Microbiological and molecular analysis of bacterial communities of an urban soil

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Diplom-Biologin
Burga Braun
aus Braunschweig

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Berichter:	Prof. Dr. Ulrich Szewzyk
Berichterin:	PD Dr. Elisabeth Grohmann
Berichter:	Dr. Andreas Farnleitner

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Zwei Dinge sind zu unserer Arbeit nötig: Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit gesteckt hat, wieder wegzuwerfen.

Albert Einstein

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Abbreviations

℃	degrees celsius
µ	micro
ANOVA	analysis of variance between groups
ARDRA	amplified ribosomal DNA restriction analysis
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair(s)
C	carbon
$CaCl_2$	calcium chloride
CARD-FISH	catalyzed reporter deposition fluorescence in situ hybridization
CFU	colony forming units
cm	centimeter
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
Cy3	5,5´-disulfo-1,1´di(carbopentyl-)-tetramethyl-inolocarbocyanin
DAPI	4´,6-diamidino-2-penylindol dihydrochloride
DGGE	denaturing gradient gel electrophoresis
DNA	desoxynucleic acid
E	east
EPS	extracellular polymeric substances
EtOH	ethanole
FISH	fluorescence in situ hybridization
g	gram
GC	Guanine + Cytosine
h	hour(s)
H_2O	water
HRP	horseradish peroxidase
HY	hydrophobicity
l	liter
m	meter
mm	millimeter
M	molar
min	minute

mM	millimol
n	nano
N	north
NaCl	natrium chloride
OD	optical density
PAC	probe active counts
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	pondus hydrogenii
PLFA	phospholipid fatty acid
rDNA	ribosomal desoxynucleic acid
RNA	ribonucleic acid
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
s	second(s)
SDS	sodium dodecyl sulfate
TAE	tris-acetate-EDTA
T-RFLP	terminal restriction fragment length polymorphism
Tris	tris-(hydroxymethyl)-aminomethane
Tris/HCl	tris-(hydroxymethyl)-aminomethane/ hydrochloric acid
TSA	trypticse soy agar
TSB	trypticase soy broth
Tween 40	polyoxyethylenesorbitan monopalmitate
Tween 80	polyoxyethylenesorbitan monooleate
U	unit
UPGMA	unweighted-pair group method using average linkages
V	volt
w/v	weight/volume
WDPT	water drop penetration time
wt	weight

1 Introduction

Bacteria in soil systems

Bacteria exist for more than three billion years and inhabit nearly every environment. They are even prevalent in very extreme habitats like glaciers, deserts or black smokers. Soil represents one of the most complex natural habitats for bacteria (Grundmann & Debouzie, 2000; Ellis *et al*., 2003), containing about 10^9 bacterial cells per gram soil. It consists of mineral particles, plant roots, soil biota and decomposed organic matter as well as soil water, soil gases and dissolved minerals. The environmental conditions (e.g. availability of carbon sources, pH, temperature or water content) within the soil may be subject to rapid changes. Due to this the soil represents a structured, heterogeneous and discontinuous environment, which is dominated by a solid phase. Soils are characterized by distinct layers. These horizons, composing the soil profile, are grouped as organic horizon (O horizon), eluvial horizon (A horizon), illuvial horizon (B horizon) and rock horizon (C horizon). At the top of the profile is the O horizon, which is primarily composed of organic matter. It contains fresh litter at the surface. Beneath the O horizon, the A horizon marks the beginning of the true mineral soil. In this horizon organic material mixes with inorganic products of rock weathering. It is typically a dark colored horizon due to the presence of organic matter. The eluviation process, the removal of inorganic and organic substances from a horizon by leaching, occurs in this horizon. Beneath the A horizon lays the B horizon, a zone of illuviation where downward moving, especially fine material, is accumulated. As a result the B horizon is a dense layer in the soil. The C horizon as the deepest horizon represents the soil parent material, either created in situ or transported into its present location. Inside this stratification soil texture, soil moisture, soil organic matter or oxygen vary vertically at micrometer, millimeter or centimeter scale. Within this physicochemical gradient in this structured system distinct microhabitats harboring microorganisms are defined (Stotzky, 1997). These microhabitats represent locations with distinct biological, chemical or physical characteristics e.g. moisture content, pH or organic matter content. They can be defined functionally as a volume of soil which influences the behavior of bacteria or populations that, in turn, exert and influence the environment. Microhabitats occur in soil aggregates as well as on particle surfaces, containing single cells, small colonies

or mixed bacterial communities. The inhabiting microorganisms are adapted to the microhabitats and live together in consortia by interacting with each other as well as other soil biota. Their activity and community structure can be affected by several factors, such as carbon source, nutrients, available water, pH, temperature, surface properties and interactions between the microorganisms (Stotzky, 1997; Sylvia *et al*., 1999; Nannipieri *et al*., 2003). As these factors can change markedly, the microhabitats are dynamic systems in soil. Within the oligotrophic bulk soil, microhabitats can serve as so called “hot spots”. These are locations with a high bacterial activity, which can be ephemeral and responsive to fluctuating substrate availability. Hot spots are zones with distinct physico-chemical properties and e.g. accumulated organic matter (Parkin, 1987). Soil aggregates, rhizosphere or preferential flow paths can act as such habitats (Bundt *et al*., 2001; Smalla *et al*., 2001). Hot spots such as preferential flow path may permit a better nutrient and substrate supply for microorganisms than bulk soil (Bundt *et al*., 2001), due to greater proportion of living or decayed roots in the flow path (Bundt *et al*., 2000). An often investigated hot spot is the soil rhizosphere, as plant growth influences the microbial community and vice versa. Microbial populations of the rhizosphere may have a neutral, negative (e.g. for pathogens), or positive (e.g. for associated or symbiotic nitrogen fixers) impact on plant growth. Plant roots modify the surrounding soil by root exudates, removal of soil water by plant uptake and release of CO_2 and O_2. As the quality and quantity of substrates of the rhizosphere microhabitat differ from those of bulk soil, populations in the rhizosphere compartment can differ in numbers and structure from bulk soil populations. Rhizosphere microorganisms modify the rhizosphere environment by producing extracellular enzymes as e.g. plant growth factors (Costacurta & Vanderleyden, 1995). The rhizosphere compartment is an example of a natural formed gradient, as e.g. the amount of available carbon decreases with distance from plant roots. Beside the vertical gradients in soil texture, moisture, structure or composition, variations along horizontal gradients also exist. This horizontal variability can result in spatial heterogeneity of microhabitats even within one distinct soil horizon.

In this dynamic environment microorganisms are responsible for most of the biological activity. Soil microbial populations reflect a great diversity, whereby diversity is defined as species richness present in soil. Bacterial communities are involved in different soil processes, such as decomposition of organic matter, humus

production, nutrient release or nitrogen fixation (Beare *et al*., 1995) and in enhancing the bioavailability of nitrates, sulfates, phosphates and essential metals. They therefore heavily influence soil structure and soil fertility (O`Donnell *et al*., 2001). Some of these soil processes have fundamental roles in soil fertility as they result in nutrient availability for plants. During the decomposition process, microorganisms assimilate organic substances and release inorganic nutrients needed for plant growth. The decomposition of organic matter is a major process in soil as it involves a wide range of substrates metabolized by microorganisms widespread throughout the bacterial and archaeal kingdoms and has a high functional redundancy.

The soil seems to be characterized by a redundancy of soil functions as e.g. no relationship between microbial diversity and decomposition of organic matter exists (Brookes, 1995; Andrén & Balandreau, 1999; Nannipieri *et al*., 2003; Griffiths *et al*., 2004). The group of Griffiths did not detect any consistent effect of biodiversity on a range of soil processes measured (incorporation of thymidine and leucine, nitrification potential, nitrate accumulation, respiratory growth response, community level physiological profile and decomposition) by inoculating sterile agricultural soil with serially diluted soil suspensions prepared from the parent soil (Griffiths *et al*., 2004). A reduction in any group of bacteria has only little effect on overall processes because other microorganisms can take over this function. This has been shown by Wertz et al. who investigated the sensitivity to diversity erosion for ammonia oxidizers, denitrifiers and heterotrophs with molecular fingerprint techniques (Wertz *et al*., 2006). Their results indicated that the enormous diversity of the soil microbiota makes the ecosystem functioning and largely insensitive to biodiversity erosion.

Most of the soil bacteria are functionally redundant, but the functional characteristics of single species and the number of species are at least as important for maintaining essential processes (Andrén & Balandreau, 1999). Therefore biodiversity stabilizes the ecosystem function by providing a reservoir of physiological diversity that allows adaptation to changing conditions (Loreau, 2000). Loreau et al. (2001) postulated the so called “insurance hypothesis” which means that a minimum number of species (low biodiversity) is essential for ecosystem functioning in steady state systems and a large number of species (high biodiversity) is essential for maintaining stable processes in changing environments. For a high physiological diversity, either a high diversity requiring more specialized functional organisms, or a

low biodiversity requiring very versatile organisms with a broad metabolic potential, is needed.

Bacteria may exist in soil either free in the interstitial water films, or attached on solid surfaces; according to Hattori (1973) almost 80-90% of the soil inhabiting microorganisms are attached. They are associated with various particles, live in water films surrounding soil particles or inside aggregates (Stotzky, 1997) as single cells, colonies or in biofilms. Biofilms are composed of bacteria embedded in a slime matrix which consists of extracellular polymeric substances (EPS) (Wingender *et al*., 1999). The EPS mainly consist of a mixture of polysaccharides and proteins, but also nucleic acids and lipids (Flemming & Wingender, 2000) and vary in their composition and hence in their chemical and physical properties. Sutherland (2001) noted that EPS may be hydrophobic, although most types of EPS are both hydrophobic and hydrophilic.

Water repellency of soil

Water repellency is a widespread phenomenon that can occur naturally as reported by Dekker and co-workers (1999) or fire induced (Doerr *et al*., 1996; Dekker *et al*., 1999). It has been documented in most continents and in a variety of land uses and climatic conditions. Water repellency is a dynamic soil property, which results from complex interactions between many physical and chemical properties. As opposed to soil wettability, water repellency defines the ability of the soil to intake water. It is accepted that repellency is affected by hydrophobic organic compounds deposited on soil minerals or aggregate surfaces (Wallis & Horne, 1992; Doerr *et al*., 2000). Morley et al. (2005) have recently shown that the amount of organic compounds is not related to the degree of water repellency and they speculated that the presence of polar compounds of high relative molecular mass is necessary for the exhibition of water repellency. Another common cause of water repellency is the accumulation of hydrophobic waxes or humic substances on soil particles (McKenna *et al*., 2002; Franco *et al*., 2006) or hydrophobic compounds released from plant roots (Doerr *et al*., 2000). Water repellency can also be caused by the growth of microorganisms, as Hallet et al. (2001) found that stimulating the microbial biomass with nutrients greatly enhances the repellency of agricultural soils. Soil water content is considered to be one of the most important factors in temporal variations in water repellency (Doerr *et al*., 2000). It becomes more severe in dry soils and declines as soil water content increases (King, 1981). Most of the soils are not permanently water

repellent. They become water repellent when they desiccate during dry periods in summer, with water repellency often vanishing when the soil becomes wet in winter times (Dekker & Ritsema, 1994). Wetting and drying cycles might also be a factor for water repellency. They led to a decrease in water repellency, as wetting and drying affect the soil structure by disrupting interparticle bonds (Czarnes *et al*., 2000). A greater amount of carbon in soil solution and its heterogeneous distribution might also influence soil wetting properties (Zhang *et al*., 2006).

Water repellency mainly impacts surface runoff and can lead to reduced wetting rates of the soil. Repellent soils tend to reduce water infiltration into the soil, enhance overland flow and cause uneven wetting patterns and preferential flow (Ritsema *et al*., 1993; Täumer *et al*., 2006). Preferential flow paths cause much faster transport of water and solutes and therefore can create a greater risk for groundwater contamination. In addition, water repellency leads to a reduction in plant available water and limits solute transport in repellent soil spots. The desiccation of soils also lowers microbial activity and can select for spore forming bacteria (Sylvia *et al*., 1999). Soil water regime plays also an important role in soil biota dynamics (Young & Ritz, 2000).

Due to this negative impact phenomenon, a number of strategies have been developed to ameliorate water infiltration into the soil. These include e.g. the addition of lime to the soil (Roper, 2005), the application of selected wax degrading bacteria to the soil (McKenna *et al*., 2002; Roper, 2004) and even the application of chemical surfactants (Kostka, 2000). Bacterial amelioration of water repellency originates by breaking down hydrophobic compounds (McKenna *et al*., 2002; Roper, 2004). Bacteria may also contribute to wetting properties of the soil by the formation of biofilms and aggregates. Stable aggregates are known to improve water infiltration into the soil and the soil water holding ability. The bacterial EPS form a highly hydrated gel in a three-dimensional network structure (Danese *et al*., 2000) which mediates the formation of cell aggregates (e.g. biofilms and flocs) in natural environments. These polysaccharides have been shown to interact with clay particles (Chen, 1998; Lünsdorf *et al*., 2001) and can persist even after cell death (Chen, 1998). Lünsdorf et al. (2001) have shown in a simple system, that a composite biofilm consists of a dense lawn of clay aggregates arranged in the form of hutches harboring the bacteria.

Due to the great impact of bacteria on e.g. soil function, plant fertility and nutrient cycling, a lot of studies have been conducted to investigate the influence of stress on bacterial communities. The effects of different stress factors such as metals, soil moisture, or soil temperature on bacterial soil populations have been investigated (Wenderoth *et al*., 2001; Bergsma *et al*., 2002; Papatheodorou *et al*., 2004). Changes in soil wettability may also represent a significant source of stress for microbial communities and is known to alter the composition and growth of soil bacterial communities (Denef *et al*., 2001). Therefor, studies on the influence of water stress on bacterial communities induced by drying and rewetting of soils have also been carried out for many years (Binstock, 1984; Bloem *et al*., 1992; Lundquist *et al*., 1999a; Lundquist *et al*., 1999b; Mamilov & Dilly, 2002; Griffiths *et al*., 2003; Fierer *et al*., 2003b; Whiteley *et al*., 2004; Pesaro *et al*., 2004; Steenwerth *et al*., 2005). To my knowledge investigations on the influence of water stress due to water repellency on microbial communities are rare.

Monitoring microorganisms in natural ecosystems

The characterization of bacterial communities is one of the major objectives in microbial ecology. Species diversity consists of species richness, total number of species, species evenness and species distribution (Ovreas, 2000). Many different methods and approaches have been applied to gain access to microorganisms residing in soil and to assess the microbial diversity. The methods to measure microbial diversity can be divided into two groups: (i) cultivation based methods and (ii) cultivation-independent methods. In the past, microbial diversity has been quantified by plate counting methods and isolation of bacterial cells followed by identification by a variety of methods addressing only the culturable diversity. Methods for microbial characterization can be based on isolation and cultivation followed by tests on cell wall composition and the occurrence of specific enzymes or catabolic analysis. However, approximately 99% of bacteria observed under a microscope are not cultivable under standard conditions (Torsvik *et al*., 1990; Atlas & Bartha, 1998; Hill *et al*., 2000). This problem is related to the lack of knowledge of the real conditions under which most bacteria are growing in their natural habitat. To overcome this problem there have been recent attempts to develop new culture media to maximize the recovery of diverse microbial groups (Balestra & Misaghi, 1997; Sait *et al*., 2002; Janssen *et al*., 2002; Joseph *et al*., 2003). Recent advances in the development of culture media and culture methods for soil bacteria (Sait *et al*.,

2002; Böckelmann *et al*., 2003; Janssen, 2006) could significantly improve plate counting as an even more useful technique for assessing the soil community.

Therefor, culture-independent techniques for community analysis have become widely applied methods, which generally involve the direct or indirect extraction of nucleic acids from soil. These methods have become a valid support to traditional techniques, especially tools including the sequencing of 16S rRNA genes (e.g. Muyzer *et al*., 1993; Amann *et al*., 1995). Molecular methods can overcome problems associated with cultivation of bacteria from natural samples. They give an overview of microbial diversity and help to monitor alterations in microbial composition after stress or changes in environmental factors by generation of genetic fingerprints. However, the approach of determining microbial diversity by traditional cultivation techniques should not be neglected as culturable bacteria may have an ecological significance in soil (Bakken, 1997) and are available for in depth studies of their physiology.

The molecular characterization of bacterial communities is based on ribosomal RNA (rRNA) or the rRNA gene which contains variable and conserved regions. For comprehensive information on the species composition, polymerase chain reaction (PCR)-based fingerprinting techniques as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA) and phospholipid fatty acid (PLFA) analysis have been developed and applied (Torsvik & Øvreås, 2002).

With the PCR-DGGE method, Muyzer et al. (1993) presented a method to generate genetic fingerprints of complex microbial communities with partial 16S rRNA gene sequences. This method separates sequences of the same length but different base composition based on the melting behavior in denaturing gradients. A GC rich clamp of about 40 to 45 bases at the 5`end prevents complete melting of both strands. This frequently used fingerprinting method allows a rapid comparison of samples and is generally used to detect shifts in spatial or temporal variations in populations (Heuer *et al*., 1997; Nakatsu *et al*., 2000; Maarit *et al*., 2001; Agnelli *et al*., 2004).

However, to understand the role of bacterial communities in soil it is essential to take into account the functional diversity. As the utilization of available carbon is the key factor in microbial growth in soil, Garland and Mills (1991) developed a technique based on the BIOLOG method to monitor microbial functional diversity. This method

has been successfully applied for assessing the metabolic potential of contaminated soils (Konopka *et al*., 1998), plant rhizospheres (Ellis *et al*., 1995; Garland, 1996; Smalla *et al*., 1998), soils treated with herbicides (El Fantroussi *et al*., 1999) or bulk soils (Widmer *et al*., 2001).

An important qualitative and quantitative tool in molecular ecology is the fluorescence in situ hybridization (FISH). Specific fluorescently labeled oligonucleotide probes target segments of the 16S rRNA. As this method can be applied in situ, it provides informations on spatial distribution of the cells as well as changes in their abundance and to a certain amount their activity.

Despite the large number of methods available to monitor microbial communities it is not easy to establish a link between structure and function of these communities due to difficulties in accurately determining microbial diversity. Soil function is determined by the measurement of microbial processes without knowing the microbial species effectively involved in these processes. Furthermore, a central problem is to understand the relation between genetic diversity and community structure and function. To get insights into the relation between microbial diversity and soil function several studies have been carried out (Degens & Harris, 1997; Griffiths *et al*., 2001; Griffiths *et al*., 2004; Wertz *et al*., 2006). And it has been shown by a lot of studies that a polyphasic approach is necessary when studying microbial community structure (Fritze *et al*., 2000; Bundt *et al*., 2001; Blume E. *et al*., 2002; Garcia-Pichel *et al*., 2003; Ekelund *et al*., 2004; Agnelli *et al*., 2004; Braun *et al*., 2006).

2 Sampling and methodical repertoire

This section gives a short introduction to the sampling strategy and an overview of the main methods used in this study.

Soil sampling

The experimental site is located in the urban park Tiergarten in the center of Berlin, Germany (52°30`N and 13°21`E) and represent s a heterogenic habitat for microorganisms due to the spatial distribution of water repellency. The park was founded in 1833 and the total area is about 780 hectares.

The sampling site within the park is located on a public area, which is marked by anthropogenic influences, like fertilization, sprinkling and pollutants. The yearly

rainfall of about 580 mm is evenly distributed. The climatic water balance becomes negative from April to September.

Bulk soil samples were collected from a medium sized fluvial sandy soil, with a thick humus layer, which can reach a height of up to 40 cm. Nineteen soil samples from the identical water repellent and wettable areas were taken from 10-30 cm depth in April, July and October 2002, January, April, July and October 2003 and in April 2004 in three parallels. The samples were immediately transported to the laboratory and processed the same day or after overnight storage at 4 ℃. From 1000 g of each soil sample, 20 g were homogenized and subsamples of 5 g were taken for further analysis. The pH was determined in $CaCl_2$ (0.01M) (DIN ISO 10390), soil / $CaCl_2$ solution-ratio 1:2.5 and in H_2O (DIN ISO 38404). Organic carbon content was measured by drying the sample at 550 ℃ for 5 h following DIN EN 12879. Water content was defined according to DIN EN 12880. Because of the high variability of water repellency in the field, samples of actually wettable and water repellent samples, which were located directly side by side at a distance of 10 to 20 cm, were compared.

Extraction of total community DNA and PCR

Total community DNA was directly extracted from soil by using the FastDNA® Spin Kit for Soil as described by the manufacturer (Qbiogene, Carlsbad, California).

For the study of the bacterial communities of the soil profiles primer pair 63f (Marchesi *et al*., 1998) and 1525r (Lane, 1991) was chosen for amplification of the 16S rRNA genes (Paper I).

For the study of the bacterial communities of wettable and non wettable soils two different primer pairs hybridizing to the evolutionary conserved regions of the 16S rDNA were chosen. These were primer pair F984 and R1378 (Heuer *et al*., 1997) covering the V6-V8 region of the 16s rRNA gene and primer pair p2 and p3 (Muyzer *et al*., 1993) for amplifying the V3 region of the 16S rDNA. The best resolution was achieved using primer pair p2 / p3. For comparison of DGGE fingerprint patterns the V3 region of rDNA was chosen (Paper II). DGGE gels generated by DNA fragments amplified with the F984 / R1378 primer pair resulted in fingerprint patters too complex for digital image analysis. Specific amplification of the V3 fragment of the 16S rDNA of total soil DNA was carried out using a hot start protocol, which has proven to be effective in minimizing unspecific annealing of primers to non-target DNA.

Separation of DNA fragments by ARDRA

The ARDRA technique was used to get community profiles for comparing the bacterial consortia within the soil profile. The 1500bp DNA fragments, generated by PCR were purified with MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and digested at 37 ℃ for at least 5 h in a final volume of 50 µl with the restriction enzymes *Hae* III and *Hinf* I (Fermentas, St. Leon-Rot, Germany). The 16S rDNA restriction fragments were purified with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and eluted to a final volume of 8 µl. Separation of the digested PCR fragments was performed by polyacrylamide gel electrophoresis (PAGE) with the Multiphor® II system (Pharmacia Biotech, Uppsala, Sweden). Electrophoresis was carried out on 12.5% Multiphor II Precast gels (Pharmacia Biotech, Uppsala, Sweden) for 90 min at 600 V, 50 mA, 20 W and 15 ℃. The DNA was stained according to the silver staining procedure described by Bassam et al. (1991).

Fluorescent in situ hybridization of soil samples

To study the distribution of different microbial groups within the three different soil layers, the FISH method has been applied. Bacteria were extracted from soil samples and the detached cells were hybridized. The focus was on the phylogenetic groups of α-, β- and γ- *Proteobacteria* and the *Eubacteria*. For the hybridization of the α-, β- and γ-subclass of *Proteobacteria* indocarbocyanine (cy3) labeled probes ALF1b, BETA42a and GAM42 were used (Manz *et al*., 1992). Probe EUB 338 (Amann *et al*., 1990) was used to detect members of the *Eubacteria*.

PAC method

For the determination of potentially active cells the probe active count method was used (Kalmbach *et al*., 1997). Bacterial cells were activated by supplying glucose and yeast extract as a substrate. To prevent cell division the antibiotic pipemidic acid was added as gyrase inhibitor.

DNA fragment separation by DGGE

The DGGE analysis was performed with a DCode® gel electrophoresis system (Bio-Rad, München, Germany) according to the manufacturers instructions using an 8% polyacrylamid gel with a parallel chemical denaturing gradient adapted to 40% (6% [w/v] acrylamide-bisacrylamide [37.5:1], 18% deionized formamide, 3.1 M urea)

to 65% (6% [w/v] acrylamide-bisacrylamide [37.5:1], 26% deionized formamide, 4.5 M urea).

Time travel experiment

To establish the optimal electrophoresis time for maximum resolution for the different DNA fragments, a time course separation experiment of PCR products obtained with primers p2 and p3 from one soil sample was performed. Samples were electrophoresed for 1, 2, 3, 10, 16, 18, and 19 h at 80 V and 60℃ on an 8% polyacrylamid gel with a denaturing gradient adapted to 40% to 65% as described in paper II.

Digital image analysis

ARDRA and DGGE profiles were analyzed for similarities by digital image analysis using the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). For the similarity matrix calculation the band based Dice correlation index was used. This calculation method compared the number of corresponding bands of the lanes with the total number of bands in the lanes. Analyzing ARDRA gels background subtraction was applied and the Dice correlation index was used to calculate the similarity matrix with a tolerance and optimization of 1%. The clustering of patterns was calculated using the unweighted-pair group method using average linkages (UPGMA).

DGGE fingerprint patterns relatedness was determined using the similarity Dice correlation coefficient of bands common in two samples. After background subtraction, the profiles were normalized using a species standard as a reference (the standard consists of 16S rDNA fragments from different isolates, see Paper II). For generating dendrograms the Dice correlation index was combined with the clustering method UPGMA.

