.00	3.00	1.99	0.07
	F	I	5	50.00	3.14	1.56	0.09
	F	I	14	64.67	2.73	1.85	0.04
	F	D	5	48.33	5.21	1.30	0.06
	F	D	14	76.25	5.36	2.11	0.18
	M	W	5	45.00	2.58	1.16	0.02
	M	W	14	62.00	2.27	1.89	0.11
	M	I	5	55.33	2.67	1.41	0.05
	M	I	14	70.00	8.76	1.94	0.20
	M	D	5	81.25	4.17	1.63	0.09
	M	D	14	90.25	4.33	1.81	0.13
	C	W	5	65.50	7.71	1.74	0.09
	C	W	14	106.75	3.94	2.71	0.14
	C	I	5	87.50	6.90	2.02	0.13
	C	I	14	104.33	1.20	2.03	0.13
	C	D	5	129.50	0.87	2.34	0.02
	C	D	14	105.67	5.04	2.20	0.11

fungal diversity

				Chao-1		Shannon_H	
	particle size	matric potential	time [days]	average	standard error	average	standard error
soil	NA	NA	0	487.67	26.03	4.64	0.07
					0.00		0.00
inoculum fung-bac exp	NA	NA	0	452.40	26.32	4.39	0.20
					0.00		0.00
fungal-bacterial exp	F	W	5	71.25	23.02	2.35	0.22
	F	W	14	59.00	12.02	1.50	0.16
	F	I	5	55.50	4.26	2.03	0.08
	F	I	14	38.00	4.24	2.21	0.16
	F	D	5	49.75	6.17	1.82	0.07
	F	D	14	188.00	61.52	2.76	0.10
	M	W	5	55.25	2.43	2.26	0.14
	M	W	14	44.75	10.03	0.88	0.51
	M	I	5	47.67	8.67	0.86	0.05
	M	I	14	42.33	10.89	1.23	0.14
	M	D	5	35.00	1.77	1.11	0.23
	M	D	14	30.00	4.78	0.90	0.16
	C	W	5	38.25	1.88	1.42	0.10
	C	W	14	38.00	4.29	1.29	0.17
	C	I	5	32.00	3.98	0.70	0.14
	C	I	14	46.33	2.99	1.55	0.04
	C	D	5	36.50	3.09	1.18	0.28
	C	D	14	48.50	10.36	1.43	0.11

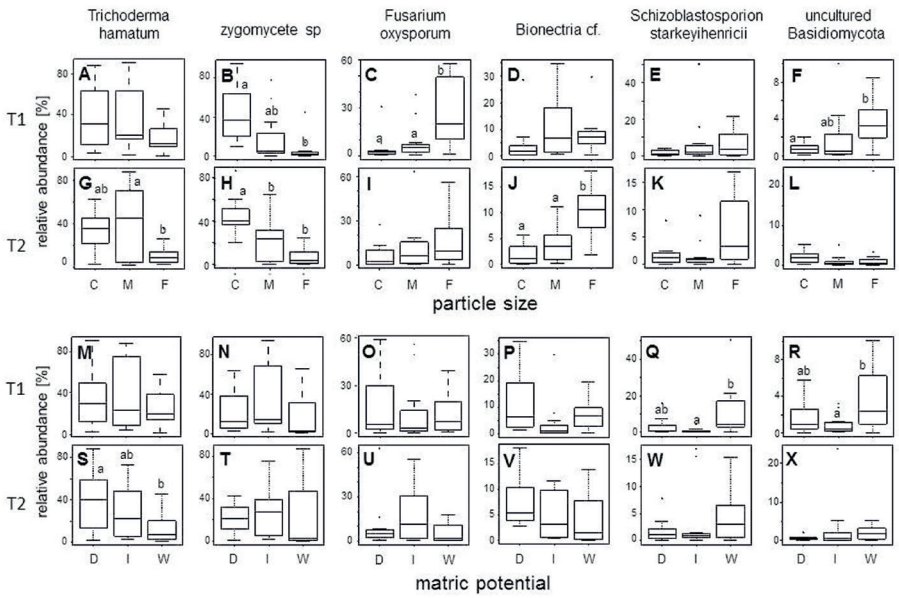


Figure 4. Overview of the 6 most abundant fungal taxa. Boxplots depict the relative abundances of 6 dominant fungal groups at the different particle sizes (A-L) and matric potentials (M-X) at T1 and T2. Different small letters (in A-H) indicate statistical differences based on bonferroni ($P < 0.05$), no significant

differences were found in B-H). The different particle sizes are given by C= coarse, M= medium and F= fine, and matric potentials are given as D= dry, I= intermediate and W= wet. The median is indicated at the midpoint, the hinges indicate the upper and lower quantities, and the lines represent the spread. Statistically different values within a panel are denoted by different letters.

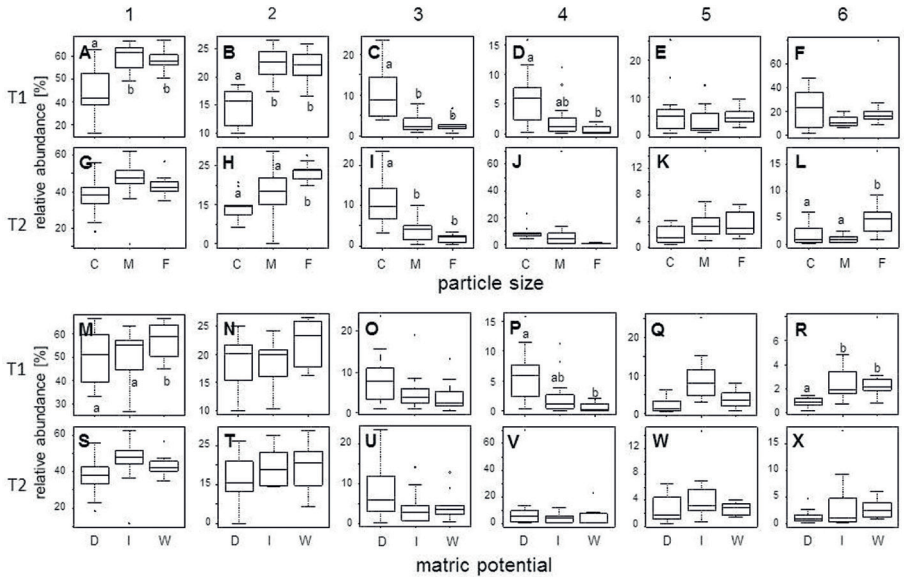


Figure 5. Overview of the 6 most abundant bacterial taxa in the fungal-bacterial experiment. Boxplots depict the relative abundances of 6 dominant bacterial groups in the fungal-bacterial experiment at the different particle sizes (A-L) and moistures (M-X) at T1 and T2. 1: Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas, 2: Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Other, 3: Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Other, 4: Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Other;Other, 5: Bacteria;Firmicutes;Bacilli;Bacillales;Paenibacillaceae;Paenibacillus, 6: Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Flavobacterium. Different small letters (in A-D, H, I, M, P and R) indicate statistical differences based on bonferroni ($P < 0.05$), no significant differences were found in G, J, K, N, O, Q, and S-X). The different particle sizes are given by C= coarse, M= medium and F= fine, and matric potentials are given as D= dry, I= intermediate and W= wet. The median is indicated at the midpoint, the hinges indicate the upper and lower quantities, and the lines represent the spread. Statistically different values within a panel are denoted by different letters.

Overall, clustering of bacterial communities according to treatment was much stronger in the fungal-bacterial experiment as compared to the bacterial experiment. This suggests an effect of fungi on structuring the composition of bacterial communities. A comparison of bacterial OTU richness (Chao1) and Shannon diversity by ANOVA followed by bonferroni correction revealed no significant effect of matric potential on bacterial richness and diversity and no effect of particle size on diversity for T1 and T2 in the bacterial experiment. Particle size did, however, have a significant effect on OTU richness at T1 and T2 (greater richness in coarse treatments). Moreover, no significant interactive effect was found between matric potential and particle size on OTU richness and Shannon diversity (Tab. S2).

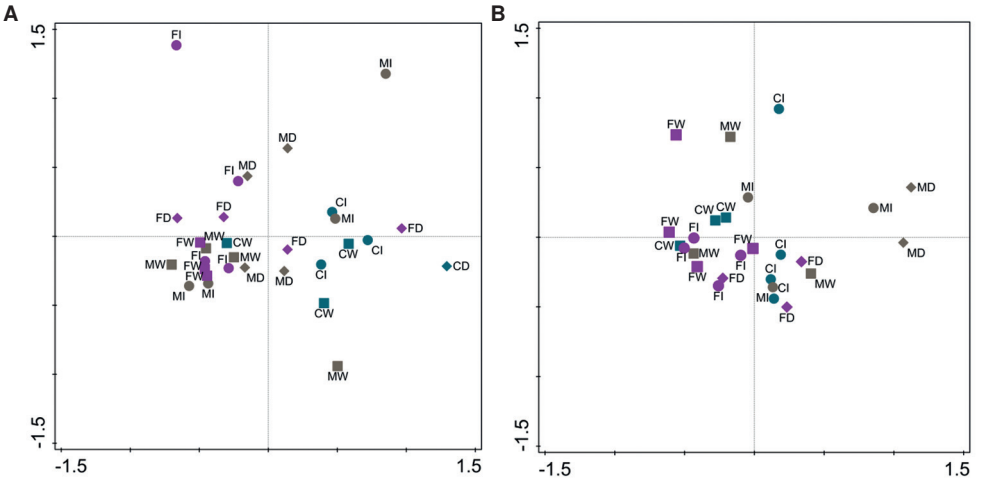


Figure 6. PCA plots of bacterial phyla in the bacterial experiment at T1 (A) and T2 (B) as determined by pyrosequencing. T1: PC1 explains 28.8% and PC2 explains 20.1%, T2: PC1 explains 21.6& and PC2 explains 12.6%.

5.4 Discussion

In the current study, we investigated the effect of habitat connectivity, as determined by particle size (*i.e.* pore size distribution) and matric potential, on the phylogenetic composition and diversity of bacterial and fungal soil communities using soil-like microcosms in conjunction with next-generation sequencing. We observed that both bacterial and fungal diversity and community structure were affected by connectivity. Interestingly, bacterial diversity was greatest in poorly connected soils with coarse particles and low moisture, while we found a contrasting effect on fungal diversity with greater diversity in more connected soils with fine particles and high moisture content. We also found that specific bacterial and fungal taxa were affected differently by particle size, e.g. some were more abundant in fine treatments, while others were more abundant in coarse treatments. Additionally, our experiments showed that, in general, particle size had a greater impact than matric potential on the structure, richness and diversity of both bacterial and fungal communities.

In our experiments, CO_2 production started approximately at day 2 when both bacteria and fungi were present and at around day 4 when the community consisted of only bacteria (Fig. S1). This difference in the onset of CO_2 production may be attributed to the fact that the community needed to adapt to the new environmental conditions after being introduced into the sand microcosms. There was more total microbial biomass in the fungal-bacterial inoculum, which may explain why CO_2 production started earlier in the

fungal-bacterial experiment. This may also have contributed to the greater total efflux of CO_2 from the fungal-bacterial communities. CO_2 concentrations in the headspace of microcosm plateaued at day 6 in most treatments in both the bacterial as well as in the fungal-bacterial experiment (Fig. S1). By day 5, the all added nutrients were consumed. This data confirms that we sampled an active community at T1 (= 5 days), whereas the sampling at T2 (= 14 days) served to determine the maintenance of diversity after the original nutrients were depleted.

We identified that particle size had a significant effect on the richness and diversity of both bacterial and fungal communities, whereas moisture only significantly affected fungal diversity and richness, but not bacterial (except diversity of bacteria in the fungal-bacterial experiment at T1) (Figs. 2 and 3). This was surprising, as we had expected little effect of moisture on fungal diversity due to their ability to bridge air gaps, which makes them less dependent on water than bacteria. However, low fungal diversity in dry habitats might be attributed to fewer species being particularly well adapted to drought conditions or may be a result of a selection for species that are most efficient in extending and exploiting their hyphal networks.

Overall, the impact of particle size was greater than the impact of matric potential. Soil connectivity is a function of both soil structure (pore size distribution) and matric potential. Coarse soils contain more large pores that are air-filled at most matric potentials, with water occurring predominately as disconnected water films. Our results support the hypothesis that increased bacterial diversity may be maintained by increases in (micro-)habitat fragmentation, which would result in reduced interspecific competition. Furthermore, limitation of substrate diffusion in poorly connected environments, which maintains a range of nutrient gradients resulting in a variety of micro-niches, may be a mechanism by which high diversity is maintained (Long & Or, 2009). Moreover, the diffusion of nutrients, which is determined by an interaction between pore size and moisture, is reduced in poorly connected soils, thus making nutrients less “reachable” for more competitive species, thereby potentially promoting the survival of less-competitive species. Coarse soils thus provide more disconnected water films in soil, which may increase diversity according to the spatial isolation hypothesis (Zhou *et al.*, 2002).

Our findings are in line with other studies providing experimental evidence that decreased connectivity stimulates bacterial diversity by allowing inferior competitors, which would otherwise be outcompeted in well-connected soils, to persist in low connected soils (Carson *et al.,* 2010). By modifying the pore size distribution and water potential of an unsterilized soil, these authors examined the impact of decreasing connectivity on community structure. T-RFLP analysis indicated that drier conditions resulted in more diverse bacterial communities. Chau and colleagues (2011) also observed that bacterial species richness, again determined via T-RFLP, increased significantly with soil coarseness (quantified

as % sand), which was attributed to the increased number of isolated water films that are likely to occur in coarser soils. In our study, we used a well-defined experimental system, instead of real soil, which allowed us to manipulate matric potential and pore size distribution independently. Thus, the observed responses of the microbial communities could be directly attributed to the variation of the specific parameter under study and not to other soil characteristics that might inadvertently be co-manipulated, which is unavoidable when working with natural soils. Also, the next-generation sequencing approaches applied allowed us to examine the community structures in depth across multiple biological replicates over time. Furthermore, our experimental design is highly replicated, providing the necessary statistical power to examine impacts on community structure and diversity. Overall, our findings suggest that habitat connectivity plays an important role in structuring of soil-borne microbial communities and that low connectivity indeed promotes bacterial diversity, as predicted in our initial hypothesis.

We observed contrasting effects of connectivity on bacteria and fungi: while bacterial diversity and richness was greater in less connected microcosms, fungal richness and diversity was greater in fine, *i.e.* more connected, habitats at both time points (Figs. 2 and 3). A possible explanation may be that fungi cannot access the finest pores due to the diameter of their hyphae. Very fine soils may therefore be more connected for bacteria, but actually provide less habitat variety for fungi. Habitat conditions with a lower proportion of water-filled pores may also provide a relatively strong selection for desiccation-resistant fungi, therefore resulting in lower fungal diversity under these conditions.

We found bacterial richness and diversity to be greater in the mixed fungal-bacterial communities as compared to communities only containing bacteria ($P_{richness} < 0.001$ and $P_{diversity} < 0.001$). We also observed greater variation of bacterial community composition between replicates when no fungi were present (see Figs. S5 and S6). Furthermore, bacterial community structure was only significantly influenced by particle size in the presence of fungi. These findings suggest a “stabilizing” effect of fungi on the composition of the bacterial community. Fungi may influence bacterial mobility, as bacterial may be able to travel along fungal hyphae (Warmink *et al.*, 2011b). Fungi may also influence the availability of nutrients, thereby creating less variation among pores, resulting in less variation within the bacterial community. It should be noted that our fungal-bacterial experiment also contained other microorganisms, whose dynamics were not followed during our experiment. Thus, one cannot rule out the possibility that other microorganisms also had an impact on bacterial diversity and variability.

Additionally, our experiments demonstrated that several bacterial and fungal OTUs were affected differently by the pore size-classes and matric potentials examined in our study (Figs. 4 and 5). For instance, some fungal species were most abundant in systems with coarse pores (e.g. *Trichoderma hamatum* and zygomycete sp.), whereas others were most abundant in environments with fine pores (e.g. *Fusarium oxysporum*). An explanation may

be that different species prefer the specific environmental conditions provided in these pore size distributions, e.g. the preferred pore size may depend on the diameter of the hyphae or ability to negotiate a given pore matrix. As observed for the total communities, we found that pore size had a greater impact than matric potential on the relative abundance of the most abundant bacterial OTUs. Similar to fungi, several specific bacterial OTUs were either more or less abundant in specific pore sizes (Fig. 5).

We harvested our microcosm systems at two different time points in an effort to compare highly active communities with those that had experienced a nutrient depleted situation for some time. In general, we found similar trends at both time points, with particle size being more important than matric potential to microbial respiration, the relative abundance of the most dominant bacterial and fungal taxa and the total structure and diversity of the microbial communities, although total community composition and diversity did show some development over time. Given the focus on habitat connectivity on the maintenance of biodiversity, longer term studies in such systems represent an important future research priority. Furthermore, our experimental system holds potential to study the impact of other environmental factors such as different nutrients on diversity, as well as testing ecological theories such as the intermediate disturbance hypothesis.

5.4.1 Conclusions

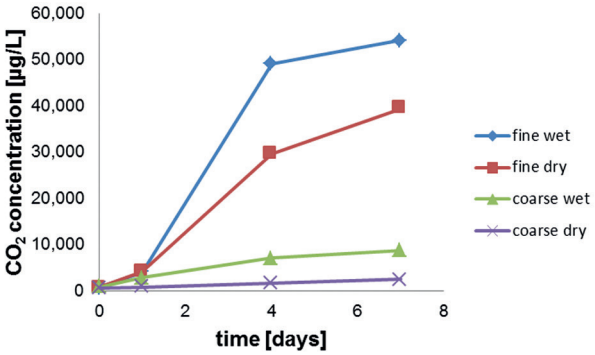
Both pore size and matric potential have a significant role in determining the composition, richness and diversity of soil bacterial and fungal communities, and these factors also influence how communities change over time. Our findings demonstrate that habitat connectivity, as determined by pore size and matric potential can have a large role in shaping microbial communities and provide a potentially important mechanism for the maintenance of high levels of soil-borne bacterial diversity. This research represents the first systematic study to examine the effects of habitat connectivity on bacterial and fungal diversity. We observed contrasting patterns of bacterial and fungal diversity in response to differences in habitat connectivity. While the greater diversity of bacterial communities in low-connected soils may be attributed to spatial separation, further studies on the underlying mechanisms that support fungal diversity in differently structured soils are needed.

5.5 Acknowledgements

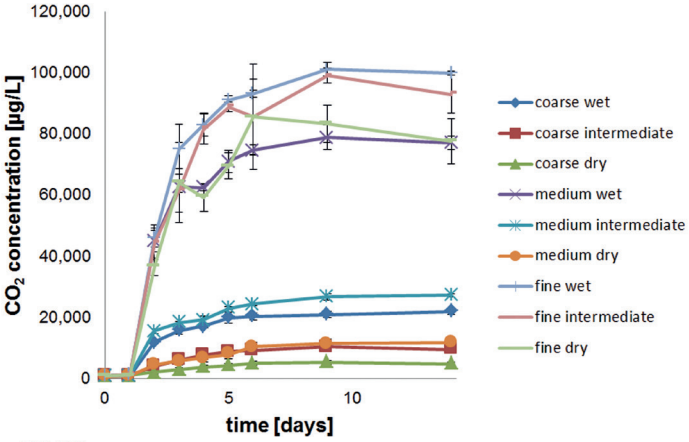
We thank Agata Pijl for assistance with the molecular work and Mattias de Hollander for bioinformatics support.

5.6 Supplementary information

(a)



(b)



(c)

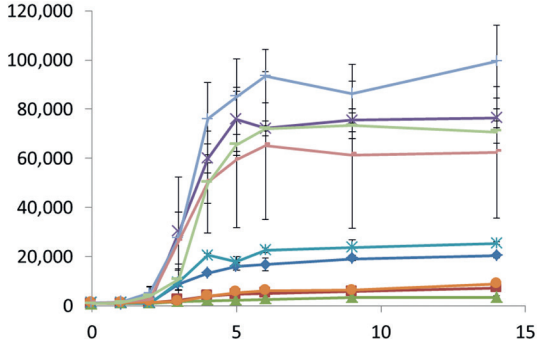


Figure S1. CO_2 concentrations in the headspace of the microcosms in the pilot study (a), fungal-bacterial experiment (b) and bacterial experiment (c). Error bars in (b) and (c) depict the standard error. (a) pilot study (n= 1), (b) fungal-bacterial experiment (n= 3), (c) Bacterial experiment (n= 3).

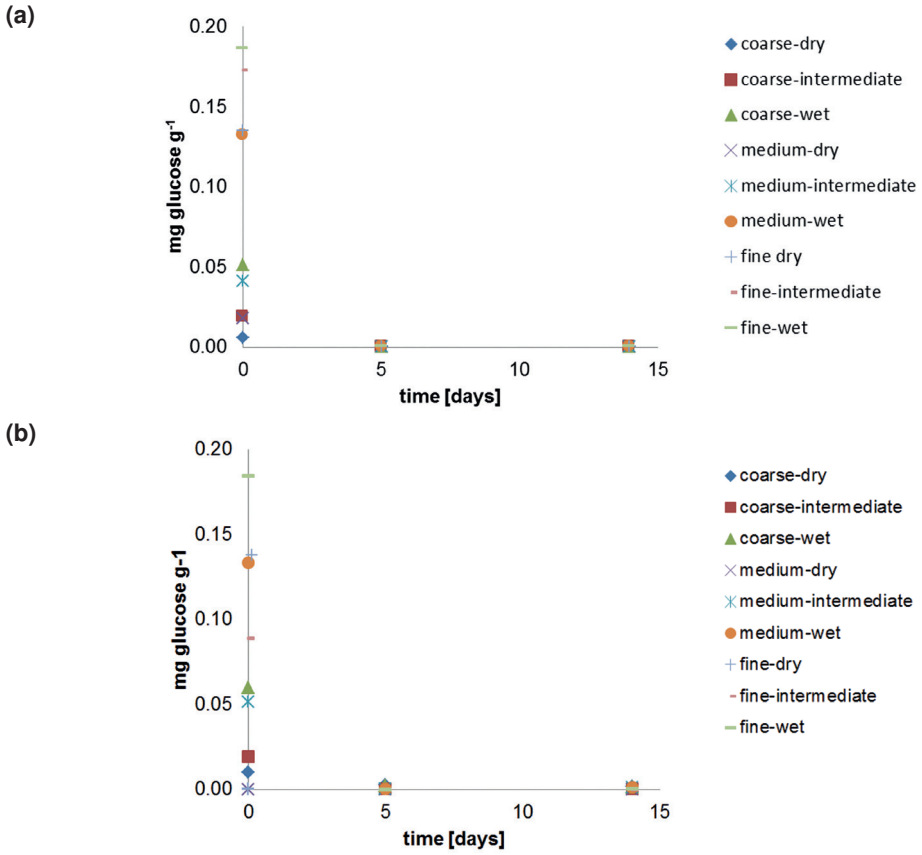


Figure S2. Glucose concentrations at T0, T1 and T2 in the different treatments in the bacterial experiment (a) and fungal-bacterial experiment (b) (n=2). (a) Fungal-bacterial experiment, (b) Bacterial experiment.

