Determinants of the Bacterial Diversity in Manipulated and Natural Soils

Delita Zul

München 2008

Determinants of the Bacterial Diversity in Manipulated and Natural Soils

Dissertation
Fakultät für Biologie
Ludwig-Maximilians-Universität München

vorgelegt von

Delita Zul
aus Pariaman, West Sumatera

München, Januar 2008

1. Gutacher: Prof. Dr. Jörg Overmann, LMU München

2. Gutacher: Prof. Dr. Dirk Schüler, LMU München

Tag der Abgabe: 29. Januar 2008

Tag des Promotionskolloquiums: 13. März 2008

Dedicated to:

my husband (Tony) and my daughter (Uthie)

ACKNOWLEDGEMENTS

First, I would like to thank Prof. Dr. Jörg Overmann for giving me the opportunity to learn about the amazing bacterial diversity in soil and science, for his supervision of this work and a financial support through the last months of my thesis. I thank Prof. Dr. Dirk Schüler for accepting the role as the second referee.

I would like to express my appreciation to all AGO for a good atmosphere in the lab that has made me feel at home. Many thanks to Dr. Karin Schubert (for her guidance on spectrophotometer), Evelyn (for her guidance on quantitative PCR and "*dirndl*"), Roland (sorry for always stealing your stapler), Jonny (the respiration guy), Isabella, Martina, and Annemarie (for their emotional support). Special thanks to Kajetan for always "being available".

I am grateful to the Technological and Professional Skills Development Sector Project (TPSDP) for a financial support. My dear colleagues in Department of Biology, Faculty of Mathematics and Natural Sciences, University of Riau, Pekanbaru, Indonesia are appreciated for giving me the opportunity to leave my duty for almost 3.5 years.

Furthermore, I am eternally grateful to my family for giving me the truth and encouragement. Most of all I gratefully acknowledge my husband, Anthony Hamzah, for always being there and his endless love (*dan untuk semuanya*). I do not know how I could have finished my work without his support and patience. Very special thanks to my little daughter, Putri Nazeeya Anita (5 yr), for giving me cheerfulness and support through her question "When will you go home, mom?" (*Maafkan mama Uthie. Mama berjanji tidak akan pernah meninggalkan Uthie lagi. Mama sayang uthie sebanyak bintang di langit*).

München

January 2008

PUBLICATIONS ORIGINATING FROM THIS THESIS

CHAPTER 2

Zul, D., S. Denzel, A. Kotz, and J. Overmann. 2007. Effects of plant biomass, plant diversity and water content on the bacterial communities in soil lysimeters: implications for the determinants of bacterial diversity. Appl. Environ. Microbiol. **73**:6916-6929

CHAPTER 3

Zul, D., G. Wanner, and J. Overmann. 2008. *Massilia brevitalea* sp. nov., a novel betaproteobacterium isolated from lysimeter soil. Int. J. Syst. Evol. Microbiol. (in press)

CHAPTER 4

Zul, D., E. Romann, B.U. Fösel, and J. Overmann. 2008. Determinants of the diversity and activity of a bacterial community in Namibian Savanna Soils. (manuscript)

CONTRIBUTIONS OF THE AUTHORS

CHAPTER 2

Profiling of the bacterial community compositions were performed by Delita Zul (*Alpha-, Betaproteobacteria, Bacteroidetes, Verrucomicrobia,* and *Actinobacteria,* species), Sabine Denzel (*Acidobacteria* and *Archaea* species), and Andrea Kotz (*Chloroflexi* species). Delita Zul (employing the MicroDrop technique) and Sabine Denzel (employing the dilution technique of soil slurry) performed cultivation of the soil bacteria. Delita Zul, Andrea Kotz, and one diploma student (Petra Fritsche) conducted screening of the cultures obtained. Moreover, Delita Zul quantified the abundance of phylotype beta10 by the real-time PCR. In addition, the diploma student (Monika Anna Janys) profiled the composition of *Firmicutes* and *Planctomycetes* species.

CHAPTER 3

Massilia brevitalea strain byr 23-80 was isolated, pheno- and genotypically analyzed by Delita Zul. Prof. Dr. Gerhard Wanner took the electron microscopy pictures and wrote the corresponding parts of the paper.

CHAPTER 4

Delita Zul carried out the soil respiration measurements, the soil exoenzyme assays, and cultivation of the soil bacteria. She also did the screening of the cultures obtained and quantified the abundance of nine major (sub)phyla of *Bacteria* in soil by use of the real-time PCR. Moreover, Delita Zul profiled the community composition of *Acidobacteria* and *Actinobacteria* species. Eva Romann counted the total cell numbers. She also did the clone library construction and analyzed the library data together with Bärbel U. Fösel.

I hereby confirm the above statements

Delita Zul Prof. Dr. Jörg Overmann

TABLE OF CONTENTS

CHAPTER 1
Introduction

1.1 Microbial diversity and its implications

Most natural environments harbor highly diverse bacterial communities. Bacteria are ubiquitous and cosmopolitan and they occupy almost all habitats on Earth. They reside not only in temperate habitats such as rice field soil (Chin *et al.* 1999), lake water (Bruns *et al.* 2003a), and agricultural soil (Stevenson *et al.* 2004), but also in extreme habitats such as black smokers (Baross and Deming 1983), hot springs (Hugenholtz *et al.* 1998a), hot deserts (Chanal *et al.* 2006), and cloud water (Amato *et al.* 2007). The total number of bacteria on Earth is estimated to be about $(4\text{-}6)\cdot10^{30}$, consisting of $1.2\cdot10^{29}$ cells in the open ocean, $3.5\cdot10^{30}$ in oceanic, $(0.25\text{-}2.5)\cdot10^{30}$ cells in the terrestrial subsurface, and $2.6\cdot10^{29}$ cells in soil. Totally, the bacterial cells are estimated to contain 350-550 Pg carbon (up to 60-100% of the estimated total carbon found in plants), 85-130 Pg of nitrogen and 9-14 Pg of phosphorus (Whitman *et al.* 1998). As determined by DNA-DNA reassociation, the number of bacteria in 1 g of soil can amount to 50,000 (Sandaa *et al.* 1999), or even up to millions of bacterial species (Gans *et al.* 2005).

Understanding why soil microorganisms are so diverse and which factors control their community composition is of importance because they not only play key roles in environmental processes such as decomposition and recycling of nutrients (Atlas and Bartha 1993), but also produce novel natural products, which are of interest for medicine and industry (Daniel 2004). In addition, soil microorganisms are also essential for controlling CO_2 flux from the soil surface to the atmosphere through microbial respiration (Ryan and Law 2005) and provide other atmospheric gases such as CH_4, H_2, N_2O and NO (Conrad 1996). The total global production of CO_2 from soils is recognized as one of the largest fluxes in the global carbon cycle (Rustad *et al.* 2000). The extent of CO_2 flux from soils estimated is about 76.5 Pg C/yr (Raich and Potter 1995). A small change in soil respiration rate therefore may have impact on atmospheric CO_2 budgets.

Bacterial community composition is linked to environmental factors (e.g., biotic, abiotic, temporal and spatial heterogeneity). It has been observed that alteration of one or more factors influence microbial diversity (Johnsen *et al.* 2001, Fraterrigo *et al.* 2006). However, it is still poorly understood whether functional redundancy or a multitude of ecological niche and adaptive mechanisms modify the abundance and community composition of bacteria in soil. Microbial communities are thought to exhibit a high degree of functional redundancy with several different species occupying a particular niche and a loss of one or a few species exerting little effect on overall environmental processes (Nannipieri *et al.* 2003). In particular, soil ecosystem functions may therefore be robust to changes in functional diversity (Micheli *et al.* 2005). In contrast to plant and animal communities, the functional redundancy of microorganisms has not been well studied to date (Yin *et al.* 2000). However, previous studies indicate that the diversity and functional redundancy of organisms in soil (Yin *et al.* 2000) or oceans (Micheli *et al.* 2005) may be coupled. Bacterial functional redundancy increased from disturbed (tin mine site) to undisturbed land as shown by the increase in the number and diversity of active bacteria (Yin *et al.* 2000). Loss of bacterial species did not interrupt carbon mineralization, denitrification and nitrification processes as observed in sterile soil microcosms that were inoculated with a different dilution factor of bacteria (Wertz *et al.* 2006), suggesting that functional redundancy of bacteria in soil is high. The study of Fuhrman *et al.* (2006) illustrated that biotic and abiotic factors significantly influenced the distribution and abundance of bacteria. Diversity patterns were reproducible over 4.5 years of observation at the southern California coast and a significant subset of bacteria exhibited low levels of functional redundancy. Taken together, functional redundancy of bacteria may therefore play an essential role in maintaining and modify ecosystem functions of soil and oceans.

The term diversity is defined as the number of species present (richness) and their relative abundance (evenness) in the system (Nannipieri *et al.* 2003) and has been defined in different manners. In terms of bacteria, it describes the number of different types of microorganisms occurring together in a community. In molecular-ecological terms, it illustrates the number and distribution of different sequence types

present and/or detected in the genomic DNA extracted from the community in the habitat (Garbeva *et al.* 2004). Species richness refers to the quantitative variation among species, while evenness (also known as equitability) refers to the distribution of individuals among these species. The bacterial diversity in a given habitat can be described by calculating the diversity index. The Shannon-Weaver index is a widely used measure of diversity that covers both species richness and evenness (Atlas and Bartha 1993).

The term "species" is used to describe a unit of biodiversity. So far, different species concepts (taxonomic, evolutionary, and ecological) exist for prokaryotes. A consistent and universal definition of a species is not available in microbiology (Roselló-Mora and Amann 2001). In the case of cultured bacteria, species are currently differentiated by a polyphasic taxonomic approach including pheno- and genotypic analyses (Vandamme *et al.* 1996) and with an emphasis on DNA-DNA hybridization. Presently, a prokaryotic species is defined a group of strains the purified DNA of which show at least 70% DNA-DNA hybridization and over 97% gene sequence identity for 16S rRNA (Stackebrandt and Goebel 1994).

In 1987, Woese introduced a phylogenetic classification system for prokaryotic species based on the nucleotide sequences of small subunit ribosomal RNA (SSU rRNA) molecules. By using SSU rRNA analysis, the number of prokaryotic taxa increased significantly over the past decade. Until 2003, 52 phyla were described (Fig. 1) consisting of 28 established phyla and 24 candidate phyla. Cultured and non-cultured organisms represent established phyla, although most of the latter such as *Acidobacteria, Verrucomicrobia,* and *Chloroflexi* are poorly represented by sequences from cultured organisms (Hugenholtz *et al.* 1998b). Candidate phyla consist only of non-cultured organisms. The number of phyla increased to at least 80 phyla in 2004 where non-cultured organisms still dominated most of the member of those phyla (Fox 2005). The current number of 16S rRNA gene sequences from different environments in GenBank is 471,792 sequences (as of January 2008; RDP-II database, http://rdp.cme.msu.edu), including the sequences from cultured bacteria (7,142 validly named species as of January 2008; Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], http://www.dsmz.de).

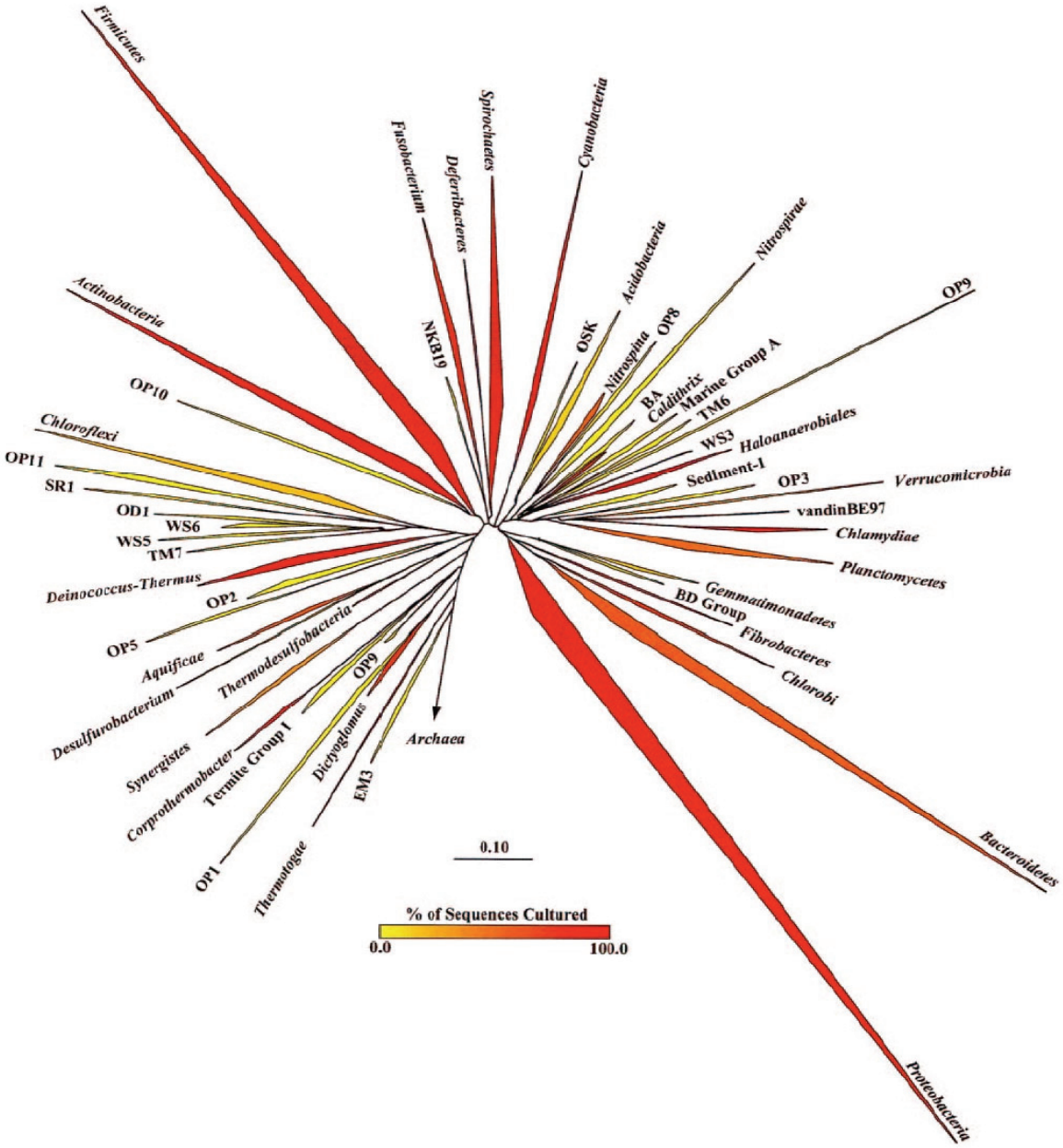


Figure 1. Phylogenetic tree of the *Bacteria* show 28 established phyla (Latin names in italics) and 24 candidate phyla. The vertex angle of each wedge indicates the relative abundance of sequences in each phylum; the length of wedge indicates the range branching depth found in that phylum (Adopted from Handelsman 2004)

1.2 Soil as a habitat and ecosystem

Soil is the most complex and dynamic biological ecosystem and is dominated by a high solid phase that is surrounded by aqueous and gaseous phases. Due to the presence of different soil aggregate sizes, physiochemical conditions are

heterogeneous (Stotzky 1997). This heterogeneity therefore creates different microenvironments, occupied by specific bacteria (Ranjard and Richaume 2001); even at a very fine scale (Nunan *et al.* 2002). Besides protozoa, bacteria and fungi are the most important inhabitants of soil as the total amount of their carbon can attain up to 91.5% (fungi represent ≈ 1.5%), whereas the amount of carbon found in protozoa and nematode range from 1.3 to 9.3% and 0.3 to 0.6%, respectively (as measured in grassland soil; Hassink *et al.* 1993). Bacteria and fungi constitute the largest part of the biomass in soil, e.g., in a temperate grassland soil their biomasses are about 1-2 and 2-5 t ha^{-1}, respectively (Killham 1994). They also determine the decomposition of organic matter, control nutrient cycles of carbon, nitrogen, sulfur and phosphorus (Tate 1999) and maintain soil structure (Dodd *et al.* 2000).

Bacteria are particularly abundant and active in soil surfaces, usually in the O horizon (primarily composed of organic material) and the A horizon (the mineral soil surface where organic material mixes with inorganic products of weathering and dark colored) (Tate 1999). Bacteria either are attached to solid surfaces (approximately 80-90% of the microorganisms in soil; Hattori 1973) or occur freely in water films surrounding soil particles. Soil particle size is divided into three categories: particles between 2 mm and 50 µm (sand), particles between 50 µm and 2 µm (silt) and particles smaller than 2 µm (clay). These particles are arranged and bound together into different size, shape and stability of soil aggregates. With soil particles, bacteria are associated as complexes of clay-organic matter inside soil aggregates (Stotzky 1997). In the sandy soil, bacteria locate inside aggregates as wells as on the surface, but they are more concentrated on the surface of clay aggregates (Chenu *et al.* 2001). They can be found as single cells or small colonies, separated from other cells or colonies by compacted clay mineral layers or by their own extracellular polysaccharides (Foster 1988). By employing a culture-independent approach, it was found that *Gemmatimonadetes* and *Actinobacteria* were highly abundant inside microaggregates (diameter less than 250 µm), while *Acidobacteria* were relatively rich in macroaggregates (diameter > 250 µm) (Mummey *et al.* 2006).

Soil may represent nutrient poor habitats compared to habitats such as skin, oral, gastrointestinal and urinary tracts. However, soil microorganisms are found in

high number and diversity than in other non-human habitats (Stotzky 1997). Numbers of bacteria found in 1 g of soil range between 10^6 and 10^9 (Atlas and Bartha 1993), most of them are heterotrophic as soil contains higher organic matter. Among the culturable fraction of bacteria, nine genera are dominant in soils, namely *Arthrobacter* (5-60%), *Bacillus* (7-67%), *Pseudomonas* (3-15%), *Agrobacterium* (up to 20%), *Alcaligenes* (2-12%), and *Flavobacterium* (2-10%) (Alexander 1977). In contrast, 16S rRNA analysis revealed that nine phyla, namely *Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes,* and *Firmicutes* are dominant in soils (Janssen 2006). Members of phylum *Acidobacteria* and *Proteobacteria* are the most dominant components in soils ranging from 5 to 46% and 10 to 77% of all *Bacteria*, respectively (Janssen 2006). *Proteobacteria* are dominant in wheat fields (Smit *et al.* 2001) and fertilized grassland (McCaig *et al.* 1999), whereas *Acidobacteria* are dominant components in subtropical Australian forest soil (He *et al.* 2006) and arid soils in northern Arizona (Dunbar *et al.* 1999).

1.3 Determinant factors of microbial diversity

1.3.1 Plant diversity and water content

Plants established various interactions with soil microorganisms. These interactions can be advantageous or disadvantageous to both the plants and the soil microorganisms (Tate 1999). Microorganisms in soil are often limited by the availability of organic carbon (Zak et al 1994), so that their community structure is expected to closely correlate to aboveground plants, as a source for those substrates. Bacteria occupy about 4 to 10% of the actual surface of plant roots (rhizoplane), and most of them are associated with soil surrounding plant roots (rhizosphere) (Atlas and Bartha 1993). Bacteria in rhizosphere utilize root exudates released by plants. Root exudates contain sugars, amino acids, organic acids, vitamins, and polysaccharides (Kozdroj and van Elsas 2000), thereby creating unique environments for bacteria in the rhizosphere. Equally, bacteria can influence which compounds the roots exude, thus generating a more favourable environment. Moreover, the quantity and quality of root exudates are different according to plant species (Sauer *et al.* 2006) and development growth stage of plant (Baudoin *et al.* 2002). By means of the

chemical composition of root exudates, plant species select bacterial communities in the rhizosphere (Grayston *et al.* 1998, Smalla *et al.* 2001). Populations of *Burkholderia cepacia* at five development stages of maize rhizosphere (between 3 to 18 weeks after planting) demonstrated a decreasing diversity over time (Di Cello *et al.* 1997). In addition, decomposition of plant litter also determines the presence and composition of the bacterial communities in soil (Dilly *et al.* 2004).

In the beginning, studies about effects of plant species/plant diversity on bacterial community composition have concentrated on the rhizosphere (Kleeberger *et al.* 1983, Lawley *et al.* 1983, Miller *et al.* 1989). More recently, studies have also focused on correlations between the plant species and plant diversity, and the bacterial community in bulk soil (soil free of roots). Different environmental conditions affect the bacterial diversity and functions in bulk soil and the rhizosphere. Generally, bulk soil is characterized by more oligotrophic conditions as it contains less organic substrates than the rhizosphere (Foster 1988). Bulk soil bacteria are less abundant (Smalla *et al.* 2001) and active (Söderberg and Bååth 2004) compared with the rhizosphere bacteria. However, it was reported that composition of the bacterial community in bulk soil differs between unplanted and planted fields (Kowalchuk *et al.* 2002, Smalla *et al.* 2001).

Different effects of plant species composition and plant diversity levels on the bacterial community composition have been reported (Fierer *et al.* 2006, Nunan *et al.* 2005, Grüter *et al.* 2006). Soil liming and nitrogen fertilization but not plant species composition influenced the bacterial community significantly in upland acidic grassland (Kennedy *et al.* 2004). In contrast, plant diversity (Carney and Madison 2005) and plant functional groups (Johnson *et al.* 2003) were shown to exert significant effects in other studies. A higher diversity of plants may produce a more heterogeneous mix of root exudates (i.e., the diversity of carbon present in soil) and thereby influence the composition of soil microorganisms (Hooper *et al.* 2000). Specifically, it has been shown that increases in substrates diversity may support a more diverse microbial community (Grayston *et al.* 1998). On the other hand, there is a possibility that these C substrates interact with each other. When between one to eight of different carbohydrates were added to the soil, no consistent effects either on

soil processes or on soil microbial functional diversity could be discerned (Orwin *et al.* 2006). In line with this, most studies did not show significant effects of plant diversity on the diversity of bacterial community in soil (Table 1). Nevertheless, Zak *et al.* (2003) reported that plant diversity indirectly modify the structure and diversity of bacterial communities via increases of plant biomass production that occurred with greater species diversity. Yet, the implications of plant diversity for microbial communities are not well understood.

Table 1. Summary of studies correlating plant species/diversity and microbial diversity in bulk soil

Plant diversity and ecosystem type descriptions	Methods used	Response of microbial diversity	Reference
Old field planted with different plant diversity levels of grasses and forbs mixture	FAME	No effect of plant diversity	Broughton and Gross 2000
Grassland planted with an increasing plant diversity from monoculture to mixtures of 32 species of grasses, forbs and legumes	BIOLOG	Plant species/diversity stimulates the activity and functional diversity of culturable soil microbia	Stephan *et al.* 2000
Experimental field (Meyendel experiment) treated with bare soil, monoculture of *Cynoglosum offinale* or *Cirsium vulgare*	PCR-DGGE	No significant effect of plant species	Kowalchuk *et al.* 2002
Limestone grassland microcosms treated with bare soil, a 12 species mixture of grasses and forbs, monoculture of *Carex flacca* or *Festuca ovina*	PCR-DGGE	No effect of plant diversity, but plant functional type has a strong effect on the bacterial diversity	Johnson *et al.* 2003
Experimental site planted with 1, 2, 4, 8, and 16 species of grasses, legumes, forbs, and trees	PLFA	No direct effect of plant diversity	Zak *et al.* 2003
Experimental site planted with 1, 3, 5 and >25 plant species of *Cedrela odorata* for monoculture, herbs, palms and hardwoods	PLFA	Significant effect of plant diversity on microbial community composition	Carney *et al.* 2005
Experimental site planted with monoculture of different grasses or forbs employing sandy and chalk soil	PLFA	Significant effect of plant species on microbial community composition in the chalk soil	Bezemer *et al.* 2006
Grassland planted with 1, 2, 3 and 4 combination of grasses and herbs	BIOLOG	Plant diversity affects the activity and diversity of culturable soil microbia	Loranger-Merciris *et al.* 2006

As another factor, soil water content also affects the type of bacteria that dominate in soil. Soil bacteria can tolerate a large range of moisture regime as demonstrated in a study where a mix of soil bacteria was able to maintain the respiration between -8 and -30 bar, but not at -50 bar (Tate 1999). In general, the tolerance of soil bacteria varies in different levels of moisture (Atlas and Bartha 1993). Previous studies did not yield consistent results with respect to the effect of water regime on the diversity and activity of bacteria in soil. A study of Griffiths *et al.* (2003) revealed that bacterial communities in grassland soil (including cultured bacteria as well as total community composition) showed no obvious changes in the diversity in dried, rewetted, or dried and rewetted regimes as revealed by DGGE pattern, whereas the physiological state of cultured bacteria changed. Another study found that soil water content is a major determinant of microbial community composition in microcosm of soil as revealed by phospholipids fatty acid (PLFA) pattern (Drenovsky *et al.* 2004), and drying and rewetting regimes cause decreasing of microbial biomass (Vangestel *et al.* 1993).

1.3.2 Land use and soil properties with special emphasis on Namibia

The effect of land use on biodiversity is a current issue, which has significant impacts on ecosystem functions and processes. Anthropogenic land use changes such as agricultural cultivation, grazing, logging, fire, or fertilizer/manure greatly affect the microbial community composition and alter soil properties. Cultivation, grazing, and logging can cause soil compaction that finally results in increased bulk density and reduced pore spaces (Johnson *et al.* 1991). These changes may influence the abundance and activity of microbial community and subsequently reduce the cycling of several elements, including carbon, nitrogen, and phosphorus (Waldrop *et al.* 2000, Cleveland *et al.* 2003). Long-term effects of past land use (cultivated or logged areas) in southern Appalachian (USA) forest on microbial communities revealed that logging did not influence the major functional group of microbial communities, whereas cultivation altered the communities as compared with reference (i.e., no disturbance history) (Fraterrigo *et al.* 2006). The long-term impact of cultivation also reduced significantly the proportion of *Alphaproteobacteria, Betaproteobacteria,* and *Actinobacteria* (Buckley and Schmidt 2001).

In Namibia, land usage is divided in three distinct categories. Approximately 44% of the country is farmland with freehold tenure, 41% is utilized as communal areas, and 15% is state land including conservation areas (Verlinden and Kruger 2007). Land management systems are also variable. Farmland comprises different types of communal and commercial management. Commercial farming systems have been introduced by European settlers and presently represent the dominating type of land use (Ward *et al.* 1998). In the Kavango region, land is used for dry land cropping and livestock grazing without competition between the two. The cultivated areas are continuously planted according to soil fertility followed by fallow periods of several years (more than 20 year) when soil fertility has vanished. Villagers usually move to find new cultivated areas and choose inter-dune areas with clay soils (**ndombe** = strong soils) because of the longer water holding capacity. Figure 2 shows several land use types in the Kavango region. Organic fertilizers are applied to increase soil fertility, whereas chemical fertilizers are usually not applied due to high cost and long distance. **Mahangu** (millet – *Penisetum glaucum*) is the main crop; about 75% of the cultivated area is used for its production. In general, this subsistence agricultural system is labor intensive. Apart from ox-drawn ploughing most fieldwork is done manually or with very simple tools (knives, axes, hoes) (Jones and Cownie 2001, Michael Proepper personal communication). Fire is also a problem in the Kavango region since villagers sometimes use fire to clear a new land for cultivation. In 1997, the Kavango region represented the largest area affected by fires (IFFN 2000).

Most previous studies that have been conducted in Namibia focused on impacts of land management practices or land use pressures on economics, the diversity of macroorganisms or the environment as a whole (Zeidler *et al.* 2002, Leggett *et al.* 2003, Hillyer *et al.* 2006, Verlinden and Kruger 2007; among others). So far, no study exists on the effect of land use pressures and soil properties on the bacterial community composition in such a subtropical area. It has been shown that land management practices and overutilization of land in Namibia reduced soil nutrients until critical levels (Zeidler *et al.* 2002). Another study did not show any effects of low or heavy grazing on plant cover, seed germination, plant growth and

soil quality in communal or commercial farms in the village of Otjimbingwe (about 200 km north-west of Windhoek) (Ward *et al.* 1998). A study of Uhlmann *et al.* (2004) also revealed that the Arbuscular mycorrhizal fungal (AMF) in villages of Mile 46, Mutompo, Toggekry 250, and Otjiamongombe West 44, Namibia did not seem to be influenced by land management systems, but rather by vegetation cover or rainfall regime. However, the bacterial community composition in the respective soil sampling sites was not investigated. So far, it has remained unclear how land use and soil properties are related to community composition and activities of bacteria in subtropical semiarid soils like those in the Kavango region.

Figure 2. Land use types in the Kavango region, Namibia. **A.** Pristine area, **B.** Grazing area, **C.** Fallow area, **D.** Cultivated area predominantly planted with **mahangu** (millet – *Penisetum glaucum*). White arrow indicates cultivated area next to fallow area (Courtesy of Prof. Dr. Jörg Overmann)

1.4 Cultivation of the non-cultured

Despite the high numbers of bacteria present in natural environments (Whitman *et al.* 1998), typically less than 1% and occasionally up to 19.0% bacteria usually can be cultured in the laboratory (Table 2). The remaining bacteria dominate and are viable, but fail to form visible colonies on laboratory media. These latter bacteria have been described as viable but non-culturable (VBNC). The term VBNC was coined to illustrate a physiological state in which bacteria fail to grow with standard culturing techniques. Recently, the VBNC terminology has been extended to cover the vast majority of environmental microbiology (Bloomfield *et al.* 1998). The VBNC state may represent a survival strategy of bacteria as a consequence of environmental stress conditions such as starvation, changes in temperature, salinity, solar illumination, and oxygen tension (McDougald *et al.* 1998). Bacteria entering the VBNC generally endure a reduction in size, for example, cells of *Vibrio vulnificus* are 3 μm long at log phase (actively growing), but are coccoid about 0.6 μm in diameter during the VBNC state. During the reduction of size, significant changes in membrane structure, protein composition, ribosomal content, or possibly even DNA arrangement occur. The levels of synthesis of DNA, RNA, and protein decrease dramatically (Nilsson *et al.* 1991), and also dramatic decreases in membrane fatty acid composition (Linder and Oliver 1989), nutrient transport and respiration rates. However, not all synthesis has ceased. Protein synthesis seems to be essential for entry the VBNC state, and under this state *V. vulnificus* produces 40 new proteins not seen during growth at normal conditions (McGovern and Oliver 1995). However, bacteria in the non-culturable state that survive and multiply in their natural habitats grow in the laboratory under suitable and favorable conditions. Medium composition is one important factor to resuscitate the cells in non-culturable state (Mukamolova *et al.* 1998a). The resuscitation-promoting factor (Rpf), a protein secreted by *Micrococcus luteus* stimulates growth and cell division and can be used to stimulate the resuscitation of non-cultured bacteria if added in small amounts to growth media (Mukamolova *et al.* 1998b). Kaeberlein *et al.* (2002) argued that providing not only the chemical components of their natural environment but also chemical signals from other bacteria could help to isolate non-cultured bacteria.

Since the full physiological potential of bacteria so far can only be assessed in defined laboratory cultures, they ultimately need to be isolated. Hence, improvements of culturing techniques are mandatory. By modifying the traditional culturing techniques, culturability could be increased and novel bacteria could be recovered from different natural environments. These modifications include the use of different media formulations, pH, gelling reagents (Tamaki *et al.* 2005), media with low nutrients concentration and long incubation times (Janssen *et al.* 2002), and the use of additional signaling compounds (Bruns *et al.* 2003a). High throughput methods by employing microcapsules to trap bacteria (Zengler *et al.* 2002), or single cell manipulation techniques such as optical tweezers and laser microdissection (Fröhlich and König 2000), or MicroDrop microdispenser system (Bruns *et al.* 2003b) are promising cultivation techniques to obtain target isolates. Although culturability increases by such modification of the culturing techniques, the available cultures still represent only a minor part of the bacteria existing in natural environments (Sait *et al.* 2002).

Table 2. Culturability of non-cultured bacteria from several habitats (Modified from Amann *et al.* 1995)

Habitat	Culturability (%)[a]	Reference(s)
Seawater	0.001-0.1	Ferguson *et al.* 1984, Kogure *et al.* 1979, Kogure *et al.* 1980
Freshwater	0.25	Jones *et al.* 1977
Mesotrophic lake	0.1-1	Staley *et al.* 1985
Unpolluted estuarine waters	0.1-3	Ferguson *et al.* 1984
Activated sludge	1-15	Wagner *et al.* 1993, Wagner *et al.* 1994
Sediments	0.25	Jones *et al.* 1977
Soil	0.3	Torsvik *et al.* 1990
Pasture soil	1.4-14.1	Janssen *et al.* 2002
Pasture soil	2.4-19.0	Sait *et al.* 2002
Contaminated soil	0.08-2.2	Ellis *et al.* 2003
Agricultural soil	4-7	Stevenson *et al.* 2004
Freshwater sediment	0.1-1.3	Tamaki *et al.* 2005
Canadian permafrost	0.02	Steven *et al.* 2007

[a] Determined as a percentage of CFU compared to total cells count.

1.5 Quantifying microbial diversity and activity

1.5.1 Microbial diversity

Initially, bacterial diversity was quantified based on cultivation and isolation of bacteria using viable count techniques like the plate count and the Most Probable Number (MPN) techniques. The viable count techniques employ various media and incubation conditions according to the type of bacteria targeted (Atlas and Bartha 1993). The diversity can then be determined by a number of phenotypic tests. These methods are laborious, time consuming, and often select for microorganisms with fast growth rates. Another method that also relies on the cultivation of bacteria but directly analysis the potential activity of soil bacteria communities is the community-level physiological profile (CLPP) as determined in multiwell Biolog plates (Garland and Mills 1991). In principle, the bacterial community is inoculated into each well to obtain information about the potential functional diversity of bacterial population through utilization of specific carbon sources. Culture-dependent methods have a limitation as only a small fraction of bacteria is culturable and accessible to study (Torsvik *et al.* 1990).

Culture-independent methods can overcome problems associated with the limitation of culture-dependent methods and permit to capture a greater fraction of the entire bacterial community. A number of culture-independent approaches have been developed to study microbial diversity and are mostly based on the analysis of small subunit ribosomal RNA (SSU rRNA). In these methods, genomic DNA or RNA is extracted from the environmental samples and purified. The 16S rRNA gene fragments of target DNA are amplified by polymerase chain reaction (PCR) using universal or group-specific primers. The PCR products can be used for preparing clone libraries or are separated by different DNA fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) or amplified ribosomal DNA restriction analysis (ARDRA) (Kirk *et al.* 2004). Among the DNA fingerprinting techniques, DGGE is probably the most widely used for characterizing bacterial communities in environmental samples, as it is reliable, reproducible, rapid and multiple samples can be analyzed at the same time (Muyzer *et al.* 1993).

DGGE was originally developed to detect point mutations in DNA sequences. Muyzer *et al.* (1993) introduced the PCR-DGGE to profile genetic fingerprints of complex microbial communities based on partial 16S rRNA gene. This method separates sequences of the same length but different base composition according to their melting behavior in denaturing gradients. A GC rich clamp of about 40 bases at the 5´end prevents a complete melting of the strands such that at least part of the DNA remains double stranded. By sequencing the DNA bands, one can obtain information about the specific microorganisms in the community. Numerous studies have been conducted to analyse the diversity of bacterial community composition using DGGE. Shifts in microbial community compositions in response to environmental disturbances or defined treatments like seasons (Krave *et al.* 2002), perturbation by agriculture (Øvreas *et al.* 1998), by aboveground plant species (Kowalchuk *et al.* 2002), or water stress (Griffiths *et al.* 2003) could be observed. Soil microbial communities are too complex to be sufficiently analyzed with universal bacterial primers, even though PCR-DGGE is able to detect members of the community that constitute 1% of the population (Muyzer *et al.* 1993). Therefore, group-specific PCR-DGGE can be applied to increase PCR-DGGE resolution (Overmann *et al.* 1999).

Culture-independent methods have facilitated the study of environmental communities without cultivation. These methods provide a possibility to quantify bacterial diversity in natural environment. However, only a combination of culture-independent and culture-dependent approaches will ultimately provide a reasonable understanding of the interaction between the microorganisms and their natural environment (Garbeva *et al.* 2004).

1.5.2 Microbial activity

The activity of soil microbiota can be determined by measuring N mineralization rate, soil respiration rate and soil exoenzyme activities (Taylor *et al.* 2002), and monitoring their gene expressions. Gene expression can be monitored following extraction of soil mRNA by using several methods such as DNA microarrays, real time PCR, competitive RT-PCR, stable isotope probing (SIP) or reporter genes (Saleh-Lakha *et al.* 2005).

Soil respiration is a typical gross parameter of the soil biological activity and positively correlated with soil organic matter, reflecting the intensity of carbon cycling (Ryan and Law 2005). Soil respiration consists of the combined respiration of roots (autotrophic) and soil microorganisms (heterotrophic) in rhizosphere and bulk soil, respectively (Raich and Schlesinger 1992). Controlling respiration rate are soil temperature and soil moisture (Kaur *et al.* 2007, Raich and Schlesinger 1992, Raich and Potter 1995), land use (Frank *et al.* 2006), management practices (Raich and Potter 1995), population and community dynamics of the belowground flora and fauna and aboveground vegetation (Raich and Schlesinger 1992). Soil respiration is very sensitive to various environmental factors and its changes (Rustad et al 2000), hence it can be employed to determine the effect of ecosystem disturbances on the soil microbial communities and ultimately the soil quality. Soil respiration can be quantified (in laboratory or *in situ*) by measuring either CO_2 production or O_2 consumption or both. The microbial respiration rate measured by determining O_2 consumption is mainly analyzed under defined conditions in the laboratory.

Exoenzymes in soil are mainly of microbial origin, but may also originate from plants or animals. These enzymes will hydrolyze extracellular substrates to produce nutrients or organic carbon substrates needed by microorganisms, to detoxify environmental compounds, or to modify the microenvironments of the living cells. Soil exoenzyme activities are often closely related to soil organic matter, soil physical properties, and other microbial activities (Tate 1999). In general, soil enzyme activities change much faster than other parameters, thus providing early indications of changes in soil quality (Bergstrom *et al.* 1998). Disturbance of soil microbial activity, as shown by changes in levels of metabolic enzymes can serve as an estimator of ecosystem disturbance. This relationship has been demonstrated when soil is polluted with heavy metals such as Cd, Zn, Cu and Pb which resulted in the soil exoenzyme activities being lower in polluted soils (Lee *et al.* 2002). The exoenzyme activities in soil can be quantified by the addition and incubation of the soil slurries with fluorogenic enzyme substrates 4-methylumbelliferone (MUF) or 7-amino-4-methylcoumarin (MCA) (Marx *et al.* 2001). Alternatively, colorimetric substrates (remazol brilliant blue), *p*-nitrophenol, or tetrazolium salts coupled with

specific compounds of interest can be used (Wirth and Wolf 1992). The activities of exoenzymes in peatland and agricultural soils were found to be higher at surface layers (Freeman *et al.* 1995, Taylor *et al.* 2002). The enzyme activities revealed strong correlation between exoenzyme activities and organic matter content.

1.6 Aims of the present study

The main objectives of the present study were:

- to compare the bacterial community composition in bare soil and soil planted with different plant diversity levels,
- to analyse water content effects on the diversity of soil bacteria,
- to observe effects of land use and soil properties on the bacterial community composition in soil,
- to study the microbial activity in order to identify the soil functions which were subjected to different land use as a result of anthropogenic activity,
- to cultivate and isolate soil bacteria by using different media combinations in order to retrieve the bacterial key species.

This study focused on the interrelation of soil bacterial communities and functions with the following environmental factors:

1. The effect of plant diversity and water content on the bacterial diversity was studied in manipulated soil from lysimeters at the Ecological Botanical Garden of the University of Bayreuth, Germany.
2. The effects of land use (anthropogenic alterations) and soil properties on the bacterial diversity and activity were studied using natural soil from the Kavango region, Namibia.

Several studies had been conducted to study effects of plant diversity on bacterial diversity (Kowalchuk *et al.* 2002, Stephan *et al.* 2000, Zak *et al.* 2003). Most studies utilized soil samples from natural sites where different plants and soil fauna exist and soil abiotic conditions vary. As a result, no clear interdependence between plant community structure and soil bacterial community may be observed. Consequently, soil lysimeters that had been planted with different plant diversity levels were employed in the present study and environmental conditions were highly

controlled. The effects of plant diversity, water content and seasonal shifts on the bacterial community were studied (Chapter 2). In order to determine which types of bacteria depend on the presence or absence of plants, high throughput cultivation method employing MicroDrop technique was performed and a representative isolate was characterized in detail (Chapter 3).

The land use effects on bacterial communities subtropical semiarid is largely unknown. Soil sample from villages in the Kavango region, Namibia, were therefore employed in a case study. Impacts of land use on soil bacteria were measured by profiling the diversity and community composition of bacteria and determining bacterial activity. The bacterial activity was determined by measuring soil respiration and exoenzyme activities, respectively to predict the effects of current and future land use in the Kavango region, Namibia (Chapter 4).

1.7 References

1. **Alexander, M.** 1977. Introduction to soil microbiology. Wiley&Sons, New York.

2. **Amann, R.I., W. Ludwig, and K-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**: 143-169.

3. **Amato, P., M. Parazols, M. Sancelme, P. Laj, G. Mailhot, and A. Delort.** 2007. Microorganisms isolated from the water phase of tropospheric clouds at the Puy de Dôme: major groups and growth abilities at low temperatures. FEMS Microbiol. Ecol. **59**:242-254.

4. **Atlas, R.M. and R. Bartha.** 1993. Microbial Ecology: Fundamentals and Applications. Benjamin Cummings, USA.

5. **Baross, J.A. and J.W. Deming.** 1983. Growth of 'black smoker' bacteria at temperatures of at least 250°C. Nature **303**:423-426.

6. **Baudoin E., E. Benizri, and A. Guckert.** 2002. Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. Appl. Soil Ecol. **19**:135-145.

7. **Bergstrom, D.W., C.M. Montreal, and D.J. King**. 1998. Sensitivity of soil enzyme activities to conservation practices. Soil Sci. Soc. Ame. J. **62**:1286-1295.

8. **Bezemer, T. M., C. Lawson, K. Hedlund, A.R. Edwards, A.J. Brooks, J.M. Igual, S.R. Mortimer, and W.H. Van der Putten.** 2006. Plant species and functional groups effect on abiotic and microbial properties and plant-soil feedback responses in two grasslands. J. Ecol. **94**:893-904.

9. **Bloomfield, S.F., G.S.A.B. Stewart, C.E.R. Dodd, I.A. Booth, and E.G.M. Power.** 1998. The viable but non-culturable phenomenon explained? Microbiology **144**:1-2.

10. **Broughton, L.C. and K.L. Gross**. 2000. Patterns of diversity in plant and soil microbial communities along a productivity gradient in a Michigan old-field. Oecologia **125**:420-427.

11. **Bruns, A., U. Nübel, H. Cypionka, and J. Overmann.** 2003a. Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. Appl. Environ. Microbiol. **69**:1980-1989.

12. **Bruns, A., H. Hoffelner, and J. Overmann**. 2003b. A novel approach for high throughput cultivation assays and the isolation of planktonic bacteria. FEMS Microbiol. Ecol. **45**:161-171.

13. **Buckley, D.H. and T.M. Schmidt.** 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microb. Ecol. **42**:11-21.

14. **Carney, K.M., and P.A. Matson.** 2005. Plant communities, soil microorganisms, and soil carbon cycling: does altering the world belowground matter to ecosystem functioning? Ecosystems **8**:928-940.

15. **Chanal, A., V. Chapon, K. Benzerara, M. Barakat, R. Christen, W. Achouak, F. Barras, and T. Heulin.** 2006. The desert of Tataouine: an extreme

environment that hosts a wide diversity of microorganisms and radiotolerant bacteria. Environ. Microbiol. **8**:514-525.

16. **Chenu, C., J. Hassink, and J. Bloem.** 2001. Short-term changes in the spatial distribution of microorganisms in soil aggregates as affected by glucose addition. Biol. Fertil. Soils **34**:349-356.

17. **Chin, K.J., D. Hanh, U. Hengstmann, W. Liesack, and P.H. Janssen.** 1999. Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. Appl. Environ. Microbiol. **65:**5042-5049.

18. **Cleveland, C.C., A.R. Townsend, S.K. Schmidt, and B.C. Constance**. 2003. Soil microbial dynamics and biogeochemistry in tropical forests and pastures, Southwestern Costa Rica. Ecol. Appl. **13**:314-326.

19. **Conrad, R.** 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O and NO). Microbiol. Rev. **60**:609-640.

20. **Daniel, R.** 2004. The soil metagenome - a rich resource for the discovery of novel natural products. Curr. Opin. Biotechnol. **15**:199-204.

21. **Di Cello, F., A. Bevivino, L. Chiarini, R. Fani, D. Paffetti, S. Tabacchioni, and C. Dalmastri.** 1997. Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. Appl. Environ. Microbiol. **63**:4485-4493.

22. **Dilly, O., J. Bloem, An Vos, and J.C. Munch.** 2004. Bacterial diversity in agricultural soils during litter decomposition. Appl. Environ. Microbiol. **70**:468-474.

23. **Dodd, J.C., C.L. Boddington, A. Rodriguez, C. Gonzalez-Chavez, and I. Mansur.** 2000. Mycellium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. Plant Soil **226**:131-151.

24. **Drenovsky, R.E., D. Vo, K.J. Graham, and K.M. Scow**. 2004. Soil water content and organic carbon availability are major determinant of soil microbial community composition. Microb. Ecol. **48**:424-430.

25. **Dunbar, J., S. Takala, S.M. Barns, J.A. Davis, and C.R. Kuske**. 1999. Levels of bacterial diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. **65**:1662-1669.

26. **Ellis, R.J., P. Morgan, A.J. Weightman, and J.C. Fry**. 2003. Cultivation-dependent and –independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl. Environ. Microbiol. **69**:3223-3230.

27. **Ferguson, R.L., E.N. Buckley, and A.V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47**:49-55.

28. **Fierer, N**. **and R.B. Jackson**. 2006. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. USA **103**:626-631.

29. **Foster R.C.** 1988. Microenvironments of soil microorganisms. Biol. Fertil. Soils **6**:189-203.

30. **Fox, J.L.** 2005. Ribosomal gene milestone met, already left in dust. ASM News **71**:6-7.

31. **Frank, A.B., M.A. Liebig, and D.L. Tanaka.** 2006. Management effects on soil CO_2 efflux in northern semiarid grassland and cropland. Soil Till. Res. **89**:78-85.

32. **Fraterrigo, J.M., T.C. Balser, and M.G. Turner.** 2006. Microbial community variation and its relationship with nitrogen mineralization in historically altered forests. Ecology **87**:570-579.

33. **Freeman, C., G. Liska, N.J. Ostle, S.E. Jones, and M.A. Lock**. 1995. The use of fluorogenic substrates for measuring enzyme activities in peatlands. Plant Soil **175**:147-152.

34. **Fröhlich, J. and H. König.** 2000. New techniques for isolation of single prokaryotic cells. FEMS Microbiol. Rev. **24**:567-572.

