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Microbial community structure and function is shaped by microhabitat characteristics in soil

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Eidesstattliche Erklärung

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Esslingen, den 10.11.2015 Franziska Ditterich

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1 Summary

Soil microorganisms play a key role in degradation processes in soil, such as organic matter decomposition and degradation of xenobiotics. Microbial growth and activity and therefore degradation processes are influenced by different ecological factors, such as substrate availability, pH and temperature. During soil development different microhabitats are formed which differ in their physiochemical properties. There is some evidence that mineral composition is a driver for specific microbial colonization. Thereby, the heterogeneity of soils with differences in mineral composition and substrate availability can lead to a spatial distribution of soil microorganisms. At the soil-litter interface, a biogeochemical hot spot in soil, the abundance and activity of soil microorganisms increases due to high substrate availability, and degradation processes such as pesticide degradation are enhanced. This thesis aimed to clarify the influence of habitat properties on the structure and function of the microbial community in soil. In particular, focus was on mineral-microbe interactions that result from the mineral composition and substrate availability in an artificial soils system. Furthermore this thesis was designed to increase our understanding of the bacterial and fungal roles in pesticide degradation at the soil-litter interface using 4-chloro-2-methylphenoxyacetic acid (MCPA) as a model xenobiotic.

These two aspects of the thesis were examined in three studies. The first study focused on the succession of microbial communities and enzyme activities in an artificial soils system with varying mineral composition and substrate availability over a period of 18 months. In the second study a microcosm experiment was used to study the bacterial pathway of MCPA degradation at the soil-litter interface. Over a period of 27 days the succession of bacterial degraders was followed. The third study focused on the degradation of MCPA in soil by nonspecific fungal enzymes, through the addition of fungal laccases as well as litter during 42 days of incubation. Both studies indicated the involvement of fungi in MCPA degradation and the importance of the ecological behavior of different degraders as a function of substrate availability.

In all three studies real-time quantitative PCR (qPCR) was used to determine the abundance of the bacterial community by analyzing the 16S rRNA genes, and of the fungal community by analyzing the fungal ITS fragment. Also, the abundances of the functional genes (*tfdA* and *cadA*) involved in the MCPA degradation were analyzed using qPCR. To get a broader picture of MCPA mineralization and degradation, the CO_2 production of non-labeled or ^{14}C labeled MCPA

was measured and the MCPA content was analyzed by HPLC. Analyses of enzyme activities, phospholipid fatty acid (PLFA) content and qPCR analyses of seven taxa provided more general information about microbial activity and abundance.

Results of the first study indicated that the microbial community was affected by mineral properties under high substrate availability and by the availability of beneficial nutrients at the end of incubation when substrate had become limited. The measured enzyme activities provided clear evidence that microbial community structure was driven by nutrient limitation during incubation. In the presence of easily available organic substrates at the beginning of the experiment, the soil microbial community was dominated by copiotrophic bacteria (e.g. *Betaproteobacteria*), whereas under substrate limitation at the end of incubation, more recalcitrant compounds became important to oligotrophic bacteria (e.g. *Acidobacteria*), which then became dominant. The results of the second study indicated that the contribution of the potential degraders to degradation of MCPA differed, and this was also seen in the succession of specific bacterial MCPA degraders. Added litter stimulated MCPA degradation due to the availability of litter-derived carbon and induced a two-phase response of fungi. This was seen in the development of pioneer and late stage fungal communities. Both fungal communities were probably involved in MCPA degradation. Therefore, the third study focused on the fungal pathway. These results indicated that the fungal laccases used had no direct influence on degradation and were as efficient as litter in providing additional nutrient sources, increasing MCPA degradation by bacteria and fungi. The observed differences between litter and enzyme addition underscored the observation that the enzyme effect was short-lived and that substrate quality is an important factor in degradation processes.

In conclusion, this thesis demonstrated that soil microbial communities and therefore degradation processes are driven by mineral composition as well as substrate availability and quality. In addition, this thesis extends our understanding of degradation processes such as the degradation of xenobiotics, with MCPA as model compound, in soil. The combined insights from all three studies suggest that the use of a simple system such as the artificial soil system can increase our understanding of complex mechanisms such as degradation of pesticides.

2 Zusammenfassung

Bodenmikroorganismen spielen im Boden eine Schlüsselrolle bei Abbauprozessen, wie Zersetzung von organischem Material und Abbau von Xenobiotika. Wachstum und Aktivität von Mikroorganismen und somit auch Abbauprozesse werden durch verschiedene ökologische Faktoren, wie Substratverfügbarkeit, pH und Temperatur beeinflusst. Während der Bodenentwicklung werden verschiedene Mikrohabitate mit unterschiedlichen physiochemischen Eingenschaften geformt. Es gibt einige Hinweise, dass die Mineralzusammensetzung ein Einflussfaktor für die spezifische mikrobielle Kolonisation ist. Dabei kann die Heterogenität von Böden mit unterschiedlicher mineralischer Zusammensetzung und Substratverfügbarkeit zu einer räumlichen und zeitlichen Verteilung von Bodenmikroorganismen führen. An der Boden-Streu Grenzfläche, einem biogeochemischen Hotspot, ist die Menge und Akivität von Bodenmikroorganismen auf Grund von hoher Substratverfügbarkeit erhöht und Abbauprozesse, wie Pestizidabbau verbessert. Ziel dieser Arbeit war es, den Einfluss von Habitateigenschaften auf Struktur und Funktion von mikrobiellen Gemeinschaften in Böden zu klären. Insbesoders lag der Fokus dieser Arbeit auf Mineral-Mikroorganismen Interaktionen im Bezug auf Mineralzusammensetzung und Substratverfügbarkeit in künstlichen Böden. Darüber hinaus wollte diese Arbeit unser Wissen über die bakterielle und pilzliche Rolle im Pestizidabbau an der Boden-Streu Grenzfläche, mit 4-Chlor-2-Methylphenoxyessigsäure (MCPA) als Modell-Xenobiotika, erweitern.

Die zwei Aspekte dieser These wurden in drei Studien untersucht. Die erste Studie konzentrierte sich auf die zeitliche Abfolge der mikrobiellen Gemeinschaften und Enzymaktivitäten in künstlichen Böden, mit unterschiedlicher mineralischer Zusammensetzung und Substratverfügbarkeit über einen Zeitraum von 18 Monaten. In der zweiten Studie wurde ein Mikrokosmenexperiment verwendet um den bakteriellen Weg des MCPA-Abbaus an der Boden-Streu Grenzfläche zu untersuchen. Über einen Zeitraum von 27 Tagen wurde die Sukzession der bakteriellen Abbauer verfolgt. Die dritte Studie konzentrierte sich auf den Abbau von MCPA im Boden mittels unspezifischer pilzlicher Enzyme, durch Zugabe von pilzlichen Laccasen sowie Streu über einen Zeitraum von 42 Tagen. Beide Studien deuten die Beteiligung von Pilzen am MCPA-Abbau und die Bedeutung von ökologischem Verhalten verschiedener Abbauer in Abhänigkeit von Substratverfügbarkeit an.

In allen drei Studien wurde die Abundanz der bakteriellen Gemeinschaft an Hand der 16S rRNA Gene und die Abundanz der pilzlichen Gemeinschaft an Hand des pilzlichen ITS Fragmentes mittels quantitativer Echtzeit PCR (qPCR) bestimmt. Außerdem wurden die Abundanzen der am MCPA-Abbau beteiligten funktionellen Gene (*tfdA* und *cadA*) mittels qPCR untersucht. Um ein umfassenderes Bild über MCPA Mineralisierung und Abbau zu erhalten, wurde die CO_2 Produktion von nicht markiertem oder ^{14}C markiertem MCPA gemessen und der MCPA Gehalt mittels HPLC untersucht. Analysen der Enzymaktivitäten, des Gehalts an Phospholipid-Fettsäuren (PLFA) und qPCR Analysen von sieben Taxa, lieferten allgemeine Informationen über mikrobielle Aktivität und Menge.

Die Ergebnisse aus der ersten Studie deuteten an, dass die mikrobielle Gemeinschaft durch Mineraleigenschaften unter hoher Substratverfügbarkeit und durch die Verfügbarkeit von förderlichen Nährstoffen am Ende der Inkubation unter Substratlimitierung beeinflusst wurden. Die gemessenen Enzymaktivitäten liefern einen klaren Hinweis, dass die mikrobielle Gemeinschaft durch Nährstofflimitierung während der Inkubation gelenkt wurde. In Anwesenheit von einfach verfügbaren organischen Substanzen wurde die mikrobielle Bodengemeinschaft von copiotrophen Bakterien (z.B. *Betaproteobakterien*) dominiert, wohingegen am Ende der Inkubation unter Substratlimitierung schwer abbaubare Komponenten für oligotrophe Bakterien (z.B. *Acidobakterien*) entscheidend wurden, welche daraufhin dominiereten. Die Ergebnisse der zweiten Studie deuteten darauf hin, dass der Beitrag der potentiellen Abbauer MCPA abzubauen unterschiedlich war, was auch in der Sukzession der spezifischen bakteriellen MCPA Abbauer zu sehen war. Zugabe von Streu stimuliert den MCPA Abbau in Abhängigkeit von verfügbarem streubürtigen Kohlenstoff und induziert eine zwei-Phasen Antwort der Pilze. Dies war in der Entwicklung von frühen und späten pilzlichen Gemeinschaften zu sehen. Beide Pilzgemeinschaften waren vermutlich am MCPA Abbau beteiligt. Aus diesem Grund konzentrierte sich die dritte Studie auf den pilzlichen Abbauweg. Die Ergebnisse deuten an, dass die verwendeten pilzlichen Laccasen keinen direkten Einfluss auf den Abbau hatten und dass sie genauso effektiv waren zusätzliche Nährstoffquellen bereitzustellen wie Streu, um den MCPA Abbau durch Bakterien und Pilze zu fördern. Die beobachteten Unterschiede zwischen Streu- und Enzymzugabe unterstreicht die Beobachtung, dass der Enzymeffekt kurzlebig war und dass Substratqualität ein wichtiger Faktor in Abbauprozessen ist.

Zusammenfassend zeigte diese Arbeit, dass die mikrobielle Gemeinschaft durch die Mineralzusammensetzung sowie Substratverfügbarkeit und Qualität angetrieben wird. Darüber hinaus erweitert diese Arbeit unser Wissen über Abbauprozesse in Böden, wie den Abbau von Xenobiotika, mit MCPA als Modelkomponente. Die Einblicke von allen drei Studien deuten an, dass ein einfaches System, wie das System der künstlichen Böden hilfreich sein kann, um unser Wissen über komplexe Mechanismen, wie Pestizidabbau zu erhöhen.

3 General Introduction

3.1 Soil microorganisms

Soil microorganisms play a central role in biogeochemical cycles, driving soil ecosystem functions such as degradation of organic matter through decomposition and degradation of xenobiotics. They take part in about 90% of soil processes (Nannipieri et al., 2003). Knowledge of the diversity and structure of soil microorganisms can provide insight into ecosystem function. Both abiotic and biotic environmental factors regulate abundance, diversity and function of soil microorganisms. Substrate availability, substrate quality and complexity are important for the diversity of microbes and therefore the growth of specific bacteria (Semenov et al., 2012). Availability of substrate is influenced by its sorption of organic matter (OM) and therefore organic carbon (OC), which in turn contribute to stabilization of OM and OC in soils. Furthermore, both availability and quality of substrates vary during litter decomposition and consequent input of easily available or recalcitrant compounds, leading to a succession of microorganisms (Poll et al., 2008).

An ecological life strategy model for microorganisms which is similar to that of higher organisms was proposed by Fierer and colleagues (2007). In this model ecological attributes likely correspond to oligotrophic (*Acidobacteria*) and copiotrophic (*Betaproteobacteria*) groups of bacteria. In general, oligotrophic bacteria are slow growing bacteria adapted to environments with low nutrient availability due to high substrate affinity, whereas copiotrophic bacteria can maximize their growth rate under high substrate availability (Fierer et al., 2007). Based on soil C content and availability, the following bacteria were categorized according to the life strategy model of Fierer and colleagues (2007): *Alphaproteobacteria*, *Betaproteobacteria* and *Bacteroidetes* are found in C rich environments, whereas *Acidobacteria* are found in C poor environments (Fierer et al., 2007; Nemergut et al., 2010). In addition, Nemergut and colleagues (2010) demonstrated that multiple aspects of soil C and N cycling in environments vary in concert with the abundance of specific phylogenetic groups. It has been proposed that N-fertilization might be the main driver for shifts at the bacterial phyla or class levels, whereas community changes at lower taxonomical levels are induced by organic amendments (Cederlund et al., 2014).

Recent advances in molecular techniques have provided new opportunities to examine the ecological characteristics of soil microbes. Lauber et al. (2009) demonstrated that pH-value is a driver for shifts in the bacterial community, with *Acidobacteria* dominant in soils with low pH in contrast to *Actinobacteria*, which were positively correlated with high soil pH. Dry- and wet-cycles influenced the bacterial community as well, with strong responses of several groups, especially *Acidobacteria*, which increased with rewetting in contrast to *Actinobacteria*, which increased with desiccation (Barnard et al., 2013). It has also been proposed that different minerals support different microbial communities due to specific surface properties or chemical composition of the minerals (Roberts, 2004; Gleeson et al., 2005, 2006; Boyd et al., 2007; Carson et al., 2007, 2009; Uroz et al. 2012). Pesticides such as xenobiotics also influence the microbial community and its activity. A review by Gianfreda and Rao (2008) showed that there are three basic pesticide-based events which affect soil population dynamic: first, "the death of sensitive organisms with the consequent utilization of organic residues by surviving populations"; second, "the direct utilization of pesticides by organisms that are able to degrade or to metabolize them"; and third, "the development of microbial populations that depend on secondary nutrient sources, e.g., metabolites produced from the decomposition of the pesticide". At normal field rates pesticides have only a weak effect on soil microbes (Johnsen et al., 2001; Gianfreda & Rao, 2008). But some microbial species may be inhibited, whereas others rapidly appear (Bollag & Liu, 1990; Schuster & Schröder, 1990).

3.2 Soil enzymes

Soil microbial function relies on the exudation of enzymes which enable microbes to degrade complex substrates to low molecular weight compounds. Therefore, enzyme activities provide a linkage between resource availability, microbial structure and function, and ecosystem processes (Sinsabaugh et al., 2002). Enzyme production and enzyme activity yield information about C degradation and release of nutrients from complex compounds (Sinsabaugh et al., 1993; Allison & Vitousek, 2005). Specific enzyme activities can be used to assess functional diversity between and within nutrient cycles due to substrate specificity of enzymes (Caldwell, 2005). For example, enzymes involved in the C-cycle are cellulolytic enzymes such as β-glucosidase, and ligninolytic enzymes such as phenol- and peroxidase, whereas urease is involved in the N-cycle, and phosphatase in the P-cycle (Caldwell, 2005). The allocation of enzymes reflects the microbial demand on nutrients and allows microbes to acquire these limiting nutrients from complex

compounds available in soils (Allison & Vitousek, 2005; Pabón-Pereira et al., 2014). Besides substrate availability, enzyme activities depend on enzyme stabilization, which is regulated by several biotic and abiotic factors, such as minerals. Because of the heterogeneity of soils, with different mineral compositions and organic compounds, a variety of responses of soil enzymes occur (Sinsabaugh, 1994; Allison, 2006). For example, Allison (2006) demonstrated that urease activity was unaffected by ferrihydrite, while phosphatase showed a slight enhancement. Changes in a specific enzyme activity can be due to different factors, e.g. substrate availability, but is not necessarily connected to shifts in the microbial community (Stone et al., 2014).

Enzyme activities also play an important role in biochemical processes such as degradation, including pesticide degradation. Transformation efficiency is related to the presence of enzymes. Several studies have researched the influence of various pesticides on enzyme activities in soil (Reddy et al., 1997; Sannino & Gianfreda, 2001; Pizzull et al., 2009). Some factors which influence enzyme activities in the presence of pesticides are pesticide concentration, toxicity and also the presence of different enzymes which change over time (Gianfreda & Rao, 2008; Riah et al., 2014). Furthermore, the adsorption of xenobiotics or enzymes to soil colloids influences the interaction between xenobiotics and enzyme activities. To study the influence of enzymes on pesticide degradation, it is helpful to use model chemicals, such as 2-methyl-4-chlorophenoxyacetic acid (MCPA). MCPA is successfully transformed by dioxygenase produced by bacteria (Fukumori & Hausinger, 1993; Vallaeys et al., 1996; McGowan et al., 1998). Furthermore it has been shown that fungal enzymes are also involved in the degradation of pesticides such as MCPA (Valli & Gold, 1991; Reddy et al., 1997; Castillo et al., 2001). For example, Castillo et al. (2001) reported an increase of peroxidase in the present of MCPA.

3.3 Soil microhabitats

Soils consist of heterogeneous mixtures of different mineral, organic and biological compounds and are associated in complex hierarchical structures. During soil development micro- and macro-aggregates are formed which differ in physiochemical properties and therefore in the spatial allocation of habitats for microorganisms. Environmental factors such as substrate availability and habitat properties such as mineral composition shape both the structure and function of soil microorganism (Chenu et al., 2001; Roberts, 2004; Gleeson et al., 2005, 2006; Carson et al., 2007, 2009). The spatial distribution of soil microorganisms is of interest in

ecology because it can provide insight into underlying processes that shape biodiversity (Philippot et al., 2009). Microhabitat characteristics determine the function of microhabitats and influence therefore biogeochemical processes by supporting different microorganisms.

Minerals form ecological niches which play an important role in biogeochemical cycles (Uroz et al., 2012) and are frequently attached to by soil microorganisms, but little is known about the influence of mineral composition on microbial community composition and function. It has been proposed that different minerals support different microbial communities due to specific surface or electrostatic properties or chemical composition of the minerals (Roberts, 2004; Gleeson et al., 2005, 2006; Boyd et al., 2007; Carson et al., 2007, 2009). Comparing a spruce plagioclase with a spruce apatite, the plagioclase was more colonized by *Acidobacteria* and *Actinobacteria*, while in contrast *Betaproteobacteria* were enriched in the presence of apatite (Uroz et al., 2012). In general, microbes are negatively charged and preferentially colonize positively charged mineral surfaces, whereas less electrostatically favorable minerals are colonized when beneficial elements like Fe or non-toxic elements like Al are available (Roberts, 2004). When minerals containing elements such as K, Mg, P or Ca, which are rare or non-existent in the surrounding soil, the bacterial communities are affected to a greater extent than without these elements (Rogers & Bennett, 2004; Carson et al., 2009). In addition, Gleeson and colleagues (2005, 2006) proposed that the elemental composition of minerals is the driver for specific ribotypes. In general, bacteria following different life strategies prefer minerals of differing compositions depending on specific properties of the mineral, such as surface charge or chemical composition. Compared to the complexity of natural soils, the use of artificial soils with defined composition offers a unique opportunity to study, in a simplified model, the link between mineralogy and soil biota as well as soil function (Zhang et al., 2011; Pronk et al., 2012; Ding et al., 2013; Vogel et al., 2014; Wei et al., 2014a, 2014b).

Microhabitats with high input of nutrients form microbial hot spots. The soil-litter interface is such a hot spot with high availability of soluble litter compounds which are transported to the adjacent soil (Poll et al., 2008, 2010a). These hot spots exhibit high microbial activity, growth and C turnover (Poll et al., 2008). Other soil processes, such as pesticide degradation, are strongly linked to growth conditions (Cederlund et al., 2007; Fredslund et al., 2008) and pesticide degradation is stimulated in the presence of litter (Duah-Yentumi & Kuwatsuka, 1980; Poll et al.,

2010a). Therefore, the soil-litter interface is an excellent model for studying degradation processes such as MCPA degradation.

3.4 MCPA degradation in soil

In agricultural ecosystems, soils are polluted by phenoxyacetic acids, which are used to reduce yield losses or to ensure crop health. Two of the most heavily used phenoxy acid herbicides are MCPA and 2,4-dichlorophenoxyacetic acid (2,4-D), both of which are used to control dicotyledonous plants in cropland. Both are also used as model compounds to study the degradation of chlorinated xenobiotics. Since the 1980s the degradation pathways of MCPA and of related compounds have been extensively studied (Beadle & Smith, 1982; Shailubhai et al., 1983). Worldwide, microbial phenoxy herbicide degraders exhibiting a high diversity of microbial strains have been identified in different soils (Fulthorpe et al., 1995; McGowan et al., 1998; Bælum et al., 2006; Rodriguez-Cruz et al., 2010). The bacterial degradation pathway at the organism level is well known and the involved functional genes, such as *cadA* and *tfdA* have been characterized (Don & Pemberton, 1981; Don et al., 1985; Streber et al., 1987; Fukumori & Hausinger, 1993; Ka et al., 1994; Fulthorpe et al., 1995; Tonso et al., 1995; Kitagawa et al., 2002). The degradation rate is related to the quantity and activity of degraders (Torstensson & Stenström, 1986; Cederlund et al., 2007); differences in degradation activity have been explained by uneven distribution of the degrader community (Vieublé-Gonod et al., 2003; Batıoğlu-Pazarbaşı et al., 2012).

Further studies have shown that litter addition increased the MCPA degrading community and therefore MCPA degradation was stimulated (Duah-Yentumi & Kuwatsuka, 1982; Poll et al., 2010a). But although the bacterial degrading pathway of MCPA is well known, less is known about the fungal degradation pathway of phenoxy acid herbicides, although earlier studies have reported that several fungi are involved in the degradation of pollutants like 2,4-D or MCPA (Reddy et al., 1997; Vroumsia et al., 1999, 2005; Castillo et al., 2001; Strong & Claus, 2011). It has been proposed that extracellular enzymes of the ligninolytic systems or intracellular enzymes are involved (Valli & Gold, 1991; Reddy et al., 1997; Castillo et al., 2001).

3.4.1 Regulation of MCPA degradation at the organism level – the diversity of functional genes

The MCPA degrading community is divided into three groups based on phylogeny and catabolic gene diversity (Kamagata et al., 1997; Itoh et al., 2002, 2004; Kitagawa et al., 2002). The first group of MCPA degraders harbors a high diversity of *tfdA* genes which encode for an α-ketoglutarate-dependent dioxygenase and consist of *Beta-* and *Gammaproteobacteria* (Vallaeys et al., 1996; McGowan et al., 1998; Rodriguez-Cruz et al., 2010; Zaprasis et al., 2010). The initial step of MCPA degradation driven by an enzyme encoded by *tfdA* (α-ketoglutarate-dependent dioxygenase) is the cleavage of the ether-bonded acetate side chain of MCPA to form MCP (4-chloro-2-methylphenol). The second group consists of oligotrophic *Alphaproteobacteria* harboring *cadA* or *tfdAα* genes with no sequence similarity of the *cadA* gene to the *tfd* genes (Itoh et al., 2004). Kitagawa et al. (2002) reported that the *cadA* gene encodes for an oxygenase that are homolog to benzoate dioxygenase BenA (about 34% identity) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) oxygenase TftA (about 46% identity) which facilitates the conversion from phenoxyacetate to phenol (Danganan et al., 1994). The third group belongs to *Alphaproteobacteria* harboring *cadA* or *tfdA-like* genes which consist of copiotrophic fast growing *Sphingomonas* strains (Itoh et al., 2004). Previous studies have shown that during MCPA degradation the *cadA*-hosting microorganisms increased (Liu et al., 2011a, 2011b, 2013).

McGowan et al. (1998) found differences in the *tfdA* sequence of the degraders belonging to the first group and suggested that this group can be subdivided into three classes. Several studies have suggested that some of these bacteria have a truncated pathway and depend on a bacterial consortium for complete MCPA degradation (Ka et al., 1994; Top et al., 1996). Melting curve analysis via quantitative real-time PCR (qPCR) revealed a shift in the *tfdA* classes during MCPA degradation (Bælum et al., 2008; Rodriguez-Cruz et al., 2010). This analysis demonstrated that the indigenous degrader community was dominated by class I, whereas class III became dominant during the degradation of MCPA. Additional studies (Bælum & Jacobsen, 2009; Batıoğlu-Pazarbaşı et al., 2012) confirmed the shift between class I and III by using a TaqMan probe-based qPCR assay. To date, not much is known about the specific functions of each of the classes. Bælum et al. (2006) have suggested that MCPA degraders belonging to class I have a truncated pathway. In general, the MCPA degraders differ in their ability to use MCPA as a single carbon-source.

3.4.2 Regulation of MCPA degradation at the cellular level – functioning of specific and nonspecific MCPA-degrading enzymes

For the initial steps in a catabolic pathway, co-substrates or energy equivalents are needed (Müller, 2007). With respect to the bacterial degradation pathways of MCPA or other phenoxyacetic acids such as 2,4-D, the enzymes involved are known as oxygenases. For the TfdA enzyme, α-ketoglutarate, which is oxidatively decarboxylated to succinate in the first step and has to be regenerated, is needed as co-substrate, and it has been proposed that this can limit degradation (Müller & Babel, 2000, 2001). Müller & Babel (2001) demonstrated the importance of the availability of α-ketoglutarate for degradation. Litter addition stimulates the degradation of MCPA (Duah-Yentumi & Kuwatsuka, 1982; Poll et al., 2010a); the transport of litter compounds has been identified as a possible regulatory process for the activity of the MCPA degrading community (Poll et al., 2010a). Possible explanations for this regulatory process are by transport of α-ketoglutarate or its metabolic precursors, and/or by the supply of energy and nutrients by litter compounds resulting in stimulation of enzyme production or bacterial activity of the degraders (Poll et al., 2010a).

In contrast to the bacterial pathway, no specific fungal pathway is known, although studies have demonstrated the ability of fungi to degrade phenoxyacetic acids (Castillo et al., 2000, 2001; Vroumsia et al., 2005; Lerch et al, 2009). Over 90 fungal strains have been tested with resulting high variances in their ability to degrade different phenoxyacetic herbicides (Vroumsia et al., 2005; Itoh et al., 2013). The proposed fungal enzymes which are able to degrade organic compounds and chlorinated aromatic pollutants are extracellular enzymes involved in lignin degradation (Valli & Gold, 1991; Reddy et al., 1997; Castillo et al., 2000, 2001). These extracellular enzymes are two types of peroxidase; manganese peroxidase (MnP), and lignin peroxidase (LiP), as well as laccases, which are involved in lignin degradation as a secondary metabolic pathway (Kirk et al., 1978; Jeffries et al., 1981; Tien & Kirk, 1983; Kuwahara et al., 1984). Under laboratory conditions, the presence of LiP, MnP/laccase activity was correlated with the degradation of MCPA (Castillo et al., 2001). Many studies working with 2,4-D have shown little effect of the tested fungal enzymes on the degradation of 2,4-D, as compared with the mineralization of the metabolite 2,4-DCP (Gianfreda et al., 1998; Sannino et al., 1999). This suggests, therefore, that the ligninolytic system is involved in degradation but not in the initial side-chain cleavage (Valli & Gold, 1991; Reddy et al., 1997). In this proposed pathway, the

chlorophenoxyacetic acids undergo ether cleavage to form corresponding chlorophenols during primary metabolic growth, and this is then catalyzed by intracellular enzymes (Reddy et al., 1997). Subsequently, once the chlorophenols are mineralized, it is proposed that ligninolytic enzymes catalyze oxidative dechlorination followed by a ring cleavage (Valli & Gold, 1991; Reddy et al., 1997). It is well known that the substrate range of the ligninolytic enzymes can be extended in the presence of a mediator (Bourbonnais & Paice, 1990; Pizzull et al., 2009). Mougin et al. (2000), for example, reported that in the presence of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) fungal laccases are able to degrade non-phenolic compounds, like di-ketone. However, to date the importance of the fungal role in MCPA degradation due to laccase production remains poorly understood.