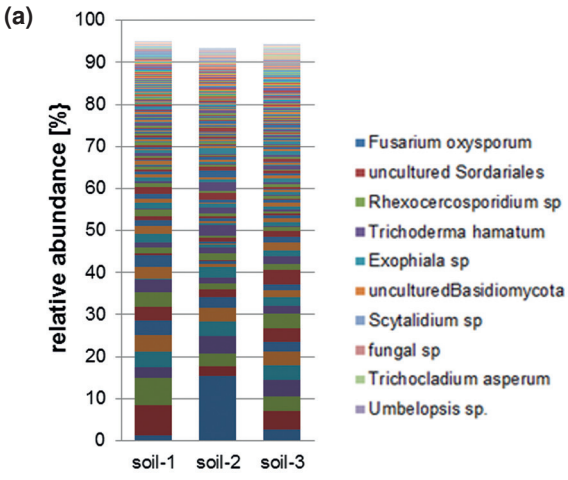
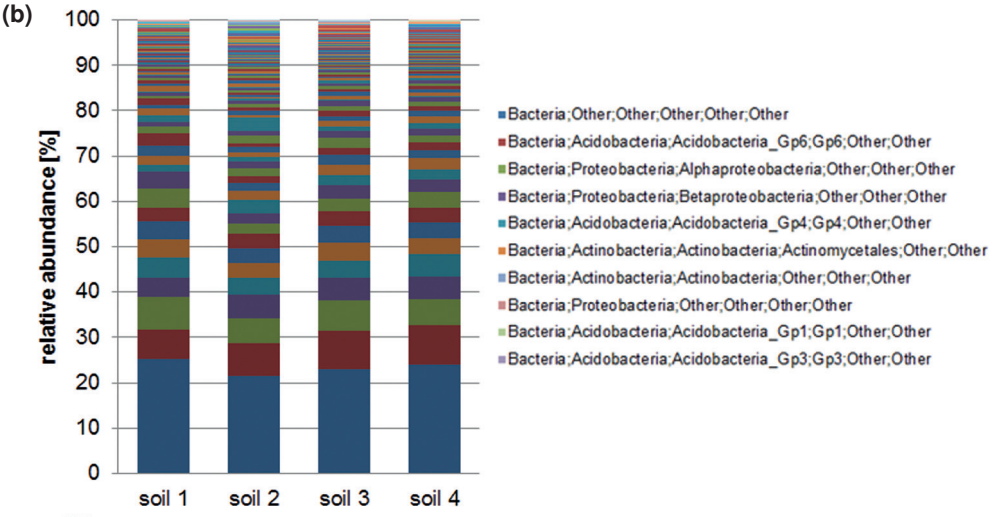
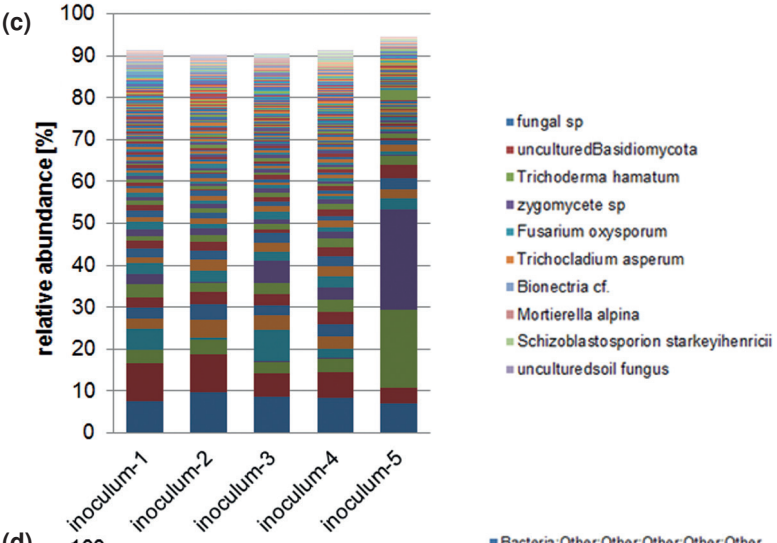
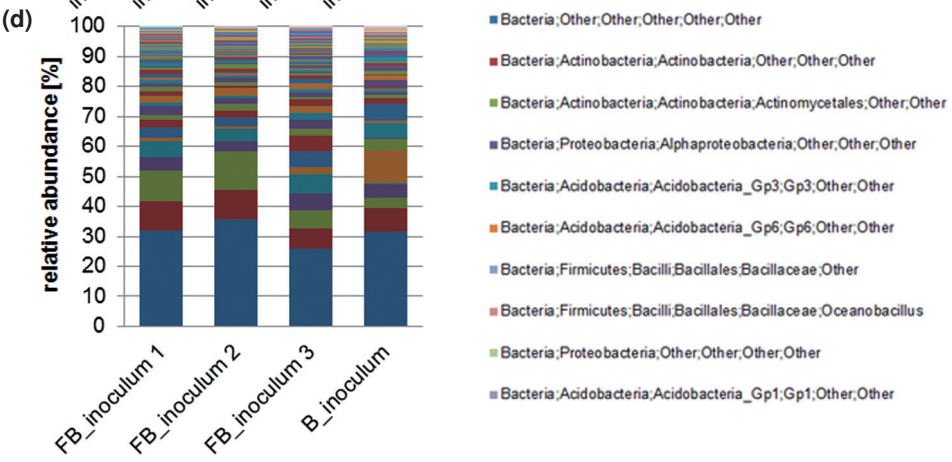
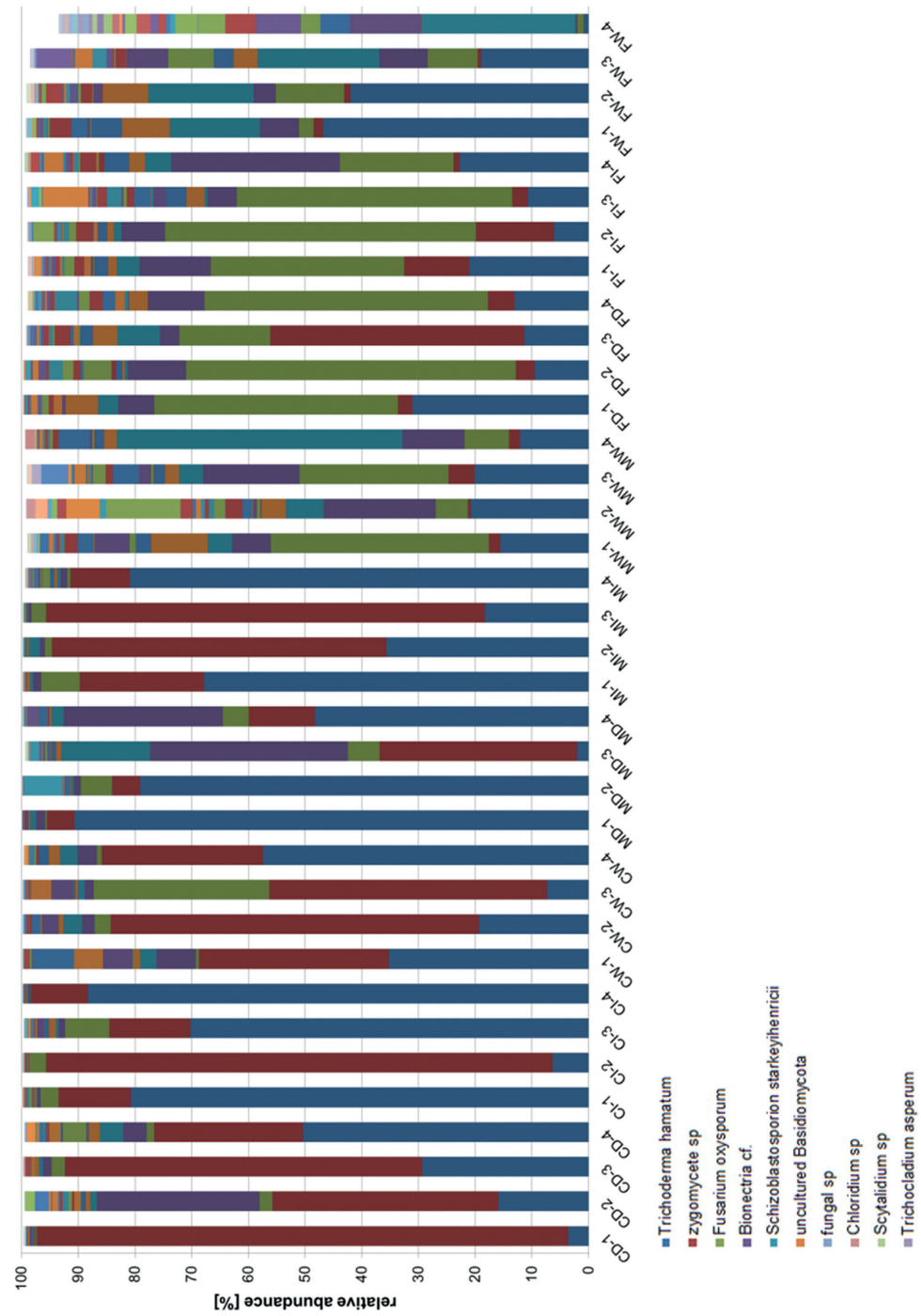


Figure S3. Relative abundance of fungal and bacterial OTUs in the soil and inoculum. (a) Fungal OTUs (minimal abundance 0.1%) in the soil that was used to prepare the inoculum in both the fungal-bacterial and bacterial experiment, (b) Bacterial OTUs in the soil that was used to prepare the inoculum in both the fungal-bacterial and bacterial experiment, (c) Fungal OTUs (minimal abundance 0.2%) in inoculum for fungal-bacterial experiment, (d) bacterial OTUs in inoculum for fungal-bacterial experiment (F) and bacterial experiment (B).


(b)
relative abundance [%]
100
90
80
70
60
50
40
30
20
10
0
soil 1
soil 2
soil 3
soil 4
Bacteria;Other;Other;Other;Other;Other
Bacteria;Acidobacteria;Acidobacteria_Gp6;Gp6;Other;Other
Bacteria;Proteobacteria;Alphaproteobacteria;Other;Other;Other
Bacteria;Proteobacteria;Betaproteobacteria;Other;Other;Other
Bacteria;Acidobacteria;Acidobacteria_Gp4;Gp4;Other;Other
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Other;Other
Bacteria;Actinobacteria;Actinobacteria;Other;Other;Other
Bacteria;Proteobacteria;Other;Other;Other;Other
Bacteria;Acidobacteria;Acidobacteria_Gp1;Gp1;Other;Other
Bacteria;Acidobacteria;Acidobacteria_Gp3;Gp3;Other;Other


(c)
relative abundance [%]
100
90
80
70
60
50
40
30
20
10
0
inoculum-1
inoculum-2
inoculum-3
inoculum-4
inoculum-5
fungal sp
unculturedBasidiomycota
Trichoderma hamatum
zygomycete sp
Fusarium oxysporum
Trichocladium asperum
Bionectria cf.
Mortierella alpina
Schizoblastosporion starkeyihenricii
unculturedsoil fungus


(d)
relative abundance [%]
100
90
80
70
60
50
40
30
20
10
0
FB_inoculum 1
FB_inoculum 2
FB_inoculum 3
B_inoculum
Bacteria;Other;Other;Other;Other;Other
Bacteria;Actinobacteria;Actinobacteria;Other;Other;Other
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Other;Other
Bacteria;Proteobacteria;Alphaproteobacteria;Other;Other;Other
Bacteria;Acidobacteria;Acidobacteria_Gp3;Gp3;Other;Other
Bacteria;Acidobacteria;Acidobacteria_Gp6;Gp6;Other;Other
Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Other
Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Oceanobacillus
Bacteria;Proteobacteria;Other;Other;Other;Other
Bacteria;Acidobacteria;Acidobacteria_Gp1;Gp1;Other;Other


(a)
relative abundance [%]
0
10
20
30
40
50
60
70
80
90
100
CD-1
CD-2
CD-3
CD-4
CI-1
CI-2
CI-3
CI-4
CW-1
CW-2
CW-3
CW-4
MD-1
MD-2
MD-3
MD-4
MI-1
MI-2
MI-3
MI-4
MW-1
MW-2
MW-3
MW-4
FD-1
FD-2
FD-3
FD-4
FI-1
FI-2
FI-3
FI-4
FW-1
FW-2
FW-3
FW-4
Trichoderma hamatum
zygomycete sp
Fusarium oxysporum
Bionectria cf.
Schizoblastosporion starkeyihenricii
uncultured Basidiomycota
fungal sp
Chloridium sp
Scytalidium sp
Trichocladium asperum

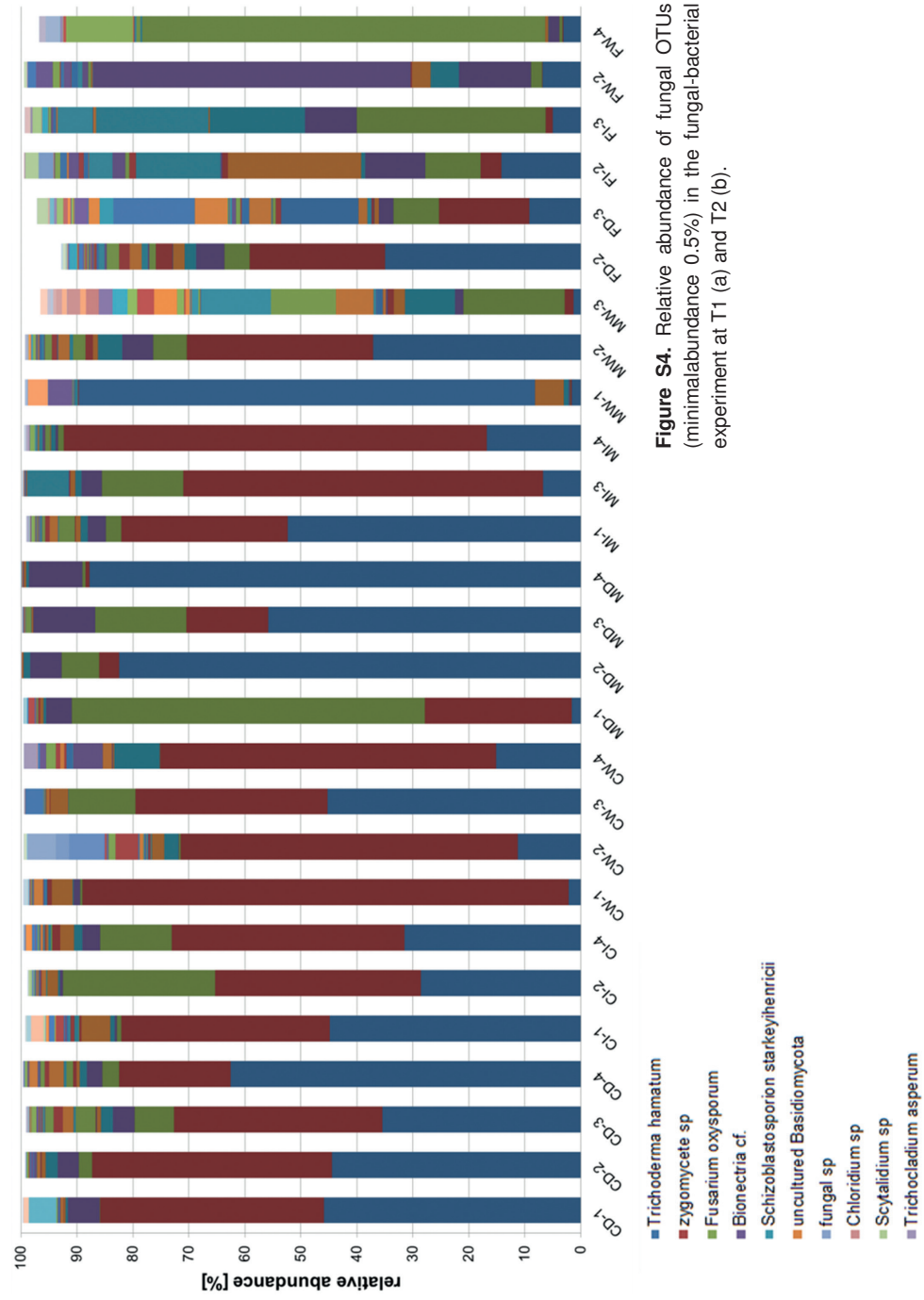
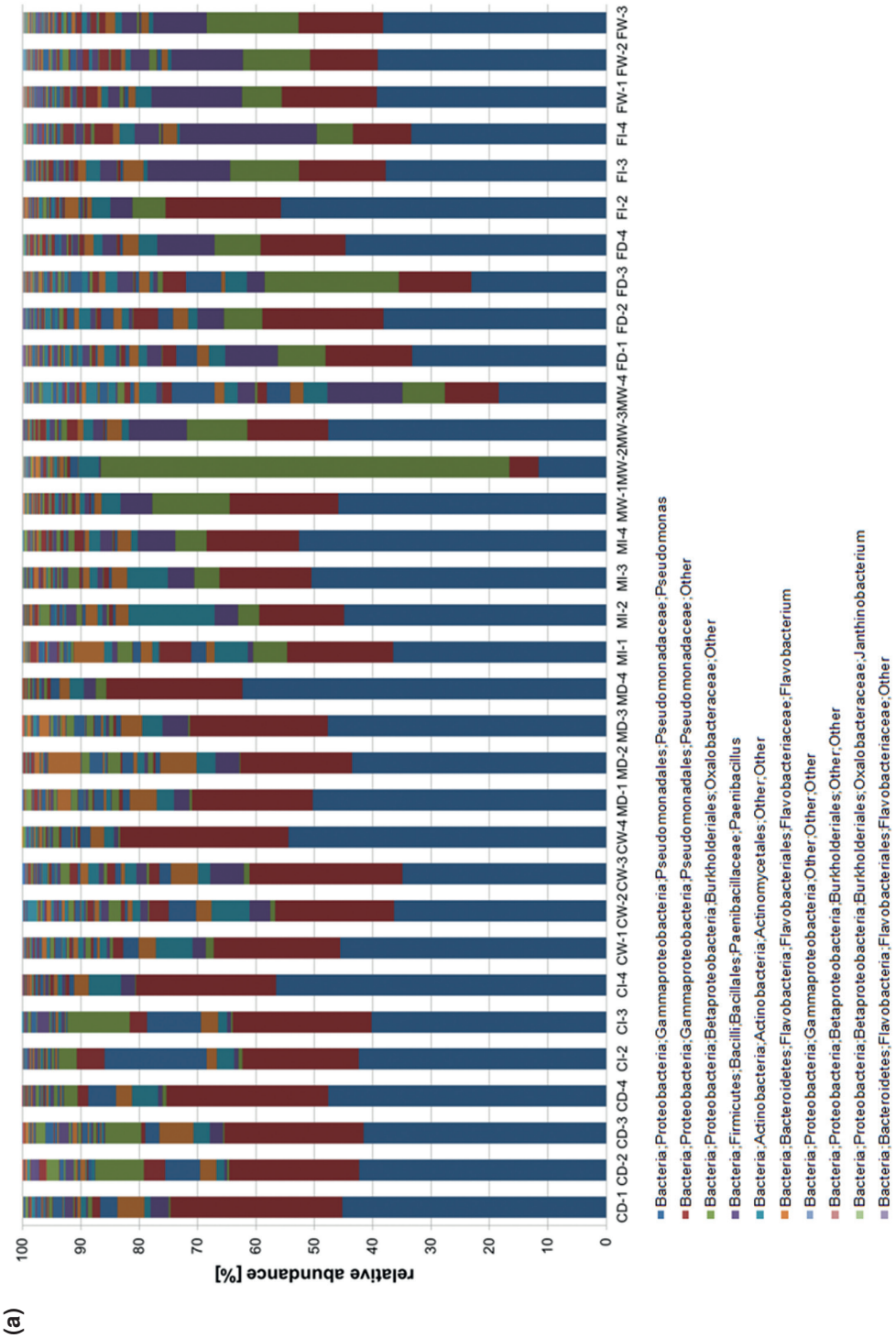


Figure S4. Relative abundance of fungal OTUs (minimalabundance 0.5%) in the fungal-bacterial experiment at T1 (a) and T2 (b).


(a)
relative abundance [%]
0
10
20
30
40
50
60
70
80
90
100
CD-1 CD-2 CD-3 CD-4 CI-2 CI-3 CI-4 CW-1 CW-2 CW-3 CW-4 MD-1 MD-2 MD-3 MD-4 MI-1 MI-2 MI-3 MI-4 MW-1 MW-2 MW-3 MW-4 FD-1 FD-2 FD-3 FD-4 FI-2 FI-3 FI-4 FW-1 FW-2 FW-3
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Other
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Other
Bacteria;Firmicutes;Bacilli;Bacillales;Paenibacillaceae;Paenibacillus
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Other;Other
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Flavobacterium
Bacteria;Proteobacteria;Gammaproteobacteria;Other;Other;Other
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Other;Other
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Janthinobacterium
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Other

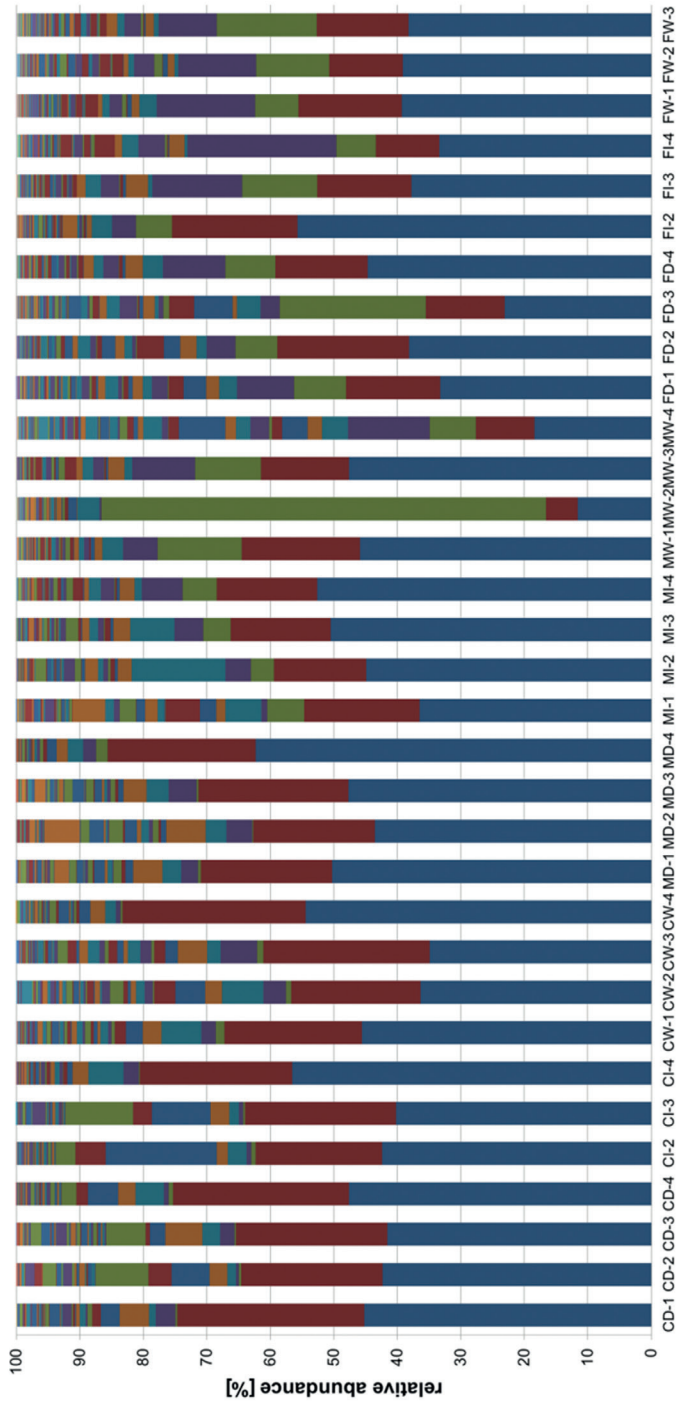
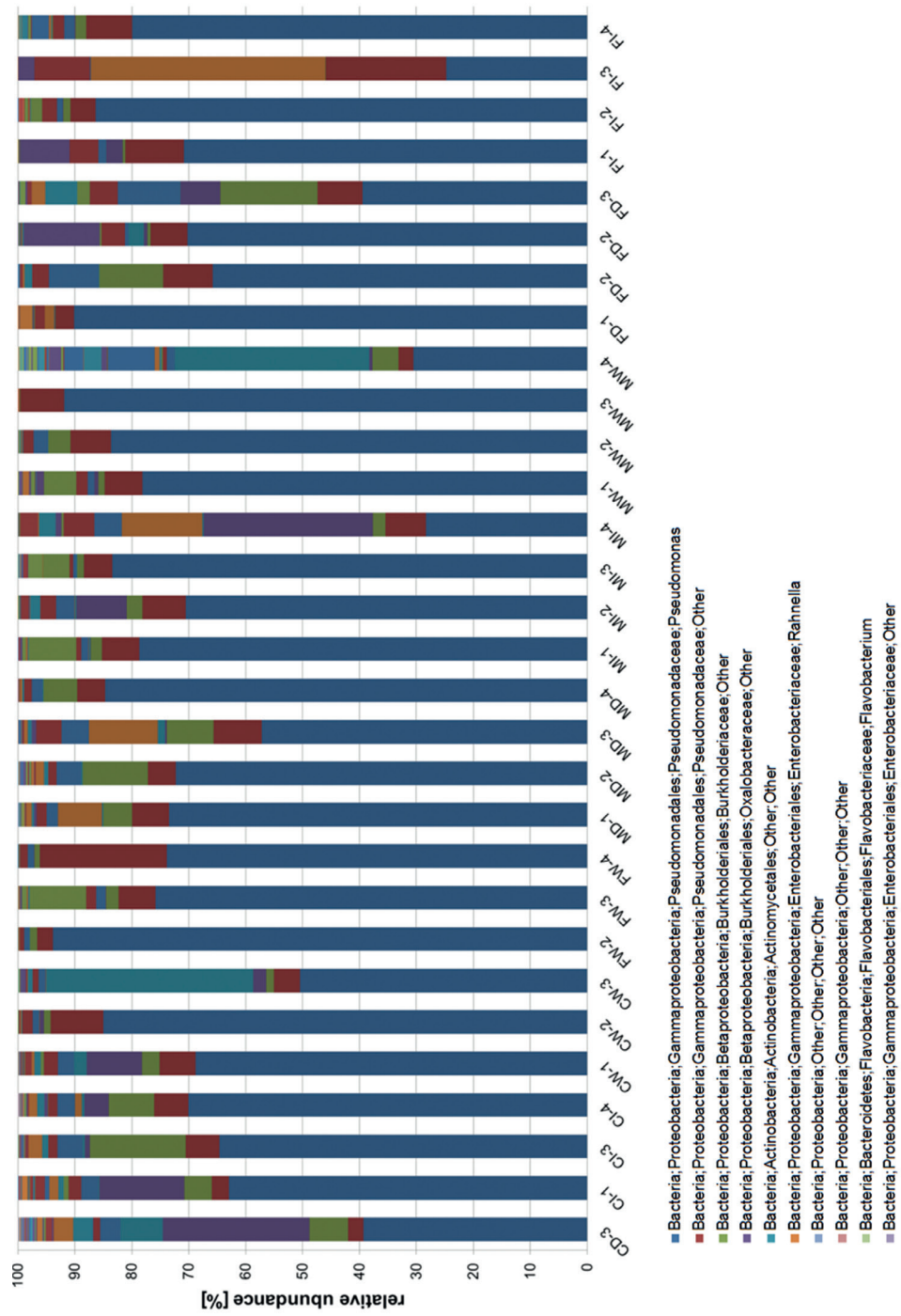


Figure S5. Relative abundance of bacterial OTUs in the fungal-bacterial experiment at T1 (a) and T2 (b). The different particle sizes are given by C= coarse, M= medium and F= fine, matric potentials are given as D= dry, I= intermediate and W= wet. 1-4 depict the replicates of each treatment. Some replicates are missing due to unsuccessful PCR amplification or low quality of sequences.