35. **Fuhrman, J.A., I. Hewson, M.S. Schwalbach, J.A. Steele, M.V. Brown, and S. Naeem.** 2006. Annually reoccurring bacterial communities are predictable from ocean conditions. Proc. Natl. Acad. Sci. USA **103**:13104-13109.

36. **Gans, J., M. Wolinsky, and J. Dunbar.** 2005. Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science **309:**1387-1390.

37. **Garbeva, P., J.A. Van Veen, and J.D. Van Elsas.** 2004. Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu. Rev. Phytopathol. **42**:243-270.

38. **Garland, J.L. and A.L. Mills.** 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole-carbon-source utilization. Appl. Environ. Microbiol. **57**:2351-2359.

39. **Grayston, S.J., C.D. Campbell, and A.C. Edwards.** 1998. Selective influence of plant species on microbial activity in the rhizosphere. Soil Biol. Biochem. **30**:369-378.

40. **Griffiths, R.I., A.S. Whiteley, A.G. O´Donnell, and M.J. Bailey**. 2003. Physiological and community responses of established grassland bacterial populations to water stress. Appl. Environ. Microbiol. **69**:6961-6968.

41. **Grüter, D., B. Schmid, and H. Brandl.** 2006. Influence of plant diversity and elevated atmospheric carbon dioxide levels on belowground bacterial diversity. BMC Microbiol. **6**:68.

42. **Handelsman, J.** 2004. Metagenomic: application of genomics to uncultured microorganisms. Microbiol. Mol. Biol. Rev. **68**:669-685.

43. **Hassink, J., L.A. Bouwman, K.B. Zwart, and L. Brussaard.** 1993. Relationship between habitable pore space, soil biota and mineralization rates in grassland soil. Soil. Biol.Biochem. **25**:47-55.

44. **Hattori, T.** 1973. Microbial Life in Soil. Marcel Dekker, New York.

45. **He, J., Z. Xu, and J. Hughes**. 2006. Molecular bacterial diversity of a forest soil under residue management regimes in subtropical Australia. FEMS Microbiol. Ecol. **55**:38-47.

46. **Hillyer, A.E.M., J.F. McDonagh, and A. Verlinden.** 2006. Land-use and legumes in northern Namibia – the value of a local classification system. Agricul. Ecosys. Environ. **117**:251-265.

47. **Hooper, D.U., D.E. Bignell, V.K. Brown, L. Brussaard, J.M. Dangerfield, D.H. Wall, D.A. Wardle, D.C. Coleman, K.E. Giller, P. Lavelle, W.H. van Der Putten, P.C. De Ruiter, J. Rusek, W.L. Silver, J.M. Tieje, and V. Wolters.** 2000. Interactions between aboveground and belowground biodiversity in terrestrial ecosystems: patterns, mechanisms, and feedbacks. Bioscience **50**:1049-1061.

48. **Hugenholtz, P., C. Pitulle, K.L. Hershberger, and N. R. Pace.** 1998a. Novel division level bacterial diversity in a Yellowstone Hot spring. J. Bacteriol. **180**:366-376.

49. **Hugenholtz, P., B.M. Goebel, and N.R. Pace.** 1998b. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180**:4765-4774.

50. **IFFN.** 2000. Fire monitoring and management in Namibia. No. 22-April 2000, p. 49-53.

51. **Janssen, P.H., P.S. Yates, B.E. Grinton, P.M. Taylor, and M. Sait.** 2002. Improved culturability of soil bacteria and isolation in pure culture of novel member of the divisions *Acidobacteria, Actinobacteria, Proteobacteria,* and *Verrucomicrobia*. Appl. Environ. Microbiol. **68:**2391-2396.

52. **Janssen, P.H.** 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. **72**:1719-1728.

53. **Johnson, C.E., A.H. Johnson, T.G. Huntington, and T.G. Siccama.** 1991. Whole-tree clear-cutting effects on soil horizons and organic matters pool. Soil Sci. Soc. Ame. J. **55**:497-502.

54. **Johnson, D., R.E. Booth, A.S. Whiteley, M.J. Bailey, D.J. Read, J.P. Grime, and J.R. Leake.** 2003. Plant community composition affects the biomass,

activity and diversity of microorganisms in limestone grassland soil. Eur. J. Soil Sci. **54**:671-677.

55. **Jones, B. and D. Cownie.** 2001. Socio-ecological survey report for the Kavango region. Every River Has Its People project. Windhoek, Namibia.

56. **Jones, J.G.** 1977. The effect of environmental factors on estimated viable and total population of planktonic bacteria in lakes and experimental enclosures. Freshwater Biol. **7**: 67-91.

57. **Jonhsen, K., C.S. Jacobsen, V. Torsvik, and J. Sørensen.** 2001. Pesticide effects on bacterial diversity in agricultural soils – a review. Biol. Fertil. Soils **33**:443-453.

58. **Kaeberlein, T., K. Lewis, and S.S. Epstein.** 2002. Isolating `uncultivable` microorganisms in pure culture in a simulated natural environment. Science **296**:1127-1129.

59. **Kaur, K., R.K. Jalota, and D.J. Midmore.** 2007. Impact of temperature and defoliation (simulated grazing) on soil respiration of pasture grass (*Cenchrus ciliaris* L.) in a controlled experiment. Agricul. Food Environ. Sci. **1**:1-9.

60. **Kennedy, N.M., D.E. Gleeson, J. Connolly, N.J.W. Clipson.** 2005. Seasonal and management influences on bacterial community structure in an upland grassland soil. FEMS Microbiol. Ecol. **53**:329-337.

61. **Killham, K.** 1994. Soil Ecology. Cambridge University Press, Cambridge.

62. **Kirk, J.L., L.A. Beaudette, M. Hart, P. Moutoglis, J.N. Klironomos, H. Lee, and J.T. Trevors**. 2004. Methods of studying soil microbial diversity. J. Microbiol. Methods **58**:169-188.

63. **Kleeberger, A., A.H. Castorph, and W. Klingmüller**. 1983. The rhizosphere microflora of wheat and barley with special reference to gram-negative bacteria. Arch. Microbiol. **136**:306-311.

64. **Kogure, K.U., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **26**:415-420.

65. **Kogure, K.U., U. Simidu, and N. Taga.** 1980. Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol. **26**:318-323.

66. **Kowalchuk, G.A., D.S. Buma, W. de Boer, P.G.L. Klinkhamer, and J.A. van Veen**. 2002. Effect of a above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Leeuwenhoek **81**:509-520.

67. **Kozdroj, J. and J.D. van Elsas**. 2000. Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. Soil Biol. Biochem. **32**:1405-1417.

68. **Krave, A.S., B. Lin, M Braster, A.M. Laverman, N.M. van Straalen, W.F.M. Röling, and H.W. van Verseveld.** 2002. Stratification and seasonal stability of diverse bacterial communities in a *Pinus merkusii* (pine) forest soil in central Java, Indonesia. Environ. Microbiol. **4**:361-373.

69. **Lawley, R.A., R. Campbell, and E.I. Newman**. 1983. Composition of the bacterial flora of the rhizosphere of three grassland plants grown separately and in mixtures. Soil Biol. Biochem. **15**:605-607.

70. **Lee, I-S., O.K. Kim, Y-Y. Chang, B. Bae, H.H. Kim, and K.H. Baek.** 2002. Heavy metal concentrations and enzyme activities in soil from a contaminated Korean shooting range. J. Biosci. Bioeng. **94**:406-411.

71. **Leggett, K., J. Fennessy, and S. Scheider.** 2003. Does land use matter in an arid environment? A case study from the Hoanib River catchment, north-western Namibia. J. Arid. Environ. **53**:529-543.

72. **Linder, K. and J.D. Oliver.** 1989. Membrane fatty acid and virulence changes in the viable but nonculturable state of *Vibrio vulnificus*. Appl. Environ. Microbiol. **55**:2837-2842.

73. **Loranger-Merciris, G., L. Barthes, A. Gastine, and P. Leadley.** 2006. Rapid effects of plant species diversity and identity on soil microbial communities in experimental grassland ecosystems. Soil Biol. Biochem. **38**:2336-2343.

74. **Marx, M.-C., M. Wood, and S.C. Jarvis.** 2001. A microplate flurometric assay for the study of enzyme diversity in soils. Soil Biol. Biochem. **33**:1633-1640.

75. **McCaig, A.E., L.A. Glover, and J.I. Prosser.** 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbial. **65**:1721-1730.

76. **McDougald, D., S.A. Rice, D. Weichart, and S. Kjelleberg.** 1998. Nonculturability: adaptation or debilitation. FEMS Microbiol. Ecol. **25**:1-9.

77. **McGovern, V.P. and J.D. Oliver**. 1995. Induction of cold responsive proteins in *Vibrio vulnificus* . J. Bacteriol. **177**:4131-4133.

78. **Micheli, F. and B.S. Halpern.** 2005. Low functional redundancy in coastal marine assemblages. Ecol. Lett. **8**:391-400.

79. **Miller, H.J., G. Henken, and J.A. Van Veen.** 1989. Variation and composition of bacterial populations in the rhizosphere of maize, wheat, and grass cultivars. Can. J. Microbiol. **35**:656-660.

80. **Mukamolova, G.V., N.D. Yanopolskaya, D.B. Kell, and A.S. Kaprelyants.** 1998a. On resuscitation from the dormant state of *Micrococcus luteus.* Anthonie Leewenhoek **73**:237-243.

81. **Mukamolova, G.V., A.S. Kaprelyants, D.I. Young, M Young, and D.B. Kell.** 1998b. A bacterial cytokine. Proc. Natl. Acad. Sci. USA **95**:8916-8921.

82. **Mummey, D., W. Holben, J. Six, and P. Stahl.** 2006. Spatial stratification of soil bacterial populations in aggregates of diverse soil. Microb. Ecol. **51**:404-411.

83. **Muyzer, G., E.C. de Waal, and A.G. Uitterlinden**. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695-700.

84. **Nannipieri, P., J. Ascher, M.T. Ceccherini, L. Landi, G. Pietramellara, and G. Renella.** 2003. Microbial diversity and soil functions. Eur J Soil Sci. **54**:655-670.

85. **Nilsson, L., J.D. Oliver, and S. Kjelleberg**. 1991. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J. Bacteriol. **173**:5054-5059.

86. **Nunan, N., K.J. Wu, I.M. Young, J.W. Crawford, and K. Ritz.** 2002. In situ spatial patterns of soil bacterial populations, mapped at multiple scales, in an arable soil. Microb. Ecol. **44**:296-305.

87. **Nunan, N., T.J. Daniell, B.K. Singh, A. Papert, J.W. McNicol, and J.I. Prosser**. 2005. Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. Appl. Environ. Microbiol. **71**:6784-6792.

88. **Orwin, K.H., D.A. Wardle, and L.G. Greenfield.** 2006. Ecological consequences of carbon substrate identity and diversity in a laboratory study. Ecology **87**:580-593.

89. **Overmann, J., M.J.L. Coolen, and C. Tuschak.** 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch. Microbiol. **172**:83-94.

90. **Øvreas, L., S. Jensenet, F.L. Daae, and V. Torsvik.** 1998. Microbial community changes in a perturbed agricultural soil investigated by molecular and physiological approaches. Appl. Environ. Microbiol. **64**:2739-2742.

91. **Raich, J.W. and W.H. Schlesinger.** 1992. The global carbon dioxide flux in soil respiration and its relationship to vegetation and climate. Tellus **44B**:81-99.

92. **Raich, J.W. and C.S. Potter.** 1995. Global patterns of carbon dioxide emissions from soils. Global Biogeochem. Cycles **9**:23-36.

93. **Ranjard, L. and A. Richaume.** 2001. Quantitative and qualitative microscale distribution of bacteria in soil. Res. Microbiol. **152:**707-716.

94. **Roselló-Mora, R. and R. Amann.** 2001. The species concept for prokaryotes. FEMS Microbiol. Rev. **25**:39-67.

95. **Rustad, L.E., T.G. Huntington, and R.D. Boone.** 2000. Controls on soil respiration: implication for climate change. Biogeochemistry **48:**1-6.

96. **Ryan, M.G. and B.E. Law.** 2005. Interpreting, measuring, and modeling soil respiration. Biogeochemistry **73**:3-27.

97. **Sait, M., P. Hugenholtz, and P.H. Janssen**. 2002. Cultivation of genetically distinct soil bacteria from phylogenetic lineages previously only detected in culture-dependent surveys. Environ. Microbiol. **4**:654-666.

98. **Saleh-Lakha, S., M. Miller, R.G. Campbell, K. Schneider, P. Elahimanesh, M.M. Hart, and J.T. Trevors**. 2005. Microbial gene expression in soil: methods, applications and challenges. J. Microbiol. Methods. **63**:1-19.

99. **Sandaa, R.A., V. Torsvik, O. Enger, F.L. Daae, T. Castberg, and D. Hanh.** 1999. Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. FEMS Microbiol. Ecol. **30**:237-251.

100. **Sauer, D., Y. Kuzyakov, and K. Stahr.** 2006. Spatial distribution of root exudates of five plant species as assessed by ^{14}C labeling. J. Plant Nutr. Soil Sci. **169**:360-362.

101. **Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer, and G. Berg.** 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. **67**:4742-4751.

102. **Smit, E., P. Leeflang, S. Gommans, J. Van Den Broek, S. Van Mil, and K. Wernars**. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. **67**:2284-2291.

103. **Söderberg, K.H., and E. Bååth.** 2004. The influence off nitrogen fertilization on bacterial activity in the rhizosphere of barley. Soil Biol. Biochem. **36**:195-198.

104. **Stackebrandt, E. and B.M. Goebel.** 1994. Taxonomic note: *a* place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44**:846-849.

105. **Staley, J.T. and A. Konopka**. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. **39**:321-346.

106. **Stephan, A., A.H. Meyre, and B. Schmid**. 2000. Plant diversity affects culturable soil bacteria in experimental communities. J. Ecol. **88**:988-988.

107. **Steven, B., G. Briggs, C.P. McKay, W.H. Pollard, C.H. Greer, and L.G. Whyte**. 2007. Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. FEMS Microbiol. Ecol. **59**:513-523.

108. **Stevenson, B.S., S.A. Eichorst, J.T. Wertz, T.M. Schmidt, and J.A. Breznak.** 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl. Environ. Microbiol. **70:**4748-4755.

109. **Stotzky, G.** 1997. Soil as an environment for microbial life. Van Elsas, JD., Trevors, JT., and Wellington, EMH. (ed), in Modern Soil Microbiology. Marcel Dekker, Inc. New York pp. 441-477.

110. **Tamaki, H., Y. Sekiguchi, S. Hanada, K. Nakamura, N. Nomura, M. Matsumura, and Y. Kamagata.** 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. Appl. Environ. Microbiol. **71**:2162-2169.

111. **Tate, R.L., III.** 1999. Soil Microbiology. John Wiley & Sons, Inc.

112. **Torsvik, V., J. Goksoyr, and F.L. Daae.** 1990. High diversity of DNA of soil bacteria. Appl. Environ. Microbiol. **56**:782-787.

113. **Taylor, J.P., B. Wilson, M.S. Mills, and R.G. Burns**. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. Soil Biol. Biochem. **34**:387-401.

114. **Uhlmann, E., C. Görke, A. Petersen, and F. Oberwinkler.** 2004. Arbuscular mycorrhizae from semiarid regions of Namibia. Can. J. Bot. **82**:645-653.

115. **Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kersters, and J. Swings.** 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. **60**:407-438.

116. **Vangestel, M., R. Merckx, and K. Vlassak**. 1993. Microbial biomass and activity in soils with fluctuating water contents. Geoderma **56**:617-626.

117. **Verlinden, A. and A.S. Kruger.** 2007. Changing grazing systems in central north Namibia. Land Degrad. Develop. **18**:179-197.

118. **Wagner, M., R. Amann, H. Lemmer, and K-H. Schleifer.** 1993. Probing activated sludge with proteobacteria-specific oligonucleotides: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59**:1520-1525.

119. **Wagner, M., R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi, and K-H. Schleifer.** 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. **60**:792-800.

120. **Waldrop, M.P., T.C. Balser, and M.K. Firestone**. 2000. Linking microbial community composition in a tropical soil. Soil Biol. Biochem. **32**:1837-1846.

121. **Ward, D., B.T. Ngairorue, J. Kathena, R. Samuels, and Y. Ofran.** 1998. Land degradation is not a necessary outcome of communal pastoralism in arid Namibia. J. Arid Environ. **40**:357-371.

122. **Wertz, S., V. Degrange, J.I. Prosser, F. Poly, C. Commeaux, T. Freitag, N. Guillaumaud, and X. La Roux**. 2006. Maintenance of soil functioning following erosion of microbial diversity. Environ. Microbiol. **8**:2162-2169.

123. **Whitman, W.B., D.C. Coleman, and W.J. Wiebe.** 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. USA **95**:6578-6583.

124. **Wirth, S.J. and G.A. Wolf.** 1992. Micro-plate colourimetric assay for endo-acting cellulase, xylanase, chitinase, 1,3-beta-glucanase and amylase extracted from forest soil horizons. Soil Biol. Biochem. **24**:511-519.

125. **Woese, C.R.** 1987. Bacterial evolution. Microbiol Rev. **51**:221-271.

126. **Yin, B., D. Crowley, G. Sparovek, W.J. De Melo, and J. Borneman.** 2000. Bacterial functional redundancy along a soil reclamation gradient. Appl. Environ. Microbiol. **66**:4361-4365.

127. **Zak, D.R., D. Tilman, R.R. Parmenter, C.W. Rice, F.M. Fisher, J. Vose, D. Milchunas, and C.W. Martin.** 1994. Plant production and soil microorganisms in late-successional ecosystems: a continental-scale study. Ecology **75**:2333-2347.

128. **Zak, D.R., W.E. Holmes, D.C. White, A.D. Peacock, and D. Tilman**. 2003. Plant diversity, soil microbial communities, and ecosystem function: are there any links? Ecology. **84**:2042-2050.

129. **Zeidler, J., S. Hanrahan, and M. Scholes.** 2002. Land-use intensity affects range condition in arid to semi-arid Namibia. J. Arid Environ. **52**:389-403.

130. **Zengler, K., G. Toledo, M. Rappe, J. Elkins, E.J. Mathur, J.M. Short, and M. Keller.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA **99**:15681-15686.

CHAPTER 2

Effects of Plant Biomass, Plant Diversity and Water Content on the Bacterial Communities in Soil Lysimeters: Implications for the Determinants of Bacterial Diversity

2.1 Abstract

Soils may comprise tens of thousands to millions of bacterial species. It is still unclear whether this high level of diversity is governed by functional redundancy or by a multitude of ecological niches. In order to address this question, we analyzed the reproducibility of bacterial community composition after different experimental manipulations. Soil lysimeters were planted with four different types of plant communities, and the water content was adjusted. Group-specific phylogenetic fingerprinting by PCR-denaturing gradient gel electrophoresis revealed clear differences in the composition of *Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, Chloroflexi, Planctomycetes,* and *Verrucomicrobia* populations in soils without plants compared to that of populations in planted soils, whereas no influence of plant species composition on bacterial diversity could be discerned. These results indicate that the present of higher plant species affects the species composition of bacterial groups in a reproducible manner and even outside of the rhizosphere. In contrast, the environmental factors tested did not affect the composition of *Acidobacteria, Actinobacteria, Archaea,* and *Firmicutes* populations. One-third (52 out of 160) of the sequence types were found to be specifically and reproducibly associated with the absence or presence of plants. Unexpectedly, this was also true for numerous minor constituents of the soil bacterial assemblage. Subsequently, one of the low-abundance phylotypes (beta10) was selected for studying the interdependence under particular experimental conditions and the underlying causes in more detail. This so-far-uncultured phylotype of the *Betaproteobacteria* species represented up to 0.18 % of all bacterial cells in planted lysimeters compared to 0.017 % in unplanted systems. A cultured representative of this phylotype exhibited high physiological flexibility and was capable of utilizing

major constituents of root exudates. Our results suggest that the bacterial species composition in soil is determined to a significant extent by abiotic and biotic factors, rather than by mere chance, thereby reflecting a multitude of distinct ecological niches.

2.2 Introduction

Soils harbor highly diverse bacterial communities with up to 50,000 (Sandaa *et al.* 1999) or even up to millions (Gans *et al.* 2005) of different 16S rRNA gene sequences. So far, it has remained unclear whether functional redundancy or a multitude of ecological niches and adaptive mechanisms governs the composition of soil bacterial communities (Griffiths *et al.* 2001, Wertz *et al.* 2006). In the present study, this question was assessed by analyzing the reproducibility of bacterial community composition with different experimental manipulations of soil lysimeters and by subsequently studying the bacteria associated with particular environmental conditions by use of cultivation-based methods. The variables selected for environmental manipulations were the absence or presence of higher plant species, plant diversity, and water content.

Aboveground plant diversity in particular has long been suggested to drive belowground microbial diversity. Thus, the diversity of bacterial communities in grassland soil samples has been shown to be affected by the numbers of plant species present (Grüter *et al.* 2006). In the rhizosphere, plants select for a specific composition of bacterial communities (Kowalchuk *et al.* 2002, Kuske *et al.* 2002, Small *et al.* 2001) depending on the type and amount of organic root exudates and of nutrients released from senescent or dead roots (Jaeger *et al.* 1999, Rangel-Castro *et al.* 2005). However, plant rhizosphere effects have been found to be of little significance for the composition of total community structure in grasslands whereas liming and nitrogen addition alters overall soil bacterial community structure (Kennedy *et al.* 2004). In addition, a change of tillage and crop residue management practice has been observed to lead to pronounced changes in the composition of the soil bacterial community as documented by fatty acid methyl ester analysis (Pankhurst *et al.* 2002). Finally, soil bacterial diversity was found to be dependent on

soil pH but to be unrelated to site temperature, latitude, organic carbon content, C : N ratio or plant diversity in a recent continent-scale study (Fierer and Jackson 2006).

Several of the above-cited studies were found to have been limited by the resolution of the culture-independent methods used (clone libraries of limited coverage, ribosomal DNA-fingerprinting using eubacterial primers) (Griffiths *et al.* 2001). Supporting this conclusion, a significant correlation between particular plants and soil bacterial populations could be detected when the diazotrophic community was analyzed separately and at high resolution by amplifying the specific functional gene *nifH* (DembaDiallo *et al.* 2004); *Rhizobium* spp. were found to be less diverse in pastures planted with soybeans (Coutinho *et al.* 1999). As an additional point of concern, different factors often do not act independently in the natural setting; e.g., the presence of different plant species also causes differences in soil chemical properties (Bezemer *et al.* 2006). Besides requiring high-resolution monitoring techniques, studies of bacterial diversity therefore need to assess different environmental factors independently and under highly controlled experimental conditions.

In the present study, we conducted a systematic and high-resolution survey of soil bacterial diversity and its interdependence with environmental factors. Specifically, the effects of soil water content, season, absence or presence of higher plant species, and diversity of higher plant species on 10 bacterial groups were studied under highly controlled conditions in soil lysimeters. Of the numerous low-abundance bacterial phylotypes which exhibited a reproducible pattern in lysimeters, we selected and isolated a representative bacterium and investigated the reasons underlying its interdependence with higher plant species.

2.3 Material and methods

2.3.1 Lysimeter design and sample collection

Soil samples were obtained from lysimeters which had been in continuous operation since 2001 in the Ecological-Botanical Garden of the University of Bayreuth. The lysimeters were built with 1-m-deep concrete walls enclosing a surface area of 1.3 by 1.3 m. The walls had been smoothed with metal brushes and sealed twice using

Inertol 49W coating material. The lysimeters were filled with endostagnic cambisol soil obtained from nearby grassland soils after homogenization of the soil by a rotor tiller and steaming at 100°C over 12 h for sterilization (Kossmann 2005). Lysimeters were seeded in the year 2001 at a density of 2,000 shoots per m^2 with perennial grasses and forbs in order to create four different plant communities of increasing diversity: (i) lysimeters devoid of higher plants which consisted of soil partially covered with mosses; (ii) lysimeters planted with the two grass species *Holcus lanatus* (Yorkshire fog) and *Arrenatherum elatius* (tall oat grass); (iii) lysimeters planted with the two grass species *H. lanatus* and *A. elatius* and the two forb species *Plantago lanceolata* (ribwort) and *Geranium pratense* (meadow crane's-bill); (iv) lysimeters planted with the four grass species *H. lanatus, A. elatius*, *Alopecurus pratense* (foxtail grass)*, Anthoxanthum odoratum* (sweet vernal grass) and the four forb species *P. lanceolata*, *G. pratense, Ranunculus acris* (tall buttercup) and *Taraxacum officinale* (dandelion blow balls). For each type of plant community, six lysimeters were set up in parallel. Weeding was performed over the growing season in one-month intervals. Following the first sampling campaign in June 2004, the water content of the soils was controlled in half of the plots of each lysimeter type in order to study the effect of different water content percentages on bacterial diversity. Irrigation was performed regularly, thereby maintaining the water content of the lysimeters at 15% (Reuter 2005). In the absence of irrigation, the water content of the soils dropped to 5% (Bremer 2007). Accordingly, four different types of treatment were available in June 2004, whereas eight different types of treatment were analyzed afterwards (four different plant covers times two types of water supply). For each treatment, three parallel lysimeters were set up. Lysimeters were mown twice a year to remove aboveground plant biomass. To compensate for the nutrients with plant biomass, "Blaukorn" fertilizer was added at 50 kg · ha^{-1}.

Sampling of the upper 10 cm of each soil was conducted on 9 June, 2 August, and 1 September 2004. This yielded a total of 20 different types of samples (4 from June and 8 each from August and September). In order to account for spatial nonhomogeneity within individual lysimeters, three subsamples were collected from each lysimeter by coring different locations between the plants. Each core yielded

between 107 and 172 g wet weight of soil. In order to account for variations between similar lysimeters, three parallel lysimeters of the same treatment group were sampled. Subsequently, all nine-soil samples of the same lysimeter type were pooled and sieved through a 0.5 mm mesh sieve. Each pooled sample was then split in half. One half was wrapped in sterile aluminum foil and immediately frozen in liquid N_2 for molecular analysis. The other half of the sample was transferred to a sterile plastic bag and kept at 4°C for subsequent enumeration and isolation of bacteria.

2.3.2 Total bacterial cell counts

Soil slurries were prepared as 1:100 (w/v) dilutions in a buffer consisting of 10 mM HEPES, 10 mM pyrophosphate and 0.08% Tween (final concentrations). Aliquots of the slurries were subsequently fixed with 2% glutaraldehyde and 30 µl of each sample was diluted in 5 ml of buffer and stained with SYBR Green II (Molecular Probes, Eugene, Oregon) for 10 minutes in the dark. Subsamples were then filtered onto polycarbonate filters (Nuclepore Track-Etch Membrane; Whatman, Springfield Mill, United Kingdom) (0.1 µm pore size, 25 mm diameter), and the filters were dried, embedded in DABCO antifading solution (25 mg of 1,4-diazabicyclo [2.2.2]octane in 1 ml of PBS buffer plus 9 ml of glycerol), and subsequently examined by epifluorescence microscopy (Zeiss Axiolab microscope) (lamp, HBO 50; filter set, Zeiss, Ex 450-490, FT 510, and LP 515) at a magnification of x1,000. At least 20 fields were counted for each sample.

2.3.3 DNA extraction and purification

Soil DNA was extracted using an UltraClean Mega Prep Soil DNA Kit (Mo Bio Laboratories, Inc., Solana Beach, USA) according to the instructions of the manufacturer, but including a sonification step (3 min at continuous mode; Branson Sonifier B15 Cell Disruptor). The eluate was precipitated with ethanol and further purified using a Genomic tip 100/G (QIAGEN GmbH, Hilden, Germany). Finally, the DNA was dialyzed using a Centricon-50 dialysis filtration unit (Millipore, Amicon, Bedford, MA) and washed twice using 2 ml Tris-EDTA buffer. DNA concentrations were determined by dye binding with PicoGreen (Molecular Probes, Eugene, OR).

For extraction of chromosomal DNA from cultured bacteria, cells in microtiter plates were harvested by centrifugation (20 min at 15,000xg, 4°C). Cell pellets were lysed by six cycles of freezing and thawing with each cycle consisting of an incubation for 3 min at -80°C followed by an incubation for 3 min at 100°C. Aliquots of 1 to 3 µl of the resulting crude extracts were directly used in PCR amplifications.

2.3.4 PCR amplification, enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and denaturing gradient gel electrophoresis (DGGE) fingerprinting

The 16S rRNA gene fragments of members of nine phyla or subphyla of the *Bacteria* and of members of the *Archaea* were amplified by PCR employing the primers and cycling conditions listed in Table 1. For the amplifications of 16S rRNA genes of some of the phylogenetic groups, primer mixtures had to be employed (see wobble positions in the primers listed in Table 1). By using DNA of pure laboratory cultures, we tested whether amplification with these primer mixes resulted in multiple fingerprints of one phylotype; however, multiple bands were never observed (compare also reference Overmann *et al.* 1999).

Amplification reactions were performed with a Gene Amp PCR 9700 system (Applied Biosystems, Foster City, CA). The reaction mixture contained the following ingredients per 50 µl: 100 ng of soil DNA, 5 µl of 10x PCR buffer containing 15 µM $MgCl_2$ (QIAGEN GmbH, Hilden, Germany), 5 µl of Q solution (QIAGEN), 40 µg of bovine serum albumin (Sigma-Aldrich, Steinheim, Germany), 500 nM $MgCl_2$, 200 nM deoxynucleoside triphosphates, a 1 µM concentration of each primer and 1.25 units of *Taq* polymerase (QIAGEN). For each primer set targeting 16S rRNA gene sequences, one of the primers contained a 40-bp-long GC clamp at its 5´ end (Table 1) in order to obtain stable melting behavior of the generated DNA fragments during subsequent DGGE. The genetic diversity of isolated cultures was studied by ERIC-PCR (Versalovic *et al.* 1991). PCR products were analyzed by standard agarose electrophoresis.

Table 1. Summary of primer set used and cycling conditions

Primer pair[a]	Target	Primer sequence (5′→ 3′)	Cycling conditions[b]	Reference
GC517f Alf968r	*Alphaproteobacteria* *(some Gammaproteobacteria)*	GTGCCAGCAGCCGCGG GGTAAGGTTCTGCGCGTT	30 s at 94°C, 45 s at 68°C, 1 min at 72°C (10 cycles); 30 s at 94°C, 45 s at 62°C, 1 min at 72°C (20 cycles)	Lane 1991 Neef 1997
GC1055r Beta680f	*Betaproteobacteria*	AGCTGACGACAGCCAT CRCGTGTAGCAGTGA	30 s at 94°C, 1 min at 56°C, 1 min at 72°C (10 cycles); 30 s at 94°C, 1 min at 51°C, 1 min at 72°C (20 cycles)	Angle *et al.* 1991 Overmann *et al.* 1999
GC341r Acido31f	*Acidobacteria*	CTGCTGCCTCCCGTAGG GATCCTGGCTCAGAATC	30 s at 94°C, 1 min at 62°C, 1 min at 72°C (10 cycles); 30 s at 94°C, 1 min at 57°C, 1 min at 72°C (20 cycles)	Muyzer *et al.* 1993 Barns *et al.* 1999
GCAB1165r 926f	*Actinobacteria*	ACCTTCCTCCGAGTTRAC AAACTCAAAGGAATTGACGG	30 s at 94°C, 45 s at 62°C, 1 min at 72°C (10 cycles); 30 s at 94°C, 45 s at 57°C, 1 min at 72°C (20 cycles)	Lüdemann and Conrad 2000 Lane 1991
GC344f ARCH785r	*Archaea*	ACGGGGNGCAGCCGCGA GGACTACVSGGGTATCTAAT	20 s at 96°C, 45 s at 54°C, 12 s at 72°C (35 cycles)	Stahl and Amann 1991 Lane 1991
GCCFB319f 907r	*Bacteroidetes*	GTACTGAGACACGGACCA CCGTCAATTCCTTTGAGTTT	30 s at 94°C, 45 s at 65°C, 1 min at 72°C (10 cycles); 30 s at 94°C, 45 s at 60°C, 1 min at 72°C (25 cycles)	Jasper *et al.* 2001 Muyzer and Ramsing 1995
GC1340r GNSB941f	*Chloroflexi*	CGCGGTTACTAGCAAC AGCGGAGCGTGTGGTTT	30 s at 94°C, 45 s at 58°C, 1 min at 72°C (10 cycles); 30 s at 94°C, 45 s at 53°C, 1 min at 72°C (25 cycles)	Gich *et al.* 2001
GCLGC354f 907r	*Firmicutes*	GCAGTAGGGAATCTTCSR CCGTCAATTCCTTTGAGTTT	30 s at 94°C, 45 s at 58°C, 1 min at 72°C (10 cycles); 30 s at 94°C, 45 s at 53°C, 1 min at 72°C (20 cycles)	Meier *et al.* 1999 Muyzer and Ramsing 1995
GCEUB338IIf Pla886r	*Planctomycetes*	ACACCTACGGGTGGCTGC GCCTTGCGACCATACTCCC	30 s at 94°C, 45 s at 69°C, 1 min at 72°C (10 cycles); 30 s at 94°C, 45 s at 64°C, 1 min at 72°C (20 cycles)	Daims *et al.* 1999 Neef 1997
GCEUBIII338r Ver40f	*Verrucomicrobia*	GCTGCCACCCGTAGGTGT CGGCGTGGWTAAGACATGCA	30 s at 94°C, 45 s at 63°C,1 min at 72°C (10 cycles); 30 s at 94°C, 45 s at 58°C, 1 min at 72°C (20 cycles)	Sangwan *et al.* 2005 This study
ERIC1R ERIC2	Enterobacterial repetitive consensus sequences	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG	30 s at 94°C, 1 min at 52°C, 8 min at 70°C (30 cycles)	Versalovic *et al.* 1991
Mas1139F Mas1281R	Betaproteobacterium strain byr23-80	TTGAGCACTCTAATGAGAC CTACGATACACTTTCTGGG	qPCR: 30s at 95°C, 45s at 56°C, 1 min at 72°C (40 cycles), melt curve from 55 to 95°C	This study

[a] GC primers contain a 40 bp-GC clamp (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC) at the 5′-end

[b] Each protocol included a single 4 min cycle at 96°C at the beginning and terminated with a 10 min cycle at 72°C

Denaturing gradient gel electrophoresis was carried out in an Ingeny phorU system (Ingeny International BV, Goes, The Netherlands) employing 6% (wt/vol) polyacrylamide gels in 1x Tris-acetate-EDTA (pH 8.0). Denaturing gradients ranged from 30 to 70% (for *Archaea, Alphaproteobacteria, Betaproteobacteria*, *Bacteroidetes, Firmicutes,* and *Planctomycetes* species), from 30 to 80% (for *Acidobacteria* species), from 40 to 80% (for *Chloroflexi* and *Verrucomicrobia* species), or from 55 to 80% (for *Actinobacteria* species), where 100% denaturant is defined as 7 M urea and 40% (vol/vol) formamide (Muyzer *et al.* 1993). Gels were stained for 45 minutes with SYBRGold (MoBiTec, Göttingen, Germany) (1:10,000 dilution), visualized on a UV transilluminator (LTF Labortechnik, Wasserburg), and photographed (Visitron Systems GmbH, Puchheim). DNA bands were excised from the gel with a sterile scalpel and transferred to a 1.5 ml Eppendorf tube containing 25 µl of 10 mM Tris-HCl buffer (pH 8.0), and the DNA was eluted for 2 hours at 65°C. Reamplifications were conducted using the corresponding primers (but without a GC clamp). PCR products were separated from free PCR primers by use a QIAquick Spin kit (QIAGEN).

2.3.5 Quantitative analysis of bacterial diversity

The generated DGGE profiles were analyzed using the ONE-Dscan electrophoresis analysis software (Scananalytics, Billerica, MA). After automated background subtraction, the lanes were normalized against each other. For each DNA band, the relative intensity value and position was recorded and incorporated in a matrix. Similarities were calculated with the SIMGEND program of the NTSYS-pc software package (Exeter Software, NY) and expressed as Nei coefficients (Nei 1972). Based on these values, dendograms were constructed using the SAHN program of the package and applying the unweighted pair-group method using average linkages. The relative intensity values of DGGE bands were also utilized to calculate the diversity of each bacterial target group established in the lysimeters soil employing the Shannon-Weaver index of diversity H' according to the following formula (Shannon and Weaver 1963): $H' = -\Sigma p_i \ln p_i$, where the relative intensity value assigned to each individual band i was used as an indicator for the relative abundance p_i of the corresponding melting type.

2.3.6 Sequencing and phylogenetic analysis

Double-pass sequencing of the 16S rRNA gene fragments was performed, employing the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems GmbH, Weiterstadt, Germany) and an ABI Prism 310 genetic analyzer (Applied Biosystems). In cases in which DNA bands from DGGE gels yielded multiple sequences, the latter were separated by cloning using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). After being plated on selective LB agar plates, recombinants were picked randomly and the plasmids were extracted from an overnight culture in liquid LB media with a QIAprep Spin Miniprep kit (QIAGEN). Plasmids were then differentiated by enzymatic digestion with EcoRI (Fermentas GmbH, St. Leon-Rot, Germany), and different clones were subsequently sequenced. All 16S rRNA gene sequences obtained in the present study were checked for possible chimeras by use the CHIMERA-CHECK online analysis program of the RDP-II database (Maidak *et al.* 2001).

The 16S rRNA sequences were analyzed using the ARB software (http://www.mikro.biologie.tu-muenchen.de). Sequences of the closest relative were retrieved from the GenBank database by use of BLAST 2.0.4 software (Altschul *et al.* 1997) and imported into the ARB database. Through the use of the integrated Fast Aligner Version 1.03 tool, the sequences were automatically aligned and the alignment was corrected manually according to secondary-structure information. First, only sequences longer than 1,300 bp were used to construct a tree by employing the maximum likelihood algorithm (Fast DNA_ML). Afterwards, the shorter environmental sequences as obtained from the DGGE bands were inserted without changing the overall tree topology, employing the parsimony interactive tool implemented in the ARB software package. Bootstrap values were calculated from 100 bootstrap resamplings.

2.3.7 qPCR

Quantitative PCR (qPCR) was conducted to quantify the abundance of *Betaproteobacterium* sp. strain byr23-80 in the different lysimeters. Oligonucleotide primers specific for this phylotype were generated using ARB software; specificity was confirmed by using the RDP-II Probe Match software (http://rdp.cme.

msu.edu/probematch/search.jsp), by using PCR assays with different other *Betaproteobacteria* species as negative controls, and finally by sequencing qPCR products generated from soil DNA. qPCR was conducted in an iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA), using 250 nM concentrations each of primers Mas1139F and Mas1281R (Table 1), 5 to 10 ng of DNA template, 20 µg of bovine serum albumin (Sigma-Aldrich), and 12.5 µl of iQ SYBR green Supermix (Bio-Rad) in a volume of 25 µl. The optimized cycling protocol (Table 1) resulted in a highly specific 142-bp amplicon. Genomic DNA of strain byr23-80 was used to generate a standard curve ranging from 1.6 to $1.6 \cdot 10^{-5}$ ng per well. All determinations were run in triplicate, and cell numbers of strain byr23-80 were estimated based on a mean content of $4.7 \cdot 10^{-15}$g DNA·cell^{-1}.

2.3.8 Cultivation and isolation of soil bacteria

The culturability of soil bacteria was assessed in samples from August 2004. Since we were mainly interested in the effects of plant diversity on bacterial culturability, irrigated and nonirrigated lysimeter soil samples of each lysimeter type were mixed in equal portions for this purpose. Soil-solution-equivalent medium (Angle *et al.* 1991) (pH 7.0) buffered with 10 mM HEPES was employed. One liter of this basal medium was supplemented with artificial root exudates (Kozdrój and van Elsas 2000), yeast extract (0.01% wt/vol), and inducers (a 10 µM concentration each of cyclic AMP, AMP, *N*-oxohexanoyl-DL-homoserine lactone (OHHL) and L-homoserine lactone; see reference Bruns *et al.* 2003). Basal medium without substrates served as a control.

High-throughput cultivation was performed in sterile 96-well polystyrene microtiter plates (Corning Inc., Corning, NY). Each well received 180 µl of growth medium and 50 bacterial cells. In the first approach, the plates were inoculated by employing the MicroDrop AutoDrop microdispenser system version 5.50 (MicroDrop GmbH, Norderstedt, Germany) as described previously (Bruns *et al.* 2003, Gich *et al.* 2005). In order to prevent clogging of the microdispenser pipette, soil suspensions (see above) were prefiltered through 12-µm pore-size polycarbonate filters prior to inoculation. The second approach was used to cultivate bacteria firmly

associated with particles of more than 12 µm. In this case, cultures were set up by manually inoculating liquid cultures with aliquots of the soil suspensions by use of a conventional multipipette. On each microtiter plate, 12 wells were left without inoculation and served as controls for contamination. Microtiter plates were incubated at 15°C for 6 to 8 weeks, with monitoring by visual inspection of turbidity.

Bacterial strains were isolated by streaking selected liquid cultures onto the medium described above solidified by use of 8 $g \cdot l^{-1}$ gellan gum (Sigma-Aldrich) (Janssen *et al.* 2002). Strains were characterized by biochemical and physiological tests including growth tests with 51 individual sugars, 36 organic acids, 4 ketoacids, 22 amino acids, 8 alcohols and 4 complex substrates as described previously (Gich and Overmann 2006).

2.3.9 Nucleotide sequence accession numbers

The 16S rRNA gene sequences have been deposited in the GenBank database under accession numbers AM749495 through AM749665 and EF546777.

2.4 Results

2.4.1 Total cell numbers and culturability of soil bacteria

Within the study period, cell numbers in the lysimeters ranged from $(0.9 \pm 0.2) \times 10^9$ to $(9.1 \pm 2.7) \times 10^9$ cells$\cdot g^{-1}$ (Table 2). In June 2004, the highest values were determined for samples obtained from the lysimeters which were devoid of higher plant species or from lysimeters planted with two plant species (Table 2). Two months later, cell counts had decreased and were similar in all lysimeter samples. In September 2004, bacterial numbers had increased in lysimeters planted with four or eight plant species. In this month, an increased water supply resulted in significantly increased bacterial cell numbers in all lysimeters stocked with higher plant species compared to those seen in the nonirrigated parallels (*t* test; $P < 0.05$). With respect to the soil pH, only minor differences were observed between the different lysimeters. No significant differences in bacterial cell volumes were detected (data not shown).

Table 2. Soil parameters, total bacterial cell counts, and culturability values for the different lysimeters

No. of plant species and lysimeter condition[a]	$H_2O/CaCl_2$ pH values for 2004 sampling date[b]			Root biomass[c] [g C·m^{-2}] (% of total organic C) value for sampling date 3 June 2004	Total soil organic C[c] [g C·m^{-2}] value for sampling date 3 June 2004	Total cell counts [x 10^9 cells · (g soil^{-1})] value for 2004 sampling date:			% Culturability value for 2 August 2004 sampling date	
	9 June	2 August	1 September			9 June	2 August	1 September	MicroDrop	Soil slurry dilution
0										
Preirrigation	5.6/4.8			0(0)	3,239 ± 36	6.5 ± 2.5			0.021 ± 0.015	0.010 ± 0.010
-H_2O		5.9/4.8	6.2/5.1				1.7 ± 0.4	1.5 ± 0.2		
+H_2O		5.7/4.6	5.9/4.7				1.2 ± 0.2	1.2 ± 0.2		
2										
Preirrigation	5.8/4.7			296 ±15 (7.7)	3,860 ± 69	9.1 ± 2.7			0.328 ± 0.061	0.085 ± 0.030
-H_2O		6.2/4.7	6.2/5.2				1.3 ± 0.3	1.5 ± 0.3		
+H_2O		6.3/4.8	6.2/5.1				0.9 ± 0.2	2.7 ± 0.3		
4										
Preirrigation	5.8/4.7			349 ± 22 (8.8)	3,973 ± 104	4.1 ± 1.0			0.010 ± 0.010	0.267 ± 0.055
-H_2O		6.2/4.7	6.3/5.2				1.5 ± 0.3	2.4 ± 0.3		
+H_2O		6.3/5.2	6.4/5.3				1.6 ± 0.5	4.1 ± 0.9		
8										
Preirrigation	5.8/4.8			318 ± 13 (7.9)	4,042 ± 138	2.3 ± 0.6			1.606 ± 0.160	0.042 ± 0.021
-H_2O		6.1/4.9	6.1/5.2				1.8 ± 0.4	2.6 ± 0.6		
+H_2O		6.2/4.9	6.4/5.4				1.6 ± 0.6	4.8 ± 0.9		

a –H_2O, nonirrigated lysimeters; +H_2O, irrigated lysimeters
b pH for $CaCl_2$ determined after suspension of soil in 10 mM $CaCl_2$
c Reported in reference Reuter 2005

The cultivation efficiency determined for the August 2004 samples ranged between 0.01 and 1.6 % of total cell counts (Table 2). No consistent trend was observed with respect to plant cover or the two cultivation techniques used.

2.4.2 Comparative analysis of bacterial diversity in the different lysimeters

The compositions of the prokaryotic communities in the 20 different soil samples were compared by phylogenetic fingerprinting employing PCR-DGGE. In order to increase the resolution of this analysis (Gich *et al.* 2005, Overmann *et al.* 1999), 16S rRNA gene fragments were selectively amplified for each of the 10 target groups of bacteria. For the specific amplification of *Verrucomicrobia* species, we initially applied published primers. However, all primer combinations tested were found to be too unspecific since they also yielded sequences of *Planctomycetes, Bacteroidetes,* and *Proteobacteria* species (data not shown). Consequently, a novel primer suitable for the PCR-DGGE approach was designed (primer Ver40f; Table 1) which, in combination with primer EUBIII338R, permitted a selective amplification of *Verrucomicrobia* sequences.

Adding up the group-specific fingerprints detected in the 20 samples, our PCR-DGGE analysis yielded total numbers of different melting types between 16 and 71 for the 10 target groups (three examples are depicted in Fig. 1; all results are summarized in Table 3). The number of fingerprints was highest for members of the phyla *Chloroflexi* (Fig. 1A) and *Verrucomicrobia*, with 71 and 54 distinguishable melting types, respectively. In contrast, between 23 and 38 melting types were detected for the *Alphaproteobacteria*, *Betaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Planctomycetes* species. Of all groups analyzed, the *Firmicutes* and the *Archaea* species yielded the lowest numbers of distinct melting types (19 and 16, respectively) (Table 3). The signal intensity of DNA fingerprints was evenly distributed for some groups, like the *Actinobacteria* species (Fig. 1C), whereas fingerprint patterns were dominated by some bands in other cases (Fig. 1B). In order to account not only for the total number of bands but also for the evenness of this intensity distribution, the DGGE fingerprint patterns of the individual groups were analyzed by densitometry. After determination of the relative signal intensities of the fingerprints in each lane, the Shannon-Weaver index of

diversity was calculated from the number of fingerprints and their relative signal intensities. The results confirmed that *Chloroflexi* species reached the highest diversity, while the *Bacteroidetes* and *Archaea* species were the least diverse among all groups investigated (Table 4).

