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**The use of an artificial rhizosphere model system to identify drivers for microbial
community structure at the plant-soil interface**

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Publication

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My contribution to the publication:

I was involved in the planning of the experimental set up and I carried out all experiments. Furthermore, the manuscript is mainly based on my input.

Abstract

The rhizosphere is a microbial hot spot in soil. Root exudates released by the plant root increase microbial biomass and activity at the plant-soil interface. This soil compartment is highly dynamic in space and time due to root exudation, but also due to abiotic factors such as soil temperature and soil pH. It is thus a challenge to study key drivers of microbial communities in nature. Although root exudation has been simulated by several models, it has not been taken into account that also the surface of the plant root affects microbial colonization.

In this thesis an artificial rhizosphere model (ARM) was created, which offers both a surface and compounds acting as root exudates to stimulate microbial colonization. The model consisted of a glass slide covered with agarose containing an embedded artificial exudate mix commonly found in plants. The model was incubated in soil and bacteria were allowed to colonize the glass slide. This was shown by Fluorescence *In Situ* Hybridization (FISH) analysis by the visualization of bacteria attached to the ARM. To test whether the bacterial community of ARM was different to the bacterial population in bulk soil, bacterial communities were analyzed by the molecular fingerprinting method Terminal Restriction Fragment Length Polymorphism (T-RFLP) based on 16S rRNA genes. Over a period of 9 days, the bacterial population of ARM compared to bulk soil revealed distinct structural differences, such as the increased abundance of the typical rhizosphere bacterial genus *Pseudomonas* in ARM. Compared with the real rhizobacterial community of the plant *Arabidopsis thaliana* prior to flowering, we discovered several similarities, but also differences in the microbial community patterns which reveal the highly complex nature of a real plant root.

Several influences affect microorganisms at the plant-soil interface. The most important ones among them are root exudates which shape a specific microbial community. To investigate the effect of exudates which have been reported to influence bacteria, single compounds (glucose, malic acid or serine) were omitted from the ARM. Surprisingly, only the subtraction of the organic acid malic acid lead to a significant bacterial community shift in the early bacterial population development of ARM, whereas omitting glucose as an easy degradable compound or the amino acid serine did not result in significant differences in the bacterial community structure pattern.

Besides natural factors such as root exudates, we investigated anthropogenic-driven influences on rhizobacteria. Manure is a widely used crop fertilizer in agriculture and often

carries high loads of antibiotics applied to the farm animals. This practice introduces not only nutrients to the soil, but also antibiotics and antibiotic resistant bacteria which then might get in contact with the soil microflora, especially in a densely populated microhabitat as the plant-soil interface. Therefore, this environment could be an important habitat to spread antibiotic resistances in the environment. In this study, sulfadiazine (SDZ), as an example for a veterinary antibiotic, was co-applied with manure to the soil. The bacterial community structure was mainly affected by manure and exudates, respectively, but not additionally by SDZ application. However, in the presence of SDZ and manure, the abundance of SDZ-resistance genes significantly increased in the artificial rhizosphere when SDZ was additionally applied on the ARMs surface. Moreover, piggery manure used in this experiment was an important carrier of these genes. Besides the transmission of resistance genes, manure-originated *Clostridium* persisted 1 week in the ARM and soil-borne *Pseudomonas*, which was highly abundant until 2 weeks in the ARM under the selective pressure of SDZ.

Zusammenfassung

Die Rhizosphäre ist ein Bodenkompartiment, das von der Pflanzenwurzel beeinflusst wird. Durch die Abgabe von Wurzelexsudaten wird die mikrobielle Biomasse und Aktivität in der unmittelbaren Nähe der Wurzel stark erhöht. Sowohl die Menge und Zusammensetzung dieser Exsudate, die jedoch sehr variabel sind, als auch die Oberfläche der Pflanzenwurzel sind wichtige Faktoren für die mikrobielle Besiedelung dieses dynamischen und komplexen ökologischen Lebensraums. Daher ist es wichtig, mit Hilfe von Modellen die Rhizosphäre zu simulieren, um Interaktionen zwischen Pflanzenwurzel und Mikroorganismen zu untersuchen. Das Ziel dieser Doktorarbeit war es, ein Rhizosphärenmodell (ARM) zu entwickeln, das im Gegensatz zu früheren Modellen sowohl eine Oberfläche für die mikrobielle Kolonisierung bietet als auch Substanzen enthält, die Wurzelexsudate simulieren. Dieses Modell bestand aus einem Glasobjekträger, der mit einer künstlichen Agarose-Exsudatmischung beschichtet wurde. Dieser Standard-Exsudatmix beinhaltete 8 Komponenten, die typischerweise von Pflanzen ausgeschieden werden. Zunächst wurde die bakterielle Gemeinschaft, die sich auf der ARM-Oberfläche gebildet hatte, mittels Fluoreszenz *In situ* Hybridisierung (FISH) analysiert. Um zu untersuchen, ob die Bakteriengemeinschaft strukturell unterschiedlich zu der im Boden war, wurden diese über einen Zeitraum von 9 Tagen mit Hilfe der molekularen Fingerprintingmethode Terminalen Restriktion Fragment Längen Polymorphismus (T-RFLP) basierend auf der Diversität von 16SrRNA Genen analysiert. Es konnte gezeigt werden, dass besonders die Gattung *Pseudomonas*, die häufig in der Rhizosphäre vorkommt, im ARM im Vergleich zum Boden akkumulierte. Um zu untersuchen, ob sich eine der natürlichen Rhizosphärengemeinschaft ähnliche Population entwickelt, wurde die Modellpflanze *Arabidopsis thaliana* verwendet. Es konnten sowohl Übereinstimmungen als auch Unterschiede zwischen dem Modell und Pflanze nachgewiesen werden, was vermutlich in der höheren Komplexität der Pflanzenexsudate begründet liegt.

Exsudate stellen einen der wichtigsten pflanzlichen Einflussfaktoren auf Mikroorganismen in der Rhizosphäre dar. In einem weiteren Experiment wurden bei dem künstlichen Standard-Exsudatmix vom ARM einzelne Komponenten (Glukose, Malat oder Serin) weggelassen. Hierbei konnte gezeigt werden, dass nur das Weglassen von der organischen Säure Malat auf die frühe Entwicklung der Bakterienpopulation einen signifikanten Einfluss hatte. Überraschenderweise führte das Abwesenheit von schnell abbaubarer Glukose und auch der Aminosäure Serin zu keinem signifikanten Unterschied in der bakteriellen Gemeinschaft im Vergleich zu dem ARM mit dem Standard-Exsudatmix.

Neben natürlichen Faktoren hat auch die Landwirtschaft einen wichtigen Einfluss auf das mikrobielle Leben im Boden. In einem weiteren Experiment wurde die Rolle von Gülledüngung und der damit oftmals einhergehende Eintrag von resistenten Bakterien und Antibiotikarückständen auf Bakterien in der Rhizosphäre untersucht. Aufgrund der hohen Anzahl von Mikroorganismen in diesem Habitat, könnte besonders hier eine Ausbreitung von Antibiotikaresistenzgenen in der Umwelt stattfinden. Um dies zu untersuchen, wurde der Boden mit Schweinegülle und dem in der Tiermedizin weitverbreiteten Sulfadiazin (SDZ) vorinkubiert. Es zeigte sich, dass die Struktur der bakteriellen Rhizosphärengemeinschaft hauptsächlich durch Gülle und Exsudate beeinflusst wurde, hingegen das zusätzlich eingebrachte SDZ keinen zusätzlichen Einfluss hatte. Des Weiteren wurde beobachtet, dass die benutzte Gülle ein wichtiger Träger von Resistenzgenen war und diese Gene sich in der Bakterienpopulation unter zusätzlicher SDZ-Zugabe auf der ARM-Oberfläche innerhalb von zwei Wochen signifikant ausgebreitet haben. Sowohl Bakterien aus dem Boden als auch aus der Gülle wurden unter dem Antibiotikaselektionsdruck in dem Rhizosphärenmodell angereichert. *Pseudomonas* aus dem Boden war über einer Dauer von 2 Wochen hochabundant, *Clostridium* aus der Gülle war bis zu einer Woche in großer Anzahl vorhanden.

1 Introduction

1.1 Soils as a highly structured environment for microbial life

Soils reveal to be a major habitat for microorganisms in the environment. There are estimates that in one gram of soil up to 10^6 bacterial species exist (Gans *et al.* 2005). The enormous amount of microorganisms is not equally spread throughout the soil, it is rather distributed in zones and areas with accumulated microbial biomass. This is due to soils' heterogeneous character based on its texture and nutrient distribution. Soils consist of an organic fraction like living plants, animals, and microorganisms. Another source of organic material is dead biomass at different decomposition stages, which is called humus. This soil fraction can be further divided in fulvic acid, humic acid, and humin, which are high molecular compounds. Humus is a source for dissolved organic matter (including carbon (DOC), nitrogen (DON), phosphate (DOP), and sulphur (DOS)) and plays an important role in the element cycling in soil, since it can be metabolized by microorganisms and plants. Besides the soil organic matter, soils consist of a non-organic fraction composed of minerals, which are classified according to their particle size as sand (2-0.05 mm), silt (0.05–0.002 mm), and clay (< 0.002 mm). This solid phase – the so-called soil matrix – is pervaded with pores filled with either water or air and thus enabling water and nutrient cycling through the soil matrix.

Microbial distribution in soil mainly depends on the availability of nutrients and water, but also on gas diffusion (Ranjard and Richaume 2001). Regarding small scale distribution, soil microorganisms mainly colonize pores, which depends on the water/gas level and the diameter of the pores. Bacteria rather inhabit small pores with a diameter less than 5 μm , probably because these pores retain water in dry soils better than larger pores, and in addition to that they are also protected from predators (Foster 1988). Especially in deeper soil layers, Nunan *et al.* (2003) showed that bacterial densities are the highest in close vicinity to pores. Besides soil pores, microorganisms can attach to soil particles, an interaction which depends on the properties of the bacterial cell surface and the type of soil particles (Huysmann and Verstraete 1993).

Concerning larger scales, soils are divided in specific horizons formed by biogeochemical processes. The deepest layer of the soil profile is the stone level or R-horizon. On top of that follows the C and then the B horizon. They are subsoil layers in contrast to the topsoil layer, the A-horizon. Finally, the O-horizon is the soil layer on the surface. Due to biogeochemical processes, nutrients decrease with rising depths which goes along with a decrease in microbial abundance (Taylor *et al.* 2002; Fierer *et al.* 2003). In contrast, the top layer (O-horizon) and the following A-horizon are major pools for soil microorganisms, which is due to the high amount of nutrients in these soil layers.

High loads of nutrients enter the soil in form of dead organic material, but also by the release of plant roots anchored in the soil. Green plants fix atmospheric carbon dioxide (CO₂) by photosynthesis and transform it into glucose which is then incorporated into plant biomass. Carbon can be released by respiration in form of CO₂ or as rhizodeposits to the surrounding soil. Up to 40 % of the net primary carbon production by photosynthesis can be allocated in soil (Lynch and Whipps 1990). Rhizodeposits consist of mucilage secreted from the root tip and border cells, and also of root exudates. Root exudates are low molecular weight compounds such as sugars, organic acids, amino acids and secondary metabolites like phenolic acids (Vancura and Hovadik 1965). As a passive mode of action, most low molecular compounds are released by diffusion along a concentration gradient to the surrounding soil (Bertin *et al.* 2003).

In 1904, Lorenz Hiltner defined the soil influenced by the plant root as rhizosphere. The root shapes the surrounding soil not only by the excretion of Carbon-rich nutrients, but also by influencing the soil structure, pH, water and oxygen level (Sorensen and Sessitsch 2006). These processes lead to a highly dynamic habitat at the plant-soil interface. As well as the surrounding soil, the plant root surface itself is colonized by various microorganisms (Morris and Monier 2003; Ramey *et al.* 2004; Danhorn and Fuqua 2007; Rudrappa *et al.* 2008). Therefore, the amount of microbial cells is 10 to 100 fold higher in the rhizosphere compared to bulk soil (Lugtenberg and Kamilova 2009), and also the microbial activity is increased in this soil compartment. This is described by the rhizosphere effect (Sorensen 1997) and thus, the rhizosphere displays a hot spot for microbial life in soil.

1.2 The microbiome of the rhizosphere

The unity of rhizosphere-associated microorganisms is entitled as the rhizosphere microbiome. The whole microbial community was termed as the rhizosphere zoo by Buée *et al.* (2009) including bacteria, archaea, phages, and fungi. Other important members are nematodes, algae, and protozoa (Mendes *et al.* 2013). However, microbiome studies often focus on the bacterial fraction, and do not include other microorganisms.

In the past, culture-dependent methods largely underestimated the number and abundance of microorganisms in soil because only approximately 1 % of them are cultivable in laboratories. Modern methods based on cultural-independent technologies, such as metagenomic studies performed with Next-generation sequencing, gave new insight in this hidden world (Bulgarelli *et al.* 2012; Lundberg *et al.* 2012). Rhizobacterial communities are selected from the vast amount of bulk soil microorganisms (Buée *et al.* 2009). It was shown that up to 30,000 procaryotic species (Egamberdieva *et al.* 2008) inhabit this soil compartment. Since the rhizo-microbiome originates from the surrounding soil with the enrichment of a few certain phyla, the species richness is therefore higher in bulk soil (Uroz *et al.* 2010). Regarding the species variety, it was thought that especially r-strategists such as Pseudomonades are attracted by the plant root and its rhizodeposits. Interestingly, besides Proteobacteria, there are also typical k-strategists like Actinobacteria dominantly present in the rhizosphere. This was shown in a study by Bulgarelli *et al.* (2012) who analyzed the microbiome of *Arabidopsis thaliana* plants by 16S rRNA gene pyrosequencing and detected Actinobacteria, Bacteroidetes, and Proteobacteria as the dominant phyla. However, large fractions with up to 16 % belong to unclassified bacteria (Mendes *et al.* 2011), which reflect the huge diversity of soil microbiota even more.

Besides taxonomic categories, bacteria can be divided into plant-beneficial and plant pathogenic ones, but also human pathogens.

1.2.1 Plant growth promoting rhizobacteria

Plant-beneficial bacteria are the so-called plant growth promoting bacteria (PGPR). There are different mechanisms how they enhance plant performance (Lugtenberg and Kamilova 2009), in direct ways which include nutrient acquirement, rhizoremediation and plant stress control, or in an indirect manner by soil pathogen suppression.

Soil bacteria - besides mycorrhiza - are key drivers in the element cycles in soil and provide plants with various nutrients essential for their growth. Plants are only able to take up ammonium (NH_4^+) and nitrate (NO_3^-) by special transport mechanisms (Jackson *et al.* 2008). Therefore, other nitrogen compounds have to be converted into bioavailable forms by processes mainly driven by bacteria. An example is the *Rhizobium*-leguminose symbiosis in which bacteria fix molecular nitrogen (N_2), transfer it into NH_4^+ and make it bio-available for the plant (Mylona *et al.* 1995). Phosphate (P) is also an important element present in nucleic acid and is present in the environment mainly in the insoluble form. Bacteria are able to solubilise P and make it available for plants (Gyaneshwar *et al.* 2002; Vassilev *et al.* 2006). A mechanism for nutrient acquisition is the secretion of siderophores by bacteria. These compounds are able to bind insoluble ferric-iron in soil and make it bioavailable for plants (Bar-Ness *et al.* 1992).

Soil pollutants which inhibit plant growth can be degraded by soil microorganisms. This process of rhizo-mediation depends on energy sources released by plant roots in form of exudates. To enhance the degradation of contaminants in natural systems, genetically modified bacteria were constructed which carry genes encoding catabolic pathways. For example, the rhizobacterium *Pseudomonas putida* was transformed with a plasmid carrying genes encoding proteins for naphthalene degradation. These strains were able to increase contaminant-removal *in situ* (Fernandez *et al.* 2012).

Phytohormones are substances that stimulate plant growth and can be produced by plants or microorganisms to stimulate plant growth (Ortiz-Castro *et al.* 2009). One famous example is indole-3-acetic acid (IAA) from the auxin group. P ikyl *et al.* (1985) showed that *Pseudomonas* isolates from the rhizosphere of maize and pea produced IAA in liquid culture. IAA plays an important role in root architecture in a dose-dependent manner triggering the stimulation of primary root elongation, or increasing lateral root and hair root formation (Vacheron *et al.* 2013). The phytohormone ethylene mediates inhibitory responses in plants under stresses. By the bacterial production of the enzyme ACC deaminase, the ethylene precursor ACC is degraded and therefore positively affects the plant performance (Glick *et al.* 2007).

Indirect beneficial bacterial effects on plant performance are mediated via biocontrol mechanisms against plant pathogens. Bacteria produce various antibiotics which act against plant pathogens. Mavrodi *et al.* (2012) detected the broad spectrum antibiotic phenazine in the rhizosphere of wheat plants. Levels of this antibiotic substance were above subinhibitory

levels and were able to inhibit growth of sensitive organisms *in situ*. Plants are able to defend against a pathogen attack by a phenomenon called induced systemic resistance (ISR). This resistance mechanism is regulated by the plant hormones jasmine acid and ethylene which lead to bacterial responses that trigger ISR in plants. Pieterse *et al.* (1998) could show ISR in *Arabidopsis* is triggered by the non-pathogenic *Pseudomonas fluorescens* after a pathogenic *Pseudomonas syringae* attack.

1.2.2 Plant pathogenic bacteria

There are also plant pathogenic bacteria which impair the plant performance or even cause the death of the plant. In agricultural systems, these organisms lead to decreased crop yield and therefore cause immense economically damage. Several traits are needed to cause a disease in plants. First, bacterial cells have to attach and enter the host. Secondly, the pathogen has to overcome the immunodefense of plants, and third, it has to elicit the disease (Oku *et al.* 1994). In 2012, Mansfield and co-workers published a list of the 10 most economically and scientifically important bacterial plant pathogens. Number one voted was *Pseudomonas syringae* which causes infections in trees and crop plants, for example tomato. This organism has been intensively studied in several plant hosts like the model plant *Arabidopsis thaliana* for its pathogenic traits, for example mobility, type III secretion system, and toxins (Collmer *et al.* 2000; Geng *et al.* 2012; Ichinose *et al.* 2013). Further knowledge about its infectious capabilities were obtained by whole genome sequencing of *P. syringae* pv *tomato* DC3000 (Buell *et al.* 2003). *Ralstonia solanacearum* causes major damage to crop plants such as potato, tomato, bananas, tobacco. This fact makes this bacterium an important model organism to study plant pathogenic interactions and was one of the first plant pathogenic bacteria of which the genome was completely sequenced (Salanoubat *et al.* 2002). Another important plant pathogen is the renowned *Agrobacterium tumefaciens* causing crown gall disease in crop plants and hence economically damage (Zupan and Zambryski 1995). This bacterial species is well studied and especially its ability for genetic transformation of the host makes it very interesting model organism for molecular plant biology (Tzfira and Citovsky 2006).

1.2.3 Human pathogenic bacteria

A third group of rhizobacteria belongs to the group of human pathogens. These bacteria are adapted to the human body, but several examples show that they are also able to survive outside humans and persist in soil and attach to or infect plants. If human-pathogenic bacteria colonize crop plants, they can return to the human host via food products. The strain *Escherichia coli* O157:H7 which causes the haemolytic-uremic syndrome was isolated from contaminated human food products (Michino *et al.* 1999; Chapman *et al.* 2001). Jiang *et al.* (2002) observed that this species was able to survive in soil for about 6 months after manure application. A study by Gagliardi *et al.* (2002) investigated the persistence of this bacterium in plant roots. They showed that this bacterial species can survive up to 96 days on rye roots. However, the cell concentration decreased after soil amendment probably due to the competition with soil microorganisms. It was shown that *E. coli* O157:H7 was able to infect leaf lettuce by entering the plant via the root system and survive inside the plant tissue (Solomon *et al.* 2002).

Besides these “real” human pathogens, there are opportunistic bacterial pathogens. They are only able to infect immunocompromised patients, for example patients after chemotherapy or HIV-patients. Berg *et al.* (2005) listed potentially pathogenic bacteria found in the rhizosphere of plants. They argued that bacteria which adapt to the rhizosphere might have traits which could also be helpful to infect humans, for example the adherence to the host or the overcoming of the humans’ immunosystem. *Burkholderia cepacia* was detected in the majority of investigated rhizosphere samples. This bacterial complex consists of a large group of different bacteria with different effects on other organisms: They can cause plant disease, but they can also be beneficial for plants. Furthermore, they can be harmful to animals and humans, especially in the case of cystic fibrosis (CF) patients (Mahenthalingam *et al.* 2008). Bevivino *et al.* (2002) compared *B. cepacia* isolates from CF patients and the rhizosphere of maize for their virulence patterns. They detected differences in pathogenic characteristics, but also showed that certain pathogenic traits are also spread in environmental strains.

1.3 Influences on the dynamics in the rhizosphere microbiome

The rhizosphere microbiome is affected by several factors, such as the plant itself and its exudate patterns, but also by the surrounding soil. More and more, human impact was discovered to influence the population structure of soil microorganisms.

1.3.1 Natural influences: Plants, root exudates, and the surrounding soil

Plant species have the ability to create their specific bacterial rhizosphere community. This was shown by Smalla *et al.* (2001) by the comparison of bacterial rhizosphere populations of strawberry, potato and oilseed plants by barcoding of 16S rRNA gene amplicons and subsequent denaturing gradient gel electrophoresis (DGGE). They detected differences between the bacterial species composition of the different cultivars. However, not only plant species but also plant genotypes create their own specific rhizobacterial communities (van Overbeek and van Elsas 2008, Micallef *et al.* 2009). In a larger scale, the diversity of plant communities in an ecosystem is an important driver for the soil microbial population structure (Lamb *et al.* 2011). Furthermore, the plant age is an important factor, as shown in a study by Houlden *et al.* in 2008, demonstrating shifts of microbial population depending on the developmental stage of field crops. The influence of the plant health status was investigated by Zhang and co-workers in 2011 on the example of cotton (*Gossypium* sp.) at different growth stages, showing that bacterial populations differ between healthy and diseased plants. Also, plant nutrient stress can cause changes in the exudation pattern as it was reported in a study with alfalfa seedlings under phosphate limitation from Lipton *et al.* in 1987. The induction of systemic acquired resistances in plants to defend against pathogens was also shown to alter the rhizobacterial community structure (Hein *et al.* 2008).

In a smaller scale, the root exudates play a key role in mediating plant-microbial interactions. The amount and composition of these organic carbon compounds depend on various plant factors and is highly dynamic. By this, plants are able to shape their specific microbial community inhabiting the rhizosphere. The root exudation pattern varies in a time-dependent manner. It was shown on the example of alfalfa, that the amount of root exudates decreased with plant age, but increased again before flowering (Hamlen *et al.* 1972). Regarding particular exudate compounds, malic acid was discovered to be responsible to attract the plant growth promoting bacteria *Bacillus subtilis* in *Arabidopsis* rhizosphere (Rudrappa *et al.* 2008b).

Regarding spatial scales, the root hair zone creates a huge surface allowing contact with the surrounding soil, which results in a strong exchange of compounds (exudates) between soil and root. Generally, young, metabolic active parts of the root as the root tip and the elongation zone have greater losses of carbon than mature parts (Jones *et al.* 2009). Moreover, single compounds are exudated in different root zones. For citrate, it was shown that in maize plants it was released mainly 5 cm above the root cap (Pineros *et al.* 2002). During their passage through the soil, root-derived compounds are metabolized or absorbed. Hence, their concentration decreases with distance from the root and the concentration is considered to be the highest on the root surface. Therefore, and due to the fact that the surface of the plant root itself facilitates bacterial colonization, the plant root is colonized by various attached microorganisms. Watt *et al.* (2006) analyzed native bacteria attached to field-grown wheat plant roots by FISH. They detected a heterogeneous distribution of microorganisms along the root surface, with the highest accumulation at the root cap and lowest bacterial cell densities at the root elongation zone. Cluster-forming bacteria were found in 40 % of the root samples. A study by Dandurand *et al.* (1997) also reported an uneven distribution pattern of *Pseudomonas fluorescens* along the root of pea seedlings. They observed high amounts of bacteria especially at the root seed junctions.