4 Overview of the thesis

It is known that microorganisms are spatially distributed due to the heterogeneity of soils (Phillipot et al., 2009) and that microorganisms are attached to mineral surfaces or organo-mineral complexes. Furthermore, the abundance and activity of soil microorganisms are influenced by biotic and abiotic factors. But little is known about the influence of mineral composition on microbial community composition and function. It is well established, however, that the soil-litter interface is a biogeochemical hot spot in soil, where the abundance and activity of soil microorganisms is high and soil organic matter turnover as well as the degradation of organic chemicals, such as MCPA, is enhanced (Poll et al., 2010a, 2010b). The bacterial degradation pathway of MCPA has been well studied since the 1980s (Beadle & Smith, 1982) whereas little is known about the fungal pathway. This thesis, therefore, focused on the influence of microhabitat characteristics on the structure and function of soil microorganisms. The goal of the first study was to clarify the influence of organo-mineral complexes on the succession of microbial communities due to mineral composition and substrate availability in an artificial soil system. A further aim of the thesis was to increase our understanding of pesticide degradation at the soil-litter interface, a microhabitat with high microbial activity. In particular, the focus of the thesis was to clarify the role of specific MCPA degraders and to gain further insight into direct and indirect effects of fungal laccases on MCPA degradation (studies 2 & 3).

In three studies the two aspects of the thesis were examined. In the first study, a unique artificial soils system was used to study mineral-microbe interactions. The hypothesis was that different minerals as well as charcoal select for specific colonizers and that initial colonization is influenced by the complexity of mineral composition. In particular it was hypothesized that the incubation time and therefore substrate availability influences microbial colonization and that microbial communities follow the ecological strategies of higher organisms. Therefore samples from an artificial soils system (Pronk et al., 2012) were used to determine the structure and activity of the microbial community. To identify the response of different microbial communities to mineral composition, abundances of seven different taxa were quantified using qPCR.

The second study focused on MCPA degradation at the soil-litter interface and followed the dynamics of the MCPA degrading community. It was hypothesized that microorganisms harboring the three different *tfdA* gene classes or the *cadA* gene differ in their ability to use

MCPA as a carbon source and that they respond differently to litter addition. Therefore, over a period of 27 days, a microcosm experiment was performed which simulated the soil-litter interface. The abundance of 16S rRNA genes, *tfdA*, *cadA*, and the fungal ITS fragment sequences were quantified using quantitative PCR (qPCR) to determine the effects of litter and MCPA addition on total bacterial and fungal communities as well as on the MCPA degrader communities. A TaqMan probe assay was used to quantify the succession of *tfdA* harboring bacteria.

The second study indicated that fungi are involved in the degradation of MCPA. The third study therefore focused on the fungal contribution to MCPA degradation due to the presence of fungal laccases which were added as active and inactivated mixtures. A second microcosm experiment was performed over a period of 42 days. To study the impact of fungal laccases on MCPA degradation, phenoloxidase activity as well as the abundance of the fungal ITS fragment were analyzed. The abundance of the functional genes *tfdA* and *cadA* as well as the 16S rRNA genes was quantified to serve as a control of the bacterial component.

5 Succession of soil microbial communities and enzyme activities in artificial soils[1]

[1] Ditterich et al. (2016); Succession of soil microbial communities and enzyme activities in artificial soils; Pedobiologia: DOI: 10.1016/j.pedobi.2016.03.002

5.1 Abstract

Soil microorganisms are frequently attached to mineral surfaces or organo-mineral complexes, yet little is known about the microbial colonization of different soil minerals. The use of artificial soils that differ only in their mineral composition (illite, montmorillonite, ferrihydrite, boehmite) and the presence of charcoal, but not in soil texture and organic composition, offered a unique opportunity to study composition, function and succession of soil microorganisms colonizing newly exposed organo-mineral surfaces. Artificial soils were incubated with a microbial inoculum from an arable topsoil at constant temperature (20 °C) and moisture conditions for up to 18 months. The succession of enzyme activities involved in C-, N- and P-cycling gave clear evidence that nutrient limitation drove microbial community structure during the incubation independent of mineral composition. Discriminant analyses of principal components of PLFAs showed that microbial community structure changed over a period of 18 months towards similar communities for all artificial soils at the end of incubation. This was supported by a shift in the soil microbial community from dominance of specific phyla like *Betaproteobacteria*, which is often referred to as copiotrophic organisms, during the first 6 months of the incubation, toward systems with a higher dominance of e.g. *Acidobacteria* which are suggested to follow the oligotrophic life-strategy. The effect of mineral surface properties on enzyme activities was pronounced during the first 6 months of incubation. Microbial colonization and succession on mineral surfaces was likely affected by mineral properties such as surface charge and, at the end of incubation, availability of beneficial nutrients. Charcoal affected the microbial community only during the first 6 months of incubation with slightly increased colonization by bacteria which are often described as oligotrophic organisms. In contrast, illite and montmorillonite probably provided nutrient rich environments with montmorillonite supplying more exchangeable cations. The artificial soils experiment clearly showed that changes in substrate availability as well as mineral properties are important drivers for the development of microbial communities.

5.2 Introduction

Soils are heterogeneous mixtures of mineral, organic and biological compounds which are frequently associated in complex hierarchical structures. During the development of soils, aggregates are formed. The surfaces of micro and macro aggregates differ in their physicochemical properties and provide habitats for soil microorganisms. Abundance, diversity and function of soil microorganisms are regulated by environmental factors such as substrate and water availability (Killham et al., 1993; Chenu et al., 2001; Monard et al., 2012), habitat properties such as particle and pore size distribution (Ranjard et al., 2000; Sessitsch et al., 2001; Strong et al., 2004) and mineral composition (Roberts, 2004; Gleeson et al., 2005, 2006; Carson et al., 2007, 2009). Previous studies have provided evidence that differences in particle and pore size distribution, in organic matter (OM) quantity and quality as well as mineral composition of soils may select for specific bacterial communities (Ranjard et al., 2000; Sessitsch et al., 2001; Davinic et al., 2012). After physical fractionation of soil samples, more bacteria were found in micro aggregates than in macro aggregates, and the distribution of bacteria depended on the content of organic carbon (OC) and clay (Ranjard et al., 2000). There is also evidence that particle size as well as mineral composition of soils drive not only specific microbial colonization of organo-mineral surfaces, but also modify microbial functions (Kandeler et al., 1999; Stemmer et al., 1999; Sessitsch et al., 2001; Poll et al., 2003).

Minerals form ecological niches which play an important role in biogeochemical cycles (Uroz et al., 2012). However, little information is available regarding the influence of mineral composition on microbial community composition and function. Recent developments in molecular techniques have created new opportunities to study the interactions between minerals and microbial communities, as well as the microbial role in soil functional processes. Previous studies have provided evidence that different soil minerals and their specific surface properties influence microbial colonization and select for different bacterial communities (Roberts, 2004; Gleeson et al., 2005, 2006; Boyd et al., 2007; Carson et al., 2007, 2009); e.g. positively charged mineral surfaces attracted negatively charged microbes, whereas negatively charged surfaces were less colonized. In addition to the electrostatic properties of mineral surfaces, their roughness and chemical composition also impact initial colonization; the colonization of negatively charged silicate surfaces increased with increasing Fe and decreasing Al content of the mineral, as Fe is a nutrient and Al is toxic (Roberts, 2004). Under P and Fe limitation, microorganisms

preferentially colonized feldspar containing the limiting nutrients P and Fe (Rogers and Bennett, 2004). Similarly, Carson et al. (2009) reported that mica, basalt and rock phosphate selected for specific bacterial communities depending on differences in their elemental and nutrient concentrations. The presence of specific ribotypes was connected with specific minerals and was driven by the elemental composition of the mineral (Gleeson et al., 2005, 2006).

The complexity of natural soils makes it difficult to find a direct link between mineralogy and soil biota. Therefore, artificial soils offer a unique opportunity to study microbial colonization and functioning of organo-mineral surfaces in a simplified model system (Zhang et al., 2011; Pronk et al., 2012; Ding et al., 2013; Vogel et al., 2014; Wei et al., 2014a, 2014b). Over the last few years, many studies on artificial soils have been based on a microcosm experiment of Pronk et al. (2012) in which artificial soils were composed of quartz, manure as the OM source, and a microbial community extracted from a natural arable soil, with 8 different mixtures of montmorillonite, illite, ferrihydrite, boehmite and charcoal. Besides studies on aggregation and chemistry of these artificial soils (Heister et al., 2012; Pronk et al., 2012, 2013) some results are available on the early microbial colonization of different organo-mineral complexes (Babin et al., 2013; Ding et al., 2013). Over a period of 3 months, Ding et al. (2012, 2013) demonstrated that the diversity of the microbial communities for all artificial soils were lower than for the inoculum used. In addition, soil minerals as well as charcoal shaped the community composition, and the bacterial community structure of charcoal-containing soils differed greatly from other soils at all taxonomic levels studied (Ding et al., 2013). Vogel et al. (2015) found that the mineralization did not correlate with the surface area of the clay minerals used in the artificial soils system. Much less information is available however, about the succession of microbial communities in relation to microbial abundance, diversity and function during prolonged incubation of artificial soils (Steinbach et al., 2015; Vogel et al., 2014).

Therefore, the objective of this study was to determine the succession of microbial communities on organo-mineral complexes, in relation to mineral composition and substrate availability over a period of 18 months. We hypothesized that 1) different minerals and/or charcoal select for specific colonizers. In particular, we hypothesized that 2) incubation time and therefore substrate availability influences microbial colonization of different mineral surfaces, and that 3) microbial succession follows copiotrophic and oligotrophic strategies based on different nutritional needs. To test our hypotheses, we used samples from an artificial soils experiment (Pronk et al., 2012).

The structure and activity of the microbial community were determined using phospholipid fatty acid (PLFA) and enzyme analyses. The abundance of 16S rRNA genes, fungal ITS fragment as well as the abundances of seven different taxa were quantified with quantitative PCR (qPCR).

5.3 Material and Methods

Experimental design

A series of eight different artificial soils was produced as described in detail by Pronk et al. (2012). The artificial soils covered a wide range of complexity (two-component systems to three- to four-component systems), but were restricted to eight combinations most likely found in nature. Quartz (Q; quartz sand, silt-sized quartz and clay-sized quartz) was mixed with one or more combinations of the model components montmorillonite (MT), illite (IL), ferrihydrite (FH), boehmite (B) and charcoal (CH) (Table 5.1); each treatment was prepared in triplicate.

Table 5.1 Composition of the artificial soils in % mass contribution of each model component (100% excluding manure). Manure was added at a rate of 1.5% of mass of mineral components, i.e. 45 g kg^{-1}.

	soil composition							
model component	MT	IL	FH	MT*IL	MT*CH	IL*FH	IL*B	IL*FH*CH
quartz sand	41.7	40.0	41.4	40.8	41.7	40.0	40.0	40.0
silt-sized quartz	52.0	52.0	52.0	52.0	52.0	52.0	52.0	52.0
montmorillonite	6.3	-	-	3.2	4.3	-	-	-
illite	-	8.0	-	4.0	-	7.0	7.0	5.0
clay-sized quartz	-	-	5.6	-	-	-	-	-
ferrihydrite	-	-	1.0	-	-	1.0	-	1.0
boehmite	-	-	-	-	-	-	1.0	-
charcoal	-	-	-	-	2.0	-	-	2.0
manure	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5

All artificial soils had similar texture with only slight variations in the sand and clay mineral or clay-sized quartz fractions. This derived from variable additions of the model compounds and clay-sized quartz to keep the mass of the size fraction <6.3 µm constantly at 5.6%. %. Ferrihydrite, boehmite and charcoal were considered as part of this fraction because of their high reactivity and specific surface area. Dried and sterilized horse manure was added as a nutrient and substrate source for microbial growth. The soils were inoculated (60 mL inoculum to each batch of 1 kg) with a microbial community extracted from the topsoil of an Eutric Cambisol (pH 6.5) obtained from the $Ca(NO_3)_2$ treatment of a long-term field trial at Ultuna, Sweden. The inoculum was prepared by shaking a soil suspension (soil (g) to water (mL) ratio of 1:2) for 2 h with gravel and centrifugation at 1000 *g* for 12 min. The supernatant was then centrifuged at 4000 *g* for 30 min and the precipitate was re-suspended in water. Soils were incubated in the dark at a temperature of 20 °C and a constant water content of 60% of maximum water holding capacity. Subsets of batches were sampled after 3, 6, 12 and 18 months and stored at -20°C for later analysis.

Enzymatic activity analysis

The activities of β-glucosidase, β-xylosidase, N-acetyl-glucosaminidase and acid phosphatase were determined using fluorogenic substrates according to Marx et al. (2001). The substrates containing the fluorescent compound 4-methylumbelliferone (4-MUF) were obtained from Sigma-Aldrich (USA). Substrates and buffer were prepared according to Poll et al. (2006). Fifty mL autoclaved water was added to 2 g fresh soil, dispersed by ultrasonication for 2 min with 50 J s^{-1} and 50 µL of the soil suspension were transferred into a 96-well microplate (PP F black 96 well; Greiner Bio-One GmbH, Germany). The soil suspension was mixed with 50 µL buffer (MES-buffer: 2-[N-Morpholino] ethanesulfonic acid; pH 6.1; 0.1M) and 100 µL substrate solution. Measurements were performed with three analytical replicates. Standards were mixed with 50 µL soil suspension and buffer to give final concentrations of 0, 0.5, 1, 2.5, 4 and 6 µM. The plates were incubated for 3 h at 30 °C, and fluorescence was measured after 30, 60, 120 and 180 min in a microplate reader (FLX 800, Microplate Fluorescence Reader, Bio-Tek Instruments Inc., USA). Enzyme activity corresponded to an increase in fluorescence and was calculated according to the standards in nmol g^{-1} soil h^{-1}.

Urease activity was measured according to Kandeler and Gerber (1988) with some modifications. To measure urease activity, 0.3 g moist soil was mixed with 1.5 mL of 0.08 M urea substrate solution (Merck, Germany) and incubated at 37 °C for 2 h. The released NH_4^+ was extracted with 12 mL of a 1 M KCl/0.01 M HCl solution and determined by a modified Berthelot reaction. Urease activity was calculated as µg N g^{-1} $2h^{-1}$.

PLFA analysis

Phospholipid fatty acids were extracted from 2 g soil and fractionated and quantified as described by Bardgett et al. (1996). This procedure is based on the method of Bligh and Dyer (1959) and modified by White et al. (1979). A Bligh and Dyer (chloroform, methanol, citrate buffer (pH 4; 1:2:0.8; v/v/v)) mixture was used as extraction solvent. The fractionation of the lipids was performed with silica acid columns (0.5 g silicic acid, 3 mL Varian Medical Systems, Inc., USA) and the PLFAs were eluted with methanol. The methanolysis of the PLFAs was conducted with 0.2 M methanolic KOH. Samples were stored at -20 °C until analysis. Fatty acid methyl-esters were identified by chromatographic retention time and comparison with a standard mixture of qualitatively defined fatty acid methyl-esters ranging from C11 to C20 (Sigma Aldrich, Germany). The abundances of individual fatty acid methyl-esters for each sample were expressed per unit dry weight. The sum of the following PLFAs represents the bacterial biomass: i15:0, a15:0, i16:0, 16:1ω7, i17:0, cy17:0, 18:1ω7, cy19:0 (Frostegård et al., 1993; Zelles, 1999). The PLFAs i15:0, a15:0, i16:0, and i17:0 are predominantly found in Gram-positive bacteria, cy17:0 and cy19:0 in Gram-negative bacteria. The PLFAs 18:1ω9 and 18:2ω6,9 were used as markers of fungal biomass (Frostegård and Bååth, 1996).

DNA extraction

DNA was extracted from 0.3 g soil using the FastDNA spin Kit for soil (MP Biomedicals, Germany). The concentration of the extracted DNA was measured with a NanoDrop ND 2000c Spectrophotometer (Thermo Scientific, Germany) at a wave length of 260 nm. The purity of the DNA was determined using the quotients of the intensities at 260 and 280 nm and at 230 and 260 nm, respectively.

Standards for qPCR

Standards for qPCR of 16S rRNA genes and the fungal ITS fragment as well as for 7 different taxa were prepared using pure strains (Table 5.2) from the DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany). After re-cultivation, DNA was extracted and amplified using specific primers (Table 5.3). PCR products of each strain were ligated into pGEM-T Easy Vector, and *E. coli* JM 109 was transformed according to the manual of Promega (Promega, Germany). After cultivation of the clones, plasmid DNA was extracted and the insert sequenced (GATC Biotech AG, Germany). After the correct sequence was identified, the plasmid DNA was linearized and a dilution series of 10^2 to 10^8 copies μL^{-1} was then prepared for each standard. The primer specificity is given in Fierer et al. (2005) and Ditterich et al. (2013).

Table 5.2 Pure strains for the standard production for the qPCR

DSMZ	Strain	Target sequence	Reference
11244	*Acidobacterium capsulatum*	*Acidobacteria*	Fierer et al., 2005
2117	*Arthrobacter crystallopoietes*	*Actinobacteria*	Fierer et al., 2005
10	*Bacillus subtilis subsp. subtilis*	*Firmicutes*	Fierer et al., 2005
1565	*Hyphomicrobium facile sub. facile*	*Alphaproteobacteria*	Fierer et al., 2005
645	*Variovorax paradoxus*	*Betaproteobacteria*	Fierer et al., 2005
14586	*Gemmatimonas aurantiaca*	*Gemmatimonadetes*	Philippot et al., 2009
4136	*Verrucomicrobium spinosum*	*Verrucomicrobia*	Philippot et al., 2009
		16S rRNA genes	Ditterich et al., 2013
1334	*Saccharomyces cerevisiae*	fungal ITS fragment	Ditterich et al., 2013

Quantitative PCR assay

The quantification of 16S rRNA genes, fungal ITS fragment and the seven taxa were carried out with an ABI Prism 7500 Fast (Applied Biosystems, Germany) using SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Germany). The primer sets and temperature program are described in Table 5.3. Each SYBR Green reaction contained 0.75 µL of each primer (5 µM), 7.5 µL SYBR Green PCR master mix, 0.375 µL T4gp32 (MP Biomedicals, Germany), 4.125 µL H_2O and 1.5 µL of diluted soil DNA (5 ng μL^{-1}).

Statistical analyses

Significant differences for enzyme activities, PLFAs and qPCR results were tested by two-factorial ANOVA in R (R version 3.0.3, R Core Team, 2014) with soil and incubation time as categorical predictors. For the PLFAs, MANOVA and discriminant analyses of principal components (DAPC) using the *adegenet* package (Jombart, 2008; Jombart and Ahmed, 2011) for R software were also conducted. If necessary, data were log transformed.

Table 5.3 Primers and conditions for PCR and quantitative PCR

Target sequence	Primer[a]	PCR conditions	qPCR conditions[b]	Reference
16S rRNA genes	341F: CCT ACG GGA GGC AGC AG	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 60°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 60°C, 30 s at 70°C, 30 s at 75°C (m.o.f.)	López-Gutiérrez et al., 2004
	515R: ATT ACC GCG GCT GCT GGC A			
fungal ITS fragment	ITS 3F: GCA TCG ATG AAG AAC GCA GC	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 55°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 55°C, 30 s at 70°C, 30 s at 76°C (m.o.f.)	Manerkar et al., 2008 White et al., 1990
	ITS 4R: TCC TCC GCT TAT TGA TAT GC			
Acidobacteria	Acid31: GAT CCT GGC TCA GAA TC	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 55°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 55°C, 30 s at 70°C, 30 s at 81°C (m.o.f.)	Fierer et al., 2005
	Eub518: ATT ACC GCG GCT GCT GG			
Actinobacteria	Actino235: CGC GGC CTA TCA GCT TGT TG	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 60°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 60°C, 30 s at 70°C, 30 s at 82°C (m.o.f.)	Fierer et al., 2005
	Eub518: ATT ACC GCG GCT GCT GG			
Firmicutes	Lgc353: GCA GTA GGG AAT CTT CCG	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 60°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 60°C, 30 s at 70°C, 30 s at 79°C (m.o.f.)	Fierer et al., 2005
	Eub518: ATT ACC GCG GCT GCT GG			
Alphaproteobacteria	Eub338: ACT CCT ACG GGA GGC AGC A	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 60°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 60°C, 30 s at 70°C, 30 s at 79°C (m.o.f.)	Fierer et al., 2005
	Alfa685: TCT ACG RAT TTC ACC YCT AC			
Betaproteobacteria	Eub338: ACT CCT ACG GGA GGC AGC A	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 55°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 55°C, 30 s at 70°C, 30 s at 80°C (m.o.f.)	Fierer et al., 2005
	Bet680: TCA CTG CTA CAC GYG			
Gemmatimonadetes	Gem440: TTC GGR KTG TAA ACC ACT G	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 60°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 58°C, 30 s at 70°C, 30 s at 78°C (m.o.f.)	Philippot et al., 2009
	Eub518: ATT ACC GCG GCT GCT GG			
Verrucomicrobia	Ver349: GYG GCA SCA GKC GMG AAW	600s at 95°C, Cycle (35): 30 s at 95°C, 30 s at 60°C, 45 s at 72°C, (end) 300s at 72°C	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 60°C, 30 s at 70°C, 30 s at 78°C (m.o.f.)	Philippot et al., 2009
	Eub518: ATT ACC GCG GCT GCT GG			

[a]M=A+C, R=A+G, W=A+T, S=G+C, Y=C+T, K=G+T
[b]m.o.f. = measurement of fluorescence

5.4 Results

Enzyme analyses

Acid phosphatase activity ranged between 6.62 and 46.4 nmol g^{-1} h^{-1} and was significantly affected by incubation time ($F_{3,64}$ = 85.7, P < 0.001) with a strong increase during the incubation (Fig. 5.1a). The timing of this initial increase was significantly affected by the mineral composition of the soils ($F_{21,64}$ = 3.42, P < 0.001). Soils containing metal oxides, especially ferrihydrite, showed a strong increase in phosphatase activity from 3 to 6 months, whereas the other soils showed this strong increase between 6 and 12 months. The β-glucosidase, xylosidase and N-acetylglucosaminidase activities were low and showed no consistent pattern (data not shown).

Urease activity ranged from 12.2 to 97.3 µg g^{-1} $2h^{-1}$ (Fig. 5.1b). During the incubation, a significant decrease in urease activity was detected ($F_{3,64}$ = 19.8, P < 0.001) except in the two treatments with charcoal (MT*CH and IL*FH*CH), and the ferrihydrite treatment without addition of a clay mineral (FH), which showed low urease activity throughout the experiment (soil x time: $F_{21,64}$ = 3.95, P < 0.001).

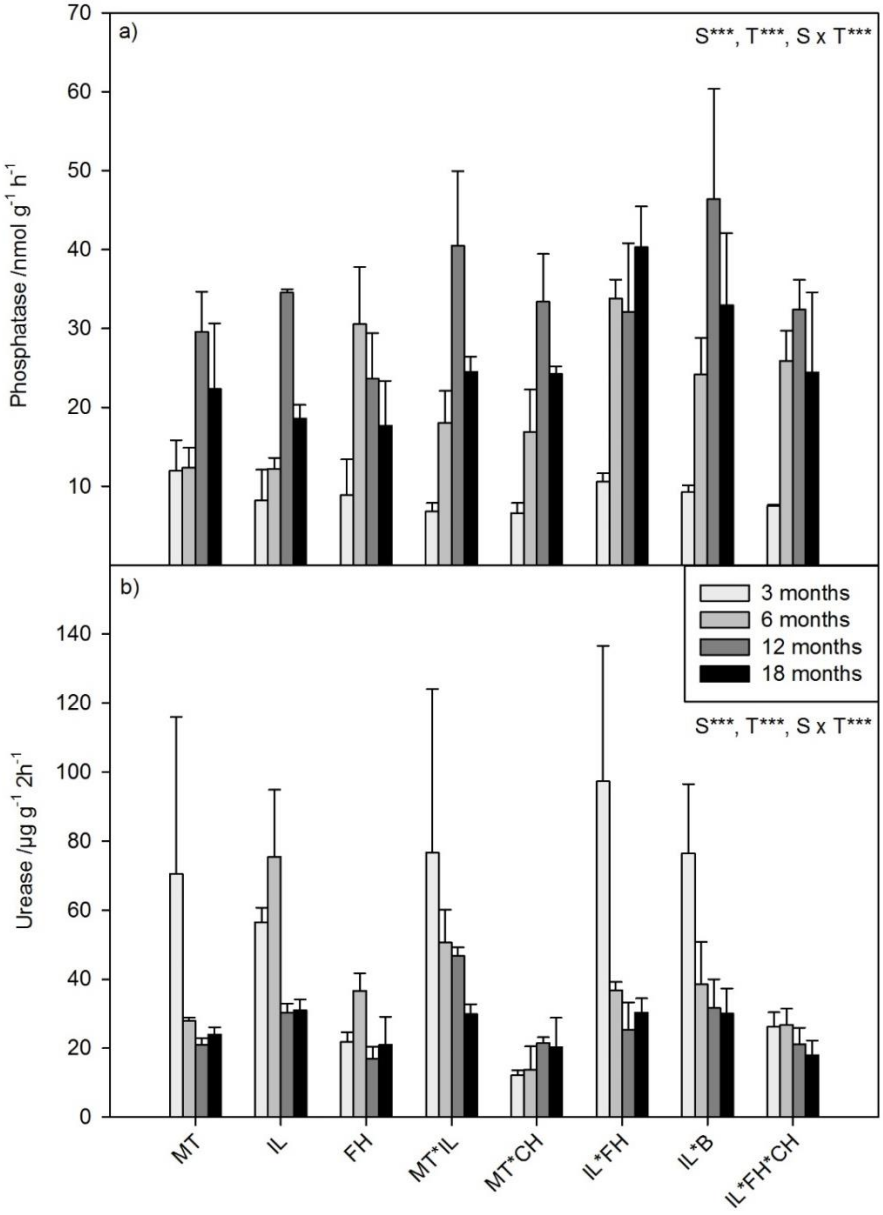

Figure 5.1 Enzyme activities for phosphatase (a) and urease (b) for all artificial soils at four sampling dates. Error bars indicate the standard deviation of three replicates. Asterisks indicate statistically significant differences (*P < 0.05; **P < 0.01; ***P < 0.001) between the treatments (two-factorial ANOVA with S = soil and T = time)

PLFAs

The total amount of bacterial PLFAs ranged from 13.7 to 28.4 nmol g^{-1} for all soils and sampling times (Fig. 5.2a). It increased during the first 6 months of incubation followed by a decrease in all treatments ($F_{3,63}$ = 67.2, P < 0.001). A significant interaction between the factors soil and time ($F_{21,63}$ = 3.35, P < 0.001) was due to higher bacterial abundance in the two-component systems FH and MT up to 6 months, whereas no differences among the artificial soils were detected after 12 and 18 months. The response of Gram-positive and Gram-negative bacteria to incubation time and mineral composition of artificial soils was similar to the response of the whole bacterial community (data not shown).

The total amount (0.53–1.39 nmol g^{-1}) of the fungal PLFAs (Fig. 5.2b) showed similar temporal dynamics to the bacterial PLFAs with a slight increase after 3 months and a decrease after 6 months ($F_{3,63}$ = 33.2, P < 0.001). On average, the content of fungal PLFAs was significantly affected by the mineral composition of the soils ($F_{7,63}$ = 6.10, P < 0.001); MT*CH had the lowest amounts, whereas MT had the highest amounts of fungal PLFAs. The content of fungal PLFAs of the different treatments showed no significant interaction between the factors soil and time. The ratio of fungal to bacterial PLFAs showed only small variation with no consistent pattern.