For each of the 10 target groups, the fingerprint patterns generated from the set of 20 different lysimeter samples resembled each other (see Fig.1A to C), indicating that the manipulation of plant communities in the lysimeters did not lead to completely altered bacterial communities. However, our analysis also revealed a differential response of some bacteria to the presence or absence of higher plant species. For example, DGGE profiling of the *Chloroflexi* species (Fig. 1A) yielded several DNA fragments which were detected exclusively (bands 11, 17, 22) or at higher abundance (bands 2, 6) in barren lysimeters whereas others (bands 9, 10) were detected at higher signal intensities in the planted lysimeters. Similarly, bands 8 and 10 of the *Alphaproteobacteria* species (Fig. 1B) were detected exclusively or at a higher signal strength in the barren lysimeters whereas bands 7, 9 and 20 of the *Actinobacteria* species (Fig. 1C) were found in planted lysimeters. Only few fingerprints, e.g., band no. 2 of the *Alphaproteobacteria* species, were unique to single soil samples.

Figure 1. Three examples of the DGGE analyses of PCR-amplified 16S rRNA gene fragments. Lanes 0, 2, 4 and 8 indicate samples from lysimeters with the respective numbers of higher plant species; the $-H_2O/+H_2O$ lanes correspond to the nonirrigated and irrigated lysimeters; lanes June, August, and September correspond to the three soil sampling dates. For each bacterial group, circles denote the bands which were excised and sequenced, and arrows with consecutive numbering indicate the different melting types analyzed. **A.** *Chloroflexi* species, **B.** *Alphaproteobacteria* species, **C.** *Actinobacteria* species. Negative images of SYBR gold-stained DGGE gels are shown.

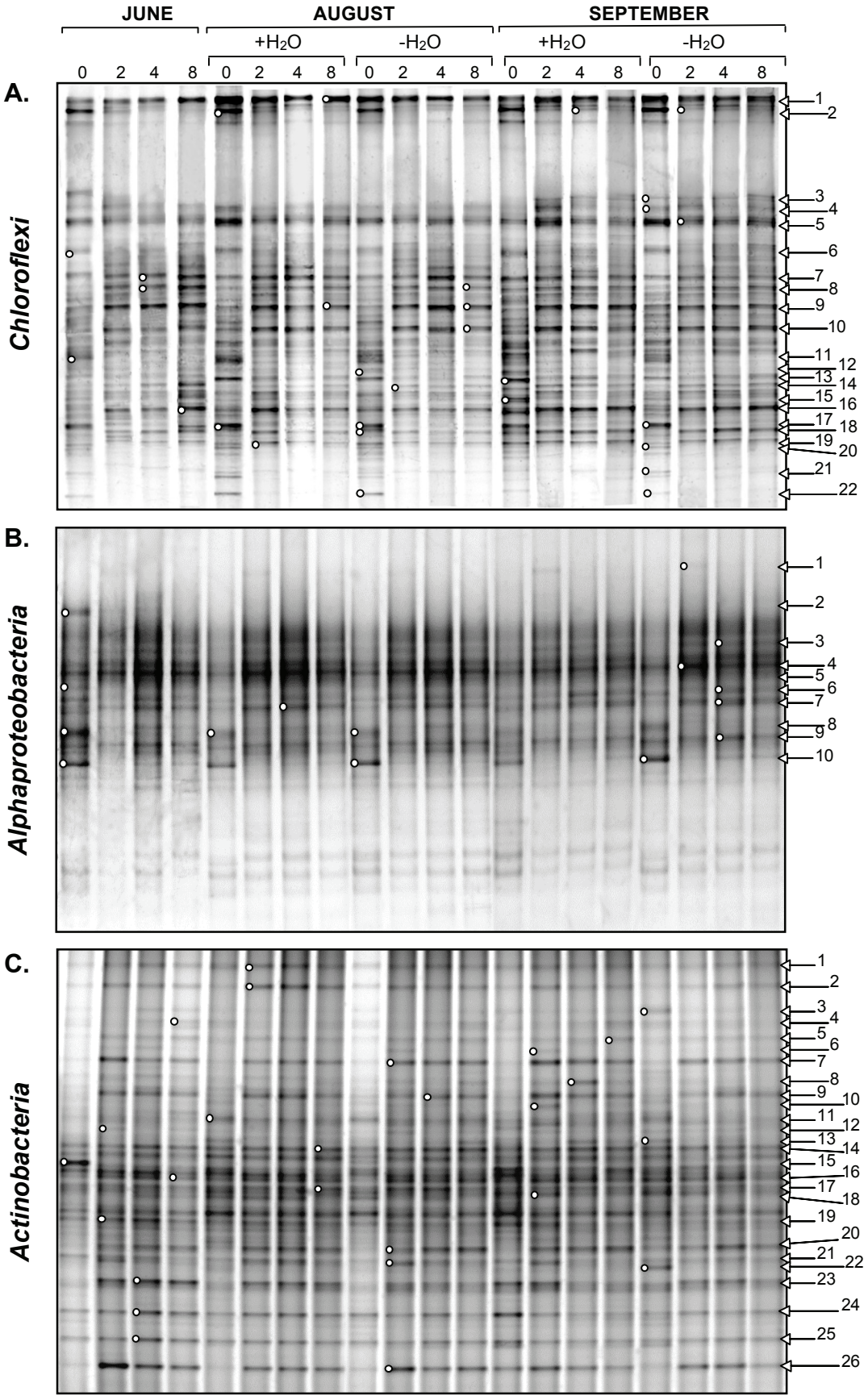

JUNE
AUGUST
SEPTEMBER
+H2O
-H2O
+H2O
-H2O
0 2 4 8 0 2 4 8 0 2 4 8 0 2 4 8
A.
Chloroflexi
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
B.
Alphaproteobacteria
1 2 3 4 5 6 7 8 9 10
C.
Actinobacteria
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Table 3. Summary of the analysis of environmental and cultured DGGE-melting types and phylotypes

Target group	Environmental samples							Cultured samples		
	Total no. of melting types detected	No. of DNA bands sequenced [b]	No. of melting types analyzed (no. of multiple sequences [c])	No. of phylotypes recovered	No. of sequences matching sequences in the databases at a similarity of:			No. of melting types	No. of melting types matching environmental melting types	No. of phylotypes matching environmental phylotypes
					> 97%	97% - 93%	< 93%			
Chloroflexi	71	29	22 (3)	27	7	14	6	0	0	0
Acidobacteria	23	20	20 (0)	20	8	12	0	3	0	0
Verrucomicrobia[a]	54	10	10 (4)	17	5	10	2	12	1	0
Planctomycetes[a]	36	11	11 (3)	14	3	10	1	8	3	0
Alphaproteobacteria	24	15	10 (0)	10	7	3	0	6	4	0
Betaproteobacteria	29	14	10 (0)	10	8	2	0	6	2	1
Bacteroidetes	26	22	12 (0)	12	9	3	0	8	0	0
Actinobacteria	38	26	26 (0)	26	14	8	4	17	1	1
Firmicutes	19	13	12 (0)	12	6	6	0	6	2	0
Archaea	16	14	12 (0)	12	6	6	0	0	0	0
Total	336	174	145 (10)	160	73	74	13	66	13	2

[a] Several DGGE bands generated for the *Verrucomicrobia* and *Planctomycetes* species were found to contain up to four different 16S rRNA gene sequences which had to be separated by cloning prior to sequencing. Therefore, the phylogenetic analysis of these two groups was limited to the 10 major bands, which yielded a total of 17 and 14 phylotypes, respectively.

[b] Corresponding to bands denoted by circles in Fig. 1.

[c] Indicated by numbered arrows in Fig. 1.

Table 4. Mean values and ranges of the Shannon-Weaver index of diversity (H´) calculated for the bacteria of 10 bacterial groups present in the lysimeter soil

Bacterial group	H`	
	Mean	Range
Chloroflexi	3.47	2.75 – 6.37
Acidobacteria	2.37	1.85 – 2.70
Verrucomicrobia	2.67	2.39 – 2.86
Planctomycetes	2.78	2.56 – 3.01
Alphaproteobacteria	2.09	1.38 – 2.65
Betaproteobacteria	2.07	1.77 – 2.36
Bacteroidetes	1.87	1.42 – 2.24
Actinobacteria	2.72	1.53 – 3.04
Firmicutes	2.57	2.31 – 2.78
Archaea	1.95	0.52 – 2.44

2.4.3 Factors influencing bacterial diversity in lysimeters

In order to identify the factors affecting bacterial community composition, pairwise similarity values were calculated from the fingerprints patterns by employing the Nei coefficient. A cluster analysis was then performed for each bacterial target group. For 6 of the ten groups, namely, the *Chloroflexi*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* species (Fig. 2A), the populations established in the absence of higher plant species (labeled "0" in Fig. 2A) were more similar to each other than to the populations in other lysimeters. In the case of the *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* species, populations in the lysimeters lacking higher plant species formed the most distant subcluster of all. In contrast to the six groups named above, no distinct clustering, and hence no obvious dependence on any of the factors tested, could be observed for the *Actinobacteria*, *Acidobacteria*, *Firmicutes* or *Archaea* species (Fig. 2B).

A comparison of the values for the Nei coefficients across all 10 bacterial groups revealed that the most pronounced changes in composition occurred in the phylum *Bacteroidetes* (maximum Nei coefficient of 2.17; Fig. 2A). DNA fingerprinting of the *Firmicutes* species yielded the smallest changes in the community composition for all lysimeter treatments (maximum value of the Nei coefficient, 0.15; Fig. 2B), suggesting a highly stable composition of this group and only marginal effects of the experimental manipulations.

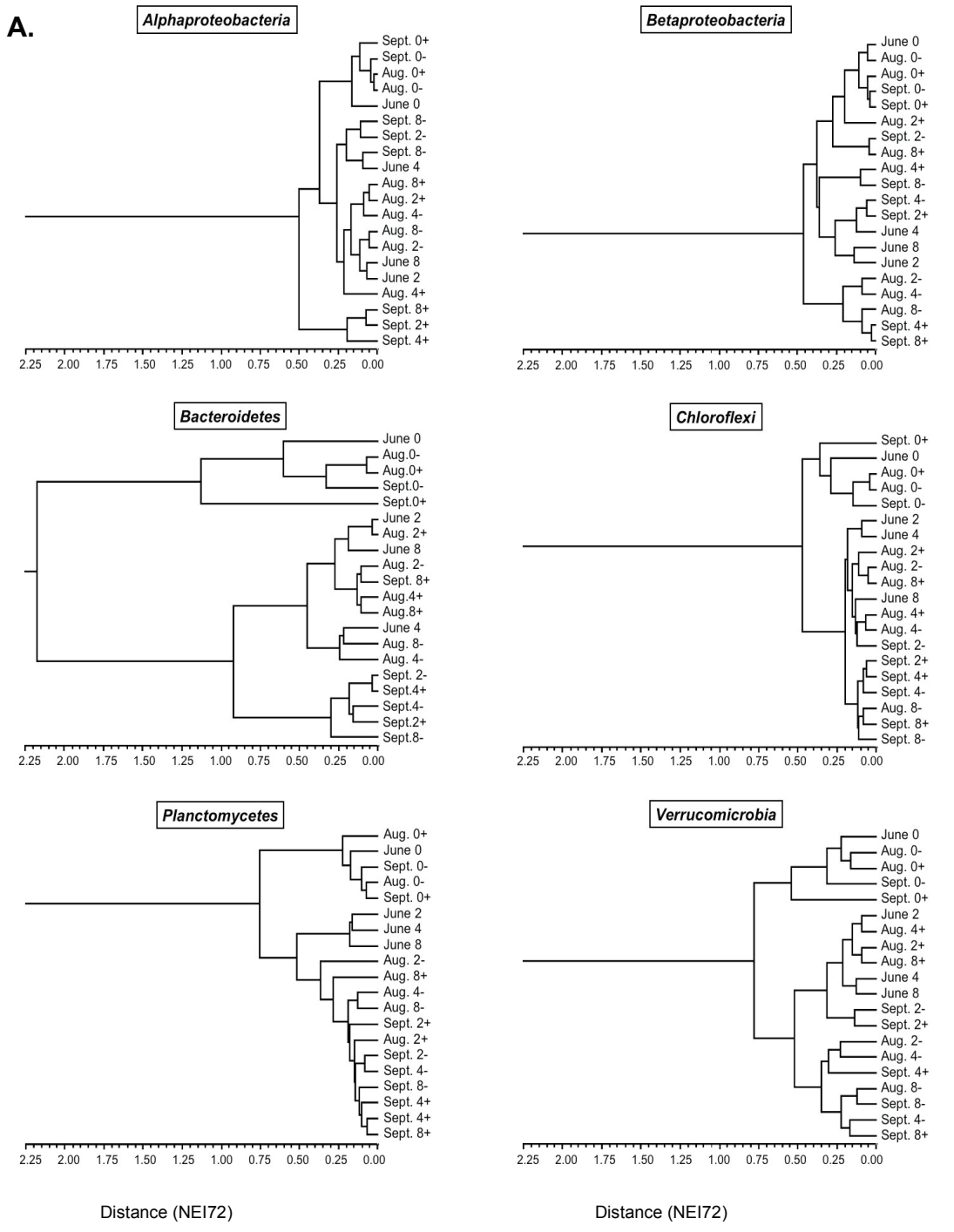


Figure 2. Similarity of the bacterial populations of individual (sub)phyla established under different experimental conditions in the lysimeters. The analyses are based on a comparison of the PCR-DGGE fingerprint patterns. **A.** Populations of *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes, Chloroflexi, Planctomycetes* and *Verrucomicrobia* species compared for each (sub)phylum separately. **B.** Populations of *Acidobacteria*, *Actinobacteria*, *Firmicutes* or *Archaea* species.

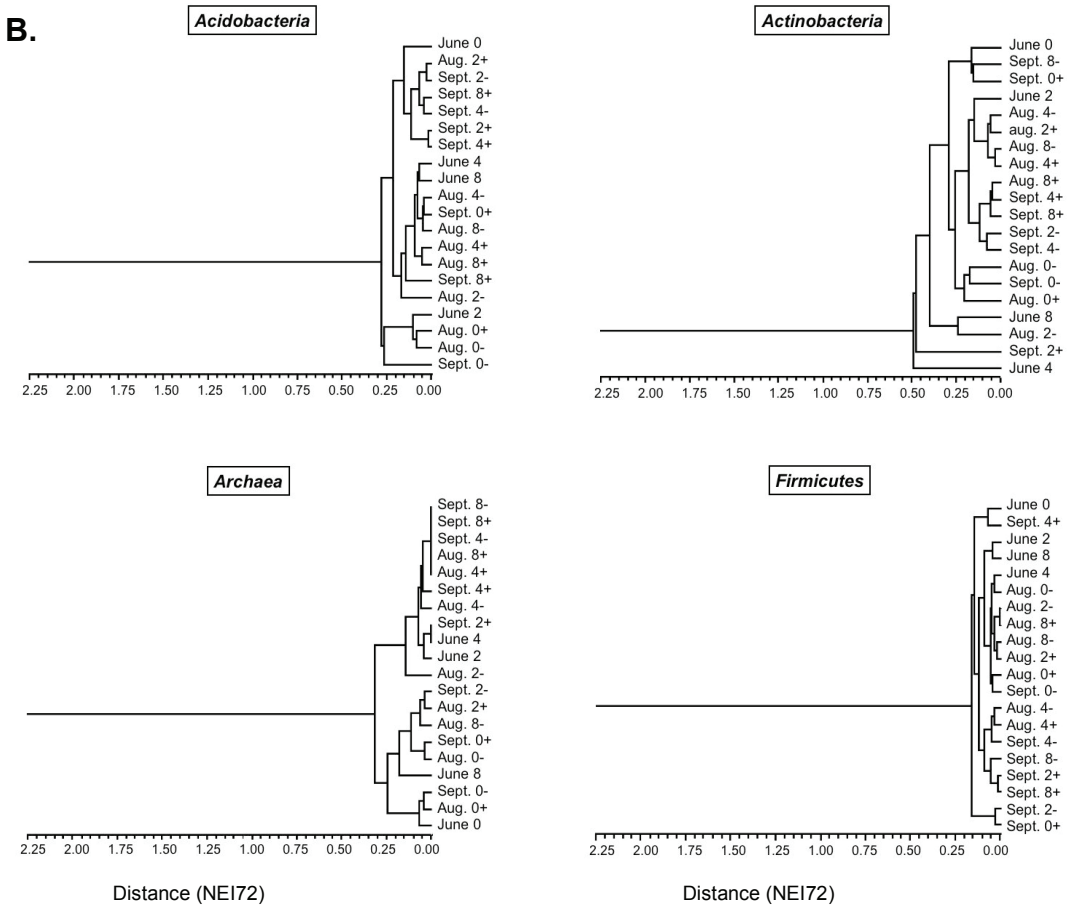


Figure 2. Continued

Through combination of the intensity profiles of fingerprints of all 10 groups, an analysis of the overall similarity of the different prokaryotic communities was conducted (Fig. 3). Two main clusters were observed which separated at a cutoff value of the Nei coefficient of 0.57. Cluster 1 comprised the populations, which had developed only in the planted lysimeters and cluster 2 those present in the lysimeters without higher plant species. Cluster 1 was composed of two subclusters, with subcluster 1a comprising the populations in the planted lysimeters sampled in September and subcluster 1b those in the planted lysimeters sampled in June and August. Our combined analysis therefore supports the conclusion that the presence or absence of higher plant species exerts the most pronounced effect on the overall composition of the prokaryotic community. Season was identified as a secondary control factor. In contrast, no effect of the other two factors tested, namely, the plant diversity or the water content, was observed.

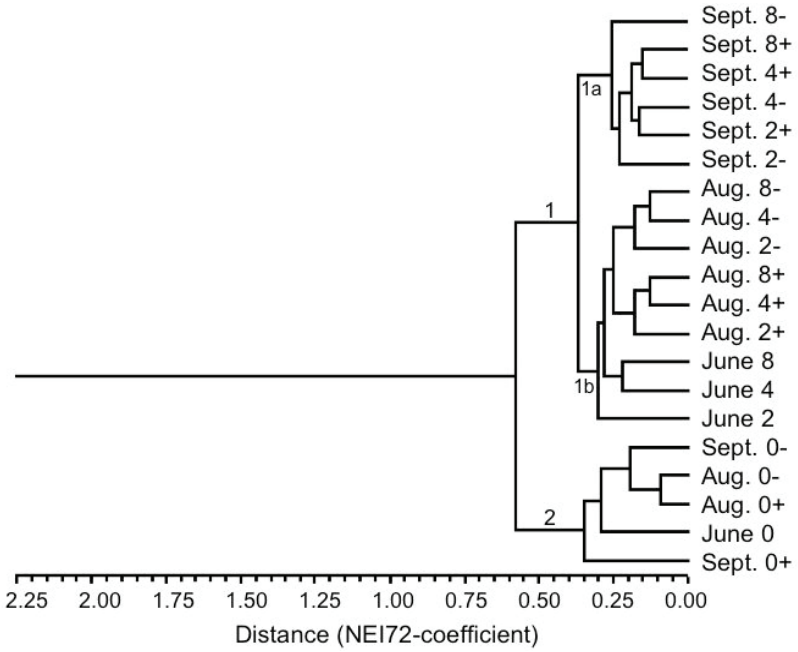


Figure 3. Similarity of the bacterial communities established under different experimental conditions in the lysimeters. The cluster analysis is based on a comparison of the combined PCR-DGGE fingerprint patterns of all 10 (sub)phyla which were investigated in the present study.

2.4.4 Phylogenetic analysis of the 16S rRNA gene fingerprints

The DNA bands generated by PCR/DGGE were excised and sequenced in order to resolve the phylogenetic affiliation of the bacteria occurring in the lysimeters and to investigate whether they represented known or novel phylotypes. In order to focus on the main fingerprints and on those associated with particular experimental conditions, we selected 145 out of the total of 336 melting types (Table 3). For some melting types, several DNA bands were analyzed in order to study the reproducibility of the fingerprinting approach. Overall, 174 DNA bands were thus analyzed by sequencing.

At the outset, we investigated whether DNA bands with the same melting behavior usually contained the same 16S rRNA gene sequences. In all 29 cases tested (e.g., case 2 of the *Chloroflexi* species and case 10 of the *Alphaproteobacteria* species; Fig. 1), bands with identical melting behaviors yielded identical 16S rRNA gene sequences. We also tested whether a single melting type comprised more than one 16S rRNA gene sequence. For 7 of the 10 target groups, each DNA band

analyzed yielded only a single 16S rRNA gene sequence (i.e., the number of melting types analyzed equaled the number of phylotypes recovered; Table 3). In contrast, some (14%) of the DGGE bands generated from *Chloroflexi* species and between 27 and 40% of the bands generated from the *Planctomycetes* and *Verrucomicrobia* species were found to contain multiple sequences which had to be separated by an additional cloning step prior to sequencing.

The presence of the same melting behavior for different 16S rRNA gene sequences masks changes in their relative abundance and hence limits the assessment of microbial diversity based on DGGE fingerprinting as described above. Based on our sequences analysis, it can be concluded that this limitation did not apply to our analysis of bacterial diversity for the seven phylogenetic groups and was only of minor importance in the case of *Chloroflexi* species (where only 14% of the bands contained multiple sequences). However, it is likely that fingerprinting of the *Verrucomicrobia* and *Planctomycetes* species did not reveal all of the differences which existed between the different lysimeters.

Out of a total of 336 detectable melting types, 145 were analyzed which (due to the presence of multiple sequences in the same band; see above) yielded 160 unambiguous 16S rRNA gene sequences (Table 3). Based on the comparison with the GenBank database, the majority of sequences of the *Chloroflexi*, *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes* species were only distantly related to those of known phylotypes. Phylogenetic analyses revealed that only 32 of the 160 phylotypes were most closely related to cultured bacteria (Fig. 4, also see Fig. S1A to S1J in the supplemental material). Members of the phylum *Actinobacteria* were exceptional in this respect, since half of the sequences from the lysimeters were affiliated with cultured phylotypes. However, the majority of the 16S rRNA gene sequences were found to be affiliated to environmental sequences, mostly originating from soil samples.

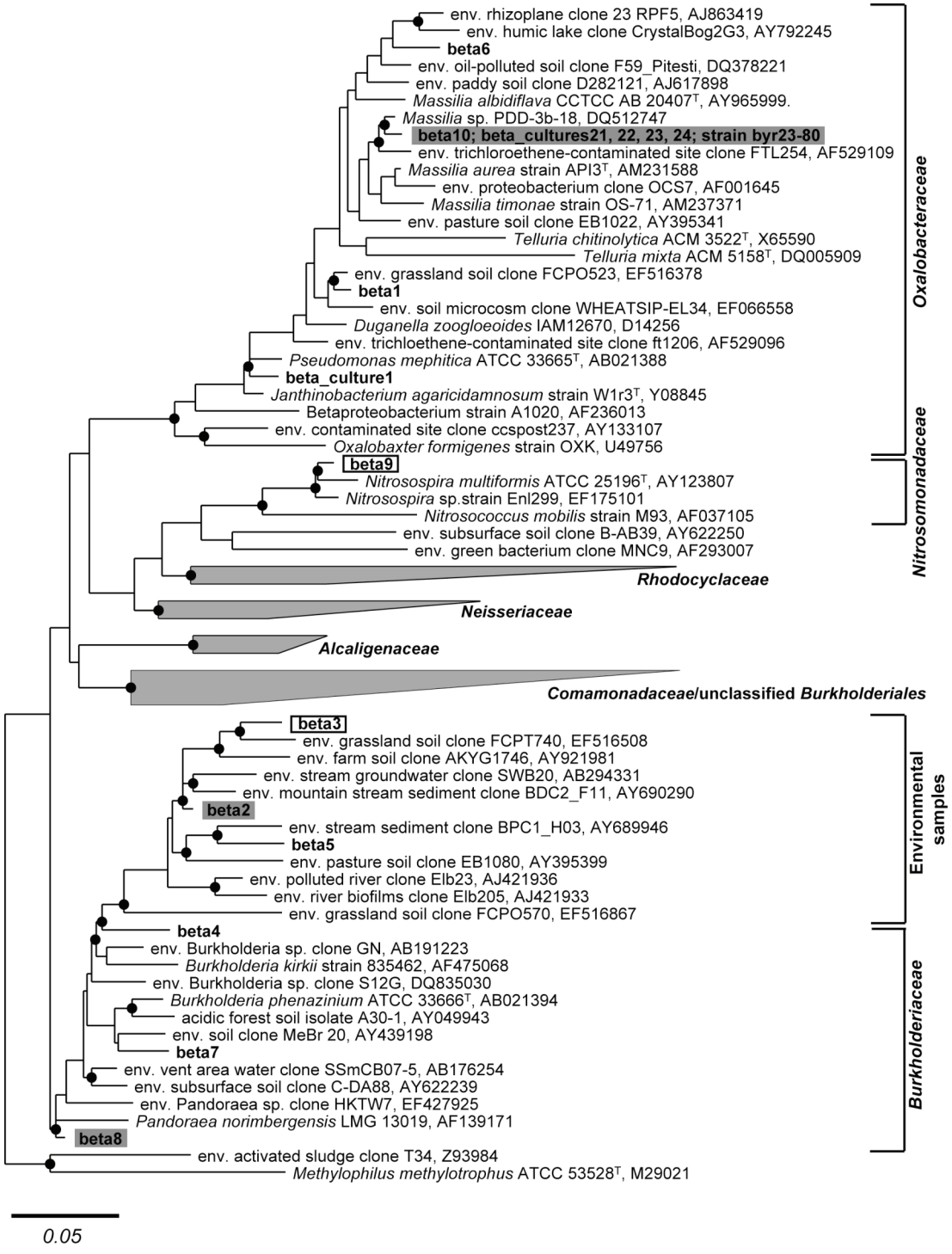


Figure 4. Maximum likelihood phylogenetic analysis of the 16S rRNA gene sequences of *Betaproteobacteria* species obtained in the present study (shown in bold face). Sequences obtained from barren lysimeters are shown in boxes; sequences recovered only from planted lysimeters are shaded in gray. Sequences detected in both types of lysimeters depicted in boldface only. Bar represents 0.05 fixed-point mutations per nucleotide. Nodes with a bootstrap support of ≥ 50% (1000 bootstrap resamplings) are denoted by black dots.

2.4.5 Diversity of cultured bacteria and comparison with environmental phylotypes

In an attempt to isolate some of the phylotypes associated with particular experimental conditions in the lysimeters, improved cultivation media were inoculated by two complementary techniques using the soil samples from August. This yielded a total of 217 bacterial cultures. For rapid screening, sets of five cultures were pooled, the cells lysed, and the 16S rRNA gene fragments of each set were amplified, employing the different group-specific primers. PCR products were analyzed by DGGE, running them besides representative amplificates of the environmental samples (Fig. 5).

Overall, 66 different DGGE melting types were detected among the 217 bacterial cultures (Table 3). Whereas no representatives of *Chloroflexi* and *Archaea* species were retrieved, the numbers of melting types detected for the other eight phylogenetic groups ranged between 3 and 17. Thirteen of the DGGE fingerprints corresponded to those of the natural community and originated from *Verrucomicrobia*, *Planctomycetes, Alphaproteobacteria*, *Betaproteobacteria, Actinobacteria* and *Firmicutes* species. Sequence analyses of these 13 melting types revealed that one melting type of the *Betaproteobacteria* species (Fig. 5) and one of the *Actinobacteria* species were identical to those of phylotypes detected by the culture-independent approach (Table 3). In the case of the *Betaproteobacteria* species, environmental sequence beta10 was detected in several culture sets (sets 21 through 24; Fig. 5). This sequence was affiliated at a sequence similarity of 99.2% to *Massilia* sp. strain PDD-3b-18 and at 99.1% to environmental clone FTL254 from a trichloroethene-contaminated site (Fig. 4). The sequences of the two actinobacterial cultures 5 and 38 matched the sequence of environmental HGC23 and were most closely related to that of *Arthrobacter* strain Ellin178 at a similarity of 97.6% (see Fig. S1G in the supplemental material).

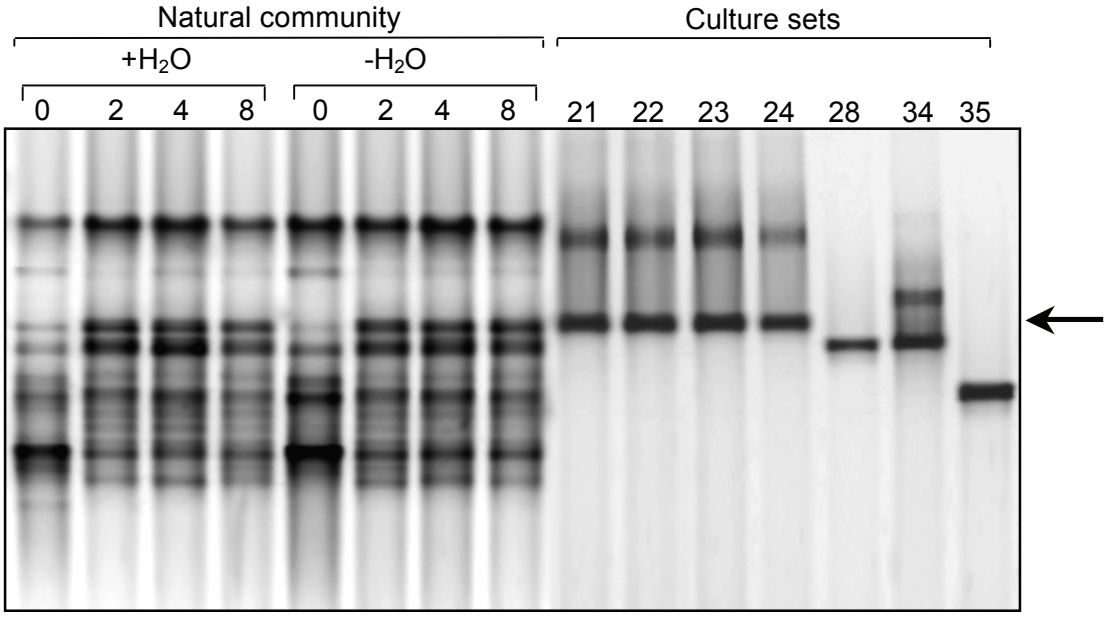


Figure 5. Comparison of DGGE fingerprints of *Betaproteobacteria* species of seven different culture sets (five cultures combined per set) isolated from August 2004 samples with fingerprints detected in the bacterial communities in lysimeters in the same month. A negative image of a SYBR gold-stained DGGE gel is shown. The arrow indicates melting position of phylotype byr23-80.

Whereas HGC23 represented a phylotype which occurred under most experimental conditions (Fig. 1C), the intensity of the fingerprints of phylotype beta10 clearly correlated with the presence of plants (Fig. 5). Consequently, beta10 was chosen for subsequent isolation and characterization of the corresponding bacterial strains.

2.4.6 Physiology and abundance of the beta10 phylotype

After different liquid cultures were streaked onto solid media, four different isolates of phylotype beta10 were recovered. All isolates exhibited the same cell morphology and were similar with respect to genome structure as revealed by genomic ERIC-PCR fingerprinting (Fig. 6). Therefore, one of the isolates was chosen for subsequent characterization. Strain byr23-80 is a 0.7- to 1.0-μm-wide and 1.5- to 2.0 μm-long motile short rods. Test results showed that it was gram negative, oxidase negative and weakly positive for catalase. The isolate is an obligate aerobe with a range of growth conditions of 4 to 30°C, pH 6 to 10, and up to 2% NaCl (wt/vol). Optimum

growth was observed at 15°C and pH 7 to 7.5. Strain byr23-80 utilized 9 out of 51 sugars or sugar derivatives (D-cellobiose, D-erythrose, L-erythrulose, D-galactose, glucose, glucose 1-phosphate, glucose 6-phosphate, *N*-acetylglucosamine and maltose). A total of 19 of 36 organic acids tested (acetate, adipate, butyrate, crotonate, fumarate, caproylate, caproate, β-hydroxybutyrate, isovalerate, lactate, levulinate, malate, caprylate, oxaloacetate, propionate, protocatechuate, pyruvate, succinate and valerate), and 3 ketoacids (α-ketoisocaproate, α-ketoglutarate and α-ketovalerate) as well as 13 different amino acids [L(+)alanine, L-alanylglycine, L-asparagine, L(+)asparaginate, L(+)cysteine, L(+)glutamate, L(+)isoleucine, L(+)leucine, L(+)lysine, L(+)phenylalanine, L(+)serine, L(+)threonine and L(+)tyrosine] were utilized as single carbon and energy source of growth. None of the eight alcohols tested served as a growth substrate. In addition, the isolate was capable of hydrolyzing Tween 20, Tween 80, starch and casein.

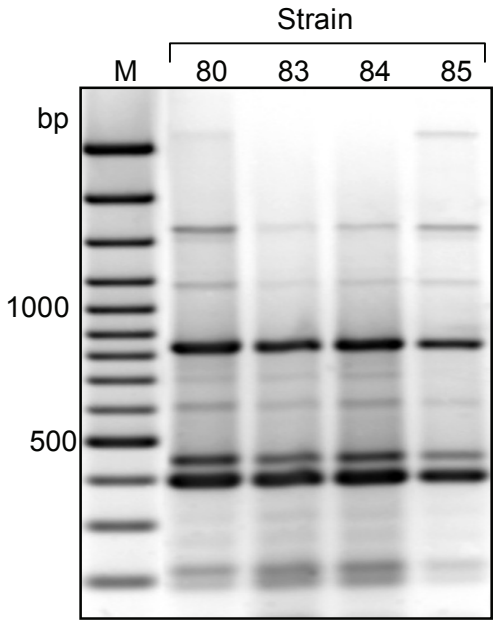


Figure 6. Genomic fingerprints of four strains of phylotype beta10 isolated from different liquid microtiter plate cultures. Genomic fingerprints were generated by ERIC-PCR. PCR products were separated in an agarose gel and stained with ethidium bromide. M, molecular size marker (100 bp-ladder).

Group-specific PCR/DGGE provides a rapid and sensitive yet semi-quantitative means of determining the composition of complex bacterial communities. We therefore used qPCR as an independent method of greater precision to quantify the abundance of phylotype beta10 and to confirm its interdependence with the presence of higher plant species. The complete sequence of the representative isolate byr23-80 was used to establish a specific qPCR protocol for its phylotype. Based on our qPCR results, cell numbers of phylotype beta10 ranged between $1.67 \cdot 10^5$ and $4.09 \cdot 10^6$ $(\text{g soil})^{-1}$ and in most cases were significantly elevated in planted lysimeters (Fig. 7A). Phylotype beta10 thereby constituted a fraction of $1.7 \cdot 10^{-4}$ to $1.8 \cdot 10^{-3}$ (i.e., 0.017 to 0.18%) of total bacterial cells (Fig. 7B).

2.5 Discussion

2.5.1 Representation of the bacterial diversity by group-specific PCR-DGGE fingerprinting

PCR-DGGE analyses which target *Bacteria* species as a single group typically yield about 30 individual melting types (Murray *et al.* 1996) and are capable of detecting phylotypes only when they constitute at least 1 to 9% of all phylotypes present in a bacterial community (Muyzer *et al.* 1991, Straub *et al.* 1998). The total number of phylotypes detected by the 10 different primer sets employed in the present study was 336. By use of the group-specific PCR-DGGE protocol, phylotype beta10 could be discerned in samples where this phylotype constituted as little as 0.017% of all bacterial cells (compare the leftmost lane in Fig. 5 with the corresponding value in Fig. 7B). This detection limit corresponded to 3,170 cells or 15 pg of genomic DNA of the beta10 phylotype per PCR. Thus, the approach used in the present study was suitable to rapid assessment of the structure of bacterial communities at an improved resolution. It should be emphasized that, since our PCR-DGGE analyses based on the amplification of 16S rRNA genes (and not rRNA), they provide information on changes in the composition of the entire bacterial community rather than focusing on differences in the physiologically active fraction of soil bacteria.

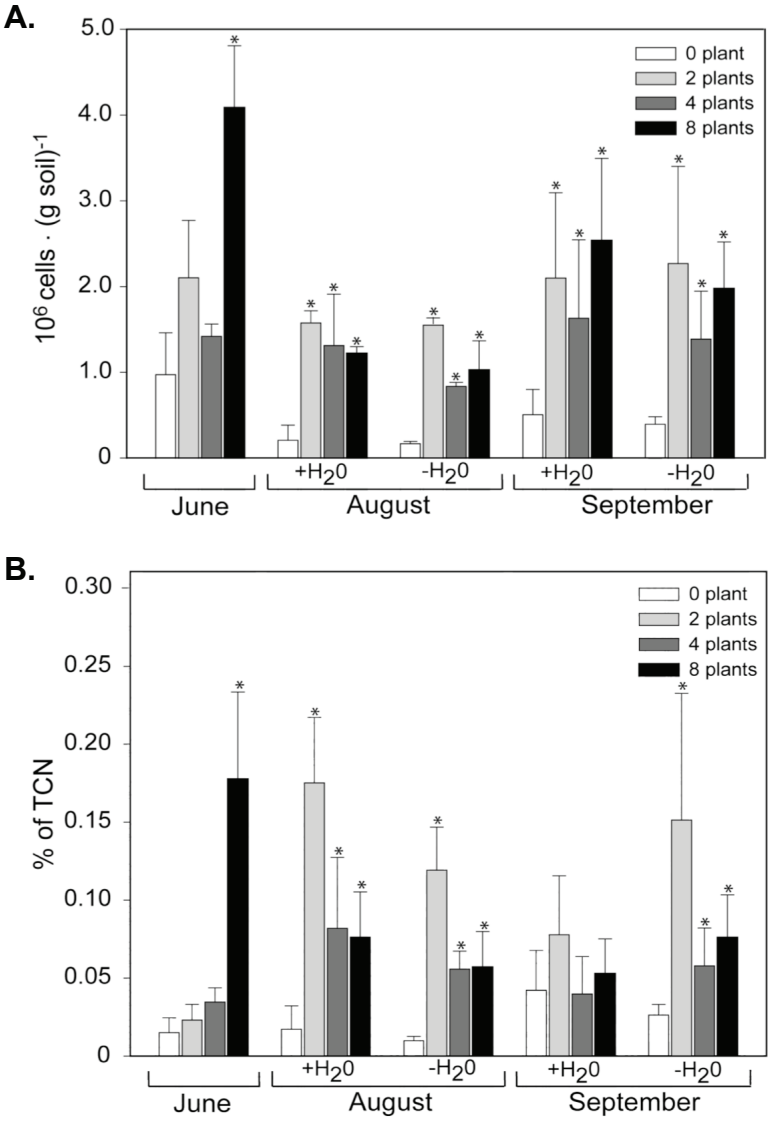


Figure 7. Abundance (**A**) and Relative abundance (**B**) (per total bacterial cell numbers [TCN]) of phylotype beta10 in the different lysimeters as quantified by specific quantitative PCR. Vertical lines indicate 1 standard deviation. *, significant differences compared to lysimeters devoid of plants at a significance level of $P < 0.05$.

2.5.2 Bacterial diversity in the lysimeters

None of the phylotypes detected in the present study matched 16S rRNA gene sequences in the databases. The sequences recovered from four of the target groups (*Chloroflexi*, *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes* species), were only distantly related to any of the sequences available in the databases. Many phylotypes occurred in different lysimeters as well as in the same lysimeter on different sampling dates and in addition were found to be most closely related to other environmental sequences from soil. Together, these observations indicate that the bacteria identified by our phylogenetic fingerprinting are members of bacterial clades which are indigenous and typical for the soil environment. Although different grassland bacterial communities have been studied extensively over the past decade (see, e.g., references Barns *et al.* 1999, Buckley and Schmidt 2001, Costello and Schmidt 2006, Janssen *et al.* 2002, Kuske *et al.* 2002, Lüdemann and Conrad 2000, McCaig *et al.* 2001, and Smalla *et al.* 2001), a significant fraction of the indigenous bacterial community obviously remains to be discovered. Based on our results, this applies in particular to the *Chloroflexi* and *Verrucomicrobia* species. Our data are in line with the recent discovery of numerous previously unknown *Chloroflexi* species in other soil ecosystems (Costello and Schmidt 2006). Although the *Chloroflexi* and *Verrucomicrobia* species typically represent rather small fractions of soil bacterial communities (7 and 3.2 % of all cloned 16S rRNA genes, respectively; see reference Janssen 2006), they represented the most diverse of the 10 groups investigated in the current study. Only 16 phylotypes of *Archaea* species were recovered by PCR-DGGE, indicating a low diversity of this group in soil. These results correspond to the low diversity of *Archaea* species in various soils of Norway and Indiana (Nakatsu *et al.* 2000).

2.5.3 Factors controlling soil bacterial diversity

Microbial biomass has been shown to significantly correlate with plant diversity in experimental fields planted with 1 to 16 plant species (Zak *et al.* 2003). This correlation was attributed to the higher level of primary production associated with higher plant species diversity. In our study, no interdependence of bacterial cell numbers and plant diversity was observed. Also, total bacterial cell counts did not

correlate with root biomass or total soil organic carbon numbers (see values for June 2004 in Table 2). Accordingly, our study focused on the interrelation of bacterial community composition with the presence or absence of plants, plant diversity, season, and water content.

Numerous previous studies did not reveal a correlation between the species composition of plants and the bacterial diversity in soils (Kowalchuk *et al.* 2002). No effect of plant community composition on the relative abundances of bacterial phyla on a Michigan long-term ecological research site (Buckley and Schmidt 2001) and a Dutch grassland site (Felske *et al.* 2000) was detected. Similarly, other studies showed that plant species composition had little direct effect on the bacterial community composition (Kennedy *et al.* 2004, Nunan *et al.* 2005), while the diversity of bacterial community in another study showed a correlation to the plant diversity in grassland soils (Grüter *et al.* 2006). Our work confirms that a coupling between the overall diversity of soil bacteria and the diversity of aboveground plant communities does not exist. This indicates that the interdependence between the absence or presence of plants and the abundance of various bacteria which we observed in the lysimeters features rather low specificity.

Specific associations between particular plant species and soil bacterial populations have so far been documented for a few individual groups, like diazotrophic (DembaDiallo *et al.* 1999, Héry *et al.* 2005) or dissimilatory (Héry *et al.* 2005) nitrate reducing bacteria. In the current study, however, 20 out of the 160 sequence types analyzed were found at increased abundance in lysimeters devoid of higher plant species. In contrast, the abundance of 32 phylotypes coincided with the presence of higher plant species in the lysimeters. Of these 32 phylotypes, only very few were closely related to known rhizosphere bacteria. Changes in the composition were observed for six different bacterial (sub)phyla. Furthermore, our analyses were limited to bulk soil. Root exudates and the microenvironments created by plants primarily affect the diversity of bacterial communities in the rhizosphere (Kowalchuk et al 2002, Smalla *et al.* 2001). Based on the fact that about one-third of the bacterial sequence types in bulk soil were found to correlate with the absence or presence of plants, the influence of plants must extend significantly beyond the

rhizosphere and must be of relevance to many different and previously unknown types of soil bacteria. These results are in contrast to observations in other grassland systems where plant rhizosphere effects were of little significance in the composition of total community structures (Kennedy *et al.* 2004). Our different results can be attributed to the higher resolution of the methods for diversity assessment employed in the current study.

Most notably, the DGGE fingerprint patterns observed for many of the minor constituents of the soil bacterial assemblage correlated with particular environmental conditions within the lysimeters. This observation also suggests that even low-abundance bacterial phylotypes reproducibly occupy particular ecological niches in soil. This indicates that the composition of soil bacterial communities is determined to a considerable extent by environmental conditions rather than being mostly the result of mere chance. Using a large-scale cultivation approach, we targeted a representative of the low-abundance phylotypes, quantified its abundance in response to environmental conditions, and obtained further insight into its ecological niche in order to test this hypothesis.

2.5.4 Insights from the culture-based studies

Analyses of the 16S rRNA gene sequences of the 217 bacterial cultures revealed that only about 3% represented environmental phylotypes detected by the culture-independent approach. In several previous studies, none of the environmental phylotypes could be recovered by cultivation (Dunbar *et al.* 1999, Lipson and Schmidt 2004, Smit *et al.* 2001). Various cultivation approaches have been shown to selectively favor the growth of *Actinobacteria* species over those of the dominant *Proteobacteria*, *Acidobacteria* or *Verrucomicrobia* species (Dunbar *et al.* 1999, Smit *et al.* 2001). Similarly, the diversity of *Actinobacteria* species was overrepresented in our culture collection in comparison to the bacterial community composition in the lysimeters. Yet sequences of two of the phylotypes cultured did match sequences detected in the natural bacterial community. Obviously, dominant phylotypes grow less readily in artificial growth media than rare ones. Consequently, the number of cultures established per soil sample needs to be increased in order to improve the chances of isolating representative bacteria from the soil environment.

The betaproteobacterial isolate byr23-80 was studied in detail, since the corresponding beta10 sequence represented that of one of the low-abundance phylotypes which displayed a distinct response towards the presence of higher plant species. Strain byr23-80 was identified as a novel lineage within the genus *Massilia*. In another study, addition of fresh plant organic matter to a calcareous silty-clay soil resulted in a pronounced and specific stimulation of *Beta-* and *Gammaproteobacteria* over *Actinobacteria*, *Cyanobacteria*, *Gemmatimonadetes* and *Planctomycetes* species (Bernard *et al.* 2007). Remarkably, the largest number of additional sequences detected in that study after the addition of the fresh organic matter belonged to the *Massilia* group. However, the correlation between the abundance of phylotype beta10 and the presence of living plants suggests a closer interaction between the two. Physiological characterization revealed that isolate byr23-80 is a highly versatile bacterium capable of utilizing a wide spectrum of organic compounds as single carbon and energy sources. Root exudates consist of organic acids, amino acids, and sugars. Strain byr23-80 was found to be capable of using at least eight (glucose, fumarate, succinate, malate, glutamate, alanine, leucine and serine) of the 21 (Kozdrój and van Elsas 2000) major constituents of root exudates. Using culture-independent stable isotope techniques, bacteria of the genus *Massilia* have been shown to be active in soil and to rapidly respire glucose, but not phenol, naphthalene or caffeine (Padmanabhan *et al.* 2003).

2.5.5 Implications for the assessment of soil microbial diversity

DNA reassociation studies indicate that soil bacterial communities harbor up 50,000 (Sandaa *et al.* 1999) or even up to millions (Gans *et al.* 2005) of different 16S rRNA gene sequences. From these large numbers it has to be deduced that the diversity of complex microbial communities resides mostly in low-abundance species. Indeed, thousands of low-abundance populations were found to account for most of the unexpectedly high diversity of deep-sea bacterioplankton communities (Sogin *et al.* 2006).