Besides the plant and its secreted substances, the surrounding soil has a large influence on microorganisms in the rhizosphere. Several studies showed that soil types have a significant influence on the bacterial community structure. Marschner *et al.* (2001) tested different soils (sandy soil, sandy loam, and clay) and reported that the microbial species composition was strongly affected by the soil type. A study by Lauber *et al.* (2009) analyzed the soil bacterial community structure from different sites in North and South America by pyrosequencing. They revealed that soil pH was a significant driver for the bacterial community structure. Other soil inhabitants, such as predators like nematodes are also attracted by the rhizosphere and graze on the microfauna (Djigal *et al.* 2004). Thereby, they play an important role by controlling bacterial population dynamics.

1.3.2 Anthropogenic influences: Agricultural practices

Modern agriculture, including monocultural crop fields and tillage practices as well as fertilizing procedures, has a strong impact on the living soil. Tillage leads to increased oxygen levels, water content, temperature and introduction of fertilizers in deep soil layers (Kladivko

2001). It was shown that these conditions select for aerobic bacteria and nitrifiers (Doran 1980). As mineral fertilizers often contain high loads of N and P, they interfere with key element turn-over processes in soil mainly driven by bacteria and hence affect their diversity (Sarithchandrea *et al.* 2001), also in long-term aspects (Chu *et al.* 2007). Manure from husbandry is also widely used as a fertilizer for crop plants.

Antibiotics have been used in large amounts in animal husbandry to treat bacterial infections or as prophylaxes. Furthermore, they have been given as feed additives in subinhibitory doses to increase animals' growth (Dibner and Richards 2005). The latter mentioned practice has been banned in the European Union since 2006, but outside the EU it is still common. In the animal gut, antibiotics select for resistant bacteria both commensales or pathogens and they are excreted along with antibiotic residues. It is estimated that around 75 % of the applied antibiotics are excreted by animals (Elmund *et al.* 1971). It was shown that manure samples from antibiotic treated animals contained up to 20 mg sulfonamide per kg liquid manure (Haller *et al.* 2002). Several studies showed the high load of manure with antibiotic-resistant bacteria (Munir and Xagorarakis 2011) and antibiotic residues (Haller *et al.* 2002). In agricultural practice, manure is stored or directly applied on arable land as fertilizers. Along this pathway, antibiotics and resistant bacteria get in contact with the soil microbiota and also reach the ground water (Christian *et al.* 2003; Sengelov *et al.* 2003). This might lead to selective pressure which selects for antibiotic resistance among bacteria and hence its further spreading in the environment (Heuer *et al.* 2011). For bulk soil, it was shown that manure and the veterinary antibiotic sulfadiazine (SDZ) lead to an increase in the abundance of antibiotic resistance among the bacterial community (Heuer and Smalla 2007). Horizontal gene transfer (HGT) is an important mechanism to spread antibiotic resistance traits among bacteria. Plasmids – mobile genetic elements often carrying resistance genes – are transferred from one cell to another by conjugation. Several factors, such as elevated microbial activity and high cell densities are thought to be crucial factors for HGT and therefore the rhizosphere could be an ideal environment for the occurrence of HGT (Dröge *et al.* 1999).

1.4 Studying plant-microbial interactions in the rhizosphere

The plant root and its exudation patterns are variable both in time and space, which makes the rhizosphere a very dynamic and heterogeneous habitat for microorganisms, as discussed in the section above. Furthermore, plant-derived DNA originating from mitochondria interferes with molecular analyses targeting bacterial 16S rRNA genes. This results in a bias towards plant-derived DNA and low abundant microorganisms may not be detected. Therefore, using models facilitates studying microbial populations and their drivers in the rhizosphere.

Several root models were developed to address plant-microbial interactions and to elucidate the role of exudates within this relation. Some studies used natural root exudates sampled from hydroponically grown plants (Delavault *et al.* 1998; Mark *et al.* 2005; Broeckling *et al.* 2008). In contrast, other researchers created artificial root mixtures containing main groups of exudates such as sugars, carbohydrates and amino acids. These solutions were added directly to the soil in single (Eilers *et al.* 2010) or repeated inputs (Shi *et al.* 2011) or applied in a continuous manner. The latter was performed in a study by Griffith *et al.* in 1999 by a wick conducted to a peristaltic pump allowing a flow of artificial exudate solution to the soil. Pearce *et al.* in 1997 invented a model which conducts the exudate solution via a hollow fibre to the growth chambers and studied the influence of exudates on *Enterobacter cloacae* isolated from rhizosphere. A further technique to study bacteria living in soil was developed by Paterson *et al.* in 2007. They placed an artificial root model in the soil offering a permeable surface and continuous exudate flow. To study bacteria at the plant-soil interface, a model was invented by Parkinson *et al.* in 1971. The buried slide technique is a very simple but efficient method consisting of a glass slide which is directly applied to the soil and bacteria are allowed to attach to its surface. This technique was further developed and modified to allow colonization by soil-born bacteria to agar-coated glass slides from soil slurry (Lunsdorf *et al.* 2000; Burmolle *et al.* 2007).

1.5 Objectives

This doctoral thesis was funded by the Helmholtz association to gain insight into plant-microbial interactions and to detect key drivers of microbial population dynamics at the plant-soil interface. The rhizosphere is densely populated by microorganisms which are highly active. The development of these microbial communities depends on a variety of factors, for example root exudates, which are thought to play a major role in plant-microbial interactions. Besides this natural influence, agricultural practices have effects on soil. By fertilizing crop land with manure, xenobiotic compounds such as antibiotics and resistant bacteria can be transmitted to the soil. Because the rhizosphere is a bacterial hotspot in soil, this habitat could favour the spreading of antibiotic resistance genes in the environment.

As the rhizosphere is highly dynamic, it has been a challenge to study its microbiome under standardized and controlled conditions. The aim of the thesis was to develop a model both offering a surface for microbial attachment and compounds serving as root exudates to attract soil microorganisms. Therefore, we developed an artificial rhizosphere model (ARM) which was based on the buried slide technique (Parkinson *et al.* 1971) and consisted of glass slides covered with an agarose matrix to allow bacterial colonization on the surface. As natural root exudation patterns are highly variable, a simple artificial exudate mix of 8 compounds (carbohydrates, organic acids, amino acids) commonly found in plants was added on the glass surface. The model was evaluated and the role of single root exudates as natural drivers and antibiotic-loaded manure as an anthropogenic driver of the bacterial community structural development in an artificial rhizosphere were investigated.

Following hypotheses were tested:

- (i) The bacterial community of the artificial rhizosphere model is reproducible and distinctly differing from the bacterial community in bulk soil.
- (ii) The bacterial community of the artificial rhizosphere model is comparable to the natural rhizosphere community of a real root. As a model plant, *Arabidopsis thaliana* Col0 is used.

- (iii) Omitting single root exudates of the standard exudate mix affects the bacterial community structure in the artificial rhizosphere model. Glucose as an easily degradable compound will have the strongest effect on the bacterial community structure.

- (iv) Application of manure and the veterinary antibiotic sulfadiazine affects the community structure and increases the abundance of sulfadiazine resistance genes in the bacterial population of the artificial rhizosphere model.

2 Material and Methods

2.1 Soil sampling

Soil sampling took place at the research farm Scheyern of Helmholtz Zentrum München in South Germany (Bavaria, Germany). Soil was taken from the A-horizon in a depth between 0-20 cm of an arable field (A02) which was characterized as a cambisol with a loamy surface layer (see Figure 2.1). Soil parameters are summarized in Table 2.1. Sampling took place in December 2009, May 2010, January 2011, and January 2012. In 2009, potatoes were grown on the site A02. In 2010, winter wheat, in 2011 sunflowers, and 2012 lucerne-clover-grass was planted as the main crop. After sampling, the soil samples were dried and sieved with a 2 mm mesh to homogenize particle sizes and stored at 4 °C until further usage.

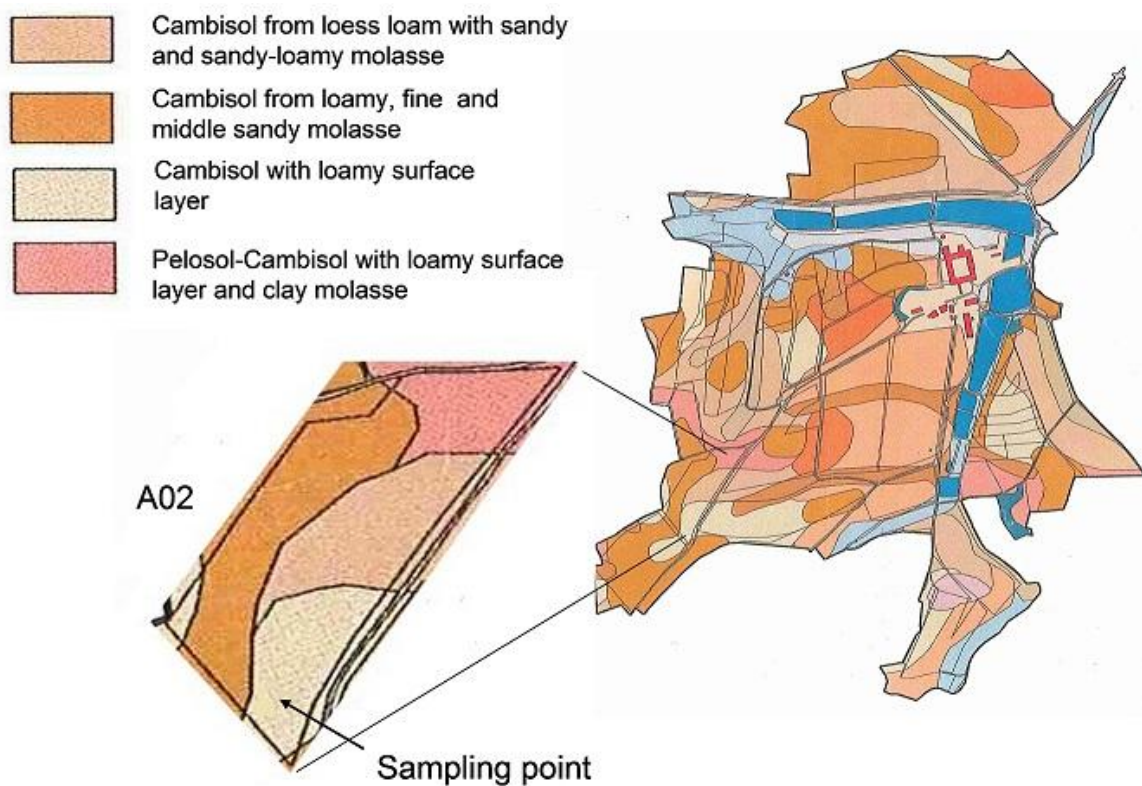


Figure 2.1 Sampling site at the research farm in Scheyern (Bavaria, Germany). Samples were taken at the area A02. Soil was characterized as Cambisol with a loamy surface layer.

Table 2.1 Soil parameters of the sampling site A02 in Scheyern (Bavaria, Germany). WHC_{max} = maximum water holding capacity.

pH	organic	total	WHC_{max} (%)	Soil texture		
	Carbon (%)	Nitrogen(%)		Sand (%)	Clay (%)	Silt (%)
6.0	1.3	0.1	50.4	45	18.2	36.8

2.2 Experimental designs

2.2.1 The artificial rhizosphere model (ARM)

300 g of dried and sieved (2 mm) soil was put in pots (9 x 9 x 9.5 cm). The glass slides (Assistant, Germany) were sandblasted on both sides to enhance the attachment of the agarose matrix. Before covering, the glass slides were sterilized. For the matrix preparation, a 1 % solution of low melting agarose (Serva, Germany) was mixed with a sterile filtrated artificial root exudate solution (Griffiths *et al.* 1999) shown in Table 2.2. This standard exudate mix resembles compounds commonly found in higher plants and contained in total 15 mg ml⁻¹ of carbon and 900 µg ml⁻¹ of nitrogen. The concentration was based on the assumption of a daily carbon input of 50-100 µg g⁻¹ to the surrounding soil (Trofymow *et al.* 1987) and that the soil with a maximum distance of approximately 1 cm to the glass slide is influenced by the exudates' diffusion. After slowly pouring the viscose solution on each side of the glass slide and air drying, the prepared slides were put in the pots with soil as shown in Figure 2.2. All pots were irrigated with tap water twice per week to maintain 50 % of the maximum water holding capacity (WHC_{max}). Based on the relatively slow bacterial generation time in soil, the experimental duration of maximum 2 weeks was chosen to monitor the development of bacterial communities under the influence of the artificial root exudate mixture of the ARM. Each biological replicate consisted of 2 glass slides per pot and each treatment was done in 5 replicates.

Table 2.2 Composition of the standard exudate mix of ARM after Griffiths *et al.* (1999)

Carbohydrates (each 300 mM)	Glucose	Fructose	Sucrose
Organic acids (each 150 mM)	Malic acid	Succinate	
Amino acids (each 75 mM)	Arginine	Cysteine	Serine



Figure 2.2 Image of the artificial rhizosphere model (ARM). The ARM consists of glass slides covered with an artificial exudate/agarose mix incubated in soil.

To sample the bacterial population, the glass slides were gently pulled out of the soil and carefully dipped in 1 x PBS to wash away loosely adherent soil particles. The agarose matrix with attached microorganisms was harvested with sterile cell scrapers and stored at -20 °C until further usage. To release the microorganisms, the agarose was digested by the agarase enzyme (Fermentas, Germany) according to the manufactures protocol. For efficient digestion, each sample was divided in 2 x 200 mg portions. The agarose was melted for 15 min at 70 °C, and digested with 2 U of agarase in each vial. Finally, the divided sample was reunited for genomic DNA extraction.

10 x PBS Buffer (Phosphate Saline Buffer)

NaCl	8.0 g
KCl	0.2 g
Na ₂ HPO ₄	1.42 g
Na ₂ HPO ₄ * 2H ₂ O	1.78 g
KH ₂ PO ₄	0.27 g
Volume was adjusted to 1L with Aqua _{dest}	

2.2.2 Evaluation of ARM and comparison of the bacterial communities of ARM, bulk soil, and the rhizosphere of *Arabidopsis thaliana*

Visual assessment of the bacterial community of ARM after one week of incubation was done by Fluorescence *In Situ* Analysis (FISH) as described in section 2.3.1.

Reproducibility, time dependent shifts of the bacterial communities of ARM and their comparison to bacterial populations of bulk soil and the rhizosphere of *A. thaliana* were performed as follows:

ARMs were prepared as described in section 2.2.1. Samples were taken after 2, 5, and 9 days of incubation. Each bulk soil sample consisted of 0.5 g of soil obtained from a pot without ARMs but also watered to keep WHC_{max} at 50 %. Each treatment was done in 5 replicates from independent pots. Bulk soil samples were taken after 0, 2, 5 and 9 days of incubation.

To evaluate if ARM enables the development of a bacterial community comparable to natural systems, it was compared to *Arabidopsis thaliana* Columbia 0. This plant was chosen, because it is a well studied model organism in plant biology, and has a relatively short life cycle as well as a high reproducibility. To grow *A. thaliana*, 300 g of the sieved soil from Scheyern was added to pots (9 x 9 x 9.5 cm) and 50 % of WHC_{max} was adjusted with tap water twice per week. The pots were incubated at room temperature (RT) with 12 h photoperiod per day. To yield enough rhizosphere material per sample for further analyses, 5 plants per pot were grown. Therefore, soil was covered with aluminium foil which had 5 holes to avoid moss and algae growth as seen in Figure 2.3. In each hole, several *A. thaliana* seeds were placed with sterile toothpicks. The pots were covered with plastic foil to maintain a high humidity until seeds germinated. Then, seedlings were removed that only 5 plants per pot remained. After approximately four weeks of growth (prior to flowering), *Arabidopsis* plants were harvested using sterile forceps soaked in 70 % ethanol. The rhizosphere samples were obtained by carefully pulling the plants out of the soil and cutting the above-ground plant from the roots. The roots were dipped in 1 x PBS Buffer to remove attached soil particles. One rhizosphere sample consisted of 0.3 g of *Arabidopsis* roots of several plants. In total, there were 5 replicates which were stored in a humidity chamber at 4 °C until further usage.

The bacterial communities of ARM, bulk soil, and the rhizosphere of *A. thaliana* were harvested and analyzed with T-RFLP based on 16S rRNA gene fragments (see section 2.3.5). Single high abundant 16S rRNA gene fragments derived from ARM were cloned and sequenced for species identification (see section 2.3.4).

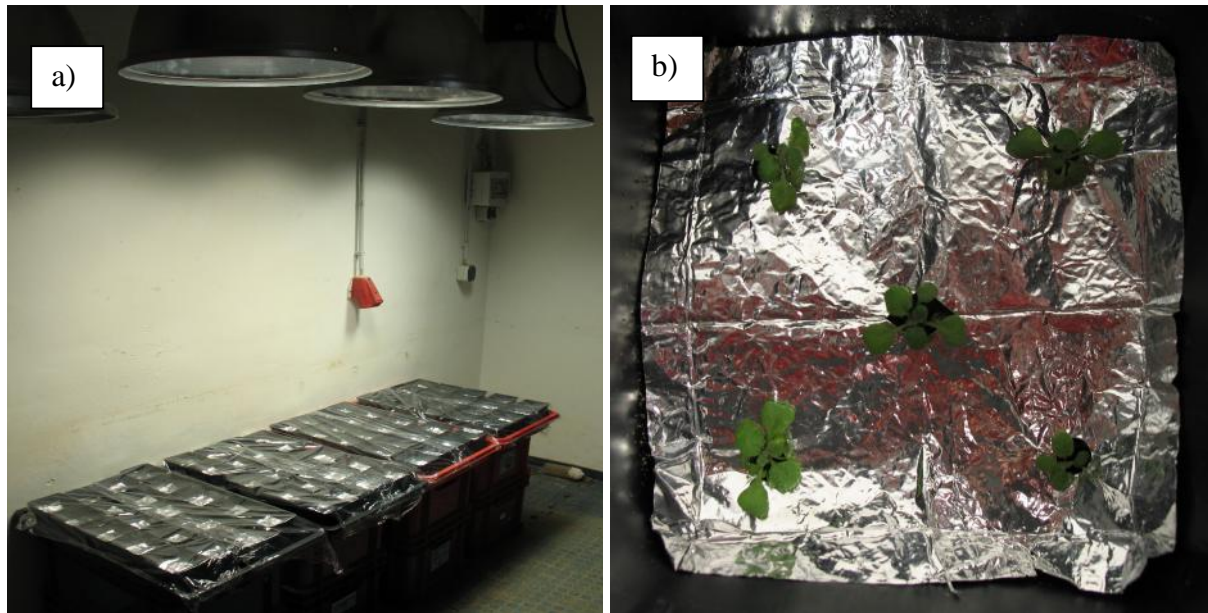


Figure 2.3 Growth conditions of *Arabidopsis thaliana* plants. A) Before seedling germination, the pots were covered with plastic foil to keep humid conditions. B) Soil was covered with aluminum foil to avoid moss and algae growth.

2.2.3 Influence of single root exudates on the structural development of the bacterial community of ARM

Plant root exudates serve as a nutrient source for microorganisms in soil and play therefore an important role in plant-microbial interactions. We tested the influence of the three main exudate component groups which have been reported to influence bacteria: carbohydrates (glucose) and organic acids (malic acid) as carbon sources, and amino acids (serine) as a nitrogen source.

The tested compound was omitted from the standard exudate mix of ARM, which resulted in three treatments: Glu(-), Mal(-), Ser(-), as shown in Table 2.3. As controls, glass slides were covered with the standard exudate mix (Ex) or only agarose (Co). The experiment took place in a room with a temperature of 14 °C in the dark. To detect changes in the temporal development of the bacterial communities, samples were taken after 2, 5 and 20 days of incubation of the ARM. The last sampling time point was chosen to detect how long bacteria are affected by the artificial exudates of the ARM. Molecular analysis of the bacterial communities of ARM were done by T-RFLP based on 16S rRNA gene fragments (see section 2.3.5)

Table 2.3 Composition of the exudate solutions to test the influence of single exudate compounds on the bacterial communities of ARM. Treatments were defined by the application of agarose and a) the standard exudate mix (Ex), b) without exudates (Co), c) the standard exudate mix without glucose (Glu(-)), d) malic acid (Mal(-)), or e) serine (Ser(-)) on ARM.

ARM Treatment	Carbohydrates			Organic acids		Amino acids		
	Fructose	Glucose	Sucrose	Malic acid	Succinate	Arginine	Cysteine	Serine
Ex	+	+	+	+	+	+	+	+
Co	-	-	-	-	-	-	-	-
Glu (-)	+	-	+	+	+	+	+	+
Mal (-)	+	+	+	-	+	+	+	+
Ser (-)	+	+	+	+	+	+	+	-

2.2.4 Influence of SDZ and manure on the structural development and abundance of SDZ resistance genes of the bacterial community of ARM

Antibiotics are common contaminants in arable soils as a result of manure application used as fertilizers to the fields. We tested the influence of the veterinary antibiotics sulfadiazine (SDZ) together with piggery manure on the bacterial community structure and the development of the bacterial SDZ resistance genes *sul1* and *sul2* in comparison to the total bacterial population by 16S rRNA genes.

Manure was obtained from an organic farm in northern Bavaria. To our knowledge, the pigs had not been treated with antibiotics. Manure was mixed with soil to a final concentration of 40 mg manure kg⁻¹ soil. Additionally, SDZ was applied to the soil (final concentration 10 mg SDZ kg⁻¹ soil). As controls, only piggery manure or water, respectively was applied to the soil. For preincubation, 300 g of the mixed soils were put in pots and kept in 14 °C in darkness and incubated for 30 days. WHC_{max} was kept at 50 % by irrigation with tap water. Bulk soil samples of the preincubated samples were taken before the ARMs were added to the soil. ARMs were covered with agarose and the standard exudate mix (section 2.2.1) and a SDZ-solution with a final concentration of 4.4 mg SDZ 100 ml⁻¹. Control ARMs were covered with agarose and the standard exudate mix (Ex) or without exudates (Co). After soil preincubation, the prepared glass slides were added to the pots and incubated for one or two weeks, respectively. The experimental work flow is shown in Table 2.4.

In a first experiment, the development of bacterial communities of ARM was analyzed by T-RFLP (see section 2.3.5). A high abundant 16S rRNA sequence of ARM and manure was cloned and sequenced for species identification (see section 2.3.4). In a second approach with the same experimental set up, 16S rRNA gene abundance and SDZ resistance gene abundances was analyzed using quantitative Real Time PCR (see section 2.3.7).

Table 2.4 Experimental set up to analyze the influence of SDZ and manure on the bacterial communities of ARM. ARMs were covered with agarose and a) SDZ and the standard exudate mix (SDZ + Ex), b) the standard exudate mix (Ex), and c) without exudates (Co).