(a)
relative ubundance [%]
0
10
20
30
40
50
60
70
80
90
100
CD-3
CI-1
CI-3
CI-4
CW-1
CW-2
CW-3
FW-2
FW-3
FW-4
MD-1
MD-2
MD-3
MD-4
MI-1
MI-2
MI-3
MI-4
MW-1
MW-2
MW-3
MW-4
FD-1
FD-2
FD-2
FD-3
FI-1
FI-2
FI-3
FI-4
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Other
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae;Other
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Other
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Other;Other
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Rahnella
Bacteria;Proteobacteria;Other;Other;Other;Other
Bacteria;Proteobacteria;Gammaproteobacteria;Other;Other;Other
Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Flavobacterium
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Other

(b)

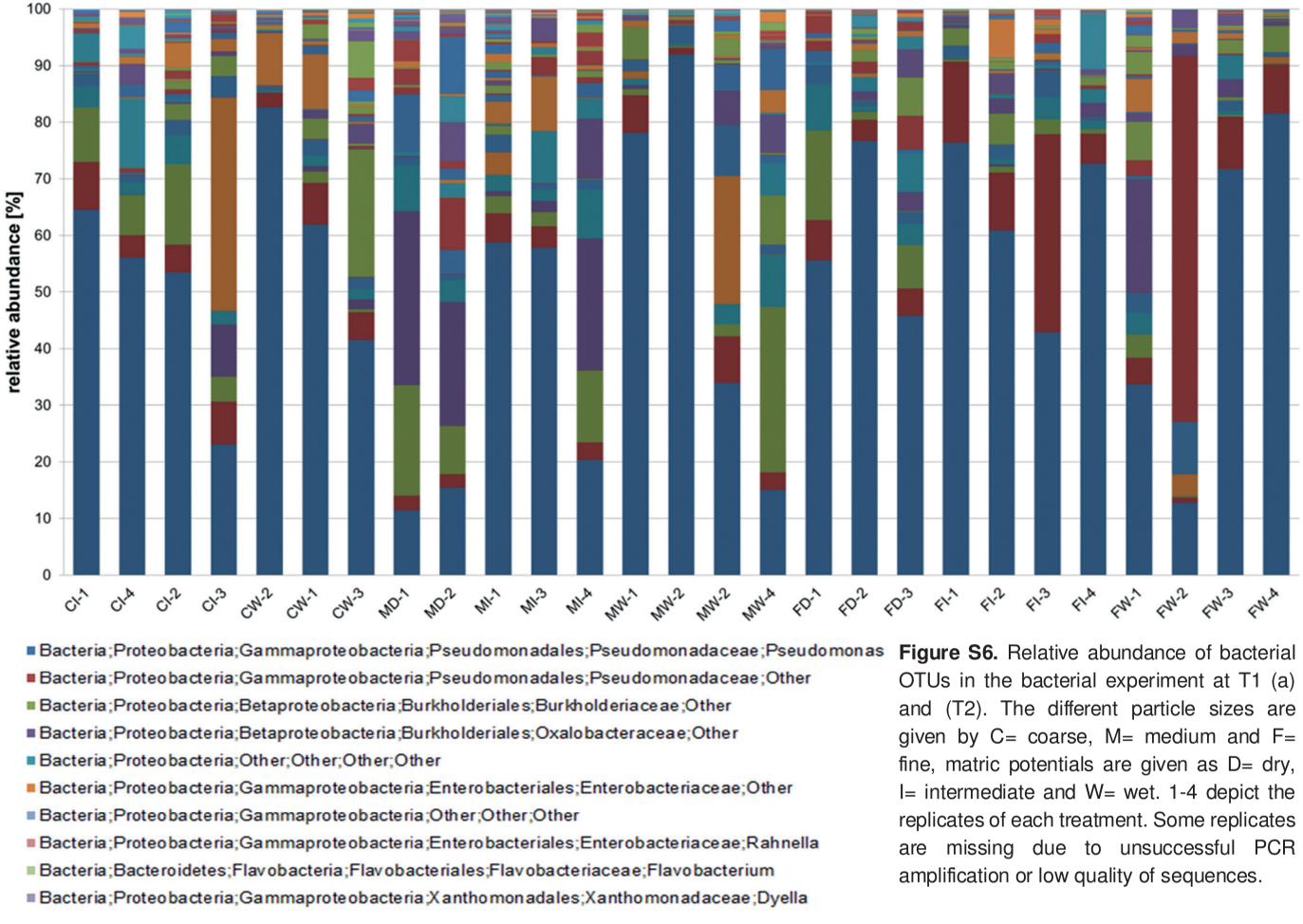


Figure S6. Relative abundance of bacterial OTUs in the bacterial experiment at T1 (a) and (T2). The different particle sizes are given by C= coarse, M= medium and F= fine, matric potentials are given as D= dry, I= intermediate and W= wet. 1-4 depict the replicates of each treatment. Some replicates are missing due to unsuccessful PCR amplification or low quality of sequences.

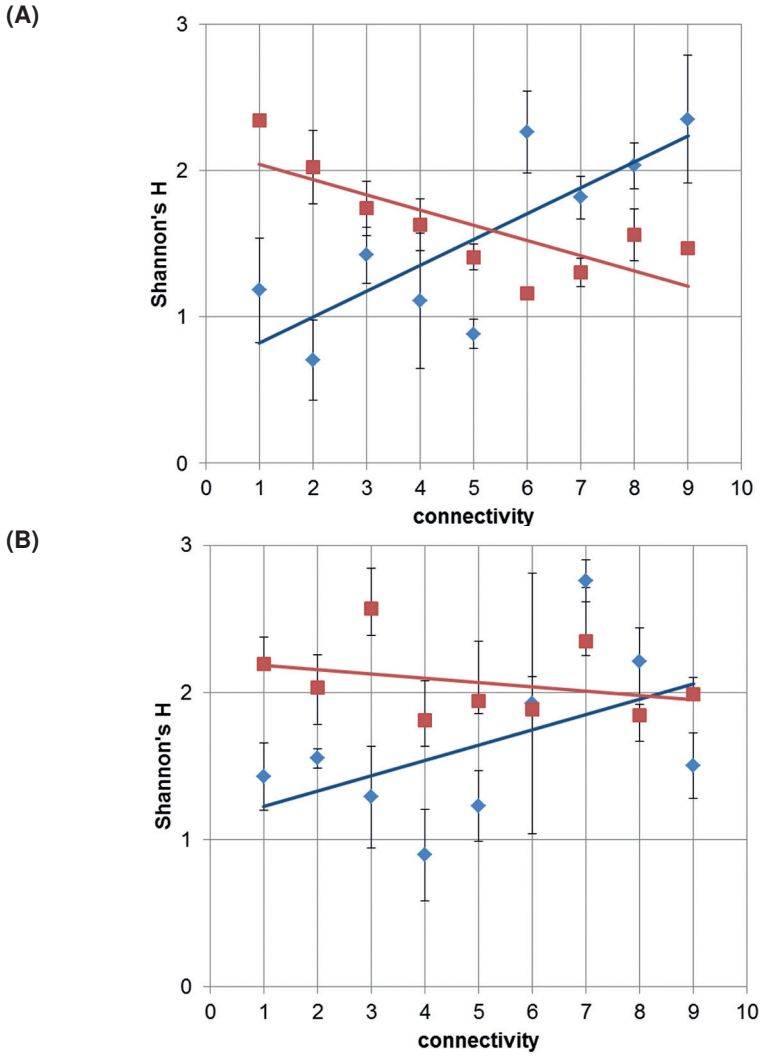


Figure A7. Fungal (blue) and bacterial diversity (red) in the fungal-bacterial experiment at T1 (A) and T2 (B) at different connectivities. Fungal diversity is greatest in well connected soils, especially at T1. In contrast, bacterial diversity is gretest in poorely connected soils.

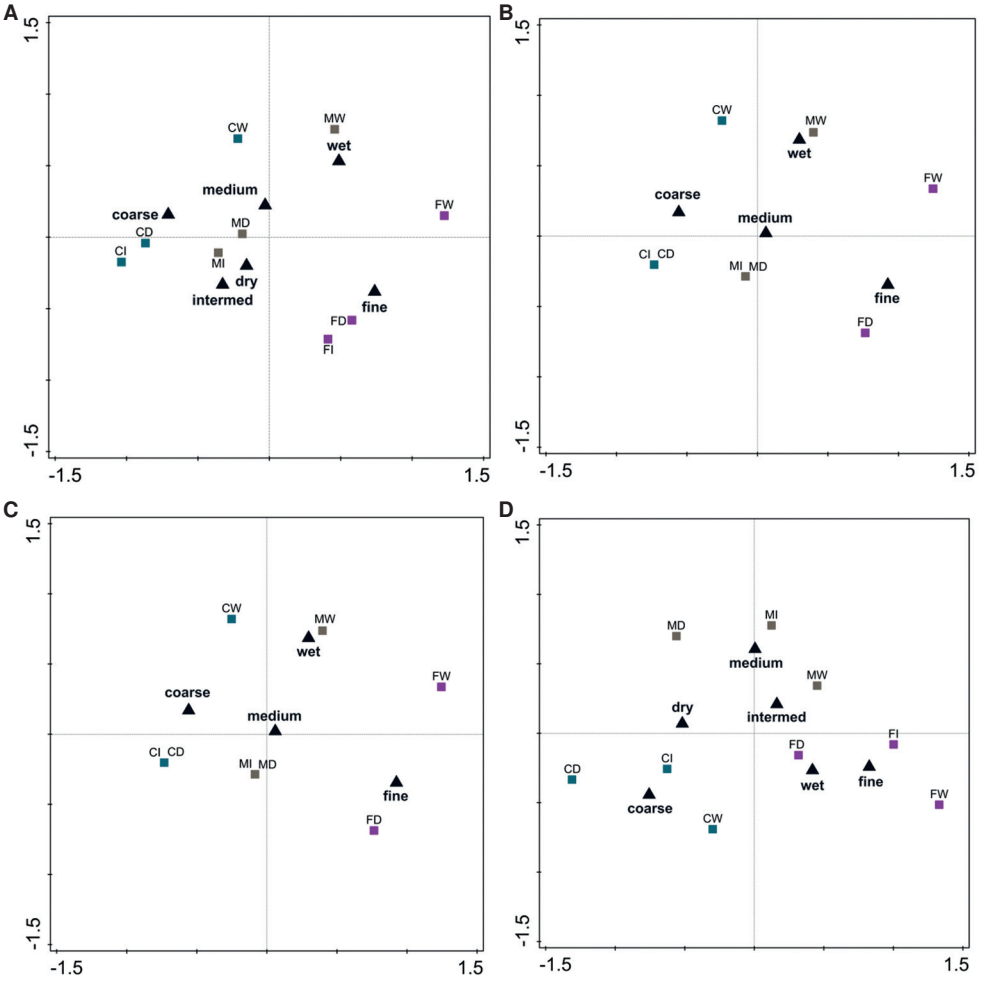


Figure S8. RDAs fungal and bacterial communities in the fungal-bacterial experiment at T1 and T2. RDA T1 fungi: Symmetric scaling of ordination scores, species scores were divided by standard deviation and species were centered. Fungal community composition is significantly correlated with fine particle sizes (P=0.002) and "wet" matric potential (P= 0.002). T2 fungi: The effect of particle size and matric potential explains a significant proportion of the variation in the dataset with a significant correlation between fungal communities and fine particle sizes (P= 0.008) and "wet" matric potentials (P= 0.004). T1 bacteria: The effect of particle size and matric potential explains a significant proportion of the variation in the dataset with a significant correlation between bacterial communities and coarse treatments (P= 0.002) and dry treatments and the community composition (P= 0.002). T2 bacteria: The effect of particle size and matric potential explains a significant proportion of the variation in the dataset with a significant correlation between bacterial communities and coarse and medium treatments (both P= 0.002).

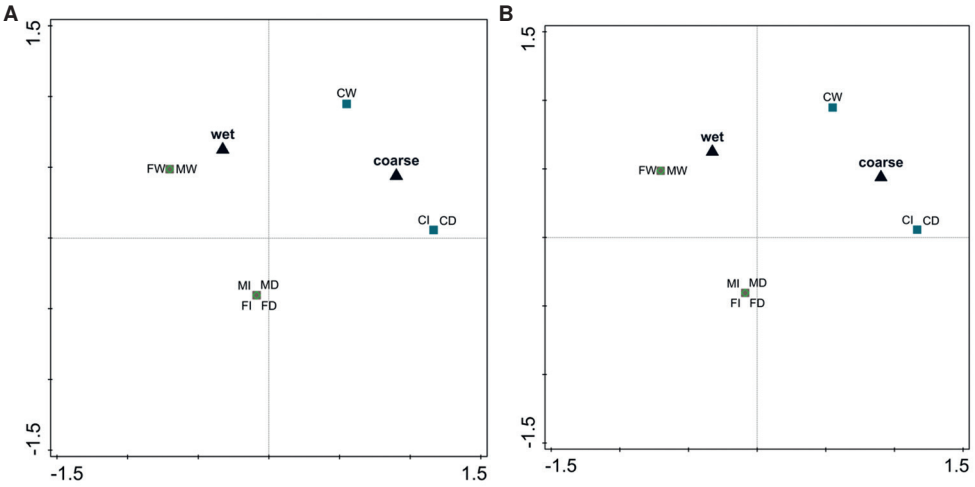


Figure S9. RDAs of bacterial communities in the bacterial experiment for T1 and T2. T1 bacteria: The effect of particle size and matric potential explains a significant proportion of the variation in the dataset with a significant correlation between bacterial communities and coarse treatments (P= 0.01) and wet treatments (P= 0.022). T2 bacteria: The effect of particle size and matric potential explains a significant proportion of the variation in the dataset with a significant correlation between bacterial communities and fine treatments (P= 0.01) and dry treatments (P= 0.002).

Table S1. Gravimetric water content, C addition per g of soil, total C that was measured in the microcosm headspace and percentage of originally added C that was respired at T1 and T2 days in the different treatments in the bacterial and fungal-bacterial experiment.

			bacterial experiment				Fungal-bacterial experiment			
treatment	gravimetric H_2O content	C addition per g soil [mg]	total C in microcosm headspace [mg]		% of C respired		total C in microcosm headspace [mg]		% of C respired	
			T1	T2	T1	T2	T1	T2	T1	T2
FW	25,7%	0,600	1,16	1,36	19,33	22,60	1,24	1,36	20,73	22,72
FI	24,9%	0,581	0,81	0,85	13,98	14,66	1,21	1,27	20,87	21,84
FD	18,9%	0,441	0,89	0,96	20,25	21,86	0,95	1,06	21,51	23,99
MW	20,2%	0,471	1,04	1,04	21,98	22,10	0,97	1,05	20,59	22,31
MI	6,7%	0,156	0,24	0,35	15,62	22,24	0,31	0,37	20,10	23,79
MD	3,4%	0,079	0,07	0,12	9,26	15,19	0,11	0,16	13,71	20,20
CW	5,9%	0,138	0,22	0,28	15,80	20,09	0,27	0,30	19,69	21,68
CI	3,5%	0,082	0,06	0,10	7,85	11,85	0,12	0,13	14,66	15,85
CD	2,0%	0,047	0,03	0,05	6,38	9,95	0,06	0,07	12,56	14,17

Table S2. ANOVA table on bacterial and fungal richness (Shannon_H) and diversity indices (Chao) in the bacterial and fungal-bacterial experiment at T1 and T2.

	bacterial experiment				fungal-bacterial experiment			
	T1		T2		T1		T2	
	Shannon_H	Chao	Shannon_H	Chao	Shannon_H	Chao	Shannon_H	Chao
particle size	0.363	<0.001*	0.138	0.002*	<0.001*	<0.001*	0.006*	<0.001*
matric potential	0.233	0.160	0.353	0.277	<0.001*	<0.001*	0.211	0.003*
PSxM	0.751	0.290	0.796	0.288	0.004*	<0.001*	0.160	0.087

	fungal diversity			
	T1		T2	
	Shannon_H	Chao	Shannon_H	Chao
particle size	<0.001*	0.031*	0.005*	0.012*
matric potential	<0.001*	0.212	0.945	0.182
PSxM	0.038*	0.862	0.015*	0.006*

* indicate statistical significance (P< 0.05).

Table S3. p.adj (pairwise comparison with bonferroni correction) of bacterial richness and diversity indices.

		bacterial experiment				fungal-bacterial experiment			
factor		T1		T2		T1		T2	
		Shannon_H	Chao	Shannon_H	Chao	Shannon_H	Chao	Shannon_H	Chao
particle size	F-C	0.450	<0.001*	1.000	0.003*	<0.001*	<0.001*	0.075	<0.001*
	M-C	1.000	0.089*	0.750	0.738	<0.001*	0.002	0.008*	<0.001*
	M-F	1.000	0.102	0.120	0.041*	1.000	0.455	1.000	0.850
matric potential	I-D	1.000	1.000	1.000	1.000	1.000	0.074	1.000	0.690
	W-D	0.470	1.000	0.540	1.000	0.120	0.003	0.760	0.600
	W-I	0.750	1.000	1.000	0.720	0.470	0.661	0.330	1.000

		fungal diversity			
factor		T1		WT2	
		Shannon_H	Chao	Shannon_H	Chao
particle size	F-C	<0.001*	0.022*	0.043*	0.103
	M-C	0.492	0.765	1.000	1.000
	M-F	0.020*	0.295	0.020*	0.085
matric potential	I-D	1.000	1.000	1.000	0.800
	W-D	0.034*	0.320	1.000	1.000
	W-I	0.006*	0.670	1.000	1.000

* indicate statistical significance (P< 0.05).

Table S4. ANOVA table on 6 most abundant fungal taxa at T1 and T2 in the fungal-bacterial experiment.

T1		Trichoderma hamatum		zygomycete sp		Fusarium oxysporum		Bionectria cf.		Schizoblastosporion starkeyihenricii		uncultured Basidiomycota	
	DF	F	P	F	P	F	P	F	P	F	P	F	P
particle size	2	2.0	0.162	7.6	0.003*	14.4	<0.001*	1.8	0.184	1.7	0.206	6.0	0.008*
moisture	2	0.8	0.477	0.3	0.291	0.5	0.591	1.6	0.225	3.7	0.040*	5.1	0.014*
PxM	4	1.2	0.325	1.3	0.290	7.5	<0.001*	1.9	0.142	0.7	0.602	1.4	0.272
total	25												

T2		Trichoderma hamatum		zygomycete sp		Fusarium oxysporum		Bionectria cf.		Schizoblastosporion starkeyihenricii		uncultured Basidiomycota	
	DF	F	P	F	P	F	P	F	P	F	P	F	P
particle size	2	6.2	0.006*	19.5	<0.001*	1.4	0.261	16.874	<0.001*	3.9	0.034*	0.5	0.605
moisture	2	4.2	0.026*	1.2	0.309	1.9	0.172	2.859	0.075	1.0	0.380	1.1	0.349
PxM	4	0.9	0.499	5.5	0.003*	3.4	0.023*	0.879	0.490	0.9	0.503	0.8	0.532
total	25												

* indicate statistical significance (P< 0.05).

Table S5. p.adj from bonferroni test (6 most abundant fungal taxa).

T1

factor		Trichoderma hamatum	zygomycete sp	Fusarium oxysporum	Bionectria cf.	Schizoblastosporion starkeyihenricii	uncultured Basidiomycota
particle size	F-C	0.280	0.003*	0.002*	0.900	0.590	0.016*
	M-C	1.000	0.060	1.000	0.270	0.360	0.789
	M-F	0.310	0.705	0.015*	1.000	1.000	0.237
moisture	I-D	1.000	0.820	1.000	0.210	1.000	1.000
	W-D	1.000	1.000	1.000	0.770	0.146	0.163
	W-I	0.720	00.320	1.000	1.000	0.034*	0.026*

T2

factor		Trichoderma hamatum	zygomycete sp	Fusarium oxysporum	Bionectria cf.	Schizoblastosporion starkeyihenricii	uncultured Basidiomycota
particle size	F-C	0.083	<0.001*	0.480	<0.001*	0.073	1.000
	M-C	1.000	0.034*	1.000	0.631	1.000	1.000
	M-F	0.012*	0.076	1.000	<0.001*	0.057	0.950
moisture	I-D	0.711	1.000	0.820	0.570	1.000	0.460
	W-D	0.036*	1.000	1.000	0.480	0.550	1.000
	W-I	0.441	1.000	0.280	1.000	1.000	1.000

* indicate statistical significance (P< 0.05).

Table S6. ANOVA table on 6 most abundant bacterial taxa in the fungal-bacterial experiment at T1 and T2.

T1		1		2		3		4		5		6	
	DF	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
particle size	2	18.1	<0.001*	33.3	<0.001*	13.6	<0.001*	13.7	<0.001*	1.3	0.302	4.3	0.027*
matric potential	2	4.5	0.027*	8.1	0.002*	3.8	0.039*	14.5	<0.001*	9.1	0.001*	8.7	0.002*
PS x MP	4	2.8	0.051	0.4	0.778	1.3	0.289	3.0	0.040*	2.4	0.081	4.9	0.005*
total	23												

T2		1		2		3		4		5		6	
	DF	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
particle size	2	2.0	0.159	12.6	<0.001*	18.4	<0.001*	1.9	0.175	2.9	0.077	12.1	<0.001*
matric potential	2	1.1	0.336	3.1	0.062	5.4	0.012*	1.5	0.255	2.0	0.154	4.7	0.019*
PS x MP	4	1.5	0.235	1.8	0.158	0.9	0.483	2.1	0.121	3.5	0.022*	5.3	0.004*
total	23												

* indicate statistical significance (P< 0.05).

1: Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas,

2:Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Other,

3: Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Other,

4: Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Other;Other,

5: Bacteria;Firmicutes;Bacilli;Bacillales;Paenibacillaceae;Paenibacillus,

6: Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Flavobacterium.

Table S7. p.adj from bonferroni test (6 most abundant bacterial taxa in the fungal-bacterial experiment).

T1							
factor		1	2	3	4	5	6
particle size	F-C	0.002	<0.001*	<0.001*	0.005*	1.000	1.000
	M-C	<0.001*	<0.001*	0.002*	0.096	0.720	0.270
	M-F	1.000	1.000	1.000	0.633	1.000	0.270
moisture	I-D	1.000	1.000	0.600	0.111	0.002	0.046*
	W-D	0.360	0.240	0.250	0.006*	1.000	0.023*
	W-I	0.470	0.230	1.000	0.623	0.026*	1.000

T2							
factor		1	2	3	4	5	6
particle size	F-C	0.710	<0.0001*	<0.0001*	0.450	0.510	0.025*
	M-C	0.200	0.162	<0.0001*	1.000	0.190	1.000
	M-F	1.000	0.038*	1.000	0.320	1.000	0.005*
moisture	I-D	0.770	0.760	0.250	0.710	0.360	0.310
	W-D	1.000	0.600	0.310	0.800	1.000	0.810
	W-I	0.460	1.000	1.000	1.000	0.430	1.000

* indicate statistical significance (P< 0.05).

Table S8. ANOVA table on 6 most abundant bacterial taxa in the bacterial experiment at T1 and T2.

T1		1		2		3		4		5		6	
	DF	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
particle size	2	0.3	0.765	2.3	0.126	1.2	0.347	2.6	0.099	1.2	0.31	0.1	0.885
matric potential	2	0.0	0.991	0.8	0.444	4.7	0.021*	1.2	0.311	2.1	0.146	2.5	0.104
PS x MP	4	0.4	0.802	0.4	0.815	1.6	0.209	3.4	0.027*	0.7	0.589	0.7	0.571
total	21												

T2		1		2		3		4		5		6	
	DF	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
particle size	2	3.4	0.059	3.4	0.061	1.8	0.204	8.5	0.003*	1.4	0.280	2.3	0.137
matric potential	2	1.9	0.186	2.0	0.168	1.7	0.216	7.3	0.006*	7.5	0.005*	1.8	0.196
PS x MP	4	0.4	0.731	0.7	0.542	0.8	0.491	5.7	0.007*	0.3	0.835	0.3	0.875
total	21												

* indicate statistical significance (P< 0.05).
1: Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas,
2: Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Other,
3: Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae;Other,
4: Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae;Other,
5: Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Other;Other,
6: Bacteria;Proteobacteria;Other;Other;Other;Other.

Table S9. p.adj from bonferroni test (6 most abundant bacterial taxa) (bacterial experiment).

