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Microbial community composition during degradation of organic matter

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Abbreviations

16S	16S subunit of the prokaryotic ribosomes
alkB	gene coding for an alkane monooxygenase
b.d.l.	below detection limit
bp	Base pair
C	carbon
cDNA	complementary deoxyribonucleic acid
CT	conventional tillage
DNA	deoxyribonucleic acid
dw	dry weight
F. sylvatica	*Fagus sylvatica* L.
fwd	forward strand
HPLC	high-pressure liquid chromatography
K	potassium
MT	minimal tillage
mV	millivolt
N	nitrogen
n.a.	not applicable
NGS	next generation sequencing
OTU	operational taxonomic unit
P	phosphorus
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
rep-PCR	PCR amplifying repetitive sequence elements
rev	reverse strand
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SOM	soil organic matter
sp./spp.	species (sg./pl.)
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
Z. mays	*Zea mays* L.

List of publications and contributions

Publications in peer-reviewed journals

I. Töwe, S; Wallisch, S; Bannert, A et al. (2011): Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. *J Microbiol Methods* 84 (3). pp. 406–412. DOI: 10.1016/j.mimet.2010.12.028.

II. Fuka, MM; Wallisch, S; Engel, M et al. (2013): Dynamics of bacterial communities during the ripening process of different Croatian cheese types derived from raw ewe's milk cheeses. *PLoS ONE* 8 (11). pp. e80734.

III. Wallisch, S; Gril, T; Dong, X et al. (2014): Effects of different compost amendments on the abundance and composition of *alkB* harboring bacterial communities in a soil under industrial use contaminated with hydrocarbons. *Front Microbiol* 5. pp. 1–10. DOI: 10.3389/fmicb.2014.00096.

My contributions to the publications

I. I carried out the extractions according to the original and modified protocol of DNA and RNA co-extraction based on Griffiths et al. (2000), the separation of DNA and RNA via enzymatic digestion and spin columns. Regarding the manuscript, I was involved in writing the first draft of the material and methods part and the results.

II. I carried out the NGS data analysis including generation of graphs and figures. Additionally, I was writing the corresponding parts of the material and methods as well as the results of the manuscript.

III. I was mainly responsible for NGS data analysis and writing the first draft of the manuscript including the introduction, material and methods, results and the discussion.

Abstract

Litter degradation in terrestrial ecosystems accounts for one of the major nutrient inputs of organic matter that affect soil quality. The degradation is mainly driven by soil microbes that metabolize the organic material and provide essential nutrients, which in turn provide the basis for plant growth mainly in non-fertilized environments. Whereas it is well accepted that fungi drive litter degradation, the role of bacteria in this process is still under debate. Major drivers for the dynamics of bacterial communities colonizing litter material as well as their functional traits are still not well-described. Here, the bacterial community structure was studied based on molecular markers using next generation sequencing (NGS). Two genes were used as molecular markers, including the small subunit of the prokaryotic ribosome (*16S rRNA* gene) and the *alkB* gene, which codes for an alkane monooxygenase that catalyzes the degradation of plant-derived alkanes. With these two genetic markers the structure of bacterial communities could be identified both addressing dynamics in time and habitat. Three experiments were performed to identify the influence of (i) different litter types, (ii) different soil types, (iii) different field management and (iv) compost residues for triggering alkane degrading bacteria in petroleum-contaminated Technosols. A field experiment was conducted with maize (*Zea mays* L.) litter. Litter bags were incubated for up to 8 weeks in arable soil, which differed in the tillage practice. The samples were embedded in 10 and 40 cm soil depths respectively, reflecting the tillage horizon in the tilled soils. Bacterial diversity in leaf material was analyzed by using *16S rRNA* gene fragments. Traditional fingerprinting techniques based on *16S rRNA* gene fragment analysis indicate that the bacterial community composition depends mainly on the incubation time of the litter in the soil. After two weeks of incubation, the different tillage management practices did no longer affect the bacterial community changes detected on leaf litter. The driving bacterial key players could be identified by NGS, being *Bifidobacteriaceae*, *Lactobacillaceae* or *Kineosporiaceae* on fresh leaf material. Bacterial specialists occurring solely on *Z. mays* leaf litter were members assigned to many families known to be involved in the nitrogen (e.g. *Alcaligenaceae*, *Bacillaceae*, *Clostridiaceae*, *Streptomycetaceae*) and carbon cycles (e.g. *Beijerinckiaceae*, *Cellulomonadaceae*, *Clostridiaceae*, *Lactobacillaceae*). In a microcosm experiment, perennial beech (*Fagus sylvatica* L.) litter was incubated in litter bags up to 30 weeks under controlled conditions in two different soil types, Cutanic Luvisol and Calcaric Regosol. Just as in the experiment before, chemical and physical soil parameters were measured and bacterial diversity was analyzed by using *16S rRNA* gene fragments. The results indicate that at initial stages of litter degradation mainly those bacteria contributed which were already present on the litter material before leaf abscission. Over time, soil microbes colonized leaf litter, so that the bacterial community structure subsequently adapted to the specific soil type. Many bacteria solely typical for forest soil

(e.g. *Burkholderiaceae*, *Acidobacteraceae*, α-proteobacterial families) and known for their participation in the carbon turnover (e.g. *Acidobacteraceae*, *Holophagaceae*, *Sinobacteraceae*) as well as plant pathogens (e.g. *Coxiellaceae*, *Enterobacteriaceae*) were identified. Independently from leaf litter input, bacterial taxa designated as generalists were identified, such as bacteria assigned to proteobacterial families (e.g. *Acetobacteraceae*, *Bradyrhizobiaceae*, *Caulobacteraceae*, *Opitutaceae*, *Pseudomonadaceae*, *Rhizobiaceae*), *Burkholderiales* and *Verrucomicrobia*. In addition to that, bacterial families containing human (*Enterobacteriaceae*) or plant (e.g. *Xanthomonadaceae*, *Pseudomonadaceae*) pathogens were also identified on both litter types. Bacterial richness, diversity and evenness showed an overall increase over time in most cases and seemed to depend on the surrounding soil habitat, as these parameters only remained unchanged on *F. sylvatica* leaf litter incubated in Calcaric Regosol. Compost amendment to petroleum-contaminated soil which originated form an industrial area was used to investigate the effects of compost addition on the abundance and diversity of bacteria harboring the *alkB* gene for up to 36 weeks in microcosms. Compared to soil without amendments (control soil), a greater richness and diversity of *alkB*-harboring prokaryotes was observed in the soil with amendments. Phylogenetic analysis suggested that compost addition stimulated the abundance of *alkB*-harboring *Actinobacteria*, γ-*Proteobacteria* such as *Shewanella* or *Hydrocarboniphaga* as well as *Cytophaga* (*Microscilla*) and α-*Proteobacteria* (*Agrobacterium*). Overall, the data has shown the importance of organic matter quality for bacterial community structure development.

Zusammenfassung

Der Abbau von Blattstreu in terrestrischen Ökosystemen bildet einen der wichtigsten Nährstoffeinträge von organischem Material, die sich auf die Bodenqualität auswirken. Der Abbau wird zu einem Großteil von Bodenmikroorganismen vorangetrieben, die stufenweise das organische Material im Boden verstoffwechseln und essentielle Nährstoffe für Pflanzenwachstum auf ungedüngten Boden bereitstellen. Während viel über den Einfluss von Pilzen auf den Streuabbau bekannt ist, wird der Einfluss von Bakterien derzeit immer noch diskutiert. Die Ursachen der Änderungen von bakteriellen Gemeinschaften auf dem Blattstreu sowie die Funktionen der Schlüsselorganismen wurden bisher nicht ausreichend beschrieben. In dieser Doktorarbeit wurde die Struktur der bakteriellen Gemeinschaft mit Hilfe von molekularen Markern mittels Next Generation Sequencing (NGS) untersucht. Es wurden zwei Gene als molekulare Marker verwendet. Zum einen wurde das Gen, das für die kleine Untereinheit des prokaryotischen Ribosoms codiert (*16S rRNA* Gen), ausgewählt. Als weiteres Marker-Gen wurde *alkB* herangezogen, das für eine Alkan-Monooxygenase codiert und den Abbau von pflanzlichen Alkan-Derivaten katalysiert. Mit den beiden genetischen Markern konnte die Zusammensetzung der bakteriellen Gemeinschaften identifiziert und die darüber hinaus resultierenden Änderungen der mikrobiellen Gemeinschaft bezüglich Zeit und Habitat bestimmt werden. Es wurden drei Experimente durchgeführt, um den Einfluss von (i) unterschiedlichen Arten von Blattstreu, (ii) unterschiedlichen Bodentypen, (iii) unterschiedlichen Feldbewirtschaftungen und (iv) einem Kompost-Inoculum in einem mit Rohöl verseuchten Industrieboden (Technosol) auf die Alkan-abbauende Bakterien zu untersuchen. Es wurde zunächst ein Freiland-Experiment mit Blattstreu von *Zea mays* L. durchgeführt. Streubeutel wurden in unterschiedlich tief gepflügtem Ackerboden bis zu 8 Wochen lang in 10 cm und 40 cm Tiefe inkubiert, was den jeweiligen Pflugtiefen entspricht. Die bakterielle Diversität in Blattmaterial wurde mittels *16S rRNA* Genfragmenten analysiert. Traditionelle Fingerprinting-Methoden basierend auf der *16S rRNA*-Genfragment-Analyse ließen Veränderungen der bakteriellen Gemeinschaft erkennen, die hauptsächlich abhängig von der Inkubationszeit des Blattstreus im Boden sind. Nach zwei Wochen wirkte sich die unterschiedliche Feldbearbeitung nicht weiter auf die Zusammensetzung der bakteriellen Gemeinschaft aus. *Bifidobacteriaceae*, *Lactobacillaceae* oder *Kineosporiaceae* konnten mittels NGS typischerweise auf frischem Blattmaterial identifiziert werden. Zahlreiche bakterielle Familien, die in den Stickstoffkreislauf (z.B. *Alcaligenaceae*, *Bacillaceae*, *Clostridiaceae*, *Streptomycetaceae*) und den Kohlenstoffkreislauf (z.B. *Beijerinckiaceae*, *Cellulomonadaceae*, *Clostridiaceae*, *Lactobacillaceae*) involviert sind, wurden ausschließlich auf Blattstreu von *Z. mays* identifiziert. In einem Mikrokosmen-Versuch wurde mehrjähriges Blattstreu von *F. sylvatica* in Streubeuteln bis zu 30 Wochen lang unter kontrollierten Bedingungen in zwei unterschiedlichen

Bodentypen (Parabraunerde und Pararendzina) inkubiert. Wie im vorherigen Experiment wurden auch hier chemische und physikalische Parameter gemessen und die bakterielle Diversität mittels *16S rRNA* Genfragment-Analyse bestimmt. Die Ergebnisse zeigen, dass in der Anfangsphase des Abbaus von Blattstreus hauptsächlich solche Bakterien beteiligt sind, die bereits vor dem Abtrennen des Blattes präsent waren. In darauffolgenden Zersetzungsstadien wurde das Blattstreu von Bodenmikroorganismen besiedelt, wodurch sich die bakterielle Gemeinschaft nach und nach an den spezifischen Bodentyp anpasste. Viele Bakterien, die typisch für Waldböden sind (z.B. *Burkholderiaceae*, *Acidobacteraceae*, α-Proteobacteria-Familien), sowie Bakterien, die eine Rolle im Kohlenstoffkreislauf (z.B. *Acidobacteraceae*, *Holophagaceae*, *Sinobacteraceae*) und als Pflanzenpathogene spielen (z.B. *Coxiellaceae*, *Enterobacteriaceae*), konnten identifiziert werden. Unabhängig von der Art des zugegebenen Blattstreus konnten ubiquitär vorkommende Bakterien identifiziert werden, wie etwa Familien von Proteobakterien (z.B. *Acetobacteraceae*, *Bradyrhizobiaceae*, *Caulobacteraceae*, *Opitutaceae*, *Pseudomonadacea, Rhizobiaceae*), *Burkholderiales* und *Verrucomicrobia*. Darüber hinaus wurden bakterielle Familien auf beiden Streutypen identifiziert, die human- (*Enterobacteriaceae*) oder pflanzenpathogene (z.B. *Xanthomonadaceae*, *Pseudomonadaceae*) Species enthalten. Artenreichtum, Diversität und Evenness der Bakterien stieg insgesamt bei fast allen Proben mit der Zeit an und schien abhängig vom umgebenden Bodenhabitat zu sein, da diese Parameter nur bei Blattstreu von *F. sylvatica* inkubiert in Pararendzina gleich blieben. Die Zugabe von Kompost zu einem Petroleum-kontaminierten Boden eines Industriegebiets wurde genutzt um Bakterien, die das *alkB*-Gen tragen, über einen Zeitraum bis zu 36 Wochen in Mikrokosmen zu untersuchen. Verglichen mit einem Boden ohne Kompost-Zugabe (Kontrollboden), wurde in Böden mit Kompost eine höhere Artenvielfalt und Diversität von *alkB*-tragenden Prokaryoten beobachtet. Phylogenetische Analysen deuten darauf hin, dass die Kompost-Zugabe die Abundanz von *alkB*-tragenden *Actinobacteria*, γ-*Proteobacteria* wie etwa *Shewanella* oder *Hydrocarboniphaga* sowie *Cytophaga* (*Microscilla*) und α-*Proteobacteria* (*Agrobacterium*) erhöht. Die Ergebnisse zeigen, welche Bedeutung die Qualität von organischem Material für die Entwicklung der Struktur bakterieller Gemeinschaften hat.

1 Introduction

1.1 Soil functions and soil organic matter dynamics

Soils are essential for life on earth, as they provide ecosystem services such as food and natural resources for all living organisms. The chemical and physical soil structures regulate the drainage, flow and storage of water as well as solutes in these habitats. Soils also store, moderate and release nutrients like carbon, nitrogen and phosphorus as well as other elements and pollutants. Water flow and biogeochemical processes drive the chemical transformation of these substances into forms that are available for plants. Soils also provide filtering and buffering systems that are involved in the bioremediation of toxic substances. Additionally, soils serve as habitat for a broad range of organisms which are part of the soil food web and are thus also involved in nutrient cycling (Harris et al., 1996).

Soil organisms and soil quality are directly connected with each other and hence are influencing the nutrient cycle in this habitat. Plant growth is directly linked to those nutrients available. Carbon represents the most abundant element in plants, whereas nitrogen is the limiting nutrient for plant growth in most cases. Nitrogen and carbon turnover in soil therefore has a prominent role resulting in the C/N ratio which is used as an indicator for nitrogen availability for both plants and microorganisms. The lower the C/N ratio in the soil, the more N is available for plants (Bausenwein et al., 2008; Kögel-Knabner et al., 2008). Hence, the degradation of plant material contributes to a major part to the cycling not only of carbon, but also of nitrogen and other essential nutrients. To prevent nutrient bleaching, the input of litter and organic waste is necessary for a balanced nutrient level in the soil habitat. Plant litter is one of the major inputs resulting in soil organic matter (SOM). SOM can be seen as the sum of carbon-containing substances from plant and animal residues in diverse stages of decomposition, from substances synthesized chemically or microbiologically from the breakdown products, and the living and dead microorganisms including their remains (Schnitzer, 1999). An average of above-ground input of leaf litter is estimated to be in the range of 100-400 g dry mass m^{-2} y^{-1} (boreal coniferous forests) to 600-1200 g dry mass m^{-2} y^{-1} (tropics) depending on latitude (Kögel-Knabner, 2002). Its quantity, composition and properties highly influence the formation of SOM as well as of subsequent humification processes in the soil (Swift et al., 1979). Hence, SOM and associated microbes affect the global carbon cycle tremendously (Gougoulias et al., 2014).

The availability of the main components of the organic substances, such as nitrogen and carbon, are vital for the plants. On a global level, vegetation is estimated to store 5-7×10^2 Pg C and 1×10^3 Tg N. C and N are estimated to contribute to SOM with 1.5×10^3 Pg C and 1.3×10^5 Tg N respectively (Nieder

and Benbi, 2008). During decomposition of plant residues, the carbon is transformed to organic matter which has an essential impact on the soil quality. For maintaining the soil fertility, amendments, such as organic wastes (e.g. plant residues), play an important role in providing sources of nutrients for plant growth and enhancing the physical, chemical and biological soil quality (Diacono and Montemurro, 2010). Inputs of plant, animal and microbial residues are the main constituents of the organic matter, which is set off against the decomposition rate of substrate mineralization of both the added and the already existing organic matter. Decomposition of organic matter is highly influenced by numerous abiotic factors such as temperature, soil moisture or pH, which directly affect the metabolism of the soil microorganisms and thereby also the cycling and release of nutrients to the plants (Murphy et al., 2007). Organic matter amendments increase the organic carbon stock in the soil which represents the largest reservoir for organic carbon on a global scale (Schlesinger, 1995). Due to the negative charge of the organic matter, it leads to nutrient retention and facilitates nutrient uptake by the plants (Weber et al., 2007; Kaur et al., 2008).

1.2 Microbial contribution to organic matter degradation

Soils harbor a multitude of different organisms, which also include a tremendously high number of microorganisms participating in nutrient cycling. Soil organisms are therefore organized in complex soil food webs comprising several trophic levels and which play a vital role in terrestrial ecosystem functions such as nutrient cycling and organic matter degradation (Gessner et al., 2010). It is remarkable, that a reduction of specific species groups has only little effect on overall processes, because other microorganisms take on their functions (Nannipieri et al., 2003). Functional redundancy of vital processes is widespread under most soil microorganisms, for example for ubiquitous biogeochemical processes such as mineralization of carbon compounds during litter decomposition. This stands in contrast to specialized functions such as nitrogen fixation, which are carried out only by specific bacterial groups (Schimel, 1995; Bell et al., 2005). In addition, physic-chemical processes and vegetation also influence these processes. Figure 1 illustrates schematically the soil micro-food web in the context of climatic and physical conditions as well as plant growth. The role of fauna and its impact on the nutrient cycle on higher trophic levels is well understood and has resulted in detailed soil food web models. The capability of nutrient mineralization and thus the decomposition of plant litter mainly depends on the decomposing microbial community (Swift et al., 1979; Hättenschwiler and Vitousek, 2000; Wardle et al., 2004). With regard to soil microorganisms, the ubiquitous fungal contribution to plant material decomposition has been well analyzed in the past (Setälä and McLean, 2004, van der Wal et al., 2013, 2013; A'Bear et al., 2014; Boberg et al., 2014).

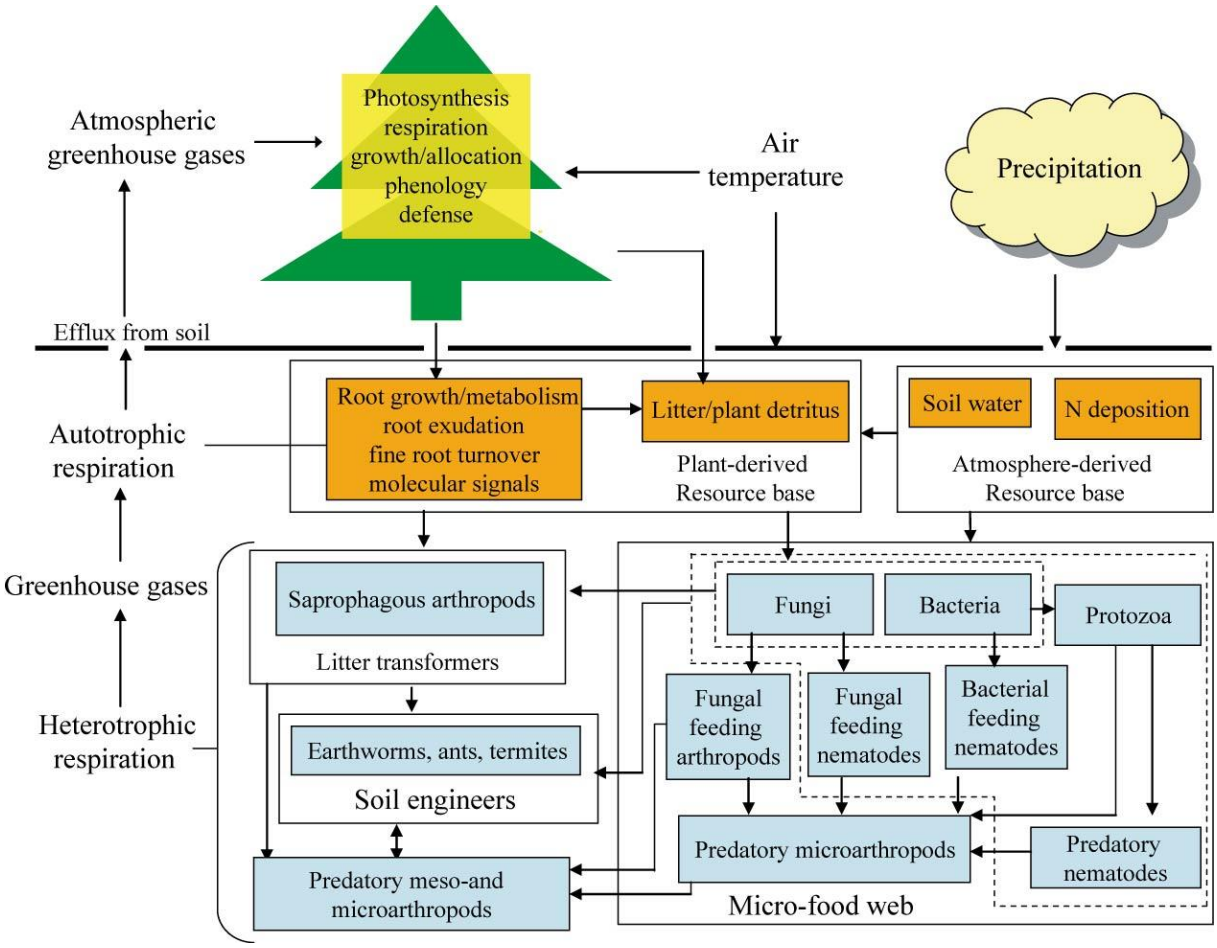

Figure 1: Soil food web. Scheme adapted from Pritchard (2011)

Soil microorganisms interact strongly with the plant roots and decomposing plant material, which results in advantages for both sides. While plants take up the nutrients provided by the microbes, the latter benefit from using the plant litter as a substrate for their own metabolism. Most of the plant cell wall components are assigned to hemi-/celluloses, lignin, lipids, proteins and cutin covered with waxes (Kögel-Knabner, 2002). Apart from that, also water-soluble phenolic compounds and sugars are also degraded by a variety of soil microbes. The more recalcitrant nutrients are preferably catabolized by fungi, in contrast to the bacterial decomposers that focus mostly on the small and easily degradable molecules. Therefore, besides the fungal participation in plant detritus, bacteria are also mainly involved in decomposition processes (Fægri et al., 1977; Boer et al., 2005).

Several studies demonstrated that litter decay rates varied under identical environmental conditions (Wardle et al., 1997; Cornelissen and Thompson, 1997). These differences were attributed to variation in litter traits, such as nitrogen, lignin and polyphenol concentrations as well as C/N and lignin/nitrogen ratios (Hättenschwiler et al., 2011). Lignin is one of the major compounds of plant material and is comparably resistant against microbial decomposition. In angiosperms, its precursors such as *p*-coumaric acid and ferulic acids, were identified. In previous studies, a ratio of 56:40:4 -

referring to coniferyl:sinapyl:*p*-coumaryl alcohol - was measured in beech, whereas in grass a ratio of 1:1:1 was detected (Kögel-Knabner, 2002). With regard to the decomposition of recalcitrant substances, Fontaine et al. (2003) reported a priming effect due to the amendment of introduced organic matter. According to this concept, a huge variety of soil microbes is present, but most of them are in dormant stages due to the lack of appropriate organic resources. By adding fresh organic matter, these dormant microorganisms are triggered to activity and are able to metabolize the specific substrates provided by the amendment. The members of this initial triggered bacterial community are classified as r-strategists which are adapted to rapid intervals of growth depending on the availability of the substrates. Subsequently, these r-strategists perish or become dormant again until enough nutrient input is available again. Many of the r-strategists are members of ubiquitous soil bacterial phyla, such as *Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia* and others, which make up 92% of the soil libraries analyzed by Janssen (2006). In contrast to that, the K-strategists are microorganisms that mainly metabolize the soil organic matter. These K-strategists have a slower growth rate due to metabolizing the more complex molecules which need more energy for degradation, e.g. for nitrifying (*Nitrosomonas* sp., *Nitrobacter* sp., etc.) or for methane oxidizing (*Methylocystaceae*, *Methylococcaceae*, etc.) bacteria (Kolb, 2009; Ettwig et al., 2010; Attard et al., 2010). This leads to the fact that they have their own niche beside the r-strategists. K-strategists are responsible for metabolizing the complex and insoluble compounds of carbon residues that may induce the so-called priming effect, i.e. the stimulation of SOM mineralization (Bingeman et al., 1953). This stimulation is enhanced due to the catabolites produced by the r-strategists. These catabolites are then subsequently metabolized by the K-strategists to build up the SOM.

1.3 Anthropogenic influences affecting leaf litter degradation in soil

Cultivated soils are dominating the terrestrial environment in agriculture. The naturally given physical soil properties are strongly influenced by the fertilization and tillage field management of arable soils. Hence, tillage has an intense effect on the soil biota in general, for example meso- and macrofauna, as well as on the microorganisms colonizing the soil and interacting with the plants. Different ploughing systems are disturbing the physical and chemical soil properties and thus have an intense effect on the soil-borne microorganisms (Kladivko, 2001; Dominy and Haynes, 2002) as well as on nutrient contents. The influence of tillage management on soluble organic carbon and soluble organic nitrogen has therefore been investigated intensively (e.g. Karlen et al., 1994; Frede et al., 1994; Lal and Kimble, 1997; van den Bygaart et al., 2002). According to Wardle et al. (1997), SOM as well as organic C and N were slightly reduced in tilled soil environments compared to untilled sites. Yin et al. (2010) detected different actinobacterial groups depending on field management. To enhance microbial communities in the soil for positively affecting crop growths, crop rotation can be

carried out. To increase crop yield, enhance soil fertility and prevent soil erosion, crop rotation has been applied for a long time in agricultural history. It has been shown that the cultivation of different types of crops in the same area in sequential seasons positively affects the microbial biomass and soil enzyme activities when compared to continuously monocultured fields. This approach, for example, also minimizes the level of *Fusarium* (Dick and Tabatabai, 1987), a known fungal plant pathogen (Enjalbert et al., 2005) that also contaminates the soil with pathogenic toxins (Schollenberger et al., 2006). Crop rotation increases the level of microbial biodiversity, which in turn enhances antagonistic microorganisms. Besides pest control, the alternation of root crops and cereals enhances diverse nutrients to preserve a well-balanced proportion of carbon and nitrogen. In addition to natural fertilizing, the adding of further N, P and K in dissolved forms ready for take up by the plant, an increase of the harvesting yield will be achieved and will also influence the microbial community of the soil bacteria. For example, fertilization with animal manure also increases the number of bacteria under continuous cropping systems (Dick, 1992). Additionally, mechanical land management in forms of tillage leads to consistent mixture of the mineral soil material and plant residues, and thus enhances the microbial activity which fosters the nutrient supply for microorganisms and plants. Besides these various impacts on the agricultural sector, the last 200 years of industrial revolution have seen an ongoing environmental contamination of soil. Apart from various chemicals such as herbicides (Schellenberger et al., 2012) and fungicides (Torgeson, 1967), heavy metals and petroleum derivatives (Huesemann, 1994; Giller et al., 2009) also increasingly put stress on microbial soil communities that have to deal with the contamination. Another anthropogenic influence can be seen in the contamination of the environment due to petroleum. Since the 1850s, industry has focused more and more on petroleum and petroleum-based products as a major source of energy (Speight, 2002). Serious environmental pollution is caused when soil gets contaminated by leaks or accidental spills during oil exploration, industrial manufacturing processes and transport. Linear and branched alkanes, cycloalkanes and other aromatic compounds which are part of petroleum are then released to the environment, leading to the contamination of terrestrial and aquatic ecosystems. As these chemical substances are inert, alkanes with ≥ 25 carbon residues in particular are associated with serious problems when it comes to the restoration of oil-contaminated soil or water (Ji et al., 2013). In this context, microorganisms play an important role with regard to bioremediation. Many bacteria and fungi are known to produce enzymes for the degradation of alkanes for using them as source of carbon and energy. Alkanes are also naturally produced by many living organisms such as plants and animals, but also by microorganisms. Alkane derivatives are part of naturally built waxes (Eglinton et al., 1962), pheromones (Mori, 2007) or fungal spores (Oró et al., 1966; Fisher et al., 1972) and many organisms have established a metabolic degradation pathway adapted to those substances. Different types of alkane hydroxylases have been described so far, depending on the chain length of the alkane

used as an input source. The alkane monooxygenase of bacteria (van Beilen et al., 2001; Kloos et al., 2006) uses alkanes with a medium carbon chain length (C_5-C_{11}), whereas alkanes with shorter and longer chain lengths are metabolized by other enzymatic pathways. The AlkB family of alkane hydroxylase mainly uses C_5-C_{16} *n*-alkanes as a substrate (Rojo, 2009). Many petroleum-contaminated soils have only limited potential to bioremediation, which can be attributed to an overall low microbial biomass due to reduced nutrient input and sparse vegetation (Scalenghe and Ferraris, 2009). Hence, nutrient input has been added to contaminated soils for increasing the bioremediation processes. Several studies have analyzed the effect of organic matter addition on petroleum-contaminated soil. Positive effects in alkane degradation after organic matter addition to the soil have been reported so far (Beaudin et al., 1999; van Gestel et al., 2003; Schulz et al., 2012) but it has remained unclear, whether these effects were due to a general shift of microbial community or an additional input of alkane-degrading microorganisms together with the plant residues. Also, only very few studies have revealed the community composition of the *alkB*-harboring microorganisms that are potentially involved in the bioremediation of petroleum-contaminated soils (Pérez-de-Mora et al., 2011; Giebler et al., 2013a).

1.4 Aims and hypotheses

Plant residues and their degradation highly influence the chemical soil properties. This nutrient input also affects the microbial soil community. Litter degradation in soil corresponds considerably to the type of litter, to the physical and chemical conditions in the soil environment, and to a high degree also to the microorganisms that contribute to the nutrient transformation. In the past, numerous studies have focused on the fungal participation during plant liter degradation and reported community shifts of the microbes involved. Many studies have analyzed shifts of bacterial community composition under different conditions based on the diversity of *16S rRNA* gene fragments using T-RFLP or DGGE for molecular fingerprinting (Sessitsch et al., 2004; Kennedy et al., 2004; Enwall and Hallin, 2009; Berthrong et al., 2013). In this context, microbial shifts have been reported, but phylogenetic classification was not at high resolution. Only very limited information about the driving bacterial key players is available so far. In this work, the bacterial key players at different leaf litter degradation stages have been identified using next generation sequencing based on *16S rRNA* gene fragments. Thereby, the distinct identification of bacterial key players up to genus level can be achieved in order to gain a deeper insight into the bacterial community composition as well as the interaction of specific bacterial groups depending on different degradation stages and the surrounding soil environment.

In this context, the following hypotheses have been proposed and tested:

- During different stages of leaf litter degradation, metabolic versatile bacterial groups such as *Proteobacteria* or *Acidobacteria* occur regardless of the surrounding soil habitat and nutrient input.
- The occurrence of specific bacterial groups such as *Nitrosomonas* sp. or *Methylocystaceae* during leaf litter degradation is linked to the specific soil and different leaf litter types.
- The co-occurrence of bacterial groups is influenced by the different leaf litter types.
- The addition of compost will enhance alkane-degrading bacterial communities in petroleum-contaminated industrial soil due to the introduction of new microbe and/or new substrates.

To verify these hypotheses, three different experiments were set up. In a first, short-term experiment of up to 8 weeks, a field trial was conducted to observe the bacterial colonization of *Z. mays* L. leaf litter in arable soil. With this approach, bacterial community composition on leaf litter was analyzed within the same soil type, but under different ploughing field management practices, such as conventional and minimal tillage. In a second long-term experiment of up to 30 weeks under controlled conditions in microcosms, bacterial colonization on *F. sylvatica* leaf litter was analyzed over time in two different soil types. For both experiments, leaf material was sewed in litter bags and molecular methods were used to characterize the bacterial community composition colonizing the degrading leaf material. With traditional fingerprinting techniques, shifts in bacterial community composition can be uncovered and subsequently linked to next generation sequencing data to identify the bacterial key players in different degradation stages cross-linked to different tillage treatments and soil types respectively. In a third experiment, practical implementation was conducted by analyzing the bacterial community colonization in a petroleum-contaminated Technosol which was enriched with compost comprising annual and perennial plant material. Here, the alkane-degrading microorganisms were analyzed as they could be enhanced using alkanes as a substrate for their metabolism. These data can help to identify the key players that are possibly involved in the decontamination of these soils.

My contribution to this work

For experiment A (Monitoring the dynamics of microbial communities during leaf litter degradation of *Z. mays* under different tillage management practices), I was responsible for planning and performing the experiment and sampling, including sample preparation for further analyses. Within these processes, I was also mainly involved in optimizing the nucleic acid extraction protocol (Töwe et al., 2011). I also processed and analyzed the samples on the molecular level, including subsequent data analysis and development of a data analysis pipeline for next generation sequencing of the *16S rRNA* gene amplicon fragments. I evaluated the degradation rates. I was mainly involved in preparing the analyses of the physical and chemical parameters (dry mass, sugars, amino sugars and starch) and subsequently performed the data analysis.

For experiment B (Monitoring the dynamics of bacterial communities during leaf litter degradation of *F. sylvatica* incubated in different soil types) I processed and analyzed the samples on the molecular level, including subsequent data analysis. I evaluated the degradation rates and performed the data analyses of the physical and chemical parameters (dry mass, sugars, amino sugars and starch).

For experiment C (Monitoring of *alkB*-harboring bacterial communities during compost degradation in petroleum-contaminated soil), I performed the NGS data analysis of *alkB* gene fragments as well as the manuscript preparation.

2 Material and methods

2.1 Experimental setup

2.1.1 Experiment A: Monitoring the dynamics of microbial communities during leaf litter degradation of *Z. mays* under different tillage management practices

To analyze the dynamics of the bacterial communities colonizing maize, a field experiment using arable soil was performed. The experiment was conducted at the research farm Scheyern, Germany (48°30' 8" N, 11° 26' 41" E) at 448-514 m above sea level. At this location the annual mean air temperature is 7.4°C, and mean precipitation is 833 mm y^{-1} related to 30-year normals (Auerswald and Kainz, 1990). The experiment was conducted on the plot IC, which is part of the long-term experiment "Integrierter Systemversuch" in the northeastern area of the research farm (Figure 2). This plot is hallmarked by a mean slope of 3% exposed to the southeast. The main soil parameters and details of the tillage treatment are summarized in Table 1.

Table 1: Main soil parameters of the field experiment with leaf litter of *Z. mays* (taken from http://fam.weihenstephan.de/indexalt.html)

tillage type	ploughing depth	sampling depth	Soil type	Clay	pH ($CaCl_2$)	C_{org}	N_{total}	C/N
Conventional (CT)	25-30 cm	30 cm	Cambisol	20%	6.0	1.3%	0.15%	8.7
Minimal (MT)	5-10 cm	10 cm	Cambisol	20%	6.5	1.2%	0.32%	9.2

The plot was cultivated with *Z. mays* L. (main crop, cultivar: Lacta) until 10.10.2009, followed by harvesting the maize. At the plot, different types of tillage management were compared using a split plot design: Since 1992, half of the plots were subjected to conventional tillage (25-30 cm depth), whereas for the other half minimal tillage was used (5-10 cm depth). Final tillage took place on 21 October 2008, with sowing on 17 April 2009. At the end of the vegetation period (October 2009), *Z. mays* leaves were cut off the stems and cut into pieces of approx. 1 cm^2. While aliquots of fresh leaves were stored at -80°C until further analysis, additional 36 litter bags with fresh leaf material were prepared for a leaf litter degradation experiment on the plots subjected to different tillage incubation in the agricultural soil. For this purpose, leaf material was sewed into 5×5 cm nylon bags (Nylon Net Filter, mesh size 40 µm, Millipore, Billerica, USA) to prevent meso- and macrofauna from participating in degradation during the incubation time in soil.