Multivariate statistical analyses of PLFA data indicated a significant separation of the artificial soils after 3 months of incubation (Fig. 5.3a) ($F_{7,16}$ = 2.9, P < 0.001). Soils containing illite but no charcoal clustered together, whereas the treatment IL*FH*CH was intermediate between this group and the mineral mixture with MT*CH. The two-component systems FH and MT were clearly separated from all other treatments. After 12 months of incubation, mineral composition no longer influenced microbial community composition based on PLFA signature molecules (Fig. 5.3b).

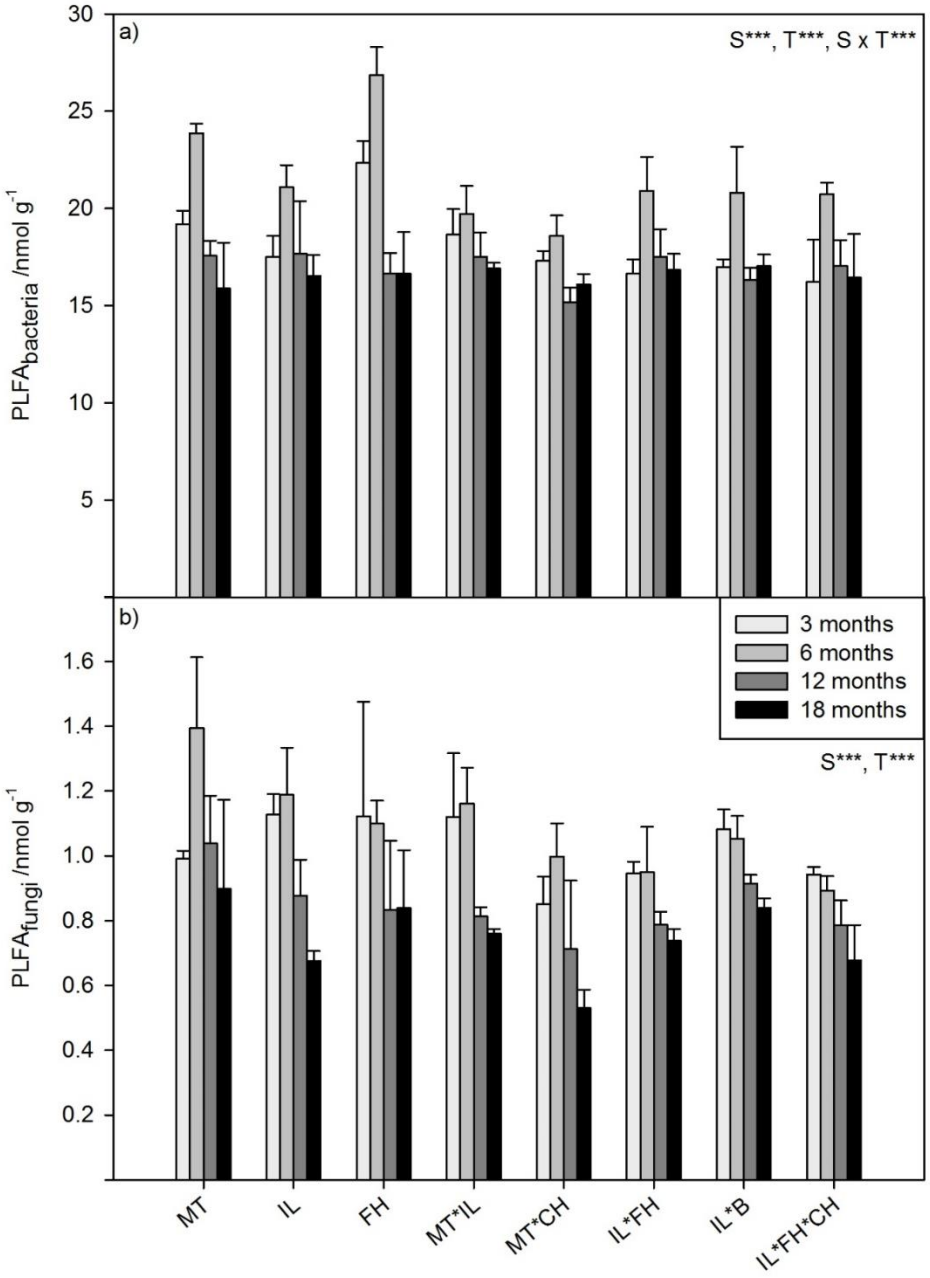

Figure 5.2 PLFA content in all artificial soils for a) bacterial PLFA and b) fungal PLFA for all four sampling dates. Error bars indicate the standard deviation of three replicates. Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) between the treatments (two-factorial ANOVA with S = soil and T = time)

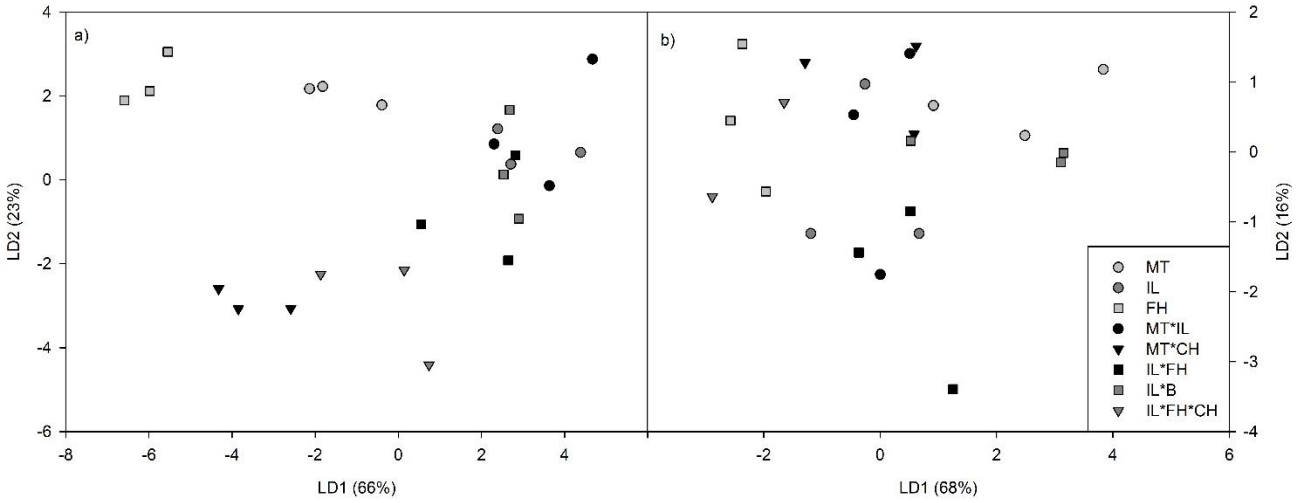

Figure 5.3 Discriminant analysis of principal component (DAPC) for $PLFA_{total}$ after (a) 3 months and (b) 12 months of incubation. In this analysis axis LD1 ((a) 66%, (b) 68%) and LD2 ((a) 23%, (b) 16%) explain most of the variance in the data cumulatively.

qPCR analyses

16S rRNA gene/fungal ITS fragment

Copy numbers of the 16S rRNA gene sequences were within the range of 2.69×10^{10} to 6.67×10^{10} copy numbers g^{-1} for all soils and sampling dates (Fig. 5.4a). During incubation, the abundance of the 16S rRNA gene decreased in most of the artificial soils ($F_{3,64} = 7.03$, $P < 0.001$); only MT*CH and IL treatments showed a different pattern with either lower (MT*CH) or constant abundance (IL), although the soil x time interaction was not significant..

Abundance of the fungal ITS fragment ranged from 1.76×10^{9} to 4.27×10^{10} copy numbers g^{-1} for all soils and sampling dates (Fig. 5.4b). Incubation time significantly affected ($F_{3,64} = 4.17$, $P < 0.01$) copy numbers of the ITS fragment without a consistent pattern. Differences between the artificial soils were significant ($F_{7,64} = 8.89$, $P < 0.001$), but no significant effect of the mineral composition on the temporal development of the ITS gene abundance was detected.

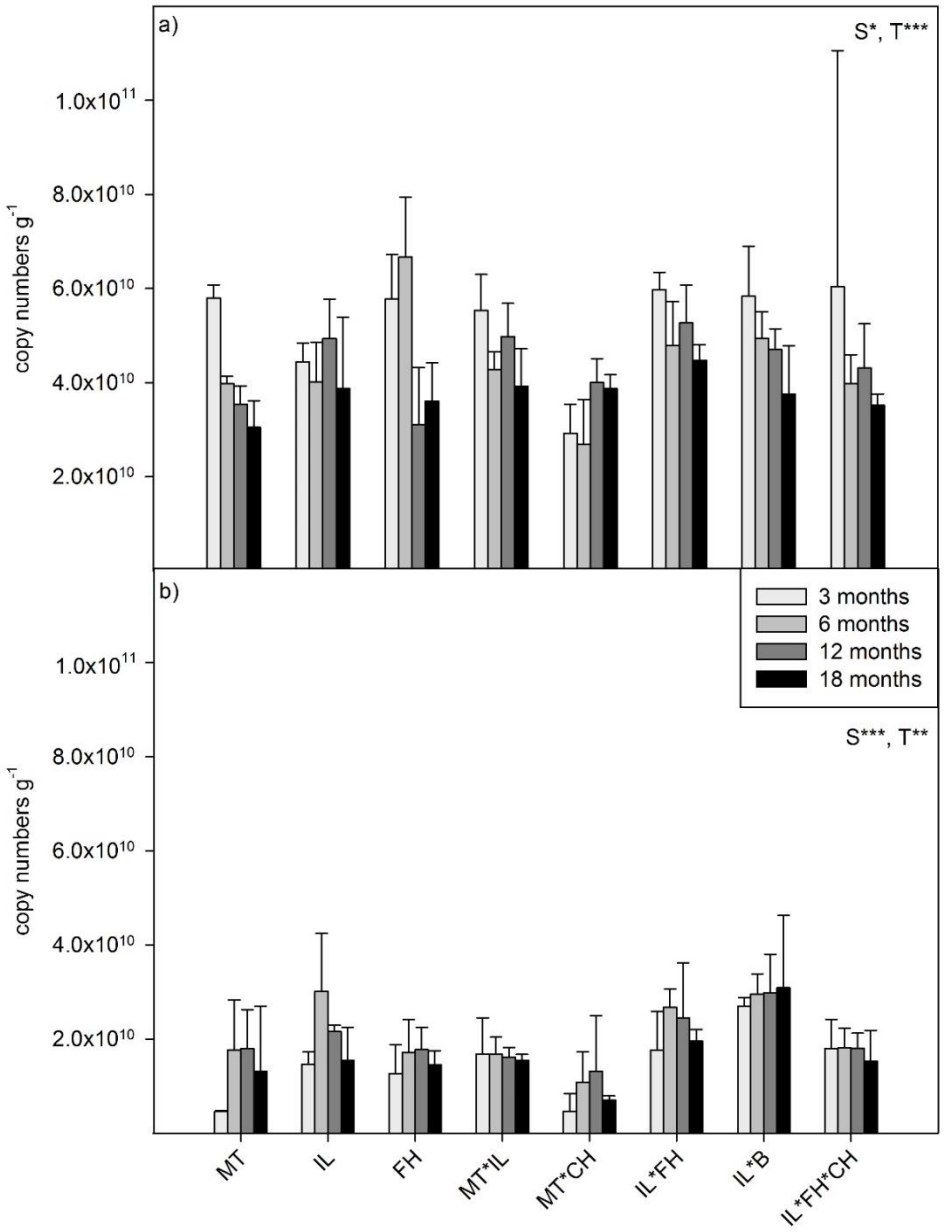

Figure 5.4 Quantities of the 16S rRNA genes (a) and the fungal ITS fragment (b) for all artificial soils at four sampling dates. Error bars indicate the standard deviation of three replicates. Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) between the treatments (two-factorial ANOVA with S = soil and T = time)

Taxa specific genes

Initially, *Acidobacteria* were lowest in abundance, but they strongly increased after 6 months ($F_{3,64}$ = 628, P < 0.001) (Fig. 5.5a). Accordingly, the microbial community composition shifted towards a higher dominance of *Acidobacteria* over time (2.49×10^8 to 3.98×10^{10} copy numbers g^{-1}). The increase after 6 months was less pronounced in soils containing montmorillonite (soil x time: $F_{21,64}$ = 1.65, P = 0.07), which had the lowest abundances after either 12 or 18 months.

The *Actinobacteria* were one of the most abundant phyla, with copy numbers ranging from 1.05×10^{10} to 4.3×10^{10} copy numbers g^{-1} for all treatments and sampling times (Fig. 5.5b). The abundance of *Actinobacteria* showed distinct temporal patterns (time: $F_{3,64}$ = 18.9, P < 0.001) for specific artificial soils (soil x time: $F_{21,64}$ = 1.85, P < 0.05): *Actinobacteria* in the two-component system FH were highest in abundance after 6 months while in soils containing illite (IL, MT*IL, IL*FH, IL*B, IL*FH*CH), copy numbers were highest after 12 months; in the soils MT and MT*CH the abundance of *Actinobacteria* increased until 18 months.

Abundance of *Alphaproteobacteria* was within a range of 1.47×10^9 to 1.35×10^{10} copy numbers g^{-1} (Fig. 5.5c). Incubation time significantly affected their abundance ($F_{3,64}$ = 14.9, P < 0.001). After 3 months, the abundance of the *Alphaproteobacteria* was lowest and an increase was observed in most of the soils after that. In the soils containing illite without metal oxides (IL and MT*IL), copy numbers increased up to the end of the incubation, whereas in soil mixtures containing metal oxides (FH, IL*FH, IL*B and IL*FH*CH) the highest abundances were detected after 6 months (soil x time: $F_{21,64}$ = 3.57, P < 0.001).

Copy numbers of *Betaproteobacteria* were within the range of 1.55×10^9 to 2.56×10^{10} copy numbers g^{-1} (Fig. 5.5d). Incubation time significantly affected their abundance ($F_{3,64}$ = 63.3, P < 0.001), but this was also significantly influenced by the mineral composition of the soils ($F_{21,64}$ = 4.12, P < 0.001). Soils containing metal oxides and/or charcoal (FH, MT*CH, IL*FH, IL*B and IL*FH*CH) showed a strong increase from 3 to 6 months and a strong decrease after 6 months. In contrast, a continuous decrease during incubation was detected for soils containing only clay minerals (MT, IL and MT*IL).

Incubation time significantly affected the abundance of *Firmicutes* ($F_{3,64}$ = 4.83, P < 0.001), but no consistent temporal pattern was observed. The *Firmicutes* showed no significant differences in

their copy numbers (2.4×10^9 to 1.52×10^{10} copy numbers g^{-1}) between the different soil mixtures (Fig. 5.5e).

The abundance of *Verrucomicrobia* ranged between 1.29×10^9 to 4.59×10^9 copy numbers g^{-1} (Fig. 5.5f). Their abundance was initially low and increased slightly during incubation ($F_{3,64}$ = 14.7, $P < 0.001$). The timing of the growth of this bacterial group was affected by the mineral composition of the soils ($F_{21,64}$ = 3.73, $P < 0.001$). In soils containing metal oxides and/or charcoal (FH, MT*CH, IL*FH, IL*B and IL*FH*CH), copy numbers were highest after 6 or 12 months and decreased towards the end of the incubation, whereas soils containing only clay minerals (MT, IL and MT*IL) had highest abundances at the end of the experiment.

The *Gemmatimonadetes* were one of the groups with the lowest abundance (2.7×10^8 to 2.43×10^9 copy numbers g^{-1}) (Fig. 5.5g). This was particularly true after 3 months of incubation, even though after the first sampling, copy numbers increased for all soils ($F_{3,64}$ = 62.7, $P < 0.001$). In most of the soils, the abundance of *Gemmatimonadetes* reached a plateau at the end of the experiment except for the two-component system FH, which showed a strong decrease compared to their abundance after 3 months (soil x time: $F_{21,64}$ = 4.12, $P < 0.001$).

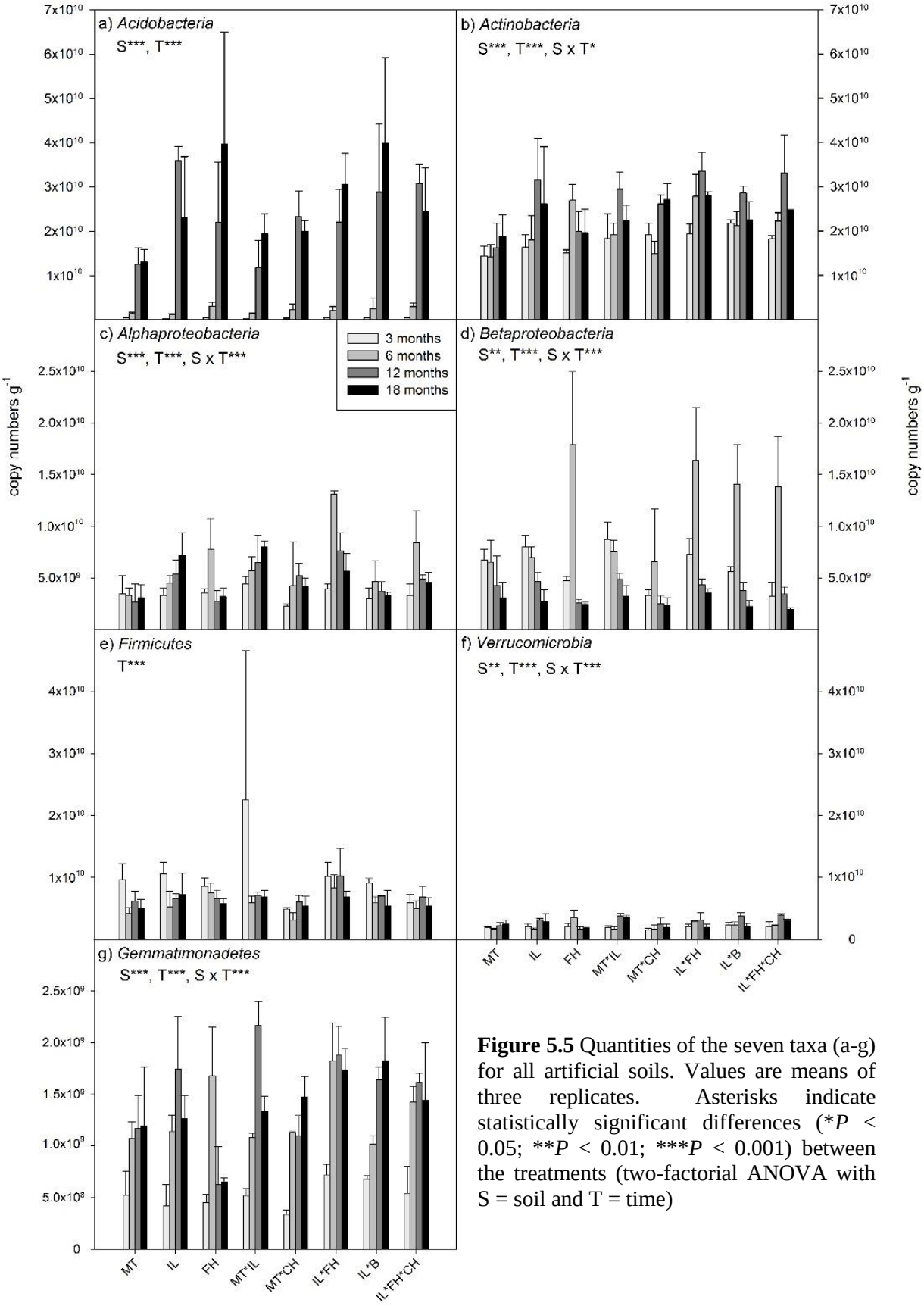

Figure 5.5 Quantities of the seven taxa (a-g) for all artificial soils. Values are means of three replicates. Asterisks indicate statistically significant differences (*P < 0.05; **P < 0.01; ***P < 0.001) between the treatments (two-factorial ANOVA with S = soil and T = time)

5.5 Discussion

The use of artificial soils gave us the opportunity to study the development of the microbial community during the formation of organo-mineral associations on newly exposed secondary mineral surfaces. Our results showed that microbial functioning and succession of microbial communities on mineral surfaces was affected by both mineral composition and substrate availability, which is in accordance with previous studies (Allison, 2006, Gleeson et al., 2005, 2006, Carson et al., 2007, 2009; Lammirato et al., 2010; Uroz et al., 2012; Turner et al., 2014). Other environmental factors which typically influence soil microorganisms are pH, water content and temperature (Fierer and Jackson, 2006; Fierer et al., 2012; Jones et al., 2009). Pronk et al. (2012) did not find any significant differences in CO_2 production, pH, temperature, moisture, OC content and C/N ratio among the artificial soils except for the two treatments with charcoal, which both had higher OC content. Therefore, differences in the microbial community structure of the artificial soils were probably due to specific physicochemical properties of the mineral surfaces such as surface charge or elemental composition. Slight differences in soil texture may also explain our findings; however, mass of model compounds and clay-sized quartz varied only between 10.8% and 12.5% and the fraction < 6.3 µm was 5.6% for all artificial soils. These differences are much smaller than for mineral properties like specific surface area (Pronk et al., 2012) and probably explain microbial functioning and succession in the artificial soils only to a small extent.

Succession of microbial community composition and function in artificial soils

In our study, the same amount of manure was added to all treatments, and both OC content and C/N ratio decreased in all samples over time (Pronk et al., 2012). The loss of OM was accompanied by decreasing numbers of macro aggregates after 12 months, probably due to the stabilizing effect of OM. The decrease in OC content was accompanied by the depletion of substrates for microbial nutrient mineralization, which we measured by different potential enzyme activities. Urease activity, which is an indicator of eutrophication (Dick and Tabatabai, 1992, Kandeler et al., 1994), strongly decreased after 3 to 6 months in most of the artificial soils, indicating a decrease of available N over time. In contrast, phosphatase activity generally increased for up to 12 months, which may have been a result of initial product inhibition due to high amounts of available phosphate in the added manure (Allison and Vitousek, 2005; Pabón-

Pereira et al., 2014). After 3 months, available phosphate may have been depleted and microorganisms could have started to produce phosphatases to mineralize organic P resources (Allison and Vitousek, 2005).

During the incubation, we also observed a succession in microbial community composition. In previous studies, changes in bacterial community composition, diversity and richness were detected along with soil and ecosystem development (e.g. Tarlera et al., 2008; Uroz et al., 2014). In our study, the incubation period was much shorter and the experiment was not intended to simulate the development of natural soils. It was previously shown, that the microbial community in the artificial soils developed within the first 3 months and differed from that in the inoculum (Ding et al., 2013). On a larger time scale, the microbial community structure showed, based on the PLFA data, an initial separation of microbial populations due to direct and indirect (e.g. via aggregation) effects of the initial mineral composition which disappeared after 12 months of incubation and was accompanied by a decrease in bacterial biomass. Initially, soil microorganisms were neither nutrient nor substrate limited, and the main driver of microbial succession in artificial soils were the properties of mineral surfaces (see *Influence of mineral composition*). Towards the end of the incubation, nutrient and substrate limitation, as indicated by the enzyme activities, became evident in all soils as the main drivers of microbial succession.

Whereas PLFA measurements gave information about microbial community changes at low taxonomic resolution, results of qPCR measurements indicated shifts of different bacterial taxa and phyla. Several studies have shown that ecological strategies may be conserved at a high taxonomical level including the oligotrophic life-strategy for *Acidobacteria* and *Verrucomicrobia* as well as the copiotrophic life- strategy for *Betaproteobacteria* (Fierer et al., 2007; Nemergut et al., 2010). *Betaproteobacteria*, often related to as rapidly growing copiotrophic bacteria found in nutrient rich environments (Fierer et al., 2007), showed the highest abundance in the first 6 months of our experiment when substrate availability was high. In contrast, *Acidobacteria* are known as oligotrophic bacteria and as specialists in nutrient poor areas, being less reliant on easily available substrates (Fierer et al., 2007). We observed an increase in the abundance of *Acidobacteria* over the duration of our experiment, which could be explained by increasing limitation of easily available substrates. *Actinobacteria*, *Verrucomicrobia* and *Gemmatimonadetes* exhibited similar behaviors with generally highest abundances after 12 or 18 months. The congruency in the temporal patterns of these three taxa and the *Acidobacteria*

suggests that they followed the oligotrophic life-strategy in our experiment. In contrast to Fierer et al. (2007), our data indicated an oligotrophic nutritional preference of the *Actinobacteria*. Differences between our study and the literature with regard to the life strategies of soil bacteria may be explained by differences in pH, temperature, substrate and water availability between the experiments (Barnard et al., 2013; Castro et al., 2010; Lauber et al., 2009). Cederlund et al. (2014) reported that N-fertilization may be the main driver for changes in the abundance of bacterial phyla or classes, whereas community changes induced by organic amendments are manifested at lower taxonomic levels. Furthermore, not all members of a phylum necessarily follow the same nutritional strategies, or the copiotroph–oligotroph spectrum may not apply to certain taxa (Fierer et al., 2007).

In summary, we observed a shift in microbial community structure and activity which was related to substrate limitation at the end of the incubation. However, separation of microbial PLFA profiles after 3 months and distinct temporal patterns in the urease activity of different artificial soils clearly indicate an interaction between substrate availability and mineral composition.

Influence of mineral composition as well as charcoal on the microbial colonization of artificial soils

Previous studies have indicated that microbial colonization of mineral surfaces depends on the specific surface area, the charge density of the mineral surface and the chemical composition of the mineral (Roberts, 2004; Gleeson et al., 2005, 2006; Uroz et al., 2012). In our study, the effect of minerals as well as charcoal on enzyme activities and the abundance of different taxa were most pronounced during the first 6 months of the experiment. In line with our hypothesis that soil minerals affect bacterial colonization of surfaces, we detected distinct temporal patterns in enzyme activities and the abundance of different taxa for specific artificial soils.

Since the actual specific surface area (SSA) was low and similar for all artificial soils (2.5-3.7 m^2 g^{-1}, Pronk et al., 2012), we suggest that microbial colonization and function of the artificial soils depended on the complexity of mineral composition and/or other mineral properties like surface charge, element composition and cation exchange capacity (CEC).

Recent studies have reported that the activities of soil enzymes are influenced by mineral composition and content as well as the availability of substrates (Allison and Vitousek, 2005;

Boyd et al., 2007; Lammirato et al., 2010). In our study, urease activity was positively affected by the presence of clay minerals at the beginning of the experiment, whereas ferrihydrite did not change urease activity, which is in accordance with Allison (2006). Since neither the hydrolysable N fraction nor ammonia concentration differed between the FH and other treatments (Pronk et al. 2013), differences in substrate concentration (urea) and released product (ammonium) could not explain the low urease activity in these treatments. Nevertheless, we could link our result to the low protein content of the FH treatment: The relatively low urease activity in the FH treatment may have been caused by low stabilization of proteins (including enzymes) in the ferrihydrite treatments (Pronk et al. 2013). This agrees with Lammirato et al. (2010) who reported that enzyme activity was affected by minerals due to the adsorption of enzymes, which was controlled by the charge density of the mineral surface. In contrast to urease activity, we detected an increase in phosphatase activity in the presence of both metal oxides. This result is in accordance with Allison (2006) who also demonstrated a slight enhancement of phosphatase activity in the presence of ferrihydrite. Contrary to these findings, Turner et al. (2014) showed that phosphatase activity was positively correlated with clay content and negatively with that of oxalate-extractable Fe and Al. The contrasting results in the literature may be due to differences in experimental conditions such as pH or C/P and C/N ratios. The opposing patterns of urease and phosphatase mirrored the availability of C, N and P in the different artificial soils, in accordance with Allison and Vitousek (2005).

