Micro-scale determinants of bacterial diversity in soil
Michiel Vos1, Alexandra B. Wolf1, Sarah J. Jennings1 & George A. Kowalchuk1,2,3

1Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands; 2Department of Ecological Science, Free University of Amsterdam, Amsterdam, The Netherlands; and 3Institute of Environmental Biology, Utrecht University, Utrecht, The Netherlands

Abstract
Soil habitats contain vast numbers of microorganisms and harbor 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 soilborne microorganisms. This review focuses 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.

Introduction
Soils are among the most vast (Whitman et al., 1998) and biodiverse microbial habitats on Earth (Quince et al., 2008). Soilborne 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. Delmont et al., 2012; Roesch et al., 2007) have been instrumental in rekindling our appreciation of soilborne 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 that seek to examine factors that drive and maintain this diversity. The typical soil sample is huge compared with the actual habitats of individual microorganisms, 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
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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 (Baas Becking, 2006; O’Malley, 2007). With advances in environmental sequencing, the topic of microbial biogeography has undergone a marked resurgence in this century (Baas Becking, 2006; O’Malley, 2007). 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.

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 larger eukaryotes, the effect of habitat on microbial biogeography was greater than that of distance. As 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 covaries with geographical distance. This indicates that microorganisms 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 microhabitat (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 harbor 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., 2007b), fertilizer 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, 2008a). 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, 2008a).

Remarkably, little is known about spatial patterns of bacterial diversity below the scale of meters. Oda et al. (2003) found that Rhodopseudomonas palustris populations sampled from marsh sediments were highly similar one centimeter apart, but that this similarity rapidly decreased so that populations nine meters apart were highly disparate. 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, 2006). Similarly, heavy metal contamination and bacterial metabolic potential 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 microsamples, but that diversity is organized in patches (Ranjard
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 (Vogels 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 x 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, 2009a). Furthermore, these isolates exhibited extensive variation in gliding motility (Vos & Velicer, 2008b), 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 unfavorable 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 favor 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 soilborne 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.

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,
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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 vs. 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., 2008b). 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.

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 have 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, 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 et al. (2001) were able to reveal patches of NO₂ oxidizers in the 250 μm range. A promising method of particularly high spatial resolution utilizes micro-manipulator-guided tungsten rods ‘stamped’ upon a soil sample, after which microorganisms adhering to the 0.013 mm² 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.

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 three-dimensional structure 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-computed tomography (μCT) (Tippkotter et al., 2009; Sammartino et al., 2012). The most common form of this technique allows visualization of soil structure in three dimensions at μm resolution, based upon the bending of X-rays by electrons in the sample, although other energy sources such as neutrons may be used (Moradi et al., 2011). 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 deconvolution of soil pores from the soil matrix, and occasionally the ratio between air- and water-filled soil pores (Sammartino et al., 2012) (Fig. 1d). Neutron tomography is one of the few techniques that allow in situ calculation of water distribution; however, care should be taken with this technique as it has the potential of creating radioactive samples (Sutton, 2008).

Nuclear magnetic resonance (NMR) or magnetic resonance imaging (MRI) is another potential nondestructive technique for the three-dimensional analysis of the soil environment, especially for localizing hydrogen nuclei (i.e. water). This method has been for instance 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 1H, 2H, 13C, 14N, 15N, and 31P (Haynes, 2012). Conventional (medical) NMR typically provides a resolution of ~3 μm (for 1H). 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 (1H) and chemistry (13C, 15N). Both μCT and NMR are nondestructive 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 predominantly 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), redox potential (Schmidt et al., 2011), 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. 1a) 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. 1b). 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, 2009; Ehrhardt et al., 2009)(Schmidt et al., 2012).

Infrared 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., 2006a, b; Tatzber et al., 2012). Although the use of this method to study micro-scale soil samples is still in its infancy, a variety of commercial probes for in situ measurements are becoming available (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 are 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 (Mue1ler 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 (He1ster et al., 2012) and to determine the distribution of organic matter and 15N in single intact aggregates (Mueller et al., 2012; Remusat et al., 2012).

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 IRS (Tatzber et al., 2012), NanoSIMS (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 localization 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 labeled with a gold particle (Kenzaka et al., 2005, 2009; Ehrhardt 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 visualization of soil bacteria within the context of the chemical environment in which they reside.

Microcosm experimentation

To gain insight into ecological mechanisms, it is necessary to go beyond purely descriptive studies of soil micro-habitats via the design of experiments in which soil parameters are systematically manipulated to examine their impacts on bacterial communities. To this end, a range of experimental systems are now 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. Rupams et al., 2011), sieved (sterilized e.g. Wright et al., 1995) or unsterilized soil (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 to preserve soil structure. An exciting development in this respect is the use of porous media consisting of transparent particles that allow for nondestructive, three-dimensional microscopic observation and tracking of the 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. 1c). This method holds great promise, as it combines relatively realistic soil conditions with nondestructive 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 coevolutionary dynamics between Pseudomonas and an associated bacteriophage were found to be very different in nonsterile soil microcosms 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 setups that are completely artificial, but can provide unprecedented insights into the behavior of individual cells in real time (Keymer et al., 2006, 2008; Kim et al., 2008a; 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.

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 microorganisms at a mere $10^{-6}$ (Young & Crawford, 2004).

As 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., 2007a) 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.