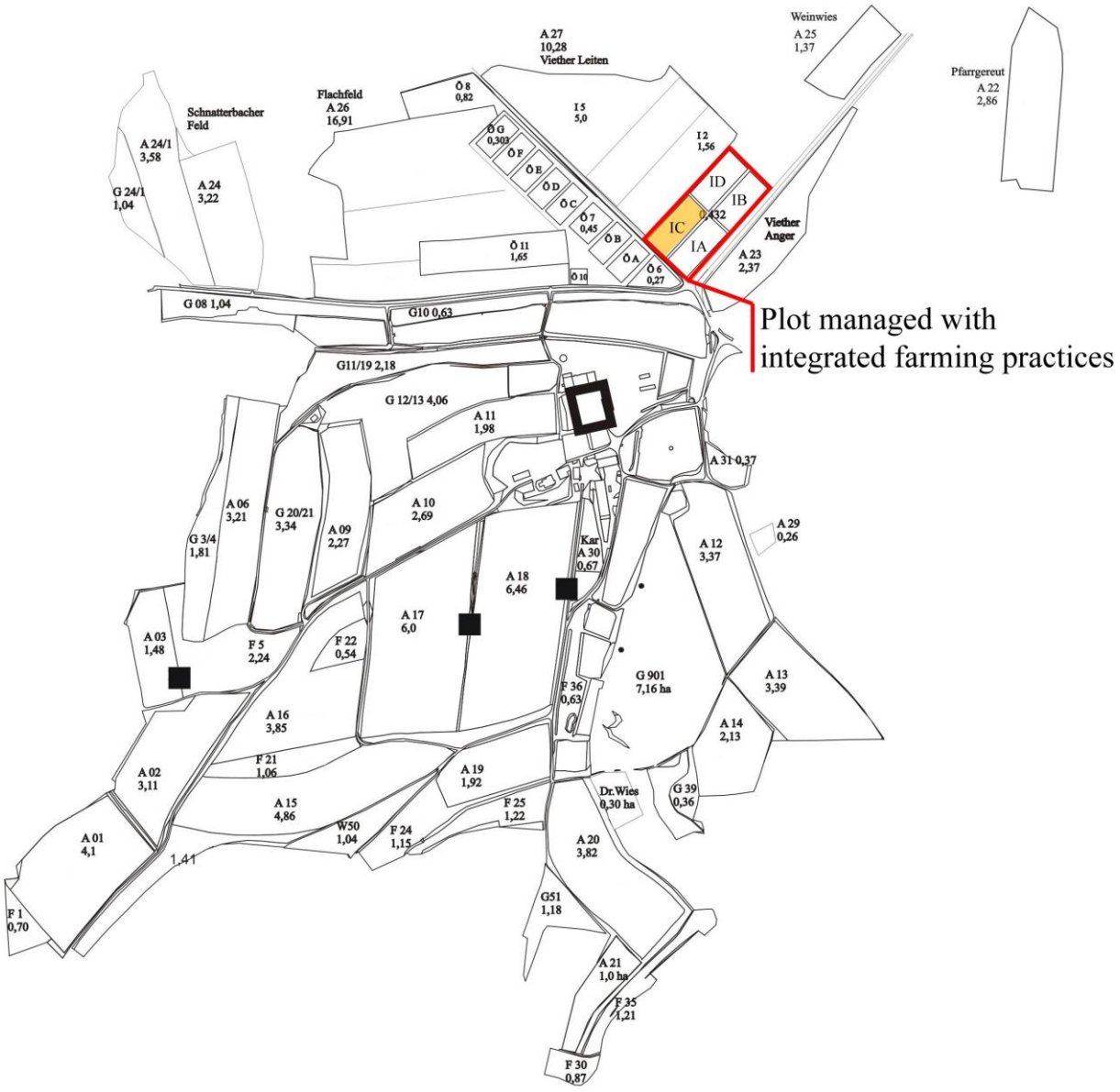

Figure 2: Map of the research farm Scheyern (taken from http://fam.weihenstephan.de/indexalt.html). Experimental lot highlighted in orange

In total, 18 litter bags were embedded in ca. 30 cm depth at the conventional tilled section, whereas another 18 litter bags were embedded in ca. 10 cm depth, which was the minimal tilled section of the plot. Each bag was placed circularly (Ø 1.5 m) in the boundary between the tilled and untilled layers (Table 2 and Figure 3).

Table 2: Sampling dates and soil temperature of *Z. mays* leaf material

Date	Activity	Soil temperature
16.09.2009	placement of samples	16°C
30.09.2009	1st sampling (6 litter bags)	14°C
14.10.2009	2nd sampling (6 litter bags)	5°C
11.11.2009	3rd sampling (6 litter bags)	3°C

Figure 3: Placement of litter bags filled with *Z. mays* leaf material.

Sampling took place after two, four and eight weeks of leaf litter incubation in the soil. At each sampling time point, six leaf litter bags were excavated both from the conventional tilled as well as from the minimal tilled site. The content of each of the six leaf litter bags was separately shock frozen with dry ice and stored at -80°C until further analysis and preparation. The six litter bags per sampling time point were used as follows: The content of one litter bag was used for determination of the dry mass and testing purposes. The content of two more litter bags was used for chemical analysis of the carbohydrates. The content of the last remaining three litter bags was used for molecular analyses.

For this experiment, molecular methods were implemented, including a randomized PCR, a gene specific PCR and NGS. Additionally, physical and chemical analyses were performed including the determination of dry mass and carbohydrates (Figure 4):

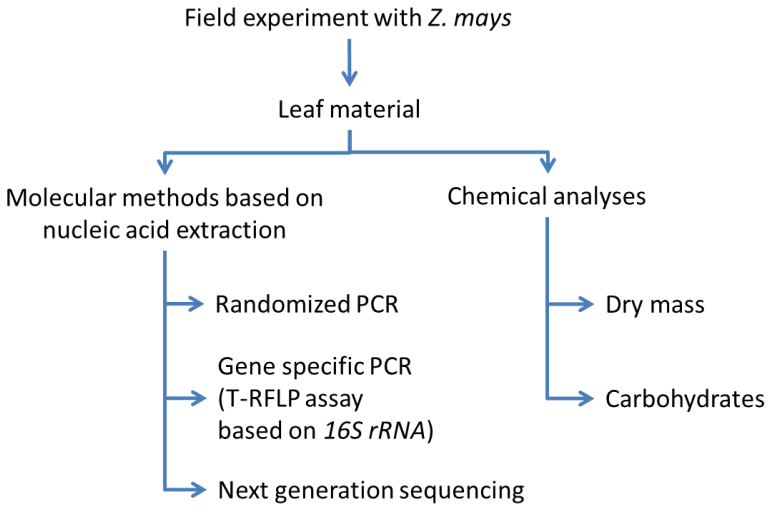

Figure 4: Methodological approach for experiment A with *Z. mays* leaf litter

The amounts of sugars, amino sugars and starch were analyzed by Frank Fleischmann[1] as previously described by Appuhn et al. (2004) and Fleischmann et al. (2009). All data of sugars, amino sugars and starch are summarized in Table A1. Starch was determined as glucose equivalents after enzymatic digestion of the remaining plant pellet with amylase and amyloglucosidase Maximal amounts of soluble sugars (99 mg $g^{-1}dw^{-1}$), trisaccharides (34 mg $g^{-1}dw^{-1}$), sucrose (36 mg $g^{-1}dw^{-1}$) and glucose (17 mg $g^{-1}dw^{-1}$) were present in fresh leaf material. The litter samples contained significantly lower concentrations of soluble sugars (18-24 mg $g^{-1}dw^{-1}$), trisaccharides (3-5 mg $g^{-1}dw^{-1}$), sucrose (7-10 mg $g^{-1}dw^{-1}$) and glucose (1-3 mg $g^{-1}dw^{-1}$ or below the detection limit of 0.3 mg g^{-1} dw^{-1}), with no effect caused by the tillage treatment. Amounts of starch were present in constantly low amounts (0.5 to 1.5 glucose equivalents g^{-1} dw^{-1}) throughout all samples, but increased towards the end of the experiment and with no effect caused by the tillage treatment (Figure 5).

[1] Sample preparation and measurements of the sugars, amino sugars and starch was performed by Dr. Frank Fleischmann (Section Pathology of Woody Plants, Technical University Munich, Weihenstephan, Germany).

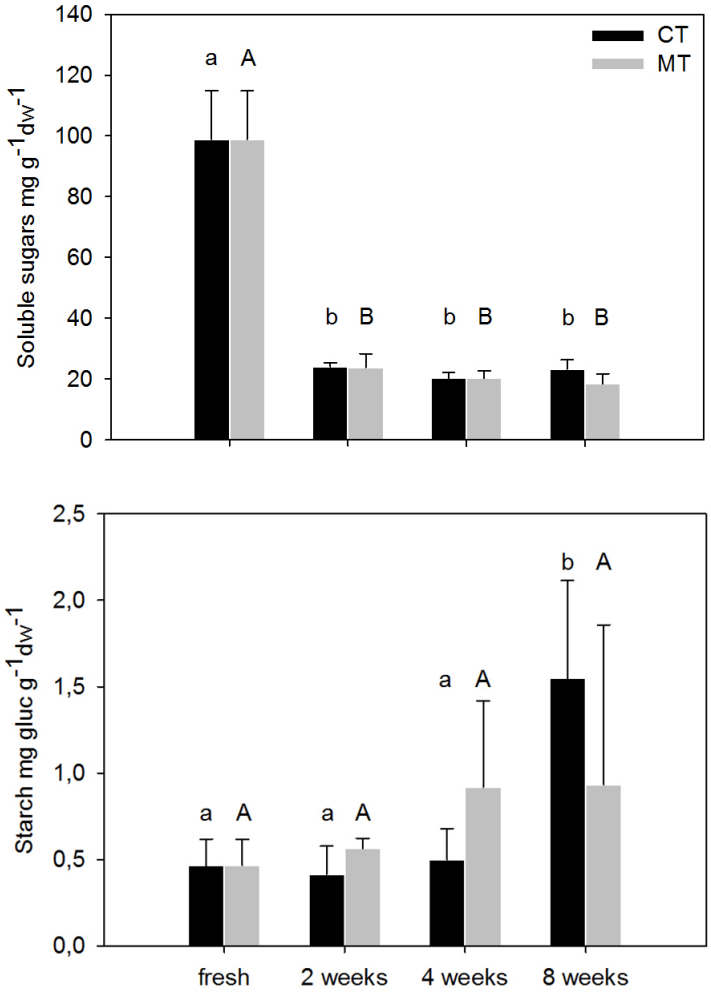

Figure 5: Soluble sugars and starch in leaf material of *Z. mays* incubated in arable soil. Mean and standard deviation are shown (n = 3). Significant different data are indicated in lower case for conventional tillage (CT) and upper case letters for minimal tillage (MT) according to Tukey multiple comparison test (p-value ≤ 0.05)

Fructose (12 mg $g^{-1}dw^{-1}$) was present in fresh material only, whereas tetrasaccharides were detected in litter samples only with significantly higher (p-value ≤ 0.05) amounts after 2 weeks (ca. 11 mg $g^{-1}dw^{-1}$), compared to all later sampling time points and again unaffected by tillage management (5-8 mg $g^{-1}dw^{-1}$). The amino sugars galactosamine and muramic acid were present only in litter samples with no significant differences. Whereas galactosamine concentration increases towards the later sampling time points by up to 2 mg $g^{-1}dw^{-1}$, muramic acid is at a constantly low level of ca. 0.3 mg $g^{-1}dw^{-1}$. Glucosamine occurred in low concentration in fresh material (ca. 3 mg $g^{-1}dw^{-1}$), and concentrations increased significantly in litter samples (12-17 mg g^{-1} dw^{-1}).

2.1.2 Experiment B: Monitoring the dynamics of bacterial communities during leaf litter degradation of *F. sylvatica* incubated in different soil types[2]

The aim of this experiment was to follow the microbial population development over time on the leaf litter embedded in different soils. A microcosm experiment was set up with leaf litter from a beech forest (*Fagus sylvatica* L.) near Helmsheim, Germany (49° 5' 39" N, 8° 40' 55" E, ca. 240m above sea level) and two different soil types (Cutanic Luvisol and Calcaric Regosol). Leaves were sampled directly from the trees in September 2008. Cutanic Luvisol and Calcaric Regosol soil (both from Ah horizon) were sieved (2 mm mesh size) and stored at 4°C in darkness until setup of the microcosms. 4 g of air-dried beech leaf litter was cut to ca. 1 cm^2 and sewed into 10×10 cm nylon bags (Nylon Net Filter, mesh size 40 µm, Millipore, Billerica, USA) to prevent any degradation by meso- and macrofauna. Important soil parameters (Sommer, 2002) are summarized in Table 3. The two soil types vary in $CaCO_3$ content and thus in humic acid enrichment and pH, which affects biota at the specific sites.

Table 3: Main characteristics of the Ah soil horizon at the two sampling sites at Helmsheim, Germany. Taken from Sommer (2002). b.d.l. = below detection limit

Soil type	Horizon	Depth	pH ($CaCl_2$)	$CaCO_3$	C_{org}	N_{total}	C/N
Cutanic Luvisol	Ah	0-6 cm	5,1	b.d.l.	5,7 %	0,34 %	17
Calcaric Regosol	Ah	0-11 cm	7,0	4,7 %	3,7 %	0,31 %	12

For each sampling time point after 1, 2, 4, 8, 16 and 30 weeks, five replicates for each soil type were set up. In total, 60 microcosms (ø 15 cm, 19 cm height) per soil were prepared in total. As a control, per soil type five additional replicates without litter bags were set up over the whole time period of 30 weeks. Figure 6 illustrates exemplarily a scheme and the final set-up of one of the microcosms. All microcosms were filled with 1946 cm^3 soil (1 g cm^{-3} bulk soil density), and the leaf litter bags were embedded below 3 cm of the soil surface. The top of the microcosms contained a removable cap for regular watering in order to maintain a constant water content of 40.0% (Cutanic Luvisol) and 38.5% (Calcaric Regosol), which corresponds to the field capacity of the two soils. A constant temperature of 15°C and darkness were guaranteed for all microcosms throughout the whole experiment. The sampled leaf litter was immediately frozen as replicates at -80°C until further processing.

[2] Setup of the microcosms was performed by Dr. Felix Haesler (Institute of Soil Ecology, Helmholtz Zentrum München, Oberschleißheim, Germany).

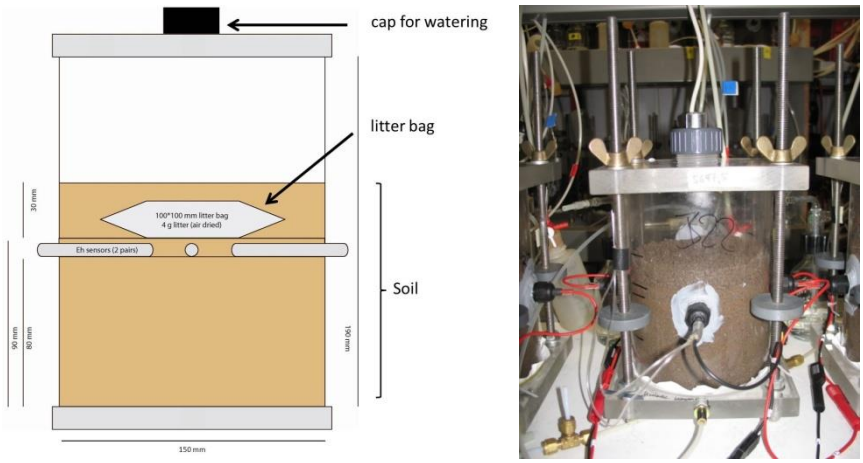

Figure 6: Microcosms with *F. sylvatica* leaf litter. Scheme (left) and final setup (right) of microcosms

To test the oxygen conditions in the microcosms, redox measurements with two pairs of redox sensors (Figure A1, constructed by Dr. Felix Haesler, Helmholtz Zentrum München, Oberschleißheim, Germany) were performed in 10 control microcosms without any leaf litter. The redox sensors were placed right below the leaf litter bags at a depth of 3 cm below the soil surface. After 5-10 days, stable redox potentials were observed ranging between 550-650 mV (Calcaric Regosol) and 550-800 mv (Cutanic Luvisol) (Figure A2). These results indicate well-aerated conditions during the incubation time in all the microcosms.

For this experiment, molecular methods were implemented, including a gene-specific PCR and NGS. Furthermore, physical and chemical analyses were performed including the determination of dry mass and carbohydrates (Figure 7):

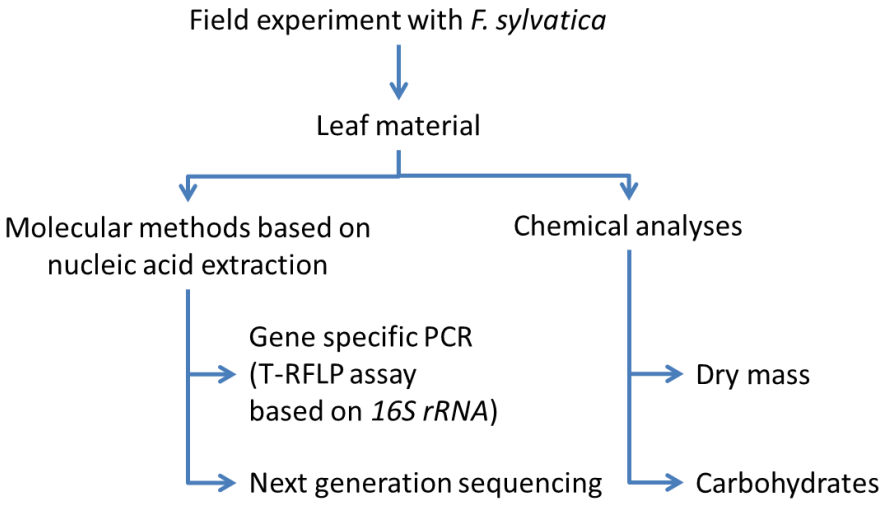

Figure 7: Methodological approach for experiment B with *F. sylvatica* leaf litter

For determining the decay of leaf litter, the amounts of sugars, starch as well as the mass loss of dry matter were determined as described in Chapter 2.1.1. The data of all sugars, amino sugars and starch is summarized in Table A2. Starch was determined as glucose equivalents after enzymatic digestion of the remaining plant pellet with amylase and amyloglucosidase. The total sugar content in leaf litter of *F. sylvatica* started at a low level (ca. 4 mg g^{-1} dw^{-1}) and significantly increased to ca. 6 mg g^{-1} dw^{-1} after 8 weeks (Cutanic Luvisol) and 16 weeks (Calcaric Regosol) respectively, and increased until the end of the experiment to a maximum of 11.9 (Cutanic Luvisol) and 9.2 (Calcaric Regosol) mg g^{-1} dw^{-1}. The amount of starch ranged in between 0.35 and 0.88 glucose equivalents mg^{-1} dw^{-1} with no significant trends. The amount of all soluble sugars in total as well as starch is visualized in Figure 8.

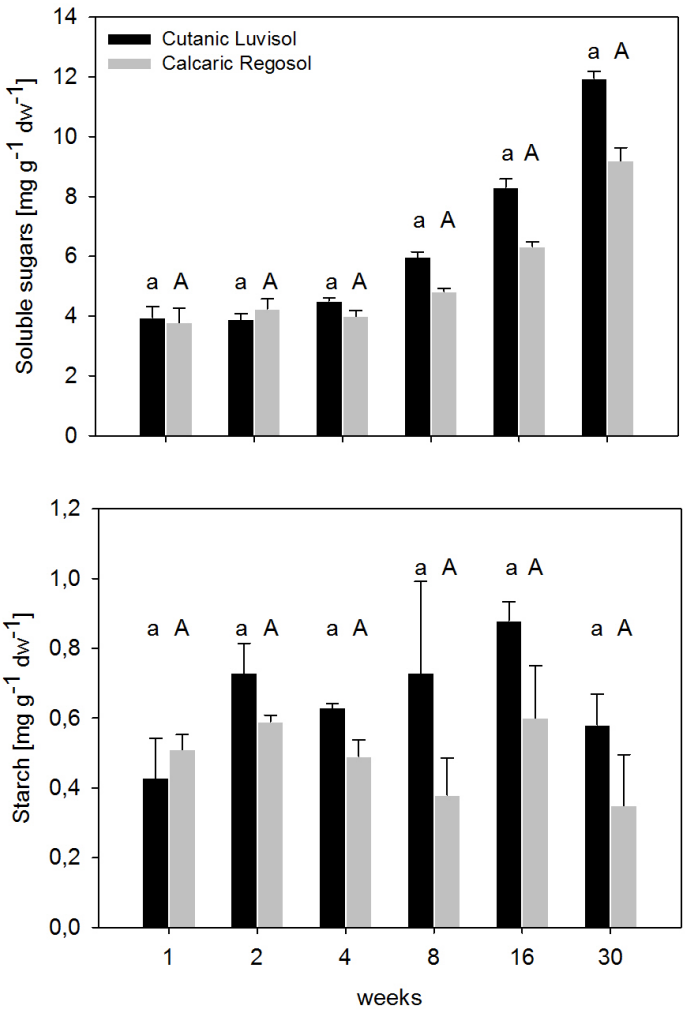

Figure 8: Soluble sugars and starch in leaf material of *F. sylvatica* incubated in two different soil types. Mean and standard deviation are shown (n = 5). Statistical significances within one soil type are indicated with upper case (Calcaric Regosol) and lower case (Cutanic Luvisol) letters according to Tukey multiple comparison test (p-value ≤ 0.05)

Whereas tetrasaccharides were only present within the first two weeks in leaf litter incubated in Cutanic Luvisol (0.12 to 0.22 mg g^{-1} dw^{-1}), tetrasaccharides in leaf litter incubated in Calcaric Regosol occurred throughout the whole experiment, but in slightly lower amounts (0.10 to 0.16 mg g^{-1} dw^{-1}) with a significant minimum after 4 and 16 weeks (0.04 mg g^{-1} dw^{-1}). The amounts of trisaccharides and fructose were below the detection limit of 0.2 and 0.3 mg g^{-1} dw^{-1} in all samples. With the exception of after one week of incubation in Calcaric Regosol soil, sucrose was omnipresent in low concentrations (0.06 and 0.58 mg g^{-1} dw^{-1}) with a significant increase towards the end of the experiment. In contrast to that, glucose was only present after 4 weeks (0.14 mg g^{-1} dw^{-1}) in Calcaric Regosol soil. While glucosamine concentrations in leaf litter incubated in both soil types were at a low level for up to 4 weeks (2.3-2.9 mg g^{-1} dw^{-1}, Cutanic Luvisol) and 8 weeks (2.2 to 2.5 mg g^{-1} dw^{-1}, Calcaric Regosol) respectively, this amino sugar increased later to 3.7 (Cutanic Luvisol) and 4.4 mg g^{-1} dw^{-1} (Calcaric Regosol). Galactosamine steadily increased from 0.2 to 3.7 mg g^{-1} dw^{-1} in leaf litter samples incubated in Cutanic Luvisol, whereas the increase of galactosamine from 0.3 to 2.6 mg g^{-1} dw^{-1} in leaf litter samples incubated in Calcaric Regosol soil was interrupted after 8 weeks of incubation (1.5 mg g^{-1} dw^{-1}). Muramic acid was present in all samples at a very low level between 0.01 and 0.04 mg g^{-1} dw^{-1} near detection limit.

2.1.3 Experiment C: Monitoring of *alkB*-harboring bacterial communities during compost degradation in petroleum-contaminated soil[3]

This experiment was conducted using soil from an industrial zone in Celje, Slovenia (46.2335°(N), 15.2764°(E)). Since 1945, heavy industry including chemical and steel industry has been located at this site, and the soil is thus known to have been contaminated with hydrocarbons for decades. The soil from the sampling site was characterized according to the ISO-referenced standard methods, resulting in loamy sand containing 8.7% C_{org} (ISO 10692:1995), 1.2% N_{tot} (ISO 11261:1995) and possessing a pH of 7.3 (ISO 10390:2005). Soil sampling (0-20 cm deep) took place in June 2009.

Two different compost types were used in this experiment. One compost type (C1) consisted of shredded shrubs and trees. The second compost type (C2) was a mix of organic kitchen waste, grass clippings, shredded shrubs (including other woody material), and vegetable and flower residues. During one year of processing of C1 in a composting plant (AHA Hanover-Lahe, Germany), temperature development was up to 65°C and windrow was turned regularly on a weekly basis for the first 8 weeks. This was followed by a decreasing frequency of turning depending on the temperature, water and oxygen content (≥ 15% (vol.)). Typically for this type of compost, stable

[3] Experimental set up was performed by Dr. Tjasa Gril (Institute of Soil Ecology, Helmholtz Zentrum München, Munich, Germany).

nutrient concentrations developed (14% C_{org}, 1.01% N_{org}, 203 mg kg^{-1} N_{min}, 715 mg kg^{-1} P, 4006 mg kg^{-1} K, pH 7.3) in C1. Compost C2 was processed for only two weeks in a model compost windrow ($2m^3$), in which temperatures of up to 72°C were measured. This windrow was turned weekly. During the first phase of this intense composition of C2, carbon loss of the raw material started with 33.4% C_{org} and 0.8% N_{org} whereas after 14 days 29.3% C_{org} and 0.94% N_{org} were measured respectively. The respective C/N ratio of 41 at the beginning decreased after 2 weeks to a C/N ratio of 31. Nutrient concentrations in C2 were measured as follows: 20.8 mg kg^{-1} N, 597 mg kg^{-1} P and 3259 mg kg^{-1} K.

The 36 microcosms used for this experiment consisted of stainless steel cylinders (ø 10 cm, 13 cm high) and were hand-packed with 120 g of fresh, homogenized and sieved (5 mm) soil, which is equivalent to ca. 100 g dry soil. A field bulk density of 1.3 g cm^3 was adjusted in each microcosm and pre-incubation was conducted for one week at the regional annual mean temperature of 14°C with a constant water holding capacity (WHC) of 80%. Subsequently, three different treatments were set up: 1) original soil (Soil), served as a control, 2) soil, mixed with 10 g of stable compost (Soil+C1), and 3) soil mixed with 10 g of young compost (Soil+C2). Composts were added to the soil in pots after soil conditioning, and thoroughly mixed with the soil. The control soil was also mixed, however, without any addition of amendments. During the whole duration of the experiment, all pots were continuously kept at 14°C and covered with perforated cups. Soil water adjustments of 80% WHC as well as aeration were performed at regular time intervals. Sampling was performed at four time points: on the day of the experiment setup (week 0) as well as after 6, 12 and 36 weeks after the compost amendment. The experiment was performed in 3 technical replicates for each treatment and sampling time point. Samples were transferred to dry ice immediately after sampling and stored at -80 °C.

N-alkanes were extracted[4] from the soil samples using a Soxhlet extractor (Labline Thermo Scientific, Dubuque, USA) following the test methods for evaluating solid waste, physical/chemical methods of the United States Environmental Protection Agency (EPA 3540C, 1996), and measured by using a gas chromatograph coupled to a mass spectrometry detector (HP 6890 Series, Hewlet-Packard, Waldbron, Germany) equipped with an Agilent DB-5MS (30 m × 0.25 mm × 0.25 mm) capillary column. Alkane concentrations in the soil decreased from initially ca. 800-850 mg/kg at the beginning of the experiment to 642 (Soil), 573 (soil+C1) and 502 mg kg^{-1} (Soil+C2) after 12 weeks (Table A3).

[4] Sample preparation, measurements and data evaluation of the *n*-alkane concentration was performed by Dr. Ester Heath (Jožef Stefan Institute, Ljubljana, Slovenia).

For this experiment the following methods were carried out (Figure 9):

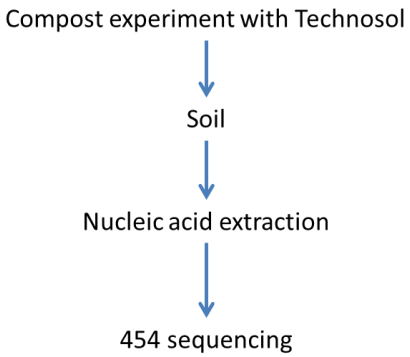

Figure 9: Methodological approach for experiment C with petroleum-contaminated Technosol

2.2 Methods

2.2.1 Measurement of the degradation rate

In order to determine the decomposition of the plant material, the loss of dry mass was analyzed. This was determined following the DIN ISO 11465 for determining the dry residues and water content in five replicates per sample. In short, 0.5-1.0 g fresh leaf and soil material in aluminum cups was incubated at 100°C, and the remaining dry mass was weighed. During the weighing procedure, the detraction of air moisture was minimized by retaining the samples in exsiccators filled with silica gel (VWR, Darmstadt, Germany). After 3 days of incubation no more mass loss was observed and a stable weight was determined.

2.2.2 Nucleic acid extraction from leaf material

Nucleic acid extraction from *Z. mays* leaf material based on the protocol published by Griffiths et al. (2000). This protocol was modified to optimize the simultaneous extraction of DNA and RNA from one single sample (Töwe et al., 2011). Extractions were performed in triplicates for each sample. Using a phenol-chloroform mix followed by a subsequent column-based separation of DNA and RNA, this method allows a straightforward extraction avoiding an enzyme digest with DNAse and RNAse respectively, in order to obtain pure DNA and RNA separately. In advance, five solutions were prepared or purchased (Table 4) for the extraction.

Table 4: Solutions for DNA/RNA coextraction. All chemicals from Sigma-Aldrich, Munich, Germany

Solution A	A.1 10% Hexadecyl trimethyl ammonium bromide and 4% sodium chloride and 0.1 vol % di-ethyl pyrocarbonate ad 100 ml of deionized water were prepared. Solution was incubated under permanent stirring over night at room temperature and subsequently autoclaved. A.2 94 ml of 1 M K2HPO4 and 6 ml of 1 M KH2PO4 were mixed and adjusted to pH 8. This mix was diluted with deionized water to a 240 mM buffer. 0.1 vol % of di-ethyl pyrocarbonate was added and solution was autoclaved. A.1 and A.2 were prepared and mixed in equal volumes. Directly before use, 10 µl β-mercaptoethanol was added per 10 ml of solution A.
Solution B	Phenol : chloroform : isoamyl alcohol (25:24:1), pH 8 and equilibrated with Tris(hydroxmethyl)aminomethane
Solution C	Chloroform : isoamyl alcohol (24:1)
Solution D	10% Polyethylen glycol (molecular weight 8000) and 1.2 M NaCl ad 100 ml of deionized water were prepared and 0.1 vol % of di-ethyl pyrocarbonate was added and solution was autoclaved.
Solution E	99.5% Ethanol was diluted with nuclease free water to a final concentration of 70%

Prior to nucleic extraction, all frozen leaf material was mechanically disrupted by grinding into powder with mortar and pestle under liquid nitrogen to keep the sampling material frozen during this procedure. For nucleic acid extraction, 0.5 g of the frozen leaf material together with 0.5 ml of solution A and 0.5 ml of solution B was mechanically disrupted with a Precellys homogenizer (30 s at 5.5 m s^{-1}) using tubes with ceramic balls (ø 1.4 mm) (PEQLAB, Erlangen, Germany). After centrifugation (5 min at 16,100 g; 4°C) the aqueous phase was sampled in a new reaction tube and the same volume of solution C was added so that the chloroform could bind the contaminants in the organic phase. This was repeated once, and the supernatant was finally transferred to a new reaction tube and the same volume of solution D was added. Samples were subsequently incubated for 2 hours on crushed ice ending up with a centrifugation step (10 min at 16,100 g; 4°C). The supernatant was discarded and the pellet was washed with an ice-cold solution E (70% ethanol). A final centrifugation step (10 min at 16,100 g; 4°C) was performed to discard all supernatant, the pellet was air-dried for 30 min and dissolved in nuclease-free water. After incubation at room temperature for 30 min, nucleic acids of the DNA/RNA mix were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Spin-column based separation of DNA and RNA was performed using the AllPrep DNA/RNA Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions. DNA was diluted in 100 µl EB buffer and RNA in 50 µl nuclease free-water (Promega, Mannheim, Germany). RNA was subsequently transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, USA) according to the manual provided. With regard to *F. sylvatica* leaf litter, the DNA of each sample was

extracted with the commercially available kit FastDNA Spin Kit for Soil (MP Biomedicals, Wolferstadt, Germany). The quantity and purity of all extracts were tested spectrophotometrically using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) with emission wavelengths set to 260 nm and 280 nm respectively. The recovery of purified nucleic acids from *Z. mays* leaf material resulted in 879-4716 (DNA) and 357-1907 µg g^{-1} dw^{-1} (RNA) with purity values of 0.9 to 2.0 (DNA) and 0.8 to 2.3 (RNA) based on 260 nm/280 nm emission ratio. Nucleic acid yield of *F. sylvatica* leaf litter samples incubated in microcosms filled with Cutanic Luvisol was between 21 and 176 µg DNA g^{-1} dw^{-1}, and for litter incubated in Calcaric Regosol soil it was between 25 and 191 µg DNA g^{-1} dw^{-1}. Purity values were between 1.5 and 2.7 (260 nm/280 nm ratio). All nucleic acid extracts were stored at -20°C until further analysis.

2.2.3 Amplification of *16S rRNA* gene fragments

A polymerase chain reaction (PCR) was performed to verify the success of the nucleic acid extraction and also to prove the absence of any DNA residues in the RNA extracts after the separation of DNA and RNA. Therefore, the extracts served as a template for a PCR with the specific primers 968f and 1401r in order to amplify fragments of the *16S rRNA* gene (Table 5). The PCR reaction mix and the conditions of the thermal cycler are summarized in Table 6.

Table 5: Primers used for amplification of *16S rRNA* gene fragments. In contrast to Heuer et al. (1997), primer 968f was used without the GC clamp.

primer	5‘ -> 3‘ sequence	reference
968f	AAC GCG AAG AAC CTT AC	Heuer et al. (1997)
1401r	CGG TGT GTA CAA GGC CCG GGA ACG	Heuer et al. (1997)

Table 6: PCR reaction mix and thermocycling conditions to amplify *16S rRNA* gene fragments (433 bp). 1 µl of template corresponds to 380-1430 ng of nucleic acids in the samples

Reaction mix

Reagent	Per reaction	
Buffer	1.000	x
$MgCl_2$	1.500	mM
dNTPs	0.100	mM
10 µM Primer fwd	0.100	pmol
10 µM Primer rev	0.100	pmol
DMSO	5.000	%
BSA	0.300	%
Taq-Polymerase	0.013	U
Template	1.000	µl
H_2O	ad 25.000	µl

Thermocycling conditions

Step	Time	Temperature	
Denaturation	5 min	95°C	
Denaturation	1 min	94°C	25x
Annealing	1 min	54°C	
Elongation	1 min	72°C	
Elongation	10 min	72°C	

2.2.4 Randomized PCR

For monitoring changes in bacterial colonization on leaf material, a randomized PCR was used to determine the community composition on a broad level by a PCR based on repetitive sequence fragments (rep-PCR). This approach was used due to the fact that this type of PCR analysis does not rely on a specific gene, and it is thus ideal for considering a widespread range of organisms. Hence, a comprehensive monitoring of the microbial community changes is guaranteed with that approach. The reaction was performed on a thermal cycler (Biometra GmbH, Germany). A master mix of 25 µl final volume was prepared containing 1× Buffer, 2 mM $MgCl_2$, 0.15 mM dNTPs, 10% DMSO, 0.025 U Taq-Polymerase, 0.3 pmol of each primer (Table 7) and 100-350 ng DNA.

Table 7: Primers used for rep-PCR

primer	5‘ -> 3‘ sequence	reference
ERIC2	AAG TAA GTG ACT GGG GTG AGC G	Versalovic et al. (1991)
ERIC1R	ATG TAA GCT CCT GGG GAT TCA C	Versalovic et al. (1991)

The reaction was performed slightly adapted from Versalovic et al. (1991) under the following conditions: initial denaturation for 5 min at 95°C, 30 cycles of denaturation for 30 s at 90°C, annealing for 1 min at 52°C and elongation for 5 min at 65°C, followed by a final elongation for 15 min at 65°C. PCR products were applied on a 3% QA agarose gel for high resolution (MP Biomedicals, Solon, USA) and electrophoretically separated. Subsequently, the gel was stained with 1% Ethidium bromide and the distribution of PCR fragments was analyzed optically. Therefore, the gel picture was analyzed using the software TotalLab 100 (biostep, Wolferstadt, Germany) in order to identify all relevant bands for further data analysis.

2.2.5 Terminal restriction fragment length polymorphisms

Terminal restriction fragment length polymorphisms (T-RFLP) were used as a fingerprinting technique to monitor the development of changes in bacterial community composition based on the *16S rRNA* gene. The amplification of *16S rRNA* gene fragments was performed as described by Sakai et al. (2004) with a semi-nested PCR using the primers listed in Table 8. For the second PCR, the forward primer was labeled with 6-Carboxyfluorescein (6-FAM). By using this semi-nested PCR approach, primer binding to partial sequences of chloroplasts and mitochondrial nucleic acids was minimized. Both reactions of the semi-nested PCR were performed in 50 µl (final volume) mixtures (Table 9).