The microbial community responded differently to the presence of clay minerals and metal oxides. The main difference between clay minerals on the one hand and metal oxides on the other hand became obvious in the timing of the highest abundance of specific taxa. For *Alphaproteobacteria, Betaproteobacteria* and *Verrucomicrobia*, growth was accelerated in all soils containing metal oxides compared to soils containing only clay minerals; for *Actinobacteria* and *Gemmatimonadetes* this was observed only for the soil with ferrihydrite alone compared to all other soils, which contained at least one clay mineral. The slightly alkaline pH (~7.6) of the artificial soils was close to the point of zero charge of synthetic ferrihydrite (Pronk et al. 2012). As the net charge of ferrihydrite was low, microbial colonization of the ferrihydrite surface and adsorption of dissolved organic compounds were unfavorable. We hypothesize, therefore, that low binding of easily available substrates to the surface of ferrihydrite induced an initially high substrate availability followed by faster substrate depletion compared to montmorillonite or illite.

This hypothesis is supported by the response of the copiotrophic *Betaproteobacteria*, which showed the strongest response of all taxa to the presence of metal oxides after 6 months of incubation, followed by a strong decrease. Therefore, ferrihydrite initially provided an environment with sufficient substrate followed by fast substrate depletion due to negligible adsorption and thus stabilization of substrates by ferrihydrite under the experimental conditions. Besides the availability of substrate, Roberts (2004) demonstrated that positively charged metal oxides were colonized by microbes to a greater extent than silicate surfaces. Since the ferrihydrite in our experiment was almost neutral and probably less OM was associated with ferrihydrite than with clay minerals (Heister et al., 2012), we could not find a generally pronounced microbial colonization of soils containing ferrihydrite.

Both clay minerals (montmorillonite and illite) were predominantly associated with OM (Heister et al., 2012) and did not differ in their microbial colonization of different taxa and phyla during the early phase of development. However, towards the end of the incubation montmorillonite decreased the abundance of *Acidobacteria* and delayed the growth of *Actinobacteria*. Macht et al. (2011) and Heister et al. (2012) showed that montmorillonite had a higher CEC with higher amounts of Ca, Mg, Na and K than illite, although total contents of some cations did not differ between artificial soils due to the addition of manure. Most soil microorganisms are attached to mineral surfaces and therefore the local availability of nutrients driven by the CEC and loading of the mineral surface is more important than the total content. This difference between both clay minerals may become important under substrate limitation at the end of incubation. Both *Acidobacteria* and *Actinobacteria* followed the oligotrophic life-strategy in our study, and we therefore suggest that survival of copiotrophic microorganisms was improved in soils containing montmorillonite. This probably increased the competition between copiotrophic and oligotrophic organisms resulting in lower abundances of *Acidobacteria* and *Actinobacteria*. In soils without montmorillonite, reduced survival of copiotrophic organisms relieved oligotrophic microorganisms from competitive restrictions and consequently, higher abundances of *Acidobacteria* and *Actinobacteria* were detected. This agrees with Roberts (2004), who reported that both the abundance and diversity of microorganisms were influenced by toxic or beneficial elements present at the mineral surface. Furthermore, Glesson et al. (2005, 2006) demonstrated that different minerals supported distinct bacterial and fungal populations, whereas community structure was driven by the elemental composition of the mineral. Carson et al. (2007) reported

that microbial community structure in nutrient poor environments depended on the addition of minerals with beneficial nutrients.

In contrast to the clay minerals and metal oxides, the effect of charcoal on microbial community structure and function of the artificial soils was weak and more pronounced after up to 6 months of incubation. The charcoal used was relatively strongly charred; therefore Pronk et al. (2012) suggested that its transformation rate was relatively low. Although addition of charcoal increased the C content, urease activity was low in the presence of charcoal, indicating poor substrate availability. This may be explained by the porous structure of charcoal in which substrates can diffuse, and by strong adsorption of OM to charcoal (Lehmann et al., 2011) or by lower protein content in the treatments containing charcoal, indicating low stabilization potential and therefore lower enzyme activity (Pronk et al. 2013). Further, more organic N was bound to charcoal at the beginning of the experiment and therefore less substrate was available in the charcoal containing treatments compared with ferrihydrite (Pronk et al., 2013). In general, the effect of charcoal on bacterial abundance was negative, especially at the beginning of the experiment. This may have been due to an initial short-term toxic effect of the added charcoal on the microbial community based on an enrichment of toxic compounds during manufacturing (Lehmann et al., 2011; Domene et al., 2015). For up to 6 months of incubation, *Betaproteobacteria* showed a slightly lower and *Acidobacteria* a slightly higher abundance in the presence of charcoal compared to the other artificial soils. The response of both taxa to charcoal addition indicated that charcoal initially provided a nutrient poor environment; overall, however, this response was weak and no specific patterns for oligotrophic or copiotrophic bacteria could be detected. Ding et al. (2013), who used the same artificial soils for up to 90 days of incubation, also showed that the relative abundances of *Sphingomonas* and *Streptomyces*, two genera which are present in oligotrophic environments, were significantly higher in the artificial soil MT*CH. During incubation, the effect of charcoal on the microbial community disappeared and no significant differences in microbial community structure based on the PLFA patterns were found. This may have been a result of fast adsorption of OM to charcoal, leading to a significant reduction in the amount of available charcoal surfaces due to occlusion of the surface within the first days of incubation (Pronk et al., 2012).

In summary, we observed a preferential colonization of specific artificial soils by different taxa due to availability of substrates. The main factor affecting the succession of bacterial taxa in

artificial soils was the presence or absence of metal oxides. Generally, soils containing metal oxides allowed fast growth of most of the bacterial taxa, probably due to low capacity to bind substrates under the experimental conditions. Higher CEC of montmorillonite compared to illite probably favored growth of copiotrophic bacteria towards the end of the incubation, whereas charcoal only had minor impact on the initial colonization of the artificial soils.

5.6 Conclusions

Our study demonstrated that the colonization of the artificial soils was shaped by minerals, charcoal and the availability and quality of substrate. Whereas at the beginning of the experiment mineral composition and therefore differences in mineral properties such as surface charge and adsorption of OM were the main drivers of colonization, substrate limitation was the driving factor for microbial community structure and function in artificial soils at the end of incubation. In general, microbial community structure changed from a dominance of copiotrophic bacteria over a period of 18 months towards oligotrophic bacteria and to more similar communities due to substrate depletion. Charcoal had only a slight initial negative effect on bacterial community abundance, whereas the succession of the bacterial community was mainly affected by the presence or absence of metal oxides and clay minerals. The artificial soil experiment clearly showed that it is helpful to use a simple system to study the interaction between minerals and microbes. However, further studies using such model systems are necessary to clarify interactions between mineral surfaces and other environmental factors like pH, which were kept constant in our experiment. This will help identify the main drivers of microbial activity at the habitat scale and foster our understanding of microbial colonization of soils.

6 Succession of bacterial and fungal MCPA degraders at the soil-litter interface[2]

[2] Ditterich et al. (2013); Succession of bacterial and fungal MCPA degraders at the soil-litter interface; FEMS Microbiology Ecology; DOI: 10.1111/1574-6941.12131

6.1 Abstract

Phenoxyacetic acids can be degraded by diverse soil microorganisms. Nevertheless, we miss information about the succession of MCPA (4-chloro-2-methylphenoxyacetic acid) degraders in micro-environments of soils as well as specific functions of different microbial groups during MCPA degradation. We studied MCPA degradation at the soil-litter interface in a microcosm experiment and followed the succession of different degrader populations by quantifying the abundance of 16S rRNA genes as well as, the fungal ITS fragment, and the functional genes *tfdA* (in total and divided into three classes) and *cadA*. Adjacent to the litter layer a dynamic depletion zone of MCPA indicated that the litter effect on MCPA degradation depends on substrate availability and the affected soil volume. The increase of the *tfdA* class III and *cadA* genes were linked to MCPA mineralization. Total abundance of *tfdA* genes was dominated by class I MCPA degraders and did not reflect MCPA degradation potential of the soil. Litter addition induced the development of pioneer and late stage fungal communities, which were probably both involved in MCPA degradation. The results underline the importance of the ecological behavior of different degrader populations for the understanding of herbicide degradation in soils.

6.2 Introduction

In agricultural ecosystems pesticides are applied to reduce the yield losses or to ensure crop health. MCPA (4-chloro-2-methylphenoxyacetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) are the most heavily used phenoxy acid herbicides for control of dicotyledonous plants in cropland. The degradation of both herbicides and their related compounds has been studied intensively since the 1980s (Beadle & Smith, 1982; Shailubhai et al., 1983). Microbial phenoxy herbicide degraders have been identified in different soils worldwide; the diversity of the strains has also been shown (Fulthorpe et al., 1995; McGowan et al., 1998; Bælum et al., 2006; Rodriguez-Cruz et al., 2010). Regulation of the bacterial degradation processes at the organism level has been studied by characterization of the functional genes involved (Don & Pemberton, 1981; Don et al., 1985; Streber et al., 1987; Ka et al., 1994; Fulthorpe et al., 1995; Tonso et al., 1995; Kitagawa et al., 2002; Bælum et al., 2010). The initial degradation step is catalyzed by oxygenases that are either encoded by *cadA* (Kitagawa et al., 2002) or by *tfdA* genes (Streber et al., 1987; Fukumori & Hausinger, 1993). Both oxygenases cleave the ether-bonded acetate side chain of MCPA during the first step of degradation. MCPA and 2,4-D degraders are divided into three groups based on their phylogeny and catabolic gene diversity (Kamagata et al., 1997).

The first group consists of *Beta-* and *Gamma-* subdivisions of *Proteobacteria* harboring a high diversity of *tfdA* genes that encode for an α-ketoglutarate-dependent dioxygenase (Vallaeys et al., 1996; McGowan et al., 1998; Rodriguez-Cruz et al., 2010; Zaprasis et al., 2010). McGowan et al. (1998) suggested that this group can be subdivided into three classes based on differences in their *tfdA* sequences. Using melting curve analyses it was shown that the indigenous degrader community was dominated by class I, whereas class III became predominant during 2,4-D and MCPA degradation (Bælum et al., 2008; Rodriguez-Cruz et al., 2010). This shift could be confirmed by using a TaqMan probe-based quantitative real-time PCR (qPCR) assay (Bælum & Jacobsen, 2009; Batıoğlu-Pazarbaşı et al., 2012). To date, the specific function of each of the classes is not well understood. However, it seems that not all *tfdA* harboring bacteria have the same potential to degrade MCPA. Some of these bacteria have a truncated pathway and rely on bacterial consortia for complete MCPA degradation (Ka et al., 1994; Top et al., 1996). Bælum et al. (2006) suggested that bacteria possessing a truncated pathway belong to the class I MCPA degraders. MCPA degradation by bacterial consortia reduces the energy gain of each single population (Müller & Babel, 2001). Therefore, an increase in the supply of external energy

sources (i.e. soluble litter compounds) might increase the activity of bacterial consortia and their contribution to MCPA degradation.

The second group consists of oligotrophic *Alphaproteobacteria* harboring *cadA* or *tfdAα* genes (Itoh et al., 2004). Kitagawa et al. (2002) suggested that *Alphaproteobacteria* possessing *cadA* genes degrade 2,4-D using an enzyme that is similar to 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) oxygenase and that facilitates the conversion from phenoxyacetate to phenol (Danganan et al., 1994). However, the *cadA* gene shows no sequence similarity to the *tfd* genes (Itoh et al., 2002).

The third group consists of copiotrophic fast growing *Sphingomonas* strains belonging to *Alphaproteobacteria* harboring *cadA* or *tfdA-like* genes (Itoh et al., 2004).

Previous studies have shown that fungi are able to degrade MCPA and 2,4-D as well (Castillo et al., 2001; Vroumsia et al., 2005; Lerch et al., 2009). In contrast to bacterial degradation, however, no specific fungal pathway for phenoxy acid herbicide degradation is currently known. Castillo et al. (2001) have suggested that non-specific enzymes like laccase or peroxidase are involved in the degradation process. We have preliminary evidence that MCPA derived carbon is incorporated into fungal phospholipid fatty acids.

Although huge efforts have been made to identify novel 2,4-D and MCPA degraders, their contribution to degradation activity and their regulation by environmental conditions remains unclear. For example, an increased soil organic matter content increases the sorption of 2,4-D and lowers its availability for microbes resulting in reduced degradation rates (Greer & Shelton, 1992). Furthermore, high observed variability in degradation activity can be explained by the uneven distribution of the degradation community and the necessary C-substrate for co-metabolic degradation of 2,4-D or MCPA (Vieublé Gonod et al., 2003; Liu et al., 2011b). Fredslund et al. (2008) found that degradation is more strongly linked to growth conditions than to the initial abundance of the degradation community. This is supported by Cederlund et al. (2007), who found that MCPA mineralization was N-limited. Other studies have shown that adding plant residues increases MCPA degradation (Duah-Yentumi & Kuwatsuka, 1980; Poll et al., 2010b). The soil adjacent to the litter, the detritusphere, is a hot-spot of MCPA degradation due to the high availability of soluble litter compounds (Poll et al., 2010b). Reduced degradation rates in deeper soil layers might reflect differences in the abundance of degraders or their functional

genes, and there might be also a correlation with organic matter content (Batıoğlu-Pazarbaşı et al., 2012).

Comprehensive studies on the function of different populations of MCPA degraders and their succession during the degradation process are scarce. The objective of this study was, therefore, to determine which group of MCPA degraders contributes to MCPA degradation at the soil-litter interface, one of the most important sites of microbial activity in soils. We hypothesized that i) microorganisms harboring the three different *tfdA* gene classes or the *cadA* gene differ in their ability to use MCPA as a carbon source, and that ii) these degrader communities respond differently to litter addition. In particular, we hypothesized that iii) class I MCPA degraders contribute to MCPA degradation in the presence of litter as an external energy source. To test these hypotheses we performed a microcosm experiment in which we studied the degradation of MCPA at the soil-litter interface over a period of 27 days at 20 °C. We quantified the abundance of 16S rRNA genes, the fungal ITS fragment, *tfdA* and *cadA* sequences to determine the effect of litter and MCPA addition on total bacterial, fungal, and MCPA degrader communities. The succession of *tfdA* harboring bacteria was quantified using a TaqMan probe assay.

6.3 Materials and Methods

Soil and plant residues

We used topsoil from an agricultural field, which was not treated with MCPA or 2,4-D since 1998. The soil was collected in July 2008 at the experimental farm Scheyern, north of Munich (Germany, 48°30´N, 11°2´E). The soil (pH [$CaCl_2$] 5.3, total C content 13.6 g kg^{-1}, total N content 1.32 g kg^{-1}) was classified as a loamy Luvisol (World Reference Base for Soil Resources). The soil was sieved (<2 mm) and stored in the dark at -20 °C. Soil was defrosted in the dark for one day at 20 °C in a climate chamber. It was then homogenized and dried to a gravimetric water content of 22 % before adding MCPA. Drying took one week in order to allow the soil to acclimate. To increase the initial number of MCPA degraders above the detection limit, soil was pre-incubated with 20 mg MCPA kg^{-1} for 8 weeks at 20 °C (Bælum et al., 2008; Poll et al., 2010b), until no more MCPA was extractable.

Maize leaf litter and stems were air dried and shredded into pieces of 2-10 mm.

Experimental design

The experimental treatments were as follows: (I) control, (II) addition of MCPA, (III) addition of litter and (IV) addition of MCPA and litter. After pre-incubation, the soil was homogenized and half the soil was spiked with 50 mg kg^{-1} MCPA. We applied a rather high MCPA dose to stimulate a clear response of the degrader community, which allows identification of underlying mechanisms of MCPA degradation at the soil-litter interface. Gravimetric soil water content was adjusted to 28.8 % (pF 1.8). Cylinders (diameter = 5.6 cm, height = 4 cm) were then filled with the soil which was compacted to a bulk density of 1.2 g cm^{-3} (height of the soil core was 3 cm). Each soil core was placed in an airtight microcosm (Poll et al., 2010b) on a ceramic plate applying a pressure of -63 hPa (corresponding to a matrix potential of pF 1.8) at the lower boundary. For the litter treatments, 0.25 g leaf litter and 0.25 g stem per cylinder were added. Before application to the soil cores, litter was rewetted with 2 mL of a 0.01 M $CaCl_2$ solution for 4 hours. The microcosms were incubated at 20 °C for 27 days in a climate chamber and were sampled after 3, 6, 10, 15 and 27 days. For each treatment and sampling date three replicate microcosms were prepared. During the incubation, the samples were irrigated once a week: After one day with 4 mL and the subsequent three times with 3 mL 0.01 M $CaCl_2$ solution.

Respiration

During the incubation, the CO_2-C production was measured 12 times at regular intervals. The evolved CO_2 was trapped in a small bin filled with 3 mL of a 1 M NaOH solution and fixed to the lid of the microcosm. For titrimetric measurement, a 0.5 mL aliquot from each trap was added to 0.5 mL 0.5 M $BaCl_2$ solution to precipitate carbonates and the trapped CO_2 was titrated with 0.1 M HCl.

Sample preparation

After incubation, the litter was removed and the soil cores were frozen at -80 °C. The frozen soil cores were cut into thin slices using a cryostat microtome (HM 500 M, MICROM International GmbH). The slices were taken at 0-1, 1-2, 2-3, 3-4, 4-6, 6-10 and 10-20 mm distance to the soil-litter interface and kept frozen at -20 °C until analysis.

MCPA content in soil

A soil suspension of 1.5 g soil mixed with 7.5 mL of methanol/water (1:1) was homogenized on a horizontal shaker at 420 rpm for 10 min, then heated in a water bath for 30 min at 50 °C. Afterwards, the mixture was centrifuged for 10 min at 2500 g min^{-1} and 2 mL of the supernatant were removed and filtered (0.45 µm pore size). Extraction recoveries of MCPA were 96%. MCPA concentration was determined by HPLC (System Gold, Beckman Instruments) using a UV detector (228 nm) according to the method of Moret et al. (2006). Solutes were separated on a 150 mm x 3 mm column packed with 3 µm Kromasil 100 C18 material (MZ-Analysentechnik GmbH) at 20 °C using a flow rate of 0.5 mL min^{-1}.

DNA Extraction

DNA was extracted from 0.3 g soil using the FastDNA spin Kit for soil (MP Biomedicals Germany). The concentration of the extracted DNA was measured with a NanoDrop ND 2000c Spectrophotometer at a wave length of 260 nm and the purity of the DNA was determined by use of the quotients of 260/280 and 230/260 (Thermo Scientific).

Standards for qPCR

Standards for the qPCR of 16S rRNA genes and the fungal ITS fragment were prepared using pure strains (Table 6.1) from the DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures Braunschweig, Germany). After re-cultivation, DNA was extracted and amplified using primer sets specific for 16S rRNA genes and the fungal ITS fragment, respectively (Table 6.2). The primers for the fungal ITS fragment binds in the regions 5.8S and LSU (White et al., 1990). Specificity was tested by Manerkar et al. (2008) using several bacterial and fungal strains. Nikolcheva et al. (2005) tested the primer pair with DNA from plants, diatoms, algae, bacteria, animals and fungi and obtained a PCR-product only for DNA from *Ascomycetes*, *Basidiomycetes*, *Chytridiomycetes*, *Zygomycetes* (fungi) and *Oomycetes* (stramenopiles). PCR products of each strain were ligated into pGEM-T Easy Vector and E. coli JM 109 was transformed (Promega, Germany). Clones for the preparation of standards for the *tfdA* gene classes and for total *tfdA* were obtained from Bælum et al. (2008), clones for the *cadA* gene from Liu et al. (2011a). With the exception of *cadA* clones, the clones were cultivated and plasmid DNA was extracted. Plasmid DNA of all clones was send to GATC for sequencing

(GATC Biotech AG Konstanz, Germany). After sequences were correctly identified, the plasmid DNA was linearized by using the restriction enzymes SalI for bacterial and fungal clones and NotI for the *tfdA* clones. Standards of *cadA* were obtained by amplifying the insert of a *cadA* containing pGEM-T vector with M13-primers flanking the multiple cloning site of the vector (Liu et al., 2011a). A dilution series of 10^2 to 10^8 copies μL^{-1} was prepared for each standard. The efficiency and the detection limits for the single qPCR assays are provided in Table 6.3.

Table 6.1 Pure strains for the standard production for the qPCR.

Target sequence	Name	DSMZ	Reference
16S	*Verrucomicrobium spinosum*	4136	
fungal ITS fragment	*Saccharomyces cerevisiae*	1334	Fierer et al., 2005

Table 6.2 Primers and conditions for quantitative PCR.

Target sequence	Primer*	qPCR conditions	Reference
16S rRNA genes	341F: CCT ACG GGA GGC AGC AG 515R: ATT ACC GCG GCT GCT GGC A	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 60°C, 30 s at 72°C, 30 s at 75°C (m.o.f.)	López-Gutiérrez et al., 2004
Fungal ITS fragment	ITS 3F: GCA TCG ATG AAG AAC GCA GC ITS 4R: TCC TCC GCT TAT TGA TAT GC	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 55°C, 30 s at 70°C, 30 s at 76°C (m.o.f.)	Manerkar et al., 2008 White et al., 1990
tfdA	F: GAG CAC TAC GCR CTG AAY TCC CG R: GTC GCG TGC TCG AGA AG	600 s at 95°C, Cycle (40): 15 s at 95°C, 30 s at 64°C, 30 s at 72°C, 30 s at 81°C (m.o.f.)	Bælum et al., 2006
cadA	F: AAG CTG CAR TTT GAR AAY GG R: MGG ATT GAA ATC CTG RTA	600 s at 95°C, Cycle (40): 15 s at 95°C, 30 s at 55°C, 30 s at 72°C, 30 s at 80°C (m.o.f.)	Kitagawa et al., 2002
tfdA class I	F: GAG CAC TAC GCR CTG AAY TCC CG R: SAC CGG MGG CAT SGC ATT TaqManProbe: FAM-TTGCGCTTCCGAATAGTCGGTGTC-BBQ	300 s at 95°C, Cycle (40): 30 s at 95°C, 90 s at 62°C (m.o.f) 420 s at 72°C	Bælum & Jacobsen, 2009
tfdA class II	F: GAG CAC TAC GCR CTG AAY TCC CG R: SAC CGG MGG CAT SGC ATT TaqManProbe: YAK-CGTTGACTTTCAGAATACTCTGTGTCG CCA-BBQ	300 s at 95°C, Cycle (40): 30 s at 95°C, 90 s at 62°C (m.o.f) 420 s at 72°C	Bælum & Jacobsen, 2009
tfdA class III	F: GAG CAC TAC GCR CTG AAY TCC CG R: SAC CGG MGG CAT SGC ATT TaqManProbe: FAM-TTGACTTTCAGAATAGTCCGTATCGCCAAG-BBQ	300 s at 95°C, Cycle (40): 30 s at 95°C, 90 s at 62°C (m.o.f) 420 s at 72°C	Bælum & Jacobsen, 2009

m.o.f., measurement of fluorescence.
*M =A or C, S = G or C, R = A or G, Y = C or T.

Table 6.3 qPCR information about efficiency and detection limits.

Gene	Efficiency	R^2	Lowest std	C_t-value	C_t-value sample	C_t-median	C_t-mean
16S	92.1	0.994	e4	25.9	16.5–20.1	17.36	17.36
Fungal ITS fragment	92.7	0.997	e4	26.77	14.5–28.5	18.71	18.17
tfdA	99.3	0.999	e2	28.54	25.9–38.5	31.18	31.11
cadA	100.8	0.995	e2	28.46	30.5–38.1	32.87	32.92
tfdA cI	106.9	0.999	e3	28.07	22.8–35.9	27.17	27.78
tfdA cII	112.6	0.998	e2	27.80	24.3–49.8 or n.d.	32.1	32.65
tfdA cIII	112.6	0.998	e2	27.83	23.6–49.5	30.25	30.55

Quantitative PCR assay

The quantification of the total bacterial (16S rRNA genes), total fungal (fungal ITS fragment), *cadA* and total *tfdA* DNA was carried out with an ABI Prism 7500 Fast (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Germany). The three different *tfdA* gene classes were quantified with TaqMan probes according to Bælum & Jacobsen (2009) on the ABI Prism 7500. The primer sets and the temperature program were previously described (Table 6.2). Each SYBR Green reaction contained 0.75 µL of each primer (5 µM), 7.5 µL SYBR Green PCR master mix, 0.375 µL T4gp32 (MP Biomedicals, Germany), 4.125 µL H2O and 1.5 µL of diluted soil DNA (5 ng μL^{-1}). For total *tfdA* and *cadA* quantification 2 µL DNA template and 3.625 µL water were used. For quantification of *tfdA* gene classes the master mix was prepared using the Taq all inclusive kit (Promega, Germany). The class I sequence was quantified in a single reaction, class II and III sequences were quantified in a duplex reaction. The single and the duplex reaction contained 2.5 µL Buffer Y, 0.5 µL dNTPs, 0.25 µL Taq, 0.04 µL ROX reference dye (Invitrogen, Germany), 1 µL of each primer (5 µM), 2 µL of each probe (1µM), and 2.5 µL template DNA. Water was added to reach a final reaction volume of 20 µL (10.21 µL and 8.21 µL, respectively).

PCR amplification of internal transcribed spacer (ITS) fragment for denaturing gradient gel electrophoresis (DGGE) analysis

Fungal community was analyzed by DGGE fingerprints of amplified ITS fragments. Based on the quantification of the fungal ITS fragment we assumed that differences in the fungal community were pronounced in the first layer (0-1 mm). Therefore, a nested PCR approach was performed with DNA samples from the first layer (0-1 mm) of the soil cores using primer pairs ITS1F/ITS4 and ITS1FGC/ITS2 (Anderson & Cairney, 2004). PCR conditions were described by Weinert et al. (2009) previously but here 0.025 U μL^{-1} Taq Polymerase (TrueStart Kit,

Fermentas, Germany) and 0.1 mg mL^{-1} BSA (Fermentas, Germany) were used in the first PCR which was run for 35 cycles and 0.05 U μL^{-1} Taq Polymerase (Stoffel Fragment, Applied Biosystems, Germany) in the second PCR which was run for 25 cycles. For both reactions 2 % (vol/vol) DMSO and 0.2 µM primer were used.

DGGE

The DGGE of PCR-amplified fungal ITS fragments was performed in an Ingeny PhorU system (Ingeny, Goes, the Netherlands) using a denaturant gradient of 23 to 58 % (Weinert et al., 2009). Three microliter of each PCR-product were applied to the gel. Joint ITS GC-amplicons from 16 fungal strains differing in electrophoretic mobility were used as marker. DGGE run was performed at 60 °C, 100 V for 18 h in 1x Tris-Acetat-EDTA buffer and the gel was afterwards silver stained according to Heuer et al. (2001).

Statistics

The results were calculated based on oven-dried soil. Significant differences for 16S rRNA genes, the fungal ITS fragment, *tfdA*, *cadA* and the *tfdA* classes were tested by repeated measures ANOVA (STATISTICA 6.0) (Tulsa, OK, USA) with the four different depths (0-1, 1-2, 2-3, 6-10 mm) as the within-subjects factor and litter, MCPA and time as the categorical predictors. For cumulative CO_2 production we also used the repeated measures ANOVA but in this case the within-subjects factor was time and the categorical predictors litter and MCPA. The results for the MCPA concentration were also compared by one-way ANOVA with repeated measures for each time point (within-subjects factor: depth, categorical predictor: litter).

The fungal DGGE community profiles were analyzed using Pearson correlation for calculating similarity coefficient values per lane, and the unweighted pair group method with arithmetic mean (UPGMA) algorithm was applied for cluster analysis (GelCompare II 6.5, AppliedMaths, Belgium).