DGGE gel band isolation, cloning, sequencing and phylogenetic analysis

Selected single bands were isolated from the stained DGGE gels and the DNA was extracted from the gel. The eluted DNA was reamplified and cloned into the pCR®4-TOPO vector (Invitrogen, Carlsbad, CA, USA). 190 clones, five clones from each of the 38 excised bands containing inserts, were chosen for 16S rDNA sequencing. Obtained sequences were processed with the BLAST program (Altschul

et al., 1997) to determine the closest known relatives derived from the partial 16S rDNA sequences.

Generating metabolic fingerprints using the BIOLOG system

The BIOLOG system provides a tool to analyze the metabolic potential of microbial communities with regard to utilization of different carbon sources. This method shows the potential catabolic abilities of a community under culture conditions. It has been used to show differences between microbial communities in different kinds of soils (Garland & Mills, 1991; Girvan *et al*., 2003) or different soil layers (Braun *et al*., 2006). This method has been considered a simple method to reveal bacterial metabolic fingerprints. But it has been shown by Preston-Mafham et al. (2002) that it is difficult to adapt this method to analyze microbial communities in soil samples. To reduce biases of this method the collected soil samples were treated according to Böckelmann et al. (2003) to improve the detachment of particle associated bacteria

Wilhelmy Plate Contact Angle

The Wilhelmy Plate method allows determination of the contact angle of flat surfaces. During immersion, a balance records the development of the effective sample weight, and the contact angle is calculated considering the water surface tension and the buoyancy. The measurements were conducted with a Dynamic Contact Angle Tensiometer (DCAT 21, Dataphysics, Filderstadt, Germany), and the data were evaluated with the respective software (SCAT Version 2.3.8).

Sessile Drop Contact Angle

For the determination of the contact angle by the sessile drop method three drops of water (50 µl) were placed on the soil sample. The time was measured that was necessary for the drops to infiltrate into the soil. The shape of the drop was documented with a digital camera Nikon COOLPIX 5400 (NIKON, Düsseldorf, Germany). The contact angle was calculated from the parameters of the ellipse fitted to the sessile drops.

Microcosm experiments

To investigate the alteration of soil communities by drying and wetting of soil, microcosm experiments were conducted in pots containing 350 g of homogenised soil. Soil samples were subjected to different watering regimens to study community

adaptation to moisture limitation. Replicated composite soil samples were randomly collected from the upper 15 cm soil layer (after removing the top 2–3 cm) from water repellent and wettable soil. Soil samples derived from wettable soil spots were dried at a temperature of 28° C to achieve a gravimetric water content of 1.2%. Whereas samples from non wettable soil spots were adjusted to a gravimetric water content of 48% at room temperature. This water content corresponds to twice the water holding capacity of this soil. Control samples from water repellent and wettable soil samples were kept at room temperature at their initial water content. The initial water content was 20% for wettable soil samples and 5.4% for non wettable soil samples. All microcosm experiments were run in three replicates.

All microcosms were sampled regularly at weekly intervals over a 16-weeks period for microbial, molecular and soil pysico/chemical investigations. The total bacterial community was analyzed by total cell counts and genetic diversity was analyzed with 16S rDNA based denaturing gradient gel electrophoresis of total community DNA (rRNA genes). The physiological response was monitored by plate counts and 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) staining.

3 Objectives of this study

Little is known about the effects of water repellency on soil bacterial communities and *vice versa*. The aim of this study was to reveal such possible effects of water repellency in an urban soil and to get information on differences in soil wettability affect bacterial community structure and their metabolic potential. Furthermore, the possibility to ameliorate soil wettability by the application of bacterial isolates, selected on the basis of their cell wall hydrophobicity, to soil samples should be investigated.

Specific objectives :

- Analysis of total bacterial numbers and colony forming units within the soil profile and in wettable and water repellent soil spots.
- Investigation of the effect of water repellency on the bacterial consortium of the different soil spots by creating DGGE community fingerprint patterns.
- Studying the impact of increasing soil depth on soil bacterial communities of water repellent soil samples.

- Determination of alterations in the bacterial metabolic potential caused by soil water repellency and soil depth.
- Gaining insights how biofilms may affect surface properties of soil samples.
- Monitoring the molecular diversity and active bacterial population subjected to water stress.

4 Results and Discussion

The main aim of this study was to gain a more profound insight into the bacterial community structure of wettable and water repellent soils. The horizontal and vertical distribution of the bacterial population was analyzed with regard to soil water repellency by combining traditional microbiological techniques and molecular methods. Finally, the effects of hydrophilic and hydrophobic bacterial biofilms on soil wettability were investigated.

Water repellency is an important problem in soil systems around the world, as it causes uneven water infiltration into the soil (Edwards *et al*., 1993; Täumer *et al*., 2004; Täumer *et al*., 2006). It influences soil chemical and physical properties which are important for many functions in ecosystems. Therefor, insights into influences of water repellency on bacterial communities are important. As water repellency can result in preferential flow paths, small scale heterogeneity of bacterial communities may occur. For this reason, in paper I: “Polyphasic characterization of the bacterial community in an urban soil profile with in situ and culture dependent methods” the bacterial community in a soil profile of water repellent soil from an urban site was investigated.

Changes in soil wettability represent a significant source of stress for microbial communities and alter the composition, growth and activity of soil microorganism communities (Denef *et al*., 2001). Effects of water stress due to water repellency were evaluated in paper II: “Effects of water repellency on bacterial communities of urban soil” where the bacterial community structure and their functional diversity was analyzed.

As reduced plant growth, poor germination of crops or pastures occur due to reduced water infiltration into the soil, many strategies have been employed to ameliorate water repellency. As water repellency can be e.g. caused by waxy coatings on soil particles, soil wettability could be improved by inoculation with wax degrading bacteria (McKenna *et al*., 2002; Roper, 2005). In paper III: “Influence of biofilms on the water repellency of urban soil samples” amelioration of soil wettability

by inoculating soil samples with bacterial isolates of different cell wall hydrophobicity was investigated.

Analyzing the bacterial consortium of wettable and water repellent soil samples no significant alterations due to water repellency could be detected in total bacterial cell counts. Total bacterial cells accounted for about 2 x 10^8 (g dry wt soil)$^{-1}$ both for wettable soil areas and water repellent soil samples. These observations fit well with findings of Bloem et al. (1992) who also did not reveal any changes in total cell numbers, when subjecting an arable soil to water stress by drying and rewetting. Also Griffiths et al. (2003) found no changes in total cell counts after drying and wetting of an upland pasture soil in microcosm experiments. They revealed total cell numbers consistently around 10^8 per g of soil. This is in agreement with our microcosm results, where the number of DAPI stained cells in dried and wetted soil samples also accounted for approximately 1 x 10^8 (g dry wt soil)$^{-1}$. However, a significant decrease in bacterial cell numbers of one order of magnitude was found within the soil profile. Total cells counts showed a decline from 2 x 10^8 (g dry wt soil)$^{-1}$ in the 15 cm soil layer to 1 x 10^8 (g dry wt soil)$^{-1}$ in the 30 cm layer and 4 x 10^7 (g dry wt soil)$^{-1}$ in the deepest soil layer (90 cm). A decrease of bacterial total cell counts with increasing soil depth has also been found by investigating a Podzol profile by Fritze et al. (2000) and in two Mollisol profiles analyzed by Fierer et al. (2003a).

Kell et al. (1998) postulated that one factor affecting the growth of bacteria on culture media can be water repellency, because culturability is related to the bacterial physiological status. This points to the assumption that cellular physiology altered by water stress can result in the loss of culturability. Hattori et al. (1997) as well as Bakken (1997) hypothesized that culturable bacteria represent the ecologically relevant portion of the soil bacterial community, which indicates that bacteria forming colonies on culture media display an important function in soil. Contrarily, it has been demonstrated by Felske et al. (1997) that uncultured bacteria were active in soil by applied the RT-PCR method in grassland soil. Furthermore, some groups of bacteria, (dwarft cells or ultramicrobacteria) which may also have significant functions in soil are not cultivable, as they can not form colonies on media (Nannipieri *et al*., 2003). The importance of microbial communities in soil functioning, such as transformations and mineralisation of natural compounds, has led to the development of methods for assessing microbial community characteristics including biomass, activity and

measures of functional, taxonomic and genetic diversity (Zwart *et al*., 1998; Griffiths *et al*., 2003).

The number of culturable bacteria (CFU) revealed a difference for wettable and water repellent soil samples and also showed a decline from topsoil to subsoil. The numbers determined were about 1 x 10^6 (g dry wt soil)$^{-1}$ for water repellent samples and about 4 x 10^6 (g dry wt soil)$^{-1}$ for wettable samples. Alteration in culturability of bacteria could also be found between the different soil layers as shown in paper I. CFU counts varied from 1 x 10^6 (g dry wt soil)$^{-1}$ in the upper soil horizon (15 cm) to 6 x 10^5 (g dry wt soil)$^{-1}$ in the subsoil (90 cm). Results of the microcosm experiments showed no differences in CFU after drying or wetting of the soil samples, as they were in the range of 10^5 (g dry wt soil)$^{-1}$ for water repellent and 10^6 (g dry wt soil)$^{-1}$ wettable samples. This is in contrast to microcosm experiments performed by Griffiths et al. (2003), who found a decrease in culturability of bacteria after drying of soils and a significant increase after wetting of soils.

Alterations of the bacterial consortium could be observed by generating a community level physiological profile based on patterns of substrate utilization. The functional diversity of the soil community, as displayed by the number of different substrates utilized, was altered by water repellency and soil depth. The number of catabolized substrates, was reduced in water repellent soil samples compared with wettable soil samples and declined with soil depth. As shown in paper II, the communities of wettable soil samples metabolized approximately 84% (26 from 31) of the different C sources present in BIOLOG EcoPlates®. In contrast, populations of water repellent soil samples degraded only 61% (19 of 31) of the offered substrates. Furthermore, a general trend that the capability of utilizing different substrates decreased with soil depth was observed (paper I). In a study investigating whether the BIOLOG assay reflects the catabolic potential of the used inoculum, Smalla et al. (1998) found the fast growing γ-*Proteobacteria* numerically dominant in the wells. They hypothesized that this group was primarily responsible for the observed substrate utilization patterns. In our study the decline in substrate utilization was also consistent with a decrease of γ-*Proteobacteria* from topsoil to subsoil.

The BIOLOG system generates a community level physiological profile based on patterns of substrate utilization, which can differentiate between communities. It has been used for analyzing microbial communities of different habitats in more than 200 different investigations since Garland and Mills introduced it 1991 into microbial

ecology studies (Garland & Mills, 1991). In this study, CFU counts and the BIOLOG technique also resulted in discrimination between water repellent and wettable soil samples, as well as between the different soil horizons.

The BIOLOG method is based on bacterial culturability and shows the catabolic potential of the culturable community, but does not reflect the in situ function of the microbial consortium. It has been demonstrated that the obtained metabolic profiles can reveal differences between microbial communities that come from different kinds of soil or soil fractions (Winding, 1994). This method has been considered to be a simple and fast technique. But, many technical problems can occur and it is still not easy to adapt the BIOLOG system to study soil microbial communities. As this culture dependent method relies on colour development after substrate utilization and growth, artifacts due to soil particles of the inoculum should be avoided. A common approach is to let the particles of the soil suspension settle down by sedimentation (Guckert *et al*., 1996; Mayr *et al*., 1999). Further limitations of this technique are selecting for only culturable organisms (Garland & Mills, 1991) and sensitivity to inoculum density (Garland, 1996). Another problem is that the number of bacteria needed to obtain reproducible results is not known. But it has been shown that the inoculum cell density has to be as high as possible, as the higher the cell density the greater the number of positive wells (Calbrix *et al*., 2005). However, Garland as well as Haack and colleagues found that colour development in BIOLOG wells was better linked to actively respiring cells than to total cell density (Haack *et al*., 1995; Garland, 1996). As colour development depends on both, number of cells and their activity, it reflects also the physiological state of the cells (Preston-Mafham *et al*., 2002). But species inactive or representing only a minor population in situ may have a competitive advantage in the BIOLOG well and the obtained metabolic fingerprints possibly overestimate the role of these species in situ (Smalla *et al*., 1998). However, Kennedy and Gewin (1997) also regarded the BIOLOG approach ecologically more relevant than taxonomic diversity approaches. Furthermore, the BIOLOG culture conditions are thought to have a harmful effect on the inoculated populations. The microbial diversity of the plates decreases during increasing incubation time (Heuer & Smalla, 1997) and changes in proportions of the microbial community during incubation have been observed (Smalla *et al*., 1998). Contrarily, in a study of Calbrix et al. (2005) who determined the effects of different conditions of extracting bacteria

from soil and incubation on microbial fingerprints no significant changes in the community occurred.

Nevertheless, for comparisons of bacterial communities of different sites, the BIOLOG technique provides a potential insight into the functional ability of the community (Preston-Mafham *et al*., 2002). Lawlor and co-workers considered the assessment of the bacterial potential activity by BIOLOG and CFU counts to be a suitable tool to measure the effect of water repellency on bacterial communities and not only the presence or absence of cells (Lawlor *et al*., 2000). The effect of soil moisture on the functional bacterial diversity in a Greek Mediterranean grassland (a lithic leptosol) has recently been investigated by Papatheodorou et al. (2004).

However, bearing in mind that the BIOLOG system is a very biased representation of the metabolic potential of the soil community, it is a useful tool for comparison of soil communities of two sites under culture conditions, as it provides insight in the potential degrading abilities. Differences may be apparent even if they are due to a part of the community in situ.

The observation that the total bacterial population seemed to be largely unaffected by water repellency and water stress was confirmed by DGGE analysis as shown in paper II and in microcosm experiments (Puttrich, 2005). Subjecting bacterial communities to water stress by drying and wetting of soil samples revealed no changes in the community structure obtained by DGGE. The obtained fingerprint after drying and wetting of the soil samples revealed different clusters with about 93% identity for dried samples and 98% for wetted samples, respectively. Furthermore, by PCR-DGGE fingerprints differentiation between soil samples of different water repellency was not possible as similarity analysis resulted in three different groups where community fingerprints of water repellent and wettable soil samples clustered differently (see paper II). The fingerprints indicated that to a large extent the community consists of stable dominant populations. Prominent bands of the total community were similar for most of the samples. Small variations between replicate samples were particularly present in the wettable soil sample lanes. This variability in microbial community composition of parallel samples could be explained by methodological biases as e.g. biases in PCR as described by von Wintzingerode (1997). Furthermore, preferential amplification of target DNA from some bacteria can occur and chimera molecules could be formed during the amplification process (Reysenbach *et al*., 1992). Another limitation of the DGGE approach is, that complete

separation of PCR products obtained from very complex mixtures of bacteria such as those which might be found in soil might be not possible (Torsvik *et al*., 1990). Likewise some of the variations between the profiles may be caused by different intensity of the staining, which was probably a result of different amounts of DNA in each lane. Furthermore, changes in band intensity can occur if the bands result from a cluster of populations with similar nucleotide sequence content that separate when exposed to different gradients. However, this technique has recently been successfully used in analyzing bacterial communities subjected to water stress (Griffiths *et al*., 2003), as they revealed no changes in DGGE analysis of the bacterial community related to moisture regimen.

The phylogenetic affiliations of the bacterial community were investigated by sequencing 16S rDNA fragments of individual DGGE bands. Nearly all the sequenced fragments showed highest similarity to 16S rDNA of uncultivated soil bacteria of five different phylotypes, which indicates that there are many yet-unknown microbes inhabiting the soil. In 10 cases where five clones were obtained from a single band, all five represented a different phylotype. This indicates that DNA fragments with different nucleotide sequence may have the same mobility in the polyacrylamid gel. Therefore, one band may not necessarily represent only one species (Gelsomino *et al*., 1999). Furthermore, one bacterial species may also give rise to multiple bands because of multiple 16S rRNA genes with slight differences (Gelsomino *et al*., 1999). Some bands contained multiple co-migrating sequences belonging to different bacterial species. In this study it has also been observed that bands of different mobility revealed the same closest relative. This is caused by sequence heterogeneities of 16S rDNA operons (Nübel *et al*., 1996; Klappenbach *et al*., 2000), where more than one fragment can be PCR amplified from one single bacterial strain. This can lead to an overestimate of the number of bacterial species/strains present in the community. Another limitation of the direct rRNA gene based approach is that sequence data do not give direct evidence of function of the organism from which the sequence has been derived.

As indicated by Dice correlation index, the differences in community structure between the two upper soil layers and the C-horizon were more pronounced than the spatial variability between wettable and water repellent soil samples. Soil water repellency did not allow distinction between the bacterial community fingerprints while

examination of vertical soil profiles revealed significantly different community patterns.

Nutrient availability is influenced by soil moisture and therefore differs between soil spots with different water repellency. The capability of the bacterial community to utilize different substrates varied between wettable and water repellent soil samples as well as between the soil horizons. Substrate utilization patterns changed within the soil profile as the number of degradable substrates declined with increasing soil depth. Changes in the metabolic potential of the bacterial community in the soil profile may be caused by moisture content or by the amount of organic matter. The effect of moisture content on bacterial populations was analyzed by investigating wettable and water repellent soil samples: differentiation among environments with distinct water repellency based on substrate utilization patterns was possible. As soil physico-chemical properties of wettable and water repellent spots were not different, alterations of the bacterial physiological status are only caused by water repellency.

Investigations of the soil profile with the ARDRA technique revealed different bacterial community patterns for the soil horizons. FISH analysis of the soil horizons with group specific probes resulted in differences in population structures and a varying dominance of different bacterial groups. The number of α- and β-*Proteobacteria* remained stable within the soil profile, whereas the number of γ-*Proteobacteria* showed a significant decline with increasing soil depth. The two upper soil layers displayed no significant variations in community structure and composition in contrast to the C-horizon. These differences are caused by differing physico-chemical soil properties and nutrient supply as the subsoil is characterized by a lower water and organic matter content. This fits well with the results of Tiedje et al. (2001) who explained a large microbial diversity in surface soil by the presence of a greater variety and content of organic compounds there than in deeper soil layers. The bacterial community inhabiting this deep soil horizon is able to utilize less of the offered substrates as shown by BIOLOG metabolic fingerprints. The reduced metabolic activity of this population could be confirmed by applying the FISH method where rRNA is used as target and the PAC method. To be detectable with FISH, soil microbes must have a sufficient content of ribosomes and possess cell walls sufficiently permeable to allow penetration of the probe (Amann *et al*., 1995). The hypothesis that detection using FISH is directly related to the metabolic state of the cells has seldom been assessed for natural communities. Kemp and Laroche (1993)

demonstrated in steady state cultures, that in this case FISH depends on the physiological state of the target cells, as fast growing or highly active cells tend to have more ribosomes (Bouvier & del Giorgio, 2003). But also cells with low activity may have rRNA at a level sufficient to yield a detectable fluorescent signal. It has been shown by Fukui et al. (1996) that the limit of detection of cells (after FISH with probe EUB338) was reached when the rRNA content of the cells was less than 8% of the rRNA content of growing cells. Kramer and Singelton (1992) e.g. concluded that 20% to 30% of the minimum value of rRNA still persists in starved cells. Also the changes in the relative abundance may represent changes in the population or in their RNA content. Unless sequenced genes are present in high copy numbers, they will not be detected by FISH. Another problem is that intrinsic differences in activity or rRNA content of phylogenetic groups could influence the capacity to detect cells. This relies on different copy numbers of the rRNA operon, leading to intrinsic differences in the number of potential targets. Nevertheless, there seems to be a relationship between bacterial metabolic rates and the capacity to detect the cells by the FISH method (Bouvier & del Giorgio, 2003). To overcome the problem of weak fluorescent signals due to low ribosome content the CARD-FISH method seems to be a powerful tool (Pernthaler *et al*., 2002). Catalyzed reporter deposition (CARD) in combination with FISH is an assay using a horseradish peroxidase (HRP) labeled oligonucleotide probe in combination with fluorochrome-labeled tyramides. The first experimental step is the permeabilization of the bacterial cell walls. In the second step, the hybridization, the HRP labeled probe binds to it's target site the rRNA in the bacterial cells. In the following third step, the tyramide signal amplification (TSA), numerous fluorescent molecules can be introduced at the hybridization site in situ. This approach results in greatly enhanced FISH sensitivity compared to the standard FISH with single fluorochrome.

In this study the bacterial community could be activated by applying the PAC method (Kalmbach *et al*., 1997) resulting in a larger number of FISH detectable cells as shown in paper I. The number of FISH-detectable *Eubacteria* nearly doubled after activation with glucose and yeast extract as substrate in topsoil and decreased with increasing depth. This suggests that deeper soil layers have a greater proportion of dormant populations or species incapable of growth on soil extract agar.

As no changes in community structure could be observed by DGGE fingerprints, whereas BIOLOG patterns revealed differences, water repellency may cause a shift

of the community from growth and production of biomass to physiological maintenance. Another reason may be the presence of a viable but non culturable slow growing population in water repellent soils that persists upon drying. These results lead to the assumption that the bacterial population is similar for all investigated soil spots but soil water repellency affects bacterial functional diversity. This results in uneven decomposition, degradation processes and nutrient availability and furthermore reduced plant growth. Bacteria may contribute to the wetting properties of soil samples by formation of biofilms. Bacterial EPS reveal strong water-holding properties (Flemming & Wingender, 2000) and therefore increases the water retention of clay minerals or sands. Different possibilities to reduce water repellency have been applied. Roper (2004) was able to reduce water repellency of a sandy soil by inoculating soil with wax-degrading bacteria. The ability of bacterial biofilms to hydrophilize or hydrophobize soil samples was examined in paper III. The results show that bacterial biofilms are able to affect surface characteristics and hydrophilize hydrophobic surfaces (paper III). Inoculating water repellent soil samples with hydrophilic bacterial isolates improved soil wettability and the inoculation of wettable soil samples with hydrophobic bacterial isolates reduced soil wettability.

5 Conclusion

The major conclusions of this study are:

1. Water repellency did not largely affect the bacterial population as water repellency specific bacterial patterns could not be detected by comparison of DGGE profiles.
2. Dominant bands of the total community assessed by DGGE were similar for all wettable and water repellent soil samples.
3. Many yet-unknown microbes inhabit the soil examined by sequencing of 16S rDNA fragments from DGGE gels.
4. Microbial functional diversity monitored by BIOLOG is altered by water repellency.
5. The bacterial communities of this urban soil seem to be adapted to different moisture conditions by regulating their physiological cell activity.
6. Variations in community structure as well as in community function and activity were observed within the soil profile.
7. Biofilm growth can have a pronounced effect on water repellency of soil samples as biofilms are able to hydrophilize or hydrophobize soil samples.

8. Bacterial biofilms show a promising potential for the amelioration of soil water repellency in the field.

Summary

Soil water repellency is a common phenomenon which occurs throughout the world and is influenced by physical, chemical and biological factors. It prevents water from infiltrating into the soil and influences soil properties. Changes in soil wettability may represent a significant source of stress for microbial communities and affect bacterial composition and soil microbial processes. Up till now, little is known about the effects of water repellency on soil bacterial communities and *vice versa*.

The aim of this study was to gain a more profound insight into the bacterial community structure of wettable and water repellent soils. The horizontal and vertical distribution of the bacterial population was analyzed with regard to soil water repellency. Furthermore, the molecular diversity and active bacterial population subjected to water stress was monitored in microcosm experiments. Finally, the effects of hydrophilic and hydrophobic bacterial biofilms on soil wettability were investigated.

For determination of alterations in the bacterial structure and their metabolic potential cultivation- and molecular methods were combined. The application of cultivation independent methods did not allow to distinguish between water repellent and wettable soil samples. Whereas determination of CFU and metabolic fingerprints resulted in differences between wettable and water repellent soil areas. Variations in community structure as well as in community function and activity were observed within the soil profile. These data indicated that water repellency did not have a significant effect on the total genetic diversity present but affected the physiological status, so that the bacteria capable of responding to laboratory culture methods were altered in activity without changes in phylogenetic distribution. Therefore, the bacterial communities of this urban soil seemed to be adapted to different moisture conditions by regulating their physiological cell activity. Microcosm experiments revealed no effects of water stress on the bacterial community, as no moisture related changes in the bacterial population were observed.

To gain insights how biofilms may affect surface properties of soil, soil samples were inoculated with bacteria of different cell surface hydrophobicity: *Bacillus sphaericus* (hydrophilic), *Variovorax paradoxus* (hydrophobic) and an α-*Proteobacterium* (hydrophobic). The results demonstrated the effect that bacterial biofilms can have on soil wettability, as biofilms were able to hydrophilize or hydrophobize soil samples.

Zusammenfassung

Nicht benetzbare Böden stellen ein allgemeines und weltweites Problem dar, welches durch physische, chemische und biologische Faktoren beeinflusst wird. Die Benetzungshemmung der Bodenoberfläche hat einen Einfluss auf die Wasserinfiltration und die räumliche Verteilung der Bodenfeuchte. Änderungen in der Benetzbarkeit des Bodens können einen bedeutenden Stressfaktor für bakterielle Bodengemeinschaften darstellen und die bakterielle Zusammensetzung sowie mikrobielle Prozesse im Boden beeinflussen. Über die Auswirkungen von nicht benetzbaren Böden auf die bakterielle Bodengemeinschaft und umgekehrt ist bisher nur wenig bekannt.