Table 8: Primers used for semi-nested PCR for T-RFLP

primer	5'->3' sequence	reference
63f	CAG GCC TAA CAC ATG CAA GTC	Marchesi et al. (1998)
1492r	GGC TAC CTT GTT ACG ACT T	Lane (1991)
783r	CTA CCV GGG TAT CTA ATC CBG	Sakai et al. (2004)

Table 9: Reaction mix and cycler protocol for semi-nested PCR according to Sakai et al. (2004) to exlude Chloroplasts and Mitochondria

Reaction mix

Reagent	Per reaction	
Buffer	1.000	x
$MgCl_2$	2.000	mM
dNTPs	0.200	mM
10 µM Primer fwd	0.200	pmol
10 µM Primer rev	0.200	pmol
DMSO	5.000	%
BSA	0.300	%
Taq-Polymerase	0.025	U
Template	20-40	ng
H_2O	ad 50.000	µl

Thermocycling conditions

Step	Time	Temperature	
Denaturation	5 min	95°C	
Denaturation	1 min	94°C	27x
Annealing	45 sec	55°C	
Elongation	3 min	72°C	
Elongation	10 min	72°C	

Subsequently, PCR amplicons were purified with the MinElute PCR Purification Kit (QIAgen, Hilden, Germany). A preliminary *in-silico* digestion of the PCR products was performed (http://rocaplab.ocean.washington.edu/tools/repk, (Collins and Rocap, 2007), which identified the restriction enzymes endonuclease MspI (Fermentas, Germany) as being ideal for generating T-RFs. Digestion of the purified PCR products was conducted according to the manufacturer's instructions. Prior to the sequencing run, the internal standard MapMarker 1000 labeled with 6-Carboxyl-X-Rhodamine ROX (Eurogentec, Germany) in a 1:300 dilution with Hi-Di™ Formamide (Applied Biosystems, Germany) was added to each sample. Fragments were separated by an ABI 3730 sequencer, and data was analyzed with GeneMapper v.4.0 software (all Applied Biosystems, USA) and T-REX v.1.12 (http://trex.biohpc.org/, Culman et al. (2009)).

2.2.6 Amplicon sequencing and data analysis

16S rRNA gene fragment amplicons were sequenced in order to identify the bacterial community composition. *alkB* gene fragment amplicons were sequenced[5] in order to identify the *alkB*-carrying prokaryotes (bacteria and archaea). Both approaches used the 454 GS FLX Titanium Series (Roche, Penzberg, Germany), and all steps were performed according to the sequencing protocol for amplicons provided by the manufacturer. In brief, an amplicon library was prepared encompassing the variable regions V6 to V9 of the *16S rRNA* gene and the central region of the *alkB* gene (Kloos et al., 2006) respectively, by using amplicon fusion primers recommended by Roche. The template specific parts of the primers are listed in Table 10.

Table 10: Primers used for next generation amplicon sequencing

target gene	primer	5‘ -> 3‘ sequence	reference
16S rRNA	926f	AAA CTY AAA KGA ATT GAC GG	Lane (1991)
16S rRNA	630r	CAK AAA GGA GGT GAT CC	Juretschko et al. (1998)
alkB	alkB-1f	AAY ACI GCI CAY GAR CTI GGI CAY AA	Kloos et al. (2006)
alkB	alkB-1r	GCR TGR TGR TCI GAR TGI CGY TG	Kloos et al. (2006)

Fusion primers were composed as follows: Template specific primers were linked to a unique Multiplex Identifier (MID) for multiplexing purpose, followed by a four-base library key and an adaptor site (Figure 10) for bidirectional amplicon sequencing using the Lib-A kit (Roche, Penzberg, Germany).

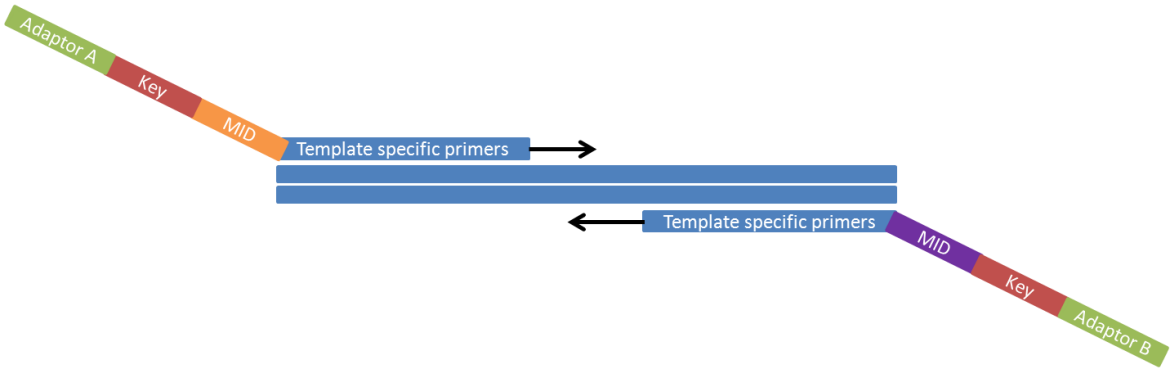

Figure 10: Scheme of fusion primers for 454 multiplex sequencing (adapted from TCB No. 013-2009, Roche, Germany)

Per sample, amplicons were generated in triplicates using the FastStart High Fidelity PCR System (Roche, Penzberg, Germany). Reaction mixes and cycler protocols for both sequencing runs are listed in Table 11 and Table 12.

[5] The laboratory work of the amplicon sequencing of the *alkB* gene fragments was performed by Dr. Xia Dong (Research Unit of Environmental Genomics, Helmholtz Zentrum München, Munich, Germany).

Table 11: Reaction mix for NGS amplicon fragment sequencing

reagents	*16S rRNA* gene fragment amplicons	*alkB* gene fragment amplicons
buffer incl. 1.8mM $MgCl_2$	1.0 ×	1.0 ×
dNTPs	0.2 mM	0.2 mM
High-Fidelity polymerase	2.5 U	2.5 U
primer forward	0.5 µM	0.5 µM
primer reverse	0.5 µM	0.2 µM
template	50.0 ng	100.0 ng
nuclease-free H_2O	ad 25.0 µl	ad 50.0 µl

Table 12: Thermocycling conditions for NGS amplicon fragment sequencing

step	*16S rRNA* gene fragment amplicons	*alkB* gene fragment amplicons
initial denaturation	95°C for 5 min	95°C for 10 min
denaturation	94°C for 1 min	94°C for 45 sec
annealing	50°C for 1 min } 22×	50°C for 60 sec } 30×
elongation	72°C for 1 min	72°C for 45 sec
final elongation	72°C for 10 min	72°C for 10 min

Triplicates were pooled after amplification, and all PCR products were purified with AMPure Beads (Beckman Coulter, Krefeld, Germany) and subsequently quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. With a Bioanalyzer 2100 device using a DNA 7500 chip (Agilent Technologies, Böblingen, Germany), average amplicon lengths of 707 bp (*16S rRNA*, *Z. mays*), 708 bp (*16S rRNA*, *F. sylvatica*) and 633 bp (*alkB*) were determined. For each experiment, all samples were then pooled in equal concentration. For sequencing the *16S rRNA* gene fragments, the subsequent emulsion PCR of the pool was slightly adapted to long-length amplicons with 94°C for 4 min, followed by 50 cycles of 94°C for 30 s and 60°C for 10 min each. Emulsion PCR and emulsion breaking for *alkB* gene fragments followed the recommendations of the manufacturer. The number of beads successfully enriched with amplicons was quantified with a Coulter Counter Multisizer Z2 (Beckman Coulter, Krefeld, Germany). All enriched beads were applied to a quarter of a picotiter sequencing plate and analyzed on a 454 GS FLX Titanium platform. An initial signal processing for amplicons was performed with the software GSRunProcessor v.2.6 (Roche, Penzberg, Germany). Sequencing output was then processed with the software mothur v.1.27.0 (Schloss et al., 2009) for reducing sequencing errors. Each flowgram was thereby separated according to each barcode and primer, and the sequences were set to a minimum length of 200 bp. Subsequently, data was de-noised and primers, barcodes and homopolymers were removed. Further data analysis of *16S rRNA* gene amplicon fragments differed from that of *alkB* gene amplicon fragments.

Subsequent data analysis of the *16S rRNA* gene amplicon fragments was performed with mothur as follows. In the subsequent step, an alignment was generated using a dataset of the SILVA database provided by mothur, and chimeric sequences were excluded. For the latter, an additional python script implementing the UCHIME algorithm (Edgar et al., 2011) was created by Thomas Rattei (Division for Computational Systems Biology, University of Vienna, Austria) to identify the false-positive chimeric sequences. Subsequently, all non-chimeric sequences were assigned to specific taxa based on the reference sequences of the publicly-released version 9 of the database deposited in the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/misc/rel9info.jsp) (Wang et al., 2007). Any chloroplast or mitochondrial sequences were eliminated. For analyzing sequencing data with reverse primers, the reverse complement was also considered. For sample comparison, rarefaction curves were calculated. For beta diversity analysis, the number of all operational taxonomic units (OTUs) was normalized for each of the samples. Therefore, for *Z. mays* the number of OTUs was restricted to 809 (forward data set) and 1158 (reverse data set). For *F. sylvatica*, only the forward data set was considered for analysis, resulting in a restriction of 2126 OTUs. The normalization was conducted to ensure an appropriate analysis of the richness, diversity and evenness measurements. Diversity and evenness measurements were based on the Shannon-Wiener index (Williamson et al., 2011). Richness was calculated following the Chao1 index (Chao et al., 2005). Using the software R v.2.15.1 (http://www.R-project.org), a heat map of the most abundant OTUs containing at least 50 sequences was calculated in order to determine which OTUs were part of the bacterial communities of each sample.

Data analysis of the *alkB* gene amplicon fragments was subsequently processed with the online tool FunGene (http://fungene.cme.msu.edu//FunGenePipeline/ as of 2013-07-01, Cole et al. (2009)). Therefore, only the forward sequencing data were considered. Duplicate sequences were removed from the data set by using the dereplicator tool provided by FunGene. Chimeric sequences were identified by using UCHIME (Edgar et al., 2011) implemented in the FunGene pipeline, and were ignored for further analysis. Subsequently, DNA sequences were translated into amino acid sequences and frame shift errors were corrected. Amino acids were aligned by using the HMMER3 algorithm based on the profile Hidden Markov Model (Finn et al., 2011), and rarefaction curves were created. The sequences were clustered to Operational Taxonomic Units (OTUs) on 97% sequence identity, and representative amino acid sequences for each cluster were calculated. Evenness and richness were calculated according to the Shannon index and the Chao1 index. Using the software R v.2.15.1 (http://www.R-project.org), a heat map of the most abundant OTUs containing at least 50 sequences was calculated in order to determine which OTUs were part of the bacterial communities of each sample. Representative amino acid sequences of the OTUs presented in the heat map were identified via a NCBI BLASTP search against the non-redundant protein sequences GenBank database.

All NGS sequencing results were submitted to the sequence read archive (SRA) of GenBank and can be found under the accession numbers SRP035887 (experiment A), SRP040259 (experiment B) and SRP029181 (experiment C).

2.2.7 Generating phylogenetic trees

To visualize the classification of the query sequences, phylogenetic trees were calculated using ARB v5.3 (Ludwig et al., 2004). All *16S rRNA* gene sequencing data were processed as follows: To gain longer sequences for a more precise phylogenetic classification, forward and reverse reads were considered. The corresponding forward and reverse reads within each single sample were assembled using the software SeqMan v9 (DNAStar, Madison, USA). The assembly was performed with an overlap of at least 400 bp (≥ 99% sequence identity), and the sequences were clustered on 90% sequences identity level which resulted in 1414 OTUs. Finally, all the sequences were aligned using the online tool SINA (http://www.arb-silva.de/aligner/ (Pruesse et al., 2012). The alignment was imported into and ARB manually corrected before it was included into the phylogenetic tree based on the SILVA reference database SSURef_NR99 release 115 using the parsimony algorithm implemented in ARB. In order to check the correct position of the OTUs in the tree, a maximum-likelihood tree was additionally calculated. All *alkB* gene sequencing data were processed as follows: The phylogenetic data analysis based on the reference database provided by FunGene, which contains 2012 (as of 2013-08) reference sequences for the *alkB* gene, as well as a phylogenetic tree on amino acid level were constructed by using the maximum parsimony algorithm implemented in the software package ARB. In a subsequent step, the representative sequences were included into this phylogenetic tree. Only those 1380 reference sequences that are flanked by the *alkB* primers alkB-1f and alkB-1r were considered. Representative sequences of each OTU except those consisting of singletons were aligned to the reference sequences and added to the tree using the parsimony algorithm implemented in ARB. To make the tree more concise, sequences closely related to reference sequences were grouped in clusters. If no reference sequence was included into a specific cluster, amino acid sequences were identified performing a BLASTP search as described in section 2.2.6.

2.3 Statistical analyses

All statistical analyses were performed with R v.2.15.1 (http://www.R-project.org) using the packages vegan, ade4 and GeneNet as well as the software SPSS version 11.5 (IBM Deutschland GmbH, Ehningen, Germany). All data fulfilled the assumptions of normal data distribution and homogeneity of variances. This was tested by histograms and the Kolmogorov-Smirnov test with a significance level of p-value ≤ 0.05, unless otherwise specified. T-RF data were analyzed using between group

analysis (BGA, experiment A) based on correspondence analysis and principal component analysis (PCA, experiment B) with data transformed by the Hellinger coefficient (Ramette, 2007) and a dissimilarity matrix according to Yue and Clayton (2005). Statistical analysis of the NGS sequencing data based on OTUs clustered to 80%, 90% and 97% sequence identity level. Significant shifts in bacterial community were determined by using BGA based on correspondence analysis (experiment B) and PCA (experiment C) with data transformed by the Hellinger coefficient and a dissimilarity matrix according to Yue and Clayton (2005). Significant differences were calculated using multifactorial ANOVA with corresponding p-values. Graphical gaussian models were generated for revealing positive or negative correlations of the OTUs (90% sequence similarity) assigned to specific bacterial taxa. Therefore, all OTUs occurring 6 times or less were ignored. A partial correlation matrix with a cutoff of 0.2 was calculated, which also reveals indirect dependencies among the bacterial taxa identified on different sampling time points and treatments or soil type. Descriptive discriminant analyses were based on relative abundance data and were performed to correlate the OTUs (90% sequence similarity) assigned to different bacterial taxa relating to the various sampling time points and treatments or soil type. Again all OTUs occurring 6 times or less were ignored.

3 Results

3.1 Nucleic acid extraction from leaf material

In order to extract DNA and RNA from the same sample, the optimized protocol based on Griffiths et al. (2000) was used for maize leaves (Töwe et al., 2011). For the leaf material from *F. sylvatica*, only DNA was extracted with a commercially available kit. The nucleic acid yield ranged from ca. 1630 to 3320 (DNA) and ca. 550 to 1350 (RNA) µg g^{-1} extracted from *Z. mays* leaf litter (Figure 11). Lower amounts of DNA were extracted from *F. sylvatica*, ranging between 46 to 188 µg g^{-1} (Figure 12). Interestingly, the total DNA extracted from *F. sylvatica* leaf material increased significantly towards the later sampling time points. By contrast, no significant changes were detected when comparing the nucleic acid extraction obtained from *Z. mays* leaf material over time, due to the high level of standard deviation (all $p>0.05$ based on ANOVA). The transcription of RNA to cDNA was successful when using cDNA for fingerprinting methods T-RFLP and rep-PCR. With primers used for NGS of the *16S rRNA* gene fragment, no bands could be amplified with cDNA as template. Thus, all further experiments focused on DNA only.

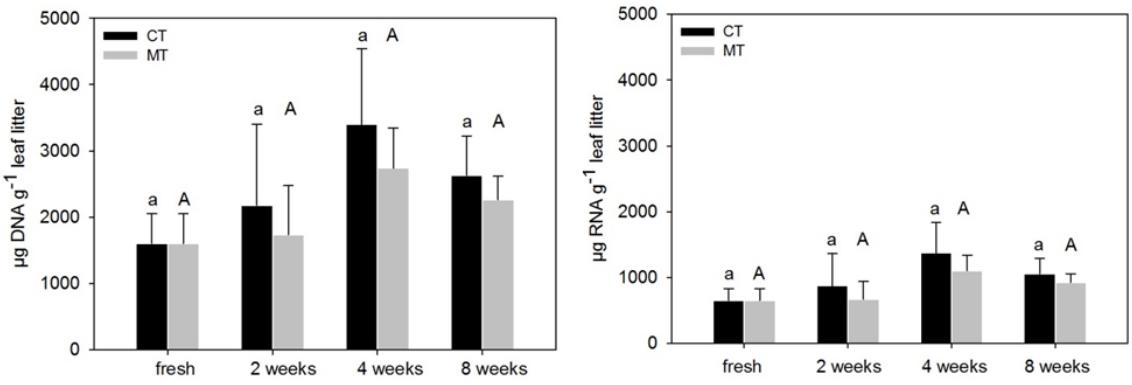

Figure 11: Nucleic acids extracted from *Z. mays*. Amounts of DNA (left) and RNA (right) after various time points during degradation. Mean and standard deviation of n = 3 are given. Significantly different data are indicated with lower case for conventional tillage (CT) and upper case for minimal tillage (MT)

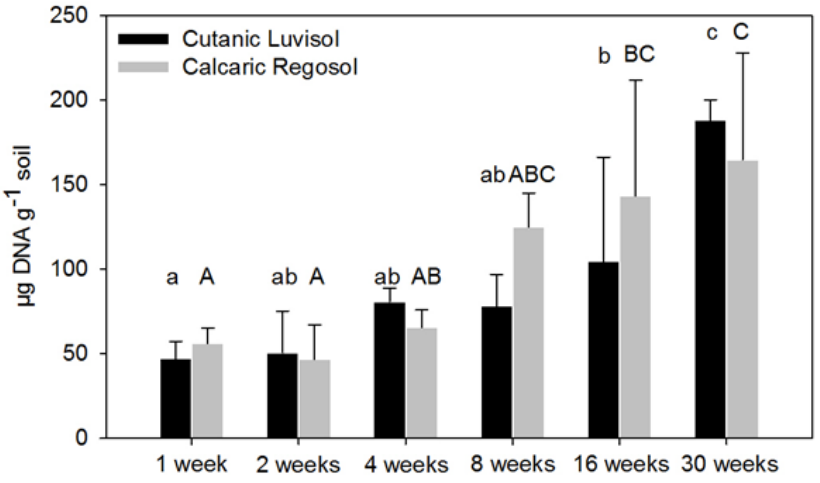

Figure 12: Nucleic acids extracted from *F. sylvatica*. Significantly different data indicated with lower case (Cutanic Luvisol) and upper case (Calcaric Regosol) letters (n = 5)

For measuring the purity of the extracted nucleic acids, extinction was analyzed spectrophotometrically, and extinction ratios of $A_{260/280}$ and $A_{260/230}$ were used as an indicator for the purity of the nucleic acid extractions. $A_{260/280}$ ratios lower than 1.8 (DNA) and 2.0 (RNA) indicate contamination of the nucleic acid extract with protein (mainly aromatic amino acids). $A_{260/230}$ ratios lower than 2.0 indicate contamination of the nucleic acid extract with phenolate ions, thiocyanates or other similar organic compounds. For *Z. mays* DNA and RNA, the mean of $A_{260/230}$ ratios was 2.31 ± 0.02 in the nucleic acid extracts. Significant lower ratios around 2.19 ± 0.05 were measured only in leaf litter samples incubated for 2 weeks in arable soil. For *F. sylvatica*, both ratios were lower ($A_{260/280}$: 1.74 ± 0.055; $A_{260}/_{230}$: 0.63 ± 0.335), indicating contamination of the DNA extract (Figure 13).

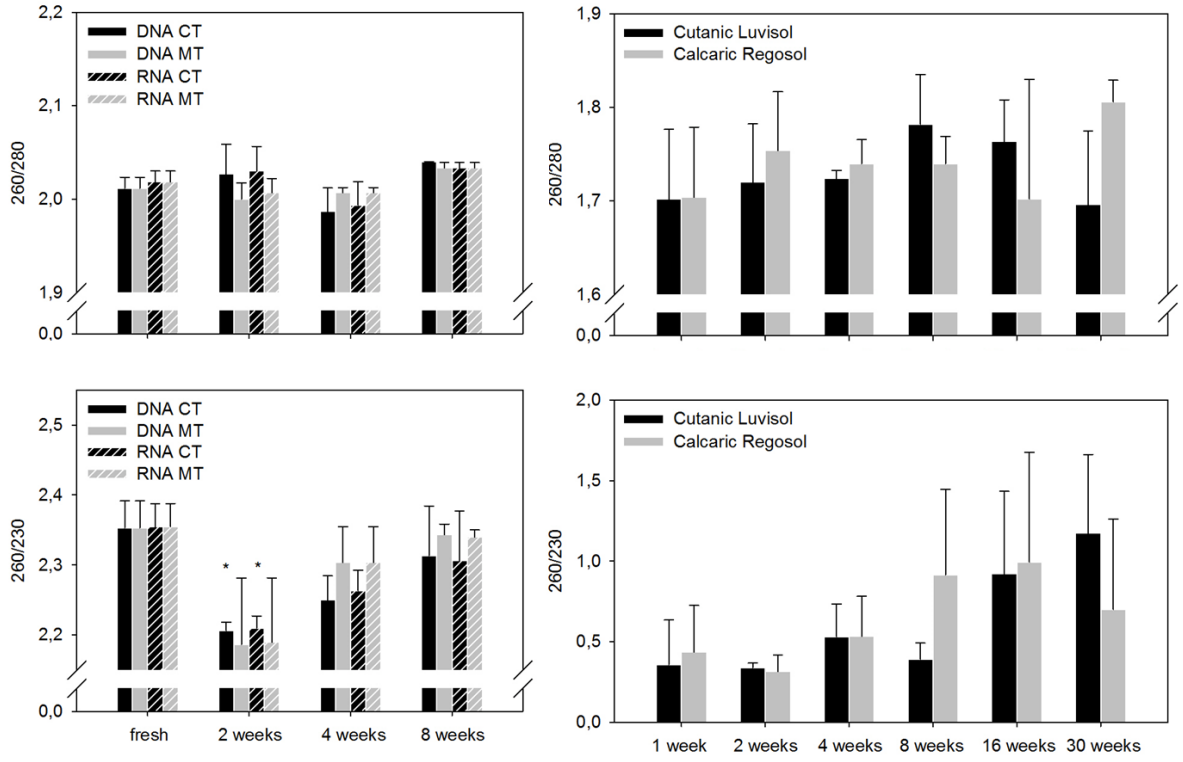

Figure 13: Purity of nucleic acids extracted from *F. sylatica* leaf litter. 260 nm/280 nm (top) and 260 nm/230 nm (bottom) extinction ratios measured spectrophotometrically after total nucleic acid extraction from *Z. mays* (left, n = 3) and *F. sylvatica* (right, n = 5). Mean and standard deviation are given.

3.2 Microbial diversity during different stages of Z. mays leaf litter degradation

Changes in the bacterial community composition colonizing *Z. mays* leaf litter were monitored in a field experiment of up to 8 weeks. Leaf material in litter bags was embedded in the boundary layer right between the tilled and untilled cambisol soil. Bacterial community shifts during leaf litter degradation were determined via molecular fingerprinting techniques. Subsequently, bacterial key players were identified by using high throughput sequencing and linked to degradation rates as well as chemical analyses of the leaf material.

3.2.1 Degradation of *Z. mays* leaf material

The dry mass of the leaves resulted in a steady decrease from 24.4% to 12.5% related to fresh weight (Figure 14). A significant decrease of mass loss of leaf material was detected after 2 and 4 weeks (p-value ≤ 0.05) with a subsequent stagnation after 30 weeks of incubation in the soil. Leaf litter in the boundary layer under minimal tillage showed a higher degradation rate after 4 and 8 weeks compared to the leaf material incubated in the boundary layer under conventional tillage.

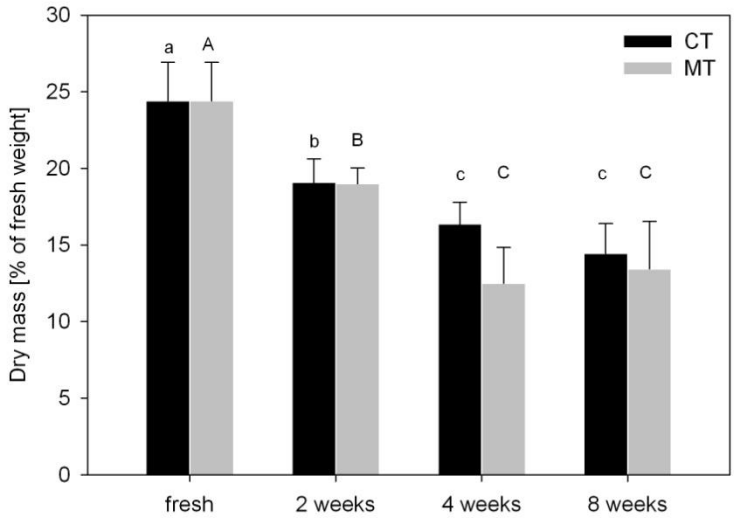

Figure 14: Dry mass of *Z. mays* leaf material exposed in litter bags incubated in arable soil. Mean and standard deviation are shown (n = 3). Significant differences are indicated in lower case for conventional tillage (CT) and upper case letters for minimal tillage (MT) according to Tukey multiple comparison test (p-value ≤ 0.05)

3.2.2 Bacterial community composition on *Z. mays* leaf material

Two molecular fingerprinting techniques were carried out to monitor the change in the bacterial community composition during the degradation process. The first method was based on a PCR which amplifies ubiquitous repetitive sequence fragments that are widespread and common among microorganisms, whereas the second method was based on a specific housekeeping gene (*16S rRNA* gene). The rep-PCR uses the low primer-binding temperature during PCR to amplify repetitive sequence elements originating from numerous organisms resulting in different amplicon patterns. Microbial shifts in the microbial community composition liked to *Z. mays* leaf material was then visualized by gel electrophoresis (Figure 15). A clear clustering of the DNA fingerprints of each leaf litter sample according to the incubation time in soil was observed based on an Euclidian distance dissimilarity matrix. According to the data analysis, rep-PCR amplicon fragment pattern of fresh leaf material clearly separated from all leaf litter samples. Additionally, all leaf litter samples incubated for 8 weeks clustered together. All other patterns from samples after 2 and 4 weeks clustered with no distinct differentiation. In these samples, three dominant amplicons between 400 and 500 bp were detected. According to the analysis, different tillage treatments only slightly influenced the pattern distribution of sequence fragments.

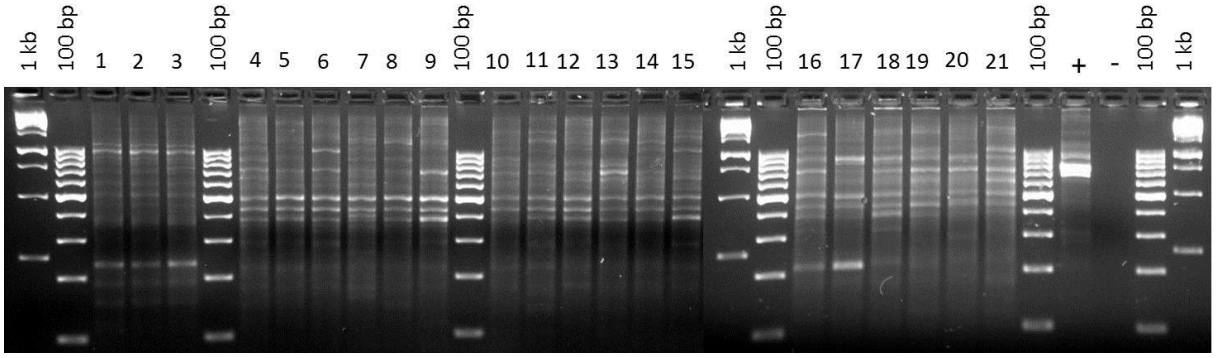

Figure 15: rep-PCR fragment distribution on a 3% agrarose gel. 1-3: Fresh leaves of *Z. mays*; 4-6: 2 weeks CT; 7-9: 2 weeks MT; 10-12: 4 weeks CT; 13-15: 4 weeks MT; 16-18: 8 weeks CT; 19-21: 8 weeks MT; +: positive control (*H. frisingense*); -: negative control; 1 kb and 100 bp: Standard

Terminal restriction fragment length polymorphism (T-RFLP) analysis of *16S rRNA* gene fragments was performed to detect changes in bacterial community composition during litter degradation. Compared to the more general rep-PCR approach, T-RFLP focuses specifically on the *16S rRNA* gene. T-RFs contributing ≥ 5% to the total community richness are summarized in Table A4. The number of T-RFs in fresh leaf material was low (17 (DNA) and 27 (cDNA)), and increased in leaf litter samples up to 28 (DNA) and 40 (cDNA). In fresh leaves of *Z. mays,* the total relative community richness was driven by two major T-RFs (112 bp and 114 bp). Together with one more T-RF, 342 bp (DNA) and 117 bp (cDNA), these T-RFs account for a community richness of 53% (DNA) and 40% (cDNA). Typical for all leaf litter samples was an 87 bp long T-RF after 2 and 4 weeks of litter incubation. Most dominant after 4 and 8 weeks was a 399 bp long T-RF, accounting for 16-25% (DNA) and 20-24% (cDNA) of the total relative community richness in all samples. Between group analysis of T-RFLP data of DNA and cDNA revealed very similar results (Figure 16).

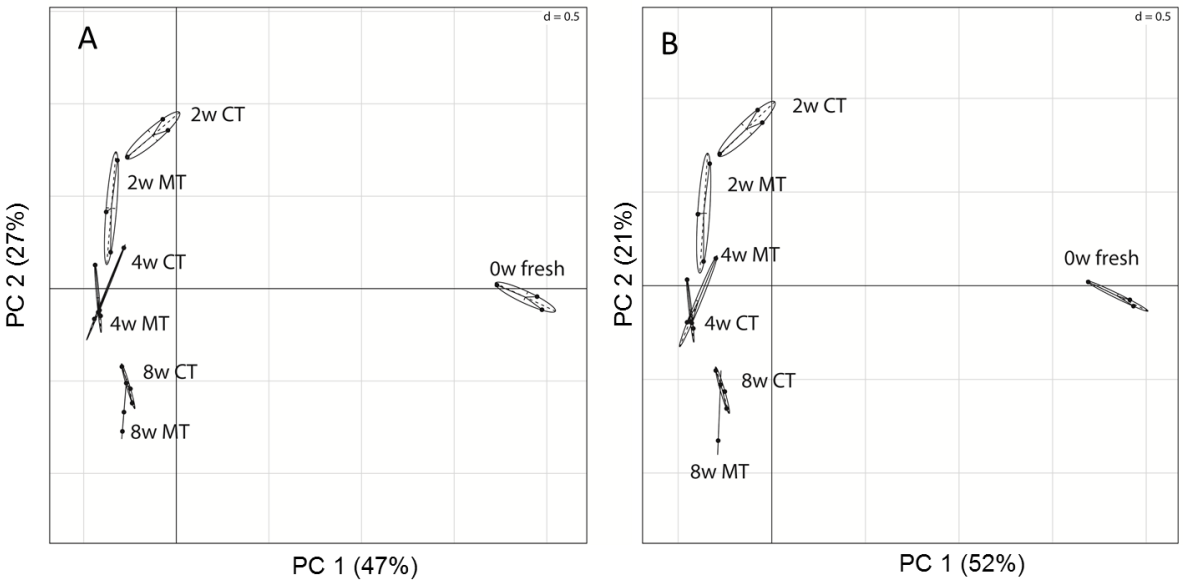

Figure 16: T-RFLP distribution during leaf litter degradation of *Z. mays*. (A) DNA level and (B) cDNA level. Between group analysis based on data transformed by the Hellinger coefficient (n = 3)

The impact of the two factors incubation time in soil and tillage treatment is summarized in Table 12. Multivariate ANOVA revealed that the incubation time in soil had a significant effect on the bacterial community, whereas the tillage treatment did not. Also, no significant effect on the interaction of both factors was measured. For both, the DNA- and the cDNA-based data set, the bacterial community composition on fresh leaf material differed significantly from that on litter (p-value ≤0.001). The leaf litter samples were influenced significantly by the incubation time in the soil. Interestingly, different tillage treatments significantly influenced the bacterial community composition on leaf litter after 2 weeks (p-value ≤ 0.05), which was not true for any later sampling time points.

Table 12: Statistical evaluation of incubation time and tillage treatment affecting the bacterial communities detected on fresh leaves and leaf litter of *Z. mays* using T-RFLP by multifactorial ANOVA. Significant impacts are marked with an asterisk (p-value ≤ 0.05)

	DNA		cDNA	
	all samples	leaf litter only	all samples	leaf litter only
incubation time	0.001*	0.001*	0.001*	0.001*
treatment	0.112	0.153	0.284	0.368
incubation time x treatment	n.a.	0.071	0.167	0.198

3.2.3 Comparison of ecological indices

A more detailed characterization of the bacterial community changes was achieved by using next generation sequencing of *16S rRNA* gene fragments covering the regions V6 to V9, which resulted in more than 225,000 detected sequences. An initial data quality check was performed with the software GSRunBrowser from Roche. This resulted in 157.736 reads, which correspond to 1092 to 8819 sequences per sample with an average length of 525 bp (±29 bp). The reads were used as input for further data processing with the software mothur. With mothur, rarefaction curves with different sequence identity levels of 80%, 90% and 97% were calculated to determine the sampling richness (Figure 17). Therefore, the data of replicates were pooled. Comparing the rarefaction curves, a similar sampling richness for both the forward and the reverse data set was achieved with *16S rRNA* gene fragment amplicon NGS. The saturation of the rarefaction curves was more often reached with lower sequence identity levels, whereas the number of OTUs decreased (Table 13).

Table 13: Number of OTUs that clustered under different sequence identity levels

sequence identity	number of OTUs	
	forward data set	reverse data set
97%	141-473	122-370
90%	90-193	33-129
80%	12-44	10-26

The results revealed an increasing richness over time and the saturation was not always reached at sequence similarity levels of 90% and higher. In total, ca. 3800-13200 (forward data set) and ca. 2100-9600 (reverse data set) reads were detected, resulting in 10-473 OTUs per sampling time point and treatment. Within leaf litter, an increasing number of OTUs was observed over time, starting with up to ca. 153-280 OTUs after 2 weeks and resulting up to 373-473 OTUs after 8 weeks of incubation (Table 14).

Table 14: Number of OTUs per replicate (*Z. mays*) at different sampling time points

number of OTUs	forward data set	reverse data set
fresh	23-141	19-140
2 weeks	13-280	10-153
4 weeks	24-318	14-241
8 weeks	27-473	20-373

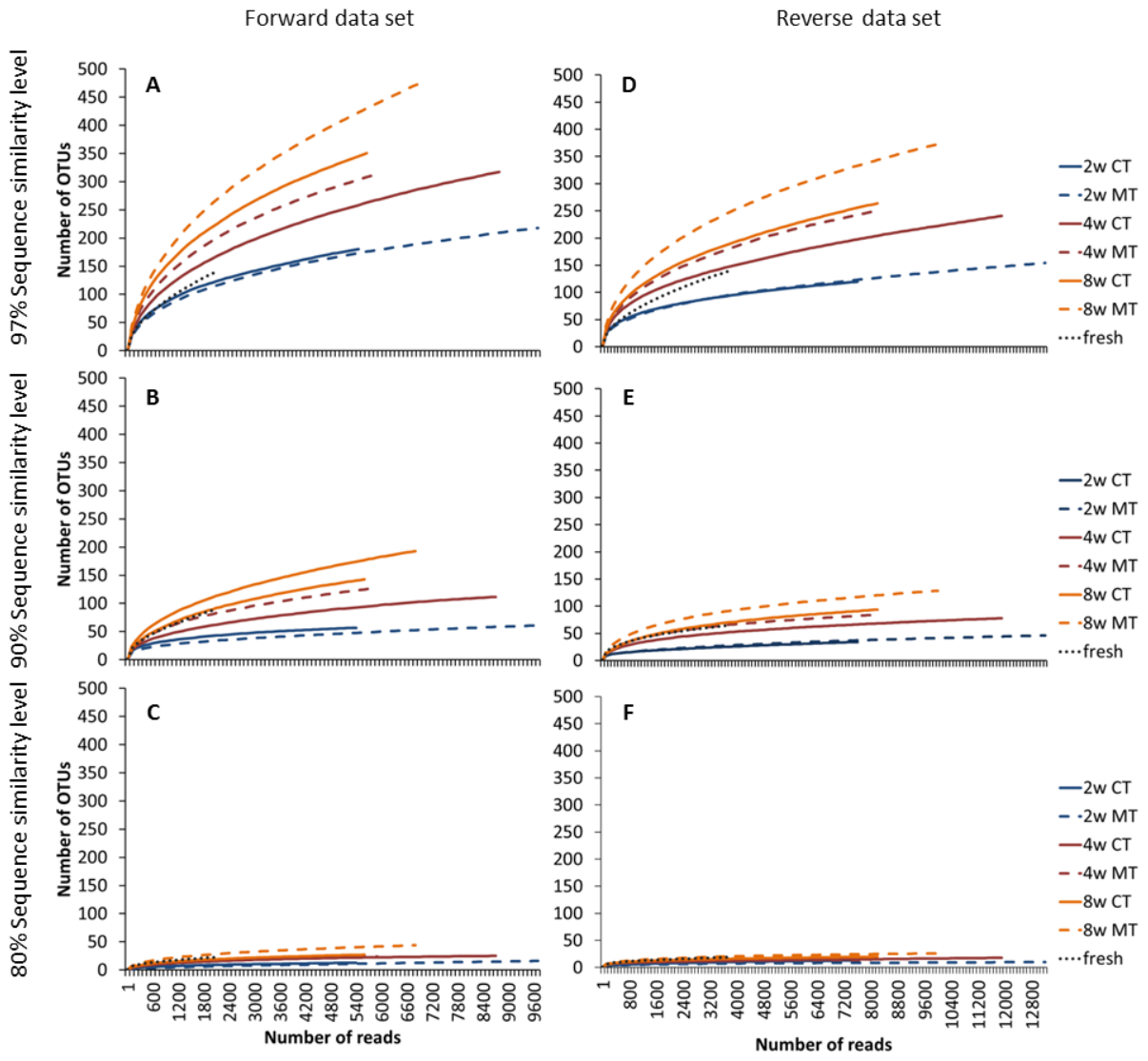

Figure 17: Rarefaction curves (*Z. mays*) based on the forward (A-C) and reverse (D-F) data sets. Different sequence similarity levels are shown at 97% (A, D), 90% (B, E) and 80% (C, F) sequence identity levels

Figure 18 illustrates the bacterial richness (Chao1 Index), the bacterial diversity (Shannon index) and the evenness (based on Shannon index) based on OTUs calculated at 97%, 90% and 80% sequence identity levels. For OTUs at 97% and 90% sequence identity levels, richness, diversity and evenness calculated based on the forward data set were always higher compared to the indices based on the reverse data set, with only one exception after 2 weeks regarding the evenness. The statistical analysis revealed zero (richness and evenness) or marginally (diversity) significant differences at the 80% sequence identity level.