T1							
factor		1	2	3	4	5	6
particle size	F-C	1.000	0.180	0.700	0.190	0.400	1.000
	M-C	1.000	1.000	0.840	0.720	0.950	1.000
	M-F	1.000	0.230	1.000	1.000	1.000	1.000
moisture	I-D	1.000	1.000	0.222	1.000	1.000	0.257
	W-D	1.000	1.000	0.045	1.000	0.380	0.095
	W-I	1.000	1.000	1.000	0.630	0.170	1.000

T2							
factor		1	2	3	4	5	6
particle size	F-C	1.000	0.311	1.000	1.000	1.000	1.000
	M-C	0.435	1.000	0.790	0.170	0.580	0.310
	M-F	0.051	0.074	0.25	0.050*	1.000	0.170
moisture	I-D	1.000	0.510	0.610	0.340	0.004*	0.600
	W-D	0.500	1.000	0.460	0.120	0.002	0.280
	W-I	1.000	0.770	1.000	1.000	1.000	1.000

Chapter 6

Early colonizers of new habitats represent a minority of the soil bacterial community

Authors:
Alexandra B. Wolf*, Max-Bernhard Rudnick*,
Wietse de Boer, George A. Kowalchuk

* These authors contributed equally to this work

Abstract

In order to understand (re-)colonization of microhabitats and bacterial succession in soil, it is important to understand which bacterial members of soil microbial communities are most motile in the porous soil matrix. To address this issue, we carried out a series of experiments in sterilized soil microcosms. Using two different model strains, *Pseudomonas fluorescens* and *Collimonas fungivorans*, we first determined the influence of nutrient availability on bacterial expansion rates. Based on these results, we then conducted similar microcosm experiments to examine microbial mobility within natural soil bacterial communities under a single nutrient regime. The expansion of bacterial populations within the community was assayed by quantitative PCR and pyrosequencing of 16S rRNA gene fragments. We observed that only a relatively small subset of the total community was able to expand to an appreciable distance (more than 2 cm) within 48 hours, with the genera *Undibacterium*, *Pseudomonas*, and *Massilia* and especially the family Enterobacteriaceae dominating the communities more distant from the point of inoculation. These results suggest that (re-) colonization of open habitats in soil may be dominated by a few rapidly moving species, which may have important consequences for microbial succession.

Keywords: bacterial diversity – high-throughput tag sequencing – microcosms – soil structure – motility – succession

6.1 Introduction

The soil environment is highly heterogeneous with sporadic availability of easily degradable energy resources for the soil inhabiting microbial community. The ability to access these spatially distributed resources may contribute to the success of microbial species within the soil environment. Some bacterial species are able to actively move towards energy resources and have evolved a variety of different motility mechanisms, often relying on flagellar movement, while others rely on passive dispersal via water flow or passing invertebrates. Soil hydration status is a major factor determining bacterial colonization of new habitats, as both passive and active motility depend on the presence of water-filled pores or water films covering the surfaces of solid particles (Abuashour *et al.*, 1994, Jiang *et al.*, 2006, Dechesne *et al.*, 2010). Despite the obvious importance of water content for bacterial movement, most studies that have examined the motility of soil-borne bacteria have not taken these factors into account. Active bacterial motility is typically investigated on agar plates (Harshey, 2003, Wang *et al.*, 2004, Caiazza *et al.*, 2005) or on sterile, porous ceramic surfaces, either by experimental (Dechesne *et al.*, 2010) or modeling approaches (Long & Or, 2009). Although such studies have provided valuable insight into the mechanisms of bacterial movement, they are highly artificial and do not mimic the *in situ* conditions of the soil environment. Studies that follow the fate of specific bacterial populations (e.g. genetically modified or pathogens) in soil (Trevors *et al.*, 1990, van Elsas *et al.*, 1991, Huysman & Verstraete, 1993, Abuashour *et al.*, 1994) are generally conducted under the assumption that bacteria are passively translocated, without consideration of active motility. Furthermore, previous studies have focused solely on tracking individual strains, without taking community processes such as microbial interactions into account. Nevertheless, it is known that microbial interactions can have pivotal influences on bacterial motility phenotypes (Garbeva & Boer, 2009).

To avoid the limitations of gel assays, the current study utilizes a quartz sand microcosm system that mimics important soil features. This system is well-defined, easy to manipulate and allows bacterial motility to be investigated in a community context (Wolf *et al.*, 2013).

In the present study, we first examined the movement of two individual model soil bacterial strains, *Pseudomonas fluorescens* and *Collimonas fungivorans*, in the sand microcosms. These initial experiments were performed in order to examine the influence of substrate availability on bacterial expansion and to determine suitable conditions for subsequent inoculation of microcosms with a complex bacterial community. In the complex community experiment, total bacterial community expansion over time was followed by qPCR, and bacterial community structure was determined as a function of distance from point of inoculation by high-throughput pyrosequencing of bacterial 16S rRNA gene fragments. Using this approach, we could identify bacterial taxa most successful in colonizing new (micro-)habitats, thereby gaining insight into patterns of microbial habitat (re-)colonization.

6.2 Methods

6.2.1 Single-strain inoculation experiments

In order to examine the impact of substrate availability on bacterial expansion and to determine suitable conditions for the community experiment, we tested the expansion of two single soil bacterial strains*, Pseudomonas fluorescens* Pf0-1 (Compeau *et al*., 1988) and *Collimonas fungivorans strain* Ter331 (de Boer *et al*., 2004), under different nutrient levels. Strains were inoculated in the center of sand microcosms and sampling was conducted at different distances from the inoculation point at different time points (see below). These strains were chosen as representatives of bacterial genera well known to be able to colonize roots in a soil environment (Lugtenberg & Dekkers, 2001, Kamilova *et al*., 2007). Both strains were pre-grown overnight, individually, in liquid 10% liquid tryptic soy broth (TSB), washed in 10 mM MES (morpholineethanesulfonic acid) buffer (pH 5.8) containing 1 gL^{-1} KH_2PO_4 and 1 gL^{-1} $(NH_4)_2SO_4$. Microcosms were established in glass petri dishes (diameter 9 cm) containing 50 g acid-washed sea sand (Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany) sterilized by autoclaving and oven-drying. The moisture content was adjusted to 7.5% (w/w) by adding the appropriate volume of liquid growth medium (either 10% or 1%TSB, pH 5.8). Microcosms were inoculated with 5 µL bacterial suspension at the center of the petri dish, sealed with parafilm, incubated at 20°C and sampled after 7, 24 and 48 hours with a multi-pronged sampling device as described by (Wolf *et al*., 2013), which provides samples at 2 mm intervals.

6.2.2 Experimental design and sampling for complex soil community experiments

Microcosms were established as described in the previous section, adjusted to 7.5% moisture (w/w) with 1% TSB and inoculated at the center of the petri dish with 5 µL soil suspension (4 replicates per time point). The soil suspension inoculum was prepared by dispersing 50 g field wet soil collected from a former arable field site located near Ede, the Netherlands (52°04′N, 5°45′E; see van der Putten *et al*., 2000) for a detailed description of the soil characteristics) in 450 mL 10 mM phosphate buffer (pH 5.8) by shaking for 30 min and sonicating (Branson 5210 ultrasonic bath) twice for 1 min. The suspension was filtered sequentially through filters with successively smaller pore diameter (11, 8, 6 and 3 µm; Whatman filter papers 1 Cat No 1001-150, 102-150, 1003-150, and Whatman Cellulose Nitrate Membrane Filters 7193-002) to exclude most eukaryotic organisms. After inoculation, the microcosms were sealed with parafilm to prevent moisture loss and incubated at 20°C in the dark. Samples were taken after 24 and 48 h by pushing the wide-end of a sterilized 1 mL pipet tip (inner diameter: 8 mm, outer diameter: 10 mm) at appropriate distances into the sand, thereby sampling at different, defined, distances from the inoculation zone (Fig. S1). We destructively harvested 5 samples (distances 1-5,

with 1 being closest and 5 furthest to the inoculation zone) along the radius of the sand microcosm, at 24 and 48 h. 24 and 48 h samples were taken from different microcosms. Each sample contained approximately 0.3 g sand. A proportion of these sand samples were used for isolation of bacteria (see below) and the rest was and was stored at -20°C until DNA isolation.

6.2.3 Identification of dominant colony form expansion zones

In order to isolate the bacteria from the motility zones of the microcosms, we dissolved 0.3 g sand samples (see above) of distances 1 and 2 in 1 ml MES-buffer and plated 50 µL samples in a dilution series from 1:10 to 1:1000 on 10 % TSB agar plates. Isolated bacteria were subjected to colony PCR using the primers 27f and 1492r (Weissburg et al 1991) with the following reagents & settings: 18.14 µl H2O, 2.5 µl 10x PCR-buffer containing 2 mM MgCl2 (Roche Scientific, Woerden, the Netherlands), 0.2 mM of each dNTP (Roche Scientific, Woerden, the Netherlands) and 0.4 µM of each Primer, 1 U Fast Start High Fidelity Polymerase (Roche Scientific, Woerden, the Netherlands) and 1 µl template. Cycling conditions consisted of a pre-denaturation step of 10 min at 95°C to break the cells open, an initial denaturation of 94°C for 2 min, followed by 34 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 90 sec with a 1 sec increment per cycle and a final elongation step at 72°C for 10 min. PCR products were examined by a standard (1.5 %) agarose electrophoresis and subsequently Sanger-sequenced with primer 1492r by Macrogen (Amsterdam, the Netherlands).

6.2.4 DNA isolation, quantitative PCR and high-throughput pyrosequencing

For each sand sample (about 0.3 g; 2 time points x 5 distances x 4 replicates), total DNA was extracted using the MOBIO PowerSoil DNA isolation kit following the manufacturer's protocol with the modification of heating the sample to 60°C for 10 min after the addition of solution C1, and the adding of 100 µL each of solutions C2 and C3 simultaneously.

To estimate bacterial density after 24 and 48 h across the sampling transect, we determined 16S rRNA gene copy numbers as a proxy of cell numbers via a quantitative real-time PCR (qPCR) approach. Briefly, 5 µL DNA template was added to a master mix consisting of 12.5 µl SYBR green mix (GC Biotech), 2.5 µl BSA (4mg/mL) and 2.3 µl milliQ water. To this, 1.25 µl (5 pmol/µL) each of the *Eub338* (forward) and Eub518 (reverse) primers were added (Lane, 1991). qPCR calibration curves (gene copy number versus the cycle number at which the fluorescence intensity reached the set cycle threshold value) were obtained using serial dilutions of pure-culture genomic DNA carrying a single 16S rRNA gene sequence (8 calibration points ranging from 1 to 4,171,775 copies/µL). All reactions were performed in duplicate. The qPCR was carried out in a Rotor-Gene Q (Qiagen, Venlo, the Netherlands). The PCR cycling conditions included 45 cycles of 5 seconds at 95°C, 10 seconds at 53°C, and 20 seconds at 72°C. Fluorescence data was recorded at the end of each 72°C step.

DNA dissociation profiles were subsequently run from 72°C to 95°C with a ramp of 1°C/5 seconds to confirm product integrity.

For pyrosequencing, the V4 region of the 16S rRNA gene was amplified from the extracted DNA using composite forward and reverse primers, consisting of primer A from 454 Life Sciences, a 10 base sample-specific barcode, a linker sequence GT and primer 515f and primer B from 454 Life Sciences, a 10 base sample specific barcode, linker sequence GG and the primer 806r (Vos *et al.*, 2012). Each sample and replicate received a unique barcode sequence. PCR amplifications were performed using 2.5 µl PCR buffer, 2.5 µl DNTP (2 mM), 0.2 µl Fast start DNA polymerase, 1 µL forward primer (5 uM), 1 µL reverse primer (5 uM), 1µL DNA template, and PCR grade H_2O to a total volume of 25 µL. Thermalcycling (C1000 Touch™ Thermal Cycler, Bio-Rad) conditions were as follows: 5 min at 95°C, 30 cycles of 30 sec at 95°C, 1 min at 53°C, and 1 min at 72°C, followed by 10 min at 72°C). The PCR products were verified by 1.5 % agarose gel electrophoresis and then purified with the Qiaquick PCR purification kit (Qiagen, Venlo, the Netherlands). Equal amounts of amplicon from each sample were mixed together and subjected to 454 sequencing on a GS FLX Titanium 454 pyrosequencing platform (Macrogen Europe, Amsterdam, the Netherlands).

6.2.5 Bioinformatics and statistical analyses

Sequence data and quality information was transferred to the Galaxy interface (Goecks *et al.*, 2010) using the SFF converter tool. Sequences were then de-multiplexed and further analyzed with the QIIME pipeline version 1.6 (Caporaso *et al.*, 2010). In the first step, sequences with a maximum of 6 ambiguous bases, 6 homopolymer runs, zero primer mismatches, a maximum of 1.5 errors in the barcode sequence and passed a quality score window of 50 were binned according to sample id and the barcodes were removed. Further, the DENOISER algorithm version 1.6.0 was used to correct for sequencing errors, and chimeras were removed by USEARCH (Edgar, 2010). Sequences were then aligned by PyNAST (Caporaso *et al.*, 2010) and UCLUST (Edgar, 2010) and assigned to OTUs (Operational Taxonomical Units), using a minimum sequence identity cutoff of 97%. From all OTU clusters, the most abundant sequence was selected as a representative for taxonomy assignment by using the SILVA database (release 108 SSU) with a minimum identity value of 75%. The relative abundance of different bacterial groups was calculated in each sample by comparing the number of sequences classified as belonging to the specific bacterial groups versus the number of classified bacterial sequences per sample. The Shannon Wiener index was used to calculate diversity in the different samples. Final graphs generated using the program MEGAN (Huson *et al.*, 2007).

All mentioned significant differences are the result of a t-test with appropriate variance distributions as determined by an f-test. Tests for significance were performed in Excel (Microsoft Corp.).

6.3 Results

6.3.1 Single-strain inoculation experiments

After 7 hours, *Collimonas fungivorans* Ter331 had moved a distance of 14 mm in sand microcosms for both nutrient levels (1% and 10% TSB). After 24 and 48 hours, we could observe significant differences (P< 0.05) in movement between 1% TSB (average ~28 mm and 40 mm, respectively) and 10% TSB (average ~12 mm and ~24 mm, respectively) (Fig. 1). An opposite pattern was observed for *Pseudomonas fluorescens*, which moved faster at higher nutrient levels (Fig. 1). *Pseudomonas fluorescens* had already colonized almost the entire microcosm at 7 h at 10% TSB, but not at 1% TSB. The lower nutrient level was therefore chosen for subsequent experiments, because it provided the appropriate range of expansion in the microcosm setup and was more representative of the nutrient poor conditions that are typical for most soils. We chose sampling times of 24 and 48 h, as this provided information on the rate of colonization during the period required for full expansion throughout the microcosm.

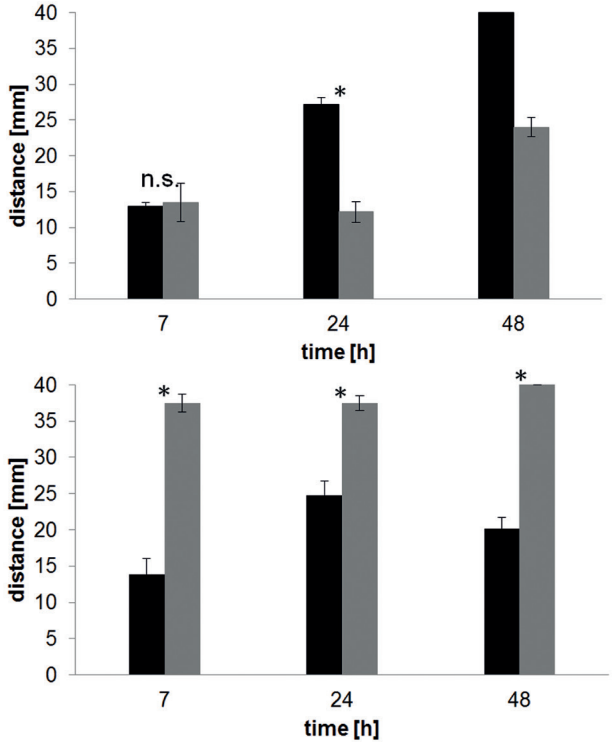


Figure 1. Expansion of (a) *Collimonas fungivorans* strain Ter331 and (b) *Pseudomonas fluorescens* strain Pf0-1 in sand microcosms under different nutrient concentrations at 7, 24 and 48 h after inoculation. Error bars depict the standard errors. * indicates statistically significant differences (P< 0.05). Black bars = 1% TSB, grey bars = 10% TSB.

6.3.2 Tracking total bacterial community expansion by qPCR

At 24 h, we found on average $7.9*10^3$ 16S rRNA gene copies per gram sand at distance 1 (0.5 - 1.5 cm away from the microcosm center) and $2.8*10^3$ at distance 2 (1.5 - 2.5 cm away from the microcosm center). At distances 3-5 (2.5 - 5.5 cm away from the microcosm center), bacterial gene copy numbers were below the level of detection. At 48 h, there were on average $5.7*10^6$ bacterial ribosomal gene copies at distance 1, $4.4*10^6$ at distance 2, $1.6*10^6$ at distance 3, $2.5*10^5$ at distance 4, and $4.8*10^3$ at distance 5 (Fig. 2). Thus, the expansion of bacteria was about 0.5 - 1.5 cm after 24 h, whereas after 48 h, nearly the whole sand microcosm was colonized.

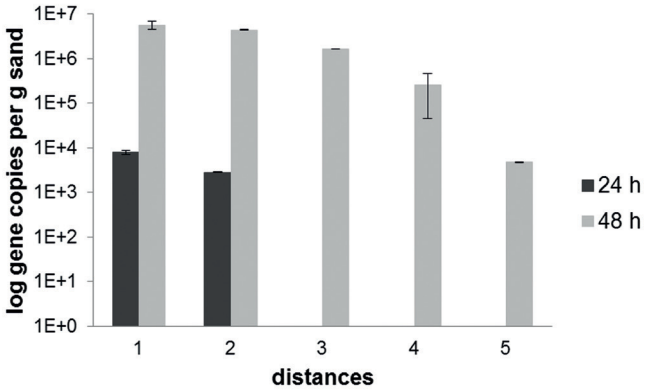


Figure 2. Copy numbers of 16S rRNA genes at different distances from the bacterial community inoculation center in sand microcosms at 24 and 48 h as determined by qPCR. Error bars depict the standard deviation and * indicate statistically significant differences ($P< 0.05$). Distances between sampling spots1 to 5 and the inoculation spot are indicated in Fig. S1.

6.3.3 Tracking community expansion via 16S rRNA gene pyrosequencing

Pyrosequencing of the V4 region of bacterial small subunit (16S) ribosomal RNA genes was performed for samples taken at 48 h, where bacteria were found to be present at all sample distances from the inoculation center. Pyrosequencing yielded 112,198 reads that could be classified to the kingdom bacteria. The obtained reads were grouped into a total of 199 Operational Taxonomical Units (OTUs). Read distribution varied substantially among samples (Tab. 1 and S1), and since samples belonging to replicate F only yielded between 0 and 108 reads in total, we decided to exclude all samples from this replicate from further analyses. Distance 5 from replicate H was also excluded because we could not obtain replicated data for that distance.

Table 1. Relative abundance of OTUs assigned to different taxa in the soil bacterial inoculum that was added to the center of sand microcosms (distance=0) and in sampling spots at distances 1 to 4, that were colonized during 48 h. Two replicates for the inoculums (1 and 2) and 3 replicates (E,G and H) for the further distances are shown.

sample	ino 1	ino 2	replicate E				replicate G				replicate H			
distance	0	0	1	2	3	4	1	2	3	4	1	2	3	4
taxa														
Enterobacteriaceae	5.61	1.46	88.11	89.82	95.88	99.80	56.78	80.01	99.96	97.44	74.82	72.08	99.96	99.13
Pseudomonas	6.49	6.35	5.30	2.74	0.24	0.00	38.27	15.96	0.00	0.00	5.26	0.12	0.02	0.49
Massilia	0.00	0.01	4.42	6.95	3.49	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Undibacterium	0.00	0.01	0.56	0.21	0.18	0.00	0.13	0.00	0.01	1.92	16.79	27.54	0.01	0.10
Luteibacter	0.03	0.00	0.56	0.07	0.02	0.00	3.08	1.03	0.00	0.00	0.57	0.01	0.00	0.10
Paenibacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.83	3.00	0.01	0.00	0.00	0.00	0.00	0.07
Actinobacteria	11.69	16.60	0.32	0.00	0.07	0.00	0.03	0.00	0.00	0.00	0.14	0.00	0.00	0.03
Phenylobacterium	1.54	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sphingomonas	0.03	2.44	0.16	0.14	0.01	0.00	0.44	0.00	0.00	0.00	0.28	0.00	0.00	0.00
Neisseriaceae	3.44	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Caulobacter	1.68	0.00	0.08	0.00	0.00	0.00	0.36	0.00	0.00	0.00	0.00	0.00	0.01	0.03
Prevotella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Pontibacter	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chlamydiae	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Opitutus	0.00	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spartobacteria	2.83	10.45	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Verrucomicrobiaceae	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chloroflexi	3.02	13.68	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cyanobacteria	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Deinococcus	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Elusimicrobia	0.85	1.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Holophagae	3.66	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fibrobacteraceae	0.66	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tumebacillus	1.04	1.30	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacillaceae	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Brochothrix	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Granulicatella	1.43	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Leuconostoc	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Weissella	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Streptococcus	4.95	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Erysipelotrichaceae	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Fusobacteria	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gemmatimonadetes	2.17	7.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nitrospira	4.87	1.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Brevundimonas	2.69	0.87	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Beijerinckiaceae	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nitrobacter	0.52	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rhodoplanes	0.00	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Methylobacterium	1.65	0.64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Rhodobium	0.00	1.39	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pseudolabrys	6.13	4.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetobacteraceae	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dongia	0.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Skermanella	0.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Candidatus Captivus	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Holospora	0.52	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sphingobium	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Alcaligenaceae	1.73	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Burkholderia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.85	0.00	0.00	0.03
Cupriavidus	0.77	0.80	0.00	0.07	0.01	0.00	0.00	0.00	0.00	0.00	0.28	0.25	0.00	0.00
Comamonadaceae	3.63	1.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.64	0.00	0.00	0.00	0.00
Nitrosomonadaceae	9.82	17.46	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rhodocyclaceae	0.30	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Desulfobacterales	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nannocystineae	2.23	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aquicella	3.52	9.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Legionella	1.79	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pseudospirillum	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pasteurellaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Acinetobacter	3.66	0.07	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Enhydrobacter	0.00	0.13	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dokdonella	1.04	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dyella	0.00	0.03	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crenarchaeota	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Halobacteriaceae	0.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

The spatial distribution of the four most abundant bacterial OTUs that had expanded from the center inoculation spot during 48 h is given in Fig. 3 (average of all microcosms) and in Fig. S2 (individual microcosms). A single OTU that could be classified within the Enterobacteriaceae increased strongly in relative abundance with increasing distance from the point of inoculation (from 3.5% relative abundance in the inoculum to, 73%, 81%, 99% and 99% at distances 1 to 4, respectively). At distances 1 and 2, other bacteria that were detected in variable abundances in addition to the Enterobacteriaceae, and these include members of the genera *Pseudomonas*, *Massilia* and *Undibacterium* (Fig. S2). At distances further away from the point of inoculation (3 and 4), the bacterial community was consistently dominated by apparently fast moving Enterobacteriaceae in all replicates (98.6% and 98.8% relative abundance on average, respectively). In all dilutions that were prepared from fresh soil taken at different distances, colonies with of orange coloring, which are a characteristic for bacteria of the genus *Pantoea* (family Enterobacteriaceae), were found. Sequencing of this most dominant isolate and comparison with results obtained by nucleotide Blast search of a 550bp DNA stretch confirmed our isolated bacterium to be *Pantoea agglomerans*.

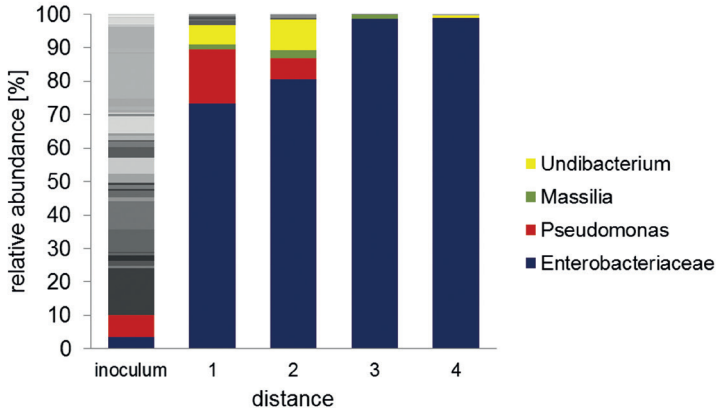


Figure 3. Relative abundance of the bacterial phyla at distances 1-4 at 48 h and in the inoculum. The 4 most abundant are displayed in color. Each bar depicts the average of 3 replicate samples.

Bacterial diversity calculated using the Shannon Wiener index was found to be much greater in the microbial inoculum spot (H = 2.9 ± 0.54) than in the zones occupied by colonizing bacteria (P≤ 0.05) (Tab. S1). Diversity indices significantly decreased with increasing distance from the central inoculum spot (Fig. 4 and Tab. S1).

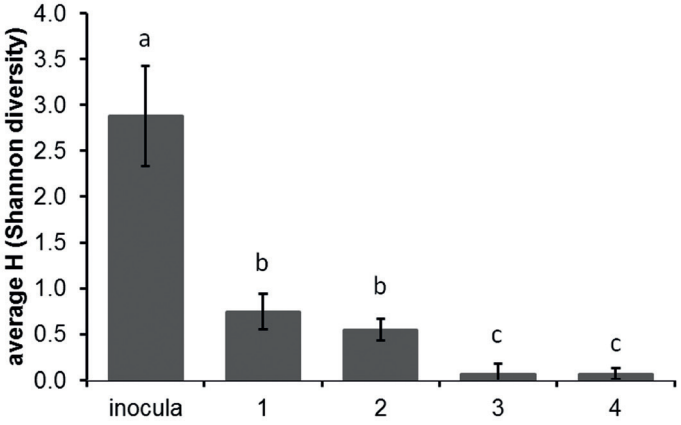


Figure 4. Comparison of bacterial diversity between the different sampling points as calculated by the Shannon Wiener diversity index (H). Error bars depict the standard deviation. Different letters indicate significant differences ($P<0.05$).