Ziel dieser Dissertation ist zum einen den Einfluss der Benetzungshemmung des Bodens auf die bakterielle Bodengemeinschaft zu ermitteln, sowie die Charakterisierung von Bakterien in einem Bodenprofil. Weiterhin sollte der Einfluss von Wasserstress auf die bakterielle Gemeinschaft durch Trocknung und Wiederbefeuchtung in Mikrokosmosversuchen untersucht werden. Letztlich wurde der Einfluss von hydrophilen und hydrophoben Biofilmen auf die Benetzbarkeit des Bodens ermittelt.

Um Änderungen in der bakteriellen Zusammensetzung und ihres physiologischen Potentials zu bestimmen, wurden kultivierungsabhängige und –unabhängige Methoden verwendet. Mit der Anwendung von kultivierungsunabhängigen Methoden war keine Unterscheidung von benetzbaren und nicht benetzbaren Bodenproben möglich. Die Erzeugung von metabolischen Fingerabdrücken, sowie die Bestimmung der Kolonie bildenden Einheiten zeigte jedoch Unterschiede zwischen benetzbaren und nicht benetzbaren Bodenbereichen. Innerhalb des Bodenprofils konnten Variationen in der Zusammensetzung und des physiologischen Potentials, sowie der Aktivität ermittelt werden. Diese Ergebnisse weisen darauf hin, dass die Benetzungshemmung von Böden keinen signifikanten Einfluss auf die gesamte genetische Diversität hat, den physiologischen Status der Gemeinschaft jedoch beeinflusst. Die Untersuchungen von Wasserstress in Mikrokosmosexperimenten zeigten keinen Einfluss auf die bakterielle Bodengemeinschaft, da keine feuchtigkeitsabhängigen Veränderungen beobachtet werden konnten. Daher scheinen die bakteriellen Gemeinschaften dieses urbanen Bodens an die unterschiedlichen Bodenfeuchten angepasst zu sein, indem sie ihre physiologische Zellaktivität regeln.

Um Einblicke zu gewinnen, wie Biofilme Oberflächeneigenschaften von Böden beeinflussen können, wurden Bodenproben mit Bakterien verschiedener Hydrophobizität der Zelloberfläche beimpft: *Bacillus sphaericus* (hydrophil), *Variovorax paradoxus* (hydrophob) und ein α- *Proteobacterium* (hydrophob). Die Ergebnisse zeigten, dass Biofilme einen Einfluss auf die Benetzbarkeit des Bodens haben können, da sie in der Lage sind Bodenproben zu hydrophilisieren bzw. zu hydrophobisieren.

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Paper I [1]

Polyphasic characterization of the bacterial community in an urban soil profile with in situ and culture dependent methods.

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Polyphasic characterization of the bacterial community in an urban soil profile with in situ and culture-dependent methods

Burga Braun, Uta Böckelmann *, Elisabeth Grohmann, Ulrich Szewzyk

Department of Environmental Microbiology, University of Technology, Berlin, Germany

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Abstract

Bacterial communities of urban soils have not been thoroughly investigated up to now. Therefore, soil samples from the urban park Tiergarten in the centre of Berlin were taken from a profile in 15, 30 and 90 cm depth. The total number of bacteria (4′,6-diamidino-2-phenylindole (DAPI) counts) as well as biomass declined one order of magnitude from topsoil to subsoil. Soil texture changed comparably and water content and amount of organic matter dropped 3–10-fold. The number of culturable bacteria (colony forming units = CFU) also decreased with increasing soil depth. Amplified ribosomal DNA restriction analysis (ARDRA) revealed similar bacterial communities in the two upper soil layers in contrast to the deepest layer. The number of bacterial cells which were detected with probe EUB338 in relation to total cell counts differed between 43 and 35% in the three soil layers. With the probe active count method (PAC) this number could be increased up to 72% of total cell counts in topsoil whereas activation of cells declined with increasing depth. In relation to total cell counts (DAPI) α-*Proteobacteria* and β-*Proteobacteria* are equally distributed in all three depths, whereas γ-*Proteobacteria* declined within the soil profile. With the BIOLOG system we observed the general trend that the capability of utilizing diverse substrates decreased with soil depth whereas a few substrates, such as Tween 40 and Tween 80 could be utilised by the bacteria of all soil depths.

Keywords: Soil profile; Bacterial community; FISH; ARDRA; PAC; BIOLOG; Urban soil

1. Introduction

Soil microbial diversity exceeds that of aquatic environments, and is a great resource for biotechnological exploration of novel organisms, products and processes (Torsvik and Ovreas, 2002). Soil prokaryote communities represent microbial assemblages with wide physiological heterogeneity at small spatial scales (Whiteley et al., 2003). Over the past 10 years, the approach to analyse soil microbial communities has changed dramatically. Many new methods and approaches are now available allowing soil microbiologists to gain access to more microorganisms residing in soil and allowing better assessment of microbial diversity. Traditionally, the analysis of soil microbial communities has relied on culturing

* Corresponding author at: Technische Universität Berlin, Fachgebiet Umweltmikrobiologie, Sekretariat FR 1-2, Franklinstraße 29, D-10587 Berlin, Germany. Tel.: +49 30 314 21721; fax: +49 30 314 73673.
E-mail address: uta.boeckelmann@tu-berlin.de (U. Böckelmann).

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techniques. However, only a small fraction, less than 0.1% of the soil microbial community has been accessible with this approach (Torsvik et al., 1990; Atlas and Bartha, 1998; Hill et al., 2000). To overcome this problem there have been recent attempts to develop new culture media to maximize the recovery of diverse microbial groups (Balestra and Misaghi, 1997; Mitsui et al., 1997). Nevertheless, culture-independent techniques for community analysis become widely applied methods to identify bacteria within their natural environment. These in situ methods mainly detect nucleic acids as important cell component molecules. Most useful is the determination of the sequence of 16S ribosomal RNA genes as described by Ward et al. (1992).

Polymerase chain reaction (PCR)-based fingerprinting techniques as well as phospholipid fatty acid (PLFA) analysis, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and amplified ribosomal DNA restriction analysis (ARDRA) provide comprehensive information on the species composition (Torsvik and Ovreas, 2002). ARDRA has often been applied to analyse soil isolates (e.g. Cheneby et al., 2000; Lucas et al., 2003; Nazaret et al., 2003) but is also a valuable tool for analysing microbial community structures (Massol-Deya et al., 1995; Heyndrickx et al., 1996). The latter is termed ‘‘community ARDRA’’ since the bands on the gels should reflect the population of all restriction fragments for at least the most abundant members in the community (Massol-Deya et al., 1995). Smit et al. (1997) used ARDRA for assessing the effect of copper contamination on the microbial community and followed the seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field (Smit et al., 2001). Ovreas and Torsvik (1998) investigated the bacterial population of two different agricultural soils combining ARDRA and DGGE analysis. Herbicide-induced bacterial community changes of a clay soil were examined with ARDRA by Crecchio et al. (2001). The relationship between plant and soil microbial communities along a successional gradient in a chalk grassland in north-western France was recently analysed by Chabrerie et al. (2003) using ARDRA technique.

Fluorescent in situ hybridization (FISH) can be used to visualize soil microorganisms that have not yet been cultured, and is useful for studying the distribution of microorganisms, e.g. specific detection of phylogenetic groups throughout diverse habitats (Amann et al., 1995). In order to understand concepts of functionality and redundancy in soil ecology, and how they are linked to soil processes, determination of the active populations within a sample must be a key requirement. Since the signal intensity obtained by hybridization with rRNA-targeted probes depends on the content of ribosomes, FISH gives indications of the physiological state of the cells (Moller et al., 1996). Consequently, Christensen et al. (1999) quantified the active soil bacteria in a sandy loamy soil with a FISH technique based on binding of a rhodamine-labelled oligonucleotide probe to 16S rRNA.

To investigate the catabolic potential of active bacterial soil communities the so called community-level physiological profiling has been demonstrated to be effective at observing spatial changes in microbial communities (Staddon et al., 1997; Buyer et al., 2002). Garland and colleagues (1991) were the first who used the BIOLOG system to characterize microbial communities in soil. The correlation between soil type and active bacterial consortia of arable soils was recently investigated with BIOLOG by Girvan et al. (2003).

Little is known about the nature of the microbial communities found throughout the soil profile. Most studies in soil microbiology have focused exclusively on the surface 25 cm of soil where the density of microorganisms is highest. However, soil profiles are often many meters deep and large numbers of microorganisms reside in subsurface horizons (Blume et al., 2002). Using PFLA analysis Fierer et al. (2003) examined the variations in microbial community composition from the soil surface down to 2 m in depth from two unsaturated Mollisol profiles near Santa Barbara. Both, microbial biomass and activity throughout a forest soil profile were examined by Agnelli et al. (2004) assessing changes with increasing depth.

In the context of an interdisciplinary research group we investigated soil samples which were collected from an urban site. In a polyphasic approach soil chemical, geophysical and biological data, obtained by the different subgroups should be combined to elucidate the dynamics of water and materials. Our aim was to characterize the bacterial communities

within the three soil horizons. We combined culture-dependent and molecular techniques such as ARDRA analysis, FISH and BIOLOG.

2. Material and methods

2.1. Soil sampling and soil characteristics

The experimental sites are located in the urban park Tiergarten in the center of Berlin, Germany (52°30′N and 13°21′E). The sampling site is located on a sunbathing area, which is marked by anthropogenic influences, like fertilization, sprinkling and the soil is compacted due to mowing. The site is characterized by a small-scale heterogeneity due to different anthropogenic substances as pollutants. Bulk soil samples were collected from a medium-sized fluvial sandy soil, with a thick humus layer, which can reach a height up to 40 cm.

The yearly rainfall of about 580 mm is evenly distributed. The climatic water balance becomes negative from April to September. The daily mean temperature at the sampling time was about 0.5 °C and the humidity about 42%.

Three separate, randomly chosen plots (0.8 m × 0.8 m) were selected within the field. Within each plot three samples from each soil horizon were taken. For the enumeration of total cell counts the samples were taken in seven parallels. The soil samples collected in April 2003 derived from the Ah-horizon (15 cm depth), B-horizon (30 cm depth) and C-horizon (90 cm depth). The samples were randomly taken using a soil corer (3 cm × 100 cm) hammered into the soil to a depth of 100 cm. Twenty grams of each sample were homogenized and subsamples of 5 g were taken for analysis. The pH was determined in $CaCl_2$ (0.01 M) (DIN ISO 10390), soil/$CaCl_2$ solution ratio 1:2.5 and in H_2O (DIN ISO 38404). Organic carbon content was measured by drying the sample at 550 °C for 5 h following DIN EN 12879. Water content was defined according to DIN EN 12880.

2.2. Total cell counts and colony forming units (CFU)

Total cell counts of soil bacteria were determined with 4′,6-diamidino-2-phenylindole (DAPI). Soil samples (10 mg) were taken from each soil layer in seven parallels. The samples were extracted according to Böckelmann et al. (2003) and 320 μl of the fixed supernatant were incubated with 80 μl DAPI (10 μg ml^{-1}) for 20 min in the dark. Then the samples were collected by filtration onto a 0.2 μm pore-size black polycarbonate filter (diameter 24 mm; Millipore, Billerica, USA). This filter was mounted on a glass slide and examined under a fluorescence microscope (Zeiss Axioskop, Oberkochen, Germany). At least 20 microscopic fields (100 μm × 100 μm) were chosen randomly and a minimum of 1000 cells was enumerated microscopically. Total cell counts were calculated for 1 g dried soil. Dry soil mass was determined as described by Öhlinger (1993).

For the analysis of microbial communities in soil we used several culture media to increase the culturability of soil bacteria. For determination of colony forming units, 10 mg of soil were shaken in sodium pyrophosphate buffer ($Na_2HPO_4 \times 2H_2O$ 4.0 g; $NaH_2PO_4 \times H_2O$ 0.22 g; aqua dest. add to 200 ml; pH 6.0 (Trolldenier, 1993)) for 45 min to detach the bacteria from soil particles. Serial dilutions of the supernatant (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) were plated (3× each) on four different media which were as follows: TSA medium (Becton Dickinson, New York, USA), GSP medium (starch 20.00 g; agar 15.00 g; sodium glutamate 10.00 g; K_2HPO_4 2.00 g; $MgSO_4 \times 7H_2O$ 0.5 g; phenol red 0.36 g; aqua dest. 1.0 l; pH 7.2), CF medium (casitone 3.0 g; $CaCl_2 \times H_2O$ 1.36 g; yeast extract 1.0 g; agar 15.0 g; aqua dest. 1.0 l) and soil agar (supernatant of 400.0 g soil sterilized in 1.0 l H_2O; 15.0 g agar, pH 7.0). The colony forming units were enumerated after 1–3 days of incubation at 23 °C in the dark. CFU were calculated for 1 g dry weight of soil.

2.3. DNA extraction and PCR amplification of 16S rRNA genes

Total community DNA was directly extracted from soil by using the FastDNA® Spin Kit for soil as described by the manufacturer (Qbiogene, Carlsbad, California, USA).

PCR was performed with the personal cycler (Biometra, Göttingen, Germany). Amplification of DNA from environmental samples of 15 and 35 cm soil depth was peformed in a final volume of 50 μl

containing 2 U DyNAzyme® EXT DNA Polymerase (Finnzymes, Espoo, Finland), 1× DyNAzyme® EXT reaction buffer supplied by the manufacturer, 160 nM of each primer, 200 μM of each desoxynucleotide and 100–200 ng of purified DNA extracted from soil. For the amplification of DNA from 90 cm soil depth, 200 nM of each primer and 200 ng of purified DNA were employed. To the reaction mixture 2% DMSO was added.

Primer pair 63f (Marchesi et al., 1998) and 1525r (Lane, 1991) was chosen for amplification of the 16S rRNA genes. PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 10 cycles each consisting of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min, followed by 35 cycles 95 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min plus 10 s per cycle, terminated by a final extension step at 72 °C for 7 min.

2.4. Amplified ribosomal DNA restriction analysis

The ARDRA technique was used to get community profiles for comparing the bacterial consortia within the soil profile. The PCR-amplified DNA was purified with MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and digested at 37 °C for at least 5 h in a final volume of 50 μl with the enzymes *Hae* III and *Hin* fI (Fermentas, St. Leon-Rot, Germany). The 16S rDNA restriction fragments were purified with the MinElute PCR Purification Kit (Qiagen) and eluted to a final volume of 8 μl. Separation of the digested PCR fragments was performed by polyacrylamide gel electrophoresis (PAGE) with the Multiphor® II system (Pharmacia Biotech, Uppsala, Sweden). Electrophoresis was carried out on 12.5% Multiphor II Precast gels (Pharmacia Biotech) for 90 min at 600 V, 50 mA, 20 W and 15 °C. The DNA was stained according to the silver staining procedure described by Bassam et al. (1991).

2.5. Fluorescent in situ hybridization of soil samples

The fluorescent in situ hybridization technique has been used to study the distribution of different microbial groups within the three different soil layers.

Five milligrams of freshly collected soil were extracted as described elsewhere (Böckelmann et al., 2003). After 4 h of fixation, each sample was shortly shaken by hand and rested for 10 min for settling of soil particles. The supernatant was transferred into a fresh tube and centrifuged (13,000 × *g*, 5 min). The precipitate was resuspended in 2 ml phosphate-buffered saline/ethanol. An aliquot of 500 μl was applied to hybridization. A dehydration process was used where the pellet was resuspended in 50, 80 and 96% ethanol after centrifugation (13,000 × *g*, 5 min). The pellets were then air dried to evaporate the ethanol and resuspended in 300 μl of hybridization solution (0.9 M NaCl, 20 mM Tris/HCl (pH 8.0), 0.01% SDS) containing 35% formamide (Roth, Karlsruhe, Germany). The respective indocarbocyanine (cy3)-labelled probes EUB338 (Amann et al., 1990) and ALF1b (Manz et al., 1992) were added to each sample with a concentration of 4.5 ng μl^{-1}. Hybridization with cy3-labelled BETA42a and GAM42 (Manz et al., 1992) probes was carried out with the competitive unlabelled probes GAM42 and BETA42a (Manz et al., 1992). Hybridization took place at 46 °C with continuous rotation for 4 h. Then the samples were washed with 500 μl washing solution (20 mM Tris/HCl, 0.01% SDS, 88 mM NaCl) for 20 min at 46 °C. The washing solution was replaced by 500 μl PBS buffer containing 1 μg DAPI and incubated for 10 min in the dark. Then the samples were collected by filtration onto a 0.2 μm pore-size black polycarbonate filter (diameter 24 mm; Millipore). The filter was mounted on a glass slide and examined under a fluorescence microscope (Zeiss Axioskop).

2.6. Probe active counts (PAC) of soil bacteria

The determination of potentially active cells was performed by the probe active count method developed by Kalmbach et al. (1997). The antibiotic pipemidic acid was used as gyrase inhibitor to prevent cell division. The performance of pipemidic acid was monitored in all activation experiments by enumeration of total cell counts before and after activation. Hundred milligrams of freshly collected soil were incubated with pipemidic acid (Sigma, Deisenhofen, Germany) at a final concentration of 10 mg l^{-1} in 30 ml of 0.5× R2A medium (yeast extract 0.5 g; proteose peptone 0.5 g; casamino acids 0.5 g; glucose 0.5 g; soluble starch 0.5 g; sodium pyruvate 0.3 g, K_2HPO_4 0.3 g; $MgSO_4 \times 7H_2O$ 0.05 g; distilled water 1000 ml) for 8 and 16 h. The soil samples were

extracted as described by Böckelmann et al. (2003). The fixed supernatant was shortly shaken by hand and rested for 10 min for settling of soil particles. The supernatant was transferred into a fresh tube and centrifuged (13,000 × *g*, 5 min). The precipitate was resuspended in 2 ml PBS/EtOH. The enumeration of metabolic active cells was performed after in situ hybridization using the eubacterial probe EUB338 (Amann et al., 1990) and DAPI staining as described above. Then the samples were collected by filtration onto a 0.2 μm pore-size black polycarbonate filter (diameter 24 mm; Millipore). The filter was mounted on a glass slide and examined under a fluorescence microscope (Zeiss Axioskop).

2.7. Substrate utilisation analysis

According to Griffith et al. (2003) 9 ml of a 2% (w/v) soil suspension were prepared and washed twice by dilution to 50 ml in sterile PBS, mixing and centrifugation for 5 min at 4000 × *g*. Following the cell washes, pelleted cells were resuspended in 20 ml of sterile PBS and 150 μl aliquots corresponding to 10^3 CFU per well (enumerated by plate counts, Engelen et al., 1998) were dispensed into each of the 96 wells of the BIOLOG EcoPlate® (Oxoid, Hampshire, England). Plates (3 × 3 parallels of each soil horizon) were incubated at 28 °C and manually scored after 8 days to determine the number of substrates utilized after 8 days. For each reading, a well was scored as positive based on visual inspection of color change.

2.8. Statistical evaluation

The data were analysed with the StatsDirect software package (CamCode, Ashwell, Herts, UK) using one-way ANOVA and the Tuckey–Kramer test. The BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyse the bacterial community fingerprints of the ARDRA gels with a slight modification of normalization settings. Background subtraction was applied and the band-based Dice correlation index was used to calculate the similarity matrix with a tolerance and optimization of 1%. Dice coefficient; $S_D = (2n_{AB})/(n_A + n_B)$, where n_A is the total number of bands in lane A, n_B is the total number of bands in lane B, and n_{AB} is the number of bands common to lane A and B. The clustering of patterns was calculated using the unweighted-pair group method using average linkages (UPGMA).

3. Results

3.1. Soil characteristics

Soil samples were taken from the urban park Tiergarten, which is located in the centre of Berlin. It was founded in 1833, the total area is 780 ha. The general characteristics of this anthropogenically affected sandy soil are shown in Table 1. With increasing depth water content dropped up to one-third and organic matter content up to one-tenth, whereas the pH declined only weakly. Soil texture and particle size differed between the deepest and the two upper layers. The amount of silt and clay dropped up to one-fourth in the C-horizon, whereas the sand fraction increased up to 96.7%, compared with 85.5% in the Ah-horizon.

3.2. Total cell counts and colony forming units

The bacterial total cell counts as well as the bacterial biomass decreased significantly ($P < 0.0002$) with increasing soil depth. The amount of culturable bacteria (CFU) on all media showed also a decline from topsoil to subsoil. The bacterial numbers declined one order of magnitude on CF and soil media, 1.5 orders of magnitude on GSP medium but only slightly on TSA medium (Table 2). Phylogenetic characterization of the isolated

Table 1
General characteristics of three soil horizons of the Tiergarten park in April 2003

Sampling depth (cm)	Water content (%)	pH (H_2O)	Organic matter (mass%)	Textural classification (%)		
				Clay <0.002 mm	Silt 0.002–0.063 mm	Sand 0.063–2.00 mm
15	9.59	5.98	3.79	4.8	9.7	85.5
30	7.37	5.76	2.51	4.9	9.1	86.0
90	3.06	5.19	0.36	0.9	2.4	96.7

Table 2
Biological features of three soil horizons of the Tiergarten park in April 2003

Sampling depth (cm)	TSA medium CFU/g soil	GSP medium CFU/g soil	CF medium CFU/g soil	Soil medium CFU/g soil	Total bacterial cell counts/g soil (DAPI)	Bacterial biomass[a] (g/g soil)
15	9.9×10^5	1.3×10^6	1.1×10^6	2.5×10^6	2.4×10^8	3.1×10^{-4}
30	4.6×10^5	6.2×10^5	4.5×10^5	1.2×10^6	1.1×10^8	1.5×10^{-4}
90	6.0×10^5	5.2×10^4	2.2×10^5	1.0×10^5	4.0×10^7	5.2×10^{-5}

[a] Bacterial biomass was determined as described by Dunger and Fiedler (1997).

bacteria with FISH revealed α-*Proteobacteria* and β-*Proteobacteria* as abundant in the 30 cm soil layer. In contrast to this, most γ-*Proteobacteria* isolates were obtained from topsoil and subsoil. Members of the *Cytophaga–Flavobacteria* and high GC group were not represented within the isolated bacteria (data not shown).

3.3. Amplified ribosomal DNA restriction analysis

To study the vertical shift in the composition of the bacterial soil population, ARDRA patterns of three soil layers were analysed. The whole community ARDRA pattern of the three soil horizons showed differences in the microbial consortia (Fig. 1a). A variety of different bands indicated a high bacterial diversity in the 15 and 30 cm soil layer. Only small community shifts (statistically not significant) were detected in these upper soil horizons. In both layers the ARDRA pattern formed a separate cluster when UPGMA was used to create a dendrogram describing the similarities between these patterns (Fig. 1b). In contrast to this, the number of bands declined in the deepest soil layer and the pattern appeared to be totally different (statistically significant), which indicates a distinct shift in the bacterial community. This was shown by UPGMA, whereby the subsoil pattern formed an own cluster. Some new bands were present within the soil profile, whereas some of the bands, characteristic of the carbone-rich upper horizons, disappeared in the pattern of the deepest soil layer.

3.4. Fluorescent in situ hybridization of soil samples

As demonstrated in Table 3 total cell counts as well as *Eubacteria* including α-, β- and γ-subclasses of *Proteobacteria* declined with increasing depth. Eubacterial counts declined significantly from 102×10^6 to 14×10^6 ($P < 0.0001$). The overall counts of α-*Proteobacteria* decreased significantly from 16×10^6 to 3×10^6 ($P < 0.0001$). The majority of the cells were coccoid or small rods. The total counts of γ-*Proteobacteria* showed the same trend, declining from 22×10^6 to 2×10^6 with increasing depth ($P < 0.0001$). The predominant cell morphology were tiny rod-shaped bacteria. In our study the overall counts of β-*Proteobacteria* accounted for 14×10^6 to 2×10^6. Microscopical observations revealed tiny rods single or in pairs but no chains of *Sphaerotilus*- or *Leptotrix*-like cells were detected.