6.4 Results

Soil respiration and MCPA degradation

The addition of litter significantly increased the cumulative CO_2-C production ($F_{1,8}$ = 681, P < 0.001), whereas adding only MCPA had no effect on respiration (data not shown). MCPA in combination with litter increased cumulative CO_2-C production relative to only litter treatments in the first 15 days and after that time almost no differences were observed between the two litter treatments (MCPA x litter x time, $F_{11,88}$ = 1.91, P < 0.05)

Litter addition significantly reduced extractable MCPA (litter, $F_{1,19}$ = 34.6, P < 0.001), depending on incubation time and distance to the litter layer (litter x time x depth, $F_{24,114}$ = 2.11, P < 0.01) (Table 6.4; Fig. 6.1 A, B). The litter effect on extractable MCPA was observed after 6 and 10 days to 6 mm depth and after 15 days to 10 mm depth.

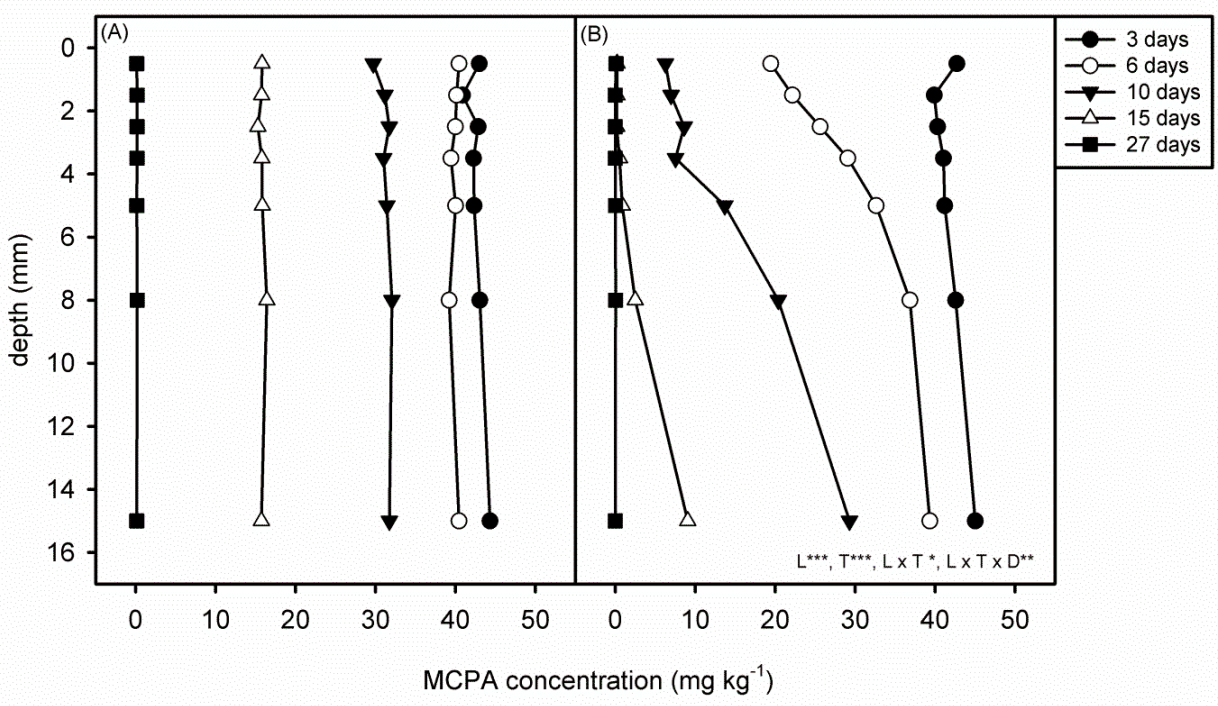

Figure 6.1 Depth profiles of the extractable MCPA at different sampling dates: (A) without litter and (B) with litter addition. Values are means of three replicates. The statistics shown in the graph refer only to the treatments with MCPA (D = depth, L = litter, T = time, MCPA = added MCPA).

Table 6.4 Statistical comparison of extractable MCPA at different sampling dates and depths for the treatment with litter addition. Only significant differences are shown.

	Depth													
	0-1 mm		1-2 mm		2-3 mm		3-4 mm		4-6 mm		6-10 mm		10-20 mm	
Time	$F_{1,4}$	P	$F_{1,4}$	P	$F_{1,4}$	P	$F_{1,4}$	P	$F_{1,4}$	P	$F_{1,4}$	P	$F_{1,4}$	P
3														
6	17.6	0.05	60.4	0.01	40.8	0.01	58.9	0.01	32.4	0.01				
10	19.5	0.05	71.1	0.01	40.5	0.01	51.5	0.01	13.4	0.05				
15	11.3	0.05	23.8	0.05	20.1	0.05	19.4	0.05	17.4	0.05	16.1	0.05		
27														

16S rRNA genes/fungal ITS fragment

The abundance of 16S rRNA gene sequences was within the range of 2×10^{10} and 3×10^{10} copy numbers g^{-1} in the two treatments without litter (Fig. 6.2A, B; Table S10.1, S10.2) with no effects of MCPA, depth, or incubation time. Adding litter significantly increased the abundance of 16S rRNA gene sequences ($F_{1,39} = 94.9$; $P < 0.001$) with copy numbers between 1.9×10^{10} and 3.9×10^{10} g^{-1}. This effect was restricted to the first 3 layers (0-3 mm) (litter × depth, $F_{3,117} = 15.7$, $P < 0.001$), whereas in the layer 6-10 mm no difference in 16S rRNA gene abundance was detected between treatments. Neither MCPA nor incubation time had an effect on 16S rRNA gene abundance in the litter treatments.

The copy numbers of the fungal ITS fragment (Fig. 6.2C, D; Table S10.1, S10.2) were within the range of 5.7×10^9 to 9.7×10^9 for the nonlitter treatments and between 1×10^{10} and 2.1×10^{11} for the litter treatments. Litter addition significantly increased the abundance of the fungal ITS fragment ($F_{1,39} = 366$, $P < 0.001$). Adding MCPA increased the abundance of the fungal ITS fragment only in the presence of litter (litter × MCPA, $F_{1,39} = 65.0$, $P < 0.001$). The magnitude of the litter effect was greatest in the first layer (0-1 mm) and decreased with increasing distance to the litter (litter × depth, $F_{3,117} = 230$, $P < 0.001$). During the incubation, the abundance of the fungal ITS fragment increased in the litter treatments (litter × time, $F_{4,39} = 10.6$, $P < 0.001$). This increase was highest in the first layer (0-1 mm) when litter and MCPA were added together (litter × MCPA × time × depth, $F_{12,117} = 2.43$, $P < 0.01$).

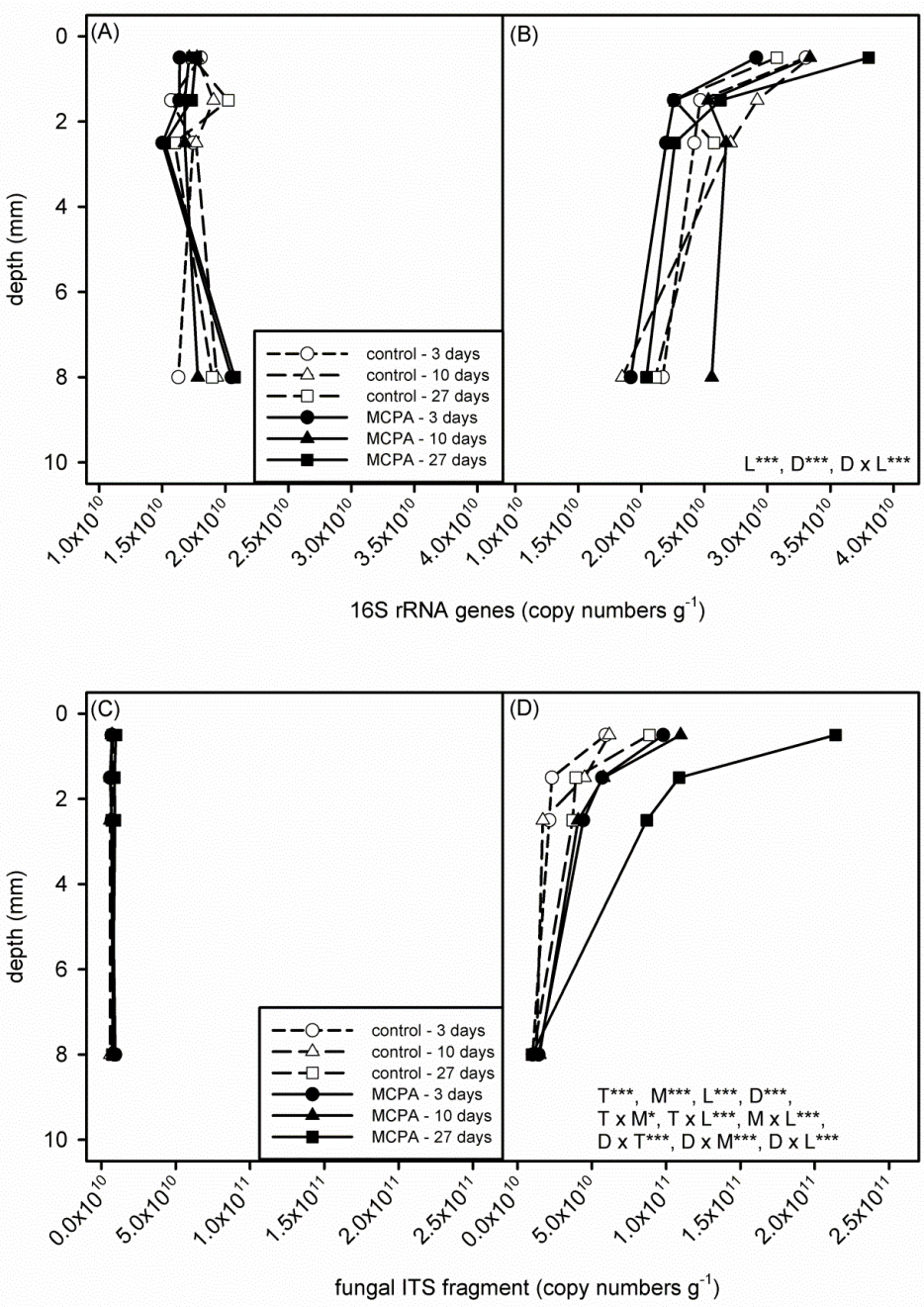

Figure 6.2 Quantities of the 16S rRNA genes (A) without litter and (B) with litter, fungal ITS fragment (C) without litter and (D) with litter addition at three sampling times over depth. Values are means of three replicates. Asterisks indicate statistically significant differences (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$) between treatments (repeated-measures ANOVA with D = depth, L = litter, T = time, M = MCPA). Significant interactions between more than three factors are not shown.

tfdA/cadA genes

Quantification of the MCPA degrading community was performed by qPCR targeting the *tfdA* or *cadA* sequences. The abundance of *tfdA* was within the range of 2.7×10^5 to 8.1×10^6 copy numbers g^{-1} (Fig. 6.3A, B; Table S10.1, S10.2). During the incubation, the *tfdA* sequences were most abundant after three days ($F_{4,39} = 3.34$, $P < 0.05$). Adding MCPA slightly increased the abundance of *tfdA* sequences ($F_{1,39} = 8.80$, $P < 0.01$), whereas litter addition strongly increased *tfdA* abundance ($F_{1,39} = 8.44$, $P < 0.01$). The litter effect was observed mainly in the layers close to the litter layer (0-2 mm) (litter × depth, $F_{3,117} = 9.94$, $P < 0.001$). This gradient was more pronounced in the samples with MCPA and litter addition (MCPA × litter × depth, $F_{3,117} = 2.21$, $P = 0.09$).

The abundance of *cadA* sequences (Fig. 6.3C, D; Table S10.1, S10.2) showed similar distribution patterns, although the range in copy numbers g^{-1} (1.2×10^4 to 1.4×10^5) was less than for *tfdA*. Adding litter ($F_{1,39} = 18.5$, $P < 0.001$) or MCPA ($F_{1,39} = 17.4$, $P < 0.001$) increased the abundance of *cadA* with a maximum after 10 days. Similar to the *tfdA* genes, copy numbers of *cadA* genes decreased with depth in the litter treatments (depth × litter, $F_{3,117} = 24.34$, $P < 0.05$). Incubation time affected *cadA* abundance as well ($F_{4,39} = 3.30$, $P < 0.05$); however, in contrast to *tfdA* genes the abundance of *cadA* genes increased during the experiment, which was most pronounced in the litter treatments (litter × time × depth, $F_{12,117} = 2.00$, $P < 0.05$).

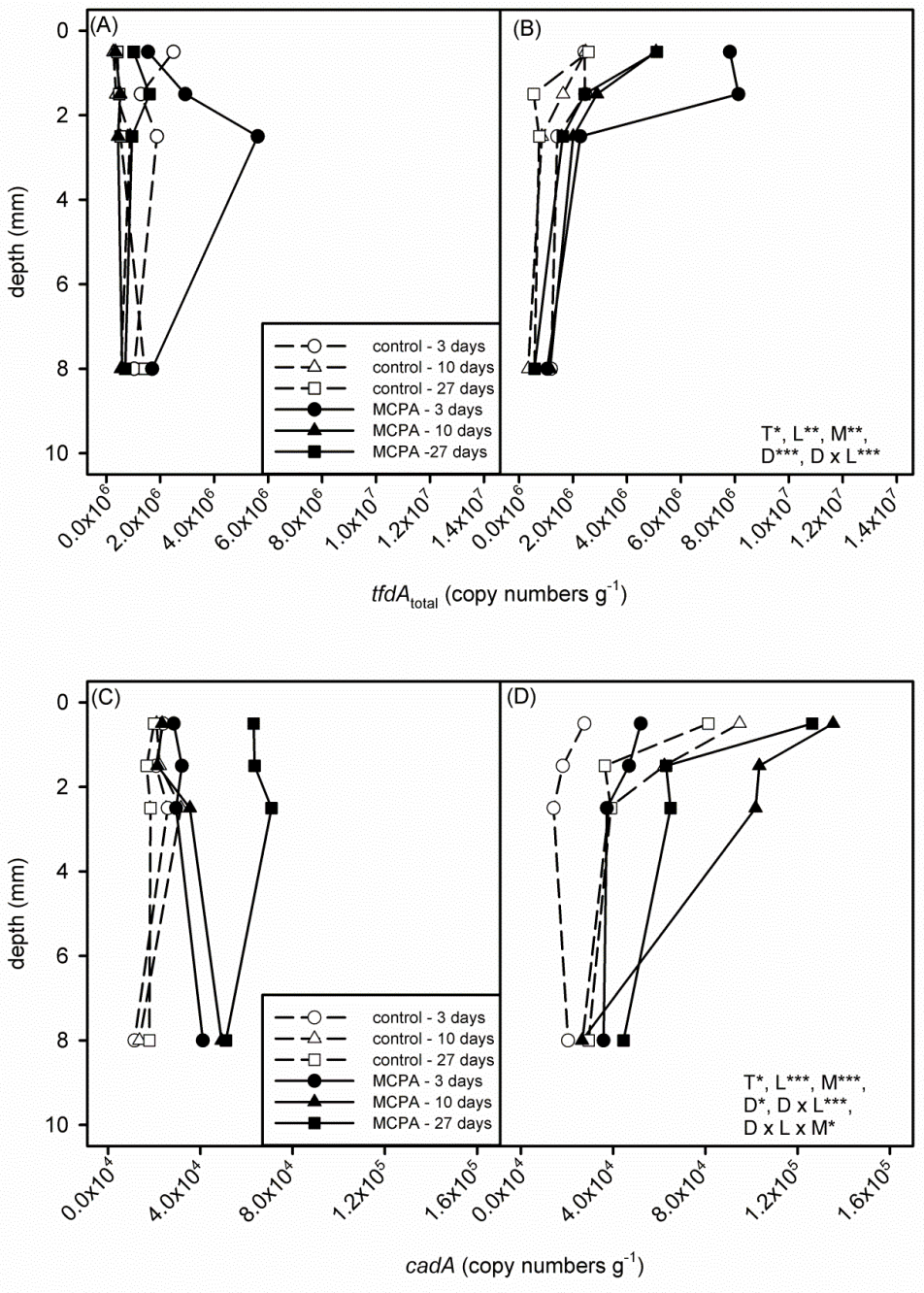

Figure 6.3 Quantities of the $tfdA_{total}$ (A) without litter and (B) with litter and *cadA* (C) without litter and (D) with litter addition at three sampling times over depth. Values are means of three replicates. Asterisks indicate statistically significant differences (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$) between treatments (repeated-measures ANOVA with D = depth, L = litter, T = time, M = MCPA). Significant interactions between more than three factors are not shown.

tfdA class specific

The three *tfdA* gene classes were analyzed using TaqMan probes. Copy numbers for the *tfdA* class II and III were near the detection limit (1×10^3 μL^{-1}), resulting in high variability. The abundance of class I genes (Fig. 6.4A, B; Table S10.1, S10.2) were in the same range (5.58×10^6 to 2.84×10^7) for the nonlitter and litter treatments with some outliers. In contrast to the total abundance of *tfdA* genes, MCPA and litter did not significantly affect the abundance of class I genes. During the incubation, the abundance of the class I genes significantly decreased, whereas at the end of the incubation there was a slight increase ($F_{4,39} = 6.59$, $P < 0.001$). The abundance of class II genes was below the detection limit in most of the samples (data not shown). Similarly, abundance of class III genes (1.13×10^3 to 8.32×10^5; Fig. 6.4C, D; Table S10.1, S10.2) was close to the detection limit or even below the detection limit in the nonlitter treatments. But at the end of the incubation the results for the nonlitter treatments stabilized and the abundance increased. Litter significantly increased the abundance of class III genes ($F_{1,40} = 20.0$, $P < 0.001$). This effect was most pronounced close to the litter layer (depth × litter, $F_{3,120} = 22.0$, $P < 0.001$). In the treatment with litter and MCPA there was a strong increase until day 10 after which abundance was stable. In the litter treatment without MCPA the abundance of class III genes increased at the end of the incubation. Adding MCPA increased the abundance of *tfdA* class III genes, although this effect was not significant ($F_{1,40} = 3.82$, $P = 0.06$).

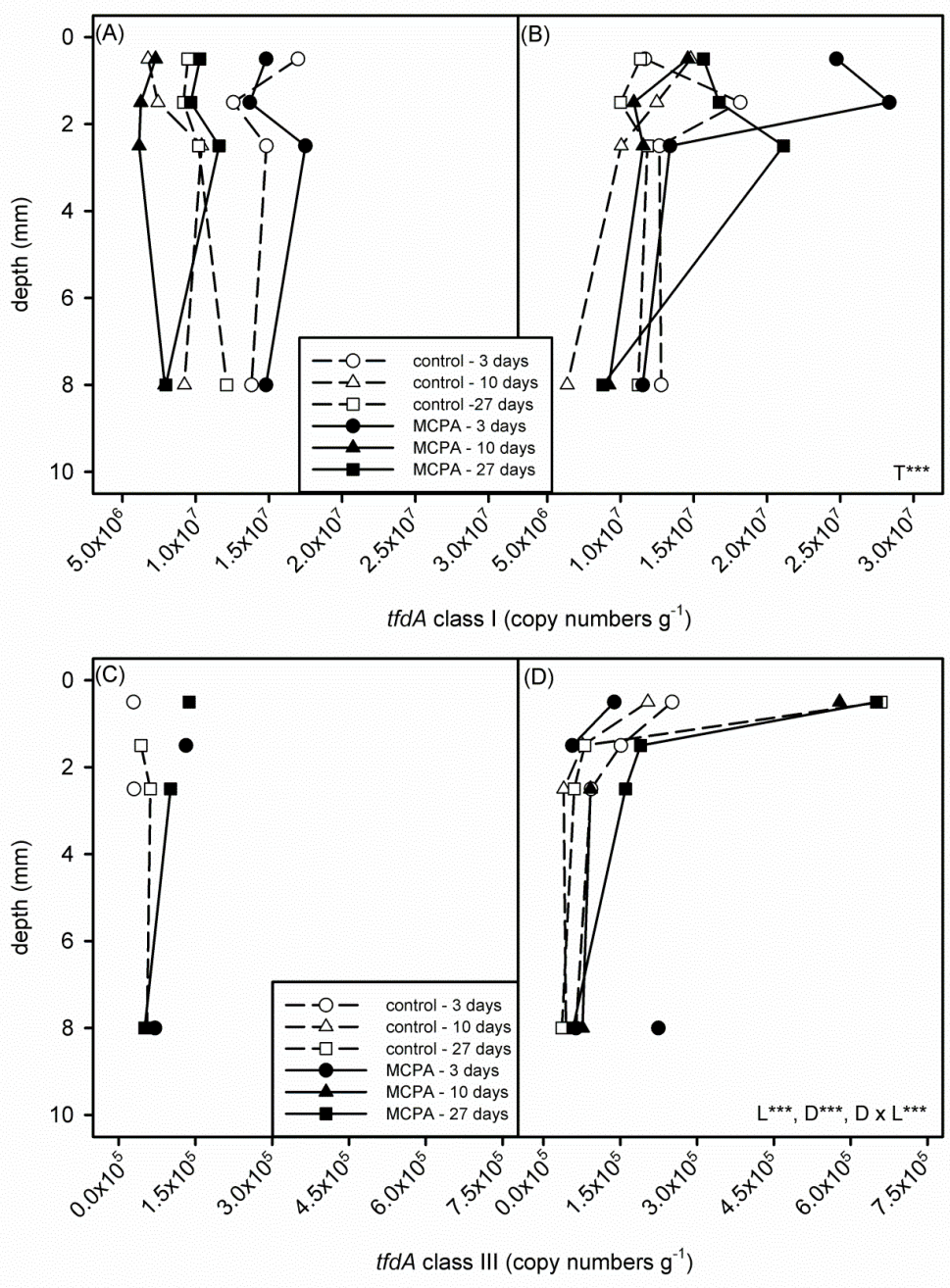

Figure 6.4 Quantities of the *tfdA* class I genes (A) without litter and (B) with litter and *tfdA* class III genes (C) without litter and (D) with litter addition at three sampling times over depth. Values are means of three replicates. Asterisks indicate statistically significant differences (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$) between treatments (repeated-measures ANOVA with D = depth, L = litter, T = time, M = MCPA). Significant interactions between more than three factors are not shown.

Analysis of the fungal community by DGGE

The DGGE profile (Fig. 6.5) showed high similarities of fungal communities between replicates. Several bands were detected in all treatments (e.g. bands A, B) but also differences in the fungal community of the first layer (0-1 mm) were observed due to litter addition. For example band C and D represent populations decreasing in abundance when litter was added. In contrast, band E represents a litter responder to all sampling times. Furthermore, a positive influence of the incubation time on fungal communities responding to litter was observed (e.g. bands F, G, H). Some populations with increased abundance in the fingerprints of MCPA added soils were observed (e.g. bands I, J).

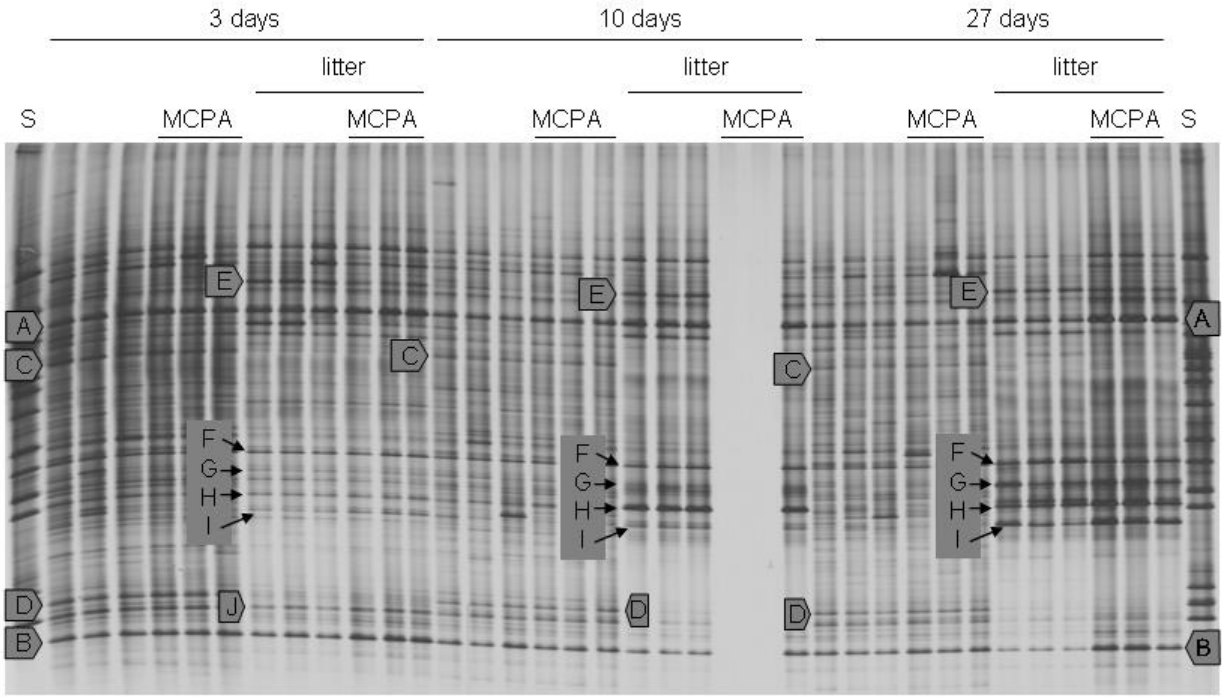

Figure 6.5 ITS based DGGE fingerprints of fungal community of the first layer (0-1 mm) from the soil cores at the sampling dates 3, 10 and 27 days with all four treatments. S: fungal standard of ITS gene fragments from 16 different fungal strains.

Cluster analysis of DGGE fingerprints (Fig. 6.6) based on UPGMA revealed that the addition of litter resulted in high differences among samples from identical sampling time. Three and 27 days after litter addition, the fingerprints of fungal communities between the nonlitter and litter treatments showed low similarities of 26.4 % and 14.9 %, respectively. After 10 days, similarity between the litter treatment and the control was higher (46.5 %). In the nonlitter treatment,

sampling days 10 and 27 clustered together, in contrast to the sampling after 3 days which showed a lower similarity to the other two sampling dates. In contrast, the litter treatments showed a higher similarity between the samplings after 3 and 10 days. The cluster analysis also showed subclusters for the added MCPA in the nonlitter and litter treatments for all sampling dates (71 to 85 %), but after 27 days and after 3 days in the litter treatment it was more striking. Overall, the importance of the factors was in the order of litter > time > MCPA.