Soil preincubation (30 days)	ARM treatment	Incubation time of ARM
SDZ + manure	SDZ + Ex	1 and 2 weeks
	Ex	
	Co	
manure	SDZ + Ex	1 and 2 weeks
	Ex	
	Co	
water	SDZ + Ex	1 and 2 weeks
	Ex	
	Co	

2.3 Molecular Analyses

2.3.1 Fluorescence *In Situ* Hybridization analysis (FISH)

To visualize the microbial communities on the surface of the ARM, a Fluorescent *In Situ* Hybridization analysis was performed after a protocol by Manz *et al.* (1992) and Amann *et al.* (1992). First, the ARMs were covered with agarose and the standard exudate mix. ARMs were incubated in soil for one week and irrigated with tap water twice per week to keep 50 % of WHC_{max}. Then, the agarose and the attached bacteria were harvested and washed in 1 x PBS. Then, the agarose was cut in pieces (diameter ca. 1 cm) and put on an epoxycoated glass slide. Samples were fixed with 4 % paraformaldehyde (PFA) or an ethanol/PBS solution (50:50). Fixation with PFA is suitable for Gram-negative bacteria, because it stabilizes their cell walls. For Gram-positive bacteria instead, ethanol is recommended as a fixation agent, because fixation with PFA would make their cell walls impermeable for FISH probes. The slides were incubated in closed petri dishes to keep the humidity for ca. 4 h at 4 °C. For hybridization, samples were dehydrated with increasing ethanol solutions (50 %, 80 %, and 100 %) and air dried. The agarose pieces were covered with prewarmed hybridization buffer containing the probes EUB388 mix (Eurofins MWG, Germany) consisting of an equimolar mix of the oligonucleotides EUB338-I, EUB338-II and EUB338-III labelled with the fluorescent dye Cy3 (red). To stain eukaryotic cells, the probe EUK516 labelled with Cy5 (blue) was used (see Table 2.5). Samples were incubated in humidity chambers at 46 °C for 2 h. Afterwards, the samples were washed with washing buffer prewarmed to 48 °C and incubated for 15 min at 48 °C. Then, the samples were rinsed with water and air dried. To avoid bleaching of the fluorescent dye, the samples were covered with Citifluor-AF1 (Citifluor Ltd. Canterbury, UK) and cover glass. Microscopic examination was performed with a confocal laser scanning microscope (Carl Zeiss, Germany).

Hybridization Buffer

5 M NaCl	360 µl
1 M Tris/HCl (pH 8.0)	40 µl
Formamid (35 %)	700 µl
Aqua _{dest}	900 µl
10 % (w/v) SDS	2 µl

Wash Buffer

5 M NaCl	700 µl
Tris/HCl pH 8.0	1 ml
0.5 M EDTA pH 8.0	500 µl
Aqua _{dest}	50 ml
10 % (w/v) SDS	50 µl

Table 2.5 Probes for FISH analysis of the microbial communities of ARM

FISH probe	Composition	Fluorescence dye	Reference
EUB338-I	5'-GCT GCC TCC CGT AGG AGT-3'	Cy3	Amann <i>et al.</i> 1990
EUB338-II	5'-GCA GCC ACC CGT AGG TGT-3'	Cy3	Daims <i>et al.</i> 1999
EUB338-III	5'-GCT GCC ACC CGT AGG TGT-3'	Cy3	Daims <i>et al.</i> 1999
EUK516	5'-ACC AGA CTT GCC CTC C-3'	Cy5	Amann <i>et al.</i> 1990

2.3.2 Extraction of genomic DNA

Genomic DNA of the microbial communities was extracted with the FastDNA Spin Kit for Soil (MP Biomedicals, Germany) according to the manufactures protocol. 0.5 g of bulk soil, 0.3 g of plant rhizosphere samples, or cell pellets derived from agarose digestion from ARM samples were used. DNA extracts were quantified and checked for purity with Nanodrop 1000 Spektrophotometer (Peqlab, Germany).

2.3.3 Gel electrophoresis

PCR reactions were analyzed by gel electrophoresis. 5 µl PCR reaction aliquots were loaded on a 1 % agarose gel with 1x TAE Buffer. The gels were stained with ethidium bromide and the amplicons were detected under UV light.

50x TAE Buffer

Tris base	242 g
EDTA	18.60 g
Glacial Acetic Acid	57.10 ml

2.3.4 Cloning and phylogenetic sequence analysis of 16S rRNA gene fragments

16S rRNA genes were amplified with PCR in a total volume of 50 µl. The primers used are listed in Table 2.6. To 1 µl DNA template, 0.2 µM of each of the universal eubacterial primer set B27f and 1401r, 2.5 U Taq Polymerase (Invitrogen, Germany), 1 x PCR Buffer, 2 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, and 0.3 % BSA were added. Each PCR cycle started with an initial denaturation step at 95 °C for 10 min and followed by 27 cycles consisting of a step at 94 °C for 1 min, annealing at 57 °C for 1 min and elongation at 72 °C for 90 s. Final elongation was done at 72 °C for 10 min.

For cloning of the 16S rRNA amplicons, the TA Cloning Kit (Invitrogen, Germany) was used according to the manufactures protocol. In summary, 16S rRNA gene amplicons were ligated in the pCR2.1 vector and products were transformed in OneShot® Competent Cells (Invitrogen, Germany). Bacterial cells were grown in SOC medium and the vectors containing 16S rRNA gene amplicons were isolated with the NucleoSpin® Plasmid Kit (Macherey-Nagel, Germany) following the manufactures protocol. After confirmation of the target insertion by *EcoR*I-digestion, a sequencing PCR was performed. A total volume of 10 µl contained 1 µM of Primer (M13 Forward, M13 Reverse or 610V), Big Dye Terminator, 1 x Big Dye Terminator Buffer, and 1 µl vector template. The sequencing PCR was performed on a T3 Thermocycler (Biometra, Germany) starting with an initial denaturation at 96 °C for 1 min, followed by 35 cycles of 96 °C for 15 s, 50 °C for 15 s and 60 °C for 4 min. The

amplicons were precipitated with ethanol and a sequencing reaction was performed on the ABI 3730 (Applied Biosystems, Germany). For further analyses, a consensus sequence was created and DNA sequences were analyzed with the basic local alignment search tool (BLAST) from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). For the phylogenetic analysis, an alignment was done using SILVA (Quast *et al.* 2013) and was further analyzed with the ARB software package (Ludwig *et al.* 2004). The phylogenetic tree was created using the Neighbor Joining method (Saitou and Nei 1987).

Table 2.6 Oligonucleotides for the sequencing PCR

Primer	Composition	Reference
B27f	5'-AGA GTT TGA TCC TGG CTC AG -3'	Orphan <i>et al.</i> 2001
1401r	5'- CGG TGT GTA CAA GAC CC-3'	Orphan <i>et al.</i> 2001
M13 Reverse	5'- CAG GAA ACA GCT ATG AC-3'	
M13 Forward (-20)	5'- GTA AAA CGA CGG-3'	
610R IV	5'- GTG CCA GCA GYY GCG-3'	

2.3.5 Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP)

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a molecular fingerprinting method widely used in environmental microbiology. This technique enables the analysis of microbial communities by a direct DNA-based PCR approach independent of cultivation. In principle (see Figure 2.4), the genomic DNA is extracted from environmental samples and genes of interest are amplified with a specific primer set. The 30S small ribosomal subunit consists of 16S rRNA which can be used as a genetic marker for total microbial community analysis because it is highly conserved among prokaryotes. One primer – usually the forward primer – is labelled with a fluorescent dye. After the amplification of the target gene by PCR, the amplicons are digested with a restriction enzyme which results in different DNA fragment sizes depending on the variable restriction sites of the bacterial taxa. Subsequently, DNA fragments are separated by size in a sequencing gel and the fluorescent-labelled 5'-end fragments are detected. Fragments are visualized in a peak profile depending on fragment length and relative fluorescence.

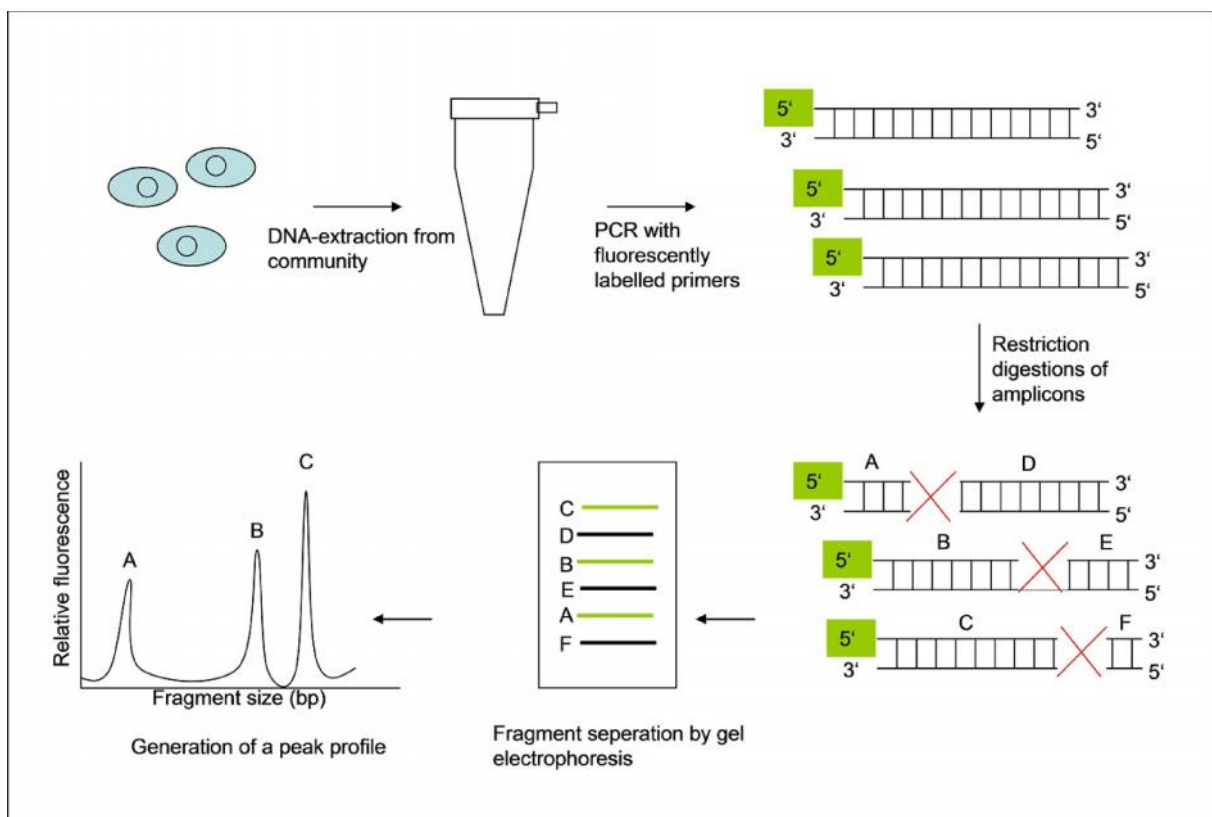


Figure 2.4 Schematic work flow of the Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP)

For the amplification of the bacterial 16S rRNA fragments, primer 63f-FAM and 783r (Sakai *et al.* 2004) were used (see Table 2.7). Each 100 μ l PCR reaction consisted of 100 ng DNA, 5U Taq Polymerase (Invitrogen, Germany), 0.2 μ M of each Primer, 1 x PCR Buffer, 2 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 0.3 % BSA. All PCR reactions were performed on a T3 Thermocycler (Biometra, Germany). Each PCR consisted of an initial denaturation step at 95 °C for 5 min, followed by 27 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 45 sec and elongation at 72 °C for 1 min. The final elongation was at 72 °C for 10 min. To confirm the specificity of the PCR, the amplicons were subjected to a gel electrophoresis with 5 μ l aliquots on a 1 % agarose gel and 1 x TAE buffer.

Table 2.7 Oligonucleotides for T-RFLP based on 16S rRNA genes

Primer	Composition	Reference
63f-FAM ^a	5'-CAG GCC TAA CAC ATG CAA GTC-3'	Marchesi <i>et al.</i> 1998
783r	5'-CTA CCV GGG TAT CTA ATC CBG-3'	Sakai <i>et al.</i> 2004

^a Primer was FAM-labeled at the 5'-end

16S rRNA amplicons were purified with the Qiaquick PCR Purification Kit (Qiagen, Germany) according to the manufacture protocol and quantified with Nanodrop 1000 Spektrophotometer (Peqlab, Germany). For the enzymatic digestion, 200 ng of purified 16S rRNA amplicons were added to a digestion reaction mixture (final volume 25 μ l) containing 5 U *Msp*1 (Fermentas, Germany) and incubated at 37 °C for 2 h. *Msp*1 was inactivated at 65 °C for 20 min. The enzymatic reaction was purified with the Minelute Reaction Clean Up Kit (Qiagen, Germany) following the manufacture protocol. 5 ng of the digested amplicons were added to 13 μ l of HiDi Formamide (Applied Biosystems, Germany) containing a 1:300 dilution of the MapMarker 1000 ROX Size standard (Bioventures, USA). After denaturation at 95 °C for 5 min, a capillary electrophoresis was carried out on an ABI 3730 (Applied Biosystems, Germany).

Primary electropherogram evaluation was performed with the GeneMapper 5.1 software (Applied Biosystems, Germany). All fragments with a peak height less than 100 relative fluorescence units or a relative abundance less than 1 % were considered as background noise. All peaks were aligned at a clustering threshold of 1 bp. To create a data matrix, T-Rex (T-RFLP analysis Expedited) software was used (Culman *et al.* 2009). The structural diversity of

the standardized T-RFLP profiles was described by richness (S), Shannon-Weaver diversity index (H) and evenness (E). Richness was the total number of T-RFLP profiles in sample.

The Shannon-Weaver diversity index was calculated as follows:

$$H' = - \sum (p_i)(\log p_i), \quad (1)$$

Where p is the proportion of an individual peak height relative to the sum of peak heights of one sample and p_i is the relative abundance of a fragment i .

Evenness was calculated as follows:

$$E = H/H_{\max}, \text{ where } H_{\max} = \log(S). \quad (2)$$

Cluster analysis of the T-RFLP data set was done by using a between-group analysis based on correspondence analysis (supervised method) under R circumstances with ade4 library (<http://pbil.univ-lyon1.fr/ADE-4/home.php?lang=eng>) kindly delivered by Dr. Gerhard Welzl. The between-group analysis aims to discriminate between sample groups and projects them in an ordinate system. The axes display the main principal components explaining the maximal variances between the sample groups of the given data set. Subsequently, the significance of the influence factors has to be tested with a permutation test.

2.3.6 Standard preparation for quantitative real time PCR (qPCR)

Plasmids R388 and RSF1010 were received from the German Collection for Microorganisms and Cell Cultures (DSMZ) in Braunschweig, Germany and were handled according to the manufactures protocol. Briefly, both *Escherichia coli* K12 strains harboring plasmid R388 or RSF1010 respectively, were inoculated in an overnight culture with LB-Medium containing Trimethoprim (final concentration: 50 µg / ml) for R388 and LB-Medium with Streptomycin (final concentration 25 µg / ml) for RSF1010. Overnight cultures were subjected to a plasmid extraction with the Nucleospin Plasmid Kit (Macherey-Nagel, Germany) according to the manufactures protocol. Plasmids containing the 16S rRNA gene were obtained from the laboratory.

LB Medium

Peptone 10 g

NaCl 10 g

Yeast Extract 5 g

Volume was adjusted to 1 L and pH 7.5

Table 2.8 Standards used for quantitative real time PCR targeting 16S rRNA genes and *sul* genes

Plasmid	Microorganism	Gene	Source
R388	<i>E.coli</i> K12	<i>sul1</i>	DSMZ
RSF1010	<i>E.coli</i> K12	<i>sul2</i>	DSMZ
pCR2.1	<i>E.coli</i> K12	16S rRNA gene	<i>Clavibacter michiganensis michiganensis</i>

2.3.7 Quantitative real time PCR (q RT PCR)

Quantitative real time PCR (q RT PCR) is a method combining an ordinary PCR with the quantification of a target gene. The SYBR GREEN based qPCR approach includes the fluorescent dye SYBR GREEN which binds only to double stranded nucleic acids strains and therefore allows the detection and quantification of the accumulated amplicons. In principle, the range of detection threshold has to be determined with a series of diluted standard DNA amplicons. This enables the generation of a standard curve to which the detected amplicons of the samples can be compared and the amount of amplicons can be calculated. The efficiency of the PCR reaction is calculated using the slope of standard curve. The reaction cycle at which the amplification curve trespasses the baseline is the so-called threshold cycle (C_T). This is a relative measure for the initial amount of DNA present in the samples. During the exponential phase of the PCR, there is an optimal proportion between DNA template and reaction reagents which leads to a duplication of the amount of template at each cycle. Thereafter, the PCR reaction reaches a plateau when all substrate is depleted.

Quantification of SDZ resistance genes *sul1* and *sul2* was performed with a SYBR GREEN-based q RT PCR. First, the optimal template concentration was determined to avoid PCR-inhibition by co-extracted inhibiting substances, for example humic acids. Primers for *sul1* and *sul2* were applied as described by Heuer and Smalla (2007) shown in Table 2.9. For amplification of *sul1*, primer sulF1 and sulR1 and as standard, plasmid R388 (DSMZ, Germany) were used. The gene *sul2* was targeted by the primer pair sulF2 and sulR2 and as standard the plasmid RSF1010 (DSMZ, Germany). For both target genes, each reaction mix contained 0.2 μ M of each primer, 50 % SYBR[®] Green PCR Master Mix containing SYBR Green I Dye, AmpliTaq Gold[®] DNA Polymerase, dNTPs with DUTP, passive reference and optimized buffer components (Applied Biosystems, Germany), 0.3 % BSA and 2 μ l of the diluted DNA templates in a total volume of 25 μ l. For both genes, an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s. Each PCR ended with one final cycle of 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Bacterial 16S rRNA genes were targeted with primers FP 16S rDNA and RP 16S rDNA by Bach *et al.* (2002). Primers were added at 0.4 μ M each to the reaction mix containing 50 % SYBR Green, 0.3 % BSA and 2 μ l of the diluted DNA template. The PCR reaction started with an initial denaturation step for 10 min at 95 °C, then 40 cycles for 20 s at 95 °C and 1 min at 62 °C. One final cycle followed at 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s.

All reactions were performed on a 7300 Real Time PCR System (Applied Biosystems, Germany).

Table 2.9 Oligonucleotides for quantitative real time PCR

Target gene	Primer	Composition	Reference
<i>sul1</i>	sulF1	5'-CTG AAC GAT ATC CAA GGA TTY CC-3'	Heuer and Smalla 2007
	sulR1	5'-AAA AAT CCC ATC CCC GGR TC-3'	
<i>sul2</i>	sulF2	5'-CTC AAT GAT ATT CGC GGT TTY CC-3'	Heuer and Smalla 2007
	sulR2	5'- AAA AAC CCC ATG CCG GGR TC-3'	
16S rRNA gene	FP 16S rRNA	5'- GGT AGT CYA YGC MST AAA CG-3	Bach <i>et al.</i> 2002
	RP 16S rRNA	5'- GAC ARC CAT GCA SCA CCT G-3	

Quantitative real time PCR runs were analyzed with 7300 System SDS Software (Applied Biosystems, Germany). For amplification specificity, the melting curve was checked for the presence of a single peak. The efficiency of each q RT PCR run was calculated based on the slope of the standard curve as follows:

$$E = (10^{-1/\text{slope}} - 1) * 100$$

Further statistical analyses were performed using SPSS Statistics software (IBM SPSS, USA). First, variables were tested for normal distribution and homogeneity of variances by Kolmogorov-Smirnov test and the Levene test. If normal distribution of the data was not given, log-transformation was performed. To evaluate significant differences among tested factors (significance level: $p < 0.05$), univariate ANOVA and Post-hoc tests were calculated.

3 Results

3.1 Analysis of the microbial community of ARM by FISH

In this thesis, a model was developed to study plant-microbial interactions at the plant-soil interface. The artificial rhizosphere model (ARM) consisted of glass slides coated with agarose and an artificial exudate mix to attract soil microorganisms. In the first part of the thesis, the ARM was evaluated. Microbial communities attached to the ARM were analyzed by Fluorescence *In Situ* Hybridization analysis (FISH). The images revealed that bacterial cells (red) and eukaryotic cells (blue) were present on the surface of ARM after 1 week of incubation in soil. Gram-negative bacteria which were fixed with PFA were mainly rod shaped (Figure 3.1a). To target Gram-positive and Gram-negative bacteria, samples were prepared with ethanol as a fixation agent. This preparation revealed the presence of filamentous bacterial cells on the ARM (Figure 3.1b). Besides the bacterial colonization of the surface of ARM, fungal hyphae (in blue) were also present.

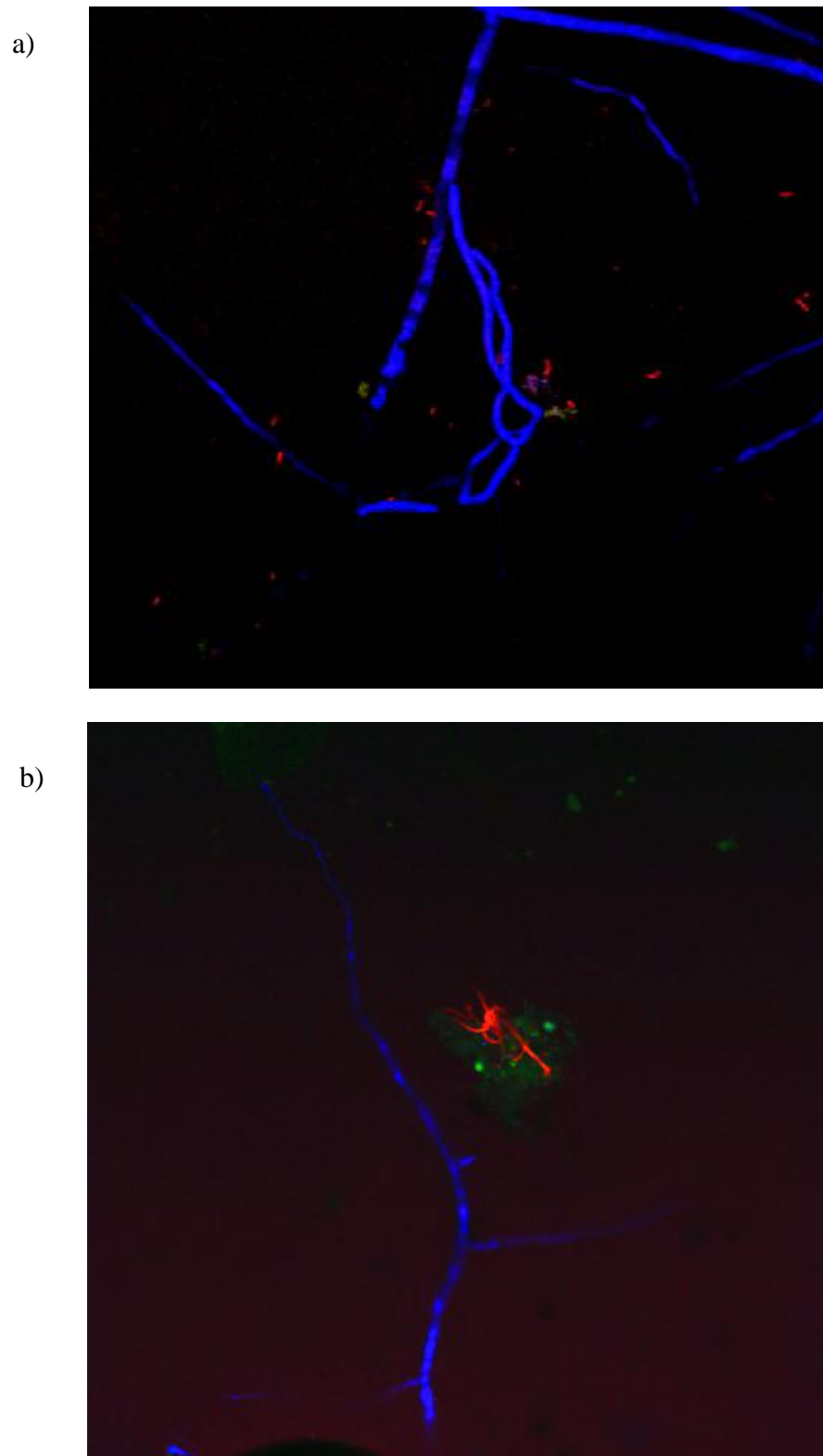


Figure 3.1 FISH analysis of the surface of ARM. The rRNA probes EUB338-cy3 mix (red) for bacterial cells and EUK516-cy5 for eukaryotes (blue) were used. Cell fixation was performed with a) PFA for Gram-negative bacteria and b) ethanol/PBS solution for Gram-positive and Gram-negative bacteria of ARMs.