3.5. Probe active counts

The number of bacterial cells detected with probe EUB338 in comparison to total cell counts ranged from 43% in topsoil to 38% in the 30 cm soil layer to 35% in subsoil. The determination of potentially active cells was performed using the probe active count method as described in Section 2.6. Activation was carried out by addition of yeast extract and glucose. Pipemidic acid was used as gyrase inhibitor to prevent cell division. As shown in Fig. 2 small and tiny cells (Fig. 2a) turned to large elongated cells after activation (Fig. 2b). Total cell numbers did not change significantly ($P > 0.0002$) in any of the experiments, clearly indicating effective suppression of cell division by this antibiotic. The amount of FISH-detectable

Table 3
Counts [$\times 10^6$ (g soil, dry wt.)$^{-1}$] of soil samples obtained after fluorescence in situ hybridisation with group-specific probes and DAPI staining

Sampling depth (cm)	Probes				
	DAPI	Alf1b	Bet42a	Gam42a	EUB338
15	242 ± 31	16 ± 6	14 ± 1	22 ± 1	102 ± 22
30	113 ± 15	7 ± 2	7 ± 2	8 ± 4	43 ± 16
90	40 ± 7	3 ± 1	2 ± 1	2 ± 1	14 ± 2

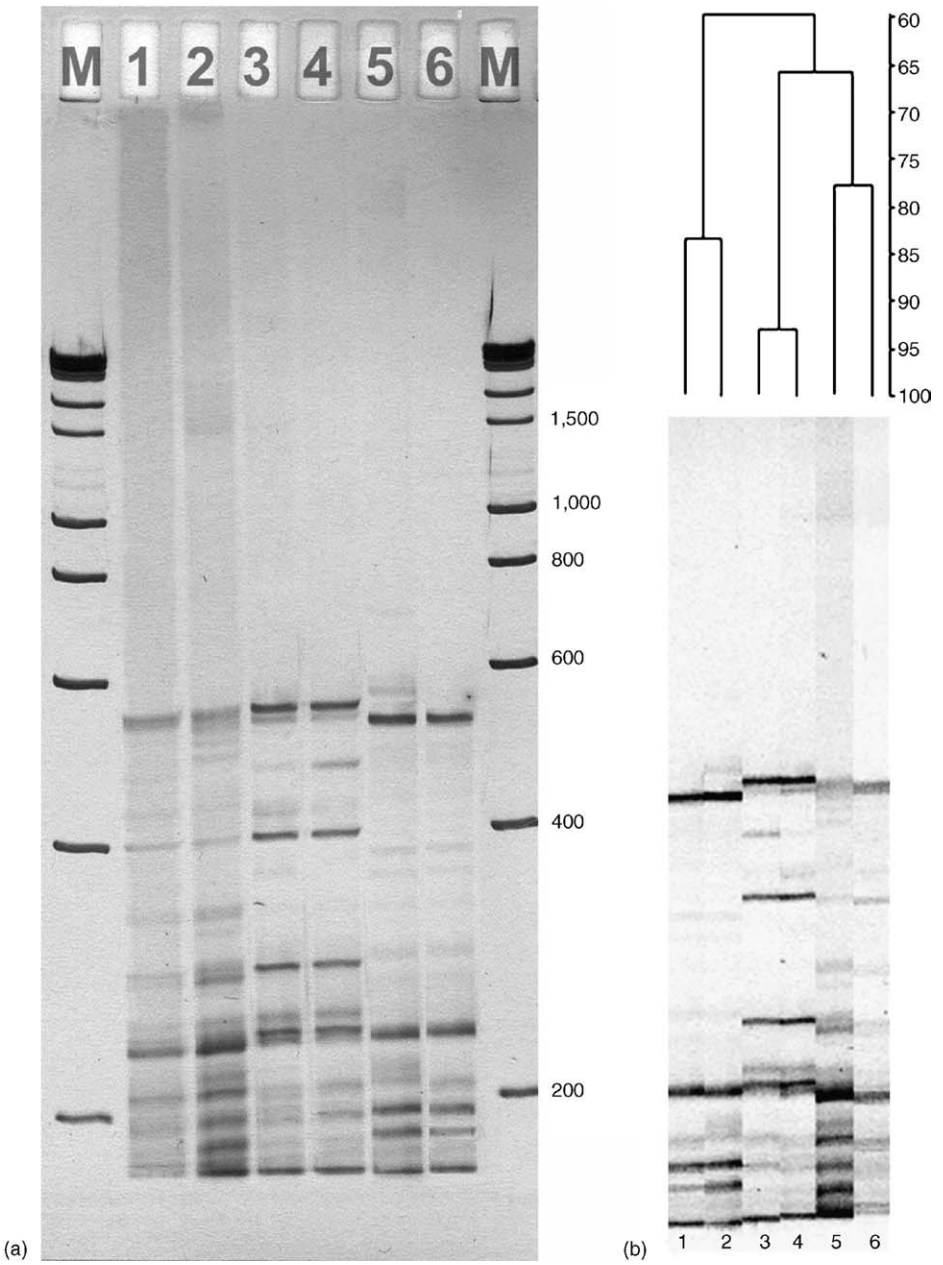

Fig. 1. (a) Whole community ARDRA pattern of two exemplary soil profiles from three different horizons each generated with *Hin* fI and *Hae* III. Lane M: marker, molecular weight in base pairs (Hyperladder I, Bioline, Luckenwalde, Germany); lane 1: 15 cm sample A; lane 2: 15 cm sample B; lane 3: 30 cm sample A; lane 4: 30 cm sample B; lane 5: 90 cm sample A; lane 6: 90 cm sample B. (b) ARDRA analysis of the 16S rRNA gene amplified with PCR and evaluated by digital image analysis. Bacterial soil communities within the soil profile. Similarity was calculated with band-based Dice correlation index as described in Section 2.8. 1–6: samples as described in the legend of (a). Scale bar indicates similarity [%].

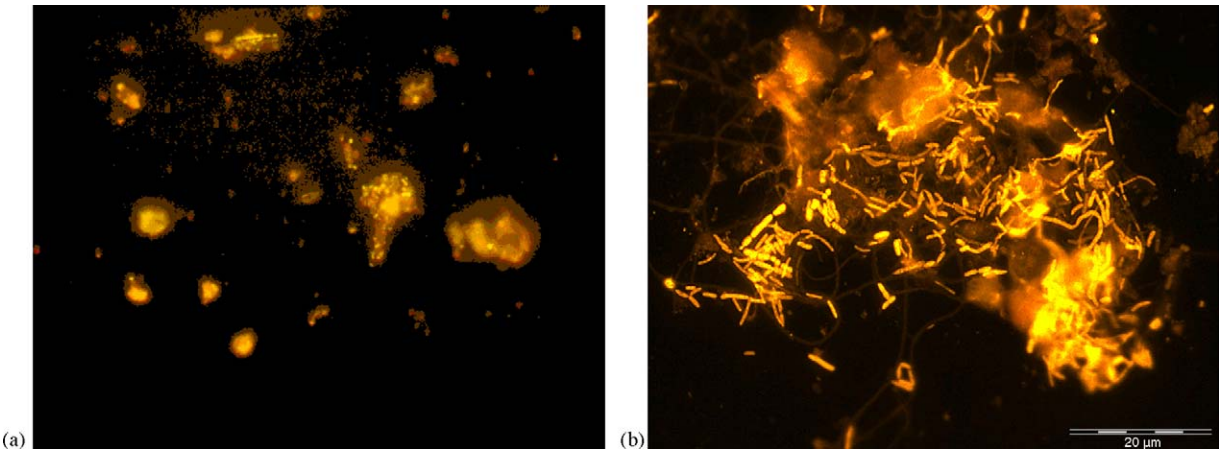

Fig. 2. Epifluorescence micrographs of bacteria in a soil sample detected by FISH with Cy3-labeled probe EUB338. (a) Small and tiny cells before activation. (b) Elongated cells after activation. All pictures were done at a magnification of 1000×. The scale bar equals 20 μm.

cells (EUB338) increased from 43 to 72% of total cell counts in topsoil whereas activation of cells was less with increasing depth. The number of cells yielding a bright hybridization signal with the EUB338 probe increased in the 30 cm soil layer from 38 up to 50% and activation of cells in the subsoil increased the signal from 35 up to 40%.

3.6. Physiological potential of the bacterial soil community

The physiological potential of the bacterial soil community was analysed with BIOLOG EcoPlate® (Fig. 3). The capability of utilizing different substrates decreased with soil depth. Carbohydrates served as a suitable substrate for the bacterial communities in the two upper soil layers, whereas the subsoil bacteria degraded only a minor part of the offered carbohydrates. Interestingly, the bacterial consortia of all soil depths failed to degrade glucose-1-posphate. The utilization of carboxy acids also declined with increasing soil depth. The bacterial community of the Ah-horizon utilized all offered carboxy acids, whereas the bacteria of the C-horizon degraded only a few. Aminoacids seemed to be the preferred substrate for topsoil microorganisms, but not for the bacterial communities of the other soil layers. The polymer glycogen and the polyoxyethylene-sorbitanes Tween 40 and Tween 80 served as good nutrients for the bacteria of all soil depths. In contrast to this α-cyclodextrin was non-degradable for all bacteria. The offered ester and amines were utilized by the bacterial communities of the two upper soil layers but were less suited for the bacteria of the subsoil. Moreover the bacterial consortium of the subsoil failed to degrade putrescine, and DL-α-glycerol phosphate was not utilised by any population of all soil horizons.

4. Discussion

4.1. Urban site characterization

Urban soil development is not caused by natural weathering. Therefore, in agreement with Craul (1992) and Lorenz et al. (2006) urban soils are characterized as a mixture of materials transformed strongly by anthropogenic activity. The special features of the urban park Tiergarten in the centre of Berlin are that until the 18th century it was a forest, which was then converted to a park. In the second world war the park was destroyed and used as agricultural area. During the rearrangement in the postwar period building rubber was used for landscape gardening. In addition, our sampling site was repeatedly refilled with compost rich in mould.

4.2. Bacterial soil communities are dependent on soil conditions

Bacterial communities of different soil horizons were analyzed with various methods providing information on different levels.

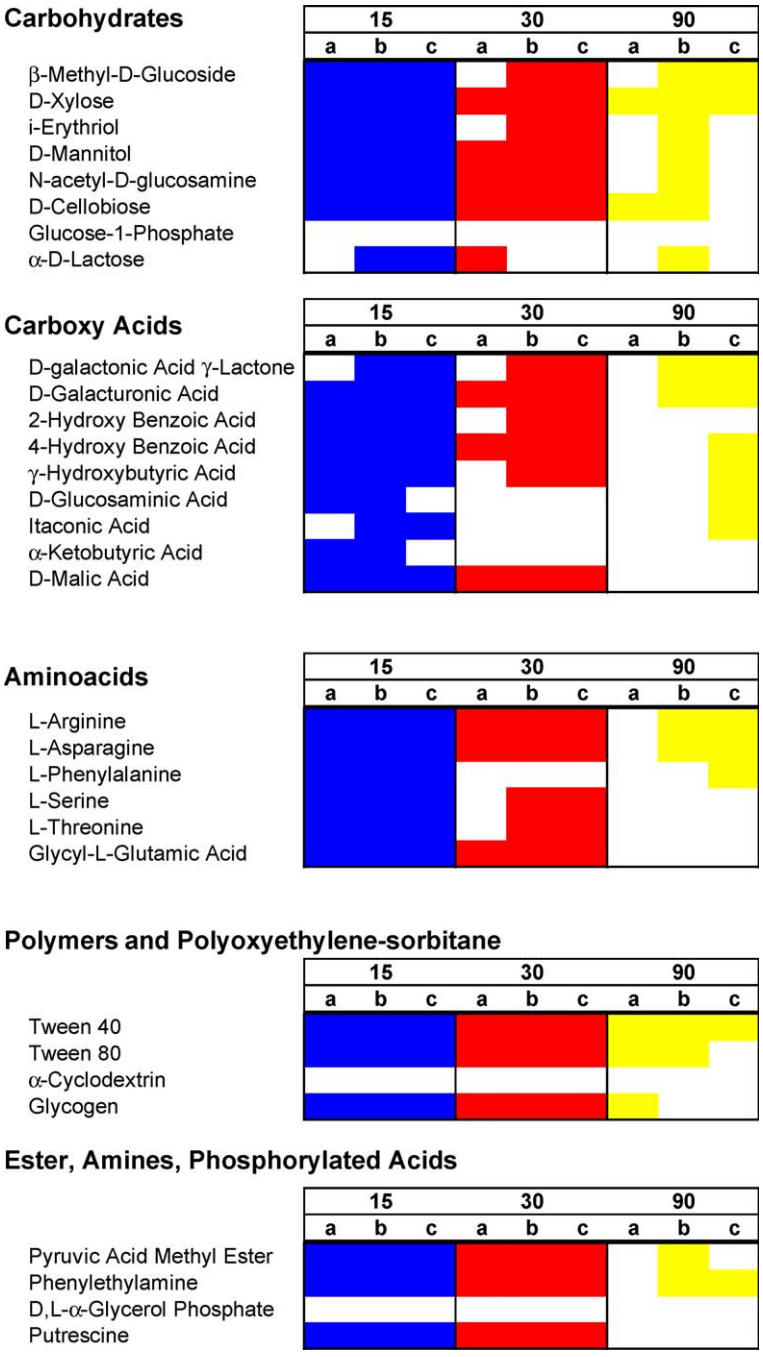

Fig. 3. BIOLOG data of the bacterial soil community of three different soil horizons. Soil depth 15 cm (blue), 30 cm (red) and 90 cm (yellow); a, b, c indicate parallel samples.

The decline of bacterial total cell counts and bacterial biomass with increasing soil depth is in good agreement with the results of Fierer et al. (2003) and Fritze et al. (2000). Water and especially organic matter content dropped strongly from topsoil to subsoil and might be the cause for these observations. According to Agnelli et al. (2002), Taylor et al. (2002), Fierer et al. (2003) the scarce availability of organic matter in the deeper soil horizon can be considered as the main factor controlling structure and activity of the microbial communities. In contrast to this, other authors regard the soil particle size as the highest impact on microbial diversity and community structure (Sessitsch et al., 2001). This also fits with our results as soil texture changed strongly with increasing depth. Obviously, the pH seemed to have no impact on the investigated bacterial population. Although the pH remained nearly stable, the microbial community changed.

ARDRA analysis revealed a complex banding pattern in the upper two soil layers. Contrarily, ARDRA analysis of the subsoil showed a less diverse pattern. These bacterial community composition changes within the soil profile agree with the overall features of the soil (Table 1) and the results of Fritze et al. (2000), Griffith et al. (2002), Fierer et al. (2003). These authors used the PLFA technique to describe differences between soil layers (Fritze et al., 2000; Fierer et al., 2003). They detected significant differences between the humus layer and the C-horizon, but only small changes between the two upper soil layers. Our results show that ARDRA is a useful tool for community analysis if the community harbors dominant members. The ARDRA pattern in Fig. 1 reveals a clear pattern of distinct bands. In non-dominant populations too many bands are generated causing difficulties in their analyses.

We performed FISH analysis with a variety of oligonucleotide probes. The bacterial cell numbers detected with EUB338 differed between 43 and 35% of total cell counts in all three soil layers. This is in contrast to other environmental habitats, e.g. activated sludge, marine- and freshwater environments where EUB counts accounted up to 80% of total cell counts (e.g. Böckelmann et al., 2000). But the results fit well with other microbial soil studies in which EUB counts formed less than 50% of total cell counts. Zarda et al. (1997) detected 36% of DAPI-stained cells with probe EUB, Chatzinotas et al. (1998) 40% and Haruta et al. (2002) 48%.

In the FISH analysis, we focused on the main phylogenetic groups of α-, β- and γ-*Proteobacteria* within the *Eubacteria*, because (i) they represent a major fraction of soil bacteria (Kent and Triplett, 2002) and (ii) members of the *Cytophaga–Flavobacteria* cluster, high GC group and *Planctomycetes* were only detected in negligible numbers (<1.0% of total cell counts) in our soil samples. The overall number of α-*Proteobacteria*, decreased with increasing depth ($P < 0.0001$). As total cell counts also declined to the same extent from topsoil to subsoil this means that the amount of α-*Proteobacteria* remained stable within the soil profile. They accounted for $6 \pm 2\%$ of DAPI-stained cells. This fits with the results of Zarda et al. (1997) and Morgan and Dow (1985). The latter regarded this class as ''oligotrophic'' showing modest growth at very low carbon concentrations. Moreover Sessitsch et al. (2001) showed that large particles, such as sand were dominated by bacteria belonging to the α-*Proteobacteria*. β-*Proteobacteria* dominate bacterial communities of freshwater environments (Zwart et al., 1998) but are not prevalent in soil samples (Zarda et al., 1997; Haruta et al., 2002; Torsvik and Ovreas, 2002). In this study β-*Proteobacteria* showed the same trend as the α-*Proteobacteria*. They accounted for $6 \pm 1\%$ in the upper soil layer to $5 \pm 1\%$ in the deepest soil horizon compared to DAPI.

The overall number of γ-*Proteobacteria* as well as the percentage of this group in relation to the decreasing DAPI counts showed a decline with increasing depth ($P < 0.0001$). They accounted for $9 \pm 1\%$ in the topsoil to $4 \pm 2\%$ in the subsoil. γ-*Proteobacteria* from various environmental habitats were preferentially isolated on rich nutrient media (e.g. Wagner et al., 1994). In agreement with this our results suggest that soil with a high content of readily available nutrients (upper soil layer) shows positive selection for this phylum.

4.3. Physiological potential of the bacterial soil community

The reason for the overall low number of FISH-detectable cells in soil might be due to the poor availability of nutrients in soil and therefore the majority of microorganisms might be dormant or

quiescent (Hahn et al., 1992; Fischer et al., 1995). Cells were generally smaller and cell walls relatively thick under these conditions (Fischer et al., 1995). This fits well with our microscopic observation of large amounts of very tiny cells in all soil layers in contrast to high cell morphological diversity of bacteria in, e.g. activated sludge or lotic aggregates. To be detectable with FISH soil microbes must be metabolically active and possess cell walls sufficiently permeable to allow penetration of the probe (Amann et al., 1995). To address this problem, we applied the PAC-method (adding simultaneously nutrients to stimulate microbial activity and pipemidic acid to prevent cell division). We could show that the amount of FISH-detectable cells (EUB338) increased up to 72% of DAPI counts. This might indicate that the major fraction of the bacterial soil community exists in a dormant state but can immediately be reactivated when a suitable substrate is available. But the question remains open if the substrates supplied (here glucose, yeast extract) were the adequate substrates for all soil bacteria. The similar question arises with regard to the BIOLOG test, since substrates provided in commercially available BIOLOG plates are not necessarily ecologically relevant and most likely do not reflect the diversity of substrates found in the environment (Konopka et al., 1998). Interestingly, in our habitat the polyoxyethylene-sorbitanes Tween 40 and Tween 80 served as good nutrients for the bacteria of all soil depths. These non-ionic surfactants are common supplements to biological solutions and buffers, but to our knowledge atypical components in soils. The results might indicate that the majority of the soil bacteria possess the enzymatic equipment to degrade such substances. On the other hand DL-α-glycerol phosphate, glucose-1-phosphate, and α-cyclodextrin were non-degradable for all tested bacterial communities. The two latter substrates commonly appear as products of starch decomposition.

In agreement with the biochemical characterization of urban soil profiles by Lorenz et al. (2006) and Lorenz and Kandeler (2005) we discovered metabolically active bacteria even in the deepest investigated soil layer. Nevertheless, we observed the general trend that the capability of utilizing different substrates decreased with soil depth. In a study investigating whether the BIOLOG assay reflects the catabolic potential of the used inoculum, Smalla and coworkers (1998) found the fast growing γ-*Proteobacteria* numerically dominant in the wells. They hypothesized that this group was primarily responsible for the observed substrate utilization patterns. In our study the decline in substrate utilization was also consistent with a decrease of γ-*Proteobacteria* from topsoil to subsoil.

5. Conclusions

In this study we used a polyphasic data set for the bacterial community analysis of an urban soil profile. Culture-independent nucleic acid techniques, culturing methods and physiological potential analyses revealed insights into the bacterial soil populations. The total number of bacteria and biomass declined from topsoil to subsoil. ARDRA and BIOLOG data revealed similar bacterial communities in the two upper soil layers in contrast to the deepest layer. The number of FISH-detectable *Eubacteria* nearly doubled after activation with a suitable substrate, and the overall number of α-, β- and γ-*Proteobacteria* varied within the investigated soil profile. But we are aware that all the methods used always address only a part of the whole community leaving the uncertainty of whether the biological characteristics of the soils have been adequately described. In future we will focus on screening our bacterial isolates for dominant in situ members scanning their physiological potential and interaction ability with the abiotic soil components.

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Paper II

Effects of water repellency on bacterial communities of urban soil

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Braun, B., Böckelmann, U., Grohmann, E. and U. Szewzyk.

Effects of water repellency on bacterial communities of urban soil

Burga Braun*, Uta Böckelmann, Elisabeth Grohmann, Ulrich Szewzyk

Department of Environmental Microbiology, University of Technology Berlin, Franklinstrasse 29, 10587 Berlin, Germany

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Corresponding author:

* Burga Braun, Department of Environmental Microbiology, Institute of Environmental Technology, University of Technology Berlin, FR 1-2 Franklinstrasse 29; 10587 Berlin. Email: burga.braun@tu-berlin.de

Fon +49(30) 31473566 Fax +49(30) 31473673

Abstract

Soil water repellency is a common phenomenon which occurs throughout the world. It prevents water from infiltrating into the soil and influences soil properties. Changes in soil wettability may represent a significant source of stress for microbial communities and affect bacterial composition and soil microbial processes. We analysed the bacterial community of an urban soil which is characterised by a distinctly developed heterogeneity of water repellency. The influence of water stress on microbial composition and activity was investigated by cultivation- and molecular methods, such as BIOLOG and denaturing gradient gel electrophoresis (DGGE). The application of cultivation independent methods did not allow to distinguish between water repellent and wettable soil samples. Total cell counts showed no significant differences between both areas. Similarity analysis of DGGE profiles of the soil community from wettable and repellent zones revealed three distinct clusters of about 78%, 80% and 85% similarity, respectively. Fingerprints of water repellent and wettable samples did not cluster together. In contrast, determination of CFU and generation of metabolic fingerprints resulted in differences between wettable and water repellent soil areas. CFUs in wettable zones were significantly higher than in water repellent zones. Substrate utilization patterns of wettable and water repellent areas differed from each other.

1 Introduction

Soil water repellency has been recognised as a severe problem throughout the world (Franco *et al*., 2000; Scott, 2000; Täumer *et al*., 2004). During the last few years, millions of ha of water repellent soils have been identified and recent studies suggest that water repellent soils are the rule rather than the exception in many regions. Water repellency is an important phenomenon in soil systems as it prevents water from infiltrating into the soil. Non homogeneous infiltration and wetting front instabilities can lead to preferential flow paths (Edwards *et al*., 1993; Täumer *et al*., 2004).

Soil water content seems to be one of the main factors influencing the current water repellency (Ritsema *et al*., 1998; Quyum *et al*., 2002). It influences important chemical and physical properties of soil required for a well functioning ecosystem. Soil is the natural habitat for many microorganisms. Bacteria inhabiting soil live free or attached to surfaces, inside aggregates and also in water films surrounding the soil particles (Stotzky, 1997). Denef et al. (2001) showed that changes in soil wettability may represent a significant source of stress for microbial communities and alter the composition and growth of soil microorganisms by selecting for microbes that can survive rapid changes in water potential (Fierer *et al*., 2003).

Due to the high relevance of water repellent soils, a great number of studies have been conducted on possible causes of water repellency. Numerous studies on the influence of water stress on bacterial communities induced by drying and rewetting of soils have been carried out (Bloem *et al*., 1992; Lundquist *et al*., 1999a; Lundquist *et al*., 1999b; Mamilov & Dilly, 2002; Fierer *et al*., 2003; Steenwerth *et al*., 2005). To our knowledge investigations on the influence of water stress due to water repellency on microbial communities are rare. Hallett and Young (1999) found an increase in microbial activity in field and laboratory studies as a consequence of increased water repellency. Bundt et al. (2001) investigated soil communities in two distinct but spatially close compartments due to a preferential flow path. They detected a significantly larger microbial biomass in preferential flow paths than in the soil matrix. But the microbial fingerprints of the community structure of both compartments were the same.

In addition to its detrimental and often costly implications for plant growth, soil water repellency has substantial hydrological and geomorphological repercussions. These include the reduced infiltration capacity of soils, enhanced overland flow and accelerated soil erosion, uneven wetting patterns, development of preferential flow and the accelerated leaching of agrichemicals (Doerr *et al*., 2000).

Polymerase chain reaction (PCR)-based molecular fingerprint techniques such as denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), as well as terminal restriction fragment length polymorphism (T-RFLP) and amplified ribosomal DNA restriction analysis (ARDRA) allow a thorough investigation of the species composition (Torsvik & Øvreås, 2002). DGGE has been applied for the investigation of bacterial communities of different soil types: Øvreås and Torsvik (1998) detected differences in the microbial communities in two different agricultural soils. Our recent study on a soil profile ranging from 15- 90 cm depth on the sampling site in the Tiergarten Park Berlin with ARDRA revealed similar bacterial communities in the two upper soil layers (15 cm and 30 cm) in contrast to the deepest layer (90 cm) (Braun *et al*., 2006). Griffiths et al. (2004) revealed effects of manipulation of the bacterial community structure in an upland pasture soil by fumigation and irradiation-reinoculation.

To understand the role of microbial communities in different environments, information on functional diversity is essential. The functional diversity is represented by the catabolic potential of the community. Garland and Mills (1991) were the first who used the BIOLOG system to assess the catabolic potential of bacterial soil consortia. This community-level physiological profiling has been demonstrated to allow monitoring of spatial changes in microbial communities (Staddon *et al*., 1997; Buyer *et al*., 2001). El Fantroussi et al. (1999)

assessed the impact of phenylurea on soil microorganisms with BIOLOG plates in conjunction with DGGE.