6.4 Discussion

In this study, we used a well-defined and easy to manipulate sand microcosm system that mimics many important soil features, such as soil structure, moisture and nutrient levels, allowing us to study bacterial motility in an ecological relevant setting. We applied this system to investigate the colonization patterns of single bacterial strains as well as different bacterial taxa in a complex bacterial community.

Results of the single-strain experiments yielded contrasting patterns of colony expansion for the two model strains examined. *Collimonas fungivorans* exhibited greater expansion at lower nutrient levels, whereas *Pseudomonas fluorescens* moved faster at higher nutrient levels. Thus, the impact of nutrient levels on bacterial motility in our microcosm setup was strain dependent. Results of the complex community experiment showed that only few bacterial cells had moved after 24 h and only a small radius around the inoculation zone was initially colonized (Fig. 1). At 48 h, the whole dimension of the microcosm was colonized, with cell densities declining with increasing distance from the inoculation zone (Fig. 1). Not only did cell densities decrease with increasing distance to the inoculation zone, bacterial diversity was also highest close to the inoculation zone and decreased with distance from that point. To our knowledge, this is the first study that examines the movement of bacteria within a complex natural community under semi-natural conditions (*i.e.* low nutrient conditions and relatively low moisture content (7.5%, which is approximately 35.7% of the water holding capacity which is approximately 21%). The fact that colonization of the entire microcosm occurred within 48 hours under our experimental conditions makes it likely that we observed active movement of bacteria, as

opposed to passive dispersal as may occur in experimental designs with higher moisture contents and shorter distances (Wertz *et al*., 2007).

Based upon sequence recovery by high-throughput pyrosequencing, bacteria belonging to the genera *Undibacterium*, *Pseudomonas*, and *Massilia*, and especially the family of Enterobacteriaceae, were most successful in expanding through the microcosm habitat (Figs. 3 & A3). Relative recovery of sequences from these bacteria increased sharply with distance from the inoculation zone, whereas as many other taxa found in the original inoculum were no longer detected in samples more distant from the point of inoculation. The bacterial genera that were most frequently detected at the more distant sample locations are all known to possess flagella and are often abundant in the rhizosphere and on plant roots (Lugtenberg & Dekkers, 2001, Chunga *et al*., 2005, Ofek *et al*., 2012).

The by far most dominant member of the mobile community was an OTU belonging to the family *Enterobacteriaceae* (γ-*Proteobacteria*). Based upon analysis of the dominant colony form recovered from the motile zones of this experiment, this OTU was tentatively assigned to the species *Pantoea agglomerans*. The family *Enterobacteriaceae* is widespread and commonly associated with eukaryotic hosts like humans, animals or plants. Animal manure and dung naturally contains high amounts of *Enterobacteria,* and manure-derived strain have the ability to spread though and establish themselves within soil environments (Jiang *et al*., 2002; van Elsas *et al.* 2007). Interestingly, while *E. coli* O157:H7 strain T was unable to invade soil microbial communities with high diversity communities, it could quickly colonize soils with less diverse communities (Van Elsas et al., 2012). Motility is considered an important trait in the lifestyle of *Enterbacteriaceae* (Yang *et al*., 2004), and the sterilized soil conditions used in our microcosm experiments evidently created the necessary habitat availability required for enterobacterial expansion.

Bacterial motility is of importance to re-colonization of soils. For instance, strong and sudden disturbances may result in a drastic reduction of biomass (Postma *et al*., 1989) and even sterilization, e.g. in the event of a forest fire (Prieto-Fernandez *et al*., 1998, Neary *et al*., 1999). Motile microorganisms, obviously, have an advantage in re-colonizing disturbed soils or soils with low biomass, especially in the early stages of re-colonization, as they are the first to reach these habitats. This may be of particular importance in microbial succession, given the fact that priority affects are often important in determining the success of bacterial populations to invade new territory (Remus-Emsermann *et al*., 2013). Microbial re-colonization might be an essential mechanism that helps to stabilize functional redundancy, and therefore an important parameter when considering the restoration of disturbed (microbial) soil systems (Bodelier, 2011). Identifying the (most) motile strains thus holds potential to predict and control re-colonization succession of sterilized soils or soils of reduced microbial biomass.

Here, we demonstrate the applicability of our microcosm system to study bacterial motility in a soil-like environment. We show that different bacterial taxa colonize the sand

microcosms at different speeds and we identify the most motile taxa under one moisture- and one nutrient level. Further additional research priorities would include examination of how other biotic and abiotic soil features, such as pH and disturbance, impact relative mobility, and the presented microcosm system might be ideal for such an examination (Wolf *et al*., 2013).

6.5 Acknowledgements

We thank Wouter Lokhorst and Peter Veenhuizen for their assistance with the experimental work. This work was sponsored by the VICI grant of GAK from the Dutch Science Foundation (NWO) entitled “Crossing the frontiers of microbial ecology”.

6.6 Supporting Information

Table S1. Overview of number of reads, Shannon Wiener diversity index and species richness in the samples taken at 24 and 48 h at the different distances from the inoculation zone.

type	distance	replicate	timepoint (h)	reads (total n)	shannon diversity (H)
inoculum	0	1	0	3637	3.27
inoculum	0	2	0	8759	2.50
sample	1	A	24	2255	2.54
sample	1	B	24	3979	1.99
sample	1	C	24	8729	0.84
sample	2	C	24	9876	0.41
sample	1	D	24	2184	1.71
sample	2	D	24	782	2.56
sample	1	E	48	1245	0.53
sample	2	E	48	1424	0.41
sample	3	E	48	8448	0.20
sample	4	E	48	992	0.02
sample	1	F	48	108	1.41
sample	2	F	48	0	0.00
sample	3	F	48	24	0.98
sample	4	F	48	62	2.53
sample	1	G	48	3836	0.90
sample	2	G	48	3201	0.62
sample	3	G	48	19580	0.00
sample	4	G	48	469	0.13
sample	1	H	48	703	0.82
sample	2	H	48	11501	0.62
sample	3	H	48	12604	0.00
sample	4	H	48	2873	0.06
sample	5	H	48	4927	0.39

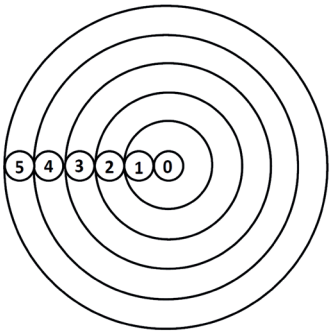


Figure S1 Schematic overview of the sampling points in the microcosms. The microcosms were inoculated in the center (distance 0) and samples were taken with sterilized pipet tips (~1 cm diameter) at distances 1-5 (approximately 0.5 - 1.5; 1.5 - 2.5; 2.5 - 3.5; 3.5 - 4.5; 4.5 - 5.5 cm away from the center, respectively) .

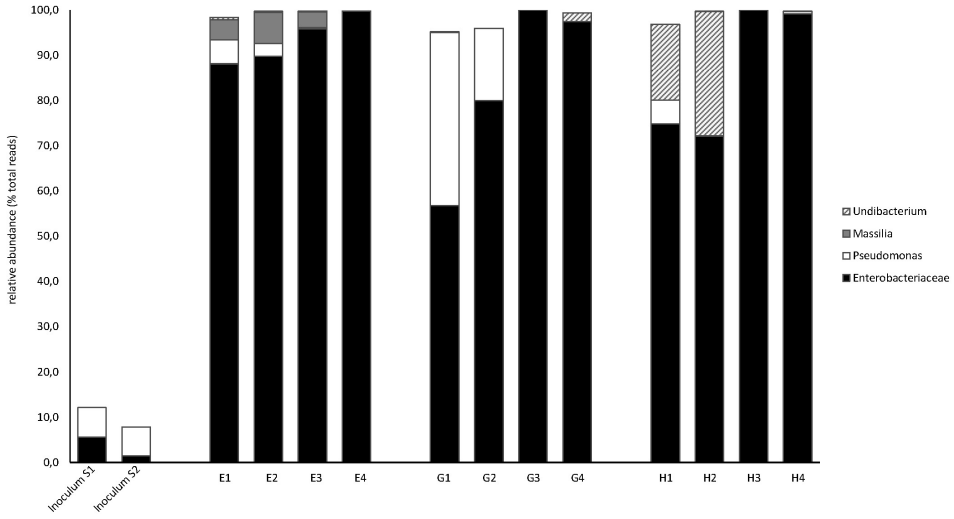


Figure S2. Comparison of bacterial community composition between the inoculum and the different sampling distances. Stacked column graph represent the relative distribution of the four most abundant phyla in the different samples.

Chapter 7

General discussion

The general aim of this thesis was to increase our understanding of the forces that allow for the establishment and maintenance of the tremendous diversity of soil-borne microbial communities. Specifically, I developed a sand microcosm system, which I then used to investigate the effects of habitat connectivity on microbial interactions and the structure and diversity of bacterial and fungal communities. In this discussion, I examine the possibilities and limitations of using such microcosm experimentation in conjunction with next-generation molecular techniques and explore the new insights that can be gleaned into the drivers of soil-borne microbial community structure and diversity.

Below, I provide an outline of this general discussion. Experimental microcosm systems are a powerful tool to investigate microbial interactions in a more realistic environment, as compared to agar plate or liquid media setups that have typically been used to investigate microbial interactions. In section 7.1 of this general discussion, I discuss how microcosm-based experimentation can be used to gain new insight into the relation between specific soil characteristics, microbial interactions and community structure and diversity, as well as to investigate relative bacterial colonization potential. Such microcosm-based approaches are compared to next-generation sequencing approaches that examine drivers of microbial communities from a survey perspective. One major finding of this thesis is that the hyphal growth form provides a competitive advantage in poorly connected soils (Chapters 3 and 4). In section 7.2, I discuss how the filamentous growth form of actimomycetes, as well as that of fungi, may provide a competitive advantage over non-filamentous bacteria in soils of low hydraulic connectivity, *i.e.* soils containing a large proportion of air-filled pores. The second major finding of this thesis is the contrasting effect of habitat connectivity on the diversity of bacterial and fungal communities in soil (Chapter 5). Poorly connected soils (coarse pores and low moisture levels) supported the greatest amount of bacterial diversity, while the opposite was found for fungi with less diverse communities in poorly connected soils and greatest diversity in well-connected habitats. The potential drivers of these contrasting diversity patterns are discussed in section 7.3. In section 7.4, I discuss the importance of microbial motility in the exploration of new micro-habitats in soil. To this end, I reflect on the application of microcosm experimentation to study bacterial motility both as individual strains and complex communities (Chapter 6). Finally, section 7.5 offers general conclusions and provides perspectives for future research priorities.

7.1 Microcosm experimentation as a tool in microbial ecology

Soil microbial communities represent a tremendous reservoir of phylogenetic and functional diversity. Despite the key role of microorganisms in numerous important soil processes, only a small fraction (<1%) of microbes has been cultured in the laboratory (Hugenholtz *et al.*, 1998)1998, and consequently their metabolic roles are still only partially understood. The in-depth determination of community composition has recently been facilitated by advances in sequencing techniques, and metagenomic studies are without doubt a powerful tool to reveal the diversity and structure of microbial communities. However, several important hurdles remain in our ability to examine soil-borne microbial communities. First of all, it is important to keep in mind that only the pool of extracted DNA is examined by any molecular method. Given the fact that no current DNA extraction method provides access to the complete soil metagenome (Delmont *et al.*, 2011), it remains difficult to relate the recovery of DNA sequences to exact gene numbers *in situ*. Furthermore, there is typically no distinction made between DNA extracted from active and inactive cells (Hirsch *et al.*, 2010). Given the fact that a large majority of the community may be inactive or dormant at any given time (Lennon & Jones, 2011), the link between recovered sequence information and actual activity remains tenuous at best. In addition, numerous unknown biases remain in the amplification, sequencing and downstream processing of DNA sequence information.

More importantly, in-depth sequencing by itself is typically not an appropriate approach to address ecological questions, and given the limited links with *in situ* physiology (see above), high-throughput sequencing methodologies do not eliminate the need for culture-based studies. Indeed, cultivation-independent and -dependent approaches remain complementary, with the former helping to explore the breadth of diversity and the latter helping to assign functions to the vast number of unknown genes that can be recovered from soil communities. Furthermore, microbial interactions are key to shaping community structure (Tiedje *et al.*, 2001) and these interactions are not revealed by metagenomic approaches, which lump entire (meta-)communities into a single gene pool (Vos *et al.*, 2013). Thus, culture-dependent studies and studies that experimentally manipulate microbial communities are still needed to link interactions to community composition.

Several important questions in soil microbial ecology are related to determining the factors that influence microbial diversity and the relative abundance of specific microbial groups (from strains to phyla). The ultimate goal is to relate phylogenetic and functional diversity to the diverse ecosystem functions to which microbes contribute, such as biogeochemical cycling and the maintenance of soil structure and quality, pathogen control and plant productivity (van der Heijden *et al.*, 2008). Instead of focusing solely on sequencing based methods, which essentially provide an inventory of diversity and community structure, approaches combining traditional, cultivation-based ecological research with state-of-the-

art molecular ecology may ultimately be better suited to answering specific ecological questions and to linking microbial community composition, diversity and interactions with ecosystem functioning.

Next-generation sequencing approaches have typically been used in field surveys to describe the composition of microbial communities in their *in situ* habitats, but such methods have rarely been used to test specific hypotheses. In this thesis, I show that the application of next-generation sequencing to controlled experimental conditions with proper replication and statistical power can lead to robust and detailed results, yielding new insights into the drivers of microbial community structure. Next-generation sequencing does thus not eliminate the need for experimental work, but rather traditional microbiological and novel high-throughput sequencing approaches should be regarded as highly complementary.

In the framework of this thesis, I developed an experimental sand microcosm system that allows one to manipulate specific soil parameters, such as pore size distribution, moisture and nutrient levels, independently. In this system, I tested specific hypotheses regarding bacterial interactions and the ecology of microbial communities using both traditional culture-dependent microbiological methods as well as high-throughput sequencing techniques. Furthermore, this soil-like system was a useful tool to study bacterial motility in soil. I investigated both how bacteria possessing different motility mechanisms differ in their ability to colonize new soil environments at different moisture levels and nutrient concentrations and motility dynamics within complex bacterial communities.

In Chapter 3, I used my soil-like microcosm system specifically to study resource competition between filamentous and non-filamentous bacteria. However, this system is applicable to study a range of other microbial interactions such as interference competition, mutualism, altruism, and quorum sensing. Also, in the context of this thesis, I examined the phylogenetic composition of complex microbial communities. However, this system also holds the potential to investigate questions regarding the link between phylogenetic and functional diversity. One does, however, have to keep in mind that even though the microcosm systems used in this thesis provide realistic soil conditions, they are still an oversimplification of the soil environment. It should be possible to increase the complexity of such microcosm systems to better reflect actual soil conditions. Such added complexity would help to translate laboratory results to explain patterns and activities in the field, yet might also complicate the interpretation of specific laboratory experiments by introducing potential confounding factors. In conclusion, the microcosm systems developed and used in this thesis provide a good simulation of actual soil environments. Thus, this system is well suited for testing the results obtained in simple, artificial laboratory systems, such as liquid culture and agar plates. Furthermore, this system could be applied to validate data obtained from modelling approaches or to generate data to help parameterize models of bacterial growth and interaction.

7.2 Filamentous growth forms provide a competitive advantage in soils of low connectivity

In Chapter 3, I investigated resource competition between filamentous and non-filamentous bacteria, using *Streptomyces* and *Bacillus* strains as examples, respectively. In Chapters 4 and 5, I examined the interactions between bacteria and fungi in complex communities. In all cases, these interactions were tracked in response to changes in the soil parameters pore size distribution and matric potential, which determine habitat connectivity, in defined quartz sand microcosms. I found bacterial biomass to be highest in fine pores and fungal biomass to be highest in coarse pores, which is in agreement with previous observations in natural soils (Hattori, 1988, Killham, 1994). Both filamentous bacteria and fungi have the ability to grow hyphae into and across air spaces, potentially giving them the ability to bridge air-gaps. A major difference between bacterial and fungal hyphae is the size; fungal hyphae typically have a diameter of 3–10 µm, and bacterial hyphae are only approximately 0.5–1 µm in diameter. This difference is size may thus affect spread within the soil matrix. However, we found that both fungi (Chapter 4 and 5) and filamentous bacteria (Chapter 3) have a relative competitive advantage over non-filamentous bacteria in poorly connected soils. I attributed this relative advantage to their ability to bridge air-gaps and thus explore new micro-habitats that may remain inaccessible to most non-filamentous bacteria. An interesting corollary to these differences in growth form is that filamentous organisms would be expected to be more susceptible to the physical disturbance of soil. The microcosm systems developed here would also be useful in the examination of this proposed relationship, by introducing different disturbance regimes into the experimental design.

7.3 Contrasting effects of habitat connectivity on bacterial and fungal diversity

In Chapter 5, I demonstrate that decreased habitat connectivity supports greater bacterial diversity and richness. These findings are in agreement with my original hypothesis, suggesting that low connectivity promotes physical separation of potentially competing species, thereby allowing for the maintenance of a greater level of bacterial diversity. Such patterns have also been observed in systems using natural soils (Carson *et al.*, 2010) and support my interpretation of the findings presented in Chapter 5. Fungal communities, however, displayed the opposite trend with greater diversity and richness in well-connected soils (fine pores and high moisture content). A possible explanation may be that less connected, and thus drier, soils support a smaller range of fungal species, as these conditions may impose a strong selection for relatively few species that are well adapted to “drought” conditions. Thus, the selection for desiccation resistant fungi in least

connected systems may result in an overall lower level of fungal diversity. However, further research is needed to address these issues in greater detail.

I also discovered that pore size distribution generally had a greater impact than matric potential in structuring the microbial communities. This result was consistent across a number of microcosm experiments presented in this thesis and was observed in both PLFA and DNA sequence datasets. Interestingly, this trend was observed for both bacterial and fungal communities, despite the contrasting patterns of diversity discussed above. I had initially expected bacteria to be more affected by soil moisture than fungi, due to their dependence on soil water to gain access to resources etc. However, this initial expectation was not met. Lastly, I observed that specific bacterial phyla and fungal species were affected differently by different pore size distributions and matric potentials. This may provide hints into the life history strategies of specific bacterial and fungal groups and potentially provide insight into the evolutionary depth at which specific adaptations to particular environmental conditions occur. Future experiments that examine the relative success of different microbial lineages in response to various environmental manipulations might offer a novel means of examining these issues experimentally. Interestingly, the presence of fungi promoted bacterial diversity in my microcosm experiments. The exact mechanism behind this interaction is not yet clear and demands future investigation.

7.4 Bacterial motility in sand microcosms

Little is known about how the heterogeneous soil environment influences the ability of bacteria possessing different motility mechanisms to explore and colonize new habitats. Previous experimental work has mostly been performed under rather artificial conditions, such as on agar plates (Harshey, 2003, Wang *et al*., 2004, Caiazza *et al*., 2005) or rough surfaces (Dechesne *et al*., 2010). Other data has principally been generated by using modeling approaches (Long & Or, 2009, Wang & Or, 2010). In Chapters 3 and 6, I therefore investigated the effect of nutrient availability on the motility of bacteria differing in motility mechanisms as well as bacterial motility within a complex community in sand microcosms. These microcosms mimic important soil features, such as soil structure, moisture and nutrient availability, more faithfully than previously used systems, thereby allowing for a more realistic examination of motility patterns. The results presented in this thesis demonstrate that different bacterial strains exhibit highly disparate colonization patterns within the sand microcosm system. For instance, increased nutrient availability decreases the spread of the model species *C. fungivorans,* yet increases the spread of *Pseudomonas fluorescens* (Chapter 6). Furthermore, only a relatively minor subset of the total community was able to expand to an appreciable distance (more than 2 cm) within 48 hours of inoculation at a given moisture and nutrient level, with the genera *Undibacterium*, *Pseudomonas*, and *Massilia* and especially the *Enterobacteriaceae* dominating the communities most distant from the

point of inoculation. Such motility experiments could be expanding in the future to include different pore size distributions, moisture levels, nutrient conditions and starting inoculum.

7.5 Conclusions and future perspectives

The work presented in this thesis demonstrates that microcosm experimentation is a useful tool to test specific hypotheses regarding the effect of soil characteristics on microbial interactions and community structure, especially when coupled to detailed community analysis as afforded by next-generation sequencing approaches. My microcosm system provides a good simulation of a structured soil habitat, while, in contrast to real soils, being easy to manipulate. Habitat connectivity was observed to be an important factor in determining microbial interactions, such as resource competition, and the structure and diversity of bacterial and fungal communities. Hyphal growth could be identified as an important feature that provides actinomyces and fungi with a relative advantage over non-filamentous bacterial in low-connected soils due to their ability to bridge air-gaps. Overall, soil structure had a greater impact on microbial community structure than moisture, and was observed for both bacterial as well as fungal communities, using multiple community analysis methods. However, I found that soil connectivity had a contrasting impact on patterns of bacterial and fungal diversity. Low habitat connectivity supported the greatest bacterial diversity, while high connectivity habitats supported the greatest levels of fungal diversity. These patterns may be related to reductions in interspecific competition amongst bacteria and the selection of specific desiccation tolerant fungi, but future research is still needed to shed light on the mechanisms that lead to these contrasting patterns of bacterial and fungal diversity.

The results of this thesis demonstrate that specific soil characteristics (*i.e.* pore size distribution and matric potential) impact microbial interactions as well as the structure and diversity of soil-borne microbial communities. I realize that many more factors impact microbial communities than the ones studied in this thesis. However, studies such as presented in this thesis contribute to our mechanistic understanding of microbial community diversity and activity in complex soil communities.

In future research, this microcosm system, or variations thereof, could be applied to examine other microbial interactions than the ones examined in this thesis. A further aim should be to develop experimental systems that allow one to track microorganisms *in situ* and in real time, thereby providing greater spatial information related to microbial interactions and more detailed information on microbial population dynamics. Also, future research should thus focus on relating phylogenetic diversity to ecosystem functioning with the ultimate aim of predicting and controlling the link between community structure and ecosystem processes.

References

A

Abuashour J, Joy DM, Lee H, Whiteley HR & Zelin S (1994) Transport of Microorganisms through Soil. *Water Air Soil Poll* **75**: 141-158.

Allison SD (2005) Cheaters, diffusion and nutrients constrain decomposition by microbial enzymes in spatially structured environments. *Ecol Lett* **8**: 626-635.

Alonso D, Etienne RS & McKane AJ (2006) The merits of neutral theory. *Trends Ecol Evol* **21**: 451-457.

Anderson JP & Domsch KH (1973) Quantification of Bacterial and Fungal Contributions to Soil Respiration. *Arch Mikrobiol* **93**: 113-127.

Antunez-Lamas M, Cabrera-Ordonez E, Lopez-Solanilla E, Raposo R, Trelles-Salazar O, Rodriguez-Moreno A & Rodriguez-Palenzuela P (2009) Role of motility and chemotaxis in the pathogenesis of Dickeya dadantii 3937 (ex Erwinia chrysanthemi 3937). *Microbiology* **155**: 434-442.

Asensio D, Owen SM, Llusia J & Penuelas J (2008) The distribution of volatile isoprenoids in the soil horizons around Pinus halepensis trees. *Soil Biol Biochem* **40**: 2937-2947.

Ashelford KE, Day MJ & Fry JC (2003) Elevated abundance of bacteriophage infecting bacteria in soil. *Appl Environ Microb* **69**: 285-289.

B

Baas Becking LGM (1934) *Geobiologie of inleiding tot de milieukunde*.

Baileya VL, McCuea LA, Fanslera SJ, Boyanov MI, DeCarloc F, Kemnerb KM & Konopka A (2013) Micrometer-scale physical structure and microbial composition of soil macroaggregates. *Soil Biol Biochem* **6**: 60–68.

Baldrian P (2008) Ecology of Saprotrophic Basidiomycetes. Vol. 28 (Boddy L, Frankland JC & West Pv, *eds*.), pp 19–41. British Mycological Society Symposia Series.

Baltz RH & (2007) Antimicrobials from Actinomycetes. Back to the future. *Microbe* **2**: 125-131.

Bapiri A, Baath E & Rousk J (2010) Drying-Rewetting Cycles Affect Fungal and Bacterial Growth Differently in an Arable Soil. *Microb Ecol* **60**: 419-428.

Barnard RL, Osborne CA & Firestone MK (2013) Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. *Isme J* **7**: 2229-2241.

Barron GL (1988) Microcolonies of Bacteria as a Nutrient Source for Lignicolous and Other Fungi. *Can J Bot* **66**: 2505-2510.

Bartram AK, Lynch MD, Stearns JC, Moreno-Hagelsieb G & Neufeld JD (2011) Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads. *Appl Environ Microb* **77**: 3846-3852.

Baudoin E, Benizri E & Guckert A (2003) Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biol Biochem* **35**: 1183-1192.

Becker JM, Parkin T, Nakatsu CH, Wilbur JD & Konopka A (2006) Bacterial activity, community structure, and centimeter-scale spatial heterogeneity in contaminated soil. *Microb Ecol* **51**: 220-231.

Bell T (2010) Experimental tests of the bacterial distance-decay relationship. *Isme J* **4**: 1357-1365.

Berg G & Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *Fems Microbiol Ecol* **68**: 1-13.