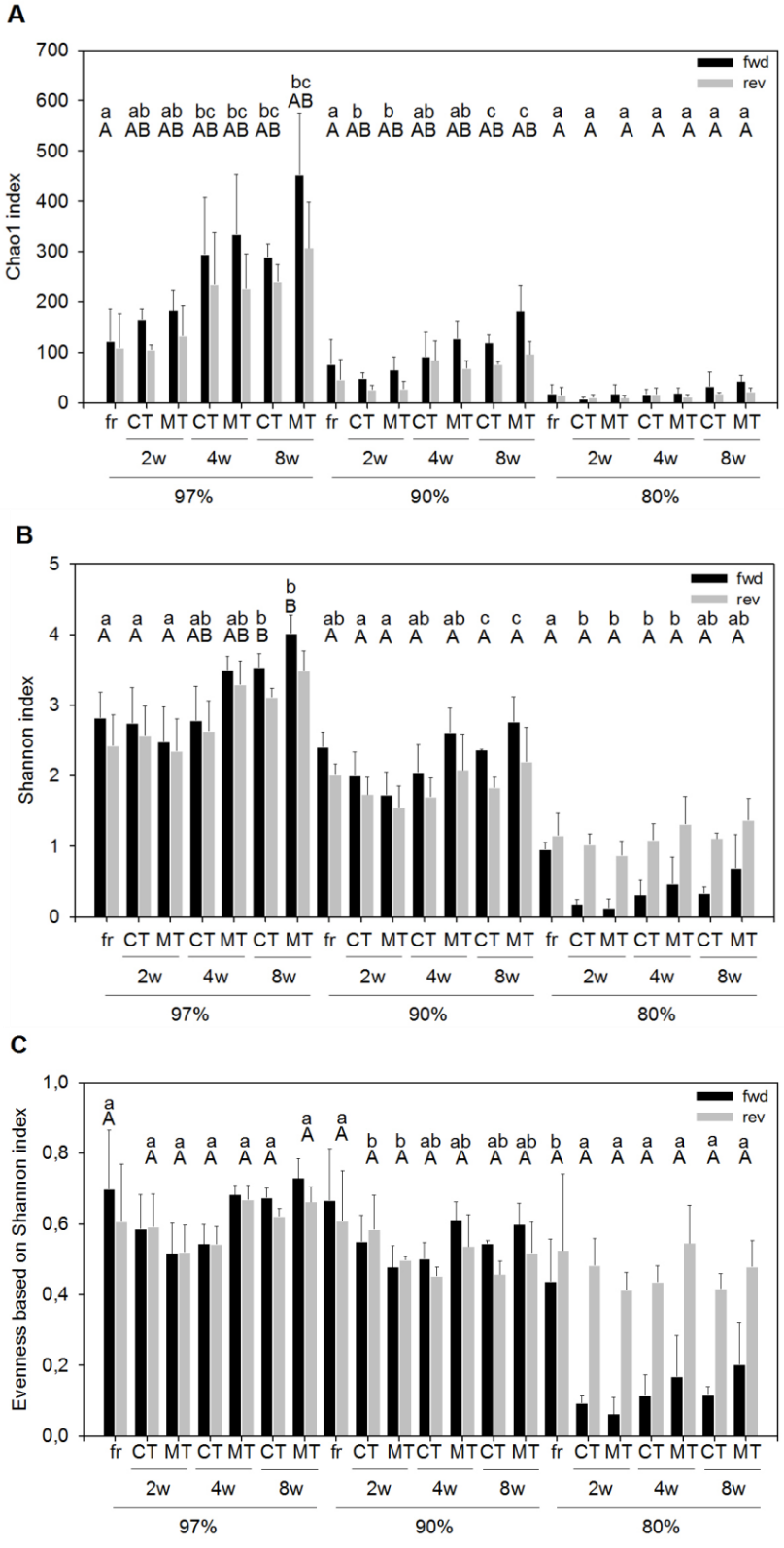

Figure 18: Bacterial richness, diversity and evenness on *Z. mays* leaf litter. Indices based on Chao1 index (A) and Shannon index (B and C) of bacterial OTUs calculated at 97%, 90% and 80% sequence identity levels. Significant differences (n = 3, p-values < 0.05 based on ANOVA) within each sequence identity level were indicted with lower case (fwd) and upper case (rev) letters

During the experiment, an overall increase in bacterial richness was observed for OTUs clustered at a 97% sequence similarity for both tillage types. At 90% and 80% sequence identity levels, an initial decrease in richness was observed after 2 weeks, followed by a subsequent increase towards the end of the experiment. Bacterial diversity tended to decrease after 2 weeks, with a subsequent increase for all later sampling time points. For CT field management, a constant level was observed after 2 and 4 weeks (97% and 90% sequence identity levels) as well as after 4 and 8 weeks (80% sequence identity level). Bacterial evenness also increased towards the sampling point after 8 weeks. For the CT field management, a decreased evenness until 4 weeks was observed, followed by an increase again after 8 weeks (97% and 90% sequence identity levels). For the MT field management, a lower evenness after 2 weeks was observed, followed by an increase after 4 weeks and later (97% sequence identity level), or stable evenness values (90% sequence identity level). OTUs clustered at the 80% sequence identity revealed a decreasing evenness within samples of MT field management, whereas under CT field management no significant differences were observed. In most of the samples, the forward primer set reached the same or even higher indices compared to the reverse primer set based at 90% and 97% sequence identity levels. Higher evenness values for the reverse data set were noticed after 2 weeks at 90% (CT and MT) and 97% (CT and MT). At 80% sequence similarity, a higher diversity and evenness was observed for the reverse data set in all samples. An analysis of the corresponding taxa is visualized in Figure 20 to Figure 25.

With regard to OTUs clustered at the 97% sequence identity level (Figure 20 and Figure 21), 49%-74% unclassified taxa and 7%-25% taxa <1% abundance at the genus level were observed. Samples of the reverse data set contained a higher proportion of low abundant genera (1%-2%), compared to the forward data set. Overall, 22 (forward) and 27 (reverse) OTUs could be assigned to specific genera, sharing 15 taxa in common. 7 (forward data set: *Aurantimonas, Caulobacter, Devosia, Kaistia, Massilia, Rhizobium, Stenotrophomonas*) and 13 (reverse data set: *Aeromicrobium, Camelimonas, Clostridium III, IV and sensu strictu, Comamonas, Delftia, Luteolibacter, Microbacterium, Phenylobacterium, Pigmentiphaga, Plantibacter, Xanthomonas*) OTUs were assigned to specific genera typical for each of the data sets.

With regard to OTUs clustered at the 90% sequence identity level (Figure 22 and Figure 23), the proportion of unclassified taxa (9%-53%) or taxa <1% abundance (up to 8%) was lower compared to OTUs clustered at the 97% sequence identity level. Overall, 12 (forward) and 15 (reverse) OTUs could be assigned to specific classes, sharing 12 taxa in common. In addition to these 12 shared taxa, 3 OTUs could be assigned to taxa typical for the reverse data set (*Holophagae*, *Methanomicrobia* and *Opitutae*). Whereas OTUs of the forward data set were mostly assigned to *α-Proteobacteria* and

Actinobacteria, OTUs of the reverse data set were mostly assigned to *Clostridia* and α-P*roteobacteria* in all samples.

With regard to OTUs clustered at the 80% sequence identity level (Figure 24 and Figure 25), 17%-70% of the unclassified taxa but no taxa <1% abundance were detected. Overall, 9 (forward) and 11 (reverse) OTUs could be assigned to specific phyla, sharing 8 taxa in common. 1 (forward data set: Thermotogae) and 3 (reverse data set: *Deinococcus-Thermus*, *TM7* and *Verrucomicrobia*) OTUs were assigned to specific phyla typical for each of the data sets. An analysis of the taxa identified revealed a higher diversity which is more evenly distributed on the phylum level for the reverse data set compared to the forward data set. On the other hand, the forward data set represents fewer but more abundant phyla. These data explain the higher levels of the reverse data set with respect to diversity and evenness, especially at the 80% sequence identity level.

Between group analysis was performed with data based on OTUs at the 90% sequence identity level from the forward data set (Figure 19). According to the results, bacterial community composition on fresh *Z. mays* leaves is significantly different (p-value ≤ 0.001) from that on degraded litter material. Furthermore, bacterial community composition on leaf litter significantly (p-value ≤ 0.05) shifts according to the incubation time in soil, whereas the field management did not have a significant influence.

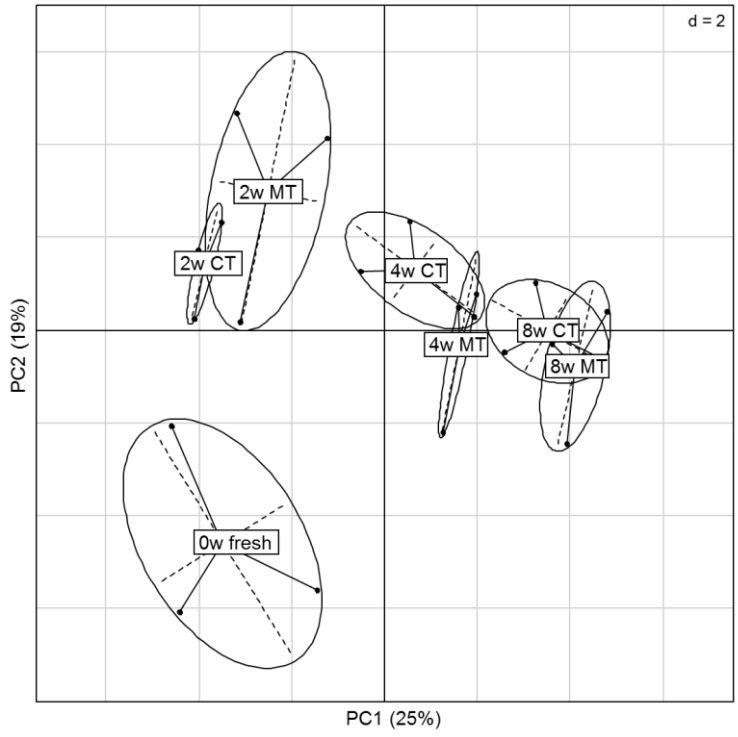

Figure 19: Bacterial community shifts on *Z. mays* leaf material. Distribution of OTUs (90% sequence identity) over time detected on fresh leaves (fresh) and leaf litter incubated for 2 (2w), 4 (4w) and 8 (8w) weeks in arable soil. Between group analysis based on data transformed by the Hellinger coefficient (n = 3)

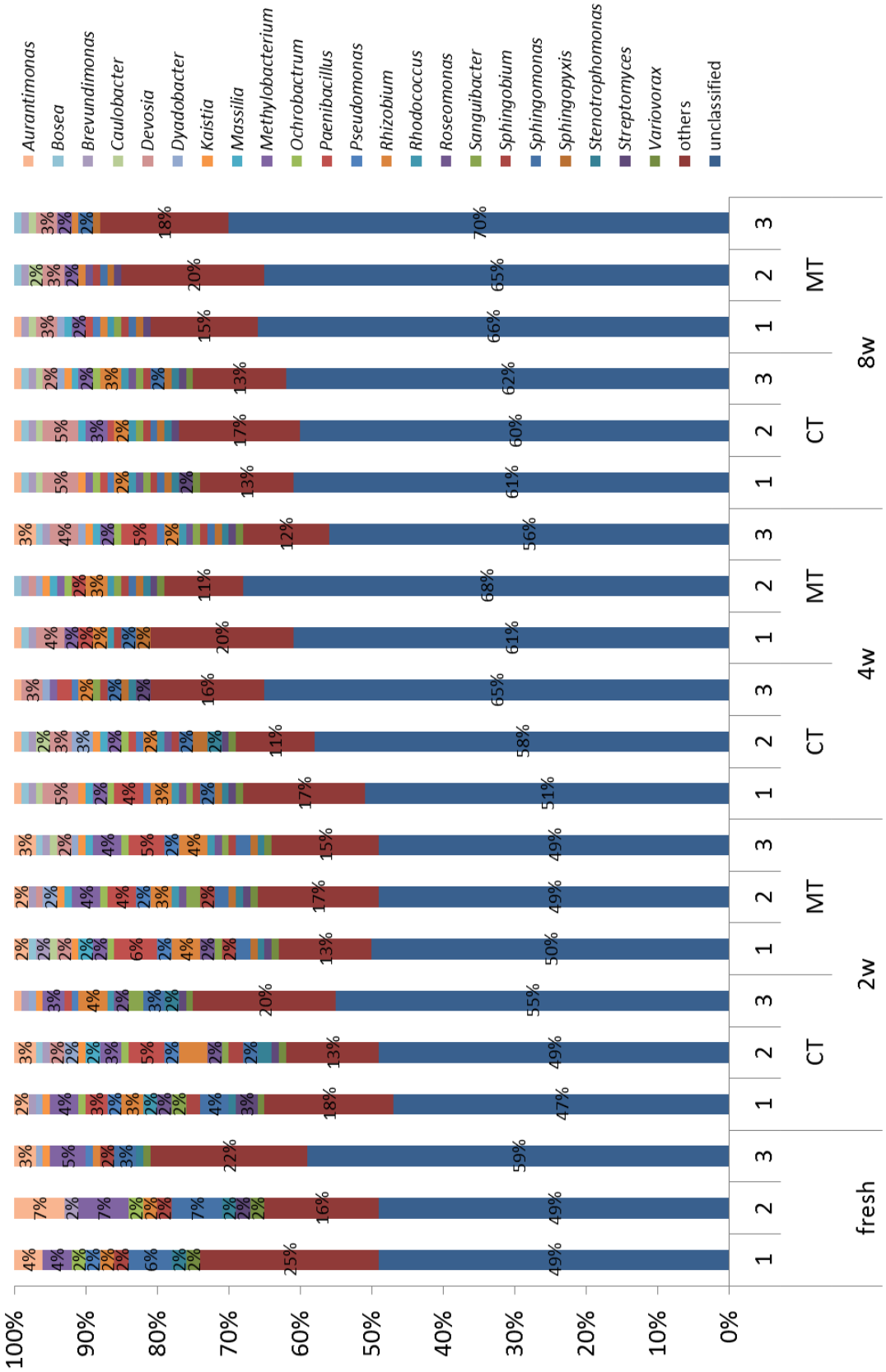

Figure 20: Bacterial composition of OTUs on *Z. mays* leaf material based on 97% sequence similarity of the forward data set (genus level). All data <1% are summed up as "others". Data of 1% abundance are simply indicated by color bars

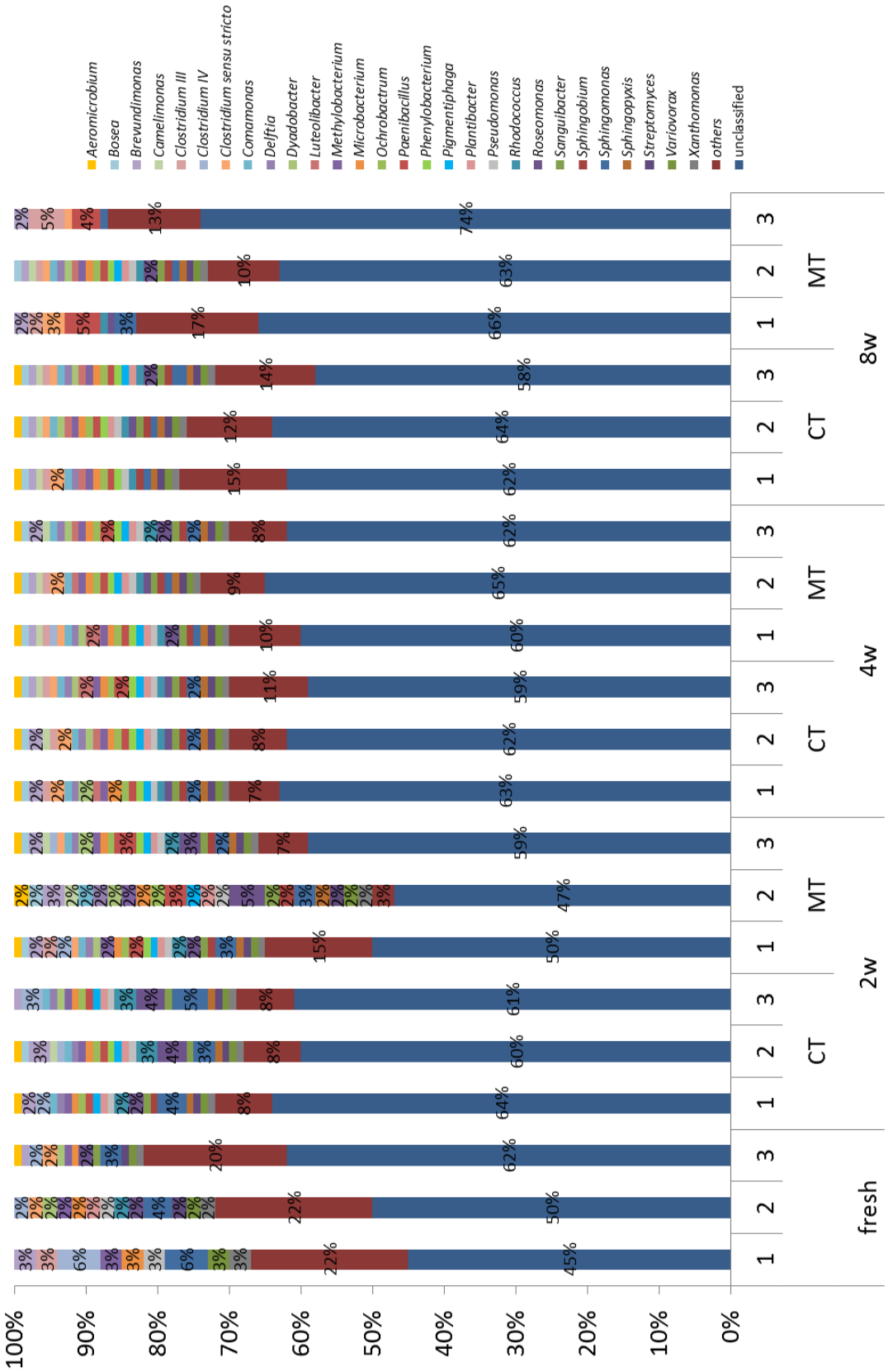

Figure 21: Bacterial composition of OTUs on *Z. mays* leaf material based on 97% sequence similarity of the reverse data set (genus level). All data <1% are summed up as "others". Data of 1% abundance are simply indicated by color bars

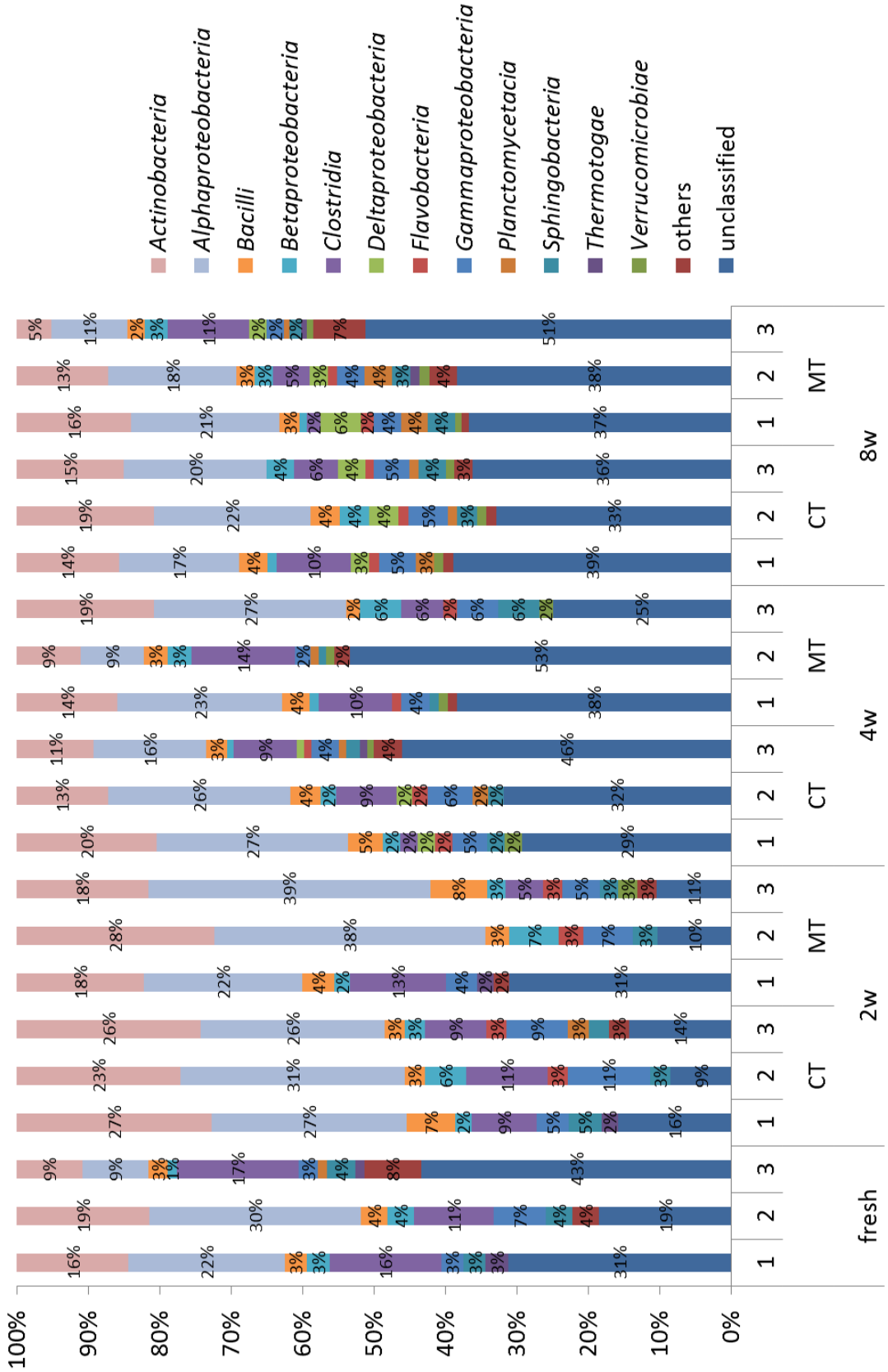

Figure 22: Bacterial composition of OTUs on *Z. mays* leaf material based on 90% sequence similarity of the forward data set (class level). All data <1% are summed up as "others". Data of 1% abundance are simply indicated by color bars

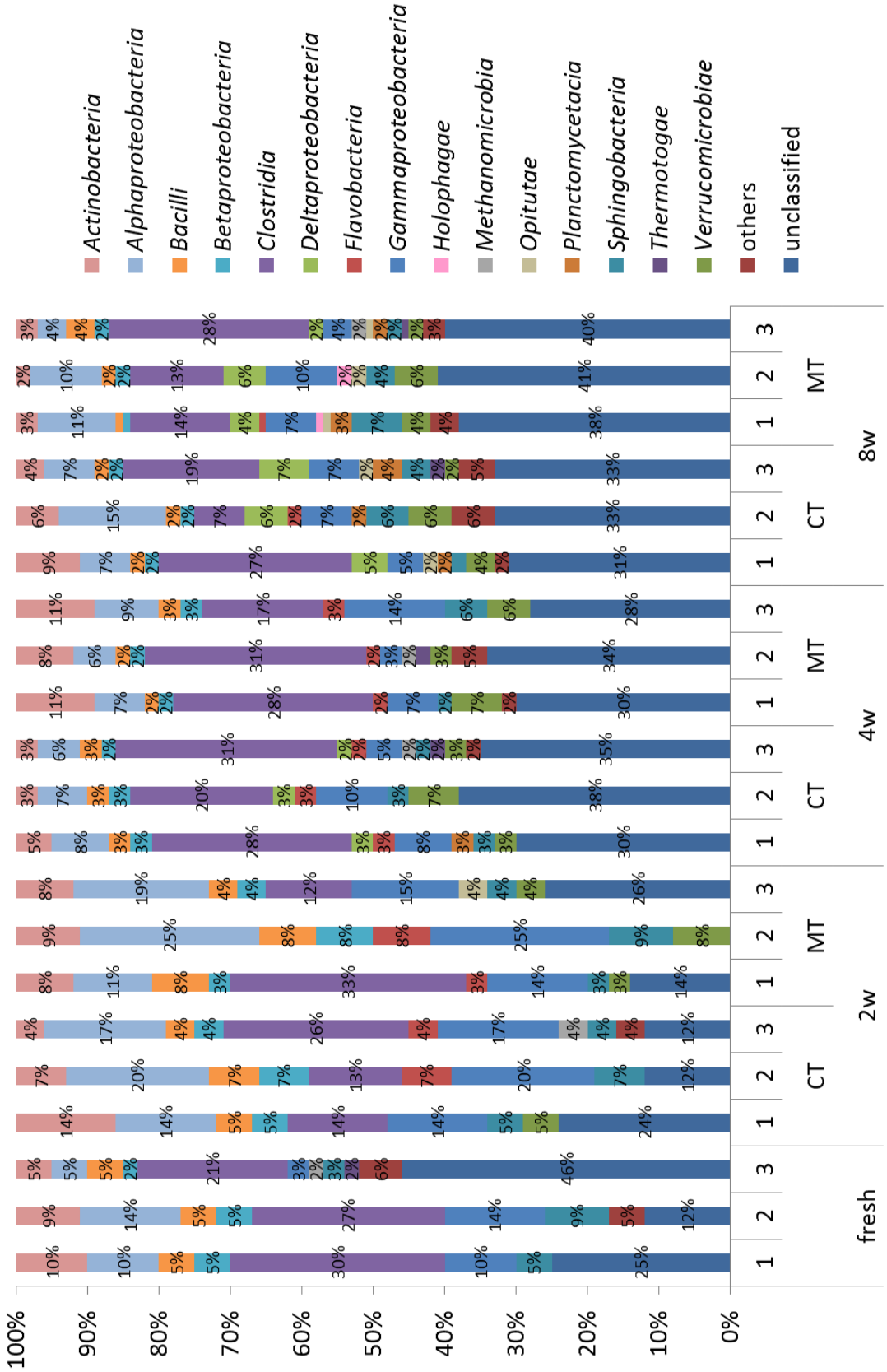

Figure 23: Bacterial composition of OTUs on *Z. mays* leaf material based on 90% sequence similarity of the reverse data set (class level). All data <1% are summed up as others. Data of 1% abundance are simply indicated by color bars

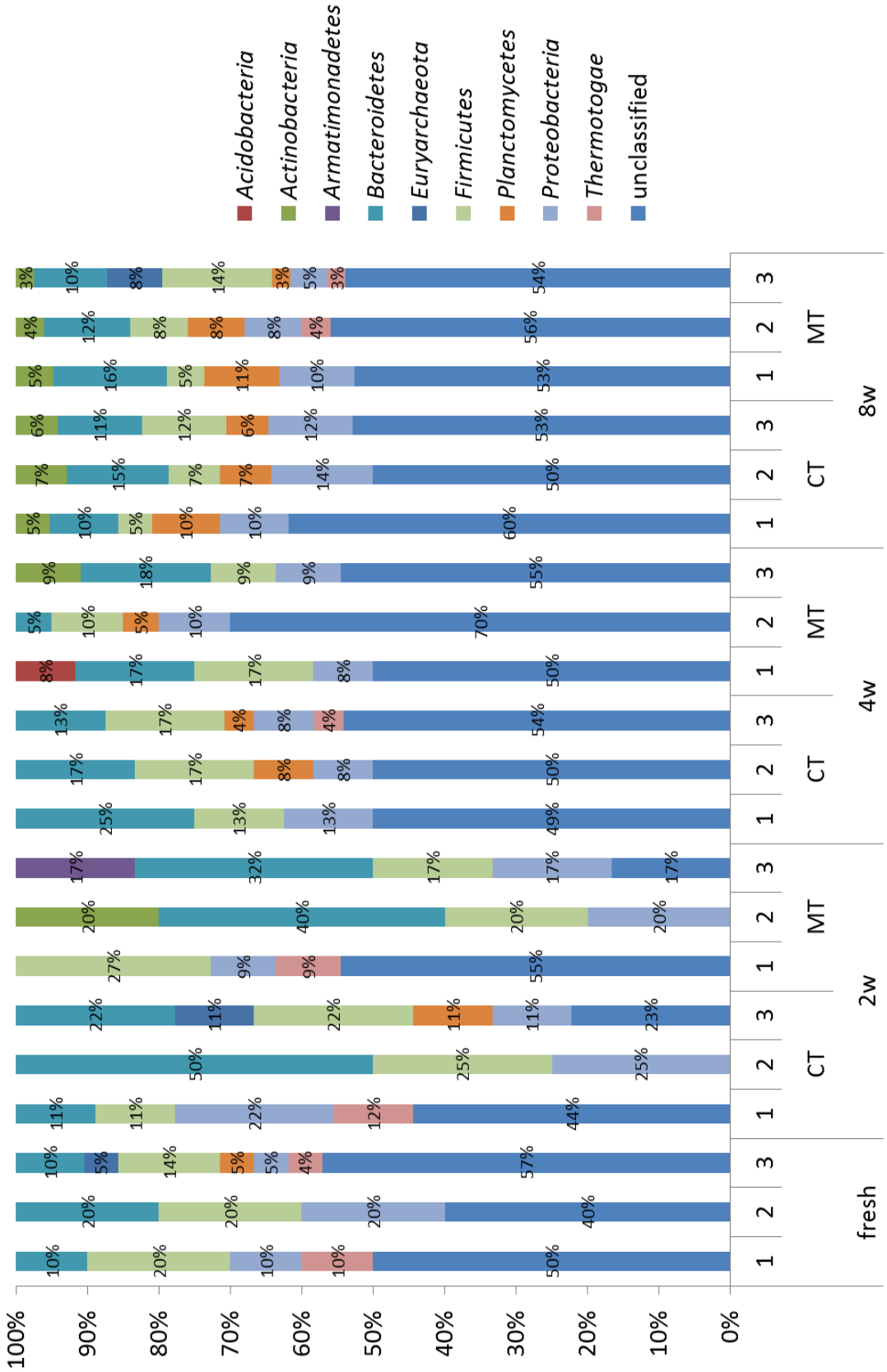

Figure 24: Bacterial composition of OTUs on *Z. mays* leaf material based on 80% sequence similarity of the forward data set (phylum level).

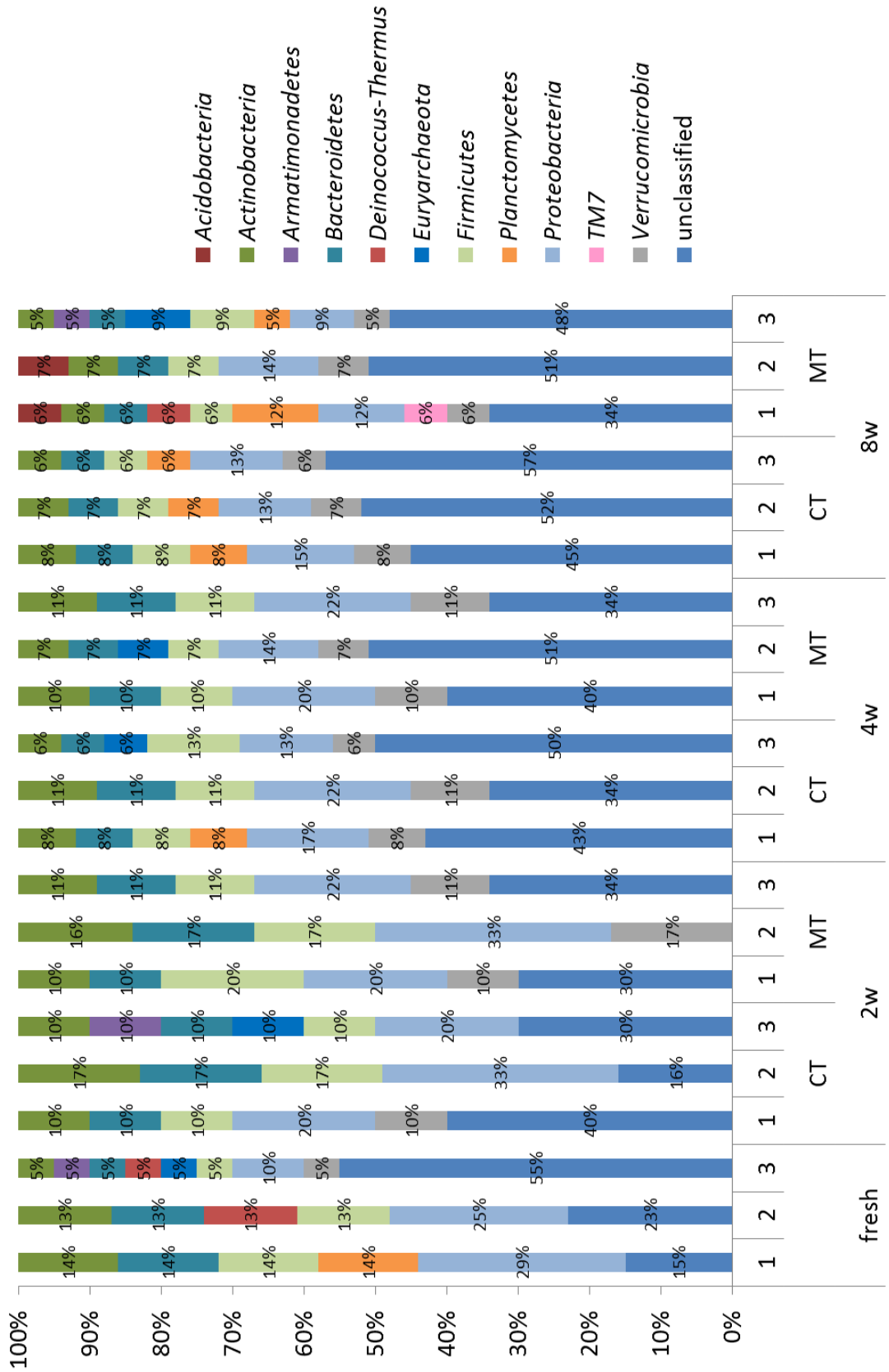

Figure 25: Bacterial composition of OTUs on *Z. mays* leaf material based on 80% sequence similarity of the reverse data set (phylum level).

3.2.4 Phylogenetic classification

Next generation sequencing was performed to identify the driving key players which are part of the different community structures. Bacterial taxa which are responsible for the changes in the community composition during the field experiment were identified by using combined sequences of the corresponding forward and reverse reads to ensure robustness of the data quality. A detailed look at the sequencing results reveals a change in distribution at the order level (Figure 26 and Table A5).