3.2 Analysis of the bacterial community of ARM by T-RFLP

To analyze the development of the bacterial community structure attached to the surface of the ARM, the agarose on the glass slides was harvested after 2, 5, and 9 days of ARM-incubation in soil. In the first step, genomic DNA was extracted from the agarose and levels significantly increased ($p < 0.05$) with proceeding incubation time of the ARM from 247 to 430 ng per fresh weight of agarose (Figure 3.2).

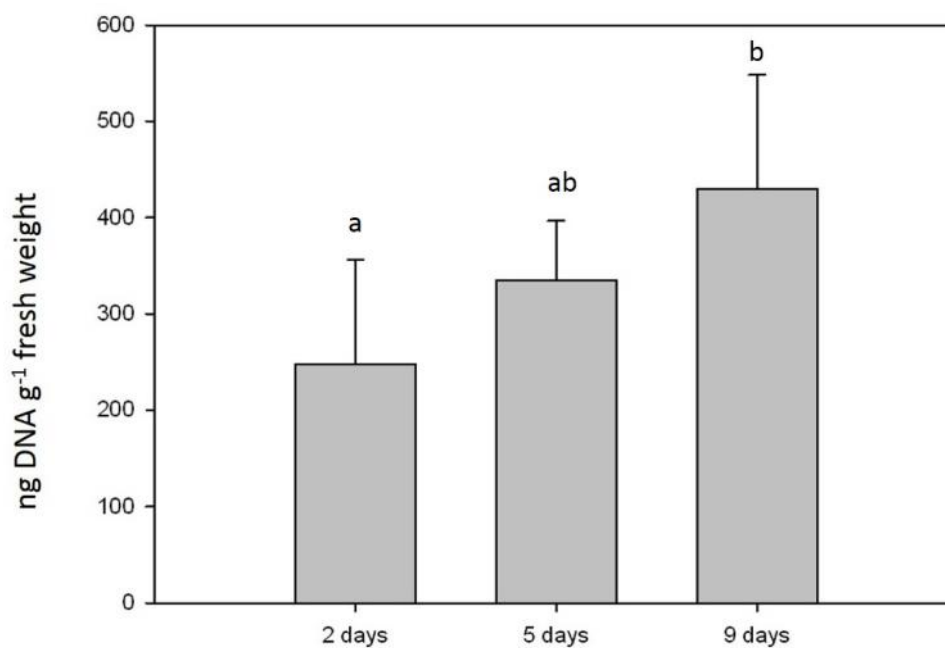


Figure 3.2 Concentration of the genomic DNA extracted from ARM. Samples were taken after 2, 5 and 9 days of incubation. Fresh weight refers to agarose harvested from the ARMs. Error bars represent standard deviation of means ($n = 5$). Significant differences ($p < 0.05$) are indicated by small letters.

Subsequent, the bacterial community attached on the ARM was analyzed by the molecular fingerprinting analysis T-RFLP. This method based on 16S rRNA genes includes PCR, enzymatic digestion, DNA purification, and gel electrophoresis. In the first step, genomic DNA extracts of ARM were conducted to a PCR targeting 16S rRNA genes and PCR specificity was confirmed by an agarose gel electrophoresis. From all ARM samples, bands were of similar thickness and at the expected size of 708 bp (see Figure 3.3).

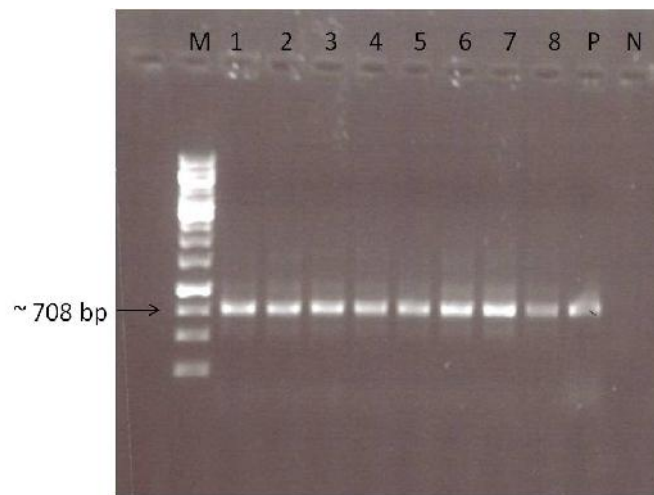


Figure 3.3 Gel electrophoresis of PCR-amplified 16S rRNA gene fragments of ARM. Lanes: M = 1kb molecular weight marker, 1-8 = ARM samples, P = positive control (genomic DNA of *Achromobacter denitrificans*), N = negative control (water).

To evaluate the reproducibility of the bacterial populations colonizing the ARM, T-RFLP peak profiles of the replicates ($n = 5$) at each sampling time point were compared (see Figure 3.4) and revealed high agreement: after 2 days of incubation, 19 TRFs of a total of 23 TRFs were common in at least 3 replicates; after 5 days of ARM incubation 19 of 21 TRFs were present in minimum 3 replicates and after 9 days of incubation 21 of 24 TRFs were detected in at least 3 replicates.

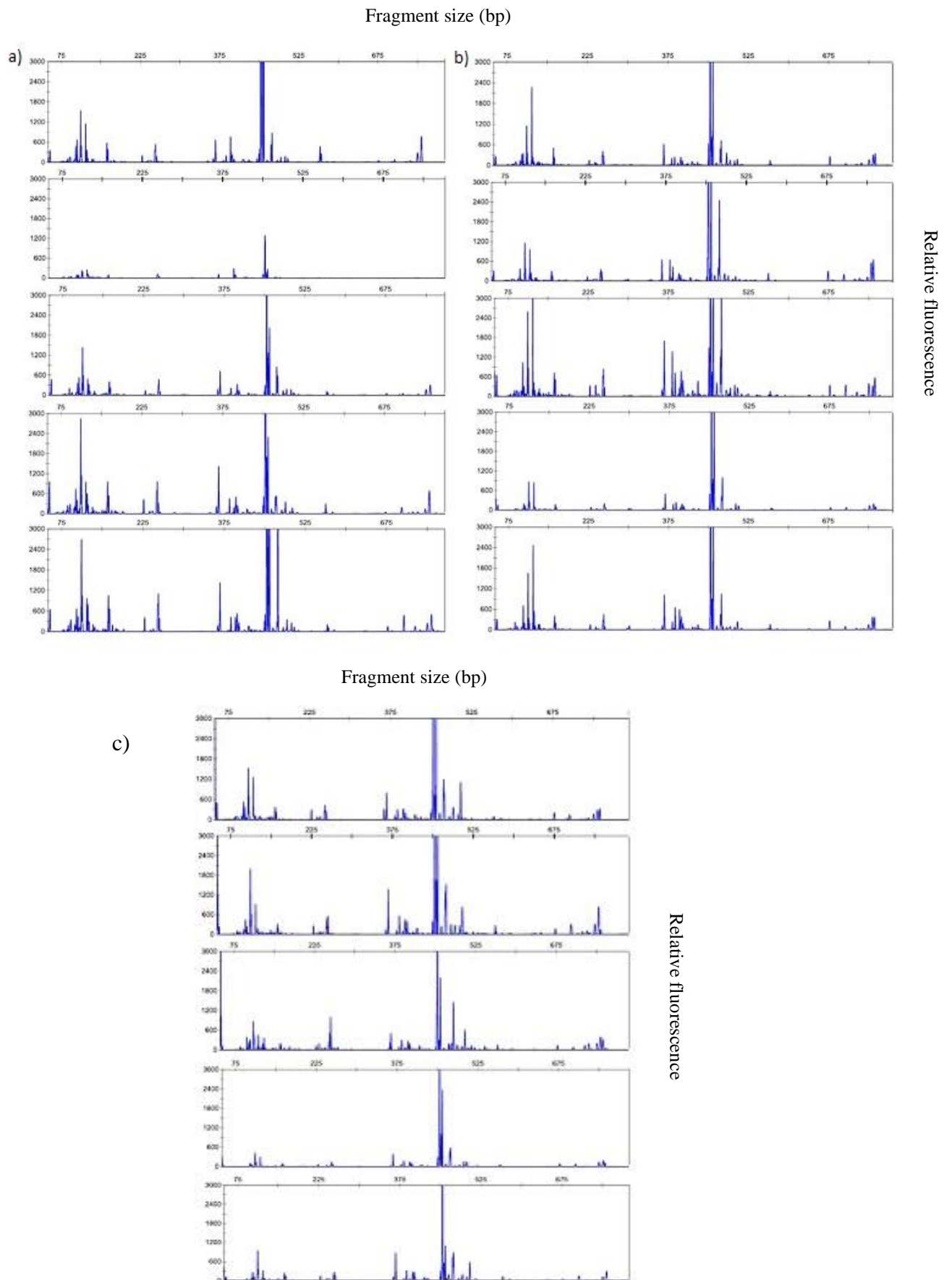


Figure 3.4 Comparison of the bacterial community of ARM-replicates. Peak profiles were based on the T-RFLP data set (16S rRNA genes) digested with *Msp*I. For each sampling time point 5 replicates were done. ARMs were incubated for a) 2 days, b) 5 days, and c) 9 days of incubation.

To detect structural changes in the bacterial community of the ARM between 2, 5, and 9 days of incubation, TRF patterns were compared. Overall, 3 dominant TRFs (more than 5 % relative abundance) were present at each sampling time point (see Figure 3.5). TRF 454B was the dominant TRF at each sampling time point with a relative abundance between 28 to 40 %. TRF 112B remained relatively constant at a level of 9-12 % and TRF 459 increased with incubation time (relative abundance: 11-28%). TRF 474 was only high abundant at day 2 and 5 and decreased at day 9 below 5 %. TRF 457 was high abundant only at day 2 and TRF 121 only at day 5. All TRFs determined high abundant at one sampling time point were detected in all ARM samples, however they not always reached the threshold of 5 %.

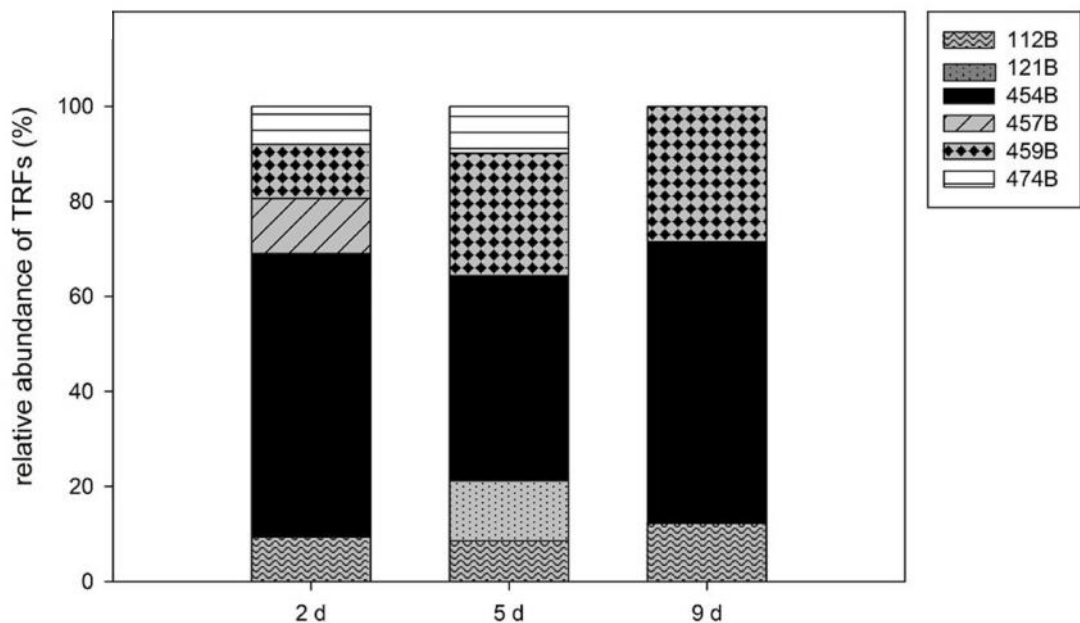


Figure 3.5 Development of the bacterial community structure of ARM. Relative abundance of major TRFs (relative abundance > 5 %) of T-FRLP analysis based on 16S rRNA genes of the ARM harvested after 2, 5 and 9 days of incubation.

3.3 Assignment of the high abundant TRF of ARM by phylogenetic sequence analysis

To assign the dominant TRF 454B of ARM, a cloning experiment with subsequent phylogenetic analysis was performed based on 16S rRNA genes. After the amplification of 16S rRNA gene fragments of the extracted genomic DNA of ARM samples, the PCR products were ligated into the vector pCR2.1. To confirm that the vector contained the 16S rRNA gene fragment with the correct size, an *EcoR1* digestion and a gel electrophoresis were performed. Clone 1, 6 and 17 carried the correct 16S rRNA fragment of the size of ca. 1400 bp as seen in Figure 3.6.



Figure 3.6 Gel electrophoresis of pCR2.1 vectors containing 16S rRNA gene fragments of ARM after *EcoR1* digestion. Lanes: M = 1 kb molecular weight marker, 1-20 = samples.

After sequencing, the clones which carried the vector pCR2.1 with the 16S rRNA amplicon insert were conducted to T-RFLP to assure the right peak size (454 bp) of the cloned 16S rRNA gene fragment (see Figure 3.7).

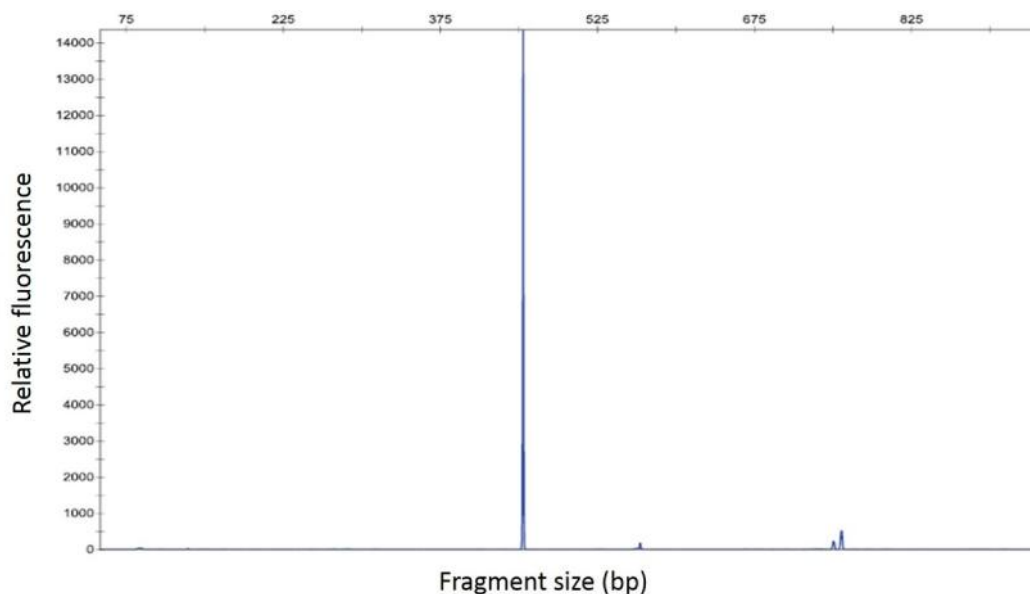


Figure 3.7 T-RFLP peak profile of the high abundant 16S rRNA gene fragment of ARM cloned into pCR2.1 vector for sequence analysis. DNA fragment was amplified (primer set 63f-FAM, 783r) and digested with *Msp1*. The dominant peak has a size of 454 bp.

The sequence of the 16S rRNA gene fragment (assigned to TRF 454B) was conducted to a phylogenetic analysis. The calculated phylogenetic tree (see Figure 3.8) revealed that the analyzed sequence (ARB_1B083E4D) showed a high similarity to species of the genus *Pseudomonas*.

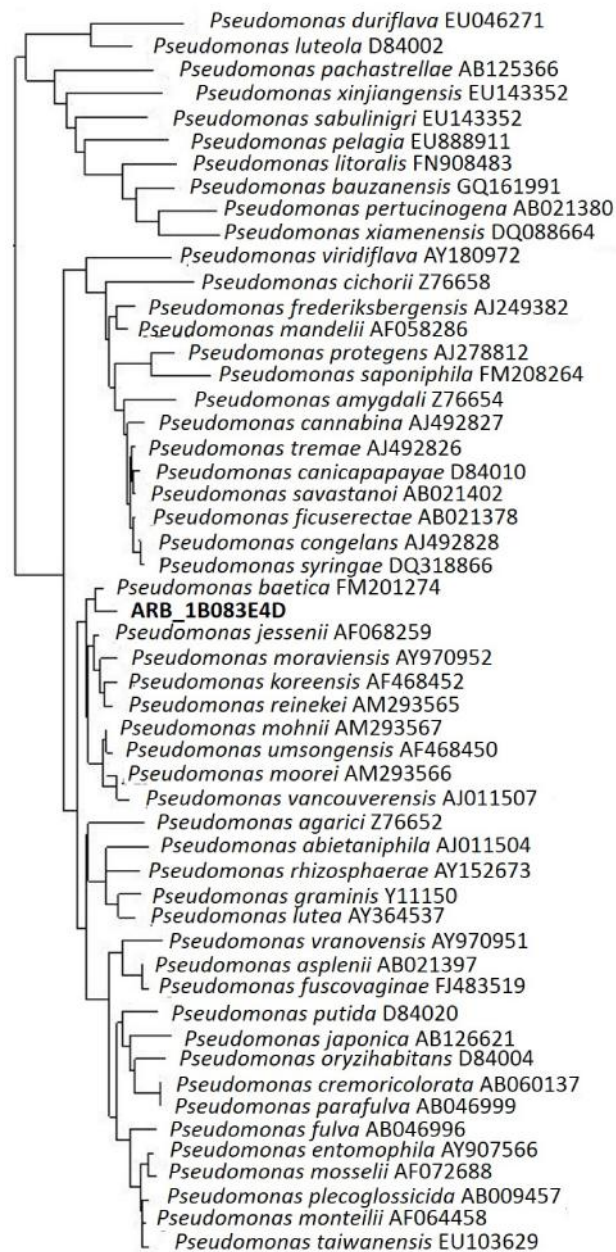


Figure 3.8 Neighbor Joining tree of the high abundant 16S rRNA gene fragment of ARM. The analyzed sequence ARB_B858C6EA had a length of ~1400 bp. The bar represents one nucleotide substitution per 100 nucleotides.

3.4 Comparison of the bacterial communities of ARM, bulk soil, and the rhizosphere of *Arabidopsis thaliana*

To evaluate the bacterial community attached on the surface of ARM, it was compared to the microflora of bulk soil and *Arabidopsis thaliana* Col0 rhizosphere by T-RFLP based on 16S rRNA genes. Samples of ARM and bulk soil were taken after 2, 5, and 9 days. Bulk soil was additionally sampled at the beginning of the experiment (day 0). The rhizosphere of *A. thaliana* was harvested after 30 days of growth prior to flowering.

At every sampling time point (2, 5, and 9 days), TRF richness, diversity, and evenness were lower in ARM samples than in bulk soil (see Table 3.1). In the rhizosphere of *A. thaliana*, species richness, diversity, and evenness values in the range of bulk soil at day 0 or day 9, respectively.

Table 3.1 Comparison of the biodiversity parameters of bacterial communities of ARM, bulk soil, and the rhizosphere of *A. thaliana*. Richness (S) with standard deviation, Shannon diversity index (H') and evenness (E) were determined based on the data set of 16S T-RFLP of the ARM. Sampling of ARM was done after 2, 5 and 9 days of incubation and bulk soil additionally at the beginning of the experiment (0 days). *A. thaliana* rhizosphere was harvested after 30 days of growth. All samples were done in 5 replicates.

Incubation (days)	Sample type	S	H'	E
0	Bulk soil	29 (\pm 5)	3.2	0.94
2	ARM	23 (\pm 4)	2.2	0.71
	Bulk soil	24 (\pm 0)	2.8	0.88
5	ARM	21 (\pm 2)	2.4	0.80
	Bulk soil	26 (\pm 5)	2.9	0.88
9	ARM	24 (\pm 4)	2.7	0.83
	Bulk soil	29 (\pm 2)	3.1	0.92
30	<i>A. thaliana</i>	30 (\pm 4)	3.1	0.93

In the following part, the T-RFLP profiles of the bacterial community of the different systems were compared. As an example, we chose ARM and bulk soil samples after 9 days of incubation, because we assumed that at this sampling time point the bacterial population should be well established. Samples of the rhizosphere of *A. thaliana* were harvested prior to flowering. TRF pattern looked more similar between ARM and *A. thaliana* than ARM and bulk soil (see Figure 3.9). The peak with the size of 302 bp (TRF 302B) was only present in the *Arabidopsis* sample, but not in ARM and was assigned to plant-mitochondrial DNA.

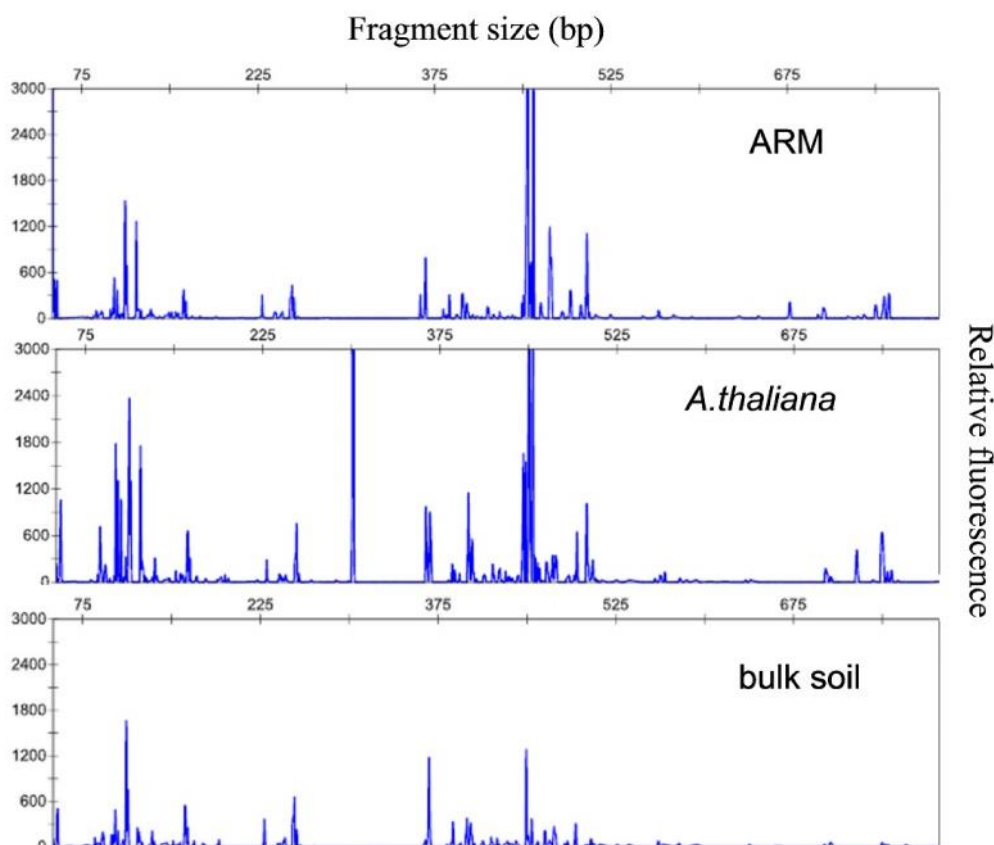


Figure 3.9 Comparison of the bacterial communities of ARM, the rhizosphere of *A. thaliana*, and bulk soil by T-FRLP peak profiles. Peak profiles of T-FRLP data set based on 16S rRNA genes derived from ARM and bulk soil samples after 9 days of incubation, and *A. thaliana* prior to flowering after 30 days of growing.