Our study aims to elucidate the influence of water repellency on the microbial composition of an urban soil. Other studies on this soil have shown considerable heterogeneity in wetting behaviour over very small distances (Müller *et al*., 2003; Hurraß & Schaumann, 2006). We investigated the bacterial population of wettable and water repellent soil samples. To assess the impact of water repellency on the microbial community composition and activity, cultivation-based (CFU, BIOLOG) and culture-independent molecular techniques for community analysis, such as DAPI staining (total cell counts) and DGGE were combined.

2 Material and Methods

2.1 Sampling and characterisation of sampling site

The experimental site is located in the urban park Tiergarten in the center of Berlin, Germany (52°30`N and 13°21`E) and represents a heterogenic habitat for microorganisms due to the spatial distribution of water repellency. The park was founded in 1833 and the total area is about 780 hectares.

The sampling site within the park is located on a public area, which is marked by anthropogenic influences, like fertilisation, sprinkling and pollutants. The yearly rainfall of about 580 mm is evenly distributed. The climatic water balance becomes negative from April to September.

Bulk soil samples were collected from a medium sized fluvial sandy soil, with a thick humus layer, which can reach a height up to 40 cm. Nineteen soil samples from the identical water repellent and wettable areas were taken from 10-30 cm depth in April, July and October 2002, January, April, July and October 2003 and in April 2004 in three parallels. The samples were immediately transported to the laboratory and processed the same day or after overnight storage at 4 °C. From 1000 g of each soil sample, 20 g were homogenised and subsamples of 5 g were taken for further analysis. The pH was determined in $CaCl_2$ (0.01M) (DIN ISO 10390), soil / $CaCl_2$ solution-ratio 1:2.5 and in H_2O (DIN ISO 38404). Organic carbon content was measured by drying the sample at 550 °C for 5 h following DIN EN 12879. Water content was defined according to DIN EN 12880. Because of the high variability of soil water repellency in the field, samples of actually wettable and water repellent samples, which were located directly side by side at a distance of 10 to 20 cm were compared.

2.2 Determination of water repellency

Actual water repellency was determined with the Water Drop Penetration Time (WDPT) test as described by several authors (e.g. Krammes & DeBano, 1965; Dekker & Jungerius, 1990). In order to establish the actual repellency three drops of distilled water were placed on the smoothed surface of a field moist soil sample using a standard glass pipette. The time that elapsed before the drops were absorbed was measured. A repellency index was applied allowing a quantitative description of the severity of water repellency as described by Dekker and Jungerius (1990). Samples with WDPT class 0 and 1 (< 5 sec – 60 sec) were regarded as wettable and samples with WDPT class 3 to 6 were regarded as water repellent (10 min - > 6 h).

2.3 Microscopic and plate counts

Total cell counts of soil bacteria were determined with 4´,6-diamidino-2-phenylindole (DAPI, Sigma, Deisenhofen, Germany). The samples were treated according to Böckelmann et al. (2003). 320 µL of the fixed supernatant were incubated with 80 µL DAPI (10 µg mL^{-1}) for 20 min in the dark. The samples were collected by filtration onto a 0.2 µm pore-size black polycarbonate filter (diameter 24 mm, Millipore, Eschborn, Germany). This filter was mounted on a glass slide and examined under a fluorescence microscope (Zeiss Axioskop, Oberkochen, Germany). At least 20 microscopic fields (100 by 100 µm) were chosen randomly and a minimum of 1,000 cells were enumerated microscopically. Total cell counts were calculated for 1 g dried soil.

For determination of colony forming units (CFU), 10 mg of soil were shaken in sodium pyrophosphate buffer (Na_2HPO_4 x 2 H_2O 4.0 g; NaH_2PO_4 x H_2O 0.22 g; distilled water was added to 200 mL; pH 6.0) for 2 h to detach the bacteria from soil particles. After 30 min of settling of soil particles, 20 mL of the supernatant were removed and the cells were pelleted by centrifugation at 4,000 x *g* for 10 min. The pellets were washed twice with 30 mL PBS buffer and finally resuspended in 20 mL PBS buffer. 100 µL of serial dilutions of the suspension (10^0, 10^{-1}, 10^{-2}) were plated (3 x each) on 10% tryptic soy agar (TSA) (Becton Dickinson, NY, USA). Bacterial growth at 23 °C in the dark was monitored for three days. CFUs were then enumerated and calculated for 1 g dry weight of soil.

2.4 Substrate utilisation patterns

20 mL of a 1% (w/v) soil suspension were prepared as described above and washed twice in 50 mL sterile PBS by mixing and centrifugation for 10 min at 4,000 x *g*. Following the cell washes, pelleted cells were resuspended in 20 mL of sterile PBS and 150 µL aliquots containing approximately 10^3 cells (enumerated by plate counts), were dispensed into each of

147 the 96 wells of the BIOLOG EcoPlate® (Oxoid, Hampshire, England). Plates were incubated
148 at 28 °C and manually scored after 8 days to determine the substrates utilised after 8 days. For
149 each reading, a well was scored as positive based on visual inspection of colour change.

150 *2.5 DNA extraction from soil samples*

151 Total community DNA was directly extracted from soil by using the FastDNA® Spin Kit
152 for Soil as described by the manufacturer (QBiogene, Carlsbad, CA, USA).

153 *2.6 Amplification of 16S rDNA fragments by PCR*

154 To study the bacterial community of urban soils, the hypervariable V3 region of the
155 bacterial 16S rDNA molecule was chosen. For the amplification, the universal primer pair p2
156 and p3 (Muyzer *et al.*, 1993) was used, which is located in conservative sequence regions
157 covering the variable V3 fragment. This primer pair amplifies a 190 bp fragment, which
158 corresponds to the position 338 to position 534 in the 16S rDNA of *E. coli*. In addition, a GC-
159 rich sequence was attached to the 5´end of the forward primer for subsequent DGGE analysis.
160 Due to the incorporation of the 40 bp GC clamp at the 5´end of the p3 primer, the total size of
161 the fragment increases to 230 bp.

162 Specific amplification of the V3 fragment of the 16S rDNA of total soil DNA was carried
163 out using a hot start protocol. The reaction was performed in a final volume of 50 µL using
164 the SurePRIME® & Go Mastermix (QBiogene). It consists of 2.5 mM $MgCl_2$, 50 mM KCl, 1
165 x PCR buffer and 1 U of SurePRIME® DNA polymerase (QBiogene). Deoxynucleosid
166 triphosphates (0.16 µM each, QBiogene), primer (0.16 µM each, SIGMA-Genosys,
167 Steinheim, Germany), 0.3 mg/mL BSA (Promega, Madison, WI, USA). Approximately 200
168 ng of target DNA were added to each reaction. The PCR was performed in a thermocycler
169 (TGradient, Biometra, Göttingen, Germany) using the following conditions: 95 °C for 15 min
170 (for enzyme activation), followed by 27 cycles of 94 °C for 45 sec, 56 °C for 45 sec, 72 °C for
171 45 sec and according to Janse et al. (2004) a final extension at 72 °C for 30 min to eliminate
172 artifactual double bands. To estimate the amount of the PCR products, 8 µL of each PCR
173 reaction were mixed with 3 µL loading dye (0.25% xylene cyanol; 40% (w/v) sucrose) and
174 loaded onto a 1% agarose gel containing 0.5 µg/mL ethidium bromide. The gel was run in 0.5
175 x TAE buffer at 80 V for 20 min and DNA was visualized with UV light.

176 *2.7 Preparation of a reference-marker*

177 To allow comparative analysis of DGGE patterns, a marker containing 16S rDNA products
178 amplified with primer pair p2 and p3 (Muyzer *et al.*, 1993) from different bacterial species

was designed. To this purpose, 12 bacterial strains from the laboratory collection, isolated by our group from different soil habitats, were tested. A colony of each isolate was suspended in 50 µL lysis buffer (0.05% SDS; 0.03 M NaOH) and incubated for 15 min at 95 °C. 450 µL of distilled water were added and samples were centrifuged for 5 min at 13,000 rpm. 1 µL of each supernatant was used for performing PCR reactions as described in 2.6. 15 µL of each PCR product were run on a DGGE gel in a single slot to estimate the running position of the amplicons. DGGE gel running conditions were the same as described in 2.8. Eight amplicons with a migration distance covering the range of soil sample patterns were chosen for the reference marker. Equivalent 16S rDNA amplicon concentrations were mixed, which were obtained from the following isolates, *Acinetobacter iweffi*, *Bacillus pumilus*, strain F8 (Böckelmann *et al*., 2006), *Rhizobium sp*., *Curtobacter sp*., *Variovorax paradoxus*, *Dyadobacter sp*. and *Arthrobacter sp*.; subsamples were stored at -20 °C until usage.

2.8 Analysis of 16S rDNA fragments by DGGE

The DGGE analysis was performed with a DCode® gel electrophoresis system (Bio-Rad, München, Germany) according to the manufacturers instructions using an 8% polyacrylamid gel with a parallel chemical denaturing gradient adapted to 40% (6% (w/v) acrylamide-bisacrylamide [37.5:1], 18% deionized formamide, 3.1 M urea) to 65% (6% (w/v) acrylamide-bisacrylamide [37.5:1], 26% deionized formamide, 4.5 M urea). To each 40 µL of PCR reaction, 10 µL loading dye (0.1 M EDTA, pH 8.0, 40% sucrose (w/v), 0.05% bromphenol blue (w/v), 0.5% SDS (w/v)) were added and an appropriate volume of the PCR product was loaded onto a gel. The gels were subjected to a constant voltage of 80 V for 18 h at 60 °C and after electrophoresis they were stained for 20 min in 1 x TAE buffer containing 1 x (final concentration) SYBR Green I (Molecular Probes, Leiden, Netherlands) followed by documentation with a GelDOC 2000 System (Bio-Rad, München, Germany).

2.9 Examination of DNA from DGGE gels

After the DGGE analysis, selected DGGE bands were punched out of the gel with sterile pipette tips. Each piece was then transferred into 50 µL of distilled water, ground with a sterile pistil and incubated overnight at 37 °C to allow diffusion of the DNA. 1 µL of the eluted DNA was thereafter used for the re-PCR, run at the same conditions as described in 2.6. To check the purity and correct running position of each fragment, each re-PCR product was run in a single slot alongside the according PCR product from total soil DNA on further DGGE gels as described in 2.8. Only products that migrated as a single band and at the same position with respect to the control were amplified and used for cloning and sequencing.

For cloning, 1 µL of the eluted DNA was applied to re-PCR with the non GC-clamp primer pair p1 and p2 (Muyzer *et al*., 1993) and the PCR conditions were as described in 2.6. 1.5 µL of the re-PCR amplicons were then ligated into the pCR®4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen) as described by the manufacturer. All together 190 clones, five clones each of 38 excised bands containing inserts, were chosen for sequencing.

2.10 Sequence analysis

The sequences were edited and aligned with the ARB software package. They were aligned with the Aligner tool of the software and manually corrected according to primary and secondary structure similarities. Searches in GenBank with the BLAST program (Altschul *et al*., 1997) were performed to determine the closest known relatives of the partial 16S rDNA sequences obtained.

2.11 Statistical evaluation

The data were analysed with the StatsDirect software package (StatsDirect Ltd., Cheshire, UK) using one-way ANOVA and the Tuckey-Kramer test. The BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyse the bacterial community fingerprints of the DGGE gels. Relatedness of microbial communities was determined using similarity coefficients of bands common in two samples. The working definition was that two bands are equivalent, if they migrate the same distance in a gel. First, the total number of different bands was determined for the samples being compared. Then each sample was scored based on the presence or absence of each band in its profile compared to the profile of each of the other samples. Dice coefficient of similarity [SD = (2nAB)/(nA+nB)] was used to make pair wise calculations of bands shared between samples. In this equation, nA is the total number of bands in lane A, nB is the total number of bands in lane B, and nAB is the number of bands common in lane A and B. The similarity analysis of patterns was calculated using the unweighted-pair group method applying average linkages (UPGMA).

3 Results

3.1 Physicochemical analysis of soil samples

Soil samples T1004, 1005, 1013, 1014, 1025, 1026, 1045 and 1046 belong to WDPT class 0, T1038 and T1040 were affiliated to WDPT class 1. The water repellent soil sample T1002

belongs to WDPT class 3, T1001 and 1016 to WDTP class 5, whereas T1015, 1023, 1037, 1039, 1043 and 1044 were affiliated to WDPT class 6. Additional physicochemical characteristics of this anthropogenically affected sandy soil are shown in Table 1.

Soil pH of the experimental sites was not different for wettable and water repellent soil samples. The pH was in the range of 4.3 ± 0.4 for water repellent zones and 4.8 ± 1.3 for wettable soil areas. The water content differed between 10% ± 2.1% for water repellent and 28% ± 10.2% for wettable samples. Soil organic matter as well as organic C content did not vary significantly among the sampling points.

3.2 Bacterial culturability and total cell counts

The effect of water repellency on the total number of culturable bacteria (CFU) was investigated on 10% TSA agar. This medium was applied as in former studies with the same soil samples CFU numbers were higher than on rich media (NB, 100% TSA). Plate count analysis revealed a significant discrepancy ($p < 0.05$) in the number of bacteria that could be cultured from water repellent and wettable soil samples. The cultured bacterial numbers were higher in wettable soil samples than in water repellent ones. Water repellent soil samples revealed an amount of culturable bacteria in the range of $1 \times 10^6 \pm 7 \times 10^5$ (g dry wt soil)$^{-1}$. The CFU values for wettable soil areas were about $4 \times 10^6 \pm 3 \times 10^6$ (g dry wt soil)$^{-1}$. Microscopic enumeration of the total number of bacteria after DAPI staining revealed no significant differences ($p > 0.05$) for water repellent and wettable soil samples. Total bacterial cells accounted about $2 \times 10^8 \pm 6 \times 10^6$ (g dry wt soil)$^{-1}$ for wettable soil areas and about $2 \times 10^8 \pm 7 \times 10^7$ (g dry wt soil)$^{-1}$ for water repellent soil samples, which are two orders of magnitude higher than those observed from cultivation based enumeration.

3.3 Metabolic fingerprints

To assess the potential functional diversity of the bacterial community sole source carbon utilization patterns were generated using BIOLOG EcoPlates®. The inoculated populations were monitored over a time of eight days for their ability to utilize the different substrates. For comparison between water repellent and wettable soil areas, we analysed substrate utilisation patterns at a single time point (8 days). The parallelism of replicate plates of the soil samples exhibited a high fidelity. The capability of utilizing different substrates varied between wettable and water repellent soil samples. Differentiation between soils with distinct water repellency was possible (Tab. 3). The number of substrates utilized was higher for the bacterial consortium of wettable soil areas. These communities metabolised approximately 84% (26 from 31) of the different C sources present in BIOLOG EcoPlates®. In contrast,

populations of water repellent soil samples degraded only 61% (19 of 31) of the offered substrates.

Carbohydrates served as a suitable substrate for the bacterial consortia of wettable and water repellent soils. Interestingly, the bacterial community of all soil samples failed to degrade glucose-1-posphate and D,L-α-glycerol phosphate. The microbial population of the wettable soil samples utilized most offered carboxy acids, whereas the bacteria of the water repellent zones degraded only a few. Itaconic acid and α-ketobutyric acid were not degradable at all. Utilization of N-containing compounds such as amino acids showed the same trend. Bacterial populations of water repellent soil samples were able to use less of the offered amino acids as carbon source. The polymers glycogen and the polyoxyethylene-sorbitanes Tween 40 and Tween 80 served as good nutrients for the bacteria of all soil samples. In contrast to this, α-cyclodextrin was not degradable for nearly all investigated bacterial communities. The offered amines were utilized by the bacterial consortia of wettable soil samples, whereas the bacterial population of water repellent soil areas failed to degrade them. The two phenolic compounds, 4-hydroxy benzoic acid and 2-hydroxy benzoic acid were utilized by the bacterial population of water repellent and wettable soil areas.

3.4 DGGE profiles of the bacterial community

To which extent bacterial populations are influenced by small scale heterogeneity of water repellent soil surfaces, was investigated. For this examination, total community DNA was recovered from wettable and water repellent soil samples. 16S rDNA fragments amplified from DNA extracted directly from soil samples at each sampling time were compared, running them in parallel on DGGE gels. DGGE analysis of 16S rDNA fragments from both areas revealed a relatively high similarity of the DGGE patterns obtained for each of the replicates per time point, suggesting a low degree of variability caused by sampling, DNA extraction, PCR amplification and DGGE analysis. The band based information of the DGGE profiles was used to determine similarities using the Dice correlation coefficient. The matrix containing similarities between the tracks was clustered using the UPGMA algorithm. For statistical comparison between wettable and water repellent soil samples, the similarity of the DGGE profiles within the different soil areas and between the different soil areas was analysed.

At all sampling times, DGGE patterns of wettable and water repellent soil samples consist of some stronger bands and a large number of less intense bands (Fig. 1a). This indicates that only a small number of organisms dominate the population, while many bacteria which were less abundant represent a diverse background population. The patterns of the different

sampling times and sampling points resulted in similar, but not identical DGGE profiles. With digital image analysis it was not possible to correlate distinct DGGE patterns with the wettability of the soil samples. The band based similarity calculation clearly revealed three distinct clusters with about 78%, 80% and 85% similarity, respectively (Fig. 2). Fingerprints of wettable and water repellent soil samples only partly clustered together. An altered composition of the bacterial community in response to soil water repellency could not be detected. In addition, no significant differences were revealed when profiles obtained from the different sampling dates were compared with each other.

3.5 Diversity of 16S rDNA gene fragments from DGGE gels

Selected DGGE bands were excised, cloned, sequenced and subjected to phylogenetic analysis to get further information on the bacterial community in the different soil areas of varying water repellency. They gave short DNA sequences, which were processed to give a rough phylogenetic affiliation based on 190 bp of the V3 region of the 16S rRNA gene. The criteria for selection of bands were that (i) they appear as a single band in the pool of lanes, (ii) they represent bands in high abundance in the community or (iii) they were relatively low abundant in the DGGE pattern. For all excised bands (Fig. 1b), multiple clones were generated to assess the degree to which single DGGE bands harboured heterogeneous mixtures of rRNA gene sequences from more than one population. In total we sequenced 190 different clones, 115 from wettable soil samples and 75 from water repellent samples, corresponding to 38 excised DNA fragments. Re-amplification products were screened by DGGE analysis of the respective 16S rDNA fragment. Parallel analysis of the re-amplified DGGE band and the soil sample on the same gel allowed us to check whether the DNA fragments comigrated with the band of the corresponding community pattern.

Analysing the selected bands confirmed that the most intense bands in all lanes were identical and that some fainter bands were also common in all lanes of water repellent and wettable samples. This indicates that the populations were dominated by the same phylotypes in wettable and water repellent soil samples. It is also worth mentioning that in some cases where multiple clones were generated from a given excised band, more than one phylotype was detected from that band. In fact, in 10 out of 38 cases where five clones were generated from a single band, each of them represented a different phylotype. The analysis of comigrating bands of water repellent and wettable samples revealed the same phylogenetic affiliation. The similarity of the closest relatives of the partial 16S rDNA fragments of all sequenced bands ranged between 91% to 100%. Sequencing of the chosen DGGE bands showed that nearly all sequences are highly similar to cloned DNA from other soil

environments (Tab. 2). The sequences of the comigrating bands from water repellent samples A1, C1 and from wettable soil samples V1, W1, X1, Z1 matched closely that of an uncultured *Oscillatoriales cyanobacterium* soil clone named T26 (Stafford *et al*., 2006). The analysed bands E1, G1 (water repellent samples) were related to *Bacillus spec*. Eur1 9.5 (Steven & Whyte, 2006) with an identity of 99%. Sequences A2 and D2 derived from water repellent soil samples matched with an uncultured *Actinomycetales* (Rheims *et al*., 1996). Sequencing of DNA fragments detected in water repellent (A6) and wettable samples (V4, X4, W2) revealed an uncultured *Burkholderiales* clone named clone T13 as closest relative (Stafford *et al*., 2006). The closest match of the analysed bands V3, X3 and Z3 was also the uncultured *Burkholderiales* clone T13. Excised DGGE bands A3, B2, C2, F2 (water repellent samples) and X2, Y2 (wettable samples) matched with an uncultured *Chloroflexi* bacterium clone F07_WMSP1 (Costello & Schmidt, 2006). DNA fragments V2 and Z2 from wettable soil samples which melt at a different particular position in the gel were also closely related to an uncultured *Chloroflexi* bacterium clone (Costello & Schmidt, 2006). DGGE bands from water repellent soil samples G3, H3 matched closely with an *Actinobacterium* clone C32.19SM (Chow *et al*., 2002). The selected DGGE bands were shown to belong to five different taxonomical classes, *Cyanobacteria*, bacilli, *Actinobacteria*, ß- *Proteobacteria* and *Chloroflexi*.

4 Discussion

Cultivation techniques as well as culture-independent methods, such as determination of CFUs and the BIOLOG system, DAPI staining and PCR-DGGE were used to study the effect of water repellency on bacterial communities.

The total soil bacterial consortium as determined by total cell counts was not affected by water repellency. These findings fit well with the results of Bloem et al. (1992) who also did not reveal changes in total cell numbers due to water stress caused by drying and rewetting of an arable soil. No significant differences in epifluorescence microscopy counts of the total bacterial community between water repellent and wettable soil samples in contrast to differences in the proportion of culturable bacteria were found. Plate count analysis revealed a discrepancy in the number of bacteria that could be cultured from wettable and water repellent soil samples. The culturability was significantly increased in the samples where water repellency was reduced. This finding could not be based on less detachment of bacterial cells from water repellent soil samples, as determination of CFUs and total cell counts were performed using identical soil suspensions. Culturable bacteria are sometimes considered to

be the most active members in a given sample and so provide a useful means of assessing biological responses to environmental changes (Bakken, 1997a; Bakken, 1997b).

The BIOLOG system generates a community level physiological profile based on patterns of substrate utilization, which can differentiate between communities. Since introduction into microbial ecology by Garland and Mills (1991) BIOLOG has been used for the analysis of microbial communities from different habitats in more than 200 different studies. In our work, the functional diversity of the soil community, as displayed by the number of different substrates utilized, was higher in wettable soil samples than in water repellent ones. The microbial community in wettable soil samples was able to utilize more than three quarters of the offered carbon sources (81%), whereas water repellent soil samples showed metabolization of more than half of the substrates (61%). Like CFU counts, this cultivation technique also resulted in discrimination between water repellent and wettable soil samples. The BIOLOG method is based on bacterial culturability and shows the catabolic potential of the soil community but does not reflect the in situ function of the microbial consortium. Further limitations of this technique are selecting for only culturable organisms (Garland & Mills, 1991) and sensitivity to inoculum density (Garland, 1996). Garland (1996) as well as Haack et al. (1995) found that colour development in BIOLOG wells was better linked to actively respiring cells than to total cell density. It is obvious that the activity of the cells reflects the physiological state of the cells (Preston-Mafham *et al*., 2002). Lawlor et al. (2000) considered the assessment of the bacterial activity by BIOLOG and CFU counts to be a suitable tool to measure the effect of water repellency on bacterial communities and not only the presence or absence of cells. Kennedy and Gewin (1997) also regarded the BIOLOG approach ecologically more relevant than taxonomic diversity approaches.