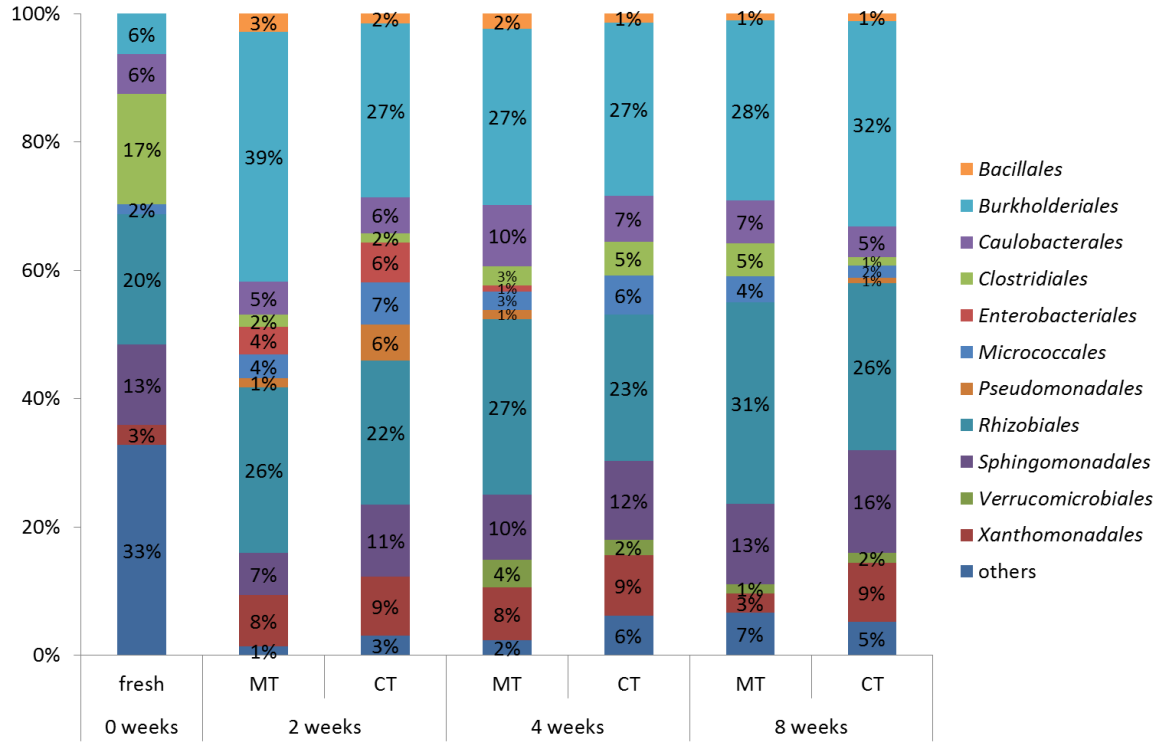

Figure 26: Distribution of bacterial groups based on assembled forward and reverse amplicon reads from *Z. mays* leaf material. Classification of the OTUs based on 90% sequence identity (order level). CT: conventional tillage, MT: minimal tillage. All OTUs contributing ≤ 1% are summed up as "others"

OTUs assigned to *Rhizobiales* (20-31%), Burkholderiales (6-39%) *Sphingomonadales* (7-16%) and *Caulobacterales* (5-10%) were detected in all samples. Characteristically for fresh leaf material were OTUs assigned to *Clostridiales* (17%) as well as a relatively high portion of OTUs occurring at only ≤1% which were summed up as "others" (33%). An increased number of OTUs assigned to *Burkholderiales* (including the families *Alcanigenaceae*, *Comamonadaceae*, *Oxalobacteraceae*) and *Xantomonadales* (*Xanthomonadaceae* only) were detected in leaf litter, compared to fresh leaf material. Furthermore, OTUs assigned to *Bacillales* (*Bacilli*, *Paenibacillaceae, Planococcaceae*), *Verrucomicrobiales* (*Verrucomicrobiaceae* only) and *Enterobacteriales* (*Enterobacteriaceae only*) were detected exclusively in leaf litter samples. In detail, OTUs assigned to *Enterobacteriales* were observed on leaf

material at the beginning of the incubation after 2 and 4 weeks, whereas OTUs assigned to *Verrucomicrobiales* were detected towards the end of the incubation experiment after 4 and 8 weeks. Nevertheless, typical for leaf litter were OTUs assigned to *Pseudomonadales* (*Pseudomonadaceae, Moraxellaceae*), but not in every sample.

Most dominant and ubiquitous in all samples were OTUs assigned to *Comamonadaceae* (ca. 20%), *Rhizobiaceae* (ca. 12%) and *Sphingomonadaceae* (ca. 11%). Characteristic for litter material were OTUs assigned to *Alcaligenaceae*, *Beijerinckiaceae*, *Enterobacteriaceae*, *Hyphomicrobiaceae*, *Oxalobacteraceae*, *Paenibacillaceae*, *Pseudomonadaceae* and *Verrucomicrobiaceae*. Interestingly, OTUs assigned to *Hyphomicrobiaceae* increased, whereas OTUs assigned to *Enterobacteriaceae* and *Pseudomonadaceae* decreased after 2 and 4 weeks. Considering the different tillage treatments, an uneven distribution of OTUs assigned to specific families was observed after 2 weeks of incubation. At that sampling time point OTUs assigned to *Enterobacteriaceae*, *Microbacteriaceae*, *Pseudomonadaceae* and *Sphingomonadaceae* were more abundant on leaf litter embedded in conventional tilled soil. In litter samples embedded in minimal tilled soil, OTUs assigned to *Alcaligenaceae*, *Comamonadaceae*, *Hyphomicrobiaceae* and others (i.e. *Bacillaceae*, *Planococcaceae*, *Rhodocyclaceae*, all <5% abundance) dominated in most of the cases. For all later sampling time points, no clear tendencies of OTUs which could be assigned to any specific bacterial families were observed. Furthermore, OTUs assigned to bacterial taxa involved in the nitrogen and carbon cycles were identified. Typically for the nitrogen cycle, *Rhizobiaceae* and *Alcaligenaceae* were identified as ubiquitous taxa in all litter samples. Towards the end of the experiment, *Mycobacteriaceae* and *Bradyrhizobiaceae* were detected after 4 and 8 weeks, whereas *Clostridiaceae* were typical at the beginning of the experiment. Various species of these genera are known for their role in denitrification and nitrogen fixation. With respect to the carbon cycle, OTUs assigned to genera such as *Cellulomonadaceae* and *Oxalobacteraceae* were detected which are linked to cellulolytic and/or ligninolytic activity.

Sequences from both the forward and reverse data sets were assembled, and thus 1414 sequences were used as input for creating a phylogenetic tree. Figure 27 illustrates the distribution of the bacterial OTUs (90% sequence identity level) in a phylogenetic tree built with ARB. Most OTUs are assigned to α-*Proteobacteria* (632 OTUs) followed by the β-*Proteobacteria* (414 OTUs) and the γ-*Proteobacteria* (145 OTUs).

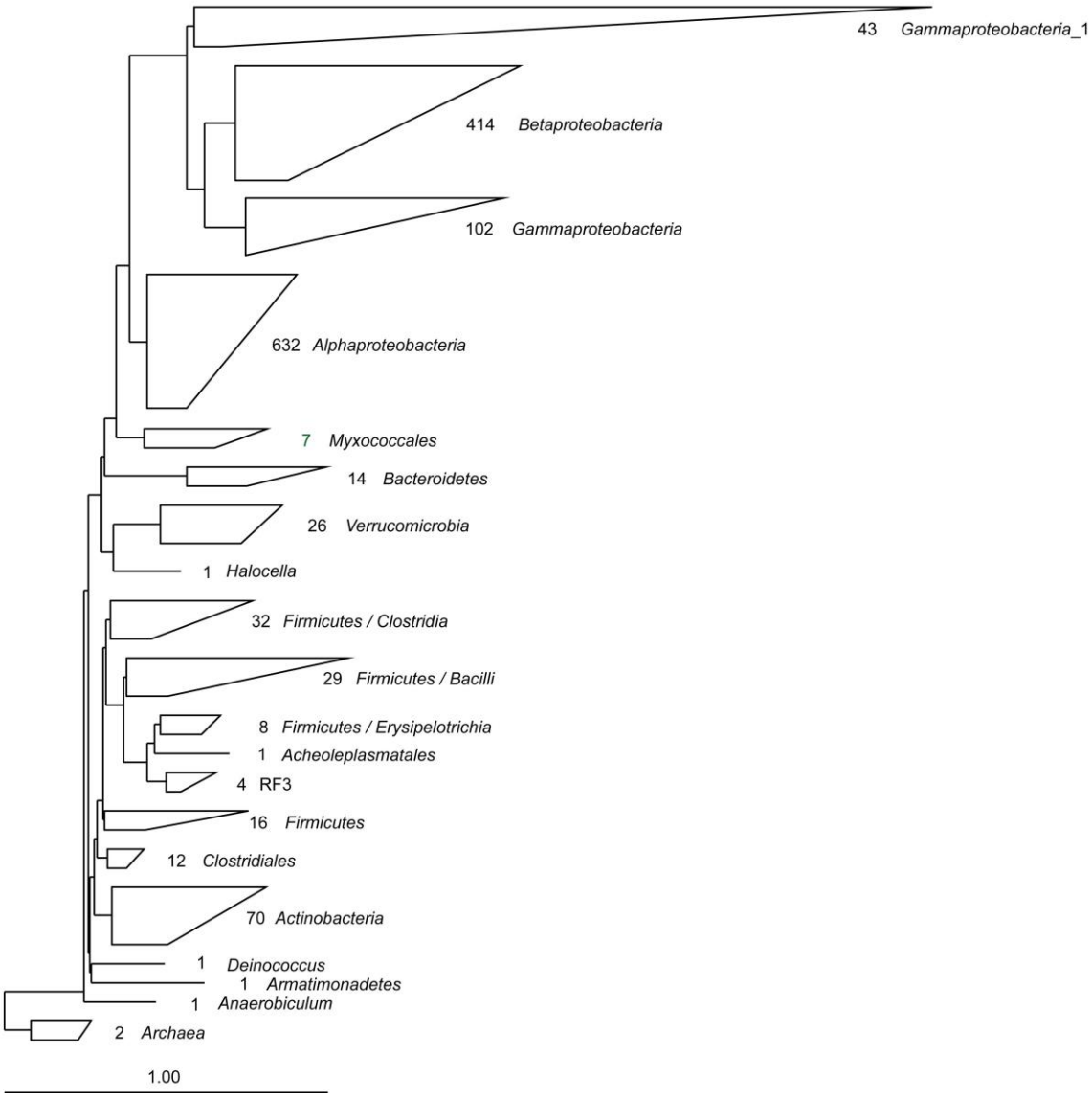

Figure 27: Phylogenetic tree based on bacterial *16S rRNA* gene fragments amplified from *Z. mays* leaf material. Archaeal sequences were used as outgroup. The number of OTUs (90% sequence identity level) is indicated for each cluster, based on the assembly of corresponding forward and reverse amplicon fragments

When applying the graphical gaussian model on the NGS sequencing data to all samples, mutual interactions concerning the appearance of OTUs assigned at the family level could be measured (Figure 28). A very strong positive correlation was detected between OTUs assigned to *Sphingomonadaceae* and *Bradyrhizobiaceae* as well as *Pseudomonadaceae* and *Enterobacteriaceae*. There was also a strong positive correlation discovered between OTUs assigned to members of the families *Aurantimonadaceae/Alcaligenaceae*, *Verrucomicrobiaceae/Caulobacteraceae* and *Methylobacteriaceae/Cytophagaceae*. A weak positive correlation was measured between OTUs assigned to *Erythrobacteraceae/Erysipelotrichaceae* as well as *Rhizobiaceae/Comamonadaceae*.

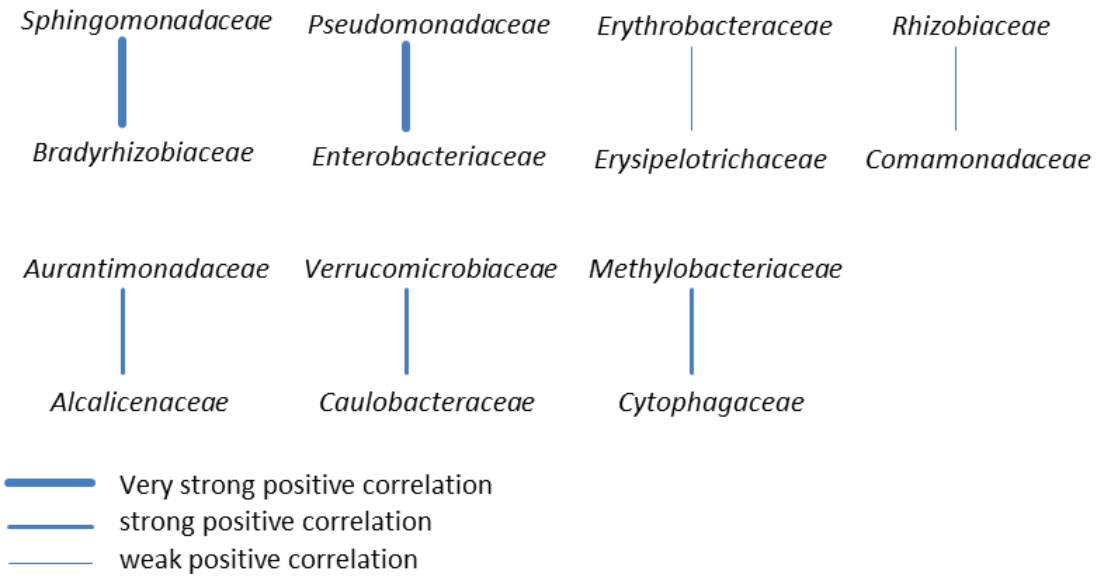

Figure 28: Co-occurrence of bacterial families involved in degradation of *Z. mays* leaf litter. Graphical gaussian model was used to visualize the correlations of OTUs (90% sequence identity) assigned to bacterial families

The descriptive discriminant analysis visualizes which OTUs (90% sequence similarity level) assigned to specific bacterial families occur at what sampling time point and tillage management (Figure 29). The significant differences of fresh leaf material compared to leaf litter of *Z. mays* was due to the presence or absence of OTUs assigned to the families *Aurantimonadacea*e, *Cytophagaceae, Clostridiaceae, Methylobacteriaceae* and *Ruminococcaceae*. After 2 weeks of incubation, OTUs assigned to *Pseudomonadaceae* and *Enterobacteriaceae* correlated positively to leaf litter embedded in minimal tilled soil. At later sampling time points, the presence or absence of *Oxalobacteraceae, Rhizobiaceae, Paenibacillaceae, Alcaligenaceae, Beijerinckiaceae, Comamonadaceae, Syntrophomonadaceae, Erythrobacteraceae, Hyphomicrobiaceae,* Verrucomicrobiaceae, *Bradyrhizobiaceae* and *Sphingomonadaceae* is crucial for significant differences.

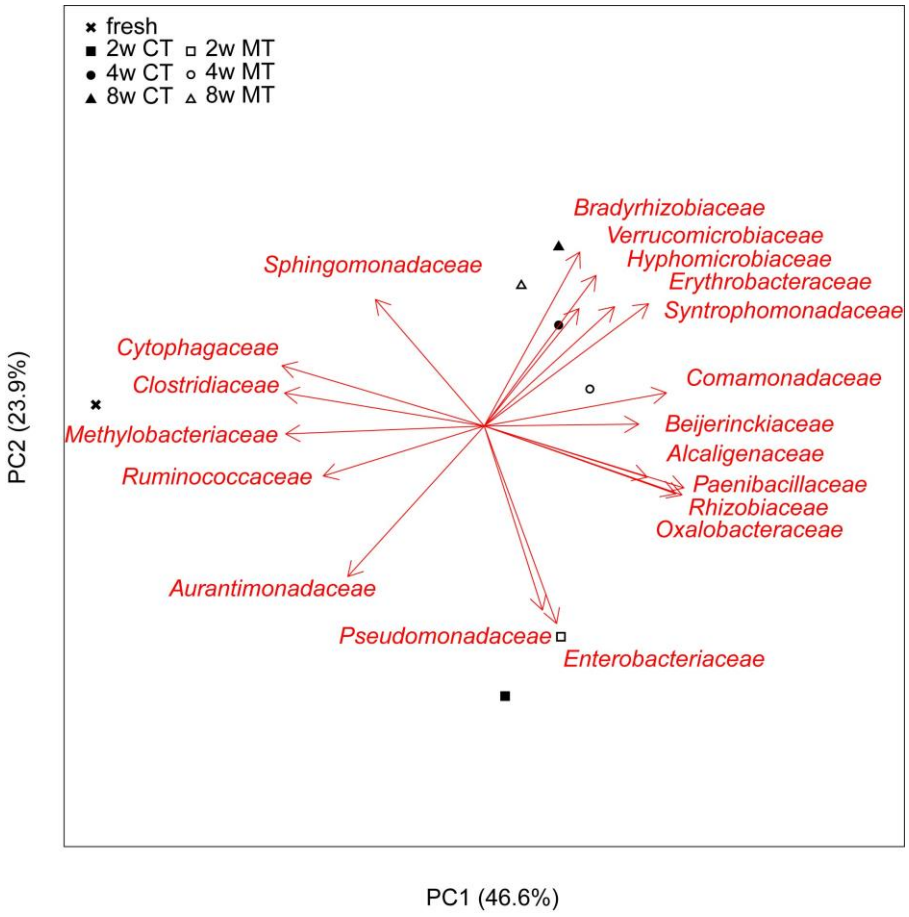

Figure 29: Occurrence of bacterial families depending on the incubation time of *Z. mays* in soil. Descriptive discriminant analysis of OTUs (90% sequence similarity). Principal components (PC) 1 and 2 are indicated

3.3 Microbial diversity during different stages of F. sylvatica leaf litter degradation

Changes in the bacterial community composition on leaf litter of *F. sylvatica* were monitored in a microcosm experiment under controlled conditions for up to 30 weeks. Leaf material in litter bags was embedded in the two different soil types Cutanic Luvisol and Calcaric Regosol, which are typical for this habitat. Bacterial community shifts on the leaf litter were determined based on molecular fingerprinting techniques. The key players were determined with high throughput sequencing and linked to degradation rates and chemical analyses of the leaf material.

3.3.1 Degradation of *F. sylvatica* leaf material

The dry mass of the leaves resulted in a continuous decrease from 36.0% (Calcaric Regosol) and 34.7% (Cutanic Luvisol) to 27.0% (Calcaric Regosol) and 26.0% (Cutanic Luvisol), in relation to fresh weight (Figure 30). With regard to the microcosms filled with Calcaric Regosol soil, a significant mass

loss of leaf litter was observed after 2 weeks of incubation, followed by stagnation after 4 weeks and reduced values after 8 weeks. Finally, another significant decrease in dry mass was measured after 30 weeks. With regard to the microcosms filled with Cutanic Luvisol, a continuous significant mass loss was detected at each of the sampling time points up to 4 weeks of leaf litter incubation, followed by stagnation after 8 and 16 weeks. A significant mass loss was again measured in the last sampling time point after 30 weeks.

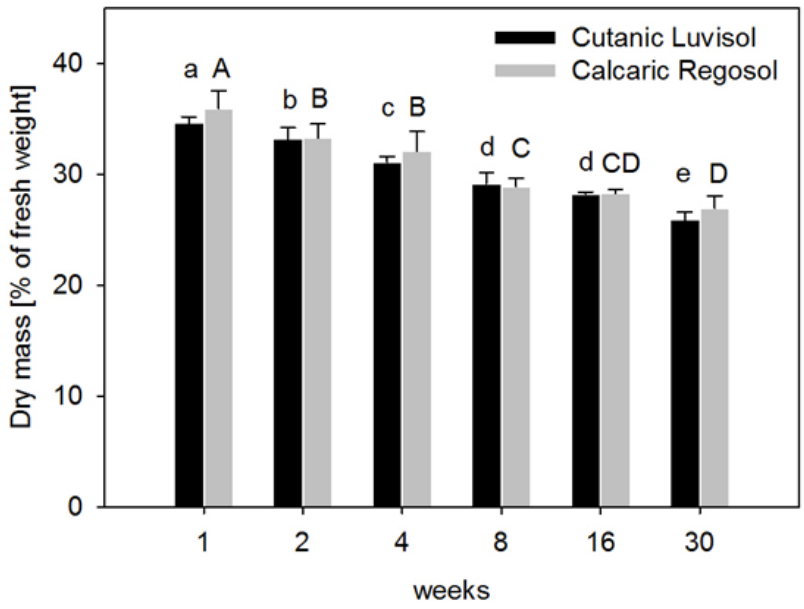

Figure 30: Dry mass of *F. sylvatica* leaf material incubated in Calcaric Regosol and Cutanic Luvisol soil. Mean and standard deviation are shown (n = 5). Significant differences are indicated in lower case (Cutanic Luvisol) and upper case (Calcaric Regosol) letters according to Tukey multiple comparison test (p-value < 0.05)

3.3.2 Bacterial community composition on *F. sylvatica* leaf material

As in the experiment with *Z. mays* leaf material, T-RFLP was used as fingerprinting technique to generate an overview of bacterial changes in leaf litter samples of *F. sylvatica* by using primers to generate *16S rRNA* gene fragments. Principal component analysis of the T-RFLP results indicated that the soil type had a significant influence on the bacterial community composition in leaf material (Figure 31). While at the beginning of the experiment a similar bacterial community was observed, the incubation time clearly leads to a further significant (all p-values ≤ 0.001) differentiation of the bacterial communities on leaf litter incubated within a specific soil after 4 weeks (Calcaric Regosol) and 2 weeks (Cutanic Luvisol).

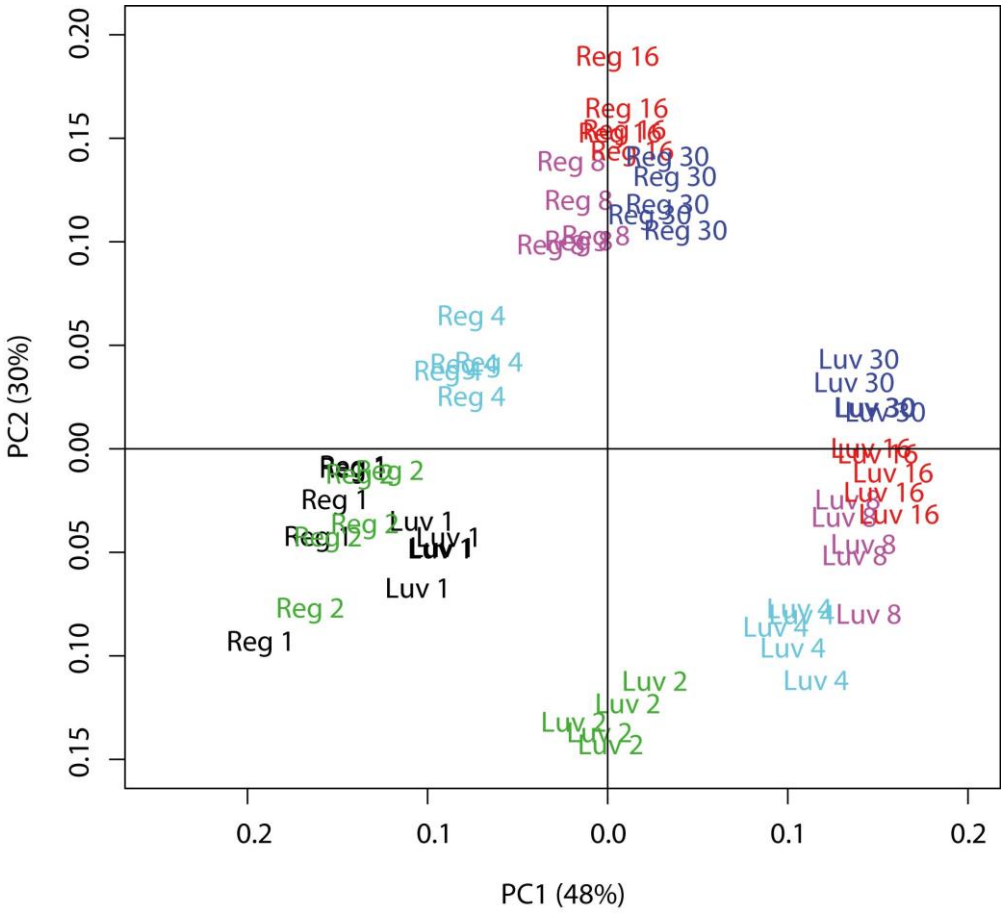

Figure 31: Principle component analysis of T-RFs from DNA of *F. sylvatica* leaf litter incubated in two different soils (n=5). Luv: Cutanic Luvisol; Reg: Calcaric Regosol; numbers indicate the weeks of incubation

Based on the results of T-RFLP, amplicon sequencing was performed in four replicates per sampling time point to identify the bacterial key players contributing to the changes in the community composition. An initial quality check with the GSRunBrowser software resulted in 254,000 reads. This number of reads corresponds to 3567-4995 (Cutanic Luvisol) and 2418-9431 (Calcaric Regosol) sequences per sample with an average length of 368 bp after a subsequent quality check with the software mothur with which was further downstream data processing performed. Rarefaction curves were generated from the forward data set to determine whether the number of sequences covers the diversity (Figure 32) based on OTUs clustering at the 90% sequence identity level. While for leaf litter samples incubated in Calcaric Regosol soil rarefaction curves nearly met the saturation phase, no saturation was observed for those samples incubated in Cutanic Luvisol. In leaf litter samples incubated in Calcaric Regosol soil a higher number of reads was observed. This also leads to an increased number of OTUs (203 to 593 OTUs) compared to samples incubated in Cutanic Luvisol soil (183 to 316 OTUs).

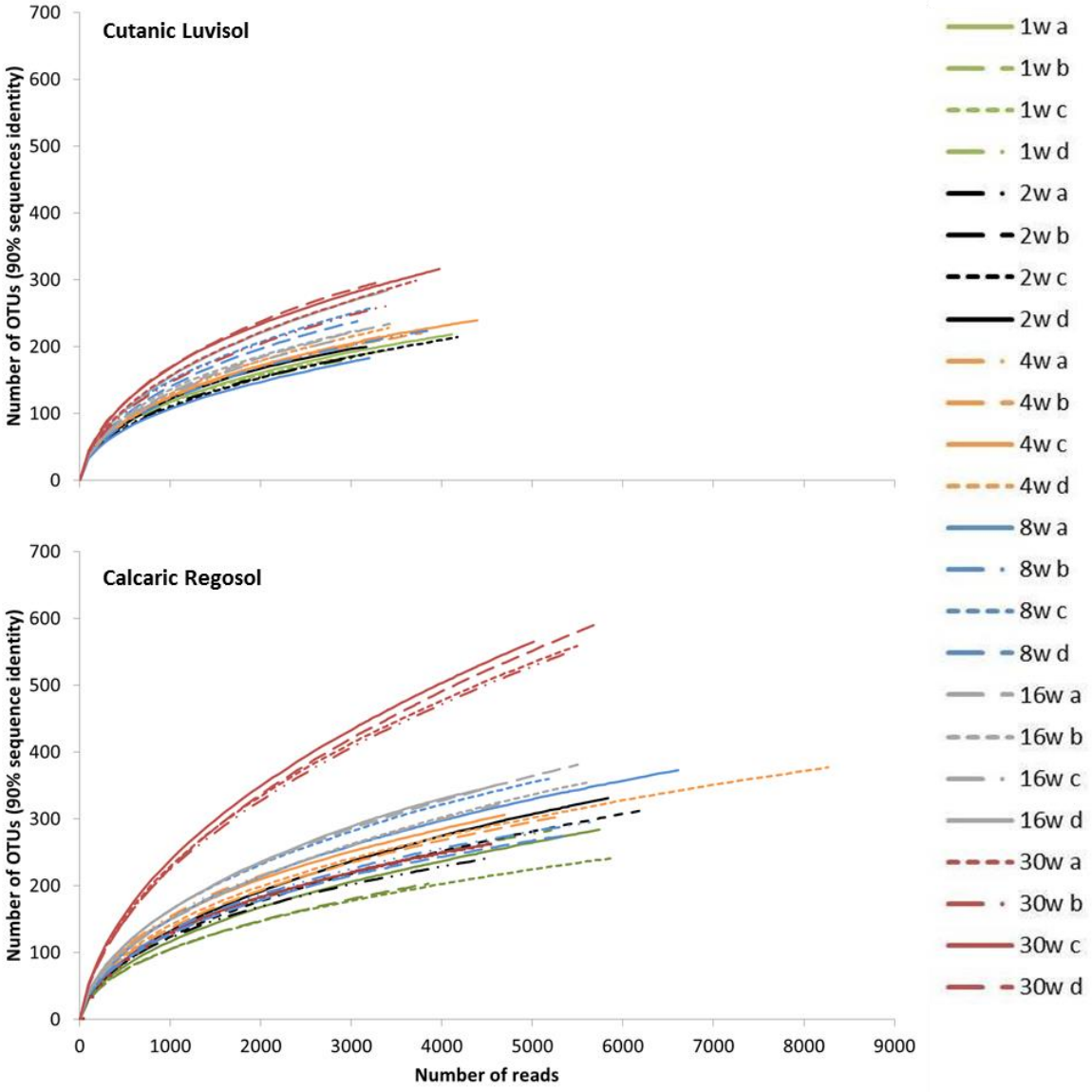

Figure 32: Rarefaction curves (*F. sylvatica*)for determination of the sampling richness of bacteria colonizing on the leaf litter during incubation of 1 to 30 weeks in the two different soils Calcaric Regosol and Cutanic Luvisol. The letters a to d indicate the replicate. OTUs were clustered at 90% sequence identity level

According to the results from the experiment with *Z. mays* leaf material, indices for richness (Chao1 index), diversity and evenness (based on the Shannon index) were calculated with OTUs clustered at the 90% sequence similarity level (Figure 33). The same bacterial richness detected on leaf litter incubated in Cutanic Luvisol was observed throughout the duration of the experiment. A significant increase (p-value ≤ 0.05) in bacterial richness was verified after 30 weeks in leaf litter, which was incubated in Calcaric Regosol soil. With regard to the evenness, no significant changes in the bacterial community were observed in samples incubated in Cutanic Luvisol. In contrast to that, evenness increased over time with significant changes after 4 and 30 weeks (p-value ≤ 0.05) in samples

incubated in Calcaric Regosol soil. In both soils, the same changes were seen with regard to the diversity.

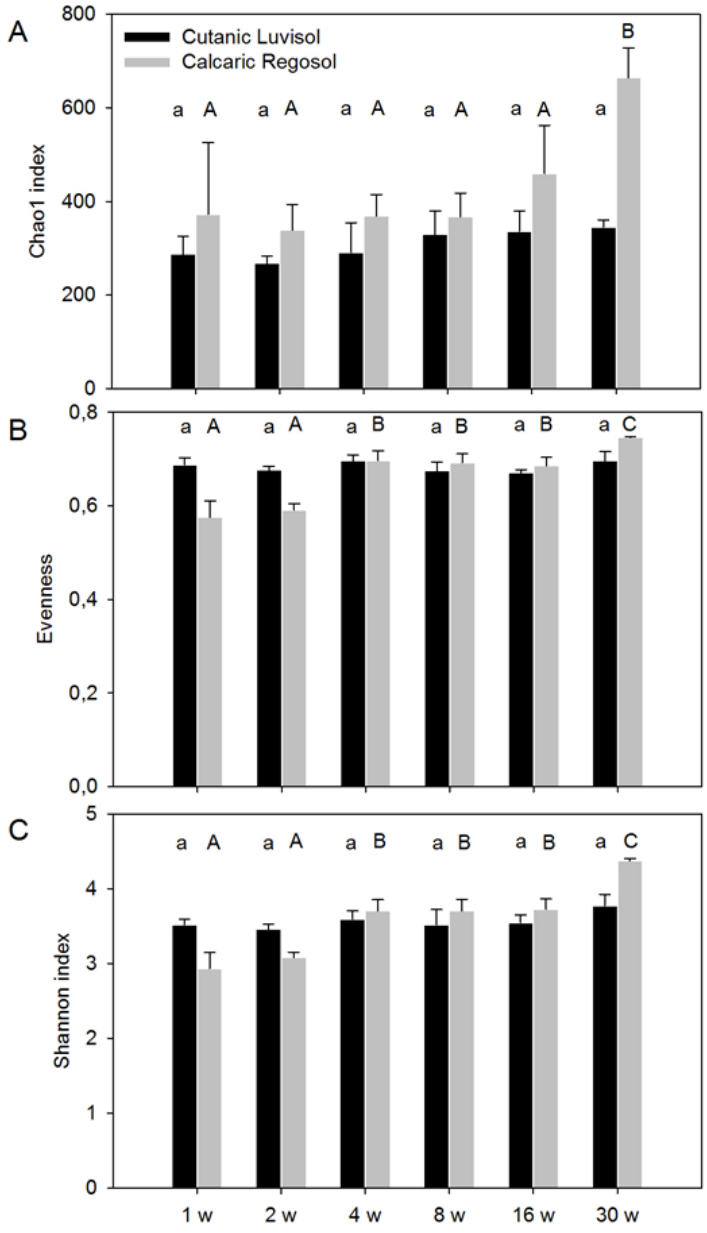

Figure 33: Bacterial richness, evenness and diversity on *F. sylvatica* leaf litter. Indices based on Chao1 index (A) and Shannon index (B and C) of bacterial OTUs. Mean and standard errof of n = 4 are given. Significant differences are indicated in lower case (Cutanic Luvisol) and upper case (Calcaric Regosol) letters (p-value < 0.05)

3.3.3 Phylogenetic classification

In order to identify the bacterial key players involved in the litter degradation of *F. sylvatica*, the *16S rRNA* gene fragments were assigned to specific bacterial taxa. Analyzing the forward data set, OTUs (90% sequence identity level) assigned to the phyla *Proteobacteria* (76%), *Acidobacteria* (2%), *Planctomycetes* (2%), *Verrucomicrobia* (2%), *Armatimonadetes* (1%) and *Firmicutes* (1%) were most dominant throughout all samples. OTUs that could not be assigned to any specific phylum are categorized as "unclassified" (15%). A detailed overview of the families identified is given in Table A6. Figure 34 gives an overview of the distribution of these phyla for each single sample. Rare phyla contributing <1% were summed up as "others" (1%). During the experiment, a maximal proportion of *Proteobacteria* was detected after 4 weeks (81% (Cutanic Luvisol), 79% (Calcaric Regosol)) of incubation in leaf material with a subsequent reduction (76% (Cutanic Luvisol), 62% (Calcaric Regosol)). The number of *Planctomycetes* and *Verrucomicrobia* increased steadily from 1% to 3% towards the end of the experiment. *Acidobacteria* were more frequent after 1 as well as after 4 weeks of leaf litter incubation in Cutanic Luvisol (4%) compared to Calcaric Regosol soil (2-3%). The proportion of Armatimonadetes steadily increased from 0% to 2% in samples incubated in Calcaric Regosol, whereas in Cutanic Luvisol samples it was only possible to verify up to 1% mainly after 16 and 30 weeks.