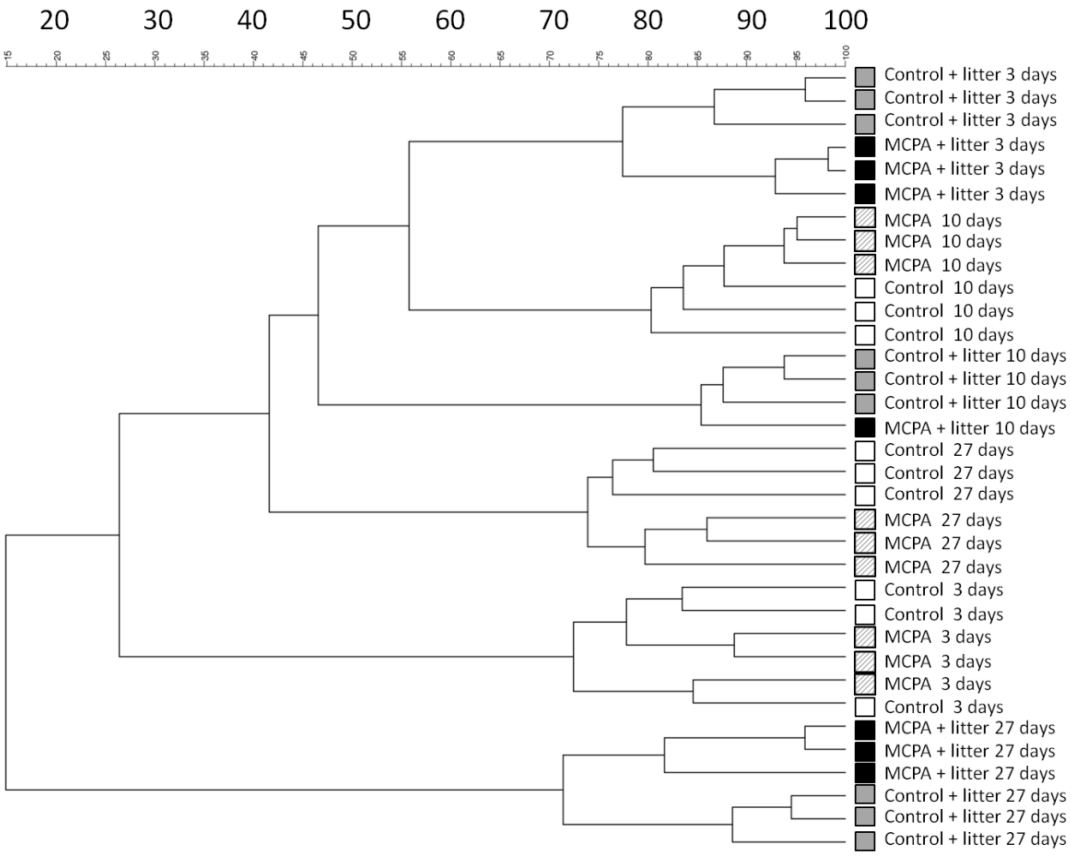

Figure 6.6 Dendrogram constructed with the fungal community fingerprints of the four treatments at three different sampling dates (3, 10 and 27 days) based on Pearson correlation indices and cluster analysis by the UPGMA.

6.5 Discussion

MCPA degradation in soil

A microcosm experiment using soil cores with a litter layer on top was conducted to clarify the role of specific MCPA degraders during the decay of MCPA at the soil-litter interface. Previous studies have suggested that different bacterial functional genes (*tfdA* and *cadA* genes) as well as fungal genes might change in their relative importance during the degradation of MCPA (Vroumsia et al., 1999; Castillo et al., 2001; Bælum et al., 2006, 2008; Liu et al., 2011a). We followed the succession of MCPA degraders and linked the abundances of functional genes to the dynamics of MCPA degradation in this microbial hot spot. The MCPA mineralization potential differed between the nonlitter and litter treatments. The observed accelerated degradation of MCPA in the litter treatment is in accordance with previous studies (Duah-Yentumi & Kuwatsuka, 1982; Poll et al., 2010b). This depletion zone of MCPA was restricted to the layers extending to 6 mm depth after 6 days of incubation and to 10 mm depth after 10 days of incubation. Therefore, the dimension of the detritusphere was slightly larger than described for other experiments (Poll et al., 2008, 2010b) and depended on the migration depth of soluble litter compounds (Poll et al., 2006). The extension of the detritusphere depends on convective transport and diffusion on the one hand and the substrate input and consumption rate on the other (Poll et al., 2008; Pagel et al., 2014). The input of soluble litter compounds is highest during the initial phase of the incubation (Ingwersen et al., 2008). This has implications for the overall effect of litter on MCPA degradation, indicating that the effect of litter depends on the amount of available substrates and the litter-affected soil volume.

16S rRNA gene copy numbers and the copy numbers of the fungal ITS fragment differed between nonlitter and litter treatments, indicating that litter changed bacterial and fungal abundances in the detritusphere. In accordance with Poll et al. (2010b) we found that litter addition increased bacterial and fungal abundances in the layers directly influenced by litter and that this effect was more pronounced for the abundance of fungal ITS fragment than for 16S rRNA genes. However, the abundances of fungal ITS fragment, 16S rRNA genes and the *tfdA* genes differed as compared to an earlier experiment using the same soil (Poll et al., 2010b). In our experiment the abundances of 16S rRNA genes and *tfdA* genes were lower, whereas the abundance of the fungal ITS fragment was higher than in the previous experiment. Mortensen

and Jacobsen (2004) found that freezing of soils for 51 h didn't change the mineralization potential. However, we stored the soil for more than 3 years at -20 °C and, therefore, the extent to which the indigenous soil microbial community was affected by damaged microbial cells might have been greater (Zelles et al., 1991; Shishido & Chanway, 1998). After thawing, fungi were probably more competitive than bacteria, resulting in reduced bacterial and increased fungal abundances. As a consequence of storage conditions, plasmids harboring functional genes like *tfdA* might have been lost. The reduced abundance of *tfdA* genes and the observed slow MCPA degradation during pre-incubation compared to the study of Poll et al. (2010b) supported this view. The abundance of the fungal ITS fragment, on the other hand, was increased by MCPA in the presence of litter. Therefore, we suggest that not only microorganisms harboring functional genes (*tfdA* and *cadA*) are able to degrade MCPA, but also fungi. This is in accordance with studies by Reddy et al. (1997), Vroumsia et al. (1999), Castillo et al. (2001) and Lerch et al. (2009) showing that fungal populations are involved in the degradation of phenoxyacetic acid herbicides.

Previous studies have reported that not only microorganisms harboring the *tfd* genes are able to degrade 2,4-D or MCPA, but also microorganisms carrying *cad* genes are involved in the degradation process (Kitagawa et al., 2002; Itoh et al., 2002, 2004; Zakaria et al. 2007; Liu et al., 2011a). These studies showed that MCPA mineralization is highly related to *tfdA* and *cadA* gene copy numbers, thus suggesting that the abundance of both functional genes may determine MCPA mineralization potential. The contribution of *cadA* gene hosting organisms to MCPA degradation was also demonstrated by Liu et al. (2011a), thus indicating that *Alphaproteobacteria* were involved in MCPA degradation. 16S rRNA based stable isotope probing with the same soil utilizing fully 13C-labelled MCPA likewise identified *Alphaproteobacteria* as major MCPA degraders (Liu et al., 2011b). Bælum et al. (2006, 2008) reported a strong response of *tfdA* after adding MCPA. In our study, MCPA increased the abundance of *tfdA* as well as *cadA* genes, particularly in the treatments with litter. Previous studies (Vallaeys et al., 1996; McGowan et al., 1998; Rodriguez-Cruz et al., 2010) showed that the *Beta-* and *Gammaproteobacteria* harboring *tfdA* genes could be subdivided into three gene classes based on differences in their *tfdA* sequences and that microbes harboring the *tfdA* gene classes differ in their ability to degrade MCPA (Bælum et al., 2008; Nielsen et al., 2011). In our study, the sum of all three *tfdA* classes was partly higher than the measured total abundance of

tfdA genes. We explain this difference by the use of two different qPCR approaches (TaqMan vs. SYBR green) and primer pairs (amplifying sequences of 81bp vs. 215bp) for detection of *tfdA* classes and *tfdA* total abundance, respectively. We found that the abundance of *tfdA* gene class I dominated the total abundance of *tfdA* genes, but was not influenced by neither MCPA nor litter. This is in accordance with Nielsen et al. (2011) who found that the MCPA mineralization rate was related to the growth of class III MCPA degraders, whereas in soils with *tfdA* genes associated only with class I the mineralization rate of MCPA was either very low or even zero. The effect of MCPA addition on the *tfdA* class III genes indicated that microorganisms harboring the class III genes could use MCPA as carbon source. This is in accordance with Bælum et al. (2008) who reported a shift from the *tfdA* gene class I to the class III during MCPA degradation. The differences in microorganisms' ability to use MCPA as a carbon source could be an indication of a truncated pathway. Müller et al. (2000) demonstrated that some microorganisms exhibit incomplete metabolism for herbicide degradation. These microorganisms have to operate as a consortium during degradation (Ka et al., 1994; Top et al., 1996). Bælum et al. (2006) suggested that bacteria harboring the class I *tfdA* gene are unable to degrade MCPA completely. This is in accordance with our results, in which the abundance of the class I *tfdA* genes was not influenced by MCPA addition alone. We therefore hypothesized that MCPA degradation by class I MCPA degraders is energy limited and that an external energy source like litter could stimulate both the growth of class I MCPA degraders and their contribution to MCPA degradation independent of the presence of bacterial consortia. However, class I *tfdA* genes were not influenced by MCPA in the presence of litter, which indicates that for these bacteria the degradation of MCPA does not depend on easily available carbon sources. The absence of any response of microorganisms harboring the *tfdA* class I gene to the combined amendment with litter and MCPA indicates either that they are unable to perform the initial degradation step, or that the availability of substrates plays only a minor role for the activation of their truncated pathway. This is in accordance with Bælum et al. (2010) who reported differences in the class I *tfdA* genes. Based on both the nucleic acid sequence and the amino acid sequence they suggested that the class I *tfdA* genes encode for two different enzymes and should be divided into two subclasses. Both subclasses could degrade 2,4-D very rapidly, but degraded MCPA either slowly or not at all, with the exception of three species that degrade MCPA rapidly (Bælum et al., 2010). In our case it seems that the *tfdA* class I genes were obtained from bacteria belonging to families that were unable to degrade MCPA.

In accordance to Bælum et al. (2008) and Nielsen et al. (2011), we could not detect the *tfdA* class II gene. The low abundance of *tfdA* class II genes could be explained by their scarce distribution within the phylogenetic tree; they are found only in *Burkholderia* branches (McGowan et al., 1998; Bælum et al., 2010; Liu et al., 2011b). One explanation for this narrow taxonomical distribution could be the absence of horizontal gene transfer due to the chromosomal location of the *tfdA* class II genes. Interestingly, taxa affiliating with *Burkholderiaceae* were likewise not detectable as MCPA degraders in the same soil during a previous 16S rRNA SIP study, suggesting little relevance of such taxa for MCPA degradation (Liu et al., 2011b). Our results confirm the hypothesis that microorganisms harboring the three different *tfdA* gene classes differ in their ability to use MCPA as carbon source; we could not, however, confirm that class I MCPA degraders contribute to MCPA degradation in the presence of an external energy source.

Succession of MCPA degraders

We hypothesized that MCPA degraders respond differentially to litter addition. Possible reasons for this are different growth strategies of MCPA degraders (Itoh et al., 2004; Ingwersen et al., 2008), or the succession of litter compounds during litter decomposition (Poll et al., 2008); the latter could activate different parts of the MCPA degrading community. Previous studies have focused on the total abundance of *tfdA* genes to investigate MCPA degradation (Gonod et al., 2006; Poll et al., 2010b; Liu et al., 2011a). They were based on the assumption that *tfdA* harboring bacteria dominate MCPA degradation and are more abundant than other degrader populations, such as *cadA* harboring bacteria. However, as shown above, MCPA degradation via the *tfd* pathway was dominated by bacteria carrying *tfdA* class III genes even though *tfdA* class I genes dominated the abundance of *tfdA* genes in our experiment. The total abundance of *tfdA* genes, therefore, did not reflect the degradation potential of the microbial community. For that reason it is necessary to quantify *tfdA* gene classes and to compare *tfdA* class III genes with *cadA* genes to investigate the succession of MCPA degraders at the soil-litter interface. Both genes reacted similarly to MCPA addition in the nonlitter treatments, although the response of *cadA* was more pronounced and class III MCPA degraders were initially below the detection limit. Litter addition accelerated the growth of both degrader populations, which coincided with an increased MCPA degradation rate. The differences in the abundances of the functional genes between the litter and nonlitter treatment could be due to growth induced by the additional available litter compounds, whereas the slight decrease at the end of the incubation could be

related to the advanced decomposition of litter and depletion of MCPA in the litter plus MCPA treatment. Hotopp and Hausinger (2001) identified a variety of non-phenoxyacetate compounds as potential substrates for the 2,4-Dichlorophenoxyacetate/α-ketoglutarate dioxygenase (TfdA). This property of the TfdA enzyme could explain the growth of MCPA degraders in the presence of litter. Both degrading communities (bacteria harboring *tfdA* class III and *cadA*) showed the same ecological behavior; a clear response to litter addition, and a concordant temporal pattern. Based on the comparable copy numbers of both sequences we suggest that both degradation pathways significantly contribute to MCPA degradation.

The increase in fungal abundance in response to litter addition was separated into two phases: an initial increase, and a second increase towards the end of the incubation. This is in accordance with Poll et al. (2008, 2010a) who detected two different fungal communities that are dependent on the succession of the released litter substrates. During the initial phase, easily available litter compounds are released and used by pioneer fungal colonizers, whereas during the late stage of incubation specific fungi degrade more complex litter compounds. To get a first indication of fungal MCPA degraders and to see a shift in the fungal community we applied the DGGE technique. Treatment dependent changes in the relative abundance of fungal populations investigated by DGGE analysis of ITS fragments showed both temporal and litter dependent differences in the fungal community composition. The added MCPA continuously affected fungal community composition and increased the abundance of the fungal ITS fragment in the litter treatment. We therefore suggest that both fungal communities (pioneer and late stage) benefit from MCPA addition in the presence of litter. Two possible explanations for the response of the fungal community to the added MCPA are: 1) a toxic effect of MCPA on the bacterial community, providing a competitive advantage for the fungi; or 2) particular fungi could benefit from MCPA in the presence of litter by the activity of litter degrading unspecific enzymes. With respect to the first explanation, if MCPA is toxic to a significant part of the bacterial community, it should be observed in both nonlitter and litter treatments. However, we could not observe a toxic effect of MCPA on the abundance of 16S rRNA gene sequences or an increase in fungal abundance in MCPA amended soil without litter addition. We therefore suggest that litter addition promotes production of litter degrading enzymes that are capable of MCPA degradation as well. This explanation agrees with previous studies in which fungal MCPA degradation by unspecific enzymes has been suggested (Castillo et al., 2001; Vroumsia et al., 2005). Pioneer

colonizers are thought to use easily available litter compounds where no oxidative enzymes are needed. However, Vroumsia et al. (2005) reported that *Mortierellaceae*, which belong to the pioneer fungal community at the soil-litter interface (Poll et al., 2010a), could degrade 2,4-D. To get a better understanding of the fungal involvement in MCPA degradation and to identify specific fungal degraders more detailed analyses (e.g. pyrosequencing) and experiments are necessary. Our results clearly show the effect of litter addition on the succession of MCPA degraders at the soil-litter interface; homogeneous behavior by bacterial MCPA degraders, and fungal MCPA degradation that likely depends on the production of unspecific enzymes in the presence of litter.

6.6 Conclusion

Our study demonstrates the stimulating effect of litter on the degradation of MCPA in the detritusphere and clarifies the succession of the MCPA degrading community at the soil-litter interface. During incubation up to 27 days the zone of accelerated MCPA degradation increased in the litter-treated soil cores. This indicated that the positive influence of litter on MCPA degradation depends on the availability of litter-derived substrates and the litter-affected soil volume. The MCPA degradation was dominated by bacteria harboring *cadA* and *tfdA* class III genes. However, the total abundance of *tfdA* genes was dominated by *tfdA* class I genes which were not influenced by MCPA or litter. Therefore, the activity of the degrading community was not linked to the total abundance of the *tfdA* genes. These findings have obvious implications for modelling approaches that use the abundance of the *tfdA* gene as a proxy for the MCPA degrader population (Pagel et al., 2014). However, the results of the present study indicate that also *tfdA* gene classes and *cadA* genes should be considered. We suggested that both *tfdA* class III and *cadA* harboring bacteria are significantly involved in MCPA degradation. Furthermore a two-phase response of fungi was observed in the litter treatments with the development of pioneer and late stage fungal communities. Both fungal groups are probably involved in MCPA degradation in the presence of litter. The succession of specific bacterial MCPA degraders as well as unspecific fungal MCPA degraders was clearly shown. Future experiments in our lab will show whether growth and/or expression of unspecific enzymes of fungi are the main reason for the fungal contribution to MCPA degradation. We recommend considering the possibility of both,

bacterial and fungal degradation of pesticides in future ecotoxicological, environmental and modelling studies (Pagel et al., 2014).

7 Degradation of phenoxyacetic acid pesticides by nonspecific fungal enzymes in soil - insights from a microcosm experiment

7.1 Abstract

Phenoxyacetic acid pesticides are used worldwide to control dicotyledonous plants in croplands. Bacteria involved in the degradation of phenoxyacetic acid pesticides have been intensively studied, whereas biochemical pathways of fungal degradation remain largely unclear. Nevertheless, nonspecific fungal extracellular enzymes, in particular laccases, may play a crucial role in the degradation of phenoxyacetic pesticides, which are known to oxidize a wide range of xenobiotic substances. We performed a microcosm experiment for up to 42 days at 20 °C to study the impact of added laccases in combination with litter on the degradation of 4-chloro-2-methylphenoxyacetic acid (MCPA, ring- and side-chain ^{14}C labeled) in soil. The ratio between ring- and side-chain labeled ^{14}C-MCPA was equal, but more side-chain labeled ^{14}C-MCPA evolved during the incubation. Although added active laccases maintained their activity during the experiment, no difference in MCPA degradation between active and inactive laccases was detected. Our results suggest rather that the application of both active and inactive laccases primarily acted as an additional, easily available nutrient source, which stimulated biodegradation of MCPA by bacteria and fungi. However, active laccases probably affected the turnover of MCPA-derived organic substrate. In comparing litter and enzyme additions, the enzyme effect was short-lived. Consequently, substrate quality played a pivotal role in the temporal pattern of stimulated MCPA degradation. Our results showed clearly that considering substrate quality is an important issue for remediation applications. This study also indicated the potential value of using well-studied model compounds like MCPA for investigating principal phenomena, such as priming effects, in soil.

7.2 Introduction

Worldwide, agricultural soils are continuously exposed to phenoxyacetic acids, which are used for control of dicotyledonous plants in cropland. Among these herbicides, 4-chloro-2-methylphenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are most frequently applied. The biodegradation of both herbicides and other phenoxyacetic acid herbicides has been studied intensively (Beadle &Smith, 1982; Shailubhai et al., 1983) and much is known about bacterial degradation pathways as well as the functional genes involved, such as *cadA*, *tfdA* or *r/sdpA* genes (Don & Pemberton, 1981; Don et al., 1985; Streber et al., 1987; Ka et al., 1994; Fulthorpe et al., 1995; Tonso et al., 1995; Kitagawa et al., 2002; Bælum et al., 2010, Liu et al. 2013). These functional genes encode for oxygenases catalyzing the cleavage of the ether-bonded acetate side chain of MCPA and 2,4-D during the initial step of degradation.

Previous studies have suggested that fungi are also involved in the degradation of phenoxyacetic acids (Castillo et al., 2001; Vroumsia et al., 2005; Lerch et al., 2009). But in contrast to bacterial degradation, no specific fungal pathway is known. Fungi differ in their ability to degrade phenoxyacetic acids (Reddy et al., 1997). For example, Vroumsia et al. (2005) tested 90 fungal strains for their ability to degrade 2,4-D or 2,4-dichlorophenol (2,4-DCP) and they showed that 20 and 54 strains degraded more than 20% of the applied herbicides within 5 days. Sixty-nine fungi from a Vietnamese soil were tested for their ability to degrade phenoxy acid herbicides and 33 isolates were found to degrade phenoxy acid herbicides, whereas only eight isolates degraded 2,4-D (Itoh et al., 2013). Furthermore, herbicide metabolism may occur mainly at the end of the fungal growing phase and seems to be correlated with the production of oxidative enzymes such as laccase (Mougin et al., 2000).

The fungal enzymes that may be involved in the degradation of phenoxyacetic acid pesticides are extracellular enzymes responsible for lignin degradation (Castillo et al., 2000, 2001; Valli & Gold, 1991; Reddy et al., 1997). The white-rot basidiomycetes, *Phanerochaete chrysosporium*, produces two types of extracellular peroxidases, manganese peroxidase (MnP) and lignin peroxidase (LiP), which are involved in the degradation of lignin as a secondary metabolic pathway (Tien & Kirk, 1983; Kuwahara et al., 1984). Castillo et al. (2001) detected an increase in LiP, MnP/laccase activity contemporaneous to the degradation of MCPA and bentazon under laboratory conditions.

In pure cultures, the ligninolytic system has been shown to be involved in the degradation of the chlorinated aromatic ring but not in the side-chain cleavage (Reddy et al., 1997). They proposed a pathway in which the chlorophenoxyacetic acids undergo ether cleavage to form corresponding chlorophenols, which then undergo dechlorination by peroxidases followed by ring cleavage. Another possible degradation pathway of chlorophenols, by removal of the chlorine atoms through an oxidative reaction by either LiP or MnP as the initial step followed by a ring cleavage, was suggested by Valli and Gold (1991). Besides peroxidases, other oxidative enzymes like laccases, which are also known to be part in the ligninolytic system, (Kirk et al., 1978; Jeffries et al., 1981), may contribute to chlorophenoxyacetic acid degradation. They belong to the multi-copper oxidase family and oxidize electron-rich compounds such as phenols to free radicals or quinones which then undergo polymerization (Dec and Bollag, 1994; Shradda et al., 2011). In the presence of mediators, the substrate range of ligninolytic enzymes such as laccases could be extended (Bourbonnais & Paice, 1990; Pizzul et al., 2009). Fungal laccases are able to degrade non-phenolic compounds, such as di-ketone, in the presence of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Mougin et al., 2000). In particular, laccases have been reported to oxidize a wide range of xenobiotic substances (reviewed by Rabinovich et al., 2004; Strong & Claus, 2011), but less is known about the influence of fungal growth and laccase activity on the degradation of MCPA.

To shed light on the impact of fungal laccases on MCPA degradation, we performed a microcosm experiment using soil amended with active and inactive fungal laccase mixtures in combination with plant litter as well as ring and side-chain labelled ^{14}C-MCPA. We hypothesized that 1) fungal laccases enhance MCPA degradation and that 2) laccases preferentially catalyze the mineralization of the aromatic moiety of MCPA. Further we hypothesized that 3) litter stimulated laccase- driven MCPA degradation by providing co-substrates and/or mediators for laccases. To test our hypothesis, we measured laccase activity and quantified the abundance of the fungal ITS fragment to determine the impact of laccase on fungal degradation of MCPA. The possible direct and indirect effects of fungal laccases were tested by adding active and inactive laccase mixtures. Additionally, the abundance of 16S rRNA genes and the functional *tfdA* and *cadA* sequences were quantified to follow the bacterial component of MCPA degradation.

7.3 Material and methods

Soil and plant residues

In October 2012 the topsoil of a loamy Luvisol [pH ($CaCl_2$) 5.3, total C content 13.6 g kg^{-1}, total N content 1.32 g kg^{-1}] was collected from an agricultural field in Scheyern, north of Munich (Germany, 48°30′N, 11°2′E). The soil had not been treated with MCPA or 2,4-D since 1998. Soil was sieved (< 2 mm) and stored in the dark at -20 °C. Before use, the soil was defrosted in the dark for 1 day at 20 °C, homogenized and dried to a gravimetric water content of 20 %. The plant residues used were maize (C/N ratio 40) leaf litter and stems which were air-dried and shredded into pieces of 2-5 mm.

Chemicals

4-Chloro-2-methylphenoxyacetic acid (MCPA) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The $^{14}C_6$ ring labeled (chemical purity: 95%, radiochemical purity: 95%) and ^{14}C side-chain (carboxyl) labeled (chemical purity: 99%, radiochemical purity: 97%) MCPA were obtained from Izotop (Institute of isotopes Co., Ltd.; Budapest, Hungary). The laccases from *Trametes versicolor* and *Pleurotus ostreatus* were obtained from Sigma Aldrich (Germany). Laccase *Cγ* from *Trametes spec*. was obtained from ASA Spezialenzyme GmbH (Wolfenbüttel, Germany). Selection of enzymes was based on a preliminary test measuring MCPA degradation in solutions amended with different oxidative enzymes. The selected enzymes showed degradation activity of 10 % to 15 % within 19 days. To test whether the laccases had a direct or indirect effect the laccases were added as active or inactive mixtures. To inactivate the laccases they were autoclaved at 121 °C for 20 min at 2 bar.

Experimental design

The experimental treatments were as follows: (1) control, (2) active laccase mixture, (3) inactive laccase mixture, (4) litter, (5) litter + active laccase mixture and (6) litter + inactive laccase mixture (Table 7.1). The soil was homogenized and all treatments were spiked with 50 mg kg^{-1} unlabeled MCPA. Two parallel setups with ^{14}C side-chain and ring labeled MCPA were prepared to account for differences in the contribution of laccases to the mineralization of either the side-chain or ring moiety. For each treatment and sampling date four replicates were prepared as well as four replicates with ^{14}C side-chain or ring labeled MCPA. After spiking, the soil was divided

into three groups and the liquid laccase mixtures (3 U g^{-1} dry soil Laccase C_γ + 20 U g^{-1} dry soil Laccase from *T. versicolor* + 20 U g^{-1} dry soil Laccase from *P. ostreatus*; the mixture being either active or inactive) or the same amount of water (control) were homogeneously added. Gravimetric soil water content was adjusted to 29 %. Soil equivalent to 10 g dry soil was put into a preserving jar (500 mL, J. Weck GmbH u. Co. KG, Germany) and for the litter treatments 0.05 g litter was separately mixed into each sample. The preserving jars were closed airtight, but vented three times per week. The soil was incubated at 20 °C for 42 days in a climate chamber and sampling was done after 2, 22 and 42 days of incubation, when mineralization of ^{14}C labeled MCPA started to slow down.

Table 7.1 Experimental treatments

No enzymes	Active laccase mixture	Inactive laccase mixture
(1) MCPA[1]	(2) MCPA[1]	(3) MCPA[1]
(4) MCPA[1] + litter	(5) MCPA[1] + litter	(6) MCPA[1] + litter

[1]MCPA or MCPA ^{14}C ring-labeled or ^{14}C side-chain-labeled

Soil respiration

During the incubation, ^{14}C-CO_2 production was measured at regular intervals three times a week. The evolved ^{14}C-CO_2 was trapped in 3 mL of 1 M NaOH solution, which was added to a small bin fixed to the lid of the preserving jar. To determine the ^{14}C content, a 1.5 mL aliqout from each trap was transferred to a 5 mL scintillation vial (LDPE) and mixed with 4.5 mL scintillation fluid (Rotiszint eco-plus, Carl Roth GmbH & Co. KG). After the vials were shaken, ^{14}C was analyzed for 10-20 min using a Wallac 1411 Liquid Scintillation System (Perkin Elmer Life Sciences). The ^{14}C activity was calculated and corrected for quenching using standard calibration (Poll et al., 2010b).

MCPA content in soil

For determination of extractable MCPA, 1.5 g soil was mixed with 7.5 mL of methanol/water (1:1). The soil suspension was homogenized on a horizontal shaker at 420 r.p.m. for 10 min and afterwards heated in a water bath for 30 min at 50 °C. After heating, the soil mixture was centrifuged for 10 min at 2500 g and 2 mL of supernatant was removed and filtered (0.45 µm pore size). The MCPA concentration was determined by HPLC (System Gold; Beckman

Instruments) using a UV detector (228 nm) according to the method of Moret et al. (2006). Separation of the solutes was done on a 150 mm x 3 mm column packed with 3 µm Kromasil 100 C18 material (MZ-Analysentechnik GmbH) at 20 °C using a flow rate of 0.5 mL min^{-1}.