The high diversity of soil bacterial communities could be (i) due to a multitude of ecological niches and adaptive mechanisms (Dykhuizen 1998, Jaspers and Overmann 2004) and/or (ii) caused by high functional redundancy (Griffiths *et al.*

2001, Wertz 2006) of the soil bacteria. One-third (52 out of 160) of the sequence types analyzed in the present study showed a distinct response to a single environmental factor tested, namely, the presence or absence of plants. As demonstrated for one of them (beta10), at least some of these phylotypes constitute only a very small fraction (0.017 to 0.18% of total cell numbers) of the soil microbial community in the lysimeter samples. Still, the occurrence and response of these phylotypes followed a reproducible pattern in independent lysimeters. Our results suggest that the bacterial species composition in soil is determined to a significant extent by abiotic and biotic factors rather than mere chance. The observation of a high reproducibility of bacterial fingerprint patterns in independent lysimeters also contradicts the general assumption of a high functional redundancy in soil and indicates the presence of a multitude of distinct ecological niches.

2.6 Acknowledgements

We thank Petra Fritsche and Monika Anna Janys for help with data analysis. We are grateful to Barbara Reinhold-Hurek (University of Bremen) for providing the soil samples. This work was supported by grant no. BIOLOG/01LC0021 of the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie to Jörg Overmann. Delita Zul was granted a Technological and Professional Skills Development Sector Project (TPSDP) scholarship to perform this work.

2.7 References

1. **Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, W. Miller, and D.J. Lipman**. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389-3402.

2. **Amann, R., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbial. Rev. **59**:143-169.

3. **Angle, J.S., S.P. McGrath, and R.L. Chaney**. 1991. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. Appl. Environ. Microbiol. **57**:3674-3676.

4. **Barns, S.M., S.L. Takala, and C.L. Kuske**. 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Appl. Environ. Microbiol. **65**:1731-1737.

5. **Bernard, L., C. Mougel, P.-L. Maron, V. Nowak, J. Leveque, C. Henault, F. el Zahar Haichar, O. Berge, C. Marol, J. Balesdent, F. Gibiat, P. Lemanceau, and L. Ranjard.** 2007. Dynamics and identification of soil microbial populations actively assimilating carbon from ^{13}C-labelled wheat residue as estimated by DNA- and RNA-SIP techniques. Environ. Microbiol. **9:** 752-764.

6. **Bezemer, T. M., C. Lawson, K. Hedlund, A.R. Edwards, A.J. Brooks, J.M. Igual, S.R. Mortimer, and W.H. van der Putten.** 2006. Plant species and functional groups effect on abiotic and microbial properties and plant-soil feedback responses in two grasslands. J. Ecol. **94**:893-904.

7. **Bremer, C.** 2007. Impact of plantidentity, diversity and composition on diversity, composition and function of *nirK*-type denitrifying microorganisms in temperate grassland soil. Ph.D. dissertation. Universität Marburg, Marburg, Germany.

8. **Bruns, A., H. Hoffelner, and J. Overmann**. 2003. A novel approach for high throughput cultivation assays and the isolation of planktonic bacteria. FEMS Microbiol. Ecol. **45**:161-171.

9. **Buckley, D.H., and T.M. Schmidt.** 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microb. Ecol. **42:**11-21.

10. **Costello, E.K., and S.K. Schmidt.** 2006. Microbial diversity in alpine tundra wet meadow soil: novel *Chloroflexi* from a cold, water-saturated environment. Environ. Microbiol. **8**:1471-1486.

11. **Coutinho, H.L.C., V.M. Oliveira, A. Lovato, A.H.N. Maia, and G.P. Manfio.** 1999. Evaluation of the diversity of rhizobia in Brazilian agricultural soils cultivated with soybeans. Appl. Soil. Ecol. **13**:159-167.

12. **Daims, H., A. Brühl, R. Amann, K.H. Schleifer, and M. Wagner**. 1999. The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22**:434-444.

13. **DembaDiallo, M., A. Willems, N. Vloemans, S. Cousin, T.T. Vandekerckhove, P. de Lajudie, M. Neyra, W. Vyverman, M. Gillis, and K. Van der Gucht.** 2004. Polymerase chain reaction denaturing gradient gel electrophoresis analysis of the N_2-fixing bacterial diversity in soil under *Acacia tortilis* spp. *raddiana* and *Balanites aegyptiaca* in the dryland part of Senegal. Environ. Microbiol. **6**: 400-415.

14. **Dunbar, J., S. Takala, S.M. Barns, J.A. Davis, and C.R. Kuske**. 1999. Levels of bacterial diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. **65**:1662-1669.

15. **Dykhuizen, D.E.** 1998. Santa Rosalia revisited: Why are there so many species of bacteria? Ant. v. Leeuwenhoek **73:**25-33.

16. **Felske, A., A. Wolterink, R. van Lis, W.M. de Vos, A.D.L. Akkermans.** 2000. Response of a soil bacterial community to grassland succession as monitored by 16S rRNA levels of the predominant ribotypes. Appl. Environ. Microbiol. **66**:3998-4003.

17. **Fierer, N**., **and R.B. Jackson**. 2006. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. USA **103**:626-631.

18. **Gans, J., M. Wolinsky, and J. Dunbar.** 2005. Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science **309**:1387-1390.

19. **Gich, F., J. Garcia-Gil, and J. Overmann**. 2001. Previously unknown and phylogenetically diverse members of green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. **177**:1-10

20. **Gich, F., and Overmann, J.** 2006. *Sandarakinorhabdus limnophila* gen. nov., sp. nov., a new bacteriochlorophyll *a*-containing obligately aerobic bacterium isolated from freshwater lakes. Int. J. Syst. Evol. Microbiol. **56**:847-854.

21. **Gich, F., K. Schubert, A. Bruns, H. Hoffelner, and J. Overmann**. 2005. Specific detection, isolation, and characterization of selected, previously uncultured members of the freshwater bacterioplankton community. Appl. Environ. Microbiol. **71**:5908-5919.

22. **Griffiths, R.I., K. Ritz, R. Wheatley, H.L. Kuan, B. Boag, S. Christensen, F. Ekelund, S.J. Sørensen, S. Muller, and J. Bloem.** 2001. An examination of the biodiversity – ecosystem function relationship in arable soil microbial communities. Soil. Biol. Biochem. **33**:1713-1722.

23. **Grüter, D., B. Schmid, and H. Brandl.** 2006. Influence of plant diversity and elevated atmospheric carbon dioxide levels on belowground bacterial diversity. BMC Microbiology. **6**:68.

24. **Héry, M., L. Philippot, E. Mériaux, F. Poly, X. Le Roux, and E. Navarro.** 2005. Nickel mine spoils revegetation attempts: effect of pioneer plants on two functional bacterial communities involved in the N-cycle. Environ. Microbiol. **7**:486-498.

25. **Jaeger, C.H., S.E. Lindow, W. Miller, E. Clark, and M. Firestone.** 1999. Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Appl. Environ. Microbiol. **65**:2685-2690.

26. **Janssen, P.H.** 2006. Identifying the dominant soil bacterial taxa in Libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. **72**:1719-1728.

27. **Janssen, P.H., P.S. Yates, B.E. Grinton, P.M. Taylor, and M. Sait.** 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria, Actinobacteria, Proteobacteria,* and *Verrucomicrobia.* Appl. Environ. Microbiol. **68**:2391-2396.

28. **Jaspers, E., K. Nauhaus, H. Cypionka, and J. Overmann**. 2001. Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. FEMS Microbiol. Ecol. **36**:153-164.

29. **Jaspers, E., and Overmann, J.** 2004. The ecological significance of "microdiversity": identical 16S rRNA gene sequences represent bacteria with highly divergent genomes and physiology. Appl. Environ. Microbiol. **70**:4831-4839.

30. **Kennedy, N., E. Brodie, J. Connolly, and N. Clipson**. 2004. Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms. Environ. Microbiol. **6**:1070-1080.

31. **Kossmann, G.** 2005. Plant functional traits and ecosystem functions in experimental grassland stands. Dissertation Universität Bayreuth, 184 p.

32. **Kowalchuk, G.A., D.S. Buma, W. de Boer, P.G.L. Klinkhamer, and J.A. van Veen**. 2002. Effect of a aboveground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Leeuwenhoek **81**:509-520.

33. **Kozdrój, J., and J.D. van Elsas**. 2000. Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. Soil Biol. Biochem. **32**:1405-1417.

34. **Kuske, R.C., L.O. Ticknor, M.E. Miller, and J.M. Dunbar**. 2002. Comparison of soil bacterial communities in rhizospheres of three plant species and the interspaces in an arid grassland. Appl. Environ. Microbiol. **68**:1854-1863.

35. **Lane, D.J.** 1991. 16S/23S rRNA sequencing, p 115–175. *In*: E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley, Chichester, United Kingdom.

36. **Lipson, D.A., and S.K. Schmidt**. 2004. Seasonal changes in an alpine soil bacterial community in the Colorado rocky mountain. Appl. Environ. Microbiol. **70**:2867-2879.

37. **Lüdeman, H**., **and R. Conrad.** 2000. Molecular retrieval of large 16S rRNA gene fragments from an Italian rice paddy soil affiliated with the class *Actinobacteria*. Syst. Appl. Microbiol. **23**:582-584.

38. **Maidak, B.L., J.R. Cole, T.G. Lilburn, C.T. Parker, P.R. Saxman, R.J. Farris, G.M. Garrity, G.J. Olsen, T.M. Schmidt and J.M. Tiedje.** 2001. The RDP-II (Ribosomal Database Project). Nucleic. Acids. Res. **29**:173-174.

39. **McCaig, A.E., L.A. Glover, and J.I. Prosser.** 2001. Numerical analysis of grassland bacterial communitiy structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Appl. Environ. Microbial. **67**:4554-4559.

40. **Meier, H., R. Amann, W. Ludwig, and K.H. Schleifer.** 1999. Specific oligonucleotide probes for *in situ* detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. **22**:186-196.

41. **Murray, A.E., J.T. Hollibaugh and C. Orrego.** 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl. Environ. Microbiol. **62:**2676-2680.

42. **Muyzer, G., E.C. de Waal, and A.G. Uitterlinden**. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695-700.

43. **Muyzer, G., and N.B. Ramsing**. 1995. Molecular methods to study the organization of microbial communities. Water Science and Technology **32**:1–9.

44. **Nakatsu, C., V. Torsvik, L. Øvreas**. 2000. Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. Soil Sci. Soc. Am. J. **64**:1382-1388.

45. **Neef, A**. 1997. Anwendung der *in situ*-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Ph.D. thesis. Technische Universität München, Munich, Germany.

46. **Neef, A., R. Amann, H. Schlesner, and K.H. Schleifer**. 1998. Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. Microbiolology. **144**:3257-3266.

47. **Nei, M.** 1972. Genetic distance between populations. Am. Naturalist **106**:283-292.

48. **Nunan, N., T.J. Daniell, B.K. Singh, A. Papert, J.W. McNicol, and J.I. Prosser**. 2005. Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. Appl. Environ. Microbiol. **71**:6784-6792.

49. **Overmann, J., M.J.L. Coolen, and C. Tuschak.** 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch. Microbiol. **172**:83-94.

50. **Padmanabhan, P., S. Padmanabhan, C. DeRito, A. Gray, D. Gannon, J.R. Snape, C.S. Tsai, W. Park, C. Jeon, and E.L. Madsen.** 2003. Respiration of ^{13}C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of ^{13}C-labeled soil DNA. Appl. Environ. Microbiol. **69**:1614-1622.

51. **Pankhurst, C.E., C.A. Kirkby, B.G. Hawke, and B.D. Harch.** 2002. Impact of a change of tillage and crop residue management practice on soil chemical and microbiological properties in a cereal-producing red duplex soil in NSW, Australia. Bio. Fertil. Soils **35**:189-196.

52. **Rangel-Castro, J.I., K. Killham, N. Ostle, G.W. Nicol, I.C. Anderson, C.M. Scrimgeour, P. Ineson, A. Meharg, and J. Prosser.** 2005. Stable isotope probing analysis of the influence of liming on root exudate utilization by soil microorganisms. Environ. Microbiol. **7**:828-838.

53. **Reuter, A.** 2005. Ph.D. dissertation. Biomassebildung und Nährstoffvermögen der Wurzeln in experimentellen Grünlandbeständen mit unterschiedlicher Pflanzenartzusammen-setzung. Universität Bayreuth, Bayreuth, Germany.

54. **Sandaa, R. A., V. Torsvik, O. Enger, F. L. Daae, T. Castberg, and D. Hahn.** 1999. Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. FEMS Microbiol. Ecol. **30**:237-251.

55. **Sangwan, P., S. Kovac, K.E.R. Davis, M. Sait, and P.H. Janssen.** 2005. Detection and cultivation of soil *Verrucomicrobia*. Appl. Environ. Microbiol. **71**:8402-8410.

56. **Shannon, C.E., and W. Weaver**. 1963. The mathematical theory of communication. University of Illinois Press, Urbana, IL.

57. **Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer, and G. Berg.** 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. **67**:4742-4751.

58. **Smit, E., P. Leeflang, S. Gommans, J. van den Broek, S. Van Mil, and K Wernars**. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. **67**:2284-2291.

59. **Sogin, M.L., H.G. Morrison, J.A. Huber, D.M. Welch, S.M. Huse, P.R. Neal, J.M. Arrieta, and G.J. Herndl.** 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc. Natl. Acad. Sci. USA **103**:12115-12120.

60. **Stahl, D.A., and R.I. Amann**. 1991. Development and application of nucleic acid probes in bacterial systematics, pp. 205–248. *In*: E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

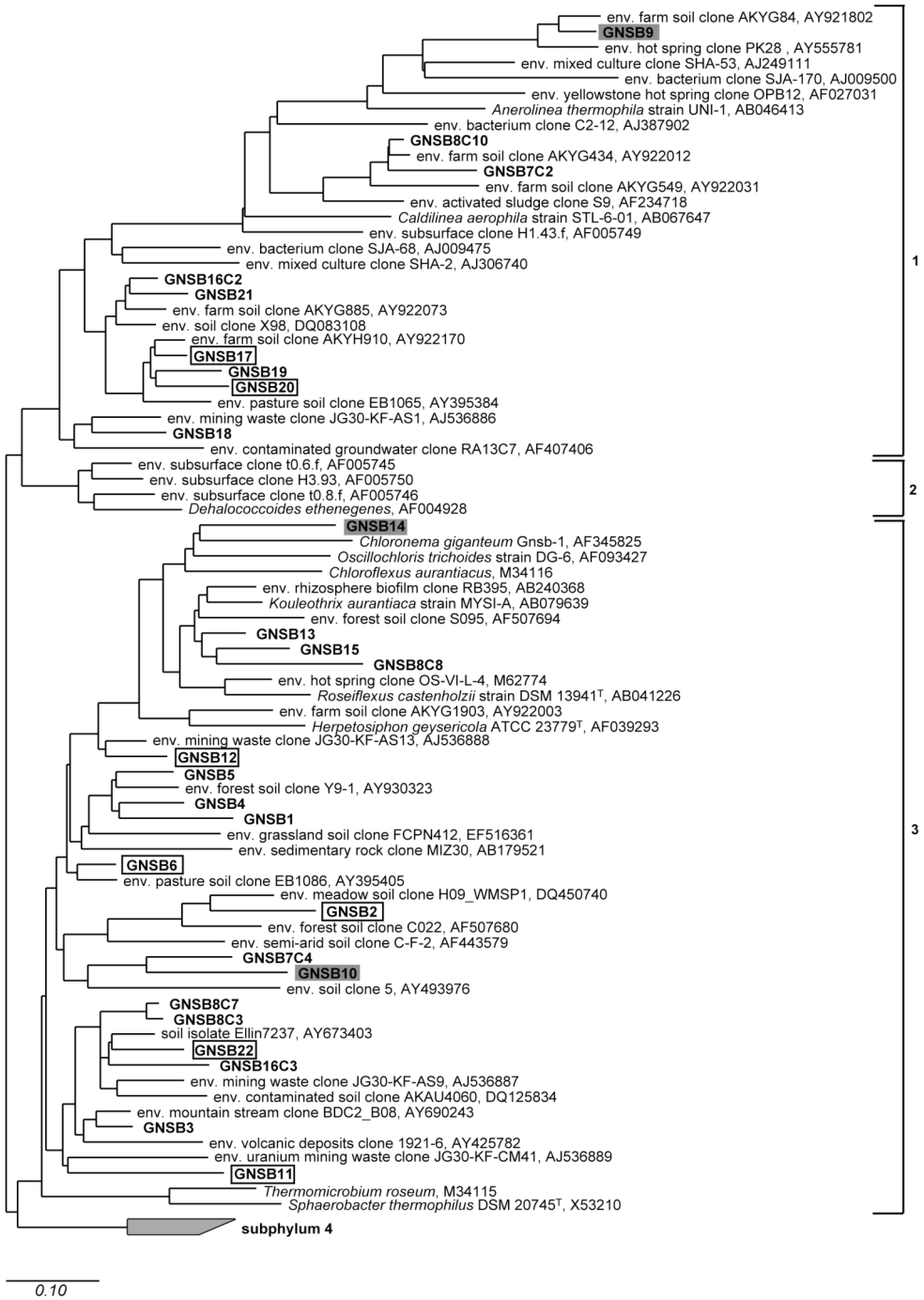
61. **Straub, K.L., and B.E.E. Buchholz-Cleven.** 1998. Enumeration and detection of anaerobic ferrous iron-oxidizing, nitrate-reducing bacteria from diverse European sediments. Appl. Environ. Microbiol. **64**:4846-4856.

62. **Versalovic, J., T. Koeuth, and J.R. Lupski.** 1991. Distribution of repetitive DNA sequences in Eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823-6831.

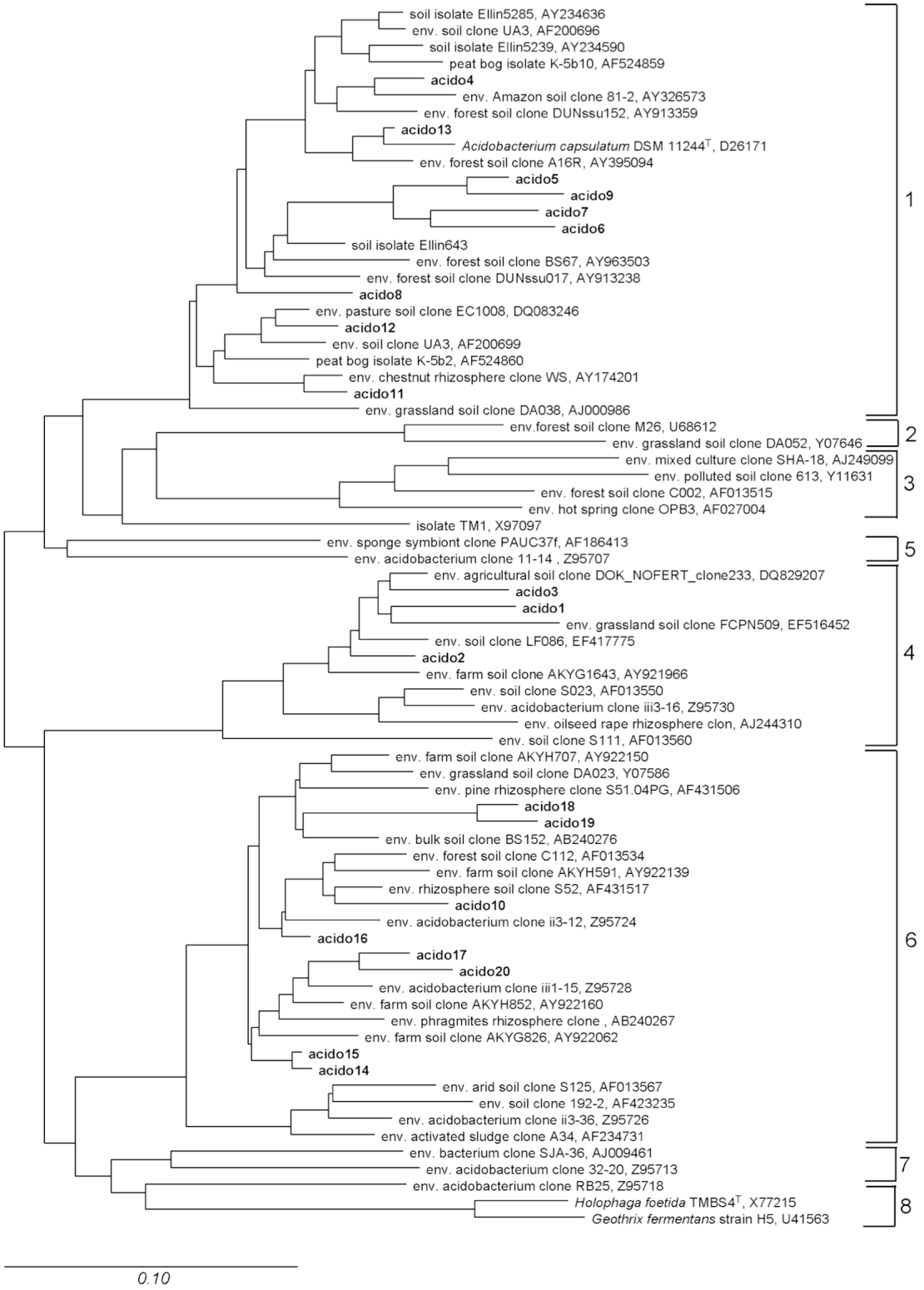
63. **Wertz, S., V. Degrange, J.I. Prosser, F. Poly, C. Commeaux, T. Freitag, N. Guillaumaud, and X. Le Roux.** 2006. Maintenance of soil functioning following erosion of microbial diversity. Environ. Microbiol. **8**:2162-2169.

64. **Zak, D.R., W.E. Holmes, D.C. White, A.D. Peacock, and D. Tilman**. 2003. Plant diversity, soil microbial communities, and ecosystem function: are there any links? Ecology. **84**:2042-2050.

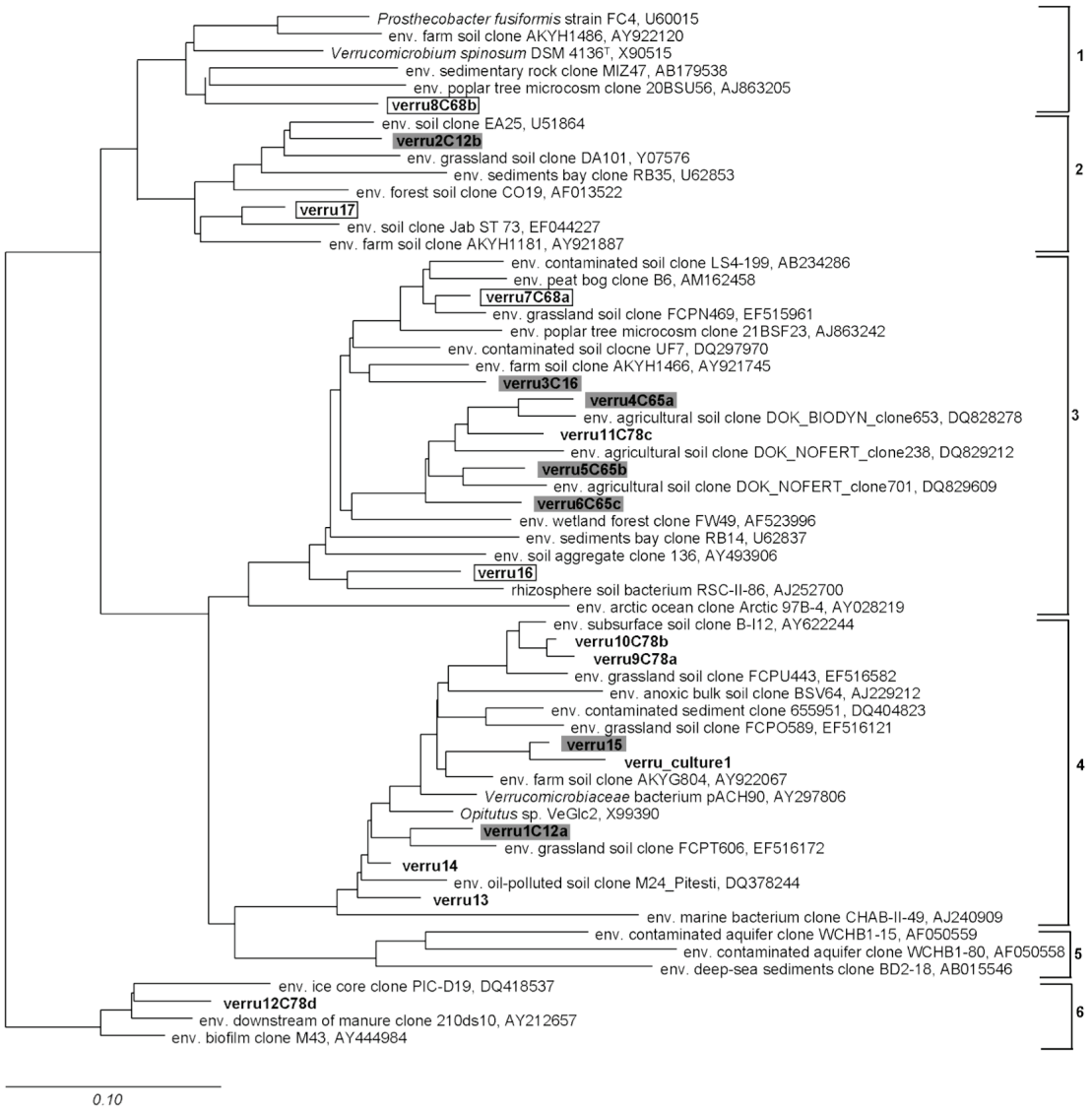
2.8 Supplementary material



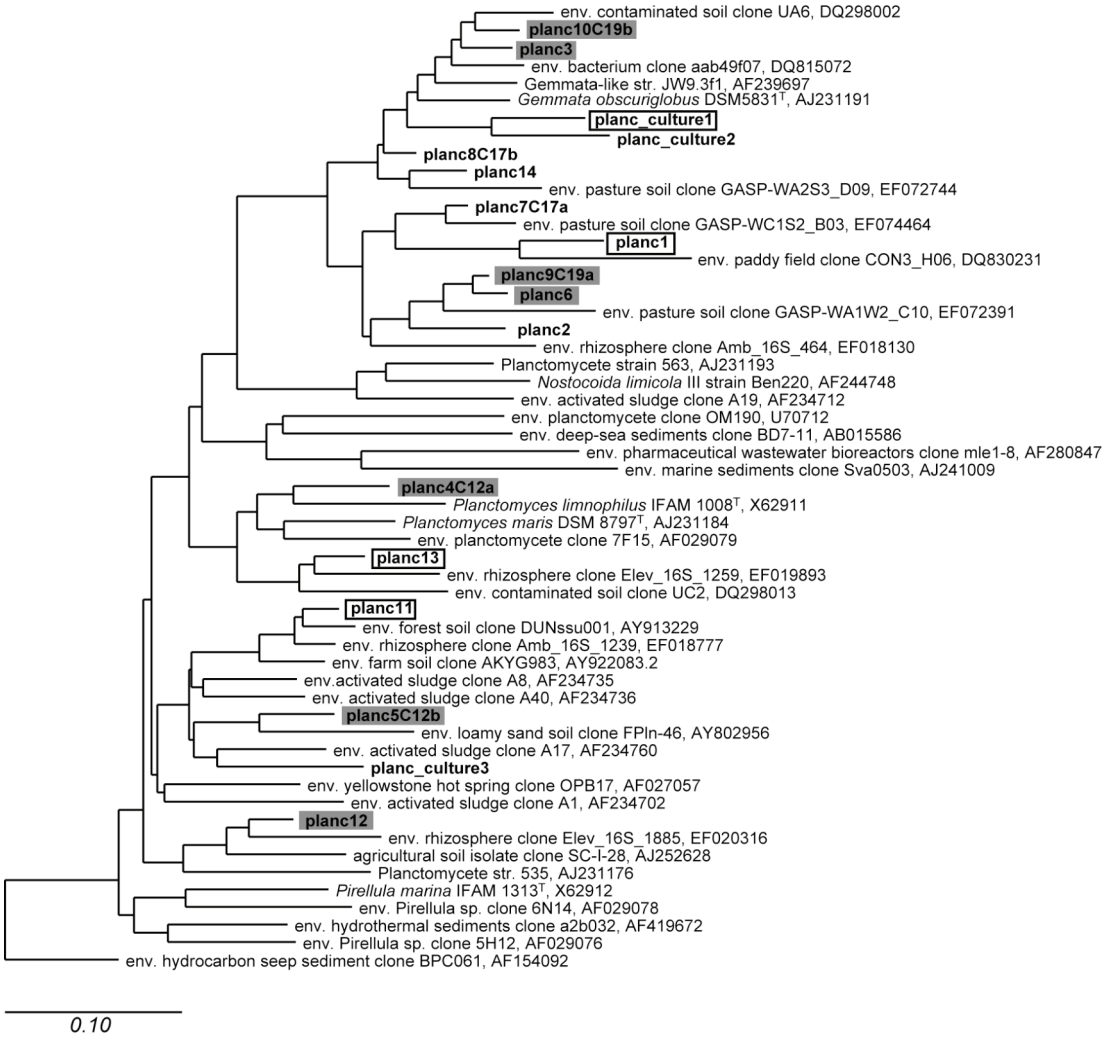
Supplementary Figure 1A. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Chloroflexi* species recovered in the present study (shown in bold face). Sequences exclusively obtained from barren lysimeters are shown in boxes, sequences recovered only from planted lysimeters are shaded in grey. Sequences detected in both types of lysimeters depicted in bold face only. Sequences denoted with an additional "C" were recovered after cloning DGGE bands. Arabic numerals to the right of the tree denote established subgroups. Bar represents 0.1 fixed point mutations per nucleotide.



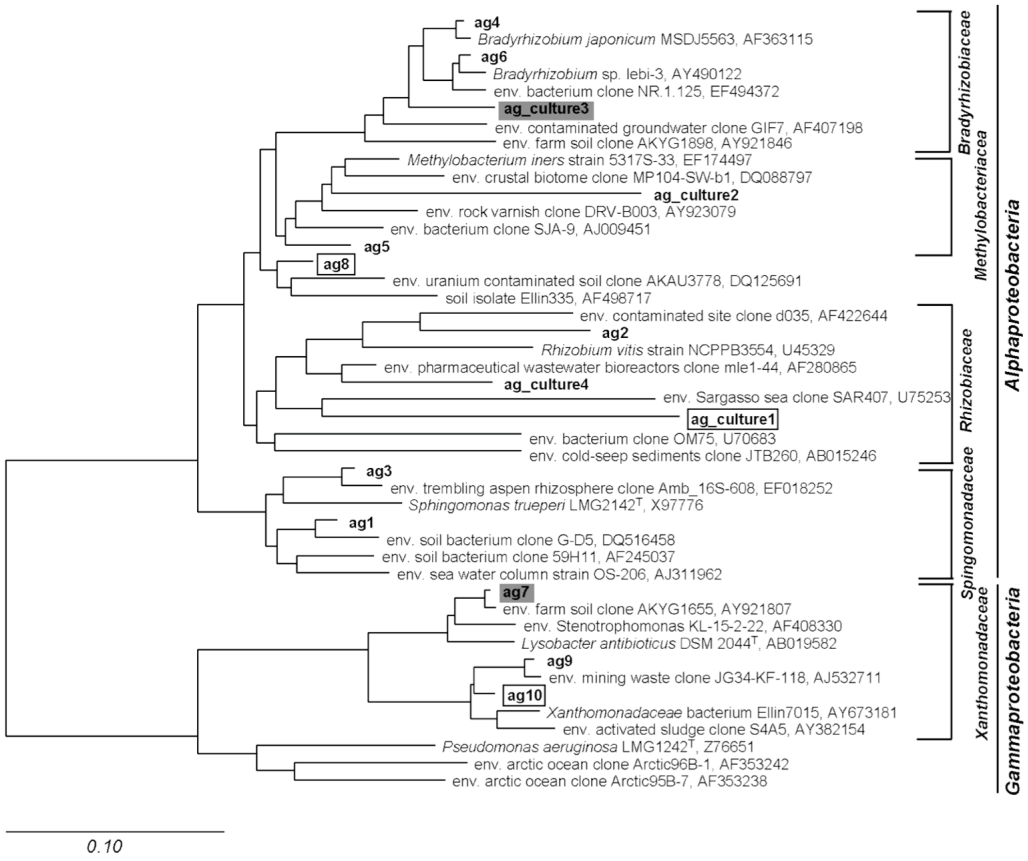
Supplementary Figure 1B. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Acidobacteria* species that all sequences were detected in barren and planted lysimeters (bold face). Arabic numerals to the right of the tree denote established subgroups. Bar represents 0.1 fixed point mutations per nucleotide.



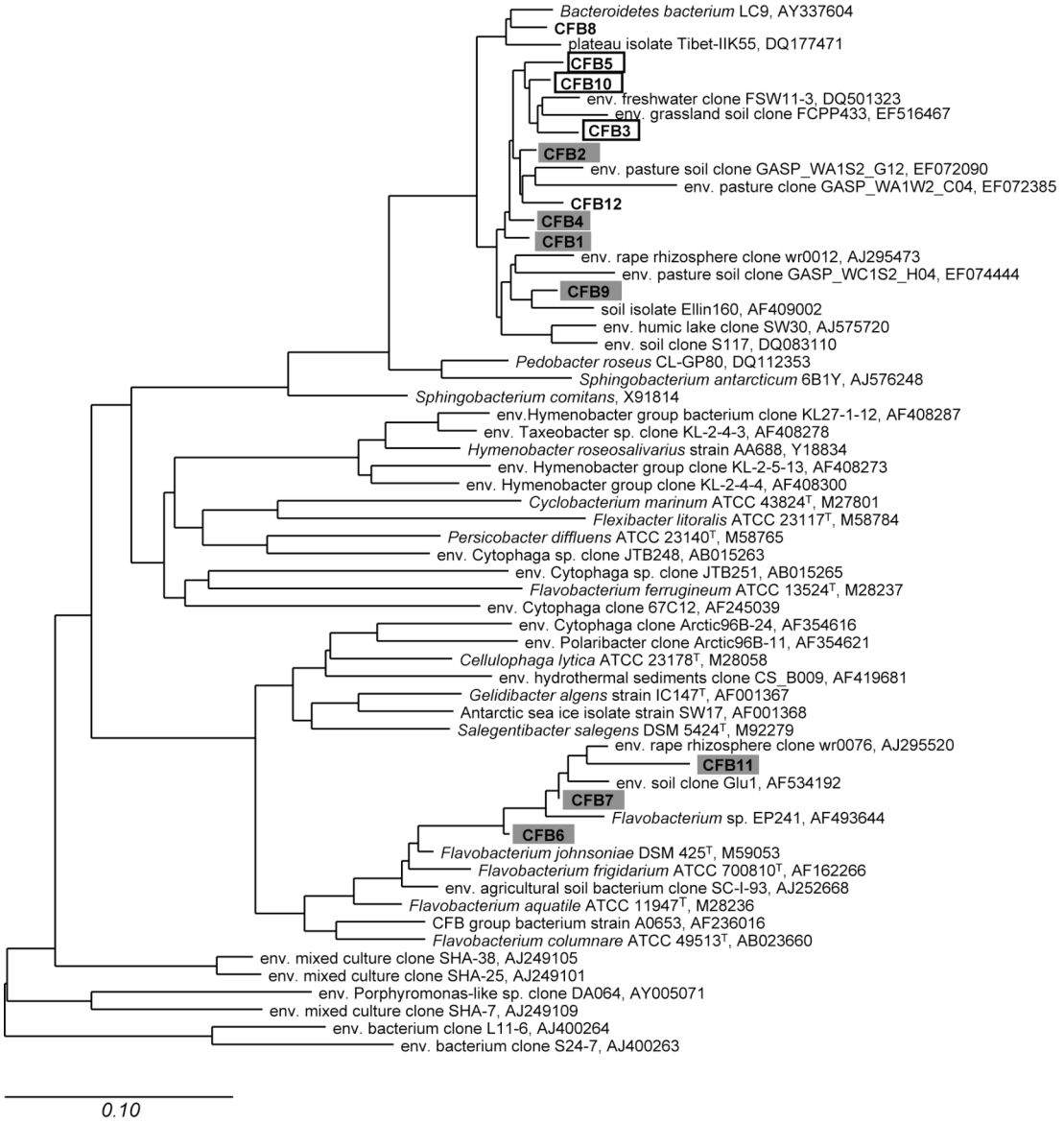
Supplementary Figure 1C. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Verrucomicrobia* species recovered in the present study (shown in bold face). Sequences exclusively obtained from barren lysimeters are shown in boxes, sequences recovered only from planted lysimeters are shaded in grey. Sequences detected in both types of lysimeters depicted in bold face only. Sequences denoted with an additional "C" were recovered after cloning DGGE bands. Arabic numerals to the right of the tree denote established subgroups. Bar represents 0.1 fixed point mutations per nucleotide.



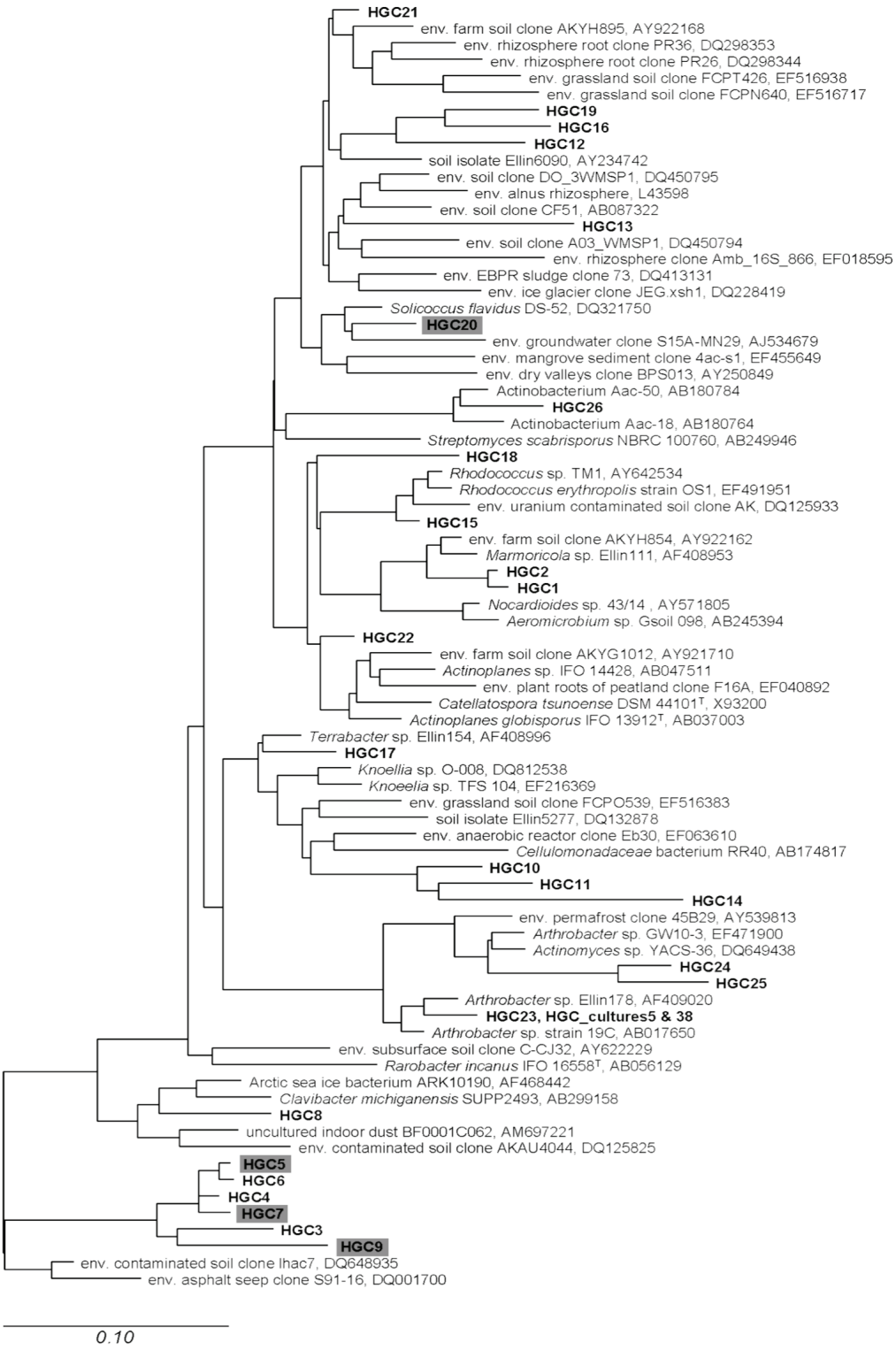
Supplementary Figure 1D. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Planctomycetes* species recovered showing in bold face. Sequences exclusively obtained from barren lysimeters are shown in boxes, sequences recovered only from planted lysimeters are shaded in grey. Sequences detected in both types of lysimeters depicted in bold face only. Sequences denoted with an additional "C" were recovered after cloning DGGE bands. Bar represents 0.1 fixed point mutations per nucleotide.



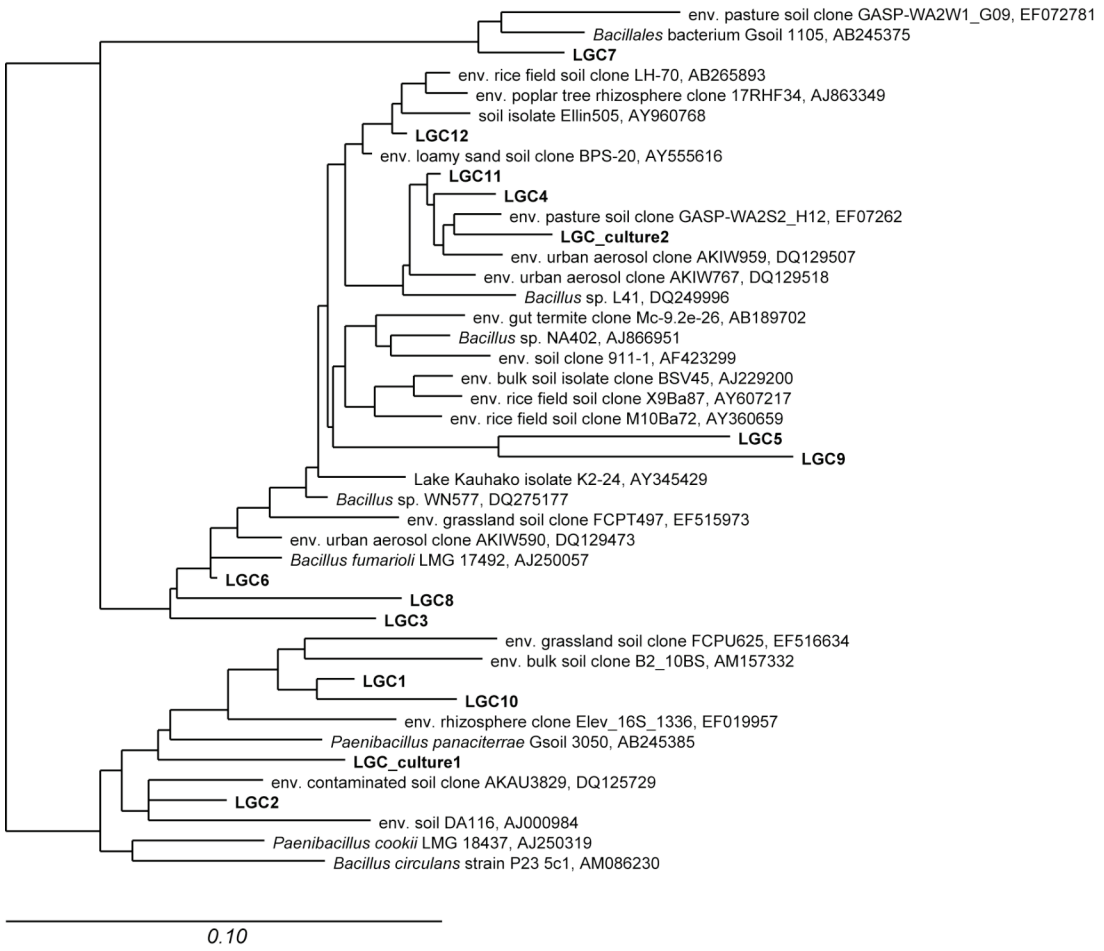
Supplementary Figure 1E. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Alphaproteobacteria/Gammaproteobacteria* species recovered in the present study (shown in bold face). Sequences exclusively obtained from barren lysimeters are shown in boxes, sequences recovered only from planted lysimeters are shaded in grey. Sequences detected in both types of lysimeters depicted in bold face only. Bar represents 0.1 fixed point mutations per nucleotide.



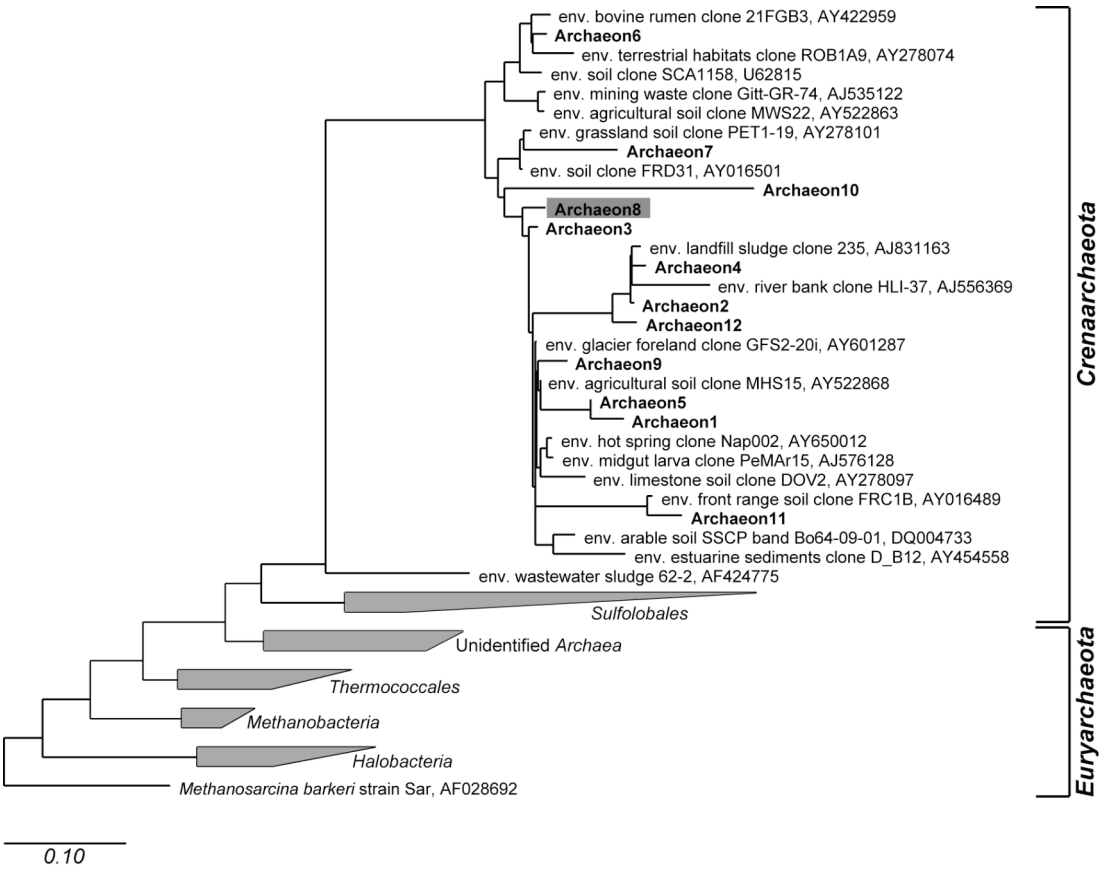
Supplementary Figure 1F. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Bacteroidetes* species recovered in the present study (shown in bold face). Sequences exclusively obtained from barren lysimeters are shown in boxes, sequences recovered only from planted lysimeters are shaded in grey. Sequences detected in both types of lysimeters depicted in bold face only. Bar represents 0.1 fixed point mutations per nucleotide.