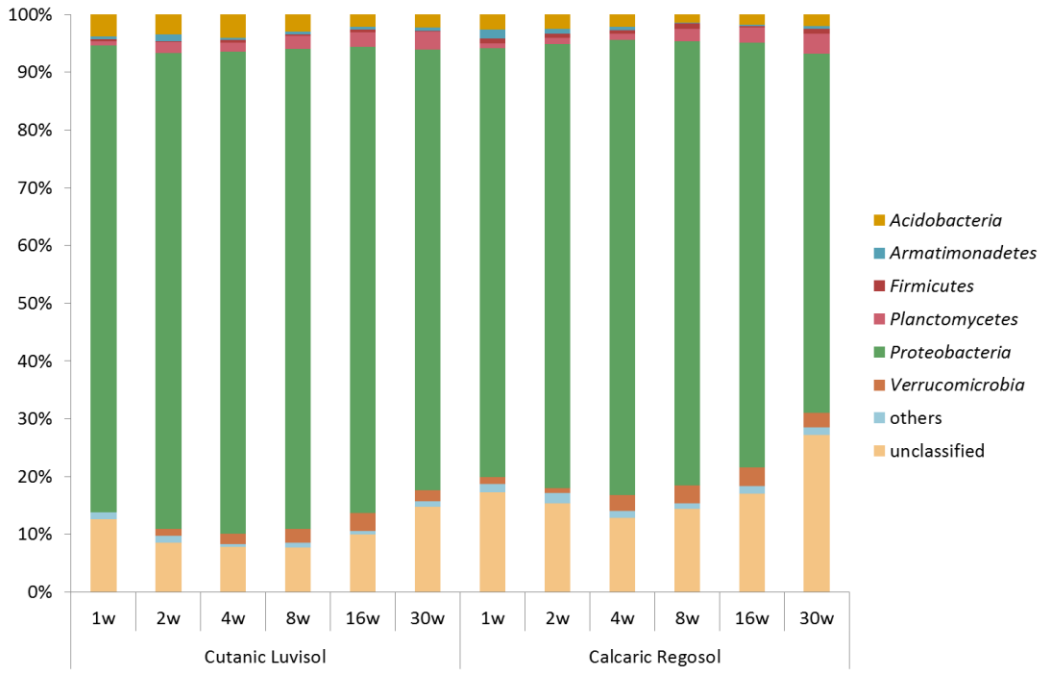

Figure 34: Distribution of phyla identified in leaf litter samples of *F. sylvatica* incubated for up to 30 weeks in the two different soils Cutanic Luvisol and Calcaric Regosol. Rare phyla contributing <1% are summed up as "others"

Figure 35 illustrates the distribution of OTUs assigned to α-, β-, γ- and δ-*Proteobacteria* in each sample. In litter samples incubated in Cutanic Luvisol soil, an increase was detected over time for OTUs assigned to α-*Proteobacteria* with a minimum after 2 weeks, whereas a decrease of OTUs assigned to β-*Proteobacteria* was measured. The proportion of OTUs assigned to γ-*Proteobacteria* was stable throughout the experiment and only few or even no OTUs could be assigned to δ-*Proteobacteria*. In litter samples incubated in Calcaric Regosol soil, the proportion of OTUs assigned to α-*Proteobacteria* showed no specific trend throughout the experiment. OTUs assigned to β-*Proteobacteria* tended to decrease over time, and the portion of OTUs assigned to γ- and δ-*Proteobacteria* increased slightly towards the later sampling time points. The proportion of OTUs assigned to *Proteobacteria* that could not be related to any class also increased over time independently from the soil type the litter was incubated in. Noticeably, δ-*Proteobacteria* occurred more frequently in litter samples incubated in Calcaric Regosol soil, whereas in samples in Cutanic Luvisol only up to 5 reads could be detected.

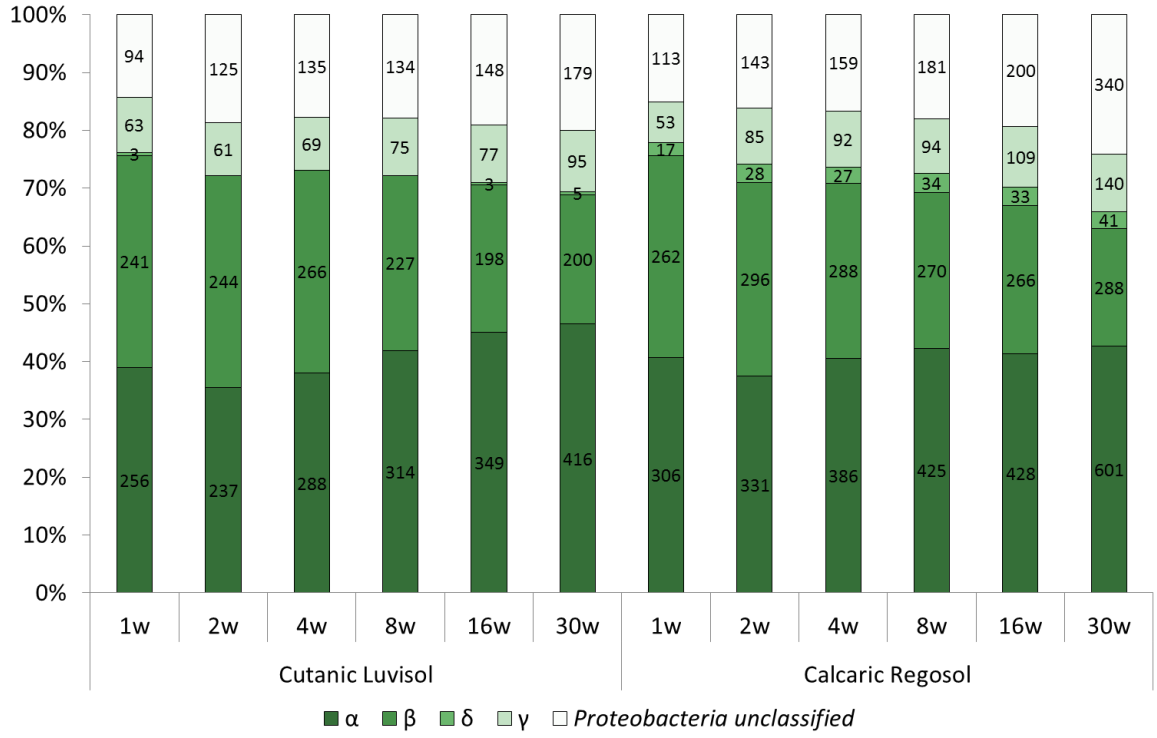

Figure 35: Distribution of proteobacterial classes detected on degrading leaf litter of *F. sylvatica* in Cutanic Luvisol and Calcaric Regosol (n=4). Absolute numbers of reads contributing to OTUs (90% sequence identity level) are given for each proteobacterial class per sample

In order to visualize the differences in the community composition of the most abundant OTUs (containing at least 50 sequences) with 90% sequence similarity among each of the samples, heatmaps were created at the family level (Figure 36).

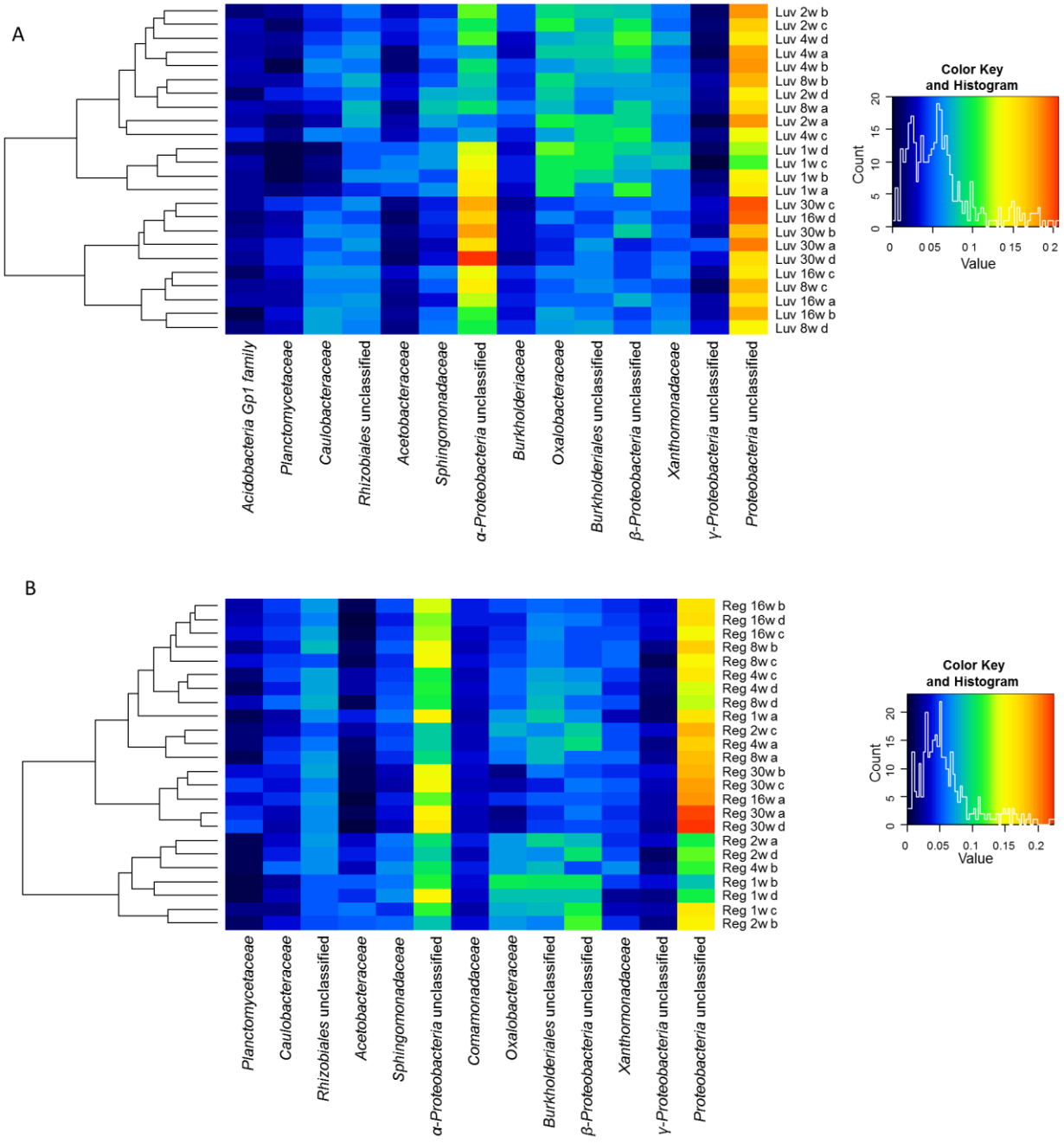

Figure 36: Heatmap showing the most abundant bacterial groups in leaf litter samples of *F. sylvatica* leaf litter. OTUs (90% sequence identity) were assigned at family level. Samples were incubated 1 (1w), 2 (2w), 4 (4w), 8 (8w), 16 (16w) and 30 (30w) weeks in (A) Cutanic Luvisol (Luv) and (B) Calcaric Regosol (Reg) soil with 3 replicates (a, b, c). Only OTUs with ≥ 50 sequences were considered

For leaf material incubated in Cutanic Luvisol soil, separate clusters for leaf litter incubated for 1 week and for 30 weeks were observed. For samples incubated in Calcaric Regosol soil, no clear

clustering corresponding to the sampling time points was visible. OTUs assigned to numerous taxa at the family level were common in samples incubated in both soil types (*Acetobacteraceae*, *Caulobacteraceae*, *Oxalobacteraceae*, *Planctomycetaceae*, *Sphingomonadaceae*, *Xanthomonadaceae*, α-/β-/γ-*proteobacterial* families). Nevertheless, OTUs assigned to the families *Acidobacteraceae*, *Burkholderiales* (Cutanic Luvisol) and *Comamonadaceae* (Calcaric Regosol) could be related to specific soil types. All these families are detected at a low level, either being stable (*Acidobacteria*, *Comamonadaceae*) or with a slight decrease towards the later sampling time points (*Burkholderiales*). For both soil types, a decrease was also observed for OTUs assigned to *Acetobacteraceae* and *Oxalobacteraceae* as well as to families related to *Burkholderiales* and β-*Proteobacteria* at later sampling time points. The abundance of OTUs assigned to *Planctomycetaceae* and families related to α-*Proteobacteria* showed an increase over time. The abundance of OTUs assigned to *Sphingomonadaceae* and *Xanthomonadaceae* as well as families related to γ-*Proteobacteria* was observed to be at a steadily low level throughout the experiment. Only low abundance of OTUs assigned to *Caulobacteraceae* and families related to *Rhizobiales* was detected throughout the experiment. Comparing the litter samples incubated in both soil types, replicates of samples in Cutanic Luvisol soil clustered more strictly related to the sampling time points, whereas in Calcaric Regosol the bacterial community structures of specific sampling time points were more heterogeneous, with the exception of the sampling time point after 30 weeks.

When applying the graphical gaussian model (GGM) on the NGS sequencing data of OTUs based on 90% sequence similarity (forward data set), mutual interactions based on the appearance of OTUs assigned at family level could be visualized (Figure 37). The presence of *Pasteuriaceae* strongly correlates with the presence of *Rhodospirillaceae*, and they also correlate very strongly to the presence of acidobacterial families as well as they coincidentally negatively correlated with the presence of *Spartobacteria* families. The presence of *Verrucomicrobiaceae* has a very strong positive correlation to the presence of *Caulobacteraceae*, but a negative correlation to the presence of α-*proteobacterial* families, which in turn are positively linked to the presence of *Hyphomicrobiaceae*. The presence of *Sphingomonadaceae*, *Acetobacteraceae* and *Pseudomonadaceae* correlate mutually in a strongly positive manner. OTUs assigned to *Holophagaceae* correlate positively to *Planctomycetaceae* as well as to *Neisseriaceae*. A strong positive correlation was also detected between *Enterobacteriaceae* and *Armatimonadaceae*. *Coxiellaceae* and some members of the acidobacterial families correlate only slightly in a positive way, whereas *Oxalobacteraceae* and *Xanthomonadaceae* correlate weakly in a negative manner.

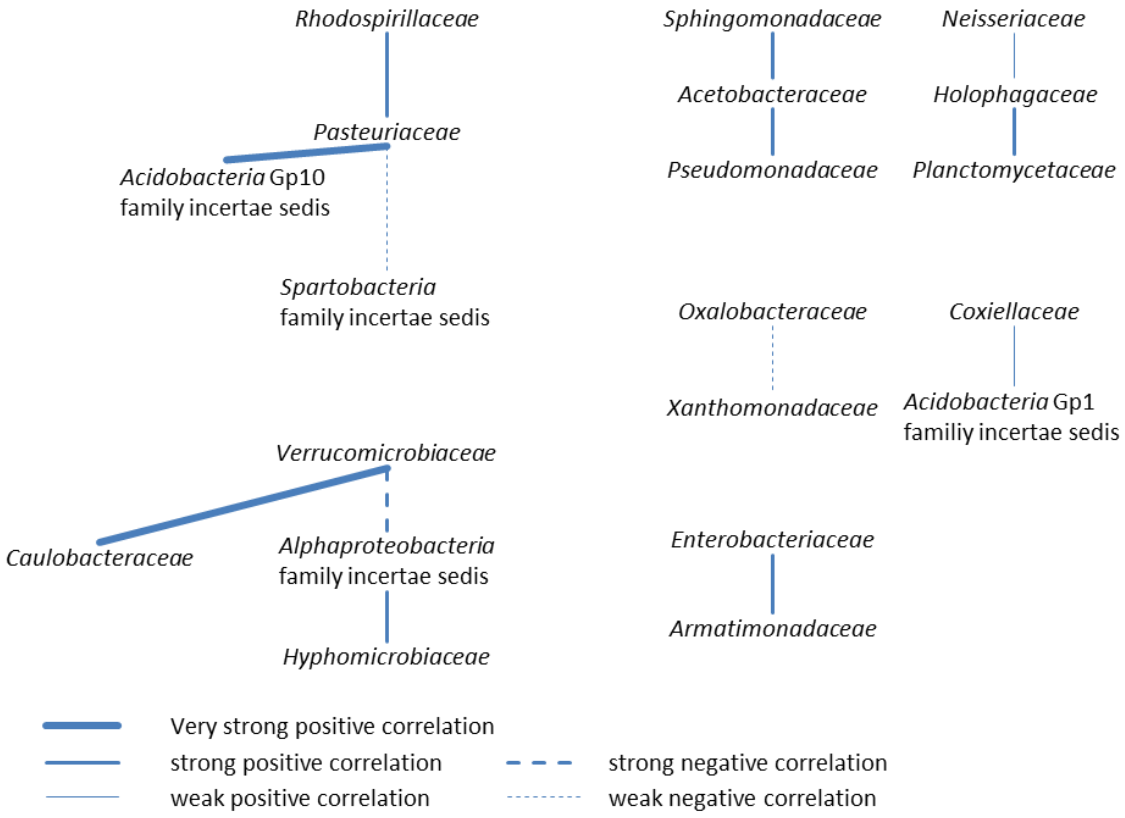

Figure 37: Co-occurrence of bacterial families involved in degradation of *F.sylvatica* leaf litter. Graphical gaussian model performed to visualize positive and negative correlations of OTUs (90% sequence identity) assigned to bacterial families detected on leaf material

The descriptive discriminant analysis visualizes which OTUs (90% sequence similarity level) assigned to specific bacterial families occur at which sampling time point (Figure 38). On leaf litter incubated for up to 2 weeks in both soil types, OTUs assigned to the bacterial families *Acetobacteraceae*, *Deinococcaceae*, *Methylobacteriaceae*, *Oxalobacteraceae* and *Pseudomonadaceae* formed the main part of the community structure. OTUs assigned to *Sphingomonadaceae* (1 week) and *Burkholderiaceae* (2 weeks) were detected on leaf litter only incubated in Cutanic Luvisol only as being part of the bacterial community structure, whereas OTUs assigned to some members of acidobacterial families (group 1) were detected in Calcaric Regosol within the first two weeks. In contrast, acidobacterial families (group 1) were part of the bacterial community structure after 30 weeks of leaf litter incubated in Cutanic Luvisol. After 4 weeks and later, leaf litter in both soils shared OTUs assigned to the bacterial families *Bradyrhizobiaceae*, *Caulobacteraceae*, Comamonadaceae, Coxieallaceae, *Hyphomicrobiaceae*, *Methylophilaceae*, *Planctomycetaceae*, *Rhodospirillaceae* and *Xanthomonadaceae*. OTUs assigned to α-*proteobacterial* families were detected only on leaf litter in Cutanic Luvisol, whereas *Opitutaceae*, *Pasteuriaceae* and *Rhizobiaceae* were measured only on leaf litter in Calcaric Regosol.

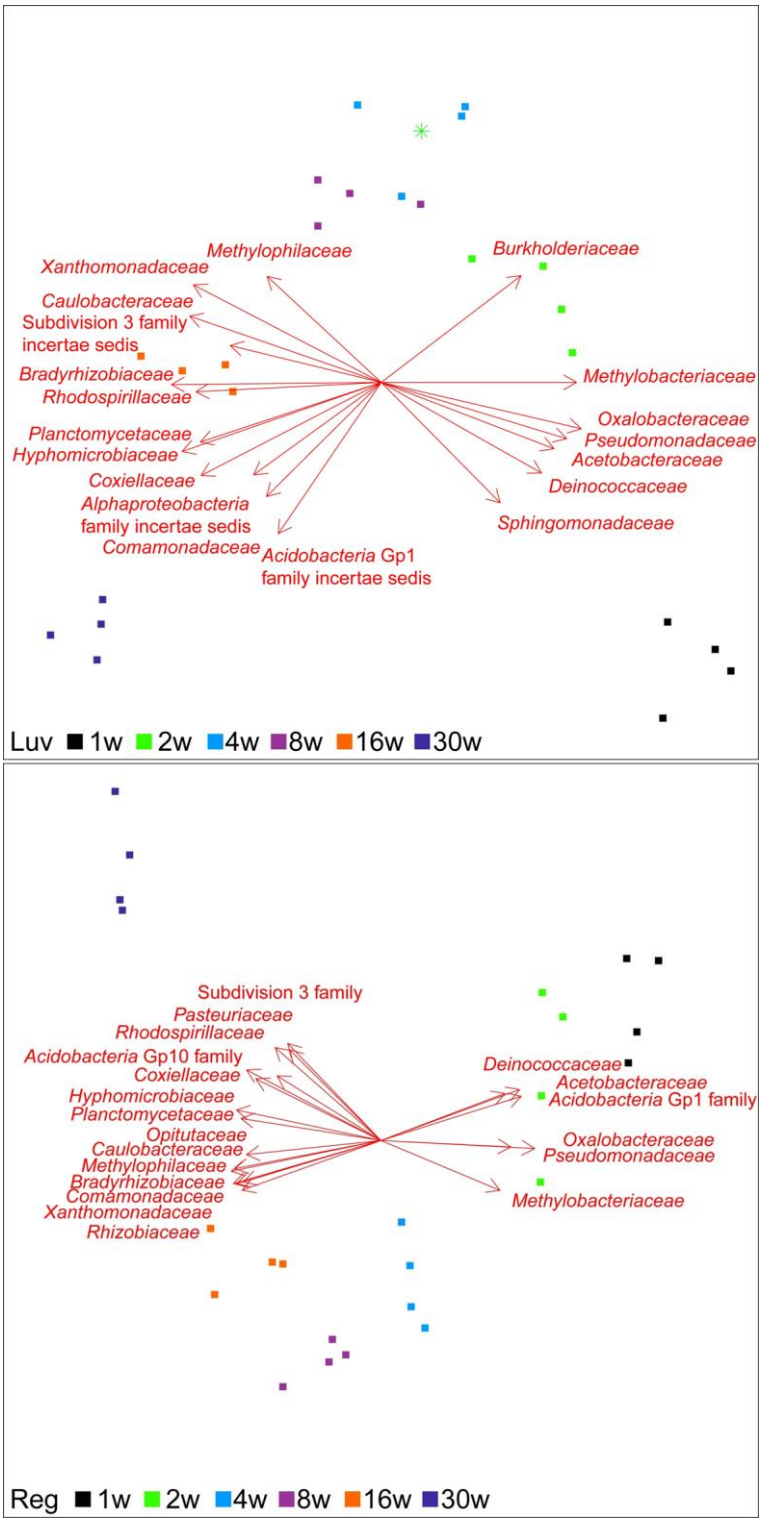

Figure 38: Occurrence of different bacterial groups during leaf litter degradation of *F. sylvatica*. Descriptive discriminant analysis of the OTUs (90% sequence similarity) assigned to bacteria at family level showing the impact on each sampling time point. Samples were incubated in Cutanic Luvisol (top) and Calcaric Regosol (bottom)

3.4 Influence of compost amendments on the diversity of alkane degrading bacteria in petroleum-contaminated soil

For a better understanding of the bacterial community structure of alkane-degrading bacteria in petroleum-contaminated soils, compost residues were mixed with soil and incubated in a microcosm experiment for up to 36 weeks. Therefore, the *alkB* gene harboring bacterial key players were identified via next generation sequencing.

3.4.1 Diversity of *alkB*-harboring bacteria in soil

After an initial quality check with the software GSRunBrowser (Roche) and data processing with the software mothur, 29,822 high-quality *alkB* gene fragment sequences were obtained (828 sequences per sample on average). Rarefaction curves were calculated to determine the sampling richness (Figure 39). Compared to the control samples (400-500 OTUs), a higher number of OTUs was observed in soil amended with young and old compost, resulting in a maximum of ca. 800 OTUs after 36 weeks. The saturation of the rarefaction curves was not reached, which indicates that not all species could be covered due to the enormous taxonomical richness in environmental samples.

A constantly high evenness of *alkB* gene fragments was observed throughout all samples (0.95 to 0.99) irrespective of the sampling time points (Figure 40). Significant differences were only measured in soil amended with young compost. In these samples, evenness was higher after 0, 6 and 12 weeks, but decreased after 36 weeks compared to the other treatments.

Figure 41 illustrates the similarity of *alkB* gene harboring bacterial communities based on principle component analysis. Community composition was similar from the beginning of the experiment up to 6 weeks. At later sampling time points in particular, principle component (PC) 1 separates the control soil from those amended with compost. PC 2 differentiates between soil amended with young and old compost. Bacterial diversity was significantly influenced by the incubation time and the compost type (p-values ≤ 0.001 based on ANOVA).

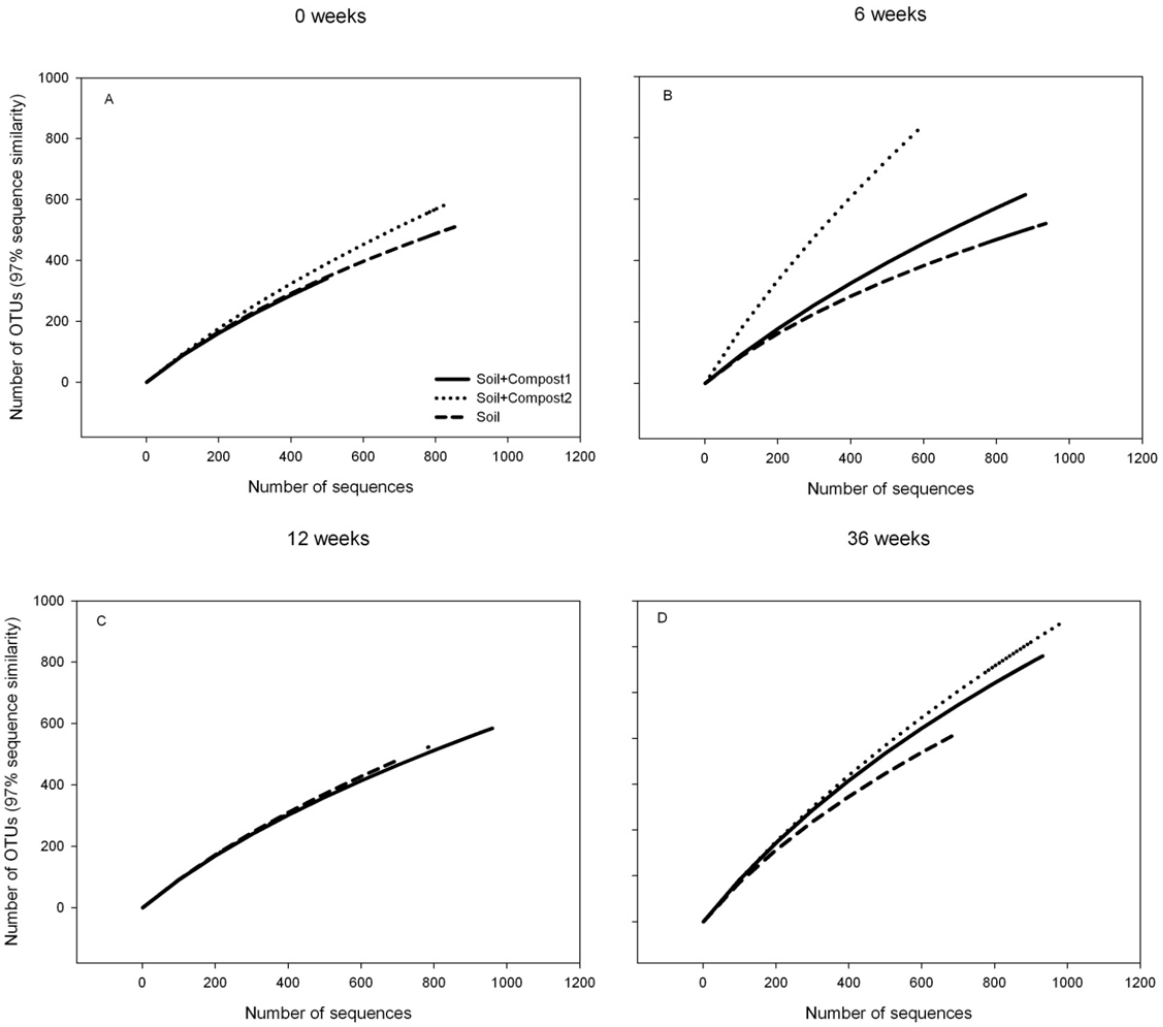

Figure 39: Rarefaction curves of *alkB* gene fragments for determination of the sampling richness based on OTUs at 97% sequence identity level at the beginning of the experiment (A), after 6 (B), 12 (C) and 36 (D) weeks (published in Wallisch et al. (2014))

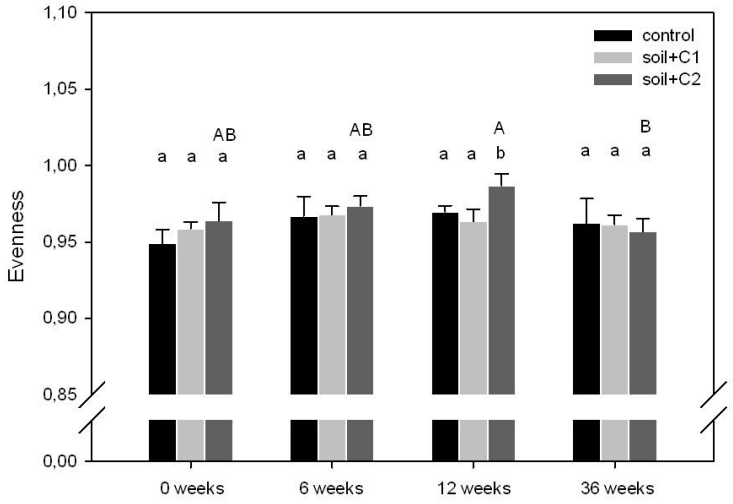

Figure 40: Evenness of *alkB* gene harboring soil bacteria. Significant different treatments within one sampling time point are indicated with different lower case letters; the upper case letters indicate significant differences over time within one treatment (n=3, p-value <0.05, error bars indicate the standard deviation). (published in Wallisch et al. (2014)

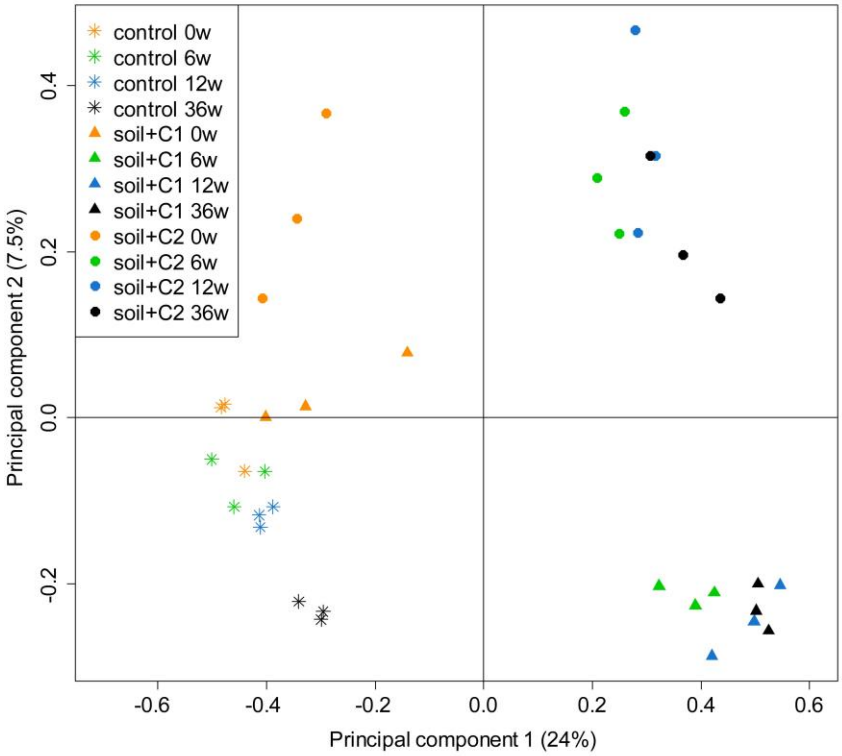

Figure 41: Changes in community composition of *alkB*-harboring bacteria during compost degradation. Principle component analysis based on a dissimilarity matrix of the most abundant OTUs. Data transformed according to Hellinger (Ramette, 2007). Only OTUs (>97% sequence identity) containing >10 sequences were considered (n = 3). The number behind the sample name indicates the incubation time in weeks (published in Wallisch et al. (2014))

3.4.2 Phylogenetic classification

For the phylogenetic classification of *alkB*-harboring soil bacteria, 3124 OTUs (97% sequence identity level) were built from high-quality sequencing data. With these OTUs, a phylogenetic analysis was performed using the ARB software package. The majority of the OTUs were assigned to *Actinobacteria* (30%), followed by γ-*Proteobacteria* (19%), *Bacilli* (17%), α-*Proteobacteria* (9%) and *Bacteroidetes* (1%) (Table 15). Thus, a large number of OTUs (47%) could be assigned to Gram-positive bacteria members of *Actinobacteria* and *Bacilli*. About 24% (749 OTUs) were assigned to uncultured bacteria. Whereas in the control soils the sequences assigned to Gram-positive *Actinobacteria* were at a stable low level, an increase was observed in soil amended with compost. In contrast to that, the highest numbers of sequences assigned to the Gram-positive *Bacilli* were detected at the beginning of the experiment throughout all samples. Sequences assigned to the Gram-negative *Bacteroidetes* were detected exclusively in soil amended with C2, but with a strong decrease over time. For soils amended with both compost types, an increase in sequences assigned to the Gram-negative α- and γ-*Proteobacteria* was measured, leading to a maximum after 6 weeks of incubation.

Table 15: Distribution of *alkB* gene fragments in all samples. Only fragments accounting for ≥ 1% of the phyla are listed (published in Wallisch et al. (2014))

		Actino-bacteria	*Bacilli*	*Bacteroi-detes*	*α-Proteo-bacteria*	*γ-Proteo-bacteria*	uncultured bacterium	Total
control	0 weeks	33 (1%)	117 (3%)			84 (3%)	100 (3%)	334 (10%)
	6 weeks	16 (1%)	87 (3%)			83 (3%)	79 (2%)	265 (9%)
	12 weeks	15 (1%)	57 (2%)			71 (2%)	52 (2%)	195 (7%)
	36 weeks	38 (1%)	28 (1%)		21 (1%)	29 (1%)	61 (2%)	177 (6%)
soil+C1	0 weeks	21 (1%)	53 (2%)			19 (1%)	50 (2%)	143 (6%)
	6 weeks	109 (3%)	17 (1%)		50 (2%)	63 (2%)	81 (3%)	320 (11%)
	12 weeks	153 (4%)			40 (1%)	66 (2%)	34 (1%)	293 (8%)
	36 weeks	139 (4%)			37 (1%)	24 (1%)	40 (1%)	240 (7%)
soil+C2	0 weeks	15 (1%)	117 (3%)	24 (1%)		48 (1%)	87 (3%)	267 (9%)
	6 weeks	123 (4%)	30 (2%)		35 (1%)	54 (2%)	65 (2%)	307 (11%)
	12 weeks	38 (1%)						38 (1%)
	36 weeks	232 (8%)			48 (2%)	39 (1%)	95 (3%)	414 (14%)
Total		932 (30%)	506 (17%)	24 (1%)	231 (8%)	580 (19%)	744 (24%)	3017 (99%)

Figure 42 illustrates the most abundant OTUs at the 97% sequence similarity level containing > 50 sequences, which reveals a high similarity of the replicates within one sample. 27 clusters could be defined using the tree-building software ARB (Figure 43). Detailed information about the distribution of the sequences in the phylogenetic tree is summarized in Table A7 and Table A8. Sequences detected in the control soil are present in 20 clusters, with low numbers of sequences assigned to the clusters B, E (*Rhodococcus* sp.), F (*Sagittula* sp.) and J (*Pseudomonas* sp.), which increased when

compost was added. The clusters D (*Shewanella sp.*), L (*Microscilla sp.*), Q, R (*Agrobacterium sp.*), S (*Hydrocarboniphaga sp.*), T and V (uncultured bacteria) contained sequences exclusively detected in soil amended with compost. 500 sequences in five clusters (P1, P2, P3, Q and T) grouped around cluster S, which contains the reference sequence of the alkane degrader *Hydrocarboniphaga sp..* This cluster consists of sequences from soil amended with young compost C2 exclusively. A database query of GenBank revealed amino acid sequence similarity (70-100%) to *Singularimonas variicoloris* (cluster P1), *Alcanivorax sp.* (cluster P2), uncultured bacterium (cluster P3) and *Agrobacterium sp.* (clusters Q and T). Whereas in clusters P1, P2 and P3 sequences from all soil treatments were detected, in the clusters Q and T it was only possible to find sequences from soil mixed with compost. A specific influence on the different compost types was only monitored in cluster L, which contains sequences only from soil samples amended with compost C2.