Bezbaruah AN & Zhang TC (2002) Fabrication of anodically electrodeposited iridium oxide film pH microelectrodes for microenvironmental studies. *Anal Chem* **74**: 5726-5733.

Borcard D, Legendre P & Drapeau P (1992) Partialling out the Spatial Component of Ecological Variation. *Ecology* **73**: 1045-1055.

Boswell GP, Jacobs H, Davidson FA, Gadd GM & Ritz K (2002) Functional consequences of nutrient translocation in mycelial fungi. *J Theor Biol* **217**: 459-477.

Brockhurst MA, Buckling A & Rainey PB (2006) Spatial heterogeneity and the stability of host-parasite coexistence. *J Evolution Biol* **19**: 374-379.

Bronick CJ & Lal R (2005) Soil structure and management: a review. *Geoderma* **124**: 3-22.

Buckling A, Maclean RC, Brockhurst MA & Colegrave N (2009) The Beagle in a bottle. *Nature* **457**: 824-829.

Buckling A, Harrison F, Vos M, Brockhurst MA, Gardner A, West SA & Griffin A (2007) Siderophore-mediated cooperation and virulence in Pseudomonas aeruginosa. *Fems Microbiol Ecol* **62**: 135-141.

Burmølle M, Hansen LH & Sørensen SJ (2007) Establishment and early succession of a multispecies biofilm composed of soil bacteria. *Microb Ecol* **54**: 352-362.

Burmølle M, Kjøller A & Sørensen SJ (2011) Biofilms in Soil. *Encyclopedia of Agrophysics* **8**: 70.

Buttner D & Bonas U (2010) Regulation and secretion of Xanthomonas virulence factors. *Fems Microbiol Rev* **34**: 107-133.

C

Caiazza NC, Shanks RMQ & O'Toole GA (2005) Rhamnolipids modulate swarming motility patterns of Pseudomonas aeruginosa. *J Bacteriol* **187**: 7351-7361.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL & Knight R (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**: 266-267.

Caporaso JG, Kuczynski J, Stombaugh J*, et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335-336.

Carson JK, Campbell L, Rooney D, Clipson N & Gleeson DB (2009) Minerals in soil select distinct bacterial communities in their microhabitats. *Fems Microbiol Ecol* **67**: 381-388.

Carson JK, Gonzalez-Quinones V, Murphy DV, Hinz C, Shaw JA & Gleeson DB (2010) Low Pore Connectivity Increases Bacterial Diversity in Soil. *Appl Environ Microb* **76**: 3936-3942.

Castro HF, Classen AT, Austin EE, Norby RJ & Schadt CW (2010) Soil Microbial Community Responses to Multiple Experimental Climate Change Drivers. *Appl Environ Microb* **76**: 999-1007.

Chang C-W, Laird DA, Mausbach MJ & Hurburgh CR (2001) Near-Infrared Reflectance Spectroscopy–Principal Components Regression Analyses of Soil Properties. *Soil Sci Soc Am J* **65**: 480-490.

Chao A (1984) Nonparametric-Estimation of the Number of Classes in a Population. *Scand J Stat* **11**: 265-270.

Chau J, Bagtzoglou A & Willig M (2011) The effect of soil texture on richness and diversity of bacterial communities. *Environmental Forensics* **12**: 333-341.

Chenu C & Cosentino D (2011) Microbial regulation of soil structural dynamics. (Ritz K & Young I, eds), pp 37–70 CABI, Wallingford, UK.

Chowdhury N, Marschner P & Burns RG (2011a) Soil microbial activity and community composition: Impact of changes in matric and osmotic potential. *Soil Biol Biochem* **43**: 1229-1236.

Chowdhury N, Marschner P & Burns R (2011b) Response of microbial activity and community structure to decreasing soil osmotic and matric potential. *Plant Soil* **344**: 241-254.

Compeau G, Al-Achi BJ, Platsouka E & Levy SB (1988) Survival of rifampin-resistant mutants of Pseudomonas fluorescens and Pseudomonas putida in soil systems. *Appl Environ Microb* **54**: 2432-2438.

Connell JH & Slatyer RO (1977) Mechanisms of Succession in Natural Communities and Their Role in Community Stability and Organization. *American Naturalist* **111**: 1119-1144.

Crawford JW (1994) The Relationship between Structure and the Hydraulic Conductivity of Soil. *Eur J Soil Sci* **45**: 493-502.

Crawford JW, Harris JA, Ritz K & Young IM (2005) Towards an evolutionary ecology of life in soil. *Trends Ecol Evol* **20**: 81-87.

Cruz-Martinez K, Suttle KB, Brodie EL, Power ME, Andersen GL & Banfield JF (2009) Despite strong seasonal responses, soil microbial consortia are more resilient to long-term changes in rainfall than overlying grassland. *Isme J* **3**: 738-744.

Curtis TP, Sloan WT & Scannell JW (2002) Estimating prokaryotic diversity and its limits. *P Natl Acad Sci USA* **99**: 10494-10499.

Czaban J, Gajda A & Wroblewska B (2007) The motility of bacteria from rhizosphere and different zones of winter wheat roots. *Polish Journal of Environmental Studies* **16**: 301-308.

Czaran TL, Hoekstra RF & Pagie L (2002) Chemical warfare between microbes promotes biodiversity. *P Natl Acad Sci USA* **99**: 786-790.

D

D'Costa VM, Griffiths E & Wright GD (2007) Expanding the soil antibiotic resistome: exploring environmental diversity. *Curr Opin Microbiol* **10**: 481-489.

D'Costa VM, McGrann KM, Hughes DW & Wright GD (2006) Sampling the antibiotic resistome. *Science* **311**: 374-377.

Daane LL, Molina JAE, Berry EC & Sadowsky MJ (1996) Influence of earthworm activity on gene transfer from Pseudomonas fluorescens to indigenous soil bacteria. *Appl Environ Microb* **62**: 515-521.

Danielson RE & Sutherland PL (1986) Porosity. *Methods of soil analysis Physical and mineralogical methods,*(Klute A, *ed.*). Amer Soc Agron, Madison, Wisconsin, USA.

Davelos AL, Xiao K, Samac DA, Martin AP & Kinkel LL (2004) Spatial variation in Streptomyces genetic composition and diversity in a prairie soil. *Microb Ecol* **48**: 601-612.

Davenport JR & Jabro JD (2001) Assessment of hand held ion selective electrode technology for direct measurement of soil chemical properties. *Commun Soil Sci Plan* **32**: 3077-3085.

De Beer D, Schramm A, Santegoeds CM & Kuhl M (1997) A nitrite microsensor for profiling environmental biofilms. *Appl Environ Microb* **63**: 973-977.

de Boer W, Leveau JHJ, Kowalchuk GA*, et al.* (2004) Collimonas fungivorans gen. nov., sp nov., a chitinolytic soil bacterium with the ability to grow on living fungal hyphae. *International Journal of Systematic and Evolutionary Microbiology* **54**: 857-864.

de Boer W, Folman LB, Summerbell RC & Boddy L (2005) Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiol Rev* **29**: 795-811.

de Wit R & Bouvier T (2006) 'Everything is everywhere, but, the environment selects'; what did Baas Becking and Beijerinck really say? *Environmental Microbiology* **8**: 755-758.

Dechesne A, Pallud C, Debouzie D, Flandrois JP, Vogel TM, Gaudet JP & Grundmann GL (2003) A novel method for characterizing the microscale 3D spatial distribution of bacteria in soil. *Soil Biol Biochem* **35**: 1537-1546.

Dechesne A, Pallud C & Grundmann GL (2007) Spatial distribution of bacteria at the microscale in soil. *(Franklin R & Mills A, eds), pp 87-107, 2007, Springer, Dordrecht, The Netherlands.*

Dechesne A, Or D & Smets BF (2008) Limited diffusive fluxes of substrate facilitate coexistence of two competing bacterial strains. *Fems Microbiol Ecol* **64**: 1-8.

Dechesne A, Wang G, Gülez G, Or D & Smets BF (2010a) Hydration-controlled bacterial motility and dispersal on surfaces. *P Natl Acad Sci USA* **107**: 14369-14372.

Dechesne A, Owsianiak M, Bazire A, Grundmann GL, Binning PJ & Smets BF (2010b) Biodegradation in a Partially Saturated Sand Matrix: Compounding Effects of Water Content, Bacterial Spatial Distribution, and Motility. *Environ Sci Technol* **44**: 2386-2392.

DeGrood SH, Claassen VP & Scow KM (2005) Microbial community composition on native and drastically disturbed serpentine soils. *Soil Biol Biochem* **37**: 1427-1435.

Dekas AE & Orphan VJ (2011) Identification of Diazotrophic Microorganisms in Marine Sediment Via Fluorescence in Situ Hybridization Coupled to Nanoscale Secondary Ion Mass Spectrometry (Fish-Nanosims). *Method Enzymol* **486**: 281-305.

Delmont TO, Robe P, Cecillon S, Clark IM, Constancias F, Simonet P, Hirsch PR & Vogel TM (2011) Accessing the Soil Metagenome for Studies of Microbial Diversity. *Appl Environ Microb* **77**: 1315-1324.

Delmont TO, Prestat E, Keegan KP*, et al.* (2012) Structure, fluctuation and magnitude of a natural grassland soil metagenome. *Isme J* **6**: 1677-1687.

Dennis PG, Miller AJ, Clark IM, Taylor RG, Valsami-Jones E & Hirsch PR (2008) A novel method for sampling bacteria on plant root and soil surfaces at the microhabitat scale. *J Microbiol Meth* **75**: 12-18.

Dexter AR (1988) Advances in Characterization of Soil Structure. *Soil Till Res* **11**: 199-238.

Diggle SP, Griffin AS, Campbell GS & West SA (2007) Cooperation and conflict in quorum-sensing bacterial populations. *Nature* **450**: 411-U417.

Doerr SH, Shakesby RA & Walsh RPD (2000) Soil water repellency: its causes, characteristics and hydro-geomorphological significance. *Earth-Sci Rev* **51**: 33-65.

Drigo B, Pijl AS, Duyts H, Kielak AM, Gamper HA, Houtekamer MJ, Boschker HTS & Bodelelier PLE (2010) Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO2. *Proc Natl Acad Sci USA* **107**: 10938-10942.

E

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**: 2460-2461.

Effmert U, Kalderas J, Warnke R & Piechulla B (2012) Volatile Mediated Interactions Between Bacteria and Fungi in the Soil. *J Chem Ecol* **38**: 665-703.

Ehrhardt CJ, Haymon RM, Sievert SM & Holden PA (2009) An improved method for nanogold in situ hybridization visualized with environmental scanning electron microscopy. *J Microsc* **236**: 5-10.

Eickhorst T & Tippkotter R (2008) Detection of microorganisms in undisturbed soil by combining fluorescence in situ hybridization (FISH) and micropedological methods. *Soil Biol Biochem* **40**: 1284-1293.

Ellis RJ (2004) Artificial soil microcosms: a tool for studying microbial autecology under controlled conditions. *J Microbiol Meth* **56**: 287-290.

Ettema CH & Wardle DA (2002) Spatial soil ecology. *Trends Ecol Evol* **17**: 177-183.

F

Faegri A, Torsvik VL & Goksoyr J (1977) Bacterial and Fungal Activities in Soil - Separation of Bacteria and Fungi by a Rapid Fractionated Centrifugation Technique. *Soil Biol Biochem* **9**: 105-112.

Falconer RE, Bown JL, White NA & Crawford JW (2007) Biomass recycling: a key to efficient foraging by fungal colonies. *Oikos* **116**: 1558-1568.

Falconer RE, Houston AN, Otten W & Baveye PC (2012) Emergent Behavior of Soil Fungal Dynamics: Influence of Soil Architecture and Water Distribution. *Soil Sci* **177**: 111-119.

Ferrari BC & Gillings MR (2009) Cultivation of fastidious bacteria by viability staining and micromanipulation in a soil substrate membrane system. *Appl Environ Microb* **75**: 3352-3354.

Fierer N, Strickland MS, Liptzin D, Bradford MA & Cleveland CC (2009) Global patterns in belowground communities. *Ecol Lett* **12**: 1238-1249.

Fierer N & Lennon JT (2011) The Generation and Maintenance of Diversity in Microbial Communities. *Am J Bot* **98**: 439-448.

Fierer N, Lauber CL, Ramirez KS, Zaneveld J, Bradford MA & Knight R (2012) Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *Isme J* **6**: 1007-1017.

Finlay RD (2006) The fungi in Soil. *Modern soil microbiology*,(Van Elsas J, Jansson JK & Trevors JT, *eds*), CRC Press.

Franklin RB & Mills AL (2003) Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. *Fems Microbiol Ecol* **44**: 335-346.

Franklin R & Mills A (2007) *The spatial distribution of microbes in the environment*. Springer.

Franklin RB & Mills AL (2009) Importance of spatially structured environmental heterogeneity in controlling microbial community composition at small spatial scales in an agricultural field. *Soil Biol Biochem* **41**: 1833-1840.

Frey SD, Elliott ET & Paustian K (1999) Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. *Soil Biol Biochem* **31**: 573-585.

Frisch RL & Rosenberg SM (2011) Antibiotic Resistance, Not Shaken or Stirred. *Science* **333**: 1713-1714.

Frostegard A & Baath E (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertil Soils* **22**: 59-65.

Frostegard A, Tunlid A & Baath E (1993) Phospholipid Fatty-Acid Composition, Biomass, and Activity of Microbial Communities from 2 Soil Types Experimentally Exposed to Different Heavy-Metals. *Appl Environ Microb* **59**: 3605-3617.

G

Garbeva P & de Boer W (2009) Inter-specific Interactions Between Carbon-limited Soil Bacteria Affect Behavior and Gene Expression. *Microb Ecol* **58**: 36-46.

Garbeva P, Silby MW, Raaijmakers JM, Levy SB & de Boer W (2011a) Transcriptional and antagonistic responses of Pseudomonas fluorescens Pf0-1 to phylogenetically different bacterial competitors. *Isme J* **5**: 973-985.

Garbeva P, Hol WHG, Termorshuizen AJ, Kowalchuk GA & de Boer W (2011b) Fungistasis and general soil biostasis - A new synthesis. *Soil Biol Biochem* **43**: 469-477.

Ge Y, He JZ, Zhu YG, Zhang JB, Xu ZH, Zhang LM & Zheng YM (2008) Differences in soil bacterial diversity: driven by contemporary disturbances or historical contingencies? *Isme J* **2**: 254-264.

Goecks J, Nekrutenko A, Taylor J & Team G (2010) Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biology* **11**.

Gomez P & Buckling A (2011) Bacteria-Phage Antagonistic Coevolution in Soil. *Science* **332**: 106-109.

Gong P, Guan X & Witter E (2001) A rapid method to extract ergosterol from soil by physical disruption. *Applied Soil Ecology* **17**: 285-289.

Gonod LV, Chenu C & Soulas G (2003) Spatial variability of 2,4-dichlorophenoxyacetic acid (2,4-D) mineralisation potential at a millimetre scale in soil. *Soil Biol Biochem* **35**: 373-382.

Gonod LV, Chadoeuf J & Chenu C (2006) Spatial distribution of microbial 2,4-dichlorophenoxy acetic acid mineralization from field to microhabitat scales (vol 70, pg 64, 2006). *Soil Sci Soc Am J* **70**: 710-710.

Goodrich-Blair H & Clarke DJ (2007) Mutualism and pathogenesis in Xenorhabdus and Photorhabdus: two roads to the same destination. *Mol Microbiol* **64**: 260-268.

Gordon H, Haygarth PM & Bardgett RD (2008) Drying and rewetting effects on soil microbial community composition and nutrient leaching. *Soil Biol Biochem* **40**: 302-311.

Green J & Bohannan BJM (2006) Spatial scaling of microbial biodiversity. *Trends Ecol Evol* **21**: 501-507.

Greenfield J & Bigland CH (1971) Comparison of Rates of Motility of Salmonella with Those of Other Enteric Bacteria. *Applied Microbiology* **21**: 240-&.

Griffin DM (1985) A comparison of the roles of bacteria and fungi. *Bacteria in Nature,* Vol. 1 (Leadbetter ER & Poindexter JS, *eds.*), pp 221-255. Academic Press, London.

Griffiths BS, Ritz K, Ebblewhite N & Dobson G (1999) Soil microbial community structure: Effects of substrate loading rates. *Soil Biol Biochem* **31**: 145-153.

Griffin AS, West SA & Buckling A (2004) Cooperation and competition in pathogenic bacteria. *Nature* **430**: 1024-1027.

Griffiths RI, Thomson BC, James P, Bell T, Bailey M & Whiteley AS (2011) The bacterial biogeography of British soils. *Environmental Microbiology* **13**: 1642-1654.

Grundmann GL (2004) Spatial scales of soil bacterial diversity - the size of a clone. *Fems Microbiol Ecol* **48**: 119-127.

Grundmann GL & Normand P (2000) Microscale diversity of the genus Nitrobacter in soil on the basis of analysis of genes encoding rRNA. *Appl Environ Microb* **66**: 4543-4546.

Grundmann GL, Dechesne A, Bartoli F, Flandrois JP, Chasse JL & Kizungu R (2001) Spatial modeling of nitrifier microhabitats in soil. *Soil Sci Soc Am J* **65**: 1709-1716.

Guenet B, Leloup J, Hartmann C, Barot S & Abbadie L (2011) A new protocol for an artificial soil to analyse soil microbiological processes. *Appl Soil Ecol* **48**: 243-246.

H

Hammer Ø, Harper DAT & Ryan PD (2001) Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**: 9.

Hanski I (1998) Metapopulation dynamics. *Nature* **396**: 41-49.

Hanson CA, Fuhrman JA, Horner-Devine MC & Martiny JBH (2012) Beyond biogeographic patterns: processes shaping the microbial landscape. *Nat Rev Microbiol* **10**: 497-506.

Harshey RM (2003) Bacterial motility on a surface: Many ways to a common goal. *Annu Rev Microbiol* **57**: 249-273.

Harris RF (1980) Effect of water potential on microbial growth and activity. *Water Potential Relations in Soil Microbiology,* Vol. Special Edition #9 (Parr JF & Gardner WR, *eds.*), pp 23–95. Soil Science Society of America, Madison.

Hartmann A, Schmid M, van Tuinen D & Berg G (2009) Plant-driven selection of microbes. *Plant and Soil* **321**: 235-257.

Hattori T (1988) Soil aggregates as microhabitats of microorganisms. *Rep Inst Agric Res* **37**: 23–36.

Haynes RJ & Swift RS (1990) Stability of Soil Aggregates in Relation to Organic-Constituents and Soil-Water Content. *J Soil Sci* **41**: 73-83.

Haynes WM (2012) Chapter 9: Molecular Structure and Spectroscopy. (Haynes WM, *ed.*). CRC Press/ Taylow and Francis, Boca Raton, FL.

Heister K, Hoschen C, Pronk GJ, Mueller CW & Kogel-Knabner I (2012) NanoSIMS as a tool for characterizing soil model compounds and organomineral associations in artificial soils. *J Soil Sediment* **12**: 35-47.

Herrmann AM, Ritz K, Nunan N, Clode PL, Pett-Ridge J, Kilburn MR, Murphy DV, O'Donnell AG & Stockdale EA (2007) Nano-scale secondary ion mass spectrometry — A new analytical tool in biogeochemistry and soil ecology: A review article. *Soil Biol Biochem* **39**: 1835-1850.

Hesselman MC, Odoni DI, Ryback BM*, et al.* (2012) A multi-platform flow device for microbial (co-) cultivation and microscopic analysis. *PloS one* **7**: e36982.

Heynen CE, Vanelsas JD, Kuikman PJ & Vanveen JA (1988) Dynamics of Rhizobium-Leguminosarum Biovar Trifolii Introduced into Soil - the Effect of Bentonite Clay on Predation by Protozoa. *Soil Biol Biochem* **20**: 483-488.

Hirsch PR, Mauchline TH & Clark IM (2010) Culture-independent molecular techniques for soil microbial ecology. *Soil Biol Biochem* **42**: 878-887.

Holden PA, Ritz K & Young I (2011) How do the microhabitats framed by soil structure impact soil bacteria and the processes that they regulate? (Ritz K & Young I, *eds.*), pp 37–70 CABI, Wallingford, UK.

Holland EA & Coleman DC (1987) Litter Placement Effects on Microbial and Organic-Matter Dynamics in an Agroecosystem. *Ecology* **68**: 425-433.

Horn R, Taubner H, Wuttke M & Baumgartl T (1994) Soil Physical-Properties Related to Soil-Structure. *Soil & Tillage Research* **30**: 187-216.

Huang WE, Li M, Jarvis RM, Goodacre R & Banwart SA (2010) Shining light on the microbial world the application of Raman microspectroscopy. *Advances in applied microbiology* **70**: 153-186.

Huang WE, Ferguson A, Singer AC, Lawson K, Thompson IP, Kalin RM, Larkin MJ, Bailey MJ & Whiteley AS (2009) Resolving Genetic Functions within Microbial Populations: In Situ Analyses Using rRNA and mRNA Stable Isotope Probing Coupled with Single-Cell Raman-Fluorescence In Situ Hybridization. *Appl Environ Microb* **75**: 234-241.

Hugenholtz P, Goebel BM & Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**: 4765-4774.

Hughes Martiny JB, Bohannan BJM, Brown JH, *et al.* (2006) Microbial biogeography: putting microorganisms on the map. *Nat Rev Microbiol* **4**: 102-112.

Huson DH, Auch AF, Qi J & Schuster SC (2007) MEGAN analysis of metagenomic data. *Genome Res* **17**: 377-386.

Hutchinson GE (1961) The Paradox of the Plankton. *Am Nat* **95**: 137-145.

Huysman F & Verstraete W (1993) Water-Facilitated Transport of Bacteria in Unsaturated Soil Columns - Influence of Inoculation and Irrigation Methods. *Soil Biol Biochem* **25**: 91-97.

I

Ihrmark K, Bödeker ITM, Cruz-Martinez K, *et al.* (2012) New primers to amplify the fungal ITS2 region – evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol Ecol* **82**: 666-677.

Ingham CJ, Kalisman O, Finkelshtein A & Ben-Jacob E (2011) Mutually facilitated dispersal between the nonmotile fungus Aspergillus fumigatus and the swarming bacterium Paenibacillus vortex. *P Natl Acad Sci USA* **108**: 19731-19736.

J

Jiang GM, Noonan MJ & Ratecliffe TJ (2006) Effects of soil matric suction on retention and percolation of Bacillus subtilis in intact soil cores. *Water Air Soil Poll* **177**: 211-226.

Jiang XP, Morgan J & Doyle MP (2002) Fate of Escherichia coli O157 : H7 in manure-amended soil. *Appl Environ Microb* **68**: 2605-2609.

Jones SE & Lennon JT (2010) Dormancy contributes to the maintenance of microbial diversity. *P Natl Acad Sci USA* **107**: 5881-5886.

K

Kai M, Haustein M, Molina F, Petri A, Scholz B & Piechulla B (2009) Bacterial volatiles and their action potential. *Applied Microbiology and Biotechnology* **81**: 1001-1012.

Kamilova F, Leveau JHJ & Lugtenberg B (2007) Collimonas fungivorans, an unpredicted in vitro but efficient in vivo biocontrol agent for the suppression of tomato foot and root rot. *Environmental Microbiology* **9**: 1597–1603.

Kandeler E, Tscherko D, Bruce K, Stemmer M, Hobbs PJ, Bardgett RD & Amelung W (2000) Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. *Biology and Fertility of Soils* **32**: 390-400.

Kaper J, Seidler RJ, Lockman H & Colwell RR (1979) Medium for the Presumptive Identification of Aeromonas-Hydrophila and Enterobacteriaceae. *Appl Environ Microb* **38**: 1023-1026.

Kato S, Haruta S, Cui ZJ, Ishii M & Igarashi Y (2005) Stable coexistence of five bacterial strains as a cellulose-degrading community. *Appl Environ Microb* **71**: 7099-7106.

Kawecki TJ & Ebert D (2004) Conceptual issues in local adaptation. *Ecol Lett* **7**: 1225-1241.

Kearns DB (2010) A field guide to bacterial swarming motility. *Nat Rev Microbiol* **8**: 634-644.

Kemper WD & Rosenau RC (1986) Aggregate Stability and Size Distribution. *Methods of Soil Analysis: Part 1—Physical and Mineralogical Methods.* Sssa book series: pp 425-442.

Kenzaka T, Ishidoshiro A, Tani K & Nasu M (2009) Scanning electron microscope imaging of bacteria based on DNA sequence. *Lett Appl Microbiol* **49**: 796-799.

Kenzaka T, Ishidoshiro A, Yamaguchi N, Tani K & Nasu M (2005) rRNA sequence-based scanning electron microscopic detection of bacteria. *Appl Environ Microb* **71**: 5523-5531.

Kerr B, Riley MA, Feldman MW & Bohannan BJM (2002) Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. *Nature* **418**: 171-174.

Keymer JE, Galajda P, Muldoon C, Park S & Austin RH (2006) Bacterial metapopulations in nanofabricated landscapes. *P Natl Acad Sci USA* **103**: 17290-17295.

Keymer JE, Galajda P, Lambert G, Liao D & Austin RH (2008) Computation of mutual fitness by competing bacteria. *P Natl Acad Sci USA* **105**: 20269-20273.

Killham K (1994) *Soil Ecology*. Cambridge University Press, Cambridge.

Kim HJ, Hummel JW, Sudduth KA & Motavalli PP (2007) Simultaneous analysis of soil macronutrients using ion-selective electrodes. *Soil Sci Soc Am J* **71**: 1867-1877.