**Fig. 2.** The micro-scale soil habitat. Soils appear to be a rather homogeneous habitat at larger scales (a), but extreme heterogeneity is evident at scales more relevant to microorganisms (b and c). (b) 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. (c) 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.
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 thereby influencing nutrient diffusion, aeration, redox potential, and pH. Such environmental gradients can be steep and change rapidly over time (Or et al., 2007b). Bacteria in micropores are sheltered against rapid changes in overall soil hydration, but also experience limited access to fresh resources (Ranjard & Richardum, 2001). Low water content is a common cause of biological stress (van de Mortel & Halverson, 2004) (Chowdhury et al., 2011), but many bacteria can adapt to such conditions by producing protective extracellular polymeric substances that trap water and nutrients (Or et al., 2007a; 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 for the success of soilborne bacterial populations soils (Burmølle et al., 2007, 2011).

**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 colony-forming type. However, in the absence of homogenization wrinkly and fuzzy colony morphotypes evolved to colonize the air-broth interface oxygen-deprived bottom of the flasks, respectively, with smooth morphotypes inhabiting the broth unaffected by 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 extremely 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 at 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 at 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 coevolutionary 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., 2010a) (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., 2007a). 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 nonrandom interactions between cells is expected to have a profound implications 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 codetermine 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.

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., 2007b), or actively through active swimming or swarming over hydrated surfaces away from stresses and toward resources (Kearns, 2010). For instance, Paenibacillus has been found to swarm at rates up to 10.8 mm h$^{-1}$ on agar (Ingham et al., 2011), dispersing fungal spores in the process. Conversely, Paenibacillus may be dispersed via the growth of fungal hyphae spanning otherwise insurmountable air-filled gaps in the soil matrix. Bacterial motility rates are likely lower in soils than when observed on agar surfaces due to impedance by soil structures and decreased hydration (Wang & Or, 2010). Nonmotile bacteria must rely on passive dispersal, for example, by passing invertebrates. In a very original study, it was demonstrated that earthworm movement in nonsterile microcosms not only resulted in the dispersal of bacteria, but also promoted horizontal gene transfer (HGT) 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).

Nontransitive fitness interactions

Most bacteria produce compounds that kill related strains (e.g. bacteriocins) or distantly related microorganisms (e.g. polyketides) (Riley & Wertz, 2002). One of the best understood interference competition systems is that of the Escherichia 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 not only have an inactivated toxin gene, but also 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 nontransitive 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 modeling (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, 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 signaling and motility (Yim et al., 2007; Raaijmakers & Mazzola, 2012), it is likely that many microbial war games are played out under our feet. Such nontransitive antagonistic interactions in the highly structured soil matrix thus have great potential for promoting diversity (Czaran et al., 2002).

A recent study by Zhang et al. (2011) on the evolution of antibiotic resistance is of great potential relevance to our understanding of the diversity in antibiotic warfare strategies in soil. 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 lysed 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.

Nontransitive 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 et al. (2008a) studied a system containing three different species, each performing a function key to the survival of the other two species: Azotobacter vinelandii producing amino acids by fixing nitrogen, Bacillus licheniformis degrading penicillin, and Paenibacillus cardinalanolyticus providing glucose by cleaving cellulose. 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.

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 modeling 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, 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 vs. 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 (Buttnar & 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 nonproducers 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 cooperators 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 cooperators. 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).

**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., 2010b). The fine spatial heterogeneity of soils results in a complex mosaic of gradients selecting for or against bacterial growth. Spatial structure also results in nonrandom interactions between different cell types, allowing for nontransitive interactions whereby no type out-competes all other types. Such nontransitivity 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 a range of 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 with 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 not only evolved 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 that are most relevant, 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 neighboring 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., 2009b). This finding indicates that selection exerted by coevolving species was strong enough to shape biodiversity at a scale of centimeters (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 centimeter 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 nonrandom 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 clearly also the case 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 for 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 microorganisms, 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 (Rudick & 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 micro-dish 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 labeled (or, when cell shapes are different, even unlabeled) 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 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 microscope’, 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 macroorganisms 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

Table 1. Methods to characterize bacterial microhabitats in soil

| Sampling method         | Analysis method | Micro-habitat characterization | Detection bacterial presence/activity | Single-cell resolution (< 2 μm) |
|-------------------------|-----------------|---------------------------------|---------------------------------------|----------------------------------|
| 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                                   |
| Aggregate visualization | Micro-computed tomography | Yes, physical | No                                    |
|                         | Yes, chemical   | Yes, with stain                 | Yes                                   |
|                         | Yes, chemical   | Yes, with stain                 | Yes                                   |
|                         | Yes, chemical   | Yes, with stain                 | Yes                                   |
|                         | Yes, chemical   | No                              | No                                    |
|                         | Yes, chemical   | Yes                             | Yes                                   |
|                         | Yes, chemical   | Yes, with stain                 | Yes                                   |
|                         | Yes, chemical   | Yes                             | Yes                                   |
|                         | Yes, chemical   | Yes                             | Yes                                   |
|                         | Yes, chemical   | No                              | No                                    |
|                         | Yes, chemical   | Yes                             | Yes                                   |
|                         | Yes, gold staining | No                          | Yes                                   |
|                         | Yes, physical   | Yes                             | Yes                                   |

*Metagenomics, tag sequencing, proteomics and transcriptomics.
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.

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