Our observation that the total bacterial population seemed to be largely unaffected by water repellency was confirmed by DGGE analysis. Fingerprints from directly extracted DNA showed the same dominant bands for wettable and water repellent soil samples. Differentiation between the different soil samples was not possible. Similarity analysis of the fingerprints resulted in three different groups with community fingerprints of water repellent and wettable soil samples differently clustered. The fingerprints at different sampling times and sampling points indicated that the community, to a large extent, consists of stable dominant populations. Prominent bands of the total community were similar for the majority of the samples. Specific patterns may have been covered by small variations between replicate samples that were particularly present in the wettable soil sample lanes. von Wintzingerode et al. (1997) explained this variability in microbial community composition of parallel samples

411 by methodological biases. Likewise some of the variations between the profiles may be
412 caused by different intensity of the staining, which was probably a result of different amounts
413 of DNA in each lane. Furthermore, changes in band intensity can occur if the bands result
414 from a cluster of populations with similar nucleotide sequence content that separate when
415 exposed to different gradients.
416 Numerous papers showed that water repellency is temporally variable in soil (e.g. Doerr *et*
417 *al*., 2000; Dekker & Ritsema, 2000; Feeney *et al*., 2006). GPR-measurements and excavations
418 of our test site in Berlin Tiergarten also showed temporal variability of water repellency
419 (Wessolek and Stoffregen, personal communication). The PCR-DGGE results of the present
420 study revealed no effect on the community due to changes in soil surface water repellency.
421 Fierer et al. (2003) also found no changes in the bacterial community of grasslands applying
422 culture independent T-RFLP analysis to study community changes due to water stress.
423 Griffiths et al. (2003) detected no alterations in CFU and DGGE fingerprints as bacterial
424 response to water stress in an established grassland. Nevertheless, they observed
425 physiological effects on the bacterial community related to the moisture regime. In contrast
426 Lundquist et al. (1999a) and Steenwerth et al. (2005) detected differences in microbial
427 communities exposed to water stress with culture independent techniques. Steenwerth et al.
428 (2005) found alterations of microbial communities from agricultural ecosystems by PLFA
429 after rewetting of the soil. But in contrast to our anthropogenically affected sampling site, the
430 changes were revealed in less disturbed environments.
431 To get phylogenetic affiliations of the bacterial community, individual DGGE bands were
432 excised from the gel, cloned and sequenced to determine the diversity of the 16S rDNA
433 fragments. The closest relatives derived from the sequenced fragments identified with the
434 BLAST program were nearly all sequenced clones. The phylogenetic affiliation of the 38
435 selected bands revealed members of five different phylotypes. Clones of DGGE bands
436 comigrating in the gel were present in wettable and water repellent soil samples, except
437 bacilli. DGGE bands whose sequenced clones revealed *Bacillus spec*. Eur1 9.5 as closest
438 relative were only detected in fingerprints of wettable soil samples. Analysed intense bands
439 (A1, C1, V1, W1, X1, Z1) revealed members of the *Cyanobacteria* and *Chloroflexi* class
440 (bands A3, B2, C2, X2, Y2). DNA sequences of faint bands (A6, V3, X3, Z3, E1,G1) showed
441 that soil communities of wettable and water repellent soil samples were inhabited by
442 representative soil bacterial families such as *Bacillus* and *Burkholderiales*. Nevertheless, the
443 sequences obtained from the DGGE fingerprints did not exactly match any known organism
444 in the BLAST database, which indicates that there are many as-yet-unknown microbes

inhabiting the soil. The detected *Actinobacteria* which derived from cloned bands A2, G3, H3 and D2 were only present in water repellent soil samples. In these samples they displayed an abundant group based upon the intensity of the DGGE bands. β-*Proteobacteria* are the dominating group of bacterial freshwater communities (Zwart *et al*., 1998) and are not prevalent in soil samples (Haruta *et al*., 2002; Torsvik & Øvreås, 2002). In our study we revealed sequences of *Burkholderiales* from faint bands of water repellent and wettable samples. This indicates that this diverse group of bacteria can easily adapt to various environmental conditions.

In ten cases where five clones from a single band were obtained, all five represented a different phylotype. This indicates that DNA fragments with different nucleotide sequence may have the same mobility in the polyacrylamide gel. Therefore, one band may not necessarily represent only one species (Gelsomino *et al*., 1999). Furthermore, one bacterial species may also give rise to multiple bands because of multiple 16S rRNA genes with slight differences (Gelsomino *et al*., 1999). Some bands contained multiple co-migrating sequences belonging to different bacterial species. We also found that bands of different mobility revealed the same closest relative. This is caused by sequence heterogeneities of 16S rDNA operons (Nübel *et al*., 1996), where more than one fragment can be PCR amplified from one single bacterial strain. This can lead to an overestimate of the number of bacterial species/strains present in the community. Another limitation of the direct rRNA gene based approach is that sequence data do not give direct evidence of function of the organism from which the sequence has been derived.

Although the dominant bands of the total community assessed by DGGE were similar for all samples (Fig. 1a) differences in CFU and substrate utilization patterns were observed. Our study shows that the effect of water repellency in soil as detected by DGGE fingerprints of DNA extracted directly from soil samples, was minimal compared to the effects on the culturable fraction of the bacterial community. It indicates that the bacterial soil community of this urban soil seemed to be adapted to different moisture conditions by regulating their physiological cell activity. This fits well with previous results of Kalmbach et al. (1997) and Braun et al. (2006). The later study demonstrated that bacterial cells of the same soil samples could be activated by applying the probe active counts (PAC) method (Kalmbach *et al*., 1997; Braun *et al*., 2006). The number of *Eubacteria* detected by the probe active count method (PAC) was increased twofold after addition of different carbon and nitrogen sources using the antibiotic pipemidic acid as gyrase inhibitor to prevent cell division.

The ability of bacteria to grow on culture media is affected by water repellency, because culturability is related to their physiological status (Kell *et al*., 1998). This points to the assumption that cellular physiology is altered by water stress and water stress might result in the loss of viability. Hattori et al. (1997) found that culturable bacteria represent the ecologically relevant portion of the soil bacterial community, which indicates that bacteria forming colonies on culture media display an important function in soil.

In our study water repellency specific bacterial patterns could not be detected by comparison of DGGE profiles and total cell counts, whereas soil microbial functional diversity as assessed by the BIOLOG technique and determination of CFU was able to discriminate between soils of different water repellency behaviour. These data indicate that water repellency in our habitat does not have a significant effect on the total genetic diversity present but affects the physiological status of the present population: bacteria capable of responding to laboratory culture methods were shown to be altered in metabolic activity.

To understand the system more comprehensively additional molecular methods, such as CARD-FISH and 16S rRNA-DGGE analysis could be applied. The CARD-FISH technique allows in situ detection of bacteria with low metabolic activity which represent the major part of soil microorganisms. In contrast to 16S rDNA-DGGE, 16S rRNA-DGGE profiles give information especially on the physiologically active part of the bacterial community.

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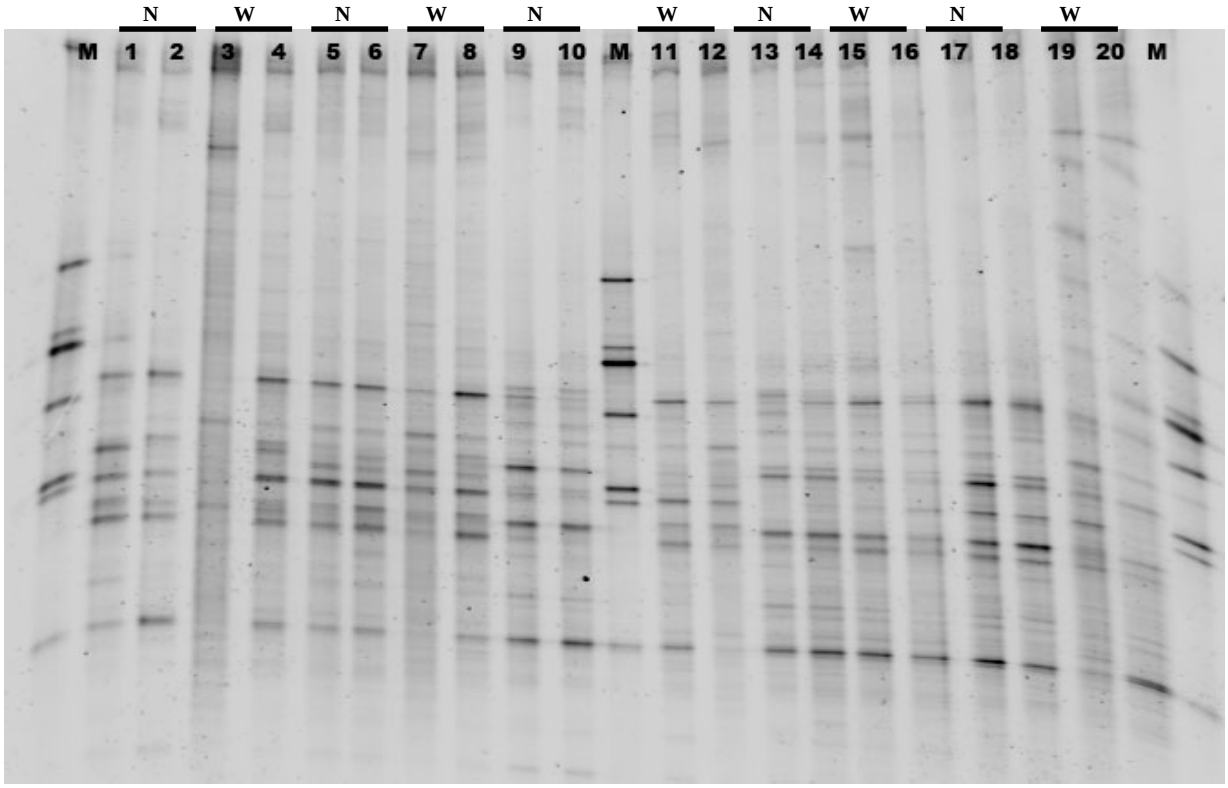

Fig. 1a. DGGE profiles showing the composition of the soil communities of wettable and non wettable soil samples. The fingerprints were generated by amplifying the V3 region of the 16S rRNA gene. M, marker lane consisting of 16S rRNA gene fragments from (listed top to bottom) *Acinetobacter iweffi*, *Bacillus pumilus*, F8, *Rhizobium sp*., *Variovorax paradoxus*, *Curtobacter sp*., *Dyadobacter sp*. and *Arthrobacter sp*. Lanes 3; 4; 7; 8; 11; 12; 15; 16; 19; 20 contain DNA fragments of wettable soil samples (W) in the following order: T 1004; T 1005; T 1013; T 1014; T 1025; T 1026; T 1038; T 1040; T 1045; T 1046, two parallels each. Lanes 1; 2; 5; 6; 9; 10; 13; 14; 17; 18 contain DNA fragments of water repellent soil samples (N) in the following order T 1001; T 1002; T 1015; T 1016; T 1023; T 1024; T 1037; T 1039; T 1043; T 1044, two parallels each.

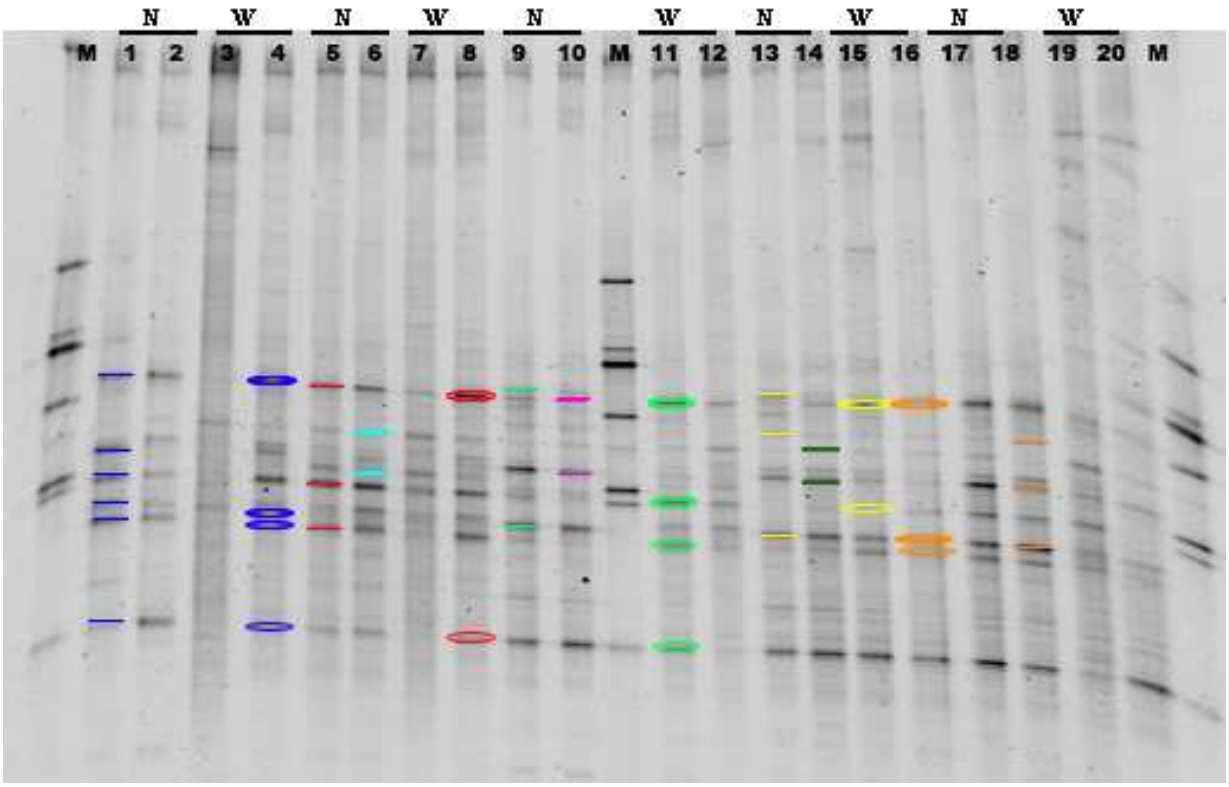

Fig. 1b. Identical DGGE profiles to Fig. 1a. Coloured lines indicate individual bands of water repellent soil samples, excised from the gel for cloning and DNA sequence analysis. Coloured ellipses indicate individual bands form wettable soil samples, excised from the gel for cloning and DNA sequence analysis. Bands derived form different lanes are marked in the following order: A= T 1001; B= T 1009; C= T 1015; D= T 1021; E= T 1023; F= T 1029; G= T 1037; H= T 1044; V= T 1005; W= T1014; X= T 1025; Y= T 1038; Z= T1040. Numbers after the letters (e.g. A1 or H3) are given from top to bottom.

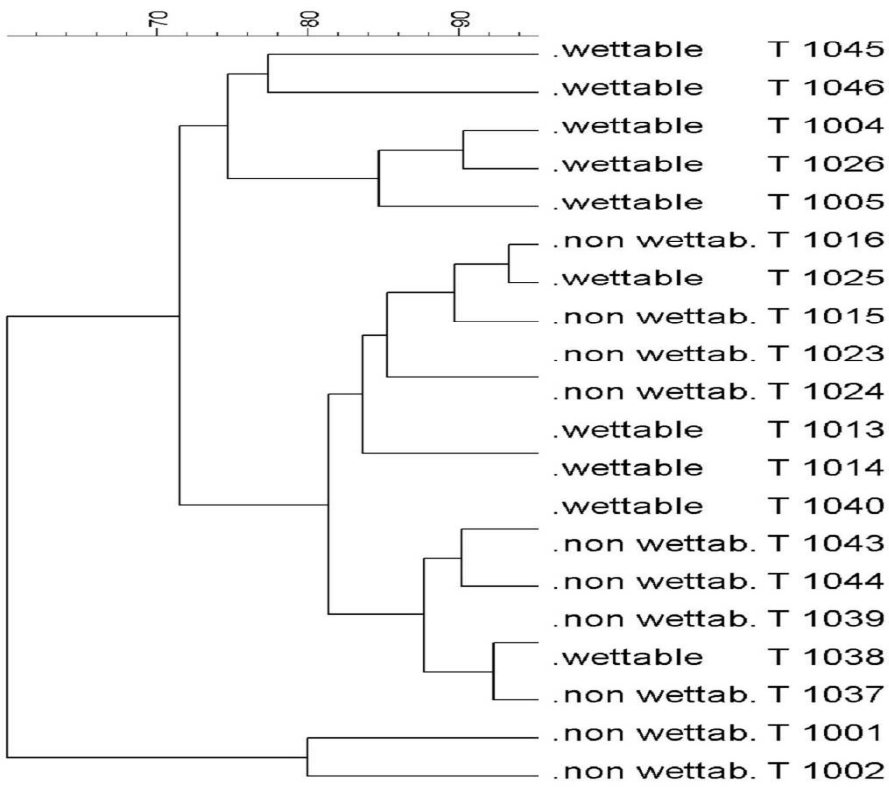

Fig. 2. Dendrogram constructed with the bacterial community fingerprints of wettable and non wettable soil samples. The difference between the profiles are indicated by similarity percentage. The dendrogram is based on the Dice coefficient and the cluster analysis on UPGMA.

Table 1 General characteristics of the soil samples from the Tiergarten Park [a]

Soil sample	Water content [%]	pH (H_2O)	C content [g soil $^{-1}$]	Organic matter [mass %]	Textural classification [%] < 0.002 mm
wettable	28 ± 10.2	4.8 ± 1.3	5 ± 2	12 ± 5	6 ± 3
non wettable	10 ± 2.1	4.3 ± 0.4	5 ± 1	11 ± 4	9 ± 11

[a] Averages were calculated out of 15 measurements; errors indicate the respective standard deviation.

Table 2 Phylogenetic affiliation of the sequenced DGGE bands based on 190 bp of the 16S rDNA.

Band[a]	Phylum or Class	Closest relative	Accession no.	Source	Similarity
A1	*Cyanobacteria*	*Oscillatoriales cyanobacterium* soil clone T26	AY827061	*Proteaceae* roots	98%
A2	*Actinobacteria*	*Actinomycetales*	X92703	"Different environments"	97%
A3	*Chloroflexi*	*Chloroflexi* bacterium clone F07_WMSP1	DQ450737	Alpine tundra wet meadow soil	100%
A6	ß- *Proteobacteria*	*Burkholderiales* clone T13	AY827053	*Proteaceae* roots	91%
B2	*Chloroflexi*	*Chloroflexi* bacterium clone F07_WMSP1	DQ450737	Alpine tundra wet meadow soil	100%
C1	*Cyanobacteria*	*Oscillatoriales cyanobacterium* soil clone T26	AY827061	*Proteaceae* roots	98%
C2	*Chloroflexi*	*Chloroflexi* bacterium clone F07_WMSP1	DQ450737	Alpine tundra wet meadow soil	99%
D2	*Actinobacteria*	*Actinomycetales*	X92703	"Different environments"	98%
E1	*Bacilli*	*Bacillus spec*. Eur1 9.5	DQ444975	Permafrost soil	99%
F2	*Chloroflexi*	*Chloroflexi* bacterium clone F07_WMSP1	DQ450737	Alpine tundra wet meadow soil	97%
G1	*Bacilli*	*Bacillus spec*. Eur1 9.5	DQ444975	Permafrost soil	99%
G3	*Actinobacteria*	*Actinobacterium* clone C32.19SM	AF431548	*Pinus contorta* rhizosphere	96%
H3	*Actinobacteria*	*Actinobacterium* clone C32.19SM	AF431548	*Pinus contorta* rhizosphere	97%
V1	*Cyanobacteria*	*Oscillatoriales cyanobacterium* soil clone T26	AY827061	*Proteaceae* roots	98%
V2	*Chloroflexi*	*Chloroflexi* bacterium clone F07_WMSP1	DQ450737	Alpine tundra wet meadow soil	93%
V3	ß- *Proteobacteria*	*Burkholderiales* clone T13	AY827053	*Proteaceae* roots	92%
V4	ß- *Proteobacteria*	*Burkholderiales* clone T13	AY827053	*Proteaceae* roots	91%
W1	*Cyanobacteria*	*Oscillatoriales cyanobacterium* soil clone T26	AY827061	*Proteaceae* roots	95%
W2	ß- *Proteobacteria*	*Burkholderiales* clone T13	AY827053	*Proteaceae* roots	91%
X1	*Cyanobacteria*	*Oscillatoriales cyanobacterium* soil clone T26	AY827061	*Proteaceae* roots	98%
X2	*Chloroflexi*	*Chloroflexi* bacterium clone F07_WMSP1	DQ450737	Alpine tundra wet meadow soil	98%
X3	ß- *Proteobacteria*	*Burkholderiales* clone T13	AY827053	*Proteaceae* roots	91%
X4	ß- *Proteobacteria*	*Burkholderiales* clone T13	AY827053	*Proteaceae* roots	93%
Y2	*Chloroflexi*	*Chloroflexi* bacterium clone F07_WMSP1	DQ450737	Alpine tundra wet meadow soil	100%
Z1	*Cyanobacteria*	*Oscillatoriales cyanobacterium* soil clone T26	AY827061	*Proteaceae* roots	98%
Z2	*Chloroflexi*	*Chloroflexi* bacterium clone F07_WMSP1	DQ450737	Alpine tundra wet meadow soil	95%
Z3	ß- *Proteobacteria*	*Burkholderiales* clone T13	AY827053	*Proteaceae* roots	92%

[a] Derivation of bands: A= T 1001; B= T 1009; C= T 1015; D= TT 1021; E= T 1023; F= T 1029; G= T 1037; H= T 1044; V= T 1005; W= T 1014; X= T 1025; Y= T1038; Z= T 1040.

Table 3 Selected BIOLOG data of the bacterial soil community of wettable and non wettable soil samples.

	substrate	wettable	non wettable
Carboxylic acids			
	D-Galacturonic Acid	[1]X	X
	D-Malic Acid	X	X
	Pyruvic Acid Methyl Ester	X	
	γ-Hydroxybutyric Acid	X	
	D-Glucosaminic Acid	X	
	Itaconic Acid		
	α-Ketobutyric Acid		
Aminoacids			
	L-Arginine	X	X
	L-Asparagine	X	X
	L-Serine	X	X
	Glycyl-L-Glutamic Acid	X	X
	L-Phenylalanine	X	
	L-Threonine	X	
Amins			
	Phenylethylamine	X	
	Putrescine	X	

[1] The cross indicates utilized substrates

Paper III[1]

Influence of biofilms on the water repellency of urban soil samples

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Influence of biofilms on the water repellency of urban soil samples

Gabriele E. Schaumann[1]*, Burga Braun[2], Dörte Kirchner[3,2], Wolfgang Rotard[3], Ulrich Szewzyk[2] and Elisabeth Grohmann[2]

[1] Department of Chemistry - Section Organic and Environmental Chemistry, Institute of Sciences, University Koblenz-Landau, Universitätsstraße 1, D-56072 Koblenz

[2] Department of Environmental Microbiology, Institute of Environmental Technology, TU Berlin, Sekr. FR 1-2, Franklinstr. 28/29, D-10587 Berlin

[1] Department of Environmental Chemistry, Institute of Environmental Technology, TU Berlin, Sekr. KF 3, Straße des 17. Juni 135, D-10623 Berlin

* Correspondence to: G. E. Schaumann, Department of Chemistry - Section Organic and Environmental Chemistry, Institute of Sciences, University Koblenz-Landau, Universitätsstraße 1, D-56072 Koblenz, email: schaumann@uni-koblenz.de.
Tel +49 (261) 287-2252 Fax +49 (261) 287-100-2252

Abstract

Water repellency is an important phenomenon in soil systems and is influenced by physical, chemical and biological factors. Studies on the influence of bacteria or surface- attached bacteria (biofilms) on soil water repellency are rare. Especially, the role of bacterial EPS in soil water repellency has not been investigated until now.

In this study, we investigated the influence of hydrophilic and hydrophobic bacteria on soil wettability. Three different soil bacteria, *Variovorax paradoxus*, *Bacillus sphaericus* and an α- *Proteobacterium*, were investigated in different states (vegetative cells and spores in the case of *Bacillus sphaericus*). The bacteria, isolated from urban soils in the Berlin Tiergarten Park and from a sewage field (in Berlin-Buch), were grown in a bioreactor on sterilized soil samples and in batch cultures on four different synthetic materials with hydrophobic and hydrophilic surfaces, to form biofilms. Surface hydrophobicity of the overgrown material was determined by the water contact angle, and cell surface characteristics of bacteria were measured using the zeta potential and a hexadecane-two-phase-system.

The α-*Proteobacterium* and *Variovorax paradoxus* were classified as hydrophobic, and *Bacillus sphaericus* was classified as hydrophilic. Contact angles of the overgrown synthetic materials showed a significant influence of the respective material, but differences between the bacteria were not significant. The differences between the materials may be due to effects of the material on biofilm growth or extracellular polymeric substances (EPS) properties. EPS form biofilm matrices and are mainly responsible for aggregate cohesion. The bacterial EPS of the hydrophobic α-*Proteobacterium* were more hydrophilic than the cell walls, while those of *Bacillus sphaericus* were less hydrophilic. In contrast to the artificial materials, differences in

contact angle for the inoculated soil sample showed significant differences between the bacterial strains. The α-*Proteobacterium* was able to hydrophobize the soil sample, while *Bacillus sphaericus* showed significant hydrophilisation. The results clearly demonstrate the effect of bacterial biofilms on soil wettability.

Keywords

soil water repellency, biofilm, contact angle, hydrophobicity, EPS, extracellular polymeric substances

Introduction

Soil water repellency is a wide spread phenomenon, which affects infiltration as well as soil water retention and plant growth. It is responsible for phenomena like surface runoff, erosion and preferential flow. Due to this high relevance, a great number of studies have been conducted on possible causes of water repellency, but the current results point to a variety of factors of influence rather than a unique mechanism.

The water content is one of the main influencing factors for the current water repellency (e.g., King, 1981; Quyum et al., 2002; Ritsema et al., 1998). Further studies point to an additional influence of time (Doerr et al., 2000; Schaumann et al., 2005), of sample history (Hurraß and Schaumann, 2006) and of drying temperature (Bayer and Schaumann, 2006) on wettability. Kinetic investigations show that the wetting mechanism may vary from location to location and is governed by either physical or chemical processes (Diehl and Schaumann, 2006).

Wettability is additionally affected by the respective composition of soil organic matter (e.g., Capriel et al., 1995), although this relation cannot be found in all locations (Hurraß and Schaumann, 2006). Aliphatic hydrocarbons and amphiphilic substances (mainly long-chain fatty acids, n-alkanes and cycloalkanes) were related to water repellency (e.g., Franco et al., 2000; Hudson et al., 1994; Roy et al., 1999), with plant-derived waxes and humic substances responsible for decreased wettability in some soils (Ma'shum and Farmer, 1985; McKenna et al., 2002).