Supplementary Figure 1G. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Actinobacteria* species recovered (shown in bold face). Sequences recovered only from planted lysimeters are shaded in grey and sequences detected in barren and planted lysimeters depicted in bold face only. Bar represents 0.1 fixed point mutations per nucleotide.



Supplementary Figure 1H. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Firmicutes* recovered in the present study. All sequences were obtained from barren and planted lysimeters (shown in bold face). Bar represents 0.1 fixed point mutations per nucleotide.



Supplementary Figure 1J. Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of *Archaea* recovered in the present study (shown in bold face). Sequences recovered only from planted lysimeters are shaded in grey. Sequences detected in barren and planted lysimeters depicted in bold face. Bar represents 0.1 fixed point mutations per nucleotide.

CHAPTER 3

Massilia brevitalea sp. nov., A Novel Betaproteobacterium Isolated from Lysimeter Soil

3.1 Abstract

A gram-negative, strictly aerobic bacterium (strain byr23-80) was isolated from lysimeter soil using a high-throughput cultivation technique. Cells were short rods, multiplied by binary fission and were motile by means of a single polar flagellum. Occasionally, 2-3 polar or lateral flagella were observed. The optimum growth temperature was 15°C and the pH optimum was 7.0-7.5. Dominant cellular fatty acids were $C_{16:1}$ ω7*c* (54.7%) and $C_{16:0}$ (21.4%). In addition, the diagnostic fatty acids $C_{10:0}$ 3-OH and $C_{12:0}$ 2-OH were detected. Q-8 was the predominant respiratory quinone. The isolate was physiologically very versatile, using a wide range of sugars, organic acids and amino acids as single carbon and energy source of growth. The G+C content of the genomic DNA was 65.3 mol%. Phylogenetic analyses supported an assignment of strain byr23-80 to the genus *Massilia* within the family *Oxalobacteraceae* of the subphylum *Betaproteobacteria*. Within the genus, strain byr23-80 was most closely related to *Massilia aurea* DSM5502^T with a 16S rRNA gene sequence similarity of 98.3 %. However, DNA-DNA hybridization revealed a pairwise similarity of the genomic DNA of only 20.1% between strain byr23-80 and DSM5502^T. The novel isolate can be distinguished from the existing species *M. timonae*, *M. dura, M. albidiflava, M. plicata, M. lutea,* and *M. aurea* by its significantly lower temperature optimum of growth and the lack of gelatinase, α-galactosidase and β-galactosidase activity. Based on these characteristics, the strain constitutes a separate species for which the name *Massilia brevitalea* sp. nov. is proposed. The type strain is byr23-80 (= DSM 18925^T, BAA-1465^{TM}).

3.2 Introduction, results, and discussion

The genus *Massilia* comprises the six species *Massilia timonae* (La Scola *et al.* 1998), *M. dura, M. albidiflava, M. plicata, M. lutea* (Zhang *et al.* 2006) and *M. aurea* (Gallego *et al.* 2006). *Massilia timonae* strains were isolated from human blood, cerebrospinal fluid and bone (La Scola *et al.* 1998, Lindquist *et al.* 2003). Bacteria of this species are straight rods, and are motile by means of a single polar flagellum or, more rarely, up to three polar or lateral flagella. They are distinguished from other aerobic nonfermentative gram-negative rods based on their 16S rRNA gene sequence, the acid production from carbohydrates and positive reactions for catalase, oxidase, RNase, pectinase, chondroitinase, starch hydrolysis, and esculin hydrolysis. Other typical characteristics are the presence of 16:0, 16:1ω7c, and 18:1ω7c as the predominant and of 3-OH-10:0 and 2-OH-12:0 as diagnostic cellular fatty acids (Lindquist *et al.* 2003). *Massilia dura, M. albidiflava, M. plicata, M. lutea* were recovered from farm soil contaminated with heavy metals and, with the exception of *M. lutea*, differed from *M. timonae* by their ability of urease production and nitrate reduction (Zhang *et al.* 2006). *Massilia aurea* was isolated from drinking water and differed from the other species by the absence of β-glucosidase and α-galactosidase (Gallego *et al.* 2006). Additional unidentified members of the genus *Massilia* utilize glucose (Padmanabhan *et al.* 2003) and have the capability to degrade phenanthrene (Bodour *et al.* 2003) or other aromatic compounds (Khammar *et al.* 2005).

Strain byr23-80 described in the present study was isolated during the investigation of bacterial communities in soil lysimeters (Zul *et al.* 2007). The high-throughput MicroDrop technique (Bruns *et al.* 2003) was employed to inoculate bacterial cells into microtiter plates containing soil solution equivalent (SSE) (Angle *et al.* 1991) which was buffered at a pH of 7.0 using 10 mM HEPES and supplemented with artificial root exudates, yeast extract (0.01% w/v) and inducers, as described previously (Bruns *et al.* 2003, Zul *et al.* 2007). Cultures were incubated at 15°C for 6 weeks. Bacterial strains were isolated by streaking liquid cultures onto the medium described above, solidified by 8 $g \cdot l^{-1}$ gellan gum (Sigma-Aldrich Chemie GmbH Steinheim, Germany). One of the cultured *Betaproteobacteria*, strain byr23-80 was selected for further characterization since it occurred in association with

higher plants (Zul *et al.* 2007). Subsequent physiological, biochemical and phylogenetic characterization revealed that the isolate represented a novel member of the genus *Massilia* within the family *Oxalobacteraceae* of the order *Burkholderiales*.

The shape and color of colonies was examined on agar solidified HD-medium (1:10 diluted; consisting of 0.05% casein peptone, 0.01% glucose and 0.025% w/v yeast extract) as well as on nutrient agar. Colonies were pale white (young cultures) to yellowish (old cultures) on HD (1:10) agar plates while they exhibited a yellow color on nutrient agar. Cells of strain byr23-80 grown for 36 h in HD (1:10) were short rods 1.5-2.0 µm in length and 0.7-1.0 µm in width (Fig. 1A); they divided by binary fission and were motile. In contrast, cells grown in undiluted HD medium became significantly elongated (up to 35 µm) and varied in shape (Fig. 1B). For scanning electron microscopy, drops of the sample were placed onto a glass slide, covered with a cover slip and rapidly frozen with liquid nitrogen. The cover slip was removed with a razor blade and immediately fixed with 2.5% glutaraldehyde in 75mM cacodylate buffer (pH 7.0), postfixed with osmium tetroxide, dehydrated in a graded series of acetone solutions, critical-point dried with liquid CO_2, mounted on stubs, and coated with 3 nm platinum with a magnetron sputter coater. The specimens were examined with a Hitachi S-4100 field emission scanning electron microscope operated at 5 kV. Most cells exhibited a single polar flagellum (Fig. 2A). Occasionally, cells with 2-3 polar or lateral flagella were observed.

Cells stained gram-negative which was confirmed by transmission electron microscopy of cells harvested in the exponential growth phase. Cells were fixed with 2.5% glutardialdehyde in 75 mM sodium cacodylate, 2 mM $MgCl_2$, pH 7.0, for 1 h at room temperature, rinsed several times in fixative buffer and post-fixed for 1 h with 1% osmium tetroxide in fixative buffer at room temperature. After two washing steps in distilled water, the cells were stained *en bloc* with 1% uranyl acetate in 20% acetone for 30 min. Dehydration was performed with a graded acetone series. Samples were then infiltrated, embedded in Spurr's low viscosity resin (Spurr 1969) and ultrathin sections (50 and 70 nm) were cut with a diamond knife and mounted on uncoated copper grids. The sections were post-stained with aqueous lead citrate (100 mM, pH 13.0). All micrographs were taken with an EM 912 Zeiss electron

microscope (Zeiss Oberkochen, Germany) equipped with an integrated OMEGA energy filter operated with zero loss mode. Investigations of exponentially growing cells confirmed the presence of an outer membrane, a thin murein layer and a cytoplasmic membrane (Fig. 2B). A striking feature of the cells is the presence of numerous protuberances of varying sizes over the entire cell surface, formed by the outer membrane (Fig. 2A,B).

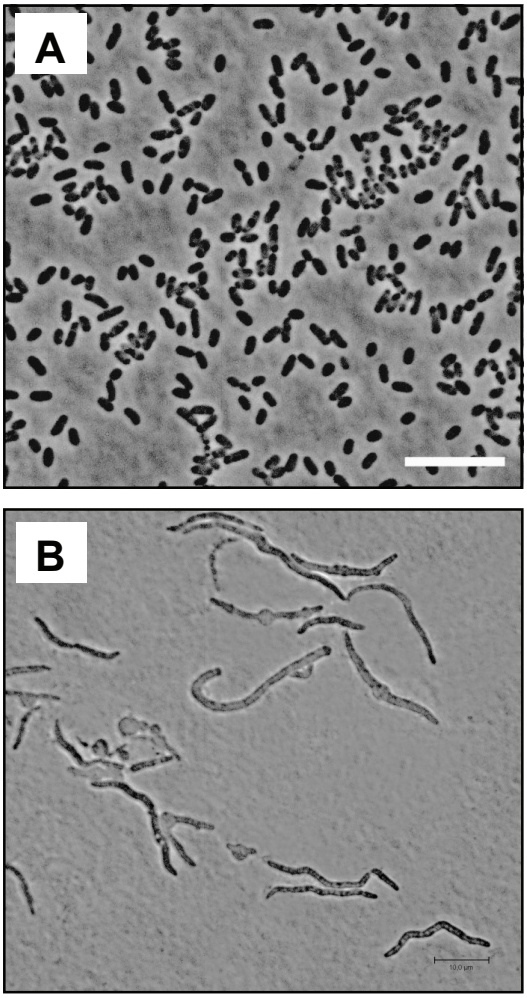


Figure 1. A. Phase contrast photomicrograph of cells of strain byr23-80 grown in 1:10 diluted HD medium at 15°C for 36 h. **B.** Morphology of cells grown in undiluted HD medium at 15°C for 36 h. Bars represent 10 µm.

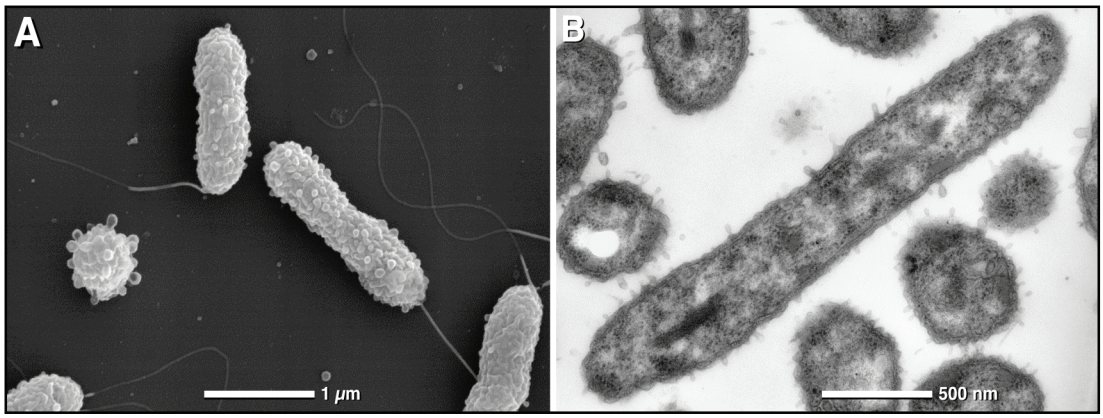


Figure 2. A. Scanning electron micrograph of cells of strain byr23-80 showing the monopolar monotrichous flagellation and numerous protuberances of the cell envelope. **B.** Transmission electron micrograph of ultrathin section of cells showing the cell envelope, typical for gram-negative bacteria, and the protuberances of the outer membrane.

Catalase and oxidase tests were performed as described by Lányi (1987) and revealed that strain byr23-80 is weakly catalase positive and oxidase negative. The temperature range and the optimum temperature for growth in HD (1:10) medium were 4 – 30°C and 15°C, respectively. While the temperature range is comparable to that of *Massilia aurea*, the optimum temperature of growth is unusual in that it is significantly lower than that of any other described *Massilia* species (Table 1). Subsequent growth tests were therefore performed at 15°C. The pH range of growth was 6.0 - 10.0 with an optimum value of 7.0 - 7.5. Salt tolerance was tested at NaCl concentrations between 0 and 10% (w/v) and demonstrated growth at concentrations ≤ 2% NaCl. Cells grew obligately aerobically and capable of reducing NO_3^- to NO_2^- as tested on nitrate broth containing 0.2 % KNO_3 (Skermann, 1967). The hydrolysis of starch was tested on agar plates containing 1% (w/v) peptone, 1% yeast extract and 0.5% soluble starch. Gelatinase production was determined on LB plates containing 3% (w/v) gelatine. The hydrolysis of casein was examined on nutrient agar supplemented with 1.5 % skim milk (Sigma). The milk coagulation and peptonization were tested on 10.0 % (w/v) litmus milk medium. Based on these assays, the cell were capable of starch and casein hydrolysis, but did not hydrolyze gelatin. This latter feature clearly distinguishes the novel isolate from all described

species of the genus *Massilia* (Table 1). Additional enzyme activities were determined by employing API ZYM galleries (API systems; bioMérieux) according to the protocol of the manufacturer and by incubation of the assays at 15°C. Strain byr23-80 had a strong positive reaction for acid phosphatase, leucine arylamidase and napthol-AS-BI-phospohydrolase. In contrast to all other *Massilia* species tested, however, the novel isolate did not exhibit α-galactosidase and β-galactosidase activity. It also tested negative for arginine dihydrolase, ornithine decarboxylase or lysine decarboxylase activity. HD broth at a 1:10 dilution was used to analyze the methyl red and Voges-Proskauer reactions by adding the methyl red and Barritt´s reagents, respectively. Indole production was examined in tryptophan broth and detected by adding Kovacs´ reagent. Urease production was determined in media supplemented with 0.5 % urea (Ederer *et al.* 1971) and H_2S production was tested on Kligler iron agar (Difco). Cells of strain byr23-80 tested negative for production of acetoin, indole and H_2S and did not exhibit urease.

Substrate utilization patterns were determined in microtiter plates filled with basal SSE-medium supplemented with individual carbon substrates (Gich and Overmann 2006). Substrate utilization was evaluated by repeatedly measuring the optical density at 620 nm in each well using a microtiter plate reader (TECAN) over an incubation period of six weeks. D-cellobiose, D-erythrose, L-erythrulose, D-galactose, glucose, glucose 1-phosphate, glucose 6-phosphate, *N*-acetylglucosamine and maltose were utilized as sole carbon and energy sources. In addition, the strain grew on 19 of 36 organic acids and on 13 of 22 amino acids tested but did not utilize any of the 8 alcohols tested (Table 1). The strain was also able to utilize α-ketoisocaproate, α-ketoglutarate and α-ketovalerate and casamino acids, yeast extract and peptone.

Antibiotics susceptibility was determined by using the disc-diffusion plate method applying the discs on HD (1:10) agar plates inoculated with strain byr23-80. The sixteen antibiotics/antibiotics combinations tested were (amounts given in µg per disc, unless otherwise stated): amikacin (30), ampicillin (25), bacitracin (10 U per disc), carbenicillin (100), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin monosulfate (30), novobiocin (5), penicillin G (10), rifampicin (5),

streptomycin sulfate (30), sulfamethoxazol + trimethoprim (23.7 and 1.2), tetracycline (30), tobramycin (10), vancomycin (30). Resistance was observed towards bacitracin, carbenicillin, chloramphenicol, erythromycin, gentamicin, novobiocin, and penicillin G.

Results of the chemotaxonomic analyses are given in Table 2 and the species description. For these determinations, cells were grown under optimum conditions on HD (1:10) at 15°C for 36 h. Respiratory lipoquinones and polar lipids were extracted from 2 and 0.1 g, respectively, of freeze dried cell material and analyzed according to Tindall (1990a, b). For fatty acid analysis, 40 mg (wet weight) of cells were scraped from Petri dishes and the fatty acid methyl esters were extracted using the method of Miller (1982) and Kuykendall *et al.* (1988) using the Microbial Identification System (MIDI, Inc.). These analyses were carried out by the Identification Services of the DSMZ, Braunschweig (Germany). The major cellular fatty acids were $C_{16:1}$ ω7*c* (54.7%) and $C_{16:0}$ (21.4%). Similar to other members of the genus *Massilia* (Lindquist *et al.* 2003), cells of strain byr23-80 contained $C_{10:0}$ 3-OH and $C_{12:0}$ 2-OH as diagnostic cellular fatty acids. The main quinone component was ubiquinone 8 (Q-8).

The G+C content of the genomic DNA was calculated from the mid point value (T_m) of the melting profile (Øvreas *et al.* 2003) using the formula as described by Mandel *et al.* (1970). Bacterial genomic DNA for the DNA G+C content determination (also for 16S rRNA analysis purpose) was isolated using the method of Marmur (1961). The DNA G+C content was 65.3 mol%. The fatty acid profile, major respiratory quinone and the DNA G+C content of strain byr23-80 all were typical for other species within the genus *Massilia* (Table 1).

Table 1. Main morphological, biochemical and physiological characteristics of strain byr23-80 compared with other species within the genus *Massilia*

Taxa: 1, strain byr23-80; 2, *M. timonae*; 3, *M. dura*; 4, *M. albidiflava*; 5, *M. plicata*; 6. *M. lutea*; 7, *M. aurea*. Data for *M. timonae* CIP 105350^T is from Lindquist *et al.* (2003) and La Scola *et al.* (1998). Data for *M. dura* CCTCC AB 204070^T, *M. albidiflava* CCTCC AB 204071^T, *M. plicata* CCTCC AB 204072^T, *M. lutea* CCTCC AB 204073^T were taken from Zhang *et al.* (2006). Data for *M. aurea* DSM 18055^T is from Gallego *et al.* (2006). +, positive; -, negative; w, weak; ND, not determined. All strains show the following phenotypic characteristic: gram negative, motile, non-spore-forming rods to short-rods, negative for arginine dihydrolase, ornithine decarboxylase, indole and H_2S production; grow in nutrient broth/agar.

Carbon substrates tested, but not utilized by strain byr23-80: *N*-acetyl-D-galactosamine, acetoin, adonitol, D-arabinose, D(+)arabitol, L(+)arabitol, ascorbate, γ-hydroxybenzoate, α-hydroxybutyrate, γ-hydroxybutyrate, isobutyrate, trimethoxybenzoate, benzoate, citrate, isocitrate, i-erythritol, α-cyclodextrin, dextrin, dulcitol, formiate, D(+)fucose, L-fucose, β-gentibiose, D-gluconate, D-glucuronate, D(+)glucosamine, glycerol, glycogen, glycolate, glyoxylate, m-inositol, βD-lactose, D-lactose, lactulose, D-lyxose, malate, D(+)mannose, D-mannitol, D(+)melezitose, D-melibiose, D-psicose, D(+)raffinose, ribitol, D-ribose, shikimate, D-sorbitol, L-sorbose, D-tagatose, tartrate, D-turanose, xylitol, D(+)xylose, ethanol, propanol, 1,2-propandiol, butanol, 1,2-butandiol, 2,3-butandiol, ethylene glycol, methanol.

Other carbon souces utilized by strain byr23-80: acetate, adipate, butyrate, β-hydroxybutyrate, caproate, caproylate, caprylate, crotonate, D-erythrose, L-erythrulose, fumarate, glucose 1-phosphate, glucose 6-phosphate, isovalerate, lactate, malate, *N*-acetylglucosamine, oxaloacetate, propionate, protochatechuate, pyruvate, succinate and valerate.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cell size (μm)	0·7-1·0 x 1·5-2·0	1·0 x 3·0	0·6-0·8 x 1·8-2·2	ND	0·6-0·7 x 1·8-2·2	ND	1·0 x 1·6-3·0
Growth characteristics:							
pH range	6·0-10·0	ND	6·5-8·5	6·5-8·5	6·5-8·5	6·5-8·5	4·0-9·0
pH optimum	7·0-7·5	ND	7·0-7·5	7·0-7·5	7·0-7·5	7·0-7·5	7·0-8·0
Temp. range (°C)	4-30	25-35	10-45	10-45	10-45	10-45	4-30
Temp. optimum (°C)	15	28	28-30	28-30	28-30	28-30	28
Flagellation	polar or lateral	polar or lateral	lateral	lateral	lateral	lateral	ND
Voges-Proskauer test	-	-	-	-	+	-	-
Methyl red test	-	-	+	-	-	-	-
Starch hydrolysis	+	+	-	+	+	+	+
Casein hydrolysis	+	ND	+	+	+	+	+
Gelatin hydrolysis	-	+	+	+	+	+	+
Nitrate reduction	+	-	+	+	+	-	-
Urease	-	-	+	+	+	-	-
Oxidase	-	+	+	+	-	+	w
Catalase	w	+	+	+	+	+	+
Milk coagulation	-	ND	-	-	-	-	ND
Peptonization	-	+	-	-	-	-	ND

Table 1. Main morphological, biochemical and physiological characteristics of strain byr23-80 compared with other species within the genus *Massilia* (continued)

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Enzyme activities:							
α-Chymotrypsin	-	ND	ND	ND	ND	ND	-
α-Fucosidase	-	ND	ND	ND	ND	ND	-
α-Galactosidase	-	ND	+	+	+	+	-
β-Galactosidase	-	ND	+	+	+	+	+
β-Glucuronidase	-	ND	+	+	-	+	-
α-Glucosidase	+	ND	+	+	+	+	+
β-Glucosidase	-	ND	+	+	+	+	-
Lipase	-	ND	-	+	+	+	-
N-acetylglucosaminidase	-	ND	+	+	-	+	-
Lysine decarboxylase	-	-	-	+	-	-	-
Utilization of:							
Glucose	+	-	+	+	+	+	+
Sucrose	-	-	+	+	+	+	-
D-Fructose	-	-	ND	ND	ND	ND	+
D-Galactose	+	-	ND	ND	ND	ND	+
L-Rhamnose	-	+	-	-	-	-	+
D-Trehalose	-	-	-	ND	-	ND	-
Malonate	-	+	-	ND	+	ND	ND
Maltose	+	+	-	ND	-	ND	+
D-Cellobiose	+	+	-	-	-	-	+
L-arabinose	-	+	-	-	-	-	-
Galacturonate	-	+	-	-	-	-	ND
Isolate source	Soil	Blood	Soil	Soil	Soil	Soil	Drinking water
DNA G+C content (%mol)	65·3	62-67	65·9	65·3	65·1	63·3	66·0

Table 2. Cellular fatty acid content (%) of strain byr23-80 and *Massilia* species

Taxa: 1, strain byr23-80; 2, *M. timonae*; 3, *M. dura*; 4, *M. albidiflava*; 5, *M. plicata*; 6. *M. lutea*; 7, *M. aurea*. Data for *M. timonae* CIP 105350^T is from Lindquist *et al.* (2003). Data for *M. dura* CCTCC AB 204070^T, *M. albidiflava* CCTCC AB 204071^T, *M. plicata* CCTCC AB 204072^T, *M. lutea* CCTCC AB 204073^T were taken from Zhang *et al.* (2006). Data for *M. aurea* DSM 18055^T is from Gallego *et al.* (2006).

Fatty acid	1	2	3	4	5	6	7
Saturated fatty acids							
$C_{10:0}$	0.5	1.0	0.6	1.3	1.4	0.5	0.4
$C_{12:0}$	5.1	8.0	5.6	6.0	7.1	2.8	4.6
$C_{14:0}$	2.1	T	3.2	2.8	1.6	1.9	0.6
$C_{15:0}$	1.2	T	-	-	-	-	-
$C_{16:0}$	21.4	25.0	25.5	22.8	25.1	22.8	36.8
Unsaturated fatty acids							
$C_{16:1}$ ω7*c*	54.7	43.0	46.7	46.4	36.9	54.7	48.3
iso $C_{17:1}$ ω9*c*	0.4	-	-	-	-	0.7	-
$C_{18:1}$ ω7*c*	7.5	10.0	5.9	7.4	11.7	10.0	2.5
Hydroxy fatty acids							
$C_{10:0}$ 3-OH	4.3	8.0	8.3	9.6	10.1	4.6	4.8
$C_{12:0}$ 2-OH	1.4	3.0	-	0.7	-	-	1.9
$C_{14:0}$ 2-OH	-	-	3.4	3.0	6.1	2.0	-

* T, trace (0.4 to 0.8%)

For a higher resolution of the relationship to described *Massilia* species, the 16S rRNA gene sequence of strain byr23-80 was amplified using primers 8f and 1492r (Lane 1991) and subsequently sequenced employing eight primers to cover the entire 16S rRNA gene (Gich *et al.* 2005). The sequence of strain byr23-80 and recently described species were retrieved from the GenBank database employing Blast 2.0.4 (Altschul *et al.* 1997), imported into the ARB database and analyzed using the ARB software package (Ludwig *et al.* 2004). The Fast Aligner V1.03 tool was used for automated sequence alignment with previously aligned sequences from the ARB database. The alignment was subsequently checked and corrected manually based on secondary structure information. Maximum likelihood and neighbour-joining methods were employed to construct phylogenetic trees and the sequence similarities were calculated using the ARB distance matrix. The robustness of the tree topology was evaluated by calculating bootstrap values (Felsenstein 1985) using 1000 resamplings.

Based on the phylogenetic tree obtained (Fig. 3), strain byr23-80 falls within the group of existing lineages of the genus *Massilia*. Similar results were obtained using the maximum likelihood method (not shown). The closest described relative of strain byr23-80 was *Massilia aurea* DSM5502^T for which a similarity value of 98.3 % was calculated. The sequence similarities to all other known *Massilia* species were ≤ 96.4 %. The 16S rRNA gene sequence divergence between strain byr23-80 and *Massilia aurea* DSM5502^T is in the range commonly used as a criterion for the separation of two bacterial species (Stackebrandt and Goebel 1994). Therefore, DNA-DNA hybridization was carried out to evaluate whether strain byr23-80 has to be considered as a novel species. DNA-DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983). The chromosomal DNA for the DNA hybridization was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite (Cashion *et al.* 1977). The similarity value between the strain byr23-80 and *M. aurea* was 20.1% and hence significantly lower than the threshold value of 70%, which is commonly accepted for the definition of bacterial species (Wayne *et al.* 1987).

Based on the phenotypic and genotypic data presented, strain byr23-80 constitutes a novel species within the genus *Massilia*, for which the name *Massilia brevitalea* is proposed.

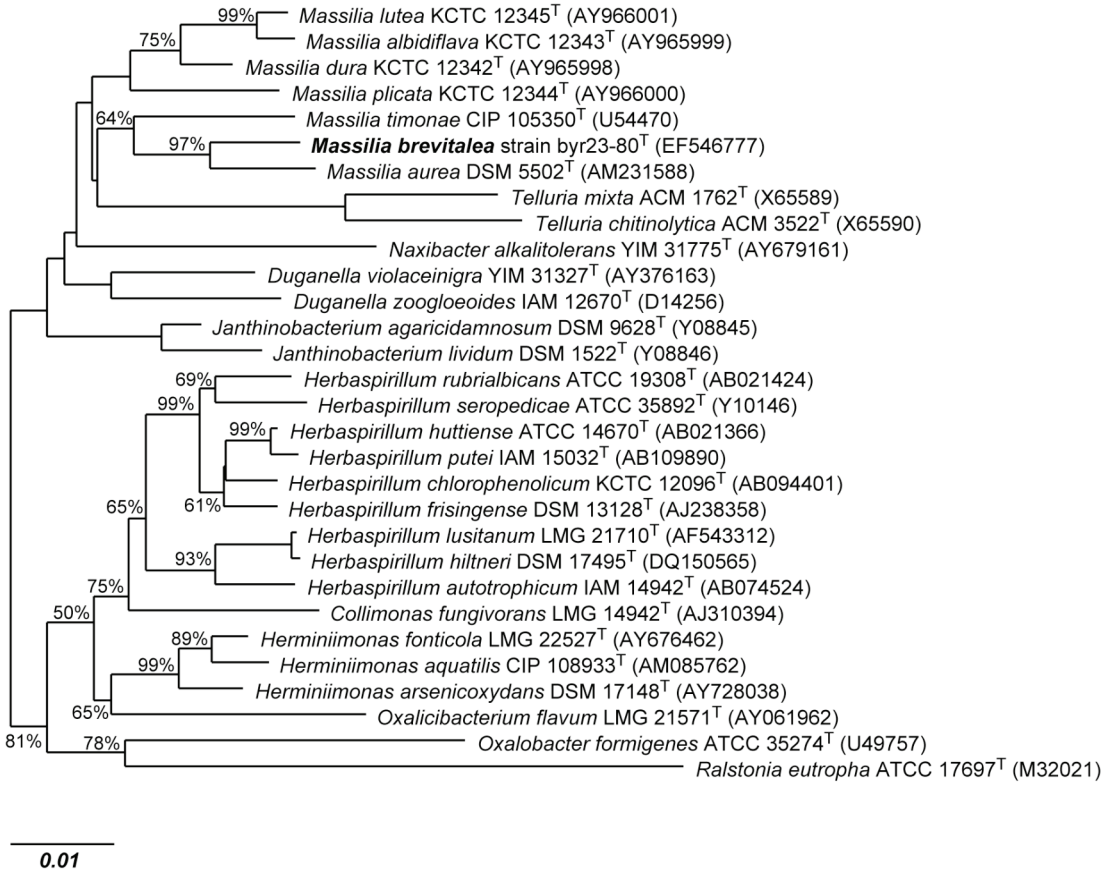


Figure 3. Neighbour-joining phylogenetic tree of 16S rRNA gene sequences of strain byr23-80 and related bacteria within the family *Oxalobacteraceae* of the β-subclass of the *Proteobacteria*. The tree was rooted using *Ralstonia eutropha* ATCC 17697^T as an outgroup. Bootstrap values obtained by 1000 bootstrap resamplings are given at branching points; only values above 50 % depicted. The bar represents 0.01 fixed point mutations per nucleotide.

3.2.1 Description of *Massilia brevitalea* sp. nov.

Massilia brevitalea (bre.vi.ta´le.a. L. adj. *brevis* short; L. fem. n. *talea* a rod; M.L. fem. n. *brevitalea* a short-rod, referring to the shape of cells).

Cells are gram-negative, short rods, 0.7-1.0 µm wide and 1.5-2.0 µm long in size, and motile by means of a single polar flagellum. Occasionally, cells with two or three polar or lateral flagella are observed. Cells are non-spore-forming, occur singly or in pairs (on 1:10 diluted HD medium at 15°C after 36 h) and are obligately aerobic. Colonies are circular, entire, convex and opaque, and pale white to yellow. Cells grow chemoorganotrophically on diluted HD-agar or nutrient agar. Oxidase-

negative and weakly positive for catalase. Temperature range for growth is 4 - 30°C, with optimum growth occurring at 15°C. The pH range for growth is 6.0 – 10.0, with optimum pH occurring between 7.0 – 7.5. Can grow at NaCl concentrations of ≤ 2% (w/v). Tween 20, Tween 80, casein and starch are hydrolyzed, but not gelatine. Positive for enzyme activities of α-glucosidase, acid phosphatase, alkaline phosphatase, leucine arylamidase, naphtol-AS-BI-phosphohydrolase and urease. The incapability of hydrolyzing gelatine and the lack of α-galactosidase and β-galactosidase activities separate *M. brevitalea* from all other known *Massilia* species. Tests negative for milk coagulation, indole production, hydrogen sulfide production, methyl red and Voges-Proskauer reactions. Reduces NO_3^- to NO_2^-. D-cellobiose, D-erythrose, L-erythrulose, D-galactose, glucose, glucose 1-phosphate, glucose 6-phosphate, maltose and *N*-acetylglucosamine are utilized as sole carbon and energy sources. Growth occurs on acetate, adipate, butyrate, crotonate, caproylate, caproate, caprylate, fumarate, lactate, malate, β-hydroxybutyrate, isovalerate, α-ketoglutarate, α-ketoisohexanoate, α-ketovalerate, oxaloacetate, propionate, protochatechuate, pyruvate, succinate, valerate and slowly on levulinate. L(+)alanine, L-alanylglycine, L-asparagine, L(+)asparaginate, L(+)cysteine, L(+)glutamate, L(+)isoleucine, L(+)leucine, L(+)lysine, L(+)phenylalanine, L(+)serine, L(+)threonine, and L(+) tyrosine are utilized. Growth occurs on casamino acid, yeast extract, peptone as complex organic substrates. The DNA base composition is 65.3 mol% G+C. Q-8 is the predominant respiratory quinone and major cellular fatty acids are $C_{16:1}$ ω7*c* and $C_{16:0}$. In addition, the diagnostic fatty acids $C_{10:0}$ 3-OH and $C_{12:0}$ 2-OH are present.

The type strain is strain byr23-80 (= DSMZ 18925^T, BAA-1465^T), which was isolated from lysimeters soil of the Botanical Garden at the University of Bayreuth, Germany.

3.3 Acknowledgements

Delita Zul is a recipient of a TPSDP (Technological and Professional Skills Development Sector Project) scholarship. The excellent technical assistance of Silvia Dobler is gratefully acknowledged. This work was supported by BMBF (Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie) grants no. BIOLOG/01LC0021A and 01LC0621C to Jörg Overmann.

3.4 References

1. **Altschul, S. F., T.L. Madden, A.A. Schäffer, J. Zhang, W. Miller, and D.J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389-3402.

2. **Angle, J.S., S.P. McGrath, and R.L. Chaney.** 1991. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. Appl. Environ. Microbiol. **57**:3674-3676.

3. **Bodour, A. A., J. Wang, M.L. Brusseau, and R.M. Maier.** 2005. Temporal changes in culturable phenanthrene degraders in response to long-term exposure to phenanthrene in a soil column system. Environ. Microbiol. **5**: 888-895.

4. **Bruns, A., H. Hoffelner, and J. Overmann.** 2003. A novel approach for high throughput cultivation assays and the isolation of planktonic bacteria. FEMS Microbiol. Ecol. **45**:161-171.

5. **Cashion, P., M.A. Hodler-Franklin, J. McCully, and M. Franklin.** 1977. A rapid method for base ratio determination of bacterial DNA. Anal. Biochem. **81**: 461-466.

6. **De Ley, J., H. Cattoir, and A. Reynaerts**. 1970. The quantitative measurement of DNA hybridization from renaturation rates. Eur. J. Biochem. **12**:133-142.

7. **Ederer, G. M., J.H. Chu, and D.J. Blazevic.** 1971. Rapid test for urease and phenylalanine deaminase production. Appl. Microbiol. **21**:545.

8. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783-791.

9. **Gallego, V., C. Sánchez-Porro, M.T. García, and A. Ventosa.** 2006. *Massilia aurea* sp. nov., isolated from drinking water. Int. J. Syst. Evol. Microbiol. **56**:2449-2453.

10. **Gich, F. and J. Overmann.** 2006. *Sandarakinorhabdus limnophila* gen. nov., sp. nov., a novel bacteriochlorophyll *a*-containing, obligately aerobic bacterium isolated from freshwater lakes. Int. J. Syst. Evol. Microbiol. **56**:847-854.

11. **Gich, F., K. Schubert, A. Bruns, H. Hoffelner, and J. Overmann.** 2005. Specific detection, isolation, and characterization of selected, previously uncultured members of the freshwater bacterioplankton community. Appl. Environ. Microbiol. **71**:5908-5919.

12. **Huss, V.A.R., H. Festl, and K.H. Schleifer.** 1983. Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst. Appl. Microbiol. **4**:184-192.

13. **Khammar, N., L. Malhautier, V. Degrange, R. Lensi, J.-J. Godon, and J-.L. Fanlo.** 2005. Link between spatial structure of microbial communities and degradation of a complex mixture of volatile organic compounds in peat biofilters. J. Appl. Microbiol. **98**:476-490.

14. **Kuykendall, L.D., M.A. Roy, J.J. O'Neill, and T.E. Devine.** 1988. Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. Int. J. Syst. Bact. **38:**358-361.

15. **Lane, D. J.** 1991. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics,* pp. 115-175. E. Stackebrandt & M. Goodfellow (ed.). Chichester: Wiley.

16. **Lányi, B.** 1987. Classical and rapid identification methods for medically important bacteria. In *Current Methods for Classification and Identification of Microorganisms*, pp. 1-69. R.R. Colwell & R. Grigorova (eds.). London: Academic Press.

17. **La Scola, B., R.J. Birtles, M.-N. Mallet, and D. Raoult,** 1998. *Massilia timonae* gen. nov., sp. nov., isolated from blood of an immunocompromised patient with cerebellar lesions. J. Clin. Microbiol. **36**:2847-2852.

18. **Lindquist, D., D. Murrill, W.P. Burran, G. Winans, J.M. Janda, and W. Probert,** 2003. Characteristics of *Massilia timonae* and *Massilia timonae*-like isolates from human patients, with an emended description of the species. J. Clin. Microbiol. **41**:192-196.

19. **Ludwig, W., O. Strunk, R. Westram, and 28 other authors,** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363-1371.

20. **Mandel, M., L. Igambi, J. Bergendahl, M.L. Dodson, and E. Scheltgen,** 1970. Correlation of melting temperature and cesium chloride buoyant density of bacterial deoxyribonucleic acid. J. Bacteriol. **101**:333-338.

21. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208-218.

22. **Miller, L.T.** 1982. A single derivatization method for bacterial fatty acid methyl esters including hydroxy acids. J Clin Microbiol **16:**584-586.

23. **Øvreas, L., F.L. Daae, V. Torsvik, and F. Rodríguez-Valera,** 2003. Characterization of microbial diversity in hypersaline environments by melting profiles and reassociation kinetics in combination with terminal restriction fragment length polymorphism (T-RFLP). Microb. Ecol. **46**:291-301.

24. **Padmanabhan, P., S. Padmanabhan, C. DeRito, A. Gray, D. Gannon, J.R. Snape, C.S. Tsai, W. Park, C. Jeon, and E.L. Madsen,** 2003. Respiration of ^{13}C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of ^{13}C-labeled soil DNA. Appl. Environ. Microbiol. **69**:1614-1622.

25. **Skermann, V. B. D.** 1967. *A guide to the Identification of the Genera of Bacteria*, 2nd edn. Baltimore: Williams & Wilkins.

26. **Spurr, A.R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct. Res. **26**:31-43.

27. **Stackebrandt, E., and B.M. Goebel,** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44**:846-849.

28. **Tindall, B.J.** 1990a. A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. Syst. Appl. Microbiol. **13**:128-130.

29. **Tindall, B.J.** 1990b. Lipid composition *of Halobacterium lacusprofundi.* FEMS Microbiol. Lett. **66**:199-202.

30. **Wayne, L. G., D.J. Brenner, R.R. Colwell, P.A.D. Grimont, O. Kandler, M.I. Krichevsky, L.H. Moore, W.E.C. Moore, R.G.E. Murray, E. Stackebrandt, M.P. Starr, and H.G. Trüper,** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37**:463-464.

31. **Zhang, Y.-Q., W.-J. Li, K.-Y. Zhang, X.-P. Tian, Y. Jiang, L.-H. Xu, C.-L. Jiang, and R. Lai,** 2006. *Massilia dura* sp. nov., *Massilia albidiflava* sp. nov., *Massilia plicata* sp. nov. and *Massilia lutea* sp. nov., isolated from soils in China. Int. J. Syst. Evol. Microbiol. **56**:459-463.

32. **Zul, D., S. Denzel, and J. Overmann.** 2007. Effects of plant biomass, plant diversity and water content on the bacterial communities in soil lysimeters: implications for the determinants of bacterial diversity. Appl. Environ. Microbiol. **73:**6916-6929.

CHAPTER 4

Determinants of the Diversity and Activity of a Bacterial Community in Namibian Semiarid Savanna Soils

4.1 Abstract

Besides natural factors like soil type or climate, anthropogenic alterations such as cultivation, logging, or fire affect soil bacterial community composition and activity. However, to date interdependence among land use, soil properties, and bacterial community compositions are poorly understood. This especially holds true for subtropical soils in semiarid climates. To elucidate determinants of soil bacterial diversity, 29 arenosols from the Kavango region, northeastern Namibia, were studied by enzymatic assays, microbial and molecular biological methods. The total cell numbers were between $(0.283 \pm 0.046) \cdot 10^9$ and $(3.940 \pm 0.019) \cdot 10^9$ (g soil)$^{-1}$ and soil respiration rates ranged from 4.03 to 23.71 nmol O_2 (g soil)$^{-1}$ min^{-1}. The lowest and the highest values were obtained in pristine sand soil and pristine dark loam soil, respectively. Exoenzyme activities greatly varied among sampling sites, but did not show a clear correlation to soil properties or land use. Quantitative PCR identified *Acidobacteria* and *Actinobacteria* as the most abundant phyla, constituting 30 and 20% of all *Bacteria*, respectively. *Alphaproteobacteria, Bacteroidetes,* and *Planctomycetes* accounted for below 10%, whereas *Betaproteobacteria, Chloroflexi*, and *Firmicutes* represented ≤ 1%. The bacterial community structure in pristine sand soil and pristine dark loam soil was quite similar, except for *Verrucomicrobia,* which showed higher abundance in sand soil. Clone library of 16S rRNA genes from the most active soil site revealed a high bacterial diversity with an estimated number of about 5600 phylotypes clustered in 13 (sub)phyla. Subsequent high-resolution phylogenetic DGGE-fingerprinting for *Acidobacteria* and *Actinobacteria* did only show minor differences in composition of the bacterial communities among sampling sites. A total of 735 bacterial cultures was recovered comprising 15 phylotypes of *Acidobacteria,* which were affiliated with subgroups 1, 4, and 6.

4.2 Introduction

Land degradation and land use have significant impacts on ecosystem functions and processes through alterations of microbial community composition (Carney *et al.* 2004). Anthropogenic land use changes such as agricultural cultivation, grazing, and logging directly or indirectly can cause soil compaction resulting in increased bulk density, reduced pore spaces (Johnson *et al.* 1991), and altered soil environments. These changes may thus influence the abundance and activity of microbial community and subsequently reduce the cycling of elements, including carbon, nitrogen, and phosphorus (Waldrop *et al.* 2000, Cleveland *et al.* 2003). It has been shown that long-term effects of cultivation caused alteration of the microbial community composition (Fraterrigo *et al.* 2006) and led to reduction in the numbers of *Alphaproteobacteria, Betaproteobacteria,* and *Actinobacteria* (Buckley and Schmidt 2001).

Besides monitoring the effects of land use or land management practices on microbial diversity and community composition (Girvan *et al.* 2003, Steenwerth *et al.* 2003, Fraterrigo *et al.* 2006), most studies also observed the effects on microbial activity through quantifying microbial respiration (Frank *et al*. 2006, Concilio *et al*. 2006) or exoenzyme activities (Dick *et al*. 1988, Gupta and Germida 1988, Kandeler *et al*. 1999, Acosta-Martinez *et al.* 2007). There is a close relationship between microbial diversity and microbial activity since variations in microbial community composition affect soil organic matter decomposition and soil respiration rate (Cleveland *et al.* 2007). Quantifying soil respiration rates and soil exoenzyme activities are employed frequently as these parameters show a rapid change and high sensitivity towards changes in the soil environment. Therefore, disturbance of soil microbial activity, as shown by changes in levels of metabolic enzymes (Bandick and Dick 1999) and respiration rates (Frank *et al.* 2006) can serve as an estimator of ecosystem disturbance.

To date very little is known about the interdependence among land use changes, soil bacterial community composition, soil respiration rates, and soil enzymes activities under subtropical conditions and no study exists so far for subtropical semiarid soils in Namibia. It has been shown that management practices

and over exploitation of soils in Namibia reduced soil fertility due to a decrease of soil nutrients until critical levels were reached (Zeidler *et al.* 2002). On the other hand, a study of Uhlmann *et al.* (2004) revealed that the diversity of Arbuscular mycorrhizal fungi (AMF) in villages of Mile 46, Mutompo, Toggekry 250, and Otjiamongombe West 44, Namibia did not seem to be governed by land management systems, but rather by vegetation cover or rainfall regime.

In the Kavango region of northeastern Namibia, farmland comprises different types of communal and commercial management (Ward *et al.* 1998). Communal land is used for rainfed and dry land cropping dominated by **mahangu** (millet – *Penisetum glaucum*) and livestock grazing, in a spatial and temporal sense. The cultivated areas are continuously planted until soil fertility has vanished and followed by fallow periods of decades (more or less 20 year) before being used again for cultivation. The subsistence cultivation is done manually using very simple tools (knives, axes, and hoes). Soil management employs traditional ox-drawn ploughing to prepare cultivated area and fire sometimes is used to clear a new land for cultivation (Jones and Cownie 2001, Michael Proepper personal communication). The villagers utilize organic fertilizers to increase soil fertility instead of chemical fertilizers, whereas pesticides are not applied. Pesticides are only applied by government agencies and only in case of emergency. The most important environmental pressures in the Kavango region are the clearing of natural land for crop cultivation (the clearing land average is about of 4% per year from 1972 to 1996) and the burning of bush that is a prominent feature of the winter months (between May and September). Heavy grazing and logging have also been shown to degrade the natural resources (Obeid and Mendelsohn 2001). The land use systems (including traditional cultivation system, burning, grazing, and logging), and overexploitation of land in the Kavango region may consequently lead to further soil acidification and loss of soil organic matter and nutrients (Gröngröft *et al.* 2006).

In the present study, the sensitivity of different semiarid subtropical soils towards anthropogenic disturbances and the relevance of other factors like soil type and soil pH were assessed. Soil samples from the Kavango region, Namibia were

chosen as a case study and were analyzed by enzymatic assays, microbial and molecular biological methods.