Taking a closer look on single TRFs present in the different systems, patterns of the dominant TRFs of ARM, bulk soil, and the rhizosphere of *A. thaliana* were analyzed. These patterns revealed differences between their bacterial community structures (see Figure 3.10). Only TRF 112B was high abundant in all samples. TRF 454B was the dominant TRF of ARM (28-40 % of relative abundance) and high abundant in *A. thaliana* rhizosphere (6.5 % of relative abundance). In bulk soil, this TRF was high abundant at the beginning of the experiment, but then decreased below 5 % of relative abundance. TRF 459B of ARM was also detected in bulk soil and the rhizosphere of *A. thaliana*, however this TRF was low abundant, whereas TRF 457B and 474B of the ARM were not detectable in bulk soil or *A. thaliana*. On the other hand, the high abundant TRFs (54B, 103B, 113B, 451B) of *A. thaliana* rhizosphere were all detected in ARM, but below 5 %. TRF 101B was not present in ARM samples. In comparison with bulk soil, all high abundant TRFs except TRF 253B and 450B were also detected in ARM, but below 5 % of relative abundance.

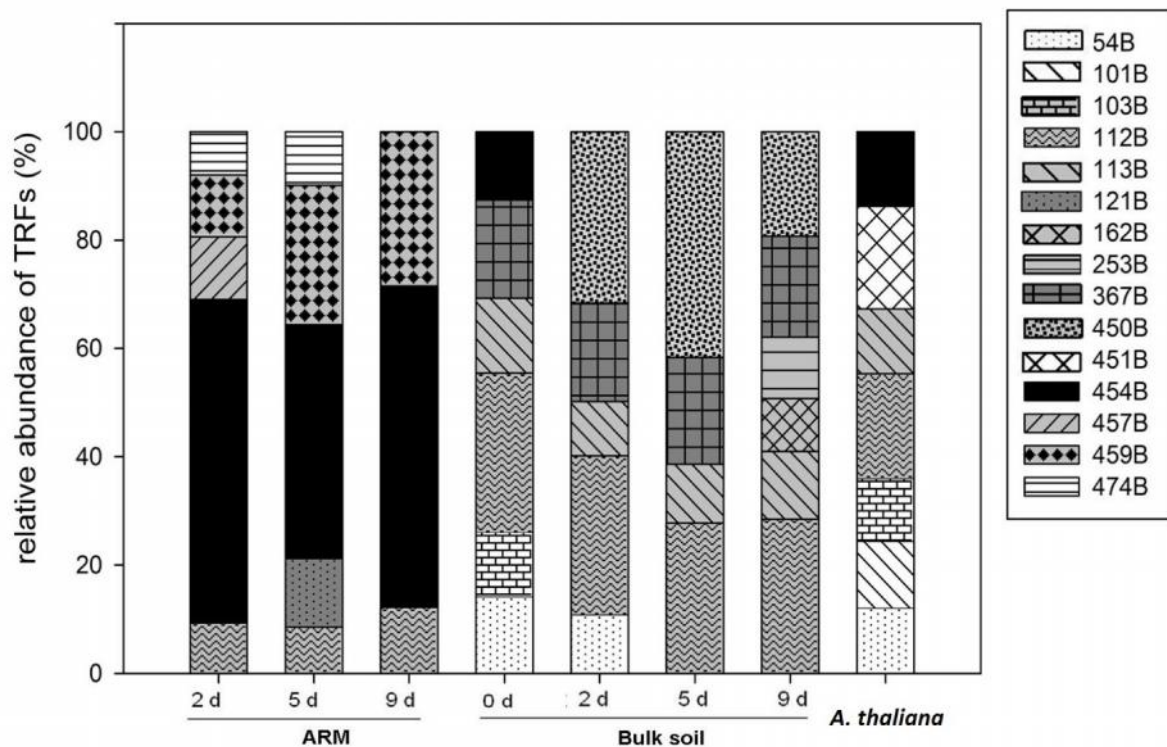


Figure 3.10 Comparison of TRF diversity patterns of ARM, bulk soil, and the rhizosphere of *A. thaliana*. Relative abundance of major TRFs (>5 % of relative abundance) of T-RFLP analysis based on 16S rRNA genes of the ARM at different incubation time points (2, 5, 9 days) in comparison with bulk soil (0, 2, 5, 9 days) and *A. thaliana* rhizosphere (prior to flowering).

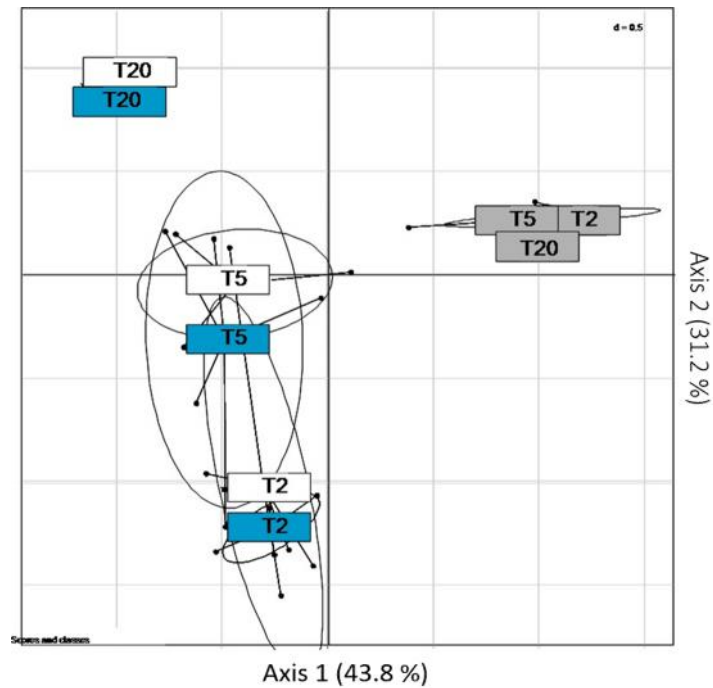
3.5 Influence of single root exudates on the structural development of the bacterial community structure of ARM

Root exudates are key drivers for plant-microbial interactions. In the following experiment, we investigated the influence of root exudates on the bacterial community in the rhizosphere by omitting single compounds of the standard exudate mix of the ARM. We chose following compounds which have been reported to influence bacterial cells: glucose (Glu(-)ARM) and malic acid (Mal(-)ARM) as representatives of carbohydrates and organic acids, and serine (Ser(-)ARM) as a representative of amino acids. We expected omitting glucose to have a strong effect on the bacterial population because this compound is utilized by a wide variety of microorganisms and hence an important energy source for soil microorganisms. As a representative for organic acid we chose malic acid as it was reported to attract plant growth promoting bacteria (Rudrappa *et al.* 2008b). For the amino acid serine, Nikata *et al.* (1992) demonstrated a chemotactic response of bacteria towards this compound. ARMs covered with the full standard exudate mix and with agarose without artificial exudates served as controls. Additional to the incubation of ARM for 2 and 5 days, the incubation period was prolonged and samples were taken after 20 days to investigate how long exudates affect bacterial populations. After the harvest, bacterial community structure was analyzed by T-RFLP based on 16S rRNA.

3.5.1 Omitting carbon sources

In this experiment, the carbon sources glucose and malic acid were omitted from the standard exudate mix, resulting in Glu(-)ARM and Mal(-)ARM. T-RFLP data sets of the ARM treatments were analyzed by a between group analysis (BGA) which explained by the first two components 75 % of the variances (see Figure 3.11). The treatments with exudates showed a time dependent community shift, whereas the control samples (CoARM) clustered at any sampling time point and were significantly different from the other treatments. Glu(-)ARM clustered with ExARM at all sampling time points (2 days, 5 days and 20 days) as shown in Figure 3.11a. After 2 days of incubation, Mal(-)ARM was separated significantly ($p < 0.05$) along the second component axis from the standard as shown in Figure 3.11b. With ongoing incubation time, Mal(-)ARM clustered again with the Glu(-) and ExARM. After 20 days of incubation, all ARMs except CoARM clustered to a higher degree than at the earlier sampling time points.

a) Glucose



b) Malic acid

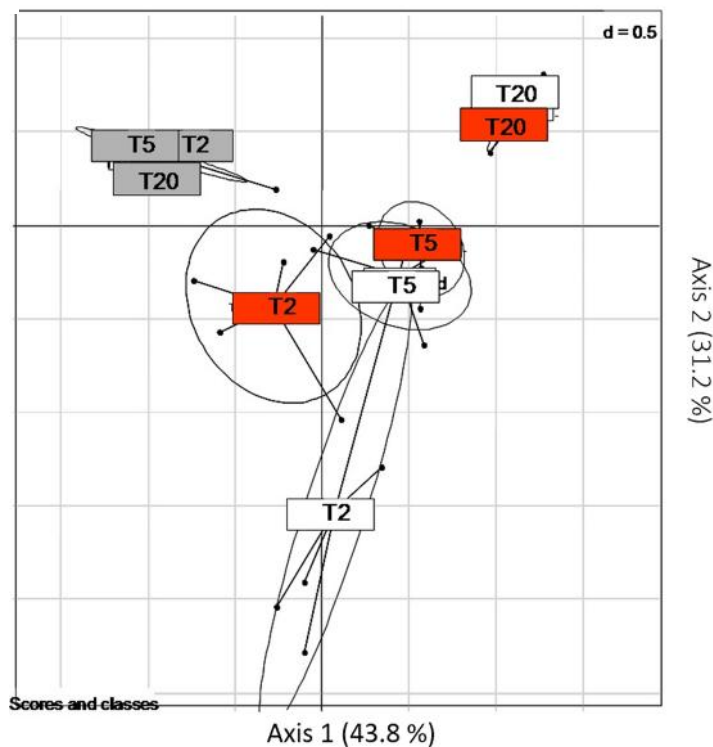


Figure 3.11 Influence of omitting carbon sources on the development of bacterial community structure of ARM. Between group analysis based on correspondence analysis of the T-RFLP data set based on 16S rRNA gene fragments of the ARM. Symbols resemble ARM covered with agarose and different exudate mixtures: the standard exudate mix without glucose (Glu(-)ARM in blue) or malic acid (Mal(-)ARM in red), the standard exudate mix ExARM (white) and the control without exudates (CoARM in grey). ARMs were incubated for 2 days (T2), 5 days (T5) and 20 days (T20). All samples were done in 5 replicates.

Regarding the abundances of single TRFs of the ARM treatments, there was a higher similarity between Glu(-), Mal(-), and ExARM than to CoARM at every sampling time point (see Figure 3.12). Over the sampling period of 20 days, TRF 454B was the dominant TRF in all treatments with exudates, however it was low abundant in CoARM (except on day 2). After 2 days of incubation, there were 6-7 dominant TRFs (more than 5 % relative abundance) detectable, whereas after 5 and 20 days, there were only 3 TRFs present which were high abundant in Glu(-), Mal(-), and ExARM.

Glu(-)ARM and ExARM were highly similar at every sampling time point, however there were different abundances of single TRFs. When ARMs were incubated for 2 days, TRF 113B was high abundant in Glu(-)ARM, but below 5 % of relative abundance in ExARM. After 5 days of incubation, except for TRF 482B, Glu(-)ARM and ExARM were similar in their TRF pattern, in contrast to CoARM. Interestingly, TRF 505B appeared after 20 days of incubation in ExARM and Glu(-)ARM (relative abundance 30 %), but this TRF was low abundant in CoARM.

Regarding Mal(-)ARM, this ARM treatment showed a higher similarity to CoARM than to ExARM after 2 days of incubation. Regarding total TRF spectrum, 20 TRFs were detected both in Mal(-)ARM and CoARM, whereas 18 TRFs were both present in Mal(-) and ExARM. In comparison, Glu(-)ARM had at the same sampling time point 22 TRFs in common with ExARM and only 13 TRFs with CoARM. Regarding high abundant TRFs, TRF 112B and 367B were both high abundant in Mal(-)ARM and CoARM, but not in ExARM after 2 days of incubation. After 5 and 20 days of incubation, the pattern of high abundant TRFs of Mal(-)ARM was highly similar to ExARM.

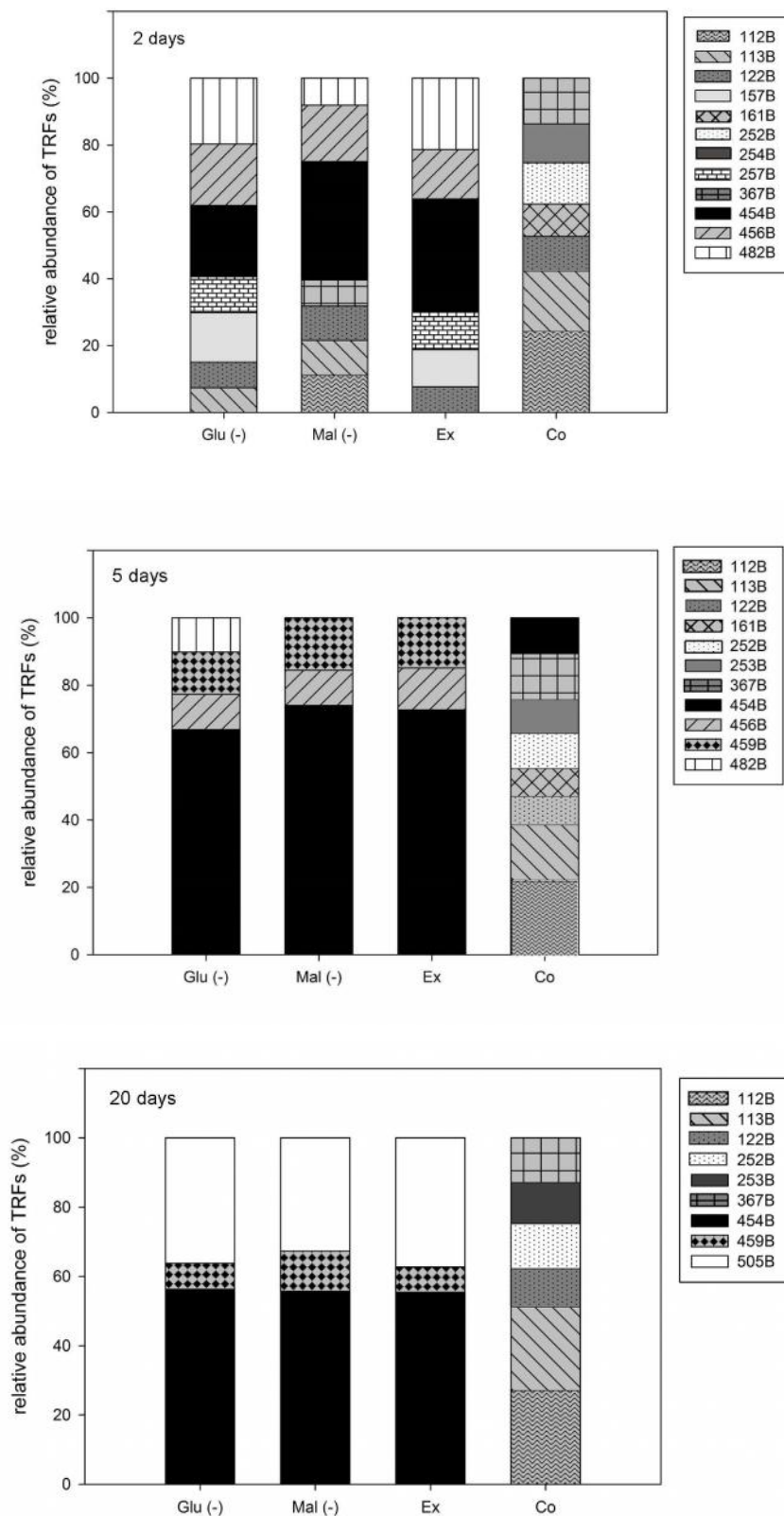


Figure 3.12 Influence of omitting carbon sources on TRF diversity patterns of ARM. Relative abundance of major TRFs (> 5 % of relative abundance) of T-RFLP analysis based on 16S rRNA genes of the ARM covered with agarose and different exudate mixtures: the standard exudate mix without glucose (Glu(-)), without malic acid (Mal(-)), the standard exudate mix (Ex), without exudates (Co)). ARMs were incubated for 2, 5 and 20 days.

3.5.2 Omitting nitrogen sources

To investigate the influence of nitrogen sources on the bacterial community at the plant-soil interface, the amino acid serine was subtracted of the standard exudate mix (Ser(-)ARM). A between group analysis of the T-RFLP data set based on 16S rRNA gene fragments explained with the first two components 74.5 % of the variances (see Figure 3.13). Ser(-)ARM and ExARM samples showed a time dependency shift, whereas the control samples clustered at any sampling time point. There were no significant differences detectable between ExARM and Ser(-)ARM treatment at any time point. Ser(-)ARM and ExARM clustered at 5 days of incubation and there was a minor shift between Ser(-)ARM and ExARM after 20 of incubation.

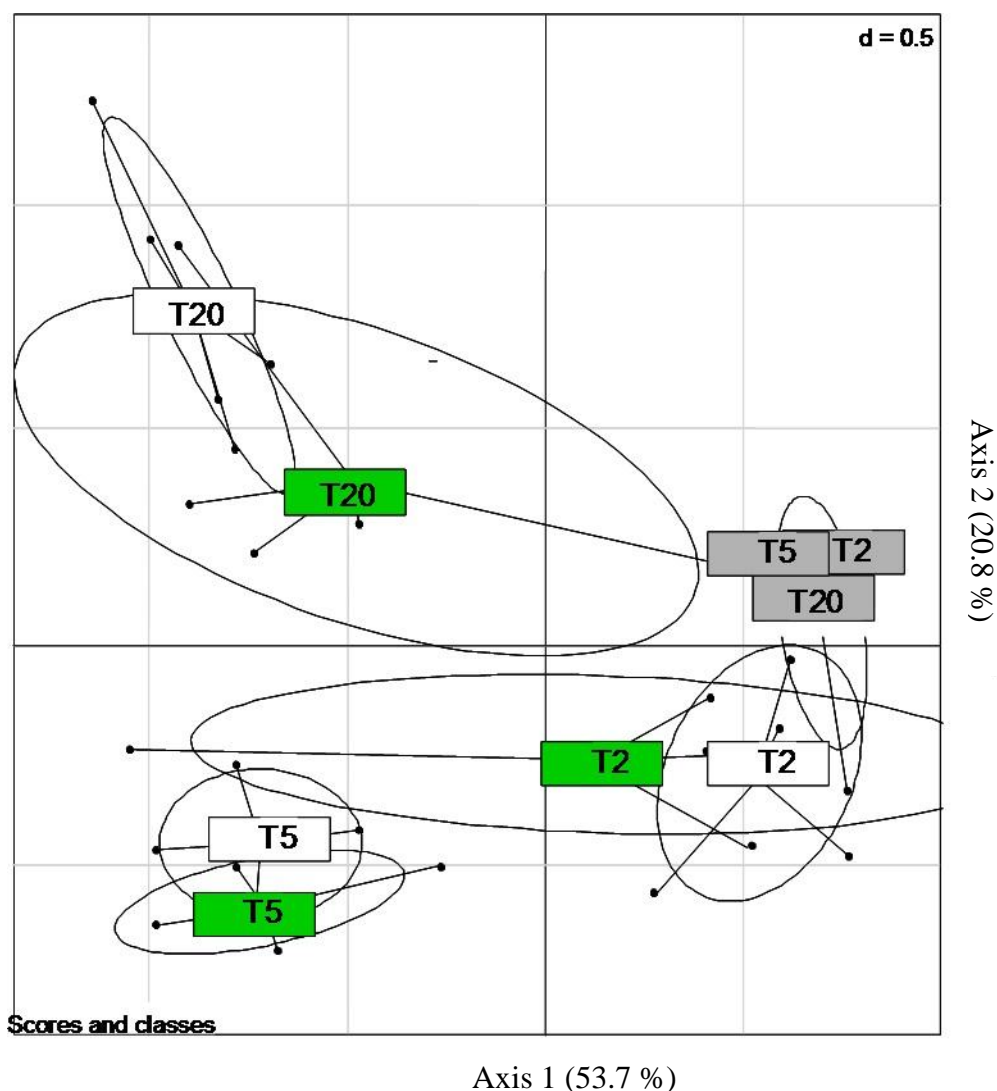


Figure 3.13 Influence of omitting a nitrogen source on the development of bacterial community structure of ARM. Between group analysis based on correspondence analysis of the T-RFLP data set based on 16S rRNA gene fragments of the ARM. Symbols resemble ARM covered with agarose and different exudates mixtures: the standard exudate mix without serine (green), the standard exudate mix (white) and without exudates (grey). ARMs were incubated for 2 days (T2), 5 days (T5) and 20 days (T20). All samples were done in 5 replicates.

Regarding dominant bacterial TRFs, Ser(-)ARM and ExARM were similar in TRF patterns and different to CoARM (see Figure 3.14). TRF 454B was dominant (between 26-40 %) in Ser(-)ARM and ExARM at every sampling time point, however it was below 5 % of relative abundance in CoARM. TRF 459B increased after 5 days of incubation (ca. 20 % of relative abundance) and TRF 505B appeared after 20 days of incubation (9-16 % of relative abundance) in both Ser(-)ARM and ExARM. Ser(-)ARM and CoARM were highly different TRF patterns in abundance and presence, for example TRF 456B and 459B were only detected in Ser(-)ARM, but not in CoARM.