Phenoloxidase activity

The phenoloxidase activity was photometrically measured by using the substrate tetramethylbenzidin (TMB) (Sigma-Aldrich, Germany) which was dissolved in dimethyl sulphoxide (DMSO), before adding autoclaved water to obtain a 60 mM stock solution (Johnsen and Jacobsen, 2008; modified by Kramer et al., 2013). Fifty mL autoclaved Na-acetate-buffer (50 mM) was added to 0.4 g fresh soil dispersed by ultrasonication for 2 min at 50 J s^{-1}, and 200 µL of the soil suspension were transferred into a 96-well microplate (Greiner Bio-One GmbH, Frickenhausen, Germany). Fifty µL TMB substrate (12 mM; TMB stock solution mixed with Na-acetate-buffer) was added to the soil suspension directly for spectroscopic measurement. For each sample a blind sample with Na-acetate-buffer instead of TMB substrate was analyzed. Measurements were performed with three analytical replicates for each sample with and without TMB. In addition, for each plate three negative controls (200 µL Na-acetate-buffer plus 50 µL TMB) were also analyzed to check the chemicals used. Measurements were done in an absorbance microplate reader (ELx 808 from BioTek, Germany) at a wavelength of 630 nm; 0, 4, 8, 12, 16 minutes after TMB addition. Between measurements the plates were incubated at 25 °C. Phenoloxidase activity was calculated as the slope of linear regression of absorbance versus time plots and the activity was expressed per g soil (dry weight (DW)) per hour [Abs 630 nm g DW^{-1} h^{-1}].

DNA extraction

DNA was extracted from 0.3 g soil using the FastDNA spin Kit for soil (MP Biomedicals, Germany). Using a NanoDrop ND 2000c Spectrophotometer (Thermo Scientific, Germany) the concentration of the extracted DNA was measured at a wave length of 260 nm and the purity of the DNA was determined by using the quotients of the intensities at 260 and 280 nm and at 230 and 260 nm, respectively.

Quantitative PCR assay

The quantification of 16S rRNA genes (total bacteria), fungal ITS fragment and total *tfdA* and *cadA* genes (for bacterial MCPA degradation) was carried out with an ABI Prism 7500 Fast (Applied Biosystems, Germany) using SYBR Green PCR master mix (Applied Biosystems, Life Technologies). The primer sets and temperature program are described in Table 7.2. Each SYBR Green reaction contained 0.75 µL of each primer (5 µM), 7.5 µL SYBR Green PCR master mix, 0.027 µL T4gp32 (Roboklon, Germany), 4.125 µL H_2O and 1.5 µL of diluted soil DNA (5 ng μL^{-1}), except for the *tfdA* and *cadA* genes were we used 2 µL diluted soil DNA and 3.973 µL H_2O. For quantification standard DNA was used according to Ditterich et al. (2013).

Table 7.2 Primers and conditions for quantitative PCR

Target sequence	Primer[a]	qPCR conditions[b]	Reference
16S rRNA genes	341F: CCT ACG GGA GGC AGC AG 515R: ATT ACC GCG GCT GCT GGC A	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 60°C, 30 s at 72°C, 30 s at 75°C (m.o.f.)	López-Gutiérrez et al., 2004
fungal ITS fragment	ITS 3F: GCA TCG ATG AAG AAC GCA GC ITS 4R: TCC TCC GCT TAT TGA TAT GC	600 s at 95°C, Cycle (35): 15 s at 95°C, 30 s at 55°C, 30 s at 70°C, 30 s at 76°C (m.o.f.)	Manerkar et al., 2008 White et al., 1990
tfdA	F: GAG CAC TAC GCR CTG AAY TCC CG R: GTC GCG TGC TCG AGA AG	600 s at 95°C, Cycle (40): 15 s at 95°C, 30 s at 64°C, 30 s at 72°C, 30 s at 81°C (m.o.f.)	Bælum et al., 2006
cadA	F: AAG CTG CAR TTT GAR AAY GG R: MGG ATT GAA ATC CTG RTA	600 s at 95 °C, Cycle (40): 15 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, 30 s at 80 °C (m.o.f.)	Kitagawa et al., 2002

[a]M =A or C, S = G or C, R = A or G, Y = C or T
[b]m.o.f. = measurement of fluorescence

Statistical analyses

Results were calculated based on oven-dried soil. For the $^{14}CO_2$ evolved from side-chain and the ring labeled ^{14}C-MCPA significant differences were tested separately. Cumulated $^{14}CO_2$ after 2, 22 and 42 days of incubation was tested by repeated measure ANOVA in R (R version 3.0.3, R Core Team, 2014) with time as a random effect and litter and enzyme addition as categorical predictors. Significant differences in MCPA concentration, enzyme activities and gene abundances were tested by three-factorial ANOVA in R (R version 3.0.3, R Core Team, 2014) with time, litter and enzyme addition as categorical predictors. If a significant interaction between time and litter and/or enzyme addition occurred, we performed a two-factorial ANOVA separately for the three time points with litter and enzyme addition as categorical predictors. The data were log transformed if the basic assumptions of normality or homogeneity of variances were not met.

7.4 Results

^{14}C-CO_2 respiration and MCPA concentration

In general, addition of fungal laccases (active or inactive forms) as well as the addition of litter increased the $^{14}CO_2$ released from side-chain or ring labeled ^{14}C-MCPA over a period of 42 days (Table 7.3). In comparison, more $^{14}CO_2$ was released from the side-chain labeled MCPA (21–40.5 %) than from the ring labeled MCPA (8–22 %) (Fig. 7.1a, b).

The effect of litter and enzyme addition on the mineralization of the side-chain of MCPA changed significantly during the incubation (Table 7.3). After 2 days of incubation the treatments with addition of laccases (active or inactive) showed a lag phase with significantly lower mineralization than the control treatments (Table 7.4). Litter addition significantly decreased mineralization in the control treatments whereas no significant litter effect was detected in the presence of laccases. After 22 and 42 days, the mineralization of the side-chain of MCPA was significantly increased by adding laccases (active or inactive) (Table 7.4) or litter, whereas in combination the effects were much lower. In the treatments with active laccases more ^{14}C was mineralized than in treatments with inactive enzymes. The mineralization of the ring labeled MCPA showed comparable patterns (Fig. 7.1b, Table 7.3, 7.4).

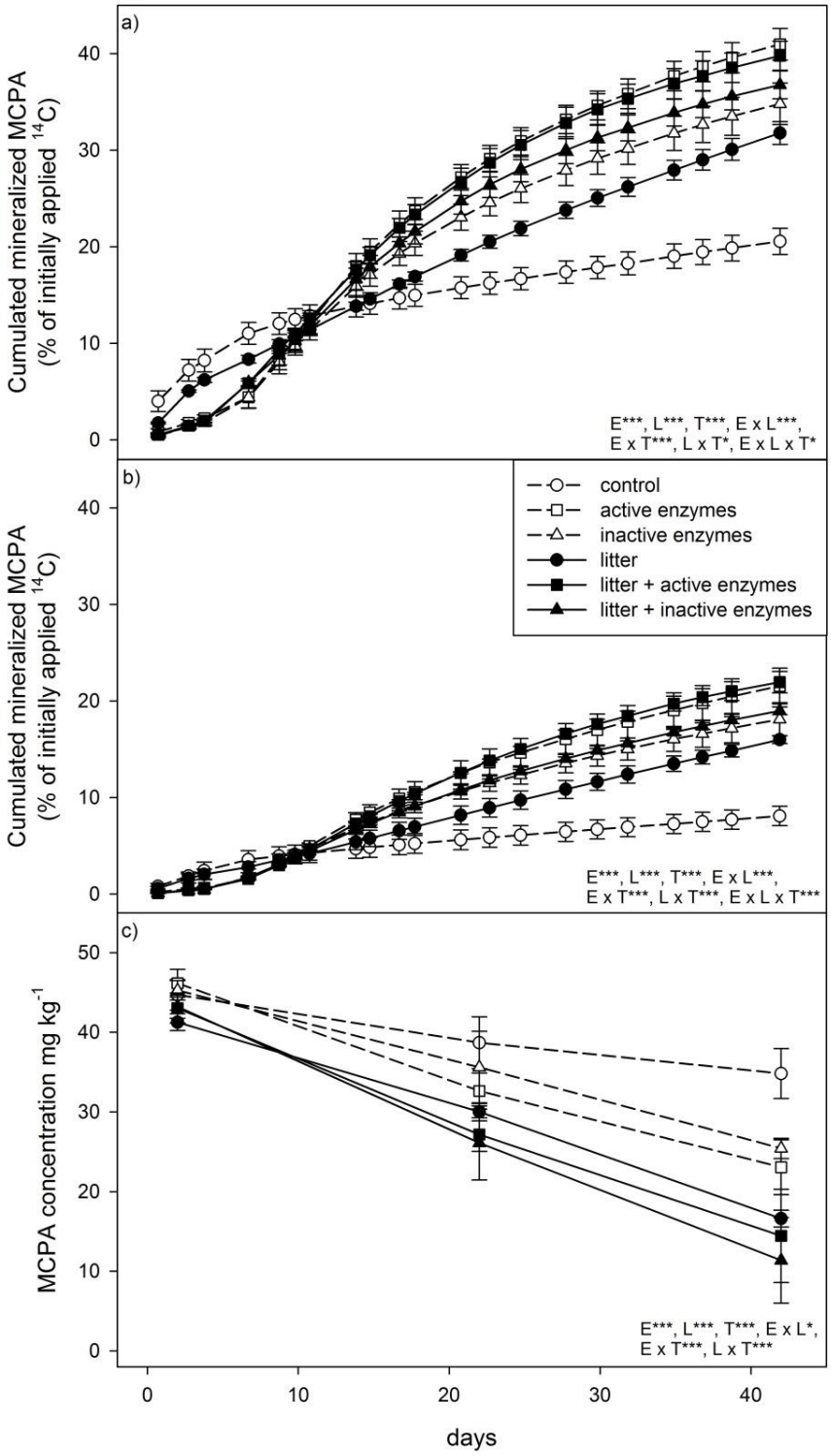

Figure 7.1 Cumulative $^{14}C - CO_2$ production of the side chain labeled ^{14}C – MCPA (a) and the ring labeled ^{14}C – MCPA (b), extractable MCPA concentration (c) at different sampling dates. Error bars indicate the standard deviation of four replicates. Asterisks indicate statistically significant differences (*P < 0.05; **P < 0.01; ***P < 0.001) between treatments (repeated measures ANOVA for the $^{14}C - CO_2$ production and three-factorial ANOVA for MCPA concentration with E = enzymes, L = litter, T = time).

Table 7.3 Statistical comparison of the evolved ^{14}C-CO_2 for the side-chain and ring labeled ^{14}C-MCPA with the factors enzyme (E), litter (L), time (T) and their interaction with time as repeated measure. Only the three sampling dates (2, 22 and 42 days) were used for the statistical analyses. Only significant differences are shown.

	E		L		T		E x L		L x T		E x T		L x E x T	
^{14}C-label	$F_{2,18}$	P	$F_{1,18}$	P	$F_{1,42}$	P	$F_{2,18}$	P	$F_{1,42}$	P	$F_{2,42}$	P	$F_{2,42}$	P
side-chain	26.0	0.001	4.92	0.05	1101	0.001	3.75	0.05	6.69	0.05	36.8	0.001	5.37	0.01
ring	99.5	0.001	22.4	0.001	1992	0.001	14.9	0.001	18.6	0.001	77.7	0.001	10.9	0.001

E = enzyme (control, active, inactive), L = litter, T = time (2, 22, 42 days)

Table 7.4 Statistical comparison of the three sampling dates (2, 22 and 42 days) of the evolved ^{14}C-CO_2 for the side-chain and ring labeled ^{14}C-MCPA and the extractable MCPA with the factors enzyme (E), litter (L) and the interaction litter x enzyme. Only significant differences are shown.

	side-chain labeled						ring labeled						extractable MCPA					
	E		L		E x L		E		L		E x L		E		L		E x L	
day	$F_{2,18}$	P	$F_{1,18}$	P	$F_{2,18}$	P	$F_{2,18}$	P	$F_{1,18}$	P	$F_{2,18}$	P	$F_{2,18}$	P	$F_{1,18}$	P	$F_{2,18}$	P
2	225	0.001	15.9	0.001	10.5	0.001	65.1	0.001							19.9	0.001		
22	144	0.001	13.7	0.01	6.92	0.01	103	0.01	10.3	0.01	6.73	0.01	4.16	0.05	35.0	0.001		
42	155	0.001	38.7	0.001	31.6	0.001	133	0.001	38.1	0.001	24.0	0.001	9.31	0.01	76.2	0.001		

E = enzyme (control, active, inactive), L = litter

Over a period of 42 days of incubation the amount of extractable MCPA significantly decreased ($F_{2,54}$ = 344, P < 0.001, Fig. 7.1c). Initially, no effect of the laccases was detected, whereas litter addition started to decrease the amount of extractable MCPA after only 2 days (Table 7.4). At the end of the incubation, the addition of laccases (active or inactive) significantly reduced extractable MCPA with final MCPA concentrations between 3.62 and 26.8 mg kg^{-1} for the treatments with added laccases and between 17 and 38.8 mg kg^{-1} for the treatments without laccases. Between the active and inactive laccase mixtures no significant differences were detected. Litter addition also significantly reduced extractable MCPA ($F_{2,54}$ = 128.5, P < 0.001) and the final MCPA concentrations varied between 18.6 and 38.8 mg kg^{-1} in the nonlitter treatments and 3.62 and 22.8 mg kg^{-1} in the litter treatments. Litter addition significantly influenced the effect of laccase addition on extractable MCPA (litter x enzyme: $F_{2,54}$ = 3.18, P < 0.05) with a pronounced response of MCPA dissipation to laccase addition in the absence of litter.

Phenoloxidase activity and abundance of fungal ITS fragment

Phenoloxidase activity ranged between 0.77 and 3.53 absorbance 630 nm g^{-1} h^{-1} (Fig. 7.2a-c) with no significant effect of litter addition. Addition of active laccases significantly increased the phenoloxidase activity ($F_{2,54}$ = 19.8, P < 0.001) with no differences for the treatments with or without inactive laccases. In general, phenoloxidase activity decreased during the incubation ($F_{2,54}$ = 13.5, P < 0.001) with the effect of active laccases decreasing towards the end of the incubation.

The initial copy number of the fungal ITS fragment in the soil was about 1.81 x 10^6 copies g^{-1} and directly after adding the laccase mixtures the abundance of the fungal ITS fragment increased up to 7.18 10^9 copies g^{-1} (data not shown). Laccase addition significantly increased copy numbers of the fungal ITS fragment from 1.5 x 10^9 to 3.4 x 10^{10} copies g^{-1} for the treatments without laccases to 3.8 x 10^{10} to 3.9 x 10^{11} copies g^{-1} for the treatments with added laccases ($F_{2,53}$ = 379; P < 0.001, Fig. 7.2d-f). Litter addition significantly increased the abundance of the fungal ITS fragment in the control treatment with no significant effect in the presence of laccases (active and inactive) (enzyme x litter: $F_{2,53}$ = 11.8; P < 0.001).

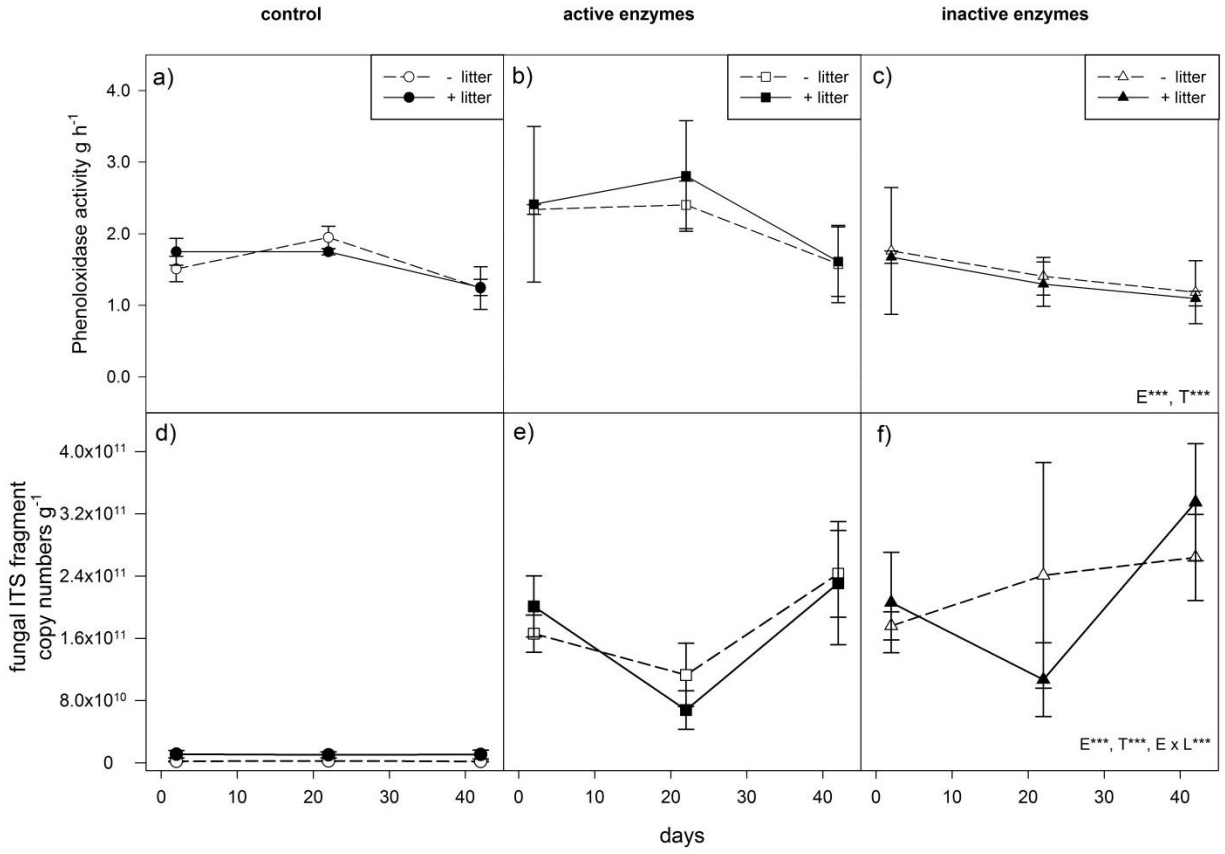

Figure 7.2 Enzyme activity of phenoloxidase for the treatments: control (a), the active enzymes (b) and inactive enzymes (c) and quantities of the fungal ITS fragment for the treatments: control (d), the active enzymes (e) and inactive enzymes (f) at three sampling dates. Error bars indicate the standard deviation of four replicates. Asterisks indicate statistically significant differences (**P* < 0.05; ***P* < 0.01; ****P* < 0.001) between the treatments (three-factorial ANOVA with E = enzymes, L = litter, T = time).

16S rRNA genes and functional genes

The abundance of 16S rRNA gene sequences was within the range of 1.6 x 10^{10} and 2.3 x 10^{11} copy numbers g^{-1} (Fig. 7.3a-c). Adding inactivated laccases significantly increased the 16S rRNA gene abundances with no significant differences between control and active laccase treatments ($F_{2,54}$ = 80.0; P < 0.001). The abundance of the 16S rRNA gene sequences significantly increased after litter addition ($F_{1,54}$ = 145; P < 0.001), with the highest 16S rRNA gene abundance in the presence of inactive laccases and almost no effect in the presence of active laccases (enzyme x litter: $F_{2,54}$ = 25.7; P < 0.001). During the incubation the abundance of 16S rRNA genes decreased in all treatments after 22 days of incubation and increased again towards the end of incubation ($F_{2,54}$ = 74.8; P < 0.001); this result was most strongly pronounced in the litter treatment with addition of inactive laccases (enzyme x litter x time: $F_{4,54}$ = 12.8; P < 0.001).

Quantification of the bacterial MCPA-degrading community was performed by qPCR targeting the *tfdA* or *cadA* sequences. The abundance of the *tfdA* gene was below the detection limit. The abundance of the *cadA* gene was within a range of 3.8 x 10^5 to 2.2 x 10^7 copy numbers g^{-1} (Fig. 7.3d-f). Adding inactive laccases significantly increased the abundance of the *cadA* gene ($F_{2,54}$ = 5.92; $P < 0.01$) whereas adding active laccases showed no effect. In addition, litter significantly increased the abundance of the *cadA* gene ($F_{1,54}$ = 7.53; $P < 0.01$).

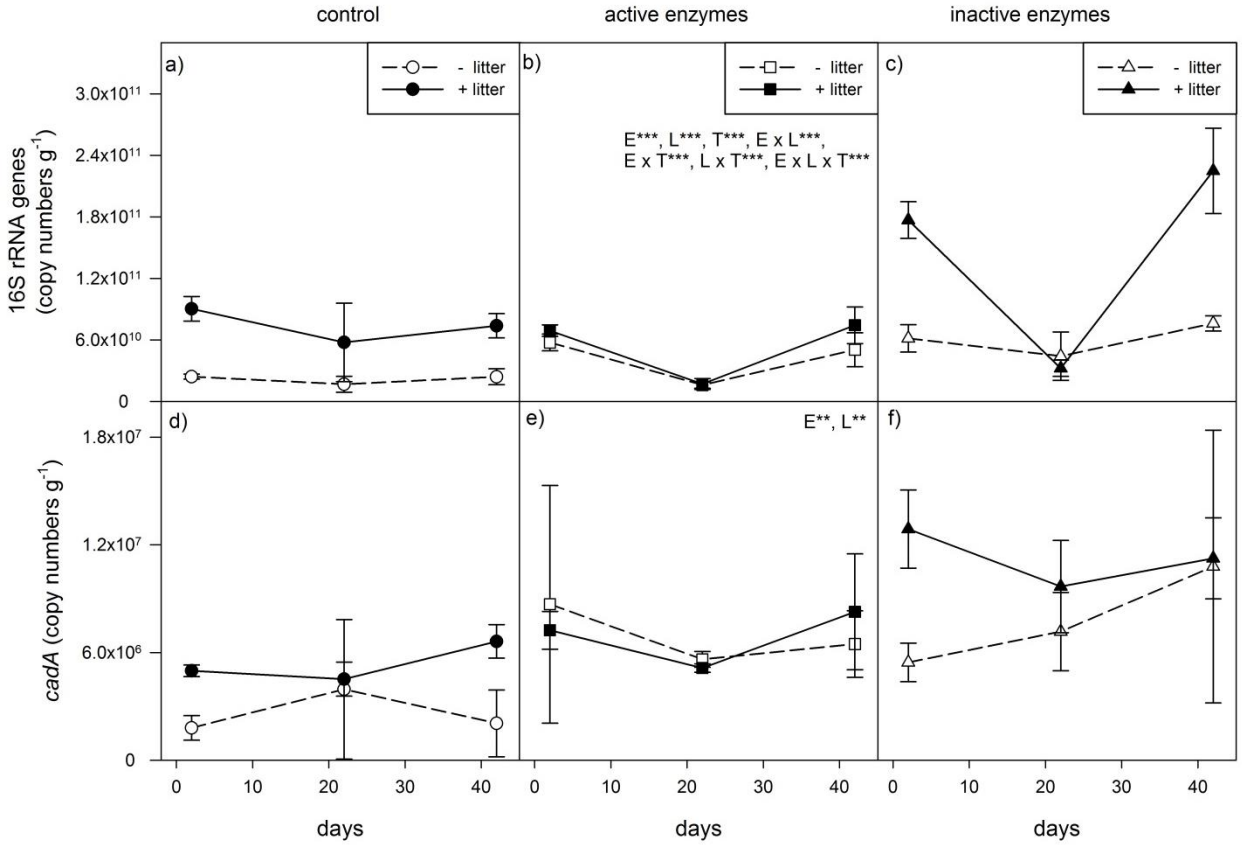

Figure 7.3 Quantities of the 16S rRNA genes for the treatments: (a) control, (b) active enzymes and (c) inactive enzymes, functional *cadA* gene for the treatments (d) control, (b) active enzymes and (c) inactive enzymes at three sampling times. Error bars indicate the standard deviation of four replicates. Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) between treatments (three-factorial ANOVA with E = enzymes, L = litter, T = time).

7.6 Discussion

Our study was established to test different mechanisms by which fungi might contribute to the degradation of phenoxyacetic acids such as MCPA and to determine whether or not addition of fungal laccases enhanced MCPA degradation. We tested possible direct (cleavage of the aromatic moiety) and indirect (use as easily available C and N source) effects of fungi by adding active or inactive laccases. The results showed a complex interaction between direct and indirect stimulation of MCPA degradation by laccase addition. In general, active as well as inactive laccases stimulated the dissipation of MCPA, and MCPA degradation was faster than in the treatments without laccases. Active enzymes were added as free enzymes and preserved their activity at least somewhat in soil as shown by increased phenoloxidase activity for more than 22 days. Phenoloxidases include various nonspecific enzymes which oxidize a broad range of substrates (Sinsabaugh, 2010). Laccases are members of this group and, therefore, the applied phenoloxidase assay provided information about the stabilization of the added laccases in our soils. The higher phenoloxidase activity together with the reduced effect on bacterial growth in the treatments with active laccases compared to treatments with inactive laccases suggested that the active enzymes were at least partially stabilized in soil and that the enzymes kept at least some of their activity after stabilization. Ahn et al. (2002) reported that the activity of free laccases quickly decreased within 3-4 days after addition to soil, whereas laccases immobilized on montmorillonite conserved their activity for more than 14 days. They further demonstrated that immobilized laccases were more efficient in degrading 2,4-DCP in soils with high organic matter, whereas in soils with low organic matter no differences between free and immobilized laccases occurred, due to enzyme inhibition by soil organic matter. Our results provided evidence that the added laccases were partially stabilized in soil and potentially able to contribute to MCPA degradation.

Speed of MCPA degradation was similar after addition of either active or inactive laccase and favored the proposed indirect pathway of degradation, suggesting that the enzyme solution might function mainly as a substrate and nutrient source. In the presence of inactive laccases the 16S rRNA genes and the functional gene *cadA* increased due to bacterial growth. In contrast, the addition of active enzymes only slightly affected the abundance of these two genes, providing evidence that the active enzymes are not used as substrate or nutrient source for bacterial growth. However, the response of fungal growth to the addition of either active or inactive laccases was

masked by high amounts of ITS fragments in the initial laccase mixtures derived from cell fragments, dormant cells or spores. Therefore, we could draw no conclusions about the response of fungal growth to the addition of this substrate and nutrient source. In general, the ability of fungal strains to degrade phenoxyacetic acids varied considerably (Vroumsia et al., 2005). Our findings are supported by results from Itoh et al. (2013), who classified different fungal strains according to their ability to degrade phenoxyacetic acid compounds such as 2,4-D or 2,4,5-trichlorlophenoxyacetic acid (2,4,5-T). Reddy et al. (1997) showed differences in the efficiency of two fungal strains to degrade 2,4,5-T. The differences in degradation potential among fungal strains may be explained by the enzymatic constitution of the specific strains. Koschorrek et al. (2008) also demonstrated that four laccase isoenzymes from *Trametes versicolor* differed in their degradation potential of PAHs due to differences in their binding sites. Furthermore, stimulation of fungal growth maintains production of fungal enzymes other than laccases but which also may be involved in degradation processes. This was further suggested by Reddy et al. (1997) who assumed that the initial step in the fungal pathway is likely to be catalyzed by an intracellular enzyme which occurs during primary metabolic growth. They reported that the first step in chlorophenoxyacetic acid degradation is the cleavage to chlorophenol, in which the ligninolytic enzymes, such as MnP, LiP and laccases are not involved. Nevertheless, MCPA degradation was stimulated by adding active or inactive laccases. The addition of enzyme solutions might initially serve as a substrate and nutrient pool for all bacteria and especially for MCPA degraders.