4.3 Material and methods

4.3.1 Site description and soil sampling

Twenty-nine soil samples were collected between 19 and 24 March 2007 at Mutompo village located in the Kavango region, the forest savanna of northeastern Namibia (Barnard 1998). The coordinates of each soil-sampling site are depicted in Table 1 and are plotted onto the Mutompo village map through Goggle Earth (Fig. 1). The soil sampling sites are parts of the BIOTA transect which extends from the Cape of Good Hope in the Republic of South Africa to the border between Namibia and Angola (http://www.biota-africa.org). The Kavango region is characterized by a semiarid climate with an average rainfall of 550 mm/yr. Most rains fall from November to March and its climate is very dry from April to October. A mean maximum temperature in the hottest month (October) is 34°C and a mean minimum temperature in the coldest month (June and July) is 6°C. The Kavango region is about 1100 m above sea level with northern Kalahari dry woodlands as the main vegetation type. Degraded dune sands of the Kalahari basin form the soil parent material and soil types are mainly dystric ferralic arenosols and eutric ferralic arenosols (Gröngröft *et al.* 2006). A mean of soil electric conductivity is quite low ranging from 6 -18 µS/cm. Soils are nutrient poor with a total N content of 0.024-0.035% and a total P content of 0.24-0.29 g/kg. Common plant species in Mutompo is *Combretum* species, *Terminalia sericea, Burkea africana, Eragrostis echinochloides,* and *Guibortia colophosperma* (Uhlmann *et al.* 2004). Detailed vegetation types in the Kavango region are described elsewhere (Strohbach and Petersen 2007). Mutompo is used for communal open-access cattle grazing with little control over stocking rates and grazing times, rainfed arable fields, and firewood collection. Twenty-nine plots were chosen representing combinations of land use (pristine, fallow, and acre), land use age (> 20 year, between 10 and 20 year, and between 3 and 10 year), and soil textures (loam [dark, brown or red] and sand). Soil temperature *in situ* at the time soil sampling conducted was 30-31°C. Detailed information about each plot of soil properties and land use are depicted in Table 1.

The soil samples were taken from the surface layer (0-15 cm) after removing the litter. Two intersecting sampling transects were marked in the field and nine soil cores were collected along both transects at 5 m distances, pooled and then homogenized. The pooled soil was sieved through a 2 mm-mesh to remove large roots and pebbles. Each pooled soil was split into three parts in the field directly, distributed into 15 or 50 ml falcon tube and transported to the laboratory at the nearby observatory, the Mile 46 Livestock Development Centre (18°18`06.4"S/ 19°15`24.6"E), within a few hours. One parallel of each soil sample was kept at room temperature (29-30°C) before utilized for isolation of bacteria and soil respiration measurement. The second parallel was kept at 4°C for enumeration of total bacteria and exoenzyme activities measurement and the third parallel was frozen immediately for later molecular analysis.

4.3.2 Total cell counts

Total cell counts were determined using a protocol as described by Lunau *et al.* (2005). Soil slurries were prepared as 1:100 (wt/vol) dilutions in 5.5 mM MES buffer pH 5.5 and subsequently fixed with 2% glutaraldehyde. Five hundred µl aliquots of the slurries were mixed with 450 µl of 100% methanol, 550 µl of 2 mM MOPS buffer pH 7.4 and subsequently incubated for 25 minutes at 35°C in an ultrasonic bath (model RK 103H, 35 kHz, 2x280 W per period; Bandelin electronic, Berlin, Germany). Five hundred µl aliquots of the cell suspensions were mixed with 10 ml MOPS buffer, stained by SYBR green I (Molecular Probes, Eugene, OR), and shaken in dark for 10 minutes. The aliquots were then filtered onto 0.1-µm-pore-size polycarbonate filters (25 mm diameter; Nucleopore Track-Etch Membrane; Whatman, Springfield Mill, UK). The filters were dried, embedded in DABCO antifading solution (25 mg of 1,4-diazabicyclo [2.2.2] octane in 1 ml of PBS buffer plus 9 ml of glycerol), and subsequently examined by epifluorescence microscopy (Zeiss Axiolab microscope) (lamp, HBO 50; filter set, Zeiss ex 450-490, FT 510, and LP 515) at a magnification of x 1,000. At least 20 fields were examined for each sample.

Table 1. Soil sampling locations, soil properties and land use types, and total cell numbers of 29 soil samples

Sample code[a]	Soil sampling locations[b]		Soil properties[c]	Land use[c]	Soil pH[d]		TCN[e] (10^9 g soil^{-1})
	Latitude S	Longitude E			10 mM $CaCl_2$	dd H_2O	
D1	18°17′14.7′′	19°17′28.3′′	**LD**	**FY**	**6.36**	**7.20**	**1.210 ± 0.129**
D2	18°17′14.6′′	19°17′20.7′′	LD	FY	6.45	7.16	0.582 ± 0.073
D3	18°17′10.4′′	19°17′26.6′′	LD	FY	6.34	7.24	0.772 ± 0.079
D4	18°16′ 0.5′′	19°15′57.2′′	**L**	**AO**	**5.84**	**6.62**	**0.419 ± 0.051**
D5	18°16′ 1.0′′	19°15′55.4′′	L	AO	6.05	6.88	0.479 ± 0.062
D6	18°16′ 4.6′′	19°15′55.5′′	**S**	**P**	**4.60**	**5.71**	**0.283 ± 0.046**
D7	18°19′11.2′′	19°18′44.7′′	LD	AO	6.51	7.14	1.370 ± 0.169
D8	18°19′ 1.0′′	19°18′39.3′′	**LD**	**AO**	**6.23**	**7.20**	**1.960 ± 0.139**
D9	18°19′ 8.6′′	19°18′35.0′′	LD	AO	5.85	6.59	2.100 ± 0.156
D10	18°19′11.3′′	19°18′45.7′′	**LD**	**FO**	**6.56**	**7.17**	**2.000 ± 0.159**
D11	18°18′27.3′′	19°15′45.8′′	**L**	**P**	**5.80**	**6.62**	**1.830 ± 0.158**
D12	18°18′25.4′′	19°15′37.5′′	L	P	5.82	6.64	1.360 ± 0.175
D13	18°18′33.5′′	19°15′45.5′′	S	P	5.23	6.57	0.429 ± 0.071
D14	18°18′28,4′′	19°16′ 5.0′′	**L**	**AY**	**6.17**	**7.14**	**0.936 ± 0.097**
D15	18°18′ 8.0′′	19°15′52.3′′	S	P	5.15	6.43	0.614 ± 0.056
D16	18°17′14.0′′	19°15′32.3′′	**LD**	**P**	**6.75**	**7.16**	**3.940 ± 0.019**
D17	18°19′10.7′′	19°19′15.5′′	**L**	**FO**	**5.73**	**6.63**	**0.964 ± 0.092**
D18	18°19′15.4′′	19°19′ 7.7′′	L	AY	5.84	6.71	0.996 ± 0.082
D19	18°19′ 7.1′′	19°19′11.8′′	L	FO	5.71	6.87	0.728 ± 0.071
D20	18°19′ 0.6′′	19°18′28.3′′	L	FO	6.85	7.26	0.942 ± 0.068
D21	18°19′ 0.5′′	19°18′30.0′′	L	AY	5.81	6.59	0.628 ± 0.083
D22	18°19′32.3′′	19°18′55.5′′	S	FY	5.76	7.01	0.686 ± 0.101
D23	18°19′38.0′′	19°18′28.7′′	**LD**	**FY**	**5.95**	**7.12**	**0.804 ± 0.099**
D24	18°19′40.9′′	19°18′30.6′′	L	FY	6.87	7.16	1.270 ± 0.143
D25	18°17′ 5.4′′	19°15′35.6′′	L	P	6.77	7.21	0.957 ± 0.092
D26	18°17′ 9.6′′	19°15′37.8′′	LD	P	6.75	7.45	1.270 ± 0.122
D27	18°19′ 0.4′′	19°18′20.5′′	LD	AM	5.48	6.81	0.735 ± 0.097
D28	18°18′59.6′′	19°18′19.8′′	S	AM	5.85	6.78	1.200 ± 0.103
D29	18°18′51.9′′	19°18′16.2′′	L	FY	5.85	6.82	0.920 ± 0.091

[a] Bold characters represent the ten main samples
[b] See Figure 1
[c] LD (loam, dark); L (loam, red or brown); S (sand); P (pristine); F (fallow); A (acre); O (old, >20 year); M (medium, between 10 and 20 year); Y (young, between 3 and 10 year)
[d] pH determined in 1:2.5 ratio of soil to 10 mM $CaCl_2$ and ddH_2O
[e] TCN (Total cell numbers)

Figure 1. Map of Mutompo village showing the location of 29 soil-sampling sites. Insert showing the location of the study area in Namibia. Colored legends and sample codes represent the ten main samples.

4.3.3 Respiration rates

Soil respiration of the ten main samples (D1, D4, D6, D8, D10, D11, D14, D16, D17, and D23) was measured within 6 h after sampling. The ten main soil samples were chosen as they represented different land use intensities and soil properties. Soil slurries were prepared by diluting two cm^3 of soil in 2 ml of 10 mM MES buffer pH 5.5. Five hundred µl of the aliquots were diluted with 1500 µl of 10 mM MES buffer and subsequently injected into the chamber of a respirometer. The soil respiration rates were also measured after the addition of a mixture of sugars, amino acids, aromatic compounds, fatty acids, or tricarboxylic acids into the soil slurries. The composition and final concentration of the substrates involved were as follow: sugars (a 5 mM concentration of each glucose, xylose, arabinose, trehalose, rhamnose, fucose, and a 2.5 mM concentration of each glucosamin, mannit, N-acetyl-D-galactosamine, turanose, □-gentibiose), amino acids (a 1.67 mM concentration of each alanine, arginine, ornithine, glutamine, glutamate, glycine, histidine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, valine, tyrosine, asparagine, isoleucine), aromatics compounds (a 5mM concentration of each natrium benzoate and natrium salicylate), tricarboxylic acids (a 2.5 mM concentration of each lactate, succinate, citrate, malate, pyruvate, α-ketoglutarate, oxaloacetate), and fatty acids (a 5 mM concentration of each formiate, acetate, citrate, propionate, butyrate, valerate). The respiration rates were determined by using an oxygraph (Hansatech version 1.00; Norfolk, UK) fitted with a Clark-type oxygen electrode, interfaced with a computer for data acquisition and analysis. Temperature was maintained between 30 and 31°C (the *in situ* temperature). The electrode was calibrated by employing air-saturated water and oxygen-free 10mM MES buffer obtained by adding a small amount of sodium dithionite to the chamber. The respiration rates were calculated from the decrease of oxygen concentration with time and express as nmol O_2 consumed $(g\ soil)^{-1}\ min^{-1}$.

4.3.4 Exoenzyme activities

The activities of the three exoenzymes alkaline phosphatase (EC 3.1.3.1), betaglucosidase (EC 3.1.21) and leucine aminopeptidase (EC 3.4.1.1) were determined by employing the soil slurries supplemented with 4-methylumbelliferone (MUF) or 7-amino-4-methylcoumarin (MCA) labeled substrate analogues. Alkaline phosphatase and betaglucosidase were tested with MUF-phosphate and MUF-β-glucoside (Sigma-Aldrich, Steinheim, Germany), while aminopeptidase was assessed with MCA-leucine (Sigma). The slurries were prepared by diluting 50 mg of soil with 5 ml final volume of sterilized tap water in 20 ml scintillation glass containing stirring bar. The enzymatic activities reaction was initiated by the addition of substrates analogue solution at final concentrations of 5, 20, 50, 100, 200 μM. For alkaline phosphatase, additional measurements were conducted with 400, 600 and 800 μM in order to obtain substrate saturation. During incubation at room temperature (about 25°C), all slurries were stirred at 200 rpm. Two different blanks were incubated in parallel. For each concentration, the first blank consisted of boiled soil slurries for 20 min in a water bath prior to addition of the substrates as controls for non-enzymatic hydrolytic cleavage of the substrate analogues. The second blank was soil slurries incubated without adding the substrate analogues. After incubation for 3 h, 1 ml of the slurries were transferred to 1.5 ml Eppendorf tubes, centrifuged for 5 min at 10,000x g and the concentrations of free dissolved fluorophores were analyzed fluorometrically by use of a Kontron SFM 25 Spectrofluorometer (AG. Switzerland). Equilibrium adsorption isotherms were determined by adding three different concentrations of MUF or MCA to the slurry aliquots of the ten main samples as adsorption controls. The rates of exoenzymatic hydrolysis were calculated from the equilibrium concentrations of the free fluorophores and from the adsorption isotherms for MUF and MCA in the respective soil samples according to the formula described previously (Coolen and Overmann 2000).

4.3.5 DNA extraction and purification

Soil DNA was extracted using an UltraClean Mega Prep soil DNA kit (Mo Bio Laboratories, Inc., Solana Beach, CA) according to the manufacturer's instructions and including additional steps as described previously (Zul *et al.* 2007). The

extracted DNA was precipitated with ethanol and subsequently purified by use of a Wizard DNA Clean-up system (Promega, Madison, WI) according to the manufacturer's instructions. The DNA was eluted using 50 µl 2 mM Tris-HCl buffer pH 8.0. Since amplification of some DNA samples was not possible because of high content of remaining humic substances, a subsequent purification was conducted using a PowerClean DNA Clean-up kit (Mo Bio Laboratories, Inc., Carlsbad, CA) as recommended by the manufacturer. The purified DNA concentrations were determined by ND-1000 Spectrophotometer (PegLab, Biotechnologie GmbH, Erlangen, Germany).

For extraction of chromosomal DNA from cultured bacteria, cells in microtiter plates were harvested by centrifugation at 15,000x g for 20 min at 4°C. Cell pellets obtained were dissolved in five µl of 2 mM Tris-HCl buffer pH 8.0 and subsequently lysed by six freeze and thaw cycles with each cycle consisting of an incubation for 3 min at -80°C followed by incubation for 3 min at 100°C. The cells lysed from ten wells of microtiter plate were combined and the resulting combined crude extracts were directly used in PCR amplifications.

4.3.6 qPCR

Samples D6 and D16 were selected for quantitative PCR (qPCR) since they represented the least and the most active soils based on the respiration results. The abundance of nine major (sub)phyla of *Bacteria* (including *Alphaproteobacteria*, *Betaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Planctomycetes*, and *Verrucomicrobia*) and all *Bacteria* was determined. The primer sets and cycling programs used are listed in Table 2. The qPCR assay was conducted in an iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). The mixture of each reaction consisted of the following: 3 µl of template containing 2 to 4 ng DNA, 20 µg of bovine serum albumin (Sigma), 240 nM of each primer, 12.5 µl of iQ SYBR green supermix (Bio-Rad), and ampuwa water (Fresenius, Bad Homburg, Germany) adjusted to 25-µl final volume. Determinations were performed in triplicate including the appropriate set of standards with the genomic DNA ranging from 2 to $2 \cdot 10^{-5}$ ng per well. The genomic

DNA representatives of the target groups, namely *Agrobacterium tumefaciens* DSM 5172^T, *Massilia brevitalea* DSM 18925^T, *Edaphobacter modestus* DSM 18101^T, *Mycobacterium phlei* DSM 43239^T, *Flavobacterium aquatile* DSM 1132^T, *Chloronema giganteum* Gnsb-1, *Bacillus subtilis* DSM 10^T, *Planctomyces maris* DSM 8797^T, *Verrucomicrobia spinosum* DSM 4136^T, and *Escherichia coli* B were utilized to generate standard curves. The specificity of qPCR products was analyzed by conducting melting curve analysis following each assay. Calibration curves (gene copy versus the cycle number at which the fluorescence intensity reaches a set cycle threshold value) were calculated by converting raw data from the iCycler into rRNA gene copy number by using an equation 1. This equation was used to determine the number of gene copies in a known amount of DNA (Ritalahti *et al.* 2006):

$$\begin{aligned}\text{gene copies} = {} & (\text{DNA concentration [ng/µl]}) \times (1\text{g}/1{,}000^3\ \text{ng}) \\ & \times (1\ \text{mol bp DNA}/660\ \text{g DNA}) \times (6.023 \cdot 10^{23}\ \text{bp/mol bp}) \\ & \times (1\ \text{copy}/16\text{S rRNA size [bp]}) \times (\text{volume of template [µl]}) \end{aligned} \quad (1)$$

Copy number estimates assume an average molecular weight of 660 for a base pair in double-stranded and one gene copy per 1502 bp-size of the respective 16S rRNA strains based on the PCR product amplified by employing eubacterial primer set (8f and 1492r; Lane 1991). The number of target genes per g of sample was determined with equation 2 (Ritalahti *et al.* 2006). The number of gene copies per reaction was determined from the appropriate standard curve; this value was multiplied by the weight (µg) of extracted DNA obtained from each sample and divided by both the µg of DNA used per reaction and the weight of sample from which the DNA was extracted.

$$\text{gene copies/g sample} = \frac{(\text{gene copies per reaction}) \times (\text{weight of DNA [µg]})}{(\text{µg DNA per reaction}) \times (\text{g sample used})} \quad (2)$$

The relative abundance of each (sub)phylum analyzed was determined as the ratio between the measured copy number for each (sub)phylum and all groups of *Bacteria* (Fierer *et al.* 2005).

Table 2. Summary of primer sets used and qPCR conditions

Target group/ Primer set	Primer sequence (5´ → 3`)	Annealing temp. (°C)[a]	Amplicons length (bp)	Reference
All *Bacteria*				
907r	CCGTCAATTCCTTTGAGTTT	60	≈ 585	Muyzer and Ramsing 1995
341f	CCTACGGGAGGCAGC			Muyzer *et al*. 1993
Alphaproteobacteria				
517f	GTGCCAGCAGCCGCGG	56	≈ 470	Lane 1991
Alf968r	GGTAAGGTTCTGCGCGTT			Neef 1997
Betaproteobacteria				
1055r	AGCTGACGACAGCCAT	58.4	≈ 395	Angle *et al.* 1991
Beta680f	CRCGTGTAGCAGTGA			Overmann *et al*. 1999
Acidobacteria				
341r	CTGCTGCCTCCCGTAGG	59	≈ 330	Muyzer *et al.* 1993
Acido31f	GATCCTGGCTCAGAATC			Barns *et al*. 1999
Actinobacteria				
AB1165r	ACCTTCCTCCGAGTTRAC	64	≈ 260	Lüdemann and Conrad 2000
926f	AAACTCAAAGGAATTGACGG			Lane 1991
Bacteroidetes				
CFB319f	GTACTGAGACACGGACCA	56.4	≈ 605	Jaspers *et al.* 2001
907r	CCGTCAATTCCTTTGAGTTT			Muyzer and Ramsing 1995
Chloroflexi				
1340r	CGCGGTTACTAGCAAC	55.5	≈ 420	Gich *et al*. 2001
GNSB941f	AGCGGAGCGTGTGGTTT			
Firmicutes				
LGC353fmix	GCAGTAGGGAATCT(TC)(CG)	55	≈ 575	Meier 1999
907r	CCGTCAATTCCTTTGAGTTT			Muyzer and Ramsing 1995
Planctomycetes				
EUB338f	ACACCTACGGGTGGCWGC	60.5	≈ 610	Daims *et al*. 1999
Pln930r	GCCTTGCGACCATACTCCC			Blackwood *et al*. 2005
Verrucomicrobia				
EUBIII338r	GCTGCCACCCGTAGGTGT	59.5	≈ 320	Sangwan *et al*. 2005
Ver40f	CGGCGTGGWTAAGACATGCA			Zul *et al*. 2007

[a] All qPCR conditions were initiated by denaturation at 94°C for 3 min (1 cycle), followed by 35 cycles of 94°C for 30 s, 1 min at the respective annealing temperature, 1 min at 72°C, and finally checked the specific PCR products by melting curve analysis in a range from 55 to 95°C.

4.3.7 16S rDNA clone library of *Eubacteria* for sample D16

Genomic DNA of sample D16 was chosen to construct a clone library in order to asses the diversity of the major bacterial (sub)phyla and compare the bacterial abundance results obtained from the qPCR assay. The 16S rRNA gene fragments of *Eubacteria* were amplified by PCR employing primers 8f (5´-AGA GTT TGA TCC TGG CTC AG) and 1492r (5´-GGT TAC CTT GTT ACG ACT T) (Lane 1991). Amplification reactions were performed with a Gene Amp PCR 9700 system (Applied Biosystems, Foster City, CA). The reaction was carried out in 50 µl mixture containing 10 ng of template DNA, 10 µl of 10x PCR buffer containing 15 µM $MgCl_2$ (QIAGEN GmbH, Hilden, Germany), 5 µl of Q solution (QIAGEN), 40 µg of bovine serum albumin (Sigma), 500 nM $MgCl_2$, 200 nM deoxynucleoside

triphosphates, a 1 μM concentration of each primer and 1.25 units of *Taq* polymerase (QIAGEN). The PCR protocol consisted of 94°C for 4 min, followed by 10 cycles of 30 s at 94°C, 45 s at 59°C, 1 min at 72°C; 20 cycles of 30 s at 94°C, 45 s at 54°C, 1 min at 72°C; and a final extension for 10 min at 72°C. The PCR product was analyzed by standard agarose electrophoresis and furthermore cloned into the pCR® II-TOPO® linear plasmid vector and then transformed into One shot TOP10 chemically competent *Escherichia coli* using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After plated on selective LB agar plates containing 100-μg ml^{-1} ampicillin and treated with X-Gal, recombinants were picked randomly. The plasmids were extracted from an overnight culture in liquid LB media by employing plasmid extraction standard method. Plasmids were differentiated by enzymatic digestion with EcoRI (Fermentas GmbH, St. Leon-Rot, Germany) and different clones were subsequently sequenced. The 16S rRNA gene sequences obtained were checked for possible chimeras using the CHIMERA-CHECK online analysis program of the RDP-II database (Maidak *et al.* 2001). The library was then normalized using the rarefaction method (Simberloff 1972) by use of a RAREFACT.FOR program written by C.J. Krebs, University of British Columbia (http://gause.biology.ualberta.ca/jbrzusto/rarefact.html). Species richness estimations (Chao1 and ACE [Chao 1984, Chao and Lee 1992]) were calculated by using the software EstimateS 7.5 (http://purl.oclc.org/estimates).

4.3.8 PCR-DGGE of 16S rRNA genes from the abundant phyla

The 16S rRNA gene fragments of *Acidobacteria* and *Actinobacteria* in 29 soil samples were amplified by PCR employing the respective primer sets (Table 2). Amplification reactions were performed with a Gene Amp PCR 9700 system (Applied Biosystems). The reaction mixtures contained per 50 μl: 10 ng of soil DNA, 10 μl of 10x PCR buffer containing 15 μM $MgCl_2$ (QIAGEN), 5 μl of Q solution (QIAGEN), 40 μg of bovine serum albumin (Sigma), 500 nM $MgCl_2$, 200 nM deoxynucleoside triphosphates, a 1 μM concentration of each primer and 1.25 units of *Taq* polymerase (QIAGEN). The cycling programs started with an initial 94°C denaturation for 4 min; 10 cycles of 30s at 94°C, 1 min and 45s (for

Acidobacteria and *Actinobacteria* species, respectively) at 62°C, 1 min at 72°C; 20 cycles of 30s at 94°C, 1 min and 45s (for *Acidobacteria* and *Actinobacteria* species, respectively) at 57°C, 1 min at 72°C; and a final extension for 10 min at 72°C. One of the primers contained a 40-bp-long GC clamp at its 5´ end in order to obtain stable melting behavior of the generated DNA fragments during the subsequent DGGE.

Denaturing gradient gel electrophoresis was carried out in an Ingeny phorU system (Ingeny International BV, Goes, The Netherlands) employing 6% (wt/vol) polyacrylamide gels in 1x Tris-acetate-EDTA (pH 8.0). Denaturing gradients ranged from 40 to 80% (for *Acidobacteria* species) or from 50 to 80% (for *Actinobacteria* species), where 100% denaturant is defined as 7 M Urea and 40% (vol/vol) formamide (Muyzer *et al.* 1993). Gels were stained for 45 min with SYBR gold (MoBiTec, Göttingen, Germany) (1:10,000 dilution), visualized on a UV trans-illuminator (LTF Labortechnik, Wasserburg, Germany) and photographed (Visitron Systems GmbH, Puchheim, Germany). DNA bands were excised from the gel with a sterile scalpel and transferred to a 1.5 ml Eppendorf tube containing 25 µl of 2 mM Tris-HCl buffer (pH 8.0). The DNA was eluted for 2 hours at 65°C. Reamplifications of the excised DGGE bands were conducted by using the corresponding primer sets (without a GC clamp) and cycling programs. PCR products were separated from free PCR primers by use of a QIAquick Spin kit (QIAGEN). The generated DGGE banding patterns were inspected visually and scored based on the melting type and the presence (coded as 1) or absence (coded as 0) of each band from each soil sample. The data obtained was integrated in a matrix. Similarities of the bacterial diversity as revealed by the DGGE banding patterns were calculated by employing the matrix with the SIMQUAL program of the NTSYS-pc (Exeter Software, New York) and expressed as DICE coefficient. The dendograms were constructed based on the coefficient and using the SAHN program with the unweighted pair-group method with mathematical average (UPMGA).

4.3.9 Sequencing and phylogenetic analysis

Sequencing of the 16S rRNA gene fragments of the clone library and the excised DGGE bands was performed with an ABI Prism Big Dye Terminator cycle sequencing ready-reaction kit (Applied Biosystems GmbH, Weiterstadt, Germany)

and an ABI Prism 310 genetic analyzer (Applied Biosystems). The 16S rRNA sequences were analyzed using the ARB software package (http://www.mikro.biologie.tu-muenchen.de). Sequences of closest relative were retrieved from the GenBank database employing BLAST 2.0.4 software (Altschul *et al.* 1997) and imported into the ARB database. Using the integrated program Fast Aligner Version 1.03 tool, the sequences were automatically aligned and the alignment was corrected manually according to secondary structure information. Sequences longer than 1300 bp were used to construct a tree by maximum likelihood. Afterwards, the shorter sequences obtained were added by employing maximum parsimony method.

4.3.10 Cultivation of soil bacteria

The cultivation of soil bacteria was also carried out in the field laboratory directly after sampling using ten of out of 29 samples. Two kinds of media were utilized as growth media employing salt-solution-equivalent (SSE; Angle *et al.* 1991) pH 5.5 buffered with 10 mM MES as basal medium. The first medium (SSE+ARE) was the basal medium supplemented with artificial root exudates containing carbohydrates (12.5 mM of glucose, 5.5 mM of arabinose, 4.1 mM of fructose, and 1.4 mM of saccharose), amino acids (0.19 mM of alanine, 0.06 mM of arginine, 0.02 mM of glutamine, 0.15 mM of glycine, 0.04 mM of leucine, 0.07 mM of serine, and 0.03 mM of valine), and organic acids (0.4 mM of benzoate, 0.7 mM of citrate, 1.0 mM of fumarate, 0.14 mM of glutamate, 0.6 mM of malate, 0.5 mM of oxalate, 0.6 mM of succinate, and 0.16 mM of tartarate) (Kozdrój and van Elsas 2000) and 0.01% yeast extract. The second medium (SSE+Cmix) was the basal medium consisting of the following (a 2 µM final concentration each of ingredient): a mixture of carbohydrates (glucose, arabinose, trehalose, rhamnose, fucose, xylose, mannit, N-acetyl-D-galactosamin, glucosamin, β-gentibiose), organic acids (lactate, succinate, citrate, malate, pyruvate, oxaloacetate, formiate, acetate, propionate, butyrate, valerate, α-ketoglutarate), aromatic compounds (sodium benzoate, sodium salicylate), and a mixture of 20 amino acids. Both media were supplemented with inducers; a 10 µM (for SSE+ARE medium) or a 2 µM (for SSE+Cmix medium) concentration each of cyclic AMP, AMP, *N*-oxohexanoyl-DL-homoserine lactone (OHHL), and *N*-butyryl-DL-homoserine lactone (BHL) (Bruns *et al.* 2003). Each well received 180 µl of

growth medium. The plates were inoculated manually by use of a conventional multipipette with 20 µl of two dilution series of soil slurries. The two dilution series (1:1,000 and 1:10,000) were prepared by diluting soil in 10 mM MES buffer pH 5.5. Twelve wells per microtiter plate were left without inoculation and served as a negative control contamination during sample manipulation. After inoculation, each microtiter plate was sealed with a silicone seal (VWR International GmbH, Darmstadt, Germany) to protect them from cross contamination and leaking out of the cultures due to transportation from the observatory in Namibia to the laboratory in München, Germany. Microtiter plates were incubated at room temperature about 30°C for 2 weeks in the observatory and incubation continued at 25°C for 4-6 weeks in München. Especially for the cultures grown in the SSE+Cmix medium, after 4 weeks incubation the carbon mixtures concentration was increased 10x by adding the carbon solutions described above into each well of microtiter plates. Growth was monitored visually by turbidity.

4.4 Results

4.4.1 Total cell numbers

The total cell numbers of 29 soil samples were determined by staining the cells with SYBR green I and counted manually under epifluorescence microscope (Table 1). The total cell numbers ranged from (0.283 ± 0.046) $\cdot 10^9$ cells (g soil)$^{-1}$ in the pristine sand soil (D6) to a maximum of (3.940 ± 0.019) $\cdot 10^9$ cells (g soil)$^{-1}$ in the pristine dark loam soil (D16). The total cell numbers from combinations of pristine/sand (D6, D13, D15) showed a tendency to be lower than that of other land use and soil property combinations. Soil pH (Table 1) did not show a clear correlation to the total cell numbers.

4.4.2 Soil respiration

The pristine sand soil (D6) exhibited the lowest respiration rate (4.03 nmol O_2 [g soil]$^{-1}$ min^{-1}), whereas other soil samples had moderately respiration rates ranging from 4.99 to 9.14 nmol O_2 (g soil)$^{-1}$ min^{-1}. In contrast, the pristine dark loam soil (D16) had a remarkably high respiration rate (23.71 nmol O_2 [g soil]$^{-1}$ min^{-1}), which was 2.5 to 6.0 times that of the other sites (Fig. 2A). These results largely

corresponded to the total cell numbers where the highest and the lowest values were also obtained from pristine sand and pristine dark loam soils, respectively. Polynomial equation best explained the relationship between the respiration rates and the total cell numbers, with $r^2 = 0.8655$ (Fig. 2B).

Generally, the addition of substrates did not increase the respiration rates. However, the addition of a mixture of either aromatic compounds or fatty acids slightly caused the increase of respiration rates of soils in sample D8, D14 and sample D1, D4, D14, D17, respectively (data not shown).

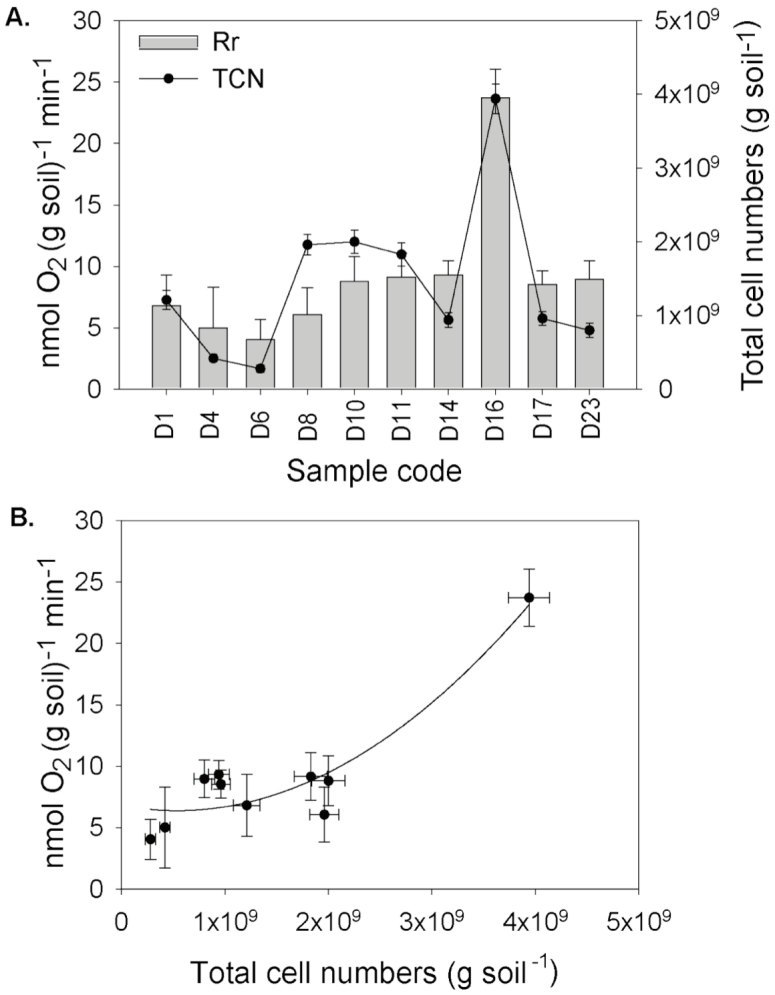


Figure 2. **A**. Soil respiration rate (Rr) and the total cell numbers (TCN) of the ten main samples, **B**. Relationship between soil respiration rates and the total cell numbers ($r^2 = 0.8655$).

4.4.3 Exoenzyme activities

Three exoenzyme activities were determined fluorometrically by the addition and incubation of the soil slurries with fluorogenic enzyme substrates 4-methylumbelliferone (MUF) or 7-amino-4-methylcoumarin (MCA). The adsorptive affinity, *K,* of MUF and MCA to the soil samples varied from $8.87 \cdot 10^{-1}$ to $1.41 \cdot 10^{-4}$ ml (g soil)$^{-1}$ with r^2 ranged from 0.88 to 0.99 (Table 3). The pristine loam soil (D11) and the old fallow loam soil (D17) had the highest affinity to MCA ($8.87 \cdot 10^{-1}$ ml [g soil]$^{-1}$) and MUF ($4.13 \cdot 10^{-1}$ ml [g soil]$^{-1}$), respectively. Sample D1, D6, D10, D11, D14, and D23 showed higher affinity to MCA than MUF (Table 3). In order to calculate Michaelis-Menten kinetics of the three exoenzymes, the substrate saturation curves were determined by the addition of various amounts of MUF-substrates (MUF-□-glucoside or MUF-phosphate) or MCA-substrate (MCA-leucine) into the slurry of ten main soil samples. Figure 3 shows representative of the substrate saturation curves from sample D16, where alkaline phosphatase required higher substrate concentration (up to 800 µM) to reach substrate saturation than betaglucosidase and aminopeptidase.

Exoenzyme activities varied among the soil sample sites, but did not show a clear correlation to the land use, land use age, and soil properties (Table 4). Generally, sample D16 (pristine dark loam) showed a trend of higher velocity and affinity for the three exoenzymes examined (Fig. 4). Alkaline phosphatase activity was obviously higher in sample D16 (15.42 µmol [g soil]$^{-1}$ h^{-1}), which was 9 to 53 times that of the other samples and lower in sample D6 (0.29 µmol [g soil]$^{-1}$ h^{-1}). The affinity values among the ten main samples were almost in the same range (ranging from 0.010 to 0.039 h^{-1}), except for sample D1 and D4 that had values 0.076 and 0.079 h^{-1}, respectively. Sample D14 (young acre loam) and sample D16 exhibited considerably higher betaglucosidase activity, which were about 4 to 44 times higher than the other samples. The lowest this enzyme activity was detected in sample D6 (0.39 µmol g [soil]$^{-1}$ h^{-1}). The activity of aminopeptidase greatly varied among the ten soil samples observed. The highest and the lowest activity of this enzyme were observed in the pristine dark loam soil (6.63 µmol g [soil]$^{-1}$ h^{-1}) and old fallow loam soil (0.07 µmol [g soil]$^{-1}$ h^{-1}), respectively.

A *Km* + *Sn* of alkaline phosphatase, betaglucosidase, and aminopeptidase were significantly higher in sample D16 (405.13 μM), D14 (214.03 μM), and D10 (345.89 μM), respectively (see Table 4). The lowest value of *Km* + *Sn* of these enzymes was detected in sample D1 (10.89 μM for alkaline phosphatase) and D4 (7.19 μM and 16.61 μM for betaglucosidase and aminopeptidase, respectively). In some cases, higher value of *Km* + *Sn* did not correspond to lower affinity, for example, a *Km* + *Sn* of betaglucosidase sample D14 was the highest, but its affinity was not the lowest.

Table 3. Parameters of the Freundlich equilibrium adsorption isotherms of the ten main samples for fluorophores

Sample code	Fluorophores	n	K (ml g^{-1})*	r^2
D1	MUF	1.498 ± 0.055	$3.635 \cdot 10^{-3} \pm 8.548 \cdot 10^{-4}$	0.9996
D4	MUF	1.116 ± 0.071	$1.569 \cdot 10^{-2} \pm 4.744 \cdot 10^{-3}$	0.9989
D6	MUF	1.979 ± 0.099	$4.739 \cdot 10^{-4} \pm 2.010 \cdot 10^{-4}$	0.9994
D8	MUF	0.966 ± 0.069	$1.090 \cdot 10^{-2} \pm 3.317 \cdot 10^{-3}$	0.9986
D10	MUF	1.074 ± 0.056	$1.907 \cdot 10^{-2} \pm 4.580 \cdot 10^{-3}$	0.9993
D11	MUF	1.453 ± 0.041	$1.098 \cdot 10^{-2} \pm 1.801 \cdot 10^{-3}$	0.9998
D14	MUF	1.540 ± 0.032	$3.520 \cdot 10^{-2} \pm 3.832 \cdot 10^{-3}$	0.9998
D16	MUF	1.286 ± 0.038	$3.766 \cdot 10^{-2} \pm 5.405 \cdot 10^{-3}$	0.9997
D17	MUF	1.035 ± 0.005	$4.126 \cdot 10^{-2} \pm 9.091 \cdot 10^{-4}$	0.9999
D23	MUF	2.047 ± 0.137	$1.408 \cdot 10^{-4} \pm 8.522 \cdot 10^{-5}$	0.9989
D1	MCA	0.709 ± 0.156	$1.615 \cdot 10^{-1} \pm 1.017 \cdot 10^{-1}$	0.9851
D4	MCA	1.050 ± 0.054	$5.260 \cdot 10^{-2} \pm 1.134 \cdot 10^{-2}$	0.9993
D6	MCA	0.784 ± 0.211	$2.000 \cdot 10^{-3} \pm 1.615 \cdot 10^{-4}$	0.9765
D8	MCA	0.358 ± 0.163	$3.179 \cdot 10^{-2} \pm 2.180 \cdot 10^{-2}$	0.8881
D10	MCA	0.991 ± 0.015	$1.680 \cdot 10^{-1} \pm 8.754 \cdot 10^{-3}$	0.9999
D11	MCA	0.632 ± 0.186	$8.874 \cdot 10^{-1} \pm 5.150 \cdot 10^{-1}$	0.9672
D14	MCA	0.602 ± 0.201	$5.105 \cdot 10^{-1} \pm 3.732 \cdot 10^{-1}$	0.9596
D16	MCA	0.569 ± 0.176	$2.983 \cdot 10^{-3} \pm 2.102 \cdot 10^{-4}$	0.9682
D17	MCA	0.901 ± 0.017	$5.117 \cdot 10^{-3} \pm 3.570 \cdot 10^{-4}$	0.9999
D23	MCA	0.639 ± 0.176	$3.449 \cdot 10^{-3} \pm 2.311 \cdot 10^{-4}$	0.9747

* The affinity coefficient (K) was calculated by employing the Freundlich equation:

$$S = K \cdot C_e^n$$

where S is the amount of a fluorophore adsorbed, C_e is the concentrations of the fluorophore remaining in solution, and n is a dimensionless exponent.

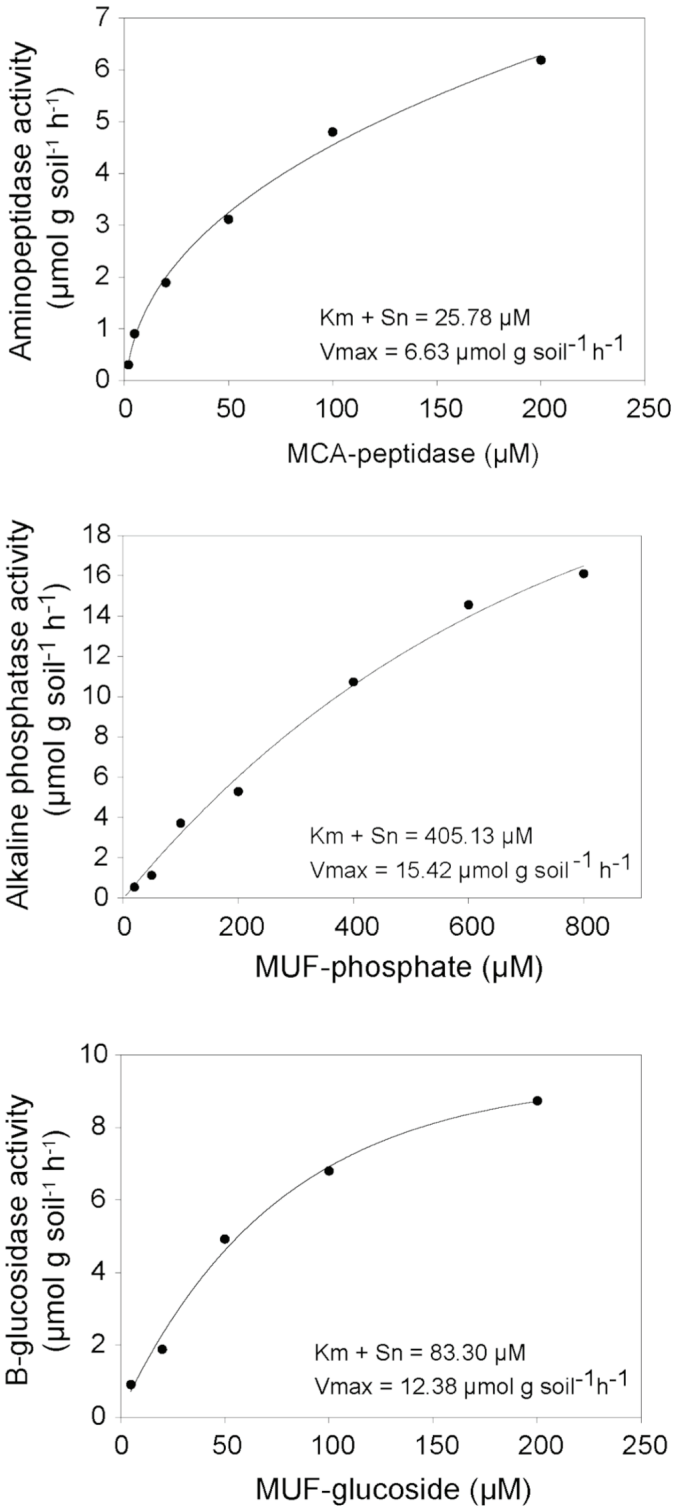


Figure 3. Michaelis-Menten kinetics of the three exoenzymes measured (aminopeptidase, alkaline phosphatase, and betaglucosidase) in the pristine dark loam soil (D16)

Table 4. The half saturation constant, $K_m + S_n$, the maximum velocity, $V_{max,}$ and affinity values of the three exoenzymes determined for the ten main samples

Sample code	Alkaline phosphatase			Betaglucosidase			Aminopeptidase		
	$K_m + S_n$ (μM)	V_{max} (μmol[g soil]$^{-1}$ h^{-1})	Affinity (h^{-1})	$K_m + S_n$ (μM)	V_{max} (μmol[g soil]$^{-1}$ h^{-1})	Affinity (h^{-1})	$K_m + S_n$ (μM)	V_{max} (μmol[g soil]$^{-1}$ h^{-1})	Affinity (h^{-1})
D1	10.89	0.83	0.076	50.83	1.94	0.038	17.06	2.18	0.128
D4	14.87	1.17	0.079	7.19	0.66	0.092	16.61	0.52	0.031
D6	15.47	0.29	0.019	39.73	0.39	0.009	136.52	1.07	0.001
D8	30.62	1.21	0.039	30.39	1.84	0.060	23.98	1.02	0.043
D10	51.25	1.07	0.021	18.23	1.99	0.109	345.89	0.40	0.001
D11	75.07	0.78	0.010	44.54	3.22	0.072	53.45	4.86	0.091
D14	60.12	0.98	0.016	214.03	17.27	0.081	30.70	3.43	0.112
D16	405.13	15.42	0.038	83.30	12.38	0.149	25.78	6.63	0.257
D17	52.86	1.66	0.031	35.58	1.53	0.047	35.67	0.07	0.002
D23	36.24	0.78	0.022	86.25	2.61	0.030	42.38	4.09	0.097

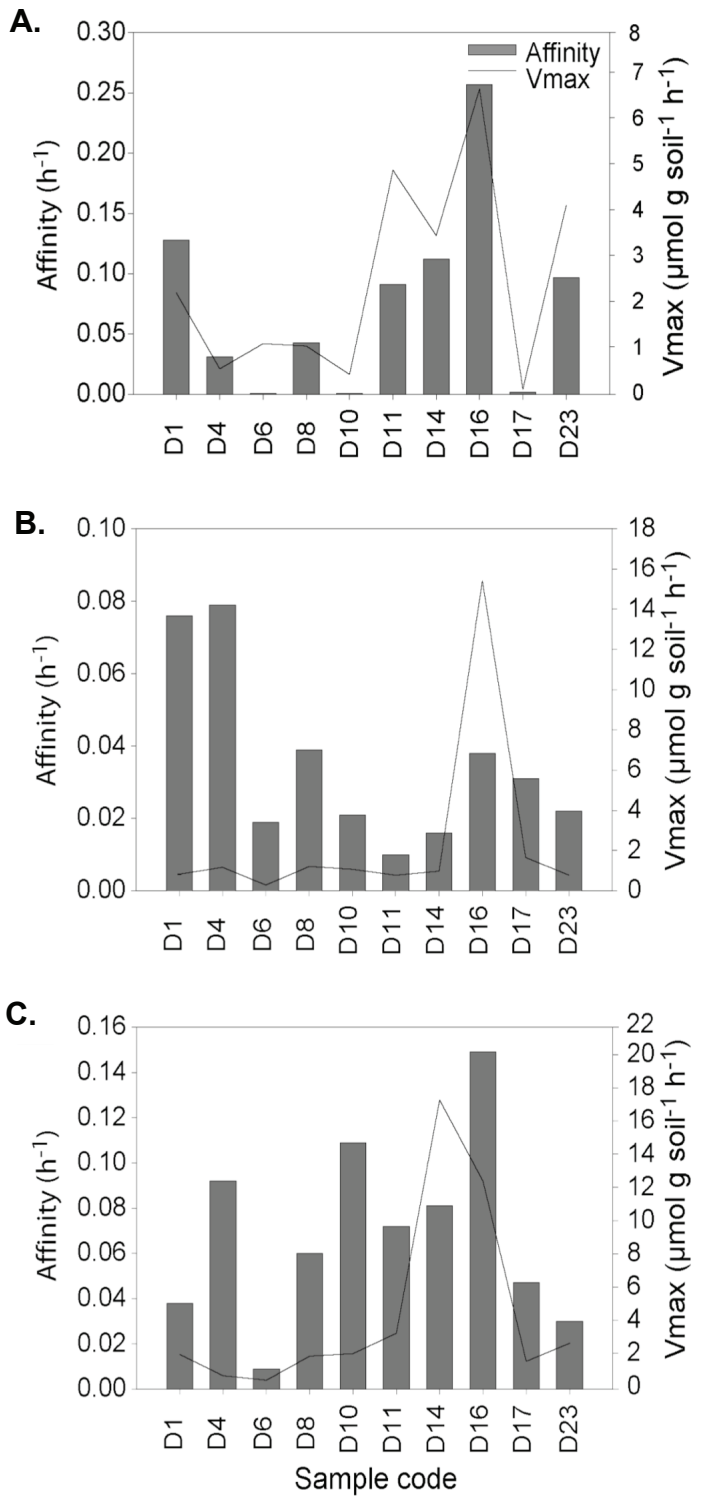


Figure 4. Relationship between affinity and velocity of three exoenzymes of the ten main soil samples, **A**. Aminopeptidase, **B**. Alkaline phosphatase, **C**. Betaglucosidase

4.4.4 Distribution and abundance of 16S rRNA

The distribution and abundance of 16S rRNA in the selected sampling sites were determined using qPCR and cloning approaches. Figure 5 (A and B) shows the estimated relative abundances of the nine target bacterial groups in the pristine sand soil (D6) and the pristine dark loam soil (D16), the least and the most active soils, respectively as determined by qPCR approach. *Acidobacteria* and *Actinobacteria* were identified as the most abundant phyla (about of 30 and 20% of all *Bacteria*, respectively) in the two sampling sites. *Alphaproteobacteria, Bacteroidetes,* and *Planctomycetes* accounted for below 10%, whereas *Betaproteobacteria, Chloroflexi*, and *Firmicutes* represented less than 1%. The bacterial community structure between the two sites was quite similar in general, only the *Verrucomicrobia* phyla showed higher abundance in D6 (12.86%) than in D16 (1.71%).