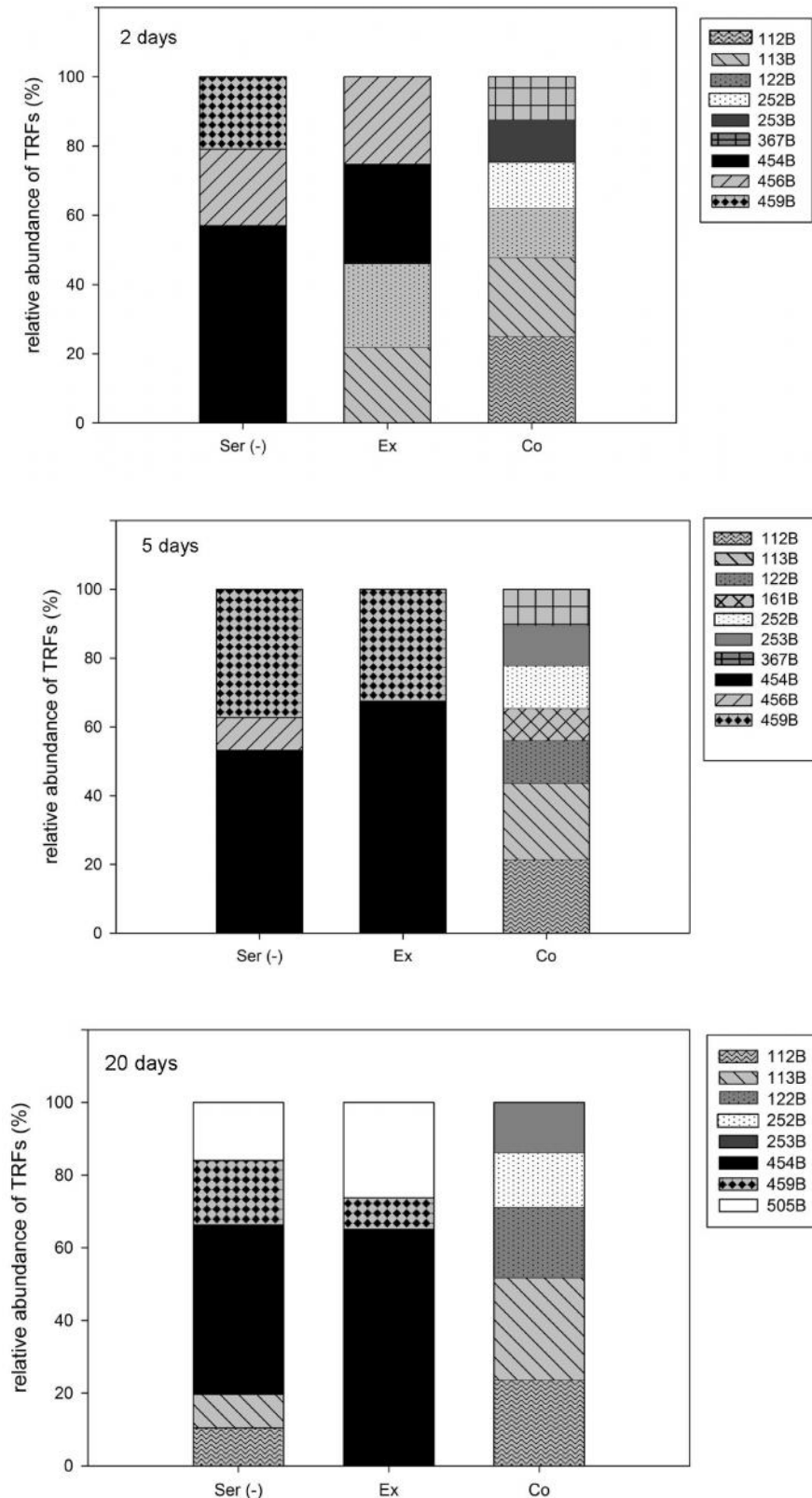


Figure 3.14 Influence of omitting a nitrogen source on TRF diversity patterns of ARM. Relative abundance of major TRFs (> 5 % of relative abundance) of T-RFLP analysis based on 16S rRNA genes of the ARM covered with agarose and different exudate mixtures: the standard exudate mix without serine (Ser(-)), the standard exudate mix (Ex), no exudates (Co) after incubation of 2, 5 and 20 days.

3.6 Influence of SDZ and manure on the bacterial communities of ARM

In the following experiment, the influence of anthropogenic impacts on rhizobacterial communities was studied. As an example of an agricultural fertilizer practice, manure was coapplied with the veterinary antibiotic SDZ to the soil. As control, only manure or water was amended to the soil. Soils were preincubated for 30 days to allow soil microorganisms to adapt. Then, ARMs were added covered with the standard exudate mix and SDZ to keep the selective pressure of the antibiotic during ARM incubation. After 1 and 2 weeks of incubation, microorganisms attached to ARM were harvested and the bacterial community structure was analyzed by T-RFLP based on 16S rRNA genes. In a second experiment with the same experimental set up, the spreading of bacterial antibiotic resistance genes after SDZ and manure soil application was investigated. To detect the abundances of SDZ-resistance genes *sul1* and *sul2*, q RT PCR was performed.

3.6.1 Influence of SDZ and manure on the bacterial community structure of ARM

To visualize structural changes in the bacterial community of the ARM, a between group analysis of the T-RFLP data set based on 16S rRNA genes was performed. In Figure 3.15a, ARM samples were analyzed based on the soil preincubation type with the first two components explaining 88.4 % of the variances. There was a significant shift in the bacterial community ($p < 0.05$) when soil was preincubated with manure in comparison to the control (water application). The additional application of SDZ to soil did not lead to structural changes in the bacterial population. Concerning the type of ARM covering (see Figure 3.15b), the BGA analysis explained 95.6 % of the variances by the first two components. There was a significant shift ($p < 0.05$) in the bacterial community when the standard exudate mix was applied on the ARM in comparison with the control (without the standard exudate mix), but also here the additional application of SDZ had no influence on the bacterial population structure. In both graphs, we observed a time-dependent bacterial community shift independent of soil preincubation and ARM coverings except for the control ARMs, which clustered at both sampling time points.

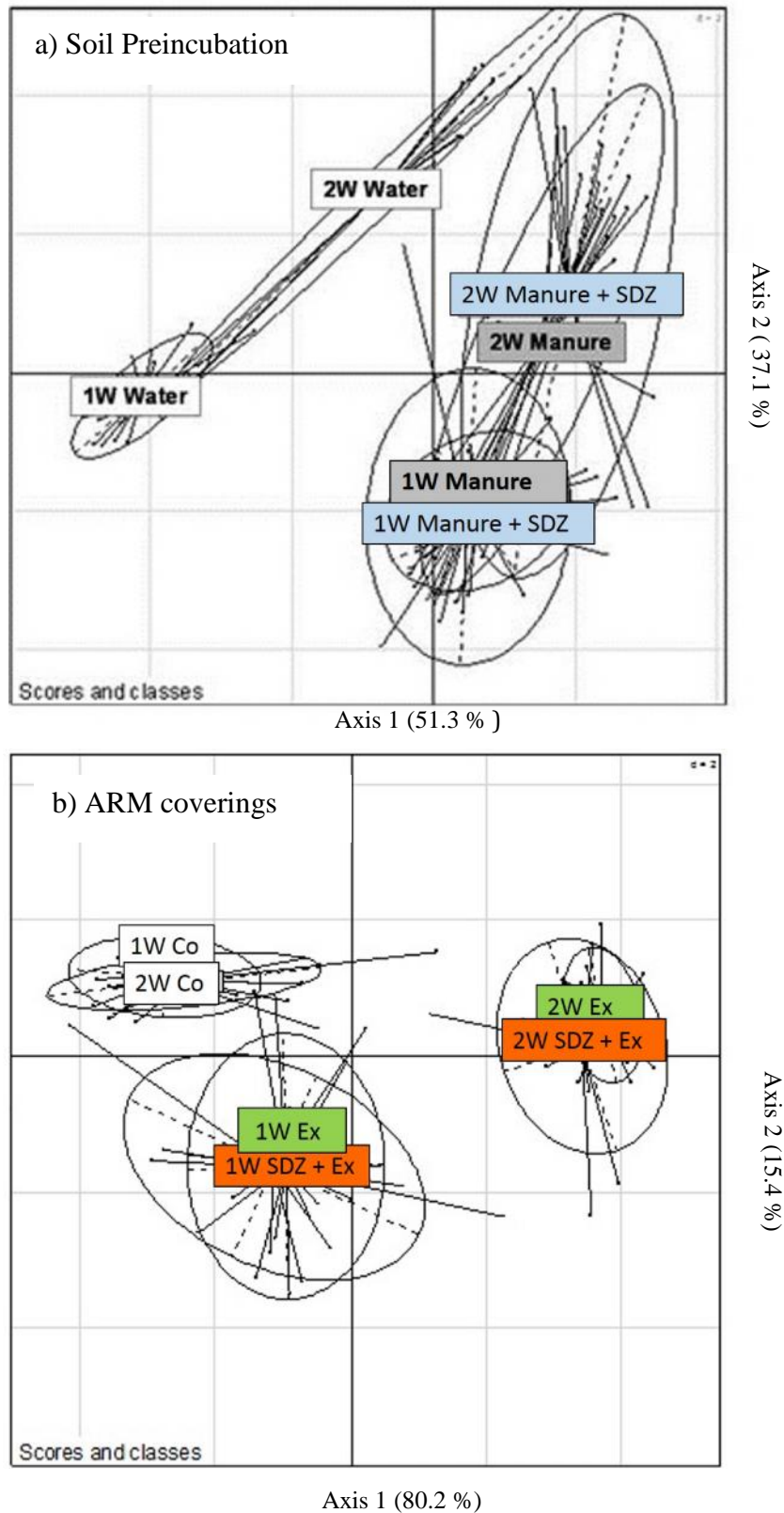


Figure 3.15 Influence of soil preincubations and ARM treatments on the development of bacterial community structure of ARM. Between group analysis based on correspondence analysis of the T-RFLP data set of 16S rRNA gene fragments of ARM. Sampling took place after 1 week (1W) and 2 weeks (2W) of incubation. A) Influence of soil preincubation for 30 days. B) Influence of ARM coverings: Co= Control, Ex = standard exudate mix, SDZ + Ex = SDZ and standard exudate mix. All samples were done in 5 replicates.

The diversity of TRFs was monitored and first the initial bacterial populations of the fresh manure and the bulk soil after 30 days of preincubation (before ARMs were added) were analyzed. Patterns of high abundant TRFs (relative abundance > 5 %) of fresh manure and soils preincubated with manure and SDZ or just manure showed lower TRF diversity compared with soil amended with water (see Figure 3.16). The most abundant TRF detected in the fresh manure (41 % of relative abundance) and bulk soil preincubated with SDZ and manure (24 % of relative abundance) or just manure (23 % of relative abundance) was TRF 486B. This TRF was not detected in the soil samples without manure application. The same distribution was observed for TRF 157B which was also high abundant in fresh manure and in both soil treatments with manure application, however this TRF was not predominant in soil preincubated with water. Only TRF 52B was solely present in fresh manure. TRF 54B, 103B, 112B, 113B, 162B, 252B, 253B, and 367B were detected in all soil samples, but not always above 5 % of relative abundance and not in fresh manure.

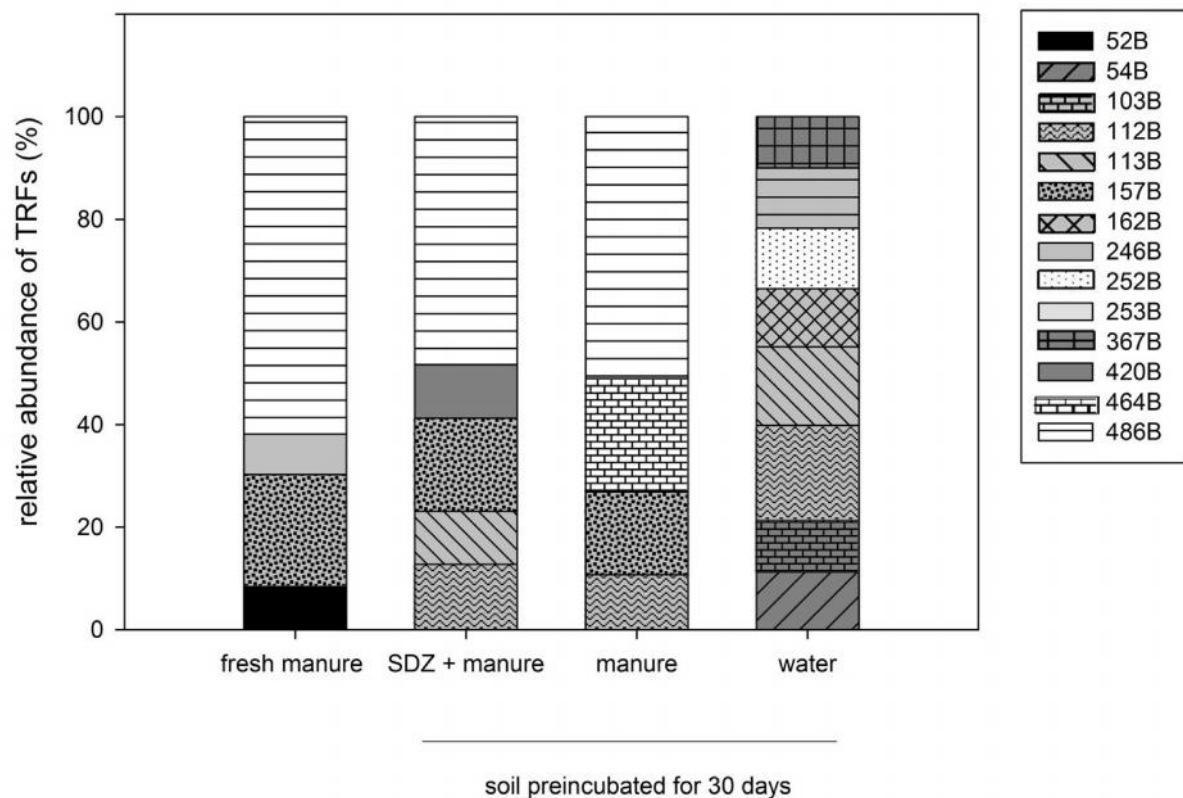


Figure 3.16 TRF diversity patterns of fresh manure and preincubated soil. Relative abundance of major TRFs (> 5 % of relative abundance) of T-RFLP analysis based on 16S rRNA genes of fresh manure and soil preincubated for 30 days with SDZ and manure, manure or water before ARMs added.

ARMs were incubated in the pretreated soils for 1 and 2 weeks, and the diversity of the major TRFs (> 5 % of relative abundance) was investigated. TRF 454B was highly abundant in all samples with exudates on ARMs independent of type of preincubations. This TRF persisted even after 2 weeks of incubation (see Figure 3.17a and b). In ARM samples without exudates applied, TRF 454B was detected, however with a relative abundance below 5 % and therefore not visible in the graph. TRF 486B was only present in samples preincubated with manure and SDZ or just manure. This TRF was only high abundant after 1 week of incubation. In the control ARMs without exudates and SDZ added, this TRF remained high abundant. A similar abundance pattern was observed for TRF 157B. TRF 505B became high abundant (14-36 % of relative abundance) at all preincubation types in ARMs with exudates after 2 weeks of incubation, but not in CoARM. Another TRF which became high abundant after 2 weeks of incubation was TRF 451B, which increased in abundance (7-14 % of relative abundance) in ARMs preincubated for 2 weeks.

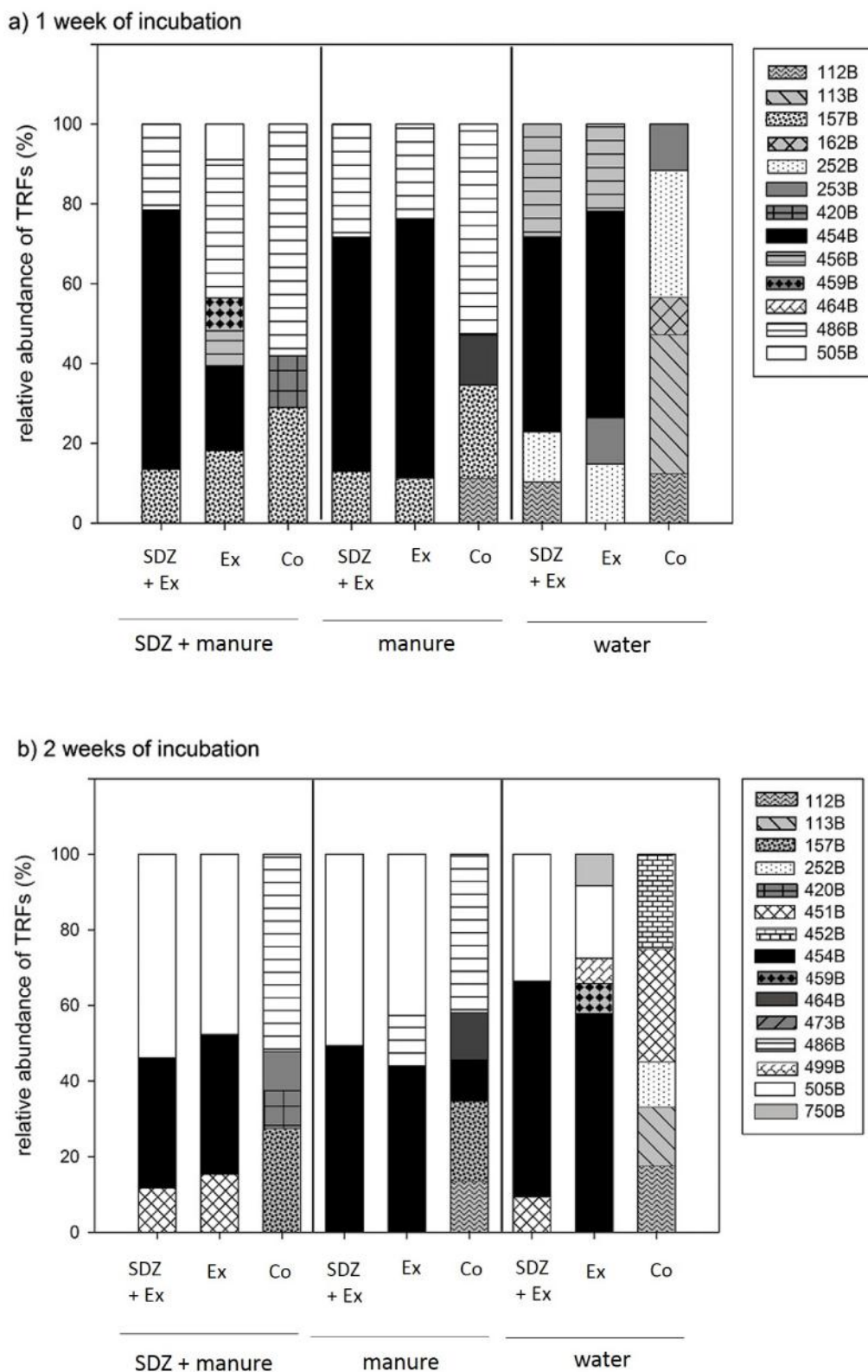


Figure 3.17 Influence of soil preincubations and ARM treatments on TRF diversity patterns of ARM. Relative abundance of major TRFs (> 5 % of relative abundance) of T-RFLP analysis based on 16S rRNA genes of the agarose-coated ARMs covered with SDZ and the standard exudate mix (SDZ + Ex), the standard exudate mix (Ex) or without exudates (Co). Soil was pretreated with SDZ and manure, manure or water after incubation of 1 (a) or 2 (b) weeks.

3.6.2 Assignment of the high abundant TRF of fresh manure by phylogenetic sequence analysis

To assign the high abundant TRF 486B detected in fresh manure which was also predominant in the soil preincubated with manure as well as in ARMs incubated in manured soil, a PCR targeting bacterial 16S rRNA genes was performed. Subsequently, PCR fragments were ligated in pCR2.1 vectors. Digestion with *EcoR*I was performed to check that the vector contain the fragment with the correct length. From 20 clones in total, 4 vectors contained the correct insert of ca. 1400 bp length, whereas the others contained fragments around 700 bp (see Figure 3.18).



Figure 3.18 Gel electrophoresis of pCR2.1 vectors containing the 16S rRNA gene fragments from fresh manure after *EcoR*I digestion. Lanes: M = 1 kb molecular weight marker, 1-20 = samples.

The plasmids with the fragment of ca. 1400 bp length were sequenced and additionally T-RFLP was performed to assure the correct peak size (see Figure 3.19).

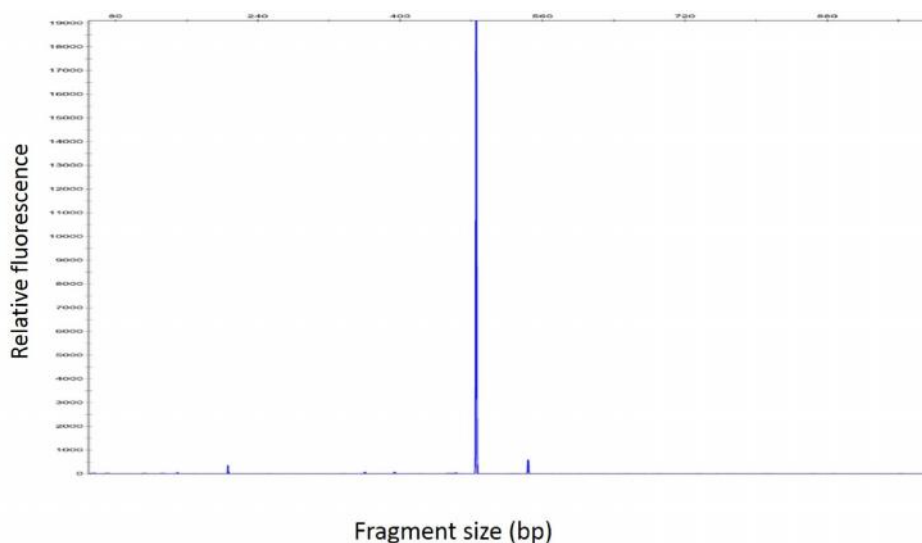


Figure 3.19 T-RFLP peak profile of the high abundant 16S rRNA fragment of manure cloned into pCR2.1 vector for sequence analysis. DNA fragment was amplified with primer (63f-FAM, 783r) and digested with *Msp*I. The main peak has a size of 486 bp.

The sequence of the 16S rRNA gene fragment (assigned to TRF 486B) was conducted to phylogenetic analysis. The calculated phylogenetic tree (see Figure 3.20) revealed that the analyzed sequence (ARB_B858C6EA) showed high similarity to species of the genus *Clostridium*.

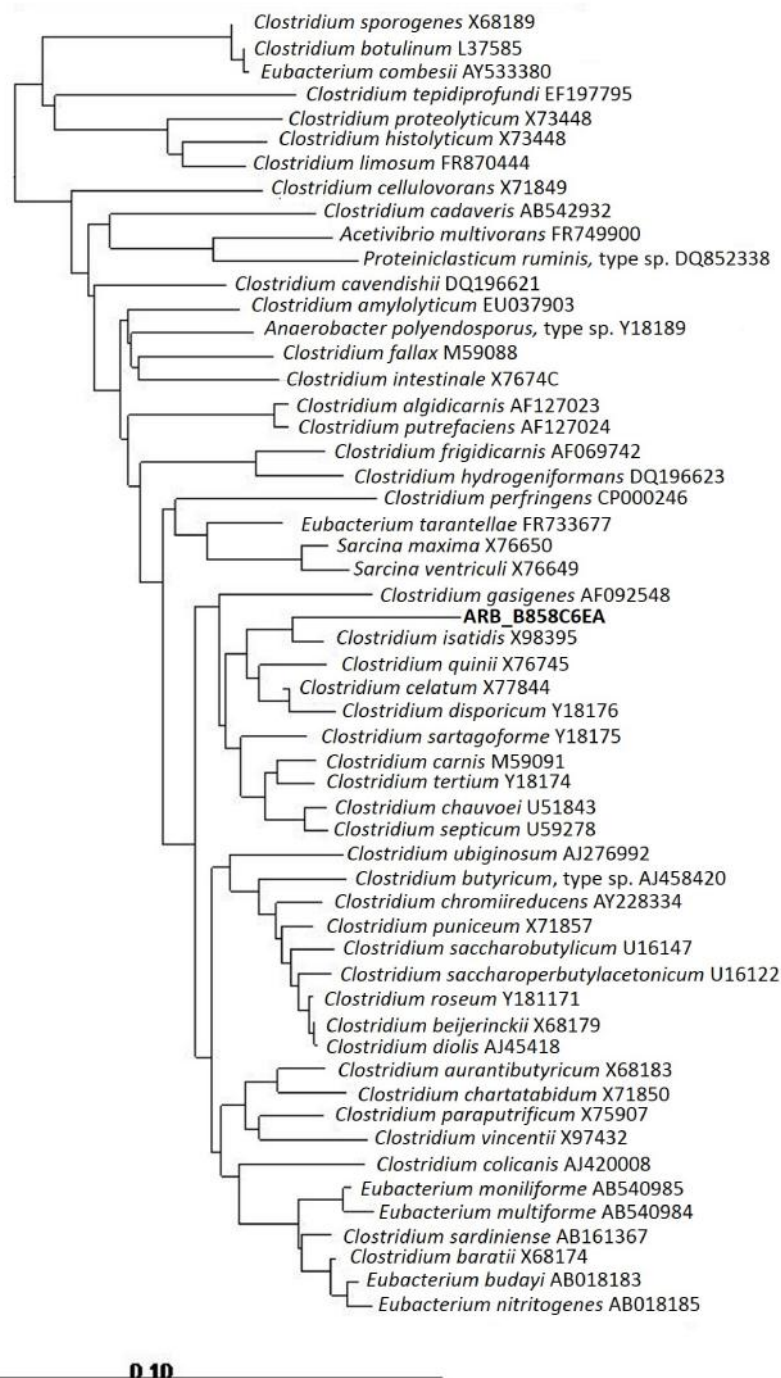


Figure 3.20 Neighbor Joining tree of the high abundant 16S rRNA gene fragment of fresh manure. The analyzed sequence ARB_B858C6EA had a length of ~ 1400 bp. The bar represents one nucleotide substitution per 100 nucleotides.