Further studies working with 2,4-D reported that fungal laccases had no effect on 2,4-D degradation but were extremely efficient in the oxidation of the metabolite 2,4-DCP (Gianfreda et al. 1998; Sannino et al. 1999). This led us to hypothesize that laccases preferentially catalyze the mineralization of the aromatic moiety of MCPA and that more generally laccases had an influence on the transformation of MCPA-C after side-chain cleavage. In our study, however, more $^{14}CO_2$ evolved from the side-chain labeled than from the ring labeled MCPA. This is in accordance with Soulas et al. (1984), who measured higher $^{14}CO_2$ emission from side-chain labelled than from ring labelled 2,4-D as well. Both results may be explained by a dominance of bacterial MCPA degradation as compared to fungal contributions. In the presence of enzyme solutions the mineralization of the ring labeled ^{14}C-MCPA also increased. This agrees with Reddy et al. (1997), who used two fungi to examine the mineralization of 2,4,5-T and showed clearly that both fungi degraded the side-chain more efficiently than the ring. Therefore,

independent of whether bacteria or fungi dominated MCPA degradation, more side-chain C was mineralized than from the ring moiety. Furthermore, a shift in the ratio of these two CO_2 sources did not indicate a shift from bacterially to fungally dominated degradation. During the incubation the quotient between mineralized side-chain and ring labeled ^{14}C-MCPA was equal for all treatments, leading us to suggest that the biochemical degradation pathways were similar and no different pathway was induced by active laccases. In contrast to the treatments without enzyme solutions, the laccase treatments (active or inactive) exhibited an initial lag phase; this can be explained that the additional laccases, MCPA and litter are in competition as substrate.

When the ^{14}C-MCPA mineralization was compared with the MCPA concentration in soil, different patterns were observed. The MCPA concentration reflected the degradation of the MCPA molecule, whereas mineralization reflected the ^{14}C transformation, which could have been evolved or adsorbed by microbes. At later time points microbial biomass turnover occurred and in addition to ^{14}C-MCPA, ^{14}C from the microbes also evolved. We suggest that microbial biomass turnover was influenced by active laccases, which could be seen in the higher ^{14}C abundances. The minor effect of the active laccases on MCPA concentration led us to assume that MCPA degradation was only indirectly stimulated by active laccases. Adding litter led to more rapid MCPA degradation and the litter effect was more pronounced than the enzyme addition.

Further studies clarified our observations that the degradation of MCPA is enhanced in the presence of litter and that litter addition increases available substrates that in turn stimulate bacterial and fungal growth (Poll et al., 2010b; Ditterich et al., 2013). We therefore used a litter- as well as litter + enzyme-treatment to determine whether a second carbon and energy source can stimulate MCPA degradation throughout a period of 42 days. In contrast to the enzyme solutions, litter consists mainly of more complex compounds which are continuously degraded and release soluble decomposition products into the soil (Poll et al., 2008). Our hypothesis was that concomitant with the addition of litter co-substrates and mediators for laccase activity were added, increasing the contribution of these enzymes to MCPA degradation. In the presence of mediators such as ABTS the substrate range of nonspecific enzymes such as fungal laccases could have been extended (Mougin et al., 2000; Pizuell et al., 2009). However, the effect of litter was strongest in the absence of laccases and we therefore assume that added litter compounds did not serve as co-substrates or mediators for laccase activity. In contrast, we found clear evidence

that litter addition stimulated the dissipation of MCPA throughout the whole period of the experiment.

7.7 Conclusion

In conclusion, litter and/or laccases addition stimulated MCPA degradation and increased the abundance of the fungal ITS fragment. The applied laccases were used as C- and N-sources and therefore only indirectly stimulate MCPA degradation. Comparing our results with the literature makes clear that more research is necessary to clarify the impact of fungal enzymes in degradation of pesticides and the mechanisms behind. Furthermore we assume that the nutritional effect of enzyme and litter addition prevailed over the effect of adding additional enzymes or co-substrates and mediators for these enzymes. *In situ* produced laccases may be more effective in MCPA degradation than indicated in our study, if the confounding effect of substrate and nutrient addition is missing. This generally questions the approach of testing laccase contribution to MCPA degradation by adding laccases, because extremely purified enzyme solutions are necessary to estimate the contribution of laccases to MCPA degradation *in situ*. However, the observed differences between the addition of simple and complex substrates with regard to intensity and longevity of the effect on MCPA degradation clearly indicate the importance of substrate quality. Further studies are necessary to clarify the role of substrate quality and nutrient availability for remediation applications or principle phenomena like priming in soils, which can be best studied using model compounds like MCPA.

8 General Conclusions

The heterogeneity of soils results in a diversity of microhabitats with different properties which shape microbial structure and function. The differing characteristic of minerals, such as surface properties or chemical composition, influence microbial communities (Gleeson et al., 2005, 2006; Uroz et al., 2012). This was confirmed by the results of the first study (chapter 5). The response of the microbial community differed in the presence of different minerals due to mineral properties such as surface charge and OM adsorption, which varied among them; this was, however, observed mainly at the beginning of the experiment. The effect of the minerals on the preferential colonization of different taxa was influenced by substrate availability due to mineral properties, but the mineral effect was masked by substrate limitation, which became the main driver of microbial structure and function by the end of the experiment. Changes in substrate availability and quality are important for the development of microbial communities due to their life strategies (Fierer et al., 2007; Nemergut et al., 2010; Cederlund et al., 2014). In the first study (chapter 5) it was shown that montmorillonite provided a nutrient-rich environment which positively affected copiotrophic bacteria, such as *Betaproteobacteria*. Subsequent substrate depletion led to a shift in the microbial community from copiotrophic to oligotrophic bacteria. Insights gained from the first study (chapter 5) provide a basis for further studies, for example, those which increase our understanding of microbial colonization of soils and degradation processes, such as pesticide degradation. Detoxification of chemical pollutants, such as MCPA, depends on different factors, such as sorption of the pollutant to mineral substrates as well as to microbial structure and function. For example, sorption of the pollutant depends on soil properties and the substance itself. The sorption of the model compound MCPA increases with decreasing pH due to the fact that deprotonated MCPA strongly interacts with the positively charged surfaces of minerals (Haberhauer et al., 2001; Waldner et al., 2012). At the same time, it is the abundance and activity of the microbial community, especially the degrader community, which influences degradation processes such as pesticide degradation (e.g. MCPA degradation). It is known that MCPA degraders belonging to the *Alpha-*, *Beta-*, and *Gammaproteobacteria* and are divided into three groups (Kamagata et al., 1997; Kitagawa et al., 2002; Itoh et al., 2002, 2004). Furthermore *Alpha-* and *Betaproteobacteria* are known as copiotrophic bacteria; they need a nutrient-rich environment. This was confirmed by the first study (chapter 5) (Fierer et al., 2007). The soil-litter interface is such an environment; a hot spot with high substrate availability

in which copiotrophic bacteria may be stimulated, and therefore two microcosm experiments were designed to study MCPA degradation in the presence of litter (study 2; chapter 6 & study 3; chapter 7).

Both studies (chapters 6 & 7) demonstrated the stimulating effect of litter on the degradation of MCPA and the degrader community. The zone of accelerated MCPA degradation increased depending on both the availability of litter-derived substrates and the soil volume which was affected by litter (chapter 6). The degrading community was stimulated, as seen in the abundance of the functional genes (*tfdA* and *cadA*). A high diversity and subdivision of the functional *tfdA* genes with different microbes harboring the *tfdA* gene classes using MCPA as a carbon source had been proposed earlier (McGowan et al., 1998; Bælum et al., 2008; Rodriguez-Cruz et al., 2010; Nielson et al., 2011). The results of study 2 (chapter 6) showed that the total abundance of the *tfdA* genes was dominated by the class I *tfdA* gene, but that *tfdA* class III or *cadA* harboring bacteria belonging to *Beta-*, *Gamma-* and *Alphaproteobacteria* dominated MCPA degradation. Fungi as well as bacteria also likely contributed to MCPA degradation, but less is known about the fungal pathway (Castillo et al., 2001; Vroumsia et al., 2005). Chapter 6 of this thesis showed that litter addition stimulated fungal growth with a two-phase response of the fungi. In the presence of litter both fungal groups, the pioneer and the late-stage fungi, appear to be involved in the degradation process of MCPA, as demonstrated by the production of nonspecific enzymes such as laccases. Therefore, the second microcosm experiment was designed to test the direct and indirect effects of fungal laccase on MCPA degradation in the presence of litter by adding active and inactive laccases (chapter 7). The added fungal laccases active or inactive) both stimulated MCPA degradation to the same degree, and were therefore used only as C- and N-sources. In the presence of a mediator, such as ABTS, the substrate range of enzymes such as laccases can be extended (Bourbonnais & Paice, 1990; Mougin et al., 2000; Pizzul et al., 2009).Therefore, litter was added to stimulated laccase driven MCPA degradation by providing co-substrate or mediators for the laccases. But litter compounds did not serve as co-substrates or mediators for the laccases used in this study, as shown in chapter 7.

In conclusion, this thesis demonstrated that the microbial community is driven by mineral composition as well as substrate availability and quality and that degradation processes, such as pesticide degradation, are influenced by the abundance and activity of the microbial community. Minerals provide, due to their properties, different habitats for microorganisms. The first study

(chapter 5) demonstrated that surface properties, such as sorption and desorption of OM, influence the colonization of microorganisms by providing nutrient-rich or poor habitats. *Beta*- and *Alphaproteobacteria* are known to live in nutrient-rich environments. Furthermore, MCPA degraders belong to the copiotrophic *Beta*- and *Alphaproteobacteria*, both of which harbor the functional genes *cadA* and *tfdA* class III. Providing nutrient-rich habitats, as was done with the artificial soils containing montmorillonite, or adding substrate such as litter, may stimulate MCPA degradation. The second and third studies (chapters 6 & 7) demonstrated that litter stimulates the bacterial MCPA degrader community and consequently the degradation of MCPA. The fungal community is also stimulated by litter addition, although the fungal role in MCPA degradation is still unclear. More research is therefore necessary to clarify the fungal role and the mechanism(s) behind it. It is also necessary to clarify the role of substrate availability and quality in detoxification processes. By combining the insights from all three studies we demonstrated that use of a simple model system can be helpful in expanding our understanding of soil processes. Using the artificial soil system to study complex mechanisms such as the fungal role in degradation of pesticides with MCPA as the model compound is a rich topic for further studies.

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10 Supporting Material

Table S10.1 Mean and standard deviation (std) of measured gene abundances in treatments without litter addition at different sampling dates.

		16S rRNA genes		ITS fragment		*tfdA*		*cadA*	
sampling date	treatment	mean	std	mean	std	mean	std	mean	std
3	control	1.81E+10	2.76E+09	6.89E+09	1.27E+09	2.49E+06	2.16E+06	2.38E+04	9.87E+03
3	control	1.57E+10	2.72E+09	6.88E+09	2.51E+09	1.28E+06	1.16E+06	2.08E+04	1.41E+03
3	control	1.75E+10	1.74E+09	6.94E+09	2.30E+09	1.88E+06	1.37E+06	2.59E+04	1.43E+04
3	control	1.63E+10	2.15E+09	8.77E+09	4.17E+09	1.04E+06	1.04E+06	1.15E+04	6.84E+03
10	control	1.78E+10	1.05E+09	7.04E+09	1.82E+09	2.70E+05	8.82E+04	2.10E+04	7.43E+03
10	control	1.91E+10	2.28E+09	7.19E+09	1.01E+09	3.70E+05	4.13E+04	2.24E+04	6.54E+03
10	control	1.77E+10	5.52E+09	5.93E+09	4.57E+09	8.84E+05	9.54E+05	3.16E+04	1.93E+04
10	control	1.93E+10	4.26E+09	6.09E+09	1.59E+09	5.52E+05	1.82E+05	1.36E+04	7.86E+03
27	control	1.74E+10	5.17E+09	7.46E+09	2.47E+09	4.13E+05	2.79E+05	2.00E+04	1.58E+04
27	control	2.03E+10	5.80E+09	7.73E+09	2.14E+09	4.81E+05	1.20E+05	1.68E+04	6.80E+03
27	control	1.60E+10	2.06E+09	6.89E+09	4.72E+09	5.35E+05	2.21E+05	1.84E+04	6.22E+03
27	control	1.90E+10	1.70E+09	8.19E+09	1.84E+09	1.42E+06	1.30E+06	1.79E+04	5.85E+03
3	mcpa	1.64E+10	3.97E+09	7.22E+09	1.91E+09	1.55E+06	1.32E+06	2.85E+04	1.58E+03
3	mcpa	1.64E+10	2.77E+09	5.74E+09	1.04E+09	2.93E+06	3.52E+06	3.20E+04	1.65E+03
3	mcpa	1.50E+10	2.47E+09	7.05E+09	2.86E+09	5.62E+06	4.13E+06	2.95E+04	1.60E+04
3	mcpa	2.05E+10	6.29E+09	9.32E+09	2.31E+09	1.70E+06	6.18E+05	4.11E+04	1.80E+04
10	mcpa	1.71E+10	2.59E+09	7.26E+09	4.90E+08	3.44E+05	1.98E+05	2.34E+04	1.17E+04
10	mcpa	1.68E+10	3.39E+09	6.15E+09	6.13E+08	5.19E+05	1.94E+05	2.14E+04	2.11E+03
10	mcpa	1.68E+10	2.60E+09	6.78E+09	1.95E+09	4.40E+05	1.26E+05	3.55E+04	1.78E+04
10	mcpa	1.78E+10	3.83E+09	8.00E+09	2.67E+09	5.93E+05	1.21E+05	4.92E+04	1.60E+04
27	mcpa	1.77E+10	1.19E+09	9.76E+09	2.17E+09	1.02E+06	6.41E+05	6.31E+04	4.45E+04
27	mcpa	1.73E+10	1.07E+09	8.76E+09	1.95E+09	1.60E+06	7.13E+05	6.36E+04	9.71E+03
27	mcpa	1.52E+10	2.17E+09	9.12E+09	3.19E+09	9.52E+05	3.64E+05	7.09E+04	4.78E+04
27	mcpa	2.07E+10	7.16E+09	7.42E+09	1.50E+09	7.04E+05	3.88E+05	5.11E+04	2.37E+04

		tfdA CI		*tfdA* CIII		*tfdA* CII	
sampling date	treatment	mean	std	mean	std	mean	std
3	control	1.70E+07	5.24E+06	2.93E+04	3.37E+04		
3	control	1.26E+07	5.94E+06				
3	control	1.48E+07	1.65E+06	3.03E+04	1.44E+04		
3	control	1.38E+07	6.79E+06				
10	control	6.75E+06	5.86E+06				
10	control	7.43E+06	3.32E+06				
10	control	1.04E+07	4.98E+06				
10	control	9.24E+06	2.46E+06				
27	control	9.47E+06	6.17E+06				
27	control	9.17E+06	1.58E+06	4.37E+04	2.83E+04	3.48E+05	4.07E+05
27	control	1.02E+07	3.17E+06	6.22E+04	4.50E+04	3.61E+05	4.72E+05
27	control	1.21E+07	3.92E+06	5.55E+04	2.92E+04	3.05E+05	2.44E+05
3	mcpa	1.48E+07	2.98E+06				
3	mcpa	1.37E+07	4.43E+06	1.32E+05	1.86E+05		
3	mcpa	1.75E+07	8.01E+06				
3	mcpa	1.48E+07	2.55E+06	7.11E+04	6.90E+04		
10	mcpa	7.27E+06	2.52E+06				
10	mcpa	6.26E+06	2.86E+06				
10	mcpa	6.14E+06	3.93E+06				
10	mcpa	7.89E+06	3.11E+06				
27	mcpa	1.03E+07	1.58E+06	1.38E+05	1.44E+05		
27	mcpa	9.67E+06	2.77E+06				
27	mcpa	1.16E+07	2.90E+06	1.01E+05	8.90E+04	2.14E+05	3.18E+05
27	mcpa	7.97E+06	2.67E+06	5.12E+04	4.98E+04		

Table S10.2 Mean and standard deviation (std) of measured gene abundances in treatments with litter addition at different sampling dates.

		16S rRNA genes		ITS fragment		*tfdA*		*cadA*	
sampling date	treatment	mean	std	mean	std	mean	std	mean	std
3	control	3.30E+10	1.02E+10	5.94E+10	2.23E+10	2.43E+06	3.66E+05	2.76E+04	3.48E+04
3	control	2.47E+10	3.45E+09	2.32E+10	4.99E+09	2.45E+06	9.22E+05	1.82E+04	2.00E+04
3	control	2.42E+10	8.39E+09	2.14E+10	4.04E+09	1.42E+06	7.25E+05	1.42E+04	1.69E+04
3	control	2.17E+10	1.99E+09	1.02E+10	1.51E+09	1.19E+06	5.23E+05	2.05E+04	1.78E+04
10	control	3.34E+10	3.74E+09	6.18E+10	1.38E+10	2.47E+06	5.76E+05	9.49E+04	4.80E+04
10	control	2.92E+10	6.33E+09	4.52E+10	1.10E+10	1.64E+06	5.70E+05	6.22E+04	1.61E+04
10	control	2.71E+10	1.71E+09	1.70E+10	8.03E+09	8.47E+05	1.55E+05	3.82E+04	6.02E+03
10	control	1.85E+10	7.09E+09	1.28E+10	7.55E+09	3.43E+05	2.01E+05	2.67E+04	1.43E+04
27	control	3.07E+10	6.57E+09	8.88E+10	2.62E+10	2.58E+06	2.37E+06	8.13E+04	7.02E+04
27	control	2.27E+10	3.29E+09	3.92E+10	1.84E+10	5.48E+05	2.31E+05	3.64E+04	1.72E+04
27	control	2.58E+10	4.54E+09	3.72E+10	2.38E+10	7.51E+05	5.27E+04	3.91E+04	1.71E+04
27	control	2.11E+10	2.03E+09	1.06E+10	1.38E+09	5.76E+05	9.87E+04	2.94E+04	1.57E+04
3	mcpa	2.91E+10	4.48E+08	9.81E+10	1.35E+10	7.82E+06	1.01E+07	5.20E+04	1.16E+04
3	mcpa	2.26E+10	2.73E+09	5.70E+10	1.07E+10	8.13E+06	1.22E+07	4.69E+04	8.43E+03
3	mcpa	2.20E+10	5.48E+09	4.43E+10	3.30E+09	2.28E+06	1.11E+06	3.73E+04	1.04E+04
3	mcpa	1.92E+10	5.56E+09	1.42E+10	4.02E+09	1.05E+06	8.29E+05	3.58E+04	7.87E+03
10	mcpa	3.34E+10	4.71E+09	1.10E+11	2.56E+10	5.08E+06	1.72E+06	1.35E+05	7.23E+04
10	mcpa	2.53E+10	4.38E+09	5.79E+10	2.30E+10	2.89E+06	5.11E+05	1.03E+05	3.95E+04
10	mcpa	2.67E+10	2.38E+09	4.08E+10	1.76E+10	2.01E+06	1.72E+05	1.02E+05	5.48E+04
10	mcpa	2.56E+10	1.02E+10	1.49E+10	5.47E+09	1.15E+06	5.66E+05	2.65E+04	1.23E+04
27	mcpa	3.80E+10	7.28E+08	2.14E+11	2.78E+10	5.11E+06	8.36E+05	1.26E+05	5.72E+04
27	mcpa	2.63E+10	1.37E+09	1.09E+11	8.92E+09	2.43E+06	1.14E+06	6.30E+04	1.38E+04
27	mcpa	2.27E+10	2.73E+09	8.70E+10	7.16E+09	1.62E+06	6.76E+05	6.49E+04	2.69E+04
27	mcpa	2.04E+10	3.36E+09	9.79E+09	8.67E+09	5.72E+05	3.68E+05	4.45E+04	7.52E+03

		tfdA CI		*tfdA* CIII		*tfdA* CII	
sampling date	treatment	mean	std	mean	std	mean	std
3	control	1.17E+07	3.10E+05	2.51E+05	5.10E+04		
3	control	1.82E+07	7.68E+06	1.51E+05	2.70E+04		
3	control	1.27E+07	3.67E+06	9.30E+04	8.28E+04		
3	control	1.28E+07	8.22E+05	6.35E+04	4.85E+04		
10	control	1.48E+07	3.74E+06	2.04E+05	2.65E+04		
10	control	1.25E+07	1.96E+06	7.67E+04	4.70E+04		
10	control	1.00E+07	2.67E+06	3.94E+04	2.90E+04		
10	control	6.36E+06	2.92E+06	4.40E+04	2.45E+04	8.40E+04	1.11E+05
27	control	1.14E+07	5.78E+06	6.59E+05	8.48E+05	1.73E+06	2.82E+06
27	control	1.00E+07	3.32E+06	8.07E+04	8.96E+04	2.17E+05	3.42E+05
27	control	1.18E+07	2.37E+06	5.99E+04	4.79E+04	1.64E+05	1.36E+05
27	control	1.12E+07	3.06E+06	3.60E+04	3.10E+04		
3	mcpa	2.47E+07	1.38E+07	1.38E+05	2.10E+04		
3	mcpa	2.84E+07	2.29E+07	5.63E+04	1.95E+04		
3	mcpa	1.34E+07	1.24E+06				
3	mcpa	1.15E+07	5.65E+06	2.25E+05	2.64E+05		
10	mcpa	1.46E+07	2.85E+06	5.78E+05	5.40E+05	2.54E+05	3.10E+05
10	mcpa	1.09E+07	1.80E+06				
10	mcpa	1.15E+07	3.31E+06	9.18E+04	5.48E+04		
10	mcpa	9.21E+06	1.81E+06	7.61E+04	5.34E+04	7.74E+04	8.54E+04
27	mcpa	1.57E+07	3.85E+06	6.50E+05	3.89E+05	2.31E+05	2.77E+05
27	mcpa	1.67E+07	7.49E+06	1.90E+05	9.80E+04	2.39E+05	2.39E+05
27	mcpa	2.11E+07	6.20E+06	1.60E+05	1.07E+05	2.17E+05	2.14E+05
27	mcpa	8.79E+06	2.76E+06	5.77E+04	4.87E+04	1.27E+05	1.15E+05

Curriculum vitae

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Education

Since 2011	PhD student at the Institute of Soil Science and Land Evaluation, University of Hohenheim/Germany, funding by the Deutsche Forschungsgemeinschaft (DFG) priority program 1315 " Biogeochemical Interfaces in Soil"
2004 –2008	University Hohenheim/Germany, studies of agrarbiology with focus on agrarbiotechnology
2002 – 2004	Berufsoberschule, Franz-Oberthür-Schule, Würzburg/Germany general qualification for university entrance (Abitur)
2000 – 2002	Staatliche Ausbildungsstätte für agrartechenische Assistentinnen, Landsberg am Lech/Germany staatlich geprüfte ATA, specialisation: agricultural economy with focus on plant production
1997 – 2000	secondary school (Realschule), Schönborn Würzburg/Germany, general certificate of secondary education (mittlere Reife)

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2008 – 2010	Landesbauernverband in Baden-Württemberg e.V., Stuttgart/Germany Agrarreferentin in der Landjugend
2008	Institute for pharmaceutical biology, University Würzburg/Germany scientific assistant

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2011	Real-Time PCR training course: basics & optimization
2008	Workshop for project manager: safety in laboratory operation
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Esslingen, den 20.09.2016 Franziska Ditterich

Publications and Presentations

Parts of the PhD thesis and other projects were published or presented on conferences as follows:

Publications

Included in the submitted dissertation

Ditterich F., Poll C., Pagel H., Smalla K., Horn M., Streck T. and Kandeler E. (2013) Succession of bacterial and fungal MCPA degraders at the soil litter interface. FEMS Microbiology Ecology 86: 85-100.

Ditterich F., Poll C., Pronk G.J., Heister K., Abhirosh C., Rennert T., Kögel-Knabner I. and Kandeler E. (2015) Succession of soil microbial communities and enzyme activities in artificial soils. Pedobiologia 59/3:93-104.

Other

Mackie K.A., Marhan S., Ditterich F., Schmidt H.P. and Kandeler E. (2015) The effects of biochar and compost amendments on copper immobilization and soil microorganisms in a temperate vineyard. Agriculture, Ecosystems and Environment 201: 58–69.

Presentations

Poster Presentations

Lang F., Poll C., Pagel H., Pronk G., Heister K., Kögel-Knabner I., Streck T. and Kandeler E. (2011) Interaktion zwischen MCPA-Abbauern und der organisch-mineralischen Oberfläche unter Verwendung von artificial soils. Annual meeting of the DBG, Berlin, Germany.

Lang F., Poll C., Pagel H., Pronk G., Heister K., Kögel-Knabner I., Streck T. and Kandeler E. (2011) Microbial regulation of MCPA degradation in artificial soils. Annual meeting of the SPP 1315, Dornburg, Germany.

Ditterich F., Poll C., Pronk G., Heister K., Kögel-Knabner I. and Kandeler E. (2012) Microbial colonization of artificial soils. Eurosoil, Bari, Italy.

Ditterich F., Poll C., Pronk G., Heister K., Kögel-Knabner I. and Kandeler E. (2012) Mikrobielle Kolonisation von künstlichen Böden. Meeting of the DBG comission III, Hohenheim, Germany.

Ditterich F., Poll C., Pagel H., Babin D., Smalla K., Horn M., Streck T. and Kandeler E. (2012) Succession of bacterial and fungal MCPA degraders at the soil-litter interface. Annual meeting of the SPP 1315, Dornburg, Germany.

Gebala A., Ditterich F., Pagel H., Streck T., Poll C. and Kandeler E. (2014). Impact of laccases on the decomposition of MCPA at the soil-litter interface – a soil microcosm experiment. DBG workshop “Soil processes – is the whole system regulated at ‘hot spots’?”, Freising, Germany.

Ditterich F., Gebala A., Pagel H., Streck T., Poll C. and Kandeler E. (2014) Impact of laccases on the decomposition of MCPA at the soil-litter interface – a soil microcosm experiment. Final symposium of the SPP 1315, Leipzig, Germany.

Ditterich F., Gebala A., Poll C., Pronk G., Heister K., Kögel-Knabner I. and Kandeler E. (2014) Succession of soil microbial communities and enzyme activities in artificial soils. Final symposium of the SPP 1315, Leipzig, Germany.

Oral Presentations

Kandeler E., Poll C., Pagel H., Ingwersen J., Lang F., and Streck T. (2012). Modeling Carbon Dynamics in Small-Scale Microbial Ecology of Soils. Conference: "Enzyme Modelling". Fort Collins, United States.

Lang F., **Poll C**., Pagel H., Streck T. and Kandeler E. (2012) Diversity of MCPA degraders at the soil-litter interface. Symposium Life in Microhabitats of Soils, Dornburg, Germany.

Ditterich F., Poll C., Pagel H., Streck T. and Kandeler E. (2012) Sukzession bakterieller und pilzlicher MCPA Abbauer in der Detritussphäre. Meeting of the DBG commission III, Hohenheim, Germany.

Pagel H., Lang F., Poll C., Ingwersen J., Babin D., Smalla K., Horn M.A., Miltner A., Kästner M., Schulz S., Wick L.Y., Schloter M., Kandeler E. and Streck T. (2012). The soil-litter interface: A hot spot for biogeochemical interactions. SPP 1315 meeting, Dornburg, Germany.

Ditterich F., **Poll C.**, Pronk G., Heister K., Kögel-Knabner I. and Kandeler E. (2013) Succession of soil microbial communities and enzyme activities in artificial soils. Goldschmidt Conference, Florence, Italy.

Poll C., Ditterich F., Pronk G., Heister K., Kögel-Knabner I. and E. Kandeler (2013) Mikrobielle Besiedlung von Mineraloberflächen. Annual meeting of the DBG, Rostock, Germany.

Pagel H., Poll C., Ingwersen J., Ditterich F., Gebala A., Kandeler E. and Streck T. (2014). Micro-scale Modeling of Pesticide Degradation Coupled to Carbon turnover in the Detritusphere. International Symposium SPP 1315, Leipzig, Germany.

Pinheiro M., Ditterich F., **Pagel H.**, Poll C., Garnier P., Streck T., Kandeler E. and Vieuble Gonod L. (2014). Water flow drives small scale biogeography of pesticides and soil microorganisms - a microcosm study using 2,4-D as a model compound. IUSS, Korea.

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