In order to compare the result obtained from qPCR approach, a clone library was constructed from the most active soil (D16; pristine dark loam) by employing the eubacterial primer set for domain *Bacteria*. The distribution and abundance of the phylogenetic groups identified in the *Bacteria* 16S rRNA gene library is depicted in Fig. 5C. Cloned of 16S rRNA genes were clustered to 13 (sub)phyla and 211 clones among 572 clone sequences affiliated with *Actinobacteria* as the most abundant phylum (36.88%), followed by *Alphaproteobacteria* (22.20%) and *Acidobacteria* (15.21%). *Betaproteobacteria, Deltaproteobacteria, Firmicutes, Gemmatimonadetes,* and *Nitrosospira* were identified ranging from 4.9% to 3.3%. The remaining (sub)phyla accounted for below 2%. Chimera checks showed that one sequence (about of 0.17%, excluding 572 sequences) was likely to be chimeric. The degree of redundancy among clones sequenced was very low and determined to be 4.7%. The distribution and abundance of (sub)phyla observed by clone library differed slightly from the qPCR results. The *Verrucomicrobia* phyla were undetected by clone library approach. The rarefaction curve was still unsaturated at 95% confidence interval (Figure 6) and the coverage of biodiversity was quite low and estimated to be 9.7%. However, the library revealed a high bacterial diversity with estimated number of about 5404 and 5600 phylotypes as determined by Chao1 and ACE richness estimators, respectively.

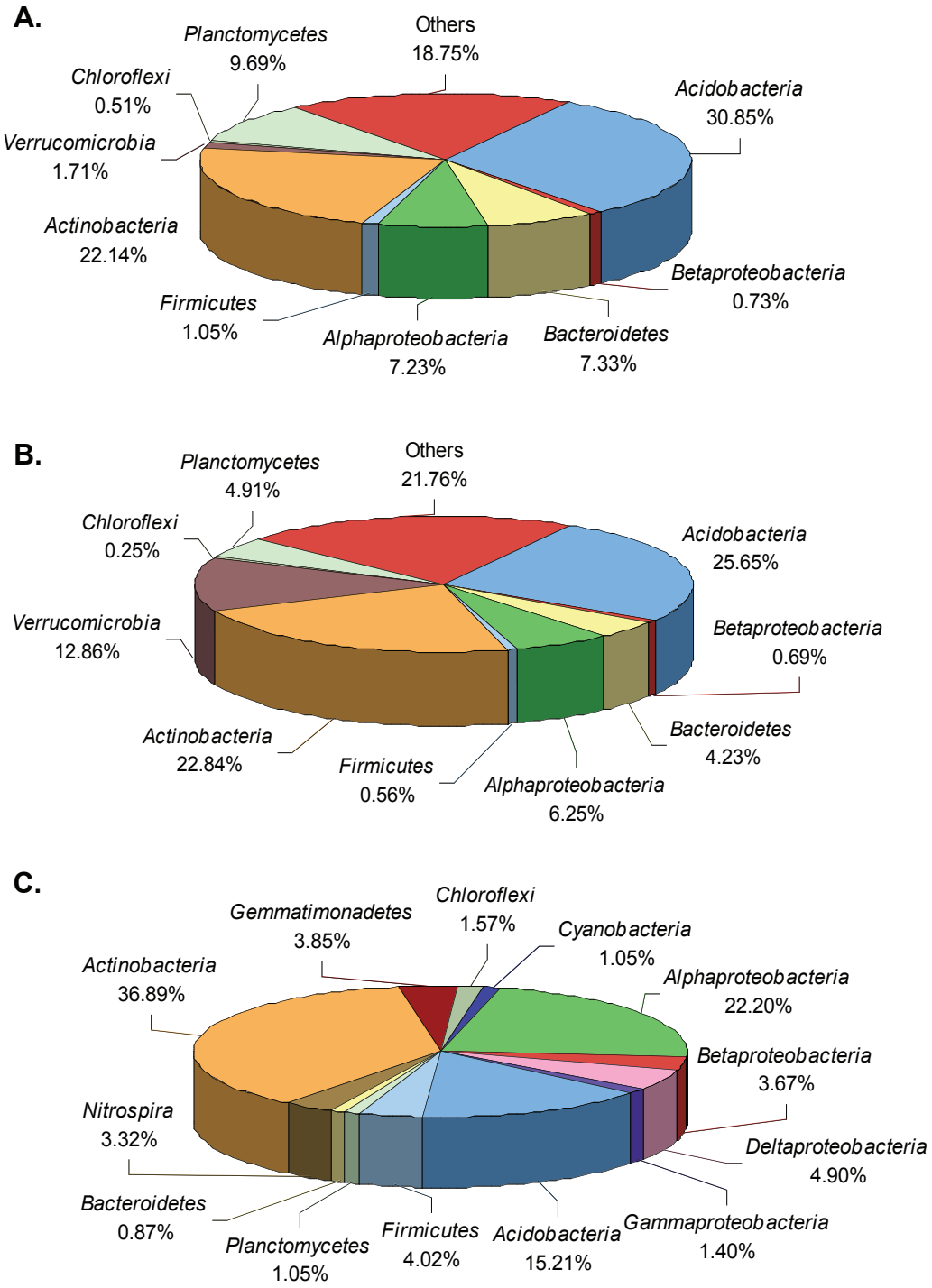


Figure 5. The relative abundances of the nine target bacterial groups as estimated by using qPCR approach in **A**. Pristine dark loam soil (D16), **B**. Pristine sand soil (D6), whereas **C.** The distributions and relative abundances of sub(phylum) observed in the pristine dark loam soil (D16) as determined by cloning of 16S rRNA gene fragments (n = 572).

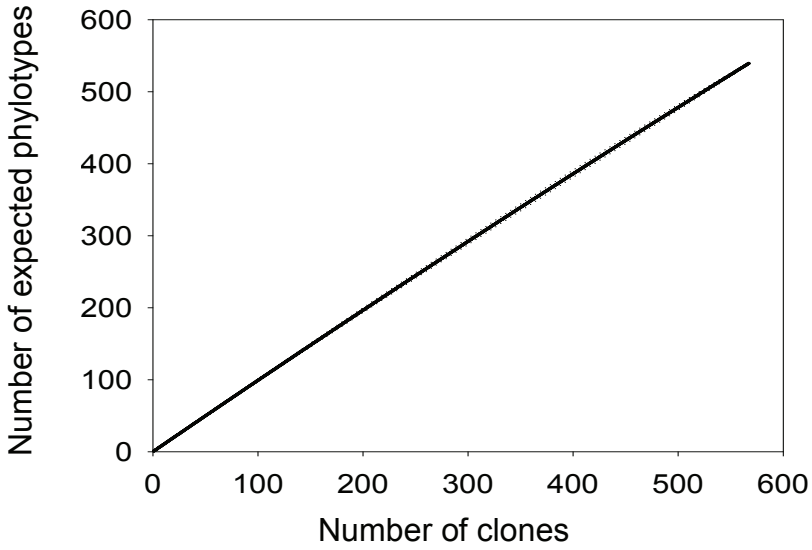


Figure 6. Predicted number of expected phylotypes based on rarefaction curve (95% confidence interval, n = 572).

The majority of the clone sequences were distantly related to cultured organisms. About 48.6% of the clones had ≥ 98% sequence similarity to environmental sequences, whereas 47.8% of them possessed the similarity to environmental sequences between 97% and 93%. Many of them were closely related to environmental bacteria from various soil types. Phylogenetic analysis indicates that all of clones in *Actinobacteria* fell into nine families. One hundred and fifty clones (71.1%) among 211 clones affiliated with this phylum were identified as members of the family *Solirubrobacteraceae, Rubrobacteraceae,* and *Thermoleophilaceae* within the subclass *Rubrobacteridae.* This subclass formed deep branching in the tree (data not shown). All of the *Alphaproteobacteria* clones were clustered into ten families, where members of the family *Beijerinckiaceae* were the most frequently encountered group (32.2%). Clones affiliated with *Acidobacteria* fell into six subgroups and most of the clones were clustered to subgroups 4 and 6.

4.4.5 Community composition of soil bacteria

Because *Acidobacteria* and *Actinobacteria* species were identified as the most abundant phyla using qPCR and 16S rRNA gene cloning approaches, subsequent high-resolution phylogenetic DGGE-fingerprinting was done to compare the

bacterial community composition in 29 sampling sites using these group-specific primer sets. The detectable melting type per sampling site was between 15 and 25 for *Actinobacteria* species (Fig. 7A) and between 10 and 23 for *Acidobacteria* species (Fig. 7B). The DGGE-fingerprint of *Actinobacteria* species was relatively consistent across all sampling sites. On the other hand, the DGGE-fingerprint of *Acidobacteria* species did only show minor differences among sites. A variety of intensity bands and additional weak bands (e.g., band 5, 16, and 18) were responsible for the differences among the sites. However, the bacterial community compositions in pristine sites were somewhat different from fallow and acre sites (refer to Fig. 7).

The dominant and unique phylotypes of *Acidobacteria* and *Actinobacteria* species (18 phylotypes for the two phyla; marked in Fig. 7) were excised and sequenced in order to determine the phylogenetic affiliation of the bacteria occurring in the sampling sites. The sequences of the *Acidobacteria* species were distantly related to known species in the GenBank database (Fig. 10), whereas 38% members of the phylum *Actinobacteria* were affiliated with cultured species. One unique phylotype (band 3, Figs. 7B and 10) was observed from the *Acidobacteria* species community that was present exclusively in D6 site (pristine sand). This phylotype was affiliated with uncultured uncontaminated surface soil clone OUT-065 (EU 122796) with 99% similarity. However, the majority of the 16S rRNA gene sequences were found to be affiliated to environmental sequences, mostly originating from soil samples. None of the sequences obtained from the excised DGGE bands were similar to the clone library sequences as revealed by the phylogenetic analysis.

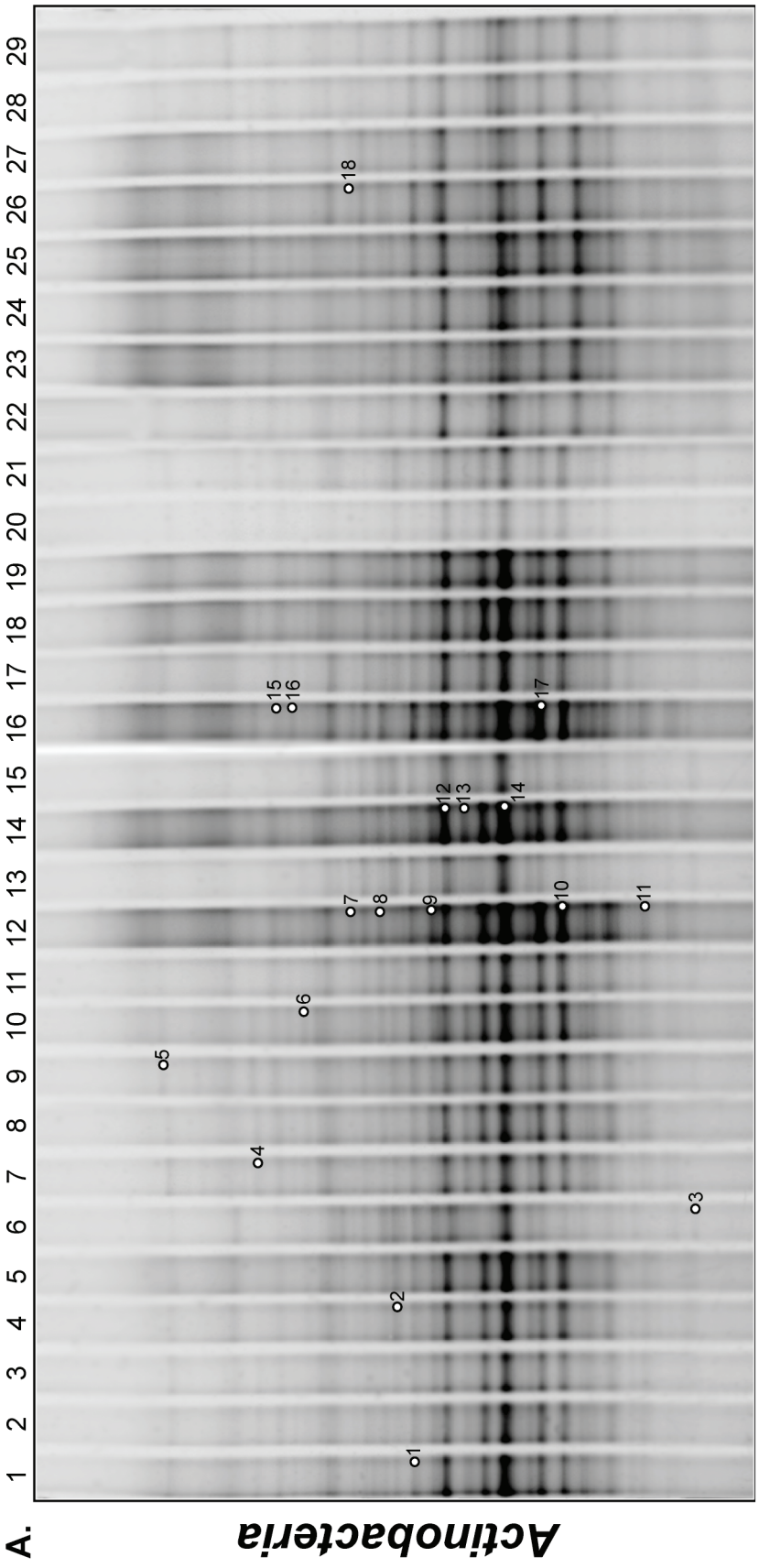
In order to determine the factors controlling the bacterial community composition, pairwise similarity was calculated from the DGGE banding patterns by employing the DICE coefficient. A cluster analysis was subsequently constructed based on the coefficient for the two bacterial groups. The dendograms formed inconsistent clusters concerning land use and soil properties (Fig. 8). However, the *Acidobacteria* species populations in pristine sand (D6, D13, and D15) were more similar and grouped together in one cluster as well as young fallow sand (D22) and acre medium sand (D28). While the populations in combinations of dark loam or loam with different land use types were clustered in several sub-clusters.

Interestingly, the populations in young fallow dark loam (D1, D2, and D3) or old acre loam (D4 and D5) were similar and clustered in small sub-clusters for the two bacterial groups (Figs. 8A and 8B). Based on the DICE coefficient values, the composition of phylum *Acidobacteria* revealed more pronounced changes than the phylum *Actinobacteria* (maximum Dice coefficient of 0.40 and 0.68, respectively; Fig. 8).

4.4.6 Culturable fractions of soil bacteria

By employing two inoculum sizes, 735 bacterial cultures were obtained from SSE+Cmix and SSE+ARE media. A combination of small inoculum size and medium supplemented with low nutrients concentration (SSE+Cmix) resulted higher culturability than a combination of big inoculum size and rich medium (SSE+ARE) (Fig. 9). Increasing tenfold of carbon sources concentration (for the cultures grown on SSE+Cmix medium after 4 weeks incubation) did not give a significant impact on the culturability values, except for sample D8 (Fig. 9A and 9B). The culturability values were ranging from 1.55% ± 0.59% to 0.06% ± 0.04% (small inoculum size) and from 0.40% ± 0.09% to 0.02% ± 0.01% (big inoculum size) for the cultures grown on SSE+Cmix medium (see Fig. 9A and 9B). Moreover, the culturability of the cultures grown on SSE+ARE medium ranged from 0.69% ± 0.19% to 0 (small inoculum size) and from 0.60% ± 0.10% to 0.03% ± 0.01% (big inoculum size) (Fig. 9C).

The cultures were subsequently screened by PCR-DGGE with *Acidobacteria* and *Actinobacteria* primer sets. *Acidobacteria* were detected only on medium with low nutrient concentrations (SSE+Cmix) in sample D1, D8, D10, D11, D16, and D17. This screening yielded 15 phylotypes of *Acidobacteria*, which were distributed on subgroups 1, 4, and 6 (Fig. 10) and distantly related to uncultured and known bacteria, so far. Screening of *Actinobacteria* species was carried out by employing the cultures recovered only from sample D6 and D16. Twelve phylotypes were observed which its sequences closely related hitherto to uncultured bacteria (7 phylotypes) and known bacteria (5 phylotypes).


A.
Actinobacteria
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

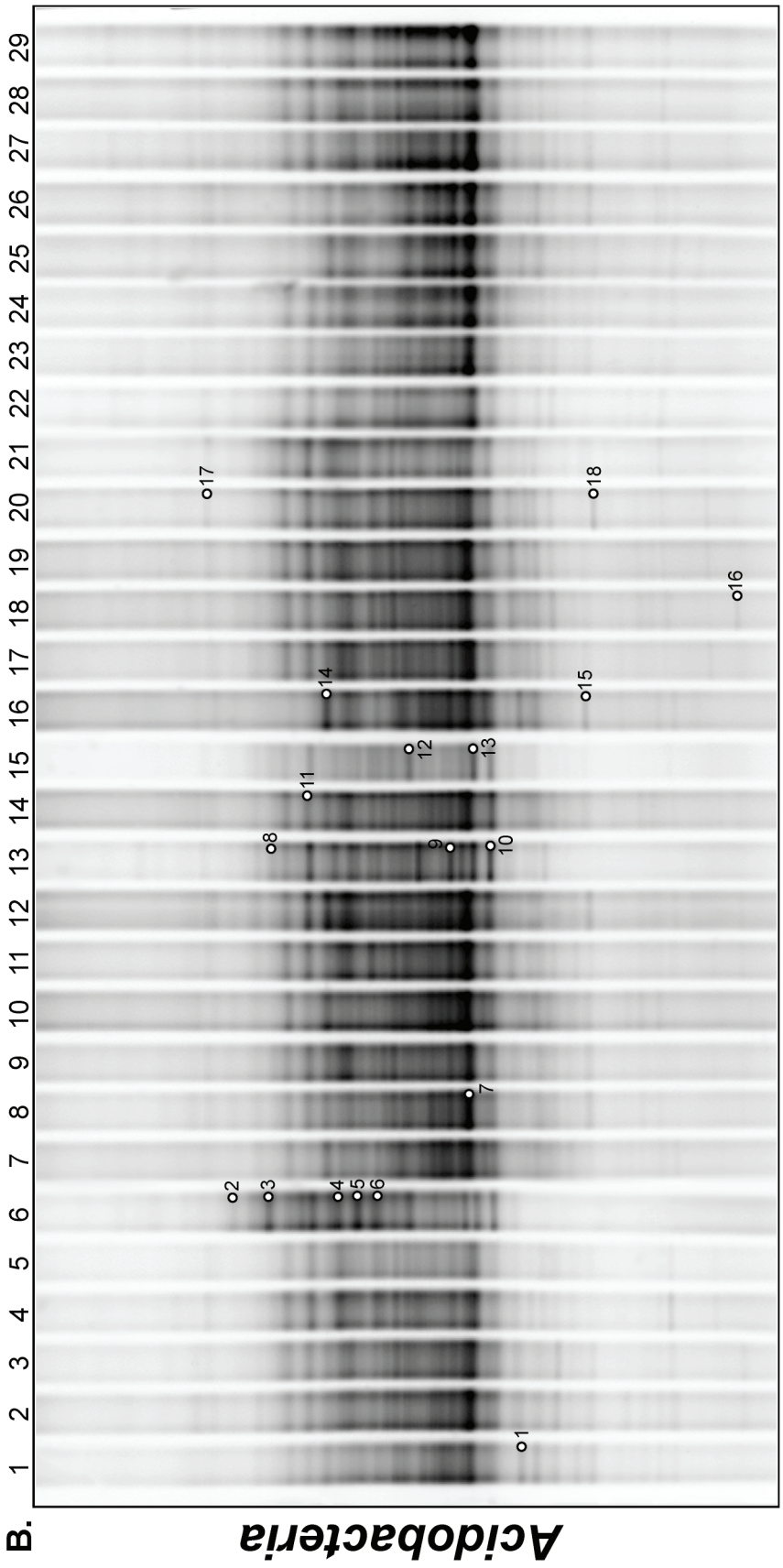


Figure 7. The DGGE analysis of PCR-amplified 16S rRNA gene fragments of **A.** *Actinobacteria* species and **B.** *Acidobacteria* species. Lanes 1 through 29 indicate soil sample designations; see Table 1 for detail information. Circles and Arabic numerals denote the bands, which were excised and sequenced. Negative image of SYBR gold-stained DGGE gels are shown.

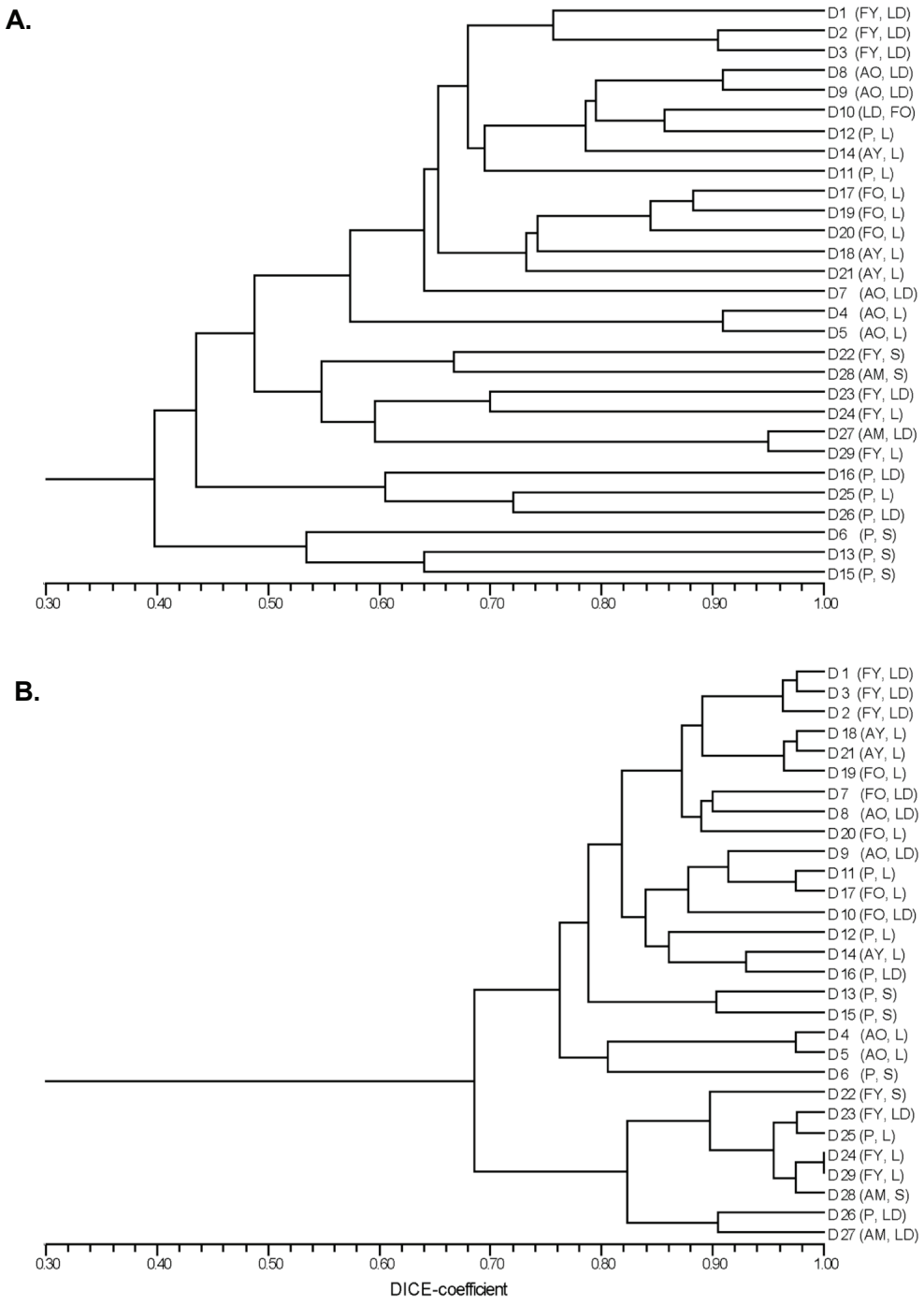


Figure 8. Similarity of the bacterial populations of **A.** Population of *Acidobacteria* species, **B.** Population *Actinobacteria* species established under different combinations of land use and soil properties. The letters in bracket represent sampling site designations as follows: P (pristine), F (fallow), A (acre), O (old), M (medium), and Y (young), LD (loam, dark), L (loam, red or brown), S (sand). The analyses are based on a comparison of the PCR-DGGE fingerprint patterns.

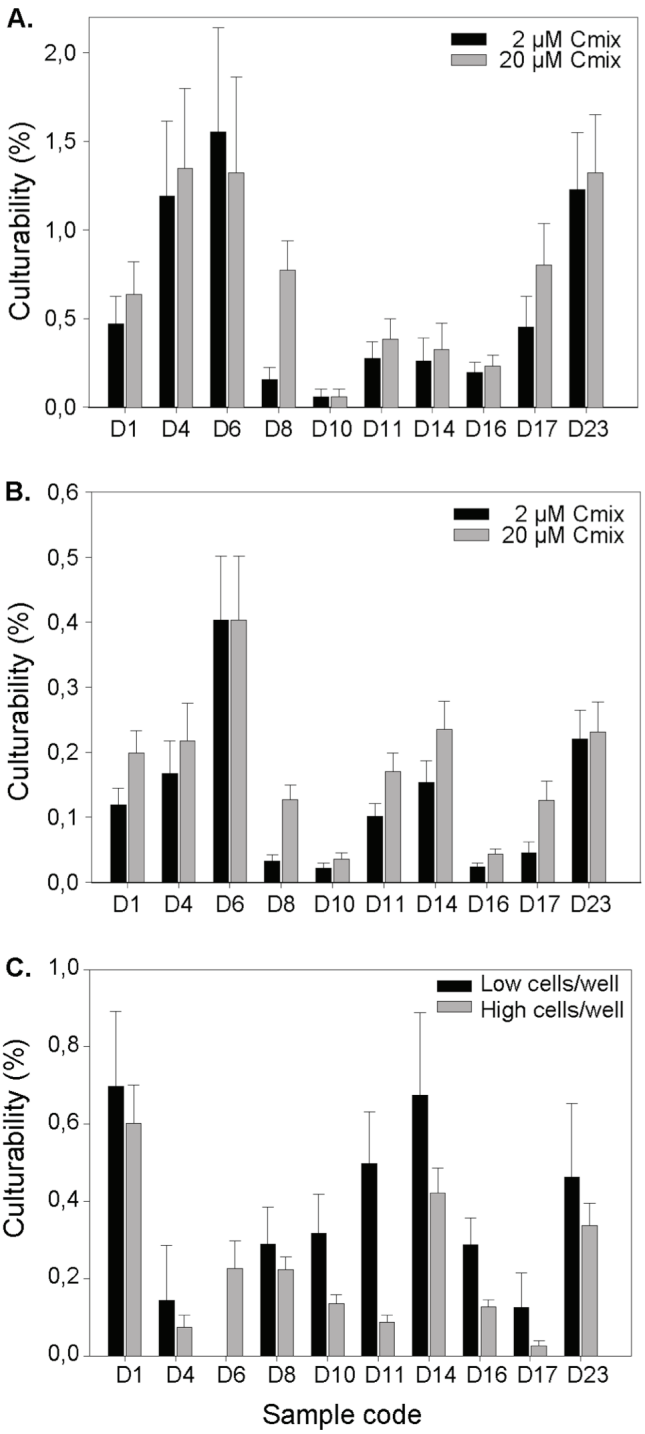


Figure 9. Culturability of the culture collections grown on **A**. SSE+Cmix/small inoculum size, **B**. SSE+Cmix/big inoculum size, **C**. SSE+ARE.

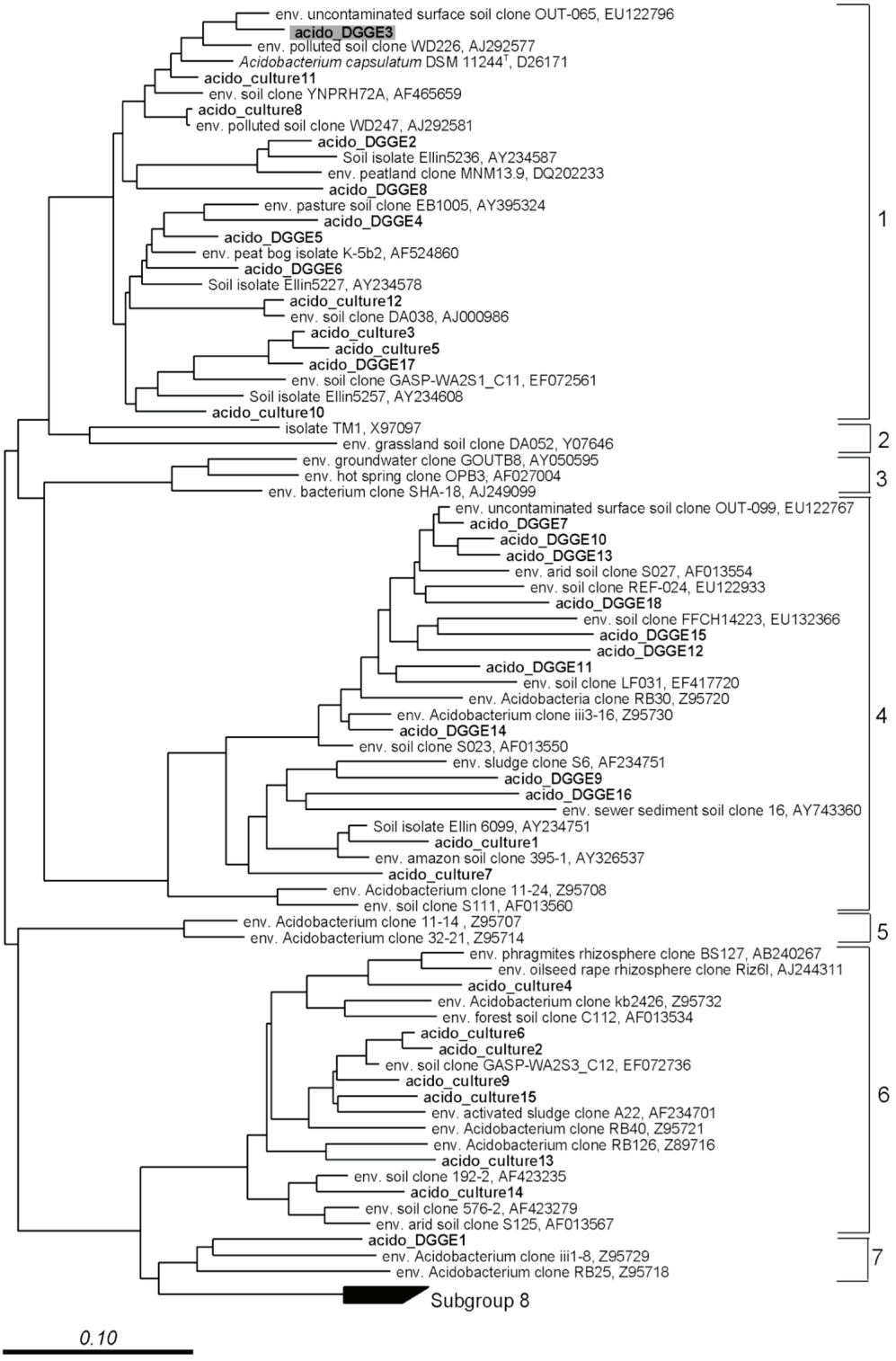


Figure 10. Maximum likelihood phylogenetic analysis of the 16S rRNA gene sequences of *Acidobacteria* species obtained in the present study (shown in bold face). Unique sequence observed is shaded in gray. Arabic numerals to the right of the tree denote established subgroups. Bars represent 0.1 fixed point mutations per nucleotide.

4.5 Discussion

4.5.1 Implications of land use and soil properties on microbial community function

It has been reported that the chemistry and amount of organic matter entering the soil correlate with microbial respiration rates (Schlesinger and Andrews 2000) and an additional dissolved organic matter rapidly stimulates respiration in the soil microcosms (Cleveland *et al.* 2007). A remarkably higher respiration rate in the pristine dark loam soil (D16) may therefore relate to the total cell numbers (Rai and Srivasta 1981) as well as possibly supported by the availability of substrates (Wang *et al.* 2003). The diversity of carbon type in soil may stimulate growth of more diverse and specific bacteria that respond to different carbon type (Padmanabhan *et al.* 2003, Cleveland *et al.* 2007), thus affects the rate of soil respiration. The substrates availability might not be a limiting factor in controlling the respiration rates in the pristine soil as well as in other soils. If it was a case, hence all the pristine soils (D6 and D11) will show similar respiration rates to D16 site. This hypothesis was supported by the fact that the addition of carbon or nitrogen sources into the soil slurries did not enhance the rate of soil respiration. The amount of organic carbon was generally low in most cases that reflect the natural conditions at Mutompo village (Gröngröft *et al.* 2006). Therefore, soil properties that interplay with the total cell numbers may hold a role in controlling the soil respiration as shown by a significant relationship between the respiration rate and the total cell numbers.

On the other hand, the soil respiration in D1, D8, D10, D16, and D23 sites (combinations of dark loam with pristine, old fallow, young fallow or old acre) revealed that the respiration rates in fallow or acre soils were markedly lower than that of in the pristine soil. It indicates that land use effects became apparent in those sites that are on the same soil properties. It has been shown that land use (Frank *et al.* 2006) and burning, thinning or a combination of the two (Concilio *et al*. 2006) affect the magnitude of soil respiration. In contrast, the soil respiration rates in D4 (old acre loam), D11 (pristine loam), D14 (young acre loam), and D17 (old fallow loam) sites only slightly differed among them. Different response of soil respiration rate regarding to the soil properties could be happened because the villagers favour utilizing dark loam soils as cultivated areas, as they are more fertile and have much

longer water holding capacity compared to loam soils (Michael Proepper personal communication). Therefore, dark loam soils (in combination with fallow and acre) may subject to a higher intensity of soil disturbance by overexploitation than loam soils. This could alter soil bulk density and soil structure (Johnson *et al.* 1991), and consequently lead to a decrease in soil respiration rate. The soil respiration results suggest that dark loam soils need longer recovery time than loam soils. The recovery time can vary from a few years to much longer (Fritze *et al.* 1993) depending on the site and type of disturbance.

In contrast to the respiration rate, the exoenzymes activities showed no clear correlation patterns to land use, soil properties, and soil pH. However, there was a trend that exoenzyme activities were higher in the pristine dark loam soil (D16), indicating that this soil is relatively more productive and biologically active than other soils. Higher enzyme activities in the pristine dark loam soil may positively correlate with greater C inputs from plant litter and the total cell numbers. The activities in the pristine sand soil (D6) tended to be lower than other pristine soils (D11, D16). In other soils, most of soil exoenzyme activities correlate to the abundance of bacteria (Taylor *et al.* 2003) and soil properties (Acosta-Martinez *et al.* 2007). The exoenzyme activities (V_{max}) in a few sites were a little bit higher than in oak canopy and open grassland soils (Waldrop and Firestone 2006) and in a subalpine forest ecosystem (Weintraub *et al.* 2007). For example, the activity of betaglucosidase measured in the pristine dark loam and the young acre loam soils were higher than those observed in oak canopy and grassland soils (about 3.9 μmol $[g\ soil]^{-1}\ h^{-1}$ and 1.75 μmol $[g\ soil]^{-1}\ h^{-1}$, respectively) and in subalpine forest (1.3 μmol $[g\ soil]^{-1}\ h^{-1}$). However, the exoenzyme activities from the three studies were within the same range, except for aminopeptidase measured were generally higher than that of in a subalpine forest. On the other hand, the adsorptive affinities of MUF and MCA to the ten main soil samples were lower than that observed to sea sediments (Coolen and Overmann 2000) and the $Km + Sn$ values were higher in several samples for the three exoenzymes than that of sediments (Coolen and Overmann 2000) and an aquatic system (Williams and Jochem 2006).

The exoenzymes betaglucosidase (Sinsabaugh *et al.* 1992) and alkaline phosphatase (Chróst 1991) are inducible by their respective substrate. These enzymes are an important extracellular enzyme involved in organic matter degradation, where glucosidase is responsible for conversion of cellobiose to glucose monomers (Eivazi and Tabatabai 1990) and alkaline phosphatase is responsible for regeneration of inorganic P (Chróst 1991). Therefore, the highest betaglucosidase activity in the young acre loam soil indicates an availability of substrates that may originate from residual of **mahangu** and organic fertilizers. **Mahangu** and organic fertilizers are likely to provide more usable organic compounds for microorganisms. Previous study showed that the activity of betaglucosidase depends on the quality of organic inputs (Eivazi and Tabatabai 1990) and this could explain the variety of betaglucosidase activity among sampling sites. The differences in vegetation cover because of soil management practices (i.e., cultivation and burning) may have had an impact on the quality of organic inputs entering the soil. Alkaline phosphatase activity was remarkably higher in the pristine dark loam soil. It may also be the result of increased microbial activity due to the availability and quality of substrate from plant litter. On the other hand, alkaline phosphatase activity was lower in other sites, even in sites where organic fertilizers added. It has been shown that phosphatase activity was higher in an experimental long-term grassland where no previous application of N fertilizer (Lovell *et al.* 1995) and varies widely due to soil management (Gupta and Germida 1988), fertilizer (Dick *et al.* 1988), tillage (Kandeler *et al.* 1999), and cultivation (Waldrop *et al.* 2000). In contrast to the activity of betaglucosidase and alkaline phosphatase, the activity of aminopeptidase is not induced by its substrate. This present study showed that aminopeptidase activity largely related to the total cell numbers.

4.5.2 Bacterial community composition in different land use and its significance

The effects of land use changes and soil properties on bacterial community composition through 29 selected sites in Mutompo village of the Kavango region were observed by employing PCR-DGGE fingerprinting. A minor shift in the microbial community composition was observed from the DGGE-fingerprint of *Acidobacteria* species. Whereas, the DGGE fingerprint of *Actinobacteria* species

was consistent through all sites, suggesting that this bacterial populations were stable in the community. Generally, the bacterial community composition of *Actinobacteria* and *Acidobacteria* showed inconsistency structure concerning land use, land use age, and soil properties. This affirmation was consistent with cluster analysis results. However, the bacterial community compositions in fallow and acre sites were different from the community in pristine sites. Changing of forest to cultivated area also did not showed consistent effects on the alteration of bacterial community composition as determined by PLFA (Waldrop *et al.* 2000), nevertheless the bacterial community composition was affected by land use, soil properties, and management practices in agricultural and grassland soils (Steenwerth *et al.* 2003).

Differences in bacterial community composition may play a role in controlling microbial activity as the soil respiration and exoenzyme activities results varied across the ten main sampling sites. Microbial community composition strongly correlated to the values of enzyme specific (Waldrop *et al.* 2000) and soil respiration rate (Cleveland *et al.* 2007). Specifically, differences in the community composition of ammonia-oxidizing bacteria (AOB) as affected by land use types were related to potential rates of nitrification (Carney *et al.* 2004).

4.5.3 Bacterial diversity and community structure in pristine soil

Based on richness estimator, the bacterial diversity in the pristine dark loam soil was estimated about of 5600 with very low coverage. The number of estimated phylotypes in this soil was higher than that of in uranium contaminated soil (123 to 250; De Santis *et al.* 2007) and lake sediments (36 -15; Bowman *et al.* 2000). The bacterial diversity in pristine soil accounted for more than 10,000 (Torsvik *et al.* 1998) or even up to millions bacterial types (Gans *et al*. 2005).

Three phyla identified as the most dominant phyla, namely *Acidobacteria, Actinobacteria,* and *Alphaproteobacteria* in the Kavango region soils as shown by the clone library, whereas by use of qPCR approach only *Acidobacteria* and *Actinobacteria* revealed as the most abundant phyla. Member of *Chloroflexi, Firmicutes, Betaproteobacteria, Bacteroidetes,* and *Planctomycetes* were less abundant. There was a discrepancy result between the clone library and qPCR

approaches that could be obtained from bias of PCR (Polz and Cavanaugh 1998) and heterogeneity in ribosomal operon number (Klappenbach *et al.* 2001, Tourova 2003). However, the results fall in the range of published data. It has been reported that *Acidobacteria, Actinobacteria,* and *Alphaproteobacteria* are generally the most abundant in soils, whereas *Firmicutes, Bacteroidetes,* and *Planctomycetes* being less abundant (Janssen *et al.* 2006). The sequences affiliated with *Acidobacteria* have been recovered from diverse environments (Barns *et al.* 1999). This group was identified as the most abundant phylum in various soil types (He *et al.* 2006, Dunbar *et al.* 1999) that consisted of 30% and 50% of the *Bacteria* in terra preta and forest soil of western Amazon, respectively (Kim *et al.* 2007). On other hand, *Proteobacteria* were the most abundant phylum (about 32% of the total number of clones) in agricultural and forest soils (USA) (Upchurch *et al.* 2007). The latter was similar to the clone library results from this present study.

4.5.4 Small inoculum size and low nutrients concentration increase the bacterial culturability

Culturability of soil bacteria was increased by employing the small inoculum size (Davis *et al.* 2005) and a medium consisted of low nutrients concentration (Janssen *et al.* 2002). Similarly, the two also resulted in the increase of culturability of bacteria from the Kavango region soils. Besides that, the culturability values remarkably varied among land use and soil properties. Soil management (Lovell *et al.* 1995), soil particle size (Sessitsch *et al.* 2001) and soil organic carbon content may contribute to differences in numbers of the culturable bacteria.

The low amount of nutrients and organic carbon in the Kavango soils (Gröngröft *et al.* 2006) may have significance for the abundance and culturability of *Acidobacteria* species. By using a medium composed of low nutrients, novel species of *Acidobacteria* were successfully isolated from pasture soil (Janssen *et al.* 2002). It has been shown that the abundance of copiotrophic and oligotrophic bacteria in soil is regulated by the abundance of labile C substrates (Fierer *et al.* 2007, Cleveland *et al.* 2007, Bernard *et al.* 2007). A study of Fierer *et al.* (2007) showed that oligotrophic group such as *Acidobacteria* were most dominant in low C condition, but copiotrophic bacteria such as *Proteobacteria* and *Bacteroidetes* increased in

relative abundance in high C soils. In line with this, *Betaproteobacteria* and *Gammaproteobacteria* species were stimulated in soil microcosms enriched with wheat-residue as determined by stable isotope probing approach (Bernard *et al.* 2007), and *Gammaproteobacteria* and *Firmicutes* species were found to be abundant as the increased of dissolved organic matter (Cleveland *et al.* 2007). This explains why no *Acidobacteria* species were detected on medium supplemented with artificial root exudates (SSE+ARE), a rich medium.

Taken together, soil properties may play a major role in controlling the diversity and activity of bacteria, whereas land use showed inconsistent effects on the diversity and activity of bacteria. Microbial activity showed a high sensitivity as soil biological parameter to anthropogenic disturbances than microbial community composition.

4.6 Acknowledgements

Alexander Gröngröft and his team (Institute for Soil Science, University of Hamburg) are gratefully acknowledged for assisting and support during collection of the soil samples. Thomas Hurek, Barbara Reinhold-Hurek (University of Bremen) and Michael Hannig (MPI for Terrestrial Microbiology, Marburg) are thanked for soil samples delivery from Namibia to München, Germany. Michael Proepper and staff of the Mile 46 Livestock Development Centre are thanked for providing the laboratory space and electricity. Susanne Mayer is thanked for help with qPCR. The government of the Republic of Namibia and landowner are appreciated for their permission to conduct the study in the Kavango region (Reference No. 14/11, No. 23001). This work was supported by BMBF (Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie) grant no. BIOLOG/01LC00621C to Jörg Overmann.

4.7 References

1. **Acosta-Martinez, V., L. Cruz, D. Sotomayor-Ramirez, and L. Pérez-Alegria.** 2007. Enzyme activities as affected by soil properties and land use in a tropical watershed. Appl. Soil Ecol. **35**:35-45.

2. **Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, W. Miller, and D.J. Lipman**. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389-3402.

3. **Angle, J.S., S.P. McGrath, and R.L. Chaney**. 1991. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. Appl. Environ. Microbiol. **57**: 3674-3676.

4. **Bandick, A.K., and R.P. Dick.** 1999. Field management effects on soil enzyme activities. Soil Biol. Biochem. **31**:1471-1479.

5. **Barnard, P.** (Ed). 1998. Biological diversity in Namibia - a country study. Namibian National Biodiversity Task Force, Windhoek, Namibia.

6. **Bernard, L., C. Mougel, P.A. Maron, V. Nowak, J. Lévêque, C. Henault, F. Z. Haichar, O. Berge, C. Marol, J. Balesdent, F. Gibiat, P. Lemanceau, and L. Ranjard.** 2007. Dynamics and identification of soil microbial populations actively assimilating carbon from ^{13}C-labelled wheat residue as estimated by DNA- and RNA-SIP techniques. Environ. Microbiol. **9**:752-764.

7. **Barns, S.M., S.L. Takala, and C.L. Kuske.** 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Appl. Environ. Microbiol. **65**:1731-1737.

8. **Blackwood, C.B., A. Oaks, and J.S. Buyer.** 2005. Phylum-and Class-specific PCR primers for general microbial community analysis. Appl. Environ. Microbiol. **71**:61913-6198.

9. **Bowman, J.P., S.A. McCammon, S.M. Rea, and T.A. McMeekin.** 2000. The microbial composition of three limnologically disparate hypersaline Antarctic lakes. FEMS Microbiol. Lett. **183**:81-88.