Kim HJ, Boedicker JQ, Choi JW & Ismagilov RF (2008) Defined spatial structure stabilizes a synthetic multispecies bacterial community. *P Natl Acad Sci USA* **105**: 18188-18193.

Kim JS, Dungan RS & Crowley D (2008) Microarray analysis of bacterial diversity and distribution in aggregates from a desert agricultural soil. *Biology and Fertility of Soils* **44**: 1003-1011.

King AJ, Freeman KR, McCormick KF, Lynch RC, Lozupone C, Knight R & Schmidt SK (2010) Biogeography and habitat modelling of high-alpine bacteria. *Nature Communications* **1**.

Klamer M & Hedlund K (2004) Fungal diversity in set-aide agricultural soil investigated using terminal-restriction fragment length polymorphism. *Soil Biol Biochem* **36**: 983-988.

Kohlmeier S, Smits THM, Ford RM, Keel C, Harms H & Wick LY (2005) Taking the fungal highway: Mobilization of pollutant-degrading bacteria by fungi. *Environ Sci Technol* **39**: 4640-4646.

Konovalova A, Petters T & Sogaard-Andersen L (2010) Extracellular biology of Myxococcus xanthus. *Fems Microbiol Rev* **34**: 89-106.

Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PGL & van Veen JA (2002) Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**: 509-520.

Kraemer SA & Velicer GJ (2011) Endemic social diversity within natural kin groups of a cooperative bacterium. *P Natl Acad Sci USA* **108**: 10823-10830.

Kraemer SA, Toups MA & Velicer GJ (2010) Natural variation in developmental life-history traits of the bacterium Myxococcus xanthus. *Fems Microbiol Ecol* **73**: 226-233.

Krug D, Zurek G, Revermann O, Vos M, Velicer GJ & Muller R (2008) Discovering the hidden secondary metabolome of Myxococcus xanthus: a study of intraspecific diversity. *Appl Environ Microb* **74**: 3058-3068.

Kuramae EE, Yergeau E, Wong LC, Pijl AS, van Veen JA & Kowalchuk GA (2012) Soil characteristics more strongly influence soil bacterial communities than land-use type. *Fems Microbiol Ecol* **79**: 12-24.

L

Lane D (1991) 16S/23S rRNA sequencing. *Nucleic acids techniques in bacterial systematics*,(Stackebrandt E GM, *ed.*). 115-175. John Wiley & Sons, West Sussex, UK.

Lauber CL, Hamady M, Knight R & Fierer N (2009) Pyrosequencing-Based Assessment of Soil pH as a Predictor of Soil Bacterial Community Structure at the Continental Scale. *Appl Environ Microb* **75**: 5111-5120.

Lawrence D, Fiegna F, Behrends V, Bundy JG, Phillimore AB, Bell T & Barraclough TG (2012) Species Interactions Alter Evolutionary Responses to a Novel Environment. *Plos Biology* **10**.

Lebuhn M, Effenberger M, Gronauer A, Wilderer PA & Wuertz S (2003) Using quantitative real-time PCR to determine the hygienic status of cattle manure. *Water Sci Technol* **48**: 97-103.

Lee WH, Lee JH, Bishop PL & Papautsky I (2009) Biological Application of Micro-Electro Mechanical Systems Microelectrode Array Sensors for Direct Measurement of Phosphate in the Enhanced Biological Phosphorous Removal Process. *Water Environment Research* **81**: 748-754.

Legendre P, Borcard D & Roberts DW (2012) Variation partitioning involving orthogonal spatial eigenfunction submodels. *Ecology* **93**: 1234-1240.

Lehmann J, Solomon D, Kinyangi J, Dathe L, Wirick S & Jacobsen C (2008) Spatial complexity of soil organic matter forms at nanometre scales. *Nature Geoscience* **1**: 238-242.

Leis AP, Schlicher S, Franke H & Strathmann M (2005) Optically transparent porous medium for nondestructive studies of microbial biofilm architecture and transport dynamics. *Appl Environ Microb* **71**: 4801-4808.

Lennon JT & Jones SE (2011) Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nat Rev Microbiol* **9**: 119-130.

Leveau JHJ, Uroz S & de Boer W (2010) The bacterial genus Collimonas: mycophagy, weathering and other adaptive solutions to life in oligotrophic soil environments. *Environmental Microbiology* **12**: 281-292.

Lipson DA, Monson RK, Schmidt SK & Weintraub MN (2009) The trade-off between growth rate and yield in microbial communities and the consequences for under-snow soil respiration in a high elevation coniferous forest. *Biogeochemistry* **95**: 23-35.

Lombard N, Prestat E, van Elsas JD & Simonet P (2011) Soil-specific limitations for access and analysis of soil microbial communities by metagenomics. *Fems Microbiol Ecol* **78**: 31-49.

Long T & Or D (2005) Aquatic habitats and diffusion constraints affecting microbial coexistence in unsaturated porous media. *Water Resources Research* **41**.

Long T & Or D (2009) Dynamics of Microbial Growth and Coexistence on Variably Saturated Rough Surfaces. *Microb Ecol* **58**: 262-275.

Lu R & Yu T (2002) Fabrication and evaluation of an oxygen microelectrode applicable to environmental engineering and science. *Journal of Environmental Engineering and Science* **1**: 225-235.

Lugtenberg BJJ & Dekkers LC (2001) What makes Pseudomonas bacteria rhizosphere competent? *Environmental Microbiology* **1**: 9-13.

Lupwayi NZ, Rice WA & Clayton GW (1998) Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. *Soil Biol Biochem* **30**: 1733-1741.

M

MacLean RC (2005) Adaptive radiation in microbial microcosms. *J Evolution Biol* **18**: 1376-1386.

Mccarthy AJ & Williams ST (1992) Actinomycetes as Agents of Biodegradation in the Environment - a Review. *Gene* **115**: 189-192.

McLean MA & Huhta V (2000) Temporal and spatial fluctuations in moisture affect humus microfungal community structure in microcosms. *Biol Fertil Soils* **32**: 114-119.

Mamin HJ, PoggioM, Degen CL & RugarD (2007) Nuclear magnetic resonance imaging with 90-nm resolution. *Nat Nano* **2**: 301-306.

Mannik J, Driessen R, Galajda P, Keymer JE & Dekker C (2009) Bacterial growth and motility in sub-micron constrictions. *P Natl Acad Sci USA* **106**: 14861-14866.

Martiny JBH, Bohannan BJM, Brown JH*, et al.* (2006) Microbial biogeography: putting microorganisms on the map. *Nat Rev Microbiol* **4**: 102-112.

Masella AP, Bartram AK, Truszkowski JM, Brown DG & Neufeld JD (2012) PANDAseq: PAired-eND Assembler for Illumina sequences. *Bmc Bioinformatics* **13**.

McBratney AB, Minasny B & Rossel RV (2006) Spectral soil analysis and inference systems: A powerful combination for solving the soil data crisis. *Geoderma* **136**: 272-278.

Mendes IC & Bottomley PJ (1998) Distribution of a population of Rhizobium leguminosarum bv. trifolii among different size classes of soil aggregates. *Appl Environ Microb* **64**: 970-975.

Mendes R, Kruijt M, de Bruijn I*, et al.* (2011) Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria. *Science* **332**: 1097-1100.

Mitra S, Rupek P, Richter DC, Urich T, Gilbert JA, Meyer F, Wilke A & Huson DH (2011) Functional analysis of metagenomes and metatranscriptomes using SEED and KEGG. *Bmc Bioinformatics* **12**.

Morgan AD, MacLean RC, Hillesland KL & Velicer GJ (2010) Comparative Analysis of Myxococcus Predation on Soil Bacteria. *Appl Environ Microb* **76**: 6920-6927.

Mueller CW, Kolbl A, Hoeschen C, Hillion F, Heister K, Herrmann AM & Kogel-Knabner I (2012) Submicron scale imaging of soil organic matter dynamics using NanoSIMS - From single particles to intact aggregates. *Org Geochem* **42**: 1476-1488.

Mummey D, Holben W, Six J & Stahl P (2006) Spatial Stratification of Soil Bacterial Populations in Aggregates of Diverse Soils. *Microb Ecol* **51**: 404-411.

Musat N, Halm H, Winterholler B, Hoppe P, Peduzzi S, Hillion F, Horreard F, Amann R, Jørgensen BB & Kuypers MMM (2008) A single-cell view on the ecophysiology of anaerobic phototrophic bacteria. *P Natl Acad Sci USA* **105**: 17861-17866.

N

Nakashima Y, Mitsuhata Y, Nishiwaki J, Kawabe Y, Utsuzawa S & Jinguuji M (2011) Non-destructive Analysis of Oil-Contaminated Soil Core Samples by X-ray Computed Tomography and Low-Field Nuclear Magnetic Resonance Relaxometry: a Case Study. *Water, Air, & Soil Pollution* **214**: 681-698.

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G & Renella G (2003) Microbial diversity and soil functions. *Eur J Soil Sci* **54**: 655-670.

Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A, Kanigan T, Lewis K & Epstein SS (2010) Use of Ichip for High-Throughput In Situ Cultivation of "Uncultivable" Microbial Species. *Appl Environ Microb* **76**: 2445-2450.

Nicolardot B, Bouziri L, Bastian F & Ranjard L (2007) A microcosm experiment to evaluate the influence of location and quality of plant residues on residue decomposition and genetic structure of soil microbial communities. *Soil Biol Biochem* **39**: 1631-1644.

Nilsson LO, Baath E, Falkengren-Grerup U & Wallander H (2007) Growth of ectomycorrhizal mycelia and composition of soil microbial communities in oak forest soils along a nitrogen deposition gradient. *Oecologia* **153**: 375-384.

Nimmo JR (2004) Porosity and Pore Size Distribution. *Encyclopedia of Soils in the Environment,* Vol. 3 (Hillel D, *ed.*), pp 295-303. Elsevier, London.

Nunan N, Young I, Crawford J & Ritz K (2007) Bacterial Interactions At The Microscale – Linking Habitat To Function In Soil. (Franklin R & Mills A, *eds*), pp 87-107, Springer, Dordrecht, the Netherlands.

Nunan N, Wu K, Young IM, Crawford JW & Ritz K (2002) In situ spatial patterns of soil bacterial populations, mapped at multiple scales, in an arable soil. *Microb Ecol* **44**: 296-305.

Nunan N, Wu KJ, Young IM, Crawford JW & Ritz K (2003) Spatial distribution of bacterial communities and their relationships with the micro-architecture of soil. *Fems Microbiol Ecol* **44**: 203-215.

O

O'Malley MA (2007) The nineteenth century roots of 'everything is everywhere'. *Nat Rev Microbiol* **5**: 647-651.

Oades JM (1984) Soil Organic-Matter and Structural Stability - Mechanisms and Implications for Management. *Plant Soil* **76**: 319-337.

Ochiai N, Dragila MI & Parke JL (2010) Three-Dimensional Tracking of Colloids at the Pore Scale Using Epifluorescence Microscopy. *Vadose Zone Journal* **9**: 576-587.

Oda Y, Star B, Huisman LA, Gottschal JC & Forney LJ (2003) Biogeography of the purple nonsulfur bacterium Rhodopseudomonas palustris. *Appl Environ Microb* **69**: 5186-5191.

Okabe S, Satoh H & Watanabe Y (1999) In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Appl Environ Microb* **65**: 3182-3191.

Or D, Phutane S & Dechesne A (2007) Extracellular polymeric substances affecting pore-scale hydrologic conditions for bacterial activity in unsaturated soils. *Vadose Zone Journal* **6**: 298-305.

Or D, Smets BF, Wraith JM, Dechesne A & Friedman SP (2007) Physical constraints affecting bacterial habitats and activity in unsaturated porous media - a review. *Advances in Water Resources* **30**: 1505-1527.

Otten W, Gilligan CA, Watts CW, Dexter AR & Hall D (1999) Continuity of air-filled pores and invasion thresholds for a soilborne fungal plant pathogen, Rhizoctonia solani. *Soil Biol Biochem* **31**: 1803-1810.

Otten W, Harris K, Young IM, Ritz K & Gilligan CA (2004) Preferential spread of the pathogenic fungus Rhizoctonia solani through structured soil. *Soil Biol Biochem* **36**: 203-210.

P

Pajor R, Falconer R, Hapca S & Otten W (2010) Modelling and quantifying the effect of heterogeneity in soil physical conditions on fungal growth. *Biogeosciences* **7**: 3731-3740.

Papendick R & Campbell G (1981) Theory and Measurement of Water Potential. *Water Potential Relations in Soil Microbiology*,(Parr J, Gardner W & Elliott L, *eds*.), pp 1-22. Soil Sci Soc Am, Madison.

Pell AN (1997) Manure and microbes: Public and animal health problem? *Journal of Dairy Science* **80**: 2673-2681.

Piccolo A, Conte P & Cozzolino A (2001) Chromatographic and spectrophotometric properties of dissolved humic substances compared with macromolecular polymers. *Soil Sci* **166**: 174-185.

Poll C, Brune T, Begerow D & Kandeler E (2010) Small-scale Diversity and Succession of Fungi in the Detritusphere of Rye Residues. *Microb Ecol* **59**: 130-140.

Postma J & Vanveen JA (1990) Habitable Pore-Space and Survival of Rhizobium-Leguminosarum Biovar Trifolii Introduced into Soil. *Microb Ecol* **19**: 149-161.

Pronk GJ, Heister K, Ding GC, Smalla K & Kögel-Knabner I (2012) Development of biogeochemical interfaces in an artificial soil incubation experiment; aggregation and formation of organo-mineral associations. *Geoderma* **189**: 585-594.

Prosser JI, Bohannan BJM, Curtis TP*, et al.* (2007) Essay - The role of ecological theory in microbial ecology. *Nat Rev Microbiol* **5**: 384-392.

Q

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J & Glockner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**: D590-D596.

Quince C, Curtis TP & Sloan WT (2008) The rational exploration of microbial diversity. *Isme J* **2**: 997-1006.

R

Raaijmakers JM & Mazzola M (2012) Diversity and Natural Functions of Antibiotics Produced by Beneficial and Plant Pathogenic Bacteria. *Annu Rev Phytopathol* **50**: 403-424.

Rainey PB & Travisano M (1998) Adaptive radiation in a heterogeneous environment. *Nature* **394**: 69-72.

Rajaniemi TK & Allison VJ (2009) Abiotic conditions and plant cover differentially affect microbial biomass and community composition on dune gradients. *Soil Biol Biochem* **41**: 102-109.

Ranjard L & Richaume AS (2001) Quantitative and qualitative microscale distribution of bacteria in soil. *Research in Microbiology* **152**: 707-716.

Reeder J & Knight R (2010) Rapid denoising of pyrosequencing amplicon data: exploiting the rank-abundance distribution. *Nat Methods* **7**: 668–669.

Reichardt W, Briones A, de Jesus R & Padre B (2001) Microbial population shifts in experimental rice systems. *Applied Soil Ecology* **17**: 151-163.

Reischke S, Rousk J & Bååth E (2014) The effects of glucose loading rates on bacterial and fungal growth in soil. *Soil Biol Biochem* **70**: 88-95.

Reller LB & Mirrett S (1975) Motility-Indole-Lysine Medium for Presumptive Identification of Enteric Pathogens of Enterobacteriaceae. *Journal of Clinical Microbiology* **2**: 247-252.

Rousk J, Baath E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R & Fierer N (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. *Isme J* **4**: 1340-1351.

Remusat L, Hatton PJ, Nico PS, Zeller B, Kleber M & Derrien D (2012) NanoSIMS Study of Organic Matter Associated with Soil Aggregates: Advantages, Limitations, and Combination with STXM. *Environ Sci Technol* **46**: 3943-3949.

Remus-Emsermann M (2012) The Ecology of Bacterial Individuality. PhD thesis Thesis, Wageningen.

Remus-Emsermann MNP, Kowalchuk GA & Leveau JHJ (2013) Single-cell versus population-level reproductive success of bacterial immigrants to pre-colonized leaf surfaces. *Environ Microbiol Rep* **5**: 387–392.

Riley MA & Wertz JE (2002) Bacteriocins: Evolution, ecology, and application. *Annual Review of Microbiology* **56**: 117-137.

Ritz K & Young IM (2004) Interactions between soil structure and fungi. *Mycologist* **18**: 52-59.

Roberts SJ, Walker A, Parekh NR, Welch SJ & Waddington MJ (1993) Studies on a Mixed Bacterial Culture from Soil Which Degrades the Herbicide Linuron. *Pesticide Science* **39**: 71-78.

Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG & Triplett EW (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. *Isme J* **1**: 283-290.

Rosenberg K, Bertaux J, Krome K, Hartmann A, Scheu S & Bonkowski M (2009) Soil amoebae rapidly change bacterial community composition in the rhizosphere of Arabidopsis thaliana. *Isme J* **3**: 675-684.

Rossel RAV, McGlynn RN & McBratney AB (2006) Determing the composition of mineral-organic mixes using UV-vis-NIR diffuse reflectance spectroscopy. *Geoderma* **137**: 70-82.

Rossel RAV, Walvoort DJJ, McBratney AB, Janik LJ & Skjemstad JO (2006) Visible, near infrared, mid infrared or combined diffuse reflectance spectroscopy for simultaneous assessment of various soil properties. *Geoderma* **131**: 59-75.

Rousk J, Demoling LA, Bahr A & Baath E (2008) Examining the fungal and bacterial niche overlap using selective inhibitors in soil. *Fems Microbiol Ecol* **63**: 350-358.

Rousk J, Baath E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R & Fierer N (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. *Isme J* **4**: 1340-1351.

Rousk J, Brookes PC & Baath E (2011) Fungal and bacterial growth responses to N fertilization and pH in the 150-year 'Park Grass' UK grassland experiment. *Fems Microbiol Ecol* **76**: 89-99.

Ruamps LS, Nunan N & Chenu C (2011) Microbial biogeography at the soil pore scale. *Soil Biol Biochem* **43**: 280-286.

Ruddick SM & Williams ST (1972) Studies on the ecology of actinomycetes in soil V. Some factors influencing the dispersal and adsorption of spores in soil. *Soil Biol Biochem* **4**: 93-103.

S

Sammartino S, Michel E & Capowiez Y (2012) A Novel Method to Visualize and Characterize Preferential Flow in Undisturbed Soil Cores by Using Multislice Helical CT. *Vadose Zone Journal* **11**.

Scheu S, Maraun M, Bonkowski M & Alphei J (1996) Microbial biomass and respiratory activity in soil aggregates of different sizes from three beechwood sites on a basalt hill. *Biology and Fertility of Soils* **21**: 69-76.

Schimel JP & Bennett J (2004) Nitrogen mineralization: Challenges of a changing paradigm. *Ecology* **85**: 591-602.

Schmidt TM (2006) The maturing of microbial ecology. *Int Microbiol* **9**: 217-223.

Schrempf H (2007) *Biology of streptomycetes. The Prokaryotes, A Handbook on the Biology of Bacteria*. Springer, New York, NY.

Sengelov G, Kowalchuk GA & Sorensen SJ (2000) Influence of fungal-bacterial interactions on bacterial conjugation in the residuesphere. *Fems Microbiol Ecol* **31**: 39-45.

Sessitsch A, Weilharter A, Gerzabek MH, Kirchmann H & Kandeler E (2001) Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. *Appl Environ Microb* **67**: 4215-4224.

Shepherd KD & Walsh MG (2002) Development of Reflectance Spectral Libraries for Characterization of Soil Properties. *Soil Sci Soc Am J* **66**: 988-998.

Shepherd KD & Walsh MG (2002) Development of reflectance spectral libraries for characterization of soil properties. *Soil Sci Soc Am J* **66**: 988-998.

Singh BK, Nunan N & Millard P (2009) Response of fungal, bacterial and ureolytic communities to synthetic sheep urine deposition in a grassland soil. *Fems Microbiol Ecol* **70**: 109-117.

Six J, Bossuyt H, Degryze S & Denef K (2004) A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. *Soil Till Res* **79**: 7-31.

Stefanic P, Decorosi F, Viti C, Petito J, Cohan FM & Mandic-Mulec I (2012) The quorum sensing diversity within and between ecotypes of Bacillus subtilis. *Environmental Microbiology* **14**: 1378–1389.

Strickland MS & Rousk J (2010) Considering fungal:bacterial dominance in soils - Methods, controls, and ecosystem implications. *Soil Biol Biochem* **42**: 1385-1395.

Stotzky G (1986) Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses. *Interactions of soil minerals with natural organics and microbes* 305-428.

Stromberger M, Shah Z & Westfall D (2007) Soil microbial communities of no-till dryland agroecosystems across an evapotranspiration gradient. *Applied Soil Ecology* **35**: 94-106.

T

Tatzber M, Stemmer M, Spiegel H, Katzlberger C, Landstetter C, Haberhauer G & Gerzabek MH (2012) 14C-labeled organic amendments: Characterization in different particle size fractions and humic acids in a long-term field experiment. *Geoderma* **177-178**: 39-48.

Tatzber M, Stemmer M, Spiegel H, Katzlberger C, Landstetter C, Haberhauer G & Gerzabek MH (2012) 14C-labeled organic amendments: Characterization in different particle size fractions and humic acids in a long-term field experiment. *Geoderma* **177–178**: 39-48.

Tiedje JM, Cho JC, Murray A, Treves D, Xia B & Zhou J (2001) Soil teeming with life: new frontiers for soil science. *Sustainable management of soil organic matter,*(Rees RM, Ball BC, Campbell CD & Watson CA, *eds*.).

Tippkotter R, Eickhorst T, Taubner H, Gredner B & Rademaker G (2009) Detection of soil water in macropores of undisturbed soil using microfocus X-ray tube computerized tomography (mu CT). *Soil Till Res* **105**: 12-20.

Tisdall JM & Oades JM (1982) Organic matter and water-stable aggregates in soils. *J Soil Sci* **33**: 141-163.

Torsvik V, Goksoyr J & Daae FL (1990) High Diversity in DNA of Soil Bacteria. *Appl Environ Microb* **56**: 782-787.

Torsvik V & Øvreås L (2002) Microbial diversity and function in soil: from genes to ecosystems. *Curr Opin Microbiol* **5**: 240-245.

Treves DS, Xia B, Zhou J & Tiedje JM (2003) A two-species test of the hypothesis that spatial isolation influences microbial diversity in soil. *Microbial Ecology* **45**: 20-28.

Trevors JT, Vanelsas JD, Vanoverbeek LS & Starodub ME (1990) Transport of a Genetically Engineered Pseudomonas-Fluorescens Strain through a Soil Microcosm. *Appl Environ Microb* **56**: 401-408.

Trujillo ME (2001) *Actinobacteria*. Chichester.

Tuller M, Or D & Dudley LM (1999) Adsorption and capillary condensation in porous media: Liquid retention and interfacial configurations in angular pores. *Water Resources Research* **35**: 1949-1964.

Tyson GW, Lo I, Baker BJ, Allen EE, Hugenholtz P & Banfield JF (2005) Genome-directed isolation of the key nitrogen fixer Leptospirillum ferrodiazotrophum sp nov from an acidophilic microbial community. *Appl Environ Microb* **71**: 6319-6324.

U

Urich T & Schleper C (2011) The “Double-RNA” Approach to Simultaneously Assess the Structure and Function of a Soil Microbial Community. *Handbook of Molecular Microbial Ecology I ,* pp 587-596. John Wiley & Sons, Inc.

V

Valaskova V, Snajdr J, Bittner B, Cajthaml T, Merhautova V, Hoffichter M & Baldrian P (2007) Production of lignocellulose-degrading enzymes and degradation of leaf litter by saprotrophic basidiomycetes isolated from a Quercus petraea forest. *Soil Biol Biochem* **39**: 2651-2660.

van de Mortel M & Halverson LJ (2004) Cell envelope components contributing to biofilm growth and survival of Pseudomonas putida in low-water-content habitats. *Mol Microbiol* **52**: 735-750.

van der Heijden MGA, Bardgett RD & van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol Lett* **11**: 296-310.

van der Putten WH, Mortimer SR, Hedlund K*, et al.* (2000) Plant species diversity as a driver of early succession in abandoned fields: a multi-site approach. *Oecologia* **124**: 91-99.

van Elsas JD, Trevors JT & Vanoverbeek LS (1991) Influence of Soil Properties on the Vertical Movement of Genetically-Marked Pseudomonas-Fluorescens through Large Soil Microcosms. *Biol Fertil Soils* **10**: 249-255.

van Elsas JD, Chiurazzi M, Mallon CA, Elhottova D, Kristufek V & Salles JF (2012) Microbial diversity determines the invasion of soil by a bacterial pathogen. *P Natl Acad Sci USA* **109**: 1159-1164.

van Genuchten M, Leij F & Yates S (1991) The RETC code for quantifying the hydraulic functions of unsaturated soils. (Agency UEP, *ed.*) .

van Genuchten MT (1980) A closed-form equation for predicting the hydraulic conductivity of unsaturated soils. *Spoil Science Society of America Journal* **44**: 892-898.

Vartoukian SR, Palmer RM & Wade WG (2010) Strategies for culture of ‘unculturable’ bacteria. *Fems Microbiology Letters* **309**: 1-7.

Velicer GJ (2003) Social strife in the microbial world. *Trends in Microbiology* **11**: 330-337.

Velicer GJ, Kroos L & Lenski RE (2000) Developmental cheating in the social bacterium Myxococcus xanthus. *Nature* **404**: 598-+.

Vogel J, Normand P, Thioulouse J, Nesme X & Grundmann GL (2003) Relationship between spatial and genetic distance in Agrobacterium spp. in 1 cubic centimeter of soil. *Appl Environ Microb* **69**: 1482-1487.