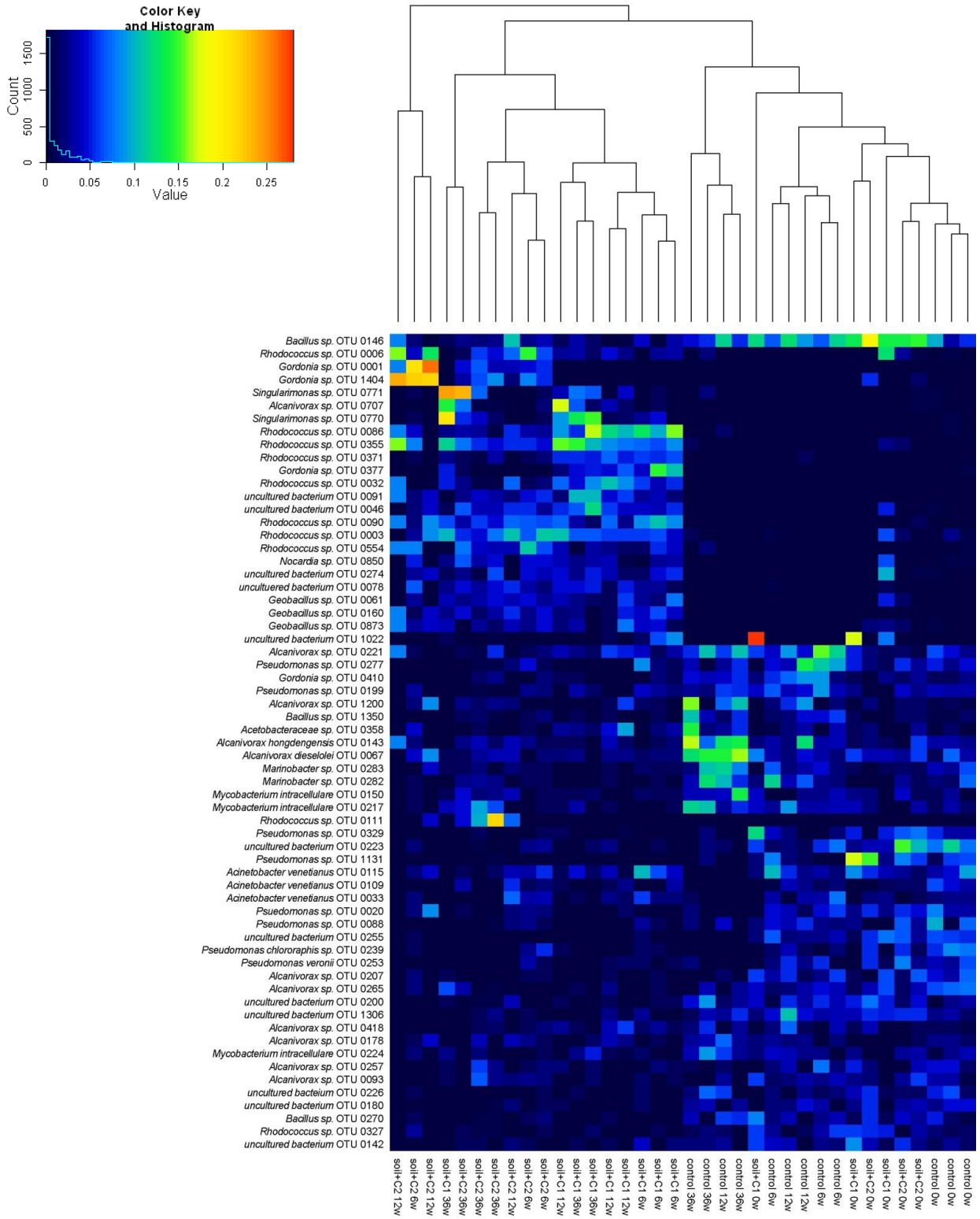

Figure 42: Distribution of *alkB*-harboring bacterial groups during compost degradation. Heatmap of OTUs based on 97% sequence identity level. The tree represents the similarity of the bacterial community composition in each sample, based on the abundance of each OTU containing > 50 sequences. Incubation time in weeks is given as number behind after each sample name. The according cluster of each OTU is specified in parentheses (published in Wallisch et al. (2014))

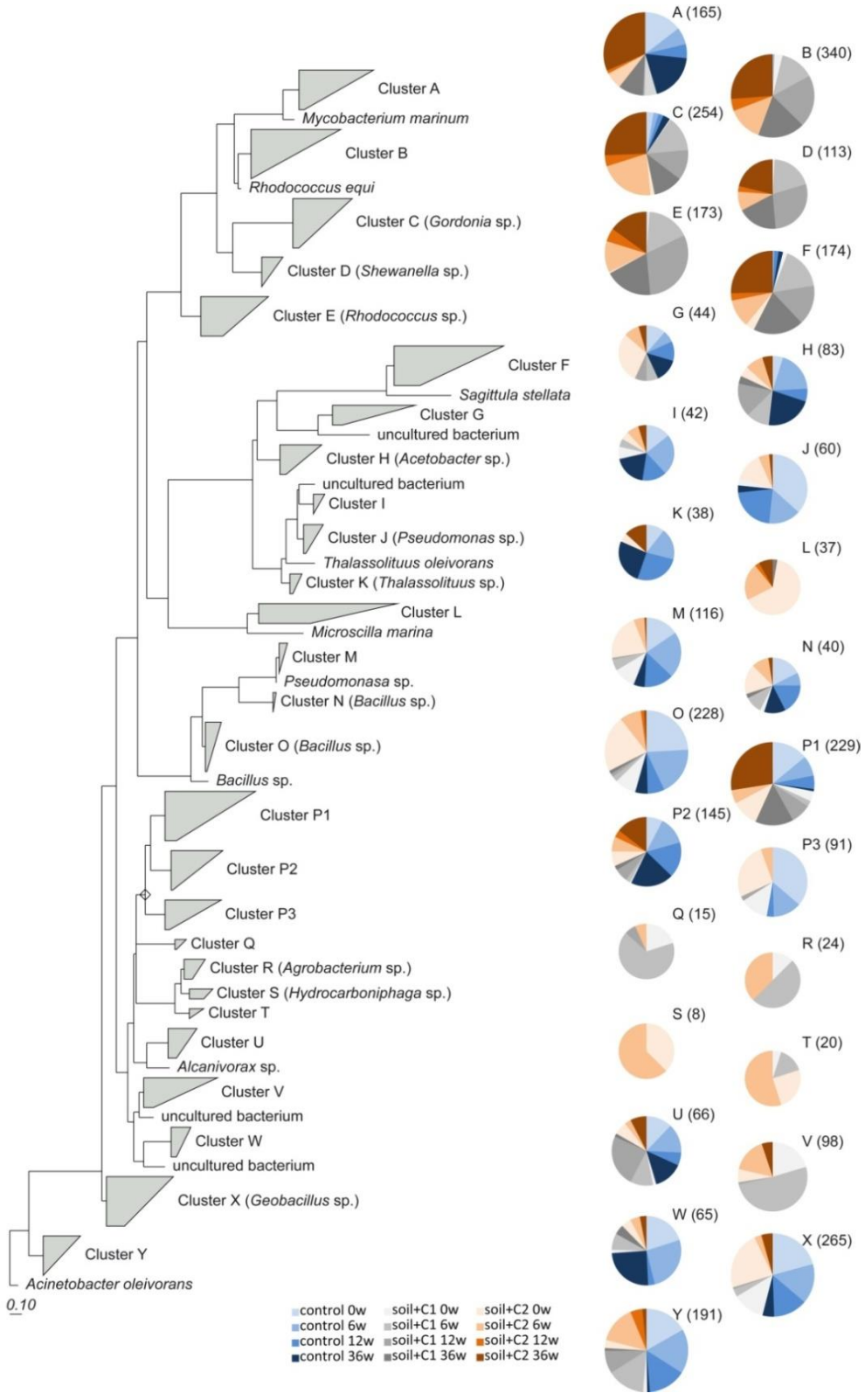

Figure 43: Phylogenetic tree including amplicon *alkB* gene fragments. Query sequences closly related to reference sequences were grouped into clusters (A-Y). For each cluster, the number of representative sequences of each sample is illustrated with pie charts. Total number of sequences (given in parentheses) is reflected by different size of the pie chart (published in Wallisch et al. (2014))

4 Discussion

The soil quality influences the primary production on a global level and greatly depends on soil ecosystem processes, which in turn are driven by a huge variety of organisms. Soil microorganisms contribute to a considerable extent to nutrient cycling in this habitat. Diversity analyses of microbial communities are the basis for understanding the ability of ecosystems to respond to changing environments. They also elucidate the need for conservation of the microbial gene pool and highlight the links between functional and physiological diversity, resilience, sustainability and ecosystem functioning (Prosser, 2002). In this study, molecular fingerprinting techniques and next generation sequencing were used to determine the influence of different litter and soil types on the bacterial community composition on *Z. mays* and *F. sylvatica* leaf material. Additionally, the input of organic compost to petroleum-contaminated Technosol on bacteria involved in alkane degradation was analyzed in order to determine whether there were any effects on bioremediation.

4.1 Comparison of methods for determining changes in bacterial community composition

Cultivation approaches for soil microbes detect only a small fraction of all microbes that actually colonize the soil in contrast to independent molecular methods based on the *16S rRNA* gene analysis, which reveals a much more heterogeneous microbial community in the soil (Wintzingerode et al., 1997; Janssen, 2006; Philippot et al., 2009). Molecular methods were used to determine bacterial community shifts on degrading leaf material in this study. The success of all molecular methods highly depends on the quality of the nucleic acid extraction performed in advance. Direct extraction of the metagenome from environmental samples allows a comprehensive insight into the biota of these habitats without the limitations of culture-dependent techniques that conceal the actual microbial coverage of the community members. Environmental experiments sometimes provide only very little sample material, and microbial composition and its activity in soil samples can vary to a high degree within very low distances in centimeter level (Torsvik and Øvreås, 2002; Trevors, 2010; Vos et al., 2013). Thus, an extraction method which allows the simultaneous extraction of both nucleic acid types out of one single sample should be preferred. Although every method is biased, there is at least the same bias for DNA and RNA. The protocol used for nucleic acid extraction from *Z. mays* leaf material was originally designed for soil samples (Töwe et al., 2011). In this study, the phenol-chloroform extraction with a subsequent column-based separation of DNA and RNA was successfully applied to plant leaf material with very good results. Compared to nucleic acid extracts of *F. sylvatica* samples that were extracted with a commercially available kit, the extracts of *Z. mays* samples had higher purity with regard to the extinction ratios $A_{260/280}$ and $A_{260/230}$. Humic substances

and other contaminants, which often remain in the extracts and impede PCR reactions, could be excluded to a high degree. This was not the case when using a commercially available extraction kit for *F. sylvatica* leaf material and the Technosol soil. The nucleic acid extracts of these samples lack purity, which may be due to co-extracted protein residues or to the fact that some chemicals (e.g. guanidine HCl) are part of the buffers supplied by the vendor. These substances adulterate the spectrophotometrical nucleic acid quantification (Pakpour, 2012). While molecular analyses based on DNA reflect the whole microbial potential, rRNA-based techniques are used to monitor the active microbial community in ecology. rRNA acts as a structural component of housekeeping catalysts such as ribosomes. Hence, the rRNA data of housekeeping genes can provide a general evidence of the relative expression of enzymes (Blazewicz et al., 2013).

However, similar community shifts of T-RFLP analysis based on DNA and cDNA extracted from *Z. mays* leaf material was seen in this study. This reflects the findings of Filion et al. (2009) and Sukenik et al. (2012), who also reported high numbers of ribosomes in dormant cells, which had also been considered during nucleic acid extraction. Additionally, highly active microorganisms with high reproductive cycles might result in similar patterns of DNA and cDNA. In order to compare microbial community changes over time, two fingerprinting methods, rep-PCR and T-RFLP, were applied in this study. While the rep-PCR approach targets the whole genome, T-RFLP focuses on a specific gene. Both methods are frequently used for rapid community analysis in soil ecology (Lynch et al., 2004; Jabłoński et al., 2011; Giebler et al., 2013a). Using the PCR conditions described by de Bruijn (1992), rep-PCR fingerprints amplify not only eubacterial but also bacteriophage, fungal or invertebrate template sequences. The low annealing temperature of 52°C, which was published by de Bruijn (1992), results in an amplification of a broad range of organisms. Nevertheless, rep-PCR is a powerful tool for reliably distinguishing between organisms and thus for determining community shifts (Gillings and Holley, 1997). On the DNA level, both methods stated different bacterial colonization on fresh leaves compared to litter samples of *Z. mays*. Regarding the leaf litter only, rep-PCR analysis points out a community shift after 8 weeks. In contrast to that, T-RFLP fingerprinting is more sensitive and provides a higher resolution. In this study, a significant influence caused by tillage field management was discovered after 2 weeks of leaf litter incubation in the soil, which was not seen using the rep-PCR method. The power of T-RFLP to detect both spatial and temporal heterogeneities in the structural composition of highly diverse communities such as those in environmental samples was also reported by Lukow et al. (2000). In concordance with the results of Lüdemann et al. (2000), between group analysis of the *16S rRNA* gene fragment T-RFLPs revealed very similar changes of the bacterial community composition for both of the nucleic acid types, the DNA and the cDNA. A closer look at the most dominant T-RFs indicates different key players. But it has to be taken into

consideration that one of the major disadvantages of T-RFLP fingerprinting is that a single T-RF could stand for more than one single organism, due to the fact that the sequence has the same length but differs in certain nucleotides.

Sequencing was used to overcome this tentativeness, and it allows a more detailed insight into the composition of the microbial communities by identifying single organisms that are involved. Because amplicon sequencing based on an initial PCR in this study, the bias of the PCR also has to be considered regarding the sequencing results. As a subsequent step, NGS is essentially based on the quality of the PCR products. Theoretically, the efficiency of amplification during PCR should be at the same level for each template. However, this is not the case. Variable amplification efficiencies were observed due to different accessibility and primer binding sites of the template sequence (e.g. caused by secondary structures), hybridization of the DNA or heterogeneity of the coding *16S rRNA* gene sequence (Suzuki and Giovannoni, 1996; Wintzingerode et al., 1997; Polz and Cavanaugh, 1998; Ishii and Fukui, 2001; Klindworth et al., 2012). Following the exponential gain of amplicons during PCR, the first cycles of this procedure are essential, as they provide enough template DNA for the following amplification cycles. Hence, NGS reveals the bias of the PCR performed beforehand.

Compared to traditional Sanger sequencing (Sanger et al., 1977), Kumar et al. (2011) showed that with NGS similar results are obtained by using primers which cover the region V7-V9 of the *16S rRNA* gene. The authors were also able to show that the Shannon index for bacterial diversity and the number of rare and abundant taxa was not affected. In many studies, analysis of the *16S rRNA* gene was used for different soils and sample types (LaMontagne et al., 2003; Sessitsch et al., 2004; Aneja et al., 2007; Rasche et al., 2011; Giebler et al., 2013a; Giebler et al., 2013b). An even more detailed insight into microbial community composition became possible using the next generation sequencing approach (Margulies et al., 2005). This bar-coded technique (Hamady et al., 2008) overcomes the financial and time-consuming limitations of clone library sequencing when using the method developed by Sanger. Amplicon pyrosequencing of *16S rRNA* gene fragments was used in many studies in order to gain a deeper insight into the composition of soil microbial communities (Roesch et al., 2007; Jones et al., 2009; Lauber et al., 2009; Kuffner et al., 2012; Davinic et al., 2012). However, differences in the variability of the variable regions of the *16S rRNA* gene have been previously reported, for example in *Pseudomonas* sp. (Bodilis et al., 2012). Within this genus, the hypervariable region V3 included up to 12 variations in the nucleic sequence of *16S rRNA* gene copies. An additional database analysis of *Pseudomonas* sp. full-length *16S rRNA* genes found the hypervariable regions V1 and V3-6 to be the most variable. Vasileiadis et al. (2012) demonstrated that environmental soil samples showed different variability within the hyper variable regions. The highest variability was seen for V1-3 and V6. The authors also reported relatively low variability of the hypervariable regions

V7 to V9. This observation is in accordance with Soergel et al. (2012), who concluded that for environmental samples no perfect primer set exists to cover all microorganisms with the same quality. Huse et al. (2008) analyzed the ambiguity of V3 and V6 within a reference database of *16S rRNA* gene sequences on different taxonomical levels. The authors came to the conclusion that for V6, on the genus level more than 2% of the sequences can still be assigned to two different genera. The primer set used in this work for amplicon fragment NGS covers the hypervariable regions V6 to V9. Including the higher variability of V6, the forward data set is therefore recommended to be used for classification. Precise taxonomic classification has been reported recently in the case of covering multiple hypervariable regions (Yarza et al., 2014). The more hypervariable regions are considered, the higher the microbial richness within one sample can be achieved. Yarza et al. (2014), for example, came to the conclusion that the first 750 nucleotides including V1 to V4 are necessary to sufficiently discriminate up to 90% of the total richness that is retained within the ranks of family, genus and species. In addition to the nucleic acid sequence, the secondary structure should also be considered for correct classification and phylogenetic ranking (Ludwig et al., 2004). In this work, only relatively short fragments (200 bp – ca. 600 bp) of the *16S rRNA* gene were considered for classification. Thus, OTUs based on a 97% sequence identity level cannot guarantee a reliable identification on species level in this case.

Here, a more general identification level such as genus level should be considered when OTUs of a 97% sequence identity level are used as input for classification. This is in accordance with the observations of Claesson et al. (2010), who analyzed the resolution that can be achieved by NGS. Whereas from phylum to family level, only moderate loss of information was apparent, on the genus level a strong decrease in correct classification was observed. For correct classification on the genus level, longer sequence fragments should be generated, e.g. by assembling the corresponding forward and reverse sequences. Nevertheless, many sequences were assigned to unclassified taxa which directly reflect the quality of the database. The total number of bacterial and archaeal species is estimated to be somewhere between 3×10^{4} and 1×10^{12}, and only ca. 11,000 of them have been classified so far (Yarza et al., 2014). Thus, with a rate of ca. 600 new descriptions per year, the quality of the sequence database repositories will increase over time, which will enhance classification in the future. However, an increased richness, diversity and evenness (with the exception of bacteria colonizing *F. sylvatica* embedded in Cutanic Luvisol) of bacteria colonizing leaf material was observed at sampling time points later in time, i.e. 8 weeks (*Z. mays*) and 30 weeks (*F. sylvatica*). This observation is supported by the findings of Dilly et al. (2004), who reported similar observations for bacteria during litter decomposition. As all data regarding the bacterial community on *Z. mays* is based on a small number of replicates (n = 3), it must be stated that statistical evidence concerning

significant differences have to be carefully interpreted. The importance of data analysis is also reflected by the loss of information regarding the indices for richness, diversity and evenness based on OTUs bundled at different sequence identity levels as performed exemplarily for bacteria colonizing *Z. mays* leaf material. The constant richness, diversity and evenness of bacterial community composition found on *F. sylvatica* incubated in Cutanic Luvisol can be explained the different chemical composition of the surrounding soil, which affects the degradation of leaf material (Hättenschwiler et al., 2005).

4.2 Bacterial key players involved in Z. mays leaf litter degradation

The decomposition of plant material can be described as a process of different steps. First, a rapid decrease of low-molecular substances such as sugars, amino acids and starch can be observed. This is followed by a slow degradation of high-molecular and recalcitrant substances such as phenolic derivatives (Horner et al., 1988). Therefore, the chemical composition of the substrate substantially impacts the degradation rates. According to that observation, in this study maximal amounts of soluble substances (glucose, sucrose, fructose, polysaccharides) were detected in fresh leaves, followed by only very small concentrations in the litter of *Z. mays*. On fresh leaves of *Z. mays*, sequences assigned to *Bifidobacteriaceae* were detected, which also were observed in cattle manure (Ventura et al., 2004). Additionally, bacterial families such as *Lactobacillacae* and *Kineosporiaceae*, which are typical for fresh leave material, were observed in this study. Members of these families were previously observed on fresh spinach leaves (Lopez-Velasco et al., 2011). The presence of sequences assigned to *Oxalobacteraceae, Flavobacteriaceae* and *Pseudomonadaceae*, besides many others which were found exclusively in litter samples supports the findings of Li et al. (2014) and thus emphasizes the importance of these genera as playing a relevant role in the soil along with the field cultivation and the turnover of organic matter. Carbon is stored temporarily in forms of starch in the chloroplasts and should result in relatively high amounts in the fresh leaves compared to litter (Jenner, 1982). This was not observed in the fresh maize leaves (ca. 0.4-1.5 mg glucose equivalents), which stands in contradiction to the observations of Fleischmann et al. (2009), who analyzed the decomposition of beech leaf litter containing ca. 10-40 mg of glucose equivalents. As with all present measurements based on leaves taken at the end of the vegetation period, this could explain the constant low amounts of starch here due to depletion or relocation in the maize-cobs and roots respectively.

Many bacteria typical for bulk soil and/or rhizosphere were identified for example members of *Bacillaceae, Microbacteriaceae, Paenibacillaceae, Enterobacteriaceae, Pseudomonadaceae* or *Xanthomonadaceae* (Liebich et al., 2007; Fierer et al., 2007; Acosta-Martínez et al., 2008; Inceoglu et

al., 2011). Members of the *Myxococcaceae* are seen as macropredators which are considered to be key players in the carbon biomass turnover in soil ecosystems, and which can be found in top soil layers (Reichenbach, 1999; Lüders et al., 2006; Zhou et al., 2014). With regard to the annual leaf material of *Z. mays*, the occurrence of some bacteria potentially involved in fungistasis, such as members of the families *Alcaligenaceae* and *Rhizobiaceae* (Zou et al., 2007), were identified as standing in positive correlation to members of *Alcaligenaceae* and *Aurantimonadaceae*. The latter seem to trigger each other, indicating that taxa involved in the soil nitrogen turnover such as *Hartmannibacter diazotrophicus* (Suarez et al., 2014) were pushed in this constellation. Also, some members of *Rhizobiaceae* are positively linked to some taxa of *Comamonadaceae*, which are involved in the soil carbon turnover as well as bioremediation of toxic and complex chemical compounds (Huang et al., 2013; Satola et al., 2013). Members of *Sphingomonadaceae* are also linked to bioremediation (Satola et al., 2013), and their occurrence also triggers members of the family *Bradyrhizobiaceae*, which for their part are involved in the soil nitrogen cycle. In *Z. mays*, members of the *Pseudomonadaceae*, which are involved in carbon and nitrogen cycle, and the *Enterobacteriaceae*, which contain numerous human and plant pathogens, trigger each other in a strong positive way. Some bacterial families involved in the nitrogen cycle were identified solely on annual leaf material, such as *Alcaligenaceae*, *Bacillaceae, Brucellaceae, Clostridiaceae, Paenibacillaceae* or *Streptomycetaceae*, supporting the findings of Bernhard (2010) who reported prokaryotes such as members of the genera *Pseudomonas*, *Alcaligenes*, *Clostridium* to be involved in nitrogen fixation as well as *Bacillus* to be involved in denitrification. Furthermore, in this study several bacterial families involved in the carbon cycle were identified in annual leaf material, such as *Beijerinckiaceae, Bifidobacteriaceae, Cellulomonadaceae, Clostridiaceae, Cytophagaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Myxococcaceae, Nannocystaceae, Patulibacteraceae, Planococcaceae, Porphyromonadaceae* and *Rhodocyclaceae*. Members of these bacteria are known to be involved in the carbon turnover, e.g. in utilizing methane (Dörr et al., 2010), possessing genes for breaking down complex carbohydrates (Eisenlord et al., 2013), participation in cellulolytic and/or saccharolytic processes (Goldfarb et al., 2011; Schellenberger et al., 2011; Felis and Pot, 2014; Schmidt et al., 2015) and degradation of charcoal (Khodadad et al., 2011; Ding et al., 2013) or phenanthrene (Regonne et al., 2013).

Over time, food web participants such as fungi degrade high molecular compounds to smaller, easily available and thus greatly demanded molecules such as sugars. The interaction of numerous organisms in the soil food web may lead to an increased diversity at later degradation stages, as a higher range of different nutrients is provided in the habitat. Fungal cell walls contain a considerable amount of glucosamine which can be seen as an indicator for *Eumycota*. The amino sugars muramic

acid and galactosamine are components of the bacterial cell wall and indicate the presence of Gram-positive bacteria and *Actinomycetes* respectively (Messner, 1997; Glaser et al., 2004; Jacobs et al., 2011). In the field trial with *Z. mays* leaf material, *Eumycota* show high abundance after 2 weeks of incubation with a steady decrease throughout all later sampling time points. The ratio of glucosamine/galactosamine constantly decreases for both tillage management types, indicating a higher proportion of *Actinobacteria* throughout the experiments.

4.3 Bacterial key players involved in F. sylvatica leaf litter degradation

In *F. sylvestris* leaf litter, *Neisseria, Holophagaceae* and *Planctomycetaceae* family members do have a positive effect on each other's occurrence. All of them are known to contain specific genera using carbon derivatives as nutrient source (Hao et al., 2011; Schellenberger et al., 2012; Schmidt et al., 2015). Furthermore, several families known for playing a role in the nitrogen turnover seem to be positively linked, such as the members assigned to *Sphingomonadaceae, Acetobacteraceae* and *Pseudomonadaceae*. In contrast, members of *Oxalobacteraceae* and *Xanthomonadaceae* seem to inhibit each other, which could indicate a reduction of plant pathogens assigned to *Xanthomonadaceae* (e.g. *Xanthomonas campestris*) in the presence of *Oxalobacteraceae*. On the other hand, family members of *Acidobacteria* Gp 1 and *Coxiellaceae* are positively correlated. This could imply that the occurrence of the latter, which are known for their human pathogenicity (e.g. *Coxiella burnetii*, which causes Q fever), could possibly be triggered by some acidobacterial taxa. Similarly, *Enterobacteria* including many human pathogens such as *E. coli* serotype O157:H7 are positively linked to *Armatimonadaceae*, some typical bacteria common in forest soil (Tamaki et al., 2011). *Verrucomicrobiaceae* and *Caulobacteraceae* are typical for the soil environment and strongly encourage the occurrence of each other, as they seem to have developed different niches for using the C and N as nutrient input. The former seem to simultaneously suppress the occurrence of certain α-*proteobacterial* members, which also play a role in the carbon and nitrogen turnover and thus compete with members of *Verrucomicrobiaceae*.

However, the occurrence of *Hyphomicrobiaceae* is positively linked to the α-*proteobacterial* members in this study. The occurrence of some bacteria of the *Pasteuriaceae* family is common for waste water. Slurry also greatly encourages the occurrence of some bacteria in the *Acidobacteria* Gp 10 family, which is linked to carbon and nitrogen turnover in soils. Furthermore, members of the *Pasteuriaceae* also trigger the occurrence of *Rhodospirillaceae*, known for their ability of nitrogen fixation, e.g. conducted by *Azospirillum* spp. or *Rhodospirillum* spp.. When analyzing the co-occurrence of these bacterial families, the physico-chemical properties such as soil moisture, micro-aggregates or temperature are also important as they influence the occurrence of single families.

Members of the bacterial families such as *Burkholderiaceae*, acidobacterial families (groups 1, 2 and 10) and α-*proteobacterial* families could be identified in *F. sylvatica* leaf litter only. This is in accordance with the findings of Landesman et al. (2014), who identified *Bradyrhizobiaceae*, *Burkholderiaceae*, *Hyphomicrobiaceae*, *Rhodospirillaceae* and *Spartobacteriaceae* amongst others as typical for forest soils. Furthermore, bacteria known for their participation in carbon turnover which are assigned to the families of *Acidobacteria, α-Proteobacteria, Holophagaceae, Neisseriaceae, Planctomycetaceae, Polyangiaceae* and *Sinobacteraceae* were identified in *F. sylvatica* leaf litter only. For example, Ward et al. (2009) analyzed the genome of different acidobacterial strains and detected similar genome-encoding cellulose synthesis genes. *Amantichitinum ursilacus* and *Chitinolyticbacter meiyuanensis* as members of the *Neisseriaceae* were recently described as chitin-degrading bacteria (Hao et al., 2011; Moss et al., 2013). Schmidt et al. (2015) revealed that *Holophagaceae* were connected to cellulose degradation. The contribution of *Planctomycetaceae*, *Polyangiaceae* and *Sinobacteraceae* to carbon turnover in soils was also reported recently (Schellenberger et al., 2012; Dallinger and Horn, 2014). The concentration of soluble sugars measured in leaf litter of *F. sylvatica* increased towards the later sampling time points.

It has to be considered that fungal hyphae were able to penetrate the meshes of the nylon litter bags and also have access to the leaf substrate. Common saprophytic soil fungi such as members of *Basidiomycota*, *Ascomycota* or *Deuteromycota* are known to be involved in these processes. They break up the bonds of more complex substances and thus release sugar molecules (Boer et al., 2005), which in turn can be used as nutrient input by the bacteria. Not only fungi, but also bacteria are be able to contribute to cutting down more complex molecules and thus providing easy degradable molecules such as sucrose and fructose (Brown and Chang, 2014) in later degradation stages. This leads to an increased diversity, richness and evenness as already demonstrated for bacterial community composition detected on litter embedded in Calcaric Regosol. The decreasing glucosamine/muramic acid ratios measured in leaf material of *F. sylvatica* revealed that Eumycota seem to be triggered after two weeks of incubation in Cutanic Luvisol with a subsequent constant decrease and thus a higher proportion of Gram-positive bacteria. In contrast to that, in leaf litter samples incubated in Calcaric Regosol soil the ratio of glucosamine/muramic acid is stable and shows a balanced microbial community of Eumycota and *Bacteria*.

4.4 Bacterial key players occurring independently from nutrient input source

In both litter types (*Z. mays* and *F. sylvatica*), common soil bacteria were identified which are known to play a role in nitrogen turnover in soils, such as members of *Acetobacteraceae, Bradyrhizobiaceae, Hyphomicrobiaceae, Opitutaceae, Oxalobacteraceae, Pseudomonadaceae, Rhizobiaceae* and *Rhodospirillaceae*. With regard to members of the families *Caulobacteraceae*, *Comamonadaceae*, *Opitutaceae* or *Xanthomonadaceae*, bacterial families known for their participation in carbon turnover in soils were also identified in both types of leaf material. For example, Li et al. (2009) list certain bacterial species such as *Caulobacter crescentus* or *Xanthomonas campestris* which produce proteins similar to fungal laccase. This leads to the assumption that soil bacteria are also able to degrade laccases. As soil represents a repository that also harbors pathogen microorganisms, it is not surprising that in this work families were identified known for causing infectious diseases in humans, plants and animals. In particular, family members of the *Enterobacteriaceae* comprise human pathogens such as *Salmonella typhii*, *Yersinia pestis* or *Shigella flexneri*. It was also possible to identify the bacterial families *Xanthomonadaceae* and *Pseudomonadaceae* in these studies. They are known to include plant pathogen strains such as *Erwinia carotovora* (Toth et al., 2006), *Xanthomonas oryzae* or *Pseudomonas syringae* (Mansfield et al., 2012). These Gram-negative bacteria are connected to soft rot disease and could prefer the leaf material as an optimal habitat. For example, *Salmonella* sp. prefers to colonize the spots on the plant leaves that have been infected prior to that by soft rot bacteria (Schikora et al., 2012).

4.5 Bacterial community composition in petroleum contaminated soils enriched with organic compost

With the input of plant material to soil, various microorganisms with special capabilities are applied to the soil habitat. These capabilities can also help to remediate contaminated soils. A wide range of soil and water derived-bacteria such as *Acinetobacter, Alcanivorax, Burkholderia, Mycobacterium, Pseudomonas, Rhodococcus* and others (van Beilen and Funhoff, 2007; Pérez-de-Mora et al., 2011) harbor *alkB*-related sequences. Many *alkB*-harboring bacteria such as *Mycobacterium* or *Nocardia* additionally carry genes (e.g. *almA*), which encode for an enzyme that is involved in the degradation of alkanes with a chain length of C_{32} and longer (Feng et al., 2007). Thus, *alkB* could serve as a marker for the degradation of alkanes with a chain length of between C_5 and C_{16} (van Beilen and Funhoff, 2005). Therefore, *alkB* can also be seen as a general indicator for alkane degradation in the environment (Rojo, 2009). Nevertheless, the number of *alkB* operons varies between different bacterial taxa. For example, for the genus *Rhodococcus* up to seven operons per cell have been

reported, whereas for other bacteria such as *Pseudomonas* the number of operons is restricted to one or two in a single organism (Heiss-Blanquet et al., 2005). This must be taken into account when interpreting the results regarding the abundance data. In order to test the potential of compost which consists of a mix of annual and perennial plant material serving as an inoculum for a petroleum-contaminated Technosol, the occurrence of alkane-degrading bacteria was monitored. In a microcosm experiment, the effects of the amendment of different compost types with contrasting maturation stages on the abundance and diversity of *alkB*-harboring bacteria were analyzed. The results of this experiment indicate that the measured effects strongly depend on the duration of the compost application to the soil. Data also indicates that rather less maturated compost, which contains high amounts of nutrients and plant-derived alkanes, has a pronounced effect on *alkB*-harboring soil bacteria. Compost amendments might provide adequate and sufficient substrate for growth stimulation of the microbes in the contaminated soil in order to degrade alkanes. This was seen with respect to the *Actinobacteria*, which became highly abundant after 6 weeks of compost inoculation regardless of the type of compost. Furthermore, no shift in bacterial diversity was detected, so that a stimulation of growth due to the amendment can be assumed hereby. Similar results were observed by Schulz et al. (2012), who measured a stimulation of the abundance of *alkB*-harboring bacterial communities in soil by litter material.

Furthermore, the nutrient amendments may stimulate the co-degradation of alkanes in soil and also change the sorption properties of alkanes to soil particles. Surfactants like Rhamnolipids are produced by several *Pseudomonas* species (e.g. *P. aeruginosa* or *P. fluorescens*), which enhance the uptake of hydrocarbons through emulsification due to decreasing the surface tension and forming micelles (Abouseoud et al., 2008; Kumar et al., 2008; Das and Chandran, 2011). Other bacterial taxa (mainly *Cytophaga*-related species) seem to be triggered only initially by the compost amendment, indicating that the microbes introduced by compost were not able to survive in this new soil environment. By contrast, other bacterial taxa, mainly assigned to proteobacteria, obviously successfully established themselves in the new soil environment. Sequencing of the *alkB* gene fragments revealed close relationship to common soil bacteria such as various species of *Agrobacterium, Bradyrhizobium, Pseudomonas* or *Acetobacter*. Some of the representative sequences are closely related to genera known for their appearance in environments rich in alkanes. For example, a number of sequences obtained from soil samples amended with compost clustered around the genus *Hydrocarboniphaga*, a member of the *δ-Proteobacteria*. This corresponds to other observations which had successfully linked the strains *Hydrocarboniphaga effusa* DSM 16095^T (Palleroni et al., 2004) and *H. daqingensis* NBRC 104238^T (Liu et al., 2011) to soil contaminated with heavy fuel oil hydrocarbons. These strains were also identified as being able to degrade alkanes with

medium chain lengths (C_9-C_{17}), which represent the major part of the alkanes quantified in the soil in this study. Furthermore, some representative sequences from soil with compost amendment were linked to the genus *Thalassolituus*. The strain *T. oleivorans* strain DSM 14913 (Yakimov et al., 2004) is linked to alkane degradation in marine environments and has recently been fully sequenced by Golyshin et al. (2013). *Alcanivorax spp., Acinetobacter spp., Acidisphaera spp., Gordonia spp., Rhodococcus spp., Nocardia spp. and Pseudomonas spp.* were also detected in this study, which have also been successfully extracted from coastal and marine sediment samples in the past (Shibata and Robert, 2009; Kuhn et al., 2009). A subset of the bacterial taxa containing *alkB* fragments were also present in original soil without any amendment, for example *Singularimonas variicoloris, Mycobacterium sp., Nocardia sp. Pseudomonas veronii* or *Acinetobacter venetianus*, for whom alkane degradation has been proven (Onaca et al., 2007; Friedrich and Lipski, 2008; Luckarift et al., 2011; Wang and Shao, 2013). However, the amendment of petroleum-contaminated soils with compost showed a pronounced effect on the abundance and diversity of *alkB*-harboring bacteria. Further studies have to prove that the inoculum will also result in enhanced stimulation of alkane degraders based on mRNA analyzes.

5 Conclusion and outlook

Organic matter input into the soil provides a mixture of nutrients including all the intermediate stages of bioavailability throughout degradation for the microorganisms involved. The analysis of the microbial key players involved in litter decomposition revealed that bacterial communities are strongly adapted to their specific soil environment and to the substrate quality provided by the litter. Different community compositions were seen for forest and agricultural soil habitats, which leads to the conclusion that the native soil environment has a considerable influence on the colonization. Bacterial specialists were identified for colonizers of exclusively fresh *Z. mays* leaf material, e.g. members of *Bifidobacteriaceae*, *Lactobacillaceae* or *Kineosporiaceae*. In contrast to that, bacteria such as *Alcaligenaceae*, *Bacillaceae*, and *Streptomycetaceae* were detected, which are found exclusively colonizing the litter embedded in arable soil. On the more recalcitrant leaf material of *F. sylvatica* it was possible to detect bacterial specialists such as *Bradyrhizobiaceae*, *Burkholderiaceae* or *Spartobacteriaceae*, which are typical for forest soil. Apart from that, bacterial generalists were identified which belong to the *Proteobacteria*, *Burkholderiales* and *Verrucomicrobia*. Examples also include *Oxalobacteraceae*, *Caulobacteraceae*, *Opitutaceae* or *Xanthomonadaceae*, which have been demonstrated to be involved in the soil nitrogen or carbon cycle. In addition, the microbial community composition also changes over time, leading to higher diversity and richness due to the complex soil food web. The participants in this food web, including fungi as well as higher organisms, metabolize high molecular components such as lignin or cellulose to small and easily degradable derivatives, such as sugars and amino acids, which in turn can be metabolized by the bacteria. In these processes, the bacterial community correlates with the chemical composition of the organic matter serving as nutrient input. On both litter types bacterial families were identified whose members are known for their participation in bioremediation processes, including families containing genera such as *Alcanivorax*, *Mycobacterium* or *Hydrocarboniphaga*. This supports the findings that compost enhances bacterial taxa using alkanes as a substrate and thus contributing to the remediation of petroleum-contaminated soils. As bacterial taxa which could cause human and/or plant diseases were also identified, the infection potential of this treatment should also be considered. Further insights into the dynamics of the organic matter degradation are necessary to identify the active key players. It might be possible to reveal the different pathways of degradation together with a detailed chemical analysis of the plant material substrate. Therefore, the sequencing of the metagenome or specific functional genes involved in the carbon and nitrogen cycle would also be helpful in enlightening the role of specific taxa and their role in the nutrient cycles. Further analysis of active key players linked to transcriptome analyses might be able to enlighten specific details of nutrient cycling in the soil habitat.