3.6.3 Influence of manure and SDZ on the abundance of 16S rRNA and bacterial antibiotic resistance genes

To evaluate q RT PCR targeting SDZ-resistance genes (*sul1*, *sul2*) and 16S rRNA genes, the slope of the log linear phase of the amplification reaction was used to calculate the efficiency of the qPCR. 100 % of efficiency means that the template is doubled after each cycle during the exponential phase. As shown in Table 3.2, efficiencies were high and ranged between 93 – 99 % for all qPCRs. The correlation coefficient R^2 indicates how well the data fits to the standard curve and should be close to 1. For all qPCRs, R^2 values were between 0.97 and 0.99.

Table 3.2 Evaluation of quantitative real time PCRs targeting *sul1*, *sul2* and 16S rRNA genes.

Target gene	Efficiency (%)	R^2
<i>sul1</i>	99	0.99
<i>sul2</i>	93	0.97
16S	96	0.99

3.6.3.1 Quantification of the 16S rRNA genes

The levels of 16S rRNA genes were measured by q RT PCR as an indicator for bacterial biomass in the samples. To quantify initial levels of 16S rRNA per ng DNA before ARMs were applied to the soil, fresh manure and the pretreated soils were analyzed. Soil preincubated with SDZ and manure or just manure showed significantly higher values (1.5×10^5 and 1.3×10^5) of 16S gene copies per ng DNA to bulk soil incubated with water (6.9×10^4 16S gene copies per ng DNA) as shown in Figure 3.21. Fresh manure was not significantly different, because of its high standard deviation. Nevertheless, there was an indication of a higher 16S ng^{-1} DNA value (3.5×10^5) in manure in comparison to soil samples with different preincubations.

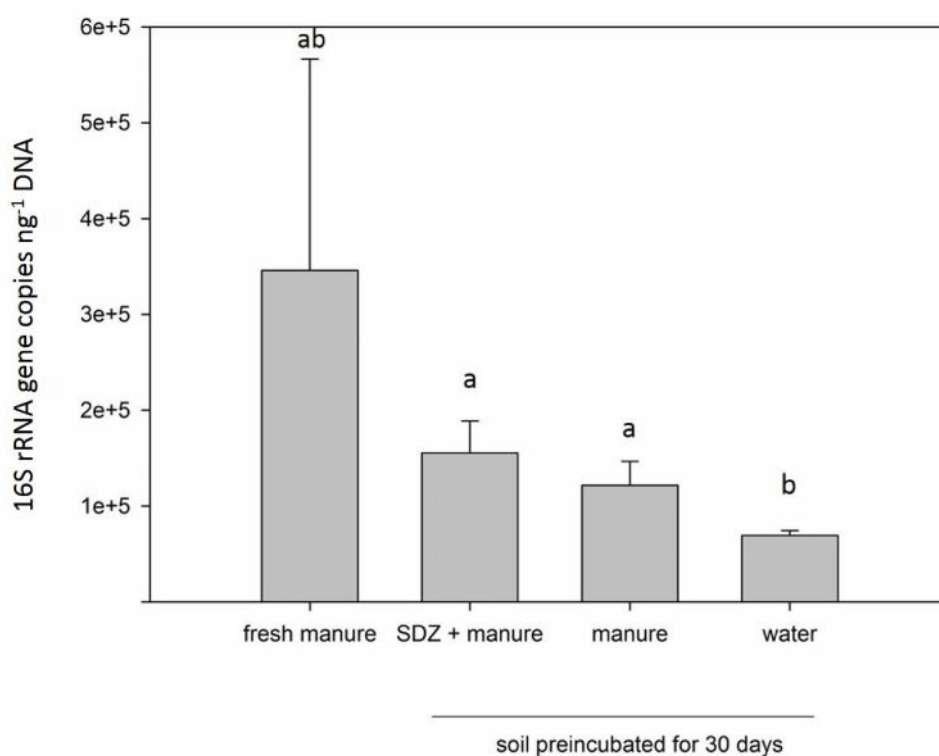


Figure 3.21 Abundance of 16S rRNA gene copies in fresh manure and preincubated soil. Before ARMs were incubated in soils, fresh piggery manure and soil preincubated with manure and SDZ, manure or water after 30 days of incubation were analyzed. 16S rRNA gene abundance was measured relative to total extracted genomic DNA. Error bars represent standard deviation of means ($n = 5$). Significant differences ($p < 0.05$) are indicated by small letters.

When ARMs were incubated in the pretreated soils for 1 and 2 weeks, respectively, the abundance of 16S rRNA genes were significantly ($p < 0.05$) influenced by the type of soil preincubation (see Figure 3.22). When soil was amended with SDZ and manure or just manure, the abundance of 16S rRNA gene copies per ng DNA of the ARMs was higher than in soil preincubated with water. The type of ARM coverings did not significantly influence the amount of 16S rRNA genes overall soil preincubations. However, independent of the type of preincubation, there was increase in 16S rRNA genes between 1 and 2 weeks of incubation when SDZ was present on the ARMs surface.

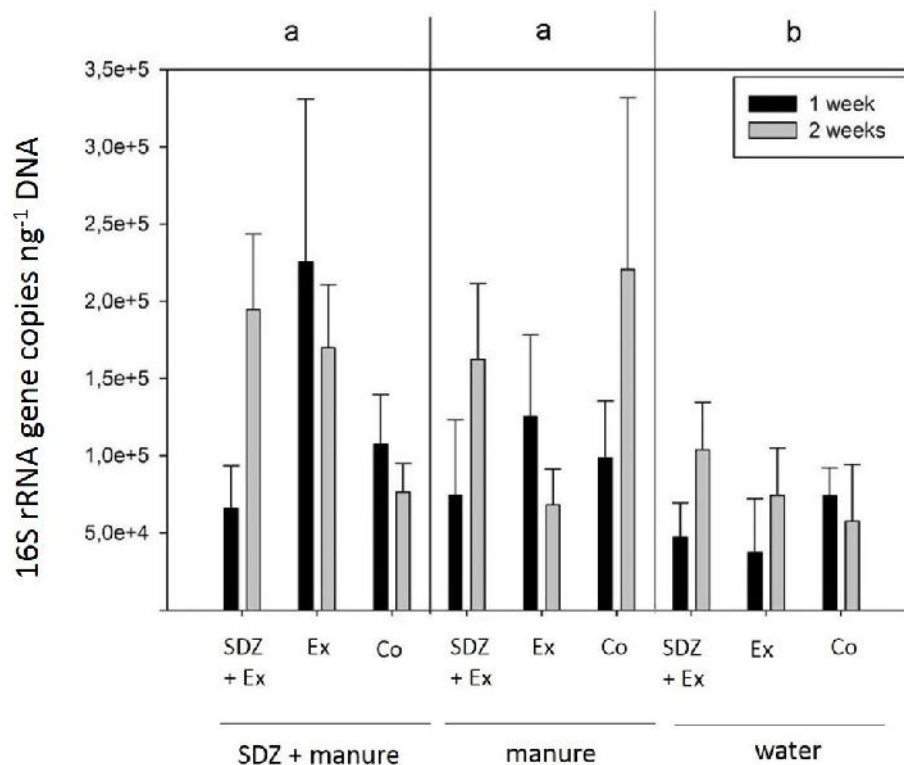


Figure 3.22 Abundance of 16S rRNA gene copies in ARM after manure and SDZ soil application. Soil was preincubated with manure and SDZ, manure or water for 30 days. Agarose-coated ARMs were covered with SDZ and the standard exudate mix (SDZ + Ex), the standard exudate mix (Ex) or without exudates (Co) and incubated for 1 or 2 weeks. 16S rRNA gene abundance was measured relative to total extracted genomic DNA. Error bars represent standard deviation of means ($n = 5$). Significant differences ($p < 0.05$) between the soil treatments are indicated by small letters.

3.6.3.2 Quantification of the SDZ resistance genes *sul1* and *sul2*

The dissemination of the SDZ-resistance genes *sul1* and *sul2* was investigated by q RT PCR. First, the amount of the *sul* genes relative to 16S rRNA gene copies was determined in fresh manure and in the soils after 30 days of preincubation (SDZ and manure, just manure or watered) before the ARMs were added. Regarding *sul1*, soil preincubated with manure or water showed significantly lower values ($4.3 \cdot 10^{-4}$ and $2.2 \cdot 10^{-4}$) of *sul1* gene copies relative to 16S gene copies in comparison to fresh manure ($3.5 \cdot 10^{-3}$) as shown in Figure 3.23. When soil was preincubated with SDZ and manure, no significant differences could be detected due to the high standard deviation. However, there was a trend that *sul1* relative to 16S gene copies was higher ($2.3 \cdot 10^{-3}$) in comparison to bulk soil incubated with manure or water.

No significant effects were detected for *sul2* (see Figure 3.23) because of the high standard deviation between the replicates. However, the trend was similar to *sul1* and fresh manure and soil preincubated with SDZ and manure showed higher values of *sul2*/16S in comparison to bulk soil preincubated with manure or water.

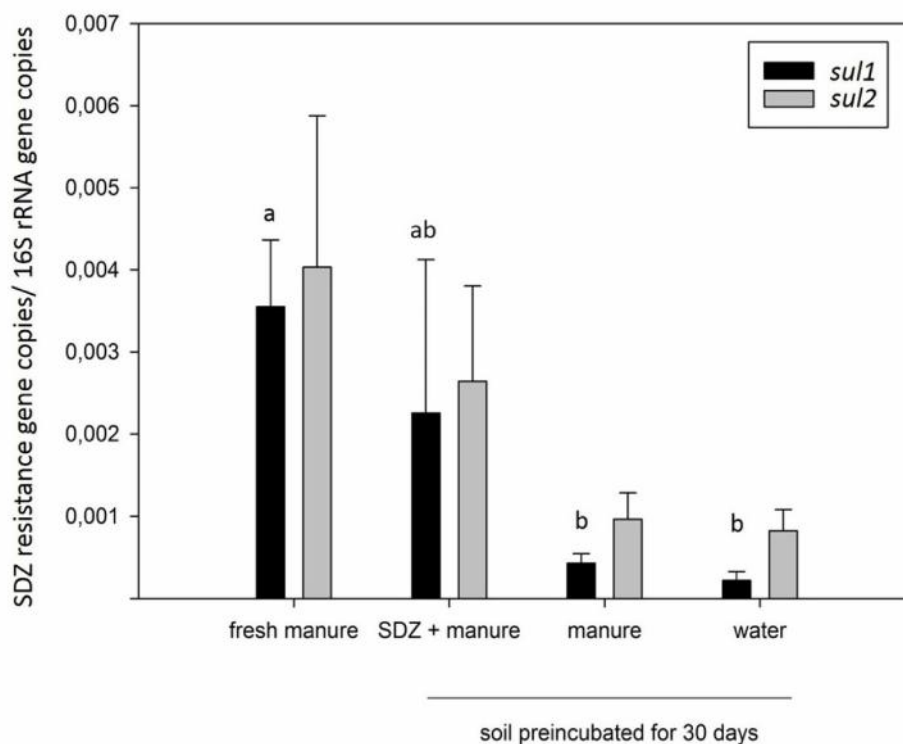


Figure 3.23 Abundances of SDZ resistance genes copies (*sul1*, *sul2*) in fresh manure and preincubated soil. Before ARMs were incubated in soils, fresh piggy manure and soil preincubated for 30 days with SDZ and manure, manure or water were analyzed. SDZ resistance genes were measured relative to 16S rRNA genes. Error bars represent standard deviation of means (n = 5). Significant differences (p < 0.05) are indicated by small letters.

After soil preincubation, the ARMs were added to the soil. After 1 and 2 weeks, respectively, the attached microorganisms of the ARM were harvested and the dissemination of the *sul* genes was analyzed (see Figure 3.24). The type of soil pretreatment strongly influenced ($p = 0.000$) the abundance of *sul1* and *sul2* of the ARMs. When the soil was incubated with manure and SDZ, the levels of *sul1* and *sul2* relative to 16S rRNA genes were significantly higher in comparison with the levels of ARMs incubated in soil pretreated with manure or water. Regarding the abundance of *sul1* and *sul2*, there was a significant increase between 1 and 2 weeks of incubation of ARM when soil was pretreated with SDZ and manure. This effect was not observed for the other soil pretreatments. Regarding *sul2*, the ratio of *sul2* gene copies relative to 16S rRNA gene copies significantly increased from 0.006 (1 week incubation) to 0.1 after 2 weeks of incubation when SDZ was present on ARMs surface. After 2 weeks of incubation, these levels were significantly lower (both 0.02) when ARMs were covered only with exudates or agarose. This trend was also seen for *sul1*, but due to the high standard deviation it was not significant.

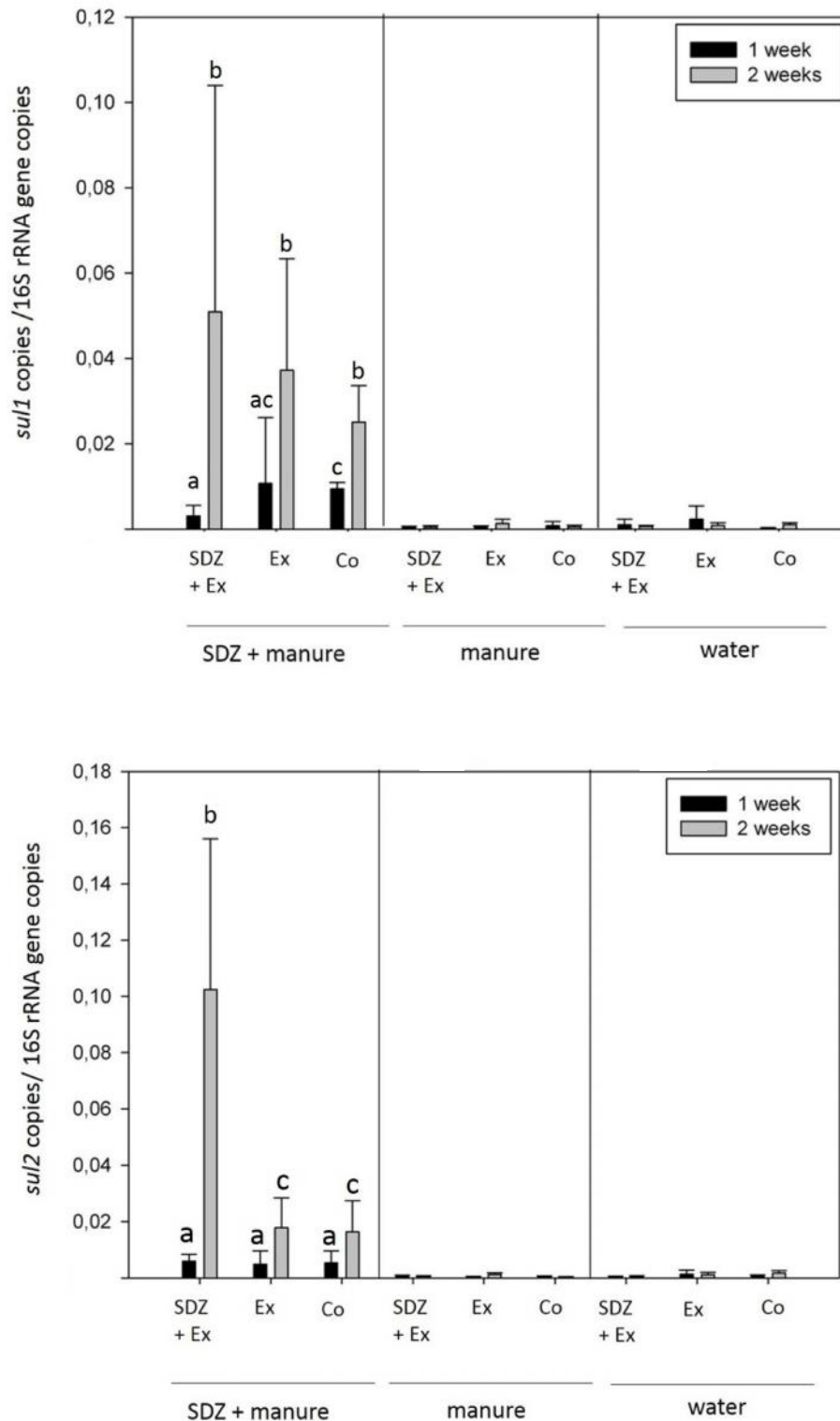


Figure 3.24 Abundance of SDZ resistance gene copies (*sul1*, *sul2*) in ARM after manure and SDZ soil application. Soil was preincubated with SDZ and manure, manure or water for 30 days. Agarose-coated ARMs were covered with SDZ and the standard exudate mix (SDZ + Ex), the standard exudate mix (Ex) or without exudates (Co) and incubated for 1 or 2 weeks. SDZ resistance genes were measured relative to 16S rRNA genes. Error bars represent standard deviation of means ($n = 5$). Significant differences ($p < 0.05$) between the samples are indicated by small letters.

4 Discussion

4.1 Comparison of the bacterial community of the artificial rhizosphere model with the microflora of other rhizosphere models, bulk soil and the rhizosphere of *Arabidopsis thaliana*

In this study, we established an artificial rhizosphere model (ARM) which combines substances simulating root exudates and a surface facilitating microbial attachment. We analyzed the development of bacterial community structure on the ARM by the molecular fingerprinting technique T-RFLP. Between replicates, T-RFLP peak profiles of the bacterial communities attached to the ARM were highly similar and thus a good reproducibility of the ARM was confirmed. The comparison of ARM with the control ARM without exudates revealed differences in their bacterial populations. Therefore, the application of artificial exudates leads to the establishment of a specific bacterial community of ARM. A FISH analysis revealed the presence of great amounts Gram-negative, rod shaped bacteria on the surface of the model. Furthermore, there was one dominant TRF over 20 days of ARM incubation which was assigned by the phylogenetic analysis based on the 16S rRNA gene sequence to the genus *Pseudomonas*. We found close relationship of our sequence to the *P. jessenii* subgroup and *P. koreensis* subgroup, which both belong to the *P. fluorescens* group described by Mulet *et al.* in 2010. Species of these groups were found in soil and water systems (Cámara *et al.* 2007; Tvrzová *et al.* 2006; Verhille *et al.* 1999). There was also close homology to *P. baetica* which was first isolated from fish (López *et al.* 2012). *Pseudomonas* is a genus of Gram-negative, rod-shaped, aerobic bacteria which belong to the gamma subclass of Proteobacteria and is ubiquitously found in great variety in natural environments. The ability of root colonization makes *Pseudomonas* an important model organism in plant-microbial interaction studies (Baharona *et al.* 2011; Oku *et al.* 2012). Finding *Pseudomonas* in high abundance in the ARM shows that this model attracts typical rhizosphere-inhabiting bacteria and thus makes it a useful model to study the plant-soil interface. Referring to plants, *Pseudomonas* can be beneficial by various mechanisms and especially *P. putida* is a species which can improve plant performance. First, *P. putida* can act as a biocontrol agent against

the fungal pathogen *Fusarium* (Scher and Baker 1982). Kloepper *et al.* (1980) showed that the production of siderophores by *P. fluorescens/putida* strains is correlated with enhanced plant growth by detracting iron levels in soil. Furthermore, the phytohormone indoleacetic acid produced by *P. putida* increases plant root growth (Patten and Glick 2002). On the other hand, there are *Pseudomonas* species which act as plant pathogens, such as *P. syringae*, which causes severe damage to crops, for example tomatoes (Zhao *et al.* 2003), and also to the rice by the pathogen *P. fuscovaginae* (Xie *et al.* 2012). Besides *Pseudomonas*, the FISH images revealed the presence of filamentous bacteria at the surface of the ARM which could belong to the group of actinomycetes. These Gram-positive and filamentous bacteria are also typical inhabitants of the rhizosphere and can exert plant growth promoting activities (Tokala *et al.* 2002; Intra *et al.* 2011).

Other studies also reported an increase of bacterial groups in nutrient-rich environments. In accordance to our results, mainly Gram-negative bacteria were found to be dominant in nutrient-rich soil environments. Griffiths *et al.* (1999) used an artificial root model to investigate soil microbial community structures under different substrate concentrations. They revealed high abundances of Gram-negative bacteria under increased substrate levels. Also a study by Paterson *et al.* (2007) showed an increase of Gram-negative bacteria when Carbon-rich substrates were applied by an artificial root model to the soil. Burmolle *et al.* (2007) used an agar-coated glass slide approach (without exudates) incubated in soil slurry for a week. They detected mainly Gram-negative bacteria attached to the glass slide by 16S rRNA gene fingerprinting analysis. Subsequent sequencing of 16S rRNA genes revealed the presence of several *Pseudomonas* species, like the rhizosphere inhabiting *P. putida*. They detected *Bacillus circulans* in the microbial consortium, as a member of Gram-positive bacteria.

We compared bacterial populations of ARM to bulk soil by T-RFLP peak profiles based on 16S rRNA genes and expected significant differences in both systems. The bacterial community of ARM was lower in TRF richness, diversity and evenness in comparison to bulk soil. This indicates a selection of microorganisms on the ARMs surface which was probably driven by exudates. This is in accordance to other studies (Smalla *et al.* 2001; Costa *et al.* 2006). The authors revealed the presence of a few dominant and numerous low abundant species in bulk soil, whereas in rhizosphere, there are more dominant species and less low abundant species. In our study, there was an increase of bacterial taxa different from bulk soil.

Especially the most abundant TRF 454B, which represents the typical rhizosphere inhabitant *Pseudomonas*, was low abundant in bulk soil.