10. **Bruns, A., H. Hoffelner, and J. Overmann**. 2003. A novel approach for high throughput cultivation assays and the isolation of planktonic bacteria. FEMS Microbiol. Ecol. **45**:161-171.

11. **Buckley, D.H. and T.M. Schmidt.** 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microb. Ecol. **42**:11-21.

12. **Carney, K.M., P.A. Matson, and B.J.M. Bohannan**. 2004. Diversity and composition of tropical soil nitrifiers across a plant diversity gradient and among land-use types. Ecol. Lett. **7**:684-694.

13. **Chao, A.** 1984. Non-parametric estimation of the number of classes in a population. Scand. J. Stat. **11**:265-270.

14. **Chao, A., and A.M. Lee.** 1992. Estimating the number of classes via sample coverage. J. Am. Stat. Assoc. **87**:210-217.

15. **Chróst, R.J.** 1991. Environmental control of the synthesis and activity of aquatic microbial ectoenzymes, p. 29-59. *In* R.J. Chróst (ed.), Microbial enzymes in aquatic environments. Springer, New York, NY.

16. **Cleveland, C.C., A.R. Townsend, S.K. Schmidt, and B.C. Constance.** 2003. Soil microbial dynamics and biogeochemistry forests and pastures, southwestern Costa Rica. Ecol. Appl. **13**:314-326.

17. **Cleveland, C.C., D.R. Nemergut, S.K. Schmidt, and A.R. Townsend.** 2007. Increases in soil respiration following labile carbon additions linked to rapid shifts in soil microbial community composition. Biogeochemistry **82**:229-240.

18. **Concilio, A., S. Ma., S.-R. Ryu, M. North, and J. Chen.** 2006. Soil respiration response to experimental disturbances over 3 years. For. Ecol. Manage. **228**:82-90.

19. **Coolen, M.J.L. and J. Overmann.** 2000. Functional exoenzymes as indicators of metabolically active bacteria in 124,000-year-old sapropel layers of the Eastern Mediterranean Sea. Appl. Environ. Microbiol. **66**:2589-2598.

20. **Daims, H., A. Brühl, R. Amann, K.H. Schleifer, and M. Wagner**. 1999. The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22**:434-444.

21. **Davis, K.E.R., S.J. Joseph, and P.H. Janssen**. 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl. Environ. Microbiol. **71**:826-834.

22. **De Santis, T.Z., E.L. Brodie, J.P. Moberg, I.X. Zubieta, Y.M. Piceno, and G.L. Adersen.** 2007. High-diversity universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb. Ecol. **53**:371-383.

23. **Dick, R.P., P.E. Rasmussen, and E.A. Kerle**. 1988. Influence of long-term residue management on soil enzyme activities in relation to soil chemical properties of a wheat-fallow system. Biol. Fertil. Soils **6**:159-164.

24. **Dunbar, J., S. Takala, S.M. Barns, J.A. Davis, and C.R. Kuske**. 1999. Levels of bacterial diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. **65**:1662-1669.

25. **Eivazi, F, and M.A. Tabatabai**. 1990. Factors affecting glucosidase and galactosidase activities in soils. Soil Biol. Biochem. **22**:891-897.

26. **Frank, A.B., M.A. Liebig, and D.L. Tanaka.** 2006. Management effects on soil CO_2 efflux in northern semiarid grassland and cropland. Soil Till. Res. **89**:78-85.

27. **Fraterrigo, J.M., T.C. Balser, and M.G. Turner.** 2006. Microbial community variation and its relationship with nitrogen mineralization in historically altered forests. Ecology **87**:570-579.

28. **Fierer, N., J.A. Jackson, R. Vilgalys, and R.B. Jackson.** 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl. Environ. Microbiol. **71**:4117-4120.

29. **Fierer, N., M.A. Bradford, and R.B. Jackson.** 2007. Toward an ecological classification of soil bacteria. Ecology **88**:1354-1364.

30. **Fritze, H., T. Pennanen, and J. Pietikäinen.** 1993. Recovery of soil microbial biomass and activity from prescribed burning. Can. J. For. Res. **23**:1286-1290.

31. **Gans, J., M. Wolinsky, and J. Dunbar.** 2005. Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science **309:**1387-1390.

32. **Gich, F., J. Garcia-Gil, and J. Overmann**. 2001. Previously unknown and phylogenetically diverse members of green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. **177**:1-10.

33. **Girvan, M.S., J. Bullimore, J.N. Pretty, A.M. Osborn, and A.S. Ball.** 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl. Environ. Microbiol. **69**:1800-1809.

34. **Gupta, V.V.S.R., and J.J. Germida**. 1988. Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation. Soil Biol. Biochem. **20:**777-786.

35. **Gröngroft, A., N. Herpel, A. Petersen, and A. Mills.** 2006. Indication of soil degradation processes in selected drylands of Southern Africa. Proceedings of the International Conference: Soil and Desertification – Integrated Research for the Sustainable Management of Soils in Drylands, 5-6 May 2006, Hamburg, Germany.

36. **He, J., Z. Xu, and J. Hughes**. 2006. Molecular bacterial diversity of a forest soil under residue management regimes in subtropical Australia. FEMS Microbiol. Ecol. **55**:38-47.

37. **Janssen, P.H., P.S. Yates, B.E. Grinton, P.M. Taylor, and M. Sait.** 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria, Actinobacteria, Proteobacteria,* and *Verrucomicrobia*. Appl. Environ. Microbiol. **68**:2391-2396.

38. **Janssen, P.H.** 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. **72**:1719-1728.

39. **Jaspers, E., K. Nauhaus, H. Cypionka, and J. Overmann**. 2001. Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. FEMS Microbiol. Ecol. **36**:153-164.

40. **Johnson, C.E., A.H. Johnson, T.G. Huntington, and T.G. Siccama.** 1991. Whole-tree clear-cutting effects on soil horizons and organic matters pool. Soil Sci. Soc. Ame. J. **55**:497-502.

41. **Jones, B. and D. Cownie.** 2001. Socio-ecological survey report for the Kavango region. Every River Has Its People project. Windhoek, Namibia.

42. **Kandeler, E., D. Tscherko, and H. Spiegel.** 1999. Long-term monitoring of microbial biomass, N mineralization and enzyme activities of a Chernozem under different tillage management. Biol. Fertil. Soils **28**:343-351.

43. **Kim, J.-S., G. Sparovek, R.M. Longo, W.J. De Melo, and D. Crowley**. 2007. Bacterial diversity of terra preta and pristine forest soil from the Western Amazon. Soil Biol. Biochem. **39**:684-690.

44. **Klappembach, J.A, P.R. Saxman, J.R. Cole, and T.M. Schmidt**. 2001. rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. **29**:181-184.

45. **Kozdrój, J., and J.D. van Elsas**. 2000. Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. Soil Biol. Biochem. **32**:1405-1417.

46. **Lane, D.J.** 1991. 16S/23S rRNA sequencing, p.115-175. *In* E. Stackebrandt & M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley, Chichester, United Kingdom.

47. **Lovell, R.D., S.C. Jarvis, and R.D. Bardgett.** 1995. Soil microbial biomass and activity in long-term grassland: effects of management changes. Soil Biol. Biochem. **27**:969-975.

48. **Lüdemann, H., and R. Conrad**. 2000. Molecular retrieval of large 16S rRNA gene fragments from an Italian rice paddy soil affiliated with the class *Actinobacteria*. Syst. Appl. Microbiol. **23**:582-584.

49. **Lunau, M., A. Lemke, K. Walther, W. Martens-Habbena, and M. Simon.** 2005. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. Environ. Microbiol. **7**: 961-968.

50. **Maidak, B.L., J.R. Cole, T.G. Lilburn, C.T. Parker, P.R. Saxman, R.J. Farris, G.M. Garrity, G.J. Olsen, T.M. Schmidt and J.M. Tiedje.** 2001. The RDP-II (Ribosomal Database Project). Nucleic. Acids. Res. **29**: 173-174.

51. **Meier, H., R. Amann, W. Ludwig, and K.H. Schleifer**. 1999. Specific oligonucleotide probes for *in situ* detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. **22**:186-196.

52. **Muyzer, G., E.C. de Waal, and A.G. Uitterlinden**. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695-700.

53. **Muyzer, G., and N.B. Ramsing**. 1995. Molecular methods to study the organization of microbial communities. Water Science and Technology **32**:1–9.

54. **Neef, A**. 1997. Anwendung der *in situ*-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Ph.D. thesis. Technische Universität München, Munich, Germany.

55. **Obeid, S.E. and J. Mendelsohn**. 2001. A Preliminary profile of the Kavango region in Namibia. Every River Has Its People project. Windhoek, Namibia.

56. **Overmann, J., M.J.L. Coolen, and C. Tuschak**. 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch. Microbiol. **172**:83-94.

57. **Padmanabhan, P., S. Padmanabhan, C. DeRito, A. Gray, D. Gannon, J.R. Snape, C.S. Tsai, W. Park, C. Jeon, and E.L. Madsen,** 2003. Respiration of ^{13}C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of ^{13}C-labeled soil DNA. Appl. Environ. Microbiol. **69**:1614-1622.

58. **Polz, M.F., and C.M. Cavanaugh.** 1998. Bias in template-to-product in multitempalte PCR. Appl. Environ. Microbiol. **64**:3724-3730.

59. **Rai, B. and A.K. Srivasta**. 1981. Studies on microbial population of a tropical dry deciduous forest in relation to soil respiration. Pedobiol. **22**:185-190.

60. **Ritalahti, K.M., B.K. Amos, Y. Sung, Q. Wu, S.S. Koenigsberg, and F.E. Löffler.** 2006. Quantitative PCR targeting 16S rRNA and reductive dehalogenase

genes simultaneously monitors multiple *Dehalococcoides* strains. Appl. Environ. Microbiol. **72**:2765-2774.

61. **Sangwan, P., S. Kovac, K.E.R. Davis, M. Sait, and P.H. Janssen**. 2005. Detection and cultivation of soil *Verrucomicrobia*. Appl. Environ. Microbiol. **71**:8402-8410.

62. **Schlesinger, W.H., and J.A. Andrews**. 2000. Soil respiration and the global carbon cycle. Biogeochemistry **48**:7-20.

63. **Sessittsch, A., A. Weilharter, M.H. Gerzabek, H. Kirchmann, and E. Kandeler**. 2001. Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. Appl. Environ. Microbiol. **67**:4215-4224.

64. **Simberloff, D.S.** 1972. Properties of the rarefaction diversity measurement. Am. Natur. **106.**

65. **Sinsabaugh, R.I., R.K. Antibus, A.E. Linkins, C.A. McClaugherty, L. Rayburn, D. Repert, and T. Weiland.** 1992. Wood decomposition over a first-order watershed: mass loss as a function of lignocellulase activity. Soil Biol. Biochem. **24**:743-749.

66. **Steenwerth, K.L., L.E. Jackson, F.J. Calderón, M.R. Stromberg, and K.M. Scow.** 2003. Soil microbial community composition and land use history in cultivated and grassland communities of coastal California. Soil Biol. Biochem. **35**:489-500.

67. **Strohbach, B.J. and A. Petersen.** 2007. Vegetation of the central Kavango woodlands in Namibia: an example from the Mile 46 livestock development centre. S. Afr. J. Bot. **73**:391-401.

68. **Taylor, J.P., B. Wilson, M.S. Mills, and R.G. Burns**. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. Soil Biol. Biochem. **34**:387-401.

69. **Torsvik, V, F.D. Daae, R.-A. Sandaa, and L. Øvreas**. 1998. Novel techniques for analyzing diversity in natural and perturbed environments. J. Biotech. **64**:53-62.

70. **Tourova, T.P**. 2003. Copy number of ribosomal operons in prokaryotes and its effect on phylogenetic analyses. Microbiology **72**:389-402.

71. **Uhlmann, E., C. Görke, A. Petersen, and F. Oberwinkler.** 2004. Arbuscular mycorrhizae from semiarid regions of Namibia. Can. J. Bot. **82**:645-653.

72. **Upchurch, R, C. –Y. Chiu, K. Everett, K. Dyszynski, D.C. Coleman, and W.B. Whitman.** Differences in the composition and diversity of bacterial communities from agricultural and forest soils. Soil Biol. Biochem. (2007), doi:10.1016/j.soilbio.2007.06.027.

73. **Waldrop, M.P., T.C. Balser, and M.K. Firestone**. 2000. Linking microbial community composition to function in a tropical soil. Soil Biol. Biochem. **32**: 1837-1846.

74. **Waldrop, M.P. and M.K. Firestone**. 2006. Seasonal dynamics of microbial community composition and function in oak canopy and open grassland soils. Microb. Ecol. **52**:470-479.

75. **Wang, W.J., R.C. Dalal, P.W. Moody, and C.J. Smith**. 2003. Relationships of soil respiration to microbial biomass, substrate availability and clay content. Soil Biol. Biochem. **35**:273-284.

76. **Ward, D., B.T. Ngairorue, J. Kathena, R. Samuels, and Y. Ofran.** 1998. Land degradation is not a necessary outcome of communal pastoralism in arid Namibia. J. Arid Environ. **40**:357-371.

77. **Weintraub, M.N., L.E. Scott-Denton, S.K. Schmidt, and R.K. Monson**. 2007. The effects of tree rhizodeposition on soil exoenzyme activity, dissolved organic carbon, and nutrient availability in a subalpine forest ecosystem. Oecologia **15**:327-338.

78. **Williams, C.J., and F.J. Jochem**. 2006. Ectoenzyme kinetics in Florida Bay: implications for bacterial carbon source and nutrient status. Hydrobiologia **569**:113-127.

79. **Zeidler, J., S. Hanrahan, and M. Scholes.** 2002. Land-use intensity affects range condition in arid to semi-arid Namibia. J. Arid Environ. **52**:389-403.

80. **Zul D., S. Denzel, A. Kotz, and J. Overmann.** 2007. Effects of plant biomass, plant diversity, and water content on bacterial communities in soil lysimeters: implications for the determinants of bacterial diversity. Appl. Environ. Microbiol. **73**:6916-6929.

CHAPTER 5
Discussion

5.1 Bacterial diversity in manipulated and natural soils

In this study, bacterial diversity in manipulated and natural soils was examined in detail by employing culture-independent and culture-dependent approaches. A combination of the two provides not only a possibility to quantify bacterial diversity in natural environment but also a reasonable understanding of the interaction between the microorganisms and their natural environment.

The culture-independent analysis of manipulated soil (lysimeters soil) revealed that the *Chloroflexi* and *Verrucomicrobia* species represented the most diverse of the 10 groups investigated, followed by the *Actinobacteria* and *Planctomycetes* species (Chapter 2). In contrast, small fractions of the *Chloroflexi* and *Verrucomicrobia* species were identified in other soils (Janssen 2006). The sequences recovered from four of the target groups (*Chloroflexi*, *Acidobacteria*, *Verrucomicrobia* and *Planctomycetes* species), were only distantly related to any of the sequences available in the databases. Many phylotypes identified are members of bacterial clades originating from the soil environment. A significant fraction of the indigenous bacterial community obviously remains to be discovered, although different grassland bacterial communities have been studied extensively over the past decade (Barns *et al.* 1999, Buckley and Schmidt 2001, Costello and Schmidt 2006, Janssen *et al.* 2002, Kuske *et al.* 2002, Lüdemann and Conrad 2000, and Smalla *et al.* 2001). Only 16 phylotypes of *Archaea* species were recovered by PCR-DGGE, indicating a low diversity of this group in soil. These results correspond to the low diversity of *Archaea* species in various soils of Norway and Indiana (Nakatsu *et al.* 2000).

The bacterial diversity in the pristine dark loam soil was estimated to amount to 5600 and hence surpasses that of lake sediments (Bowman *et al.* 2000) and uranium contaminated soil (De Santis *et al.* 2007). The bacterial diversity in another pristine soil accounted for more than 10,000 species (Torsvik *et al.* 1998). Three phyla identified as the most dominant phyla, namely *Acidobacteria, Actinobacteria,*

and *Alphaproteobacteria* in this soil as shown by the clone library, whereas by use of qPCR only *Acidobacteria* and *Actinobacteria* were revealed as the most abundant phyla. Member of *Chloroflexi, Firmicutes, Betaproteobacteria, Bacteroidetes,* and *Planctomycetes* were less abundant. Even though a discrepancy was observed between the clone library and qPCR approaches, the results fall in the range of published data. *Acidobacteria, Actinobacteria,* and *Alphaproteobacteria* are generally the most abundant in soils, whereas *Firmicutes, Bacteroidetes,* and *Planctomycetes* are less abundant (Janssen *et al.* 2006). *Acidobacteria* were identified as the most abundant phylum in various soil types (He *et al.* 2006, Dunbar *et al.* 1999) that consisted of 30% and 50% of the *Bacteria* in terra preta and forest soil of western Amazon, respectively (Kim *et al.* 2007). On the other hand, *Proteobacteria* represented the most abundant phylum (about 32% of the total number of clones) in agricultural and forest soils (USA) (Upchurch *et al.* 2007).

5.2 Factors determining the bacterial diversity

5.2.1 Plant species and plant diversity

Previous studies showed inconsistent effects of plant species and plant diversity on the bacterial community composition in bulk soil. A correlation between the species composition of plants and the bacterial diversity in soils did not establish (Kowalchuk *et al.* 2002). Other studies showed that plant species composition had little direct effect on the bacterial community composition (Kennedy *et al.* 2004, Nunan *et al.* 2005). In contrast to no effect of the plant community composition on the relative abundances of bacterial phyla in soil of a Michigan long-term ecological research site (Buckley and Schmidt 2001) and a Dutch grassland soil (Felske *et al.* 2000), the diversity of bacterial community showed a correlation to the plant diversity in northwestern Switzerland grassland soils (Grüter *et al.* 2006). The relationship between the overall diversity of soil bacteria and the diversity of aboveground plant communities also does not observe in this study, indicating the interdependence between the absence or presence of plants and the abundance of various bacteria in lysimeters shows rather low specificity.

In the current study, however, 20 out of the 160 sequence types analyzed were found at increased abundance in unplanted lysimeters. In contrast, the abundance of 32 phylotypes coincided with the presence of higher plant species in the lysimeters. Of these 32 phylotypes, only very few were closely related to known rhizosphere bacteria. Changes in the composition were observed for six different bacterial (sub)phyla. Furthermore, the analyses of this study were limited to bulk soil. Based on the fact that about one-third of the bacterial sequence types in bulk soil were found to correlate with the absence or presence of plants, the influence of plants must extend significantly beyond the rhizosphere and must be of relevance to many different and previously unknown types of soil bacteria. These results are in contrast to observations in other grassland systems where plant rhizosphere effects were of little significance in the composition of total community structures (Kennedy *et al.* 2004).

5.2.2 Water content

Previous studies did not yield consistent results with respect to the effect of water regime on the diversity of bacteria in soil. Similarly, no effect of water content on the bacterial community composition was observed in the present study. The bacterial communities in grassland soil showed no obvious changes in the diversity in dried, rewetted, or dried and rewetted regimes, whereas the physiological state of cultured bacteria changed (Griffiths *et al.* 2003). Another study found that soil water content is a major determinant of microbial community composition in microcosm of soil as revealed by phospholipids fatty acid (PLFA) pattern (Drenovsky *et al.* 2004).

5.2.3 Land use and soil properties

Land use, soil properties, and management practices affect the bacterial community composition in agricultural and grassland soils (Steenwerth *et al.* 2003). In contrast, the community composition of *Actinobacteria* and *Acidobacteria* species in the Kavango region soils (natural soil) did not show clear correlations with the effects of land use, land use age, and soil properties. A minor shift in the bacterial community composition was observed from the DGGE-fingerprint of *Acidobacteria* species. Whereas the DGGE fingerprint of *Actinobacteria* species was consistent through all

sites, suggesting that this bacterial populations were stable in the community. However, the bacterial community compositions in fallow and acre sites were different from the community in pristine sites. This present study results in agreement with previous studies where changing of forest to cultivated area also did not showed consistent effects on the alteration of bacterial community composition as determined by PLFA (Waldrop *et al.* 2000).

5.3 Implications of land use and soil properties on microbial activity

Microbial activity in soil can be monitored, for example, through quantifying soil exoenzyme activity and soil respiration. The activity of soil enzyme consists of activities associated with different soil constituents (e.g., active cells, cell debris, clay and humic colloids) that are produced by soil bacteria, fungi, protozoa and plant root (Burns 1982). Similarly, soil respiration represents the combined respiration of roots (including rhizosphere and associated mycorrhizal fungi), soil micro- and macroorganisms (Rustad *et al.* 2000).

In this study, microbial activity was monitored by measuring soil respiration rate and soil exoenzyme activities to asses the sensitivity of semiarid soils toward land use (as anthropogenic disturbances) and soil properties. The two parameters varied among sampling sites (Chapter 4). In general, the pristine dark loam soil showed higher soil respiration rate than other sites, which could be related to the total cell numbers (Rai and Srivasta 1981) and the availability of substrates (Wang *et al.* 2003). The diversity of carbon type in soil may stimulate growth of more diverse and specific bacteria (Padmanabhan *et al.* 2003, Cleveland *et al.* 2007) and consequently affects the microbial activity. The effects of land use (Frank *et al.* 2006) on the soil respiration obviously appeared in the dark loam soil. This could be the result of overexploitation of cultivated areas as compared to that of in the loam soil (Michael Proepper, personal communication).

Soil exoenzyme activities varied among sampling site, but did not show clear correlation with land use and soil properties. However, the pristine dark loam soil also showed the highest exoenzyme activities, suggesting that this soil is relatively more productive and biologically active than other soils. In contrast, the activities in

the pristine sand soil tended to be lower than other pristine soils. Higher enzyme activities in the pristine dark loam soil may positively correlate with greater C inputs from plant litter and the total cell numbers. In other soils, most of soil exoenzyme activities correlate to the abundance of bacteria (Taylor *et al.* 2003), soil properties (Acosta-Martinez *et al.* 2007), and carbon organic inputs (Eivazi and Tabatabai 1990).

The exoenzymes betaglucosidase (Sinsabaugh *et al.* 1992) and alkaline phosphatase (Chróst 1991) are inducible by their respective substrate. Therefore, the highest betaglucosidase activity observed in young acre loam indicates an availability of more usable substrates that may originate from residual of **mahangu** and organic fertilizers. Alkaline phosphatase activity was remarkably higher in the pristine dark loam site. It may also be the result of increased microbial activity due to the availability and quality of substrate from plant litter. On the other hand, alkaline phosphatase activity was lower in other sites, even in sites where organic fertilizers added. Alkaline phosphatase activity varies widely due to soil management (Gupta and Germida 1988) and fertilizer (Lovell *et al.* 1995). In contrast to the activity of betaglucosidase and alkaline, the activity of aminopeptidase largely related to the total cell numbers.

No general relationships between exoenzymes activity, land use changes, and land use age were determined, indicating interactions of anthropogenic disturbances and environmental factors involved in the present study sites are very complex. However, the lower enzyme activities in most fallow and acre sites, which reflect the low rate of decomposition process, may be the result of soil physical and chemical changes through soil management practices and overexploitation. Soil management practices i.e., ploughing may alter soil structure, increase the losses of organic matter, affect the microbial activity, and consequently decrease the soil enzyme activities (Gupta and Germida 1998). In addition, the differences in vegetation cover because of soil management practices (i.e., cultivation and burning) may have had an impact on the quality of organic inputs entering the soil.

5.4 Cultivation of soil bacteria: factors determining of bacterial culturability and phylotypes analysis

By modifications the traditional culturing techniques or employing high throughput methods, culturability of soil bacteria were found to be increased and novel bacteria were successfully captured from different natural environments (Tamaki *et al.* 2005, Janssen *et al.* 2002, Sait *et al.* 2002, Bruns *et al.* 2003b, Zengler *et al.* 2002). In this study, cultivation was done by employing the MicroDrop dispenser system (Bruns *et al.* 2003b) and inoculating liquid cultures with aliquots of the soil suspensions manually. The cultivation was performed by modifying media composition with rich (Kozdrój and van Elsas 2000) and low nutrients concentration (Janssen *et al.* 2002), additional signaling compounds (Bruns *et al.* 2003a), and low inoculum size. In total, 217 and 735 bacterial cultures were obtained from lysimeter soils (manipulated soil) and semiarid soils (natural soil), respectively. The culturability values varied among not only the treatments but also concerning the presence or absence of plant, land use, and soil properties (Chapter 2 and 4). For example, by employing rich medium (SSE+ARE), 3 phylotypes *Acidobacteria* were obtained from manipulated soil (Chapter 2) and none of them were detected on that medium from natural soils (Chapter 4). In contrast, 15 phylotypes of *Acidobacteria* were detected growing on medium with low nutrients concentration (SSE+Cmix) isolated from natural soils. As oligotrophic bacteria, *Acidobacteria* were most abundant in low C environment (Fierer *et al.* 2007), while copiotrophic bacteria such as *Proteobacteria* and *Firmicutes* were found to be abundant in high C conditions (Cleveland *et al.* 2007, Bernard *et al.* 2007). The natural conditions of the Kavango region that has low amounts of nutrients and organic carbon content (Gröngröft *et al.* 2006) may significantly correlate with the abundance and culturability of *Acidobacteria* species. Besides the amount of organic carbon, the individual plant species or functional group (Stephan et al. 2000), soil management (Lovell *et al.* 1995), and soil particle size (Sessitsch *et al.* 2001) may contribute to differences in numbers of the culturable bacteria.

Analyses of the 16S rRNA gene sequences of the 217 bacterial cultures from manipulated soil revealed that about 3% represented environmental phylotypes

detected by the culture-independent approach. In several previous studies, none of the environmental phylotypes could be recovered by cultivation (Dunbar *et al.* 1999, Lipson and Schmidt 2004). However, sequences of two phylotypes cultured did match sequences detected in the natural bacterial community. One of them, phylotype beta 10, displayed a distinct response towards the presence of higher plant species. In contrast, none of sequences of the 735 bacterial cultures from the natural soil matched by environmental phylotypes.

5.5 A novel betaproteobacterium isolated from manipulated soil

The betaproteobacterial strain byr23-80 was successfully isolated from soil lysimeters. Its sequence was similar to phylotype beta10 sequence (Chapter 2) and affiliated with uncultured bacteria, so far. This phylotype constituted only a very small fraction (0.017 to 0.18% of total cell numbers) of the soil microbial community in the lysimeter soils as quantified by qPCR. The occurrence and response of this phylotype followed a reproducible pattern in independent lysimeters. Because of its unique characteristic, strain byr23-80 was characterized in detail (Chapter 3). Physiological, biochemical and phylogenetic characterization revealed that the isolate represented a novel member of the genus *Massilia* within the family *Oxalobacteraceae* of the order *Burkholderiales*. Members of this genus can be found in diverse environments (Zhang *et al*. 2006, Gallego *et al*. 2006, La Scola *et al*. 1998, Lindquist *et al*. 2003) and are capable to degrade phenanthrene (Bodour *et al.* 2003) or other aromatic compounds (Khammar *et al.* 2005).

Several characteristics for distinguishing strain byr 23-80 from other members of described *Massilia* species were identified. For example, the temperature range of strain byr23-80 is comparable to that of *Massilia aurea*, but the optimum temperature of growth (15°C) is unusual in that it is significantly lower than that of any other described *Massilia* species. In contrast to all other *Massilia* species tested, strain byr23-80 did not exhibit α-galactosidase and β-galactosidase activity as determined by employing API ZYM galleries (API systems; bioMérieux). This strain did not hydrolyze gelatin that distinguished the novel isolate from all described species of the genus *Massilia*. On the other hand, the ability of this strain to utilize eight of the

21 (Kozdrój and van Elsas 2000) major constituents of root exudates indicates its interdependence with plant. It has been shown that members of the genus *Massilia* were active in soil and rapidly respired glucose, but not phenol, naphthalene or caffeine (Padmanabhan *et al.* 2003).

Based on the phylogenetic analysis, strain byr23-80 closely related to *Massilia aurea* DSM5502^T with a similarity value of 98.3 %. The 16S rRNA gene sequence divergence between strain byr23-80 and *Massilia aurea* DSM5502^T is in the range commonly used as a criterion for the separation of two bacterial species (Stackebrandt and Goebel 1994). Based on the DNA-DNA hybridization results, strain byr23-80 has to be considered as a novel species as the similarity value between the strain byr23-80 and *M. aurea* was 20.1%. This value was lower than the threshold value of 70% that is commonly accepted for the definition of bacterial species (Wayne *et al.* 1987). Based on the phenotypic and genotypic data presented, strain byr23-80 therefore represents a novel species within the genus *Massilia*, the subclass *Betaproteobacterium* (Chapter 3).

The results of this study suggest that the bacterial species composition in soil is determined to a significant extent by abiotic and biotic factors rather than mere chance. In conclusion, the high diversity of soil bacterial communities could be (i) due to a multitude of ecological niches and adaptive mechanisms (Dykhuizen 1998, Jaspers and Overmann 2004) and/or (ii) caused by high functional redundancy (Griffiths *et al.* 2001, Wertz 2006) of the soil bacteria.

5.6 References

1. **Acosta-Martinez, V., L. Cruz, D. Sotomayor-Ramirez, and L. Pérez-Alegria.** 2007. Enzyme activities as affected by soil properties and land use in a tropical watershed. Appl. Soil Ecol. **35**:35-45.

2. **Barns, S.M., S.L. Takala, and C.L. Kuske**. 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Appl. Environ. Microbiol. **65**:1731-1737.

3. **Bernard, L., C. Mougel, P.-L. Maron, V. Nowak, J. Leveque, C. Henault, F. el Zahar Haichar, O. Berge, C. Marol, J. Balesdent, F. Gibiat, P. Lemanceau, and L. Ranjard.** 2007. Dynamics and identification of soil microbial populations actively assimilating carbon from ^{13}C-labelled wheat residue as estimated by DNA- and RNA-SIP techniques. Environ. Microbiol. **9:** 752-764.

4. **Bowman, J.P., S.A. McCammon, S.M. Rea, and T.A. McMeekin.** 2000. The microbial composition of three limnologically disparate hypersaline Antarctic lakes. FEMS Microbiol. Lett. **183**:81-88.

5. **Bruns, A., U. Nübel, H. Cypionka, and J. Overmann.** 2003a. Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. Appl. Environ. Microbiol. **69**:1980-1989.

6. **Bruns, A., H. Hoffelner, and J. Overmann**. 2003b. A novel approach for high throughput cultivation assays and the isolation of planktonic bacteria. FEMS Microbiol. Ecol. **45**:161-171.

7. **Buckley, D.H., and T.M. Schmidt.** 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microb. Ecol. **42:**11-21.

8. **Burns, R.G.** 1982. Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol. Biochem. **14**:423-427.

9. **Bodour, A. A., J. Wang, M.L. Brusseau, and R.M. Maier.** 2005. Temporal changes in culturable phenanthrene degraders in response to long-term exposure to phenanthrene in a soil column system. Environ. Microbiol. **5**: 888-895.

10. **Chróst, R.J.** 1991. Environmental control of the synthesis and activity of aquatic microbial ectoenzymes, p. 29-59. *In* R.J. Chróst (ed.), Microbial enzymes in aquatic environments. Springer, New York, NY.

11. **Cleveland, C.C., D.R. Nemergut, S.K. Schmidt, and A.R. Townsend.** 2007. Increases in soil respiration following labile carbon additions linked to rapid shifts in soil microbial community composition. Biogeochemistry **82**:229-240.

12. **Costello, E.K., and S.K. Schmidt.** 2006. Microbial diversity in alpine tundra wet meadow soil: novel *Chloroflexi* from a cold, water-saturated environment. Environ. Microbiol. **8**:1471-1486.

13. **De Santis, T.Z., E.L. Brodie, J.P. Moberg, I.X. Zubieta, Y.M. Piceno, and G.L. Adersen.** 2007. High-diversity universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb. Ecol. **53**:371-383.

14. **Dunbar, J., S. Takala, S.M. Barns, J.A. Davis, and C.R. Kuske**. 1999. Levels of bacterial diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. **65**:1662-1669.

15. **Drenovsky, R.E., D. Vo, K.J. Graham, and K.M. Scow**. 2004. Soil water content and organic carbon availability are major determinant of soil microbial community composition. Microb. Ecol. **48**:424-430.

16. **Dykhuizen, D.E.** 1998. Santa Rosalia revisited: Why are there so many species of bacteria? Ant. v. Leeuwenhoek **73:**25-33.

17. **Eivazi, F, and M.A. Tabatabai**. 1990. Factors affecting glucosidase and galactosidase activities in soils. Soil Biol. Biochem. **22**:891-897.

18. **Felske, A., A. Wolterink, R. van Lis, W.M. de Vos, A.D.L. Akkermans.** 2000. Response of a soil bacterial community to grassland succession as monitored by 16S rRNA levels of the predominant ribotypes. Appl. Environ. Microbiol. **66**:3998-4003.

19. **Fierer, N., M.A. Bradford, and R.B. Jackson.** 2007. Toward an ecological classification of soil bacteria. Ecology **88**:1354-1364.

20. **Frank, A.B., M.A. Liebig, and D.L. Tanaka.** 2006. Management effects on soil CO_2 efflux in northern semiarid grassland and cropland. Soil Till. Res. **89**:78-85.

21. **Gallego, V., C. Sánchez-Porro, M.T. García, and A. Ventosa.** 2006. *Massilia aurea* sp. nov., isolated from drinking water. Int. J. Syst. Evol. Microbiol. **56**:2449-2453.

22. **Griffiths, R.I., A.S. Whiteley, A.G. O´Donnell, and M.J. Bailey**. 2003. Physiological and community responses of established grassland bacterial populations to water stress. Appl. Environ. Microbiol. **69**:6961-6968.

23. **Griffiths, R.I., K. Ritz, R. Wheatley, H.L. Kuan, B. Boag, S. Christensen, F. Ekelund, S.J. Sørensen, S. Muller, and J. Bloem.** 2001. An examination of the biodiversity – ecosystem function relationship in arable soil microbial communities. Soil. Biol. Biochem. **33**:1713-1722.

24. **Gröngroft, A., N. Herpel, A. Petersen, and A. Mills.** 2006. Indication of soil degradation processes in selected drylands of Southern Africa. Proceedings of the International Conference: Soil and Desertification – Integrated Research for the Sustainable Management of Soils in Drylands, 5-6 May 2006, Hamburg, Germany.

25. **Grüter, D., B. Schmid, and H. Brandl.** 2006. Influence of plant diversity and elevated atmospheric carbon dioxide levels on belowground bacterial diversity. BMC Microbiology. **6**:68.

26. **Gupta, V.V.S.R., and J.J. Germida**. 1988. Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation. Soil Biol. Biochem. **20:**777-786.

27. **He, J., Z. Xu, and J. Hughes**. 2006. Molecular bacterial diversity of a forest soil under residue management regimes in subtropical Australia. FEMS Microbiol. Ecol. **55**:38-47.

28. **Janssen, P.H.** 2006. Identifying the dominant soil bacterial taxa in Libraries of 16S rRNA and 16S rRNA genes. Appl. Environ. Microbiol. **72**:1719-1728.

29. **Janssen, P.H., P.S. Yates, B.E. Grinton, P.M. Taylor, and M. Sait.** 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria, Actinobacteria, Proteobacteria,* and *Verrucomicrobia.* Appl. Environ. Microbiol. **68**:2391-2396.

30. **Jaspers, E., and Overmann, J.** 2004. The ecological significance of "microdiversity": identical 16S rRNA gene sequences represent bacteria with

highly divergent genomes and physiology. Appl. Environ. Microbiol. **70**:4831-4839.

31. **Kennedy, N., E. Brodie, J. Connolly, and N. Clipson**. 2004. Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms. Environ. Microbiol. **6**:1070-1080.

32. **Khammar, N., L. Malhautier, V. Degrange, R. Lensi, J.-J. Godon, and J-.L. Fanlo.** 2005. Link between spatial structure of microbial communities and degradation of a complex mixture of volatile organic compounds in peat biofilters. J. Appl. Microbiol. **98**:476-490.

33. **Kim, J.-S., G. Sparovek, R.M. Longo, W.J. De Melo, and D. Crowley**. 2007. Bacterial diversity of terra preta and pristine forest soil from the Western Amazon. Soil Biol. Biochem. **39**:684-690.

34. **Kowalchuk, G.A., D.S. Buma, W. de Boer, P.G.L. Klinkhamer, and J.A. van Veen**. 2002. Effect of a aboveground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Leeuwenhoek **81**:509-520.

35. **Kozdrój, J., and J.D. van Elsas**. 2000. Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. Soil Biol. Biochem. **32**:1405-1417.

36. **Kuske, R.C., L.O. Ticknor, M.E. Miller, and J.M. Dunbar**. 2002. Comparison of soil bacterial communities in rhizospheres of three plant species and the interspaces in an arid grassland. Appl. Environ. Microbiol. **68**:1854-1863.

37. **La Scola, B., R.J. Birtles, M.-N. Mallet, and D. Raoult,** 1998. *Massilia timonae* gen. nov., sp. nov., isolated from blood of an immunocompromised patient with cerebellar lesions. J. Clin. Microbiol. **36**:2847-2852.

38. **Lindquist, D., D. Murrill, W.P. Burran, G. Winans, J.M. Janda, and W. Probert,** 2003. Characteristics of *Massilia timonae* and *Massilia timonae*-like isolates from human patients, with an emended description of the species. J. Clin. Microbiol. **41**:192-196.

39. **Lipson, D.A., and S.K. Schmidt**. 2004. Seasonal changes in an alpine soil bacterial community in the Colorado rocky mountain. Appl. Environ. Microbiol. **70**:2867-2879.

40. **Lovell, R.D., S.C. Jarvis, and R.D. Bardgett.** 1995. Soil microbial biomass and activity in long-term grassland: effects of management changes. Soil Biol. Biochem. **27**:969-975.

41. **Lüdeman, H**., **and R. Conrad.** 2000. Molecular retrieval of large 16S rRNA gene fragments from an Italian rice paddy soil affiliated with the class *Actinobacteria*. Syst. Appl. Microbiol. **23**:582-584.

42. **Nakatsu, C., V. Torsvik, L. Øvreas**. 2000. Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. Soil Sci. Soc. Am. J. **64**:1382-1388.

43. **Nunan, N., T.J. Daniell, B.K. Singh, A. Papert, J.W. McNicol, and J.I. Prosser**. 2005. Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. Appl. Environ. Microbiol. **71**:6784-6792.

44. **Padmanabhan, P., S. Padmanabhan, C. DeRito, A. Gray, D. Gannon, J.R. Snape, C.S. Tsai, W. Park, C. Jeon, and E.L. Madsen.** 2003. Respiration of ^{13}C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of ^{13}C-labeled soil DNA. Appl. Environ. Microbiol. **69**:1614-1622.

45. **Rai, B. and A.K. Srivasta**. 1981. Studies on microbial population of a tropical dry deciduous forest in relation to soil respiration. Pedobiol. **22**:185-190.

46. **Rustad, L.E., T.G. Huntington, and R.D. Boone.** 2000. Controls on soil respiration: implication for climate change. Biogeochemistry **48:**1-6.

47. **Sait, M., P. Hugenholtz, and P.H. Janssen**. 2002. Cultivation of genetically distinct soil bacteria from phylogenetic lineages previously only detected in culture-dependent surveys. Environ. Microbiol. **4**:654-666.

48. **Sessittsch, A., A. Weilharter, M.H. Gerzabek, H. Kirchmann, and E. Kandeler**. 2001. Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. Appl. Environ. Microbiol. **67**:4215-4224.

49. **Sinsabaugh, R.I., R.K. Antibus, A.E. Linkins, C.A. McClaugherty, L. Rayburn, D. Repert, and T. Weiland.** 1992. Wood decomposition over a first-order watershed: mass loss as a function of lignocellulase activity. Soil Biol. Biochem. **24**:743-749.

50. **Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer, and G. Berg.** 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. **67**:4742-4751.

51. **Stackebrandt, E., and B.M. Goebel,** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**:846-849.

52. **Steenwerth, K.L., L.E. Jackson, F.J. Calderón, M.R. Stromberg, and K.M. Scow.** 2003. Soil microbial community composition and land use history in cultivated and grassland communities of coastal California. Soil Biol. Biochem. **35**:489-500.

53. **Stephan, A., A.H. Meyre, and B. Schmid**. 2000. Plant diversity affects culturable soil bacteria in experimental communities. J. Ecol. **88**:988-988.

54. **Tamaki, H., Y. Sekiguchi, S. Hanada, K. Nakamura, N. Nomura, M. Matsumura, and Y. Kamagata.** 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. Appl. Environ. Microbiol. **71**:2162-2169.

55. **Taylor, J.P., B. Wilson, M.S. Mills, and R.G. Burns**. 2002. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. Soil Biol. Biochem. **34**:387-401.

56. **Torsvik, V, F.D. Daae, R.-A. Sandaa, and L. Øvreas**. 1998. Novel techniques for analyzing diversity in natural and perturbed environments. J. Biotech. **64**:53-62.

57. **Upchurch, R, C. –Y. Chiu, K. Everett, K. Dyszynski, D.C. Coleman, and W.B. Whitman.** Differences in the composition and diversity of bacterial communities from agricultural and forest soils. Soil Biol. Biochem. (2007), doi:10.1016/j.soilbio.2007.06.027.

58. **Wertz, S., V. Degrange, J.I. Prosser, F. Poly, C. Commeaux, T. Freitag, N. Guillaumaud, and X. Le Roux.** 2006. Maintenance of soil functioning following erosion of microbial diversity. Environ. Microbiol. **8**:2162-2169.

59. **Waldrop, M.P., T.C. Balser, and M.K. Firestone**. 2000. Linking microbial community composition to function in a tropical soil. Soil Biol. Biochem. **32**: 1837-1846.

60. **Wang, W.J., R.C. Dalal, P.W. Moody, and C.J. Smith**. 2003. Relationships of soil respiration to microbial biomass, substrate availability and clay content. Soil Biol. Biochem. **35**:273-284.

61. **Wayne, L. G., D.J. Brenner, R.R. Colwell, P.A.D. Grimont, O. Kandler, M.I. Krichevsky, L.H. Moore, W.E.C. Moore, R.G.E. Murray, E. Stackebrandt, M.P. Starr, and H.G. Trüper,** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **37**:463-464.

62. **Zengler, K., G. Toledo, M. Rappe, J. Elkins, E.J. Mathur, J.M. Short, and M. Keller.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA **99**:15681-15686.

63. **Zhang, Y.-Q., W.-J. Li, K.-Y. Zhang, X.-P. Tian, Y. Jiang, L.-H. Xu, C.-L. Jiang, and R. Lai,** 2006. *Massilia dura* sp. nov., *Massilia albidiflava* sp. nov., *Massilia plicata* sp. nov. and *Massilia lutea* sp. nov., isolated from soils in China. *Int. J. Syst. Evol. Microbiol.* **56**:459-463.

CHAPTER 6

Summary

Soils harbor highly diverse bacterial communities. It is still poorly understood whether functional redundancy or a multitude of ecological niche modify the abundance and community composition of bacteria in soil. Understanding why soil microorganisms are so diverse and which factors control their community composition is of importance because they are essential for maintaining ecosystem processes and functions. Alterations of biotic or abiotic factors as results of natural or anthropogenic disturbances are known to influence soil bacterial diversity. However, the relation of those factors on microbial diversity is not well understood. This work examined effects of several environmental factors, specifically the presence of higher plant species, water content, land use, and soil properties, on bacterial diversity by employing two different soil sources.

The reproducibility of bacterial community composition in manipulated soil was analyzed by use of group-specific phylogenetic PCR-DGGE fingerprinting. Soils were taken from lysimeters that had been planted with four different types of plant communities and the water content was adjusted. The composition of *Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, Chloroflexi, Planctomycetes,* and *Verrucomicrobia* populations were clearly different from soils without plants compared to that of populations in planted soils. In contrast, the composition of *Acidobacteria, Actinobacteria, Archaea,* and *Firmicutes* populations did not influenced by the environmental factors tested. No clear influence of plant diversity and water content could be observed. The reproducibility of bacterial composition associated with the absence or presence of plants was true, even for the low-abundance phylotypes as shown by phylotype beta10 representing up to 0.18% of all bacterial cells in planted soils compared to 0.017% in those unplanted.

A high throughput cultivation approach was performed by employing the MicroDrop and the soil slurry dilution techniques. Soil-solution-equivalent medium (pH 7.0) supplemented with artificial root exudates, yeast extract, and inducers was

utilized. From 217 cultures obtained, isolate byr23-80 showing the same sequence with phylotype beta10 was recovered and studied in detail as this phylotype displayed a distinct response towards the presence of higher plant species and its sequence affiliated with uncultured bacteria, so far. The strain exhibited high physiological flexibility and was capable of utilizing major constituents of root exudates. A polyphasic taxonomic analysis and DNA-DNA hybridization data supported an assignment of strain byr23-80 as a novel species to the genus *Massilia* within the family *Oxalobacteraceae* of the subphylum *Betaproteobacteria*, for which the name *Massilia brevitalea* is proposed.

Effects of land use and soil properties on the bacterial diversity and activity were determined by employing natural soil from the Kavango region, Namibia. Soil properties in fact controlled the soil respiration rates rather than land use as pristine dark loam soil had remarkably higher respiration rate than pristine sand soil. Exoenzyme activities greatly varied among sites, but did not show a clear correlation to one of the two factors. The quantitative PCR identified *Acidobacteria* and *Actinobacteria* as the most abundant phyla about of 30 and 20% of all *Bacteria*, respectively. *Alphaproteobacteria, Bacteroidetes,* and *Planctomycetes* accounted for below 10%, whereas *Betaproteobacteria, Chloroflexi*, and *Firmicutes* represented less than 1%. Clone library of 16S rRNA genes from pristine dark loam soil revealed a high bacterial diversity with an estimated number of about 5600 phylotypes. The PCR-DGGE fingerprinting of *Acidobacteria* and *Actinobacteria* did only show minor differences in composition of the bacterial communities among sampling sites.

This study suggests that the bacterial species compositions in soil are determined to a significant extent by abiotic and biotic factors, rather than by mere chance, thereby reflecting a multitude of distinct ecological niches.

CURRICULUM VITAE

Personal Data

Name	:	Delita Zul
Date of Birth	:	July 11, 1968
Place of Birth	:	Pariaman, West Sumatra, Indonesia
Office Address	:	Department of Biology, Faculty of Mathematics and Natural Sciences, University of Riau, Kampus Bina Widya Km. 12.5 Simpang Baru, Pekanbaru, Indonesia
Home Address	:	Jln. Puyuh Mas No. 50 Pekanbaru Riau, Indonesia 28282

Education

1975-1980	Elementary School (Padang, West Sumatra, Indonesia)
1981-1983	Junior High School (Padang, West Sumatra, Indonesia)
1984-1986	Senior High School (Padang, West Sumatra, Indonesia)
1987-1991	Undergraduate (study of Biology at the Andalas University in Padang, West Sumatra, Indonesia)
1997-1999	Master (study of Biotechnology at the Gadjah Mada University, in Jogjakarta, Indonesia)
Since 11/2004	Ph.D. student at Ludwig-Maximillians-Universität (LMU) München, München, Germany