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8 Appendix

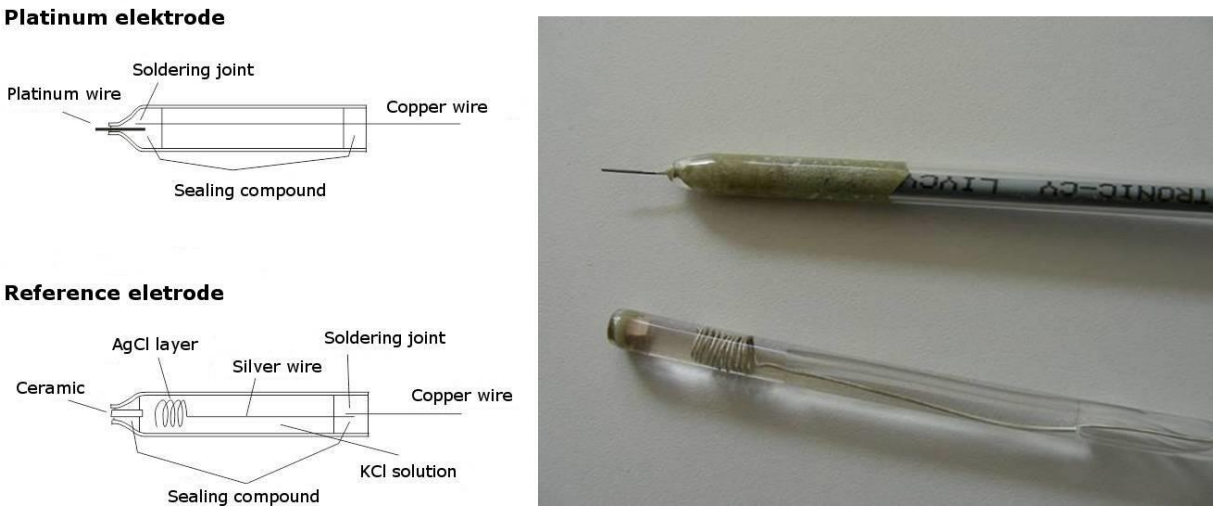

Figure A1: Eh sensor sensor pair for redox measurements. Schematic set-up (left) and picture (right) of the platinum electrode (top) and reference electrode (bottom)

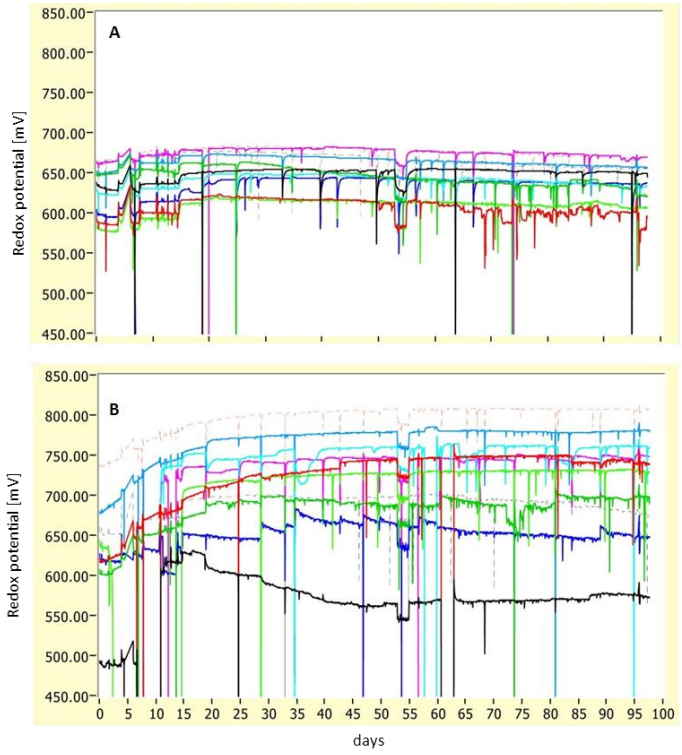

Figure A2: Redox smeasurements of the microcosms filled with Calcaric Regosol (A) and Cutanic Luvisol (B)

Table A1: Amounts of sugars, amino sugars and starch measured in leaf material of *Z. mays* embedded in agricultural soil. Starch is given as glucose equivalents g^{-1} dw^{-1}, sugars and amino sugars in mg g^{-1} dw^{-1}. Mean ± standard deviation are shown (n = 3). CT = conventional tillage. MT = minimal tillage. Different letters (upper case: MT; lower case: CT) indicate statistically significant difference between averages according to Tukey multiple comparison test (p-value ≤ 0.05)

	fresh leaves	leaf litter					
		2 weeks		4 weeks		8 weeks	
		CT	MT	CT	MT	CT	MT
Sum of soluble sugars	98.86±15.9^{aA}	23.86±4.52^{b}	24.16±1.20^{B}	20.3±2.40^{b}	20.50±1.68^{B}	18.43±3.35^{b}	23.30±3.00^{B}
Tetrasaccharides	b.d.l	10.76±0.30^{a}	11.06±1.81^{A}	7.3±0.26^{b}	5.76±0.86^{B}	8.03±0.57^{b}	6.90±0.55^{B}
Trisaccharides	34.00±0.75^{aA}	3.40±0.40^{b}	3.8±1.05^{B}	4.00±1.74^{b}	5.10±0.86^{B}	4.83±1.11^{b}	3.06±0.11^{B}
Sucrose	35.86±4.52^{aA}	10.00±1.13^{b}	9.00±1.9^{B}	9.20±0.29^{b}	8.16±1.95^{B}	8.13±1.77^{b}	6.90±2.87^{B}
Glucose	17.00±8.34^{aA}	b.d.l.	b.d.l.	b.d.l.	1.26±1.09^{B}	2.30±1.99^{b}	1.56±1.42^{B}
Fructose	12.00±4.00	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Starch	0.46±0.15^{aA}	0.56±0.05^{a}	0.41±0.16^{A}	0.91±0.5^{a}	0.50±0.18^{A}	0.93±0.92^{a}	1.55±0.56^{A}
Glucosamine	3.74±0.31^{aA}	16.31±0.86^{b}	17.65±2.31^{B}	14.2±3.01^{b}	15.64±0.99^{B}	12.54±1.14^{b}	14.96±0.68^{B}
Galactosamine	b.d.l.	1.01±0.18^{a}	0.94±0.20^{A}	0.97±0.16^{a}	1.18±0.14^{A}	1.44±0.12^{b}	2.09±0.43^{B}
Muramic acid	b.d.l	0.30±0.01^{a}	0.26±0.05^{A}	0.27±0.06^{a}	0.33±0.02^{A}	0.28±0.08^{b}	0.33±0.00^{B}

Table A2: Amounts of sugars, aminosugars and starch measured in leaf material of *F. sylvatica* embedded in two differents soils (Cutanic Luvisol, Calcaric Regosol). Starch is given as glucose equivalents g^{-1} dw^{-1}, sugars and amino sugars are given as mg g^{-1} dw^{-1}. Mean and ± standard deviation are shown (n = 5). Different letters indicate statistically significant difference between averages according to Tukey multiple comparison test (p-value ≤ 0.05)

	1 week	2 weeks	4 weeks	8 weeks	16 weeks	30 weeks
Cutanic Luvisol						
Sum of soluble sugars	3.95 ± 0.37^{a}	3.89 ± 4.51^{a}	4.51 ± 0.09^{a}	5.98 ± 0.16^{b}	8.30 ± 0.27^{c}	11.93 ± 0.25^{d}
Tetrasaccharides	0.12 ± 0.07^{a}	0.22 ± 0.04^{b}	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Trisaccharides	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Sucrose	0.06 ± 0.06^{a}	0.18 ± 0.4^{ab}	0.40 ± 0.030^{cd}	0.42 ± 0.04^{cd}	0.54 ± 0.06^{c}	0.32 ± 0.03^{bc}
Glucose	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Fructose	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Starch	0.42 ± 0.11^{a}	0.73 ± 0.63^{a}	0.63 ± 0.01^{a}	0.73 ± 0.26^{a}	0.88 ± 0.05^{a}	0.58 ± 0.08^{a}
Glucosamine	2.27 ± 0.13^{a}	2.67 ± 2.94^{ab}	2.94 ± 0.14^{bc}	3.62 ± 0.15^{bc}	3.60 ± 0.18^{bc}	3.76 ± 0.29^{c}
Galactosamine	0.22 ± 0.01^{a}	0.64 ± 1.30^{ab}	1.30 ± 0.04^{b}	2.17 ± 0.09^{c}	2.95 ± 0.18^{d}	3.70 ± 0.37^{d}
Muramic acid	0.01 ± 0.00^{a}	0.01 ± 0.01^{a}	0.01 ± 0.00^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.03 ± 0.00^{c}
Calcaric Regosol						
Sum of soluble sugars	3.79 ± 0.47^{A}	4.23 ± 3.98^{A}	3.98 ± 0.19^{A}	4.81 ± 0.11^{A}	6.33 ± 0.14^{B}	9.19 ± 0.43^{C}
Tetrasaccharides	0.14 ± 0.08^{A}	0.10 ± 0.04^{A}	0.04 ± 0.04^{B}	0.10 ± 0.05^{A}	0.04 ± 0.04^{B}	0.16 ± 0.10^{A}
Trisaccharides	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Sucrose	b.d.l.	0.32 ± 0.18^{AB}	0.18 ± 0.08^{A}	0.47 ± 0.02^{BC}	0.60 ± 0.03^{C}	0.58 ± 0.04^{C}
Glucose	b.d.l.	b.d.l.	0.14 ± 0.14	b.d.l.	b.d.l.	b.d.l.
Gructose	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Starch	0.51 ± 0.04^{A}	0.59 ± 0.49^{A}	0.49 ± 0.04^{A}	0.38 ± 0.10^{A}	0.60 ± 0.15^{A}	0.35 ± 0.14^{A}
Glucosamine	2.22 ± 0.12^{AB}	1.55 ± 2.52^{A}	2.52 ± 0.47^{AB}	4.05 ± 0.40^{AB}	4.39 ± 0.49^{AB}	4.12 ± 0.35^{B}
Galactosamine	0.27 ± 0.00^{A}	0.39 ± 1.86^{A}	1.86 ± 0.85^{AB}	1.51 ± 0.15^{AB}	1.86 ± 0.26^{AB}	2.57 ± 0.54^{B}
Muramic acid	0.02 ± 0.01^{AB}	0.01 ± 0.02^{A}	0.02 ± 0.01^{AB}	0.03 ± 0.01^{AB}	0.04 ± 0.01^{B}	0.04 ± 0.01^{B}

Table A3: Average total alkane concentrations with standard errors (n = 3). Different letters indicate statistically significant difference between averages according to Tukey multiple comparison test (p-value ≤ 0.05) (published in Wallisch et al. (2014))

Treatment	Time	Conc. mg/kg
Soil	0 weeks	854 ± 19^{a}
	12 weeks	642 ± 32^{b}
Soil + C1	0 weeks	854 ± 17^{a}
	12 weeks	537 ± 24^{c}
Soil + C2	0 weeks	795 ± 15^{a}
	12 weeks	502 ± 23^{c}

Table A4: Most abundant T-RFs of *16S rRNA* gene fragments detected in leaf material of *Z. mays* incubated in arable soil. T-RFs representing <5% of the total relative community richness are summed up as "others"

		DNA				cDNA			
Leaf material	Treat-ment	T-RF [bp]	Total relative community richness			T-RF [bp]	Total relative community richness		
0 weeks	fresh	342	22%	±	0,037	112	17%	±	0,030
		112	21%	±	0,028	117	12%	±	0,023
		114	10%	±	0,018	114	11%	±	0,020
		117	7%	±	0,016	124	6%	±	0,027
		457	7%	±	0,038	342	5%	±	0,019
		others	33%	±	0,008	others	49%	±	0,010
2 weeks	CT	454	15%	±	0,051	87	12%	±	0,031
		87	12%	±	0,020	363	9%	±	0,026
		112	11%	±	0,040	399	8%	±	0,040
		417	10%	±	0,050	165	7%	±	0,029
		367	9%	±	0,030	454	6%	±	0,044
		165	8%	±	0,014	452	6%	±	0,012
		363	5%	±	0,012	others	52%	±	0,011
		others	30%	±	0,009				
	MT	367	13%	±	0,014	399	13%	±	0,012
		87	11%	±	0,011	87	10%	±	0,025
		454	9%	±	0,015	363	9%	±	0,008
		399	8%	±	0,007	367	6%	±	0,016
		363	8%	±	0,015	452	6%	±	0,038
		165	7%	±	0,046	others	56%	±	0,010
		452	6%	±	0,073				
		428	6%	±	0,039				
		417	5%	±	0,013				
		others	27%	±	0,010				
4 weeks	CT	399	17%	±	0,019	399	22%	±	0,015
		367	15%	±	0,012	363	7%	±	0,010
		428	11%	±	0,055	428	6%	±	0,027
		417	7%	±	0,064	87	6%	±	0,013
		452	6%	±	0,056	452	6%	±	0,014
		87	5%	±	0,008	367	5%	±	0,016
		363	5%	±	0,005	417	5%	±	0,054
		others	34%	±	0,009	others	43%	±	0,009
	MT	399	16%	±	0,037	399	20%	±	0,050
		367	15%	±	0,021	363	7%	±	0,006
		363	7%	±	0,011	505	6%	±	0,043
		402	7%	±	0,023	87	5%	±	0,015
		454	7%	±	0,021	367	5%	±	0,013
		87	5%	±	0,020	others	57%	±	0,011
		428	5%	±	0,031				
		others	38%	±	0,010				

Table A4 (continued): Most abundant T-RFs of *16S rRNA* gene fragments detected in leaf material of *Z. mays* incubated in arable soil. T-RFs representing <5% of the total relative community richness are summed up as "others"

		DNA				cDNA			
Leaf material	Treat-ment	T-RF [bp]	Total relative community richness			T-RF [bp]	Total relative community richness		
8 weeks	CT	399	22%	±	0,004	399	21%	±	0,015
		112	11%	±	0,020	452	9%	±	0,021
		452	9%	±	0,018	112	6%	±	0,019
		367	8%	±	0,011	127	5%	±	0,047
		402	6%	±	0,016	505	5%	±	0,011
		others	44%	±	0,009	others	54%	±	0,011
	MT	399	25%	±	0,040	399	24%	±	0,056
		452	12%	±	0,124	452	8%	±	0,026
		112	11%	±	0,017	122	6%	±	0,037
		367	7%	±	0,008	112	5%	±	0,009
		402	7%	±	0,025	others	57%	±	0,012
		others	38%	±	0,010				

Table A5: Bacterial families on *Z. mays* leaf litter. OTUs (90% sequence identity) assigned on family level were included into a phylogenetic tree calculated with ARB. Percentages are given in parentheses. CT: conventional tillage, MT: minimal tillage

	0 weeks	2 weeks		4 weeks		8 weeks		
	fresh	CT	MT	CT	MT	CT	MT	Total
Acetobacteraceae		2 (0.14%)	1 (0.07%)	2 (0.14%)	1 (0.07%)			6 (0.42%)
Acholeplasmataceae							1 (0.07%)	1 (0.07%)
Alcaligenaceae		2 (0.14%)	18 (1.27%)	7 (0.50%)	7 (0.50%)	4 (0.28%)	4 (0.28%)	42 (2.97%)
Aurantimonadaceae	3 (0.21%)	7 (0.50%)	7 (0.50%)	2 (0.14%)	2 (0.14%)		1 (0.07%)	22 (1.56%)
Bacillaceae			1 (0.07%)		1 (0.07%)	1 (0.07%)		3 (0.21%)
Beijerinckiaceae		3 (0.21%)	2 (0.14%)	3 (0.21%)	1 (0.07%)	2 (0.14%)	7 (0.50%)	18 (1.27%)
Bifidobacteriaceae	4 (0.28%)							4 (0.28%)
Bradyrhizobiaceae				1 (0.07%)	2 (0.14%)	5 (0.35%)	6 (0.42%)	14 (0.99%)
Brucellaceae		1 (0.07%)	2 (0.14%)	2 (0.14%)	8 (0.57%)		1 (0.07%)	14 (0.99%)
Caldicoprobacteraceae							1 (0.07%)	1 (0.07%)
Caulobacteraceae	4 (0.28%)	11 (0.78%)	11 (0.78%)	15 (1.06%)	20 (1.41%)	12 (0.85%)	18 (1.27%)	91 (6.44%)
Cellulomonadaceae				2 (0.14%)				2 (0.14%)
Clostridiaceae	8 (0.57%)	2 (0.14%)	1 (0.07%)	4 (0.28%)		2 (0.14%)	7 (0.50%)	24 (1.70%)
Comamonadaceae	4 (0.28%)	33 (2.33%)	50 (3.54%)	43 (3.04%)	34 (2.40%)	62 (4.38%)	59 (4.17%)	285 (20.16%)
Cryptosporangiaceae							1 (0.07%)	1 (0.07%)
Cytophagaceae	7 (0.50%)			1 (0.07%)		1 (0.07%)	2 (0.14%)	11 (0.78%)
Deinococcaceae	1 (0.07%)							1 (0.07%)
Enterobacteriaceae		12 (0.85%)	9 (0.64%)	1 (0.07%)	2 (0.14%)			24 (1.70%)
Erysipelotrichaceae				5 (0.35%)	2 (0.14%)	1 (0.07%)		8 (0.57%)
Erythrobacteraceae				3 (0.21%)	2 (0.14%)	2 (0.14%)		7 (0.50%)
Family incertae sedis Candidatus Microthrix							1 (0.07%)	1 (0.07%)
Family XI incertae sedis Sedimentibacter				1 (0.07%)			2 (0.14%)	3 (0.21%)
Flavobacteriaceae		1 (0.07%)	1 (0.07%)					2 (0.14%)
Halanaerobiaceae				2 (0.14%)				2 (0.14%)
Hyphomicrobiaceae		2 (0.14%)	9 (0.64%)	15 (1.06%)	17 (1.20%)	27 (1.91%)	35 (2.48%)	105 (7.43%)
Kineosporiaceae	1 (0.07%)	2 (0.14%)						3 (0.21%)
Lachnospiraceae					1 (0.07%)			1 (0.07%)
Lactobacillaceae	6 (0.42%)							6 (0.42%)
Methylobacteriaceae	9 (0.64%)	3 (0.21%)	2 (0.14%)	4 (0.28%)	1 (0.07%)		7 (0.50%)	26 (1.84%)
Microbacteriaceae	1 (0.07%)	13 (0.92%)	6 (0.42%)	11 (0.78%)	6 (0.42%)	4 (0.28%)	11 (0.78%)	52 (3.68%)
Moraxellaceae						2 (0.14%)		2 (0.14%)
Mycobacteriaceae						1 (0.07%)		1 (0.07%)
Myxococcaceae							2 (0.14%)	2 (0.14%)
Nannocystaceae						1 (0.07%)		1 (0.07%)
Opitutaceae							1 (0.07%)	1 (0.07%)
Oxalobacteraceae		18 (1.27%)	15 (1.06%)	7 (0.50%)	16 (1.13%)	14 (0.99%)	13 (0.92%)	83 (5.87%)

Table A5 (continued): Bacterial families on *Z. mays* leaf litter. OTUs (90% sequence identity) assigned on family level were included into a phylogenetic tree calculated with ARB. Percentages are given in parentheses. CT = conventional tillage, MT = minimal tillage

	0 weeks	2 weeks		4 weeks		8 weeks		
	fresh	CT	MT	CT	MT	CT	MT	Total
Paenibacillaceae		3 (0.21%)	4 (0.28%)	3 (0.21%)	3 (0.21%)	2 (0.14%)	3 (0.21%)	18 (1.27%)
Patulibacteraceae						1 (0.07%)		1 (0.07%)
Phyllobacteriaceae						2 (0.14%)	1 (0.07%)	3 (0.21%)
Planococcaceae			1 (0.07%)		1 (0.07%)			2 (0.14%)
Porphyromonadaceae	1 (0.07%)							1 (0.07%)
Pseudomonadaceae		11 (0.78%)	3 (0.21%)		3 (0.21%)			17 (1.20%)
Rhizobiaceae	1 (0.07%)	28 (1.98%)	33 (2.33%)	21 (1.49%)	26 (1.84%)	29 (2.05%)	27 (1.91%)	165 (11.67%)
Rhodocyclaceae			1 (0.07%)			3 (0.21%)		4 (0.28%)
Rhodospirillaceae						2 (0.14%)	1 (0.07%)	3 (0.21%)
Ruminococcaceae	3 (0.21%)	1 (0.07%)	2 (0.14%)	2 (0.14%)	3 (0.21%)			11 (0.78%)
Sanguibacteraceae			1 (0.07%)			1 (0.07%)		2 (0.14%)
Sphingomonadaceae	8 (0.57%)	22 (1.56%)	14 (0.99%)	23 (1.63%)	19 (1.34%)	38 (2.69%)	34 (2.40%)	158 (11.17%)
Streptomycetaceae				1 (0.07%)				1 (0.07%)
Synergistaceae							1 (0.07%)	1 (0.07%)
Syntrophomonadaceae			1 (0.07%)	4 (0.28%)	2 (0.14%)	1 (0.07%)	4 (0.28%)	12 (0.85%)
uncultured		1 (0.07%)					4 (0.28%)	5 (0.35%)
Verrucomicrobiaceae				5 (0.35%)	9 (0.64%)	4 (0.28%)	4 (0.28%)	22 (1.56%)
Xanthomonadaceae	2 (0.14%)	18 (1.27%)	17 (1.20%)	20 (1.41%)	17 (1.20%)	23 (1.63%)	8 (0.57%)	105 (7.43%)
Identified above family level	1 (0.07%)		1 (0.07%)	2 (0.14%)	2 (0.14%)	3 (0.21%)	4 (0.28%)	13 (0.92%)
Total	64 (4.53%)	196 (13.86%)	213 (15.06%)	212 (14.99%)	208 (14.71%)	250 (17.68%)	271 (19.17%)	1414 (100.00 %)

Table A6: Bacterial families on *F. sylvatica* leaf litter. Families with <6 OTUs (90% sequencence identity) were ignored. Absolute numbers and percentages are given (published in Wallisch et al. (2014))

	Cutanic Luvisol						Calcaric Regosol						
Family	1 week	2 weeks	4 weeks	8 weeks	16 weeks	30 weeks	1 week	2 weeks	4 weeks	8 weeks	16 weeks	30 weeks	Sum
Acetobacteraceae	204 (0,20%)	72 (0,07%)	63 (0,06%)	77 (0,08%)	40 (0,04%)	36 (0,04%)	200 (0,20%)	81 (0,08%)	35 (0,03%)	11 (0,01%)			819 (0,81%)
Acidobacteria Gp1 family incertae sedis	81 (0,08%)	29 (0,03%)	43 (0,04%)	50 (0,05%)	65 (0,06%)	176 (0,17%)	102 (0,10%)	86 (0,09%)	29 (0,03%)				661 (0,66%)
Acidobacteria Gp10 family incertae sedis											11 (0,01%)	33 (0,03%)	44 (0,04%)
Acidobacteria Gp2 family incertae sedis	7 (0,01%)												7 (0,01%)
α-Proteobacteria family incertae sedis						16 (0,02%)							16 (0,02%)
Armatimonadaceae						7 (0,01%)	10 (0,01%)						17 (0,02%)
Bradyrhizobiaceae		7 (0,01%)	36 (0,04%)	63 (0,06%)	136 (0,13%)	179 (0,18%)		8 (0,01%)	73 (0,07%)	130 (0,13%)	199 (0,20%)	187 (0,19%)	1018 (1,01%)
Burkholderiaceae	665 (0,66%)	950 (0,94%)	820 (0,81%)	741 (0,73%)	451 (0,45%)	420 (0,42%)	8 (0,01%)	16 (0,02%)	19 (0,02%)	39 (0,04%)	40 (0,04%)	26 (0,03%)	4195 (4,16%)
Caulobacteraceae	17 (0,02%)	87 (0,09%)	212 (0,21%)	248 (0,25%)	377 (0,37%)	325 (0,32%)	28 (0,03%)	54 (0,05%)	201 (0,20%)	240 (0,24%)	259 (0,26%)	312 (0,31%)	2360 (2,34%)
Comamonadaceae	76 (0,08%)	47 (0,05%)	49 (0,05%)	63 (0,06%)	106 (0,11%)	158 (0,16%)	260 (0,26%)	414 (0,41%)	1225 (1,21%)	1386 (1,37%)	2007 (1,99%)	1640 (1,63%)	7431 (7,36%)
Coxiellaceae					19 (0,02%)	40 (0,04%)					10 (0,01%)	25 (0,02%)	94 (0,09%)
Deinococcaceae	15 (0,01%)						23 (0,02%)	7 (0,01%)					45 (0,04%)
Enterobacteriaceae								7 (0,01%)					7 (0,01%)
Holophagaceae		7 (0,01%)		9 (0,01%)	13 (0,01%)	25 (0,02%)							54 (0,05%)
Hyphamicrobiaceae				18 (0,02%)	72 (0,07%)	151 (0,15%)		12 (0,01%)	29 (0,03%)	30 (0,03%)	56 (0,06%)	129 (0,13%)	497 (0,49%)
Methylobacteriaceae	128 (0,13%)	66 (0,07%)	66 (0,07%)	48 (0,05%)	28 (0,03%)	11 (0,01%)	209 (0,21%)	118 (0,12%)	175 (0,17%)	131 (0,13%)	61 (0,06%)	36 (0,04%)	1077 (1,07%)
Methylophilaceae	10 (0,01%)	38 (0,04%)	141 (0,14%)	71 (0,07%)	81 (0,08%)	98 (0,10%)	17 (0,02%)	50 (0,05%)	185 (0,18%)	160 (0,16%)	211 (0,21%)	207 (0,21%)	1269 (1,26%)
Neisseriaceae	9 (0,01%)	17 (0,02%)	12 (0,01%)	17 (0,02%)									55 (0,05%)

Table A6 (continued): Bacterial families on *F. sylvatica* leaf litter. Families with <6 OTUs (90% sequencence identity) were ignored. Absolute numbers and percentages are given (published in Wallisch et al. (2014))

	Cutanic Luvisol						Calcaric Regosol						
Family	1 week	2 weeks	4 weeks	8 weeks	16 weeks	30 weeks	1 week	2 weeks	4 weeks	8 weeks	16 weeks	30 weeks	Sum
Opitutaceae					11 (0,01%)				9 (0,01%)	23 (0,02%)	59 (0,06%)	42 (0,04%)	144 (0,14%)
Oxalobacteraceae	3574 (3,54%)	2344 (2,32%)	1270 (1,26%)	865 (0,86%)	812 (0,80%)	529 (0,52%)	5443 (5,39%)	5077 (5,03%)	2899 (2,87%)	1274 (1,26%)	718 (0,71%)	114 (0,11%)	24919 (24,70%)
Pasteuriaceae												17 (0,02%)	17 (0,02%)
Planctomycetaceae		12 (0,01%)	16 (0,02%)	27 (0,03%)	72 (0,07%)	126 (0,12%)			11 (0,01%)	42 (0,04%)	48 (0,05%)	92 (0,09%)	446 (0,44%)
Polyangiaceae									9 (0,01%)	12 (0,01%)	11 (0,01%)	13 (0,01%)	45 (0,04%)
Pseudomonadaceae	42 (0,04%)	21 (0,02%)					56 (0,06%)	28 (0,03%)	24 (0,02%)	12 (0,01%)			183 (0,18%)
Rhizobiaceae		12 (0,01%)	20 (0,02%)	21 (0,02%)	47 (0,05%)	24 (0,02%)		24 (0,02%)	110 (0,11%)	157 (0,16%)	127 (0,13%)	150 (0,15%)	692 (0,69%)
Rhodospirillaceae			13 (0,01%)		21 (0,02%)	45 (0,04%)				9 (0,01%)	22 (0,02%)	134 (0,13%)	244 (0,24%)
Sinobacteraceae		8 (0,01%)	24 (0,02%)	15 (0,01%)					10 (0,01%)				57 (0,06%)
Spartobacteria family incertae sedis												7 (0,01%)	7 (0,01%)
Sphingomonadaceae	541 (0,54%)	178 (0,18%)	167 (0,17%)	264 (0,26%)	228 (0,23%)	212 (0,21%)	672 (0,67%)	532 (0,53%)	472 (0,47%)	374 (0,37%)	437 (0,43%)	335 (0,33%)	4412 (4,37%)
Subdivision3 family incertae sedis				22 (0,02%)	10 (0,01%)	17 (0,02%)							49 (0,05%)
unclassified	2374 (2,35%)	2741 (2,72%)	2510 (2,49%)	2076 (2,06%)	1966 (1,95%)	2082 (2,06%)	1336 (1,32%)	1547 (1,53%)	2172 (2,15%)	1913 (1,90%)	2002 (1,98%)	2740 (2,72%)	25459 (25,23%)
Verrucomicrobiaceae			15 (0,01%)	32 (0,03%)	25 (0,02%)				27 (0,03%)	31 (0,03%)	26 (0,03%)	28 (0,03%)	184 (0,18%)
Xanthomonadaceae	693 (0,69%)	1786 (1,77%)	2952 (2,93%)	3694 (3,66%)	3845 (3,81%)	3701 (3,67%)	36 (0,04%)	367 (0,36%)	719 (0,71%)	2441 (2,42%)	2123 (2,10%)	2025 (2,01%)	24382 (24,16%)
Sum	8436 (8,36%)	8422 (8,35%)	8429 (8,35%)	8421 (8,35%)	8425 (8,35%)	8378 (8,30%)	8400 (8,32%)	8428 (8,35%)	8433 (8,36%)	8415 (8,34%)	8427 (8,35%)	8292 (8,22%)	100906 (100,00%)

Table A7: *alkB* gene fragments of amplicon sequencing included into a phylogenetic tree on amino acid level created with ARB. Sequences closely related to reference sequences were grouped to clusters. Dashes (-) indicate sequences that could not be assigned to any specific cluster (published in Wallisch et al. (2014))

Taxon identified	Cluster	Number of representative sequences
Acetobacteraceae sp.	H	83
Acetobacteraceae sp.	-	11
Acidisphaera sp.	-	1
Acinetobacter sp.	Y	191
Aeromicrobium sp.	-	23
Agrobacterium sp.	R	24
Agrobacterium sp.	-	24
Alcanivorax sp.	U	66
Alcanivorax sp.	-	9
Bacillus sp.	N	40
Bacillus sp.	O	228
Bradyrhizobiaceae sp.	-	1
Caulobacter sp.	-	5
Cluster P1	P1	229
Cluster P2	P2	145
Cluster P3	P3	91
Cluster Q	Q	15
Cluster T	T	20
Conexibacter sp.	-	4
Dietzia sp.	-	1
Geobacillus sp.	X	265
Gordonia sp.	C	254
Gordonia sp.	-	29
Hydrocarboniphaga sp.	S	8
Methylibium sp.	-	6
Microscilla sp.	L	37
Mycobacterium sp.	A	165
Mycobacterium sp.	-	58
Nocardia sp.	-	27
Nocardioides sp.	-	4
Pedobacter sp.	-	4
Pseudomonas sp.	J	60
Pseudomonas sp.	M	116
Pseudomonas sp.	-	26
Pseudoxanthomonas sp.	-	4
Ralstonia sp.	-	27
Rhodococcus sp.	B	340
Rhodococcus sp.	E	173
Rhodococcus sp.	-	100
Rhodopseudomonas sp.	-	2

Table A7 (continued): *alkB* gene fragments of amplicon sequencing that were included into a phylogenetic tree on amino acid level created with ARB. Sequences closely related to reference sequences were grouped to clusters. Dashes (-) indicate sequences that could not be assigned to any specific cluster (published in Wallisch et al. (2014))

Taxon identified	Cluster	Number of representative sequences
Sagittula sp.	F	174
Sagittula sp.	-	3
Shewanella sp.	D	113
Stenotrophomonas sp.	-	16
Thalassolituus sp.	K	38
Uncultured bacterium	G	44
Uncultured bacterium	I	42
Uncultured bacterium	V	98
Uncultured bacterium	W	65
Uncultured bacterium	-	100
Xanthobacter sp.	-	6

Table A8: Distribution of representative sequences of *alkB* harbouring bacteria within each cluster for each sample as shown in the pie charts presented in Figure 43. Numbers are given in percentages (published in Wallisch et al. (2014))

			soil			soil+C1				soil+C2				
Taxon identified	**Cluster**	**Number of representative sequences**	**0w**	**6w**	**12w**	**36w**	**0w**	**6w**	**12w**	**36w**	**0w**	**6w**	**12w**	**36w**	**sum**
Acetobacteraceae sp.	H	83	4.8	19.3	6.0	21.7	0.0	10.8	15.7	3.6	4.8	8.4	0.0	4.8	100
Acinetobacter sp.	Y	191	16.8	17.3	14.7	1.0	1.6	14.7	8.9	1.0	3.1	14.7	4.7	1.6	100
Agrobacterium sp.	R	24	0.0	0.0	0.0	0.0	12.5	50.0	0.0	0.0	0.0	37.5	0.0	0.0	100
Alcanivorax sp.	U	66	12.1	13.6	6.1	13.6	1.5	10.6	24.2	1.5	6.1	3.0	0.0	7.6	100
Bacillus sp.	N	40	17.5	7.5	17.5	12.5	2.5	10.0	0.0	2.5	17.5	10.0	0.0	2.5	100
Bacillus sp.	O	228	24.1	18.9	6.6	4.8	8.3	2.2	1.3	1.3	21.9	8.3	1.3	0.9	100
Cluster P1	P1	229	14.0	7.9	5.2	0.9	3.9	2.2	7.9	14.8	10.5	5.2	0.0	27.5	100
Cluster P2	P2	145	7.6	13.1	16.6	20.0	0.7	2.1	6.2	2.1	6.9	6.9	3.4	14.5	100
Cluster P3	P3	91	36.3	13.2	3.3	0.0	13.2	0.0	2.2	0.0	26.4	5.5	0.0	0.0	100
Cluster Q	Q	15	0.0	0.0	0.0	0.0	20.0	66.7	6.7	0.0	0.0	6.7	0.0	0.0	100
Cluster T	T	20	0.0	0.0	0.0	0.0	5.0	15.0	0.0	0.0	25.0	55.0	0.0	0.0	100
Geobacillus sp.	X	265	20.8	15.5	13.2	4.5	12.5	3.0	0.8	0.0	22.6	2.6	0.4	4.2	100
Gordonia sp.	C	254	2.8	2.0	2.0	2.8	0.4	13.8	11.8	11.4	1.6	21.7	4.3	25.6	100
Hydrocarboniphaga sp.	S	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	37.5	62.5	0.0	0.0	100
Microscilla sp.	L	37	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.7	64.9	21.6	2.7	8.1	100
Mycobacterium sp.	A	165	14.5	6.7	5.5	18.8	4.8	0.0	0.6	9.7	6.1	0.6	1.2	31.5	100
Pseudomonas sp.	J	60	36.7	15.0	21.7	3.3	3.3	0.0	0.0	0.0	13.3	5.0	0.0	1.7	100
Pseudomonas sp.	M	116	15.5	21.6	13.8	5.2	10.3	5.2	0.9	0.0	21.6	5.2	0.0	0.9	100
Rhodococcus sp.	B	340	0.3	0.0	0.3	0.0	3.2	13.2	20.3	18.2	0.0	13.5	4.7	26.2	100
Rhodococcus sp.	E	173	0.6	0.0	0.0	0.0	0.6	16.8	30.6	18.5	0.6	12.1	5.2	15.0	100
Sagittula sp.	F	174	0.0	0.6	1.7	1.7	1.7	16.7	15.5	19.5	3.4	10.9	2.9	25.3	100
Shewanella sp.	D	113	0.0	0.0	0.0	0.0	0.9	19.5	28.3	18.6	0.0	8.8	2.7	21.2	100
Thalassolituus sp.	K	38	10.5	18.4	26.3	26.3	0.0	0.0	0.0	0.0	5.3	0.0	0.0	13.2	100
Uncultured bacterium	G	44	11.4	6.8	11.4	13.6	0.0	6.8	6.8	0.0	29.5	9.1	0.0	4.5	100
Uncultured bacterium	I	42	14.3	23.8	14.3	19.0	7.1	4.8	0.0	0.0	4.8	7.1	0.0	4.8	100
Uncultured bacterium	V	98	0.0	0.0	0.0	0.0	20.4	51.0	1.0	0.0	6.1	16.3	0.0	5.1	100
Uncultured bacterium	W	65	20.0	26.2	3.1	24.6	1.5	7.7	0.0	4.6	4.6	4.6	0.0	3.1	100