In contrast to bulk soil, we hypothesized that the bacterial community of ARM would be similar to the rhizosphere of *Arabidopsis thaliana*. The T-RFLP peak profiles of ARM and the rhizosphere of *A. thaliana* revealed a high resemblance of the bacterial community structures. However, TRF richness, evenness and diversity levels of ARM were lower compared to the plant root-derived bacterial populations and we detected differences in high abundant TRFs between ARM and the rhizosphere of *A. thaliana*. *Pseudomonas* was the dominant taxon of the ARM, and it was also highly abundant, but not as dominant in the rhizosphere of *A. thaliana*. This is in accordance with a study by Bulgarelli *et al.* from 2012 which revealed that the rhizosphere of *Arabidopsis* is mainly colonized by the phyla Proteobacteria, Bacteroidetes and Actinobacteria, whereas Pseudomonadeceae played a minor role in the rhizoflora community. In comparison with our simplified rhizosphere model which contained a mix of 8 different compounds simulating root exudates, *Arabidopsis* renders a diverse variety of compounds to the surrounding soil, e.g. all 20 proteinogenic amino acids, fatty acids, proteins and enzymes (Narasimhan *et al.* 2003) which can act in various ways on soil bacteria creating a very specific bacterial community. Besides bacteria-attracting substances, plant roots secrete compounds to defend itself against plant pathogens. Bais *et al.* (2002) reported that hairy root cultures of *Ocimum basilicum* L. release rosmarinic acid which shows antibiotic activity against *Pseudomonas aeruginosa*. A study by Teplitski *et al.* (2000) showed that pea plants secrete molecules which interfere with the quorum sensing system by mimicking the signal molecule N-acyl homoserine lactone (AHL) molecules and thereby influencing the bacterial behavior. But not only root exudates, also rhizodeposits, for example decaying root cells and mucilage, are present in a natural rhizosphere. They are considered to be of great importance to the soil C-pool (Dennis *et al.* 2010). Besides the variety of compounds, the distribution of compounds is not homogenous as in our model and results in different zones along the root surface (Badri and Vivanco 2009). For example, zones with low amounts of exudates are less attractive to r-strategists such as *Pseudomonas*.

4.2 Drivers of bacterial community structure at the plant-soil interface

4.2.1 Influence of single exudates on the bacterial community structure of the ARM

In this experiment, we omitted single exudates of the full standard exudate mix of ARM to investigate their influence on bacterial population's structure in the artificial rhizosphere during a period of 20 days. In the first experiment, we focused on carbon-rich compounds and chose glucose as a representative of carbohydrates and malic acid as a representative of organic acids.

We subtracted glucose from the standard exudate mixture (Glu(-)ARM) and expected a shift in the bacterial community structure. Glucose is an easy degradable carbon source which is utilized by a broad range of microorganisms (Paterson *et al.* 2007). We hypothesized that in the absence of glucose, less easily degradable compounds have to serve as nutrients which should result in a bacterial community shift compared to the standard exudate treatment with glucose (ExARM). After 2, 5 and 20 days of incubation of ARM, we did not detect significant shifts in the bacterial community structure between Glu(-)ARM and ExARM. As already mentioned above, ARM was densely populated by the genus *Pseudomonas*. Fonseca *et al.* (2011) determined the generation time of *Pseudomonas putida* at 10 °C to be around 5 hours. Hence, the effect of the absence of glucose on the structural development of the bacterial community could occur within the first hours of ARM incubation. Therefore, perhaps all glucose was already metabolized in the ExARM after 2 days of ARM incubation and mimicked the effect of subtracted glucose in the Glu(-)ARM treatment. Besides temporal effects, glucose can be metabolized by various bacteria and therefore small population shifts might not be detectable. Also other studies had similar results. Landi *et al.* (2006) added single exudates to analyze effects on the soil microbiota. They detected an increase in CO₂ levels which indicates elevated microbial activity. But in comparison to the tested oxalic acid, they observed smaller changes in the bacterial community during glucose application. The same effect was observed by Shi *et al.* in 2011. They added separately a mixture of organic acids or sugars to the soil. The bacterial richness and shifts in dominant taxa were more pronounced when organic acids were added compared with the application of sugars. Eilers *et al.* (2010) showed that citric acid has the strongest impact on bacterial community shifts in soil compared with the tested sugars and amino acids. In accordance to the aforementioned studies, we detected significant shifts in the early development of bacterial community when the organic acid malic acid was subtracted (Mal(-)ARM). Malic acid plays an important role

among plant root exudate compounds which are able to mediate aluminum tolerance (Delhaize *et al.* 1993; Pellet *et al.* 1995) and nutrition acquisition of plants (Dakora and Phillips 2002). Furthermore, malic acid attracts beneficial microorganisms which are able to defend against plant pathogens (Rudrappa *et al.* 2008b). Hence in ExARM, probably a fraction of bacteria was attracted by malic acid and was not selected in the Mal(-)ARM treatment which resulted in a community shift.

In a second experiment we subtracted serine from the exudate mix (Ser(-)ARM) as a representative of amino acids. After 20 days of ARM-incubation, there was a slight change in the bacterial community structure in Ser(-)ARM. Generally, soil microorganisms are carbon-limited (Lynch & Whipps 1990; Demoling *et al.* 2007). In the rhizosphere, there is a different situation due to carbon-rich root exudates and dead root cells. This leads to an increase of the microbial activity and microbial growth, and is described by the so-called rhizosphere effect. Therefore, carbon-rich compounds from the exudate mix of the ARM probably trigger an increase of microbial metabolization and growth which is correlated with a nitrogen need, since this element is essential for the protein and nucleic acid synthesis in cells. Johansen *et al.* (2002) reported a stimulation of enzymes (nitrate reductase activity) involved in nitrogen-turnover processes in the rhizosphere of barley in comparison with bulk soil which indicates the need of microorganisms for nitrogen. This could explain why the weak population shift occurred with a delay in the experiment. Also other nitrogen sources, for example cell components of dead soil microbial organisms, could serve as a nitrogen source and therefore lower the effect of subtracted serine from the full exudate mix. However, the role of amino acids delivered by the plant in natural rhizosphere systems might not be that important to microorganisms. A study on the rhizosphere of tomatoes argued that the bio-availability of amino acids is too low to support bacterial root colonization and thus the amino acid synthesis by microorganisms is essential (Simons *et al.* 1997). Therefore, other nitrogen sources such as soil ammonium (NH_4^+) and nitrate (NO_3^-) could be more important than amino acids excreted by the plant root for bacterial growth. Generally, microorganisms are said to be better competitors for nitrogen than plants (Kaye and Hart 1997; Hodge *et al.* 2000) and therefore soil nitrogen (organic and inorganic) will be first taken up by bacteria because of their fast growth, their high surface area to volume ratio and their role in nitrogen turn over processes.

4.2.2 Influence of SDZ and manure on the bacterial community structure and the abundance of SDZ resistance genes of the ARM

In the following section, we investigated the influence of manure and the antibiotic sulfadiazine (SDZ) on microorganisms in the rhizosphere. By the wide-spread fertilizing technique of manure application, antibiotic residues and antibiotic resistant bacteria can be transmitted to the soil. To simulate this effect, we preincubated soil with manure and the veterinarian antibiotic SDZ. A concentration of 10 mg SDZ kg⁻¹ soil was applied to soil as a realistic concentration introduced via manure to soil (Heuer and Smalla 2007). After an adaptation of the soil microorganisms for 30 days, ARMs with additional SDZ on their surface were applied to the soil. Bacteria attached to the ARM were harvested after 1 or 2 weeks, respectively and the bacterial community structure was analyzed by T-RFLP. In a second approach, we measured the abundances of 16S rRNA genes and the SDZ-resistance genes *sul1* and *sul2* to investigate the spreading of antibiotic resistance genes in the artificial rhizosphere after manure and SDZ soil application.

4.2.2.1 Influence on the bacterial community structure

In this experiment, the bacterial community of the ARM was significantly influenced by the application of manure and the presence of exudates on the ARMs surface. When bulk soil was preincubated additionally with SDZ, it did not induce a shift in the population structure of the ARM in comparison with the manure treatment. Also the amendment of SDZ on the surface of ARM did not result in a change of the bacterial community. It was shown that the coapplication of manure and SDZ influences the soil microbial community and affects the bacteria to fungi ratio (Hammersfahr *et al.* 2008), as well as resulting in a decrease of typical soil inhabitants such as *Pseudomonas* (Ding *et al.* 2014). These studies were performed in bulk soil, whereas in a nutrient rich environment as the rhizosphere, microbial populations could be less affected to the antibiotic due to several reasons. First, the effect of SDZ on the bacterial population of the ARM was probably overloaded by the stimulating properties of the manure and the exudates, respectively. Moreover, the bioavailability of SDZ in the rhizosphere can be lowered by various processes which have to be taken into account when studying the effects of antibiotics in this environment. Antibiotics can be absorbed by soil particles and therefore not affect soil bacteria. Sukul *et al.* (2008) reported high sorption rates

to soil particles which are increased with manure application. Sorption rates also increase with acidic pH (ter Laak *et al.* 2006; Kurwadkar *et al.* 2007). This could be an important factor in the rhizosphere because the soil pH decreases due to root exudation. Also, soil bacteria are able to degrade SDZ. Especially in a bacterial hotspot as the rhizosphere, antibiotic degradation could be an important factor. Tappe *et al.* (2013) showed that the bacterium *Microbacterium lacus* strain SDZm4 isolated from pig-manured soil degraded SDZ into 2-aminopyrimidine. SDZ can also be degraded by photolysis (Numan and Danielson 2004) which probably did not take place in this experiment, since the trays were kept in the dark. SDZ can persist for long periods in soil and its half-life in soil was determined to be 28 days (Halling-Sorensen 2000). Therefore, we added SDZ on the surface of the ARM after the soil preincubation with manure and SDZ for 30 days to keep the antibiotic selection pressure during the duration of the experiment. However, there is evidence that persistence of SDZ in soil could be even longer. A study by Förster *et al.* (2009) on the sequestration of ¹⁴C-labelled SDZ and its residues in soil transferred via pig manure revealed that after 210 days of incubation about 50 % of the radioactivity was still extractable with a harsh extraction protocol. We detected significant influences by the coapplication of SDZ and manure on the gene abundances as discussed later, and hence we presume that the applied amount of SDZ was high enough to affect bacterial populations of the ARM during the experimental duration.

In our experiment, one TRF was highly abundant in fresh manure and persisted in soil preincubated with manure and SDZ for 30 days. 16S rRNA sequence analysis revealed that this TRF belonged to the genus *Clostridium*. This genus consists of Gram-positive, obligate anaerobe, spore-forming bacteria which are ubiquitously present in soil and in the digestive tracts of mammals. Therefore, this genus was very likely transmitted via manure to the soil. This is also supported by the fact that soils applied with manure showed significantly higher amounts of 16S rRNA genes which indicates the accumulation of soil and probably manure-originated bacteria. The phylogenetic analysis of the 16S rRNA sequence of this highly abundant TRF revealed close homology to *Clostridium* species found in the gut of mammals, such as *C. carnis*, *C. disporicum*, and *C. septicum*. Also the animal pathogen *C. chauveoi* showed close homology, which causes severe infections, such as blackleg in cattle (Kuhnert *et al.* 1997). Other *Clostridium* species have clinical significance as they produce neurotoxins, like *C. botulinum*. Generally, bacteria introduced to soil are thought to be able to persist only for a short period (Acea *et al.* 1988). However, Ding *et al.* (2014) detected high levels of *Clostridium* in soil after manure and SDZ soil application up to 60 days of incubation. Similar

to the situation in the bulk soil study mentioned before, we monitored *Clostridium* in ARM when soil was preincubated with manure and SDZ. However, this genus decreased after 1 week in the artificial rhizosphere of ARM. In contrast, *Clostridium* was still high abundant up to 2 weeks when no exudates were added on the surface of ARM. This indicates that these manure-originated bacteria are outcompeted by resident bacteria in the rhizosphere and that this process is faster in the densely populated rhizosphere than in bulk soil. There are different explanations why *Clostridium* was able to persist up to 1 one week in the ARM when SDZ was applied. In consideration of the fact that this genus is strictly anaerobic, *Clostridium* probably persisted in the surrounding soil of ARM (which is supposed to be mainly aerobic) in form of dormant cells instead of being metabolically active. Hence, the cells were not affected by SDZ as it only acts on growing cells. This antibiotic inhibits bacterial growth by acting as a structural analog of the substrate para-amino-benzoe acid of dihydropteroat synthetase and thereby inhibits the folic acid synthesis of bacteria (O'Neil *et al.* 2001). If *Clostridium* remained metabolically active in oxygen-depleted zones in soil surrounding the ARM, SDZ concentrations could have been too low to inhibit bacterial growth. Carr *et al.* (1973) reported that *Clostridium* is susceptible to SDZ, but at higher levels in comparison to other bacteria. If SDZ-levels were high enough to restrict growth, antibiotic resistance was not mediated by the SDZ-resistance *sul* genes since they are constraint to Gram-negative bacteria. Several chromosomal encoded sulfonamides resistance mechanisms are reported (Skold 2001) which might have occurred in *Clostridium*.

Besides manure-originated bacteria, also soil-borne bacteria as *Pseudomonas* were able to persist in high amounts after SDZ-application on the ARM. As shown in the experiments presented before, *Pseudomonas* is well adapted to a nutrient rich environment like the artificial rhizosphere presented here. As *Pseudomonas* is a typical r-strategist and grows rapidly under nutrient rich conditions, it should be targeted by SDZ. The ability of cell aggregation is well known for several *Pseudomonas* species (Mah *et al.* 2003) which reduces susceptibility to antibiotic compounds. This increased resistance of bacterial colonies is multifactorial and several mechanisms are discussed being responsible (Anderson and O'Toole 2008). First, the matrix in which cells are embedded acts as a diffusion barrier. Second, there are different zones of oxygen and nutrient within a colony leading to alterations in the bacterial metabolism. Furthermore, due to the high cell density, the spreading of genes should be elevated by horizontal gene transfer. It was shown that on the surface of the plant root, microorganisms can attach and form microbial consortia (Morris and Monier 2003;

Ramey *et al.* 2004; Danhorn and Fuqua 2007; Rudrappa *et al.* 2008) and thus could facilitate resistance to antimicrobial substances. Furthermore, *Pseudomonas* is resistant against multiple antibiotics (Hancock and Speert 2000), and it was reported by Coward *et al.* in 1973 that a *P. aeruginosa* strain is resistant to silver-sulfadiazine. In case *Pseudomonas* carries antibiotic resistance genes, this genus could be an important reservoir for antibiotic resistance genes in the environment. Experiments by Molbak and coworkers (2003) revealed the occurrence of horizontal gene transfer between *P. putida* and indigenous bacteria in the rhizosphere of alfalfa sprouts, even without antibiotic pressure. SDZ is an antibiotic applied in veterinary medicine, but plasmids which carry SDZ-resistance genes could also contain resistance genes against antibiotics applied on humans. Since the rhizosphere was found to host various human bacterial pathogens (Berg *et al.* 2005), the transmission of antibiotic resistance genes from bacteria in the rhizosphere could increase the abundance of antibiotic resistance in human pathogens. However, *Pseudomonas* themselves are important human pathogens, such as *P. aeruginosa* which has the ability to infect humans and cause severe infections.

4.2.2.2 Influence on the abundance of SDZ-resistance genes

In the first part of the experiment, we determined the background levels of the *sul1* and *sul2* genes in fresh manure and bulk soil. In fresh manure, there was a high load of bacterial biomass measured by the amount of 16S rRNA genes, as well as surprisingly high amounts of *sul1* and *sul2* relative to 16S rRNA genes which were also enriched in bulk soil after manure and SDZ application. This leads to the assumption that the manure used in this experiment carried SDZ resistant bacteria. Also other studies identified manure as an important source of antibiotic resistance genes. Zhou and coworkers (2009) detected macrolide-resistant bacteria in manure samples from antibiotic free swine. Manure sampled from cattle farms in the USA with no prior antibiotic treatment contained *sul1* genes and also bulk soil samples without manure application revealed the presence of this antibiotic resistance genes (Munir and Xagorarakis 2011). The manure used in this experiment was obtained from pigs of an organic farm without antibiotic-treatments. However, farm pigs were allowed to go outside the stable and might have been in contact with environmental microorganisms carrying SDZ-resistance genes. In the study presented here, also bulk soil which was only amended with water contained SDZ-resistance genes. This is in accordance with the theory that antibiotic resistance genes naturally occur in the environment (D'Costa *et al.* 2006; Martinez 2008).

Brooks *et al.* (2007) estimated 2.53×10^6 to 1.06×10^7 CFU g⁻¹ of antibiotic resistant bacteria to be present in untreated bulk soil.

In the second part of this experiment, the dissemination of the *sul* genes was investigated in the artificial rhizosphere. The abundances of the *sul* genes were significantly influenced by the type of soil pretreatment. When ARMs were incubated in soils pretreated with manure and SDZ, gene levels were significantly higher in comparison with ARMs incubated in soil treated with manure or water with very low amounts of the *sul* genes.

In the following discussion, we will only focus on ARMs incubated in soil pretreated with manure and SDZ. We detected an increase of the *sul* genes in the bacterial population of ARM between 1 and 2 weeks of incubation, however there were no significant differences between ARMs with exudates (without additional SDZ) and the controls (without exudates). A study by Kopmann *et al.* (2013) compared SDZ-resistance development in bulk soil versus maize rhizosphere in a mesocosm experiment. In accordance with our study, they detected an enrichment of SDZ-resistance genes in the rhizosphere when soil was treated with manure from SDZ-amended pigs in comparison with manure from untreated pigs. However, in the plant rhizosphere, they measured lower abundances of the *sul* genes compared with bulk soil, which was accompanied by a faster degradation of the antibiotic in rhizosphere than in bulk soil. Hence, the antibiotic applied in the beginning of our experiment could have been degraded and therefore being too low to select for antibiotic resistance in the bacterial population of the ARM. When SDZ was additionally added on the surface of ARM, there was a significant enrichment in the abundance of *sul2* relative to 16S rRNA genes after 2 weeks in comparison with ARMs only covered with exudates or agarose. The level was even higher in order of 2 magnitudes in comparison with fresh manure or bulk soil preincubated with manure and SDZ. This effect was only observed if soil was pretreated with SDZ and manure, otherwise *sul* levels remained in all ARM treatments on a very low level. The same trend was observed for *sul1*, but it was not significant. These findings lead to the conclusion that the rhizosphere is an important environment for the spreading of antibiotic resistance genes if the antibiotic is present in high concentrations. The accumulation of antibiotic resistance genes in soil hot spots was also reported by Brandt *et al.* (2009). They applied artificial root exudates and an antibiotic compound to the soil and observed an increase of antibiotic resistance genes in the soil. However, levels of SDZ-resistance genes could be lower in natural rhizosphere systems in comparison with our model due to the uptake of antibiotic compounds by plants (Dolliver *et al.* 2007; Bassil *et al.* 2013) although this factor has been argued to be quite low.

In a further study, the development of *sul* gene abundances in the rhizosphere after repeatedly manure application from SDZ-treated pigs was investigated in a field experiment (Jechalke *et al.* 2013). They also observed lower amounts of *sul* genes in the rhizosphere in comparison with bulk soil, however in the rhizosphere there was a constant increase in the abundance of the antibiotic resistance genes compared with the control treatment. This effect was not observed in bulk soil, and the authors suggested that this might be due to ongoing selection pressure in this compartment.

In the presented study, the increase of SDZ resistance genes could be the result of the exchange of mobile genetic elements, an accumulation of resistant clones, or multiple antibiotic resistant copies per cell. *Sul1* is usually linked to Tn21 type integron and *sul2* is encoded on IncQ plasmids (Skold 2000), which are highly mobile and can spread within bacterial populations. Piggery manure was identified as a reservoir of transferable multiple antibiotic resistance plasmids (Binh *et al.* 2008) and it was reported that in the rhizosphere exists a prevalence of LowGC type plasmids carrying antibiotic resistance genes after manure and antibiotic application (Jechalke *et al.* 2013). Musovic *et al.* (2006) showed conjugal transfer between bacteria in the rhizosphere of barley *in situ* by a cultivation-independent method. A study by Mølbak *et al.* from 2007 investigated plasmid exchange ratios in the rhizosphere of barley. They reported 10-fold higher rates in the rhizosphere than in bulk soil. These studies support the occurrence of horizontal gene transfer as being the mechanism for the dissemination antibiotic resistance genes in the artificial rhizosphere. Thereby, also antibiotic resistance genes could be transmitted to human pathogens inhabiting this environment. Furthermore, rates of horizontal gene transfer differs by the plant species (Schwaner and Kroer 2001) which underlines the importance of using models to investigate this phenomenon in the rhizosphere.

5 Conclusion and Outlook

The plant-soil interface is a highly complex and dynamic environment, which is inhabited by various microorganisms. This field has gained strong interest, although much about drivers for rhizobacterial communities is still unknown. The ARM developed in the present study is a useful tool to investigate questions concerning microbial populations at the plant-soil interface as it attracts microorganisms typically inhabiting the rhizosphere and it allows reproducible microbial studies under defined plant root conditions, such as plant root exudation. To our knowledge, this is the first rhizosphere model combining artificial root exudates and a surface for microbial attachment. In future experiments, bacterial populations of ARM should be investigated in more detail to get better insight in the bacterial taxonomic groups inhabiting the ARM. Microbiome analyses could be addressed by Next-generation sequencing or matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS). This proteomic based technique allows rapid characterization of bacterial species and was already successfully applied to identify the microbiome in the rhizosphere of wheat (Stets *et al.* 2013). In this thesis, we demonstrated that malic acid plays a role in the early establishment of bacterial populations in the artificial rhizosphere. Besides testing compounds simulating common root exudates of plants, the application of crop specific exudate compositions on ARM could be examined to address their specific interactions with the rhizosphere bacterial community since their rhizospheric bacterial populations (Peiffer *et al.* 2013), as well as their root exudate compositions (Fan *et al.* 2001; Carvalhais *et al.* 2011), have been well studied.

Soil microbiological experts have argued whether plant or soil parameters are of greater importance for bacterial populations in the rhizosphere (de Ridder-Duine *et al.* 2005). Therefore, besides plant factors, the soil type, the soil pH or temperature and also their effect on the bacterial community of the ARM would be worth to investigate.

The rise of multi-resistant bacterial pathogens in humans have led to a great interest in understanding the spreading of antibiotic resistance genes in natural systems. The discovery of the presence of antibiotic resistant bacteria in natural environments (Linares *et al.* 2006; Martinez 2008) has been very important for understanding the natural role of antibiotics and

resistances, even before the antibiotic era. The influence of agricultural practices such as crop fertilizing by manure accompanied by antibiotic transmission on the structure of the bacterial community and the spreading of antibiotic resistance genes in the ARM was investigated in this thesis. Bacterial community structure was mainly influenced by manure application to the soil and the artificial root exudates on the ARM, whereas there was no additional effect observed by SDZ application in soil or on the ARM. However, we showed that antibiotic resistance genes disseminate in an artificial rhizosphere after manure and antibiotic application to the soil if the SDZ was applied on the surface of the ARM. Therefore, in soils with high concentration of an antibiotic, the rhizosphere can be a hotspot for the occurrence of antibiotic resistant bacteria. Whether this increase of abundance is due to horizontal gene transfer can be answered by applying the technique of exogenous plasmid isolation. We observed that *Pseudomonas* remains high abundant and that SDZ-resistance gene copy numbers increase under antibiotic selection pressure. If they are correlated could be addressed by isolation of *Pseudomonas* from the ARM and subsequent analyses of its resistome. Furthermore, it would be important to investigate if the spreading of antibiotic resistance genes in the ARM is a combined effect of the presence of exudates and SDZ or mainly due to the antibiotic. Therefore, ARM control samples only covered with SDZ (without exudates) on the glass slides should be implemented in the experimental set up. In further studies, it should be tested if the soil application of SDZ via manure influences bacterial resistance against human applied antibiotics, for example due to the spreading of multiple drug resistance plasmids. Furthermore, do other agricultural practices, such as herbicide and pesticide application trigger the spreading of antibiotic resistance by the exchange of multiple resistance plasmids?

6 References

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