There is also evidence for a biological contribution to soil water repellency. Soil fungi were found to increase soil water repellency (e.g., Bond and Harris, 1964; Chan, 1992; Dekker and Ritsema, 1996). Hallett and Young found an increase in water repellency after increase in microbial activity (Hallett and Young, 1999). The first direct evidence of the effect of fungal activity on water repellency was found by White and co-workers, who observed a strong increase in water repellency of soil after inocculation with the two fungal strains *Coriolus versicolor* and *Phanerochaete chrysosporium* (White et al., 2000). This was explained by fungal production of hydrophobic compounds (White et al., 2000). On the other hand, results reported by Feeney and co-workers do not support the assumption of a close relationship between fungal biomass and degree of water repellency (Feeney *et al.*, 2006). Some of the ambiguous results may be due to methodical problems, because the addition of fungicides or pesticides already affects soil physicochemical characteristics (Jaeger *et al.*, 2006), and the applied biocides may reveal lower effectiveness than expected (Feeney *et al.*, 2006). Therefore, there is a large need of research to separate experimental artefacts from microbial effects.

Bacteria may contribute to the wetting properties of soil samples by the formation of biofilms. Biofilms grow on nearly every surface that is in contact with

91 water and are enclosed in a slime matrix. They play an important role for the physico-
92 chemical features of soils. In most cases, they are composed of mixed communities
93 of microorganisms and their metabolic products. The predominant components of
94 these slimes are extracellular polymeric substances (EPS) (Wingender et al., 1999),
95 which account for approximately 90% of the enveloping matrix polymers (Jones et
96 al., 1969). EPS are biological polymers synthesized by microbial cells, they vary
97 greatly in their composition and hence in their chemical and physical properties. The
98 exact structure of any biofilm is defined by the environment in which the biofilm
99 develops (Sutherland, 2001). EPS mainly consist of a mixture of polysaccharides and
100 proteins, but also nucleic acids and lipids (Chappell and Evangelou, 2002; Flemming
101 and Wingender, 2000). Some of these polysaccharides are neutral or polyanionic, as
102 in the case of EPS of gram negative bacteria (Sutherland, 2001). Sutherland (2001)
103 noted that EPS may be hydrophobic, although most types of EPS are both
104 hydrophobic and hydrophilic. The EPS form a highly hydrated gel in a three-
105 dimensional network structure (Danese et al., 2000), which mediates the formation of
106 cell aggregates (e.g. biofilms and flocs) in natural environments. There are
107 indications that the EPS structure is not only a random accumulation of biopolymers,
108 but carries elements of organization and provides significant ecological advantages
109 for the inhabitants of microbial aggregates (Flemming and Wingender, 2000).

110 In soils, biofilms are represented by aggregations of bacteria attached to soil
111 particles. The EPS play an important role for bacterial attachment in soils
112 (Böckelmann et al., 2003), as they promote adherence to substrates. The
113 polysaccharides may occur as a network of strands in the interparticle porosity of
114 clay minerals and build up an organomineral network with extensive interparticle
115 bridging (Chenu, 1993). EPS furthermore reveal strong water-holding properties

(Chenu, 1993; Flemming and Wingender, 2000), and therefore increase the water retention of clay minerals or sands and reduce desiccation and rehydration rates (Chenu, 1993). The increased water repellency in sandy soils upon microbial growth (Bond and Harris, 1964), and the increase in repellency of aggregates due to enhanced microbial activity in the field and in laboratory experiments (Hallett and Young, 1999) also point to a contribution of EPS to water repellency. Microbial extracellular polysaccharides can be an important factor affecting soil structure, as they contribute to the stability of soil aggregates (Roberson et al., 1996).

Infiltration of water into non wettable soils can often be improved by the application of nonionic surfactants, which reduce the surface tension of water. Microorganisms are known to synthesize and excrete surfactants, these biosurfactants can also render the bacterial cell surface more hydrophobic (Al-Thahhan et al., 2000).

Despite the possible relevance and expected effects of biofilms on soil water repellency, there is a lack of knowledge, to which extent biofilms may affect surface properties of soil samples. The objective of this study was to obtain first information on the extent of the relevance of bacterial EPS on soil water repellency. We hypothesized that bacterial biofilms are able to either hyrophilize or hydrophobize soil samples and artificial materials depending on their own surface hydrophilicity. We measured the soil-water contact angle of three different bacterial strains (*Variovorax paradoxus*, *Bacillus sphaericus* and an *alpha- Proteobacterium*) on sterilized soil samples. The bacterial strains varied in their cell wall hydrophobicity. Although the initial situation – sterilized soil samples – does not reflect the real soil conditions in the field, it allows to judge individual bacterial contributions to overall repellency and to exclude the effect of changes in microbial communities during laboratory

experiments. In order to judge the surface properties of the biofilms, we additionally investigated the influence of the biofilms of the same bacterial isolates on the surface properties of synthetic materials. The obtained contact angles were compared with the respective cell hydrophobicity determined by water-hexadecane-distribution and with the zeta potential of the cells.

Material and Methods

Bacteria

Three bacterial isolates (71CF4, T7E1, T7TSA1) from urban sites in Berlin were used for the investigation of the influence of bacterial biofilms on soil samples. 16S rDNA sequencing of the isolated bacteria revealed strain 71CF4 as *Variovorax paradoxus* (99% identity), T7E1 as *Bacillus sphaericus* (99% identity) and T7TSA1 as an α-*Proteobacterium* (98% identity). The gram positive *Bacillus sphaericus* and the α-*Proteobacterium* were isolated from the Ah-horizon of the Tiergarten Park Berlin. *Variovorax paradoxus* (β- *Proteobacteria*) was isolated from the Ah-horizon of a former sewage field in Berlin Buch.

Batch experiments: Biofilm gowth on synthetic materials

Slides of polyethylene, polystyrene, polyvinyl chloride and glass (2 cm x 4 cm) were used as model sorbents for bacterial biofims. The contact angle of these materials was determined before and after treatment with the media (control samples). The slides were placed into batch reactors filled with 200 mL 10% Trypticase Soy Broth (TSB) (Becton Dickinson, New York, USA; pancreatic digest of casein 17.0 g; papaic digest of soybean meal 3.0 g; sodium cloride 5.0 g; dipotassium phosphate 2.5 g; dextrose 2.5 g; distilled water 1000 mL); pH =

164 7.3 ± 0.2. Each reactor was inoculated with one colony of the investigated bacterial
165 strains, respectively. The batch reactors were incubated at 28℃ on a horizontal
166 shaker at 100 rpm for seven days. Nine to fifteen replicates for each material and
167 each microbial strain were set in order to conduct statistical analyses.

168 After incubation, the slides were taken from the batch reactor, rinsed gently
169 with 1x phosphate buffered saline (PBS) (NaCl 8.0 g; KCl 0.2 g; NaH_2PO_4 1.44 g; K
170 H_2PO_4 0.24 g; distilled water 1000 mL, pH 7.4) and the contact angle was determined
171 by means of the Wilhelmy plate method (see below). Optical density of the bacterial
172 suspensions at a wavelength of 588 nm was determined to estimate bacterial growth.
173 Zeta potential was determined in 10% TSB medium and in 0.85% NaCl solution with
174 a Malvern Zetasizer (DTS 5200, Worcestershire, UK) in order to estimate the cell
175 surface charge. Control values of zeta potential were determined for pure 0.85%
176 NaCla solution and 10% TSb medium.

177 Biofilm formation on slides was examined under a fluorescence microscope
178 (Zeiss Axioskop, Oberkochen, Germany) after staining with 4´,6-diamidino-2-
179 phenylindole (DAPI, Sigma, Deisenhofen, Germany). The slides were removed from
180 the bach reactor, rinsed with 1x PBS buffer and air dried. The biofilms were
181 incubated with 10 µl DAPI (10 µg mL^{-1}) for 10 min in the dark prior to the
182 examination. Characterization of the biofilms was performed using a scanning
183 electron microscope (REM S-2700, Hitachi, Japan). The slides were taken from the
184 batch reactor and fixed in 3.7% formaldehyde for 4 h at 4℃. After fixation, the slides
185 were washed with 1x PBS buffer and dried. Prior to the examination, the slides were
186 sputtered with a thin gold layer.

187 **Soil reactor: Biofilm growth on soil sample**

188 The soil sample originated from the Berlin inner-city park Tiergarten, where a
189 strongly developed heterogeneity of water repellency was observed (Hurraß and
190 Schaumann, 2006). The soil sample (85% sand, 11% silt and 4% clay) showed
191 severe water repellency with a water drop penetration time of several hours. It was
192 taken from 10-15 cm depth and revealed a pH of 4.8. The water content of the field
193 moist sample was (8 ± 1)% related to dry mass, and the sample contained (4 ± 1)%
194 organic matter. The overall sample characteristics were comparable to those of the
195 water repellent samples from Tiergarten described in Hurraß and Schaumann (2006).
196 The sample was air-dried and sieved to 2 mm. 130 g of freshly collected soil samples
197 were filled into 2 L Schott flasks (Schott, Mainz, Germany) and sterilized by
198 autoclaving for 20 min at 121℃. The soil samples were inoculated with one of the
199 bacterial strains (*Bacillus sphaericus*, *Variovorax paradoxus*, α-*Proteobacterium*),
200 respectively and agitated during the incubation time.

201 Figure 1

202 The reactor was maintained with 10% TSB medium with a continuous flow of
203 60 mL h^{-1} throughout the incubation period. During incubation, the supernatant was
204 removed every 12 hours, after a settling period of 30 min. After seven days of
205 incubation, the soil samples were removed from the reactor. The soil was collected
206 by vacuum filtration onto a glass membrane filter and dried on silica gel in an
207 exsiccator. The collected soil was used for determination of contact angle by the
208 sessile drop method. The part of the sample, which was not fixed on the filter, was
209 used for the determination of the Wilhelmy plate contact angle. A sterile soil sample,
210 not inoculated with bacteria, served as a control. Each treated soil sample was
211 investigated by four to nine replicates.

Wilhelmy Plate Contact Angle

Method: We investigated the Wilhelmy Plate contact angle of the coated synthetic slides and of slides covered with the crushed soil . The Wilhelmy Plate method allows determination of the contact angle of flat surfaces. It is a dynamic method, which includes continuous immersion of a plate or a body into water. During immersion, a balance records the development of the effective sample weight, and the contact angle is calculated under consideration of the water surface tension and the buoyancy (see Diehl and Schaumann, 2006). The measurements were conducted with a Dynamic Contact Angle Tensiometer (DCAT 21, Dataphysics, Filderstadt, Germany), and the data were evaluated with the respective software (SCAT Version 2.3.8) and then corrected according to the procedure described in Diehl and Schaumann (2006). From preliminary experiments, we obtained a measurement error of $\Delta\Theta = \pm 5°$, which denotes the minimum error to be assumed. In the results, we give the standard deviation as long as it exceeds 5°, but report the measurement error for lower standard deviations.

Sample preparation was conducted as follows: The synthetic slides (PE, PVC, PE, glass) covered with biofilm were removed from the batch cultures and used directly for contact angle determination. In addition to the plates covered with biofilm, we investigated the original materials without pre-treatment, after cleaning with 70% ethanol and after immersion in the 10% TSB medium (control). The soil samples were fixed as thin layer on a glass slide provided with double side adhesive tape prior to measurement. As the samples were partly aggregated after incubation, we had to destroy the aggregates in order to obtain samples suitable for the Wilhelmy Plate method. Therefore, our contact angles do not represent contact angles of aggregated

soil. This might represent a source of error, and we therefore investigated the soil samples additionally with the sessile drop method (see below).

Evaluation of surface hydrophilization If the slides are not fully covered with biofilm, the contact angle of the overgrown synthetic materials is a function of the surface characteristics of the material and of the biofilms, weighted by the degree of surface coverage by biofilm. As the characteristics of both surfaces contribute to the measured contact angle, we discuss the biofilm effect in terms of surface hydrophilzation. The degree of surface hydrophilization ($\Delta\Theta_{Hydrophil}$) by the biofilms was calculated from the difference between the control contact angle and contact angle of the respective overgrown material:

$$\Delta\Theta_{Hydrophil} = \Theta_{control} - \Theta_{material} \qquad (1)$$

Sessile Drop Contact Angle

For the determination of the contact angle by the sessile drop method, soil samples fixed on glass membrane filters were used. The filters were cut into 10 x 10 mm pieces and three drops of water (50 µl) were placed on the sample. The shape of the drop was documented with a digital camera Nikon coolPix 5400 (NIKON, Düsseldorf, Germany). The contact angle was calculated from the parameters of the ellipse fitted to the sessile drops (see Diehl and Schaumann, 2006 for further details). The errors given in the tables correspond to the respective standard deviation.

Cell Surface Hydrophobicity

The cell surface hydrophobicity was determined with the Hexadecane method according to Rosenberg et al. (1980). The method bases on the distribution of the bacterial cells between the aqueous solution and the hexadecane phase or the

water-hexadecane interface, with the distribution coefficient applied as measure for the surface hydrophobicity (Rosenberg et al., 1980).

5 mL of the bacterial suspension were centrifuged at 5800 x *g* for 10 min. The pellet was washed twice with 5 mL of phosphate urea magnesium-buffer (PUM) (K_2HPO_4 22.2 g; KH_2PO_4 7.26 g; urea 1.8 g; $MgSO_4$ 0.2 g; distilled water 1000 mL; pH 7.1) and re-suspended in 5 mL PUM buffer. The optical density of the obtained suspension was measured at a wavelength of 558 nm (OD_0). Then, 1 mL of n-hexadecane was added and the suspension was allowed to settle for 10 min. The mixture was then shaken vigorously for 90 sec twice with a waiting period of 10 min between the first and second shaking event. After another 15 min, the optical density of the aqueous phase was measured for a second time (OD_H). The cell surface hydrophobicity was calculated as follows:

$$HY = \frac{OD_0 - OD_H}{OD_0} * 100\ \% \qquad (2)$$

The higher HY, the more hydrophobic are the cell surfaces. As the threshold between hydrophilic and hydrophobic is not uniquely defined in the literature, we operationally define cell walls as hydrophobic if HY is higher than 50%, i.e. more than 50% of the cells have left the aqueous phase; hydrophilic cells are related to HY values below 50%. According to van Loodsrecht et al. (1987), bacterial cells collected on a filter revealed contact angles between 60° and 90° for HY values above 50%. Therefore, hydrophobic bacterial cell walls in terms of our definitions would be related to contact angles of 60° and more.

Results and Discussion

Cell surface characteristics

Variovorax paradoxus and the α-*Proteobacterium* grow as smooth colonies on solid medium, while *Bacillus sphaericus* formed a widely ramified mycel. All three bacteria are rod shaped with sizes between 2 µm and 4 µm; only *Bacillus sphaericus* was able to form endospores. 16S rDNA sequencing revealed the α-*Proteobacterium* as a mixed culture, in which two or more microbial strains coexist in symbiosis and are not separable. Figure 2 shows SEM photographs of *Bacillus sphaericus* and *Variovorax paradoxus* grown on glass slides, covered with slime (EPS). *Bacillus sphaericus* formed chains of two or more cells, while *Variovorax paradoxus* rather formed randomly arranged aggregates.

Figure 2

Table 1 summarizes surface characteristics of bacterial cell walls and biofilms of the investigated microbial strains. According to the water-hexadecane distribution, *Bacillus sphaericus* was categorized as hydrophilic (HY = 12%), while *Variovorax paradoxus* and the α-*Proteobacterium* mixed culture were hydrophobic (HY = 65% and 85%, respectively). The zeta potentials indicated negatively charged bacterial cell walls for all strains. The zeta potential of the cells suspended in 0.85% NaCl solution showed the highest negative value for the hydrophobic *Variovorax paradoxus* (-30 mV), while the hydrophobic α-*Proteobacterium* and the hydrophilic *Bacillus sphaericus* revealed a comparable, less negative zeta potential (-12 mV and –16 mV, respectively). This is in accordance with Rosenberg and Kjelleberg (1986) who found, that most bacteria are negatively charged, but still contain hydrophobic surface components. The zeta potential values are also in accordance with those

304 reported for some bacterial strains and yeast when suspended in modified tap water,
305 e.g.: *Escherichia coli* -12 mV, *Staphylococcus aureus* -13 mV, *Bacillus subtilis* spore
306 -20 mV, and *Saccharomyces cerevisiae* was -7 mV (Kondo et al., 2005), and with the
307 general observation that bacterial cell walls are negatively charged around neutral pH
308 (e.g., James, 1957; Li and Logan, 2004; Salerno et al., 2004). Zeta potential of
309 bacterial cells is known to be affected by pH and ionic strength. The point of zero
310 charge was reported to pH=4.4 for *Mycobacterium bovis,* BCG vaccine*,* Tice
311 substrain (Zhang et al., 1988) or *Bacillus subtilis* B 213 at pH= 2.0 (Ahimou et al.,
312 2001), which is supported by the negatively charged cell walls at our experimental pH
313 (7.0).

314 The 10% TSB medium reveals a lower ionic strength than 0.85% NaCl
315 solution, and contains additional, partial colloidal organic compounds, mainly casein,
316 which explain the negative zeta potential of the control. The zeta potential of the
317 suspensions in the 10% TSB medium was more negative than in the 0.85% NaCl
318 solution, and the three bacterial strains did not react uniformly on the change in
319 solution composition. In contrast to the 0.85% NaCl solution, the most negative zeta
320 potential was observed in the 10% TSB medium for the hydrophobic *α-*
321 *Proteobacterium* (-46 mV), while neither *Variovorax paradoxus* nor *Bacillus*
322 *sphaericus* changed the zeta potential of the bacteria-free 10% TSB medium
323 significantly. This suggests that under the conditions in the 10% TSB medium, only
324 the hydrophobic α-*Proteobacterium* revealed a significant negative charge.

325 In accordance with literature, the changes in zeta potential are due to
326 physicochemical changes at the cell wall surface. They may be explained by the
327 lower ionic strength of the solution and additional interactions between the bacterial
328 cells and the organic colloids or with the phosphate ions: zeta potential was reduced

329 with increasing ionic strength (James, 1957; Uyen et al., 1989), and Na-
330 pyrophosphate altered the surface charge of bacterial cells significantly (Sharma et
331 al., 1985). It was also shown that in high ionic strength environments, bacteria with
332 rough cell walls have greater zeta potential absolute values than smooth cells, and
333 in low ionic strength, the zeta potential of both forms coincides (James, 1957). This
334 fits with our observations in 10% TSB, and the results thus suggest possibly rougher
335 cell walls for Variov*orax paradoxus* than for *Bacillus sphaericus*. Our observation that
336 changes in solution chemistry composition affect the zeta potential of the three
337 strains to different extent, point to the relevance of specific physicochemical
338 mechanisms rather than pure physical effects, which need to be investigated in more
339 detail in future studies.

340 Table 1

341 **Contact angle of biofilms and soil samples**

342 The contact angles between water and the artificial materials covered with
343 biofilms are listed in Table 1 together with the control values of the materials covered
344 with 10% TSB medium, but without biofilm. As expected, the control contact angles
345 of the organic polymers without biofilm indicate hydrophobic surfaces with contact
346 angles between 82° and 88°, and the biofilm-free gl ass-water contact angle (42°)
347 indicates hydrophilic surface characteristics.

348 The resulting contact angles of the overgrown slides range between 27° and
349 45° for all three strains and all investigated synt hetic materials. Upon air-drying for 72
350 h, the contact angles of all samples increased by 20°-50°, and this effect was
351 reversible in terms of remoistening (data not shown).

352 Differences in contact angle between the three strains were not significant (α =
353 0.05). Therefore, we combined data for the three strains for each material in order to

354 evaluate possible differences between the materials. As the slides were not fully
355 covered with biofilm, the characteristics of both surfaces contribute to the measured
356 contact angle, and we discuss the biofilm effect in terms of surface hydrophilization
357 ($\Delta\Theta_{Hydrophil}$, equation 1). $\Delta\Theta_{Hydrophil}$ is shown in Figure 3 for the four artificial materials.
358 Data of all strains were herein combined for each material. Differences between the
359 means were significant for all pairs except PP and PS ($\alpha = 0.05$), such that the
360 degree of hydrophilization increased in the order glass << PVC < PS ≈ PP: While the
361 mean contact angle was reduced by 51° and 50° on po lypropylene and polystyrene,
362 respectively, it was reduced by 42° on polyvinylchl oride, and neither a significant
363 hydrophilization nor any hydrophobization was observed for glass. The final contact
364 angles on the overgrown organic polymers were even lower than those obtained for
365 glass, which suggests more hydrophilic properties for biofilms grown on the
366 polymeric materials than grown on glass.

367 The application of the results of van Loodsrecht and co-workers (1987) to the
368 cell wall hydrophobicity determined in our study, would suggest contact angles of
369 (60 ± 10)°, (40 ± 10)°, and (10 ± 5)° for the pure cells of the *α-Proteobacterium*,
370 *Variovorax paradoxus* and *Bacillus sphaericus*, respectively. The contact angles of
371 the overgrown slides differ from the expected value at least for the *α-*
372 *Proteobacterium* and *Bacillus sphaericus*. This implicates that the surface
373 characteristics of the cells may differ from those of the biofilms. Despite the lack of
374 significant difference in contact angle between the three bacterial strains, it can thus
375 be concluded that the bacterial EPS are more hydrophilic than the respective cell
376 walls for the α-*Proteobacterium*, and less hydrophilic for the *Bacillus sphaericus*. This
377 is not unexpected, as the EPS matrix consists of different compounds than the cell
378 walls, and its surface characteristics are additionally dominated by the incorporation

379 of high amounts of water. Polysaccharides may be hydrophilic, but can also have
380 hydrophobic properties (Neu and Poralla, 1990). Polymer hydrophobicity can play an
381 important role in determining the behaviour of the polysaccharides at the cell surface
382 or at an interface. The effect of water on the contact angle is demonstrated by the
383 reversible increase in contact angle upon air-drying, which thus shows that the EPS
384 themselves are less hydrophilic than the biofilms, and the final contact angle is
385 additionally strongly dominated by the water content.

386 Exopolysaccharides are also subjected to environmental modulation with
387 respect to composition and molecular mass (Tait et al., 1986), which in turn can
388 affect their capacity to interact with other polymers and cations (Sutherland, 1990). It
389 is also well documented that bacterial cell surface phenotype can change markedly in
390 response to changes in the surrounding growth environment, particularly those
391 brought about by growth rate and nutrient limitation (Brown and Gilbert, 1993; Brown
392 and Williams, 1985).

393 Figure 3

394 The lack of significant differences between the biofilms of the three bacterial
395 strains suggests that on the artificial material surfaces, individual bacterial
396 characteristics were either not pronounced or were obscured by the high data
397 scattering. Even the difference between gram positive bacteria (e.g. *Bacillus*
398 *sphaericus*), which produce only few amounts of EPS and contain no LPS
399 (lipopolysaccharide) in their outer membrane, and gram negative bacteria like α-
400 *Proteobacteria* or *Variovorax paradoxus,* which develop EPS and LPS (Baikun and
401 Logan, 2004), could not be observed in the contact angles of the biofilms.

402 Data scattering may be due to two reasons: (i) the surface characteristics of
403 the biofilm vary strongly, or (ii) the plates are not completely covered with biofilm.

Differences in biofilm properties cannot be excluded, and SEM photographs as well as DAPI counting showed that surface coverage was heterogeneous in all cases, such that the fluctuation in contact angle is most probably due to inavoidable differences in surface coverage. These differences in surface coverage of the slides are the main characteristics of biofilms. As they are non-homogenous bacterial poly-layers, with a mushroom morphology where channels for water and nutrition transport pass through (Costerton *et al.*, 1994), biofilms could never cover a surface homogenously. It was also shown by Wimpenny and Colasanti (1997) that biofilms exist as different growth models, such as heterogeneous mosaics, structures penetrated by water channels and dense confluent biofilms. The resulting contact angle thus corresponds to the area-weighted mean of surface and biofilm contact angle, and the knowledge of the degree of surface coverage of each investigated plate is essential to determine the contact angle of the biofilm.

The differences between the overgrown artificial materials thus may indicate a significant effect of the material on biofilm growth and/or properties, and at our current state of knowledge, it is not possible to distinguish between both explanations. Methods for fast and non-destructive examination of surface coverage are however rare. DAPI counting is not applicable to estimate the biofilm coverage ratio of large areas (1-2 cm^2) of the slides. The evaluation of selected spots is impossible due to lack of representativity. For future research, a fast and non-destructive method needs to be developed, such that contact angle and surface coverage can be estimated at the same time on the same slide.