Vos M & Velicer GJ (2006) Genetic population structure of the soil bacterium Myxococcus xanthus at the centimeter scale. *Appl Environ Microb* **72**: 3615-3625.

Vos M & Velicer GJ (2008) Isolation by distance in the spore-forming soil bacterium Myxococcus xanthus. *Current Biology* **18**: 386-391.

Vos M & Velicer GJ (2008) Natural variation of gliding motility in a centimetre-scale population of Myxococcus xanthus. *Fems Microbiol Ecol* **64**: 343-350.

Vos M & Velicer GJ (2009) Social Conflict in Centimeter and Global-Scale Populations of the Bacterium Myxococcus xanthus. *Current Biology* **19**: 1763-1767.

Vos M, Birkett PJ, Birch E, Griffiths RI & Buckling A (2009) Local Adaptation of Bacteriophages to Their Bacterial Hosts in Soil. *Science* **325**: 833-833.

Vos M, Quince C, Pijl AS, de Hollander M & Kowalchuk GA (2012) A Comparison of rpoB and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity. *PloS one* **7**.

Vos M, Wolf AB, Jennings SJ & Kowalchuk GA (2013) Micro-scale determinants of bacterial diversity in soil. *Fems Microbiol Rev* **37**: 936-954.

Vreeken-Buijs MJ, Hassink J & Brussaard L (1998) Relationships of soil microarthropod biomass with organic matter and pore size distribution in soils under different land use. *Soil Biol Biochem* **30**: 97-106.

Vulic M & Kolter R (2001) Evolutionary cheating in Escherichia coli stationary phase cultures. *Genetics* **158**: 519-526.

W

Wang G & Or D (2010) Aqueous films limit bacterial cell motility and colony expansion on partially saturated rough surfaces. *Environmental Microbiology* **12**: 1363-1373.

Wang JJ & Bishop PL (2005) Development of a phosphate ion-selective microelectrode and its use in studies of the enhanced biological phosphorus removal (EBPR) process. *Environmental Technology* **26**: 381-388.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microb* **73**: 5261-5267.

Wang QF, Frye JG, McClelland M & Harshey RM (2004) Gene expression patterns during swarming in Salmonella typhimurium: genes specific to surface growth and putative new motility and pathogenicity genes. *Mol Microbiol* **52**: 169-187.

Warmink JA & van Elsas JD (2009a) Migratory Response of Soil Bacteria to Lyophyllum sp Strain Karsten in Soil Microcosms. *Appl Environ Microbiol* **75**: 2820-2830.

Warmink JA, Nazir R & van Elsas JD (2009b) Universal and species-specific bacterial 'fungiphiles' in the mycospheres of different basidiomycetous fungi. *Environmental Microbiology* **11**: 300-312.

Warmink JA, Nazir R, Corten B & van Elsas JD (2011) Hitchhikers on the fungal highway: The helper effect for bacterial migration via fungal hyphae. *Soil Biol Biochem* **43**: 760-765.

Watve MG, Tickoo R, Jog MM & Bhole BD (2001) How many antibiotics are produced by the genus Streptomyces? *Arch Microbiol* **176**: 386-390.

Wertz S, Czarnes S, Bartoli F, Renault P, Commeaux C, Guillaumaud N & ClaysJosserand A (2007) Early-stage bacterial colonization between a sterilized remoulded soil clod and natural soil aggregates of the same soil. *Soil Biol Biochem* **39**: 3127-3137.

West SA, Diggle SP, Buckling A, Gardner A & Griffins AS (2007) The social lives of microbes. *Annu Rev Ecol Evol Syst* **38**: 53-77.

Wetterlind J & Stenberg B (2010) Near-infrared spectroscopy for within-field soil characterization: small local calibrations compared with national libraries spiked with local samples. *Eur J Soil Sci* **61**: 823-843.

Whitman WB, Coleman DC & Wiebe WJ (1998) Prokaryotes: The unseen majority. *P Natl Acad Sci USA* **95**: 6578-6583.

Williams MA & Rice CW (2007) Seven years of enhanced water availability influences the physiological, structural, and functional attributes of a soil microbial community. *Applied Soil Ecology* **35**: 535-545.

Wilson JM & Griffin DM (1975) Respiration and radial growth of soil fungi at two osmotic potentials. *Soil Biol Biochem* **7**: 269-274.

Wolf AB, Vos M, de Boer W & Kowalchuk GA (2013) Impact of matric potential and pore size distribution on growth dynamics of filamentous and non-filamentous soil bacteria. *PLoS one* **8**.

Wong PTW & Griffin DM (1976) Bacterial Movement at High Matric Potentials .1. Artificial and Natural Soils. *Soil Biol Biochem* **8**: 215-218.

Woyke T, Xie G, Copeland A*, et al.* (2009) Assembling the Marine Metagenome, One Cell at a Time. *PloS one* **4**.

Wright DA, Killham K, Glover LA & Prosser JI (1995) Role of Pore-Size Location in Determining Bacterial-Activity during Predation by Protozoa in Soil. *Appl Environ Microb* **61**: 3537-3543.

Y

Yang HH, Vinopal RT, Grasso D & Smets BF (2004) High diversity among environmental Escherichia coli isolates from a bovine feedlot. *Appl Environ Microb* **70**: 1528-1536.

Yergeau E, Bezemer TM, Hedlund K, Mortimer SR, Kowalchuk GA & van der Putten WH (2010) Influences of space, soil, nematodes and plants on microbial community composition of chalk grassland soils. *Environmental Microbiology* **12**: 2096-2106.

Yim G, Wang HH & FRS JD (2007) Antibiotics as signalling molecules. *Philos Roy Soc B* **362**: 1195-1200.

Young IM & Crawford JW (2004) Interactions and self-organization in the soil-microbe complex. *Science* **304**: 1634-1637.

Young IM, Crawford JW, Nunan N, Otten W & Spiers A (2008) Microbial Distribution in Soils: Physics and Scaling. *Advances in Agronomy,* Vol. **100**: 81-121.

Yuste JC, Penuelas J, Estiarte M, Garcia-Mas J, Mattana S, Ogaya R, Pujol M & Sardans J (2011) Drought-resistant fungi control soil organic matter decomposition and its response to temperature. *Global Change Biol* **17**: 1475-1486.

Z

Zhang QC, Lambert G, Liao D, Kim H, Robin K, Tung CK, Pourmand N & Austin RH (2011) Acceleration of Emergence of Bacterial Antibiotic Resistance in Connected Microenvironments. *Science* **333**: 1764-1767.

Zhou JZ, Xia BC, Treves DS, Wu LY, Marsh TL, O'Neill RV, Palumbo AV & Tiedje JM (2002) Spatial and resource factors influencing high microbial diversity in soil. *Appl Environ Microb* **68**: 326-334.

Summary

Soils contain tremendous microbial phylogenetic and functional diversity. Recent advances in the application of molecular methods into microbial ecology have provided a new appreciation of the extent of soil-borne microbial diversity, but our understanding of the forces that shape and maintain this tremendous source of biodiversity still remain rudimentary. Microbes interact in various ways with their biotic and abiotic environment, and microbial interactions are thought to be key factors in controlling microbial community structure and diversity. In order to understand the forces shaping microbial community structure, it is crucial to take the physical structure of the soil into account. One of the most important controlling factors of microbial interactions is habitat connectivity, which is determined by the geometry of the pore network and the hydration status of the soil. Many microbial interactions are mediated by the soil water phase. While fungi can bridge air-filled pore space due to their hyphal growth form, bacteria are entirely dependent on water for their nutrient supply and motility. The impact of soil moisture and pore structure on microbial interactions, community composition and diversity are therefore the main foci of this thesis work.

The overall aim of the work presented in this thesis is to increase our understanding of the forces that allow for the tremendous amount of diversity to be maintained in soil microbial communities. The work presented has a particular focus on how soil connectivity impacts the structure, diversity and interactions within soil-borne microbial communities.

The three general research questions addressed in this thesis are as follows:

1. What are the impacts of pore size distribution and matric potential, which determine soil connectivity, on microbial resource competition and habitat utilization?
2. How does connectivity influence the structure and diversity of complex bacterial and fungal communities and is there an effect on microbial groups with different life-history strategies? Do bacteria or fungi have a relative competitive advantage under specific soil conditions?
3. How does soil moisture impact the colonization potential of bacteria with different motility mechanisms?

In situ observations of microbial communities at microbiological relevant scales are difficult due to the extreme complexity of the soil habitat. The general approach in this thesis was therefore the development of a sand microcosm system, which allows for the independent manipulation of specific soil parameters. This system was applied to study resource competition between filamentous and non-filamentous bacteria (Chapter 3), the diversity and community structure of complex bacterial and fungal communities as impacted by soil connectivity (Chapter 4 and 5) and bacterial motility in a soil-like environment (Chapter 6).

Chapter 3 synthesizes the results from a microcosm experiment investigating the effect of pore size and matric potential on the growth dynamics of filamentous and non-filamentous bacteria. Filamentous bacteria were found to have a relative competitive advantage over non-filamentous bacteria in poorly connected soils, which was attributed to their ability to bridge air-gaps and thus explore new micro-habitats. Conversely non-filamentous bacteria had an advantage in well-connected soils. Similar to filamentous bacteria, the fungal hyphal growth form provides fungi with a relative advantage over bacteria in poorly connected soils (Chapter 4), as demonstrated in a microcosm study investigating bacterial and fungal PLFAs at different pore size distributions and matric potentials. Sequencing analysis of bacterial and fungal communities revealed a contrasting effect of habitat connectivity on bacteria and fungi (Chapter 5). While decreased habitat connectivity (coarse pores and low moisture) facilitated the maintenance of higher bacterial diversity and richness, fungal communities exhibited the opposite trend with greater diversity and richness in well-connected soils (fine pores and high moisture content). We also found that habitat pore size generally had a greater impact than matric potential in structuring the microbial communities and that specific bacterial phyla and fungal species were affected differently by different pore sizes and matric potentials. Lastly, in an examination of bacterial motility, we found that different bacteria species showed contrasting impacts of habitat pore size, matric potentials and levels of nutrient availability (Chapters 3 & 6). Moreover, results showed that only a small fraction of the total bacterial species present in a natural soil community was able to expand rapidly through a soil-like sand microcosm system (Chapter 6).

In summary, the work presented in this thesis demonstrates that habitat connectivity, as determined by pore geometry and hydration status, has a great impact on microbial interactions and plays an important role in structuring bacterial and fungal communities in soil.

Samenvatting

Bodems herbergen een enorme diversiteit aan microbiële soorten en functies. Dit inzicht is vooral te danken aan recente ontwikkelingen in de toepassing van moleculair biologische methoden in de Microbiële Ecologie. Tot dusver ontbreekt echter een sluitende verklaring voor de hoge mate van diversiteit van micro-organismen in bodems. Bodem micro-organismen staan in voortdurende wisselwerking met hun abiotische en biotische omgeving. Er wordt verondersteld dat interacties tussen micro-organismen in belangrijke mate bepalend zijn voor de opbouw en diversiteit van microbiële gemeenschappen in de bodem. Deze interacties kunnen echter niet los worden gezien van de bodemstructuur. De mogelijkheid van micro-organismen om interacties aan te gaan met andere micro-organismen in de bodem is sterk afhankelijk van fysische mogelijkheden die de bodem daarvoor biedt, en wordt bepaald door de geometrie van het bodemporiën netwerk en de verdeling van het bodemvocht. Veel microbiële interacties verlopen via de water fase. De meeste bacteriën zijn voor hun voedselvoorziening en mobiliteit geheel afhankelijk van de waterfase in de bodem. Daarentegen vormen luchtgevulde poriën geen belemmering voor de mobiliteit van schimmels dankzij hun draadvormige groeivorm. Het onderzoek dat in dit proefschrift is beschreven richt zich met name op het effect van bodemvocht en poriegrootte op microbiële interacties en de samenstelling en diversiteit van microbiële gemeenschappen in bodems.

De algemene doelstelling van dit promotieonderzoek is het verkrijgen van meer inzicht in de factoren die bepalend zijn voor de enorme diversiteit van microbiële gemeenschappen in bodems. Het beschreven onderzoek heeft zich met name gericht op het effect van connectiviteit in bodems op structuur, diversiteit en interacties in bodem microbiële gemeenschappen.

De volgende drie onderzoeksvragen zijn daarbij aan de orde gekomen:

1. Wat zijn de effecten van poriegrootte verdeling en matrixpotentiaal, factoren die de connectiviteit bepalen, op competitieve interacties en benutting van micro-habitats?
2. Wat is de invloed van connectiviteit op de structuur en diversiteit van complexe bacteriële -en schimmelgemeenschappen en zijn de effecten gerelateerd aan ‘life history’ kenmerken van microbiële groepen? Zijn de bodemomstandigheden waarbij schimmels of bacteriën een relatief competitief voordeel hebben verschillend?
3. Wat is de invloed van bodemvocht op het vermogen van verschillende groepen van bacteriën om bodems te koloniseren?

Directe waarnemingen van microbiële gemeenschappen in de bodem op een schaal die relevant is voor micro-organismen wordt bemoeilijkt door de enorme heterogeniteit van de bodem. Daarom is er in dit onderzoek voor gekozen om een zand-microcosmos systeem te

ontwikkelen, waarin specifieke bodemparameters afzonderlijk kunnen worden veranderd. Dit zand-microcosmos systeem is gebruikt om (1) de competitie om voedingsstoffen tussen een draadvormige bacteriesoort en een niet-draadvormige bacteriesoort te bestuderen (Hoofdstuk 3), (2) de invloed van bodem connectiviteit op de diversiteit en opbouw van complexe gemeenschappen van bacteriën en schimmels te bepalen (Hoofdstukken 4 en 5), en (3) de bewegelijkheid van bacteriën in een model-bodem te onderzoeken (Hoofdstuk 6). In Hoofdstuk 3 worden de resultaten beschreven van een zand-microcosmos experiment waarin het effect van poriegrootte en matrixpotentiaal op de groeidynamiek van draadvormige en niet-draadvormige bacteriën is bepaald. Draadvormige bacteriën hadden een relatief competitief voordeel in bodems met een lage connectiviteit. Dit werd toegeschreven aan hun vermogen om luchtgevulde poriën over te steken en nieuwe micro-habitats te koloniseren. Niet-draadvormige bacteriën hadden een voordeel in bodem met een hoge connectiviteit. Een soortgelijk voordeel van draadvormige groei werd ook voor schimmels gevonden; schimmels hadden een relatief voordeel ten opzichte van bacteriën in bodems met een lage connectiviteit (Hoofdstuk 4), zoals werd aangetoond middels het meten van schimmel- en bacterie-specifieke fosfolipide vetzuren (PLFA) in het zand-microcosmos systeem bij verschillende poriegrootte verdelingen en verschillende matrixpotentialen. Sequentie analyse van bacterie- en schimmelgemeenschappen leverde een contrasterend effect op van bodem connectiviteit (Hoofdstuk 5). Terwijl een lagere connectiviteit (brede poriën en laag vochtgehalte) gepaard ging met een hoge diversiteit en soortenrijkdom van bacteriën, was de diversiteit en soortenrijkdom van schimmels juist het hoogst bij een goede connectiviteit (smalle poriën en hoog vochtgehalte). Tevens werd gevonden dat de opbouw van microbiële gemeenschappen sterker beïnvloed werd door de poriegrootte dan door de matrixpotentiaal. Daarnaast was het effect van poriegrootte en matrixpotentiaal verschillende voor verschillende taxa van bacteriën en schimmels. Als laatste is aangetoond dat poriegrootte, matrix potentiaal en beschikbaarheid van voedingsstoffen sterk differentiërend werkte met betrekking to het koloniserend vermogen van bacteriële soorten in het zand-microcosmos systeem (Hoofdstuk 3&6). Slechts een klein deel van de bacteriële soorten afkomstig uit een natuurlijke bodem bleken in staat tot snelle kolonisatie van het zand-microcosmos systeem (Hoofdstuk 6).

Samenvattend: Het onderzoek dat beschreven is in dit proefschrift laat zien dat bodem connectiviteit, zoals bepaald door poriegrootte en matrixpotentiaal, een sterke invloed heeft op microbiële interacties en een belangrijke rol speelt bij het structureren van bacterie- en schimmelgemeenschappen in de bodem.

Zusammenfassung

Böden beinhalten eine enorme phylogenetische und funktionelle mikrobielle Diversität. Die Weiterentwicklungen und Anwendung neuer molekularer Methoden im Gebiet der mikrobiellen Ökologie ermöglichten eine neue Wertschätzung des Umfangs dieser mikrobiellen Diversität in Böden. Dennoch bleibt unser Verständnis der Kräfte, welche diese unglaubliche Diversität formen und erhalten, rudimentär. Mikroorganismen interagieren auf unterschiedlichste Weise mit ihrer biotischen und abiotischen Umgebung, und diese mikrobiellen Interaktionen können als Schlüsselfaktoren für Struktur und Diversität mikrobieller Lebensgemeinschaften angesehen werden. Um die Kräfte, welche die mikrobiellen Lebensgemeinschaften formen zu verstehen, ist es daher essentiell die physikalische Bodenstruktur in Betracht zu ziehen. Das Ausmaß mikrobieller Interaktionen in Böden wird maßgeblich durch Habitatkonnektivität bestimmt. Diese Konnektivität ist ihrerseits von der Geometrie des Porennetzwerkes sowie des Hydrationsstatus des Bodens abhängig. Viele mikrobielle Interaktionen werden durch das Bodenwasser ermöglicht. Während Pilze durch ihr Hyphenwachstum die Möglichkeit haben, mit Luft gefüllte Poren zu überbrücken, sind Bakterien für ihren Zugang zu Nährstoffen und ihre Beweglichkeit vollständig vom Bodenwasser abhängig. Die Auswirkungen von Bodenwasser und Bodenstruktur auf mikrobielle Lebensgemeinschaften sind folglich Schwerpunkt dieser Arbeit.

Das Haupziel dieser Dissertation ist es, unser Verständnis der Kräfte, die den Erhalt der unglaublichen Vielfalt mikrobieller Lebensgemeinschaften in Böden ermöglichen, zu erhöhen. Die hier präsentierte Arbeit zielt besonders auf die Auswirkung von Bodenkonnektivität auf Struktur, Diversität und Interaktionen innerhalb mikrobieller Lebensgemeinschaften in Böden ab. Mit Bodenkonnektivität wird in diesem Kontext bezeichnet, inwieweit Mikrohabitate in Böden durch die Wasserphase hydraulisch miteinander verbunden sind.

Die drei Hauptfragestellungen sind wie folgt:

1. Was ist der Einfluss der Faktoren Größenverteilung der Bodenporen und Matrixpotenzial (ein Maß, dass alle Kräfte umfasst, die das Bodenwasser festhalten), welche zusammen die Bodenkonnektivität bestimmen, auf mikrobiellen Wettbewerb um Ressourcen und Lebensraumnutzung?
2. Wie beinflusst Konnektivität die Struktur und Diversität komplexer Bakterien- und Pilzgemeinschaften und gibt es einen Effekt auf mikrobielle Gruppen unterschiedlicher Lebensgeschichts-Strategien („life history strategies“)? Haben Bakterien oder Pilze einen relativen Wettbewerbsvorteil in bestimmten Bodenverhältnissen?
3. Wie beeinflusst Bodenfeuchte das Kolonisationspotenzial von Bakterien unterschiedlicher Bewegungsmechanismen?

In situ Beobachtungen mikrobieller Lebensgemeinschaften in einem mikrobiell relevanten Maßstab sind aufgrund der extremen Komplexität von Böden schwierig. Der grundsätzliche Ansatz dieser Arbeit bestand daher in der Entwicklung eines auf Sand basierenden Mikrokosmensystems, welches die unabhängige Manipulation spezifischer Bodenparameter erlaubt. Dieses System wurde dazu verwendet, den Ressourcen-Wettbewerb zwischen filamentösen und nicht-filamentösen Bakterien (Kapitel 3), den Einfluss von Bodenkonnektivität auf die Diversität und Struktur komplexer mikrobieller Lebensgemeinschaften (Kapitel 4 und 5) sowie die Beweglichkeit von Bakterien in einer bodenähnlichen Umgebung zu studieren (Kapitel 6). Kapitel 3 stellt die Ergebnisse der Mikrokosmenversuche, die den Einfluß von Porengrößenverteilung und Matrixpotenzial auf die Wachstumsdynamik von filamentösen und nicht-filamentösen Bakterien untersuchen, dar. Es wird gezeigt, dass filamantöse Bakterien in Böden mit niedriger Konnektivität einen relativen Wettbewerbsvorteil besitzen, was ihrer Fähigkeit Luft-gefüllte Poren zu überbrücken zugeschrieben wurde. Ähnlich den filamentösen Bakterien gibt das Hyphenwachstum Pilzen einen relativen Wachstumsvorteil in Böden mit niedriger Konnektivität (Kapitel 4). Dies wurde in einer Mikrokosmenstudie, die bakterielle und pilzliche Phospholipid-derived fatty acids (PLFA) in Böden verschiedener Größenverteilungen der Bodenporen und Matrixpotentiale untersuchte, nachgewiesen. Sequenzanalysen bakterieller und pilzlicher Lebensgemeinschaften zeigten einen gegensätzlichen Effekt von Bodenkonnektivität auf bakterielle und pilzliche Diversität (Kapitel 5). Während niedrigere Habitatkonnektivität (d.h. vorwiegend große Poren und niedriger Feuchtigkeitsgehalt) den Erhalt höherer bakterieller Diversität ermöglichten, zeigten Pilzgemeinschaften ein gegenteiliges Verhalten mit höherer Diversität und Artenvielfalt in Böden mit hoher Konnektivität (d.h. viele feine Poren und hoher Feuchtigkeitsgehalt). Des Weiteren wird gezeigt, dass die Struktur mikrobieller Lebensgemeinschaften insgesamt mehr von der Größenverteilung der Bodenporen als vom Matrixpotenzial beeinflusst wurde. Außerdem reagierten spezifische bakterielle und pilzliche Phyla und Arten unterschiedlich auf verschiedene Größenverteilung der Bodenporen und Matrixpotenziale. Schlussendlich zeigte die Untersuchung bakterieller Beweglichkeit, dass verschiedene Bakterienarten auf unterschiedliche Weise von der Größenverteilung der Bodenporen, dem Matrixpotenzial und dem Nährstoffgehalt beeinflusst werden (Kapitel 3 und 6). Des Weiteren war nur ein sehr kleiner Anteil der natürlichen Bodengemeinschaft dazu in der Lage, sich rasch in diesem bodenähnlichen Mikrokosmensystem auszubreiten (Kapitel 6).

Zusammenfassend demonstriert die in dieser Dissertation präsentierte Arbeit, dass die Habitatkonnektivität, welche durch Porengeometrie und Hydrationsstatus gegeben ist, einen maßgeblichen Einfluß auf mikrobielle Interaktionen hat und eine wesentliche Rolle in der Strukturierung von Bakterien- und Pilzgemeinschaften in Böden spielt.

Acknowledgements

I would like to express my gratitude towards Prof. George Kowalchuk for supervising my thesis and helping me investigate the small things without losing the bigger picture.

I am thankful to Michiel Vos for his enthusiasm in my project and the many discussions we had at the early stage of my PhD, which helped me develop the experimental setup.

I am grateful to Wietse de Boer for taking the time for in-depth discussions of experimental design, data and manuscripts.

Paolina Garbeva, thank you for the good collaboration on the bacterial interaction experiment, all your helpful advice and suggestions on my experimental work as well as encouragement when difficulties arose.

Annemieke van der Wal, thanks for helpful discussions, providing advice on statistical analysis and for your contribution to the microbial community experiment. I am thankful to Hans van Veen and Eiko Kuramae for their helpful discussions. I thank Mattias de Hollander for his help with the bioinformatics and for patiently answering my questions. Agaat – I greatly appreciate your help with the molecular work. I would further like to thank all technicians in the lab for always being helpful and supportive. Special thanks to Saskia for making sure everything runs smoothly in the microbiology lab where I spent many hours in the past few years.

To Sarah, my PhD project buddy - It was great working with you in the past 4.5 years. Best of luck back home in New Zealand! I am looking forward to reading your book and I hope our path will cross again!

To Emilia, thanks for your encouragement and the endless number of coffee breaks we spent chatting on the couch. Sabrina, thanks for introducing me to the best shopping and coffee places in Amsterdam. It was great being your paranymph, and it's great that you both will now also be my paranymphs and I hope to stay in touch with both of you in the years to come.

Olaf, I greatly enjoyed sharing an office and working on the microbial interaction experiment with you. Max, thank you for the good collaboration on the motility experiment.

A big thanks to my office mates for the gezellig atmosphere and fun times as well as quiet working hours in the office.

To my awesome fellow PhDs and colleagues: Thank you so much for your support, patience and encouragement and for good times inside and outside of work! The past 5 years would have been much less enjoyable without you. Special thanks to Maaike, Julia, Anne, Ruth, Sabine, Sarash, Yani, Elisabeth, Paolo, Chunxu, Thiago and Leonardo – it is not impossible that you are the most fun colleagues one can wish for!

Finally, I would like to thank my parents Mechtild and Dietrich for their never ending support. Ohne eure Unterstützung hätte ich es niemals geschafft diese Doktorarbeit zu Ende zu führen. Danke, dass ihr es klaglos hinnehmt, dass ich so selten zu Hause bin und ich hoffe, dass ihr mich bald in den USA besuchen kommt!