Figure 4 shows sessile drops on the soil samples, and Figure 5 illustrates the contact angle between water and biofilms of the α-*Proteobacterium*, *Variovorax paradoxus* and *Bacillus sphaericus* grown on polypropylene and soil in comparison

with the cell wall hydrophobicity. In contrast to the overgrown artificial materials, differences can be seen between the individual bacterial strains, and the trend in contact angle follows the trend in cell wall hydrophobicity. On a significance level of $\alpha = 0.05$, the trend is as follows:

Θ Sessile Drop: α-*Proteobacteruim* ≈ Control > *V. paradoxus* ≈ *B. sphaericus*

Θ Wilhelmy: α-*Proteobacterium* ≈ Control ≈ *V. paradoxus* > *B. sphaericus*

Figure 4

Figure 5

Sessile drop and Wilhelmy plate method indicate weak hydrophobizing characteristics for the α-*Proteobacterium* and significant hydrophilizing properties of *Bacillus sphaericus*. The sessile drop also suggests hydrophilizing characteristics of *Variovorax paradoxus*. Sessile drop method and Wilhelmy plate method thus suggest the same order of contact angle, although the absolute contact angles differ significantly between both methods. These differences are most probably of methodical character, especially due to the unknown surface roughness of the soil samples (see Diehl and Schaumann, 2006), which are currently investigated and discussed in detail by the group of Bachmann.

Conclusions

Our results demonstrate that bacterial biofilms affect the surface characteristics and are able to hydrophilize hydrophobic surfaces. These findings fit with the results of Roper (2004) who found in increase in soil wettability after inoculation soil with wax-degrading bacteria. Activating these bacteria with lime

addition to the soil also resulted in a decline of repellency of the soils (Roper, 2005). In order to investigate the effect of individual bacterial strains on biofilm surface characteristics, it is essential to determine the surface coverage.

Depending on the bacterial strain, bacterial biofilms can hydrophilize (e.g., *Bacillus sphaericus*) or hydrophobize (e.g., *α-Proteobacterium*) soil samples. The order in contact angle follows the order of cell wall hydrophobicity. The significant differences ($\alpha = 0.05$) between the bacterial strains on the soil samples suggest that biofilm growth has a more pronounced effect on water repellency of soil samples than on the artificial materials. The lower variability of contact angles on soil samples than on the synthetic materials gives rise to the assumption that the soil may represent a better growth medium than the smooth artificial surfaces. This would result in a more reproducible surface coverage in soil, such that differences between bacterial strains are not overbalanced any more by the variability in surface coverage.

The demonstrated effect of bacterial biofilms on soil water repellency shows the relevance of biological factors for the development of water repellent zones in soil. The results also show a promising potential to use certain bacteria for the improvement of soil wettability in the field.

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Table

Table 1 Cell wall hydrophobicity (*HY*) and zeta potential of bacterial cell walls, as well as contact angle of their biofilms grown on synthetic materials and soil. Errors given in the table indicate the respective maximum of measurement error and standard deviation (see materials and methods).

	α-*Proteobacterium*	*Variovorax paradoxus*	*Bacillus sphaericus*	Control
Cell Wall Hydrophobicity HY [%]				
	85 ± 3 hydrophobic	65 ± 8 hydrophobic	12 ± 1 hydrophilic	n.d. n.d.
Zeta potential [mV]				
in 10% TSB medium	-46 ± 4	-33 ± 1	-29 ± 4	-29 ± 6
in 0.85% NaCl solution	-12 ± 4	-30 ± 5	-16 ± 2	0 ± 1
Wilhelmy Plate Contact Angle of synthetic materials overgrown with biofilms [°]				
Glass	42 ± 9	45 ± 8	43 ± 7	42 ± 5
Polypropylene	27 ± 13	35 ± 11	26 ± 7	85 ± 5
Polystyrene	36 ± 13	38 ± 12	32 ± 14	88 ± 5
Polyvinylchloride	39 ± 8	43 ± 10	37 ± 7	82 ± 5
Contact Angle of soil overgrown with biofilms [°]				
Wilhelmy Plate	114 ± 5	107 ± 5	52 ± 5	103 ± 5
Sessile Drop	83 ± 7	68 ± 5	67 ± 5	80 ± 5

n.d.: not determined

Figure Captions

Figure 1 Soil reactor for biofilm growth. The reactor consisted of a 2 L Schott flask provided with 130 g of sterilized and inoculated soil. The TSB pool and the trash flask consisted of a 5 L Schott flask, respectively. The 10% TSB medium was pumped continuously with 60 mL h^{-1} into the soil reactor. The reactor was incubated for 7 days, and every 12 h, the supernatant TSB medium was removed from the reactor and then discarded.

Figure 2 Scanning electron micrographs of *Bacillus sphaericus* (A) and *Variovorax paradoxus* (B) grown on glass slides.

Figure 3 Degree of hydrophilization of artificial surfaces by bacterial biofilms in terms of difference between contact angle of the control and the respective overgrown materials (eq. 1). In this figure, the data of all three bacterial strains are combined. The boxes denote the 25% and 75% percentile; the bars denote the 5% and 95% percentile. The number of data points was 37, 31, 28 and 32 for glass, PVC, PS and PP, respectively. Differences between the means were significant for all pairs except PP and PS ($\alpha = 0.05$), such that the degree of hydrophilization increased in the order glass << PVC < PS ≈ PP.

Figure 4 Representative sessile drops (50 µL) on the control soil sample and the soil samples inoculated with the α-*Proteobacterium*, *Variovorax paradoxus* and *Bacillus sphaericus* (from top to bottom).

Figure 5 Contact angle between water and biofilms of the α-*Proteobacterium*, *Variovorax paradoxus* and *Bacillus sphaericus* grown on polypropylene and soil in comparison with the cell wall hydrophobicity. Soil (Wilh) denotes the contact angle on

642 the soil sample determined with the Wilhelmy plate method, and Soil (Sess) stands
643 for the respective contact angle determined from sessile drops. The error bars
644 characterize the respective maximum value of standard deviation and the
645 measurement error (see experimental section).

Figures

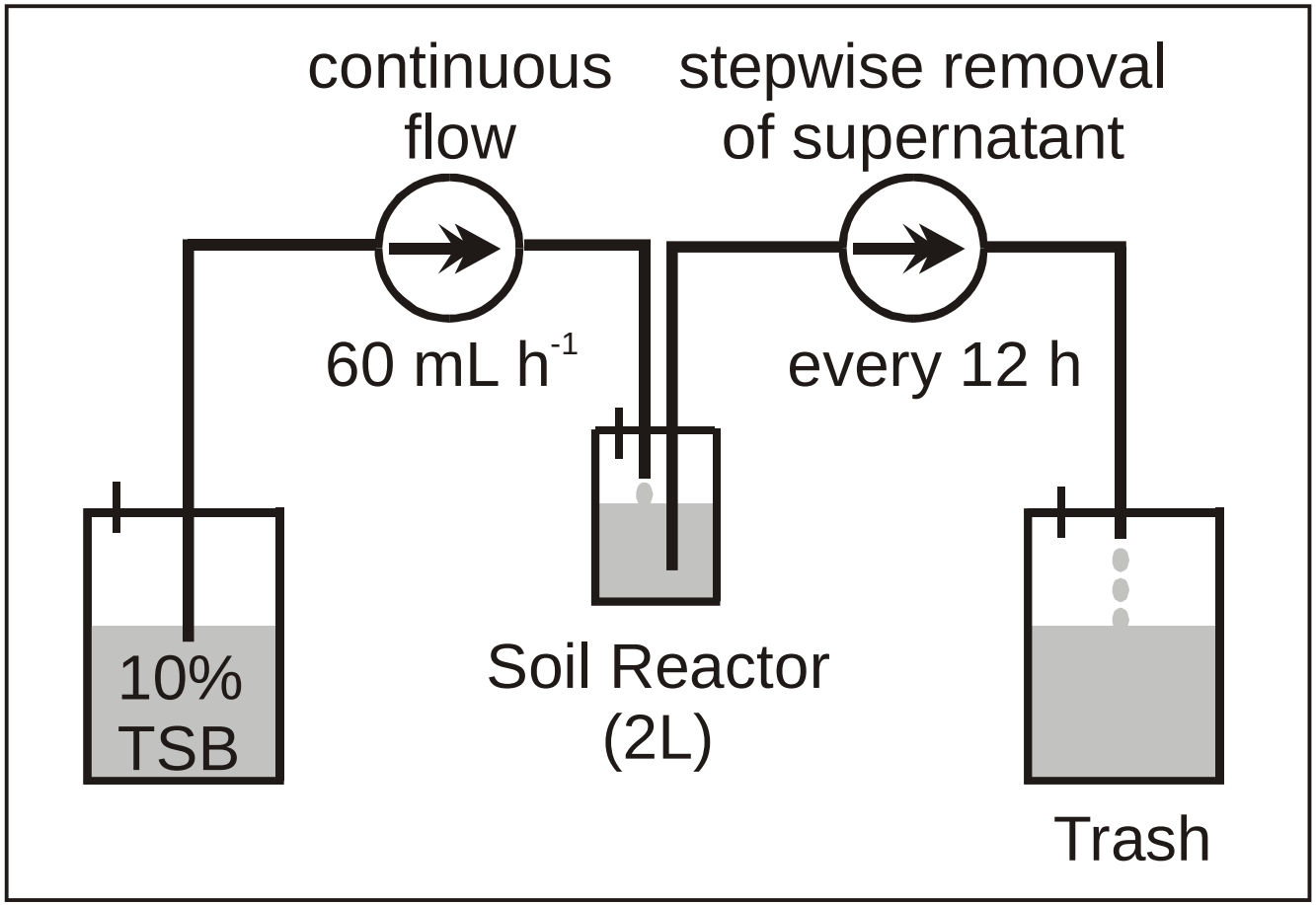
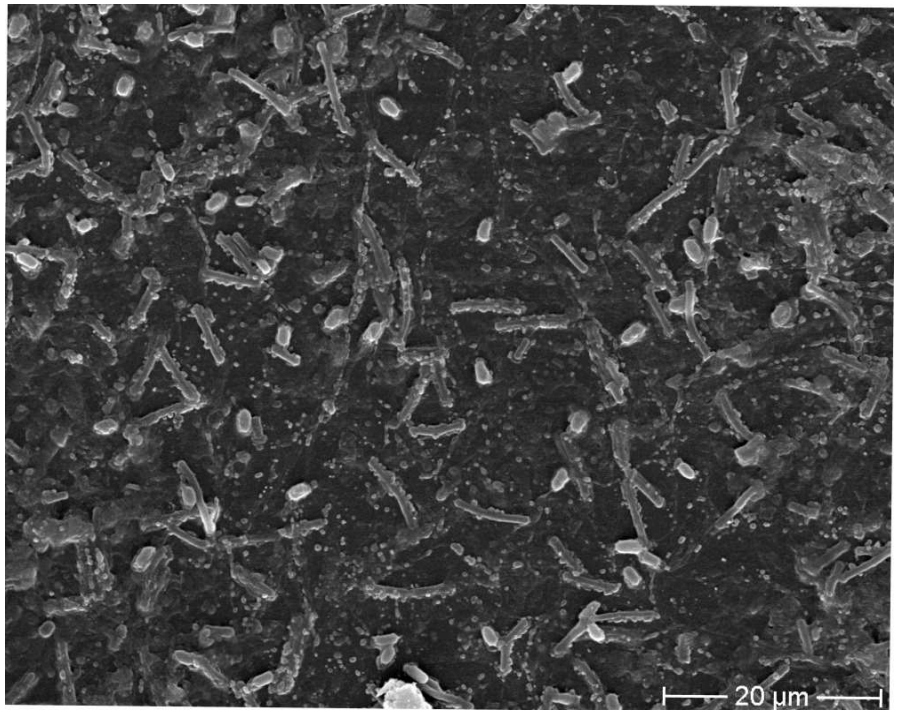

continuous
flow
stepwise removal
of supernatant
60 mL h-1
every 12 h
10%
TSB
Soil Reactor
(2L)
Trash

Figure 1

A



B

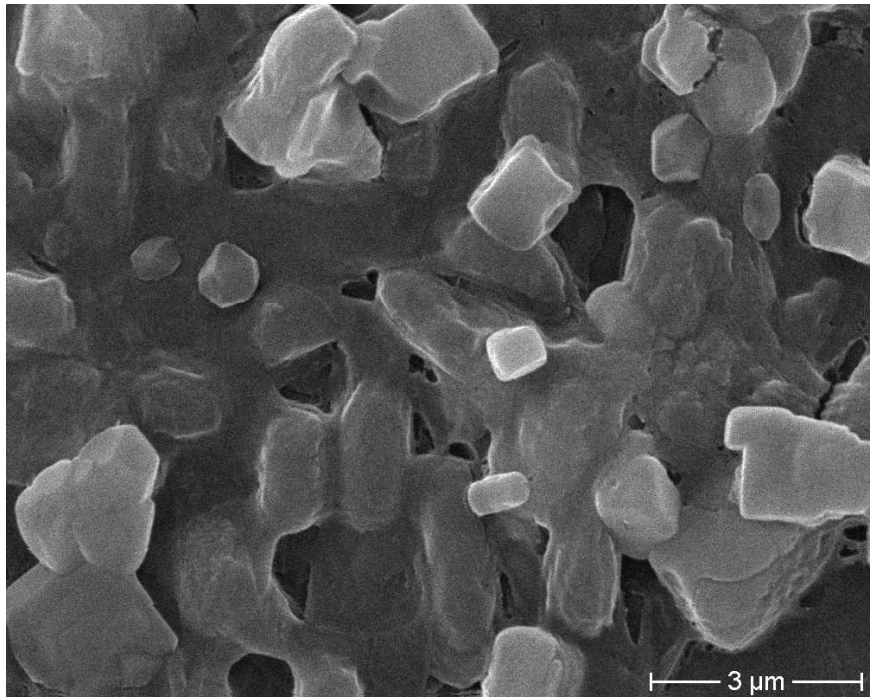


Figure 2

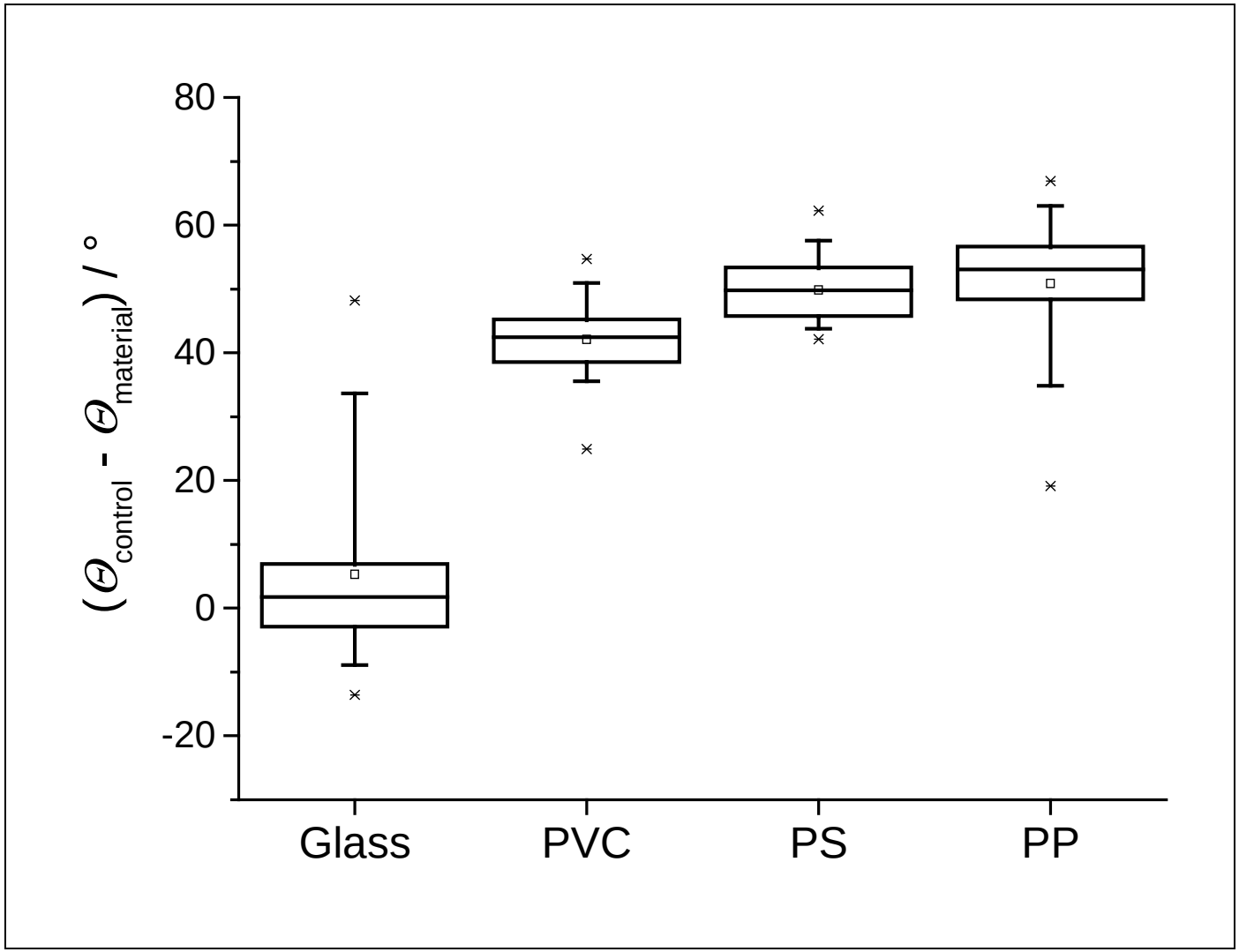


Figure 3

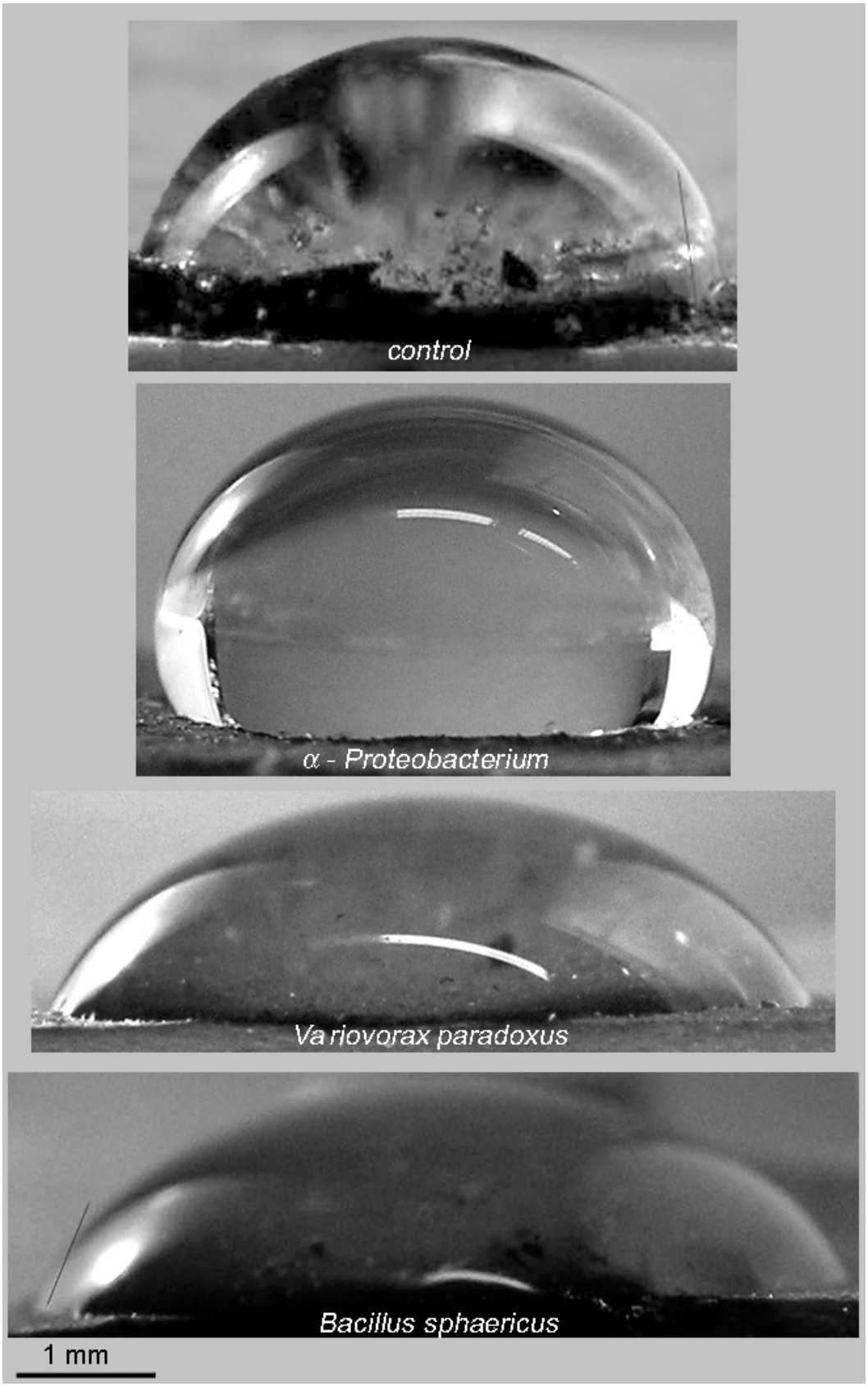

control
α - Proteobacterium
Variovorax paradoxus
Bacillus sphaericus
1 mm

Figure 4

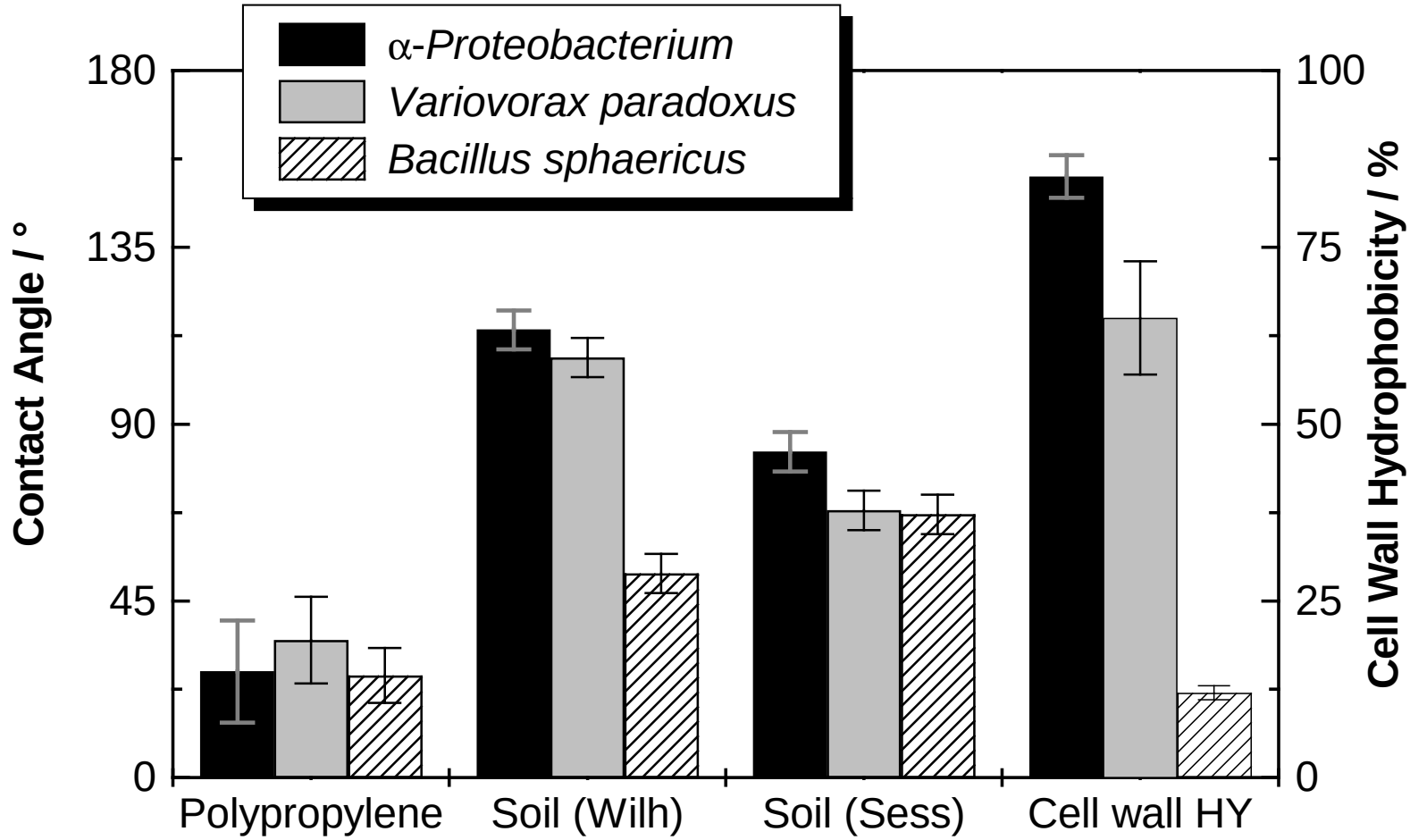

α-Proteobacterium
Variovorax paradoxus
Bacillus sphaericus
Contact Angle / °
Cell Wall Hydrophobicity / %
180
135
90
45
0
100
75
50
25
0
Polypropylene
Soil (Wilh)
Soil (Sess)
Cell wall HY

Figure 5